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Knockdown of SVCT2 impairs *in-vitro* cell attachment, migration and wound healing in bone marrow stromal cells☆

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Abstract Bone marrow stromal cell (BMSC) adhesion and migration are fundamental to a number of pathophysiologic processes, including fracture and wound healing. Vitamin C is beneficial for bone formation, fracture repair and wound healing. However, the role of the vitamin C transporter in BMSC adhesion, migration and wound healing is not known. In this study, we knocked-down the sodium-dependent vitamin C transporter, SVCT2, the only known transporter of vitamin C in BMSCs, and performed cell adhesion, migration, *in-vitro* scratch wound healing and F-actin re-arrangement studies. We also investigated the role of oxidative stress on the above processes. Our results demonstrate that both oxidative stress and down-regulation of SVCT2 decreased cell attachment and spreading. A trans-well cell migration assay showed that vitamin C helped in BMSC migration and that knockdown of SVCT2 decreased cell migration. In the *in-vitro* scratch wound healing studies, we established that oxidative stress dose-dependently impairs wound healing. Furthermore, the supplementation of vitamin C significantly rescued the BMSCs from oxidative stress and increased wound closing. The knockdown of SVCT2 in BMSCs strikingly decreased wound healing, and supplementing with vitamin C failed to rescue cells efficiently. The knockdown of SVCT2 and induction of oxidative stress in cells produced an alteration in cytoskeletal dynamics. Signaling studies showed that oxidative stress phosphorylated members of the MAP kinase family (p38) and that vitamin C inhibited their phosphorylation. Taken together, these results indicate that both the SVCT2 transporter and oxidative stress play a vital role in BMSC attachment, migration and cytoskeletal re-arrangement. BMSC-based cell therapy and modulation of SVCT2 could lead to a novel therapeutic approach that enhances bone remodeling, fracture repair and wound healing in chronic disease conditions.
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Introduction

Bone marrow stromal cells (BMSCs) are multipotent stem cells capable of differentiation into numerous cell types, including fibroblasts, bone and cartilage, muscle, and brain cells (Pittenger et al., 2002; Orlic et al., 2001; Yoon et al.,

2005; Ferrari et al., 1998; Lee et al., 2004; Oswald et al., 2004). Differentiation of BMSCs is a crucial aspect of bone formation and fracture healing. There are a number of factors that contribute to BMSC differentiation, and vitamin C (Ascorbic Acid, AA) is among the most important (Franceschi and Iyer, 1992; Sullivan et al., 1994; Harada et al., 1991). Deficiency of vitamin C leads to impaired bone matrix synthesis and abnormal bone formation and can result in delayed fracture healing (Alcantara-Martos et al., 2007; Sugimoto et al., 1998; Simon and Hudes, 2001; Mohan et al., 2005). Furthermore, vitamin C is required for hydroxylation of proline and lysine residues (Englard and Seifter, 1986; Padh, 1990; Phillips and Yeowell, 1997), which is essential for the formation of immature callus during bone healing (Day et al., 2000). In addition, it is the primary water-soluble antioxidant, serving as a powerful scavenger of reactive oxygen and nitrogen species. However, because ascorbic acid is highly water-soluble, it cannot simply diffuse across the hydrophobic lipid bilayer of the plasma membrane to gain access into these cells; specific transport systems must be present in the plasma membrane to mediate the entry process. We have previously shown that vitamin C is transported into BMSCs and bone cells with the help of a transporter known as the Sodium Dependent Vitamin C Transporter 2 (SVCT2) (Fulzele et al., 2013). Our results demonstrate that SVCT2 helps in BMSC differentiation to osteogenesis. Furthermore, SVCT2 is regulated by dexamethasone, aging and oxidative stress (Fulzele et al., 2013).

Oxidative stress plays an important role in the pathogenesis of delayed fracture and wound healing in chronic disease conditions. Oxidative stress is known to negatively influence cellular activities such as cell attachment, migration and wound healing (Castro et al., 2012; Fuseler and Valarmathi, 2012; Jiang and He, 2005), whereas supplementation of antioxidant helps in enhancing these activities (Zhou et al., 1999; Patel et al., 2012). Furthermore, oxidative stress has been shown to contribute to morphologic and cytoskeletal changes in various cells types (Malorni et al., 1991; Awai-Kasaoka et al., 2013). Based on our previous findings and the literature, we hypothesized that down-regulation of the SVCT2 transporter impairs BMSC cell attachment, migration and wound healing. Furthermore, the combination of both SVCT2 down-regulation and increased oxidative stress contributes to a destabilized/dis-organized cytoskeleton in BMSCs. We previously reported that SVCT2 is down-regulated with increased oxidative stress and aging. Wound and fracture healing proceeds normally in the majority of patients; however, it is significantly delayed by a number of conditions such as aging, diabetes, cardiovascular diseases and other chronic medical conditions. The mechanism of compromised/delayed healing is multifactorial, including increased oxidative stress, impaired cell attachment, and migration. An understanding of these aspects of the SVCT2 transporter and BMSCs is important to design a novel treatment strategy to reduce the healing period in aging and chronic disease conditions.

Materials and methods

Isolation of BMSCs from mice

BMSCs were isolated from the long bones of 6 month-old C57BL/6 mice (n = 6) as described previously (Fulzele et al., 2013). Briefly, the mice were euthanized and the femurs and humeri removed. The marrow was flushed with phosphate-buffered saline (PBS) and the cellular material harvested. The cellular material was centrifuged, the supernatants were removed 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 supernatants 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), 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,).

