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RESEARCH ARTICLE

Tumor prevention facilitates delayed transplant of stem cell-derived motoneurons

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Abstract

Objective: Nerve injuries resulting in prolonged periods of denervation result in poor recovery of motor function. We have previously shown that embryonic stem cell-derived motoneurons transplanted at the time of transection into a peripheral nerve can functionally reinnervate muscle. For clinical relevance, we now focused on delaying transplantation to assess reinnervation after prolonged denervation. **Methods**: Embryonic stem cell-derived motoneurons were transplanted into the distal segments of transected tibial nerves in adult mice after prolonged denervation of 1–8 weeks. Twitch and tetanic forces were measured ex vivo 3 months posttransplantation. Tissue was harvested from the transplants for culture and immunohistochemical analysis. **Results**: In this delayed reinnervation model, teratocarcinomas developed in about one half of transplants. A residual multipotent cell population (~ 6% of cells) was found despite neural differentiation. Exposure to the alkylating drug mitomycin C eliminated this multipotent population in vitro while preserving motoneurons. Treating neural differentiated stem cells prior to delayed transplantation prevented tumor formation and resulted in twitch and tetanic forces similar to those in animals transplanted acutely after denervation. **Interpretation**: Despite a neural differentiation protocol, embryonic stem cell-derived motoneurons still carry a risk of tumorigenicity. Pretreating with an antimitotic agent leads to survival and functional muscle reinnervation if performed within 4 weeks of denervation in the mouse.

Introduction

Pathologies characterized by motoneuron (MN) death or axonal injury lead to muscle denervation and loss of motor function resulting in impairment in quality of life and longevity.[1,2] Amyotrophic lateral sclerosis, spinal cord injuries, nerve root avulsion, and plexus injuries result in motor dysfunction, either due to MN demise[3] or irreversible denervation.

Restoring function to paralyzed muscles can be achieved with functional electrical stimulation provided the targeted muscle retains some innervation.[4] This strategy is far less

effective if the targeted muscles lack innervation because large currents are required to directly activate denervated myofibers.[5] Consequently, we and others have explored the possibility of combining cell replacement therapy with electrical stimulation to restore function to permanently denervated muscles. Collectively, these studies have shown that MNs derived from embryonic ventral spinal cord cells,[6] embryonic stem (ES) cells,[7] or induced pluripotent stem (iPS) cells[8] can functionally innervate denervated muscle fibers when transplanted into the peripheral nerve near the target muscle.[6–10] Although the innervating MNs do not fully restore predenervated contractile force, the level of force achieved when electrically stimulated is likely sufficient to provide meaningful function. For example, transplanted embryonic stem cell-derived motoneurons (ESCMNs) innervate denervated fibers and generate ~40% of the original contractile force when electrically stimulated, provided the cells were grafted immediately after muscle denervation.[7] These studies demonstrate proof of principle that cells transplanted into peripheral nerves can be stimulated to control muscle contraction.

In clinical practice, however, surgical interventions to improve denervation injuries are generally delayed to allow for spontaneous recovery.[11] The same practice would apply if transplantation procedures were to be implemented. This delay could impact transplantation outcomes because the local environment postdenervation changes as time progresses.[12] For translational purposes, it is therefore necessary to demonstrate that MNs can be transplanted after prolonged denervation and still provide functional innervation.

ES and iPS cells are alluring for cell replacement therapies: they can be expanded into large numbers and be directed to differentiate into specific neuronal types, including functional MNs.[13,14] However, ES and iPS cells have been associated with the development of untoward outcomes following transplantation, such as the development of tumors.[15,16] Tumorigenesis likely results because the differentiation protocols lead only to enrichment of a specific cell type and do not completely eliminate pluripotent progenitor cells.[17] Consequently, strategies must be developed to eliminate the risk of tumorigenesis before these cell types can be used clinically.

Here, we sought to establish a safe and effective means to transplant ESCMNs into peripheral nerves following prolonged denervation. We initially found that transplanting ESCMNs following a delay resulted in nearly half of the transplanted animals developing teratocarcinomas. We demonstrate that pretreating the ESCMNs with the alkylating agent mitomycin C eliminated residual pluripotent cells following differentiation while sparing terminally differentiated MNs. Mitomycin C treatment prevented tumor formation and led to functional reinnervation after prolonged injury similar to that seen following transplantation after acute denervation.

Materials and Methods

ESCMN cell preparation

HB9, a homeobox gene, is expressed in embryonic MNs early after differentiation from neuronal progenitors.[18,19] An Hb9-eGFP ES cell line on a pure C57Bl6 background, denoted as HBGB6, was generously provided by Dr. Craig Cox (Jackson, ME) and was used to generate MNs. ESCMNs were generated by treating free-floating clusters of ES cells known as embryonic bodies (EB) with retinoic acid (1 μmol/L; Sigma Aldrich, Oakville, ON, Canada) and smoothened agonist (500 nmol/L; Enzo Life Sciences, Farmingdale, NY, USA) as described previously.[7,13,14] Differentiated EBs were enzymatically dissociated in Tryple express (ThermoFisher Scientific, Ottawa, ON, Canada) with 0.01% (w/v) DNaseI (Sigma-Aldrich). The obtained single cell suspension was resuspended in DFK10.[14] For in vitro experiments, 10^5 dissociated cells were plated onto growth factor reduced matrigel (BD Biosciences, Rockville, MD, USA)-coated glass coverslips (ThermoFisher Scientific). In vitro cells were maintained in DFK10 supplemented with 10 ng/mL GDNF (Millipore, Etobicoke, ON, Canada) and 10 ng/mL CNTF (ThermoFisher Scientific). Media was changed every other day.

Surgery

All procedures were performed in accordance with protocols approved by the Dalhousie University Animal Care Committee, and conformed to the standards of the Canadian Council of Animal Care. Transplantation was performed as described previously.[7] In summary, under deep anesthesia, the tibial nerve of 5-week-old C57Bl6 mice (Charles River) was transected at midthigh level. Both nerve ends were ligated and the proximal end was buried into adjacent muscle to prevent spontaneous reinnervation. ESCMN transplantation was done either immediately after transection or after a delay period of 1, 2, 4, or 8 weeks posttransection. Ten thousand differentiated cells in 0.1 μL containing 0.01% DNaseI, 20 μg/mL CNTF, and 10 μg/mL GDNF were transplanted using a glass micropipette into the transected distal tibial nerve, which was subsequently proximally ligated. All distal branches except the medial gastrocnemius (MG) nerve branch were transected.

