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Effects of Na/K-ATPase and its ligands on bone marrow stromal cell differentiation

Moustafa Sayed[a], Christopher A. Drummond[a], Kaleigh L. Evans[a], Steven T. Haller[a], Jiang Liu[b], Zijian Xie[a], Jiang Tian[a,*]

[a] Department of Medicine, University of Toledo, Toledo, OH, USA
[b] Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, USA

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Abstract Endogenous ligands of Na/K-ATPase have been demonstrated to increase in kidney dysfunction and heart failure. It is also reported that Na/K-ATPase signaling function effects stem cell differentiation. This study evaluated whether Na/K-ATPase activation through its ligands and associated signaling functions affect bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) differentiation capacity. BMSCs were isolated from male Sprague–Dawley rats and cultured in minimal essential medium alpha (MEM-α) supplemented with 15% Fetal Bovine serum (FBS). The results showed that marinobufagenin (MBG), a specific Na/K-ATPase ligand, potentiated rosiglitazone-induced adipogenesis in these BMSCs. Meanwhile, it attenuated BMSC osteogenesis. Mechanistically, MBG increased CCAAT/enhancer binding protein alpha (C/EBPα) protein expression through activation of an extracellular regulated kinase (ERK) signaling pathway, which leads to enhanced rosiglitazone-induced adipogenesis. Inhibition of ERK activation by U0126 blocks the effect of MBG on C/EBPα expression and on rosiglitazone-induced adipogenesis. Reciprocally, MBG reduced runt-related transcription factor 2 (RunX2) expression, which resulted in the inhibition of osteogenesis induced by β-glycerophosphate/ascorbic acid. MBG also potentiated rosiglitazone-induced adipogenesis in 3T3-L1 cells and in mouse BMSCs. These results suggest that Na/K-ATPase and its signaling functions are involved in the regulation of BMSCs differentiation.

Introduction

Bone marrow stem cells have the capacity for self-renewal and the potential for multidirectional differentiation (Friedenstein et al., 1970; Pittenger et al., 1999; Sacchetti et al., 2007). Two types of stem cells are found in the bone stromal environment, hematopoietic stem cells (HSCs) and bone marrow stromal cells (BMSCs, also known as bone marrow derived mesenchymal stem cells) (Majumdar et al., 1998; Short et al., 2003). Induction of differentiation can be accomplished in BMSCs under special physiological or experimental conditions. Differentiation into specific functional cells, such as osteoblasts, cartilage cells, lipocytes, hepatocytes, neurons and astrocytes are critical for organ and tissue development and healing (Pittenger et al., 1999). Although, in clinical trials, BMSCs have been used to treat

Abbreviations: BMSCs, bone marrow stromal cells; C/EBPα, CCAAT/enhancer binding protein alpha; MBG, marinobufagenin; PNx, partial nephrectomy; PPARγ, peroxisome proliferator-activated receptors γ.

* Corresponding author at: Department of Medicine, University of Toledo, 3000 Arlington Ave., Mail Stop 1025, Toledo, OH, USA.
E-mail address: Jiang.Tian@utoledo.edu (J. Tian).

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cardiac, genetic, hematological, metabolic, and neurologic diseases (Chen et al., 2004; Horwitz et al., 1999, 2002; Koc et al., 2002; Strauer et al., 2002), direct evidence showing that BMSCs can be transdifferentiated into functional cells except for osteoblasts chondroblasts, hematopoiesis-support stroma and marrow adipocytes is lacking (Bianco et al., 2008, 2013). Self-renewal and differentiation of BMSCs are controlled by endogenous signaling pathways and can be regulated by extrinsic changes of the microenvironment, known as the stem cell niche (He et al., 2009; Yin and Li, 2006). Certain chemokines such as stem cell factor and other circulating factors can interact with the niche environment stimulating stem cell expansion, motivation, and homing to injury sites (Hosoda, 2012; Zhao et al., 2007). Since its relative convenience of isolation and well established differentiation protocols, BMSCs are widely used in studies of stem cell differentiation.

It has been reported that uremic conditions cause defects in BMSC self-renewal and differentiation (He et al., 2009; Noh et al., 2012). Our previous studies showed that chronic kidney disease induced by partial nephrectomy (PNx) increases the circulating level of Na/K-ATPase ligands in rat and mouse models (Kennedy et al., 2006, 2008). Na/K-ATPase ligands are a group of digitalis-like compounds, including plant-derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin (MBG) (Akera and Brody, 1976; Barry et al., 1985; Schoner, 2002). These compounds were also found to be released endogenously, known as cardiotonic steroids (CTS), in animals and humans with essential hypertension, heart failure and renal dysfunction (Balzan et al., 2001; Fridman et al., 2002; Gottlieb et al., 1992; Manunta et al., 1999; Tian et al., 2010). The production and secretion of CTS are regulated by multiple physiological stimuli including ACTH and angiotensin II (Fedorova et al., 1998; Hamlyn et al., 1991; Laredo et al., 1997; Schoner, 2002). High levels of circulating CTS are an important risk factor that contributes to uremia-induced cardiac remodeling and dysfunction (Bagrov et al., 2009; Palazzuoli and Ronco, 2011; Simoes and Flynn, 2012). Mechanistically, a role for Na/K-ATPase ligand-induced signaling has been demonstrated in the regulation of somatic cell growth and survival (Liu et al., 2012; Tian et al., 2009). However, the effects of these compounds on BMSCs differentiation are not well studied. Previous reports demonstrated Na/K-ATPase expression in undifferentiated embryonic stem cells (ESCs) as well as in ESC-derived cardiomyocytes (Otsu et al., 2005). CTS such as ouabain promote differentiation of ESCs into cardiac myocytes through an extracellular regulated kinase (ERK) signaling pathway (Lee et al., 2011). This study evaluated whether CTS-induced Na/K-ATPase signaling regulates differentiation in BMSCs.

