Activation of Notch Signaling During *Ex Vivo* Expansion Maintains Donor Muscle Cell Engraftment

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ABSTRACT

Transplantation of myogenic stem cells possesses great potential for long-term repair of dystrophic muscle. However, a single donor muscle biopsy is unlikely to provide enough cells to effectively transplant the muscle mass of a patient affected by muscular dystrophy. Expansion of cells *ex vivo* using traditional culture techniques significantly reduces engraftment potential. We hypothesized that activation of Notch signaling during *ex vivo* expansion would maintain donor cell engraftment potential. In this study, we expanded freshly isolated canine muscle-derived cells on tissue culture plates coated with Delta-1[ext]-IgG to activate Notch signaling or with human IgG as a control. A model of canine-to-murine xenotransplantation was used to quantitatively compare canine muscle cell engraftment and determine whether engrafted donor cells could function as satellite cells *in vivo*. We show that Delta-1[ext]-IgG inhibited differentiation of canine muscle-derived cells and increased the level of genes normally expressed in myogenic precursors. Moreover, cells expanded on Delta-1[ext]-IgG resulted in a significant increase in the number of donor-derived fibers, as compared to cells expanded on human IgG, reaching engraftment levels similar to freshly isolated cells. Importantly, cells expanded on Delta-1[ext]-IgG engrafted to the recipient satellite cell niche and contributed to further regeneration. A similar strategy of expanding human muscle-derived cells on Notch ligand might facilitate engraftment and muscle regeneration for patients affected with muscular dystrophy. STEM CELLS 2012;30:2212–2220

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Duchenne muscular dystrophy, the most common and severe form of muscular dystrophy, is caused by mutations in the dystrophin gene, the largest gene identified in the human genome. Transplantation of myogenic stem cells possesses great potential for long-term repair of dystrophic muscle. Indeed, intramuscular injection of adult satellite cell-derived myoblasts from a normal syngeneic donor into *mdx* mice results in the formation of dystrophin-positive muscle fibers [1–3]. Furthermore, intramuscular injection of allogeneic donor muscle-derived cells into chimeric *cxmd* canine recipients restored dystrophin expression for at least 24 weeks in the absence of post-transplant immunosuppression, indicating that cell transplantation may be a viable therapeutic option for muscular dystrophy [4].

However, multiple muscle groups within the body will need to be targeted, and a single donor muscle biopsy is unlikely to provide enough cells to effectively transplant the muscle mass of a patient affected by muscular dystrophy. Traditional means of expanding satellite cell-derived myoblasts *ex vivo* result in a dramatic loss of engraftment potential [4, 5]. The success of single muscle fiber transplantation suggests that mimicking the biochemical and biophysical signaling from the fiber may be important for maintaining engraftment potential of expanded muscle satellite cells [6, 7].

Expansion of hematopoietic progenitor cells on Notch ligand maintains their engraftment potential and it is reasonable to suggest that Notch ligand might similarly maintain muscle cell engraftment [8–12]. Skeletal muscle injury in mice results in increased expression of Delta-like-1 (Dll-1) within the niche, and activation of Notch signaling increases the number of proliferating myogenic cells and promotes

muscle regeneration after injury [13]. *In vitro*, overexpression of an activated form of Notch downregulates expression of MyoD and myogenin and inhibits myogenic differentiation in primary mouse myoblasts and C2C12 cells [13, 14]. The extracellular domain of Dll-1 fused to the Fc portion of human IgG (Delta-1[ext]-IgG) is sufficient for inhibition of differentiation in cultured C2C12 myoblasts; however, immobilization is required for effective signaling [15].

Therefore, we compared canine muscle-derived cells expanded on immobilized Delta-1[ext]-IgG to cells expanded on immobilized human IgG control. We show that activation of Notch signaling during expansion of canine muscle-derived cells inhibited myogenic differentiation. Furthermore, canine-to-mouse xenotransplantation demonstrated that activation of Notch signaling during donor cell expansion maintained engraftment potential.

MATERIALS AND METHODS

Donor Cell Isolation

The Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, approved this study. Elevated enclosed runs were used for housing, and dogs were maintained in social groups wherever possible. All dogs were enrolled in a veterinary preventative medicine program that included a standard immunization series against canine distemper, parvovirus, adenovirus type 2, parainfluenza virus, coronavirus, and rabies.

Each donor canine underwent a maximum of four skeletal muscle biopsies. For each canine-to-murine transplantation experiment, a 1 cm × 1 cm × 0.5 cm skeletal muscle biopsy was harvested from the biceps femoris muscle of the donor canine. The muscle biopsy was trimmed and cut into smaller pieces along the length of the fibers and digested with 200 U/ml collagenase type 4 (Worthington Biochemical, Lakewood, NJ, www.worthington-biochem.com) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, www.invitrogen.com) supplemented with 5 mM CaCl$_2$, 1 U/ml dispase (Invitrogen), and 0.5% bovine serum albumin (BSA) for 30 minutes at 37°C. The intact fibers and muscle pieces were rinsed in Hanks' balanced saline solution (HBSS; Invitrogen) and transferred to a new dish. The muscle fibers were chopped and digested fully with 400 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com) in DMEM (Invitrogen) supplemented with 5 mM CaCl$_2$ for 45 minutes at 37°C. The digested muscle was triturated and filtered through a series of nylon mesh filters. The resulting mononuclear cells released from the muscle were washed twice in PBS and resuspended in PBS. Mouse muscle-derived cells were isolated using the same method.

Canine Muscle Fiber Isolation

The muscle biopsies measured approximately 1 cm^3 and were from the belly of the canine biceps femoris muscle. We did not remove an entire muscle group tendon-to-tendon, as the biopsy was a survival surgery procedure. Canine muscle biopsies were cut into smaller pieces along the length of the fiber, transferred to Ham's F-12 media containing 400 U/ml of collagenase type 1 (Worthington Biochemical) and incubated at 37°C for 2 hours with regular agitation. The digest was transferred to a 10-cm plate with F-12 media supplemented with fetal bovine serum (FBS). The majority of isolated canine muscle fibers appeared hypercontracted. Fibers of longer length and smoother appearance were visible, yet constituted less than 1% of fibers (data not shown). Using a dissecting microscope, fibers displaying a smooth appearance with no signs of hypercontraction were transferred to PBS using flame-polished pasteur pipettes and prepared for injection.

