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Methylguanine DNA Methyltransferase-Mediated Drug Resistance-Based Selective Enrichment and Engraftment of Transplanted Stem Cells in Skeletal Muscle

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ABSTRACT

Cell replacement therapy using stem cell transplantation holds much promise in the field of regenerative medicine. In the area of hematopoietic stem cell transplantation, O^6-methylguanine-DNA methyltransferase MGMT (P140K) gene-mediated drug resistance-based in vivo enrichment strategy of donor stem cells has been shown to achieve up to 75%–100% donor cell engraftment in the host's hematopoietic stem cell compartment following repeated rounds of selection. This strategy, however, has not been applied in any other organ system. We tested the feasibility of using this MGMT (P140K)-mediated enrichment strategy for cell transplantation in skeletal muscles of mice. We demonstrate that muscle cells expressing an MGMT (P140K) drug resistance gene can be protected and selectively enriched in response to alkylating chemotherapy both in vitro and in vivo. Upon transplantation of MGMT (P140K)-expressing male $CD34^{+ve}$ donor stem cells isolated from regenerating skeletal muscle into injured female muscle treated with alkylating chemotherapy, donor cells showed enhanced engraftment in the recipient muscle 7 days following transplantation as examined by quantitative-polymerase chain reaction using Y-chromosome specific primers. Fluorescent in situ hybridization analysis using a Y-chromosome paint probe revealed donor-derived de novo muscle fiber formation in the recipient muscle 14 days following transplantation, with approximately 12.5% of total nuclei within the regenerated recipient muscle being of donor origin. Following engraftment, the chemo-protected donor $CD34^{+ve}$ cells induced substantial endogenous regeneration of the chemo-ablated host muscle that is otherwise unable to self-regenerate. We conclude that the MGMT (P140K)-mediated enrichment strategy can be successfully implemented in muscle. STEM CELLS *2009;27:1098–1108*

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

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Introduction

Cell replacement therapy using stem cell transplantation is currently at the cutting edge of medical research. Combined with gene therapy, stem cell transplantation is a potential treatment for conditions that affect a wide range of conditions including central nervous system disorders such as Parkinson's disease, muscle disorders such as muscular dystrophy, or cancers such as leukemia. At present, hematopoietic stem cell transplantation combined with gene therapy is the most well-established form of stem cell therapy [1–3]. The high success rate of hematopoietic stem cell transplantation is mainly due to the efficient delivery of donor cells via the circulatory system to the bone marrow. Furthermore, highly proliferative hematopoietic stem cells result in efficient in vivo expansion and substantial levels of donor cell engraftment [1]. Recent developments in hematopoietic stem cell transplantation resulted in a strategy to selectively enrich donor cells with a survival advantage in the recipient tissues by treatment with alklylating chemotherapy [2, 4].

Most chemotherapeutic agents have the undesirable side effect of myelosuppression which leads to a compromised immune system [5, 6]. Strategies for selective enrichment of donor hematopoietic stem cells to enhance engraftment were initially developed as a treatment for myelosuppression. There are many studies which have investigated various drug resistance-mediated in vivo selection enrichment strategies [7–13] among which, the most effective strategy uses O^6-methylguanine-DNA-methyltransferase (MGMT) (P140K) gene-mediated drug resistance [14–19]. MGMT (P140K) is a mutant form of the drug resistance gene MGMT. MGMT activity has the ability to repair chemotherapy-induced DNA alkylation thereby rendering chemotherapeutic drugs ineffective as a cancer treatment [20, 21]. Administration of O^6benzylguanine (O^6BG) in combination with alkylating chemotherapy, results in the inhibition of MGMT making the chemotherapeutic drugs more effective [22, 23]. In contrast, MGMT (P140K) activity is not inhibited by O^6BG [22–24]. The MGMT (P140K)-mediated donor stem cell enrichment strategy relies on the combined cytotoxic effect of alkylating chemotherapy plus O^6BG on the host's endogenous stem cells and the drug resistance exhibited by transplanted cells expressing MGMT (P140K). This enrichment strategy has been demonstrated to achieve 75%–100% donor cell engraftment within the host's hematopoietic stem cell pool [24].

Skeletal muscle is not as well-established for cell transplantation as the hematopoietic system despite its ability to undergo rapid regeneration following injury. Skeletal muscle stem cell transplantation has been studied since 1980s [25–27]. The first application of muscle stem cell transplantation was in the mouse model of Duchenne muscular dystrophy (DMD) and resulted in the formation of dystrophin expressing muscle fibers within treated muscles [28]. However, subsequent clinical application of muscle stem cell transplantation showed no significant functional improvements in DMD patients [29–33]. Inefficient engraftment was due to donor cell death immediately following transplantation as a result of immune rejection and/or competition by endogenous cells [30, 34] and the inefficient migration of donor cells from the site of injection for widespread engraftment [35, 36].

In this study, we test the feasibility of applying the MGMT (P140K)-mediated selective enrichment strategy of cell transplantation in solid organs using skeletal muscle as the organ of choice. We show that the cells with conferred drug-resistance can be selectively enriched in vitro and are protected in vivo following administration of the chemotherapeutic agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) combined with O^6BG. Syngeneic transplantation trials confirmed the survival advantage of drug-resistant donor cells and resulted in the formation of de novo muscle fibers. Furthermore, upon engraftment, the transplanted population of heterogeneous drug-resistant donor cells is able to drive endogenous regeneration of otherwise unrecoverable muscle ablated by the alkylating agent.

Materials and Methods

Cell Culture and Retroviral Transduction

C2C12 cells and human myoblasts were cultured as described previously [37]. MFG-MGMT-P140K retroviral vectors were constructed using the retroviral backbone MFG as described previously [38]. Vector supernatant was collected from PA317 cells transfected with the MFG vector plasmid and subjected to drug selection using 10 μM O^6BG (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and 100 μM BCNU (Bristol-Myers-Squibb, Noble Park North, Australia, http://www.bsma.com.au). For transduction of C2C12 cells and myoblasts, vector supernatant was used to replace culture medium overnight, before being replaced with normal growth medium the following day.

Animal Surgery—Injection of Notexin, BCNU, and O^6BG

All surgical procedures were approved by the Children's Medical Research Institute/Children's Hospital at Westmead Animal Care and Ethics Committee and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The mice were anesthetized with a Ketamine (0.1 mg/g b. wt.) and Xylazine (0.01–0.02 mg/g b. wt.) mixture and a 1-cm skin incision was made on either the anterior or posterior aspect of the hindlimb.

For the in vivo study to determine the effect of BCNU and O^6BG on the regenerating muscle, 0.2 μg of Notexin (40 μg/ml: Latoxan, Valence, France, http://www.latoxan.com) and 0.1–0.5 mg of BCNU (33.3 mg/ml) were mixed and injected intramuscularly (10 μl) into the extensor digitorum longus (EDL) muscle using a 50-μl Hamilton syringe with a 33-gauge needle (Hamilton Company, Reno, NV, http://www.hamiltoncompany.com). Following recovery from anesthesia, O^6BG (30 mg/kg b.wt. in PEG400) was injected intraperitoneally.

