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Limited regeneration in long acellular nerve allografts is associated with increased Schwann cell senescence

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Abstract

Repair of large nerve defects with acellular nerve allografts (ANAs) is an appealing alternative to autografting and allotransplantation. ANAs have been shown to be similar to autografts in supporting axonal regeneration across short gaps, but fail in larger defects due to a poorly-understood mechanism. ANAs depend on proliferating Schwann cells (SCs) from host tissue to support axonal regeneration. Populating longer ANAs places a greater proliferative demand on host SCs that may stress host SCs, resulting in senescence. In this study, we investigated axonal regeneration across increasing isograft and ANA lengths. We also evaluated the presence of senescent SCs within both graft types. A sciatic nerve graft model in rats was used to evaluate regeneration across increasing isograft (~autograft) and ANA lengths (20, 40, and 60 mm). Axonal regeneration and functional recovery decreased with increased graft length and the performance of the isograft was superior to ANAs at all lengths. Transgenic Thy1-GFP rats and qRT-PCR demonstrated that failure of the regenerating axonal front in ANAs was associated with increased levels of senescence related markers in the graft (senescence associated β -galactosidase, p16^{INK4A}, and IL6). Lastly, electron microscopy (EM) was used to qualitatively assess senescence-associated changes in chromatin of SCs in each graft type. EM demonstrated an increase in the presence of SCs with abnormal chromatin in isografts and ANAs of increasing graft length. These results are the first to suggest that SC senescence plays a role in limited axonal regeneration across nerve grafts of increasing gap lengths.

Keywords

Peripheral nerve; Acellular nerve allograft; Cellular senescence; Nerve autograft; Nerve grafting; Schwann cell senescence

Introduction

Large complex peripheral nerve injuries necessitate repair with nerve grafts. Nerve autografts are the current standard for clinical nerve reconstruction, but their use is limited by the amount of expendable tissue and donor site morbidity. An alternative approach is to

use allografts, but the necessity of systemic immunosuppression limits its application (Brenner et al., 2002; Mackinnon et al., 2001). Recent efforts have concentrated on addressing the immune challenge associated with allografts by removing the immunogenicity of the grafted tissue (Brenner et al., 2005; Fox et al., 2005a, 2005b; Hudson et al., 2004a, 2004b; Ray et al., 2010, 2011; Whitlock et al., 2010a, 2010b). A number of studies have established donor Schwann cells (SCs) as the immunogenic target in nerve allografts (Ansselin and Pollard, 1990; Lassner et al., 1989). In keeping with these findings, various thermal and chemical techniques (Fox et al., 2005a, 2005b; Gulati, 1998; Hudson et al., 2004a, 2004b; Moore et al., 2011a, 2011b; Ray et al., 2011; Sondell et al., 1998; Whitlock et al., 2010a, 2010b) have been devised to create acellularized nerve allografts (ANAs), thereby eliminating the need for immunosuppression.

Axonal regeneration in ANAs has been demonstrated to be similar to that in autografts across short gaps (Moore et al., 2011a, 2011b; Whitlock et al., 2009), but is reduced across longer defects (Whitlock et al., 2009), due to a poorly-understood mechanism. Following nerve repair with ANAs, there is early and progressive migration of SCs from both the proximal and especially the distal nerve stumps (Fornaro et al., 2001; Hayashi et al., 2007; Tseng et al., 2003; Whitlock et al., 2010a, 2010b). Host SCs provide the environment necessary for axonal regeneration in ANAs (Hall, 1986a, 1986b) through synthesis of neurotrophic factors (Bunge, 1993), adhesion molecules (Bixby et al., 1988), and axonal myelination (Bunge, 1993; Levi et al., 1994, 1997) and organization (Fansa et al., 2001). Failure of SCs to provide a positive regenerative environment in ANAs would significantly affect regeneration.

Cells undergo senescence in response to telomere shortening or dysfunction that arises from consecutive cell divisions, DNA damage, oncogenes, and/or other stressors that can cause epi(genomic) dysfunction (Campisi, 2011). The senescent state is characterized by irreversible arrest in proliferation accompanied by altered gene expression and changes in secretory profile (Campisi, 2005, 2011; Collado et al., 2007; Krtolica and Campisi, 2002; Pazolli and Stewart, 2008). Thus the presence of senescent SCs in nerve grafts would alter the local environment potentially leading to a loss of necessary support required for regenerating axons.

In this study, we investigated the role of SCs in mediating failure of axonal regeneration across long nerve grafts. Specifically, we hypothesized that longer ANAs place a greater proliferative demand on host SCs to fill longer grafts. This demand either exceeds the replication limit of SCs or creates a stressful environment causing them to undergo senescence.

Materials and methods

Animals

Adult male Lewis rats (250 g, Charles River Laboratories, Wilmington, MA) underwent sciatic nerve transection and grafting with a long 60 mm or shorter (20 mm or 40 mm) isografts or ANAs as outlined in Table 1. At the appropriate endpoint, the animals were sacrificed for assessment of grafts for neuroregeneration and senescent SCs (SenSCs). An additional cohort of Thy1-Sprague–Dawley transgenic rats (genOway, Lyon, France; Moore et al., 2011a, 2011b), which express green fluorescent protein (GFP) in axons under the control of neuron-specific Thy1 promoter, were used for in vivo visualization of axonal regeneration through long ANAs or isografts.

Surgical procedures and peri-operative care measures were conducted in compliance with the Washington University Institutional Animal Studies Committee and the National

Institutes of Health guidelines. All animals were housed in a central animal care facility and provided with food (PicoLab rodent diet 20, Purina Mills Nutrition International, St. Louis, MO) and water ad libitum. They were monitored post-operatively for signs of infection and distress.