Materials and methods

Isolation and culture of rat BMSCs

All animal experiments were conducted in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee at the University of Toledo. Sprague–Dawley (SD) rats (male, 8 weeks old) were used for BMSCs isolation as previously described (Karaoz et al., 2009; Pantoja et al., 2008). Briefly, SD rats were anesthetized with 1 mg/kg Ketamine HCl/Xylazine HCl solution and sacrificed by cervical dislocation. Under sterile conditions, both left and right femurs and tibiae from each rat were excised. Muscle and all remnant connective tissue were detached from the bones. The ends of the bones were cut away and centrifuged at 8000 *g* for 10 min. A 21-gauge needle was inserted into shaft of the bones to extrude any remaining marrow by flushing with minimum essential medium alpha (MEM-α) from Invitrogen (Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS) and 1% 1000 IU/ml penicillin–1000 μg/mL streptomycin (Cellgro, VA, USA). Marrow plug suspension was dispersed by pipetting, and then filtered through a 70-μm mesh nylon filter from BD Biosciences (Falcon, NJ). The cells from one rat were seeded onto three 10 cm^2 plastic tissue culture plates in the same medium described above and incubated at 37 °C in a humidified incubator containing 5% CO_2 for 2 days. On the second day, red blood cells and other non-adherent cells were removed by aspiration of medium followed by addition of fresh medium to allow further growth. The adherent cells grown to 70% confluence over 6–10 days were defined as passage one (P1) cells. The P1 BMSCs were washed with phosphate-buffered saline (PBS), trypsinized and split into additional plates. For each passage the cells were plated similarly and grown to a confluence of 60–80%. Passage 3 (P3) cells were used for experiments.

BMSCs isolation from sham and partial nephrectomized (PNx) mice

PNx surgery was performed on mice by ligating 2/3 of left renal artery and removal of right kidney similarly as previously described (Kennedy et al., 2008). Briefly, an incision was made in the left flank, through which the left kidney was extirpated, and the artery(ies) supplying the upper pole of the kidney were observed under a high power dissecting microscope and subsequently ligated with 6-0 Silk Sutures. This resulted in cessation of two thirds of the blood flow to the kidney. Following ligation, the kidney was observed for a characteristic color change over approximately two-thirds of the kidney tissue indicating successful interruption of blood flow to the discolored portion of the kidney. Once the color change was observed, the kidney was reinserted to the body cavity and the incision closed. After one week, the right kidney, exposed through the right flank, was decapsulated to avoid removal of the adrenal gland and subsequently the renal artery, vein and ureter were ligated, and the kidney was removed. Sham-operated mice were used as control. These mice were sacrificed 8 weeks after surgery and BMSCs were isolated from their femurs and tibiae using the same procedures as for rat BMSCs except that the first passage cells were used for experiments.

Flow cytometry analysis

Passage 3 (P3) rat BMSCs were subjected to flow cytometric analysis using a FACSCalibur machine from BD Biosciences (San Jose, California). Briefly, stem cells at passage 3 were harvested and suspended in culture medium at a concentration

of 1×10^6 cells/ml. After a brief centrifugation, cells were resuspended in PBS and 300 μl of cell suspension was incubated with FITC-conjugated antibodies for 45 min at room temperature. Three surface markers including rat antigens CD29 (Ha2/5; FITC Hamster Anti-Rat; BD CAT# 55505), CD45 (OX-33; FITC Mouse Anti-Rat; BD CAT# 554883), and CD90 (H1S51; FITC Mouse Anti-Rat; BD CAT# 55489) were used to characterize the BMSCs. The data were analyzed with Cell Quest software. Using forward and side scatter profile, debris and dead cells were removed by gating.

In vitro adipogenic differentiation

Adipogenic differentiation was performed as previously described (Wang et al., 2011). Briefly, BMSCs or 3T3-L1 cells (10,000 cells/cm^2) were seeded onto 6-well plates. After 24 h of incubation in MEM-α, these cells were pretreated with MBG or solvent control (0.1% DMSO) for 3 days. To induce adipogenic differentiation, 1 μM rosiglitazone was added to the above pretreated cells for an additional 72 h. Cells without addition of rosiglitazone were used to test if MBG alone could induce adipogenesis. Cells were then washed three times with PBS and fixed in 10% formalin for 10 min and subsequently stained with Oil-Red-O staining solution from Sigma-Aldich (0.3% Oil-Red-O in isopropanol, diluted 5.5 to 4.5 in water and filtered with a 0.22-μm filter). After staining, cells were washed three times with water. The stained colonies were counted manually using light microscopy to estimate the effect of treatment on adipogenesis. Alternatively, the Oil Red O stain was dissolved with isopropanol. The absorbance at 500 nm was measured and quantified using a standard curve generated with different concentrations of Oil-Red-O.

In vitro osteogenic differentiation

Rat BMSCs from passage 3 (10,000 cells/cm^2) were seeded onto 6-well plates. After 24 h incubation in MEM-α, these cells were treated with MBG or solvent control (0.1% DMSO) for 3 days. To induce osteogenic differentiation, 200 μM ascorbic acid and 10 mM β-glycerophosphate were then added to the medium and the cells were cultured for additional 14 days. Osteogenic medium was refreshed once a week. At the end of the second week, osteogenic differentiation was assessed by staining with alizarin red (Sigma-Aldich). Briefly, excess medium on cells was shaken off and the cells were rinsed with PBS 3 times, fixed for 10 min at room temperature using 10% formalin (w/v), and then washed twice with PBS and allowed to dry completely. Cells were then stained with alizarin red solution comprising 2% alizarin red S (pH value of the Alizarin Red S solution was adjusted to 4.1–4.3 with sodium hydroxide) for 10 min and washed with distilled water and left to dry. Absorbance was measured by dissolving the stain in glacial acetic acid and measured at 405 nm.

Measurement of plasma MBG concentrations

Plasma MBG was measured using a competitive ELISA method described previously (Kennedy et al., 2008). Briefly, 100 μl of mouse plasma extraction was suspended in TBST solution (150 mM NaCl, 50 mM Tris, 0.05% Tween-20, pH 7.6) and was incubated with anti-MBG antibody (50 μl/well) in an MBG-BSA coated plate for 1 h. A secondary HRP-conjugated anti-mouse antibody was added after washing and incubated for additional 1 h. Plates were washed again and the HRP substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) was used for color development and OD_{450} was measured after addition of 1 N H_2SO_4 to stop the reaction. MBG concentration was quantified against a standard curve.

Immunostaining of fatty acid binding protein 4 (FABP-4) and osteocalcin

Undifferentiated and differentiated BMSCs were fixed with formalin, blocked with 1% BSA and incubated overnight at 4 °C with anti-FABP-4 antibody (goat IgG, R&D, cat# AF1443) for adipogenesis or anti-Osteocalcin antibody (mouse IgG, R&D, cat# MAB1419) for osteogenesis. Fluorescent secondary antibodies were applied afterwards and a Leica confocal microscope was used to visualize the fluorescence. Differential interference contrast (DIC) images were also taken to show the BMSCs under transmitted light.

