Enhancing In Vivo Survival of Adipose-Derived Stromal Cells Through Bcl-2 Overexpression Using a Minicircle Vector

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ABSTRACT

Tissue regeneration using progenitor cell-based therapy has the potential to aid in the healing of a diverse range of pathologies, ranging from short-gut syndrome to spinal cord lesions. However, there are numerous hurdles to be overcome prior to the widespread application of these cells in the clinical setting. One of the primary barriers to effective stem cell therapy is the hostile environment that progenitor cells encounter in the clinical injury wound setting. In order to promote cellular survival, stem cell differentiation, and participation in tissue regeneration, relevant cells and delivery scaffolds must be paired with strategies to prevent cell death to ensure that these cells can survive to form de novo tissue. The Bcl-2 protein is a prosurvival member of a family of proteins that regulate the mitochondrial pathway of apoptosis. Using several strategies to overexpress the Bcl-2 protein, we demonstrated a decrease in the mediators of apoptosis in vitro and in vivo. This was shown through the use of two different clinical tissue repair models. Cells overexpressing Bcl-2 not only survived within the wound environment at a statistically significantly higher rate than control cells, but also increased tissue regeneration. Finally, we used a nonintegrating minicircle technology to achieve this in a potentially clinically applicable strategy for stem cell therapy.

INTRODUCTION

There is a pressing need for skeletal tissue regeneration in the clinical setting. Skeletal defects from trauma, congenital abnormalities, tumor resection, and degenerative disorders often require significant amounts of replacement tissue. Large, critical-sized skeletal defects, especially in the calvarial area, do not completely heal on their own [1], leading to complications [2]. Although autologous bone grafts are the gold standard for therapy, this technique is limited by a lack of donor sites along with procedural morbidity [3]. Synthetic biomaterials are widely available but are limited by infection, expense, and breakdown over time [4]. Progenitor-based stem cell therapy would be an ideal tool for regeneration, but there are significant hurdles that need to be overcome prior to widespread clinical implantation.

One of the primary barriers is the drastic difference in environmental conditions that a progenitor cell faces when taken from a defined in vitro setting and placed into an in vivo tissue injury/wound. The stem cell is transferred from a controlled environment to a wound that is often hypoxic [5], has an upregulation of inflammatory mediators [6], and is lacking in adequate blood supply [7]. Given this harsh transition, it is no surprise that a large percentage of the implanted stem cells undergo apoptosis [8] and show poor engraftment rates [9] prior to in vivo lineage differentiation and active participation in tissue formation. Although stem cell therapy has been shown to have positive effects through paracrine mechanisms [10], the ideal strategy would enhance survival of these cells, which could increase the ability of these cells to participate in de novo tissue formation.

One way to enhance stem cell survival is through modulation of the apoptosis pathways. Decreasing the mediators of apoptosis within the stem cell could confer a survival advantage that would be helpful in the critical early days following implantation. The Bcl-2 protein is a mitochondrial protein in the intrinsic apoptosis pathway that plays a key role in the regulation of apoptosis [11]. Clinically, the Bcl-2 protein has been implicated in the development of follicular lymphomas [12]. Unlike the majority of oncogenes, the...
Bcl-2 protein does not mediate its effects through proliferation, but through the prevention of apoptosis. All apoptotic pathways converge to activate the caspases—cysteinyl aspartate proteases that are the final executioners of cell death [13]. The Bcl-2 protein prevents mitochondrial outer membrane permeabilization [14] during stressful conditions, which prevents the release of mitochondrial mediators such as cytochrome c [15] and DIABLO [16]. Decreasing the activated form of these proteins leads to decreased activation of caspases, resulting in reduced cell death.

The manipulation of the Bcl-2 protein has been shown to accrue survival advantages that present it as a favorable target [17, 18]. Pang et al. demonstrated decreased apoptosis using rat mesenchymal stem cells expressing Bcl-2 with no impairment in differentiation capacity [19]. Ardhalh et al. created a line of human embryonic stem cells that constitutively expressed Bcl-2 and found that this significantly reduced disassociation-induced apoptosis and increased cell colony viability during stress while maintaining pluripotency [20]. Wang et al. demonstrated that the upregulation of Bcl-2 does not impede the differentiation capacity of mouse embryonic stem cells [21], and Li et al. showed that expression of Bcl-2 in rat mesenchymal stem cells exhibited increased recovery of cardiac function in a rat ischemic model [22]. It is still unknown whether the same principle of decreasing apoptosis through Bcl-2 overexpression can augment tissue regeneration using human stem cells and whether this can be done through a clinically applicable strategy.

