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Deficiency of heat shock protein A12A promotes browning of white adipose tissues in mice

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ABSTRACT

Browning of white adipose tissues (WAT) is critical for a variety of physiological and pathophysiological events. Given the limited understanding in molecular control of WAT browning, further research is needed. Heat shock protein A12A (HSPA12A) is a new member of multigene *Hsp70* family. This study investigated the effect of HSPA12A on the browning of WAT. WAT Browning in mice was induced by cold exposure for 5 days. We observed that nuclear HSPA12A content was increased in WAT after cold exposure, while deficiency of HSPA12A (*Hspa12a*$^{-/-}$) promoted the cold-induced browning of WAT in mice compared to wild type (WT) littermates. Accordingly, *Hspa12a*$^{-/-}$ mice showed attenuation of body temperature drop and increase of thermogenic gene expression compared to WT mice after cold exposure. However, *in vitro* experiments demonstrated that HSPA12A deficiency in primary white adipocytes did not affect their browning and thermogenic gene expression. Further loss- and gain-of-HSPA12A functional studies revealed that HSPA12A deficiency promoted whereas HSPA12A overexpression impeded M2 macrophage polarization. Importantly, the conditioned medium (CM) from *Hspa12a*$^{-/-}$ bone marrow-derived macrophages (BMDMs) enhanced the browning of primary white adipocytes when compared to the CM from WT BMDMs. The data identified macrophage HSPA12A as a novel regulator of WAT browning through a paracrine mechanism. Targeting HSPA12A might provide meaningful advances for the management of browning-associated physiological events such as hypothermia adaptation and pathophysiological disorders such as obesity and cancer-related cachexia.

1. Introduction

Adipose tissues, including white adipose tissue (WAT) and brown adipose tissue (BAT), play a key role in maintaining metabolic homeostasis [1,2]. In contrast to WAT that stores surplus energy as lipids, BAT dissipates excess energy as heat [3]. In humans, BAT is the key depot to generate heat in neonates but its distribution is diminished during postnatal aging [4]. However, a subset of inducible brown-like adipocytes, known as “beige” or “brite” adipocytes [5], is found in WAT. Beige adipocytes could be generated by a process termed as “browning of WAT” or simply “browning” through either *de novo* differentiation of a specific pool of precursors or transdifferentiation from pre-existing white adipocytes [5–7]. Browning of WAT confers numerous metabolic benefits in thermogenic adaptation and reversing obesity and its associated co-morbidities [8,9], however, it also shows dark side effects such as promoting cancer-related cachexia, atherosclerosis, and hepatic steatosis [10,11]. Therefore, modulation of WAT browning is critical for a variety of physiological and pathophysiological events. Given the limited understanding in molecular control of WAT browning, further research is needed for understanding beige adipogenesis.

Characteristically, brown adipocytes are morphologically multilocular and are packed with mitochondria for heat production *via* the expression of uncoupling protein 1 (UCP1) [12]. UCP1 is an inner

Abbreviations: HSPA12A, heat shock protein A12A; HSP70, heat shock protein 70; WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SVF, stromal vascular fraction; iWAT, inguinal subcutaneous white adipose tissue; TH, tyrosine hydroxylase

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mitochondrial membrane protein that uncouples electron transport from ATP synthesis to a predominant production of heat. Not only as the hallmark of brown adipocytes, UCP1 is also paramount to the process of browning of white fat [7]. Controlling the expression of UCP1 is therefore critical for WAT browning. Some factors such as Irisin and fibroblast growth factor 21 (FGF21) have been shown to activate UCP1 expression for WAT browning [13]. Interestingly, alternative activated (M2) of macrophages has been implicated in the UCP1 expression and WAT browning by recent studies [12,14]. Indeed, M2 macrophage markers are increased following cold exposure. Moreover, expansion of M2 macrophages facilitates WAT browning in mice [14,15]. Therefore, modulating macrophage polarization towards to M2 phenotype is beneficial for WAT browning.

Heart shock protein A12A (HSPA12A) was initially cloned from macrophages in mouse atherosclerotic lesions in 2003 and classified as a distant member of heat shock protein 70 (HSP70) family due to containing an ATPase domain [16]. A high level of *Hspa12a* mRNA was detected in the brain of both mice and humans under normal conditions [16]. Subsequent study revealed that *Hspa12a* transcripts were decreased in the prefrontal cortex of patients with schizophrenia [17]. Our recent study demonstrated that knockout of HSPA12A exaggerates the cerebral ischemic injury [18]. However, the expression profile of HSPA12A in adipose tissues and the functional roles of HSPA12A in WAT browning are both unclear.

