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Journal of Cellular Biochemistry 113:2330–2345 (2012)

Journal of Cellular Biochemistry

Intramuscular Nerve Damage in Lacerated Skeletal Muscles may Direct the Inflammatory Cytokine Response During Recovery

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ABSTRACT

The expression of inflammatory cytokines and growth factors in surgically repaired lacerated muscles over a 12-week recovery phase was investigated. We hypothesized that these expression levels are influenced by both neural and muscular damage within lacerated muscles. Microarrays were confirmed with reverse transcription-polymerase chain reaction assays and histology of biopsies at the lesion of three simulated lacerated muscle models in 130 adult rats. The lacerated medial gastrocnemius with the main intramuscular nerve branch either cut (DN), crushed but leaving an intact nerve sheath (RN); or preserved intact (PN) were compared. At 4 weeks, DN had a higher number of interleukins up-regulated. DN and RN also had a set of Bmp genes significantly expressed between 2 and 8 weeks ($P \leq 0.05$). By 12 weeks, DN had a poorer and slower myogenic recovery and greater fibrosis formation correlating with an up-regulation of the Tgf-β gene family. DN also showed poorer re-innervation with higher mRNA expression levels of nerve growth factor (Ngf) and brain-derived neurotrophin growth factor (Bdnf) over RN and PN. This study demonstrates that the inflammatory response over 12 weeks in lacerated muscles may be directed by the type of intramuscular nerve damage, which can influence the recovery at the lesion site. Inflammatory-related genes associated to the type of intramuscular nerve damage include Gas-6, Artemin, Fgf10, Gdf8, Cntf, Lif, and Igf-2. qPCR also found up-regulation of Bdnf (1-week), neurotrophin-3 (2w), Lif (4w), and Ngf (4w, 8w) mRNA expressions in DN, making them possible candidates for therapeutic treatment to arrest the poor recovery in muscle lacerations (250). J. Cell. Biochem. 113: 2330–2345, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: OLIGO cDNA MICROARRAY; QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION; RE-INNERVATION

Lacerations of the extremities are a common occurrence and often the deeper lacerations involve a partial or through-thickness muscle laceration. The common practice to repair the lacerated portion of a muscle belly is to suture the cut ends together [Garrett et al., 1984; Huard et al., 2002; Jarvinen et al., 2005; Kragh et al., 2005]. The surgically repaired muscle undergoes an immediate attempt to bridge the two cut ends by an infiltration of inflammatory cells [Huard et al., 2002]. This initiates a response dominated by the presence of neutrophils and macrophages during the acute phase [Menetrey et al., 2000; Tidball, 2005]. The inflammatory process is a critical phase of muscle regeneration with the release of various cytokines play important and diverse

Authors Contributions. ZY and TBL performed surgeries, tissue collections, and RNA isolations. TBL, KZA and HHC performed histology, immunohistochemistry, gene-array and real-time RTPCR. BPP and DTL performed statistical analysis. BPP, TBL and DTL drafted the manuscript. All authors participated in the design of the study, and read and approved the final manuscript.

Conflicts of interest: All authors have read the manuscript and have no conflict of interest to declare.

Grant sponsor: URF Tier-1; Grant number: T13-0802-P21; Grant sponsor: Biomedical Research Council; Grant number: BMRC/04/1/21/19/309.

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Manuscript Received: 8 February 2012; Manuscript Accepted: 10 February 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 22 February 2012

DOI 10.1002/jcb.24105 • © 2012 Wiley Periodicals, Inc.

roles to regulate the normal immune system in tissue damage. The acute phase involves the removal of dead tissue and cells through lysis, where after a few days macrophages then release cytokines and growth factors to initiate the regenerative repair phase. The timing of the cytokines released by the macrophages influences the rate of recovery and further regulates inflammatory cells as well as influence stem cells, satellite cells activation, and collagen synthesis to reconstitute the lesion gap [Husmann et al., 1996; Hirata et al., 2003; Pelosi et al., 2007].

Clinically, muscle repairs recovery well. However if the cut ends are not sufficiently repaired, the haematoma at the gap may take longer across the lesion resulting in increased fibrosis at the lacerated site and muscle atrophy [Garrett et al., 1984; Kragh et al., 2005]. Moreover, if the peripheral or intramuscular nerve branches were also compromised, it would have an influence on the axonal regeneration and remyelination across the lesion site to re-innervate the distal cut end. The poor recovery can become irreversible with muscle fibers being replaced by noncontractible connective tissue and adipose tissue degeneration, affecting function [Garrett et al., 1984; Irintchev et al., 1990; Gordon and Fu, 1997]. Re-innervation may likely sprout from adjacent muscles, yet, this may take more than 3–4 months and could result in an inappropriate re-innervation which might further complicated or hinder the full return and function of the muscle [Fu and Gordon, 1995; Pereira et al., 2010].

Separately, several studies on muscle recovery after denervation, supports the proposition that the integrity of the peripheral nerve plays an important role in degeneration and recovery in an injured muscle [Fu and Gordon, 1995; Borisov et al., 2001; Carlson, 2008]. Applying this knowledge to lacerated muscles the issue highlights a more complex repair and recovery phase if the intramuscular nerve is involved [Zheng et al., 2004; Lim et al., 2006; Pereira et al., 2006, 2010]. This is because apart from the myogenic regenerative response to repair the cut muscle fibers, the muscle would also require a neurogenic regenerative response. The range of recovery of the muscle mass in a muscle laceration [Garrett et al., 1984; Huard et al., 2002; Kragh et al., 2005] or in a dennervated muscle [Fu and Gordon, 1995; Borisov et al., 2001] over a period of more than 3–4 months is reported to be between 60% and 80%. In simulated muscle models where the lacerated muscle has an intramuscular nerve also cut, the recovery of muscle mass is not more than 80% even up to a period of 7 months, suggesting an irreversible process [Zheng et al., 2004; Lim et al., 2006; Pereira et al., 2006]. The early regenerative response at the lesion site of a lacerated muscle where both the muscle and nerves are damaged has not been completely characterized. It is known that after the initial inflammatory response macrophages, apart from having to activate the satellite cells for myogenic regeneration may have an additional role to ensure neurogenic regeneration are initiated. It is not clear if the damaged intramuscular nerve might influence the inflammatory response and the activation of growth factors associated with satellite cells proliferation, axonal regeneration or remylination, or fibrosis formation at the lesion site. Having knowledge of the key inflammatory responses at the early stages of repair becomes important when trying to provide a rationale to introduce biological solutions to improve muscle repair at the onset of surgical repair.

The study aims at evaluating the inflammatory cytokines and a set of growth and neurotrophic factors in lacerated muscles over 12 weeks. This covers the period where the loss of muscle mass is still reversible with the possibility of re-innervation and myogenic regeneration; to a stage where muscle damage can become irreversible [Pereira et al., 2010]. The study investigates these associations in lacerated muscle models simulated with different intramuscular nerve damage. Clinically in a muscle laceration the zone of injury is the cut lesion, however, there is a possibility that it may also include damaged to the intramuscular nerve branches. We hypothesize that the inflammatory cytokines expressed at the repair site of a lacerated muscle is further influenced by the type of intramuscular nerve damage at the zone of injury.

METHODS

ANIMAL MODEL

The study was approved by the Ethics Committee of the Animal Holding Unit at the National University of Singapore (674/04). The left medial gastrocnemius of the adult Sprague-Dawley (SD) rat ($n = 130$, mean body mass $= 313 \pm 39$ g) was chosen as the lacerated skeletal muscle model. The right limb was used as the control where no surgery was performed [Zheng et al., 2004; Pereira et al., 2006, 2010].

All rats were acclimatized were housed individually in metal cages, kept on a 12:12-h, light–dark cycle at room temperature (28–30°C) and fed with laboratory chow and water ad libitum. The animals were anaesthetized with an intraperitoneal injection of 3:2 Ketamine and Xylazine (0.2 ml/100 g). The lower limb was extended at the hip, knee, and ankle to allow the popliteal fossa to be exposed. A skin incision on the posterior aspect of the mid thigh to about 1-cm proximal to the calcaneum was made. The skin flap was dissected exposing the popliteal fat and the two bellies of the gastrocnemius muscle. The popliteal vein, artery, and the sciatic nerve and branches were isolated, exposing the nerve branches arising from the tibial nerve, to the bellies of the gastrocnemius, and soleus. The nerve to the medial belly of the gastrocnemius was seen passing obliquely to the entry point (motor point) between the proximal quarter and distal three quarters of the belly. The intramuscular nerve branches were visible on the surface under the epimysium as one main intramuscular nerve branch entering deep into the muscles before bifurcating with one set of branches going proximally and one distally.

EXPERIMENTAL GROUPS AND DESIGN

In all groups, the cut ends of the MG were surgically repaired using 4-0 Poly-propylene (Prolene, Ethicon, Somerville, NJ) Kessler core sutures and a continuous epimysial suture. The myofascia was reattached, and the skin closed. The three groups assessed up to 12 weeks post-repair were, PN ($n = 38$): where care was taken to preserve the main IM nerve intact when lacerating the muscle, simulating an innervated lacerated muscle and used as a control for comparison. RN ($n = 38$): where the main IM nerve was crushed with an artery forceps damaging the axons within while preserving the nerve sheath intact. The axonal damage was confirmed by a negative muscle response to electrical stimulation of the peripheral

branch proximal to the crush. This was done to prior to repair the lacerated muscle. This model simulates a re-innervated lacerated muscle; and DN ($n = 38$): where the main IM nerve was concomitantly cut during muscle laceration, simulating a denervated lacerated skeletal muscle. The gastrocnemius muscle of the right limb was used as a normal control, where no surgery was performed. The soleus was used as a control to assess the amount of compensation by the contra-lateral limb, since the rats were likely to function on the non-operated limb during the recovery period.

MUSCLE SAMPLES

At various time points after the animals were euthanized, the MG and the soleus from both limbs were removed and their wet weights measured. The muscle samples were snap frozen in liquid nitrogen, and stored at $-80°C$ to avoid RNA degradation.