In this study, we used human adipose-derivedstromal cells (hASCs) in order to evaluate whether the overexpression of human Bcl-2 (h-Bcl-2) could produce increased in vivo healing using human multipotent stem cells. We used hASCs because of their easy clinical accessibility through a relatively simple liposapsulation procedure [23] and the ability to harvest large quantities of stem cells per harvest [24]. In order to test this hypothesis, we used different tissue/wound healing contexts: a calvarial defect to test skeletal regeneration and stented full-thickness wounds to evaluate soft tissue regeneration. We used an adenovirus vector to demonstrate that overexpression of h-Bcl-2 decreases apoptosis in vitro and in vivo and increases implantation survival and regeneration in vivo. We used bioluminescent imaging and a high-resolution magnetic resonance imaging (MRI) cell tracking approach that allowed for precise evaluation of in vivo survival after implantation. We used micro-computed tomography (microCT) to evaluate skeletal tissue formation. Using cells with h-Bcl-2 overexpression, we were able to demonstrate significantly increased tissue regeneration in both models. Finally, we used nonviral, nonintegrating minicircle technology [25] to stably express h-Bcl-2 in our stem cells to produce a survival advantage within these cells in a manner that is clinically applicable. Our data suggest that manipulation of the apoptosis pathway is a strategy that helps to overcome the environmental challenges presented to stem cells upon implantation and could significantly augment tissue regeneration in the clinical setting.

**Materials and Methods**

**Chemicals, Supplies, and Animals**

Medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco/Life Technologies (Carlsbad, CA, http://www.invitrogen.com). ABT-737 was purchased from Selleck Chemicals (Houston, TX, http://www.selleckchem.com) and reconstituted in dimethyl sulfoxide to a working stock of 10 mM. Recombinant Bcl-2 was purchased from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.com) and used at 10 μg/ml. Staurosporine was purchased from Sigma-Aldrich and reconstituted to a working stock of 1 mM. Adenovirus vectors (green fluorescence protein [GFP] and Bcl-2) were purchased from Vector Biolabs (Philadelphia, PA, http://www.vectorbiolabs.com). All viral work was performed in a BSL-2+ approved laboratory setting under the approval of the Stanford Department of Environmental Health and Safety under APB protocol 1084. Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich. CD-1 nude female mice (Crl:CD1-Foxn1nu) were obtained from Charles River Laboratories (Wilmington, MA, http://www.criver.com). All animal work was done under protocols 9999 and 21607, approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC) with ethical animal care as stated by APLAC and Stanford University.

**Cell Harvest**

Human adipose-derived stromal cells were harvested from liposaparates of female patients between the ages of 28 and 49, with an average age of 39 and an average body mass index of 28. None of the women had any major medical comorbidities. The stem cells were isolated through a series of digestions using type II collagenase as previously described [26]. All cells were passage 1 or 2. Work with human tissue was approved through the Stanford Institutional Review Board, protocol 2188, and all work with stem cells was approved by Stem Cell Research Oversight protocol #177.

**Adenoviral and Lentiviral Transduction**

Adenovirus vectors for Bcl-2, GFP, and luciferase with cytomegalovirus promoters were purchased from Vector Biolabs. For transduction of hASCs, we plated the cells in 10-cm plates at 50%–70% confluence. For transduction medium, we used OptimEM (Gibco) with polybrene (Sigma-Aldrich) at 5 μg/ml. We used a multiplicity of infection of 400 to achieve complete transduction efficiency as measured by GFP labeling of the control cells. After 24 hours, the transduction medium was removed. The same transduction conditions were used to create a stable cell line of hASCs with silencing of the Bcl-2 protein using Bcl-2-directed short hairpin RNA (shRNA) constructs and delivered through lentiviral particles (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com). Colonies were isolated with puromycin selection at 5 μM.

**In Vitro Culture Assays**

Proliferation of hASCs was assessed by bromodeoxyuridine (BrdU) incorporation as previously described [27]. Cells were grown in 96-well plates seeded at a density of 1,000 cells per well. After 24 hours in culture, BrdU labeling was performed for 8 hours. Next, BrdU incorporation was quantified using photometric enzyme-linked immunosorbent assay (Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com). Apoptosis was induced in undifferentiated hASCs using staurosporine at a concentration of 1 μM for 6 hours using standard growth medium (Dulbecco’s modified Eagle’s medium + 10% FBS). Cell viability was evaluated using the Cell Titer 96 Aqueous One Solution assay kit (Promega, Madison, WI, http://www.stemcellstm.com ©AlphaMed Press 2013
www.promega.com) to measure metabolic activity. Measurements of cells transfected with GFP+, Bcl-2, and short hairpin RNA-Bcl-2 (sh-Bcl-2) were converted to percentage viability in comparison with untreated control cells. Activated Caspase 3/7 (Promega) was measured after apoptotic induction per the manufacturer’s instructions, and luminescence was measured using the IVIS Spectrum system (Perkin-Elmer, Waltham, MA, http://www.perkinelmer.com).

**Fluorescence-Activated Cell Sorting**

After the cells were treated with staurosporine to induce apoptotic conditions, the cells were lifted using TrypLE (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and centrifuged at 1,000 rpm for 5 minutes. hASCs were labeled using the Mitochondrial Membrane Potential/Annexin V Apoptosis Kit (Invitrogen) using the manufacturer’s protocol. The cells were then analyzed using a FACSAria II instrument (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com), and the data were analyzed with FlowJo (Tree Star, Ashland, OR, http://www.treestar.com).

**Human Apoptosis Antibody Array**

We examined the expression profile of apoptosis-related proteins using a human apoptosis array kit (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) using cell lysates from control hASCs, hASCs with Bcl-2 overexpression, and hASCs with sh-Bcl-2 after treatment with apoptotic conditions. The membranes were blocked with bovine serum albumin and then treated with lysates overnight along with the detection antibody cocktail. Using a streptavidin-horseradish peroxidase, the membrane was incubated and then detected using chemiluminescence. The membrane was developed, scanned, and then analyzed for pixel density using Photoshop (Adobe Systems Inc., San Jose, CA, http://www.adobe.com).