In vitro electrophysiology

The MG muscle together with the tibial nerve was harvested 3 months after transplantation as described

previously.[7] Forces were measured with a force transducer (FT03, Grass Technologies, West Warwick, RI, USA) connected to an AC/DC strain gage amplifier (P122, Grass Technologies). A glass suction electrode was used to provide stimuli to the MG nerve via a square pulse stimulator (S88, Grass Technologies) isolated from ground by a constant current stimulus isolator (PSIU6, Grass Technologies). EMG recordings were obtained with a polyethylene suction electrode (PE-190, Clay Adams, Sparks, MD, USA) applied over the muscle midbelly. EMG signal was amplified with a differential amplifier (EX4-400, Dagan Corporation, Minneapolis, MN, USA) and bandpass filtered between 0.3 Hz and 3 kHz. Signals were recorded via a Digidata 1320A, using Axoscope 9.2 software (Molecular Devices, Sunnyvale CA, USA). Supramaximal stimulation was performed at 1.5× the stimulus necessary to provide maximal twitch force (usually 100 μA, 0.2 msec). Tetanic stimulation was performed at 50 Hz for 500 msec. Motor unit (MU) sizes were estimated by force increments[7,20] obtained by stepwise increases of the stimulus. Motor unit number estimation was estimated by dividing the whole muscle twitch force by the averaged MU force obtained after seven force increments. The susceptibility of the muscle to fatigue was quantified by calculating the ratio between the force generated after a 2-min tetanic stimulation by the force at the onset of the stimulation as described previously.[7]

Tumor tissue harvesting and culture

Upon palpable tumor formation from ESCMN transplantation, mice were anesthetized and the tumor was dissected under sterile conditions in terminal experiments. One half of the tumor was fixed and processed for paraffin embedding and thin slice hematoxylin and eosin histochemistry. The other half was kept for cell culture. Tissue was sharply fragmented in a slurry in Ca^{2+}/Mg^{2+}-free HBSS, washed multiple times before enzymatically digested in trypsin-EDTA (1–0.25%, Sigma-Aldrich) for 30 min at 37°C with constant agitation. The cell suspension was spun and washed with DMEM supplemented with 10% fetal calf serum (Gibco) followed by trituration, washing, and filtration over a 40-μm cell filter (Millipore). In order to generate cell colonies, 10^6 cells were plated onto a feeding layer of mitomycin C-treated primary mouse embryonic fibroblasts (Stem Cell Technologies, Vancouver, BC, Canada) in ES media as previously described.[13,14] Media was supplemented with 2× penicillin–streptomycin (Gibco) and 2.5 μg/mL amphotericin B (Sigma-Aldrich). Media was changed daily for the first week. Initial colonies were passaged on the fifth day in culture and every second day thereafter. Passaging of colonies was performed as described previously for mouse ES cultures.

Immunohistochemistry

Cells were fixed in 1% paraformaldehyde in PBS for 20 min, followed by three washes in PBS, then perforated and blocked for 20 min in PBS-0.3% triton-X (v/v, Sigma-Aldrich) supplemented with 10% donkey serum before incubation with primary antibodies. Cells were washed three times in PBS between each step. Nuclei were stained with Hoechst 33342 (Sigma-Aldrich) 0.1 μg/mL for 20 min in PBS. Primary and secondary antibodies are described in Table 1.

Table 1. Antibodies.

Primary antibodies						
Antiserum	Host	Subtypes	Dilutions	Incubation	Sources	Cat. Number
GFP	Rabbit	IgG	1:1000	1 h	Millipore	AB3080
GFP	Sheep	IgG	1:1000	O/N	Novus Biologicals	NB100-62622
TUJ1	Mouse	IgG2a	1:1000	1 h	Covance	MMS-435P
SSEA-1[1]	Mouse	IgM	1:1000[1]	1 or 72 h	Cell Signaling	4744
Sox2	Mouse	IgG1	1:100	72 h	Cell Signaling	4900
Oct4A	Rabbit	IgG	1:100	72 h	Cell Signaling	2840

Secondary antibodies						
Antiserum	Host	Conjugates	Dilutions	Incubation	Sources	Cat. Number
Anti-rabbit IgG	Donkey	AF488	1:500	1 h	Thermo Fisher Scientific	A-21206
Anti-sheep IgG	Donkey	AF488	1:500	O/N	Thermo Fisher Scientific	A-11015
Anti-mouse IgG	Donkey	AF546	1:500	1 h or O/N	Thermo Fisher Scientific	A10036
Anti-mouse IgM	Goat	AF546	1:500[1]	1 h or O/N	Thermo Fisher Scientific	A-21045
Anti-rabbit IgG	Donkey	AF647	1:500	O/N	Thermo Fisher Scientific	A-31573

[1]Best staining results obtained without cell perforation, used at 1:100 for 20 min for FACS experiments, 72 h incubation performed when combined with Sox2 and Oct4A.

Mitomycin C treatment

To quantify the effect of mitomycin C on ESCMNs, differentiated EBs were incubated with increasing concentrations of mitomycin C (from 0.01 μg/mL to 10 μg/mL, Sigma-Aldrich) for 2 h followed by three washes in HBSS, dissociation as described above, and plating with neuroprogenitor cells (NPC) in an equal ratio on matrigel-coated coverslips.[21] The presence of astrocytes from NPC was essential to maintain adhesion of ESCMNs to the coverslips for more than 3 days.[22] Coverslips were fixed after 3, 5, or 7 days in vitro. SSEA-1 immunofluorescence was used to identify undifferentiated cell colonies; ESCMN GFP signal was enhanced by immunofluorescence. ESCMNs and undifferentiated cell colonies were counted by selecting five random fields per coverslip with a 10× objective (area of 2.95 mm^2).

