Stromal Cell-Derived Factor-1β Mediates Cell Survival through Enhancing Autophagy in Bone Marrow-Derived Mesenchymal Stem Cells

Samuel Herberg[1,2], Xingming Shi[5,6,7], Maribeth H. Johnson[3], Mark W. Hamrick[2,4,6,7], Carlos M. Isales[2,4,6,7], William D. Hill[1,2,4,6,7]*

1 Charlie Norwood VA Medical Center, Augusta, Georgia, United States of America, 2 Department of Cellular Biology and Anatomy, Georgia Regents University, Augusta, Georgia, United States of America, 3 Department of Biostatistics and Epidemiology, Georgia Regents University, Augusta, Georgia, United States of America, 4 Department of Orthopaedic Surgery, Georgia Regents University, Augusta, Georgia, United States of America, 5 Department of Pathology, Georgia Regents University, Augusta, Georgia, United States of America, 6 Institute of Molecular Medicine and Genetics, Georgia Regents University, Augusta, Georgia, United States of America, 7 Institute of Regenerative and Reparative Medicine, Georgia Regents University, Augusta, Georgia, United States of America

Abstract

Bone marrow-derived mesenchymal stem/stromal cells (BMSCs) hold great potential for cell-based therapy, yet the therapeutic efficacy remains uncertain. Transplanted BMSCs often fail to engraft within the bone marrow (BM), in part due to the poor survival of donor cells in response to inflammatory reactions, hypoxia, oxidative stress, or nutrient starvation. Two basic cell processes, apoptosis and autophagy, could potentially be responsible for the impaired survival of transplanted BMSCs. However, the functional relationship between apoptosis and autophagy in BMSC homeostasis is complex and not well understood. The stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) signaling axis appears to be critical in maintaining proliferation and survival of BM stem cell populations through improving cell proliferation and survival in response to stress; however, the exact mechanisms remain unclear. We recently described novel genetically engineered Tet-Off-SDF-1β BMSCs, which over-express SDF-1β under tight doxycycline-control, thus providing an ideal model system to investigate the isolated effects of SDF-1β. In this study we tested the hypothesis that SDF-1β can mediate cell survival of BMSCs in vitro through increasing autophagy. We found that SDF-1β had no effect on BMSC proliferation; however, SDF-1β significantly protected genetically engineered BMSCs from H_2O_2-induced cell death through increasing autophagy and decreasing caspase-3-dependent apoptosis. Taken together, we provide novel evidence that the SDF-1/CXCR4 axis, specifically activated by the SDF-1β isoform, plays a critical role in regulating BMSC survival under oxidative stress through increasing autophagy.

Introduction

Over the last decade, numerous studies have revealed that bone marrow-derived mesenchymal stem/stromal cells (BMSCs) hold great potential for cell-based therapy as BMSCs possess multi-lineage potential [1]. For instance, both autologous and allogeneic BMSCs have been utilized to repair or regenerate bone in experimental and clinical studies [2,3]. However, attempts to transplant BMSCs from whole bone marrow (BM), enriched peripheral blood, or highly purified low-passage cultures almost universally fail to significantly engraft within the BM when infused into the peripheral circulation of animal and human subjects, in large part due to the poor survival of donor cells [4–8]. After being transplanted, BMSCs can face a complex hostile environment with factors that may promote cell loss/death including inflammatory reactions, hypoxia, oxidative stress including reactive oxygen species, and nutrient starvation.

Two basic self-destructive cell processes, apoptosis ('self-killing', programmed cell suicide) and autophagy ('self-eating', pro-grammed cell recycling), could potentially be responsible for the poor survival and engraftment of transplanted BMSCs used in current cell therapy protocols. Apoptosis, on the one hand, is a set of well-described forms of programmed cell death, which involves the activation of proteolytic enzymes in signaling cascades leading to the rapid destruction of cellular organelles and chromatin [9,10]. On the other hand, three forms of autophagy have been described, which in general mediate highly regulated mechanisms of cell survival. Macroautophagy (hereafter referred to as autophagy) involves the bulk turnover of cytoplasmic proteins, including damaged or pathologically aggregated proteins, in a generalized fashion as part of a constitutive homeostatic recycling process. Autophagy can be increased in response to stress to provide critically needed nutrients and energy for cellular survival; however, when extreme levels of autophagy are induced,

it can also lead to "autophagic cell death" [11–13]. Furthermore, autophagy can also specifically target distinct organelles (e.g., mitochondria in mitophagy or the endoplasmic reticulum (ER) in reticulophagy), thereby eliminating supernumerary or damaged cell structures [11,12]. During autophagy, parts of the cytoplasm and intracellular organelles are sequestered within characteristic double- or multi-membrane autophagosomes and eventually delivered to lysosomes for bulk degradation [11,12]. Importantly, the functional relationship between apoptosis and autophagy in BMSC homeostasis is complex and not well understood.

Increasing evidence suggests a critical role of the stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) signaling axis in maintaining proliferation and survival of BM stem cell populations through improving cell proliferation and survival in response to stress [14–16]. It has been speculated that SDF-1 may promote cell survival through two distinct mechanisms: post-translational inactivation of the cell death machinery (e.g., increase anti-apoptotic and decrease pro-apoptotic proteins) and increased transcription of cell survival genes [15,17–20]. In contrast, very little is known about the role of autophagy in stem cells. Three recent reports revealed that MSCs possess high levels of basal autophagy and that further induction of autophagy protects stem cell populations from hypoxia and serum-deprivation-induced cell death [21–23]. However, no direct link between the survival-enhancing effects of the SDF-1/CXCR4 axis and autophagy in BMSCs has been established.