**Enzyme-Linked Immunosorbent Assay on Cell Lysates**

After the cells were placed in the appropriate culture conditions, cell lysates were harvested per the manufacturers’ protocols. Quantikine human enzyme-linked immunosorbent assay (ELISA) kits for Bcl-2, Bax (Enzo Life Sciences, Inc., Farmingdale, NY, http://www.enzolifesciences.com), cytochrome c (Invitrogen), APAF (Novatein Biosciences, Woburn, MA, http://www.novateinbio.com), DIABLO (RayBiotech, Inc., Norcross, GA, http://www.raybiotech.com), and Cleaved-Caspase-3 (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com) were used according to the manufacturers’ protocols, as previously described for in vitro experiments. Optical density was determined using a microplate reader.

**Quantitative Reverse Transcription-Polymerase Chain Reaction**

Reverse transcription was performed with 1 µg of RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was carried out using the Applied Biosystems Prism 7900HT Sequence Detection System and SYBR Green PCR Master Mix (Applied Biosystems). Specific primers for the genes examined were based on their PrimerBank sequences and are listed in supplemental online Table 1.

**Surgical Procedures**

Nonhealing, critical-sized (4-mm) calvarial defects were created in the right parietal bones of adult (60-day-old) female CD-1 nude mice as previously described (n = 4 animals per group: hASC-adenovirus [Ad]-control [Cont], hASC-sh-Bcl-2, hASC-Ad-Bcl-2) [27]. Defects of 4 mm will not heal within the lifetime of the animal and are thus defined as critical-sized. In preparation for cell engraftment, hydroxyapatite-coated poly(lactic-co-glycolic acid) scaffolds were created from 85/15 poly(lactic-co-glycolic acid) by solvent casting and a particulate leaching process as previously described [28] and seeded with hASCs 24 hours prior to implantation. Cells (500,000) were placed on scaffolds in 200 µl of medium in 96-well culture plates and incubated for 24 hours.

**Mouse Excisional Wound Healing Model**

All experiments were performed in accordance with the Stanford University Animal Care and Use Committee Guidelines and approved APLAC protocols. Female mice, age 4–6 weeks, were housed one per cage. Two 6-mm full-thickness wounds extending through the panniculus carnosus were made at the same level on the dorsum of mice, as previously described [29]. A silicone 12-mm-diameter washer (Invitrogen) was placed around the perimeter of the wound and secured with cyanoacrylate glue and interrupted Ethilon 6-0 sutures (eSutures.com, Mokena, IL, https://www.esutures.com). Mice were randomized to four groups: phosphate-buffered saline (PBS) (negative control), control Ad-Cont-hASC, Ad-Bcl-2 hASC, and sh-Bcl-2 hASC (n = 4 animals per group, eight wounds per group). Wounds treated with transduced and untreated ASCs received 1 × 10⁶ cells per wound suspended in 80 µl of PBS, injected subdermally at the four quadrants of the wound using a tuberculin syringe at the edge of the wound. Negative controls received an equivalent volume of sterile PBS injected in four quadrants in order to evaluate healing without cell-based therapy. Wounds were dressed with Tegaderm sterile dressing (3M Healthcare, St. Paul, MN, http://www.3m.com), which was changed every other day until wound closure. Digital photographs were taken at the time of surgery and every other day until closure, defined as the time at which the wound bed was completely re-epithelialized and filled with new tissue. Wound area was quantified using Photoshop (Adobe) and expressed as a ratio of wound circumference to silicone stent circumference.

**In Vivo Imaging**

MicroCT was performed on live animals (n = 4 animals per group: hASC-Ad-Cont, hASC-sh-Bcl-2, hASC-Ad-Bcl-2) in a serial manner postoperatively (through 4 weeks of healing) using a high-resolution MicroCAT II (Gamma Medica, Salem, NH, http://www.gammamedica.com) small animal imaging system and quantified as previously described [30]. All cells implanted in vivo were transduced with an adenovirus luciferase vector prior to implantation with the protocol described above. Bioluminescent imaging (BLI) was performed on the same animals for day 1, day 2, and every other day thereafter through 10 days. Animals were anesthetized by 2%–3% inhaled isoflurane and injected intraperitoneally with the reporter probe D-Luciferin at 100 mg per kg of body weight. The IVIS Spectrum system (Xenogen) was used to image the animals while they were under 2% inhaled isoflurane anesthesia. Each animal was scanned until the peak signal of
average radiance was reached, and the radiance of regions of interest in photons was recorded. Radiance was quantified in photons per second per cm\(^2\) per steradian. A separate experimental group \((n = 3)\), unlabelled hASC, hASC control, hASC-Ad-Bcl-2) underwent high-resolution MRI. This was performed on a 7 T MRI scanner (microSigna 7.0; GE Healthcare, Waukesha, WI, http://www3.gehealthcare.com; Agilent, Walnut Creek, CA, http://www.agilent.com) after cells were prelabelled with ferucarbotran as previously described [31].