To quantify apoptotic cells by FACS after mitomycin C treatment, differentiated EBs were treated with 1 μg/mL mitomycin C for 2 h, washed, and incubated for 12 h in DFK10 with 10 μg/mL GDNF and CNTF prior to dissociation. Annexin-V labeling (Thermo Fisher Scientific) was performed as per the manufacturer's protocol with minor modifications. Dissociated cells were first immunolabeled with primary antibody against SSEA-1 (Table 1) for 1 h at 4°C in cold annexin-binding buffer with constant agitation. After washing, annexin-V labeling was initiated. Secondary antibody staining for SSEA-1 was performed during the annexin-V conjugate incubation step. Cells were kept on ice until analyzed by FACS (BD FACS AriaIII) for the expression of eGFP, SSEA-1, and annexin-V.

Mitomycin C treatment for ESCMN transplantation was performed in vitro with 1 μg/mL exposure for 2 h prior to EB dissociation. EBs were washed three times with HBSS and dissociated as per the above protocols.

Imaging

Color images were obtained on an Axioplan II (Zeiss Microimaging, Thornwood, NY, USA) microscope equipped with a color Axiocam HRC camera (Zeiss). Confocal images were obtained on a Zeiss LSM710 confocal microscope running Zen software (Zeiss). Images were transferred to ImageJ (NIH) for analysis.

Statistical analysis

Results are presented as means ± standard deviations. One-way analysis of variance (ANOVA) was used to compare electrophysiological data between experimental and control groups and between immediate and delayed experimental groups. Kruskal–Wallis tests and Dunn's multiple comparisons were applied to identify significant differences. Two-way ANOVA with Bonferroni multiple comparisons was used for mitomycin C experiments. Freeman–Halton extension of the Fisher's exact probability test was used for innervation success ratios. Statistics were calculated using GraphPad Prism version 6.00 for Mac (GraphPad Software, La Jolla, CA, www.graphpad.-com).

Results

Teratocarcinoma formation following transplantation

To ask whether delayed MN transplantation studies could be successful, we used a mouse stem cell line derived from a pure C57Bl6 background (denoted as HBGB6), in which ESCMNs express GFP. Following delayed transplantation, however, there was limited evidence of reinnervation: 1 week after immediate transplantation, GFP^+ cells were seen in only two of the six mice. In a series of 17 transplants, only 5 (30%) were able to generate force at 3 months (3/10 immediate and 2/7 1 week delayed transplants). Four of the remaining 12 transplanted animals had no sign of the graft, while eight generated tumors by 1 month (Fig. 1A–C). The tumors were consistent with teratomas, with the presence of all three embryonic lineages on histology: ectoderm (neurons, Fig. 1D), mesoderm (cartilage, Fig. 1E), and endoderm (gastrointestinal mucosa, Fig. 1F). Malignant histological features were also identified: mitotic figures (Fig. 1G), nuclear atypia and hypercellularity (Fig. 1H), and necrosis (Fig. 1I), indicating the tumors were teratocarcinomas.

To confirm the diagnosis of teratocarcinoma, we next asked whether pluripotent cells remained within the tumors.[23] To do so, we isolated and dissociated the tumors, and cultured them on primary mouse embryonic fibroblasts. We found colonies typical of stem cells that could be renewed for over 1 month when passaged every second day. These colonies were positive for the pluripotent markers SSEA-1, Oct-4A, and Sox2 (Fig. 2A). Furthermore, these tumor-derived colonies could be differentiated into GFP^+ MNs by treatment with retinoic acid and smoothened agonist as previously described for mouse ES cells.[13,14] GFP expression was observed as early as the third passage, 2 days in vitro (Fig. 2B), and was maintained even following 15 passages (1 month). In addition to GFP^+ cells, βIII-tubulin$^+$/GFP^- cells were also present, demonstrating that these tumor-derived pluripotent cells, like ES cells,[13,14]