For donor cell preparation, 8–12-week-old male C57BL/6JArc wild-type or MGMT-P140K$^{tg/tg}$ transgenic mice [38] were injected with 0.1 and 0.4 μg of Notexin intramuscularly into the EDL and tibialis anterior (TA) muscles, respectively. The muscles were collected 3 days later for donor cell isolation.

Isolation of Donor CD34^{+ve} Cells

The cell isolation protocol was adopted from Montarras et al. [39] and CD34^{+ve} cell selection was performed using a magnetic EasySep-PE Cell Separation Kit (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) according to the manufacturer's instructions with anti-CD34 biotinylated antibody (eBioscience Inc., San Diego, CA, http://www.ebioscience.com; 1:50).

Transplantation of Donor CD34^{+ve} Cells in Combination with BCNU (I.V.) and O^6BG (I.P.)

Recipient female C57BL/6JArc wild-type mice (8–12-week-old) were injected intramuscularly with donor CD34^{+ve} cells (60,000 cells in 5 μl of sterile PBS) mixed with 5 μl of Notexin (40 μg/ml). In each transplant recipient, the Great saphenous vein was exposed on the posterior aspect of the hindlimb and a tourniquet was placed at the base of the hindlimb to temporarily block the blood flow [40] and BCNU (0.2 mg in 50 μl) was injected intravenously using a 33-gauge needle. Following injection, a sterile

cotton bud was placed on the puncture site and pressure applied to prevent hemorrhage, while blood flow was blocked for a further 2 minutes. Following recovery from anesthesia, O^6BG (30 mg/kg b.wt.) was delivered intraperitoneally.

Immunochemistry and Fluorescent In Situ Hybridization

Immunodetection of α-actinin-2 was performed as previously described [41] and detection of MGMT and dystrophin were performed using a mouse on mouse kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com; product number FMK-2201) according to manufacturer's instructions with the primary antibodies: anti-human MGMT (Neomarkers, Fremont, CA, http://www.labvision.com; 1:100) and antidystrophin (NCL-DYS, Novocastra, Newcastle upon Tyne, U.K., http://www.novocastra.co.uk; 1:200). Fluorescent in situ hybridization (FISH) protocol was performed as previously described [42] using Biotin labeled Y-chromosome paint probe (ID Labs Biotechnology, London, Ontario, Canada, http://www.idlabs.com). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000).

Western Blot Analysis

Western blot analysis was performed as previously described [43]. Primary antibodies used: antisarcomeric tropomyosin (Clone CH1, Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com; 1:200), anti-α-actinin-2 (gift from Alan Beggs; 1:200), anti-myogenin (Abcam, Cambridge, U.K., http://www.abcam.com; 1:100), anti-MyoD (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com; 1:200).

Fluorescence-Activated Cell Sorting Analyses

Isolated cells were fixed in 1% paraformaldehyde for 30 minutes and washed in FACS Buffer (PBS, 0.1% BSA, 0.1% Na Azide) and permeabilized in 0.1% Tween-20 for 1 hour at 37°C (for detection of intracellular markers). Cells were incubated with the following primary antibodies and incubation conditions: anti-CD34 biotinylated antibody (eBioscience Inc., San Diego, CA, http://www.ebioscience.com; 1:100) for 30 minutes at 4°C, mouse anti-human MGMT monoclonal antibody (Neomarkers, Fremont, CA, http://www.labvision.com; 1:25) for 2 hours at 4°C, anti-mouse CXCR4 FITC-conjugated antibody (R&D Systems Inc., Minneapolis, http://www.rndsystems.com; 1:5) overnight at 4°C, anti-mouse CD133 FITC-conjugated antibody (eBioscience Inc.; 1:50) overnight at 4°C, anti-mouse stem cell antigen 1 (Sca-1) (Ly-6A/E) PE-Cy5.5-conjugated antibody (CALTAG Laboratories, Burlingame, CA, http://www.caltag.com; 1:100) for 30 minutes at 4°C, anti-mouse CD45 FITC-conjugated antibody (CALTAG Laboratories; 1:100) for 30 minutes at 4°C, anti-mouse CD11b PE-conjugated antibody (eBioscience Inc.; 1:100) for 30 minutes at 4°C. For the biotinylated primary antibodies, the cells were further incubated with streptavidin-RPE (DAKO, Glostrup, Denmark, http://www.dako.com) or streptavidin-FITC fluorescein (Perkin Elmer Life Sciences, Life and Analytical Sciences, Boston, MA, http://www.perkinelmer.com) secondary antibodies. Isotype control samples for each primary antibody were included and FACS analyses were performed using a FACScan (Becton Dickinson Biosciences, Franklin Lakes, NJ, http://www.bd.com).

Quantitative-Polymerase Chain Reaction

DNA was extracted from the recipient muscles by incubating in 400 μl of TE/SDS buffer (100 mM Tris pH 8, 1 mM EDTA, 0.5% SDS) with freshly added Proteinase K (400 μg, Roche Products Pty Ltd, Dee Why, Australia, http://www.roche-australia.com) at 56°C overnight with agitation. Following digestion, 75 μl of NH_4OAc and 1 ml of absolute ethanol were added and mixed by inversion then centrifuged for 20 minutes. The supernatant was removed and the DNA pellet was washed in 70% ethanol (1 ml) and centrifuged for 10 minutes. The DNA pellet was air-dried and re-suspended in Milli-Q water (100 μl) and stored at 4°C. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers with the following sequence: Y chromosome-forward (TGG AGA GCC ACA AGC TAA CCA), Y chromosome-reverse (TCC CAG CAT GAG AAA GAT TCT TC). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used to quantify the total amount of DNA present within the samples with the following sequence: GAPDH-forward (GAA GGT GGT GAA GCA GGC AT), GAPDH-reverse (GCA TCG AAG GTG GAA GAG TG). Reaction volume (25 μl) was made up with the 2× QuantiTect SYBR Green® master mix (Qiagen, Hilden, Germany, http://www1.qiagen.com). Y-chromosome and GAPDH standards with known DNA concentrations were