### Histological Staining

After the 1- and 4-week time points, the tissue was fixed using formalin and embedded with paraffin to create 8-µm sections. Immunohistochemistry using Bcl-2 antibody (Santa Cruz Biotechnology) and human-specific nuclear antigen (Millipore, Billerica, MA, http://www.millipore.com) was performed using Vectastain Elite Kits (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) on the 8-µm formalin-fixed paraffin section per the manufacturer’s instructions. The amount of staining was quantified using an independent blinded observer using Photoshop (Adobe). In situ apoptosis was measured using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) protocol. Cells were plated overnight without antibiotic.

### Creation of Minicircle Construct

pMC A2-minicircle backbone vector was obtained from the laboratory of Dr. Joe Wu (Stanford University). Briefly, this vector contains a multiple cloning site and poly(A) tail flanked by attP and attB sites for PhiC31-integrase recombination and 32×Scel sites for bacterial backbone degradation, which is kanamycin resistant. The human Ubiquitin C (hUbC) promoter was amplified with primers that added 5’-PstI and 3’-Nhel sites. Human B-cell lymphoma 2 (Bcl-2) was amplified with primers that added 5’-Nhel and 3’-Clai sites (supplemental online Table 2). Single-step ligation with high-concentration T4 DNA ligase (Invitrogen) allowed for the addition of both hUbC and Bcl-2. Confirmation was determined by restriction enzyme digests and DNA sequencing (Pan Facility, Stanford University). DH5a subcloning efficiency technology (Invitrogen) were used to produce parental plasmid stocks of pHMC_hUbC_Bcl2. ZCY10P3S2T Escherichia coli cells were obtained from the laboratory of Dr. Joe Wu (Stanford University), and pHMC_hUbC_Bcl2 was transformed into these cells to produce minicircle. Briefly, following overnight liquid culture growth in Terrific Broth (Invitrogen) and 50 µg/ml kanamycin, minicircle induction medium (Luria-Bertani broth [MP Biomedicals, Irvine CA, http://www.mpbio.com], 0.04 N NaOH, and 0.01% l-arabinose [Sigma-Aldrich]) was added to double the culture volume. Minicircle was purified using HiSpeed MaxiPrep kits (Qiagen, Hilden, Germany, http://www.qiagen.com) and verified on a 1.5% agarose gel relative to the parental vector (data not shown).

### Nucleofection

hASCs were trypsinized, neutralized with FBS medium, and then centrifuged at 1,000 rpm for 5 minutes. Cells were then counted using a hemocytometer and spun into 1.5-ml centrifuge tubes at a concentration of 2 × 10^6 cells per sample. Cells were resuspended in nucleofection medium specialized for mesenchymal stem cells (Lonza, Walkersville, MD, http://www.lonza.com) with 3 µg of minicircle Bcl-2 DNA or minicircle control DNA and transfected with setting B-16 on a Nucleofector (Lonza, Basel, Switzerland, http://www.lonza.com) per the manufacturer’s protocol. Cells were plated overnight without antibiotic.

### Minicircle Surgical Procedure

After nucleofection, hASCs with control minicircle vector \((n = 5)\) and h-Bcl-2 \((n = 5)\) minicircle were implanted into a critical-size calvarial defect as described above. One animal in each group was sacrificed for histology at day 7, and the rest were imaged using microCT as described above.

### Explant of Scaffold Within Calvarial Defect

For this experiment \((n = 3)\), hASC-Control, hASC-Ad-Bcl-2, after the cells were seeded and implanted according to the calvarial surgical procedure stated above, scaffolds were explanted 12 hours after in vivo placement into the calvarial defect. These scaffolds were then washed with PBS and trypsinized to release the cells from the scaffold, and cells underwent a trypan blue assay to assess cell viability.

### Statistical Analysis

All data were expressed as mean ± SEM. Student’s t test and one-way analysis of variance for multiple comparisons with the Tukey post hoc test were used to determine statistical significance, which was defined by a p value <.05.

## RESULTS

### Creation of hASCs With Bcl-2 Overexpression

We set out to create human adipose-derived stromal cells that could overexpress Bcl-2 and create a stable line that silences Bcl-2 expression. In order to confirm overexpression of Bcl-2 in our hASCs and decreased expression with our lentiviral vector, we used QRT-PCR to evaluate Bcl-2 gene expression. We were able to demonstrate that hASCs with h-Bcl-2 adenovirus transduction had significantly increased levels of Bcl-2 transcript (supplemental online Fig. 1A, *, p < .05) as compared with hASCs with control transduction and hASCs with Sh-Bcl-2 silencing. We confirmed this at the protein level through an ELISA demonstrating significantly increased production of the Bcl-2 protein (supplemental online Fig. 1B, *, p < .05). This effect was also demonstrated through histology in vivo. There was higher quantifiable staining of h-Bcl-2 in the calvarial defect area as compared with control hASCs and hASCs with Sh-Bcl-2 (supplemental online Fig. 1C, *, p < .05).