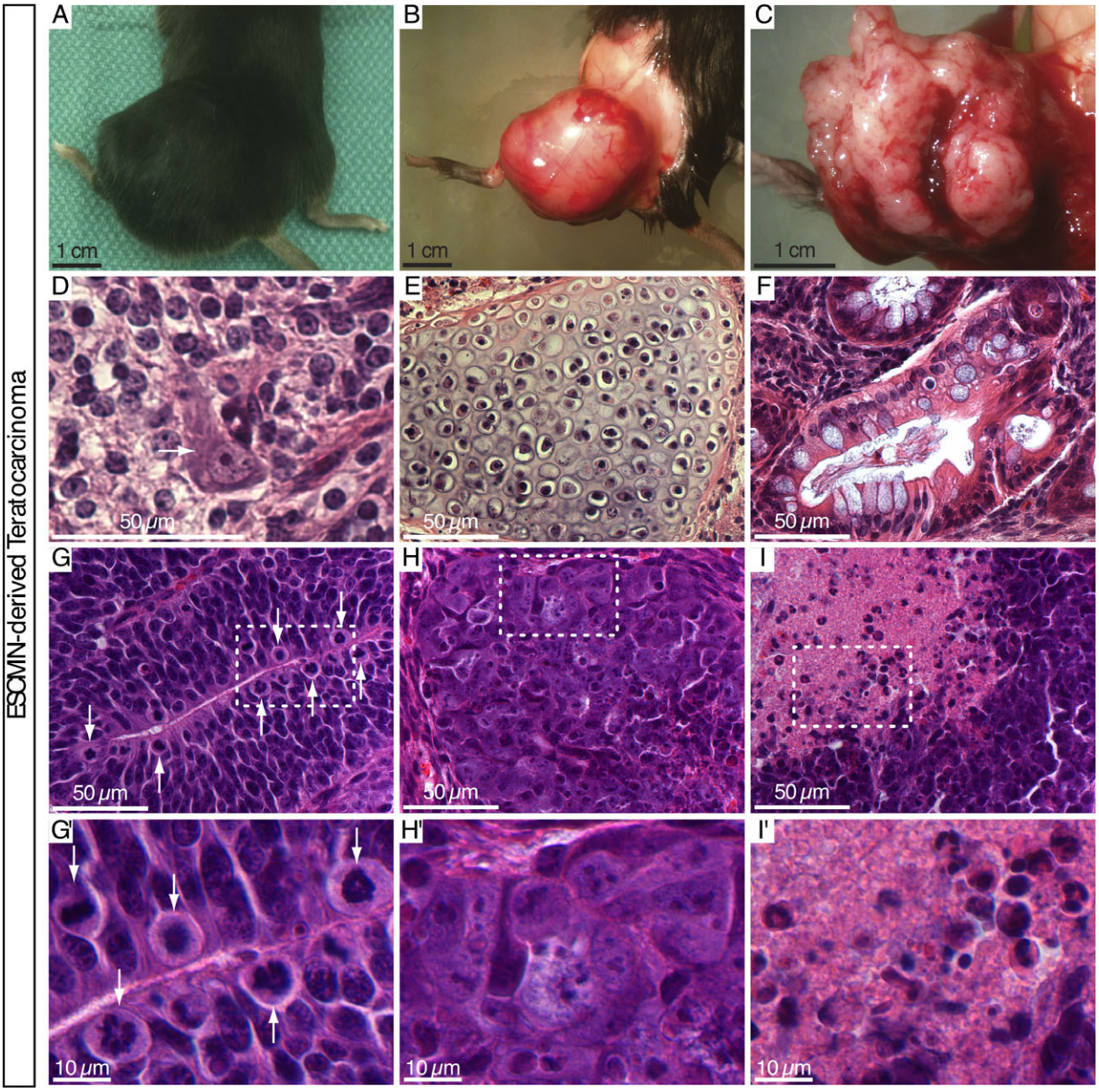

Figure 1. Embryonic stem cell-derived motoneurons (ESCMN) transplants led to formation of teratocarcinomas. (A–C) Macroscopic appearance of tumor. This first tumor appeared rapidly and unexpectedly. Once aware of this issue, we ensured that no further tumors grew larger than a palpable size of 1 cm. (D–I) Microscopic images of tumors originating from transplanted ESCMNs. All three germ lineages were present in tumors consistent with the formation of a teratomatous tumors: epidermal lineage (neuron: arrow, D), mesodermal lineage (cartilage: E), and endodermal lineage (ciliated glandular epithelium with goblet cells: F). Characteristics of a malignant teratoma (teratocarcinoma): high mitotic rate (arrows pointing at mitotic figures; G, enlarged view G'), hypercellularity with nuclear atypia (H, enlarged view H'), and intratumoral necrosis (I, enlarged view I').

differentiated into more than one postmitotic neuronal type (Fig. 2C). Taken together, these results indicate that the observed tumors contained pluripotent cells that could be differentiated into a number of neuron types, including MNs. Furthermore, this shows that the development of malignant teratocarcinomas is a substantial risk in mice when differentiated stem cells are used for transplantation.

