Int J Clin Exp Pathol 2015;8(9):10875-10886
www.ijcep.com /ISSN:1936-2625/IJCEP0013481

Original Article

Cold exposure stimulates lipid metabolism, induces inflammatory response in the adipose tissue of mice and promotes the osteogenic differentiation of BMMSCs via the p38 MAPK pathway *in vitro*

Yizhen Nie[1], Zhaoqi Yan[1], Wei Yan[1], Qingyan Xia[2], Yina Zhang[3]

[1]Physical Examination Center, The Second Affiliated Hospital of Harbin Medical University, Harbin 150001, People's Republic of China; [2]Department of Medical Imaging, Harbin Fourth Hospital, Harbin 150026, People's Republic of China; [3]Cadre Ward, The Second Affiliated Hospital of Harbin Medical University, Harbin 150001, People's Republic of China

Received July 27, 2015; Accepted August 28, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: This study was to explore the effect of long-term cold exposure on morphological changes of WAT and BAT, metabolic changes and inflammatory responses *in vivo*. We also investigated the effect of cold exposure on the osteogenic differentiation of BMMSCs and the mechanism involved *in vitro*. At the end of the animal experiments, WAT and BAT were isolated and analyzed by HE staining. The results showed that both temperature and exposure time were associated with the degree of WAT browning. Then, peripheral blood samples were collected and centrifuged to obtain serum. Serum biochemical analysis was performed. After exposure to cold air for 21 d, cyclic adenosine monophosphate (cAMP) level in BAT was greatly upregulated. cAMP in WAT and glycerol levels were slightly increased. Cold exposure decreased triglyceride (TG) level and increased the levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C). Whereas, high-density lipoprotein cholesterol (HDL-C) and free fatty acid (FFA) levels remains unchanged. Moreover, leptin and adiponectin (ADP) levels were remarkably downregulated. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations were significantly elevated. Furthermore, the results showed that cold exposure significantly elevated runt-related transcription factor 2 (Runx2), bone sialoprotein (BSP), osteopontin (OPN) and collagen I levels and promoted the phosphorylation of p38 MAPK. However, the inducing effects were greatly inhibited by p38 MAPK inhibitor SB203580. These data suggest that long-term cold exposure activate BAT, increase lipolysis rate and enhance inflammatory response in mice. Furthermore, cold exposure promoted the osteogenic differentiation of BMMSCs partially via the p38 MAPK pathway.

Keywords: Adipose tissue, cold exposure, WAT browning, lipolysis, BMMSCs, osteogenic differentiation, p38 MAPK signalling pathway

Introduction

Optimum environmental temperature is essential for us to maintain good health. Cold or hot is harmful to humans. Accumulating studies have found that lower than normal body temperature may lead to higher hospital admission, chronic lung diseases or even death in some cases [1]. Once received cold stimulation, body will undergo repetitive and involuntary skeletal muscle contractions to produce extra heat for the maintenance of constant body temperature and thermal homeostasis via shivering thermogenesis [2]. Additionally, non-shivering thermogenesis and physical activity are also involved in thermoregulatory heat production [3, 4].

White adipose tissue (WAT) and brown adipose tissue (BAT) are the two main types of adipose tissue that had been found and identified in mammals [5, 6]. They play different roles in the process of energy metabolism. WAT mainly serves as the primary energy storing organ and stores energy in the form of triglyceride (TG). Whereas, BAT is responsible for non-shivering thermogenesis and regulates energy balance and body weight [7-10]. Cold is a well-known trigger for the activation of BAT [11]. It has been shown that cold exposure could upregulate the expression of uncoupling protein 1 (UCP1) that is located in the inner mitochondrial membrane of brown adipocytes, brown WAT to increase the volume of BAT and enhance thermogenesis in BAT in rats and humans [12, 13].

Mesenchymal stem cells (MSCs) are a group of cells that possess excellent proliferation and differentiation capabilities. MSCs can be isolated from adipose tissue, bone marrow and other tissues. Researchers have demonstrated that MSCs have strong potentials to differentiate into various types of cells (chondrocytes, osteoblasts, and adipocytes) and tissues (bone, fat, cartilage, muscle and nerve) [14, 15]. The mitogen-activated protein kinase (MAPK) pathway is reported to participate in proliferation and differentiation of the cells. Moreover, MAPK pathway is also associated with the osteogenic differentiation of stem cells [14].

Here, we investigated the role of cold exposure in morphological changes of adipose tissues, lipid metabolism and inflammatory cytokine release in C57BL/6 mice. The effect of cold exposure on the osteogenic differentiation of BMMSCs *in vitro* and the mechanism involved was discussed.

Materials and methods

Animals and cold exposure

A total of 36 C57BL/6 mice (6-8 weeks old, weighting 22 g) were purchased from Charles River Laboratories (Beijing, China). The mice were housed in a temperature controlled room with a 12 h light/12 h dark cycle up to the cold stimulation study. The mice were fed standard food and water *ad libitum*. Animal care was in accordance with institutional guidelines. All animal experiments were approved by the Animal Care and Use Committee of Harbin Medical University. The mice were randomly divided into two groups (18 mice in each group): (i) the control group, the mice were kept at 20°C for 7, 14 and 21 d; and (ii) the cold exposure group, the mice were kept at 4°C. After cold exposure for 7, 14 and 21 d, the mice were euthanized and two types of adipose tissues were excised immediately. White adipose tissue (WAT) was collected from the adipose tissue in the axilla. Brown adipose tissue (BAT) was isolated from the interscapular region.

Haematoxylin and eosin (HE) staining

Adipose tissues were dehydrated in sucrose solution, embedded in optimum cutting temperature (OCT) compound and sliced to 10-μm slides. The sections were stained with haematoxylin and eosin for histological examination and then photographed under a microscope (DP73; Olympus, Tokyo, Japan).

Measurement of cyclic adenosine monophosphate (cAMP) and glycerol levels

After cold exposure, tissue homogenates of WAT and BAT were obtained to determine cyclic adenosine monophosphate (cAMP) (USCN life science, Wuhan, China) and glycerol levels (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Biochemical analysis

At the end of the animal experiments, peripheral blood samples were collected and centrifuged at 4000 rpm for 10 min to obtain serum for use. Serum levels of total cholesterol (TC) (Dongou, Wenzhou, China), triglyceride (TG) (Dongou), low-density lipoprotein cholesterol (LDL-C) (Beihua, Beijing, China), high-density lipoprotein cholesterol (HDL-C) (Dongou), free fatty acid (FFA) (Sigma-Aldrich), leptin and adiponectin (ADP) (USCN life science) were measured using commercial kits. The concentrations of TC, TG, LDL-C and HDL-C in serum were expressed as mmol/L. FFA level was expressed as μmol/L.