Primary Cell Culture

Each 10-cm tissue culture dish was coated with 50 μg of human IgG (Sigma-Aldrich) or Delta-1[ext-Ig] and incubated overnight at 4°C. The following day, the human IgG and Delta-1[ext]-IgG were removed, and the dishes were washed with 1× PBS. The dishes were blocked with 2% bovine serum albumin in 1× PBS for 1 hour at 37°C. After washing the dishes three times with 1× PBS, canine cells were plated at a density of 7.5×10^4–1×10^5 cells per dish in DMEM containing 20% FBS and 2.5 ng/ml fibroblast growth factor 2 (FGF-2) (Invitrogen). Cells were maintained in culture for 8 days, unless otherwise indicated.

Cells were removed from the dishes by incubating with 5 mM EDTA in HBSS at 37°C for 5 minutes. Cells were transferred to a 15-ml conical tube and centrifuged at 1,000 rpm for 5 minutes. The cells were washed three times before resuspending in PBS for injection.

Immunocytochemistry

Primary antibodies specific for Pax7 and myogenin (F5D) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Cultured cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in 1× PBS. Cells were blocked in 10% goat serum and incubated with primary antibody diluted in primary antibody dilution buffer (1% BSA, 0.1% cold fish skin gelatin, 0.05% sodium azide, and 1 × PBS) for 1 hour at room temperature. The cells were washed in 1 × PBS, incubated with secondary antibody for 1 hour at room temperature, washed with 1 × PBS, and mounted with ProLong Gold Anti-fade with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Photomicrographs were taken using a Nikon E800 and a CoolSnap camera.

RNA Isolation and RT-Quantitative PCR

RNA was isolated from cells using the RNeasy Kit (Qiagen, Valencia, CA, www.qiagen.com) and 1 μg reverse transcribed using SuperScript III (Invitrogen) and random primers. Quantitative PCR (qPCR) was performed using an iQ5 machine (BioRad, Hercules, CA, www.bio-rad.com), using Platinum SYBR Green qPCR Super-Mix (Invitrogen), 1/100th of the cDNA reaction mix and the following primers: Hey1-F1 tcggctctaggttccatgtc; Hey1-R1 agcagatccctgcttctcaa; HeyL-F1 gatcacttgaaaatgctccac; HeyL-R1 tacctgatgacctcggtgag; Dtx4-F1 agccgcaaaactaccaagaa; Dtx-R1 cgtgagacgctccatacaga; Pax7-F1 aagattctctgccgctacca; Pax7-R1 tcacagtgtccgtccttcag; Myf5-F1 ggcctgcctgaatgtaacag; Myf5-R1 gttgctcggagttggtgatt; musculin-F1 ggctggcatccagttacatc; musculin-R1 gcggaaacttctttggtgtc; MyoD-F1 cgattcgctacatcgaaggt; MyoD-R1 aggtgccatcgtagcagttc; CXCR4-F1 gagctccatatatacccttcagata; CXCR4-R1 ggtaacccatgaccaggatg; CD34-F1 tgacccaagtcctgtgtgag; CD34-R1 gtcttgcgggaatagctctg; cadherin11-F1 gaaccagttcttcgtgatagagga; cadherin11-R1 tgtcttggtggcatgaatgt; TIMM17B-F1 atcaagggcttccgcaatg; and TIMM17B-R1 cacagtcgatggtgggagaacag. Threshold cycle values were used to generate relative gene-specific expression values normalized to TIMM17B expression. To ensure the data was consistent, expression was also normalized to expression of TATA binding protein (TBP) (data not shown).

Fluorescence-Activated Cell Sorting

Anti-CXCR4 was obtained from R&D Systems (clone 44716; Minneapolis, MN, www.rndsystems.com) and used at 10 μg/ml for fluorescence-activated cell sorting (FACS) of 1×10^6 cells. Anti-syndecan 4 and Alexa Fluor 488-labeled anti-chicken antibody was a kind gift of D.D. Cornelison (University of Missouri). Alexa Fluor 488-labeled anti-mouse IgG2b was obtained from Invitrogen and used at 1:200. Expanded canine skeletal muscle cells dissociated from the plate were resuspended in FACS buffer (HBSS, 5% FBS) and incubated on ice with anti-

CXCR4, anti-syndecan 4, or isotype control, followed by Alexa Fluor 488-labeled secondary antibodies. The cells were washed, resuspended in FACS buffer, and sorted using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, www.bdbiosciences.com).

Cell Injection into Mice and Tissue Processing

The right hind limb of each 7–12-week-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse was exposed to 12 Gy of ionizing irradiation (Mark 1 cesium source, Shepherd and Associates, San Fernando, CA, www.jlshepherd.com), and the tibialis anterior (TA) muscle of the same hind limb was injected with 50 μl of 1.2% barium chloride immediately after irradiation. The following day, the same TA muscle was injected with 50 μl of freshly isolated canine muscle-derived cells or mouse muscle-derived cells, or cells expanded on human IgG or Delta-1^{ext}-IgG, along the length of the muscle, so as to distribute cells from the distal to the proximal end of the muscle. The injected muscle was harvested 28 days after injection, unless otherwise indicated.

The harvested mouse muscle was covered in OCT within a plastic cryomold and placed on top of an aluminum block immersed in liquid nitrogen. Frozen tissue was stored at $-80°C$. Cryosections were cut (10 μm) from the distal to the proximal end of the frozen muscle using a Leica CM1850 cryostat and adhered to Superfrost slides (Fisher Scientific, Pittsburgh, PA). Each glass slide consisted of four serial sections, and the corresponding section on the subsequent slide represented a separation of approximately 200 μm from the previous slide.

Each TA muscle normally generated 24 slides, each consisting of four serial sections. Initially, slides 6, 12, and 18 were stained for dystrophin and lamin A/C to determine the region of highest engraftment. Three more even numbered slides were chosen from the region of highest engraftment and stained for canine dystrophin and lamin A/C. Three odd numbered slides in the same region were used for Pax7 and lamin A/C costaining. In almost all cases, the region of highest engraftment was between slides 6 and 18, representing the belly of the muscle, which does not vary considerably in cross-sectional area.