SIHLER IN-TOTO STAINING

A pair of muscles from each group was used to demonstrate the intramuscular nerve distribution at 2 weeks, using the in-toto modified Sihler's staining technique, as previously described [Liu et al., 1997; Mu and Sanders, 2010]. Serial transverse 10 μm thick sections were obtained from the lesion site, using a cryostat (Leica, CM 3050 S, Leica Microsystems, Nussloch, Germany) at $-20°C$, and mounted on poly-L-lysine coated slides. Longitudinal sections were stained for haematoxylin and eosin and Masson's Trichrome.

HISTOCHEMISTRY

A portion of each tissue sample was fixed in phosphate-buffered paraformaldehyde at 4°C, dehydrated with graded series of ethanol, infiltrated with xylene, embedded in paraffin blocks, and later sectioned at a thickness of 8 μm. These sections were later dewaxed in xylene and rehydrated in descending grades of ethanol followed by a short 5-min rinse under running water. Serials were stained for hematoxylin and eosin (H&E) and Masson's modified trichrome stains to reveal the muscle architecture and fibrosis components, respectively.

RNA ISOLATION

Total RNA was extracted from tissues using Qiagen RNeasy fibrous tissue Mini kit according to the manufacturer's instructions (Qiagen, San Diego, CA). RNA concentrations were estimated on the basis of absorbance measurement at 260 and 280 nm, using a NanoDrop ND-1000 (NanoDrop, Columbus, OH). The absorbance ratio for all samples ranged between 1.8 and 2.0. The quality of RNA for all samples was confirmed by resolving them on a 1.5% formaldehyde agarose gel.

MICROARRAY ASSAYS

Cytokine gene expression was assayed using a rat common cytokines microarray (Superarray Biosciences, Bethesda, MD). The oligo/microarray blot contained 113 rat cytokine and growth factor genes and includes genes encoding interleukins (IL), interferon (Ifn), bone morphogenic proteins (Bmp), colony stimulating factors (csf), and tumour necrosis factor (Tnf) superfamily. It also included other growth factors such as fibroblast (Fgf), insulin-like (Igf), palatette-derived (Pdgf), transforming (Tgf) and vascular endothelial (Vegf) growth factors, and other cytokines families related to the regeneration process (Table I). Biotin-labelled cRNA probes were synthesized from total RNA using a TrueLabeling-AMP Linear RNA amplification kit (SABiosciences Corp., Frederick, MD). The labeled cRNA probes were hybridized to oligonucleotide fragments spotted on the gene array membranes. Membranes were washed to remove any unincorporated probe and incubated with alkaline phosphatase-conjugated streptavidin (AP-streptavidin). Relative expression levels of specific genes were detected from signals generated by chemiluminescence from the alkaline phosphatase substrate, CDP-Star. The luminizing blots were used to expose X-ray films and quantified by spot densitometry with the aid of GEArray expression analysis suite (SABiosciences Corp.). The abundance of each transcript was normalized to the normal un-operated muscle of the opposite limb, and to the housekeeping gene markers on each array (Aldoa, GAPdH, and BAS2C). The results were presented as log2-fold expression with PN used as a control, to compare the two different types of nerve injuries (RN and DN). Genes that were found to have a log2-fold expression greater than 1 (i.e., 2^1) were considered up-regulated, while genes with a log2-fold expression of -1 (i.e., 2^{-1}) were considered down-regulated relative to PN. The MIAME compliant data were curate in Miamexpress with the ArrayExpress accession: E-MTAB-755.

REAL TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ASSAYS

Reverse transcription was performed using 5 μg of total RNA with the Reaction Ready™ First Strand cDNA Synthesis Kit (SABios-

TABLE I. List of 112 Cytokines and Growth Factor Genes on the Oligo GEArray® Rat Common Cytokines Microarray (ORN-021, Superarray Bioscience)

Family	Genes
Interferons (Ifn)	Ifn-α1, Ifn-β1, Ifn-γ, Ifn-κ
Interleukins (IL)	IL1α, IL1β, IL1f10, IL1f5, IL1f6, IL1f8, IL1rn, IL2,IL3, IL4, IL5, IL6, IL7, IL9 IL10, IL11, IL12α, IL12β, IL13, IL15, IL16, IL17, IL17β, IL17f, IL18, IL19, IL21, IL24
Tumor necrosis factor (Tnf) superfamily	Tnf, Tnf-rsf11β, Tnf-sf4, Tnf-sf6, Tnf-sf9, Tnf-sf10, Tnf-sf11, Tnf-sf12, Tnf-sf13, Tnf-sf14, Tnf-sf15, Tnf-sf18
	CD70, LOC84349, Lta, Ltb
Bone morphogenic proteins (Bmp), growth development factors (Gdf), and transforming Growth factor (Tgf) family	Bmp1, Bmp2, Bmp3, Bmp4, Bmp5, Bmp6, Bmp7, Bmp15
	Gdf1, Gdf2, Gdf6, myostatin (Gdf8), Gdf9, Gdf10, Gdf11, Gdf15
	Inh α, Inh βa, Inh βb, Nodal,
	Tgf-α, Tgf-β1, Tgf-β2, Tgf- β3
Platelet-derived growth factors (PDgf) and vascular endothelial growth factors (VEGF) family	Figf, PdgfA, PdgfB, PdgfC, Vegf, VegfB
Other growth factors and cytokines	Areg, Artn, Cntf, Csf1, Csf2, Csf3, Ctf1, Dlk1, Ebaf2, Epo, Fgf10, Gas6, Grn, Hgf, Igf1, Igf2, Ik, Kitl, Lif, Mif, Mst1, Nrg1, Oit1, Osm, Pbef, Pgf, Prl, Ptn, S100a11, Scgf, Scye1, Sparc, Spp1, Thpo, Tyro3

ciences Corp.) according to the manufacturer's instructions. PCR amplification and detection of the template were carried out using gene-specific primer sets for rats for Igf-1, Igf-2, hepatocyte growth factor (Hgf), transforming growth factor beta 1 (Tgf-β1); Tgf-β2, nerve growth factor (Ngf), leukemia inhibitory factor (Lif), tumor necrosis factor (Tnf); brain-derived neurotrophin factor (Bdnf), neurotrophin-3 (NT-3); and stem cell growth factor (Scgf) for time points 1 day, 3 days, 1 week, 2 weeks, 4 weeks, 8 weeks and 12 weeks (all primers were supplied by SuperArray Bioscience). The Rat GAPD Internal normalizer was used as an endogenous invariant control (housekeeping gene). RT-PCR was performed using an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA) and the ReactionReady Hotstart Sweet PCR mix (SABiosciences Corp) with the polymerase chain reaction products were separated in a 2% agarose gel.

COMPUTERIZED GEL DENSITOMETRY

An AlphaDigiDoc RT Gel Documentation System (Alpha Innotech Corporation, San Leandro) was used to photograph the gels; and a standard densitometer program (Gel-Pro μAnalyzer version 4.5; MediaCybernetics, MD) was used to assess concentrations of the bands obtained by PCR. These were measured as total density units.

STATISTICAL ANALYSIS

The mean body mass of the animals from the groups was compared before the operation; and at the various assessment time points. The wet mass of the muscles of the operated limb was compared to that of the contra-lateral limb to obtain a value representing a percentage of the control. The paired Student's *t*-test was used to compare between the left and right sides, to determine any significance between their means. A two-way analysis of variance with the Bonferroni post-hoc multiple range test was applied to determine differences between groups for the gene and mRNA expressions based on triplicate data sets. A value of $P < 0.05$ was considered statistically significant.

RESULTS

GROSS ANATOMY

The study simulated three groups with different intramuscular nerve damage at the site of the lesion. The Sihler's in-toto staining demonstrated the extent of damage to the main intramuscular nerve at 2 weeks with retrograde damage of the intramuscular nerve branch to the peripheral branch supplied by the tibial nerve (Fig. 1). The myelinated tissue stained by Sihler's confirmed that the nerve sheath at the lesion site remained intact for RN and PN at 2 weeks. Only the PN showed the presence of intramuscular branches extending distally beyond the lesion site. Gross muscle mass changes were recorded at 2 weeks and up to 12 weeks (Fig. 2). Muscle atrophy was noted mainly in the distal segment of the muscle. At the site of the lesion, the Masson's Trichrome staining in longitudinal paraffin embedded sections, demonstrated that DN had a poorer recovery with a discontinuity at the cut ends noted at 2 weeks. The RN muscles showed that regeneration had begun with formation of a fibrotic region and some myogenic regeneration across the lesion, while PN muscles demonstrated more myogenic regeneration across the lesion site, with a reduced fibrotic region. By 12 weeks the difference between the three groups was less noticeable in terms of the presence of mononucleated cells, yet PN demonstrated a markedly reduced fibrotic region and increased in the number of mature and uniform-sized myofibers across the cut region. The mass of the medial gastrocnemius calculated as a percentage normal contra-lateral muscle showed significant changes after repair over the 12-week period. One week after repair, a similar rapid loss of muscle mass was recorded in all groups. Muscle mass improved for PN by 4 weeks, with RN and DN continuing to lose muscle mass ($P < 0.05$). At 2 weeks and 4 weeks, significant differences were noted in all groups (ANOVA, $P < 0.05$). By 8 weeks and 12 weeks, only DN showed a poor and slower return of mass recording only 48%, while RN and PN reached 80% of normal muscle mass.

LOG2-FOLD GENE EXPRESSION PROFILES BETWEEN DN, RN, AND PN MUSCLES

The tissue level gene expression of cytokines was evaluated at 2, 4, 8, and 12 weeks. Given that PN only involved a muscle laceration with the intramuscular nerve preserved, it was taken as a control for comparing the two types of intramuscular nerve injuries, RN against DN as the rationale was to observe the influence of the type of nerve injury to the release of the inflammatory cytokines and growth factors. A heat map of the gene expression of cytokines and growth factors based on triplicate data first compared PN against the sham contra-lateral limb summarizes the triplicate data to assess the influence of an innervated/lacerated muscle on the cytokine profile (Fig. 3A). Most of the cytokines in PN were up-regulated by twofold or more at 2 weeks and subsequently down-regulated gradually at 4- and 8 weeks. At 12 weeks post-repair, some up-regulated neurotrophic-related and myotrophic-related growth factors were noted suggesting re-innervation and improved muscle regeneration were taking place correlating well with the return on muscle mass. DN was compared to RN to assess the differential inflammatory cytokine profile between the two types of nerve injuries in lacerated muscles, normalized against PN to exclude the influence of the muscle laceration. The heat map divided into the cytokines protein families and growth factors (Fig. 3B) and the log2-fold gene expression, demonstrating a >2-fold difference, was used to compare between RN and DN at 2, 4, and 8 weeks post-repair (Tables IIA and B). The control genes, Aldolase (Aldoa), Glyceraldehyde-3-phosphate dehydrogenease (GapdH), and Biotinylated Artificial sequence 2 Complementary sequence (BAS2C) registered log2-fold expressions between −0.01 and −0.02 for all time points.

