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RESEARCH ARTICLE

Bioprinted Notch ligand to function as stem cell niche improves muscle regeneration in dystrophic muscle

Zewei Sun[1†], Xianlin Yue[1†], Lei Liu[1], Ying Li[1], Jie Cui[1], Dong Li[2], Lee Weiss[3], Phil Campbell[3], Yanling Mu[1*], Johnny Huard[4*], Xiaodong Mu[1*]

[1]Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, China

[2]Cryomedicine Lab, Qilu Hospital of Shandong University, Jinan, Shandong, China

[3]The Robotics Institute, Carnegie Mellon University, Pittsburgh, PA, USA

[4]Center for Regenerative Sports Medicine, Steadman Philippon Research Institute, Vail, CO, USA

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Abstract

In Duchenne muscular dystrophy, dystrophic muscle phenotypes are closely associated with the exhaustion of muscle stem cells. Transplantation of muscle stem cells has been widely studied for improving muscle regeneration, but poor cell survival and self-renewal, rapid loss of stemness, and limited dispersion of grafted cells following transplantation have collectively hindered the overall success of this strategy. Optimized mechanisms for maintaining and improving stem cell function are naturally present in the microenvironment of the stem cell niche in healthy muscles. Therefore, one logical strategy toward improving stem cell function and efficiency of stem cell transplantation in diseased muscles would be the establishment of a microenvironment mimicking some key aspects of healthy native stem cell niches. Here, we applied inkjet-based bioprinting technology to engineer a mimicked artificial stem cell niche in dystrophic muscle, comprising stem cell niche regulating factors (Notch activator DLL1) bioprinted onto 3D DermaMatrix construct. The recombinant DLL1 protein, DLL1 (mouse): Fc (human) (rec), was applied here as the Notch activator. Bioprinted DermaMatrix construct was seeded with muscle stem cells *in vitro*, and increased stem cell maintenance and repressed myogenic differentiation process was observed. DLL1 bioprinted DermaMatrix construct was then engrafted into dystrophic muscle of *mdx*/*scid* mice, and the improved cell engraftment and progression of muscle regeneration was observed 10 days after engraftment. Our results demonstrated that bioprinting of Notch activator within 3D construct can be applied to serve as muscle stem cell niche and improve the efficacy of muscle stem cell transplantation in diseased muscle.

Keywords: Muscle dystrophy, Stem cell niche, Muscle stem cell, Notch signaling

[†]These authors contributed equally to this work.

***Corresponding authors:**
Xiaodong Mu (muxiaodong@sdfmu.edu.cn)

Yanling Mu (muyanling@sdfmu.edu.cn)

Johnny Huard (jhuard@sprivail.org)

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1. Introduction

During the regeneration of injured or diseased skeletal muscle, the deposition of scar tissue (fibrosis) often dominates the healing process when stem cells become dysregulated or depleted. Indeed, the depletion of functional muscle stem cells has been

observed in both aged muscles and dystrophic muscles of patients with Duchenne muscular dystrophy (DMD)[1,2]. Therefore, transplantation of muscle stem cells has been widely studied for improving the regeneration of injured or diseased skeletal muscles[3-5]. However, poor cell survival and self-renewal, rapid loss of stemness, high occurrence of fibrogenesis, and limited dispersion of grafted cells away from the site of injection following transplantation have collectively hindered the overall success of this strategy[6-10]. Therefore, for successful clinical translation, new strategies are needed to improve the efficacy of stem cell transplantation for the treatment of diseased muscle.

In DMD patients, despite the lack of dystrophin at birth, clinical signs and symptoms of muscle weakness do not become apparent until the patient reaches the age of 4–8 years which happens to coincide with the depletion of the muscle stem cell pool[1]. These observations suggest that preventing the depletion of the stem cell pool may represent a novel approach to improve muscle strength in patients with DMD despite the lack of dystrophin expression.

Notch signaling is more activated in younger skeletal muscle, but declines as muscle ages[11-13]. Notch activity in dystrophic muscle was shown to be decreased[14]. Also, our preliminary data demonstrated decreased Notch activity in skeletal muscle of *mdx* mice compared to normal mice. Therefore, the lack of activated Notch signaling in dystrophic muscle should be considered when performing stem cell therapy for DMD.

Notch is a crucial molecular regulator of stem cell activity in skeletal muscle. In addition to maintaining proper function of the stem cell niche, Notch activation is also able to repress apoptosis[15,16] and fibrogenesis[17], and promote angiogenesis[18,19] of many cell types. Although these concepts of Notch in stem cell niches have been well established and proven in the laboratory, the practical application of these concepts has never been implemented in the treatment of muscle diseases.

Stem cell niches in skeletal muscle are "nests" of quiescent stem cells beneath the basal lamina of myofibers and are critical for other cells to interact with the stem cells to maintain them or promote their differentiation[20]. When skeletal muscle is damaged, stem cells within the niche are activated to proliferate via asymmetric division[21]. Some of these cells migrate toward the site of injury to participate in muscle regeneration while other cells remain within the niche to maintain the stem cell pool[20,21]. Maintenance of the stem cell niche therefore determines muscle regeneration potential. In diseased or aged muscles, the functions of stem cell niches are impaired, resulting in the loss of self-renewal and regeneration capacities of stem cells[20,22]. Therefore, engineering an artificial microenvironment that has some similarities to native stem cell niches could represent a promising strategy to improve the efficacy and survivability of stem cells transplanted into diseased or injured muscles, such as dystrophic muscle. The efficiency of stem cell transplantation depends largely on the number of cells that survive transplantation and maintain the regeneration potential of those cells. Notch activation is effective for repressing apoptosis[15,16] and fibrogenesis[17], promoting angiogenesis[18,19], and maintaining the self-renewal capacity of muscle stem cells[23-25]. Notch activation is also a key molecular signature of native stem cell niches in skeletal muscle and is required for the colonization of stem cells within the niche and asymmetric cell division [21,26,27]. A recent study of two exceptional Golden retriever muscular dystrophy (GRMD) dogs that escaped from the severe phenotype associated with dystrophin deficiency revealed that Jagged1, a Notch ligand, is upregulated in mildly affected dystrophin-deficient dogs[28]. Based on these observations, Notch activation seems to be a promising strategy for the healing of dystrophic muscle. In our previous studies, muscle progenitor cells (MPCs) were effectively isolated from skeletal muscle using a modified preplate technique[29], and these cells were proven to be highly effective in promoting the regeneration of multiple tissue types after transplantation in both skeletal and cardiac muscle[3,30,31].