Knockdown of SVCT2 activity using lentivirus

Knockdown of SVCT2 in BMSCs was carried out as described previously (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 brief, 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 BMSCs.

Estimation of shSVCT2 efficiency

The efficiency of shRNA activity was analyzed by real-time PCR and a vitamin C uptake assay as per our published method (Fulzele et al., 2013). For real time PCR, RNA was isolated from shSVCT2 and shControl cells using the Trizol method following manufacturer's instructions. The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). 50 ng of cDNA was amplified in each real-time PCR using published primers of SVCT2 and GAPDH (Fulzele et al., 2013). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization.

Uptake studies on BMSCs were performed as described previously (Fulzele et al., 2013). Briefly, BMSCs were seeded in 24-well plates at an initial density of 1×10^4 cells/well. Uptake of [^{14}C] ascorbic acid was measured after treatment. The medium was removed by aspiration and the cells washed with uptake buffer once. Uptake was initiated by adding 0.25 mL of uptake buffer containing [^{14}C] ascorbic acid (20 nM). Uptake measurements were made with a 15 min incubation representing initial uptake rates. Uptake was terminated by aspiration of the uptake buffer from the cells. The cell monolayers were quickly washed twice with ice-cold uptake buffer without the radiolabeled substrate. Cells were then lysed in 0.5 mL of 1% SDS/0.2 N NaOH and the radioactivity

associated with the cells was quantified. Samples were analyzed using a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA, model LS-6500) and the rate of uptake was normalized to the protein content of each well.

Cell attachment assay

The cell attachment assay was carried out using 24-well tissue culture plates. Confluent BMSCs were trypsinized and resuspended in DMEM. Three hundred microliters of cell suspension (30 K cells) was added to each well and allowed to attach for 60 min with and without treatment. The cells were washed gently with PBS twice, and fixed with 4% paraformaldehyde (EMS, Hatfield, PA) for 20 min and stained with crystal violet (Fisher, Rochester, NY) for 30 min. Then plates were washed in deionized H_2O to remove the excess stain and photographed under a microscope. For the spectrophotometric assay, the stain was recovered with 500 μL of 2% acetic acid solution and the absorbance measured at 590 nm. The data were normalized by determining the OD590 ratio of the control verses treatment groups and expressed as a percentage.

Trans-well migration assay

Assays were performed in Boyden Chambers (Trans-well, Corning, Lowell, MA) according to the manufacturer's instructions. Briefly, BMSCs were seeded in 100-mm dishes and cultured for 24 h to 80–90% confluence. After reaching 80–90% confluence the cells were starved overnight (16 h) with and without ascorbic acid. Then, the cells were trypsinized and re-suspended in serum-free medium and plated onto the top of each chamber insert and serum-containing medium was added to the bottom chamber. After 3 h, inserts were removed, washed and cells that migrated to the bottom side of the inserts were analyzed.

Wound healing assay

A monolayer scratch assay was used to compare the migratory ability of shControl BMSCs and shSVCT2 BMSC cells and also the effect of oxidative stress on these cells types. In this study, we used 3-morpholinosydnonimine (Sin-1) to induce oxidative stress on BMSCs. Sin-1 is a peroxynitrite donor that releases nitric oxide and superoxide under physiological condition (More et al., 1998). Cells were cultured on 6 well plates to confluency and monolayers were wounded with a sterile 200-μL pipette tip. The cultures were washed with PBS to remove detached cells, and stimulated with oxidative stress (Sin-1, 600 μM and 800 μM). At the same time ascorbic Acid (AA, 125 μM, Acros Organics, New jersey, USA) was administered, alone or in the same Sin-1-treated plates, as antioxidant. Photographs were taken using phase contrast microscopy at 0, 12, 16 and 20 h.

Immunofluorescence staining for F-actin

Cells were fixed using 4% paraformaldehyde solution and permeabilized by 0.1% Triton X-100 in PBS before staining. 5% BSA in PBS was applied to the cells for 30 min to block non-specific binding. F-actin was fluorescently labeled with Alexa Fluor 488® phalloidin (Invitrogen, Grand Island, NY, USA). After staining, cells were washed with PBS, and the nuclei were stained with DAPI (Sigma-Aldrich) for 5 min and then rinsed with water. Cells were analyzed by fluorescence microscopy (Axioplan 4.2, Carl Zeiss, Germany).

Western blot analysis

For western blot analysis, the cells were treated with Sin-1 (600 μM) for 30 min, 1 h, 2 h and 4 h. Cells were also treated with Sin-1 (600 μM), AA (125 μM), SB202190 (p38 inhibitor, Cayman, Chicago, IL) and a combination of Sin-1 + AA or Sin-1 + SB202190. Protein was extracted from cells using cell lysis buffer, subjected to SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding was blocked with 5% BSA in 1× TBST (Tris Buffered Saline with Tween 20). Membranes were incubated with antibody against phospho and total p38 (Cell signaling, Danvers, MA) 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.

Statistics

GraphPad Prism 5 (La Jolla, CA) was utilized to perform ANOVA with Bonferroni pair-wise comparison or unpaired t-tests as appropriate. A p-value of <0.05 was considered significant.