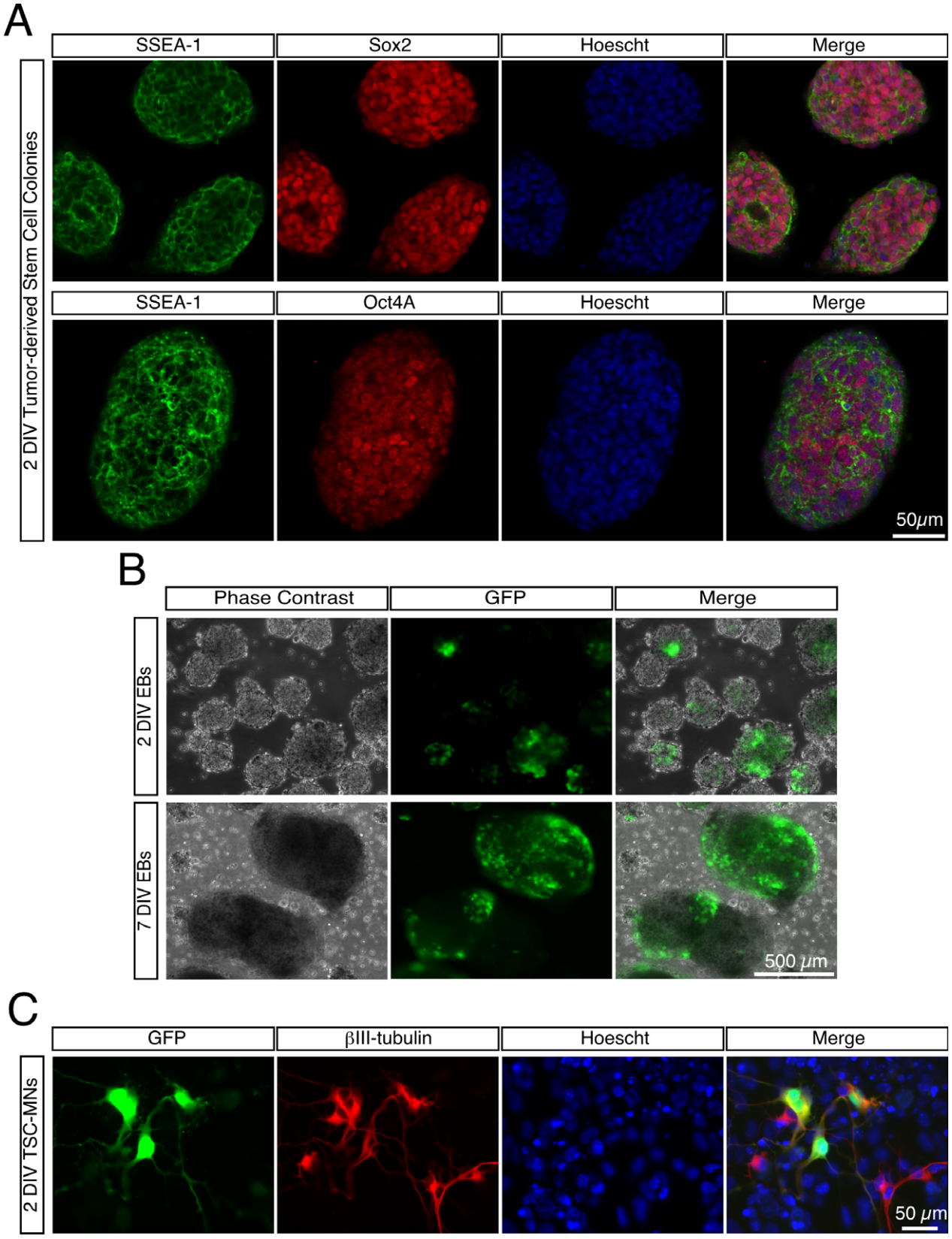

Figure 2. Pluripotent cells isolated from teratocarcinomas generated from transplanted ESCMNs can be differentiated into motoneuron (MNs) in vitro. (A) Pluripotent cells isolated from dissociated teratocarcinoma tissue formed colonies expressing the stem cell markers SSEA-1, Sox2, and Oct4A. Hoechst staining was used to visualize individual nuclei. (B) Following treatment with retinoic acid and a smoothen agonist, embryonic bodies (EBs) generated from teratocarcinoma-derived cells contain GFP^+ MNs after 2 and 7 days in vitro. (C) Dissociated and plated EBs that were treated for 5 days in vitro with retinoic acid and a smoothened agonist, contained GFP^+ MNs and βIII-tubulin$^+$ cells that were GFP^- after 2 days in vitro. ESCMNs, embryonic stem cell-derived motoneurons.

Pluripotent cells remain even after differentiation of stem cells

We next examined the extent to which pluripotent cells remained following differentiation of ES cells into MNs, prior to transplantation. We found that following differentiation, ESCMN cultures contained cells expressing the pluripotent markers SSEA-1, Oct4A, and Sox2 (Fig. 3A). By combining live cell immunolabeling with FACS analysis we found that $6 \pm 2\%$ ($n = 3$) of the cells expressed SSEA-1 after differentiation protocol. Once plated, all wells ($n > 12$) with differentiated ESCMNs formed SSEA-1-expressing colonies as early as 3 days in vitro. These data indicate that residual pluripotent cells persisted within differentiated ESCMN cultures, and suggest that these pluripotent cells were the substrate for the malignancies.

Treatment with mitomycin C eliminates pluripotent cells

Our next strategy was based on the reasoning that since neurons are mitotically inactive, pluripotent cells could be selectively eliminated while neurons were preserved by an alkylating agent. We therefore treated differentiated EBs with mitomycin C for 2 h and immunolabeled the cells with SSEA-1 and the apoptotic marker annexin-V. SSEA-1^+ cells were isolated using FACS and then further analyzed for their expressing of annexin-V and GFP. This showed that the proportion of SSEA-1^+ cells expressing annexin-V was twice as high in the mitomycin C-treated group (Fig. 3B, mitomycin C lower right quadrant) compared to control (Fig. 3B, control lower right quadrant). To test whether this increase in annexin-V was associated with a reduction in the number of pluripotent cells, we treated the differentiated dissociated EBs with mitomycin C, and then cultured them for 1 week. This eliminated pluripotent cells, as demonstrated by the absence of SSEA-1^+ cells in the mitomycin C-treated cultures (Fig. 3C). Concentrations of at least 1 μg/mL of mitomycin C were required to effectively eliminate SSEA-1^+ cells from the cultures (Fig. 3D). Thus, mitomycin C was found to be effective at eliminating undifferentiated pluripotent cells from EBs.

We next asked whether mitomycin C was toxic to MNs. Three days after differentiation and dissociation, there were fewer ESCMNs after treatment with mitomycin C compared to untreated controls. However, by 5 days in vitro, there was no difference in ESCMN numbers (Fig. 4E) with controls. At all time points, however, survival of MNs was significantly decreased when EBs were treated with ≥ 5 μg/mL mitomycin C. Of note, mitomycin C treatment also ablated glial progenitors such that no cells were labeled with glial fibrillary acidic protein (GFAP) at 1 week in vitro, a time when strong GFAP staining is normally seen in cultures of untreated differentiated ES cell lines. Thus, mitomycin C treatment of 1–2 μg/mL was effective at eliminating pluripotent cells while preserving differentiated MNs.

Mitomycin C prevents the formation of teratocarcinomas leading to successful reinnervation

Given that incubation with mitomycin C led to elimination of pluripotent cells, we next tested whether mitomycin C-treated ESCMNs formed tumors after transplantation. ESCMNs treated with mitomycin C did not engraft after immediate transplantation ($n = 6$). However, when mitomycin C-treated ESCMNs were transplanted 1–4 weeks after tibial nerve transection, none (0/20) of the animals developed tumors, indicating that mitomycin C was effective at preventing the formation of teratocarcinomas.

We next determined whether the treated ESCMNs survived and innervated muscle when transplanted into the peripheral nerve of mice. Treating the cells prior to transplantation with mitomycin C resulted in successful innervation after delayed transplantation: six of the eight animals following 1 week delay; three of the six animals after 2 weeks delay; and two of the six animals after 4 weeks delay (Table 2). None of the transplants performed after 8 weeks delay resulted in successful MG innervation. Furthermore, twitch and tetanic forces after 3 months were similar to those found after immediate transplantation of nonmitomycin-treated ESCMNs (Fig. 4A–D; as immediate transplantation after mitomycin C did not engraft, forces from immediate transplants in Fig. 4 are from nonmitomycin C-treated ESCMNs in animals that did not generate tumors). It should be noted, however, that a single pulse of electrical stimulation resulted in doublet or triplet EMG depolarizations (Fig. 4A'). These multiple depolarizations likely accounted for larger twitch amplitude (compared to smaller tetanic