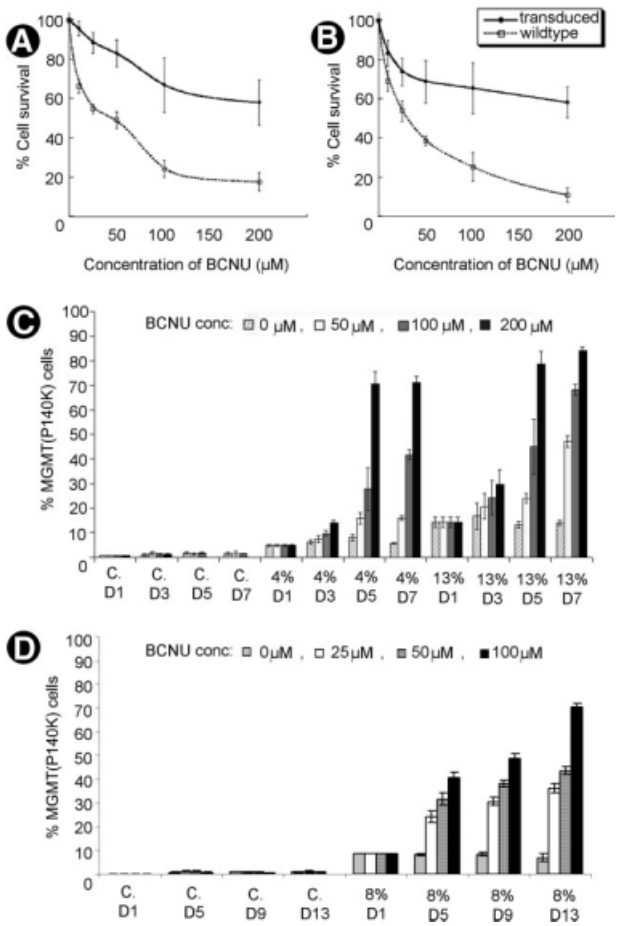

Figure 1. MGMT (P140K) expression protects against BCNU + O^6benzylguanine (O^6BG) treatment and allows selective enrichment of myoblasts. The percentage of MGMT (P140K)-transduced C2C12 myoblasts (**A**) and human myoblasts (**B**) that survive compared to wild-type myoblasts following BCNU (1-hour exposure) and 40 μM O^6BG (2-hour exposure) treatment is shown. (**C**): Selective enrichment of C2C12 cells transduced with MGMT (P140K) treated with BCNU and 40 μM O^6BG for 1, 3, 5, or 7 days (Control **[C]** D1–D7: 0% MGMT positive cells at the start; 4% D1–D7: 4% MGMT positive cells at the start; 13% D1–D7: 13% MGMT positive cells at the start). (**D**): Selective enrichment of human myoblasts transduced with MGMT (P140K) using BCNU and 40 μM O^6BG for 1, 5, 9, and 13 days (Control **[C]** D1–D13: 0% MGMT positive cells at the start; 8% D1–D13: 8% MGMT positive cells at the start). Each value is a mean ± SD of three cultures. Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MGMT, O^6-methylguanine-DNA methyltransferase.

included in the assay. A negative control using female DNA and a no template control were also included. The reaction settings were: denature (95°C for 15 minutes), cycling (40 repeats—step one at 95°C for 20 seconds, step two at 56°C for 20 seconds, and step three at 72°C for 30 seconds acquiring SYBR green signal), hold (60°C for 1 minute), melt (from 60°C to 95°C—hold 5 seconds on first step, hold 5 seconds on next steps then acquiring the SYBR green signal).

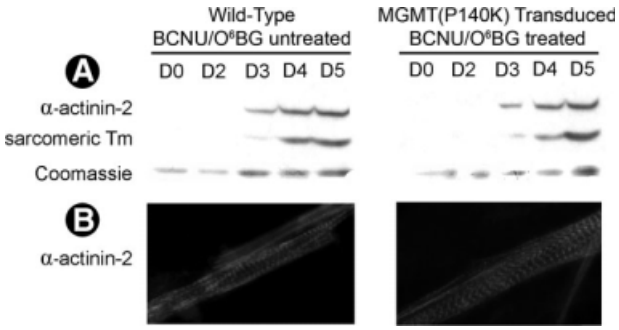

Figure 2. C2C12 cell differentiation is not affected by MGMT (P140K) transduction and BCNU + O^6BG treatment. MGMT (P140K) transduced C2C12 cells were treated with BCNU (1-hour exposure) and 40 μM O^6BG (2-hour exposure). **(A):** Western blot analysis of α-actinin-2 and sarcomeric tropomyosin (sarcomeric Tm) expression on days 0–5 (Day 0–Day 5) following incubation in differentiation-promoting medium. **(B):** Immunochemical staining for α-actinin-2 expression in C2C12 myotubes maintained in differentiation medium for 5 days. Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MGMT, O6-methylguanine-DNA methyltransferase; O^6BG, O^6benzylguanine.

Results

Selective Survival and Enrichment of MGMT (P140K) Transduced Muscle Cells with BCNU and O^6BG Selection In Vitro

We first determined whether the forced expression of MGMT (P140K) in C2C12 cells and human myoblasts confers protection from BCNU resulting in a selective enrichment of the transduced cells. When both the transduced and untransduced cells were treated with various doses of BCNU (0 μM, 50 μM, 100 μM, 200 μM) and O^6BG (40 μM), the cell proliferation assay revealed that C2C12 (Fig. 1A) and human primary myoblasts (Fig. 1B) expressing MGMT (P140K) showed greater resistance to BCNU and O^6BG compared with wild-type cells. To test the selective enrichment of MGMT (P140K)-expressing cells, C2C12 cultures containing 0, 4, or 13% MGMT (P140K) transduced cells in a background of wild-type cells, were treated with four concentrations of BCNU over a 7-day period (Fig. 1C). Similarly, human primary myoblast cultures containing either 0 or 8% MGMT (P140K)-transduced cells were treated with three concentrations of BCNU over a 13-day period (Fig. 1D). FACS analyses revealed selective enrichment of MGMT (P140K)-transduced cells over time in both cultures which correlated with the amount of MGMT (P140K)-transduced cells originally present and the dose of BCNU. For both sets of cultures, higher doses of BCNU achieved better selection than the lower doses. These results also confirm that the EF-1α promoter driving the expression of MGMT (P140K) is active both in mouse and human myogenic cells.