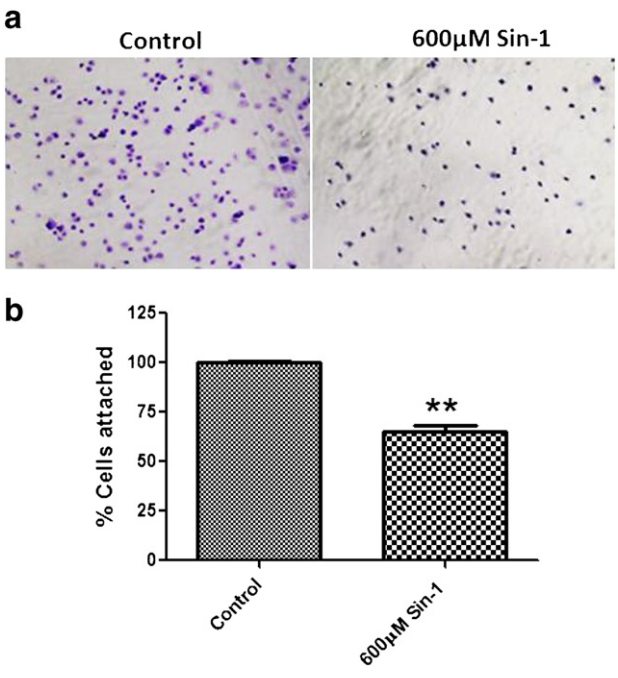

Figure 1 Effect of oxidative stress on BMSC attachment. BMSCs were cultured and treated with 3-morpholinosydnonimine (Sin-1) (600 μM) for 1 h followed by cell attachment assay. a) Representative image of crystal violet staining shows decreased cell attachment with Sin-1 treatment (scale bar 100 μm). b) Quantitative analysis of crystal violet staining as described in the Materials and methods section. Values are mean ± SE (n = 5). Data were analyzed by unpaired t-tests (*$p < 0.05$; **$p < 0.01$).

