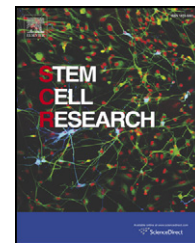Available online at www.sciencedirect.com

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The crucial role of vitamin C and its transporter (SVCT2) in bone marrow stromal cell autophagy and apoptosis

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Abstract

Vitamin C is an antioxidant that plays a vital role in various biological processes including bone formation. Previously, we reported that vitamin C is transported into bone marrow stromal cells (BMSCs) through the sodium dependent Vitamin C Transporter 2 (SVCT2) and this transporter plays an important role in osteogenic differentiation. Furthermore, this transporter is regulated by oxidative stress. To date, however, the exact role of vitamin C and its transporter (SVCT2) in ROS regulated autophagy and apoptosis in BMSCs is poorly understood. In the present study, we observed that oxidative stress decreased survival of BMSCs in a dose-dependent manner and induced growth arrest in the G1 phase of the cell cycle. These effects were accompanied by the induction of autophagy, confirmed by P62 and LC3B protein level and punctate GFP-LC3B distribution. The supplementation of vitamin C significantly rescued the BMSCs from oxidative stress by regulating autophagy. Knockdown of the SVCT2 transporter in BMSCs synergistically decreased cell survival even under low oxidative stress conditions. Also, supplementing vitamin C failed to rescue cells from stress. Our results reveal that the SVCT2 transporter plays a vital role in the mechanism of BMSC survival under stress conditions. Altogether, this study has given new insight into the role of the SVCT2 transporter in oxidative stress related autophagy and apoptosis in BMSCs.

Introduction

Vitamin C (Ascorbic acid, AA) is an essential micronutrient that acts as an important cofactor in numerous enzyme-related metabolic activities. Vitamin C is not synthesized by most mammals including humans (Naidu, 2003; Drouin et al, 2011). Animal studies have demonstrated that a deficiency of this vitamin leads to impaired bone mass, cartilage, and connective tissue (Poal-Manresa et al, 1970; Kipp et al., 1996; Miyajima et al, 1995). These musculoskeletal tissues originate from multipotent bone marrow stromal cells (BMSCs). BMSCs are progenitor cells which differentiate into osteoblasts, osteocytes, adipocytes, and cartilage (Prockop, 1997; Pittenger et al, 1999). Vitamin C plays an important role in differentiation of BMSCs into osteoblasts and osteocytes, and in collagen synthesis, cell proliferation and multilayering of osteoblastic cells (Bellows et al, 1986; Otsuka et al, 2000; Koyama et al, 2000; Wang et al, 2006; Choi et al, 2008; Weiser et al, 2009; Li et al, 2011; Kishimoto et al, 2013).

In our previous study, we reported that vitamin C is transported into BMSCs and bone with the help of the Sodium dependent Vitamin C Transporter 2 (SVCT2) (Fulzele et al, 2013). We also reported that the SVCT2 transporter helps in the in-vitro differentiation of BMSCs into osteoblastic lineage/osteogenesis and is regulated by dexamethasone and oxidative stress (Fulzele et al, 2013). We also reported that oxidants reduce BMSC cell viability and vitamin C protects cells from oxidant cytotoxicity. Autophagy and apoptosis are two complex processes through which injured cells or cell organelles are eliminated. Autophagy is an adaptive response to extracellular and intracellular stresses (Kathiria et al, 2012), including nutrient deprivation (Rikiishi, 2012), whereas apoptosis removes damaged or unwanted cells (Meijer and Codogno, 2004; Gozuacik and Kimchi, 2007; Mizushima, 2007). Autophagy is a stress adaptation process that suppresses apoptosis or alternative pathways of cell death, depending on the amount of stress on the cells (Meijer and Codogno, 2004; Gozuacik and Kimchi, 2007; Mizushima, 2007; Baehrecke, 2005). Based on these findings, we hypothesize that Vitamin C reduces oxidative stress and regulates autophagy and apoptosis in BMSCs through the SVCT2 transporter. There are two forms of SVCT transporter, SVCT1 and SVCT2. SVCT1 (SLC23A1) is mainly expressed in transporting epithelia, such as the intestine, liver and kidney, whereas SVCT2 is ubiquitously expressed in a wide variety of tissues including the placenta, liver, brain, heart, BMSCs, intervertebral disc cells and cartilage (Fulzele et al, 2013; Rajan et al, 1999; Tsukaguchi et al, 1999; Chothe et al, 2013; Bürzle et al, 2013). In this study, we examined the role of SVCT2 in oxidative-stress-induced autophagy and apoptosis in cultured BMSCs. To our knowledge, this is the first report demonstrating a relationship between the SVCT2 transporter, oxidative stress, apoptosis and autophagy in BMSCs.

Material and methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of the Georgia Health Science University.