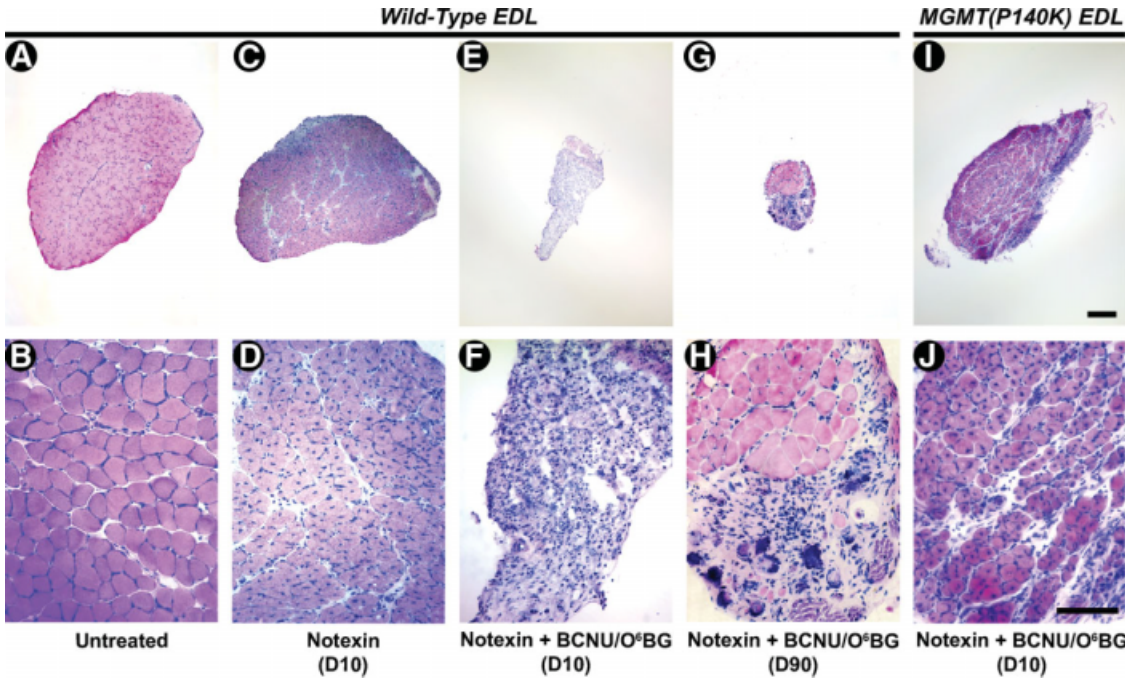

Figure 3. MGMT (P140K) transgenic skeletal muscles are resistant to BCNU + O^6BG treatment during regeneration. EDL muscles in wild-type C57BL/6JArc mice (**A–H**) or MGMT (P140K) transgenic mice (**I, J**) were either untreated (**A, B**), injected with notexin alone (**C, D**), or injected with notexin plus BCNU + O^6BG (**E–J**) and collected for analysis 10 days (**C–F, I, J**) or 90 days (**G, H**) post treatment. Cross sections were cut from the widest portion of each muscle. Images are representative of eight experiments per experimental data set. Scale bars = 200 μm (**A, C, E, G, I**), 100 μm (**B, D, F, H, J**). Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; EDL, extensor digitorum longus; MGMT, O^6-methylguanine-DNA methyltransferase; O^6BG, O^6benzylguanine.

Myoblast Differentiation Is Not Affected Following Retroviral Transduction and BCNU/O^{6}BG Treatment

To determine if the retroviral transduction of MGMT (P140K) and also BCNU/O^{6}BG treatment of myoblasts compromise their differentiation capacity, MGMT (P140K)-transduced C2C12 cells were treated with BCNU/O^{6}BG and allowed to differentiate into myotubes. The differentiation potential of MGMT (P140K)-transduced cells were compared with that of untreated wild-type C2C12 cells. The differentiated cells were analyzed for expression of the differentiation-specific proteins of the muscle thin filament, α-actinin-2 and sarcomeric tropomyosin. Levels of expression for both α-actinin-2 and sarcomeric tropomyosin were comparable between the transduced (and BCNU/O^{6}BG treated) and wild-type (and BCNU/O^{6}BG untreated) cells following the initiation of differentiation (Fig. 2A), indicating that the rate and extent of differentiation was not altered. Furthermore, immunochemical staining of α-actinin-2 revealed the normal arrangement of sarcomeres and z-line formation in MGMT (P140K) transduced cells treated with BCNU/O^{6}BG (Fig. 2B). BCNU/O^{6}BG-treated wild-type C2C12 cultures displayed substantial cell death and subsequent differentiation was impaired (data not shown).

Regeneration Is Hindered in Wild-Type Muscles Treated with BCNU + O^{6}BG While MGMT (P140K)-Expressing Muscles Are Protected

BCNU alkylates DNA within a cell irrespective of the cell cycle status; however, apoptosis that results from the DNA alkylation only occurs when the cell undergoes proliferation [44]. Skeletal muscles are postmitotic and the satellite cells, resident stem cells of the skeletal muscles, are quiescent under normal conditions, but become mitotically active during regeneration. To gain proof-of-principle evidence that MGMT (P140K) can protect against BCNU alkylation in the animal, we used a transgenic mouse line in which MGMT (P140K) is expressed in all cell types analyzed [38]. We induced regeneration in the EDL muscles of wild-type and MGMT (P140K)$^{tg/tg}$ mice by i.m. injections of the myotoxic agent notexin (0.1 μg) with and without BCNU + O^{6}BG. After 10 days of regeneration in the absence of drug selection, histological cross-sections of the wild-type muscle show regenerated muscle fibers with internal nuclei, a hallmark of normal myofiber regeneration (compare Fig. 3C, 3D with 3A, 3B). When the wild-type EDL was injected with notexin and BCNU (0.2 mg, i.m.) + O^{6}BG (30 mg/kg b. wt., i.p.), the muscle showed a complete lack of regenerated myofibers 10 days following treatment (Fig. 3E, 3F). This failure to regenerate following treatment persisted through 90 days, the latest time point examined, with a very small proportion of intact muscle fibers evident (Fig. 3G, 3H). This indicated that the ablation of resident satellite cells by BCNU/O^{6}BG results in an apparent permanent inability of the muscle to regenerate. In contrast, when the same procedure was applied to the EDL muscle of the MGMT (P140K) transgenic mouse, regenerated myofibers were clearly present 10 days following treatment (Fig. 3I, 3J). Although the extent of the regeneration in the drug-treated MGMT (P140K) transgenic muscle was diminished in comparison to wild-type muscle regenerating for the same amount of time in the absence of drugs (compare Fig. 3D with 3J), these results confirm that sufficient expression of MGMT (P140K) in cells that contribute to muscle regeneration must be achieved to account for a successful regeneration following the administration of BCNU + O^{6}BG.

Immuno-detection of human MGMT revealed a number of MGMT (P140K)-expressing cells in the regenerating muscles of the MGMT (P140K) transgenic mice (Fig. 4C, 4D) compared to untreated muscle (Fig. 4A, 4B). Because the expression of the MGMT (P140K) transgene is driven by the EF-1α promoter, which is only active in mitotically active cells [45], expression of MGMT (P140K) is absent in untreated muscle (Fig. 4A, 4B) and in the postmitotic myonuclei within the regenerated muscle fibers (Fig. 4C, 4D). Upon BCNU/O^{6}BG treatment, the regenerating MGMT (P140K) transgenic muscle has a significantly higher number of MGMT-expressing cells (Fig. 4E, 4F) indicating an expansion of these cells under the selection pressure exerted by BCNU/O^{6}BG treatment. Western blot analysis confirmed an increased amount of MGMT protein present in the MGMT