Recently, we described genetically engineered Tet-Off-SDF-1β BMSCs that conditionally express SDF-1β; with constitutive SDF-1β overexpression and doxycycline (Dox)-mediated transgene suppression [24]. Constitutive transgene expression results in significantly increased SDF-1β mRNA (30-fold) and protein levels (5-fold) relative to Dox-suppressed and Tet-Off-empty vector (EV) control BMSCs, without altering the normal basal expression of SDF-1 splice variants. Dox control of transgene expression is tightly regulated in a dose-dependent manner with full suppression at 100 ng/ml within 24 hours [24]. Hence, our model, employing both an internal control (+Dox) and a second external empty vector control, allows us to investigate the specific role of SDF-1β in different cell processes with consistent, yet adjustable, cellular expression levels, more akin to continuous endogenous exposure. This avoids potential inconsistencies associated with direct exogenous pre-conditioning, or transient genetic overexpression, where cell exposure levels vary over time or depend largely on the degree of vector transfection. SDF-1β was chosen over the more abundant splice variant SDF-1α due to its greater resistance to proteolytic cleavage suggesting that SDF-1β may be better suited for local applications directly in the BM microenvironment, especially in bone injury sites with increased inflammatory and proteolytic activity [25–27]. Given the dearth of scientific literature examining the SDF-1/CXCR4 axis in autophagy and any specific contribution of the SDF-1β isoform to BMSC proliferation and survival, we investigated the role of SDF-1β in H_2O_2-induced cell death and tested the hypothesis that SDF-1β mediates cell survival of BMSCs through increasing autophagy, as well as decreasing apoptosis in vitro.

Materials and Methods

Isolation and Culture of BMSCs

BMSCs were derived from 18-month-old male C57BL/6J mice at the Georgia Regents University Stem Cell Core Facility. Male C57BL/6 mice were purchased from the National Institute on Aging (Bethesda, MD, USA) aged rodent colony. Animals were maintained at the Georgia Regents University - Division of Laboratory Animal Services Facility. All aspects of the animal research were conducted in accordance with the guidelines set by the Georgia Regents University Institutional Animal Care and Use Committee (GRU-IACUC) under a GRU-IACUC approved Animal Use Protocol. The BMSC isolation process, retroviral transduction to express Green Fluorescent Protein (GFP), and clonal selection have been described previously [24,28,29]. In brief, six mice were euthanized by CO_2 overdose followed by thoracotomy. Whole bone marrow aspirates were flushed from femora and tibiae and BMSCs isolated by negative immunodeple-tion using magnetic microbeads conjugated to anti-mouse CD11b (cat#558013), CD45R/B220 (cat#551513) (BD Biosciences Pharmingen, San Diego, CA, USA), CD11c, and plasmacytoid dendritic cell antigen (PDCA)-1 (cat#130-092-465) (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by positive immunoselection using anti-stem cell antigen (Sca)-1 microbeads (cat#130-092-529) (Miltenyi Biotec) according to the manufac-turer's recommendations. Enriched BMSCs were labeled with GFP [24,28,29], and maintained in Dulbecco's Modified Eagle Medium (cat#10-013) (DMEM; Cellgro, Mediatech, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (cat#S11150) (FBS; Atlanta Biologicals, Lawrenceville, GA, USA). As described in detail, clone 2 was used as the parental cells for further genetic modification with the Tet-Off system at 70–80% confluence [24].

Genetic Modification of BMSCs for Conditional Expression of SDF-1β

BMSCs were transduced with retroviral Tet-Off expression vectors. The sequential protocol of retrovirus production, two-step infection, and selection to generate double-stable Tet-Off-SDF-1β BMSCs and Tet-Off-EV control BMSCs has been described previously [24]. In brief, 293GPG packaging cells [30] were transfected at passage 8 with retroviral Tet-Off expression vectors containing the SDF-1β coding sequence, or empty control (cat#632105) (Clontech Laboratories, Mountain View, CA, USA). BMSCs (clone 2) were infected at passage 10 with 2 ml of the respective retroviral supernatant containing 4 µg/ml poly-brene (cat#H9268) (Sigma-Aldrich, St. Louis, MO, USA) and 100 ng/ml doxycycline (cat#D9891) (Dox; Sigma-Aldrich) fol-lowed by selection with 400 µg/ml G418 (cat#091672548) (MP Biomedicals, Solon, OH, USA) and 2.5 µg/ml puromycin (cat#P8833) (Sigma-Aldrich). Clonally selected parental BMSCs and Tet-Off-modified BMSCs were shown to retain their multi-potent differentiation potential, including osteogenic potential, over more than 10 passages both in vitro and in vivo upon transplantation (see [24] and unpublished data). Genetically engineered BMSCs were maintained in DMEM supplemented with 10% Tet-FBS (cat#631106) (Clontech), 400 µg/ml G418, and 2.5 µg/ml puromycin. For in vitro experiments, cells at passage 16 were plated at 2.5×10^3 cells/cm^2 and then treated with Dox starting the next day. The medium was exchanged daily. To induce cell death, genetically engineered BMSCs were incubated with 1.0 mM H_2O_2 or vehicle control for 6 h.

Cell Proliferation

Cell proliferation of BMSCs in normal growth medium was measured over the course of 7 d using the Vybrant® MTT Cell Proliferation Assay Kit (cat#V13154) (Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendation. The assay involves the conversion of the water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an in-soluble formazan, which is then solubilized using DMSO and its absorbance measured at 540 nm.

Cell and Nuclear Morphology

Morphological changes of BMSCs in response to H_2O_2 treatment were visualized by phase contrast microscopy. Furthermore, the chromatin dye Hoechst 33342 was used to assess alterations in the nuclear morphology. Cells were washed with PBS, fixed with methanol for 10 min at $-20°C$, and stained with 5 µg/ml Hoechst 33342 (cat#62249) (Pierce, Thermo Fisher Scientific) for 30 min at room temperature. BMSCs undergoing cell death were visualized by standard phase contrast and fluorescence microscopy using an inverted microscope (Carl Zeiss, Jena, Germany) equipped with an Exfo X-Cite 120 fluorescence lamp (Lumen Dynamics, Mississauga, Ontario, Canada).