Experimental design

Lewis and Thy1-GFP Sprague–Dawley transgenic rats were randomized to undergo sciatic nerve transection and repair with isografts or ANAs. Lewis and Thy-1 GFP rats received isografts from Lewis and Sprague–Dawley donors, respectively. Isografts for each specific group were used as positive control for both short- and long-term studies. Lewis (*RT-1^L* MHC) and Sprague–Dawley (*RT-1^b* MHC) rat strains are known to be completely MHC incompatible and thus were used as allograft donors to one another. Sciatic nerve allografts harvested from donor rats were chemically processed and decellularized using a series of detergents as described by Hudson et al. (2004a, 2004b) and Moore et al. (2011a, 2011b). Two end time points of short term (10 weeks) and long term (20 weeks) were designated for the study. For the short term (10 weeks), 20, 40, and 60 mm nerve grafts were engrafted. For the long term (20 weeks), 40 and 60 mm grafts were implanted. Following harvest, nerves were analyzed for axonal regeneration and reinnervation using histomorphometry, in vivo imaging, and extensor digitorum longus (EDL) muscle weight and electrically evoked force measurements. Select nerves from the short term endpoint engrafted with 60 mm ANAs and isografts were assessed for presence of SenSCs: Immunohistochemistry and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were used to measure markers of SCs (S100) and cellular senescence (β -galactosidase, p16^{INK4A}, p53, and IL-6); Electron microscopy was used to examine nuclei for reorganization of heterochromatin associated with senescence (Table 1).

Surgical procedures

Surgical procedures were performed under aseptic conditions and with the aid of an operating microscope (JEDMED/KAPS, St. Louis, MO) as described previously (Moore et al., 2011a, 2011b). The animals were anesthetized with subcutaneous delivery of Ketamine (75 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and dexmedetomidine (0.5 mg/kg, Pfizer Animal Health, Exton, PA). Donor nerves were harvested as described previously (Moore et al., 2011a, 2011b). The dissection was extended proximally and distally to allow harvest of 32–35 mm of nerve, which was later trimmed to 30 mm or 20 mm lengths as necessary. Sciatic nerves were transferred to aseptic tubes to undergo acellular processing (Hudson et al., 2004a, 2004b) or immediately used as fresh nerve isografts. Donor animals were then euthanized. We employed a novel long 60 mm rodent nerve graft model by coapting two 30 mm acellularized or fresh sciatic nerves using a minimum of a single 9–0 nylon epineurial suture and fibrin sealant (Baxter Healthcare Corp., Deerfield, IL; Whitlock et al., 2010a, 2010b). For 40 mm grafts, the same model was applied using coaptation of two 20 mm nerves.

Recipient rats underwent exposure of the right sciatic nerve. The recipient nerve was transected at 5 mm proximal to the sciatic trifurcation. The defect was reconstructed with an isograft or ANA (20, 40, or 60 mm) and secured to the proximal and distal nerve stumps using a minimum of a single 9–0 nylon epineurial suture and fibrin sealant (Moore et al., 2011a, 2011b; Whitlock et al., 2010a, 2010b). For 20 mm graft, the grafted nerves were settled with “S” style at original sciatic nerve bed. For 40 and 60 mm nerve grafts, the grafted nerves were shaped like a loop and inserted into an under-skin “pocket” around the femur (Fig. 1A). Thy1-GFP rats underwent intra-operative imaging of the newly-implanted nerve graft, for the purposes of later comparison of axonal regeneration, prior to closure of incision.

At the 10- and 20-week endpoints, the animals were sacrificed for analysis of the graft for axonal regeneration and SC senescence. Thy1-GFP rats underwent *in vivo* imaging of the exposed nerve prior to harvest. The sciatic nerve was harvested en bloc ~5 mm proximal and ~5 mm distal to the interposed graft. The nerves were stored in 3% glutaraldehyde or 4% paraformaldehyde for histomorphometry and electron microscopy (EM) analysis or immunohistochemical analysis, respectively. Samples for qRT-PCR analysis were stored in RNAlater solution (QIAGEN, Valencia, CA) and stored at -80 °C prior to extraction. The EDL muscle was harvested from both the experimental and the contralateral sides and weighed to evaluate percent muscle recovery following denervation.

Histomorphometry

The recipient/donor sciatic nerve complex was processed and assessed for evidence of nerve regeneration by histomorphometric analyses as described previously (Hunter et al., 2007). Briefly, the nerve specimens were preserved in glutaraldehyde, postfixed in osmium tetroxide, embedded in epoxy, and sectioned with an ultramicrotome. An observer blinded to the experimental groups obtained all measurements. Further analyses of microscopic images were performed with an automated digital image analysis system linked to histomorphometry software to quantify the total nerve fiber count (Hunter et al., 2007).

Functional assessment

Functional neuromuscular junction formation and functional regeneration of the sciatic nerve were assessed at the 20 week end point by examining the evoked motor response in reinnervated EDL muscle upon electrical stimulation of the repaired sciatic nerve as described previously (Moore et al., 2011a, 2011b). Briefly, the animals were immobilized in an automated functional assessment station (FASt System, Red Rock Laboratories, St. Louis, MO) where the distal portion of the EDL muscle was fixed to a 5 N load cell. Elicited twitch contractions were utilized to determine the optimal stimulus amplitude (V_o) and optimal muscle length (L_o) for isometric force production in the EDL muscle. All subsequent isometric force measurements were made at V_o and L_o . Single twitch contractions were recorded, and maximum twitch force (F_t) was calculated. Tetanic contractions were recorded at increasing frequencies of stimulation (5–200 Hz), allowing two minute intervals between stimuli to prevent muscle fatigue. Maximum isometric tetanic force (F_o) was automatically calculated from the resulting sets of recorded force traces. Maximum specific isometric tetanic force was calculated as the maximum isometric force normalized to physiological muscle cross-sectional area (Urbanchek et al., 1999). Four additional Lewis rats were utilized as uninjured controls to denote normal levels of EDL function. Denervated/reinnervated EDL muscle mass was normalized to their contralateral EDL muscle to quantify the relative degree of muscle atrophy distal to repaired sciatic nerve.

In vivo imaging

Nerve grafts in Thy1-GFP rats were imaged intra-operatively immediately following graft implantation and at the 10 week endpoint prior to explantation of graft using the methods described previously (Moore et al., 2011a, 2011b). In brief, sciatic nerve was exposed and imaged using a fluorescence-enabled dissecting microscope (Olympus MVX10, Olympus America Inc., Center Valley, PA) under GFP (488 nm) fluorescent and bright field filters (Fig. 2A). The images were recorded monochromatically using MetaMorph version 7.1.0.0 (Universal Imaging Corporation, Downingtown, PA). Images were standardized according to magnification (6.3 $\times$), exposure time (100–300 ms), and orientation. All images were standardized with respect to brightness and contrast.