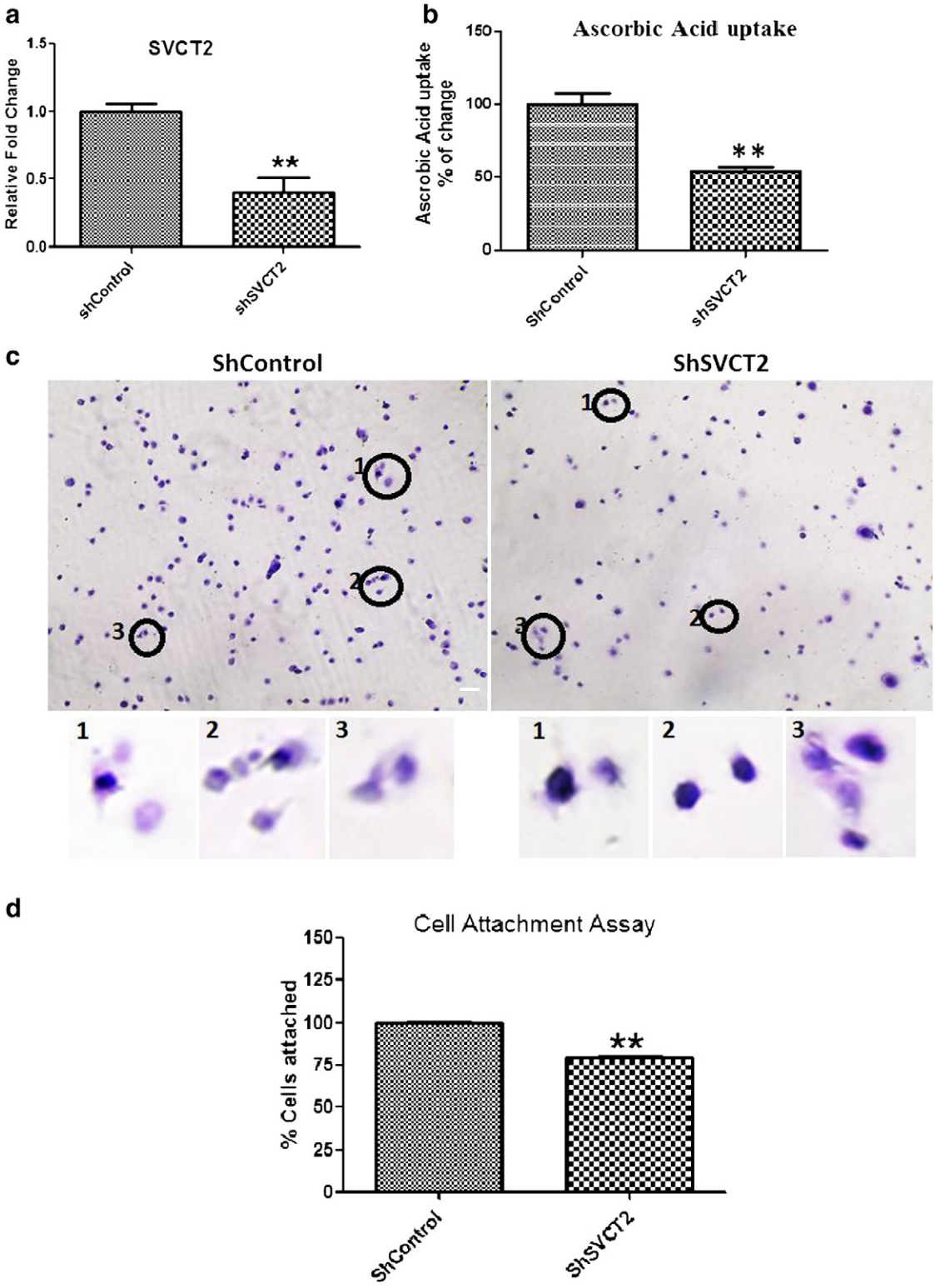

Figure 2 Effect of SVCT2 knockdown on BMSC attachment. a) Lentivirus-mediated SVCT2 knockdown. Quantitative real-time PCR analysis of total SVCT2 transcripts from stable, SVCT2 shRNA transfected and non-targeting shControl mouse BMSCs. Data for each sample were normalized with GAPDH mRNA. Data (means ± SD, n = 4) are represented as the fold change in expression compared to control. **$p < 0.01$. b) Uptake of [^{14}C]-ascorbate (20 nM) was measured for 15 min in SVCT2 shRNA and non-targeting shControl in mouse BMSCs. Uptake measured in control non-targeting shRNA (shControl) was taken as the control (100%) and uptake measured in the SVCT2 shRNA given as a percentage of this control value (mean s ± SD, n = 3). c) ShControl and shSVCT2 BMSCs were cultured for 1 h followed by cell attachment assay. Representative image of crystal violet staining showing decreased cell attachment in shSVCT2 cells (scale bar 100 μm). d) Quantitative analysis of crystal violet staining as described in the Materials and methods section. Values are mean ± SE (n = 6). Data were analyzed by unpaired t-tests. (*$p < 0.05$; **$p < 0.01$).

Results

Oxidative stress and SVCT2 knockdown decreases cell attachment in BMSCs

To determine the effects of oxidative treatment on BMSC cell attachment, we plated BMSCs with Sin-1 (600 μM) on 24 well tissue culture plates for 1 h. The plates were gently washed with PBS to remove unattached cells and to dislodge non-adherent or weakly adherent cells. Treatment with Sin-1 decreased cell attachment by ~40% compared to control (p-value, 0.001) (Fig. 1).

To test whether SVCT2 might be involved in cell attachment and adhesion, 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 an ascorbic acid (AA) uptake assay and real time PCR (Figs. 2a& b). The SVCT2 and BMSC control cells were plated and allowed to adhere to the tissue culture plate for 1 h. We found that there was ~25% decrease in cell attachment of SVCT2 knockdown cells compared to controls (p-value, 0.001) (Figs. 2c & d). Control cells also showed significantly increased cell spreading compared with the SVCT2 knockdown cells (Fig. 2c).

SVCT2 knockdown in BMSCs decreases cell migration in trans-well migration assay

To determine the role of vitamin C and its transporter (SVCT2) on BMSC migration, we performed a trans-well cell migration assay. The cells were cultured in the presence and absence of vitamin C in shControl and shSVCT2 BMSCs. We found that the supplementation of vitamin C significantly increased cell migration by 30% in shControl cells (Fig. 3). The shSVCT2 cells showed decreased cell migration compared to shControl cells. Furthermore, we observed no significant effect on shSVCT2 cell migration with the addition of ascorbic acid (Fig. 3). Taken together, these findings show that down-regulation of the SVCT2 transporter decreased cell migration and vitamin C promoted cell migration in normal BMSCs.

Effect of the redox reaction and knockdown of SVCT2 on *in-vitro* wound closing

To determine the effects of knockdown of the SVCT2 transporter and redox treatment on cell migration, we treated shControl and shSVCT2 cells with oxidant (Sin-1), antioxidant (AA) and a combination of both (Sin-1 + AA). The effect on cell migration was tested in an *in-vitro* wound healing model, in which scrape wounds were generated in confluent cell cultures. Cells with or without treatment were allowed to migrate into the denuded area for 12–20 h at 37 °C. ShControl cells started to migrate into the denuded area at 12 h after being scratched, and scratch closure was almost (~70%) complete at 20 h (Fig. 4a). In contrast, shSVCT2 cells migrated less, as indicated by fewer cells in the denuded area at 12, 16, and 20 h after scratching (Fig. 4b). The Sin-1 treated cells showed a dose dependent decrease in cell migration on both cell types, but shSVCT2 cells were more severely affected than shControl cells. Supplementation of AA to shControl cells significantly nullified the effect of Sin-1, causing increased cell migration compared to Sin-1 alone. In contrast, supplementation of AA to shSVCT2 cells showed a less significant increase than in the shControl cells. These observations indicate that knockdown of the SVCT2 transporter and an increase in oxidative stress decreased cell migration, while AA increased cell migration.

Changes in F-actin dynamics in SVCT2 knockdown cells

Actin re-arrangement is an important event in cell attachment and migration. We investigated whether this event was altered in the presence of oxidative stress and knockdown of the SVCT2 transporter in BMSC cells. We used fluorescent Phalloidin stain, which binds specifically at the interface between F-actin subunits (Small et al., 1999). Induction of oxidative stress in BMSCs resulted in a rearrangement of the actin cytoskeleton with more prominent F-actin. This reorganization was totally inhibited by supplementation of ascorbic acid (Fig. 5). Knockdown of the SVCT2 transporter in BMSCs showed some rearrangement and of F-actin, and induction of oxidative stress in these cells completely dis-organized the cytoskeleton. Under the influence of oxidative stress, supplementation of ascorbic acid to the knockdown cells partially inhibited the cytoskeletal dis-organization. Surprisingly, ascorbic acid itself caused significant re-arrangement of F-actin in the knockdown cells (Fig. 5).