Isolation of BMSCs from mice

Murine BMSCs were isolated from the long bones of C57BL/6 mice as previously described (Fulzele et al, 2013). The mice were euthanized and the femurs and humeri removed. The marrow was flushed with PBS and the cellular material harvested. The cellular material was centrifuged, the supernatant discarded and the pellet washed with PBS. The cells were plated in 100-cm^2 culture plates with DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin/streptomycin, and 2 mM L-glutamine. After 24 h, the supernatant were removed and the adherent stromal cells trypsinized for negative selection. A negative selection process was used to deplete hematopoietic cell lineages (T- and B-lymphocytic, myeloid and erythroid cells) using a commercially available kit (BD biosciences, Catalog, 51-9000794), thus retaining the progenitor (stem) cell population. The positive fractions were collected using the following parameters: negative for CD3e (CD3 ε chain), CD11b (integrin αM chain), CD45R/B220, Ly-6G and Ly-6C (Gr-1), and TER-119/Erythroid Cells (Ly-76). Next, positive selections were performed using the anti-Stem cell antigen-1 (Sca-1) column magnetic bead sorting kit (Miltenyi Biotec, Catalog, 130-093-222).

Cell cycle analysis by flow cytometry

For cell cycle analysis by flow cytometry, BMSCs were seeded in a 10 cm dish at 60% confluency. After 24 h, cells were treated for 24 h with oxidant (Sin-1 600 μM), antioxidant (AA 250 μM) and combination of both (Sin-1 600 μM and AA 250 μM). After completion of treatment, cells were trypsinized and washed twice with PBS. Cell pellets were resuspended in 0.5 ml PBS and fixed in 70% cold ethanol overnight at 4 °C. Ethanol-fixed cells were centrifuged at 1800 rpm for 5 min, washed twice with PBS to remove ethanol and resuspended in PBS-Triton X-100 (0.1%). The cells were concomitantly treated with RNaseA (Sigma-Aldrich, R4642) and stained with propidium iodide (Sigma-Aldrich, 81845) for 30 min at room temperature before analysis by flow cytometry. Cell cycle status was determined using a FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK) and analyzed using FlowJo-887 software.

MTT assay

In order to investigate the effect of redox reaction on cell survival in control BMSCs (shBMSCs) and SVCT2 knockdown BMSCs (shSVCT2), we pre-incubated the cells with oxidant (Sin-1 200 μM, 400 μM and 600 μM) (Cayman chemical, 82220), antioxidant (Ascorbic acid (AA), Acros organics, 352681000) (250 μM) and a combination of oxidant and antioxidant for 24 h and then used the cells for MTT assays. The number of viable cells was determined using a CellTiter 961 AQueous One MTS Cell Assay kit (Promega, G3580) as previously described (Fulzele et al, 2010).

Knockdown of SVCT2 using *Lentivirus*

Knockdown of SVCT2 was performed using *Lentivirus* as previously described (Fulzele et al, 2013). All work with

lentiviruses was performed under Biosafety Level 2 (BSL2) conditions. The lentiviral particles, shSVCT2 (sc-41008-V), shControl (sc-108080), Polybrene® (sc-134220) and puromycin (sc-108071), were purchased from Santa Cruz Biotechnology, Inc. USA. In short, BMSCs were plated at 30–50% confluence and transfected with appropriate dilutions of lentivirus particles and polybrene. Forty-eight hours after transfection, the cells were cultured in growth medium containing puromycin (2 μg/mL) to obtain the stable, transfected BMSC cells. The efficiency of shRNA activity was analyzed by real-time polymerase chain reaction (PCR) and vitamin C uptake assays as per our published method (Fulzele et al, 2013).

Analysis of GFP-LC3 redistribution

The GFP-LC3B plasmid (Addgene, Catalog, 22405) was used for identification of autophagic puncta formation (Fung et al, 2008). Briefly, cells were plated on an 8 chamber culture plate to reach 50% confluence for transfection with 1 μg of plasmids by using Lipofectamine 2000 reagent (Invitrogen, 11668-019). The cells were maintained in culture medium for 24 h to reach 80–90% confluence for treatment. For identification of GFP-LC3B-labeled puncta formation, cells were treated with Sin-1 600 μM, AA 250 μM and a combination (Sin-1 600 μM + AA-250 μM) for 4 h. At the end of incubation, cells were fixed with 4% paraformaldehyde and examined by fluorescence microscopy to count the cells with punctate GFP-LC3B. Cell images were also collected by confocal microscopy.

Western blot analysis

For Western blot analysis, cells were treated with Sin-1 600 μM, AA 250 μM and a combination (Sin-1 600 μM and AA 250 μM) for 4 h. Protein was extracted from cells, subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with a polyclonal antibody against LC3B (Abcam, ab48394) and p62 (Abcam, ab56416) overnight at 4 °C, followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized with an ECL Western blot detection system (Thermo Scientific, Waltham, MA). GAPDH served as the loading control. Densitometry measurements were obtained by calculating the ratio of band density of LC3B and p62 to GAPDH using Adobe Photoshop CS2.

Annexin V staining

For identification of apoptotic cells in control and SVCT2 knockdown BMSCs, cells were treated with Sin-1 (600 μM), AA (250 μM) and a combination of sin-1 and AA (Sin-1 600 μM + AA 250 μM,) for 4 h. Cells were stained with annexin V and PI, as per the manufacturer's directions (BD Pharmingen™, 559763). Briefly, cells were trypsinized and washed twice with PBS, the supernatant was discarded and the pellet was re-suspended in 95 μL annexin-V-binding buffer at a density of 0.6×10^6 cells/mL with 2.5 μg/mL annexin-V-FITC at 4 °C for 30 min. Cells were analyzed by flow cytometry on a FACScan cytometer after addition of

2.5 μg/mL PI. 10,000 events were analyzed using the FL-1 and FL-3 detector filters.