Histological analysis of cellular senescence

Harvested nerves were immunostained with S100 (1:1000, Dako, Carpinteria, CA) for markers of SCs and β -galactosidase and p16^{INK4A} (1:500, Abcam, Cambridge, MA) for cellular senescence. Explanted nerves were fixed in 4% paraformaldehyde (Polysciences, Warrington, PA) at 4 °C overnight and transferred to 30% sucrose for cryoprotection. The proximal and distal stumps were trimmed to 5 mm from the proximal and distal sutures, respectively. The graft was then equally divided into three segments (proximal, middle, and distal thirds) to enable later assessment of SC phenotype along the length of the graft (Fig. 3A). Each segment was embedded in OCT Compound (VWR Inc.) and cut into 20- μ m longitudinal-sections using a cryostat and stored at -25 °C prior to staining.

β -Galactosidase

Nerve sections were stained for presence of β -galactosidase using the Senescence Detection Kit according to the manufacturer's protocol (Abcam, Cambridge, MA) (Dimri et al., 1995). The nerve sections were counterstained for nuclei with Nuclear Fast Red Solution (Electron Microscopy Sciences, Hatfield, PA) for 1 min. Sections were then rinsed with distilled water and dehydrated in gradients of ethanol and xylene. Nerve sections were imaged at 200 $\times$ using Leitz dialux 22 microscope (Wetzlar, Germany) and PAXit version 7.5 (MIS, Villa Park, IL).

Immunohistochemical staining

To further confirm the presence of SenSCs in long grafts, nerve sections were dual-stained for S100 and a key mediator of cellular senescence, p16^{INK4A}. The primary and secondary antibodies used are outlined in Table 2. Sections were then stained for the appropriate fluorochrome-conjugated secondary antibodies (Table 2). After antibody staining was complete, the slides were covered with VECTASHIELD® mounting medium (Vector Laboratories, Burlingame, CA), which contains the nonspecific nuclei stain 4',6-diamidino-2-phenylindole (DAPI).

Quantification of senescent markers

Two locations along each third segment were examined (a total of 6 locations, see Fig. 3). For each nerve, 3 samples were imaged at each of the 6 locations. Attempts were made to capture images that consisted mostly of the endoneurial contents of the nerve. This analysis was performed on 3 serial sections from each nerve and the numbers were averaged to provide a single value for each nerve.

β -Galactosidase

Nerve sections stained for β -galactosidase were imaged at the 6 locations along the graft. The total number of presumed SCs staining positively for β -galactosidase was manually counted using Cell Counter plug-in for ImageJ by a blinded experimenter and quantified as a ratio of total cells in each image.

S100, p16^{INK4A}

Nerve sections immunostained for markers of SCs (S100), and senescence (p16^{INK4A}), were imaged using MetaMorph at the 6 locations along the graft at 200 $\times$ using an inverted fluorescence microscope (Olympus 1X81, Olympus America Inc., Center Valley, PA) under the CY3 (555 nm) and FITC (488 nm) filters as appropriate for the secondary antibody used. Captured images were analyzed with ImageJ and the median fluorescence intensity of each stain was recorded. Median intensity was chosen as parameter for analysis so as to minimize the effect of brightly-fluorescing staining artifacts on data.

Quantitative reverse transcriptase PCR (qRT-PCR)

The gene expression of markers of SCs (S100, NGF receptor p75) and senescence (p16^{INK4A}, p53, and IL-6) in isografts (n = 4) and ANAs (n = 4) was analyzed using qRT-PCR. The 60 mm long grafts and ~5 mm of proximal and distal host nerve stumps were harvested. Each graft was divided into thirds as described above. In addition, the proximal and distal host nerve stumps were divided from the graft, yielding a total of 5 segments from each nerve (Fig. 4A). Each segment was stored in RNAlater solution (QIAGEN, Valencia, CA) at -4 °C overnight and then transferred to -80 °C until RNA extraction.

Total RNA was extracted from nerves using RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's direction. Total RNA was then bound to RNeasy Mini spin column and washed. The RNA sample was subsequently purified of any genomic DNA by incubating with gDNA Wipeout Buffer at 42 °C for 2 min. RNA was then reverse transcribed into complementary DNA strands (cDNAs) using the protocol described in the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). S100, p16^{INK4A}, p53, and IL-6 transcripts were quantified using real-time PCR (Taqman; Applied Biosystems) on a Step One Plus 96-well thermocycler (Applied Biosystems). All primers were purchased from Applied Biosystems and measured values were compared to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a house keeping gene (Bangaru et al., 2012; Thellin et al., 1999). To estimate the mRNA concentrations, the differences in gene expression levels between two different samples were calculated using the comparative delta crossover threshold (Ct) method (Livak and Schmittgen, 2001). A value of 2 or greater was selected as the minimum criteria for a significant difference in expression levels between graft groups and the normal fresh nerve (Hoke et al., 2006).

Electron microscopy

We observed DAPI staining of SC nuclei that was consistent with published reports of heterochromatin reorganization associated with senescence (Narita et al., 2003) (Fig. 5A). Subsequently, one representative nerve from each of the Thy1-GFP isograft or ANA groups was chosen for EM analysis of SC chromatin matter. Glutaraldehyde preserved and toluidine blue stained specimens were embedded in Araldite 502 resin and cut into 90 nm cross sections at each 5 mm interval along the length of the graft using an LKB III microtome (Bromma, Sweden). The sections were stained with uranyl acetate and lead citrate. Ultramicrographs were taken with a Joel 1200EX electron microscope at 10,000× magnification. Abnormal heterochromatin organization was defined as clumping and central involution of the chromatin matter in SC nuclei.

Statistical analyses

Data were recorded using Microsoft Excel (Microsoft, Redmond, WA). Statistical analyses were performed using SigmaStat (Systat, Chicago, IL). A two-tailed ANOVA was performed to determine histomorphometric differences within groups using Statistica statistical software (StatSoft, Inc.). A Student–Newman–Keuls test was then performed to compare groups. A two-tailed Student's *t*-test with assumed unequal variance of data was used to compare immunohistochemical staining at multiple locations between the two groups. Significance was established at $p < 0.05$. All results are reported as mean $\pm$ standard deviation (SD).