Western blot

Treated cells were washed with PBS and lysed with RIPA lysis buffer (Santa Cruz, sc-24948) at 4 °C. Cell lysates were rotated for 10 min at 4 °C and cleared by centrifugation (10,000 ×*g* for 10 min at 4 °C). Protein lysates were resolved by SDS-PAGE and transferred to PVDF membranes (Thermo Scientific, Chicago, IL, USA). The membranes were then used to probe for C/EBPα, PPARγ, RunX2, and actin. These antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis

Data were reported as means ± SD. Data were analyzed using 2-sided t-test. A probability value of $p < 0.05$ was considered to denote statistical significance.

Results

Characterization of the BMSCs isolated from SD rats

BMSCs were characterized by their ability to adhere to plastic and proliferate (Friedenstein et al., 1970). We used P3 rat BMSCs for our experiments, this passage is reported to be nearly homogeneous (Harting et al., 2008). To confirm the phenotypic characterization of the isolated BMSCs, we also used flow cytometry to detect the membrane antigens CD29, CD90, and CD45. As shown in Fig. 1A, about 90% of the cell population expressed CD29 (90.1%) and CD90 (83.6%), two commonly used rat BMSCs surface markers. Meanwhile, less than 1% of the cell population expressed CD45, indicating that these cells are not of hematopoietic origin. These cells can also differentiate into adipocytes or osteoblasts when induced by rosiglitazone or by vitamin C/β-glycerophosphate, respectively (Fig. 1B). As shown in Fig. 1C, the capacity of differentiation into adipocytes and osteocytes were also significantly increased as indicated by increased expression of

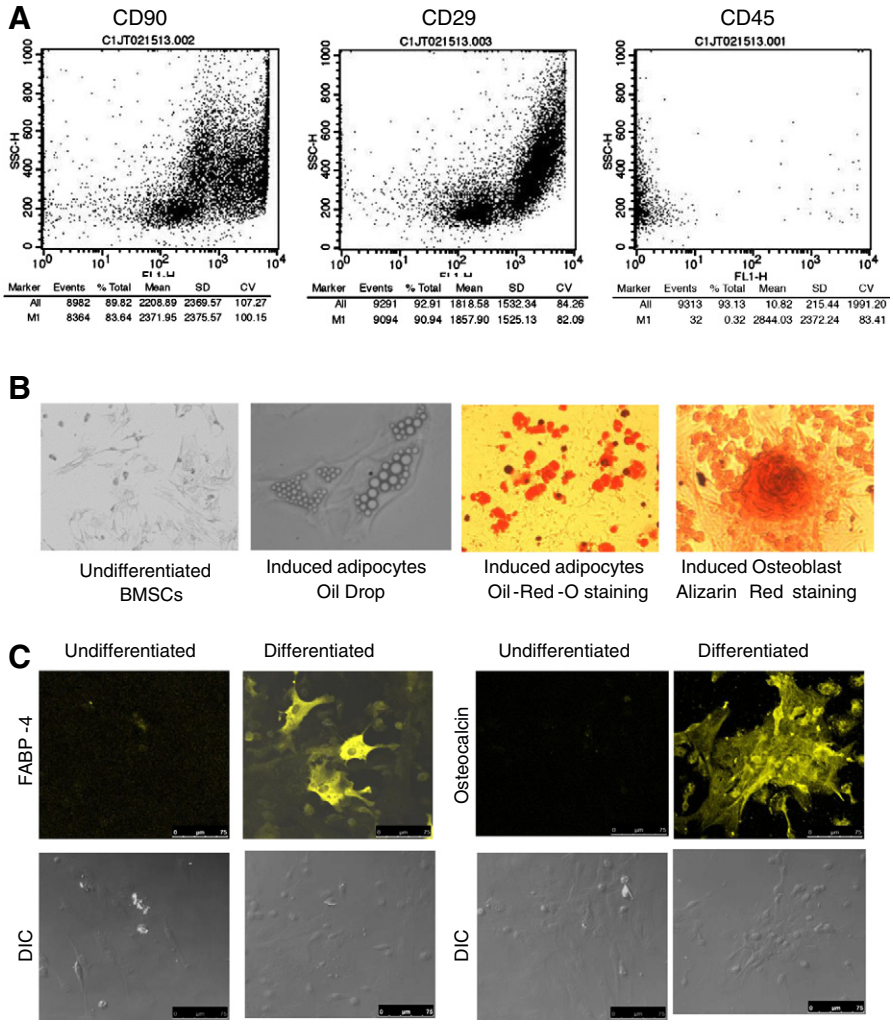

Figure 1 Characterization of bone marrow stromal cells (BMSCs). A: Expression of CD90, CD29 and CD45 in Rat BMSCs were determined by Flow Cytometry. Purified passage 3 BMSCs were immunostained with FITC-conjugated antibodies corresponding to each surface marker. B: Adipogeneic and osteogeneic differentiation of rat BMSCs. Undifferentiated and differentiated BMSCs were imaged to show the oid drop formation, Oio-Red-O staining (for adipogenesis), and Alizarin Red staining (for osteiogenesis). C: Immunostaining of adipocyte marker (FABP-4) and osteoblast marker (Osteocalcin). Undifferentiated or differentiated BMSCs were fixed and incubated with anti-FABP-4 or anti-Osteocalcin antibodies overnight at 4 °C. Fluorescent secondary antibodies were applied and a Leica confocal microscope was used to visualize the fluorescence. Differential interference contrast (DIC) images were also shown for each slide.

FABP-4 (an adipogenesis marker) or Osteocalcin (an osteogenesis marker). These data are consistent with other reports on rat BMSCs (Boxall and Jones, 2012; Harting et al., 2008).