INFLAMMATORY CYTOKINES ASSOCIATED TO THE TYPE OF NERVE INJURY (DN AND RN)

At 2 weeks, DN and RN commonly expressed IL-1f5 and IL-1f10 (Fig. 4a). IL-1f5 gene remained up-regulated in RN up to 8 weeks while in DN it was down-regulated at 4- and 8 weeks. IL-1f10 remained up-regulated in DN (at 4- and 8 weeks), but was down-regulated for RN at 4 weeks then up-regulated at 8 weeks. DN also

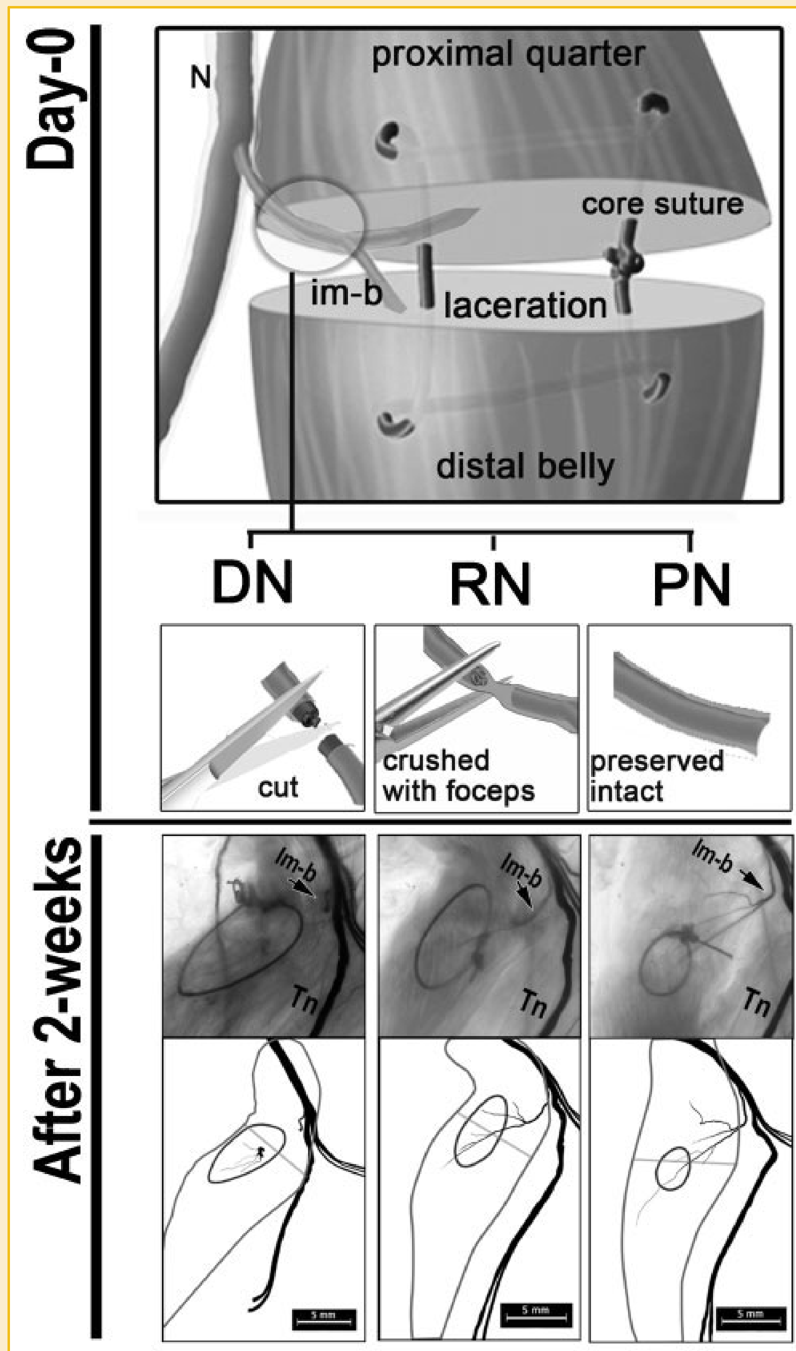

Fig. 1. The experimental lacerated skeletal muscle models. A transverse through-thickness laceration was simulated at the proximal quarter of the muscle belly just below the entry of the peripheral nerve branch supplied by the tibial nerve (N). The peripheral nerve branch enters the muscle at the epimysium and becomes the main intramuscular nerve branch (im-b). The three lacerated skeletal muscle injuries simulated were DN, a denervated/lacerated skeletal muscle, where the im-b was also cut, RN, a re-innervated/ lacerated skeletal muscle group, where the im-b was crushed with the foceps damaging the axons while leaving the epineurium intact; and PN, where the im-b was preserved intact in an innervated/lacerated skeletal muscle. All muscle belly lacerations were repaired with core sutures. At 2 weeks post-repair, the Sihler's intoto stains demonstrated the different intramuscular nerve distribution and the extent of neural damaged among the three models. The schematics outline the extent of the mylinated intramuscular nerve branches in this set of muscles. (Note: Tn = Tibial Nerve)

showed an up-regulation of IL-1β (2–8 weeks, Fig. 4b), IL-18 (at 2–4 weeks) and Tnfsf13 (2–4 weeks, Fig. 4c) with no relative expression noted in RN.

At 4 weeks, DN had the most number of up-regulated cytokines genes including Ifn-β1 (Fig. 4d), Ifn-κ (Fig. 4e), Ifn-γ, IL-1rn, IL-4, IL-10, IL-12α, IL-12β, IL-13, Tnfs11β, Tnfsf13, and CD70. Of these, Ifn-β1, IL-4, IL-12α, and IL-13 were down-regulated in RN. RN also had IL-1f6, IL-2, IL-5, IL-6 (Fig. 4f), IL-11, IL-17β, IL-17f, IL-19, IL-24, Tnf-sf5, Tnf-sf6, and Tnf-sf14 down-regulated, while these not demonstrated in DN.

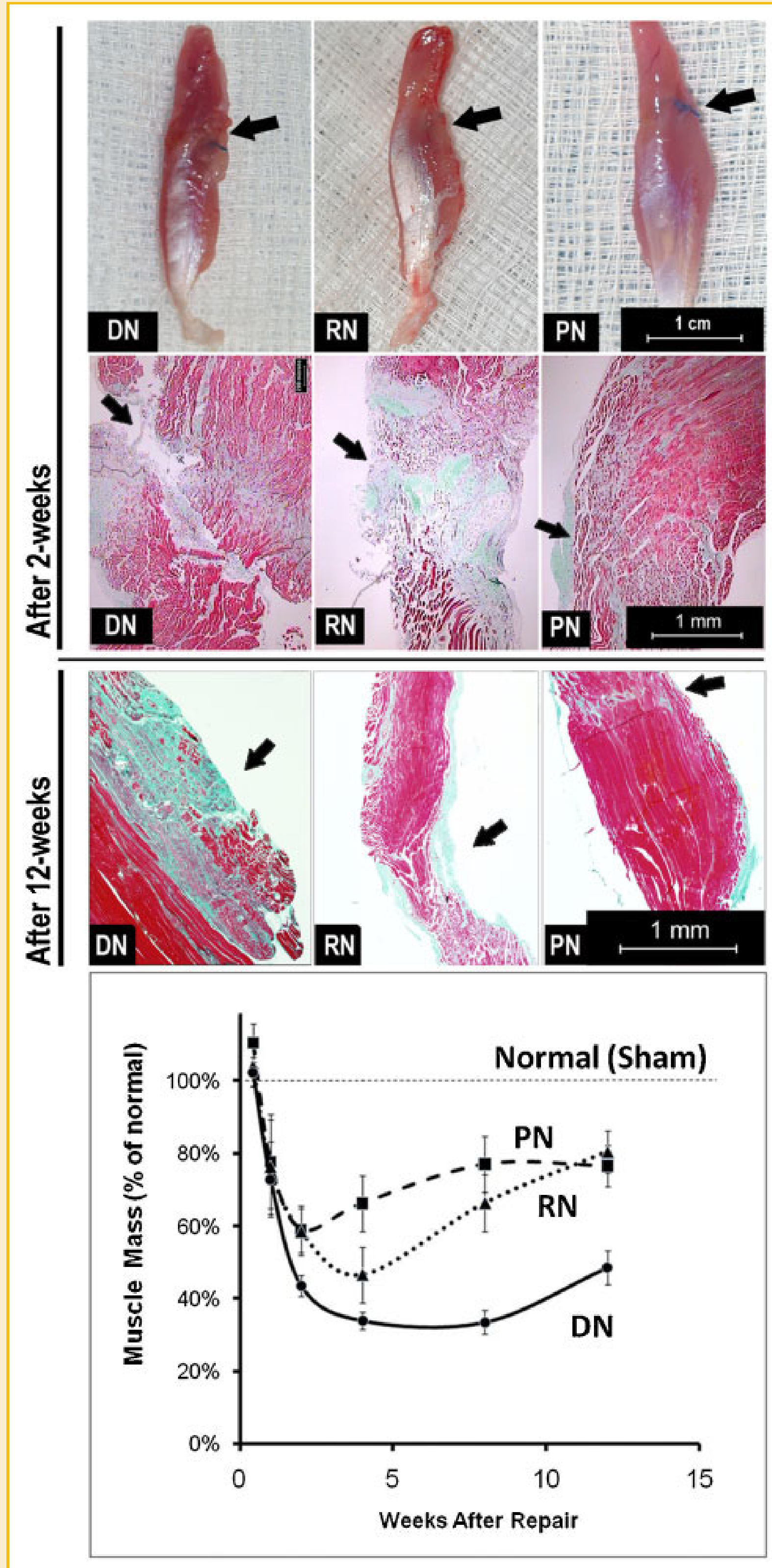

Fig. 2. At 2 weeks after repair, all muscles had a significant reduced muscle mass. Masson's Trichrome staining at the lesion site (arrows) showed the repair site at 2 weeks with DN having a discontinuity, while RN and PN having its cuts ends bridged differentially by connective and muscle tissue. At 12-weeks the cut lesion was less visible, yet DN still continued to have a poor repair (longitudinal section, original magnification 100×).