### Bcl-2 Overexpression and Apoptosis

In order to evaluate whether h-Bcl-2 overexpression confers a survival advantage for stem cells, we used an apoptosis assay using staurosporine, a tyrosine kinase inhibitor. hASCs with h-Bcl-2 overexpression showed a significant increase in viability over hASCs with control transduction and hASCs with sh-Bcl-2...
Bcl-2 Overexpression Enhances Survival

Bcl-2 Overexpression and Proliferation
We next set out to investigate whether the increased survival numbers were due to a cell proliferation effect. We used a BrdU assay with hASCs transduced with h-Bcl-2 compared with control adenovirus transduction and shRNA silencing of h-Bcl-2. hASCs with h-Bcl-2 overexpression showed a slight decrease in proliferation (Fig. 1C), whereas sh-Bcl-2 cells had a slight increase in proliferation, demonstrating that any Bcl-2 survival effects were not due to increased proliferation.

Bcl-2 Overexpression Evaluation With an Apoptosis Array
We then used an apoptosis array looking at the relative expression pattern of 35 apoptotic genes with cell lysates from hASCs with Ad-Bcl-2, Ad-GFP, and Sh-Bcl-2 treated with staurosporine (blots on left). (E): Analysis/photodensity plots of six key proteins: Bcl-2, Procaspase-3, Caspase 3, cytochrome c, Smac/DIABLO, and XIAP (*, p < .05). Abbreviations: Ad, adenovirus; Cont., control; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; hASC, human adipose-derived stromal cell; PE, R-phycoerythrin; Sh, short hairpin RNA.
with h-Bcl-2 overexpression, which would be in line with h-Bcl-2 inhibiting mitochondrial permeabilization, as both apoptotic mediators are released from within the mitochondria in response to stress or apoptotic signals (Fig. 1E, middle row). Ultimately, the overexpression of Bcl-2 significantly \((p < .05)\) decreased Caspase 3 activity (Fig. 1E, bottom row), the final mediator of apoptosis.

**Bcl-2 Overexpression Decreases Apoptotic Mediators In Vitro**

We performed quantitative real-time PCR for key apoptotic genes to look at the relative level of transcripts for hASCs with Bcl-2 overexpression versus silencing of h-Bcl-2 and control transduction hASCs. hASCs with h-Bcl-2 overexpression had significantly increased levels of transcript for the prosurvival gene XIAP (Fig. 2A, top left, \(p < .05\)). Cells with Bcl-2 overexpression had significantly \((p < .05)\) lower levels of transcript for Bcl-2-associated X protein (BAX) (Fig. 2A, top right), a competing protein of Bcl-2 that promotes mitochondrial apoptosis. Bcl-2 overexpression in hASCs not only increases the expression of prosurvival genes but also has the ability to decrease mediators of apoptosis. This was seen with the decrease in the levels of transcript of Smac/DIABLO (Fig. 2A, middle left) along with APAF-1 (Fig. 2A, middle right) in hASCs with h-Bcl-2 overexpression leading to subsequent decreased levels of Caspases 3 and 7 (Fig. 2A, bottom row, \(p < .05\)). This same principle was seen using recombinant human (rh)-Bcl-2 and ABT-737. Using ABT-737, we were able to demonstrate a decrease in h-Bcl-2 expression during apoptosis (supplemental online Fig. 2B, top left) along with an increase in BAX, Caspase 3, and Caspase 7 gene expression levels (supplemental online Fig. 2B, bottom row) during apoptosis as compared with cells treated with rh-Bcl-2 \((p < .05)\).

Next, using ELISAs, we examined the mediators of apoptosis at the protein level. hASCs with h-Bcl-2 overexpression had decreased levels of Bax (Fig. 2B, top left), demonstrating that the Bcl-2/Bax ratio was significantly \((p < .05)\) altered at the protein level to favor survival with h-Bcl-2 overexpression. There were decreased levels of APAF-1 (Fig. 2B, top right) and mitochondrial apoptotic mediators cytochrome c (Fig. 2B, middle left) and Smac/DIABLO (Fig. 2B, middle right, \(p < .05\)). Using a luminescent assay, we showed significantly decreased activity of active Caspase 3 and Caspase 7 (Fig. 2C, \(p < .05\)) with h-Bcl-2 overexpression. Collectively, these data demonstrate that h-Bcl-2 overexpression significantly decreased the mediators of apoptosis in vitro.
Increased Bone Formation and In Vivo Survival With Bcl-2 Overexpression