Mitogen-activated protein kinases (MAPK) signaling

Since MAPK signaling plays a very important role in cell growth and migration, phosphorylation of p38 MAPK proteins was examined to determine whether oxidative stress was mediated by a MAPK pathway in BMSCs. The activation of p38 MAP kinases was examined by Western blotting at

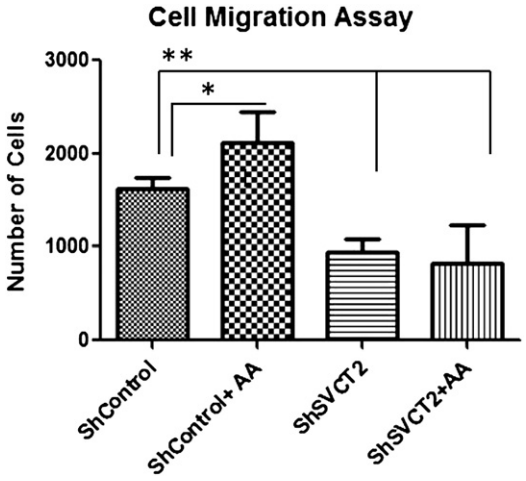

Figure 3 Decreased cell migration after SVCT2 knockdown in BMSCs. a) ShControl and shSVCT2 BMSC cells were subjected to the trans-well cell migration assay as described in the Materials and methods section. Values are mean ± SE (n = 6). Data were analyzed by ANOVA followed by Bonferroni post hoc test (*$p < 0.05$; **$p < 0.01$).

30 min, 1 h, 2 h and 4 h after treatment. In the presence of oxidative stress, the rates of phosphorylation of MAP kinases were significantly more rapid in both cells types (shControl and shSVCT2) in a time dependent manner (Fig. 6a); protein phosphorylation reached a plateau at 2 to 4 h. Supplementation of AA along with Sin-1 (2 hour treatment) significantly inhibited the activation of p38 in shControl cells (Fig. 6b). In contrast, in SVCT2 knockdown cells, AA did not significantly inhibit phosphorylation. To determine whether phosphorylation of p38 proteins is crucial in Sin-1 induced oxidative stress, the effects of specific p38 inhibitor SB202190 treated cells were assessed. Treatment with the inhibitor significantly inhibited Sin-1 induced phosphorylation in shControl cells (Fig. 6c).