Three-dimensional printing facilitates the application of scaffold-based or scaffold-free tissue and organ constructs, mini-tissues, and organ-on-a-chip model system. Using a 3D bioprinter allows for the proper distribution and positioning of biomaterials, signaling factors, and heterogeneous cells in high densities to form tissue engineering constructs (TECs). Moreover, 3D-bioprinted constructs with interconnected pores and large surface areas support cell attachment, growth, intercellular communication, and exchange of gas and nutrients. Compared with the conventional postcell seeding approach, bioprinting achieved a close connection between materials and cells, resulting in higher cell-loading efficiency and more homogenous cell distribution within the constructs[32-34]. According to different forming principles and printing materials, biological 3D printing process can be divided into vat polymerization, material extrusion, material jetting, etc. Although the extrusion-based bioprinting approach is a commonly used technique for fabrication of 3D complex tissue constructs due to its wide range of printable materials and rapid fabrication speed, the drop-on-demand material jetting approach is attractive for contactless deposition and patterning of different types of living cells and biomaterials within each layer to achieve improved cell–cell and cell–matrix interactions[35-38], which was therefore applied in our study of new strategies to improve the regeneration of dystrophic muscle.

Therefore, we applied inkjet-based bioprinting technology to create artificial stem cell niches comprised of regulators of Notch activation (Notch ligands) bioprinted into biodegradable 3D construct, and evaluated their effects with seeded MPCs by intramuscular implantation into the gastrocnemius muscle of *mdx*/*scid* mice. The efficiency of stem cell transplantation using Notch activator/3D construct delivery was compared to using 3D construct delivery alone.

2. Materials and methods

2.1. Mice model

Wild-type (WT; C57BL/10J), *mdx* and *mdx*/*scid* mice (6-month-old, males) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in groups of 4 on a 12:12-h light-dark cycle at 20°C–23°C. All mice were housed and maintained in accordance with established guidelines and protocols approved by the UTHealth Animal Welfare Committee.

At least eight mice were used in each experimental sample group. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh (IACUC-1109718).

2.2. Muscle cell isolation from skeletal muscle

The MPCs were isolated from skeletal muscle tissues of WT mice (8-week-old, male) using the modified preplate technique[29]. Mice were sacrificed in a carbon dioxide chamber according to standard protocols, and gastrocnemius muscles were collected for the isolation of MPCs. MPCs cells were cultured in culture medium specific for the MPCs (Dulbecco's Modified Eagle Medium [DMEM] supplemented with 20% fetal bovine serum [FBS], 1% penicillin-streptomycin antibiotics, and 0.5% chicken embryo extract [CEE]).

2.3. mRNA analysis via reverse-transcription polymerase chain reaction

Total RNA was obtained from the skeletal muscles of mice using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sequences of primers are shown in Table 1 for Notch1, Hes1, Jagged1, DLL1, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Regular PCR reactions were performed using an iCycler thermal cycler (Bio-Rad Laboratories, Inc.). The cycling parameters used for all primers are as follows: 95°C for 10 min for initial denaturation; PCR, 40 cycles of 30 s at 95°C for denaturation, 1 min at 54°C for annealing, and 30 s at 72°C for extension. All data were normalized to the expression of GAPDH.

Table 1. PCR primer sequences

Gene	Primer sequence
GAPDH	Forward: 5′-TCCATGACAACTTTGGCATTG-3′ Reverse: 5′-TCACGCCACAGCTTTCCA-3′
Notch1	Forward: 5′-GCCGCAAGAGGCTTGAGAT-3′ Reverse: 5′-GGAGTCCTGGCATCGTTGG-3′
Hes1	Forward: 5′-CCAGCCAGTGTCAACACGA-3′ Reverse: 5′-AATGCCGGGAGCTATCTTTCT-3′
Jagged1	Forward: 5′-ACAGTTGTTATGGGTGGCTCT-3′ Reverse: 5′-CGGCTCCTCTCACGTTCTTTC-3′
DLL1	Forward: 5′-CAGGACCTTCTTTCGCGTATG-3′ Reverse: 5′-AAGGGGAATCGGATGGGGTT-3′

2.4. Inkjet-based bioprinting system and bioprinting of Notch ligand

The custom inkjet printing system at Drs. Phil Campbell and Lee Weiss's group has been proven to be efficient for creating 3D constructs printed with protein and delivering cells into tissues[40-43]. Bioinks were printed as defined patterns on the dermal surface of 4-mm diameter discs of acellular DermaMatrix (ADM; Synthes, West Chester, PA, USA) using our custom inkjet-based biopatterning system, as previously described[40-43]. Briefly, the deposited concentrations are modulated using an overprinting strategy whereby each location on the pattern is overprinted with dilute bio-inks (sodium phosphate buffer, pH 7.4) such that the deposited concentrations increase in proportion to the number of overprinted drops. A drop-on-demand piezoelectric inkjet printhead with a 30-μm-diameter orifice (MicroFab Technologies, Plano, TX) was used for patterning. The center-to-center drop spacing was set to 75 μm. To passivate the glass surface of the inkjet, it was filled with 1 μg/mL bovine serum albumin (Sigma Chemical, St. Louis, MO) and incubated for 10 min. The jet was rinsed three times with deionized water and filled immediately with the bioink. The bioinks consisted of human DLL1 (R&D Systems, Minneapolis, MN), diluted in 10 mM sodium phosphate, pH 7.4. Deposited inks absorb into the ADM prior to drying, so patterns are created within the matrix. A square pattern of Notch ligand was printed on each disc, with 50 overprints (OPs) of 100 μg/ml bioinks. Notches were cut in the discs opposite the printed area to maintain orientation upon implantation. Delta-like protein-1 (DLL1) is important activating ligand for Notch signaling. DLL1 (mouse):Fc (human) recombinant protein (Adipogen) (i.e., the fusion of signal peptide mouse DLL1 at the C-terminus to the Fc portion of human IgG1) can function as DLL1 ligand. The DermaMatrix construct was placed on the printing stages and was held in place during printing using a vacuum chuck.