MBG potentiates rosiglitazone-induced adipogenesis in BMSCs

Since cardiotonic steroids (CTS) have been reported to regulate ESC differentiation through Na/K-ATPase-related signaling pathways (Lee et al., 2011), we reasoned that CTS may also affect the differentiation of adult stem cells such as, BMSCs. The above mentioned P3 BMSCs were used to test whether MBG, a specific ligand of Na/K-ATPase, regulates BMSCs differentiation. Cells were pretreated with MBG or solvent control (0.1% DMSO) for 3 days and then 1 μM rosiglitazone was added and the cells cultured for an additional 72 h. Cells without addition of rosiglitazone were used to evaluate whether MBG alone induces adipogenesis. The results demonstrate that MBG alone does not increase adipogenesis, but instead it enhances adipogenesis induced by rosiglitazone in these cells. As shown in Fig. 2A, the Oil-Red-O staining was higher after 72 h rosiglitazone induction in the cells pre-treated with MBG at concentrations of 10 nM, 100 nM, and 1 μM compared to rosiglitazone alone. The same result was obtained by counting the Oil-Red-O stained colonies (Fig. 2B). To test if MBG changes cell growth, BMSCs were seeded in 6-well plates at 50,000 cells/well and cultured for 24 h. The cells were then treated with 1 μM MBG or its solvent control (01% DMSO) for additional 72 h. Treated cells were trypsinized and counted with Trypan Blue exclusion. As shown in Fig. 2C, the MBG treatment did not affect the cell growth

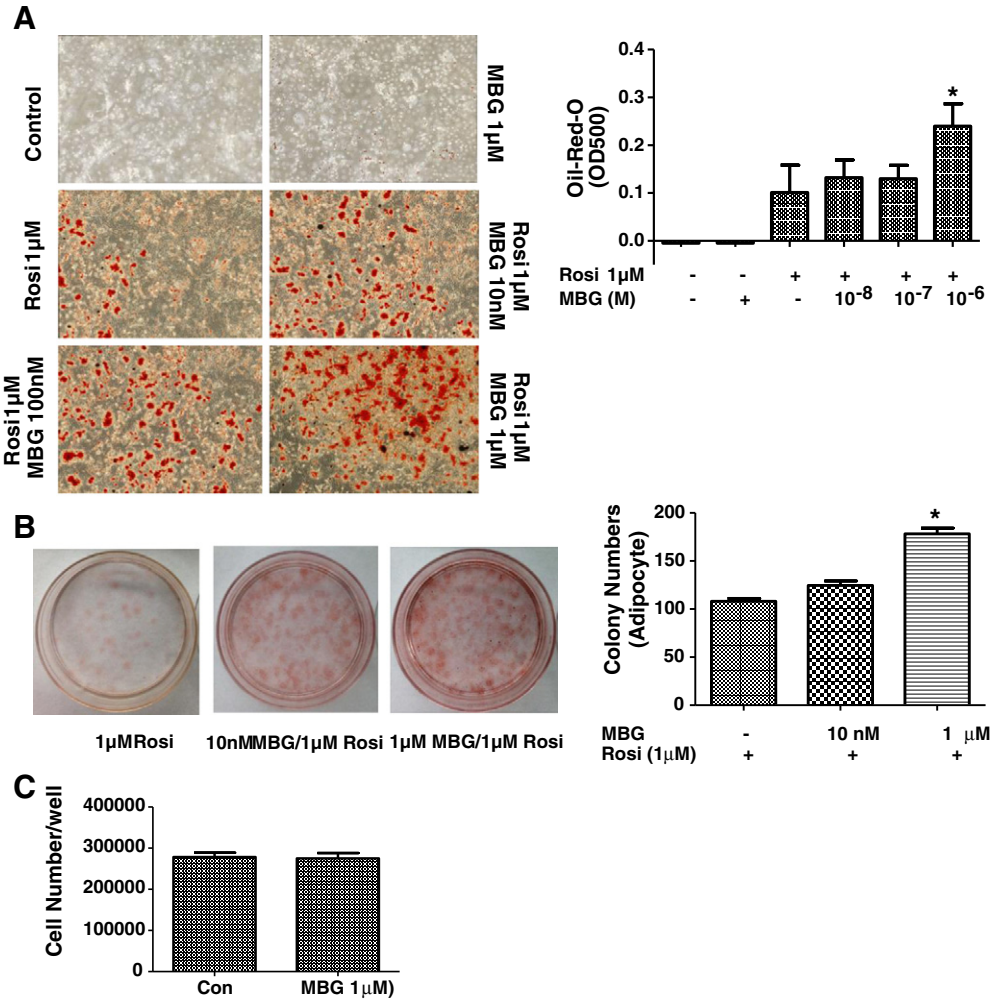

Figure 2 MBG potentiates rosiglitazone-induced BMSCs adipogenesis. Passage 3 BMSCs were pretreated with MBG or solvent control (0.1% DMSO) for 3 days and then 1 μM rosiglitazone (Rosi) was added and cultured for an additional 72 h. Cells without addition of Rosi were used to test if MBG alone induces adipogenesis. A: The treated cells were stained with Oil-Red-O and quantified by measuring absorbance at 500 nm; B: Total Oil-Red-O stained colonies of adipocyte were counted manually for each dish. C: BMSCs (50,000cell/well) were cultured for 24 h in 6-well plates and then treated with 1 μM MBG or solvent control (01%DMSO) for 72 h. Treated cells were trypsinized and cell number was counted with trypan blue exclusion. All data were obtained from 3 independent experiments and presented as mean ± SD. * indicates $p < 0.05$ vs Rosi alone treatment.

rate under this condition. These results indicate that MBG can prime the BMSCs and enhance rosiglitazone-induced adipogenesis.

MBG activates ERK and increases CCAAT/enhancer binding protein (C/EBP) expression in rat BMSCs

To understand the mechanism by which MBG potentiates rosiglitazone-induced adipogenesis, we examined MBG-induced signaling events in BMSCs. As shown in Fig. 3A, MBG treatment alone for 15 min activated ERK1/2. MBG also increased C/EBPα expression after 72 h treatment. No significant change in PPARγ was observed after 72 h treatment of MBG. In addition to MBG, another Na/K-ATPase ligand, ouabain, also increased C/EBPα expression in these cells (Fig. 3C). We also examined ERK activation, C/EBPα, C/EBPβ, C/EBPδ and PPARγ expression with the combination treatment of MBG and rosiglitazone. The results in Fig. 3D illustrated that 72 h pretreatment with MBG plus an additional 72 h of rosiglitazone treatment increases C/EBPα, C/EBPβ and C/EBPδ expression in BMSCs. The combined treatment also has a mild increase in PPARγ expression in these cells.