In this study, we observed that HSPA12A nuclear translocation was increased during the cold-induced browning of WAT in mice, while deficiency of HSPA12A promoted WAT browning and thermogenic adaptation. However, browning of primary white adipocytes is regulated by the paracrine effects of macrophage HSPA12A rather than by adipocyte HSPA12A. Our data identified HSPA12A as a novel regulator for WAT browning, and targeting HSPA12A might provide meaningful advances for the management of browning-associated physiological events such as hypothermia adaptation and pathophysiological disorders such as obesity and cancer-related cachexia.

2. Materials and methods

2.1. Reagents and antibodies

Primary antibody for GAPDH was from Bioworld Technology (Louis Park, MN). Primary antibody for UCP1, CD206 and LAMIN A/C were from Proteintech Group (Rosemont, IL). Primary antibody for HSPA12A and F4/80 was from Abcam (Cambridge, MA). Primary antibody for PGC-1α was from Millipore (Billerica, MA). Collagenase Type II, rosiglitazone, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and macrophage colony-stimulating factor (M-CSF) were purchased from Sigma-Aldrich (St. Louis, MO). Interleukin-4 (IL-4) was purchased from PreproTech (Oak Park, CA). Trizol reagent was purchased from Life Technology (Carlsbad, CA). Bovine serum albumin (BSA) was from Roche (Basel, Switzerland). Normal Goat Serum (NGS), peroxidase conjugated secondary antibody, Cy3- and 488-conjugated secondary antibody were from Jackson lmmunoResearch (West Grove, PA). Dulbecco's Modified Eagle's medium (DMEM), F12 medium and fetal bovine serum (FBS) were from Gibco (Shelton, CT). High-sig enhanced chemiluminescence (ECL) western blotting substrate was from Tanon (Shanghai, China). M-MLV reverse transcriptase was from Promega (Madison, WI). SYBR Green Master Mix was from Roche (Indianapolis, IN).

2.2. Animals

The conditional HSPA12A knockout mice (*Hspa12a*$^{-/-}$) were generated using a *Cre-LoxP* recombinant system as described in our previous study [18]. To remove the *Hspa12a* gene, the chimeric mice were crossed with EIIa-Cre transgenic mice. The mice were bred at the Model Animal Research Center of Nanjing University, and were kept at a 12 h light/dark cycle and received water and food *ad libitum*. All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011), and the principles outlined in the Declaration of Helsinki, and the international guidelines on the ethical use of animals. The animal care and experimental protocols were approved by Nanjing University's Committee on Animal Care (#XG55).

2.3. Induction of WAT browning in mice

Cold exposure was used to induce WAT browning in mice as described previously [19]. Briefly, male 8-week-old *Hspa12a*$^{-/-}$ mice and wild-type (WT) littermates were housed at 4 °C for 5 days (120 h). The mice housed at room temperature served as controls. Two mice were housed in each cage. The rectal temperatures and body weights were recorded at the indicated time points.

2.4. Adenovirus and lentivirus construction

The adenoviral and lentiviral vectors containing 3 flags-tagged coding region of mouse *Hspa12a* (NM_175199) were generated by GeneChem (Shanghai, China).

2.5. Stromal vascular fraction isolation, differentiation, transfection and browning treatment

To prepare the stromal vascular fraction (SVF), inguinal subcutaneous WATs (iWATs) were isolated from 6-week-old male mice and finely minced and digested in 0.15% type II collagenase for 30 min at 37 °C. The cell suspension was then filtered through a 50-μm nylon mesh and centrifuged at 1100 ×*g* for 10 min. The pellet was resuspended then plated in 60-mm culture dishes or 12-well culture plates supplied with DMEM/F12 containing 10% FBS. After confluent, differentiation of primary SVF cells were induced with 1 μM dexamethasone, 0.5 mM IBMX, and 5 μg/mL insulin for 2 days and then maintained in the medium containing 5 μg/mL insulin for 2 days, followed by maintaining in regular medium for 2 days. For overexpression of HSPA12A, primary adipocytes were infected with lentivirus that carrying *Hspa12a* expression sequence. The cells infected with empty virus served as normal expression controls. For browning stimulus, cells were exposed to growth medium in the absence (control) or presence of 1 μM rosiglitazone (browning treatment) for 5 days according to previous study [20].

2.6. Macrophage culture

2.6.1. Isolation and differentiation of bone marrow-derived macrophages (BMDMs)

Bone marrow cells isolated from femurs and tibias of male 8-week-old mice were cultured in DMEM plus 10% FBS for 6 h, then the non-adherent cells were collected and cultured in DMEM with 10% FBS and 20 ng/mL macrophage colony-stimulating factor (M-CSF) for 7 days.