Determination of cytokines in serum

After cold exposure, peripheral blood samples were harvested. Serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations were measured by ELISA (BOSTER, Beijing, China) according to the manufacturer's instructions.

Isolation and characterization of bone marrow-derived mesenchymal stem cells (BMMSCs)

The mice were sacrificed and the tibiae cleared of epiphysis and any connective tissue was obtained under sterile conditions. BMMSCs were isolated from the bone marrow cavity by flushing with Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies, Grand Island, NY, USA). Cell suspension was centrifuged at 1500 rpm for 10 min. Then, the cells were washed with red blood cell lysis buffer and phosphate buffered saline (PBS). BMMSCs were resuspended in DMEM supple-

mented with 10% FBS (fetal bovine serum) (HyClone, Logan, UT, USA). After passage 3, the cells were harvested for further experiments.

Characterization of BMMSCs

After passage 3, the medium was discarded and BMMSCs were washed with PBS for 3 times. After trypsinization, the cells were resuspended in 100 μl PBS (at a density of 1×10^6/ml) and incubated with fluorescein isothiocyanate (FITC)-conjugated CD44 antibody or Sca-1 antibody for 30 min at 37°C. The cells were resuspended in PBS and subjected to flow cytometry analysis (C6 model; Becton, Dickinson and Company, USA).

p38 mitogen-activated protein kinase (MAPK) inhibitor stimulation

After passage 3, the isolated BMMSCs were divided into four groups (BMMSCs in each group were incubated with the osteoblast inducing conditional media under different conditions throughout the experiment): (i) 37°C stimulation group, BMMSCs were cultured at 37°C; (ii) 37°C stimulation + SB203580 group, BMMSCs were pretreated with 10 μM p38 MAPK inhibitor SB203580 for 1 h/day for ten consecutive days at 37°C; (iii) 18°C stimulation group, BMMSCs were cultured at 18°C for 12 h/day for ten consecutive days; (vi) 18°C stimulation + SB203580 group, the cells were pretreated with SB203580 for 1 h before cold stimulation (18°C for 12 h/day). The levels of Runx2, bone sialoprotein (BSP), osteopontin (OPN) and collagen I were determined by western blot and real-time PCR. The levels of p38 MAPK and p-p38 MAPK were measured by western blot.

Western blot analysis

Total proteins were obtained from the cells in each group and the concentration was determined using bicinchoninic acid (BCA) kit (Beyotime, Haimen, China). Proteins (40 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The PVDF membranes were blocked with non-fat milk (M/V: 5%) or 1% BSA and then incubated with Runx2 antibody (1:500), BSP antibody (1:500), OPN antibody (1:1000), collagen I antibody (1:400), p38 MAPK antibody (1:500) and p-p38 MAPK antibody (1:500) at 4°C overnight. After washing with TTBS (Tween 20-Tris buffered saline), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5000) at 37°C for 45 min and the protein bands were visualized using ECL (enhanced chemiluminescence) reagent (Wanleibio). The bands were quantified using Gel-Pro-Analyzer 4.0 software (Media Cybernetics, Bethesda, MD, USA).

Real-time PCR analysis

Total RNAs were isolated from the cells in each group using a commercial kit (TIANGEN) according to the manufacturer's instruction and then reverse transcribed into cDNAs using super M-MLV Reverse Transcriptase (BioTeke, Beijing, China). Real-time PCR analysis was performed with SYBR GREEN mastermix (Solarbio, Beijing, China). The reaction was: 95°C for 10 min; 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 30 s; cooled at 4°C for 5 min. Relative gene expression levels were quantified using $2^{-\Delta\Delta Ct}$ method on Exicycler™ 96 Real-Time Quantitative Thermal Block (BIONEER, Daejeon, Korea) [16]. β-actin was used as normalized control. Primer sequences are shown below: Runx2-Forward, GGAGCGGACGAGGCAAGAGT; Runx2-Reverse, AGGAATGCGCCCTAAATCAC; BSP-Forward, GAACAAACAGGCAACGAATA; BSP-Reverse, GGTAGCCAGATGATAAGACAGA; OPN-Forward, TTCACTCCAATCGTCCCTAC; OPN-Reverse, TTAGACTCACCGCTCTTCAT; collagen I-Forward, AAAGACGGGAGGGCGAGTG; collagen I-Reverse, CCATAGGACATCTGGGAAGCAA; β-actin-Forward, CTGTGCCCATCTACGAGGGCTAT; β-actin-Reverse, TTTGATGTCACGCACGATTTCC.

Statistical analysis

GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The results are expressed as means ± SD. The data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. A value of $P<0.05$ was considered statistically significant.

Results

Effect of cold exposure on morphological changes

As shown in **Figure 1**, the results showed that WAT in mice underwent brown-like morphologi-