Cell Viability

The viability of BMSCs in response to H_2O_2 treatment was analyzed using standard trypan blue exclusion staining. Cells were washed with PBS, lifted with trypsin/EDTA, and resuspended with normal growth medium. Following 1:5 dilution, BMSC suspensions were mixed with an equal volume of 0.4% trypan blue staining solution (cat#15250061) (Gibco, Invitrogen), and counted in 5 inner squares using a hemacytometer with cover slip (Hausser Scientific, Horsham, PA, USA).

Western Blotting

Whole cell lysates of BMSCs in response to H_2O_2 treatment were prepared in Complete Lysis-M EDTA-free buffer containing protease inhibitors (cat#04719964001) (Roche Diagnostics, Indianapolis, IN, USA). Equal amounts (20 µg) of protein lysates were subjected to SDS-PAGE using 10% NuPAGE® Bis-Tris gels (cat#NP0315BOX) (Invitrogen) and transferred to 0.2 µm PVDF membranes (cat#ISEQ00010) (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk in TBST. Apoptosis and autophagy markers were detected using specific primary antibodies (anti-poly(ADP-ribose) polymerase (PARP) (cat#9532), anti-cleaved PARP (cat#9544), anti-cleaved caspase-3 (cat#9664): Cell Signaling Technology, Danvers, MA, USA; anti-beclin 1 (cat#ab16998): Abcam, Cambridge, MA, USA; anti-LC3B-II (cat#R-155-100): Novus Biologicals, Littleton, CO, USA; anti-β-actin (cat#A1978): Sigma-Aldrich, St. Louis, MO, USA) followed by HRP-conjugated secondary antibodies (D-anti-Rb cat#711-035-152; D-anti-Ms cat#715-035-150) (Jackson ImmunoResearch, West Grove, PA, USA). Bound antibodies were visualized with the ECL detection system (cat#34080) (Pierce, Thermo Fisher Scientific) on autoradiography film (cat#E3018) (Denville Scientific, Metuchen, NJ, USA). The intensity of immunoreactive bands was quantified using Photoshop CS4 v11.0 (Adobe Systems, San Jose, CA, USA).

Statistical Analysis

Experiments were performed three independent times (n = 3–6). All data are expressed as means ± SD. Analysis of variance (ANOVA) followed by Tukey's or Bonferroni's *post hoc* test were used to determine mean differences between groups. Null hypotheses were rejected at the 0.05 level. Data were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Cell Proliferation

To investigate the role of SDF-1β in cell proliferation, we cultured BMSCs in normal growth medium and measured the absorbance of DMSO-solubilized MTT formazan at 540 nm after 1, 3, and 7 days (Fig. 1). No differences in cell proliferation were
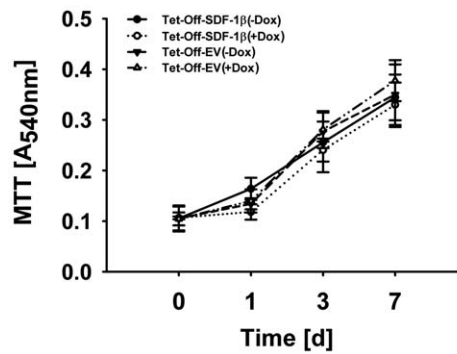

Figure 1. SDF-1β does not affect BMSC proliferation. Colorimetric quantification of DMSO-solubilized MTT formazan at 540 nm showed no differences in proliferation of Tet-Off-SDF-1β compared to Dox-suppressed and Tet-Off-EV controls (1,3, and 7 d, ±100 ng/ml Dox, n = 6, 3 independent experiments).
doi:10.1371/journal.pone.0058207.g001

found in Tet-Off-SDF-1β BMSCs compared to Dox-suppressed and Tet-Off-EV controls over the course of 7 d.

Cell and Nuclear Morphology and Viability

Next, we evaluated the potential role of SDF-1β in protecting BMSCs from cell death. The concentration of H_2O_2 necessary to deplete approximately 50–60% of BMSCs was established previously in a series of dose-response and time course studies (data not shown). BMSCs were incubated with 1.0 mM H_2O_2 for 6 h before the cell and nuclear morphology were assessed by standard phase contrast microscopy and Hoechst 33342 staining (Fig. 2 and 3). No differences in cell and nuclear morphology were found among all vehicle-treated control groups. In contrast, SDF-1β markedly protected Tet-Off-SDF-1β BMSCs from H_2O_2-induced cell death relative to Dox-suppressed (Fig. 2,3A) and Tet-Off-EV controls (Fig. 2,3B). Overall more live cells retaining the typical spindle-shaped BMSC morphology with normal round nuclei were observed compared to controls showing substantial cell loss/shrinkage and condensed nuclei, indicative of apoptosis.

These findings led us to quantify the total number of surviving cells using a standard trypan blue staining protocol (Fig. 4). In agreement with previous results, no differences in the number of trypan blue negative and positive BMSCs were found among vehicle control groups. In contrast, SDF-1β in Tet-Off-SDF-1β BMSCs significantly increased the number of surviving cells and decreased the number of dying cells in response to H_2O_2 treatment compared to Dox-suppressed (Fig. 4A) and Tet-Off-EV controls (Fig. 4B) (*trypan blue negative*: Tet-Off-SDF-1β: −Dox, $6.0×10^6±2.7×10^5$ cells, +Dox, $2.8×10^6±3.6×10^5$ cells; Tet-Off-EV: −Dox, $2.3×10^6±3.2×10^5$ cells, +Dox, $2.2×10^6±2.5×10^5$ cells; p<0.0001; *trypan blue positive*: Tet-Off-SDF-1β: −Dox, $2.3×10^6±2.5×10^5$ cells, +Dox, $5.2×10^6±4.4×10^5$ cells; Tet-Off-EV: −Dox, $5.5×10^6±4.1×10^5$ cells, +Dox, $5.4×10^6±5.4×10^5$ cells; p<0.0001).