Statistics

All experiments were performed at least three times, in duplicate or triplicate. GraphPad Prism 5 (La Jolla, CA) was utilized to perform ANOVA with Bonferroni pairwise comparison or unpaired t-tests as appropriate. A P value of <0.05 was considered significant.

Results

Effect of oxidative stress on BMSCs cell survival and cell cycle analysis

To examine the cytotoxicity of Sin-1 on BMSCs, we treated BMSCs with Sin-1 at concentrations of 600 μM, 800 μM and 1000 μM for 24 h. To quantify the effect of Sin-1 on cell survival, we performed an MTT assay. As shown in Fig. 1a, Sin-1 decreased cell number in a dose-dependent manner. In control cells with normal SVCT2 expression, 1000 μM of Sin-1 caused the largest decrease (~30%) in cell numbers, whereas 800 μM and 600 μM caused ~15% and ~5% decreases respectively (Fig. 1a). Supplementation of AA to control cells nullified the effects of Sin-1. To investigate whether cell cycle arrest is induced in BMSCs by Sin-1, we performed DNA flow cytometric analyses. As shown in Fig. 1b, Sin-1 treatment increased the population at the G1 phase by ~40%. These results indicate that Sin-1 induced G1 arrest in BMSCs.

Effect of oxidative stress on shSVCT2-BMSCs (SVCT2 knockdown-BMSCs) cell survival

To investigate the role of SVCT2 in oxidative-stress-induced decreases in BMSC survival, a lentivirus-based shRNA system was used to knockdown SVCT2 in BMSCs. The knockdown efficiency of lentivirus shSVCT2 in BMSCs was ~40–50% as assessed by both an ascorbic acid (AA) uptake assay and real time PCR (Supplementary Fig. S1) (Fulzele et al, 2013). We hypothesized that SVCT2 knockdown cells would be weaker and more susceptible to oxidative stress. The shSVCT2 cells showed a drastic reduction in cell numbers; i.e., 1000 μM of Sin-1 caused the highest decrease (~95%) in cell numbers whereas 800 μM and 600 μM caused ~60% and ~40% decreases respectively (Fig. 2). Supplementation of AA to shSVCT2 cells caused a significant improvement in survival, but much more cell death compared to control cells.

Oxidative stress induces SVCT2 dependent autophagy in BMSCs

To analyze the combined effect of oxidative stress and down-regulation of SVCT2 on autophagy in BMSCs, we performed Western blot analysis on the reliable markers for autophagy, LC3B and P62 proteins (Tang et al, 2011; Cheng et al, 2013). We observed that both LC3B and p62 protein levels were significantly up-regulated in a time dependent manner following oxidative stress (Fig. 3a) and the supplementation of
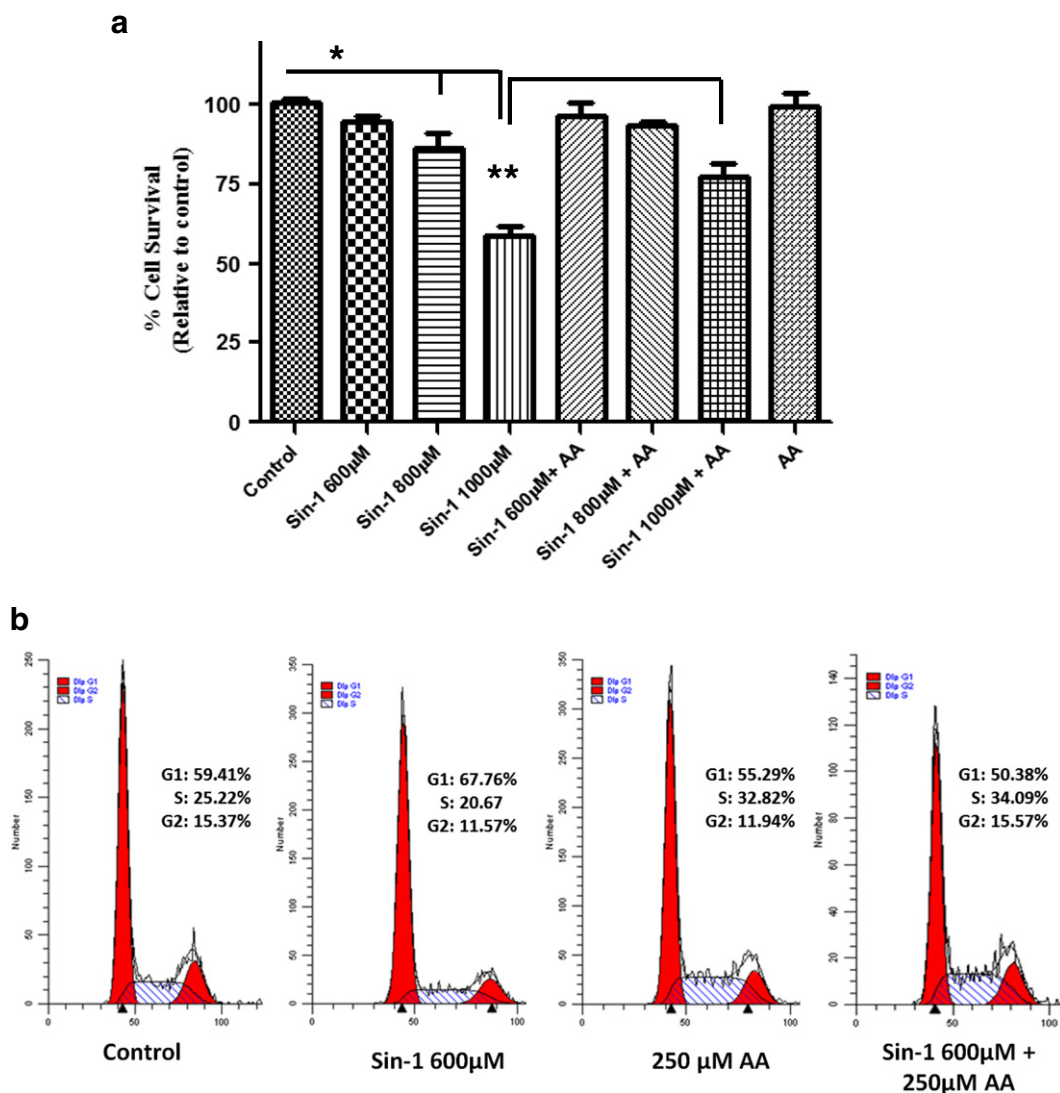
Figure 1 Effect of oxidative stress on cell survival and cell cycle progression of BMSCs. a) Cells were treated with increasing concentrations of Sin-1 (600–1000 μM), ascorbic acid (250 μM) and combination of both. Data were recorded after 24 h following treatment. b) Sin-1 induced G1 cell cycle arrest. BMSCs were treated with Sin-1 (600 μM), ascorbic acid (250 μM) and combination of both for 24 h. Cells were harvested after 24 h then they were fixed, stained and analyzed for DNA content. The distribution and percentage of cells in G1, S and G2 phase of the cell cycle are indicated. Data were analyzed by ANOVA followed by Bonferroni post hoc test (*$p < 0.05$; ** $p < 0.01$ n = 6).