Immunostaining

Anti-dystrophin (MANDYS107) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. Anti-lamin A/C (clone 636) and antidevelopmental myosin heavy chain was obtained from Vector Laboratories (Burlingame, CA, www.vectorlabs.com). Alexa fluor 488-conjugated goat anti-mouse IgG and Alexa fluor 568-conjugated goat anti-mouse IgG2b secondary antibodies, both from Invitrogen, were used at 1:200. For dystrophin and lamin A/C staining, the sections were fixed in acetone at $-20°C$ for 10 minutes, allowed to dry, and rehydrated in phosphate buffered saline (PBS). Sections were incubated in blocking buffer (2% goat serum, 1% BSA, 0.1% cold fish skin gelatin, 0.05% sodium azide, and 1$\times$ PBS) for 1 hour at room temperature, followed by primary antibody diluted in primary antibody dilution buffer (1% BSA, 0.1% cold fish skin gelatin, 0.05% sodium azide, and 1$\times$ PBS) for 1 hour at room temperature or overnight at 4°C. The sections were washed in 1$\times$ PBS, incubated with secondary antibody for 1 hour at room temperature, washed with 1$\times$ PBS, and mounted with ProLong Gold Anti-fade with DAPI (Invitrogen).

Primary antibody specific for Pax7 antibody was used at 1:10 and was obtained from the Developmental Studies Hybridoma Bank. Alexa fluor 488-conjugated goat anti-mouse IgG1 (Pax7) and Alexa fluor 568-conjugated goat anti-mouse IgG2b (lamin A/C) were used at 1:200 and were obtained from Invitrogen. For Pax7 and lamin A/C costaining, cryosections were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed with 1$\times$ PBS, followed by permeabilization with methanol at $-20°C$ for 6 minutes. The sections were washed in 1$\times$ PBS, and antigen retrieval was performed by incubating the slides twice in

10 mM citric acid (pH 6.0) at 90°C for 5 minutes. Sections were washed with 1$\times$ PBS, blocked in blocking buffer (2% goat serum, 1% BSA, 0.1% cold fish skin gelatin, 0.05% sodium azide, and 1$\times$ PBS) for 1 hour at room temperature, and incubated in primary antibody diluted in primary antibody dilution buffer (1% BSA, 0.1% cold fish skin gelatin, 0.05% sodium azide, and 1$\times$ PBS) for 1 hour at room temperature or overnight at 4°C. The sections were washed in 1$\times$ PBS, incubated with secondary antibody for 1 hour at room temperature, washed with 1$\times$ PBS, and mounted with ProLong Gold Anti-fade with DAPI (Invitrogen).

Photomicrographs were taken using a Zeiss AxioImager.Z1 as part of a TissueFaxs system (TissueGnostics, Los Angeles, CA, www.tissuegnostics.com). The images for each field of view were stitched together to form an entire cross-sectional view. The number of fibers expressing canine dystrophin, the number of nuclei expressing canine lamin A/C, and the number of nuclei expressing canine lamin A/C and Pax7 were counted from these cross-sectional views.

Expanding Canine Muscle Cells Negatively Impacts Engraftment

Currently, muscle fiber preparations and freshly isolated muscle-derived cells are considered the most effective material for muscle transplantation. To compare the engraftment efficiency of fresh fibers to freshly isolated muscle-derived cells, we transplanted each population into the TA muscle of a NOD/SCID mouse, as previously described [16]. The mouse hind limb was preirradiated with 12 Gy of ionizing radiation to prevent regeneration by host mouse satellite cells and pretreated with BaCl$_2$ to induce muscle degeneration (see Materials and Methods). On average, injection of 50,000 freshly isolated canine muscle-derived cells appeared to be equivalent to injection of five single canine muscle fibers from the same donor muscle biopsy, comparing both the number of fibers expressing canine dystrophin and the number of nuclei expressing Pax7 and canine lamin A/C (Fig. 1A, 1B). Because each isolated muscle fiber might have approximately 10 mononuclear cells capable of regeneration, our results are consistent with prior studies showing that transplanting muscle fibers show the greatest per cell regeneration potential [6, 7].

Despite the superior potential, muscle fiber preparations are not likely to yield enough transplantable material to treat all muscles of an individual affected with muscular dystrophy. Therefore, to achieve sufficient numbers of donor cells for large scale transplantation, *ex vivo* expansion will be required. However, muscle-derived cells expanded on standard tissue culture dishes displayed significantly reduced engraftment as compared to freshly isolated cells (Fig. 1C).

The donor used for the experiment in Figure 1C was not the same donor used for the experiment in Figure 1A. Therefore, the difference in the level of engraftment observed between Figure 1A and 1C likely reflects how each donor's muscle-derived cell population has a different capacity for reconstitution [16]. Moreover, the freshly isolated cells transplanted for the experiment in Figure 1A remained on ice for a longer period of time before transplant to accommodate the muscle fiber preparation, which may have had a negative impact on engraftment.