2.6.2. Growth of mouse Raw264.7 macrophages

Raw264.7 macrophages were grown in DMEM supplemented with 10% FBS.

2.6.3. Overexpression of HSPA12A (*Hspa12a*$^{o/e}$) in macrophages

Raw264.7 macrophages were infected with adenovirus (20 MOI) that carrying *Hspa12a* expression sequence for 16 h. The cells infected with empty adenovirus (20 MOI) served as normal expression controls (NC).

2.7. IL-4 treatment and conditioned medium (CM) collection

IL-4 (10 ng/mL) was introduced to BMDMs or Raw264.7

macrophages to induce M2 polarization as described previously [14,21]. Briefly, BMDMs and Raw264.7 cells were stimulated with IL-4 for 12 h followed by incubation with IL-4 free fresh medium for another 12 h. The cells were collected for protein or mRNA analysis and the medium were collected as CM.

2.8. Paracrine effect of macrophage HSPA12A on the browning of white adipocytes

2.8.1. Conditioned medium (CM) collection from macrophages

Following treated with IL-4 as mentioned above, conditioned medium was collected from macrophage cultures.

2.8.2. Paracrine effects of macrophage HSPA12A on white adipocyte browning

The SVF cells were incubated with the collected conditioned medium during browning induction by rosiglitazone (1 μM).

2.9. RNA isolation and real-time PCR

Total RNA was isolated from cultured cells or adipose tissues using Trizol Reagent. An amount of 2 μg of RNA was reverse-transcribed to cDNA using N15 random primers. The quantitative RT-PCR analysis was performed with SYBR Green Master Mix on a Step One Plus real time PCR System (Applied Biosystems). Gene expression levels were normalized to Actin. The primers used in the experiments were listed in Table 1.

2.10. Western blot analysis

Total protein extracts from tissues or cells in equivalent amounts were separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membranes were blocked in 5% non-fat milk for 1 h at room temperature then blotted with the primary antibodies at 4 °C overnight. The blots for GAPDH or LAMIN A/C were used as loading controls. After thoroughly washing, membranes were incubated with peroxidase-conjugated secondary antibodies. Signals were detected with an ECL kit and quantified by scanning densitometry. The results from each experimental group were expressed as relative integrated intensities compared with those of controls.

Table 1
Primers used in the experiments.

Gene name	Primers
Ucp1 for mus	Forward CTTTGCCTCACTCAGGATTGG
	Reverse ACTGCCACACCTCCAGTCATT
Cox7a for mus	Forward GCTCTGGTCCGGTCTTTTAGC
	Reverse GTACTGGGAGGTCATTGTCGG
Cox8b for mus	Forward TGTGGGGATCTCAGCCATAGT
	Reverse AGTGGGCTAAGACCCATCCTG
Elovl3 for mus	Forward TTCTCACGCGGGTTAAAAATGG
	Reverse GAGCAACAGATAGACGACCAC
Dio2 for mus	Forward AATTATGCCTCGGAGAAGACCG
	Reverse GGCAGTTGCCTAGTGAAAGGT
Arg1 for mus	Forward CTCCAAGCCAAAGTCCTTAGAG
	Reverse AGGAGCTGTCATTAGGGACATC
Cd206 for mus	Forward TGTTACCAACTGGGACGACA
	Reverse TGGCACTCCCAAACATAATTTGA
Th for mus	Forward GTCTCAGAGCAGGATACCAAGC
	Reverse CTCTCCTCGAATACCACAGCC
Actin for mus	Forward TGTTACCAACTGGGACGACA
	Reverse TCTCAGCTGTGGTGGTGAAG

2.11. Immunohistochemistry

Inguinal adipose tissues were harvested from mice and fixed with 4% phosphate-buffered formaldehyde for paraffin-embedded sectioning (5 μm). After deparaffinization and rehydration, antigen was retrieved using citrate buffer. After blocked with 5% normal goat serum, sections were incubated with primary antibody for UCP1 (1:100) overnight at 4 °C followed by incubation with the peroxidase-labeled secondary antibody at room temperature for 2 h. The staining was visualized with 3,3′-diaminobenzidine. The hematoxylin was used to counterstain nuclei. The stainings were examined and photographed by a light microscopy at a magnification of 400× (Zeiss Ltd., Germany).

2.12. Immunofluorescence

Immunofluorescence staining was performed on 4% PFA-fixed frozen iWAT sections. After incubation with the primary antibody for HSPA12A or F4/80 (1:100) overnight at 4 °C, Cy3- or 488-conjugated secondary antibody was applied to the sections to visualize the staining. Hoechst 33342 was used to counterstain the nuclei. The staining was observed and quantified in ten randomly selected areas of each sample using a fluorescence microscope with Cellsens Dimention 1.15 software (Olympus, Tokyo, Japan).