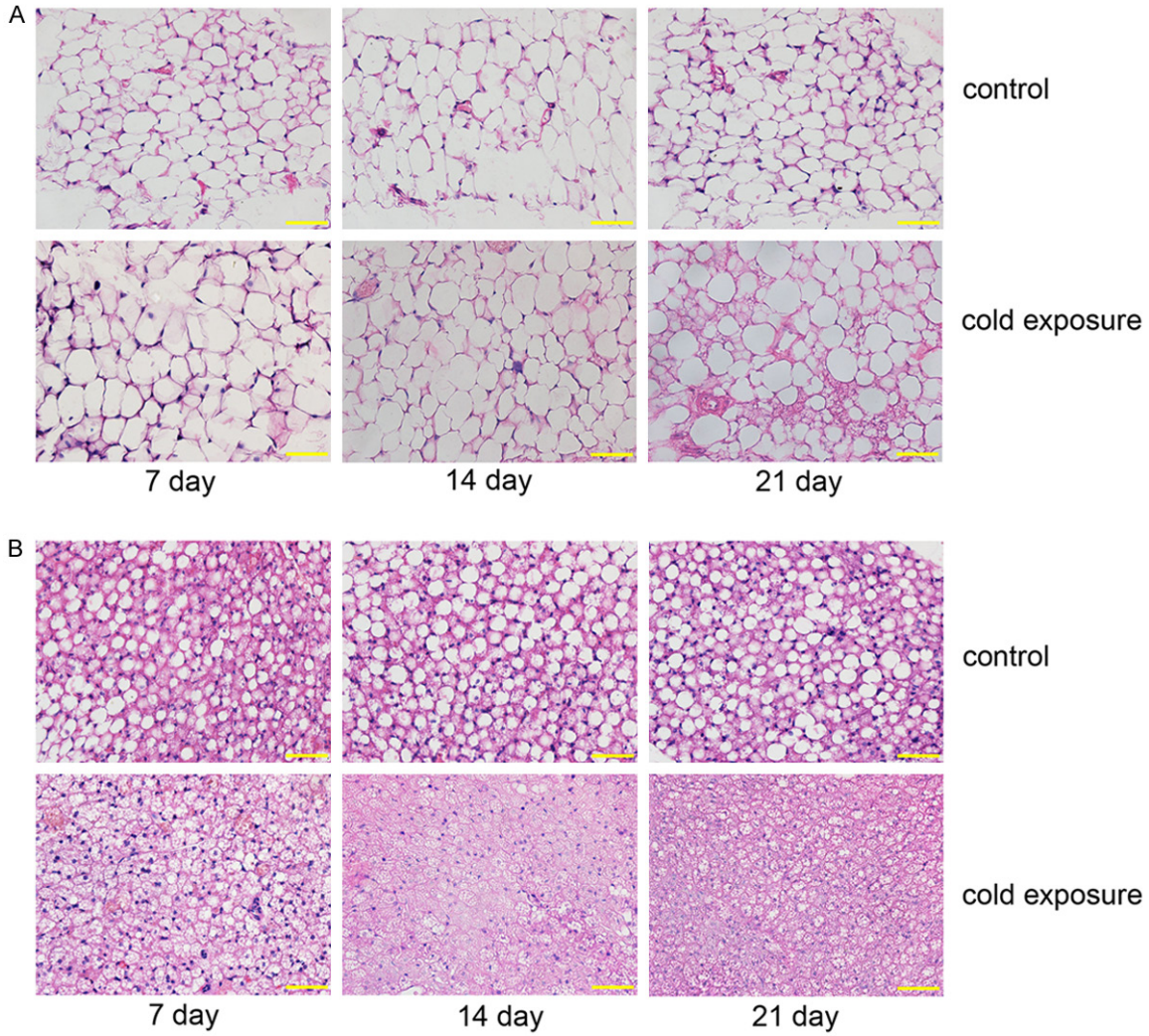

Figure 1. Micrographs of HE-stained white adipose tissue (WAT) and HE-stained brown adipose tissue (BAT) sections. A. WAT collected from the adipose *tissue* in the axilla *was* dehydrated, embedded, sectioned into 10-μm slides and stained with haematoxylin and eosin (HE) for histological examination. *Scale bar* 50 μm. B. Micrographs of HE-stained sections. BAT isolated from the interscapular region was stained with HE staining and observed under a microscope. *Scale bar* 50 μm.

cal changes (**Figure 1A**). Lipid droplets (LDs) in adipose cells of WAT became smaller and multilocular. BAT showed smaller LDs after cold exposure (**Figure 1B**).

Effect of cold exposure on cAMP and glycerol

The results showed that cold exposure did not remarkably alter the level of cAMP in WAT (**Figure 2A**). However, cold exposure led to an increase of cAMP level in BAT in a time-dependent manner (**Figure 2B**). After 21 days of cold exposure, cAMP level in BAT was significant higher than that in the control group ($P<0.01$). Moreover, cold exposure caused a slight rise (or at least maintained at the original level) in glycerol level in WAT (**Figure 2C**) and BAT (**Figure 2D**) when compared to the control group at the corresponding time points (7 d, 14 d and 21 d). However, the difference was not statistically significant.

Effect of cold exposure on serum metabolites

Serum TC (**Figure 3A**) and LDL-C concentrations (**Figure 3C**) were significantly higher in the cold exposure group than those in the control group ($P<0.01$). However, cold stimulation remarkably lowered TG level when compared to the control group (**Figure 3B**, $P<0.01$). We also

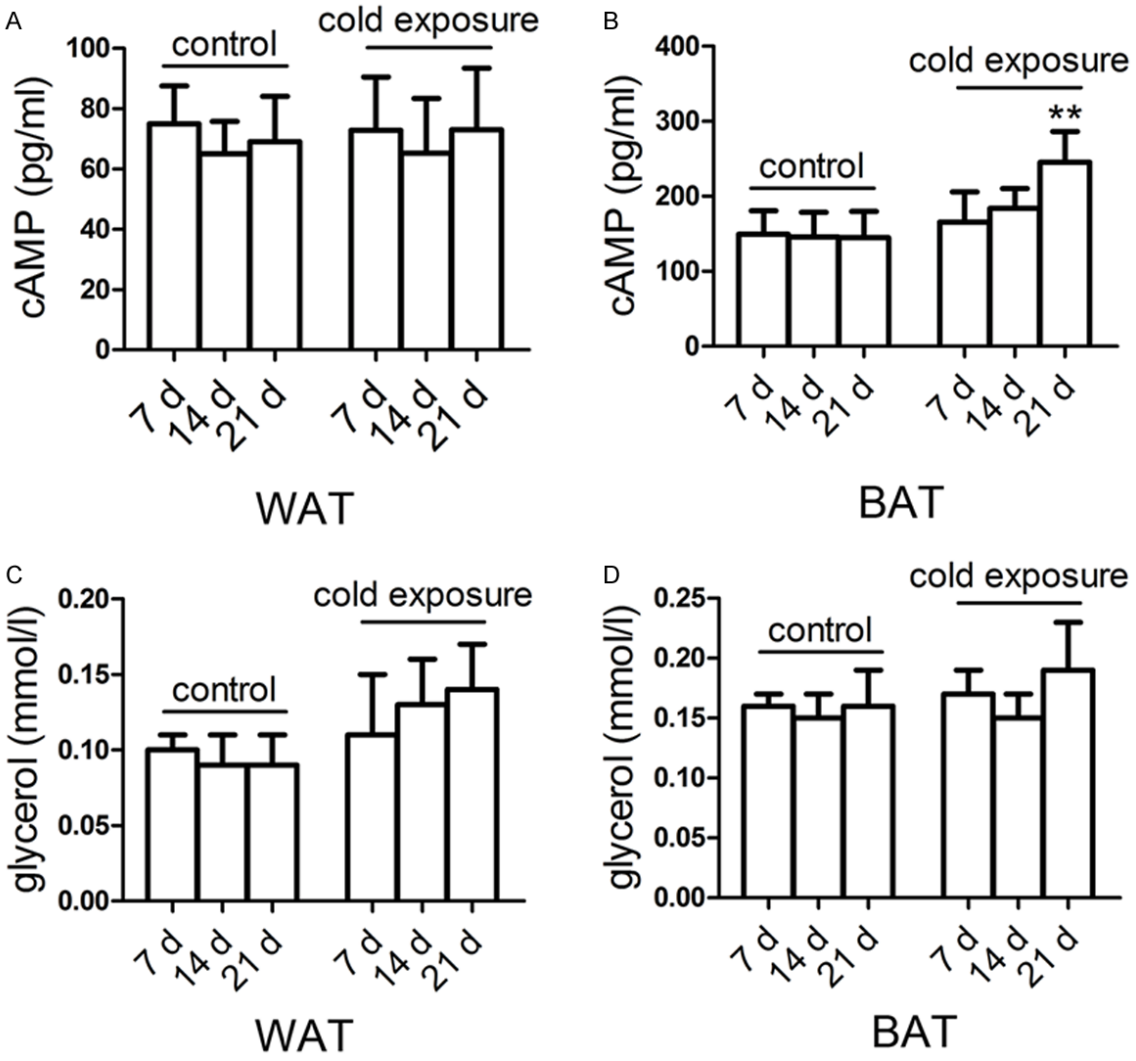

Figure 2. Effect of cold exposure on cAMP and glycerol levels. WAT and BAT were obtained. cAMP and glycerol releases were measured. Values are expressed as mean ± SD, n=6. $^{**}P<0.01$ compared to the corresponding control.

found that the levels of HDL-C (**Figure 3D**) and FFA (**Figure 3E**) were basically the same in these two groups.