Yet these results are consistent with previous studies showing that expanding myoblasts diminishes transplantation efficiency [4, 5]. Based on studies of the expansion of hematopoietic stem cells, we hypothesized that activating Notch
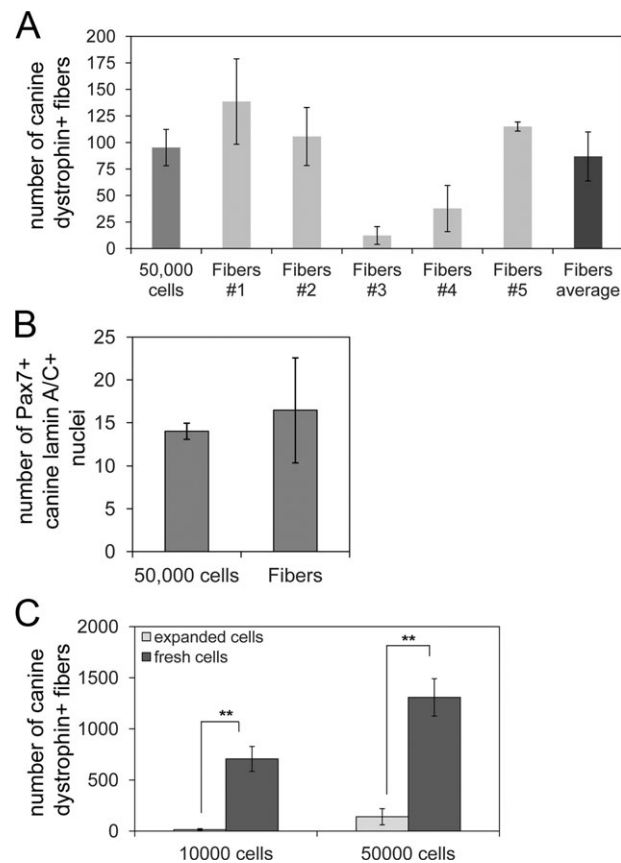
Figure 1. Cultured myoblasts display poor engraftment. Cryosections from non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse muscle injected with 5×10^4 fresh canine muscle-derived cells or five single muscle fibers were immunostained with anti-dystrophin, or anti-Pax7 and anti-lamin A/C, and fluorescently labeled secondary antibodies. The number of fibers expressing canine dystrophin **(A)** and the number of nuclei expressing Pax7 and canine lamin A/C **(B)** per cross-section were counted using cryosections surrounding the region of highest engraftment within the muscle. For the fiber transplants in (A), the bars represent the average ± SD ($n \geq 3$ cryosections per mouse). For all others, the bars represent the average of the averages ($n \geq 3$ cryosections per mouse, $n = 3$ mice per cell dose). **(C):** Cryosections from NOD/SCID mouse muscle injected with 1×10^4 or 5×10^4 fresh canine muscle-derived cells or cells expanded *ex vivo* for 8 days were immunostained with anti-dystrophin and fluorescently labeled secondary antibodies. The bars represent the average of the averages ± SD ($n \geq 3$ cryosections per mouse, $n = 3$ mice per cell dose). A Student's *t* test was used to determine statistical significance (**, $p < .01$).

signaling in muscle-derived cells during expansion would maintain engraftment potential of donor cells.

Activation of Notch Signaling Inhibits Canine Myogenic Differentiation

To mimic activation of Notch signaling, tissue culture-treated polystyrene plates were coated with Delta1ext-IgG. Control plates were coated with human IgG. Canine satellite cell-derived myoblasts, previously cultured on uncoated tissue culture plates, were cultured on Delta-1ext-IgG or human Ig-coated plates for 8 days in DMEM supplemented with 20% FBS and 2.5 ng/ml FGF. As predicted by studies with mouse myoblasts, Delta-1ext-IgG inhibited differentiation of canine myoblasts (Fig. 2A).

Similarly, exposure of freshly isolated canine muscle-derived cells to Delta-1ext-IgG inhibited differentiation (Fig. 2B) and resulted in a 6.5- to 20-fold expansion of total cell number

over 8 days (Supporting Information Table S1). Increased expression of Hey1, HeyL, and Dtx4 confirmed activation of Notch signaling in cells exposed to Delta-1ext-IgG (Fig. 3A).

Expression of musculin, an inhibitor of myogenic differentiation, was significantly increased in cells exposed to Delta-1ext-IgG. This was accompanied by a significant decrease in expression of MyoD and an increase in expression of Myf5 and Pax7 in cells expanded on Delta-1ext-IgG (Fig. 3B). Expression of myogenin was almost undetectable in cells grown on human IgG but completely absent from cells grown on Delta-1ext-IgG (data not shown), confirming immunocytochemistry results (Fig. 2). Therefore, Delta-1ext-IgG inhibited canine myogenic differentiation.

When compared with cells expanded on human IgG, expanding cells on Delta-1ext-IgG did not increase the percentage of cells expressing syndecan 4, a marker of satellite cells and satellite cell-derived myogenic cells in culture (Fig. 4B) (Table 1) [17]. In contrast, the CXCR4 receptor, which has a critical role in muscle regeneration [16], showed increased RNA and protein levels in cells expanded on Delta-1ext-IgG (Figs. 3C, 4A); however, the percentage of CXCR4 expressing cells did not increase (Table 1), indicating a higher abundance of CXCR4 per cell. Together, these data show that culture of primary muscle-derived cells on Delta-1ext-IgG promotes the expansion of Pax7- and Myf5-positive cells with enhanced CXCR4 expression.

Activation of Notch Signaling During Expansion Maintains Engraftment of Donor Cells

Engraftment of 5×10^4 cells expanded on Delta-1ext-IgG was similar to engraftment of 5×10^4 freshly isolated cells, as shown by the similar number of fibers expressing canine dystrophin, nuclei expressing canine lamin A/C, and nuclei expressing canine lamin A/C and Pax7 (Fig. 5A–5C). Approximately 80% of cells expanded on Delta-1ext-IgG is myogenic cells, as evidenced by syndecan 4 expression (Table 1), whereas less than 4% of freshly isolated cells generates myogenic cell clones in culture (data not shown).

In contrast, transplantation of cells expanded on human IgG resulted in significantly fewer fibers expressing canine dystrophin and less than one nuclei coexpressing Pax7 and canine lamin A/C per cross-section, similar to cells expanded on uncoated tissue culture plates (Fig. 1C). Therefore, Notch activation during *ex vivo* muscle cell expansion maintained engraftment potential. However, muscle-derived cells must be exposed to Delta-1ext-IgG immediately after isolation, as activating Notch activity in myoblasts previously cultured on uncoated tissue culture plates did not restore engraftment potential (Fig. 5D).

The enhanced muscle regeneration capacity of muscle cells expanded on the Notch ligand was largely due to enhanced myogenesis rather than simple cell survival, based on the ratio of donor lamin A/C+ cells to donor myofibers (Fig. 5E). For muscle injected with cells expanded on human IgG, the ratio of the number of canine lamin A/C-positive nuclei to the number of canine dystrophin-positive fibers per cross-section was 18.6; however, the ratio is 1.7 for muscle injected with cells expanded on Delta-1ext-IgG, and 1.8 for muscle injected with fresh cells. This indicates that cells expanded on human IgG survived transplantation but did not contribute as effectively to the formation of fibers expressing canine dystrophin during regeneration as compared to cells expanded on Delta-1ext-IgG or fresh cells.