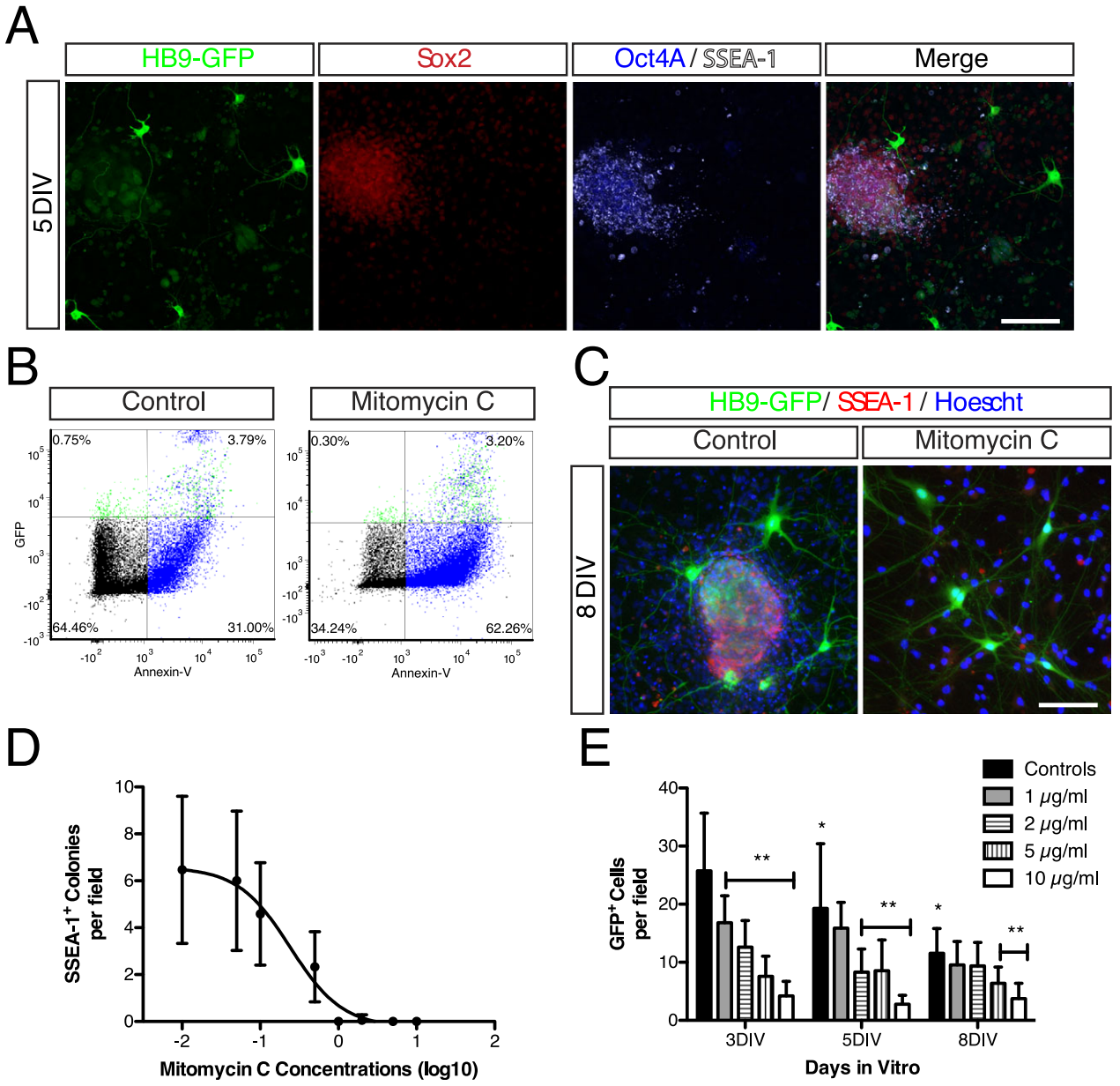

Figure 3. Residual pluripotent cells postembryonic stem cell-derived motoneurons (ESCMN) differentiation from HBGB6 are sensitive to mitomycin C. (A) Dissociated differentiated embryonic bodies (EBs) grown for 5 days in vitro on matrigel demonstrate the formation of colonies expressing pluripotent markers (SSEA-1, Oct4A, Sox2) in the absence of LIF and PMEF. Scale bar: 100 μm. (B) FACS sorting of SSEA-1$^+$ cells from dissociated and annexin-V immunolabeled EBs (previously treated with retinoic acid and a smoothened agonist) without and with pretreatment with mitomycin C (1 μg/mL for 2 h) 12 h prior to sorting. The shift to the right indicates that the majority of SSEA-1$^+$ cells expressed annexin-V (but not GFP) after mitomycin C exposure. (C) Seven days following treatment with 1 μg/mL mitomycin C, dissociated EBs did not contain any colonies of SSEA-1$^+$ cells (right), but these pluripotent cells were present in untreated EBs (left). Scale bar 100 μm. (D) Dose–response of SSEA-1$^+$ colonies 7 days in vitro after mitomycin C treatment. No colonies were found when EBs were treated with mitomycin C concentrations of 1 μg/mL or above. *$P < 0.05$ compared to controls. (E) Toxicity of mitomycin C on ESCMNs showing statistically significant effects with concentrations of 5 μg/mL or greater at all times points compared to controls. **$P < 0.01$ compared with controls of the same time point, by two-way ANOVA and Bonferonni multiple comparisons.

amplitude) in the example shown following a 4-week delay (Fig. 4A, C), and the longer than normal half-relaxation times induced by the single pulse contractions (Table 3). Interestingly, all other contractile characteristics, such as contractile speed and fatigability, were similar to normal MG muscles. The average MU force was