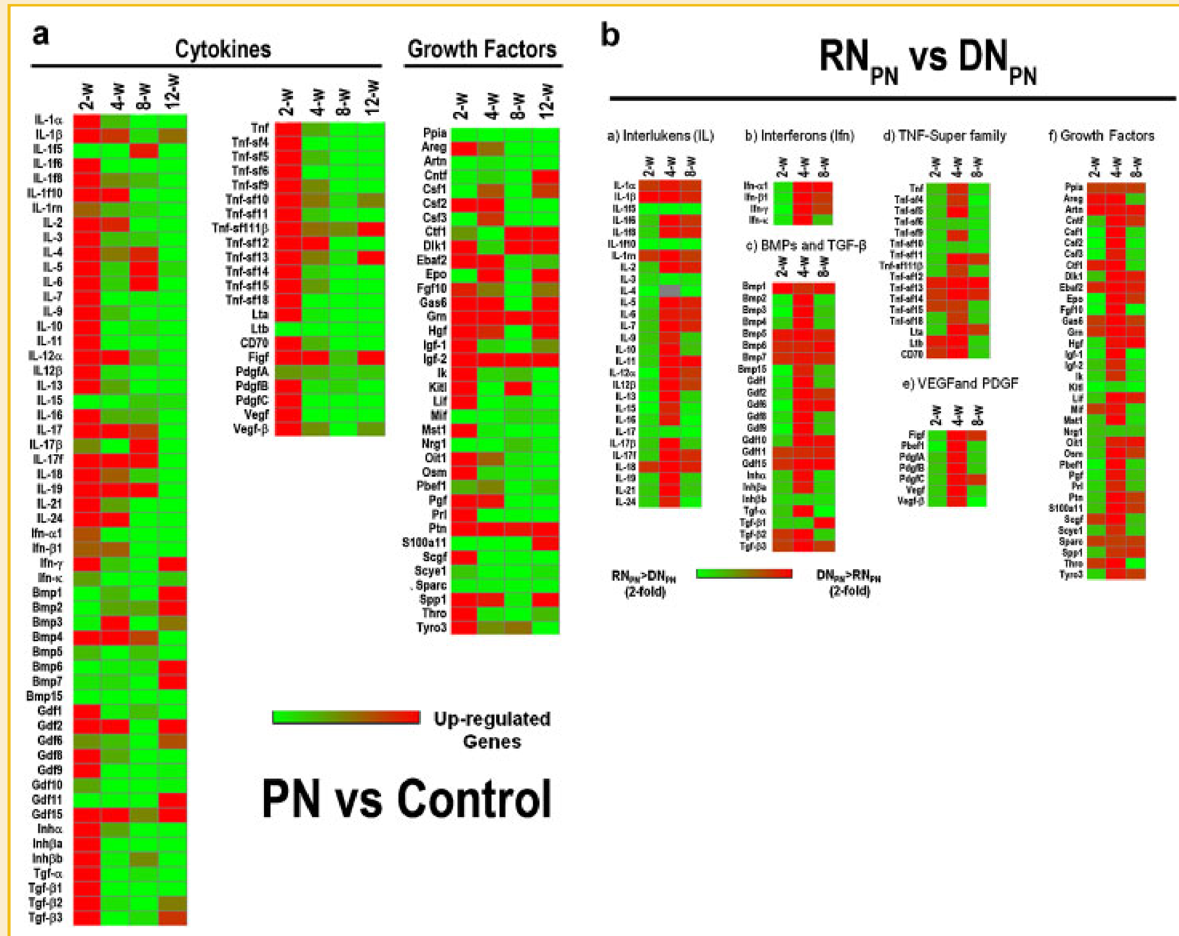

Fig. 3. a: Heat map of cytokines and growth factors (ORN021 Oligo GE Assay, Superarray Biosciences) of PN at 2,4, 8, and 12 weeks. The red (dark) boxes indicate genes with >2-fold expression levels and the green (light) boxes indicate the normal expression levels (>2-fold expression) of PN compared to the contra-lateral sham muscle. b: Heat map of cytokines and growth factors, comparing DN, and RN normalized to PN at 2, 4, and 8 weeks post-repair. The red (dark) boxes indicate highly expressed genes in DN to RN (>2-fold), while a green (light) boxes indicates an upregulated expression of genes in RN to DN (>2-fold).

At 8 weeks the common up-regulated genes for RN and DN were IL-1α (Fig. 4g), Ifn-κ, and IL-21 (Fig. 4h); while IL-6, IL-17β, and Ltb (Fig. 4i) were the common down-regulated genes. In the case of PN, IL-21 was noted to be down-regulated, and hence the comparative data for RN and DN were shown to be highly expressed with respect to PN.

PRO-FIBROTIC FACTORS

The transforming growth factor family, Tgf-β1, Tgf-β2, and Tgf-β3, which is associated with myofibrobalst and fibrosis formation, had significantly different gene expression levels at the lesion site between RN and DN Fig. 5a–c). Tgf-β1 was shown to be significantly raised only at 2 weeks for RN and DN, while Tgf-β1 (at 8 weeks), Tgf-β2 (at 4 weeks), and Tgf-β3 (at 4 weeks) were significantly raised for DN ($P < 0.05$). Similar to the gene-array data, the mRNA expression levels of Tgf-β1 (at 2–4 weeks), Tgf-β2 (at 2–4 weeks), Hgf (3 days), and Scgf (1 and 4 weeks) were significantly up in DN compared to RN and PN, while IGF-1 was not found to be significantly different (Fig. 5d–i).

GROWTH FACTORS ASSOCIATED TO THE TYPE OF NERVE INJURY (DN AND RN)

At 2 weeks, similar high log2 gene expression levels of Bmp1, Gdf9, Gas6 (Fig. 6a), Grn (Fig. 6b), Ik, and cardiotrophin-1 (Ctf1) and down-regulation of Bmp15, colony forming factors 1 (Csf1), Csf2, Fgf10 (Fig. 6c), and Areg in both RN and DN suggest a similar response. Yet RN specifically had Igf-1, Kit ligand (Kitl), a stem cell factor; and Csf3 up-regulated with Artemin (Fig. 6d), a gene associated to neural repair, down-regulated, that was not demonstrated in DN at 2 weeks. DN on the other hand had Ebaf2 gene expressions elevated and Epo (Fig. 6e), down-regulated at 2 weeks.

By 4 weeks, DN had several Bmps (Bmp1, Bmp2, Bmp3, Bmp4, Bmp5, and Bmp15) and Gdfs (Gdf1, Gdf2, Gdf6 (Fig. 6f), Gdf8 (myostatin, Fig. 6g), Gdf9, and Gdf15) up-regulated by >2-fold. While only Bmp1, Bmp3, and Bmp15 were expressed in RN. High expression levels were seen in DN for Bmp3 (2^3-fold expression), Bmp4 (2^3-fold expression), and Gdf8 or myostatin (2^2-fold expression). Common genes up-regulated in DN and RN were Artn, Csf2, and Areg; while DN had higher expression of Dlk1,

TABLE IIA. Log2-Fold Expression Levels of Up-Regulated ($\geq 2^1$) and Down-Regulated ($\leq 2^{-1}$) Inflammatory Cytokines After the Acute Repair Phase (2-, 4-, and 8-weeks) in DN and RN Relative to PN

	Log2-fold expression levels					
	Reinnervated model (RN/PN)			Dennervated model (DN/PN)		
	2-weeks	4-weeks	8-weeks	2-weeks	4-weeks	8-weeks
IL-1α			1.5			1.7
IL-1β				2.3	1.4	1.2
IL-1f5	4.2	1.8	1.6	1.9	−1.1	−1.0
IL-1f6		−1.7				
IL-1f10	3.4		2.4	2.1	−2.3	1.2
IL-1rn					4.0	
IL-2		−1.0				1.3
IL-3			1.2		−1.1	
IL-4	2.2	−5.5	1.3		>5.0	
IL-5		−2.3		−1.2		
IL-6		−1.1	−1.7			−1.2
IL-10					1.1	
IL-11		−1.0	−1.3			
IL-12α		−1.0		−2.5	2.3	
IL-12β					2.3	
IL-13		−1.1		−1.1	1.7	
IL-15		1.5			2.6	−1.0
IL-17			1.1			
IL-17β		−1.1	−1.4			−1.8
IL-17f		−1.4				
IL-18				1.0	2.0	
IL-19		−1.3				
IL-21			1.3			1.1
IL-24		−1.4	1.3			
Tnfsf5		−1.5				
Tnfsf6		−1.6				
Tnfsf9		−1.1			−1.3	
Tnfrsf11β					1.6	
Tnfsf13				1.4	2.9	
Tnfsf14		−1.3				
Ifn-α1			−1.3	−1.3		
Ifn-κ			1.1		1.9	1.1
Ifn-γ				−1.4	5.1	
Ifn-β1		−1.1		−1.5	1.0	
CD70	−1.5			−1.2	1.7	
Lta		−1.2				
Ltb		−3.2	−1.1		−1.2	−1.4

Fgf10, Ctf1, Cntf (Fig. 6h), Grn, Spp1, Gas6, and Igf1. RN conversely had a down-regulation of Tgfα, Figf, Osm, Lif (Fig. 6i), Tyro, Prl, Hgf, Thpo, and Epo.