We then set out to test the capacity of these cells for in vivo survival and tissue regeneration. We determined that there was significantly (\(\ast, p < 0.05\)) increased early survival of the hASCs with h-Bcl-2 after implantation by explanting scaffolds placed in vivo and examining cell viability; we found that approximately 50% \((n = 3)\) of hASCs were still viable, compared with 30% viable with control cells \((n = 3)\) after 12 hours. We then placed hASCs with h-Bcl-2, control vector, and silencing of Bcl-2 into the calvarial defect and evaluated for cellular survival and skeletal regeneration. Using bioluminescence, we tracked the survival of the implanted cells (Fig. 3A). The animals with \((n = 4)\) hASCs with h-Bcl-2 overexpression showed a significant \((\ast, p < 0.05)\) increase in luminescence as compared with control luciferase hASCs \((n = 4)\) and sh-Bcl-2 hASCs \((n = 4)\) (Fig. 3B) on days 2, 6, 8, and 10. Using high-resolution MRI, we were able to further specify increased retention of h-Bcl-2-overexpressing hASCs \((n = 3)\) in the scaffold compared with control hASCs \((n = 3)\). Follow-up MRI scans up to 14 days after implantation confirmed the persistence of iron-labeled T2-hypointense cells in the calvarial defect (Fig. 3C, red arrows). The iron signal, quantified as T2 relaxation rate, persisted in both groups over the course of 14 days. However, the T2 signal was significantly \((\ast, p < 0.05)\) stronger for h-Bcl-2-overexpressing hASCs (Fig. 3D), suggesting increased survival of the hASCs with h-Bcl-2 overexpression. Next, we performed imaging studies with microCT in order to evaluate bony regeneration. At 2 and 4 weeks, we were able to appreciate greater osteogenic regeneration in the hASCs with h-Bcl-2 overexpression (Fig. 3F, right, and 3E, bottom right) at 76% healing at 4 weeks compared with control hASCs (52.5%) (Fig. 3F, middle, and 3E, bottom middle, \(\ast, p < 0.05\)). These CT data correlate with the increased cell survival, with the BLI data in Figure 3B showing that increased h-Bcl-2 expression increases cellular survival in vivo, which leads to greater regeneration.

Bcl-2 Overexpression Decreases Apoptosis In Vivo

Using an apoptosis in situ assay, we were able to demonstrate significantly \((\ast, p < 0.05)\) decreased apoptosis within the hASCs with h-Bcl-2 overexpression (Fig. 4A, right) within the calvarial defect area. In addition, we were able to show increased human
cell survival with immunohistochemistry for human nuclear antigen (Fig. 4B). There was significantly ($p < .05$) increased staining for human-specific cells within the calvarial defect area (Fig. 4B, right), demonstrating an increased presence of human cells in vivo after implantation. With immunofluorescence, we were able to show a decrease in apoptosis mediators in vivo for the animals in the calvarial defect area with implanted hASCs with h-Bcl-2 overexpression. There was decreased staining of mitochondrial mediators of apoptosis cytochrome c, Smac/DIABLO (Fig. 4C, 4D) and Caspase 3 (Fig. 4E) in the cells with hASCs with h-Bcl-2 overexpression. Most importantly, we were also able to show that there was decreased staining for the final mediators of apoptosis Caspase 3 (Fig. 4E) in calvarial bone defects implanted with cells with h-Bcl-2 overexpression.

**Bcl-2 Overexpression Accelerates Wound Healing Closure**

We evaluated the ability of h-Bcl-2 overexpression in a second in vivo tissue injury: a mouse excisional soft tissue wound healing model. After tagging each group with luciferase, we were able to evaluate the presence and survival of our cells using bioluminescence (Fig. 5A). On days 2, 4, and 10 (Fig. 5B), hASCs with h-Bcl-2 overexpression had significantly ($p < .05$) more luminescence than control hASCs. This survival advantage was even more pronounced compared with hASCs with sh-Bcl-2; hASCs with h-Bcl-2 overexpression had significantly ($p < .05$) increased luminescence at every time point. More importantly, this survival advantage also correlated with accelerated wound healing rates when compared with control animals injected with PBS. Our results demonstrate that hASCs with h-Bcl-2 overexpression showed almost complete closure by day 12 (Fig. 5C, 5D) as compared with day 14 for both controls and hASCs with sh-Bcl-2.

**Creation of Bcl-2 Minicircle Vector Increases Calvarial Regeneration**

We then created a nonintegrating minicircle vector that expressed Bcl-2 only within the lifetime of the cell (data not shown). We evaluated the ability of our minicircle vector after nucleofection to effectively express h-Bcl-2. We used a double transfection strategy with a GFP-expressing marker to look for transfection efficiency in our cells and showed that 25%–35% of our cells were positive on fluorescence-activated cell sorting.

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**Figure 4.** Bcl-2 overexpression decreases apoptosis mediators in vivo. (A): Apoptosis in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (left) showing DNA-based fragmentation ($p < .05$). Apoptosis was decreased in hASCs with Ad-Bcl-2 with corresponding quantification (right) in the area of the calvarial defect. Scale bars = 50 μm. (B): Immunohistochemistry of human nuclear antigen (left) and quantification of staining (right). A higher percentage of human cells was present in the calvarial defect area in hASCs with Ad-Bcl-2 ($p < .05$) in the area of the calvarial defect. Scale bars = 50 μm. (C-E): Immunofluorescent staining of the calvarial defect by confocal microscopy (magnification, ×63) of cytochrome c (C), Smac/DIABLO (D), and Caspase 3 (E). Top rows: No primary control. Second rows: hASCs with control-Luc. Third rows: hASCs with sh-Bcl-2. Bottom rows: hASCs with Ad-Bcl-2. Left columns: Combined DAPI and immunofluorescent Alexa Fluor 488 (green). Middle columns: DAPI (blue). Right columns: Alexa Fluor 488 (green). Scale bars = 20 μm. Abbreviations: Ad, adenovirus; DAPI, 4′,6-diamidino-2-phenylindole; hASC, human adipose-derived stromal cell; Sh, short hairpin RNA.
More importantly, we examined the transcript level with QRT-PCR (Fig. 6A) and were able to demonstrate a significant (p < .05, p < .05) increase in BCL-2 gene expression in vitro with hASCs with h-Bcl-2-minicircle. Next, we used an ELISA to demonstrate that the minicircle vector effectively (p < .05) increased the protein expression of h-Bcl-2 (Fig. 6B) in vitro. We confirmed the minicircle-h-Bcl-2’s expression level in vivo by immunohistochemistry (Fig. 6C), showing increased h-Bcl-2 expression in the minicircle-h-Bcl-2. Using microCT (Fig. 6D), we were able to demonstrate that minicircle-h-Bcl-2 is an effective way to increase tissue regeneration by showing increased bone formation with the hASCs with minicircle-h-Bcl-2, with significantly (p < .05) increased skeletal regeneration (Fig. 6E) at 1, 2, and 4 weeks as compared with hASCs with minicircle control vector.