MBG potentiates rosiglitazone-induced adipogenesis in 3T3-L1 cells by increasing C/EBPα expression

To test if the effect of MBG on C/EBPα expression and adipogenesis is BMSC-specific, we employed 3T3-L1 cells as another adipogenesis model. When 3T3-L1 cells were pretreated with MBG for 72 h, potentiation of rosiglitazone-induced adipocyte formation was observed (Fig. 4A). MBG in combination with rosiglitazone also increases C/EBPα expression in 3T3-L1 cells (Fig. 4B). These results suggest that the

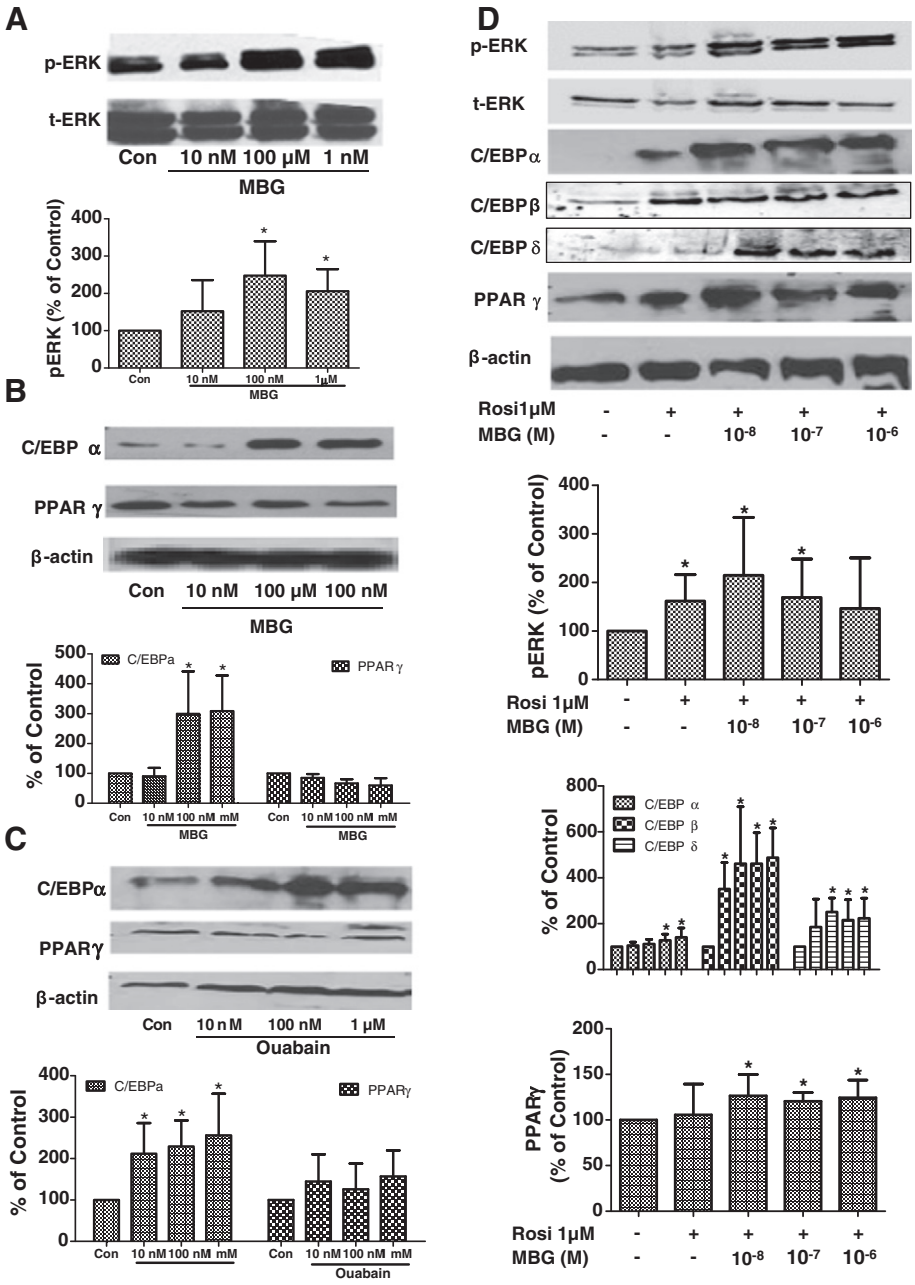

Figure 3 MBG activates ERK signaling and regulates C/EBP expression. A: Rat P3 BMSCs were treated with MBG for 15 min and cell lysates were collected in RIPA buffer to probe for phosphorylated and total ERK using Western blot; B: BMSCs were treated with MBG for 72 h and C/EBPα and PPARγ were probed by Western blot. C. BMSCs were treated with ouabain for 72 h and C/EBPα and PPARγ were probed with Western blot. D. BMSCs were pretreated with MBG for 72 h and then treated with rosiglitazone for additional 72 h. Cell lysates were then collected and probed for pERK, tERK, C/EBPα, C/EBPβ, C/EBPδ, and PPARγ. Actin was used as loading control. Experiments were repeated for 3–5 times and data were presented as mean ± SD. * indicates $p < 0.05$ vs control in each experiment.

potentiating effects of MBG-induced Na/K-ATPase signaling may play a role in more cell types than just BMSCs.

Blocking ERK activation inhibits the effect of MBG on C/EBPα expression and on rosiglitazone-induced adipogenesis

It has been reported that ERK activation can regulate C/EBP expression, which is essential for adipogenesis (Belmonte et al., 2001). As such, we examined whether MBG-induced ERK activation is required for MBG-related regulation of C/EBPα expression and subsequent adipogenesis in rat BMSCs. The U0126 compound, a specific mitogen activated protein kinase (MAPK) kinase inhibitor, was used to block ERK phosphorylation before MBG treatment. We found that pretreatment of BMSCs with U0126 (20 μM) blocked MBG-induced C/EBPα expression (Fig. 5A). In accordance with the C/EBPα expression data, U0126 also blocks MBG-potentiated adipogenesis in the presence of rosiglitazone (Fig. 5B). Since we have reported