AA further surged the autophagic flux and led to the disappearance of LC3B and p62 expression (Fig. 4). ShSVCT2 cells showed accumulation of p62, which is an indication of compromised autophagic flux (Fig. 3b), and AA supplementation failed to decrease the expression of autophagic markers and led to the accumulation of LC3B and p62 proteins (Fig. 4). We also analyzed GFP-LC3B-labeled puncta formation to focus on subcellular localization and redistribution of LC3B from cytosol to a punctate autophagosome staining, which is an indication of autophagy. Representative LC3B stainings in shControl and shSVCT2 BMSCs are shown in Fig. 5. By analyzing LC3B redistribution, we found that in shControl BMSCs, Sin-1 treatment increased the GFP-LC3B puncta autophagic vacuoles, and supplementation with AA led to gradual disappearance of LC3B puncta, an observation that was consistent with the degradation of LC3B in Western blot analysis (Fig. 4). The

ShSVCT2 cells showed even higher level of GFP-LC3B-labeled puncta formation with Sin-1 treatment, and supplementation of AA failed to rescue and consequently enhanced formation of punctate LC3B.

SVCT2 knockdown cells are more sensitive to oxidative-stress-induced apoptosis

We observed that SVCT2 knockdown cells were more sensitive to oxidative stress, with reduced cell proliferation and compromised autophagy. To further investigate the role of the SVCT2 transporter in cell death, the cells were treated either with/without Sin-1, AA, or in combination and stained with Annexin V-FITC/PI. Annexin-V binds to those cells that express phosphatidylserine on the outer layer of
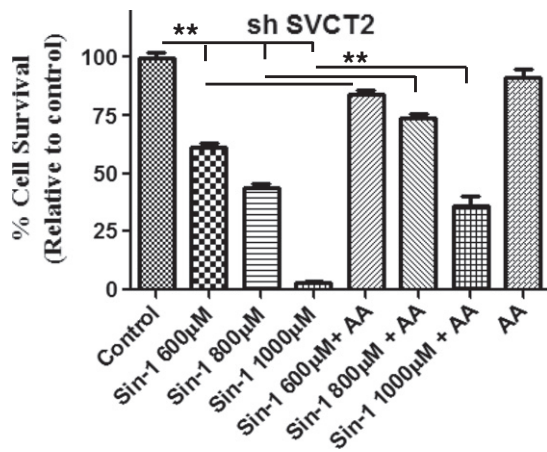
Figure 2 Effect of oxidative stress on cell survival of SVCT2 knockdown BMSCs. a) Cell were treated with increasing concentrations of Sin-1 (600–1000 μM) and ascorbic acid (250 μM) and combination of both. Data were recorded after 24 h following treatment. Data were analyzed by ANOVA followed by Bonferroni post hoc test (*p < 0.05; ** p < 0.01 n = 6).

the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with annexin-V)

and necrotic cells (stained with both annexin-V and PI). Apoptosis marker analysis (Annexin V and PI) suggested significant increases of cell death from oxidative stress in shSVCT2 cells compared to control, and AA did not rescue shSVCT2 cells efficiently from oxidative stress. We observed a significant increase in early (Annexin V staining) and late apoptosis (Annexin V and PI) in shSVCT2 cells (Fig. 6). Overall, down-regulation of SVCT2 in BMSCs hampered cell proliferation, cell survival, and anti-apoptotic properties. These basic properties are important to rescue cells from any type of stress during disease conditions.