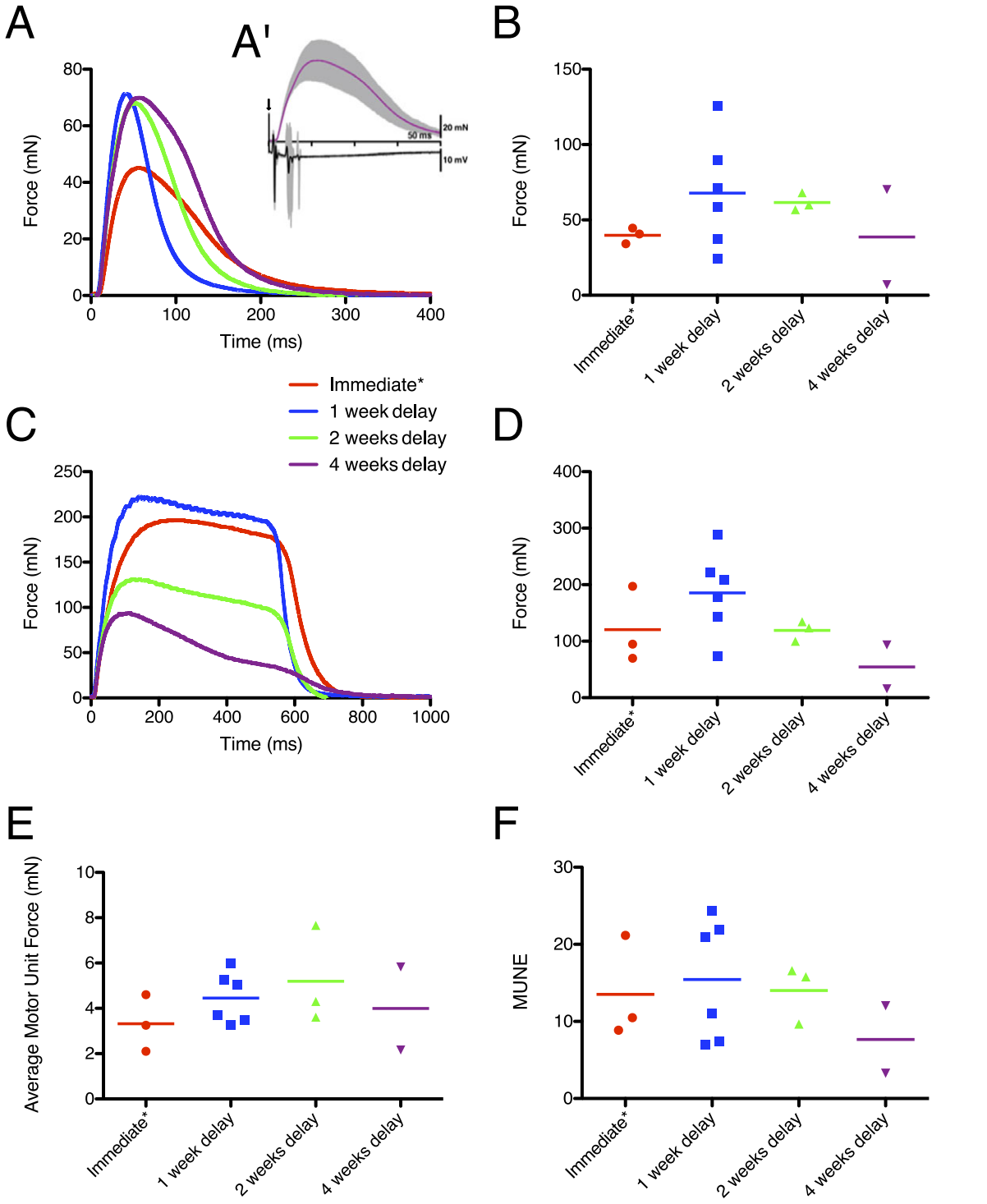

Figure 4. Reinnervation after delayed transplantation of embryonic stem cell-derived motoneurons (ESCMNs) treated with mitomycin C. (A, B) Twitch force and (C, D) 50 Hz tetanic force of the medial gastrocnemius (MG) in delayed transplantation (1, 2, and 4 weeks delay). Traces in A and C are from the same animals. No group showed a statistically significant difference compared to immediate transplants by Kruskal–Wallis test. (A') Four-week delayed transplant curve force with range (in gray) of response shown in (A) superimposed on EMG signal. Depolarization triplets can be seen in the EMG. Arrow indicates the stimulus artifact. (E) Average MU force from transplanted mice obtained by force gradation with increasing stimulus. (F) Motor unit number estimation. No statistical significant difference between groups. *Forces from immediate transplants are those of HBGB6 ESCMNs not treated with mitomycin C. MU, motor unit.

Table 2. Innervation of MG by transplanted mitomycin-treated ESCMNs after prolonged denervation.

Transplantation delay	Animals with MG contraction (%)	Transplanted animals
Immediate	0 (0%)	6
Immediate[1]	3 (50%)	6
1 week delay	6 (75%)	8
2 weeks delay	2 (33%)	6
4 weeks delay	2 (25%)	10
8 weeks delay	ND	ND

MG, medial gastrocnemius

[1]Immediate group representing HBGB6 ESCMNs transplanted without mitomycin treatment. Mitomycin-treated ESCMNs transplanted immediately after denervation ($n = 6$) did not generate contraction. Fisher's exact t-test $P = 0.12$.

increased compared to normal MUs, but was not statistically different between transplant groups (Fig. 4E, Table 3). The motor unit number estimation was ~13 (Fig. 4F), similar to the number we reported previously for immediate transplantation of untreated ESCMNs.[7] Together, these results demonstrate that incubating ESCMNs with the antimitotic agent mitomycin C not only prevented tumor formation, but also led to engraftment and innervation following delays between denervation and transplantation.

Discussion

In developing a clinically relevant model of cell transplantation for denervation injury, we found that muscle innervation decreased with increasing delays between nerve transection and ESCMN transplantation. Concurrently with this, we found that about 50% of animals developed teratocarcinomas, which arose from residual pluripotent cells within the graft. We addressed tumorigenesis by pretreating the cultures with the antimitotic agent mitomycin C, and found that this prevented cancer formation and led to successful muscle innervation from transplanted ESCMNs.

Motor force restoration

Following denervation, return of innervation can occur within a finite time window (5 weeks in mice,[24] 12 weeks in rats,[25] 12–18 months in humans[26]), beyond which functional recovery is poor.[25,27,28] In the absence of innervation, muscle contraction cannot be efficiently restored.[5]

To this end, transplantation of MNs into either the spinal cord gray matter or the peripheral nervous system has been investigated and has demonstrated that transplanted MNs can reinnervate muscle fibers.[29,30] Force generation in our transplants performed either immediately[7] or, as demonstrated here, after prolonged denervation recovered to about half of control forces, a finding that is consistently demonstrated after immediate transplantation by other groups as well.[7–10,31–33] We estimated that after transplantation, ~13–15 MUs innervated the MG, whereas the mouse MG normally contains ~50 MUs.[7,34] Given the sprouting capacity of MNs (up to five times their native innervation ratio[35]), it would be expected that all muscle fibers would be reinnervated, and that close to normal force would be restored. There are two possible explanations as to why this was not the case. First, muscle fibers could have transformed from fast to slow types and/or became smaller than normal. Second, ESCMNs could have a more limited capacity to form enlarged MUs compared to endogenous MNs. Support for the former possibility comes from our previous study,[7] which showed an increase in the number of slow

Table 3. Physiological characteristics of ESCMN-transplanted MG compared to normal MG.