2.5. Myogenesis and proliferation assays

For myogenic differentiation assay, WT MPCs were seeded as ~90% confluence in the DMEM containing 2% horse serum (HS) and allowed for differentiation for 96 h before being fixed and imaged to compare the number of myotubes formed by WT MPCs in different groups. For proliferation assay, WT MPCs were seeded as 2000 cells/cm^2 in the DMEM containing 20% FBS and 1% CEE and allowed for proliferation for 72 h before being observed to compare the number of WT MPCs in different groups.

2.6. *In vitro* migration assay

DLL1(mouse):Fc (human)(rec) was bioprinted onto DermaMatrix construct (4 × 4 × 1 mm in dimensions). Cell seeding was performed by injecting of 0.2 × 10^5 green fluorescent protein (GFP)-labeled MPCs into both the DLL1-bioprinted DermaMatrix constructs and control DermaMatrix constructs (IgG Fc) and cultured in medium. Three days after cell seeding, DermaMatrix constructs were placed in the Matrigel-coated upper chamber of an electrode-impedance-based invasion assay system (xCELLigence)[39]. The Matrigel layer was made of Matrigel dissolved in medium (1 mg/mL). The numbers of cells migrating out of the DermaMatrix constructs and through the 3D Matrigel layer were monitored.

2.7. Bioprinting of Notch ligand on coverslip

DLL1(mouse): Fc(human) (rec.) was bioprinted onto fibrin/protein G-coated coverslips, as a circular dot with a diameter of 1.5 mm. WT MPCs (0.5 × 10^4/cm^2) were then seeded on the slides and cultured for 4 days. Immunostaining with Myosin Heavy Chain antibody (MHC-MF20) were performed to track the myogenic differentiation potential of MPC.

2.8. Implantation of bioprinted constructs

The DermaMatrix construct (4 × 4 × 1 mm in dimensions) seeded with MPCs (4 × 10^4) was implanted into the gastrocnemius (GM) muscle of *mdx*/*scid* mice (6-month-old). At 10 days after implantation, cell engraftment was compared between DermaMatrix constructs with or without bioprinted DLL1.

2.9. Histology and immunohistochemistry

GM muscles of *mdx*/*scid* mice implanted with MPC-seeded DLL1-DermaMatrix constructs were harvested 10 days after implantation. Snap-frozen skeletal muscles were cryo-sectioned at a thickness of 10 microns and processed for histological analysis. Muscle stem cells seeded in DermaMatrix constructs were identified and tracked by the expression of GFP. Muscle regeneration involving the direct fusion of implanted WT-MPCs was tracked and confirmed by immunostaining of dystrophin.

2.10. Statistical analysis

Image analysis was performed with ImageJ software (version 1.32j; National Institutes of Health, Bethesda, MD, USA). Data from at least six samples from each subject were pooled for statistical analysis. Results are given as the mean ± standard deviation (SD). Statistical differences between groups in the functional assays were determined by two-way repeated analysis of variance (ANOVA). For other experiments, statistical differences were determined by Student's *t*-test (2 categories) or one-way ANOVA (>2 categories).

3. Results

3.1. Skeletal muscle cells in *mdx* mice developed lower Notch activity, and Notch reactivation in dystrophic muscle promoted stem cell proliferation

Notch activity in skeletal muscle declines with age in both mice and humans, and this decline is responsible for the depletion of functional muscle stem cells[11-13]. When compared to WT mice (6-month-old), we observed a down-regulation in the expression of key Notch signaling factors (i.e., Notch1, Hes1, Jagged1 and DLL1/ Delta-like protein-1) in the skeletal muscle of *mdx* mice (Figure 1A). To determine the effects of reactivating Notch signaling in dystrophic muscle, we then chose a recombinant protein of Notch ligand DLL1 [DLL1 (mouse): Fc (human) (rec), Adipogen] as a Notch activator to be tested in our system. DLL1 (mouse): Fc (human) (rec) was injected into the limb muscles (gastrocnemius) of *mdx* mice, and the number of Pax7-positive cells was found to be obviously increased 3 days after injection when compared to the control mice injected with inactive IgG (Figure 1B and C). Therefore, this result indicates that the reactivation of Notch signaling in dystrophic muscle is effective in promoting self-renewal of muscle stem cells. However, the effect of this local injection of soluble protein could be temporary, and we expect that the delivery of bioprinted Notch ligands on some type of biocompatible carrier could generate a much longer effect in promoting stem cell proliferation.

3.2. Treatment of muscle progenitor cells with DLL1 recombinant protein *in vitro* effectively promoted cell proliferation capacity and inhibited myogenic differentiation capacity.

We then tested the way of DLL1-Fc in affecting the function of muscle stem cells. MPCs were isolated from 8-week-old WT mice (C57BL/6J, Jackson Lab) using a modified preplate technique[29]. MPCs were then treated with DLL1 (mouse): Fc (human) (rec) (200 ng/mL) for 3 days for either the proliferation assay or the myogenic differentiation assay. Results showed that the proliferation potential of the MPCs was increased and the myogenic potential was