Results

Histomorphometric analysis of axonal regeneration

Nerve regeneration through isografts and acellular allografts was assessed via histological and histomorphometric analyses of explanted grafts. Processed nerve sections just distal to the third sciatic coaptation site (i.e. distal to the graft) were analyzed in all groups. At the 10 week end point, it was noted that increased graft length correlated with decreased axonal regeneration in both graft groups (Fig. 1B). The 20 mm isograft (ISO—20 mm) demonstrated the most robust nerve regeneration with $11,420 \pm 3114$ myelinated fibers. The remaining isografts of 40 mm (ISO—40 mm) and 60 mm (ISO—60 mm) supported 4090 ± 592 and 3262 ± 1426 myelinated fibers, respectively. In contrast the acellular allografts (ANAs) demonstrated significantly fewer myelinated axons compared to the isografts of comparable graft length. The 20 mm ANA (ANA—20 mm) supported 3603 ± 2108 myelinated fibers, while the 40 mm (ANA—40 mm) and 60 mm (ANA—60 mm) supported only 705 ± 1238 and 17.8 ± 50.4 myelinated fibers, respectively. Additionally, among the 8 rats in the ANA—60 mm group, seven lacked any myelinated fibers in the distal stump.

Analysis was performed at 20 weeks after implantation to allow for complete regeneration. The comparison of total number of myelinated fibers demonstrated a similar trend as the 10 week histomorphometric data with significantly decreased regeneration in ANA when compared to isografts of comparable length. There was significantly fewer nerve fibers in the 40 mm ANA (4579 ± 3573 , ANA—40) compared to the 40 mm isograft ($12,701 \pm 6784$, ISO—40 mm). The 60 mm isograft (ISO—60 mm) supported the regeneration of 3777 ± 1644 myelinated fibers, while there were no myelinated nerve fibers in any of the eight 60 mm ANAs (ANA—60 mm) explanted (Fig. 1C).

Representative histological sections acquired distal to the repaired nerve defect after 20 weeks are shown in Fig. 1D. The micrographs demonstrate populations of axons successfully regenerating through 40 and 60 mm fresh nerve isograft, and 40 mm ANA graft. Sections acquired 5 mm distal to implanted isografts of 40 mm and 60 mm showed robust axonal regeneration, numerous myelinated fibers, and mature nerve architecture. Sections acquired distal to 40 mm ANA graft also show nerve regeneration. In contrast, the micrograph of the 60 mm ANA grafts demonstrates a complete absence of healthy, myelinated axons.

In summary, regeneration of axons across isografts and ANAs into the distal stump decreased with increased graft length. Although the number of regenerating axons decreased with graft length, all animals treated with isografts supported axonal regeneration into the distal nerve stump at all graft lengths. In contrast, the ANA failed to consistently support axonal regeneration in graft lengths beyond 20 mm. Of the sixteen animals (six at 10 weeks and ten at 20 weeks) from the 40 mm groups, four animals demonstrated no axonal regeneration in the distal stump. Of the fourteen animals (eight at 10 weeks and six at 20 weeks) from 60 mm groups respectively, thirteen animals demonstrated no axonal regeneration in the distal stump.

EDL functional recovery

Return of muscle weight following peripheral nerve injury and muscle denervation is a corollary to functional recovery (Akassoglou et al., 2000; Aydin et al., 2004; Brown et al., 2002; Haase et al., 2003; Kobayashi et al., 1997; Lewin et al., 1997; Newman et al., 1996; Sterne et al., 1997; Terris et al., 1999; Tham et al., 1997). Measurements of EDL muscle mass were used to assess the amount of muscle atrophy and recovery due to sciatic nerve injury and muscle regeneration following reinnervation. Axonal regeneration and subsequent reinnervation of the EDL result in recovery of muscle mass. The ratio of

experimental/contralateral uninjured muscle was used to normalize and quantify this process as percent recovery. After graft reconstruction, a correlation between muscle atrophy and graft length in both isograft and allograft was observed. The observed amount of muscle recovery decreased with graft length and graft type. Animals treated with isografts demonstrated significantly increased percent EDL muscle mass when compared to ANA reconstructed animals at the same length and end points. Ten weeks after reconstruction the EDL muscle of ISO—20 mm demonstrated $65.2 \pm 5.2\%$ recovery compared to a significantly decreased $35.9 \pm 15.3\%$ in the ANA—20 mm group (Fig. 1E). As the length increased, recovery decreased at the 10 week time point. The ISO—40 mm and ISO—60 mm demonstrated $41.1 \pm 7.7\%$ and $33.6 \pm 6.9\%$ recovery, respectively, and both ANA groups (ANA—40 mm: $24.1 \pm 3.3\%$ and ANA—60 mm: $20.4 \pm 1.5\%$) demonstrated significantly less recovery over the same time period (Fig. 1E). EDL muscle recovery in the isograft treated animals improved from ten to twenty weeks after reconstruction with ISO—40 mm and ISO—60 mm groups exhibiting $74.6 \pm 4.9\%$ and $53.6 \pm 14.4\%$ recovery of the EDL. In contrast, EDL muscle recovery decreased between ten and twenty weeks for ANA—40 mm ($12.3 \pm 2.5\%$) and ANA—60 mm ($13.5 \pm 1.2\%$) (Fig. 1F). The decrease in muscle recovery of the ANA groups between 10 and 20 weeks indicates continued muscle atrophy and degeneration of the EDL muscle.

Functional motor recovery was assessed in the 40 and 60 mm graft groups 20 weeks after reconstruction by measuring the extent of evoked extensor digitorum longus (EDL) muscle force. The maximum isometric tetanic force measurement in 40 mm and 60 mm isograft groups demonstrated significant functional recovery (Fig. 1G). In comparison with the uninjured control group ($27.3 \pm 1.7 \text{ N/cm}^2$, $n = 4$), the 40 mm isograft recovered 42.5% ($11.6 \pm 4.8 \text{ N/cm}^2$) of normal force and the 60 mm isograft group recovered 50.5% ($13.8 \pm 7.1 \text{ N/cm}^2$) of normal force. In contrast, the ANA groups at both graft lengths (40 and 60 mm) exhibited no functional recovery over 20 weeks (Fig. 1G). The difference between the ANA and Isograft groups at their respective lengths was significant ($p < 0.01$ for 40 mm and 60 mm groups.).

In summary, the extent of muscle atrophy increased with length of the grafts used to reconstruct the injury. Recovery of muscle mass increased over time in all of the groups treated with nerve isografts, which indicated that the EDL muscle was functionally reinnervated. The transected nerves that were reconstructed with ANAs greater than 20 mm demonstrated increased levels of atrophy over the time course of the study. These results correlated with the muscle force analysis which indicated that the EDL was not significantly reinnervated in the groups after 20 weeks of regeneration.