Discussion

In our previous studies, we showed that SVCT2 plays an important role in BMSC differentiation, cell attachment, and migration (Fulzele et al, 2013; Sangani et al, 2014). We also demonstrated that the SVCT2 transporter is down-regulated by oxidative stress (Fulzele et al, 2013). In the continuation of this work, the present study demonstrates that oxidative stress decreased cell viability in a dose dependent manner and AA rescued cells from oxidative stress. Cell cycle analysis revealed that oxidative stress induced cell arrest in the G1 phase and AA rescued cells from G1 growth arrest. Here, we demonstrated that the effect of oxidative stress on BMSCs was associated with a specific disruption of cell cycle events and an induction of G1 arrest, which may further lead to cell
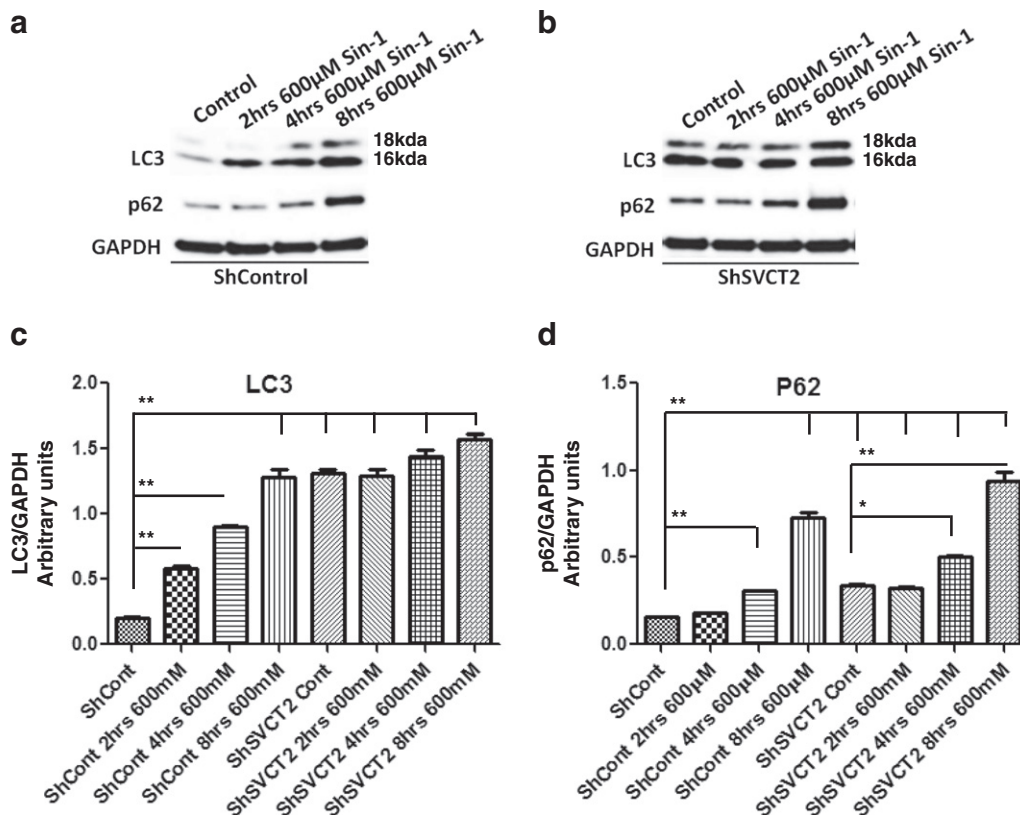

Figure 3 Regulation of autophagy in shcontrol BMSCs and SVCT2 knockdown BMSCs. Representative western blots of LC3B and p62 key players involved in autophagy pathways. a) shcontrol BMSCs treated with Sin-1 (600 μM) in time dependent manner for 2 h, 4 h and 8 h. b) SVCT2 knockdown BMSCs treated with Sin-1 (600 μM) in time dependent manner for 2 h, 4 h and 8 h. c) Densitometry quantification of LC3 and d) p62. Data were analyzed by ANOVA followed by Bonferroni post hoc test (*p < 0.05; ** p < 0.01 n = 4).
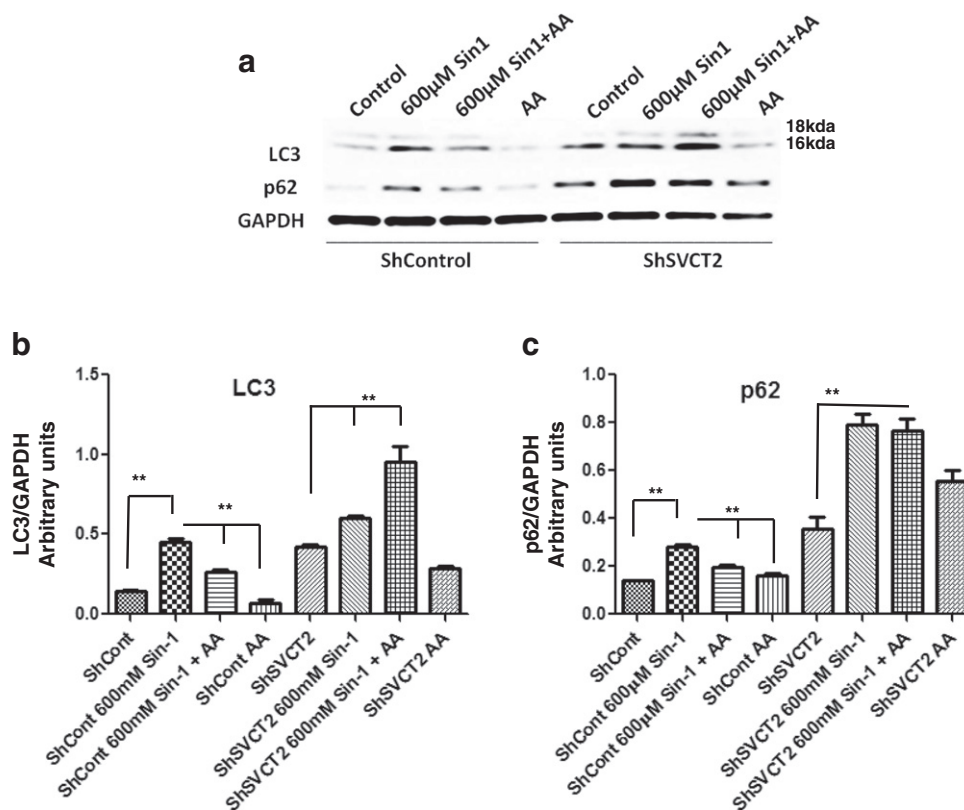
Figure 4 Vitamin C rescue shcontrol BMSCs from oxidative stress but not in SVCT2 knockdown BMSC cells. a) shcontrol BMSCs and SVCT2 knockdown BMSCs treated with Sin-1 (600 μM), ascorbic acid (250 μM) and combination of both for 4 h. Representative western blots of LC3B and p62 key players involved in autophagy pathways. b) Densitometry quantification of LC3 and c) p62. Data were analyzed by ANOVA followed by Bonferroni post hoc test (*p < 0.05; ** p < 0.01 n = 4).