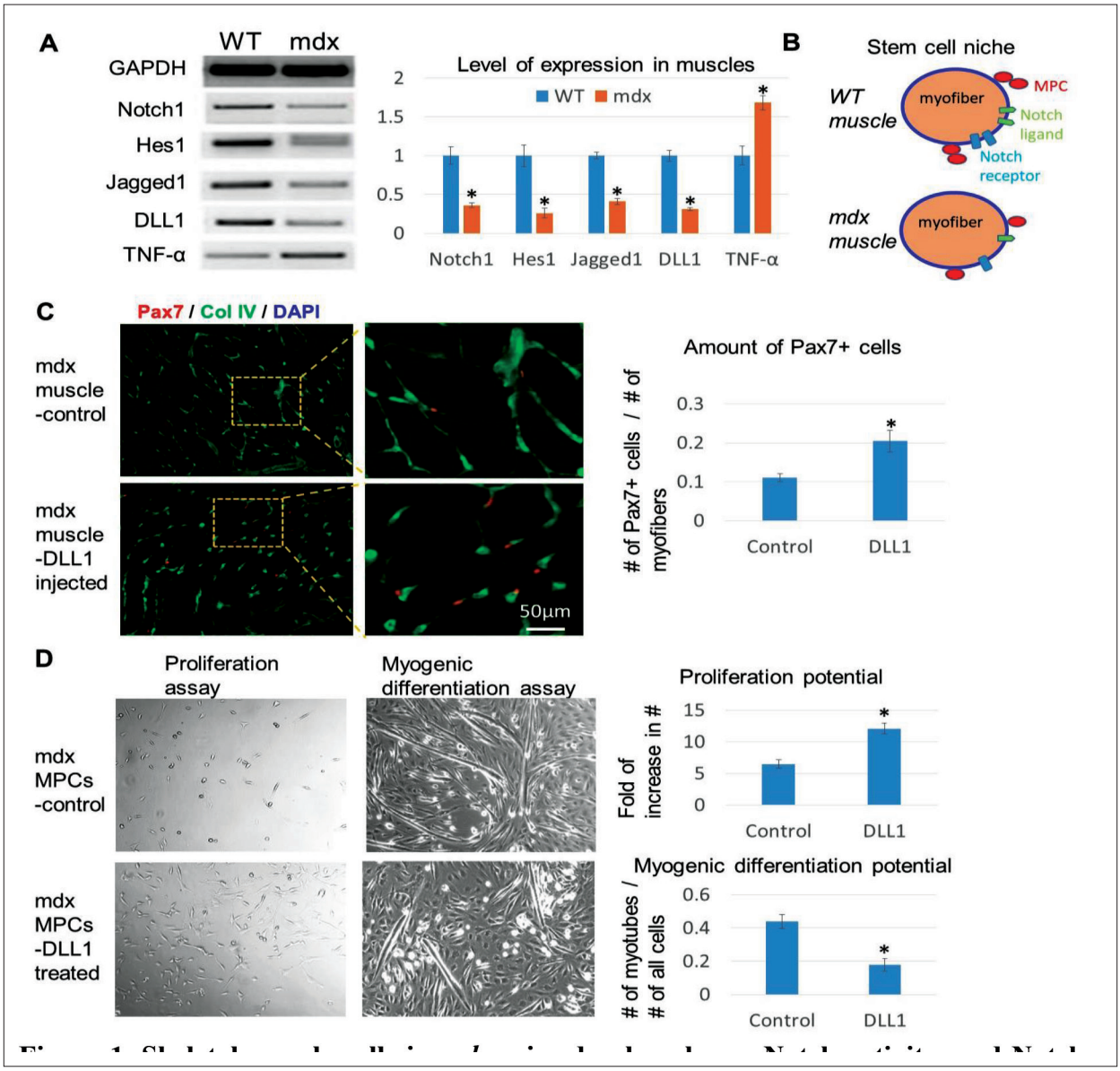

Figure 1. Skeletal muscle cells in mdx mice develops lower Notch activity, and Notch reactivation in dystrophic muscle promotes stem cell proliferation. (A) RT-PCR assay with skeletal muscles (gastrocnemius) from 6-month-old WT and *mdx* mice verified that the expression of Notch signaling factors, such as Notch1, Hes1, Jagged1 and DLL1, were generally down-regulated in mdx muscle compared to WT muscle. (B) The schematic demonstration of differential status of Notch activation in muscle stem cell niche (surface of myofibers) of WT and mdx muscle, and its effect on the number of muscle progenitor cells (MPCs). (C) To determine the effects of reactivating Notch signaling in dystrophic muscle, a recombinant protein DLL1 (mouse): Fc (human) (rec) (40 μg in 20 μL of PBS) was injected into the limb muscles (gastrocnemius) of *mdx* mice and the number of Pax7-positive cells was found to be obviously increased 3 days after injection when compared to the control mice injected with inactive IgG. (D) *In vitro* treatment of mdx MPCs with DLL1 (mouse): Fc (human) (rec) (200 ng/mL) led to increased proliferation potential and repressed myogenesis potential of mdx MPCs.

decreased (Figure 1C). These results verify that DLL1-FC is effective at regulating the activity of MPCs.

3.3. Bioprinted Notch ligand effectively maintained the undifferentiated status of muscle stem cells

DLL1 (mouse):Fc (human) (rec) was bioprinted onto fibrin/protein G-coated coverslips. MPCs were then seeded on the slides and cultured for 4 days. Immunostaining with Myosin Heavy Chain antibody (MHC-MF20) showed that there were many MHC-MF20-positive cells (myotubes) at the location in the absence of bioprinted DLL1, whereas there were almost no MHC-MF20-positive cells on printed DLL1 patterns (Figure 2, MHC-MF20 is green and DAPI is blue), indicating that the bioprinted DLL1 prevented