Apoptosis and Autophagy

To further characterize the mechanisms underlying the SDF-1β-mediated protection from cell death, we investigated key players involved in apoptosis and autophagy (Fig. 5). Western blot analysis showed that the relative levels of apoptosis markers PARP (Fig. 5C), cleaved PARP (Fig. 5D), and cleaved caspase-3 (Fig. 5E) as well as autophagy markers beclin 1 (Fig. 5F) and LC3B-II (Fig. 5G) were comparable among all vehicle control

A

Tet-Off-SDF-1β
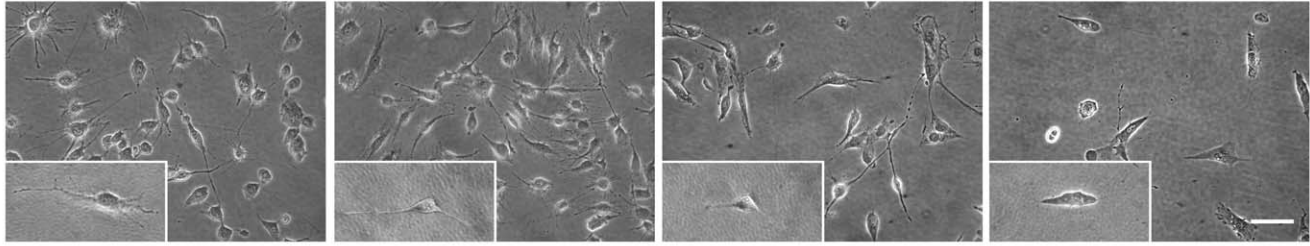
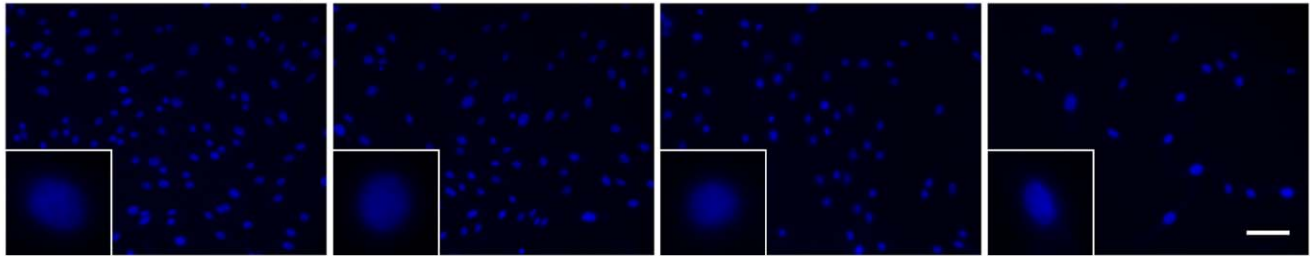| Dox | − | + | − | + |
| H₂O₂ | − | − | + | + |

B

Tet-Off-EV
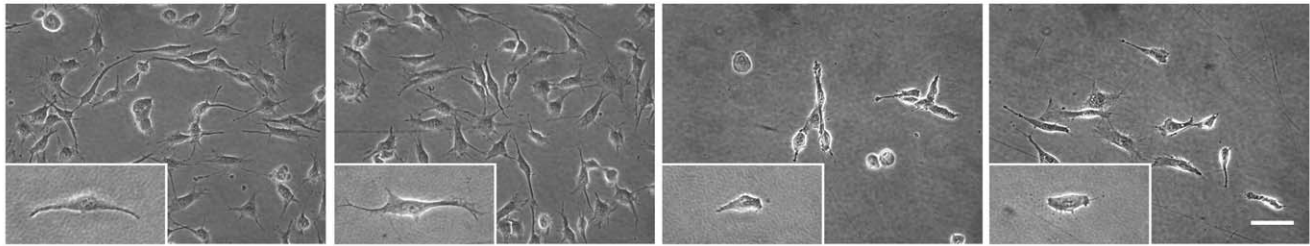
| Dox | − | + | − | + |
| H₂O₂ | − | − | + | + |

Figure 2. SDF-1β preserves BMSC morphology following exposure to H₂O₂. Representative phase contrast micrographs of A) Tet-Off-SDF-1β BMSCs and B) Tet-Off-EV control BMSCs after vehicle control or H₂O₂ treatment. Overexpression of SDF-1β in Tet-Off-SDF-1β BMSCs allows for a greater number of cells with preserved morphology after H₂O₂ treatment relative to Dox-suppressed and Tet-Off-EV controls (6 h, ±100 ng/ml Dox, ±1.0 mM H₂O₂, 20×, 40×, bar 100 μm, n = 3, 3 independent experiments).
doi:10.1371/journal.pone.0058207.g002

groups. In contrast, SDF-1β significantly increased the normalized levels of intact PARP, decreased the levels of cleaved PARP and cleaved caspase-3, and increased the levels of beclin 1 and LC3B-II in Tet-Off-SDF-1β BMSCs in response to H₂O₂ treatment (Fig. 5A–G) relative to Dox-suppressed controls (*PARP*: −Dox, 0.29±0.01, +Dox, 0.10±0.03; p<0.01; *cleaved PARP*: −Dox, 0.12±0.01, +Dox, 0.35±0.01; p<0.001; *cleaved caspase-3*: −Dox, 0.49±0.01, +Dox, 0.80±0.02; p<0.001; *beclin 1*: −Dox, 0.24±0.01, +Dox, 0.12±0.01; p<0.01; *LC3B-II*: −Dox, 0.89±0.02, +Dox, 0.53±0.02; p<0.001). No differences were found between H₂O₂-treated Tet-Off-EV control groups (Fig. 5B-G) (*PARP*: −Dox, 0.17±0.01, +Dox, 0.16±0.01; *cleaved PARP*: −Dox, 0.32±0.01, +Dox, 0.30±0.01; *cleaved caspase-3*: −Dox, 0.75±0.01, +Dox, 0.73±0.01; *beclin 1*: −Dox, 0.13±0.01, +Dox, 0.15±0.01; *LC3B-II*: −Dox, 0.70±0.01, +Dox, 0.71±0.01).