Expanded Cells Contribute to Further Regeneration

The presence of Pax7+ donor canine cells suggests that some donor cells enter a repopulating or satellite cell compartment.
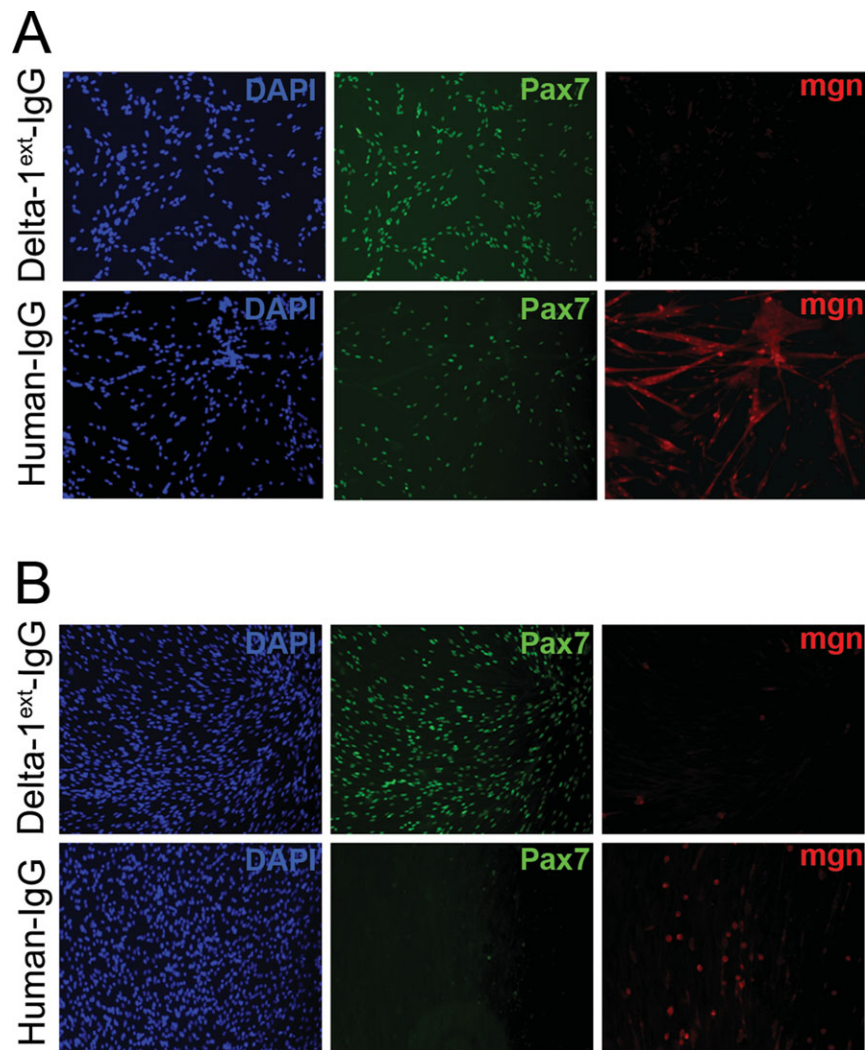
Figure 2. Delta-1^{ext}-IgG inhibits canine muscle cell differentiation. **(A):** Established canine satellite cell-derived myoblasts or **(B)** freshly isolated canine muscle-derived cells were cultured on plates coated with Delta-1^{ext}-IgG or human IgG. After 8 days, the cells were fixed and immunostained with anti-Pax7 (green) and anti-myogenin (red). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

To determine whether the engrafted donor muscle cells are capable of regeneration, mice were subjected to two additional rounds of intramuscular BaCl$_2$ injection at 4 and 8 weeks after donor cell transplant. As noted above, the initial hind limb irradiation prior to the donor cell transplantation prevents muscle regeneration from the host mouse satellite cells and the majority of muscle repair will require donor canine satellite cell activity.

Four weeks following two additional rounds of BaCl$_2$-induced regeneration, muscle injected with Delta-1^{ext}-IgG expanded cells showed a significant increase in the number of fibers expressing canine dystrophin and a consistent number of nuclei coexpressing Pax7 and canine lamin A/C (Fig. 6A–6C). Expression of a developmental form of myosin heavy chain (devMyHC), expressed in immature myofibers, indicated ongoing muscle regeneration (Fig. 6D, 6E).

To further demonstrate the ability of engrafted cells to participate in regeneration, we performed secondary transplants using cells isolated from mouse muscle injected with freshly isolated canine muscle-derived cells or Delta-1^{ext}-IgG expanded cells. All three secondary recipients of Delta-1^{ext}-IgG expanded cells displayed fibers expressing canine dystro-

phin (Fig. 6F, 6G). Nuclei coexpressing Pax7 and canine lamin A/C were detected in two recipients (Fig. 6H, 6I). However, there was no statistically significant difference in the level of engraftment between secondary recipients of fresh cells and Delta-1^{ext}-IgG expanded cells. Together, these data indicate that canine donor cells expressing Pax7 in muscle transplanted with cells expanded on Delta-1^{ext}-IgG can function similar to satellite cells and participate in muscle regeneration and maintain a Pax7^{+} population after regeneration.