Visualized nerve regeneration in long grafts

The very poor regeneration observed in the ANA-treated animals prompted our investigation into what environmental factor may contribute to the lack of axonal regeneration in long graft ANAs (>20 mm). In vivo imaging of neuroregeneration in Thy1-GFP rats (Moore et al., 2011a, 2011b) allowed visualization of the regenerating axonal front in 20, 40 and 60 mm isografts and ANAs after 10 weeks. A strong inverse correlation between graft length and degree of axonal regeneration was observed (Figs. 2B–G). Consistent with the literature and our histological observations, axonal regeneration was robust in the short 20 mm isografts and ANAs (Figs. 2B and E; Moore et al., 2011a, 2011b; Whitlock et al., 2009). In longer graft lengths, however, axonal regeneration failed to span the length of the graft in ANAs (Figs. 2F and G) and intensity of regeneration qualitatively diminished in isografts (Fig. 2D). Of note, the extent and length of axonal regeneration into the ANA was shorter in 60 mm ANAs (Fig. 2G) when compared to the 40 mm ANAs (Fig. 2F). The regenerative front of axons in the 40 mm ANA reached the middle suture line (a distance of 20 mm), while the front of axons in the 60 mm regenerated a distance of approximately 5 mm.

Cellular senescence in long grafts

Stressful cellular environments are associated with decreased nerve regeneration. Prolonged cellular stress due to chronic denervation in distal stump SCs decreases axonal regeneration (Fu and Gordon, 1995; Gold et al., 1999; Gordon et al., 2003, 2009, 2011; Hoke et al., 2002; Sulaiman and Gordon, 2000, 2009; Sulaiman et al., 2002a, 2002b; Walsh et al., 2010). ANAs require proliferation of host SCs to populate the acellular graft to support axonal regeneration (Hall, 1986a, 1986b; Hayashi et al., 2007). Based on our observations of diminished regeneration in long graft ANAs, we hypothesized that the increasing proliferative demand to populate ANAs of increasing lengths may induce a senescent state in graft SCs. To evaluate this hypothesis, we evaluated 60 mm ANAs and isografts in a sciatic nerve injury model for the presence of senescent cells.

Histological analysis of cellular senescence

Long ANAs and isografts (n = 9 per group) were stained for β -galactosidase (β -gal), which is secreted by senescent cells, and counterstained for nuclei using nuclear fast red stain. For analysis of stained sections, two locations in each third segment of the graft (proximal, middle, distal) were chosen resulting in six analysis areas (regions 1–6) (Fig. 3A). Analysis of the six graft areas revealed a significantly greater ratio of β -gal positive cells in the ANA (0.74 ± 0.18) when compared to isografts (0.50 ± 0.10) in the region 3 of the graft ($p = 0.0046$). Additionally, the difference between ratio of β -gal expressing cells in the ANAs (0.58 ± 0.17) and isografts (0.45 ± 0.14) in the region 2 of the graft approached significance ($p = 0.10$). Both of these locations correspond to the region of the ANA at which the axonal front stalled in Thy1-GFP rat model (Fig. 3B). The average number of cells per area of graft from each graft type was quantified to determine if each graft type contained a significantly different number of cells. The total number of cells in the ANA (1962 ± 478 cells/mm²) was not significantly different from the total number found in the Isograft (1959 ± 205 cells/mm², $p = 0.56$).

To further characterize the presence of senescent markers in the graft, immunohistochemical staining for S100 and p16^{INK4A}, a known mediator of senescence, was also performed. The results of median intensity measurement of p16^{INK4A} staining from 9 rats per group are shown in Fig. 3C. Median intensity of p16^{INK4A} was noted to be significantly greater in long ANAs compared to isografts in region 2 (ANA 234.1 ± 34.4 vs. ISO 192.6 ± 23.6 , $p = 0.02$). A higher median intensity of p16^{INK4A} was also demonstrated in region 3 of ANAs when compared to isografts (ANA 269.08 ± 24.34 vs. ISO 234.67 ± 39.38 , $p = 0.02$). There was no difference in median intensity of S100 staining for SCs at any of the 6 areas of analysis between ANAs and isografts (results not shown). Double labeling of graft section from ANA and isografts with S100 and p16^{INK4A} demonstrated co-localization of the two markers (Figs. 3D–E).

qRT-PCR of senescent marker expression

qRT-PCR was used to further characterize the expression of senescence related markers in the long graft ANAs and isografts. Grafts were harvested after 10 weeks with 5 mm sections of proximal and distal host nerves attached. The sections of host nerve were separated from the graft at the most proximal and distal suture lines, and the remaining graft was divided into three equal segments (Fig. 4A). The five tissue sections (proximal host, proximal graft, middle graft, distal graft, and distal host) from isografts (n = 4) and ANAs (n = 4) were then analyzed for the expression of SCs (S100, NGF receptor p75) and senescence (p16^{INK4A}, p53, and IL-6) related markers (Fig. 4B). The expression of p16^{INK4A} was up-regulated (>2 fold expression increase) in four locations in the isograft group (proximal graft, middle graft, distal graft, and distal stump) and in all locations in the ANA group (proximal stump, proximal graft, middle graft, distal graft, and distal stump). Interestingly, the location of

highest fold change in p16^{INK4A} (12 fold increase) in the ANAs was in proximal graft, which is the same vicinity of the halted regenerating front seen in the Thy1-GFP rats (Fig. 2G) and increased β -gal/p16^{INK4A} immunohistochemical staining (Figs. 3B and C). The expression of p53, a marker of senescence, was marginally increased in both isografts and ANAs (>2.5 fold increase). IL6 expression is associated with the senescent state in cells and has been implicated as a mediator of stress induced senescence (Coussens and Werb, 2002; Kuilman et al., 2008). Its expression was up-regulated (>2 fold) in three locations in the isograft (middle graft, distal graft, and distal stump) and in all five locations in the ANA (proximal stump, proximal graft, middle graft, distal graft, and distal stump).

SCs can be identified by the expression of S100 (mature myelinating SCs), glial fibrillary acidic protein (GFAP, mature non myelinating SCs) and the low affinity p75 nerve growth factor receptor (Ngfr, immature SCs). The former two markers are associated with mature SCs that are present in normal nerve and the latter marker is associated with immature SCs that are present during development and after nerve injury. The expression of the mature SC marker S100 was down-regulated (<0.5 fold expression) in all locations examined in the isograft and ANA. The expression of the immature SC marker Ngfr was up-regulated (>2 fold expression) in all locations in the isograft (proximal stump, proximal graft, middle graft, distal graft, and distal stump). The ANA lacked up-regulated expression of Ngfr in the middle and distal portions of the graft (<2 fold expression).