**Figure 5.** Bcl-2 overexpression accelerates wound healing curve. (A): Representative images of bioluminescent imaging with PBS control (top row) hASCs transduced with luciferase vector (second row), sh-Bcl-2 (third row), and Bcl-2 (bottom row). (B): Quantification of luminescence for 10 days. Values are expressed as mean ± SE, with wounds with PBS control (gray) and hASCs with Bcl2 (blue) having significantly increased luminescence (∗, p < .05) versus control (beige) and sh-Bcl-2 (red). (C): Representative images of wound closure. hASCs with Bcl-2 overexpression showed increased closure of wound healing (bottom row). (D): Percentage of open wound evaluated every 2 days postwounding. hASC-Ad-Bcl-2 produced significantly (∗, p < .05) accelerated wound healing compared with hASCs with Luc control and hASCs with sh-Bcl-2 or PBS control. Values are expressed as mean ± SE. Abbreviations: Ad, adenovirus; hASC, human adipose-derived stromal cell; PBS, phosphate-buffered saline; Sh, short hairpin RNA.

**DISCUSSION**

These data demonstrate that manipulation of the apoptosis pathway through the overexpression of Bcl-2 can increase the healing efficacy of a human multipotent stem cell. First, we were able to show that the overexpression of Bcl-2 decreases the mediators of apoptosis in vitro. The overexpression of Bcl-2 increases the ratio of Bcl-2 to Bax, which can be a determining factor in the susceptibility of a cell to undergo apoptosis [32] by decreasing mitochondrial permeabilization. We have shown that by inhibiting the mitochondrial pathway of apoptosis, we can decrease the release of apoptotic mediators cytochrome c and Smac/DIABLO into the cytosol, leading to decreased activation of Apaf-1, an apoptosome that activates the caspases [33]. The overexpression of Bcl-2 also increased, in our hASCs, the level of XIAP, another anti-apoptotic protein, which has been shown to bind and inhibit Caspases 3, 7, and 9 [34]. Together, we were able to show that the overexpression of Bcl-2 in hASCs significantly decreased the activation of Caspase 3 and Caspase 7, which are the final executioners of apoptosis [35].

Using QRT-PCR, we were able to demonstrate that the overexpression of Bcl-2 also appears to decrease the transcriptional regulation of certain apoptotic factors. Although the main effects of Bcl-2’s antiapoptosis are mostly due to the protein-protein interactions decreasing the release of apoptotic factors [11, 14, 15], there is evidence that Bcl-2 can alter gene expression patterns [36]. Bcl-2 has been shown to sequester Btf, a death-promoting transcriptional factor from activity causing decreased transcription of downfield apoptotic mediators [37]. Grimm et al. [38] demonstrated that part of Bcl-2’s antiapoptotic effect could be related to an ability to decrease nuclear factor-B through nuclear gene expression modification. It is clear that Bcl-2 has a wide range of effects that combine to decrease apoptosis and transcriptional regulation, and its exact mechanism is an area that needs further delineation in order to fully understand all of Bcl-2’s antiapoptotic effects.
In attempting to cross the bridge from the laboratory to the bedside, using the paradigm of “the seed and the soil” for regenerative medicine can be a useful way of viewing the challenges facing stem cell therapy and tissue regeneration. When the soil is inherently hostile toward tissue repair, as is the case in the vast majority of clinical wound settings, research must focus on the best way to maximize survival of the stem cell. Manipulation of the apoptosis pathway can be a powerful way to augment the “seed” in the critical early days of implantation into a hostile in vivo wound/tissue injury setting. Studies in mouse models have shown that caspase inhibition can improve hematopoietic stem cell engraftment in vivo [39] and can increase the survival rate of neural stem cells through decreased apoptosis [40]. We have demonstrated that the overexpression of h-Bcl-2 in hASCs can decrease the presence of Caspase 3 in vivo and that this correlates with an increased presence of our human stem cells in the calvarial defect area through staining with human nuclear antigen.