2.13. Statistical analysis

Data are represented as mean ± standard deviation. Comparisons between groups were assessed by unpaired two-tailed Student *t*-test or two-way ANOVA followed by Tukey's test as a post-hoc test. A P value of < 0.05 was considered as significant.

3. Results

3.1. HSPA12A is highly expressed in adipose tissues

The expression profile of HSPA12A in adipose tissues is unclear. We therefore examined HSPA12A protein expression in mice tissues by immunoblotting. Though lower than brain, adipose tissues including BAT and WAT (iWAT, visceral WAT and perirenal WAT) showed higher expression levels than heart, liver, spleen, lung, pancreas, skeletal muscle, bone, lymph node, bone marrow, and monocyte (Fig. 1 and S1).

3.2. HSPA12A expression and nuclear translocation is increased during browning of iWAT

Given that cold challenge is canonical to induce WAT browning [22], we investigated whether HSPA12A expression is altered after cold exposure. Following housed at low temperature (LT) of 4 °C for 5 days, C57BL/6 mice showed significant browning of iWAT as indicated by increase of UCP1 expression (Fig. 2A). Elevated nuclear and total HSPA12A protein level (33% and 28%, respectively) were detected in iWAT of LT-exposed mice compared to room temperature (RT) controls, although the cytosolic HSPA12A protein level was unchanged (Fig. 2B). Immunofluorescence staining also showed an increase of HSPA12A level in iWAT from LT-exposed mice (Fig. S2). However, the protein level of HSPA12A in BAT and visceral white adipose tissue (vWAT) remained unchanged after cold exposure (Fig. S3). Thus, HSPA12A showed increased protein expression and nuclear translocation in iWAT during cold-induced browning.

3.3. HSPA12A deficiency facilitates thermogenic adaptation in mice in cold ambience

WAT browning is a robust defense against hypothermia [23]. We therefore examined the effects of HSPA12A deficiency on cold-induced drop of body temperature using *Hspa12a* knockout (*Hspa12a*$^{-/-}$) mice.

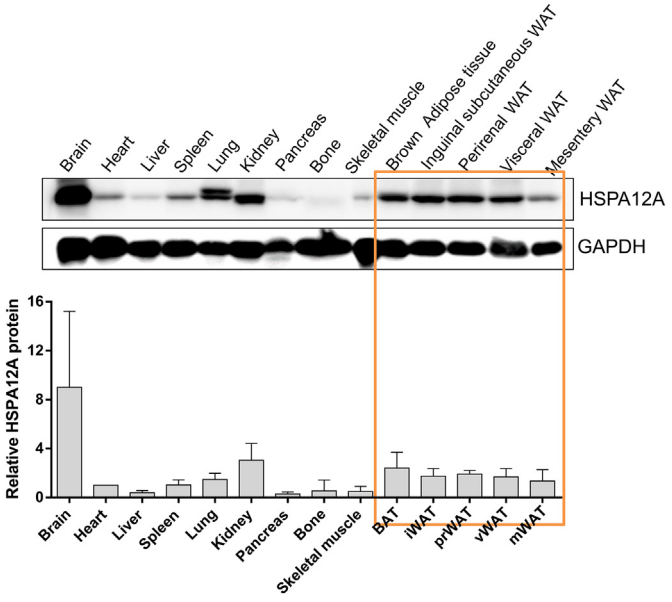

Fig. 1. HSPA12A protein was expressed at a high level in BAT and WAT.
HSPA12A protein levels were analysed in 14 different tissues that collected from 8-week-old C57BL/6 mice. The blots against GAPDH were used as loading controls. $n = 6$. iWAT, inguinal subcutaneous WAT; prWAT, perirenal WAT; vWAT, visceral WAT; mWAT, mesentery WAT.

The successful deletion of *Hspa12a* gene expression in iWAT and BAT of *Hspa12a*$^{-/-}$ mice was confirmed by immunoblotting analysis (Fig. 3A).

Body temperature showed no difference at room temperature environment between two genotypes (Fig. 3B). After exposure to cold ambience at 4 °C, the body temperatures were markedly decreased in both WT and *Hspa12a*$^{-/-}$ mice. However, the cold-induced drop of body temperature was ameliorated in *Hspa12a*$^{-/-}$ mice compared to WT controls (Fig. 3B). The body weights and food intakes were comparable between two genotypes (Fig. 3C and D). The results indicate that HSPA12A deficiency facilitates adaptive thermogenesis after cold exposure.