Effect of cold exposure on the serum levels of leptin and ADP

We then measured leptin and ADP levels in serum. As shown in **Figure 4**, leptin (**Figure 4A**) and ADP levels (**Figure 4B**) reduced significantly after cold stimulation when compared to the control. On day 21, the differences were statistically significant (leptin, $P<0.05$; ADP, $P<0.01$).

Effect of cold exposure on the release of inflammatory cytokines

Cold exposure resulted in significant elevated levels of inflammatory cytokines TNF-α (**Figure 5A**) and IL-6 (**Figure 5B**). After 21 days of cold stimulation, the levels of TNF-α and IL-6 were upregulated significantly compared to the control group ($P<0.01$).

Cell morphology and surface antigen analysis of BMMSCs

BMMSCs have a spindle-shaped and fibroblast-like morphology (**Figure 6A**). We further analyzed cell surface markers of BMMSCs. The results showed that CD44 and Sca-1 was highly expressed on BMMSCs (**Figure 6B**).

Effect of cold exposure on the expression of osteoblastic differentiation markers and its related mechanisms

Compared with the 37°C group, p38 MAPK inhibitor SB203580 did no affect the mRNA levels of osteoblastic differentiation markers,

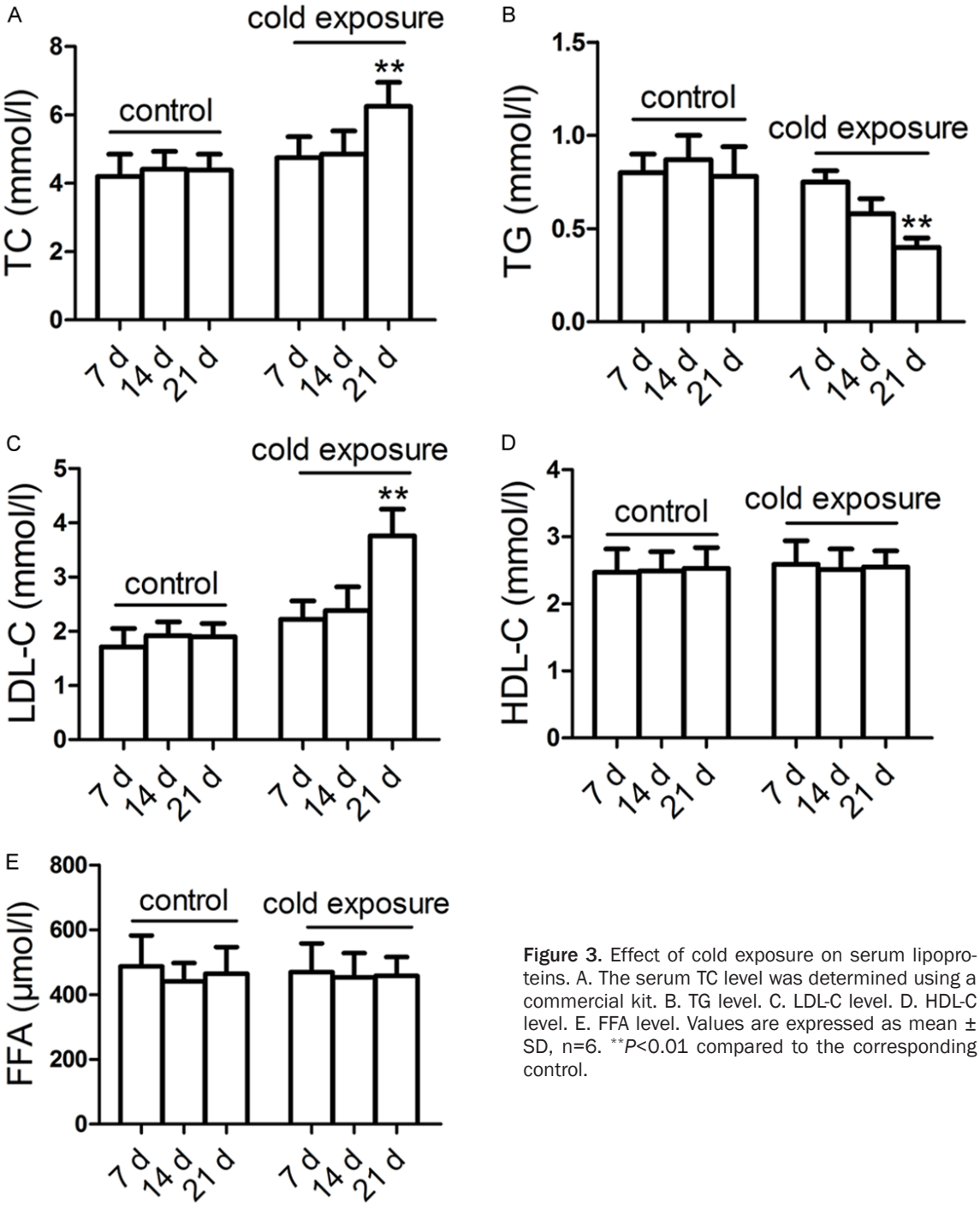

Figure 3. Effect of cold exposure on serum lipoproteins. A. The serum TC level was determined using a commercial kit. B. TG level. C. LDL-C level. D. HDL-C level. E. FFA level. Values are expressed as mean ± SD, n=6. $^{**}P<0.01$ compared to the corresponding control.

including Runx2, OPN, BSP and collagen I. As shown in **Figure 7A**, cold exposure significantly elevated the levels of Runx2, OPN, BSP and collagen I ($P<0.01$). However, SB203580 treatment greatly lowered all these markers ($P<0.01$). As shown in **Figure 7B**, the results of western blot analysis were consistent with real-time PCR ($P<0.01$). Downregulation of p-p38 MAPK levels were observed in the 37°C + SB203580 group ($P<0.05$). Additionally, cold exposure significantly induced the phosphorylation of p38 MAPK ($P<0.01$). SB203580 treatment significantly attenuated cold exposure-induced elevation of p-p38 MAPK levels.