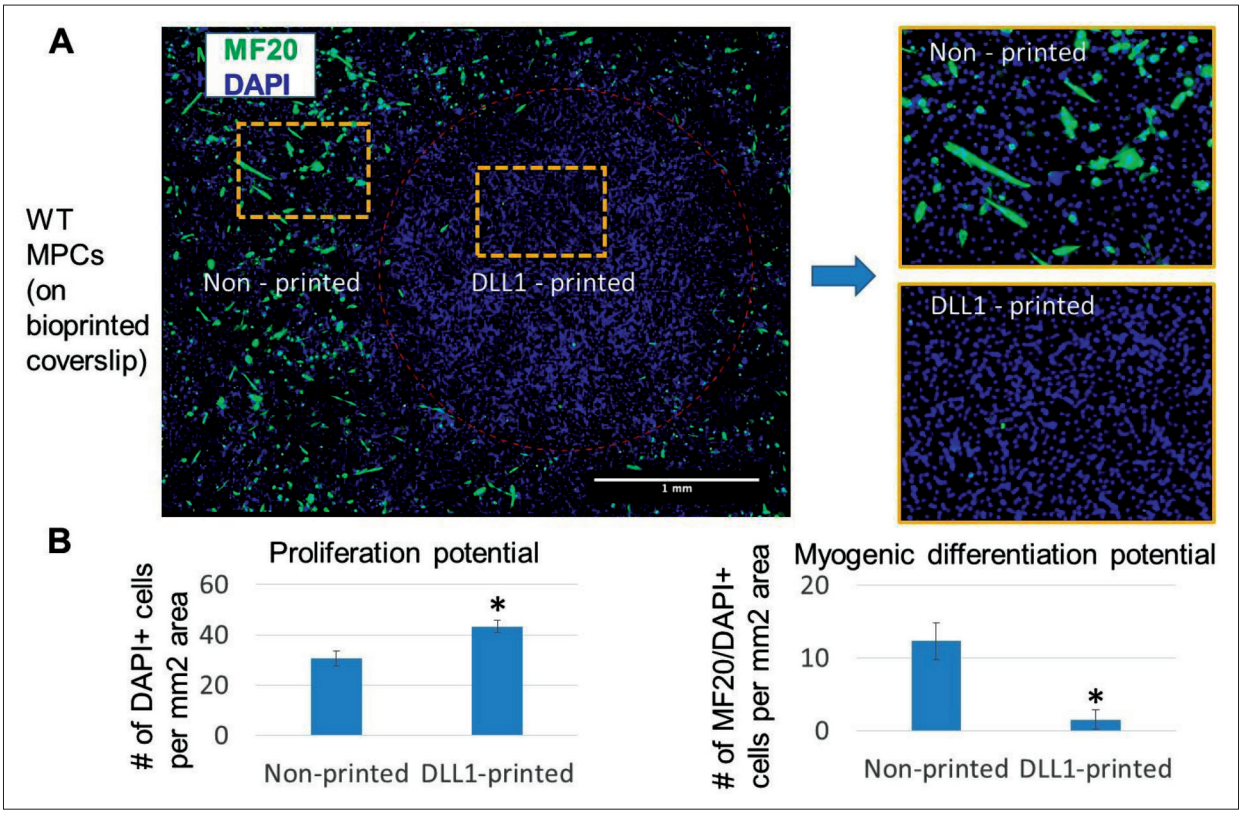

Figure 2. Bioprinted Notch ligand effectively maintained the undifferentiated status of muscle stem cell. (A) DLL1 (mouse): Fc (human) (rec.) was bioprinted onto fibrin/protein G-coated coverslips. MPCs from 6-month-old WT mice (WT MPCs) were then seeded on the slides and cultured for 4 days. Immunostaining with Myosin Heavy Chain antibody (MHC-MF20) showed that there were many MHC-MF20-positive cells (myotubes) at the location in the absence of bioprinted DLL1, whereas there were almost no MHC-MF20-positive cells on printed DLL1 patterns (MHC-MF20: green; DAPI: blue), indicating that the bioprinted DLL1 prevented the differentiation of seeded muscle stem cells, thus maintaining their undifferentiated stem cell state. (B) The statistics of proliferation and myogenic differentiation potential.

the differentiation of seeded muscle stem cells, thus maintaining their undifferentiated stem cell state.

3.4. Persistence of printed IgG Fc (a model for Fc fusion proteins DLL1-Fc we are using) on 3D construct

We performed protein binding experiments to demonstrate that Fc fusion proteins can be stably bioprinted and biopatterned on an artificial 3D DermaMatrix constructs (derived from human acellular dermis). Cy3-labeled human IgG Fc was printed onto DermaMatrix construct, and printed constructs were then rinsed with phosphate-buffered saline (PBS; 2 times for 15 min, 37°C). Fluorescent images were taken immediately postprinting (before washing with PBS for 15 min) and 5 days postprinting, rinsed, and incubated with simulated interstitial fluid (10% serum MEM culture media containing 25 mM HEPES and 0.1% sodium azide) at 37°C (Figure 3A). As it is shown in the images, increasing the number of OP (overprints / repeated prints) accordingly increased the amount of IgG Fc protein printed onto the DermaMatrix constructs. Following the removal of unbound IgG Fc, the printed pattern persisted throughout the incubation period. Therefore, by performing repeated overprints of the Fc protein, the system is effective at assuring the persistence of Fc protein on the DermaMatrix constructs. This indicates that when IgG bioprinted DermaMatrix constructs are prerinsed, ~70% of initial bound IgG remains bound after 11 days of incubation under simulated *in vivo* condition. In fact, over 18 days after rinsing and incubation, the levels of IgG bound to the DermaMatrix constructs were very consistent (Figure 3B), further validating the retention of IgG on the DermaMatrix constructs. GFP-labeled WT MPCs (GFP-WT MPCs) were seeded in the DermaMatrix constructs with DLL1-printed or nonprinted areas, and the growth potential of the two groups of cells were compared (Figure 3C), demonstrating the cells seeded in the DLL1-printed area grew faster than the cells in nonprinted areas.

3.5. More MPCs from DLL1-conjugated constructs were able to migrate through 3D-Matrigel layers in an *in vitro* migration assay

DLL1 (mouse):Fc (human)(rec) was bioprinted onto biodegradable DermaMatrix construct (4 × 4 × 1 mm in