In summary, the expression of senescence related markers (p16^{INK4A}, and IL6) was upregulated in the grafts of each experimental group. The increase in p16^{INK4A} expression in the ANA was associated with a location in the graft at which the GFP axon front halted in the transgenic rats (Fig. 2G). The expression of the nerve regeneration related Ngfr SC marker in the isograft was increased as function of distance away from the proximal stump, with the largest increase in expression in the distal graft. In contrast, the expression of Ngfr in the ANA was minimal in the middle and distal portions of the ANA.

Electron microscopy

The irreversibility of a senescent state is thought to be due to a characteristic reorganization in heterochromatin (Narita et al., 2003). Evaluations of DAPI stained nuclei from cells within long graft ANA, demonstrated clumping of chromatin that resembled this reorganization (Fig. 5A). Further investigation with electron microscopy revealed that the proximal host nerve of all grafts and short 20 mm ANAs contained SCs with evenly-dispersed chromatin along the periphery of normally-shaped nuclei (Fig. 5B). Schwann cells can be positively identified using electron microscopy by the morphological presence of double basement membrane (Mackinnon and Dellon, 1988). Within the long graft ANAs (40 and 60 mm), there was a qualitative increase in the presence of SCs with chromatin clumped in central involutions in the distal portions of the grafts (Figs. 5C–D). Such abnormally-appearing nuclei were observed very proximally in the 60 mm ANA (Fig. 5D), near the stalled axonal front. Interestingly, SCs in the 60 mm isograft also exhibited the altered nuclei, albeit more distally than the ANA and with less frequency. The distal host nerve of the 60 mm isograft contained normal SCs, while the ANA distal host continued to show abnormal chromatin organization (Fig. 5D). In summary, the accumulation of cells exhibiting senescent nuclear characteristics in nerve grafts increases with increased graft length, and is more notable in ANAs. These cells are found in the isograft at 60 mm in length but are consistently in the company of SCs with normal nuclear morphology. Further, the identification of altered heterochromatin in SCs, positively identified by double basement membrane, indicates that SCs are contributing to the increase in senescent related markers found in long graft ANAs via the other analysis techniques.

Discussion

The current study is the first to correlate the presence of senescent cells in peripheral nerve grafts with limited axonal regeneration. It demonstrates that acellular nerve allografts (ANAs), which are used clinically, support inconsistent regeneration in a rodent sciatic nerve injury model across gap lengths of 40 mm, and support no regeneration across graft lengths of 60 mm or greater. Further, the current study provides evidence that limited axonal regeneration across increasing graft lengths is associated with an increase in the presence of senescent cells within the graft. A portion of these senescent cells are SCs that express β -galactosidase and p16^{INK4A}, and their presence is extensive in the region that corresponds with stalled axonal regeneration.

Commercially available ANAs are replacing nerve conduits as an alternative to nerve autografts (Karabekmez et al., 2009; Moore et al., 2009). In the current study, three ANA graft lengths (20, 40 and 60 mm) were evaluated for their ability to support axonal regeneration. ANA demonstrated limited ability to support axonal regeneration in graft lengths up to 40 mm. Our results revealed an inverse relationship between axonal regeneration and ANA graft length, suggesting that the mechanism at play is not a threshold phenomenon. If the mechanism limiting regeneration was merely a distance threshold, one would expect that the penetration of axons into the acellular graft would be constant regardless of ANA graft length. However, the distance of penetration is not consistent, which suggests a significant change in the local regenerative environment that actively halts regeneration. Schwann cells (SCs) provide the pro-regenerative environment that facilitates nerve regeneration after injury, and ANAs depend on the proliferation of SCs from the host nerve to provide that environment within the graft (Hall, 1986a, 1986b; Hayashi et al., 2007). Based on the histomorphometric analysis and Thy1-GFP axonal imaging, it appears that the burden of increased proliferation either stressed or exhausted host SCs within the graft and negatively altered the regenerative micro-environment.

Cellular senescence is a state of permanent cellular growth arrest (Bodnar et al., 1998; Dimri et al., 1995; Hayflick and Moorhead, 1961; Itahana et al., 2007; Vaziri and Benchimol, 1998) and is a likely candidate for limiting regeneration in ANAs. No single marker is used to identify senescent cells and rather a combination of markers identifies this cellular state. We used β -gal expression, immunohistochemical analysis of p16^{INK4A}, expression of senescent associated genes, and microscopic analysis of cell nuclei to identify senescent cells in the nerve grafts. The expression of these markers was increased in both long ANAs and isografts. β -Gal expression in the 60 mm ANAs was significantly increased when compared to 60 mm isografts. Similarly, increased expression of p16^{INK4A}, a tumor suppressor protein, was verified in proximal sections of the 60 mm ANA by immunohistochemical. The expression of p53, another tumor suppressor protein, was found to be marginally up-regulated in both isografts and ANAs. The two known pathways to senescence are mediated by p16^{INK4A} and p53. Classically, the p53 pathway is implicated in replicative senescence and other sources of DNA damage. The p16^{INK4A} pathway is more characteristically activated in response to stressful stimuli. However, each pathway, while distinct, shares many similar features including triggers and intermediate factors making them difficult to separate (Campisi and d'Adda di Fagagna, 2007; Serrano and Blasco, 2001; Sharpless and DePinho, 1999). In addition to the expression of senescent markers in cells identified as SCs, senescent markers were found in cells not expressing markers for SCs. Other cellular components, including macrophages, fibroblasts and endothelial cells, are likely contributing to the senescent signal quantified in both ANAs and Isografts. Analysis of the role of senescent cells other than SCs in long grafts will be crucial to the full understanding of the regenerative environment of long graft ANAs.

A change in the organization of heterochromatin in the nucleus of cells is also associated with senescence. This change is characterized by the formation of senescence-associated heterochromatin foci (SAHFs) which are compact regions of DNA (Funayama et al., 2006; Narita et al., 2003) that can be identified by microscopic observation. We observed structures consistent with SAHFs in DAPI-stained SCs from long grafts and further characterized them under electron microscopy. This analysis revealed a strong correlation between the presence of SCs with augmented chromatin and increased graft length. The combined results of the three employed assessment techniques provide substantial evidence that limited regeneration across long grafts is associated with the increased presence of senescent cells in the graft. The identification of SAHFs in SCs suggests that SCs are a significant component of this increased senescent cell population.