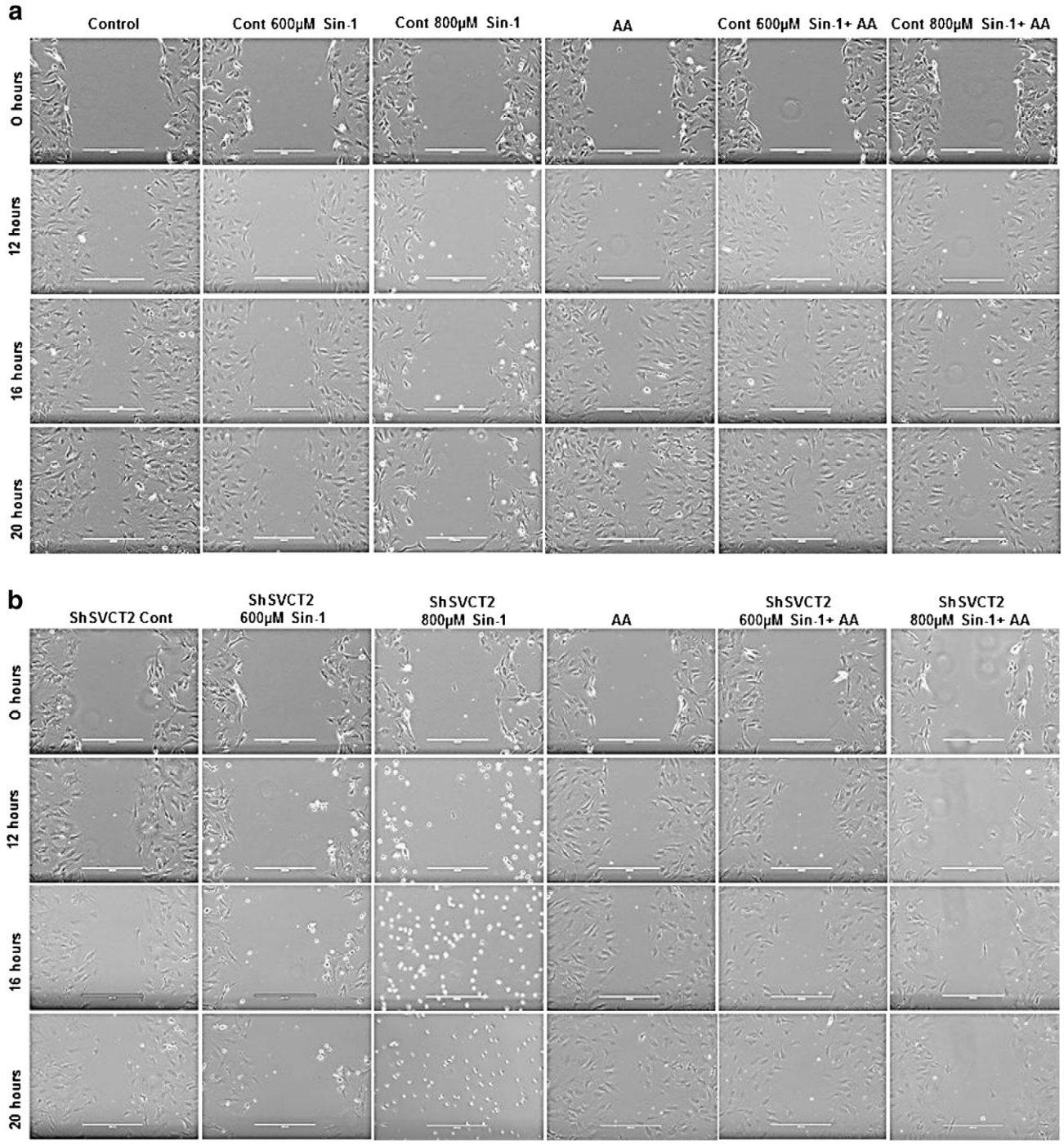

Figure 4 Effect of oxidative stress and SVCT2 knockdown on BMSC migration during *in vitro* scratch wound healing. Representative phase-contrast images (10×) show cells migrating into wounded area in an *in-vitro* scratch wound healing assay. a) ShControl and b) ShSVCT2 BMSCs were treated with Sin-1 (600 μM, 800 μM), ascorbic acid (AA, 125 μM) and a combination of both. Phase-contrast images were taken after 12 h, 16 h and 20 h.

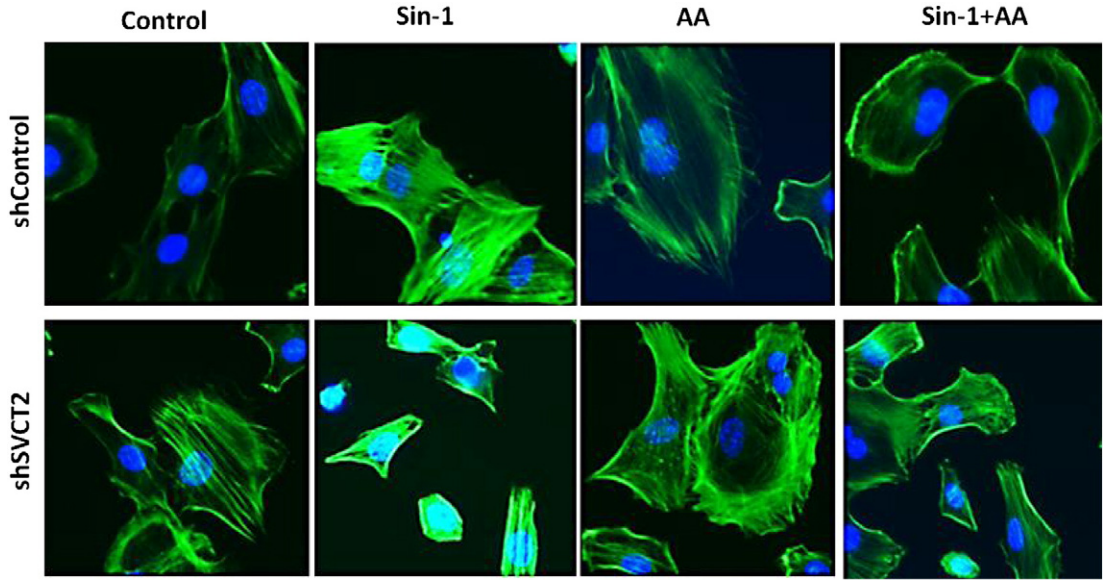

Figure 5 Effect of oxidative stress and SVCT2 knockdown on reorganization of the actin cytoskeleton. Representative fluorescence images (40×) show F-actin staining. ShControl and ShSVCT2 BMSCs were treated with Sin-1 (600 μM), ascorbic acid (AA, 125 μM) and combination of both. Fluorescence images (scale bar 50 μm) were taken after 2 h as described in the Materials and methods section.

Discussion

We previously reported that vitamin C enters into BMSCs through the SVCT2 transporter and this transporter plays a vital role in bone formation (Fulzele et al., 2013). In the present study, we elucidate the following additional role(s) of SVCT2 in *in-vitro* BMSCs: cell attachment, migration and wound healing. Cell attachment and migration are cellular activities involved in homing of cells to an injury region (Hu et al., 2011).The initial cell adhesion and spreading activities are important in wound and fracture healing (Min et al., 2004). These activities are partially regulated by oxidative stress (Abhijit et al., 2013; Liu et al., 2013). We have investigated the effects of oxidative stress and SVCT2 transporter status on initial attachment and spreading of BMSCs. We demonstrated that both oxidative stress and knockdown of SVCT2 significantly decreased BMSC attachment. In particular, control BMSCs attached in greater numbers and spread to a greater extent compared to SVCT2 knockdown cells. Our findings suggest that adequate expression of the SVCT2 transporter is important for rapid adhesion and cell spreading, indicating a potential role of the cytoskeleton. For example, oxidative stress has been reported to decreases cell attachment by an alteration in cytoskeletal organization (Castro et al., 2012; Fuseler and Valarmathi, 2012; Jiang and He, 2005; Lamari et al., 2007; Wu et al., 2013).