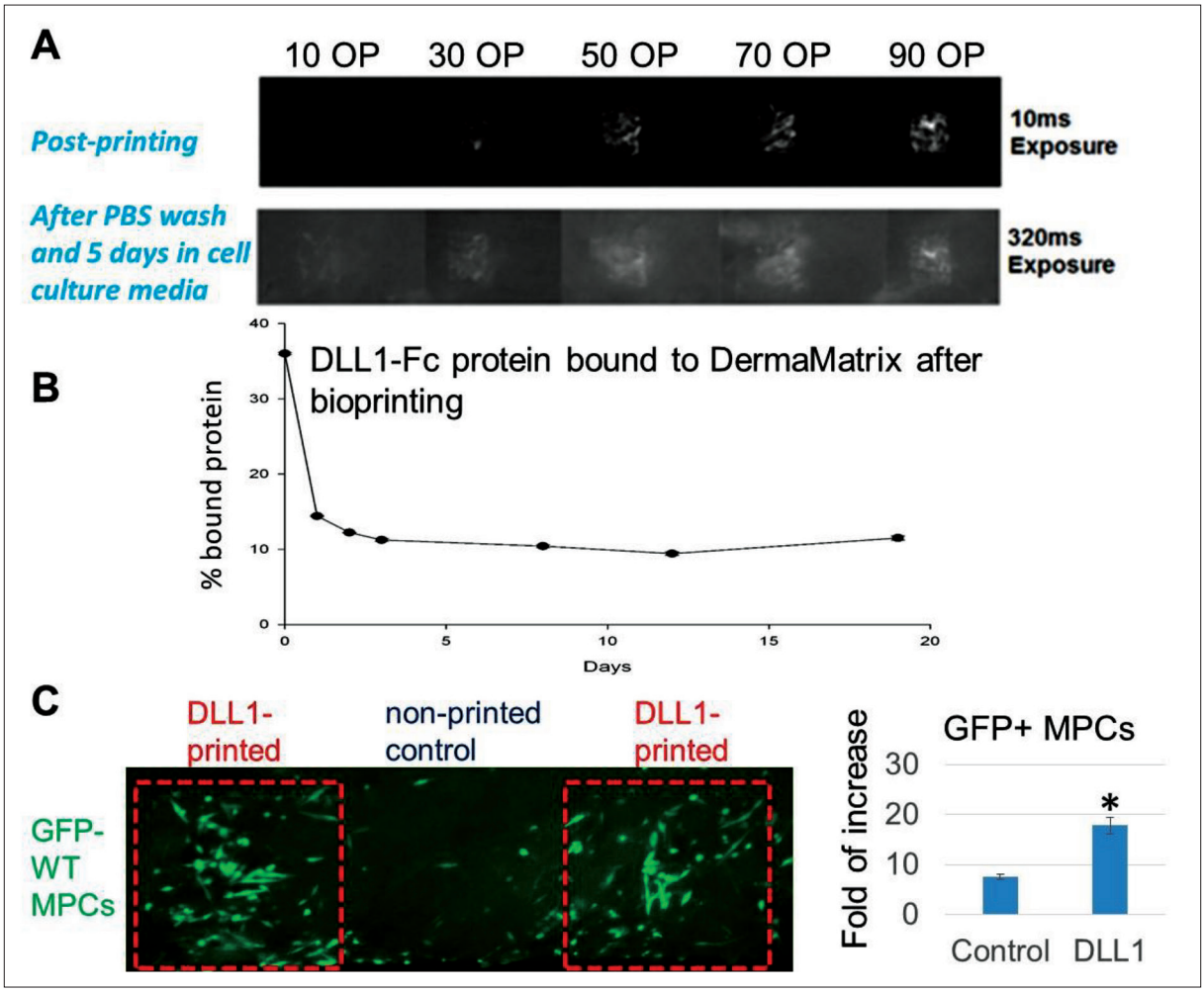

Figure 3. Persistence of bioprinted Fc fusion proteins on 3D construct. (A) We performed protein binding experiments to demonstrate that Fc fusion proteins can be stably bioprinted and biopatterned on artificial 3D construct, made of DermaMatrix. Cy3-labelled human IgG Fc was printed onto DermaMatrix construct, with varying number of OP (overprints/repeated prints). Fluorescent images were taken immediately postprinting (before washing with PBS for 15 min) and 5 days postprinting. Incubation was performed in simulated interstitial fluid (10% serum MEM culture media containing 25 mM HEPES and 0.1% sodium azide). Following the removal of unbound IgG Fc, the printed pattern persisted throughout the incubation period. (B) Over 18 days after rinsing and incubation in simulated interstitial fluid, the levels of IgG bound to the DermaMatrix construct were very consistent. (C) GFP-labeled WT MPCs (GFP-WT MPCs) were seeded in the DermaMatrix construct with DLL1-printed or nonprinted areas to compare their growth rate.

dimensions). Cell seeding was performed by injecting of 0.2×10^5 GFP-labeled MPCs into both the DLL1-bioprinted DermaMatrix constructs and control DermaMatrix constructs (IgG Fc) and cultured in medium. Three days after cell seeding, DermaMatrix constructs were placed in the Matrigel-coated upper chamber of an electrode-impedance-based invasion assay system (xCELLigence) (Figure 4A)[39]. The 3D Matrigel layer was made of Matrigel dissolved in medium (1 mg/mL). The numbers of cells migrating out of the DermaMatrix constructs and through the 3D Matrigel layer were monitored; we found that the number of cells migrating out of DLL1-DermaMatrix constructs were much higher (Figure 4B). The increased migration capacity may be beneficial for improving cell engraftment when constructs are implanted into skeletal muscle.

3.6. Implantation of GFP-MPC-seeded DLL1-bioprinted DermaMatrix constructs into *mdx* mice resulted in improved cell engraftment

MPCs were virally transfected to express GFP protein and seeded into DermaMatrix constructs with or without bioprinted DLL1 (4×10^4 cells in $4 \times 4 \times 1$ mm construct). Ten days after the implantation of GFP-MPC-seeded DLL1-DermaMatrix constructs or MPC-seeded control DermaMatrix constructs, an improved cell engraftment was observed in the muscle tissue implanted with the MPC-seeded DLL1-DermaMatrix constructs (Figure 5).