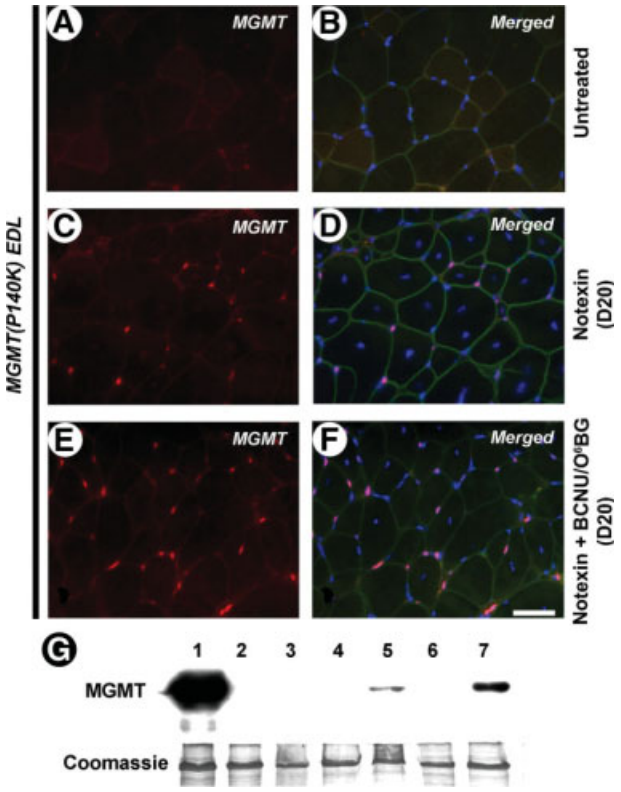

Figure 4. MGMT-expressing cells are enriched in regenerating MGMT (P140K) transgenic muscle following BCNU/O^{6}BG treatment. EDL muscles in MGMT (P140K) transgenic mice were either untreated (**A, B**), injected with notexin alone (**C, D**), or injected with notexin plus BCNU + O^{6}BG (**E, F**) and collected for analysis 20 days post treatment. Cross sections were cut from the widest portion of each muscle. MGMT-expressing cells were detected with an anti-human MGMT antibody (**A, C, E**). Merged images showing MGMT-expressing cells (pink), membranes of fibers delineated with an anti-dystrophin antibody (green) and nuclei stained with 4′,6-diamidino-2-phenylindole (blue) (**B, D, F**). Images are representative of three experiments per experimental data set. Scale bar = 40 μm (**A–F**). (**G**) Western blot analysis of MGMT expression in MGMT-P140K transgenic EDLs compared to wild-type EDLs 20 days post treatment with; Lane 1: K562 cancer cells transduced with MGMT (P140K) transgene; Lane 2: untreated wild-type EDL; Lane 3: untreated MGMT (P140K) transgenic EDL; Lane 4: wild-type EDL treated with notexin; Lane 5: MGMT (P140K) transgenic EDL treated with notexin; Lane 6: wild-type EDL treated with notexin + BCNU/O^{6}BG; Lane 7: MGMT (P140K) transgenic EDL treated with notexin + BCNU/O^{6}BG. Coomassie stain of the gel shows protein loading. Western blots are representative of three experiments per experimental data set. Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; EDL, extensor digitorum longus; MGMT, O^6-methylguanine-DNA methyltransferase; O^{6}BG, O^{6}benzylguanine.

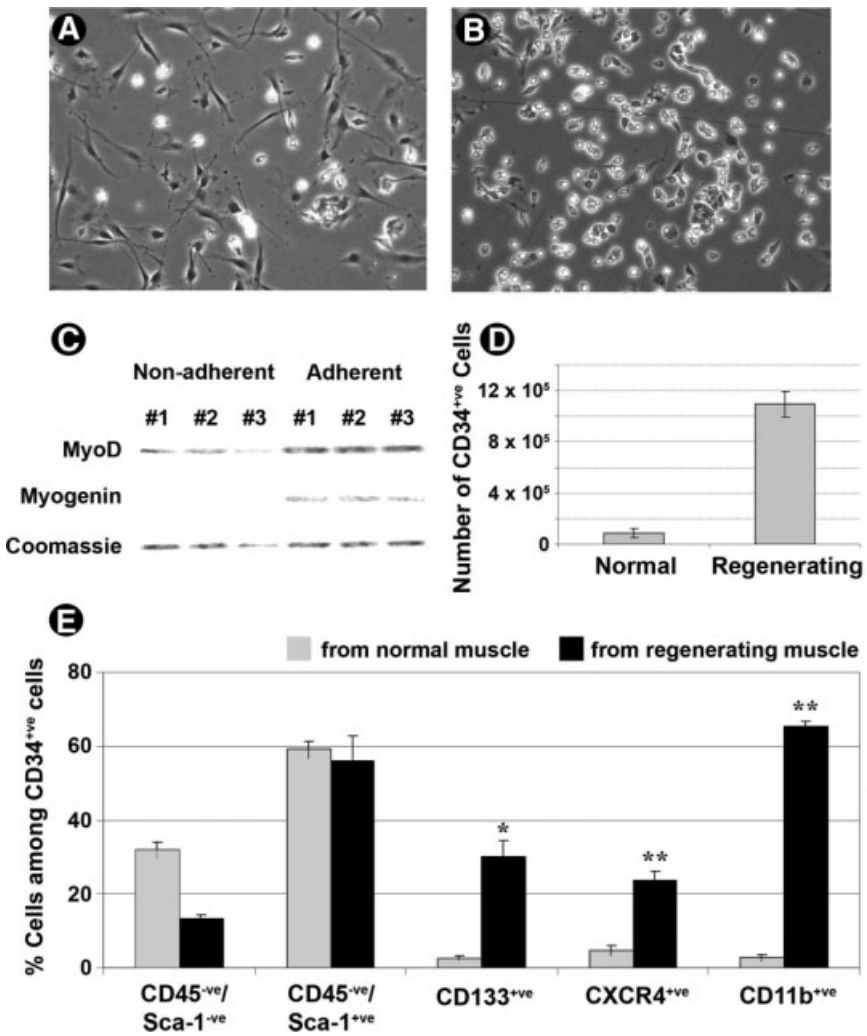

Figure 5. Characterization of $CD34^{+ve}$ donor cells isolated from normal and regenerating muscles. Representative (**A**) adherent and (**B**) nonadherent $CD34^{+ve}$ cells isolated from 3-day regenerating tibialis anterior (TA) muscles, following 24 hours in culture. (**C**): Western blot analysis of MyoD and myogenin expression in adherent and nonadherent $CD34^{+ve}$ cells from three separate samples. (**D**): Number of $CD34^{+ve}$ cells isolated from a 3-day regenerating TA compared with a normal, untreated TA. Values are mean ± SD of three samples. (**E**): Cell subpopulations present within the $CD34^{+ve}$ cells isolated from a 3-day regenerating TA compared with an unmanipulated TA. Error bars represent standard deviations of more than six different samples within a treatment group. Statistical analyses performed using *t* test. $\langle 226\rangle = {}^{*}p < .05$; $\langle 226\rangle\langle 226\rangle = {}^{**}p < .005$. Abbreviation: sca-1, stem cell antigen 1.

(P140K) transgenic EDL treated with notexin and BCNU/O^6BG (Fig. 4G, Lane 7) compared to that treated with notexin alone (Fig. 4G, Lane 5). Untreated wild-type and MGMT (P140K) transgenic muscles, and notexin injected wild-type muscles with or without BCNU/O^6BG did not contain detectable levels of MGMT (Fig. 4G, Lanes 2, 3, 4, 6).