3.4. HSPA12A deficiency promotes the cold-induced WAT browning in mice

We then investigated the direct effect of HSPA12A deficiency on WAT browning. Cold exposure increased expression of UCP1, a hallmark gene of browning, at both mRNA and protein levels in iWAT in both genotypes, when compared to their respective RT controls (Fig. 4A–C). Notably, the cold-induced increase of UCP1 expression was markedly enhanced in *Hspa12a*$^{-/-}$ iWAT compared to WT controls, as indicated by immunostaining, immunoblotting and PCR analyses (Fig. 4A–C). We also noticed that peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a critical co-activator for WAT browning [23], showed an enhancement of cold-induced expression in *Hspa12a*$^{-/-}$ iWAT compared to WT controls (Fig. 4C). Altogether, the findings suggest that HSPA12A deficiency promoted the cold-induced WAT browning in mice.

3.5. HSPA12A deficiency upregulates the expression of thermogenic genes in murine WAT in response to cold exposure

Analysis of mRNA revealed that the expression of several important thermogenic genes, including *Cox7a*, *Cox8b*, *Elovl3*, and *Dio2*, was upregulated in iWAT of *Hspa12a*$^{-/-}$ mice whereas remained unchanged in WT mice following cold challenge (Fig. 4B). Moreover, *Hspa12a*$^{-/-}$ mice showed higher levels of *Cox7a*, *Cox8b*, *Elovl3*, and *Dio2* mRNA than those in WT controls following cold challenge.

3.6. Deficiency of HSPA12A in primary white adipocytes does not affect their browning in vitro

To further determine the regulation of HSPA12A in browning of WAT, we compared *in vitro* browning of primary adipocyte precursors in isolated stroma vascular fraction (SVF) from both murine genotypes. Browning treatment markedly increased UCP1 and PGC-1α expression in the SVF of both genotypes (Fig. 5A and B). Also, browning treatment upregulated *Cox7a*, *Cox8b and Elovl3* mRNA expression in both SVFs. However, the browning treatment-induced upregulation of UCP1, PGC-1α, *Cox7a*, *Cox8b* and *Elovl3* was not different between the SVF of both genotypes (Fig. 5A and B). The data suggest that HSPA12A in white adipocytes does not affect their own browning.

3.7. HSPA12A regulates macrophage polarization

Macrophage polarization towards to M2 phenotype has been shown to play critical roles in promoting browning of WAT [15,24,25]. We therefore analysed M2 macrophage markers in iWAT of mice after cold exposure. As shown in Fig. 6A, mRNA expression of M2 markers including arginase 1 (*Arg1*) and *Cd206* was increased in iWAT of both genotypes following cold challenge, indicating low temperature activated macrophages M2 polarization. Importantly, *Hspa12a*$^{-/-}$ iWAT showed significant higher levels of *Arg1* and *Cd206* expression than WT

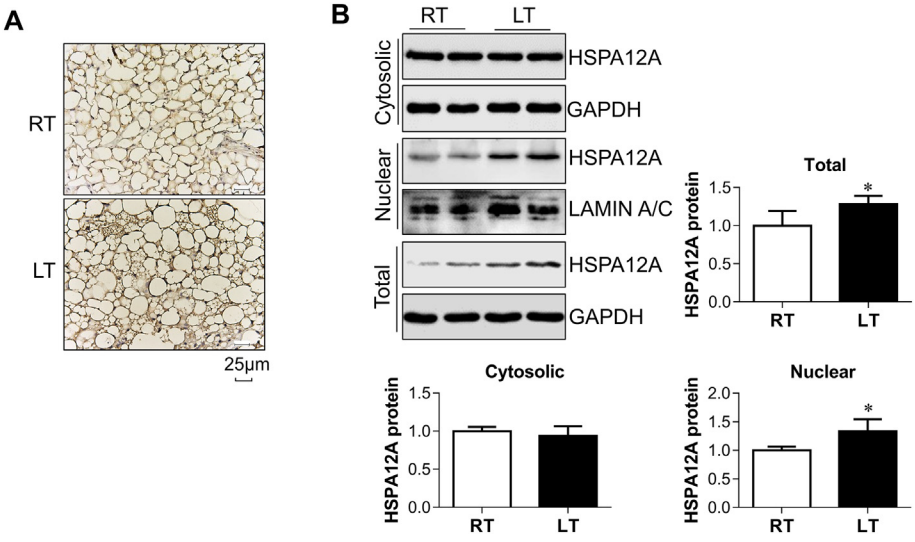

Fig. 2. HSPA12A expression and nuclear translocation was increased in iWAT of mice upon cold exposure.
Inguinal subcutaneous WAT (iWAT) was collected from C57BL/6 mice after cold exposure for 5 days (120 h). The following experiments were performed subsequently.
A. Immunostaining for UCP-1 was performed on paraffin-embedded sections. Scale bar = 25 μm. $n = 4$/group.
B. HSPA12A protein levels were analysed in cytosolic, nuclear and total fractions using immunoblotting. The blots for GAPDH or LAMIN A/C were used as cytosolic and nuclear loading controls, respectively. * $P < 0.05$, n = 4/group.
RT, room temperature; LT, low temperature.