Discussion

In the present study, our aim was to utilize genetically engineered Tet-Off-SDF-1β BMSCs, which conditionally over-express SDF-1β in absence of Dox [24] as a tool to investigate whether SDF-1β could reduce H₂O₂-induced cell death through increasing autophagy and decreasing apoptosis in BMSCs *in vitro*. In the Tet-Off system, Dox prevents binding of the Tet-controlled transactivator to the Tet-promoter on the response vector and thus suppresses transcription of the downstream gene of interest, in our case SDF-1β [31]. We previously showed that SDF-1β mRNA expression by Tet-Off-SDF-1β BMSCs was 30-fold increased compared to controls and this increase was accompanied by a similar augmentation in SDF-1β protein levels, validating our model [24]. Importantly, we utilized both an internal control (+Dox) and a second external empty vector control, which have been shown to possess comparable levels of SDF-1 splice variant
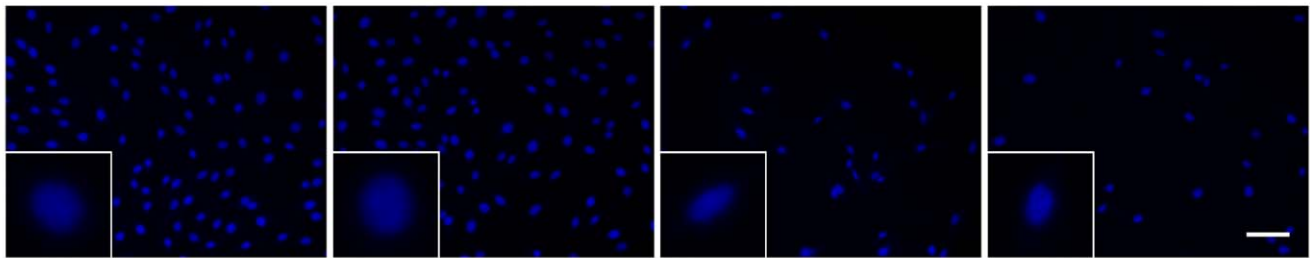
Figure 3. SDF-1β preserves BMSC nuclear morphology following exposure to H₂O₂. Representative fluorescence micrographs of Hoechst 33342-stained A) Tet-Off-SDF-1β BMSCs and B) Tet-Off-EV control BMSCs after vehicle control or H₂O₂ treatment. Overexpression of SDF-1β in Tet-Off-SDF-1β BMSCs allows for a greater number of surviving cells and cells with preserved nuclear morphology after H₂O₂ treatment compared to Dox-suppressed and Tet-Off-EV controls (6 h, ±100 ng/ml Dox, ±1.0 mM H₂O₂, 20×, 40×, bar 100 µm, n = 3, 3 independent experiments).
doi:10.1371/journal.pone.0058207.g003

expression and downstream effects when subjected to osteogenic differentiation [24].

Previous studies have suggested a critical role of the SDF-1/CXCR4 signaling axis in maintaining proliferation and survival of stem cell populations in the BM [14]. In light of the growing interest in using BMSCs for therapeutic applications, and reports that SDF-1(α) pre-treatment or over-expression promotes the proliferation and survival of rat and human BMSCs after re-oxygenation following H₂O₂-treament or exposure to the apoptosis-inducing cytokine IL-4 [15,16], we asked if SDF-1β enhances BMSCs proliferation and survival, and specifically if survival was through effects of SDF-1β on apoptotic and/or autophagic mechanisms. We showed in our system of Dox-controllable expression, that increasing SDF-1β above basal levels had no effect on BMSC proliferation over the course of 7 d compared to controls. However, we found that increasing SDF-1β significantly protected BMSCs from H₂O₂-induced cell death resulting in increased numbers of surviving cells relative to controls, which also

retained their typical spindle-shaped morphology and normal round nuclei. Hence, our studies suggest that SDF-1β-mediated protection of BMSCs against cell death could be independent from potential effects on cell proliferation.

Upon binding to its cognate receptor CXCR4, SDF-1 has been implicated in modulating the survival-enhancing PI3-kinase/Akt and MAP-kinase/Erk1/2 signaling pathways [32–35], which can be blocked by the specific CXCR4 antagonist AMD3100 [15]. In addition, it was shown that SDF-1 can increase the levels of anti-apoptotic Bcl-2 and decrease the levels of pro-apoptotic Bax [15,18]. These findings led to the investigation of the cellular mechanisms underlying the SDF-1β-dependent protection from cell death in novel Tet-Off-SDF-1β BMSCs. Therefore, we focused on key players involved in two cell death/survival processes, apoptosis and autophagy, in response to oxidative stress. Caspase activation plays a central role in the execution and completion of apoptosis. In particular, caspase-3 is critical during early apoptosis as it is involved in the proteolytic cleavage/
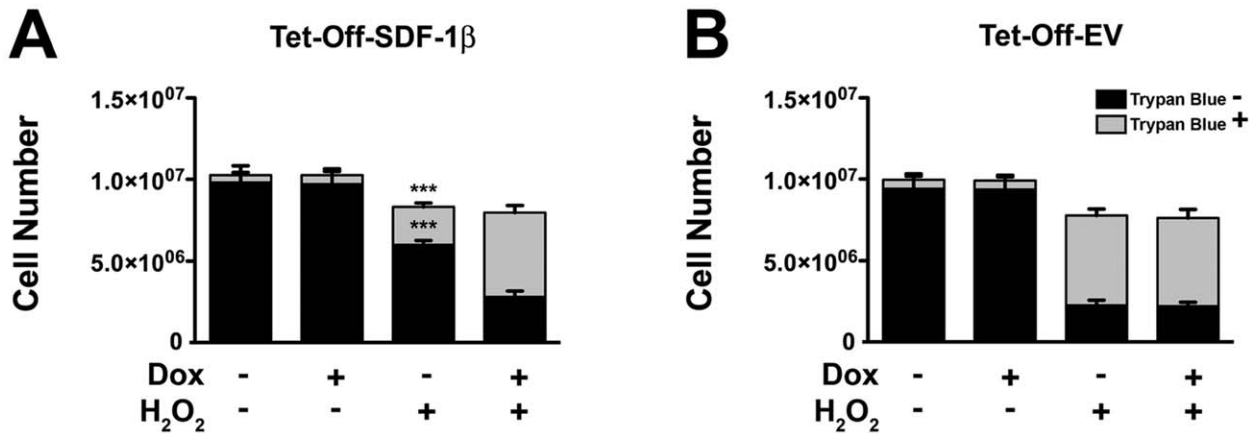
Figure 4. SDF-1β increases the number of surviving BMSCs following exposure to H$_2$O$_2$. Cell number of trypan blue-stained A) Tet-Off-SDF-1β BMSCs and B) Tet-Off-EV control BMSCs after vehicle control or H$_2$O$_2$ treatment. SDF-1β significantly increased the number of surviving cells (trypan blue negative) and decreased the number of dying cells (trypan blue positive) in response to H$_2$O$_2$ treatment compared to Dox-suppressed and Tet-Off-EV controls (6 h, ±100 ng/ml Dox, ±1.0 mM H$_2$O$_2$, ***p<0.0001, −Dox; H$_2$O$_2$ vs. +Dox; H$_2$O$_2$, n = 3, 3 independent experiments).
doi:10.1371/journal.pone.0058207.g004