Isolation and Characterization of $CD34^{+ve}$ Donor Cells from Regenerating Skeletal Muscle

The CD34 cell surface antigen was used to select a population of donor cells as many recently identified muscle stem cell populations commonly express this cell surface antigen [46]. This selection strategy allowed for the highest possible donor cell number without ex vivo expansion and the use of a single marker minimized the time required for sorting, while maximizing cell viability. We also reasoned that cellular heterogeneity in the donor cell population might recapitulate the regenerative niche as opposed to homogeneous cell populations. The number of $CD34^{+ve}$ cells that can be isolated from normal, uninjured muscle was low approximately 1×10^5 cells per TA muscle (Fig. 5D). The regenerating TA muscle, harvested 3 days following notexin injection, however, contained an order of magnitude more $CD34^{+ve}$ cells at approximately 11×10^5 cells per TA muscle (Fig. 5D). Within this expanded population of $CD34^{+ve}$ cells, two distinct subpopulations became distinguishable after overnight culture. One population of adherent cells displayed fibroblast-like morphology (Fig. 5A), whereas the second population of small, round, refractile cells was nonadherent (Fig. 5B). Western-blot analyses revealed a higher expression of MyoD, a marker of committed myogenic cells, in the adherent cells compared with that in the nonadherent cells (Fig. 5C). In addition, the adherent cells expressed detectable levels of myogenin, a marker of terminal muscle differentiation (Fig. 5C).

We further characterized the $CD34^{+ve}$ cell population using FACS analyses. A number of studies reported that muscle-derived stem and satellite cell progenitors in mouse commonly express CD34 and lack expression of CD45. Among these, a subpopulation of satellite cell progenitors does not express Sca-1 [39], whereas others such as muscle-derived stem cells [47], mesoangioblasts [48], and skeletal muscle side population (SP) cells [49] all express Sca-1. FACS analyses revealed that the $CD45^{-ve}/Sca\text{-}1^{-ve}$ cells comprised approximately 13% of the $CD34^{+ve}$ cells isolated from regenerating muscle compared with 31% from uninjured muscle (Fig. 5E). The representation of the $CD45^{-ve}/Sca\text{-}1^{+ve}$ subpopulation was similar in uninjured and regenerating muscles at 59 and 56%, respectively (Fig. 5E).

Nonmuscle cells such as endothelial progenitors, monocytes, and macrophages are attracted to the site of injury in skeletal muscle. These cells play a crucial role not only in promoting stem cell activation and proliferation, but also in

removing tissue debris and participating in revascularization [50]. To assess the representation of such supporting cells within the $CD34^{+ve}$ cell population, FACS analyses were performed to quantify the percentages of $CD133^{+ve}$ (endothelial progenitor cell marker), $CXCR4^{+ve}$ (receptor for the cytokine stromal derived factor-1 [SDF-1]) and $CD11b^{+ve}$ (marker for monocytes). Each of these three subpopulations comprised a very small portion of the $CD34^{+ve}$ cells isolated from uninjured muscle at approximately 3%–5% (Fig. 5E). However, in regenerating muscle, the percentages increase significantly within the $CD34^{+ve}$ population: 30% $CD133^{+ve}$, 24% $CXCR4^{+ve}$, 66% $CD11b^{+ve}$ cells (Fig. 5E). The presence of these supporting cells in substantial amounts within the $CD34^{+ve}$ cells from regenerating muscle may prove beneficial for propagating donor stem cells following transplantation by recapitulating the heterogeneous cellular niche that best supports cells that contribute to muscle regeneration.

Enhanced Survival of $CD34^{+ve}$ MGMT (P140K) Donor Cells and De Novo Muscle Fiber Formation Upon Transplantation into an Injured Muscle Bed Treated with BCNU and O^6BG

To test if conferred protection and selective enrichment of MGMT (P140K)-expressing cells can occur in vivo following transplantation, donor $CD34^{+ve}$ cells from regenerating muscles of male MGMT $(P140K)^{tg/tg}$ or male C57BL/6JArc wild-type mice were transplanted into EDL muscles of recipient female wild-type C57BL/6JArc mice. The donor cells were coinjected into the recipient muscle together with notexin (0.2 μg) to induce degeneration of otherwise healthy muscle. The recipient mice were subdivided into three groups with five mice or more in each group: Group A (wild-type donor cells only), Group B (wild-type donor cells with BCNU + O^6BG), Group C (MGMT $[P140K]^{tg/tg}$ donor cells with BCNU + O^6BG).

Recipient muscles were collected 7 days following transplantation and the percentage of male DNA was determined using quantitative-PCR. In Group A ($n = 5$), a small proportion of total DNA (0.39% ± 0.05% SD) was of donor origin (Fig. 6, Group A). This group represents a conventional transplantation protocol whereby donor cells are transplanted into a regenerating recipient muscle without any selection pressure. In Group B ($n = 8$), a higher proportion of DNA was donor derived (2.1% ± 0.6% SD), possibly reflecting the cytotoxic effect of BCNU/O^6BG treatment on the regenerating endogenous muscle stem cell population (Fig. 6, Group B). In this group, only donor cells which had exited the cell cycle at the time of transplantation would have survived, as proliferating wild-type donor cells were similarly exposed to the cytotoxic effects of BCNU/O^6BG administered at the time of transplant. Recipient muscles from Group C ($n = 10$) contained the highest proportion (6.4% ± 1.7% SD) of donor DNA content (Fig. 6, Group C), suggesting the protective effect of MGMT (P140K) against the cytotoxic effects of BCNU/O^6BG has resulted in selective survival and enrichment of the donor cells in a conditioned wild-type recipient muscle bed.

When the recipient muscles from Group B ($n = 5$) and C ($n = 5$) were collected at 14 days following transplantation, the muscles from the two groups showed a remarkable difference in size (compare Fig. 7A and 7D, muscles outlined for ease of comparison). Histological examination revealed an absence of the normal regeneration response in Group B recipient muscles (wild-type donors) with the majority of the tissue occupied by mononucleated cells (Fig. 7B, 7C). This suggests that the enhanced amount of wild-type donor cells in the BCNU/O^6BG-treated muscle bed does not manifest as enhanced muscle regeneration. In contrast, the Group C recipient muscles with donor cells from MGMT (P140K) transgenic mice showed a normal regeneration response, with abundant muscle fibers containing centrally aligned nuclei, indicative of fiber regeneration (Fig. 7E, 7F).