By 8 weeks, the number of genes up-regulated (5:6) and down-regulated (6:4) genes between DN and RN were reduced. Some common up-regulated (Gdf2, Inhβa, and Prl) and down-regulated (Scgf and Areg) genes were noted. The genes specific to RN (Inhβa and Mstl up-regulated; Bmp1, and Hgf down-regulated) and DN (Gdf6, Tgf-β1, and PdgfC up-regulated; and Tgf-α down-regulated) were few. At 8 weeks, RN demonstrated a similar profile to PN, suggesting that re-innervation may have been established and a more improve muscle regeneration, correlating well with the recovery of muscle mass, and with previously reported data from our group [Zheng et al., 2004; Pereira et al., 2006].

RELATIVE mRNA EXPRESSION OF GROWTH FACTORS RELATED TO MYOGENIC AND NEUROGENIC RECOVERY

In addition, we assessed the mRNA levels of a set of neurotrophic factors—Lukemia inhibitory factor (Lif, Fig. 7a); nerve growth factor (Ngf, Fig. 7b), neurotrophin-3 (NT-3, Fig. 7c), and brain-derived neurotrophin factor (Bdnf, Fig. 7d), which are retrogradely transported from a peripheral organ after the onset of a peripheral nerve damage or injury [DiStefano et al., 1992]. We noted that Lif and Ngf mRNA, two Schwann cell derived factors released to initiate axon regeneration and neural cell survival; had elevated expressions at the lesion site in DN with peaks at 3 days post-repair for Lif and at the 8 weeks for Ngf, which remaining elevated at 12 weeks The similar expression levels for Lif and Ngf at the acute repair phase in all groups suggests a similar degree of axonal damaged during this period. For PN, the Ngf expression gradually returned to a normal level by 4 weeks, while RN showed fluctuations between 2 and 12 weeks. The expression of NT-3, a neurotrophin required for the differentiation and survival of sympathetic, sensory, and motor

TABLE IIB. Log2-Fold Expression Levels of Up-Regulated ($\geq 2^1$) and Down-Regulated ($\leq 2^{-1}$) Growth Factors After the Acute Repair Phase (2-, 4-, and 8-weeks) in DN and RN Relative to PN

	Log2-fold expression levels					
	Reinnervated model (RN/PN)			Dennervated model (DN/PN)		
	2-weeks	4-weeks	8-weeks	2-weeks	4-weeks	8-weeks
Areg	−2.1	1.0	−1.3	−1.2	2.8	−1.3
Artn	−5.8	1.0	−1.1		1.8	
Bmp1	4.0	1.1	−1.0	>4.0	1.4	
Bmp2					1.4	
Bmp3		1.7	−1.4		2.7	−1.6
Bmp4					2.6	
Bmp5					1.1	
Bmp15	−1.2	1.1		−2.8	1.7	
Cntf					1.3	
Csf1	−2.2			−3.8		
Csf 2	−1.6	1.5		−2.9	3.1	
Csf3	1.7					
Ctf1	1.4			1.2	2.1	
Dlk1					1.4	
Ebaf2				1.0		
Epo		−1.0		−2.5		
Fgf10	−1.4			−2.5	1.4	−2.0
Figf		−1.2				
Gas6	1.3			1.3	1.2	
Gdf1					1.6	
Gdf2			1.4		1.2	1.6
Gdf6				−1.0	1.0	1.3
Gdf8					2.0	
Gdf9	2.0			1.3	3.3	
Gdf15					1.2	
Grn	2.5			2.9	1.7	
Hgf		−1.1	−1.6			
Igf1	1.1				1.4	
Ik	1.6			1.3		
Inhba			1.3			
Inhba			1.4			1.1
Kitl	1.9					
Lif		−1.5				
Mstl			1.2			
Osm		−1.6				
Pdgfc	1.3					1.2
Prl		−1.1	1.2			1.1
Scgf			−1.1			−1.0
Spp1					1.1	
Tgfα		−2.6				−1.6
Tgfβ1	1.0					1.1
Tgfβ2					2.8	
Tgfβ3					2.6	
Thpo		−1.0				
Tyro3		−1.2				

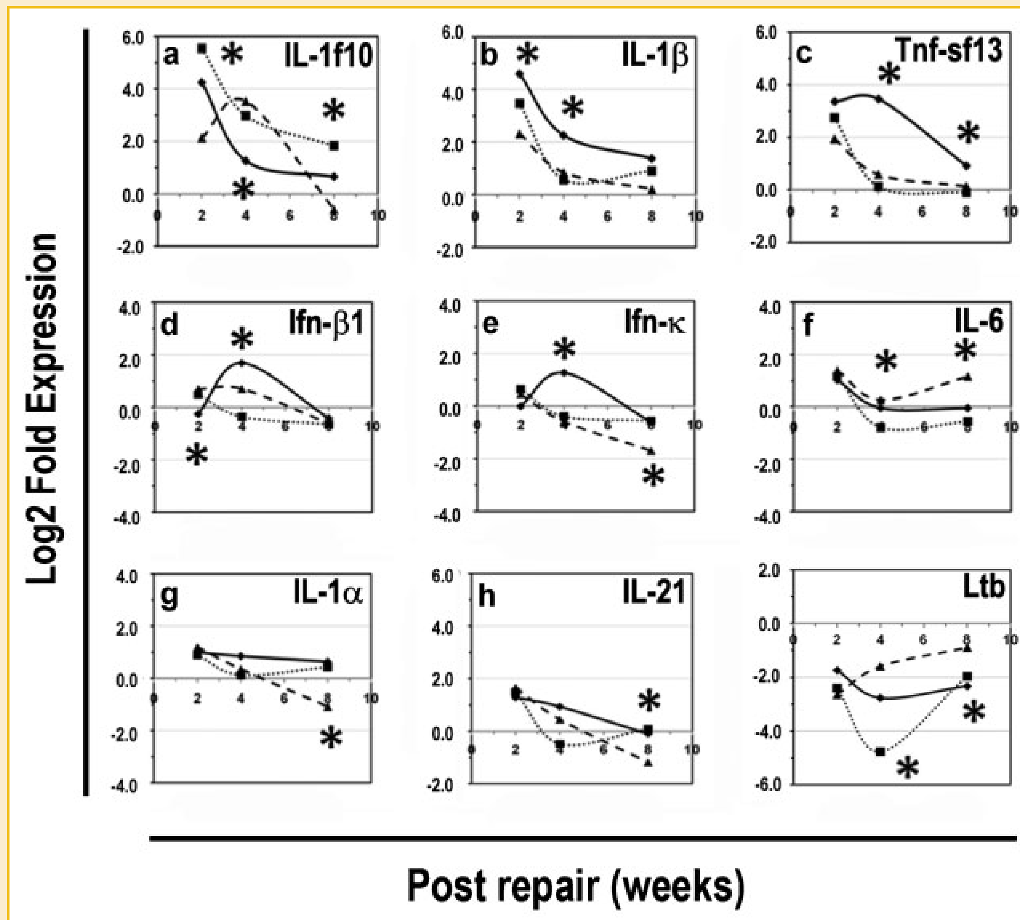

Fig. 4. Inflammatory cytokines. Relative log2-fold gene expression levels of (a) IL-1f10, (b) IL-1β, (c) Tnf-sf13, (d) Ifn-β1, (e) Ifn-κ, (f) IL-6, (g) IL-1α, (h) IL-21, and (i) Ltb at 2-, 4-, and 8-weeks. The data compares DN (bold line), RN (dotted line), and PN (dashed line) relative to the contra-lateral non-operated muscle. Note: * significant difference of at least 1-pair at each time point (ANOVA, $P < 0.05$).

neurons, was higher at the lesion site in DN muscles during the 1st week continuing to be raised up to 4 weeks. NT-3 mRNA expression for RN and PN on the other hand remained at nominal levels (ANOVA, $P < 0.05$). Bdnf mRNA was up-regulated in DN within the 1–3 day period and at 8 weeks ($P < 0.05$), compared to RN and PN. This would suggest that DN had a greater extent of axonal damage and denervation with greater requirements to repair and re-innervate across the lesion site. Compared to DN, RN had its nerve sheath conduit preserved allowing axonal regeneration and re-innervation through it as one possibility, while PN had its intramuscular nerve preserved and hence did not require as much re-innervation across the lesion site to the distal muscle belly.

DISCUSSION

Lacerated skeletal muscles with the main intramuscular nerve cut or involved, are partially denervated lacerated skeletal muscles and require a coordinated neuromuscular regenerative response during the repair phase. These severe muscle injury types are likely to result in irreversible muscle atrophy with muscle mass and function not fully returned [Garrett et al., 1984; Huard et al., 2002; Kragh et al., 2005].

Using microarray analysis with a selection of candidate genes confirmed by q-PCR, we showed a differential expression of several inflammatory cytokines and growth factors at the lesion site after the acute repair phase. These differential expressions suggest an association to the type of intramuscular nerve injury and their integrity in surgically repaired lacerated muscles. The key issues in the surgical repair of lacerated muscles are to prevent the formation of fibrosis at the lesion site, muscle atrophy, poor or inappropriate re-innervation and muscle degeneration [Garrett et al., 1984; Menetrey et al., 2000; Huard et al., 2002; Kragh et al., 2005; Tidball, 2005]. Skeletal muscles are innervated by peripheral nerves that enter the muscle belly at the so-called "motor-point," and form an orderly network of intramuscular nerve branches within the muscle belly [English et al., 1993; Liu et al., 1997; Lim et al., 2004]. In a muscle laceration these intramuscular nerve branches may also be severed resulting in sections of muscle belly being partially denervated. Our results showed differential changes to the muscle mass with poor return for DN muscles (Fig. 2). This group has its intramuscular nerve severed in our simulated model, and therefore