activation of many key proteins such as PARP and other caspases [36,37]. Therefore, we asked whether it was possible for SDF-1β to reduce caspase-3-dependent apoptosis in BMSCs. Using Western blot analysis we showed that SDF-1β over-expression significantly decreased the levels of cleaved caspase-3, resulting in decreased levels of cleaved PARP and, in turn, increased levels of
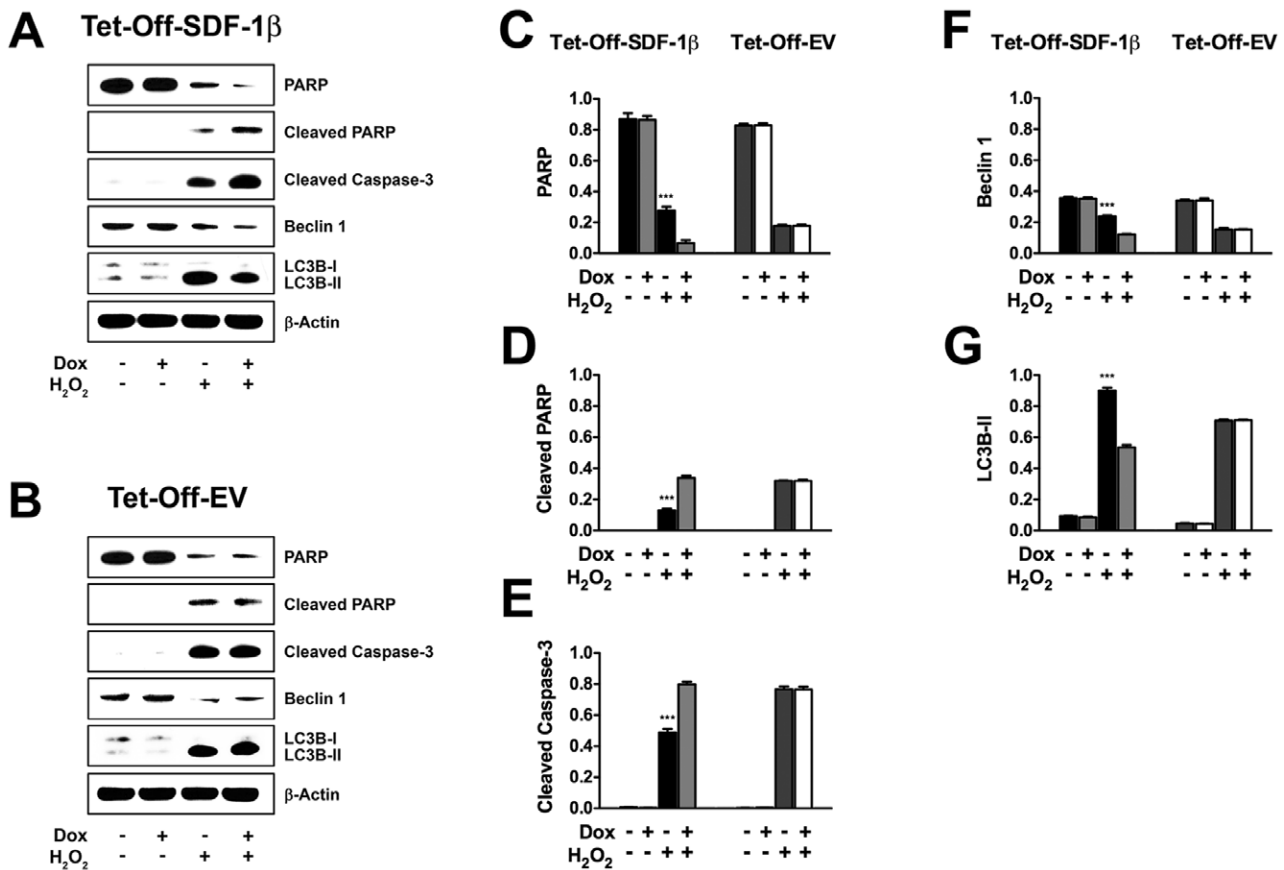

Figure 5. SDF-1β significantly increases key autophagy markers and decreases apoptotic markers during H$_2$O$_2$-induced cell death. A,B) Representative Western blots of key players involved in apoptosis and autophagy pathways. Densitometry quantification of immunoreactive bands revealed that SDF-1β significantly increased the normalized levels of C) intact PARP, decreased the levels of D) cleaved PARP and E) cleaved caspase-3, and increased the levels of F) beclin 1 and G) LC3B-II in Tet-Off-SDF-1β BMSCs in response to H$_2$O$_2$ treatment compared to Dox-suppressed and Tet-Off-EV controls (6 h, ±100 ng/ml Dox, ±1.0 mM H$_2$O$_2$, ***p<0.0001, −Dox; H$_2$O$_2$ vs. +Dox; H$_2$O$_2$, n = 3, 3 independent experiments).
doi:10.1371/journal.pone.0058207.g005

intact PARP relative to controls suggesting that SDF-1β partially blocked caspase-3-dependent apoptosis in BMSCs.