While we provide evidence for the presence of senescent SCs in long grafts, the mechanism inducing senescence in these SCs remains unclear. Excessive proliferative demand on host SCs to populate increasing acellular volumes could contribute to senescence. It is also possible that the long ANAs create an environment that is stressful to SCs, thereby inducing senescence. For example, hyperglycemia inhibits axonal regeneration and initiates a senescent-like SC phenotype characterized by arrest in proliferation, lack of apoptosis, and impaired function (Gumy et al., 2008). As the length of the graft increases so does the length of time for which SCs, particularly in the more distal regions, are denervated (i.e. not in contact with regenerating axons). The stress induced by prolonged denervation has been shown to diminish the ability of SCs to support axonal regeneration (Gordon et al., 2009, 2011; Sulaiman and Gordon, 2000, 2009). In fact, activation of p38 MAPK, a SC dedifferentiation after peripheral nerve injury (Yang et al., 2012), has been shown to induce senescence in fibroblasts (Wang et al., 2002) and has been shown to mediate the SASP in senescent cells (Freund et al., 2011). It is possible that chronic denervation and prolonged absence of trophic support from axons, induces SC senescence. In support of this, Li and colleagues found that there was a paucity of proliferating cells in the endoneurium of chronically denervated nerves, which was largely irreversible even after re-innervation (Li et al., 1997). In our long graft model, senescence in the ANA could easily be induced by proliferative pressure and delayed axonal contact. An active immune response to the ANA may also induce a stressful cellular environment. This is unlikely, however, because our histological analysis failed to demonstrate the presence of immune infiltrate and previous work has demonstrated the lack of immune response to processed ANAs (Moore et al., 2011a, 2011b).

Senescent cells undergo phenotypic changes that alter both cell function as well as the secretory profile, which affects surrounding cells (Coppe et al., 2010; Pazolli et al., 2009). IL-6 is a notable component of the change in secretory profile that has been called the senescence associated secretory phenotype (SASP). We observed significant increases in the expression of IL-6 in the long graft ANAs, which is consistent with SASP formation. Since the neurotrophic function of SCs is related to their capacity to proliferate in response to regenerating axons (Sulaiman and Gordon, 2009), it is likely that the altered SC phenotype causes a shift away from their pro-regenerative role. Accordingly, an irreversible and progressive decrease in the expression of neurotrophic factors is also observed in chronically-denervated SCs (Sulaiman and Gordon, 2009), which adversely affects axonal regeneration.

Other factors inherent to the ANA may also contribute to limited axonal regeneration. The extent of vascular perfusion of any nerve graft has an effect on nerve regeneration. Nerve graft ischemia has been shown to decrease the extent and degree of axonal regeneration after peripheral nerve injury (Best et al., 1999a, 1999b; Hoke et al., 2001; Korthals and Wisniewski, 1975; Penkert et al., 1988; Shupeck et al., 1989; Sladky et al., 1991). Studies

have shown that nerve auto/isografts vascularize through the process of inosculation, where by the existing vasculature of the graft is connected directly to the vasculature of the proximal and distal nerve stumps (Best et al., 1999a, 1999b; Penkert et al., 1988). This provides extensive vascular perfusion throughout the graft over a very short time frame. In contrast, ANAs lack existing vasculature because tissue processing removes the cellular components that make up the graft (Hudson et al., 2004a, 2004b). While it has not been demonstrated experimentally, it is likely that vascular perfusion of ANAs occurs through the process of neovascularization, or the formation of new microvascular networks within the graft (Laschke and Menger, 2012). This process is significantly slower than inosculation and may hinder the process of axonal regeneration. It is unknown how increases in graft length may affect the efficiency of neovascularization within ANAs but changes in volume have been shown to affect graft vascularity (Best et al., 1999a, 1999b). Significant increases in graft length, such as those evaluated in our study, may result in areas of ischemia within the ANA. Interestingly, ischemic events in nerve have been associated with oxidative stress in SCs (Iida et al., 2004; Qi et al., 2001; Wang et al., 2005), which is associated with the induction of senescence (Barascu et al., 2012; Chen et al., 2001; Nogueira et al., 2008; Parrinello et al., 2003). Further study is needed to establish the mechanism and extent of vascularization of long graft ANAs and the effect on regeneration and SC phenotype.

In conclusion, significant evidence is presented that demonstrates that limited axonal regeneration in long graft ANAs is associated with an increase in senescent SCs within the graft. The proliferative demand of populating a large graft and/or chronic denervation may contribute to the induction of senescence. Once senescent, SCs may adversely affect regeneration by changing their secretory phenotype negatively altering the nerve microenvironment. Future studies will be devoted to demonstrating the causal relationship between limited axonal regeneration and senescent SCs.

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Abbreviations

SCs	Schwann cells
ANAs	acellularized nerve allografts
SenScs	senescent Schwann cells
GFP	green fluorescent protein
EDL	extensor digitorum longus
DAPI	4',6-diamidino-2-phenylindole

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Fig. 1.

Evaluation of nerve regeneration in long graft model in rat. A) Two sciatic nerve grafts (lengths of >30 mm) were harvested from a single donor rat. The two nerve pieces were coapted together in a proximal–distal end to end fashion to form a graft of up to 60 mm. The coapted donor was then trimmed to the desired length (40 or 60 mm) for nerve interposition and implanted in a “pocket” under the skin. (P indicates proximal. D indicates distal. Arrows indicate suture lines). Histomorphometric analysis of regenerating nerve fibers demonstrated decreased axonal regeneration with increased graft lengths in both graft groups. The total number of myelinated nerve fibers was quantified at 10 weeks (B) and 20 weeks (C) after reconstruction. At both time points, isografts (ISO) demonstrated superior regeneration in

comparison to ANAs at all lengths. D) Representative histological sections of regenerating nerve 5 mm into the distal nerve stump at 400× magnification were taken. Sections acquired from 40 mm and 60 mm nerve isografts showed robust axonal regeneration, numerous myelinated fibers, and mature nerve architecture. The section acquired from 40 mm ANA also demonstrated some axonal regeneration. In contrast, the section acquired from 60 mm ANA graft demonstrated no healthy, myelinated axon. E, F) Assessment of recovery of EDL muscle mass demonstrated that there was a positive correlation between muscle atrophy and graft length for both isograft and ANA graft. E) EDL muscle recovery (the ratio of experimental/contralateral) after 10 weeks demonstrated superior recovery for animals reconstructed with isografts. F) Similarly, recovery of EDL muscle mass after 20 weeks demonstrated increased recovery in isograft treated groups. In contrast, groups treated with ANA exhibited increased atrophy over the time period between 10 and 20 weeks. G) Functional reinnervation after 20 weeks measured by evoked tetanic muscle force in the EDL demonstrated no significant reinnervation in the 40 mm and 60 mm ANA groups. In contrast the isograft treated groups at both lengths exhibited significantly increased tetanic muscle force. ISO: isograft. ANA: acellular nerve allograft. Tetanic SpF: Specific isometric tetanic force. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Error bars present the mean $\pm$ standard deviation (SD).