We have shown that by decreasing the mediators of apoptosis, we can significantly increase osteogenic healing in the calvarial defect model. Whether the overexpression of Bcl-2 directly stimulates osteogenesis directly remains an interesting question. Nagase et al. demonstrated that the Bcl-2 protein is critical in augmenting the anabolic ability of the parathyroid hormone with skeletal maintenance [41], and Boot-Handford et al. showed that Bcl-2 knockout mice had thinner ossification centers in the growth plate with impaired osteoblast function [42]. These studies imply that this protein does play a role in osteogenesis, but we theorize that the majority of the reason for the increase in osteogenic healing comes from the decrease in caspase 3 activity.
increased healing comes from the antiapoptotic survival effects on our hASCs. We have shown previously that the osteogenic niche of the calvarial defect is critical in controlling the behavior of implanted progenitor cells [43]. By increasing the survival of our hASCs with Bcl-2 overexpression, instead of simply undergoing apoptosis after implantation, they have a longer window not only to have paracrine effects but also to interact with the surrounding osteogenic niche provided by the context of a skeletal defect, an osteoconductive scaffold [44], and the cues from the underlying dura mater [45]. Using these environmental cues, our implanted stromal cells have an increased opportunity to participate in directed differentiation and the formation of de novo tissue.

We used a second wound healing model in order to demonstrate that the increased survival and regeneration is not merely a specific effect of the calvarial defect and the skeletal niche. We chose a stented skin wound healing model because of the critical need for replacement epithelial tissue, especially in the context of the worldwide rise of diabetes [46] and the growing problem of diabetic foot ulcers [47]. We were able to demonstrate increased survival and regeneration using our hASCs with Bcl-2 overexpression that decreased the wound closure rate by an average of 2 days over the control group. Wound healing itself is a highly evolved, coordinated process. Any acceleration of this process to increase healing can have a significant impact on the clinical outcomes for multiple disease processes that can impair wound healing. This is especially the case when the injury is of a critical size where the endogenous progenitors within the injury environment are insufficient to provide complete healing and where replacement tissue is not readily available.

Through several models of in vivo tracking, including bioluminescence and high-resolution MRI, we were able to show that implanted hASCs with Bcl-2 overexpression showed significantly increased in vivo signaling. Although there is an expected decrease in signal due to cellular apoptosis [8] and cellular turnover [48], the hASCs with Bcl-2 overexpression showed significantly increased survival compared with control and silenced Bcl-2 hASCs. Most importantly, this increased survival also correlated with increased regeneration. Through the overexpression of h-Bcl-2, we were able to demonstrate approximately 25% greater osteogenic healing in a calvarial defect model compared with control cells and accelerated healing in a skin excisional wound model.

One of the key hallmarks of cancer stem cells is the ability to avoid apoptosis [49]. In addition, Bcl-2 has been implicated in the formation of follicular lymphomas [12]. Therefore, any strategy to overexpress Bcl-2 must take into account that although decreasing apoptosis can be critical in the early days following implantation in order to increase survival, long-term overexpression could lead to the development of cells with tumorigenic potential [50]. Specifically, the ability for the minicircle vector to efficiently express the targeted protein without integrating into the genome [51] lends the minicircle a particular advantage in the manipulation of the apoptosis pathway. Any stem cell expressing Bcl-2 with this method will overexpress the potential oncogene only within the lifetime of the cell and will not pass on replicated copies overexpressing the gene. Unlike conventional plasmid backbones, minicircle DNA lacks bacterial DNA sequences for an origin of replication, bacterial resistance markers, and inflammatory sequences that could produce an immune response [52] from the host. Another possible strategy to enhance cellular survival through Bcl-2 could come from the advent of increasingly sophisticated scaffold technology capable of delivering recombinant Bcl-2 that could provide increased survival abilities without manipulating the genome.

In addition, by using the minicircle vector, we have shown the ability to express Bcl-2 effectively without using a viral vector. Although viral vectors have high transduction efficiency, clinical viral gene therapy is limited given their safety concerns [53]. We used an enrichment strategy for the Bcl-2 minicircle. By enriching a certain percentage of implanted cells with increased survival, we showed that overall expression within the heterogeneous cohort is significantly increased and that in the calvarial model this produces significantly increased bony regeneration. Any stem cell strategy involving gene therapy with an eye toward translational medicine must look toward a method to effectively express the vector in a manner that has a high probability of Food and Drug Administration approval. This can be achieved through multiple strategies, including the use of small molecules [54], nanoparticles [55], scaffold technology [56], and minicircle. This is a powerful potential advantage of using the minicircle method for enhancing stem cell survival.

CONCLUSION

The results from this study demonstrate that Bcl-2 overexpression can increase the ability of a multipotent stem cell to increase survival within the critical early days following implantation. It also shows that this increased survival is due to the effective downregulation of the mediators of apoptosis. Most importantly, we have shown that this can increase tissue regeneration and that this can be done in a way that is a clinically viable strategy. We believe that this study can serve to demonstrate the effective modulation of the apoptosis pathway in human stem cells and that this can be used for future therapeutics as a way to increase the survival and efficacy of implanted progenitor cells in the clinic.

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AUTHOR CONTRIBUTIONS

J.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; M.G.: conception and design, collection and assembly of data, data analysis and interpretation; H.N., D.L., and D.M.: collection and assembly of data, data analysis and interpretation; S.M.: collection and assembly of data, provision of study material; M.C. and A.Z.: collection and assembly of data; G.G.W.: data analysis, manuscript writing; M.L.: provision of study material; H.D.-L. and D.C.W.: financial support, provision of study material, final approval of manuscript; M.T.L.: conception and design, financial and administrative support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

M.T.L. has uncompensated consultant/advisory and stock options.
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