DISCUSSION

The ability of single muscle fibers to engraft more effectively than mononuclear cell preparations suggests that association of the satellite cell with the fiber preserves the ability of the satellite cell to participate in muscle repair. In mouse studies, physical trituration of the fibers to disrupt satellite cell-fiber interactions yields cells with significantly greater engraftment potential than cells enzymatically removed from the fiber [6]. The authors hypothesize that enzymatic disruption may cleave
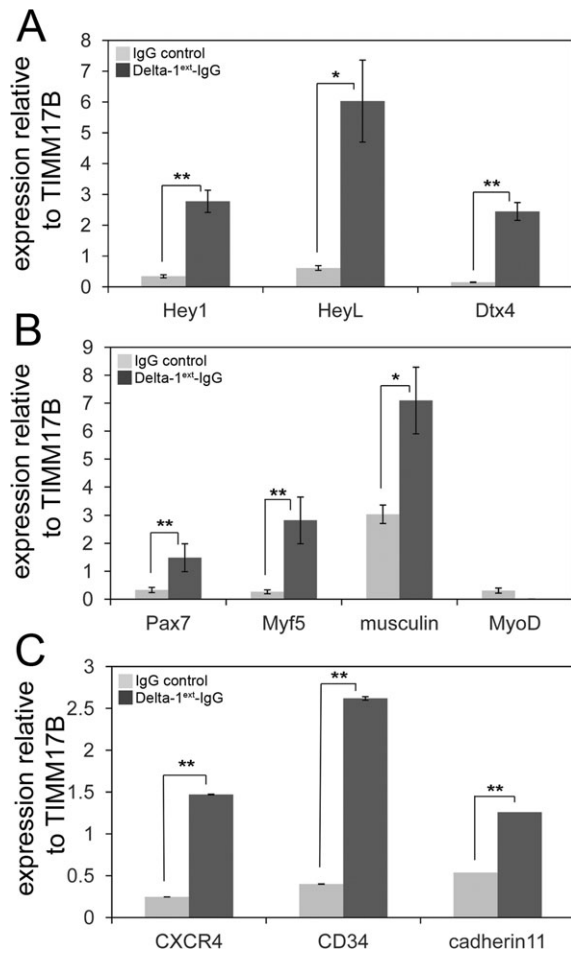
Figure 3. Notch activation alters gene expression in canine muscle-derived cells. **(A–C):** cDNA was generated from RNA isolated from canine muscle-derived cells cultured on plates coated with Delta-1^{ext}-IgG (darker bars) or human IgG (lighter bars) and used for quantitative PCR using the primers indicated. The bars represent the average expression level relative to TIMM17B $\pm$ SD ($n = 3$). A Student's t test was used to determine statistical significance (*, $p < .05$; **, $p < .01$).

cell surface proteins required for donor cell engraftment. However, it is also possible that time away from the fiber or niche has a negative effect on donor satellite cell engraftment. Indeed, culturing muscle-derived cells on a substrate with a similar stiffness to normal skeletal muscle (12 kPa) improves donor cell engraftment, indicating that biophysical signaling is important for satellite cell stemness [18, 19].

Activation of Notch signaling is required for satellite cell proliferation and muscle regeneration after injury [13]. New evidence indicates that Notch activity also plays an important role in maintenance of the satellite cell population after injury, and that expression of Notch target genes is associated with quiescent satellite cells that express high levels of Pax7 [20, 21]. The number of myogenic cells was not significantly different between cells expanded on Delta-1^{ext}-IgG and cells expanded on human IgG; however, Pax7 expression was increased in canine cells expanded on Delta-1^{ext}-IgG. This suggests that upregulating Notch activity during *ex vivo* expansion increased the number of myogenic progenitor cells that are similar to quiescent or newly activated satellite cells.

As expected from studies with mouse myoblasts, activation of Notch signaling in canine muscle-derived cells resulted in downregulation of MyoD and myogenin expression [13, 14] and an increase in Myf5, Pax7, and CXCR4 expression. Myf5 is not expressed during myogenic differentiation [22, 23], and Myf5 transcripts have been detected in quiescent and newly activated satellite cells [24–27]. Increased expression of Myf5 indicates that induction of Notch signaling with Delta-1^{ext}-IgG during *ex vivo* culture of the canine muscle-derived cells resulted in maintenance and expansion of a myogenic cell with characteristics of an early activated satellite cell.

Blocking CXCR4 receptor activity on donor cells before transplant significantly impairs donor cell engraftment [16]. In contrast, promoting CXCR4 activity by inhibiting CD26/DPP-IV degradation of SDF-1 with diprotin A enhances donor cell engraftment. Together, this suggests that CXCR4 may be a marker of donor cells that effectively participate in donor cell dependent muscle regeneration. Increased expression of CXCR4 in cells expanded on Delta-1^{ext}-IgG may provide part of the reason for the increase in engraftment compared to cells expanded on human IgG, indicating that diprotin A may have a potent effect on engraftment of cells expanded on Delta-1^{ext}-IgG.
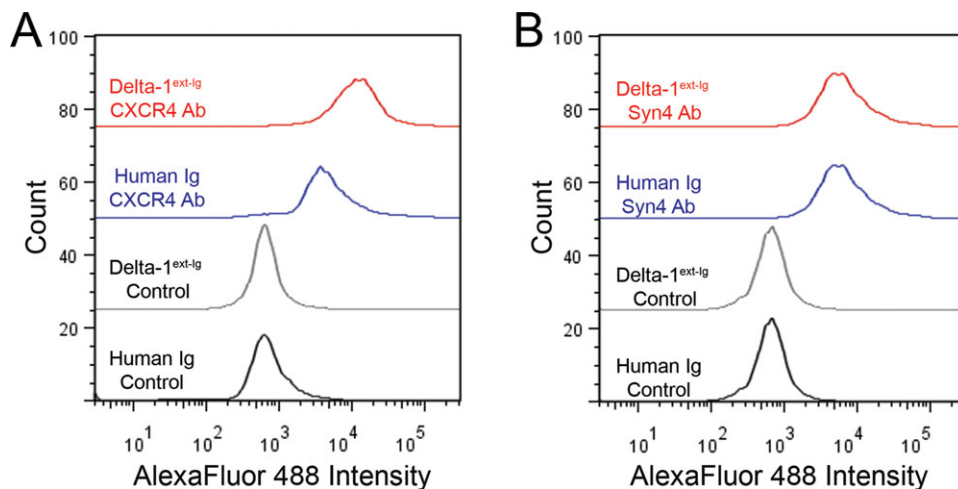

Figure 4. Expanded canine muscle-derived cells express a higher level of CXCR4. Freshly isolated canine muscle-derived cells, cultured on plates coated with Delta-1^{ext}-IgG or human IgG, were incubated with anti-CXCR4 **(A)** or anti-syndecan 4 **(B)**, and Alexa Fluor 488-labeled secondary antibody, or isotype control and AlexaFluor 488-labeled secondary antibody, and sorted using fluorescence-activated cell sorting. The resulting histograms are vertically offset and scaled to avoid overlap.