Fig. 2.

Axonal regeneration in Thy1-GFP rats. Thy1-GFP rats express green fluorescent protein (GFP) in their axons allowing for visualization of the regenerated axons in grafts after 10 weeks (A). A strong inverse relationship between the axonal regeneration and the length of ANA was observed: Both 20 mm isografts (B) and ANAs (E) were able to support axonal regeneration through the length of the graft. When the graft length was increased to 40 mm, axonal regeneration was hindered in ANAs (F) but not isografts (C); this difference in extent of regeneration was even more pronounced at 60 mm (D & G). Of note, the axons regenerated a shorter distance in the 60 mm ANA (G) than in the 40 mm ANA (F). All images were obtained at 6.3× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Immunohistochemical staining for markers of senescence. Long nerve grafts (isografts $n = 9$, ANAs $n = 9$) were stained for senescence marker, β -galactosidase (blue), and counter-stained for nuclei with nuclear fast red stain (red). A) A representative image of β -galactosidase stained isograft and ANA are shown. Each tissue section was analyzed in six regions along the length of the graft to quantify the presence of senescent cells. B) A manual count of the average ratio of nuclei staining positively for β -galactosidase at each of 6 locations along 60 mm isografts and ANAs was performed. C) Long nerve grafts (isografts $n = 4$, ANAs $n = 4$) were also immunostained for markers of SCs (S100) and senescence (p16). The median intensity of fluorescence from each marker was quantified. The results of p16^{INK4A} evaluation are shown in (C). D) A representative image of an ANA and isograft is shown, with areas staining positively for p16^{INK4A} shown in green and S100 in red. Blue areas indicate presence of cellular nuclei, which are stained with DAPI. E) A representative transverse section within the graft of an ANA was immunostained for senescent and SCs

specific markers (20× image, scale bar is 50 μm, blue staining is DAPI for cell nuclei). The same tissue section was stained for the senescence marker p16^{INK4A} (green left panel) and the SC specific marker S100 (red left panel). F) Colocalization of both markers in areas staining positively for both p16^{INK4A} (green) and S100 (red) appears yellow, and indicates the expression of the senescent marker p16^{INK4A} within SCs. (ISO: isograft. ANA: acellular nerve allograft. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Error bars present the mean $\pm$ standard deviation (SD)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Quantification of senescent gene expression in long nerve grafts. The graft and portions of the proximal and distal nerve stumps were harvested after 10 weeks from each experimental group ($n = 4$). A) The tissue was sectioned into five segments. B) Each segment (proximal host, proximal graft, middle graft, distal graft, and distal host) was processed to quantify the expression of senescent ($p16^{\text{INK4A}}$ (p16), p53, and IL6), and Schwann cell (S100, p75 (Ngfr)) related mRNA. (ISO: isograft. ANA: acellular nerve allograft. Dotted line indicates 2 fold expression. Error bars present the mean $\pm$ standard deviation (SD)).

Fig. 5.

Chromatin reorganization in senescent cells. DAPI staining revealed clumping of DNA in SC nuclei in the distal ANA (A), consistent with the presence of senescence-associated heterochromatin foci. Closer examination of nuclei of SCs for changes in the chromatin within a representative 20 (B), 40 (C), 60 (D) mm ANA and isografts was performed under electron microscopy. The analysis revealed a progression of clumping of chromatin with central involutions in the 40 and 60 mm ANA (orange outlines and arrowheads). These augmented nuclei were found in the region of stalled axonal front and persisted throughout the distal graft. The DNA clumping starkly differed from the uniform peripheral appearance of chromatin in short graft ANA and the normal proximal host (gray outline). Similar

changes were noted in the isograft but at a more distal location and only in the 60 mm graft length. All EM images were obtained at 10,000× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Groups, time points and animal numbers.

	Graft length	10 week endpoint	20 week endpoint	Analysis
Isograft	20 mm	n = 7	–	Histomorphometry
	40 mm	n = 6	n = 14	
	60 mm	n = 6	n = 6	
	20 mm	n = 2	–	In vivo imaging (Thy1-GFP rats)
	40 mm	n = 2	–	
	60 mm	n = 3	–	
	20 mm	n = 6	–	EDL muscle force/mass measurement
	40 mm	n = 6	n = 3	
	60 mm	n = 6	n = 6	
	60 mm	n = 9	–	Histology
	60 mm	n = 4	–	qRT-PCR
	60 mm	n = 1	–	EM
Allograft	20 mm	n = 7	–	Histomorphometry
	40 mm	n = 6	n = 10	
	60 mm	n = 8	n = 6	
	20 mm	n = 2	–	In vivo imaging (Thy1-GFP rats)
	40 mm	n = 2	–	
	60 mm	n = 3	–	
	20 mm	n = 6	–	EDL muscle force/mass measurement
	40 mm	n = 6	n = 3	
	60 mm	n = 6	n = 6	
	60 mm	n = 9	–	Histology
	60 mm	n = 4	–	qRT-PCR
	60 mm	n = 1	–	EM

Table 2

Antibodies used for immunohistochemical staining of grafts for senescent SCs.

Primary antibody	Host	Concentration	Secondary antibody	Host	Concentration
S100 (Dako, Carpinteria, CA)	Rabbit, polyclonal	1:1000	CY3	Goat	1:1000
p16 ^{INK4A} (Abcam, Cambridge, MA)	Chicken, polyclonal	1:250	FITC	Donkey	1:1000
Cleaved caspase-3 (Millipore, Billerica, MA)	Rabbit, polyclonal	1:500	CY3	Goat	1:1000