death. The cells arrested in G1 phase may undergo apoptosis or recover and enter into next phase of cycle (Yuan et al, 2006; Deng et al, 2004; Chen and Makino, 2004). In our previous study, we reported that SVCT2 is down-regulated by oxidative stress. To correlate the role of the SVCT2 transporter in vitamin C-mediated rescue of cells from oxidative stress, we knocked down SVCT2 expression in BMSCs cells using shSVCT2 and performed a cell survival assay. The results show that shSVCT2 cells were more sensitive to oxidative stress even at low doses, with significantly reduced cell number compared to control cells. Furthermore, AA helped the control cells to recover from oxidative stress to almost normal levels but this protection did not occur in shSVCT2 cells. We also made an important observation that oxidative stress susceptibility of BMSCs depended on cell density (Supplementary Fig. S2).

Cell survival or death is a complex process and autophagy is an integral part of this process. Autophagy plays important role in cell survival during metabolic or environmental stress (Papáčková and Cahová, 2014). In the present study, we investigated the importance of SVCT2 transporter in vitamin C dependent autophagy in BMSCs. We demonstrated that oxidative stress treated BMSC cells had increased levels of LC3B, a specific marker for autophagic vesicles, which contributed to the formation of autophagosomes. Furthermore, GFP-LC3B transfected cells with punctate GFP-LC3B distribution were markedly increased after oxidative stress, which represented the recruitment of LC3B to autophagic vacuoles.