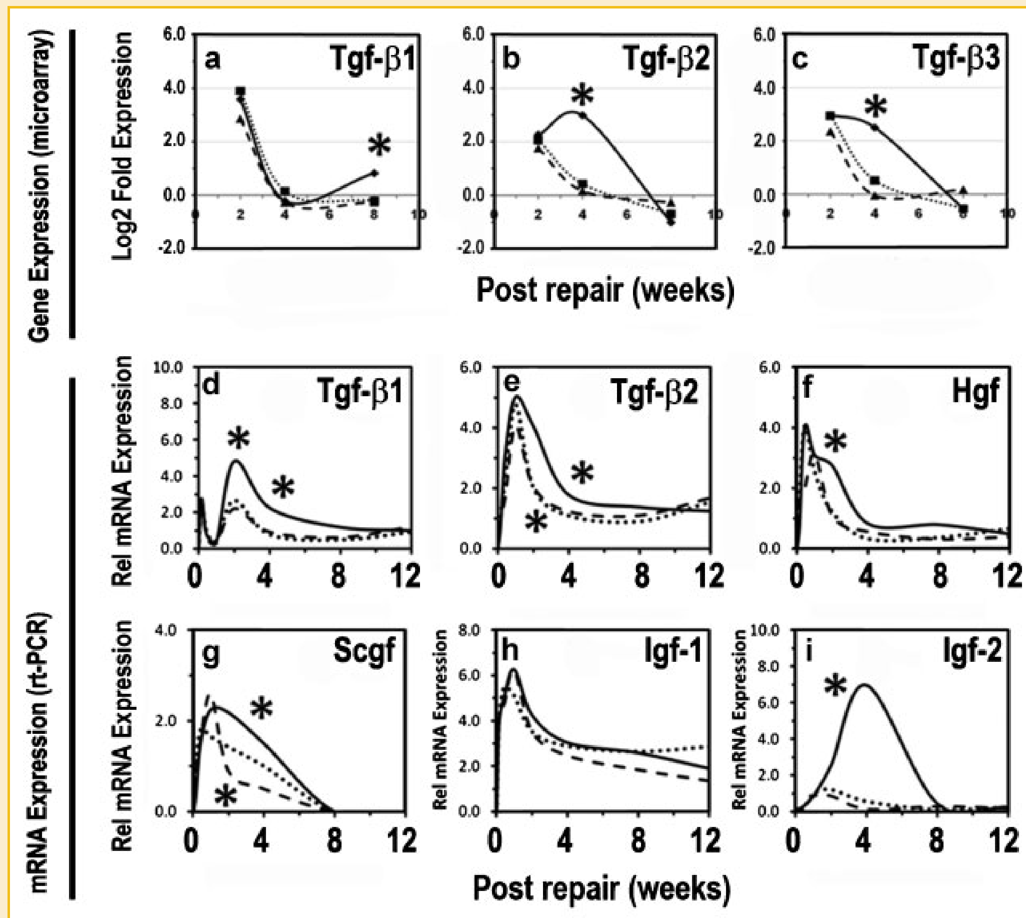

Fig. 5. Pro-fibrotic markers and growth factors. Relative log2-fold gene expression of set of the profibrotic genes (a) Tgf-β1, (b) Tgf-β2, (c) Tgf-β3 complimented with the relative mRNA expression levels from the q-PCR assays for same profibrotic markers, (d) Tgf-β1, (e) Tgf-β2 and the myogenic growth factors, (f) Hgf, (g) Scgf, (h) Igf-1, and (i) Igf-2. The data compares DN (bold line), RN (dotted line), and PN (dashed line) relative to the contra-lateral non-operated muscle. Note: * significant difference of at least 1-pair at each time point (ANOVA, $P < 0.05$).

strongly suggests that the poor results are associated to the absences of the intramuscular nerve. The gross and histological appearances at the lesion site and the changes to the intramuscular motor nerves within the muscle demonstrated by the Sihler's in-toto staining at 2-weeks highlight the differences among the 3 models (Fig. 2). The results are in-line with previous reports that showed that even up to 7 months; surgically repaired lacerated muscles that had their intramuscular nerve severed at the onset remain denervated, or poorly re-innervated [Pereira et al., 2010]. These 7 months post-operative muscles also demonstrated muscle atrophy, a persistent fibrotic region at the lesion site with poor histological results [Pereira et al., 2006]. The inappropriate re-innervation or denervation was associated to an alteration of muscle fiber typing and myosin heavy chain (MHC) profiles distal to the lacerated site leading to poor functional recovery. A similar study showed that microanastomosis of the intramuscular nerve might prove to have a better return of form and function, however, this may be technically challenging [Lim et al., 2006].

Several studies support the proposition that re-innervation of the peripheral nerves has a role in influencing the recovery of the denervated muscle [Irintchev et al., 1990; Fu and Gordon, 1995; Gordon and Fu, 1997; Carlson, 2008]. These data encourages a need to re-innervate the muscle at the early repair phase before irreversible damage is done. Most lacerated muscles are repaired only surgically [Kragh et al., 2005], there is therefore some interest in investigating the molecular mechanism and the inflammatory changes after a lacerated muscle injury to avoid an irreversible outcome post-operatively [Tidball, 2005; Pereira et al., 2010]. Several gene expression studies have looked at various muscle injuries and how certain genes may be key to regulating an improved clinical result [Hirata et al., 2003; Zhou et al., 2006; Warren et al., 2007; Zeman et al., 2009]. So far none have looked at lacerated muscles and how the integrity of the intramuscular nerve might play a role in altering the repair and recovery of lacerated skeletal muscles.

RELEASE OF INFLAMMATORY CYTOKINES IN LACERATED MUSCLE MODELS

When pro-inflammatory cytokines remain up-regulated after 1-week, there is concern that the skeletal muscle regeneration may not be

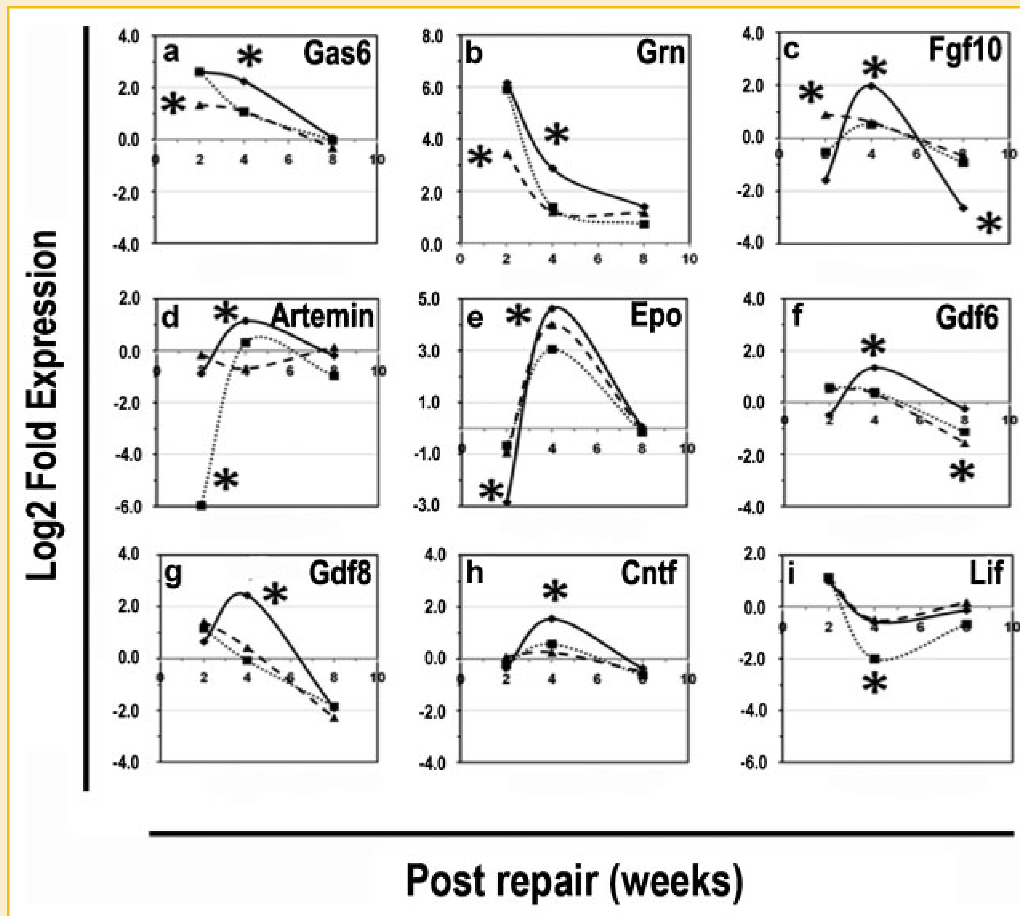

Fig. 6. Inflammatory growth factors. Relative log2-fold gene expression levels of (a) Gas-6, (b) Grn, (c) Fgf10, (d) Artemin, (e) Epo, (f) Gdf6, (g) Gdf8 (or myostatin), (h) Cntf, and (i) Lif at 2, 4, and 8 weeks. The data compares DN (bold line), RN (dotted line), and PN (dashed line) relative to the contra-lateral non-operated muscle. Note: * significant difference of at least 1-pair at each time point (ANOVA, $P < 0.05$).

progressing as planned [Ciciliot and Schiaffino, 2010]. For the DN muscle IL-1β remained up-regulated from 2 weeks to 8 weeks, while IL-18 and the tumour necrosis factor superfamily (Tnf-sf) ligands Tnf-sf11 and Tnf-sf13 remained up-regulated at 2- and 4 weeks. Pro-fibrotic cytokines produced by the T-lymphocytes Th1 cells, IL-12α, and IL-12β were also up at 4- and 8 weeks. In addition, the anti-inflammatory cytokines, IL-10 and IL-13 had >2-fold expression levels at 4 weeks. These 2 cytokines down-regulate Tnf-α, IL-1β, IL-8, and the pro-inflammatory mediator, Ifn-γ, which is known to further promote pro-inflammatory cytokines IL-2, IL-12, and IL-18. The imbalance in regulation of the anti-inflammatory cytokines, IL-10 and IL-13; and the pro-inflammatory markers with very high levels of expression, Ifn-γ (2^5-fold expression over RN), IL-12α ($>2^2$-fold expression), IL-12β ($>2^2$-fold expression), IL1β, and IL-18 ($>2^2$-fold expression); could have worked towards stimulating a continued repair phase, because at 8 weeks pro-inflammatory markers IL-1α, Il-1β were still raised, thus supporting a more pro-fibrotic pathway in DN.