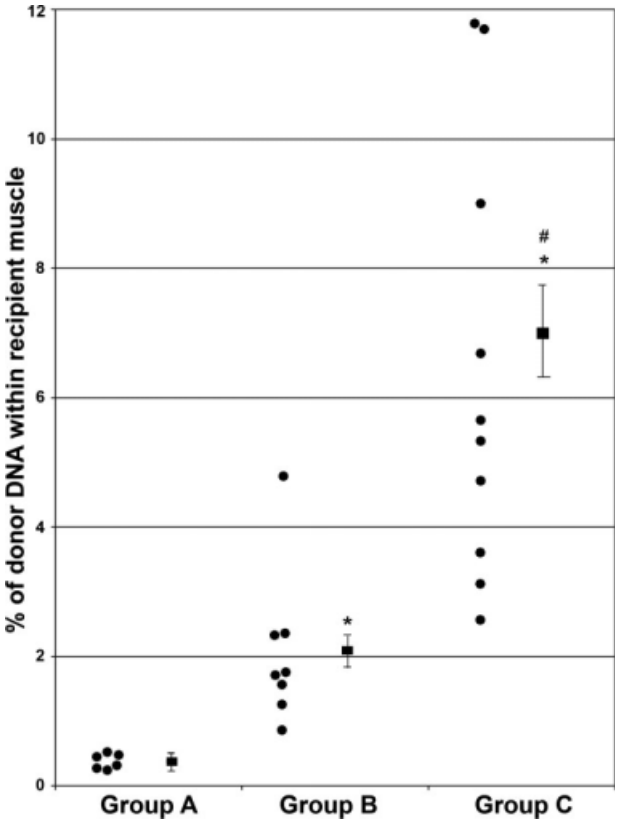

Figure 6. Enhanced engraftment of transplanted $CD34^{+ve}$ donor cells following 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) + O^6benzylguanine (O^6BG) driven selective enrichment strategy. Quantitative reverse transcriptase polymerase chain reaction was used to determine the amount of Y chromosome DNA present within the female recipient extensor digitorum longus (EDL) muscles 7 days post transplantation. Female recipient muscle treatment groups: Group A (notexin + wild-type male donor cells), Group B (notexin + BCNU/O^6BG + wild-type male donor cells), Group C (notexin + BCNU/O^6BG + O^6-methylguanine-DNA methyltransferase (MGMT) $(P140K)^{tg/tg}$ male donor cells). Black dots: percent of Y chromosome DNA in total DNA isolated from individual recipient muscles. Black squares: average amount of Y chromosome DNA in individual treatment groups. Error bars represent standard deviations of 6–10 different samples within a treatment group. Statistical analyses performed using t test. $\langle 226 \rangle = *p < .05$ compared with Group A; $\# p < .05$ compared with Group B.

To determine donor cell contribution to muscle fiber formation in the recipient muscle, the donor cells were visualized by FISH using a Y-chromosome paint probe on a longitudinal section of the Group C recipient muscle (Fig. 7G, 7H, 7I). This analysis showed aligned Y-$chromosome^{+ve}$ nuclei indicating de novo muscle fiber formation by the donor cells within the recipient muscle bed (Fig. 7I). Across the entire section, however, a significant number of nuclei was negative for the Y-chromosome marker (Fig. 7G, 7H) indicating a significant contribution to regeneration from endogenous stem cells. When quantifying the number of donor nuclei throughout the recipient muscle, we found the distal-half of the muscle contained 21% Y $chromosome^{+ve}$ nuclei (220 of 1,066 nuclei) while the proximal-half of the muscle contained 4% (46 of 1,045 nuclei). This regional difference may have resulted from the injection method used, whereby donor cells

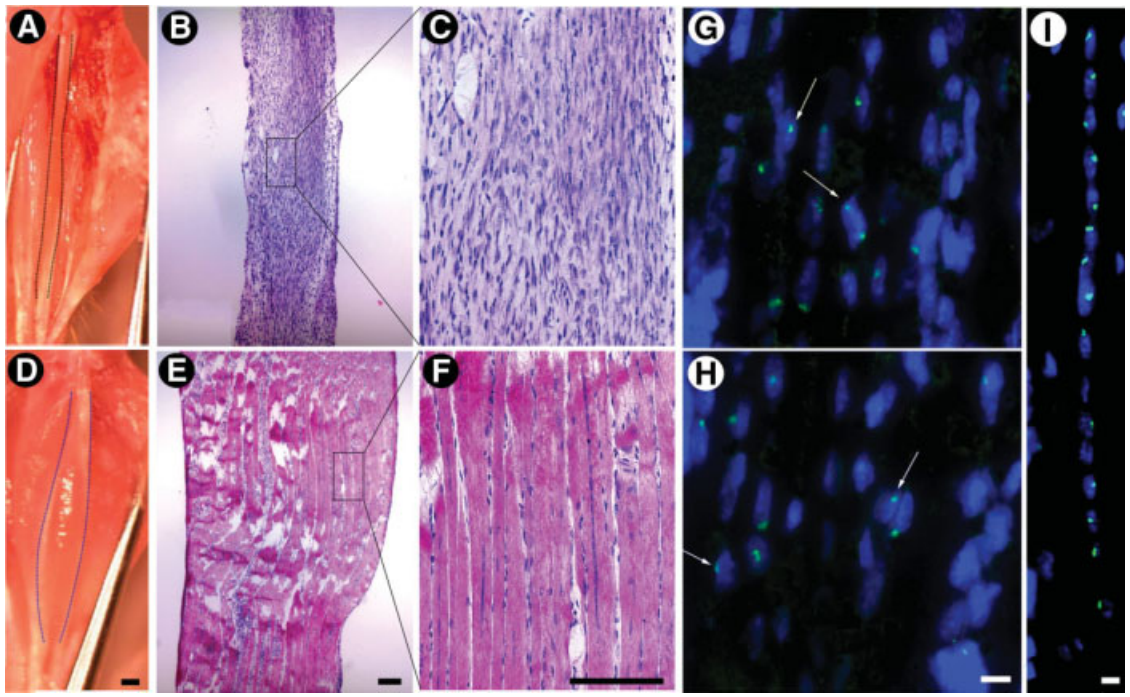

Figure 7. Participation of O^6-methylguanine-DNA methyltransferase (MGMT) $(P140K)^{tg/tg}$ donor cells in the regeneration of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) + O^6benzylguanine (O^6BG)-treated recipient muscles. Gross morphology and the number of Y-chromosome^{+ve} donor nuclei were assessed in recipient EDL muscles 14 days post-transplantation. (**A–C**): Recipient EDL muscles simultaneously received 6×10^4 wild-type $CD34^{+ve}$ donor cells, notexin, BCNU + O^6BG, (**D–F**) 6×10^4 MGMT $(P140K)^{tg/tg}$ $CD34^{+ve}$ donor cells, notexin, BCNU + O^6BG. Gross morphological examination of recipient muscles revealed a significant difference in size (compare **A** with **D**) and extent of fiber regeneration (compare **B**, **C**, with **E**, **F**). (**G–I**): Fluorescent in situ hybridization labeling of Y-chromosome^{+ve} donor nuclei (white arrows) within female recipient EDLs. Nuclei are stained with 4′,6-diamidino-2-phenylindole. (**I**): Aligned donor nuclei within the female recipient muscles. Scale bars = 1 mm (**A, D**), 200 μm (**B, E**), 200 μm (**C, F**), 10 μm (**G, H, I**).

were initially deposited into the distal end of the EDL. Nevertheless, the average donor nuclei percentage of approximately 12.5% [(4% + 21%) ÷ 2] at 14 days following transplantation indicates proliferation of donor cells when compared with the average donor DNA content of approximately 6.4% at 7 days following transplantation (Fig. 6, Group C).

Discussion

MGMT (P140K)-Mediated Donor Cell Enrichment Following Stem Cell Transplantation in Skeletal Muscle

Our results provide proof-of-concept evidence that the MGMT (P140K)-mediated BCNU/O^6BG-driven selective enrichment strategy can be successfully applied in somatic stem cell transplantation using skeletal muscle as a target organ. The strategy has been developed for in vivo enrichment of hematopoietic stem cells with proven efficacy [2, 17, 51, 52]. The only nonhematopoietic application of the strategy reported to date showed that bone marrow cells expressing MGMT (P140K) can engraft in the lung and convert into cells expressing the marker for type II pneumocytes and that the engrafted cells were enriched following alkylating agent-mediated lung injury [4]. Here, we demonstrate successful engraftment of MGMT (P140K) expressing $CD34^{+ve}$ cells following direct transplantation into an injured skeletal muscle treated with an alkylating agent. The engrafted donor cell population may include bona fide satellite cells as well as other stem cells expressing CD34 antigen at the time of donor cell isolation. Furthermore, other nonmuscle cell type(s) within the $CD34^{+ve}$ donor cell population could have provided indirect support for donor muscle stem cell engraftment and fiber formation.