Cell migration is a characteristic of BMSCs and important in wound and fracture healing (Kumar and Ponnazhagan, 2012; Schindeler et al., 2009; McIlwraith et al., 2011). We observed that supplementation of ascorbic acid helped *in-vitro* migration of BMSCs using the trans-well cell migration assay (Fig. 3). Surprisingly, SVCT2 knockdown cells showed a significant decrease in cell migration. Furthermore, supplementation of AA to SVCT2 knockdown cells did not improve their migration ability. To validate our results, we used an *in-vitro* scratch wound healing model to study cell migration and closing of the wound. This method is useful in studying the properties of single cell types without the inherent heterogeneity of *in-vivo* models. *In-vitro* scratch wound healing studies also showed similar results as the trans-well migration assay. Ascorbic acid helped to increase cell migration and accelerate wound closure, whereas oxidative stress decreased these processes (Fig. 4). Knockdown of the SVCT2 transporter decreased cell migration. Furthermore, SVCT2 knockdown cells were more sensitive to the adverse effects of oxidative stress (Fig. 4b), and supplementation of ascorbic acid did not fully rescue the cells from oxidative stress. In our previous report, we have shown that increased oxidative stress decreases vitamin C transporter activity, which leads to decreased vitamin C uptake (Fulzele et al., 2013). It is therefore possible that SVCT2 indirectly promotes cell migration rates and tissue repair by facilitating vitamin C uptake. It has been previously reported that oxidative stress negatively impacts cell migration in various cell types (Rikitake et al., 2000; Murugesan et al., 1993; San Miguel et al., 2010, 2011). Ours is the first report showing a role of the SVCT2 transporter in *in-vitro* wound healing.

Cell migration is a highly coordinated and multifactorial biological process in which cytoskeletal proteins play an important role. Actin is a highly conserved and abundant cytoskeletal protein in eukaryotic cells (van den Ent et al., 2001). Actin filaments are involved in a wide variety of cellular processes, including cell motility, cell cycle control, cellular structure and cell signaling (Zhu et al., 2005). They function in cellular processes by undergoing dynamic structural re-arrangement/reorganization or remodeling. We hypothesize that knockdown of the SVCT2 transporter and increased oxidative stress can disorganize the cytoskeleton in BMSCs. Consistent with this hypothesis, our results show that oxidative stress induced a rapid re-arrangement of the actin filaments, and vitamin C rescued the cytoskeleton from oxidative effects. These results are consistent with studies in