Discussion

Accumulating evidences have reported that cold exposure may increase human discomfort, impair cognitive function and even lead to phys-

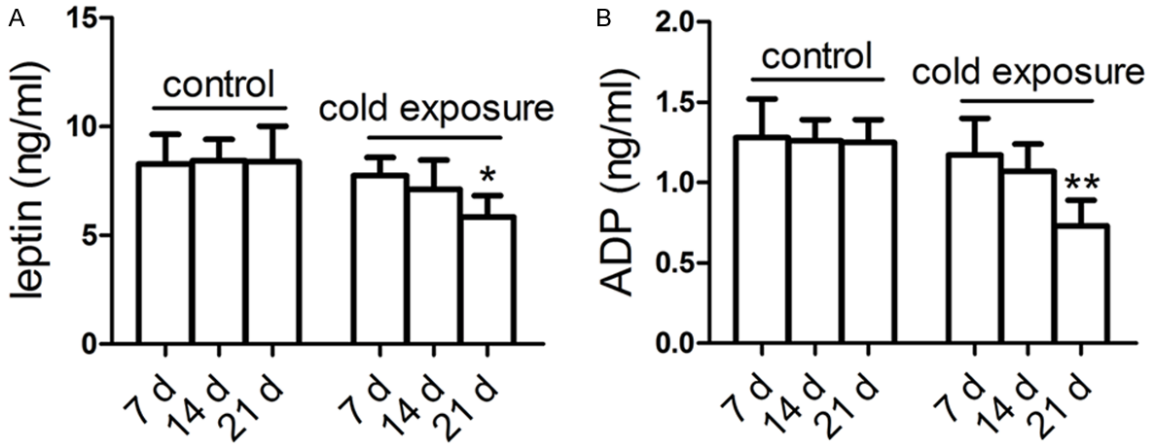

Figure 4. Effect of cold exposure on serum leptin and ADP concentrations. A. Leptin level. B. ADP level. Values are expressed as mean ± SD, n=6. $^{*}P<0.05$ and $^{**}P<0.01$ compared to the corresponding control.

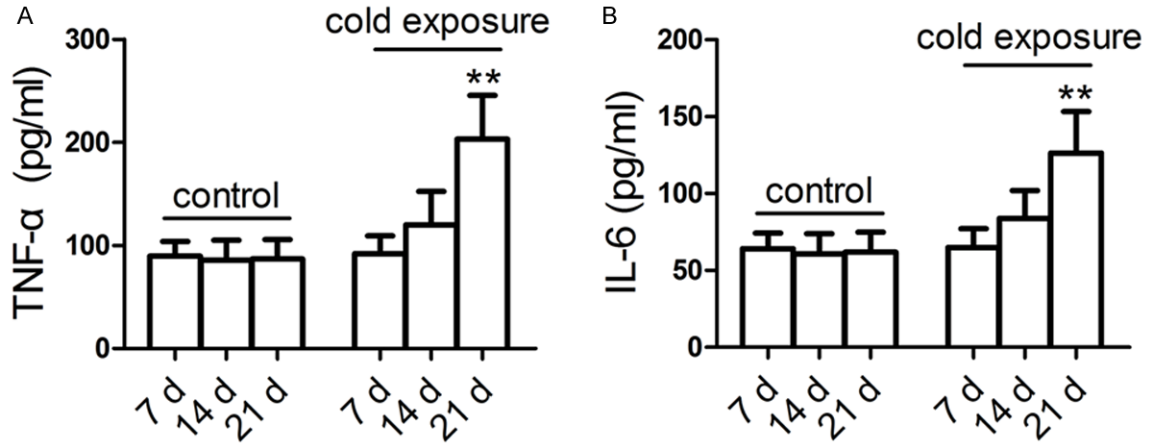

Figure 5. Effect of cold exposure on the release of inflammatory cytokines. A. TNF-α level. B. IL-6 level. Values are expressed as mean ± SD, n=6. $^{**}P<0.01$ compared to the corresponding control.

ical function disturbances [17]. Cold exposure also causes increases of metabolites including adrenaline, growth hormone and glucagon in mammals to avoid hypothermia [18]. Lipolysis is a biochemical process in which triglycerides (TG) are hydrolyzed into glycerol and free fatty acids (FFA). Generally, lipolysis occurs in various types of cells and tissues to meet the physiological needs of cells for FFA [19]. cAMP plays a major role in lipolysis and determines the rate of lipolysis in adipocytes and adipose tissues [20, 21]. Park Y *et al* have demonstrated that Plutella xylostella could express cold hardiness and accumulate glycerol in hemolymph to increase their cold tolerance [22]. Additionally, low temperature greatly increases glycerol concentration in plasma in humans and cAMP in rats [23, 24]. Here, our results demonstrated that cAMP levels were increased with increasing exposure time. Cold stimulation greatly upregulated intracellular cAMP contents in BAT after 21 d of exposure to cold, but this effect was not observed in WAT. Meanwhile, glycerol level was a little higher when exposed to 4°C than the controls (20°C). Thus, our results suggest that long-term cold exposure can increase the rate of lipolysis to some extent in adipose tissues, especially in BAT.

BAT plays a major role in TG metabolism and controls TG clearance [25]. BAT produces heat to maintain body temperature through consuming TGs that are stored in intracellular lipid droplets (LDs) [26]. Cellular cholesterol homoeostasis balances a number of interac-