Table 1. Expanding cells on Delta-1^{ext}-IgG does not alter the number of CXCR4$^+$ or syndecan 4$^+$ cells

	Isotype control	CXCR4	Isotype control	Syndecan 4
Human IgG	0.74%	78.6%	1.17%	89.5%
Delta-1^{ext}-IgG	0.78%	81.5%	3.25%	81.1%

The percent of Alexa Fluor 488-positive cells was determined from the fluorescence-activated cell sorting shown in Figure 4.
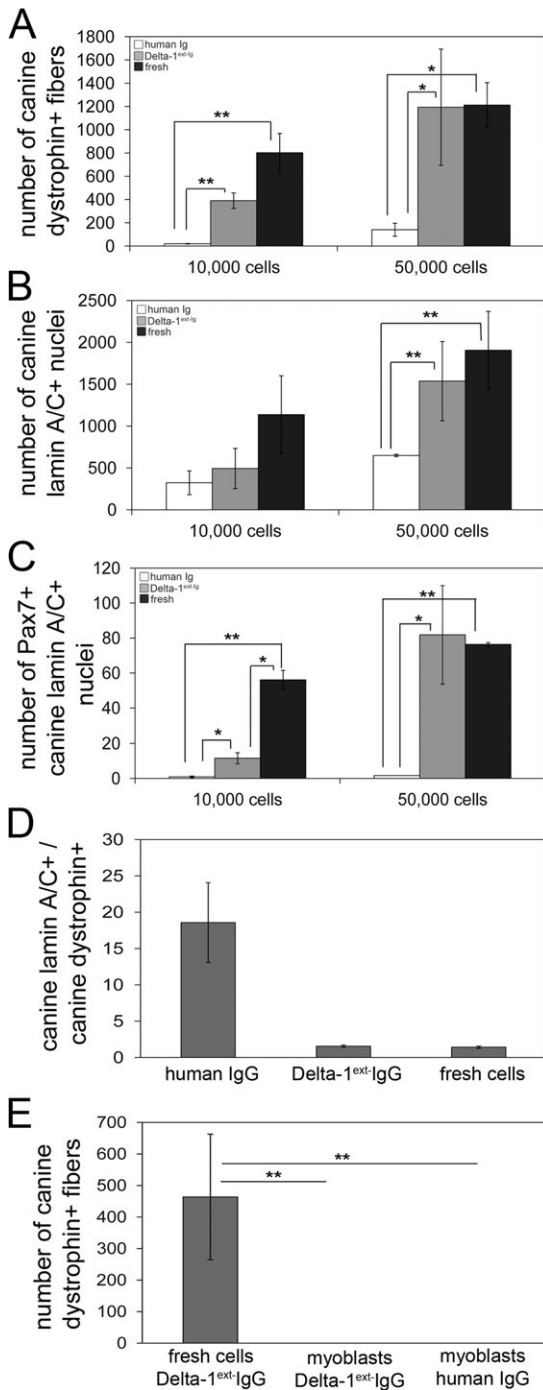
In hematopoietic transplant, short-term repopulating cells are more committed progenitors that engraft quickly; however, long-term repopulating cells are a more primitive cell capable of self-renewal. BaCl$_2$-induced regeneration in muscle transplanted with canine cells expanded on Delta-1^{ext}-IgG increased the number of fibers expressing canine dystrophin and maintained the number of donor Pax7$^+$ cells. Moreover, engraftment was detected in secondary recipients of Delta-1^{ext}-IgG expanded cells. Together, this indicates that donor cells expanded on Delta-1^{ext}-IgG that had engrafted into recipient muscle participated in muscle repair similar to satellite cells, and had the capacity to self-renew, similar to long-term repopulating hematopoietic cells. Together, these data support the hypothesis that activating Notch signaling during expansion of canine muscle-derived cells maintained a subpopulation of progenitor cells.

Effective expansion of cells *ex vivo* for transplant will require mimicking the fiber environment, both biophysically and biochemically, to maintain a large proportion of cells as stem cells. The ability to expand donor muscle-derived cells *ex vivo* will be an important step toward making cell transplantation a possible therapy for muscular dystrophies. Similarly, immobilized Delta-1^{ext}-IgG inhibits differentiation of human CD34$^+$CD38-cord blood precursors and dramatically increases the number of precursors capable of repopulating NOD/SCID mice [8, 9, 11]. A phase I clinical trial of transplantation of *ex vivo* expanded CD34$^+$CD38-cord blood precursors is currently underway in patients with high-risk leukemias and appears to successfully promote donor cell engraftment [12]. This study indicates that a similar strategy of expanding human muscle-derived cells on Notch ligand might facilitate engraftment and muscle regeneration and should be considered for future studies of human muscle transplantation.

CONCLUSIONS

Activating Notch signaling in cultured canine muscle-derived cells inhibits myogenic differentiation and increases the number of myogenic progenitor cells that are similar to quiescent or newly activated satellite cells. Importantly, cells expanded in the presence of Notch activation maintain engraftment potential, indicating the potential for therapeutic benefit. Therefore, we will translate these results to human muscle-