A different situation was seen for RN, where at 2 weeks, the anti-inflammatory cytokine, IL-4 was already up, and its purpose is to down-regulate pro-inflammatory cytokines Tnf-α, IL-1, and IL-8 cytokines. The anti-inflammatory cytokines IL-6 and IL-18 for RN were also down-regulated at 4 weeks, with IL-6 continuing to be down-regulated at 12 weeks. Interestingly, IL-6 was up-regulated in PN at 8 weeks, significantly expressed compared to RN at 4 weeks. The mRNA levels of IL-6 were noted to be low in normal muscles and were up-regulated in response to muscle injury [Kami et al., 2000]. While IL-6 expressions have been induced in models of skeletal muscle growth, functional overloads and recovery disuse [Carson et al., 2002], the lack of IL-6 expression at the early stages of repair for RN and DN is not clear. We can only postulate that the elevated gene expression of IL-6 in PN might be playing another role in myofiber regeneration, extracellular regeneration or the survival and sprouting of the motor neurons to ensure synaptic connections at the neuromuscular junction of regenerating moyfibers. Whereas the down-regulation of IL-6 in RN and DN muscles could be an inhibition by some other cytokines or growth factors in response to the type of nerve injury [Kushima and Hatanaka, 1992]. The inflammatory processes particularly if it is prolonged after 2 weeks post-operatively can either disrupt or promote muscle repair and regeneration and result in severe fibrosis formation at the lesion site. The releases and invasion of inflammatory cells at the site of injury to remove the debris can also be influenced by the type and extent of the injury.

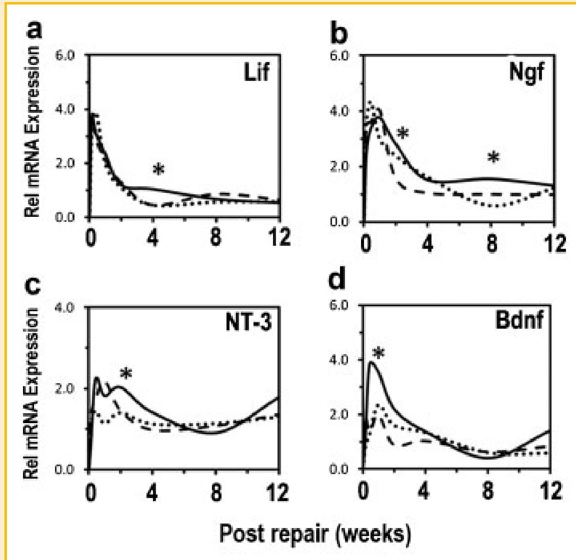

Fig. 7. Relative mRNA expression neurtrophins (a) Lif, (b) Ngf, (c) NT-3 and (d) Bdnf associated to the type of intramuscular nerve injury over a 12-week repair period as quantified by reverse-transcribed RNA PCR. Data points for this assay were taken after 1 day, 3 days, 1 week, 2, 4, 8, and 12 weeks (DN, bold line; RN, dotted line; and PN, dashed line).

The lacerated muscle model is of course a severe injury model, and in our model the laceration was a through-thickness cut and therefore would take a longer time to remodel the lesion site. The difference between different type of intramuscular nerve damage in DN and RN seems to influence the differential inflammatory cytokine profile as well as a difference in the repair process at the lesion site. In RN the nerve damage might be less severe, as axonal degeneration and regeneration is guided within the nerve sheath conduit, and therefore may evoke a different cytokine profile. The RN muscle is denervated, but recovery as seen in the return of muscle mass was far better compared to DN muscles. Therefore cytokines specifically up-regulated or down-regulated in only DN or RN suggest a possible association to the type of nerve injury. The time course differences of some cytokines seen between RN and DN demonstrates a delayed response to repair and may be associated to the difference in the extent of damage seen in DN and RN. While cytokines that are commonly expressed at the same time points for both RN and DN, these may be associated to a common injury pattern for RN and DN.

REGULATORS OF MYOGENIC REPAIR, A BALANCE BETWEEN Tgf-β AND Igf

Apart from the direct inflammatory cytokine response from the macrophages and monocytes, other genes like the bone morphogenic proteins (Bmps), growth development factors (Gdf) family and Tgf-β family and Igf-1 and Igf-2 genes are known to have an influence on myogenic, fibroblastic and neurogenic responses to muscle repair. The families of Tgf-β genes, which are pro-fibroblastic factors, were all raised in DN at various time points. The mRNA data also showed DN having elevated expression levels of Tgf-β1 and Tgf-β2 over RN and PN. This further confirmed the presence of a fibrotic region at the lesion site. We have previously demonstrated that the histopathology at the muscle belly distal to the lacerated site to be poor at 16 weeks and 28 weeks post-repair for lacerated muscles with their intramuscular nerve cut. There were also poorly organized AchE-positive end-plates spots with several multiple and poly-neural re-innervations, typical of nerve sprouting from an inappropriate source [Zheng et al., 2004; Pereira et al., 2006].

Only recently has it been shown that Bmp signaling can also maintain satellite cell proliferation and differentiation in skeletal muscles [Friedrichs et al., 2011; Ono et al., 2011]. In our model, the 2-week post-repair period showed a significant up-regulation of Bmp genes in DN muscles. Bmp1, Bmp3, and Bmp15 were commonly up-regulated in both RN and DN suggesting a similar response to encourage the survival of satellite cells. However, the higher expression of Bmp genes could be associated to the more severe type of intramuscular nerve damage seen in DN. Some of these might be positive or negative regulators of myogenic repair or associated to fibrosis formation, but this remains unclear. The high levels of Bmp gene expression in DN at 4 weeks deserves attention given that not much is known of their effect on muscle repair, and if they are promoters, inhibitors or having other roles. In addition, DN muscles also showed expression of a set of Gdf family genes and the negative regulators of muscle cell differentiation, Tgf-β1, Tgf-β2, and Tgf-β3 genes, further adding to the proposition that the extent of nerve damage influences the repair response at the lesion site. Tgf-β is also a known fibrosis mediator, and is likely to be associated to the type of fibrosis formation measured at the lesion site [Jarvinen et al., 2005], and this corresponds well to the histopathology seen at 16 weeks and 28 weeks post-repair of lacerated skeletal muscle with their intramuscular nerves damaged at the onset [Zheng et al., 2004].

Blocking the negative regulators and enhancing the positive regulators could be an alternative or supplementary approach to dealing with poor muscle function after a muscle laceration, as previously shown [Huard et al., 2002; Bedair et al., 2008; Zhu et al., 2011]. Much has been reported on the role of Igf-1 and Igf-2 in myogenic and neurogenic regeneration in injured skeletal muscles [Keller et al., 1999; Menetrey et al., 2000; Hill et al., 2003]. In our data, Igf-1 mRNA expression levels were not found to be significant higher for DN at 4 weeks likely demanding a greater amount of satellite cells to be activated given the severity at the lesion site. Igf-1 could also be involved in modulating the inflammatory cytokines and limiting fibrosis in the early acute phase as shown in some previous work. This argues that Igf-1 is necessary to accelerate the process of muscle regeneration by regulating the inflammatory cytokines, while also playing a role in activating the satellite cells and earlier proposed by Hill et al. [2003], Ciciliot and Schiaffino [2010], Pelosi et al. [2007], and Washington et al. [2011].

The Igf-2 mRNA expression was more pronounced for DN muscles at 2 weeks spiking at more than 6-times relative to the contra-lateral sham in DN muscle. Interest in Igf-2 and its beneficial effects to improve muscle recovery has been previously shown to

encourage a late regeneration in skeletal muscles by exogenous administration of Igf-2 peptides [Kirk et al., 2003]. Igf-2 administration to damaged nerves have also resulted in enhanced nerve regeneration and re-innervation, however, it was only tested positive in the early repair phases between 3 and 7 days. Our data on IGF mRNA at 1-week for DN might also be attempting to maintain the survival of the damaged axons. Thereafter, its effect might have diminished. Gardner et al. recently showed in-vitro that Tgf-β disrupts an Igf-2 autocrine release required for muscle fiber differentiation and that what is required is an exogenous or a delayed paracrine response to counter this disorder. We suspect that at 4 weeks, the DN muscle is responding to correct the imbalance between Tgf-β and Igf-2 and inhibiting the process of axonal regeneration and remyelination [Gardner et al., 2011]. This interaction between Tgf-β and Igf in muscle lacerations remains a topic for further research.

SATELLITE CELL ACTIVATION AND MYOFIBER REGENERATION—HGF, SCGF AND MYOSTATIN

Apart from Igf-1, the activation of satellite cells could be linked to other known factors such as hepatocyte growth factor (Hgf) and hematopoetic stem cell growth factors (Scgf). In an injury in-vitro muscle model, Hgf, which has been shown to reside in the intracellular domain of the muscle fibers acts as an autocrine mechanism and quickly activates the quiescent state of satellite cells [Sheehan et al., 2000]. Yet high levels of Hgf could also inhibit satellite proliferation by inducing myostatin. Moreover, myostatin might not necessarily be involved in the satellite cell activation and differentiation. Being a member of the Tgf-β family, it might be more involved in regulating fibrosis formation and regulating the muscle atrophy or hypertrophy status [Terenghi, 1999; Yamada et al., 2010]. The interaction between Hgf, and myostatin, a reported negative regulator of muscle growth is not clear and would be of interest to study. For our RN and DN lacerated muscle models at 2 weeks, Hgf was close to twofold expression level, while PN was about an order less. RN showed a decline in Hgf expression levels significantly down-regulated at 8 weeks, while DN and PN were not significantly different up to 8 weeks. Comparing their expression levels of myostatin there was a fourfold increase in DN over RN and PN at 4 weeks, and by 8 weeks the levels were not significantly different from the PN controls. The up-regulation of myostatin could be associated to the poor return of muscle mass recorded over that period for DN and RN, and not by inhibiting satellite cell activation. The RT-PCR data showed that Hgf mRNA expression levels of all groups peaked at 1 week as expected, with the expression remaining higher in DN up to 4 weeks, when all groups showed no significant difference [Ciciliot and Schiaffino, 2010]. Therefore no association was established between Hgf and myostatin for lacerated muscles over the 2- and 8-week post-repair period. For PN, with the expression levels of Hgf and Scgf up-regulated at 2 weeks, and the Hgf mRNA showing elevated expression at 3 days and 1-week, it is possible that the activation of satellite cells by Hgf might be an early response to injury and that Scgf mRNA expressions playing a role to maintain the survival of the activated satellite cells up to 4 weeks post-repair.