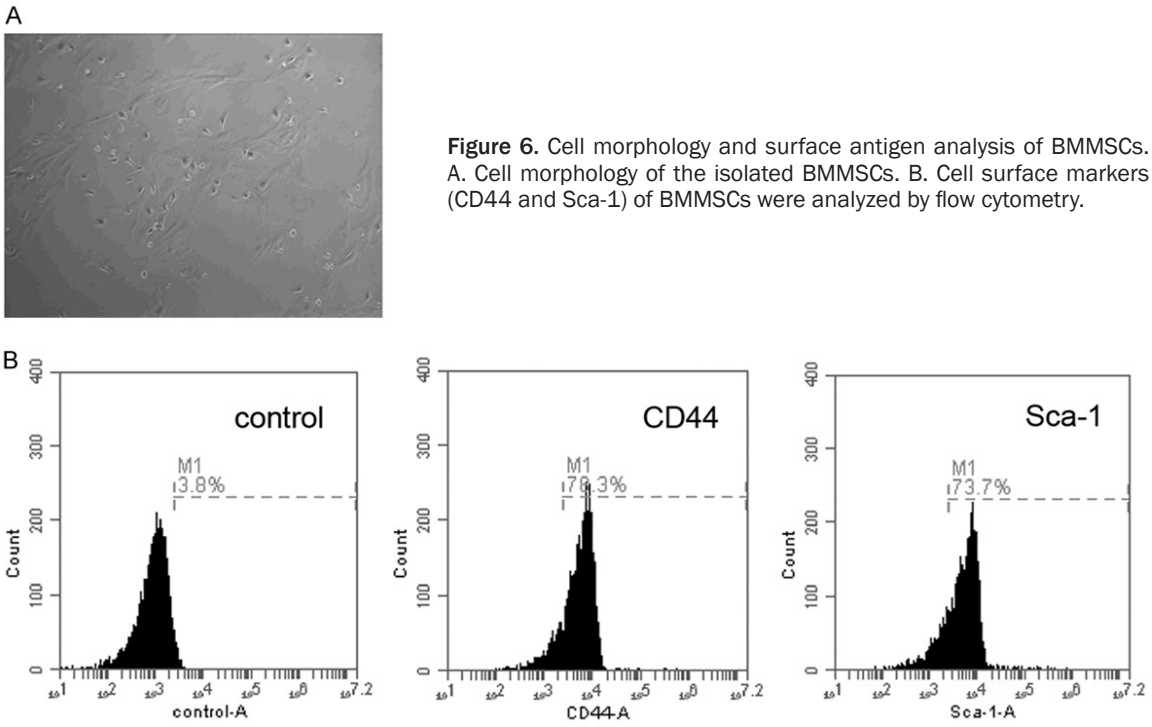

Figure 6. Cell morphology and surface antigen analysis of BMMSCs. A. Cell morphology of the isolated BMMSCs. B. Cell surface markers (CD44 and Sca-1) of BMMSCs were analyzed by flow cytometry.

tive and complex processes in body [27]. In the present study, we investigated the effect of cold exposure on serum lipids. Interesting, our data showed that long-term cold exposure (4°C for 21 d) resulted in remarkable increases in TC and LDL-C and decrease in TG. Serum HDL-C and FFA levels remained unchanged in the cold-exposed mice in relative to the control mice. The above results suggest that cold exposure enhance the metabolism of lipids.

Previous findings have reported that long-term cold stimulation caused smaller cell size of WAT in mice and the bats. Moreover, adipose cell size is positively correlated with adiponectin (ADP) in type 2 diabetic parents who have healthy non-diabetic children [28]. Leptin is found to be expressed in adipose cells and circulates in the plasma of humans and mice. The levels of leptin increase in obese people and decrease after weight loss. Previous evidences have found that, in cold environment, serum leptin level was downregulated and the thermogenic capacity of BAT was increased in Brandt's vole [29]. The reduction of ADP level in plasma that induced by cold has been detected in humans [30]. Nowadays, the therapeutic effect of leptin injection on the treatment of human obesity is being evaluated in clinical trials [31]. In the present study, we explored the relationship between cold exposure and leptin or ADP in C57BL/6 mice. Interestingly, our results consistently demonstrated that serum leptin and ADP levels in mice were reduced significantly with the increasing time of cold exposure. These results suggest that cold exposure could decrease the concentrations of leptin and ADP.

Generally, inflammation responses in adipose tissue are increased in obesity and contribute to dysregulation in adipose tissue [32]. Adding IL-6 in cultured adipose cells decreases ADP and peroxisome proliferator-activated receptor (PPAR) γ-2 levels [33]. Previous research has reported that multiple inflammatory cells, including granulocytes and alveolar macrophages, tend to infiltrate the lungs of healthy people when exposed to cold air [34]. Despite this, cold could also induce airway hyperresponsiveness and airway inflammation in dogs [35]. Recent researchers have proved that transient cold exposure (0°C) increases IL-8 and TNF-α in bronchoalveolar lavage fluid (BALF) in Wistar rats and promotes the release of IL-6 (18°C) in lung epithelial cells BEAS-2B [1]. In this study, we discussed the effect of long-term cold exposure on inflammatory cytokine release in C57BL/6 mice. We found that cold stimulation caused significant increases of TNF-α and IL-6 release after exposed to 4°C for