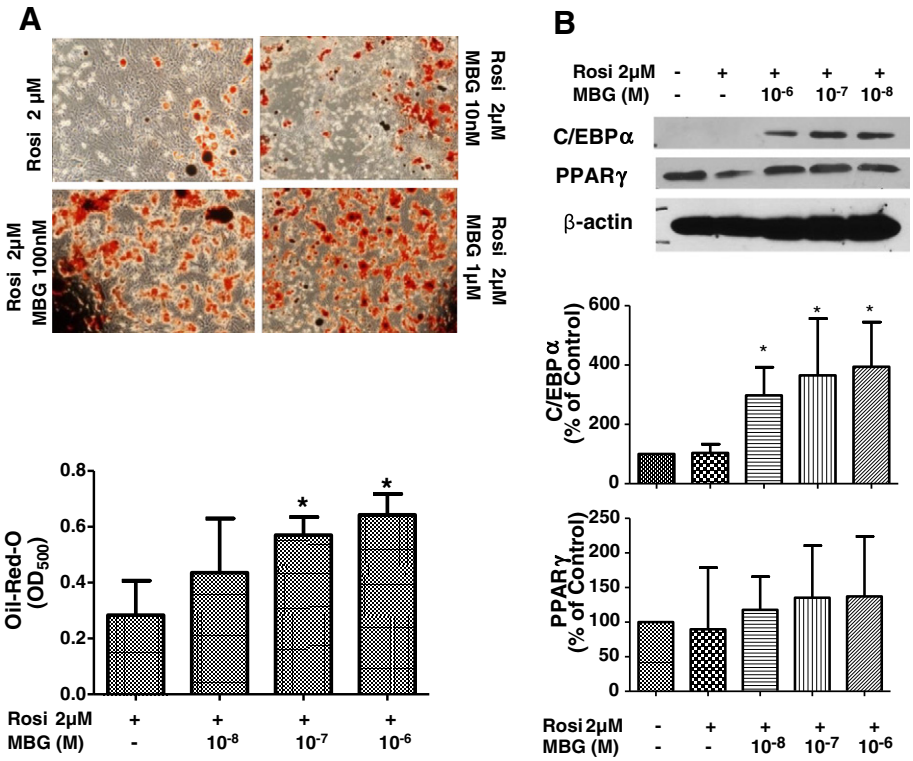

Figure 4 MBG potentiates rosiglitazone-induced adipogenesis in 3T3-L1 cells. 3T3-L1 cells were treated with 2 μM rosiglitazone alone or in combination with MBG pretreatment as described in Fig. 2. A: Adipogenic differentiation in 3T3-L1 cells was indicated by Oil-Red-O staining. Data were obtained from 3 independent experiments and presented as mean ± SD. * indicates $p < 0.05$ vs rosiglitazone (Rosi) treatment alone. B: Western blots results showed the changes in C/EBPα and PPARγ after combined treatment of MBG and rosiglitazone. Data were obtained from 3 independent experiments and presented as mean ± SD. * indicates $p < 0.05$ vs control.

that cardiotonic steroids (CTS) also activate Src and mTOR signaling pathways (Haas et al., 2002; Liu et al., 2012; Tian et al., 2006, 2009), which are upstream of ERK signaling, we then tested if inhibition of Src or mTOR affect C/EBPα expression. As shown in Figs. 5C and D, both PP2 (Src inhibitor) and rapamycin (mTOR inhibitor) pretreatment partially blocked the MBG-induced C/EBPα expression. These results suggest that MBG pretreatment primed the BMSCs through an ERK-related signaling pathway and made them ready for rosiglitazone-induced adipogenesis.

MBG pretreatment attenuates osteogenesis by reducing RunX2 expression

It is known that BMSCs can differentiate into either adipocytes or osteoblast and that these two events may be regulated in a reciprocal process (Banfi et al., 2000; Beresford et al., 1992; Li et al., 2003; Satomura et al., 2000). To test if MBG pretreatment also affects rat BMSCs osteogenesis, we induced BMSCs osteogenesis in the osteogenic medium described in the Materials and methods section with or without pretreatment of MBG. As shown in Fig. 6, pretreatment of MBG attenuates osteogenesis in these cells. We further tested the expression of RunX2, an essential transcription factor for osteogenesis (Franceschi and Xiao, 2003; Karsenty et al., 1999), in the BMSCs treated with MBG. The results demonstrated that RunX2 expression decreased after 72 h of MBG treatment, which is consistent with the notion that MBG attenuates osteogenesis in these cells.

Partial nephrectomy (PNx) on mouse BMSCs adipogenesis

Previous reports show that PNx increases circulating levels of MBG (Elkareh et al., 2007; Kennedy et al., 2006, 2008). To test if PNx regulates BMSCs differentiation through increased MBG, we isolated BMSCs from PNx-operated and sham-operated mice. Adipogenesis was induced in the first generation of BMSCs obtained from mice by rosiglitazone. As shown in Fig. 7A, PNx surgery increased plasma MBG levels as we have reported before. Meanwhile, BMSCs isolated from sham-operated mice behave just like BMSCs isolated from rats. MBG at 10 nM, 100 nM, and 1 μM potentiates rosiglitazone-induced adipocyte formation. However, BMSCs isolated from PNx mice exhibit a higher capacity for adipogenesis induced by rosiglitazone compared to the sham controls without MBG treatment. Interestingly, pretreatment of BMSCs isolated from PNx mice with MBG does not further increase adipogenesis (Fig. 7B). These results suggest that BMSCs from PNx mice may already be primed *in vivo* due to increased MBG levels and thus are less responsive to *in vitro* MBG treatment.