We also analyzed the expression of P62 following oxidative stress, antioxidant and a combination of both at the protein level in shcontrol and shSVCT2 BMSCs. Sin-1 induced oxidative stress accompanied by p62 accumulation, indicative of compromised autophagy, whereas vitamin C rescued from stress by attenuating the p62 buildup. Thus, Vitamin C promotes autophagy and protects cells from undergoing apoptosis. Interestingly, we observed that shSVCT2 cells have higher P62 expression in the oxidant-treated cells, and there was no significant effect of antioxidant. This indicates that SVCT2 knockdown cells have impaired autophagy flux and even vitamin C is not able to recover from stress. Accumulation of LC3B and p62 in SVCT2 knockdown cells even after supplementation of vitamin C indicated complete failure of autophagy mechanism. Normally, the toxic cellular waste is recognized by P62 which is then scavenged by a sequestration process (Rusten and Stenmark, 2010). Lack of autophagy regulation increases P62 accumulation, which ultimately leads to abnormal cellular stress (Sansanwal and Sarwal, 2012; Johansen and Lamark, 2011). In normal BMSCs, vitamin C helps to recover from dysfunctional autophagy. Vitamin C induces autophagy in normal BMSCs in oxidative stress condition and decreases p62 accumulation. The loss of p62 protein is a measure of the flux of autophagy and indicative of active autophagy (Patel et al, 2013; Mohammed et al, 2013). We hypothesized that failure of autophagy mechanism in SVCT2 knockdown cells may lead to increased cell apoptosis and necrotic.
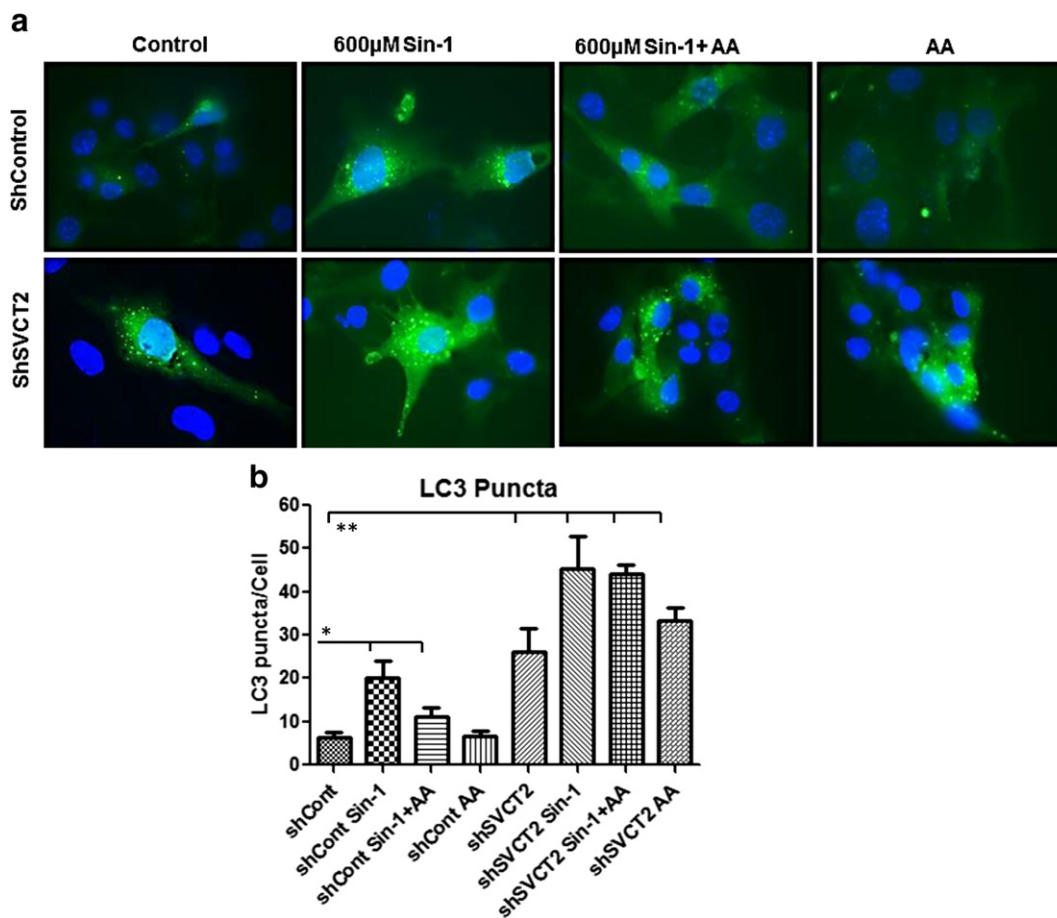
Figure 5 Effect of oxidative stress on GFP-LC3 dots in shcontrol BMSCs and SVCT2 knockdown BMSCs. Both cell types were transiently transfected with GFP-LC3B expression vector and then treated with Sin-1 (600 μM), ascorbic acid (250 μM) and combination of both for 4 h as described in material and methods. a) Representative confocal photos demonstrated that Sin-1 increase punctuate GFP-LC3B and ascorbic acid down-regulate GFP-LC3B in shcontrol BMSCs. SVCT2 knock down BMSCs showed high level of punctuate GFP-LC3B and ascorbic acid did not nullify GFP-LC3B punctuate dots. b) Determination of the average number of puncta, as distinct fluorescence green spots, per cell was performed. Data were analyzed by ANOVA followed by Bonferroni post hoc test (*p $<$ 0.05; ** p $<$ 0.01, n = 3).

To investigate apoptotic and necrotic cell death, we have used a phosphatidylserine-binding protein (Annexin-V-FITC) to detect apoptosis-dependent redistribution of phospholipid. Cells were also counter-stained with PI to distinguish apoptosis from necrosis. We found few apoptotic and necrotic cells in shcontrol BMSCs, an increase in response to oxidant, and a substantial block of this effect by Vitamin C. However, with decreased transporter levels, shSVCT2 BMSCs showed significantly higher apoptotic and necrotic cells. Moreover, supplementation with vitamin C to shSVCT2 BMSCs did not block oxidative stress-induced apoptosis and necrosis. Recently, Song et al (2014) reported that autophagy is critical for the BMSCs' survival under oxidative conditions. Furthermore, other investigators also reported that low level or acute exposure induces autophagic flux, whereas chronic or high dose oxidative treatment blocks autophagy and enhances apoptosis in a number of cell types (Song et al, 2014; Boya et al, 2005; Zhou et al, 2012). We speculate that knockdown or down-regulation of SVCT2 transporter disturbs cell anti-oxidant properties because of less availability of vitamin C which leads to weaker cell survival mechanism. Vitamin C is known for its antioxidant dependent autophagy and cell

survival role in various cell types (Bridges, 1987; Martin et al, 2002; Gruss-Fischer and Fabian, 2002; Tannetta et al, 2008; Hung et al., 2010; Singletary and Milner, 2008; Tomasetti et al, 2012). Ours is the first study to demonstrate the critical role of SVCT2 transporter in antioxidant effect of vitamin C on autophagy and cell survival in BMSCs.