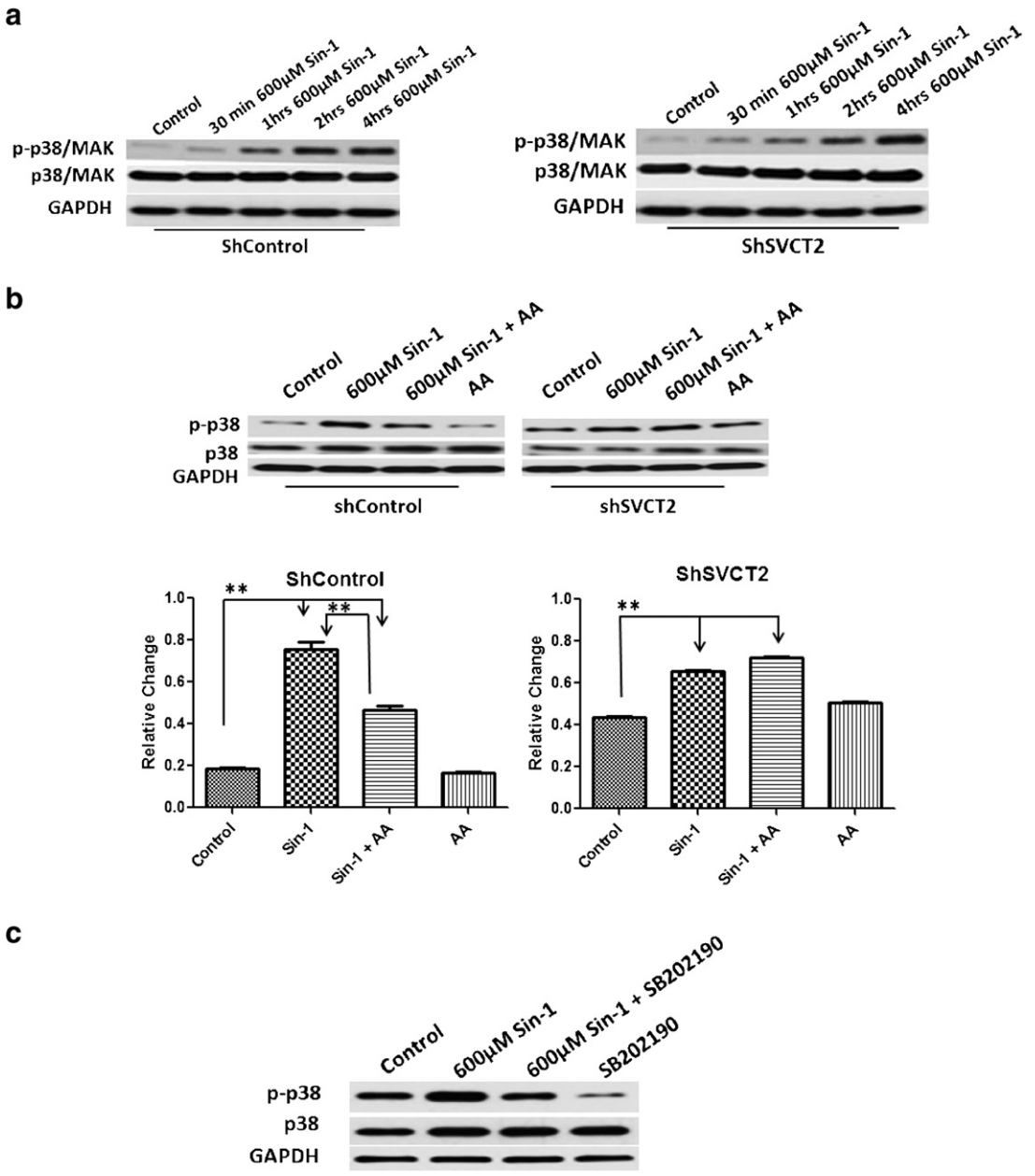

Figure 6 Redox reaction regulates phosphorylation and activation of p38 MAP kinase. a) Time-dependent activation of p38 phosphorylation induced by oxidant (Sin-1) in shControl and shSVCT2 BMSCs. b) Effect of ascorbic acid on p38 MAP kinase activation in shControl and shSVCT2 BMSCs. The cells were treated with Sin-1 (600 μM), ascorbic acid (AA, 125 μM) and combination of both. Bar graph values are mean ± SE (n = 3). Data were analyzed by ANOVA followed by Bonferroni post hoc test (*$p < 0.05$; **$p < 0.01$). r) SB202190 (p38 inhibitor) inhibited activation of p38 induced by Sin-1. The ShControl cells were treated with Sin-1 (600 μM), SB202190 and combination of both.

other cell types indicating a rapid remodeling of the structure of actin filaments upon oxidative stress (Qian et al., 2003; Zhao and Davis, 1998; Zhu et al., 2005). Unique to this study is our demonstration that induction of oxidative stress in SVCT2 knockdown BMSCs extensively increased oxidant-induced disorganization of the cytoskeleton. This could be because of increased accumulation of ROS in cells that do not optimally transport vitamin C. Farah et al. (2011) have shown that oxidative stress induces a rapid disassembly/depolarization of the actin cytoskeleton, followed by an adaptive phase that is required for subsequent re-assembly of a polarized cytoskeleton. If cells undergo continuous oxidative stress, they do not reassemble or show delayed re-assembly of the polarized cytoskeleton. Our study suggests that increased oxidative stress and down-regulation of SVCT2 leads to an alteration in cytoskeletal organization, which could affect cellular processes such as cell attachment and migration. Lee et al. (2013) and others report that cytoskeletal proteins play an important role in oxidative stress regulated cell migration.

The molecular mechanism of oxidative stress with regard to cell attachment, cell migration and cytoskeletal organization needs to be understood in detail, and due to the broad biological activity of these processes, it is difficult to draw conclusions about specific regulatory pathways involved in these cellular processes. In this study, we investigated phosphorylation of the MAPK family proteins, specifically p38, because it plays critical roles in oxidative stress, cell differentiation (Nöth et al., 2003), cytoskeletal organization (Zhu et al., 2005; Huot et al., 1997; Ingram et al., 2000), and cell attachment and migration in various cell types (Yamboliev and Gerthoffer, 2001; Laprise et al., 2002; Kavurma and Khachigian, 2003). In other investigations, it has been reported that activation of p38 destabilizes cytoskeletal proteins (Lovett et al., 2006) and reduces cell attachment (Du et al., 2010) and migration (Saika et al., 2004). Here we report that stimulation of the p38 signaling cascade was an important event in Sin-1 mediated oxidative stress in BMSCs. In control cells, phosphorylation of p38 was observed after oxidative stress treatment, and AA inhibited this activation. In SVCT2 knockdown cells, oxidative-stress-induced accumulation of ROS was extensive enough that even the antioxidant ascorbic acid was not able to totally inhibit the activation of p38. These results suggest that inhibition of p38 phosphorylation or activation after oxidative treatment might be beneficial to cells. However, our results also indicate that the antioxidant effect of ascorbic acid is not by itself sufficient to inhibit phosphorylation, but adequate expression of the SVCT2 transporter is required.

Fracture and wound healing are widely recognized as major problems in most of the chronic diseases of aging. Our study suggests that the SVCT2 transporter may play an important role in such conditions. We previously reported that the SVCT2 transporter is down-regulated with aging in bone (Fulzele et al., 2013). Michels et al. (2003) report down-regulation of the vitamin C transporter in aging rat liver. Seno et al. (2004) reported that the inflammatory cytokines (TNF-α, IL-1ß) suppress the activity of SVCT in human endothelial cells. In most chronic disease conditions, there is an increased burden of oxidative and inflammatory stress, which could form the basis for down-regulation of the SVCT2 transporter.

In summary, this study provides novel mechanistic insight into the effects of oxidative stress and the vitamin C transporter on cell attachment, migration and wound closure, which are likely contributors to the defects in wound and fracture healing often observed in aging and chronic medical conditions.

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