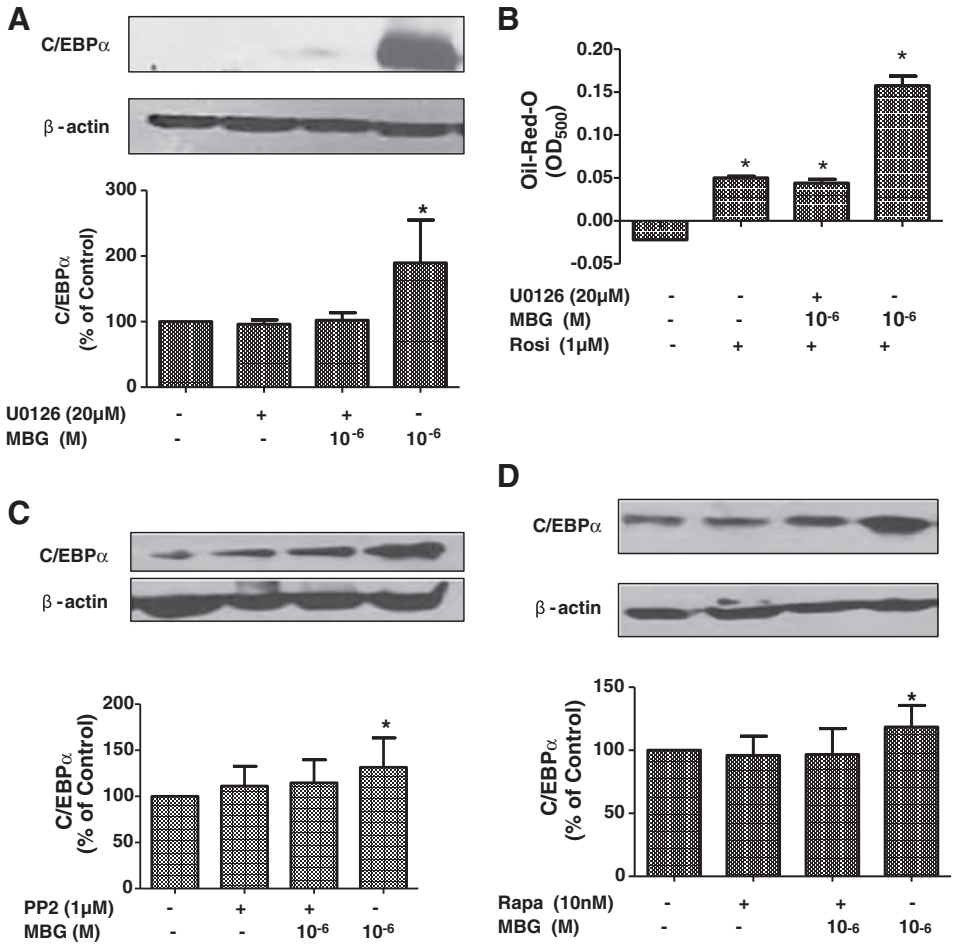

Figure 5 Inhibition of ERK signaling attenuates the regulation effect of MBG on C/EBPα and on rosiglitazone-induced adipogenesis. A: Rat BMSCs were treated with MBG alone or in combination with pretreatment of U0126 (A) for 72 h. The U0126, were added 30 min before MBG addition to the medium. Cell lysates were collected in RIPA buffer after 72 h of MBG treatment for Western blot to probe C/EBPα. B: MBG or MBG plus U0126 pretreated cells were induced for adipogenesis by adding 1 μM rosiglitazone to the medium and cultured for an additional 72 h. Differentiated cells were stained with Oil-Red-O and absorbance at 500 nm was measured as described in Materials and methods section. C & D: Rat BMSCs were treated with MBG alone or in combination with pretreatment of 1 μM PP2 (C) or 10 nM rapamycin (D) for 72 h as described in panel A. C/EBPα was probed with Western blot. Data from 3 independent experiments were presented as mean ± SD. * indicates $p < 0.05$ vs control (first column in each graph).

Discussion

The current study demonstrated the involvement of Na/K-ATPase and its ligands in stem cell differentiation, which has not been well studied in the field. Na/K-ATPase is an important membrane ion transporter existing in all mammalian cells. The function of Na/K-ATPase lies in two aspects, namely the ion transporting function and signal transducing function (Aizman and Aperia, 2003; Tian et al., 2006; Xie and Cai, 2003). The physiologic and pathologic role of Na/K-ATPase has been well studied in somatic cells such as kidney and heart cells, showing that activation of Na/K-ATPase signaling can induce cardiac hypertrophy, fibrosis, and regulate cell apoptosis under different conditions (Kennedy et al., 2006, 2008; Li et al., 2006; Liu et al., 2012; Tian et al., 2009). Only recently has the research on the effect of Na/K-ATPase in stem cell differentiation been initiated. It was reported that ouabain, another specific ligand of Na/K-ATPase, facilitates cardiac differentiation of embryonic stem cells via activation of an ERK-regulated signaling pathway (Lee et al., 2011); it also affects BMSCs differentiation by regulating the membrane potential (Sundelacruz et al., 2008). In addition to Na/K-ATPase, other ion transporting proteins such as, Ca^{2+}-activated K^+ channels (BKKCa) (Zhang et al., 2014), voltage sensitive K^+ channels (You et al., 2013) and H^+-pump (Adams et al., 2007) have been reported to regulate stem cell development and differentiation. These findings together with our current data suggest that ion transporters, traditionally considered as essential components for maintaining the homeostasis of ion concentration and electrical charge, may actually also participate in regulating cell signaling process including stem cell differentiation. However, whether the regulation is through the ion flow or signaling pathway or both remains elusive and merits further studies.

There have been reports showing the linkage between kidney dysfunction and lipid metabolization (Cignarelli and