It is well accepted that similar stressors can induce either apoptosis or autophagy in a context-dependent fashion. In some cases, a mixed phenotype of apoptosis and autophagy can be detected [38]. The induction of autophagy utilizes two ubiquitin-like conjugation systems as part of the vesicle elongation process. One pathway involves the covalent conjugation of Atg12 to Atg5 and the second pathway comprises the conjugation of phospha-tidylethanolamine to LC3/Atg8. Lipid conjugation then leads to the conversion of the soluble form of LC3-I to the autophagosome-associated form LC3-II [39]. Another key player involved in the onset of autophagy is beclin 1. Like other BH3-only proteins, beclin 1 interacts with anti-apoptotic multi-domain proteins of the Bcl-2 family via its BH3 domain, and this interaction can be competitively disrupted to liberate beclin 1 and stimulate autophagy [40–46]. Despite the well-characterized signaling pathways involved, very little is known about the role of autophagy in MSC maintenance and differentiation. In a recent study it was shown that MSCs possess high levels of basal autophagy and that suppression of autophagy through knockdown of Bcl-2-xL dramatically impairs the survival and differentiation capacities of human MSCs [22]. Furthermore, the activation of autophagy has been linked to protection of MSCs from hypoxia and serum deprivation through regulating the phosphorylation of mTOR [21,23]. In addition, a genome-wide siRNA screening revealed that under normal homeostatic conditions upregulation of autophagy requires the type III PI3-kinase, but not inhibition of mTORC1 [47]. Positive regulators of cell survival and pro-liferation including SDF-1/CXCR4 were identified to be involved in regulating autophagy in different cell types [47]. While these findings are intriguing and provide new insight in the role of basal autophagy in the maintenance of mammalian cells, thus far, no direct link between the survival-enhancing effects of the SDF-1/CXCR4 axis and autophagy in BMSCs has been established.

Thus, we asked if the observed SDF-1β-mediated reduction in caspase-3-dependent apoptosis of BMSCs coincided with an increase in autophagy. Western blot analysis revealed that SDF-1β significantly increased the levels of LC3B-II and beclin 1 compared to controls, demonstrating an increase in autophagic markers, and suggesting that SDF-1β exerts part of its cell-protection through increasing autophagy in BMSCs. To our knowledge, this is the first report of a direct interaction of the SDF-1/CXCR4 signaling axis, and specifically the SDF-1β isoform, with autophagy in BMSCs. Future studies aim to clarify the autophagic pathways involved and to investigate the *in vivo* role of SDF-1β in protecting BMSCs from cell death upon local or systemic transplantation in models of bone disorders such as fracture and osteoporosis.

Conclusion

In the present study we provide novel evidence that SDF-1β, a more potent splice variant compared to SDF-1α, plays a critical role in regulating BMSC survival under oxidative stress through, at least in part, increasing autophagy *in vitro*. Our studies may provide new opportunities to support BMSC-based therapies for improving regenerative medicine approaches to treat acute and chronic bone injuries.

Author Contributions

Conceived and designed the experiments: SH XS MHJ MWH CMI WDH. Performed the experiments: SH. Analyzed the data: SH MHJ WDH. Contributed reagents/materials/analysis tools: XS. Wrote the paper: SH XS WDH.

References

1. Caplan AI (2009) Why are MSCs therapeutic? New data: new insight. J Pathol 217: 318–324.
2. Korbling M, Estrov Z (2003) Adult stem cells for tissue repair - a new therapeutic concept? New England Journal of Medicine 349: 570–582.
3. Marcacci M, Kon E, Moukhachev V, Lavroukov A, Kutepov S, et al. (2007) Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. Tissue Engineering 13: 947–955.
4. Hou Z, Nguyen Q, Frenkel B, Nilsson SK, Milne M, et al. (1999) Osteoblast-specific gene expression after transplantation of marrow cells: implications for skeletal gene therapy. Proceedings of the National Academy of Sciences of the United States of America 96: 7294–7299.
5. Nilsson SK, Dooner MS, Weier HU, Frenkel B, Lian JB, et al. (1999) Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. Journal of Experimental Medicine 189: 729–734.
6. Pereira RF, O'Hara MD, Laptev AV, Halford KW, Pollard MD, et al. (1998) Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proceedings of the National Academy of Sciences of the United States of America 95: 1142–1147.
7. Dominici M, Pritchard C, Garlits JE, Hofmann TJ, Persons DA, et al. (2004) Hematopoietic cells and osteoblasts are derived from a common marrow progenitor after bone marrow transplantation. Proceedings of the National Academy of Sciences of the United States of America 101: 11761–11766.
8. Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, et al. (2002) Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A 99: 8932–8937.
9. Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell 116: 205–219.
10. Green DR (2005) Apoptotic pathways: ten minutes to dead. Cell 121: 671–674.
11. Shintani T, Klionsky DJ (2004) Autophagy in health and disease: a double-edged sword. Science 306: 990–995.
12. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ (2007) Potential therapeutic applications of autophagy. Nat Rev Drug Discov 6: 304–312.
13. Kroemer G, Levine B (2008) Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 9: 1004–1010.
14. Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, et al. (2004) CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. J Mol Histol 35: 233–245.
15. Liu X, Duan B, Cheng Z, Jia X, Mao L, et al. (2011) SDF-1/CXCR4 axis modulates bone marrow mesenchymal stem cell apoptosis, migration and cytokine secretion. Protein Cell 2: 845–854.
16. Kortesidis A, Zannettino A, Isenmann S, Shi S, Lapidot T, et al. (2005) Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. Blood 105: 3793–3801.
17. Suzuki Y, Rahman M, Mitsuya H (2001) Diverse transcriptional response of CD4(+) T cells to stromal cell-derived factor (SDF)-1: cell survival promotion and priming effects of SDF-1 on CD4(+) T cells. J Immunol 167: 3064–3073.
18. Teicher BA, Fricker SP (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res 16: 2927–2931.
19. Lataillade JJ, Clay D, Bourin P, Herodin F, Dupuy C, et al. (2002) Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. Blood 99: 1117–1129.
20. Broxmeyer HE, Kohli L, Kim CH, Lee Y, Mantel C, et al. (2003) Stromal cell-derived factor-1/CXCL12 directly enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i proteins and enhances engraftment of competitive, repopulating stem cells. J Leukoc Biol 73: 630–638.
21. Lee Y, Jung J, Cho KJ, Lee SK, Park JW, et al. (2012) Increased SCF/c-kit by hypoxia promotes autophagy of human placental chorionic plate-derived mesenchymal stem cells via regulating the phosphorylation of mTOR. J Cell Biochem.
22. Oliver L, Hue E, Priault M, Vallette FM (2012) Basal autophagy decreased during the differentiation of human adult mesenchymal stem cells. Stem Cells Dev 21: 2779–2788.
23. Zhang Q, Yang YJ, Wang H, Dong QT, Wang TJ, et al. (2012) Autophagy activation: a novel mechanism of atorvastatin to protect mesenchymal stem cells from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway. Stem Cells Dev 21: 1321–1332.