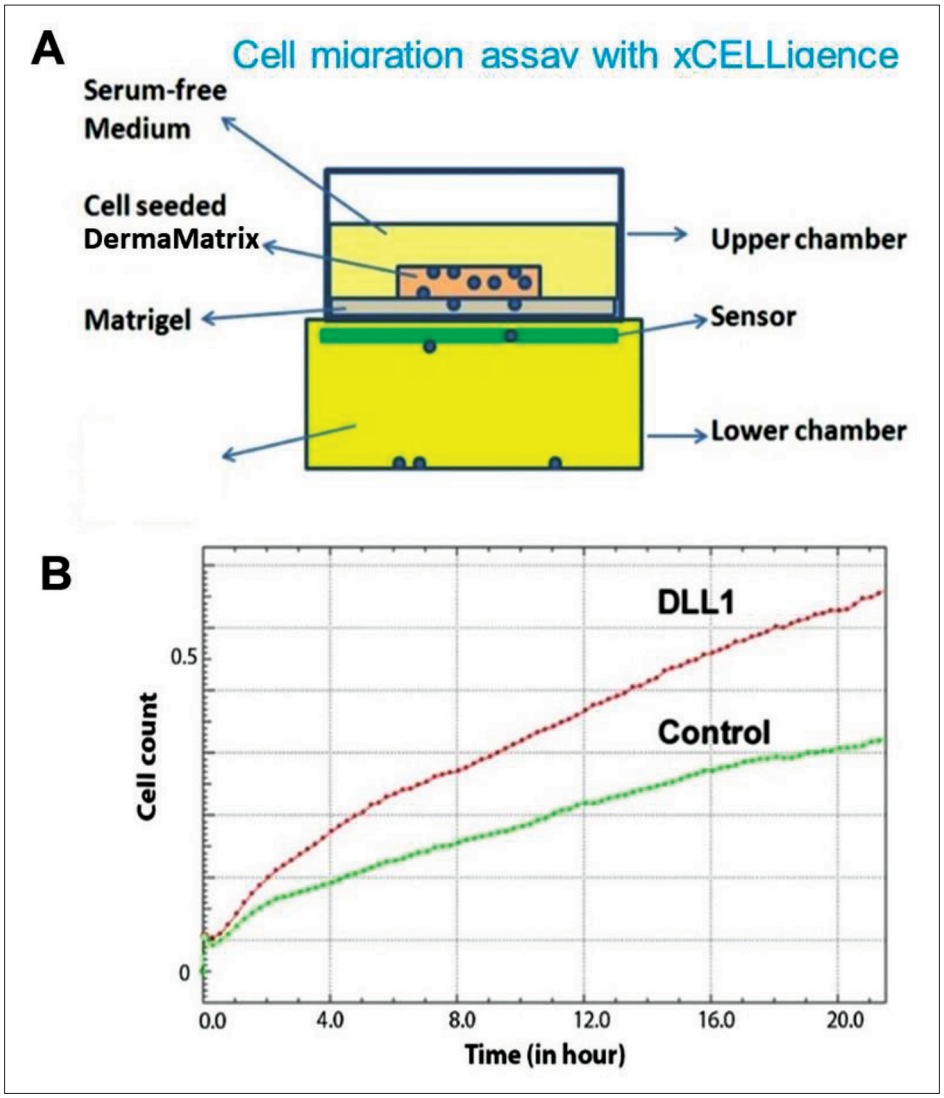

Figure 4. *In vitro* migration assay revealed that MPCs from DLL1-DermaMatrix constructs developed increased migration capacity. (A) DLL1 (mouse): Fc (human) (rec) was bioprinted onto biodegradable DermaMatrix construct (4 × 4 × 1 mm in dimensions). 2 × 10^4 GFP-labeled WT MPCs were seeded into both the DLL1-bioprinted DermaMatrix and control DermaMatrix constructs (IgG Fc) and cultured in medium. Three days after cell seeding, DermaMatrix constructs were placed in the Matrigel-coated upper chamber of an electrode-impedance-based cell migration/invasion assay system (xCELLigence). (B) The result of cell migration/invasion assay showing the increased migration capacity of MPCs from DLL1-DermaMatrix constructs.

There were more GFP-positive cells (yellow arrow head) in the DLL1 group, indicating an improved proliferation of the implanted stem cells; moreover, there were also more dystrophin-positive myofibers (white arrows), indicating an improvement in muscle regeneration.

4. Discussion

In certain situations, such as the later stages of muscle diseases when muscle stem cells have been depleted, *ex vivo* expansion and transplantation of muscle stem cells could be beneficial for patients with these conditions. Although significant improvements in the reduction of apoptosis and fibrogenesis in the diseased muscle have been recently accomplished using immunosuppressive therapy or anti-fibrotic agents[30,44-46], few studies have focused on maintaining the stemness and potent self-renewal capacity of muscle stem cells after cell transplantation. In our previous studies using the preplate technique, we were able to isolate a population of MPCs, which have great promise for cell-based therapeutic strategies in regenerative medicine, especially with regard to skeletal muscle regeneration[47,48]. Notch is a crucial molecular regulator of stem cell activity in skeletal muscle and its activation during *ex vivo* expansion has been shown to maintain donor muscle cell engraftment[49]. Notch activity