NEUROTROPHINS NGF, BDNF, AND NT-3 mRNA EXPRESSION LEVELS USED AS A MEASURE OF RE-INNERVATION

The expression of Ngf, NT-3, and Bdnf peaked before the 2 weeks post-repair period. In DN muscles, Ngf, and Bdnf mRNA expression levels were twice that of NT-3 and showed higher expressions levels for all 3 neurotrophins throughout the time course. RN muscles fluctuated in their expression levels yet maintaining levels similar to PN. Previous work confirms that these neurotrophins are known to peak early but if they continued to be expressed then there is a delayed re-innervation. Bdnf and Ngf have also been proposed as factors to assess the status of re-innervation [Michalski et al., 2008]. Based on this assumption, clearly, DN and RN in our study might be delayed, with DN having a more prolonged period to recover and reinnervate across the lesion site in lacerated skeletal muscles.

DIFFERENTIAL NEUROTROPHIC GENE EXPRESSION

We therefore looked at factors specifically elevated in RN and DN to observe for those that could be associated to the poor re-innervation. The response to the muscle repair in DN appears to be expressing more growth factor genes including Ebaf2 (at 2 weeks), Dlk1 (at 4 weeks), fgf10 (4), Cntf (4), Grn (4), Spp1 (4), Gas6 (4), and Igf-1 (4); and these could be related to the poorer axonal regeneration or denervation seen in DN. Whether this is a protective mechanism, to await a synaptic reconnection remains to be seen as the time to re-innervation for either groups is not known. But RN did show a down-regulation of Artn (at 2- and 8 weeks), Osm (4), Lif (4), Tyro3 (4), Prl (4), Hgf (4 and 8), Thpo (4), and Epo (4). All these might be neurotrophic genes that are specific to axonal regeneration and remyelination given that this might already be progressively expressed over the time course of the repair and recovery phase as seen in some other studies of peripheral nerve damage and denervated muscles [Li et al., 1996; Prieto et al., 1999; Terenghi, 1999; Batt et al., 2006; Elfar et al., 2008; Michalski et al., 2008]. These sets of growth factors become possible candidates for testing out therapeutic solutions. One possibility could be the exogenous administration of some of these factors to improve the recovery of lacerated skeletal muscle as well as to avoid irreversible damage. Gas-6 for instance is a ligand for the receptor protein kinase Tyro-3. Gas-6 is expressed in Schwann cells of motor neurons, and work by Prieto et al. suggests that this pair of Gas-6 and Tyro-3 could be a signaling pathway in the maintenance of a mature nervous system. It is also reported that Gas-6 would be up-regulated in the Schwann cells at injury [Li et al., 1996; Prieto et al., 1999]. This was seen in DN and RN muscles at 2 weeks, with DN having an extended expression levels at 4 weeks. Tyro-3 on the other hand showed up-regulated expression at 2 weeks for all groups with RN down-regulated at 4 weeks. This suggests a more prolonged state after the acute phase, in trying to encourage re-innervation through the lesion site. In a treatment study of Erthropoietin (Epo) to sciatic nerve injuries in rats, it was noted that the nerve crushed injuries had an improved neuroprotection which aided in an accelerated healing. In our study, Epo was down-regulated at 2 weeks in all groups, and then swung to a gene expression levels between 23-and 25-fold at 4 weeks, with DN having a higher expression, and RN having the lower level of expression. By 8 weeks all groups showed no expression levels. This interesting response could be a delayed autocrine expression to

sustain the neural protection of the nerve endings at the lesion site, particularly at 2 weeks. This has not been shown previously in lacerated muscles, and would be an interesting path to follow [Elfar et al., 2008]. Artemin, which was also up-regulated in both DN ($2^{1.8}$-fold) more than and RN ($2^{1.1}$-fold) when compared to the PN control at 4 weeks might have a role in the survival and maintenance in the sensory and sympathetic neurons. When compared to the non-operated contra-lateral limb, RN muscles were generally down-regulated. It is known the Artemin is a neurotrophic factor that selectively binds to the glial-derived neurotrophic factor, but this was not explored in our study.

SCHWANN CELL-DERIVED FACTORS AND THEIR EXPRESSION LEVELS

Lif and Cntf, two Schwann cells-derived factors that are release by the nerve stumps, are known to regulate axonal regeneration when damage occurs. Their expression levels after a nerve cut have been reported to contrast each other [Kami et al., 2000; Michalski et al., 2008]. At the onset of nerve damage, the stump expressed higher levels of Lif, while Cntf was down-regulated. As axonal regeneration is initiated, Lif is modulated and gradually down regulates while Cntf was raised. This was seen in our heat map for the PN muscles (Fig. 3A). Between DN and RN, the initial response was the same as in PN, with up-regulated Lif supported by the q-PCR mRNA expressions levels as well. Lif mRNA expression remain elevated up to 12 weeks, with significantly higher values at 4 weeks for DN. The data supports a greater axonal damage in DN compared to RN, and this was also shown in the elevated mRNA expression levels for Ngf and Bdnf [Terenghi, 1999]. The expression of Lif mRNA in PN reflects that some axonal damage did occur, more likely to minor intramuscular nerve branches that cross the plane of laceration. The Lif mRNA profile of DN may be due to the absence of Schwann's cells at the lesions site. Based on the model described by Jarvinen et al. [2005], we suspect the axonal regeneration might possibly be blocked by the fibrosis formation at the lesion site, and given that DN showed a disrupted junction at the lesion site at 2 weeks, could slow down the entire regeneration process. At 4 weeks the increased Lif expression levels in DN might be a delayed response associated with an attempt to encourage nerve sprouting, axonal regeneration and remyelination across the lesion site to the distal muscle belly.

REPERFUSION INJURY PATTERNS—VEGF, AND PDGF

Except at 4 weeks for DN, no differences where noted for the expression levels of Vegf and Pdgf among the 3 groups. This could be related to the myogenic response to the laceration and not so much to the difference in the type of nerve injuries. We are aware that a lacerated muscle would undergo vascular injuries and reperfusion might be an issue, however, this was not the main focus of our current study, yet is an important aspect in muscle repair, and is a focus of future work.

STUDY LIMITATIONS

The gene-array is only a quick overview analysis before narrowing down any of the factors that are related to the damaged intramuscular nerve in these lacerated models. We were interested to improve the recovery during the inflammatory stages of repair up to 12 weeks to avoid any irreversible damage and so a gene-array study allows for this. Gene-array studies are however, expensive and we will selective in our choice for time-points to study. Although we only chose 2–8 weeks as our study cohort, we supported that by confirming some genes with RT-PCR for the mRNA expression levels over a wider range of time points. Another major limitation is that the simulated lacerated muscle model used a sharp dissection, where in most clinically related laceration the injury type is a blunt dissection. We did this to avoid having too many variables during surgery and it allowed us to duplicate the animal model more consistently. A related critique is the simulation of the re-innervated model, RN. Here the crushed intramuscular nerve damaged the axons and not the nerve sheath, and this was assessed with electrical stimulation proximal to confirm axonal damage before repairing the lacerated muscle.

CONCLUSION

Generally, most muscle lacerations are surgically managed by simply suturing the cut ends together. We previously highlighted the significance of repairing the intramuscular nerve, albeit this being technically difficult. This prompted a relook for possible therapeutic and biological solutions to enhance the repair at an earlier repair phase. In particular, the study specifically looked for factors that could improve both neurogenic and myogenic recovery across the lacerated site to avoid irreversible damage. In this preliminary study, we demonstrated the temporal gene expression of various growth factors and cytokines at 2, 4, and 8 weeks post-repair that are directed by the type of denervation injury in surgically repaired lacerated skeletal muscles. If the intramuscular nerve is damaged (DN and RN) the results showed a differential influence on the inflammatory response with DN having a more severe injury pattern over RN, while a far more improved result was obtained if the intramuscular nerve was preserved intact (PN). Apart from surgically repairing the lacerated muscle, which can result in unpredictable and often irreversible damage, there is a clear need to also improve axonal regeneration and re-innervation of the regenerating myofibers across the lesion site. This preliminary study was therefore design to give a quick overview on the selection of possible candidates to study for the administration of exogenous cytokines and growth factors genes at particular time points and how it could improve re-innervation and overall muscle repair. A muscle that is lacerated as well as partially denervated would involve a major regenerative process of infiltration of neutrophils and macrophages during the first few hours up to 3 days, an anti-inflammatory response over the next 3–5 days, followed by satellite cell activation, proliferation, differentiation and fusion and a parallel yet slower nerve regeneration process beginning with the activation of Schwann cells. Synaptic re-establishment will follow depending on the extent of the damage, and remodeling and reconstitution of the whole muscle would complete the return of muscle function. The whole process might take a longer period, spanning over 3 months [Garrett et al., 1984; Jarvinen et al., 2005; Ciciliot and Schiaffino, 2010].

In our study we noted several possible candidate genes observed in the more severe damaged lacerated muscle where the intramuscular nerve was also involved (i.e., DN) at various time points and not elevated in the reinnervated lacerated muscle (RN) or the model where the intramuscular nerve was preserved (PN). Some of the likely candidates of interest include Gas-6, Artemin, Fgf10, Gdf8, Cntf, Lif, and Igf-2. Others, which were commonly seen in both DN and RN and confirmed by q-PCR include the neurotrophins, Ngf, Bdnf, and NT-3, and were likely associated to the nerve injury with expression at different time points. In addition, to encourage a better inflammatory response some possible target genes to inhibit would include the pro-inflammatory markers like the Tgf-b family and Tnf-family of genes related to fibrosis. Some of these factors and cytokines have previously been studied by others [Menetrey et al., 2000; Huard et al., 2002; Gordon et al., 2003; Hirata et al., 2003; Batt et al., 2006; Warren et al., 2007; Carlson, 2008; Michalski et al., 2008].

ACKNOWLEDGMENTS

This project was supported in part by a URF Tier-1 grant (T13-0802-P21) and a Biomedical Research Council (BMRC/04/1/21/19/309) grant, Singapore. We are grateful to Dr Zheng Ling, Dr Ren and Ms Julee Chan for their involvement in some of the bench-work.

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