24. Herberg S, Fulzele S, Yang N, Shi X, Hess M, et al. (2013) Stromal cell-derived factor-1beta potentiates bone morphogenetic protein-2-stimulated osteoinduction of genetically engineered bone marrow-derived mesenchymal stem cells in vitro. Tissue Eng Part A 19: 1–13.

25. Davis DA, Singer KE, De La Luz Sierra M, Narazaki M, Yang F, et al. (2005) Identification of carboxypeptidase N as an enzyme responsible for C-terminal cleavage of stromal cell-derived factor-1alpha in the circulation. Blood 105: 4561–4568.

26. De La Luz Sierra M, Yang F, Narazaki M, Salvucci O, Davis D, et al. (2004) Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. Blood 103: 2452–2459.

27. Marquez-Curtis L, Jalili A, Deiteren K, Shirvaikar N, Lambeir AM, et al. (2008) Carboxypeptidase M expressed by human bone marrow cells cleaves the C-terminal lysine of stromal cell-derived factor-1alpha: another player in hematopoietic stem/progenitor cell mobilization? Stem Cells 26: 1211–1220.

28. Zhang W, Ou G, Hamrick M, Hill W, Borke J, et al. (2008) Age-related changes in the osteogenic differentiation potential of mouse bone marrow stromal cells. Journal of Bone and Mineral Research 23: 1118–1128.

29. Zhang W, Yang N, Shi XM (2008) Regulation of mesenchymal stem cell osteogenic differentiation by glucocorticoid-induced leucine zipper (GILZ). Journal of Biological Chemistry 283: 4723–4729.

30. Ory DS, Neugeboren BA, Mulligan RC (1996) A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proceedings of the National Academy of Sciences of the United States of America 93: 11400–11406.

31. Gossen M, Bujard H (2002) Studying gene function in eukaryotes by conditional gene inactivation. Annual Review of Genetics 36: 153–173.

32. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, et al. (2003) Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 9: 1195–1201.

33. Xu R, Chen J, Cong X, Hu S, Chen X (2008) Lovastatin protects mesenchymal stem cells against hypoxia- and serum deprivation-induced apoptosis by activation of PI3K/Akt and ERK1/2. J Cell Biochem 103: 256–269.

34. Choi SC, Kim SJ, Choi JH, Park CY, Shim WJ, et al. (2008) Fibroblast growth factor-2 and -4 promote the proliferation of bone marrow mesenchymal stem cells by the activation of the PI3K-Akt and ERK1/2 signaling pathways. Stem Cells Dev 17: 725–736.

35. Zhang J, Cai H (2010) Netrin-1 prevents ischemia/reperfusion-induced myocardial infarction via a DCC/ERK1/2/eNOS s1177/NO/DCC feed-forward mechanism. J Mol Cell Cardiol 48: 1060–1070.

36. Fernandes-Alnemri T, Litwack G, Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J Biol Chem 269: 30761–30764.

37. Porter AG, Janicke RU (1999) Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6: 99–104.

38. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8: 741–752.

39. Tanida I, Ueno T, Kominami E (2004) LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 36: 2503–2518.

40. Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, et al. (2007) Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. EMBO J 26: 2527–2539.

41. Kessel D, Reiners JJ, Jr. (2007) Initiation of apoptosis and autophagy by the Bcl-2 antagonist HA14–1. Cancer Lett 249: 294–299.

42. Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, et al. (2004) Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. Cancer Res 64: 4286–4293.

43. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, et al. (2007) Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. Cell Death Differ 14: 146–157.

44. Oberstein A, Jeffrey PD, Shi Y (2007) Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. J Biol Chem 282: 13123–13132.

45. Maiuri MC, Criollo A, Tasdemir E, Vicencio JM, Tajeddine N, et al. (2007) BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-X(L). Autophagy 3: 374–376.

46. Nobukuni T, Kozma SC, Thomas G (2007) hvps34, an ancient player, enters a growing game: mTOR Complex1/S6K1 signaling. Curr Opin Cell Biol 19: 135–141.

47. Lipinski MM, Hoffman G, Ng A, Zhou W, Py BF, et al. (2010) A genome-wide siRNA screen reveals multiple mTORC1 independent signaling pathways regulating autophagy under normal nutritional conditions. Dev Cell 18: 1041–1052.