Animals	Contraction time (msec)	Half-width (msec)	Half-relaxation time (msec)	Sag index	Fatigue index	Twitch–tetanic ratio	Av. MU force (mN)
Normal MG	44.10 ± 1.65	39.71 ± 1.74	15.31 ± 1.45	0.99 ± 0.03	0.02 ± 0.01	0.40 ± 0.03	1.63 ± 0.20
Normal soleus	64.40 ± 10.19	113.52 ± 17.37	69.38 ± 11.60	1	ND	0.22 ± 0.02	1.83 ± 0.59
Transplanted animals							
Immediate	47.25 ± 7.83	108.32 ± 37.24[1]	76.07 ± 29.81[1]	0.97 ± 0.04	0.17 ± 0.08	0.50 ± 0.14	3.32 ± 1.25
1 week delay	48.17 ± 8.34	89.37 ± 25.59[1]	57.70 ± 19.17[1]	0.93 ± 0.07	0.11 ± 0.04	0.45 ± 0.13	4.45 ± 1.12[1]
2 weeks delay	44.22 ± 5.20	79.51 ± 9.64	49.42 ± 4.57	0.89 ± 0.07	0.08 ± 0.01	0.61 ± 0.04[1]	5.19 ± 2.16[1]
4 weeks delay	50.73 ± 0.78	104.56 ± 6.61[1]	68.78 ± 7.01[1]	0.77 ± 0.16[1]	0.12 ± 0.13	0.65 ± 0.02[1]	4.00 ± 2.59

Data presented as mean ± SD. Sag index and fatigue index as defined in Yohn et al.[7] ND: not done; MG, medial gastrocnemius; MU, motor unit

[1]Statistically significant to normal MG ($n = 5$) by ANOVA and Tukey's multiple comparisons ($P < 0.05$). No statistics performed on normal soleus ($n = 8$).

muscle fibers in the mouse MG following ESCMN innervation. Because slow fibers are smaller and less powerful than fast fibers, this conversion would result in smaller whole muscle force.[36] However, aside from having half-relaxation times similar to slow contracting muscles, all of the contractile properties measured in the present study including contraction time were typical of fast muscles (Table 3). While we have no evidence that there is a cell-autonomous reason for ESCMNs to have impaired capacity to form enlarged MUs, the lack of activity resulting from their reduced microcircuit environment may limit their ability to form the number of axonal branches required to expand their innervation ratio.[37] Indeed, while the average MU forces were higher than normal in muscles innervated by ESCMNs, they were not five times greater (Table 3). This inability of ESCMNs to form such enlarged MUs would result in a large number of muscle fibers remaining denervated, which, in turn, would lead to a loss in force.

Timing of transplantation

We found that a delay of transplantation beyond 1 week resulted in lower engraftment success, similar to previous studies.[32,38] At 1 week, the inflammatory environment resulting from the transection has likely transformed to a restorative milieu rich in neurotrophic factors, axonal growth-promoting substrates, and supportive adhesion molecules produced by activated Schwann cells.[32,39] Given that the rate of cellular death in the grafts is highest at the time of transplantation,[40] these survival signals may be essential to sustain initial transplant survival. In fact, during development, Schwann cells provide adhesion molecules and trophic support for embryonic MN survival during the critical period of programmed cell death.[41] For these reasons, mitomycin C-treated ESCMNs transplanted immediately likely failed to engraft; Schwann cells had not yet proliferated to provide adhesive and trophic support and glial cells normally present in EBs would had been ablated by the mitomycin C treatment. In fact, ESCMNs treated with mitomycin C always died by the fifth day in vitro if not cocultured with glial cells, a finding reported for embryonic neurons[22] even in the absence of mitomycin C treatment.

Tumor prevention

When considering translation of stem cell therapies, it is crucial to be able to prevent cancer formation.[16,42] Tumor formation has been seen following the transplantation of a number of cell types, including various human pluripotent cell lines.[43–47] The formation of teratocarcinomas is dependent on the transplantation environment and the host immune system,[23] but requires the presence of residual pluripotent cells remaining postdifferentiation.[43,48–51] Our previous publication used an allogeneic stem cell line[7] which may have triggered the host immune system to reject residual pluripotent cells. Introduction of the isogenic HBGB6 cell line could thus have contributed to tumorigenesis. It has been shown that as few as two undifferentiated stem cells in two million non-neoplastic cells can form tumors in 60% of transplants; this rate reached 100% of transplants when 20 undifferentiated stem cells were transplanted.[52] Given that current sorting techniques are limited to the detection of 1 in 10,000,[53] presorting of cells is currently inadequate. The use of mitomycin C – via a short 2-h exposure time prior to transplantation – prevented early tumor formation by eliminating tumorigenic cells.

Translational considerations

When considering cell-based therapies for MN loss, it is pragmatic to first consider proximal peripheral nerve and plexus injuries: these lead to significant deficits and are associated with limited functional recovery.[54] Commonly, the approach to nerve injury is to delay invasive interventions in order to identify spontaneous recovery.[11] We demonstrate that delayed transplantation of ESCMNs is possible and effective if tumor formation is prevented.

Because the neurons in this model are transplanted into the periphery, there is no connection with the central nervous system, and thus no voluntary control. To obtain control, this technique could be combined with functional electrical stimulators to activate the transplanted neurons. Alternatively, ESCMNs could perhaps be considered as "placeholders," preserving muscle fiber innervation until endogenous axons return.

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Author Contributions

P. M., V. R. F., and R. M. B. contributed to the conception and design of the study; P. M. acquired and analyzed the data; and P. M., V. R. F., and R. M. B. wrote the manuscript.

Conflict of Interest

None declared.

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