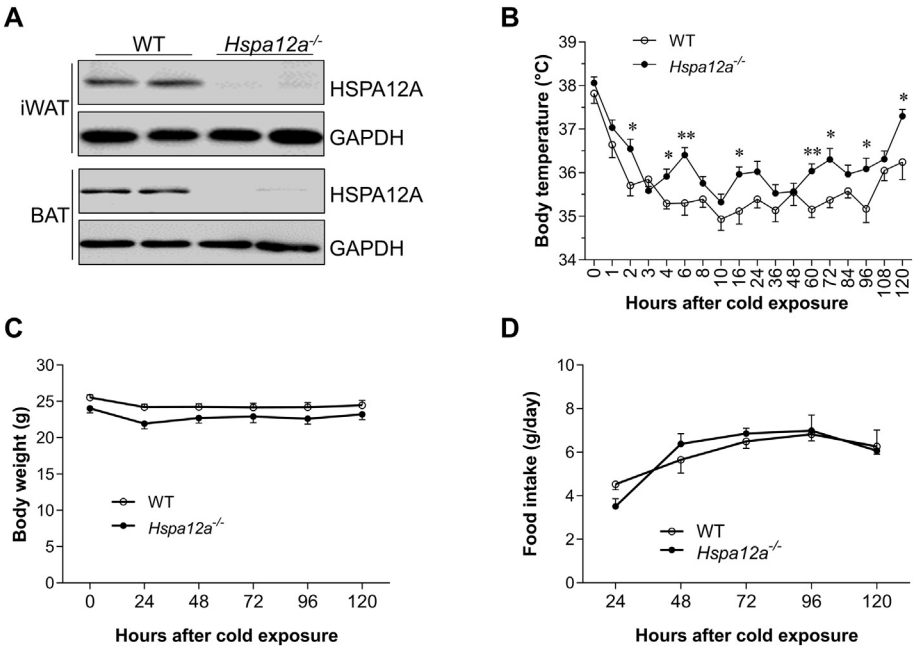

Fig. 3. *Hspa12a*$^{-/-}$ mice showed increased adaptive thermogenesis upon cold exposure.

A. HSPA12A expression. Knockout of HSPA12A in adipose tissues of *Hspa12a*$^{-/-}$ mice. Inguinal subcutaneous WAT (iWAT) and BAT were collected from WT and *Hspa12a*$^{-/-}$ mice for the immunoblotting against HSPA12A. The blots for GAPDH were used as loading controls. n = 4/group.

B. Body Temperature. Rectal temperatures were measured at the indicated times after housed in cold ambience. ** $P < 0.01$ and * $P < 0.05$ *vs.* the time-matched WT mice. $n = 7$/group.

C. Body weight. Body weight was recorded at the indicated times after cold exposure. n = 7/group.

D. Food intake. Food intake was evaluated at the indicated times after cold exposure. n = 7/group.

RT, room temperature; LT: low temperature.