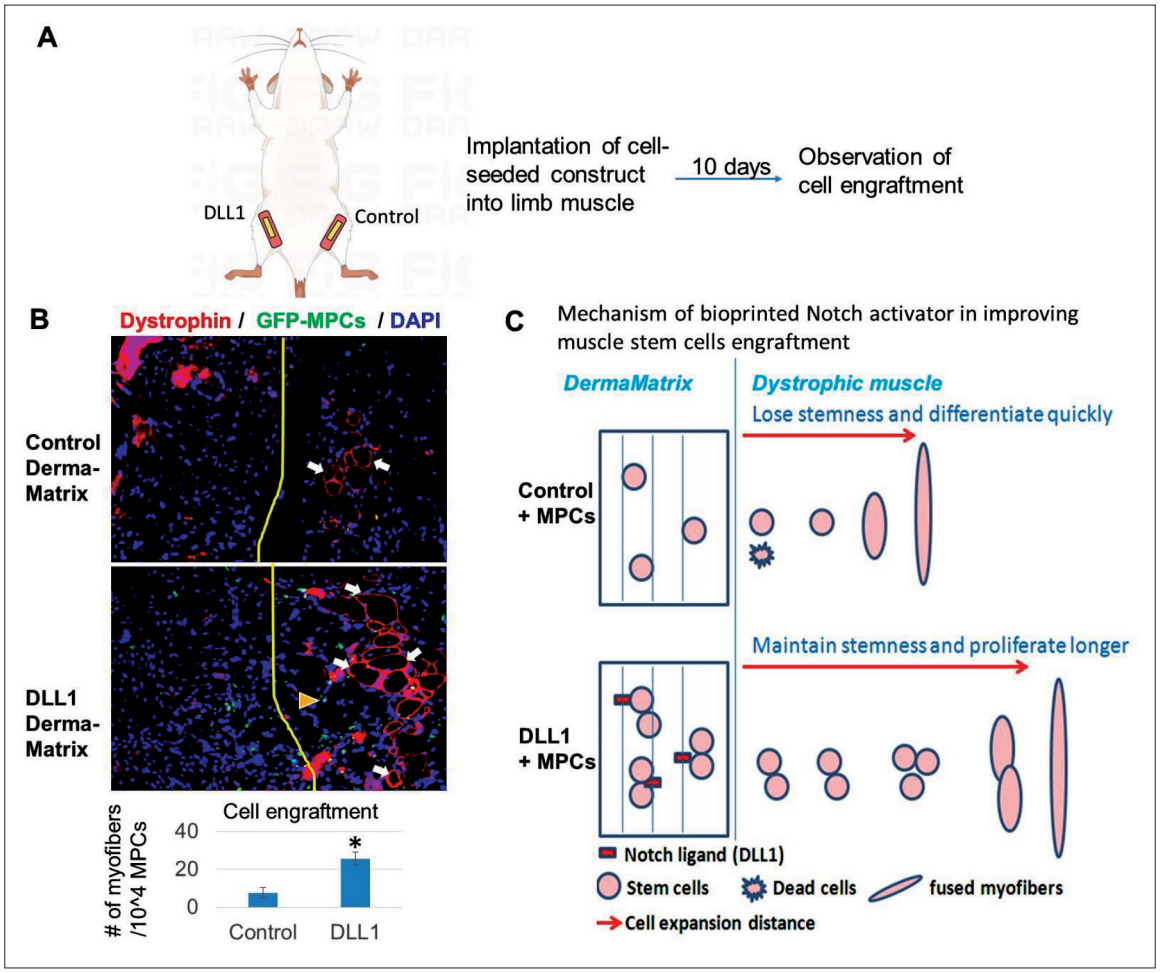

Figure 5. Implantation of GFP-MPC-seeded DLL1-DermaMatrix construct into mdx muscle resulted in improved cell engraftment. (A) Schematic demonstration of the *in vivo* experiment of the implantation of 3D constructs into mice (control and DLL1). (B) GFP-labeled MPCs from WT mice were seeded into DermaMatrix constructs with or without bioprinted DLL1 (4×10^4 cells in DermaMatrix construct of $4 \times 4 \times 1$ mm in dimensions). Ten days after the implantation of MPC-seeded DLL1-DermaMatrix or MPC-seeded control DermaMatrix constructs, an improved cell engraftment was observed in the muscle tissue implanted with the MPC-seeded DLL1-DermaMatrix constructs. There were more GFP-positive MPCs (yellow arrow head) and dystrophin-positive myofibers (white arrows) in DLL1-DermaMatrix construct group. (C) Proposed mechanism for the improved muscle stem cell engraftment by bioprinting of Notch activator in 3D construct to serve as stem cell niche.

in skeletal muscle declines with age in both mice and humans, and this decline is responsible for the depletion of functional muscle stem cells[11-13]. When compared to WT mice (6-month-old), we observed a down-regulation in the expression of key Notch signaling factors (i.e., Notch1, Hes1, Jagged1 and DLL1) in the skeletal muscle of *mdx* mice. Therefore, when performing stem cell therapy for DMD, an ECM construct containing muscle stem cells and Notch activating factors that allow for the sustainability and self-renewal of tissue-resident stem cell would be extremely attractive.

We have developed a novel strategy for repairing dystrophic muscle, by bioprinting a recombinant DLL1 protein [DLL1 (mouse): Fc (human) (rec)] into biodegradable 3D construct to serve as artificial muscle stem cell niche. DLL1-DermaMatrix construct seeded with MPCs was implanted into the limb muscles (gastrocnemius) of *mdx*/*scid* mice, and the number of dystrophin-positive myofibers was found to be obviously increased 10 days after implantation when compared to the muscles implanted with control DermaMatrix construct. Bioprinting of Notch ligands within 3D construct is expected to lead to the maintenance of stemness and improved proliferation of both resident and seeded stem cells, and the antiapoptotic effect of Notch activation is expected to increase cell survival. These stem cells can then migrate to locations distant from the site of initial implantation before they differentiate and fuse into myofibers, thus generating a larger engraftment. Also, the Notch ligands bioprinted in

3D construct can be released slowly with time into adjacent tissues, which could be beneficial for stem cell survival and persistence surrounding the implanted construct.

5. Conclusion

Our current result indicates that the reactivation of Notch signaling in dystrophic muscle is effective in promoting self-renewal of muscle stem cells, and demonstrates that bioprinting of Notch activator can be applied to serve as stem cell niche and improve the efficacy of muscle stem cell transplantation in diseased muscle. We believe that the same technology could possibly be adapted for the treatment of a wide variety of traumatic and atraumatic muscle conditions in the future.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Lee Weiss, Phil Campbell, Johnny Huard, Xiaodong Mu

Formal analysis: Zewei Sun, Xianlin Yue, Lei Liu, Ying Li

Investigation: Zewei Sun, Xianlin Yue, Lei Liu, Ying Li, Jie Cui

Methodology: Zewei Sun, Xianlin Yue, Lee Weiss, Phil Campbell, Johnny Huard, Yanling Mu, Xiaodong Mu

Visualization: Zewei Sun, Xianlin Yue, Johnny Huard, Xiaodong Mu

Writing – original draft: Zewei Sun, Xianlin Yue, Johnny Huard, Xiaodong Mu

Writing – review & editing: Zewei Sun, Xianlin Yue, Johnny Huard , Yanling Mu, Xiaodong Mu

Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh (IACUC-1109718).

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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