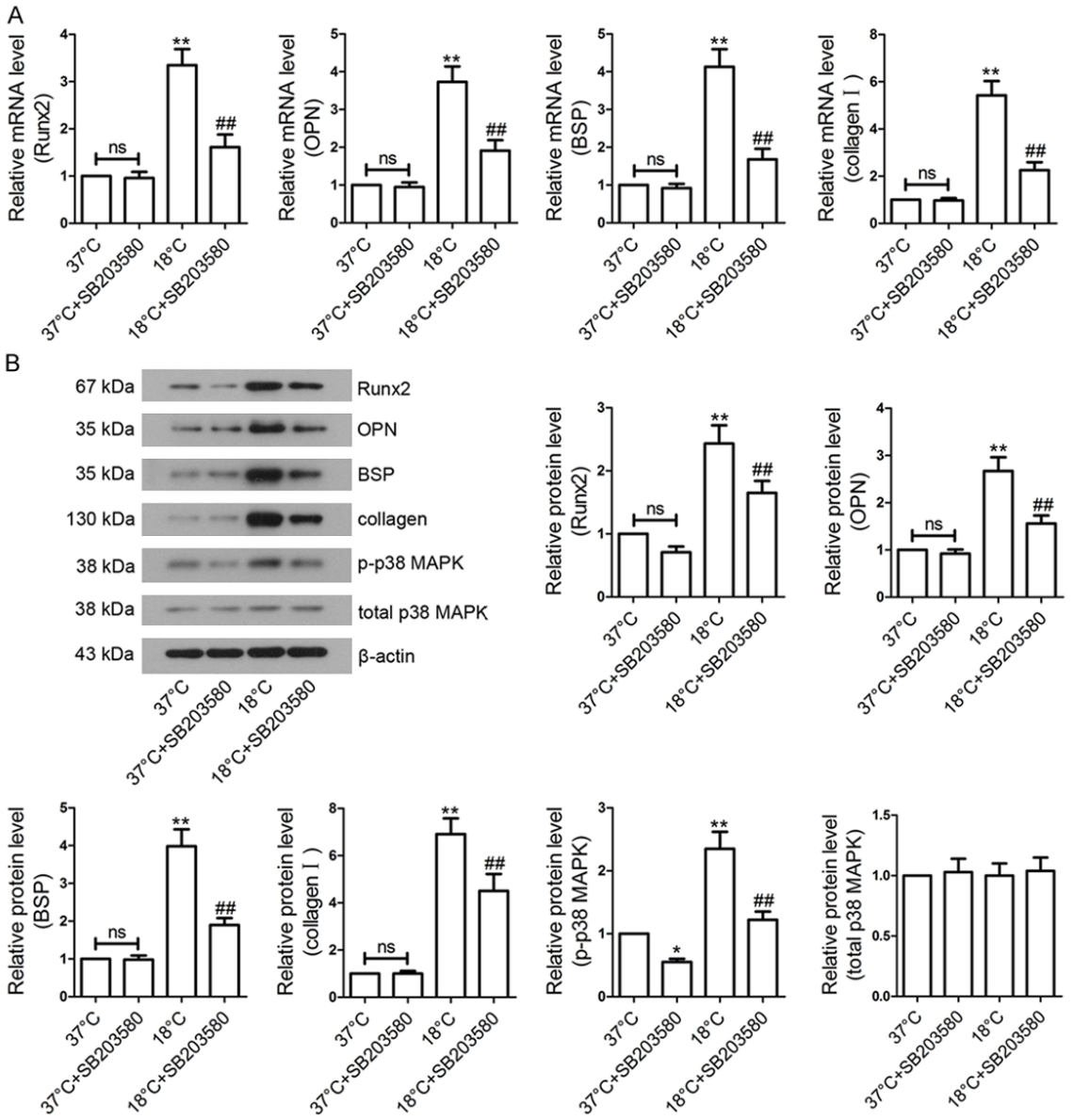

Figure 7. Effect of cold exposure on the expression of osteoblastic differentiation markers and its related mechanisms. A. Gene expression of Runx2, BSP, OPN and collagen I. All the values were normalized to β-actin. B. Protein expression of Runx2, BSP, OPN, collagen I, p38 MAPK and p-p38 MAPK. All the values were normalized to β-actin. Values are expressed as mean ± SD, n=3. $^{*}P<0.05$ and $^{**}P<0.01$ compared to the 37 °C group. $^{\#\#}P<0.01$ compared to the 18 °C group.

21 d, which is consistent with previous findings. The results indicate that cold exposure could induce inflammatory response in mice.

Bone marrow-derived mesenchymal stem cells (BMMSCs) are a population of cells that isolated from bone marrow. BMMSCs have multiple differentiation potentials and can differentiate into multiple cells, including chondrocytes, adipocytes and osteoblasts [36]. Runx2 is an osteoblast-specific transcription factor and also known as Cbfa1 (core-binding factor α 1) and PEBP2aA (polyomavirus enhancer binding protein 2 α A subunit). It is essential for osteoblast differentiation. Evidences have found that Runx2-deficient mice show arrest of osteoblast differentiation and thus lead to a complete absence of bone formation [37]. Runx2 is also reported to control the expression of bone-related genes, including OPN and BSP [38].

Accumulating studies have demonstrated that p38 MAPK pathway plays a critical role in regulating the osteogenic differentiation of BMMSCs [39]. However, the impacts of cold exposure on the expression of osteogenic genes and its possible mechanisms need to be investigated. In this study, cold exposure promoted the expression of Runx2, OPN, BSP and collagen I (a specific marker of bone matrix). Meanwhile, the phosphorylation levels of p38 MAPK were significantly upregulated. The increased levels of osteogenic genes were markedly downregulated in the present of p38 MAPK inhibitor. These results clearly suggest that cold exposure promote the osteogenic differentiation of BMMSCs by activating the p38 MAPK pathway.

In summary, our obtained results showed that long-term cold stress could induce the browning of WAT, increase the rate of lipid metabolism and activate inflammatory response. Cold exposure regulated the osteogenic differentiation of BMMSCs via the p38 MAPK signalling pathway. However, further studies are needed to verify our results.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yina Zhang, Cadre Ward, The Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Road, Harbin 150086, People's Republic of China. Tel: +86-451-86296486; E-mail: z_yina@126.com

References

[1] Luo B, Shi H, Wang L, Shi Y, Wang C, Yang J, Wan Y and Niu J. Rat lung response to PM2.5 exposure under different cold stresses. Int J Environ Res Public Health 2014; 11: 12915-12926.

[2] Gagnon DD, Gagnon SS, Rintamaki H, Tormakangas T, Puukka K, Herzig KH and Kyrolainen H. The effects of cold exposure on leukocytes, hormones and cytokines during acute exercise in humans. PLoS One 2014; 9: e110774.

[3] Putri M, Syamsunarno MR, Iso T, Yamaguchi A, Hanaoka H, Sunaga H, Koitabashi N, Matsui H, Yamazaki C, Kameo S, Tsushima Y, Yokoyama T, Koyama H, Abumrad NA and Kurabayashi M. CD36 is indispensable for thermogenesis under conditions of fasting and cold stress. Biochem Biophys Res Commun 2015; 457: 520-525.

[4] Klingenspor M. Cold-induced recruitment of brown adipose tissue thermogenesis. Exp Physiol 2003; 88: 141-148.

[5] Poher AL, Altirriba J, Veyrat-Durebex C and Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. Front Physiol 2015; 6: 4.

[6] Sethi JK and Vidal-Puig AJ. Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. J Lipid Res 2007; 48: 1253-1262.

[7] Dempersmier J, Sambeat A, Gulyaeva O, Paul SM, Hudak CS, Raposo HF, Kwan HY, Kang C, Wong RH and Sul HS. Cold-inducible Zfp516 activates UCP1 transcription to promote browning of white fat and development of brown fat. Mol Cell 2015; 57: 235-246.

[8] Cohen AW, Schubert W, Brasaemle DL, Scherer PE and Lisanti MP. Caveolin-1 expression is essential for proper nonshivering thermogenesis in brown adipose tissue. Diabetes 2005; 54: 679-686.

[9] Yao X, Shan S, Zhang Y and Ying H. Recent progress in the study of brown adipose tissue. Cell Biosci 2011; 1: 35.

[10] Campbell CS, Tabor J and Davis JD. Small effect of brown adipose tissue and major effect of photoperiod on body weight in hamsters (Mesocricetus auratus). Physiol Behav 1983; 30: 349-352.

[11] Boon MR, van den Berg SA, Wang Y, van den Bossche J, Karkampouna S, Bauwens M, De Saint-Hubert M, van der Horst G, Vukicevic S, de Winther MP, Havekes LM, Jukema JW, Tamsma JT, van der Pluijm G, van Dijk KW and Rensen PC. BMP7 activates brown adipose tissue and reduces diet-induced obesity only at subthermoneutrality. PLoS One 2013; 8: e74083.

[12] Bai Z, Wuren T, Liu S, Han S, Chen L, McClain D and Ge RL. Intermittent cold exposure results in visceral adipose tissue "browning" in the plateau pika (Ochotona curzoniae). Comp Biochem Physiol A Mol Integr Physiol 2015; 184: 171-178.

[13] Cadena V and Tattersall GJ. Body temperature regulation during acclimation to cold and hypoxia in rats. J Therm Biol 2014; 46: 56-64.