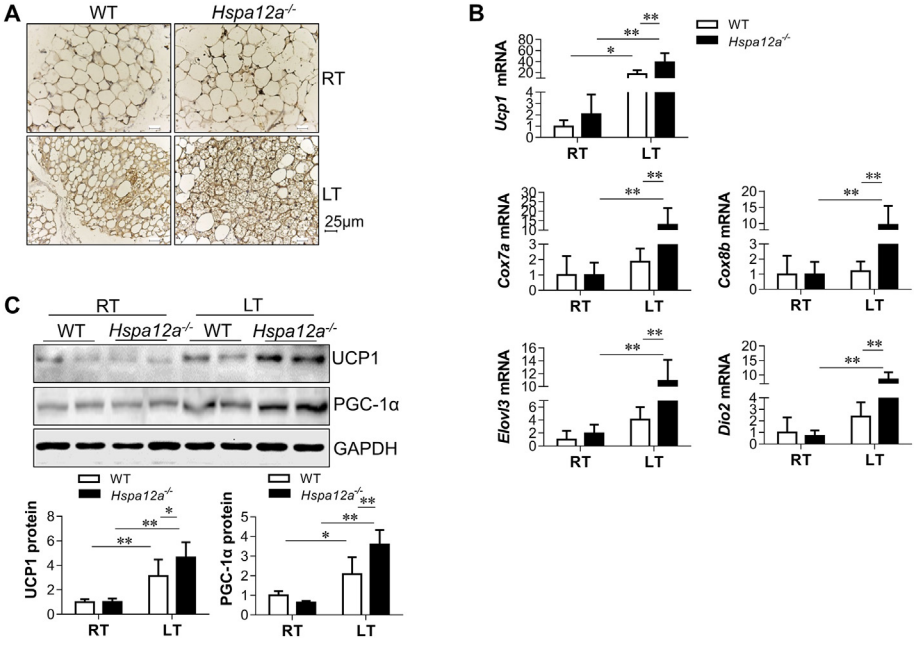

Fig. 4. The cold-induced WAT browning was promoted in *Hspa12a*$^{-/-}$ mice.

WT and *Hspa12a*$^{-/-}$ mice were housed in cold ambience for 5 days (120 h). Inguinal subcutaneous WAT (iWAT) was collected for the following experiments.

A. Immunostaining for UCP-1 was performed on paraffin-embedded sections. Scale bar = 25 μm. n = 4/group.

B. Levels of mRNA were analysed using real-time PCR. ** $P < 0.01$ and * $P < 0.05$, n = 4–7/group.

C. Protein levels were analysed using immunoblotting. The blots for GAPDH were used as loading controls. ** $P < 0.01$ and * $P < 0.05$, $n = 5$–8/group.

RT, room temperature; LT, low temperature.

controls after cold exposure (Fig. 6A), suggesting that HSPA12A deficiency promoted M2 macrophage polarization during WAT browning. Also, Immunofluorescence staining revealed that cold exposure significantly increased the expression of HSPA12A in iWAT macrophages (Fig. S4).

To further investigate the regulation of HSPA12A in macrophage polarization, we performed loss- and gain-of-HSPA12A function experiments *in vitro*. BMDMs isolated from WT and *Hspa12a*$^{-/-}$ mice were treated with IL-4. As shown in Fig. 6B and C, HSPA12A deficiency enhanced IL-4-induced M2 macrophage polarization, as indicated by the increased CD206 protein expression. By contrast, overexpression of HSPA12A (*Hspa12a*$^{o/e}$) in Raw 264.7 macrophages, which was achieved by infection with adenovirus carrying *Hspa12a* expression sequence (Fig. 6D), significantly blunted the response to IL-4-induced M2 macrophage polarization, as indicated by reduced expression of *Arg1* and *Cd206* expression in IL-4-treated Raw264.7 macrophages (Fig. 6E). Low temperature (32 °C) incubation for 24 h did not change the expression of *Arg1* and *Cd206* mRNA expression in Raw264.7 cells (Fig. S5).

3.8. Paracrine effects of macrophage HSPA12A on browning of white adipocytes

We then asked whether macrophage HSPA12A could regulate browning of white adipocytes through a paracrine mechanism. Culture experiments were designed to evaluate the interaction of macrophage HSPA12A with white adipocyte browning as follows.

3.8.1. Browning of white adipocytes was enhanced by deficiency of HSPA12A in macrophage

The conditioned medium (CM) was collected from IL-4-treated WT or *Hspa12a*$^{-/-}$ BMDMs, referred as WT CM or *Hspa12a*$^{-/-}$ CM.