In previous studies involving muscle stem cell transplantation, the poor survival of donor cells within the host niche following transplantation has been a major problem [34]. The survival and engraftment of the MGMT (P140K)-expressing donor cells shown in this study reiterates the importance of a receptive host niche in the initial survival and subsequent propagation of the donor stem cells. The concept of conditioning the host niche has led to the use of nonlethal doses of irradiation for ablating host cell activity [29, 53, 54]. However, two major draw-backs exist including the safety of applying high-dose irradiation to achieve meaningful conditioning of the niche and the nondiscriminatory nature of irradiation, leaving the donor cells equally susceptible to damage should subsequent doses of irradiation be applied. This strategy may provide an effective therapy for the treatment of diseases where specific muscles are affected, such as oculo-pharyngeal muscular dystrophy, when combined with viral transduction of donor stem cells to correct genetic mutations. Furthermore, the restricted local delivery of the alkylating agent used in this study emphasizes the safety of this delivery method targeting a specific solid organ of interest. This is encouraging when considering that cell therapy for the replacement of dysfunctional adult stem cells due to aging and in diverse genetic/degenerative disorders is being considered across many different tissue types including lung disorders, chronic liver injuries, gastrointestinal disorders, and types I and II diabetes (reviewed in ref. [55]). Our data supports the feasibility of applying the MGMT-mediated donor cell enrichment strategy in other tissue types and solid organs

where cell replacement therapy exists as a viable treatment option.

Induction of Endogenous Regeneration in a Chemo-Ablated Host Muscle Bed Following Transplantation of Donor CD34^{+ve} Cells Expressing MGMT (P140K)

The unexpected finding that endogenous cells contribute significantly to regeneration in the BCNU/O^6BG-treated recipient muscle following transplantation of MGMT (P140K)-expressing donor cells raises the question—What factors and/or cell types are marshaled by the MGMT (P140K) expressing cells and why? In the absence of transplanted MGMT (P140K)-expressing cells, this muscle is not able to self-regenerate following injury and drug treatment (this was followed up to 90 days, Fig. 3G, 3H). We postulate that factor(s) released during the formation of donor-derived myofibers attracted cells that are capable of myogenic differentiation. One such candidate is α-chemokine (SDF-1), a chemoattractant for the cells expressing its G-protein-coupled receptor CXCR4. It is well-established that SDF-1 is released from regenerating muscles [56], but more importantly, that undifferentiated muscle satellite cells express CXCR4 and respond strongly to the chemoattractant gradient provided by the presence of the SDF-1 through phosphorylation of p42/44 mitogen-activated protein kinase (MAPK) and AKT serine-threonine kinase [57].

It is therefore possible that the satellite cells residing in the muscles adjacent to the recipient EDL injected with MGMT (P140K) donor cells have migrated into this muscle and contributed to regeneration in response to SDF-1 released from fibers formed from the donor cells. However, the numbers of these cells residing in neighboring muscles would be reduced because the method of injecting BCNU, local i.v injection with a subsequent 2-minute blockade of the blood flow at the proximal base of the hindlimb, would have exposed all neighboring muscle groups (and their resident satellite cells) in the hindlimb to the alkylating effects of the BCNU. Once alkylated, the resident cells would not have been able to undergo division, thereby diminishing the contribution of satellite cells from neighboring muscles [44].

It has been documented that a single muscle fiber (containing on average seven attached satellite cells) transplanted into a radiation-ablated recipient muscle can generate over 100 new myofibers [58]. Moreover, a recent study showed that a single muscle stem cell transplanted in the same manner was able to both self-renew and give rise to myogenic progeny in successive waves following sequential muscle injuries [59]. It is therefore possible in this study, that one or a small number of satellite cells within the recipient and/or neighboring muscles was spared from alkylation, responded to signals from the engrafted donor cells and contributed the significant endogenous regeneration.

Another possibility is that the cells contributing to the endogenous muscle regeneration may have come from cells present in the circulation originating from remote sources such as bone marrow. The contribution of bone marrow cells to regenerating muscle has been documented in a number of studies [37, 49, 60, 61]. It was not clear until recently, however, whether the incorporation of bone marrow cells was the result of random fusion of inflammatory cells into the regenerating muscle or whether some cells in the bone marrow possessed the capacity to differentiate into the myogenic lineage. In a recent study, the CD45^{+ve} bone-marrow SP cells were able to give rise to CD45^{-ve}/desmin^{+ve} cells capable of myogenic differentiation [62]. These cells were able to form de novo muscle fibers within the recipient regenerating muscle injured with cardiotoxin suggesting the presence of precursors within the bone marrow that can give rise to the myogenic lineage.

The peripheral blood has long been envisioned as a "highway" for lymphoid memory cells and hematopoietic stem cells transiting through the circulation [63, 64]. With mounting evidence showing that circulating cells in the peripheral blood are able to contribute to muscle [37, 49, 60, 61], it has been hypothesized that stem cells that contribute to a variety of cell and tissue types may reside in peripheral blood as a means to maintain pools of stem cells that are readily mobilized [57]. Recently, mesenchymal stem cells (or multipotent stromal cells) have been identified as contributors to the regeneration of a range of mesenchymal tissues including bone, muscle, cartilage, tendon, and adipose tissue, with a remarkable ability to migrate and home to sites of injury (reviewed in [65]).

The potential for residual nonalkylated endogenous stem cells in neighboring muscles and/or mobilized stem cells in the peripheral blood to contribute to regeneration in response to transplanted MGMT (P140K)-expressing cells may appear to work against the strategy of selective repopulation of the recipient muscle bed. However, the potential recruitment of endogenous stem cells can be overcome and peripheral blood stem cells can be used as another source of MGMT (P140K) protected cells. The nature of the MGMT-mediated selection strategy allows further enrichment of engrafted donor stem cells by applying follow-up injections of the alkylating agent. By using this strategy, Davies et al. [24] were able to achieve 75%–100% donor cell engraftment within the host's hematopoietic stem cell compartment following transplantation of MGMT (P140K) donor cells combined with repeated treatments of alkylating chemotherapy and O^6BG. A similar study will be carried out in muscle to determine if the contribution from endogenous cells can be repressed by follow-up treatments of BCNU and O^6BG while promoting further expansion of the protected donor cells. As the MGMT (P140K) gene-mediated drug resistance-based in vivo enrichment strategy of donor stem cells was established for hematopoietic stem cell transplantation, it is conceivable that once the host's bone marrow is reconstituted with MGMT (P140K) transduced cells, they will also be able to populate peripheral blood stem cell pools and that these cells entering into regenerating muscle can be further enriched by local administration of the selective agents.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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