[14] Zhou Y, Wu Y, Jiang X, Zhang X, Xia L, Lin K and Xu Y. The Effect of Quercetin on the Osteogenesic Differentiation and Angiogenic Factor Expression of Bone Marrow-Derived Mesenchymal Stem Cells. PLoS One 2015; 10: e0129605.

[15] Kurte M, Bravo-Alegria J, Torres A, Carrasco V, Ibanez C, Vega-Letter AM, Fernandez-O'Ryan C, Irarrazabal CE, Figueroa FE, Fuentealba RA, Riedel C and Carrion F. Intravenous administration of bone marrow-derived mesenchymal

stem cells induces a switch from classical to atypical symptoms in experimental autoimmune encephalomyelitis. Stem Cells Int 2015; 2015: 140170.

[16] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.

[17] Lintu NS, Mattila MA, Holopainen JA, Koivunen M and Hanninen OO. Reactions to cold exposure emphasize the need for weather protection in prehospital care: an experimental study. Prehosp Disaster Med 2006; 21: 316-320.

[18] Shore AM, Karamitri A, Kemp P, Speakman JR, Graham NS and Lomax MA. Cold-induced changes in gene expression in brown adipose tissue, white adipose tissue and liver. PLoS One 2013; 8: e68933.

[19] Hofer P, Boeszoermenyi A, Jaeger D, Feiler U, Arthanari H, Mayer N, Zehender F, Rechberger G, Oberer M, Zimmermann R, Lass A, Haemmerle G, Breinbauer R, Zechner R and Preiss-Landl K. Fatty acid-binding proteins interact with comparative gene identification-58 linking lipolysis with lipid ligand shuttling. J Biol Chem 2015; 290: 18438-53.

[20] Wang YC, Hung HC, Feng CW, Huang SY, Chen CH, Lin YY, Chen YC, Yang SN, Su JH, Sheu JH and Wen ZH. Dihydroaustrasulfone Alcohol (WA-25) Impedes Macrophage Foam Cell Formation by Regulating the Transforming Growth Factor-beta1 Pathway. Int J Mol Sci 2015; 16: 10507-10525.

[21] Tu PT and Tawata S. Anti-obesity effects of hispidin and Alpinia zerumbet bioactives in 3T3-L1 adipocytes. Molecules 2014; 19: 16656-16671.

[22] Park Y and Kim Y. A specific glycerol kinase induces rapid cold hardening of the diamondback moth, Plutella xylostella. J Insect Physiol 2014; 67: 56-63.

[23] Koska J, Ksinantova L, Sebokova E, Kvetnansky R, Klimes I, Chrousos G and Pacak K. Endocrine regulation of subcutaneous fat metabolism during cold exposure in humans. Ann N Y Acad Sci 2002; 967: 500-505.

[24] Habara Y. Effects of cold exposure on cyclic AMP concentration in plasma, liver, and brown and white adipose tissues in cold-acclimated rats. Int J Biometeorol 1989; 33: 95-100.

[25] Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, Kaul MG, Tromsdorf UI, Weller H, Waurisch C, Eychmuller A, Gordts PL, Rinninger F, Bruegelmann K, Freund B, Nielsen P, Merkel M and Heeren J. Brown adipose tissue activity controls triglyceride clearance. Nat Med 2011; 17: 200-205.

[26] Khedoe PP, Hoeke G, Kooijman S, Dijk W, Buijs JT, Kersten S, Havekes LM, Hiemstra PS, Berbee JF, Boon MR and Rensen PC. Brown adipose tissue takes up plasma triglycerides mostly after lipolysis. J Lipid Res 2015; 56: 51-59.

[27] Ezhumalai M, Ashokkumar N and Pugalendi KV. Combination of carvacrol and rosiglitazone ameliorates high fat diet induced changes in lipids and inflammatory markers in C57BL/6J mice. Biochimie 2015; 110: 129-136.

[28] Hammarstedt A, Graham TE and Kahn BB. Adipose tissue dysregulation and reduced insulin sensitivity in non-obese individuals with enlarged abdominal adipose cells. Diabetol Metab Syndr 2012; 4: 42.

[29] Zhang Q, Lin Y, Zhang XY and Wang DH. Cold exposure inhibits hypothalamic Kiss-1 gene expression, serum leptin concentration, and delays reproductive development in male Brandt's vole (Lasiopodomys brandtii). Int J Biometeorol 2015; 59: 679-691.

[30] Iwen KA, Wenzel ET, Ott V, Perwitz N, Wellhoner P, Lehnert H, Dodt C and Klein J. Cold-induced alteration of adipokine profile in humans. Metabolism 2011; 60: 430-437.

[31] Friedman JM and Halaas JL. Leptin and the regulation of body weight in mammals. Nature 1998; 395: 763-770.

[32] Gustafson B, Gogg S, Hedjazifar S, Jenndahl L, Hammarstedt A and Smith U. Inflammation and impaired adipogenesis in hypertrophic obesity in man. Am J Physiol Endocrinol Metab 2009; 297: E999-E1003.

[33] Sopasakis VR, Sandqvist M, Gustafson B, Hammarstedt A, Schmelz M, Yang X, Jansson PA and Smith U. High local concentrations and effects on differentiation implicate interleukin-6 as a paracrine regulator. Obes Res 2004; 12: 454-460.

[34] Larsson K, Tornling G, Gavhed D, Muller-Suur C and Palmberg L. Inhalation of cold air increases the number of inflammatory cells in the lungs in healthy subjects. Eur Respir J 1998; 12: 825-830.

[35] Davis MS and Freed AN. Repeated hyperventilation causes peripheral airways inflammation, hyperreactivity, and impaired bronchodilation in dogs. Am J Respir Crit Care Med 2001; 164: 785-789.

[36] Cui H, Yi Q, Feng J, Yang L and Tang L. Mechano growth factor E peptide regulates migration and differentiation of bone marrow mesenchymal stem cells. J Mol Endocrinol 2014; 52: 111-120.

[37] Farshdousti Hagh M, Noruzinia M, Mortazavi Y, Soleimani M, Kaviani S, Abroun S, Dehghani Fard A and Mahmoodinia M. Different Methylation Patterns of RUNX2, OSX, DLX5 and BSP in Osteoblastic Differentiation of

Mesenchymal Stem Cells. Cell J 2015; 17: 71-82.

[38] Yang D, Okamura H, Nakashima Y and Haneji T. Histone demethylase Jmjd3 regulates osteoblast differentiation via transcription factors Runx2 and osterix. J Biol Chem 2013; 288: 33530-33541.

[39] Hu N, Feng C, Jiang Y, Miao Q and Liu H. Regulative Effect of Mir-205 on Osteogenic Differentiation of Bone Mesenchymal Stem Cells (BMSCs): Possible Role of SATB2/Runx2 and ERK/MAPK Pathway. Int J Mol Sci 2015; 16: 10491-10506.