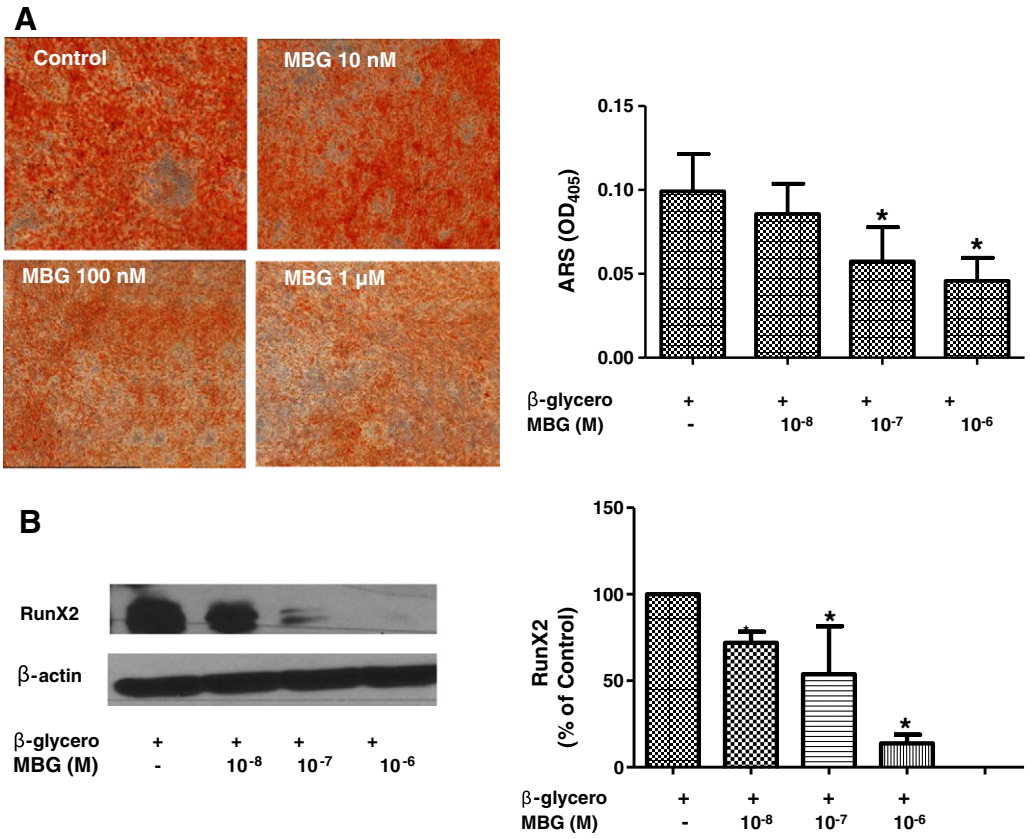

Figure 6 MBG treatment attenuates ascorbic acid (VitC)/β-glycerophosphate-induced osteogenesis. P3 Rat BMSCs were treated with 200 μM ascorbic acid/10 mM β-glycerophosphate alone or in combination with MBG pretreatment (72 h pretreatment of MBG before addition of ascorbic acid/β-glycerophosphate). A: Treated cells were stained for osteogenesis with Alizarin Red S. Left panel shows a representative image and right panel shows the quantification data from 9 experiments. Data are presented as mean ± SD. * indicates $p < 0.05$ vs control. B: Western blot for RunX2. Cell lysates from treated BMSCs were collected and probed for RunX2 using Western blot. Data were from 3 independent experiments and presented as mean ± SD. * indicates $p < 0.05$.

Lamacchia, 2007; Hall et al., 2003; Kramer, 2006; Maric-Bilkan, 2013; Wickman and Kramer, 2013). Indeed, we found that BMSCs isolated from PNx mice have higher rates of adipogenesis after induction with rosiglitazone, a synthesized PPARγ ligand, compared to cells from sham-operated control mice. On the other hand, metabolic bone disease and defective bone formation are common in chronic kidney disease (CKD) (Couttenye et al., 1999; Martin and Gonzalez, 2007). Many studies have shown that CKD-associated abnormalities of calcium, phosphorus, parathyroid hormone, and vitamin D may contribute to bone metabolic disorders. (Moe et al., 2006). Our data showed that MBG, a Na/K-ATPase ligand, can reduce osteogenesis of BMSCs. Since MBG release is increased in CKD animal models, it is conceivable that changes in circulating levels of MBG and other hormones may affect bone formation in CKD.

Rosiglitazone is a potent agonist for PPARγ and has been clinically used as a drug to treat type-2 diabetics (Baldwin and Duffin, 2004; Fidan et al., 2011; Hamm et al., 1999). Adipogenesis is greater with rosiglitazone treatment than dexamethasone treatment, a conventional method used to stimulate adipogenesis (Wang et al., 2011). Adipogenesis is coordinated by activation of PPARγ and increased expression of C/EBPα and other transcriptional factors (Christy et al., 1989; MacDougald et al., 1995; Nanbu-Wakao et al., 2000; Wu et al., 1999). Our results show that MBG alone increases C/EBPα expression but has no effect on PPARγ, which may explain the fact that MBG does not induce adipogenesis by itself. However, the combination treatment of MBG and rosiglitazone increased both C/EBPα and PPARγ compared to rosiglitazone alone, indicating cross-regulation between these transcriptional factors. On the other hand, Beresford, et al (Beresford et al., 1992) demonstrated an inverse relationship between the differentiation of adipocytic and osteogenic cells in BMSCs. Our results also indicate that MBG reduces osteogenesis while potentiates the adipogenesis. These findings are consistent with the possibility that the regulation of adipogenesis and osteogenesis can occur at the level of a common precursor.

The regulation of C/EBPα by MBG treatment is apparently mediated through the activation of ERK signaling pathways. Though the role of ERK activation on adipogenesis has been contradictory (Bost et al., 2005), a positive role for ERK in adipogenic differentiation has been found in 3T3-L1 cells (Prusty et al., 2002) and in other cellular models (Aubert et al., 1999; Belmonte et al., 2001). In addition, ERK activity is necessary for the expression of crucial adipogenic regulators such as C/EBP and PPAR (Bost et al., 2005). We showed that MBG activates the ERK pathway, and inhibition of ERK activation using U0126 results in strong inhibition of adipocyte formation (Bost et al., 2002).

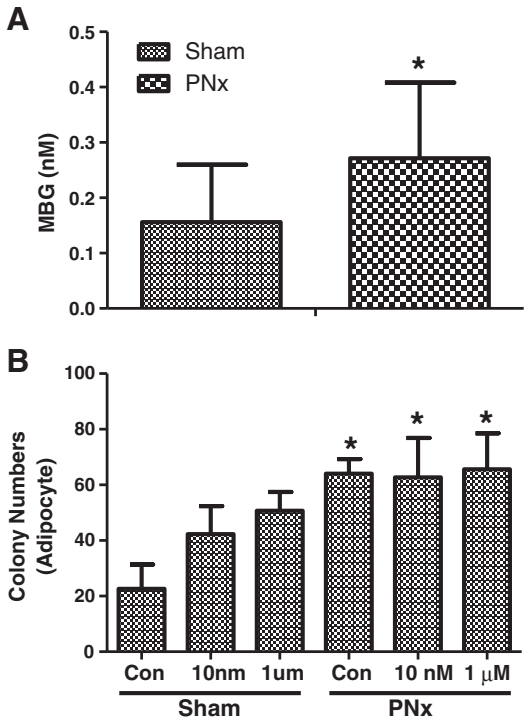

Figure 7 Partial Nephrectomy (PNx) increases circulating MBG concentration and promotes rosiglitazone-induced adipogenesis in mice BMSCs. A: Plasma MBG concentration was measured using competitive ELISA method. Plasma were collected from 13 sham mice and 9 PNx mice. B: First generation of BMSCs isolated from sham or PNx mice were treated with rosiglitazone alone or in combination with MBG pretreatment. The total colonies stained with Oil-Red-O were counted and presented as mean ± SD, n = 3 for each group. * indicates $p < 0.05$ vs sham control.

In summary, our study revealed an important role for Na/K-ATPase ligand activated signaling in the regulation of stem cell differentiation and broadened the knowledge of Na/K-ATPase signaling function in BMSCs. Since chronic kidney disease conditions often result in pathological development of excess adiposity and/or decreased bone density in individuals (Cignarelli and Lamacchia, 2007; Hall et al., 2003; Kramer, 2006; Maric-Bilkan, 2013; Wickman and Kramer, 2013), these studies may provide a potential therapeutic target to regulate BMSC differentiation and subsequent adipogenesis and osteogenesis.

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