In our previous studies, we showed that expression of SVCT2 decreases with aging (Fulzele et al, 2013) and in diabetic bone and bone marrow (Sangani et al, 2013). Therefore, decreased SVCT2 expression appears to overload the accumulation of ROS, which leads to a weakened cell survival mechanism, increased apoptotic and necrotic cell death, and a reduction in the differentiation of BMSCs into osteoblasts (osteogenesis). These events could be one of the reasons for bone loss, weaker bone structure, and increased fracture risk in aging, postmenopausal and diabetic medical conditions. The molecular mechanism of oxidative stress, supplementation of ascorbic acid, and SVCT2 transporter activity with regard to levels of autophagy and apoptosis need to be understood in detail.

In conclusion, this study demonstrates that SVCT2 plays a critical role in protecting against oxidative stress in BMSCs.
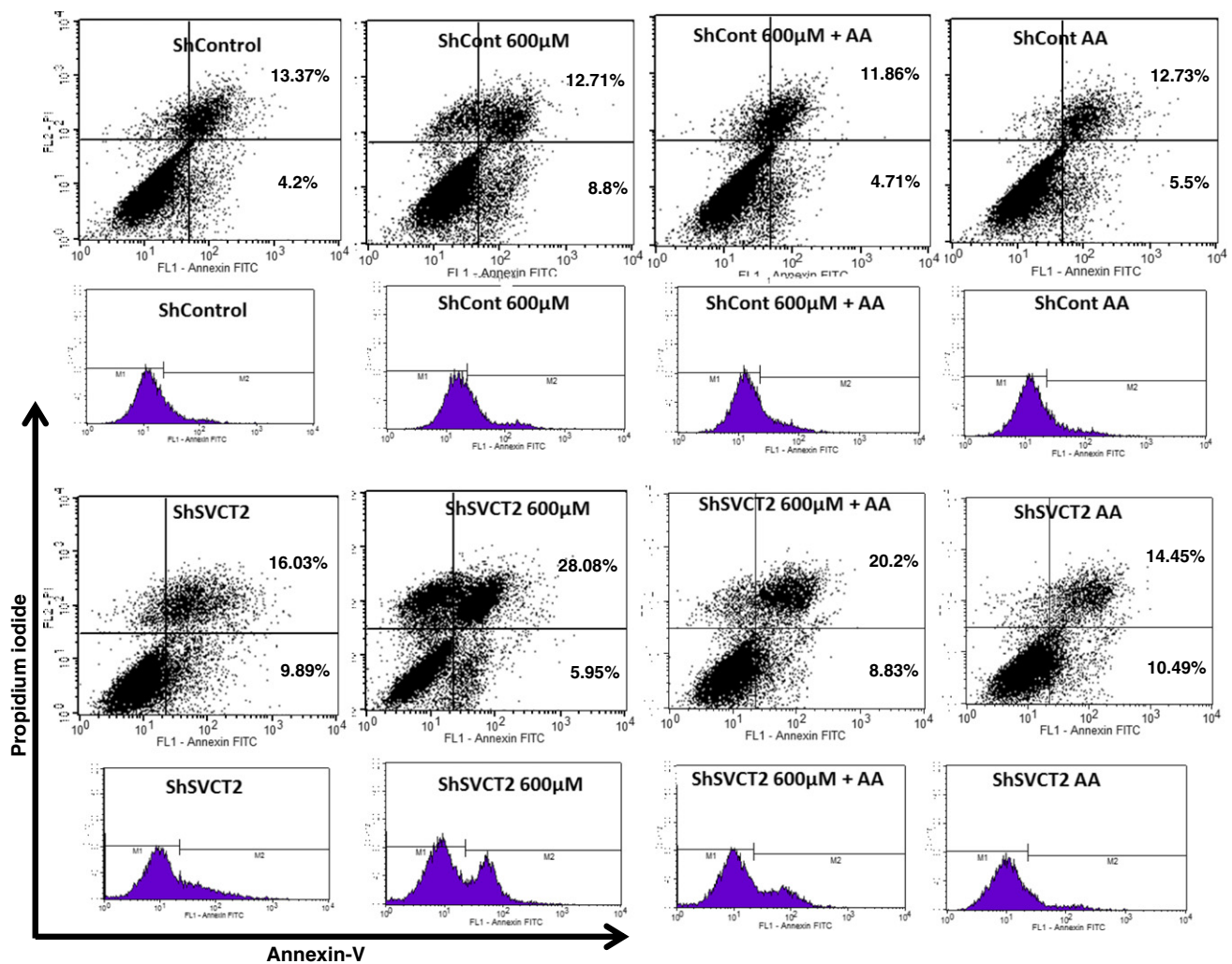
Figure 6   Effect of oxidative stress on shcontrol BMSCs and SVCT2 knockdown BMSCs. Shcontrol BMSCs and SVCT2 knockdown BMSCs were treated with Sin-1 (600 μM), ascorbic acid (250 μM) and combination of both for 4 h as described in material and methods and stained with FITC labeled Annexin-V and PI according to manufacturer's protocol and analyzed by flow cytometry. The histograms show a comparison of the distribution of annexin V and PI positive and negative cells (n = 3).

Our results give clear evidence that the optimal expression of SVCT2 is important to achieve the antioxidant property of vitamin C. Furthermore, the autophagy dependent anti-oxidative effect of vitamin C requires SVCT2 expression. The role of vitamin C and its transporter can be very important in slowing the aging process by reducing oxidative stress, modifying autophagy and the apoptotic mechanism. Future studies are required to understand the detailed mechanisms of autophagy and cell death in response to oxidative stress and its dependence upon vitamin C transporter status.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.06.002.

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