Figure 5. Cells expanded on Delta-1^{ext}-IgG maintain engraftment. **(A–D):** Cryosections from mouse muscle injected with 1×10^4 or 5×10^4 freshly isolated mixed canine muscle-derived mononuclear cells, cells expanded on Delta-1^{ext}-IgG, or cells expanded on human IgG, were immunostained with anti-dystrophin, anti-lamin A/C, and/or anti-Pax7 antibodies and fluorescently labeled secondary antibody. The number of fibers expressing canine dystrophin (A), the number of nuclei expressing canine lamin A/C (B), the number of nuclei expressing canine lamin A/C and Pax7 (C), and the ratio of the number of nuclei expressing canine lamin A/C to the number of fibers expressing canine dystrophin per cross-section (D) were determined. The bars represent the average of the averages $\pm$ SD ($n \geq 3$ cryosections per mouse, $n = 3$ mice per cell dose). **(E):** Cryosections from mouse muscle injected with 1×10^4 freshly isolated cells expanded on Delta-1^{ext}-IgG, established canine myoblasts expanded on Delta-1^{ext}-IgG, or established canine myoblasts expanded on human IgG, were immunostained with anti-dystrophin. The number of fibers expressing canine dystrophin per cross-section was determined. The bars represent the average of the averages $\pm$ SD ($n \geq 3$ cryosections per mouse, $n = 3$ mice per cell dose). For all data, a Student's *t* test was used to determine statistical significance (*, $p < .05$; **, $p < .01$).
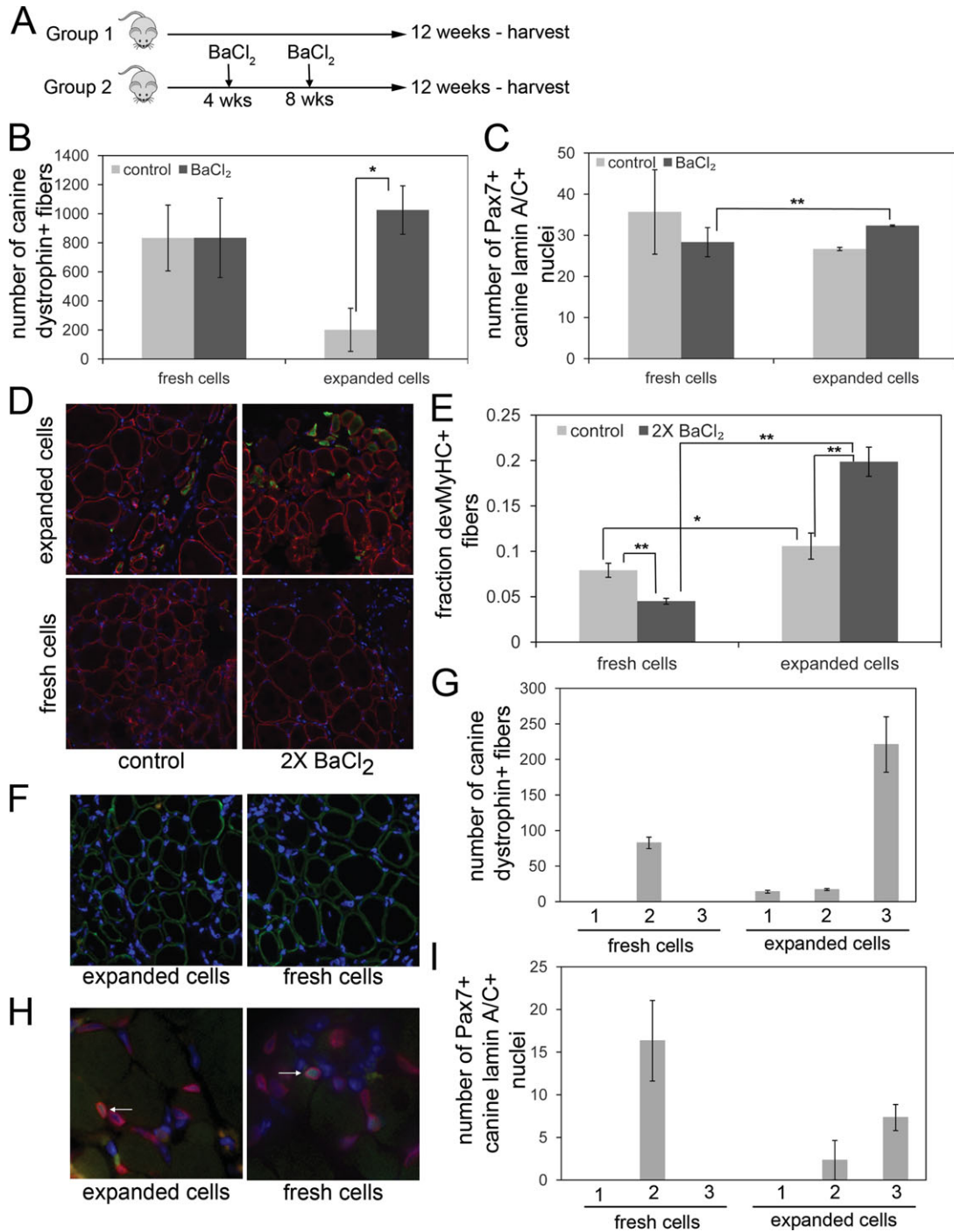
Figure 6. Delta-1^ext-IgG expanded cells function as long-term repopulating cells. **(A):** Two groups of mice were injected with 1×10^4 freshly isolated canine muscle-derived cells or cells expanded on Delta-1^ext-IgG. The transplanted muscle of group 1 was harvested 12 weeks after cell injection. The transplanted muscle of group 2 was injected with 1.2% BaCl$_2$ 4 and 8 weeks after cell injection and harvested 12 weeks after cell injection. **(B,C):** Cryosections from the experiment outlined in (A) were immunostained with anti-dystrophin, or anti-lamin A/C and anti-Pax7 antibodies, and fluorescently labeled secondary antibodies. The number of fibers expressing canine dystrophin (B) and the number of nuclei expressing canine lamin A/C and Pax7 (C) per cross-section were determined. The bars represent the average of the averages $\pm$ SD ($n \geq 3$ cryosections per mouse, $n = 3$ mice per cell dose). **(D):** Cryosections were immunostained with anti-dystrophin (red) and antidevelopmental myosin heavy chain (green), and fluorescently labeled secondary antibodies. **(E):** The fraction of canine dystrophin-positive fibers expressing developmental myosin heavy chain (devMyHC) was determined. The bars represent the average of the averages $\pm$ SD ($n \geq 3$ cryosections per mouse, $n = 3$ mice per cell dose). A Student's t test was used to determine statistical significance (*, $p < .05$; **, $p < .01$). **(F–I):** Two groups of three mice were injected with 5×10^4 freshly isolated canine muscle-derived cells or cells expanded on Delta-1^ext-IgG. The mixed population of muscle-derived cells was isolated from each injected muscle and transplanted into a secondary recipient. Muscle from the secondary recipients was harvested 4 weeks after injection, and cryosections immunostained with anti-dystrophin (green) (F), or anti-lamin A/C (red) and anti-Pax7 antibodies (green) (H), and fluorescently labeled secondary antibodies. The number of fibers expressing canine dystrophin (G) and the number of nuclei expressing canine lamin A/C and Pax7 (I) per cross-section were determined. The bars represent the average $\pm$ SD from each mouse ($n = 3$ cryosections per mouse).

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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