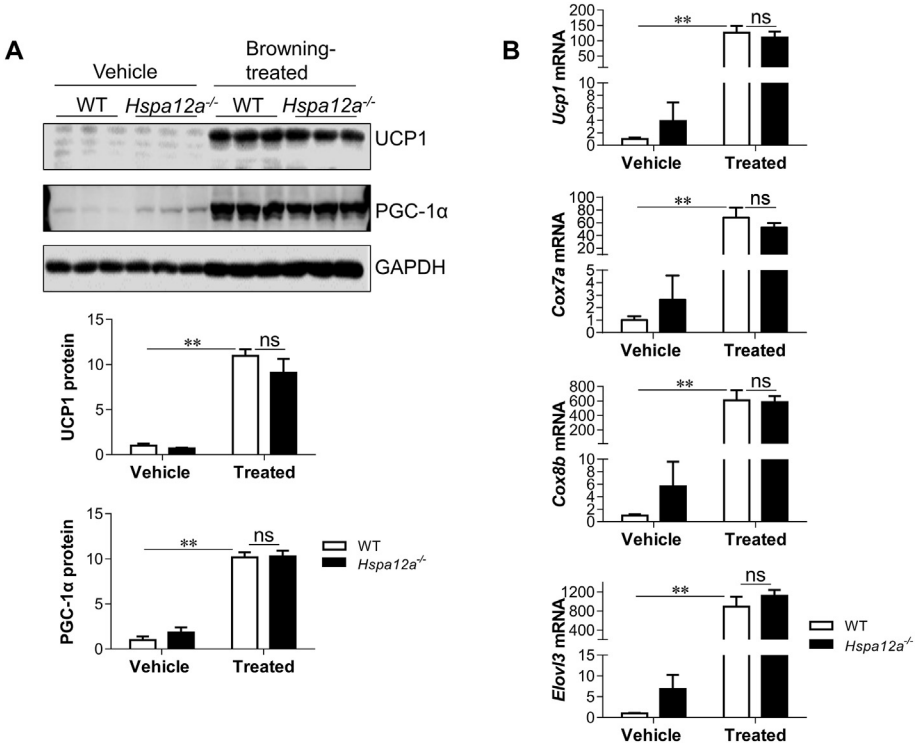

Fig. 5. Deficiency of HSPA12A in white adipocytes did not affect their browning *in vitro*.
Primary SVF from WT and *Hspa12a*$^{-/-}$ mice were induced to differentiation and browning. The levels of the indicated protein and mRNA expression were analysed using Immunoblotting (**A**) and real-time PCR (**B**). ** $P < 0.01$, $n = 3$/group.

Notably, primary adipocytes incubated with *Hspa12a*$^{-/-}$ BMDMs CM exhibited higher levels of UCP1 and PGC-1α than the cells incubated with WT CM (Fig. 7A).

3.8.2. Browning of white adipocytes was impeded by overexpression of HSPA12A in macrophage

HSPA12A was overexpressed in Raw264.7 macrophages by infection with *Hspa12a*-adenovirus, and macrophages infected with empty virus served as normal controls (NC). The CM was collected from IL-4-treated *Hspa12a*$^{o/e}$ or NC macrophage cultures, referred as *Hspa12a*$^{o/e}$

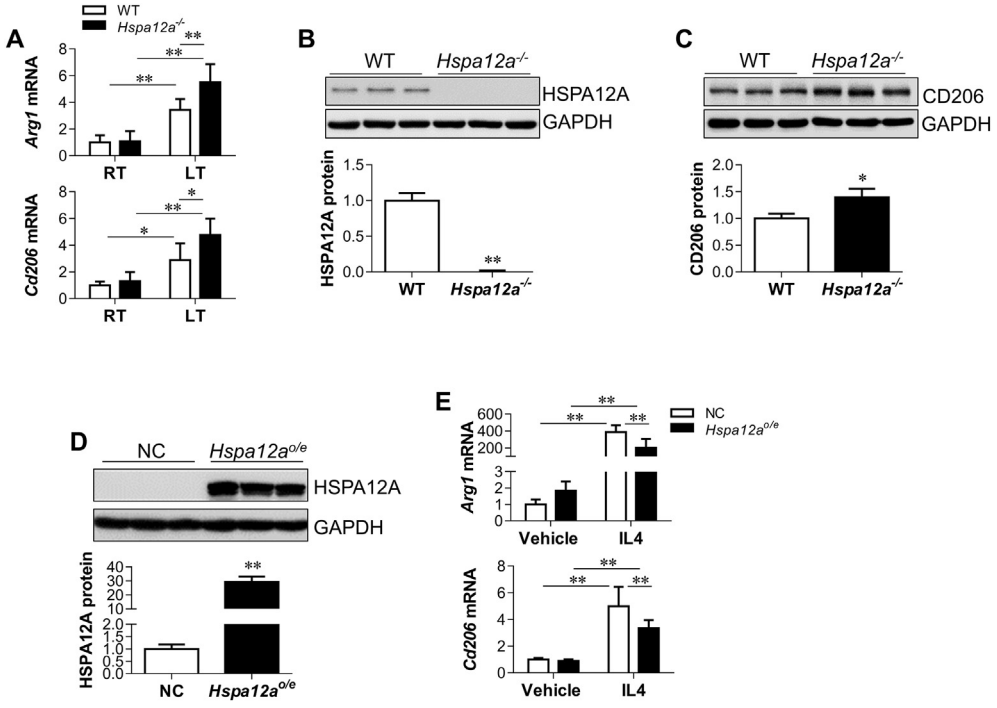

Fig. 6. HSPA12A regulated M2 macrophage polarization both *in vivo* and *in vitro*.
A. Analysing mRNA levels in murine iWAT. WT and *Hspa12a*$^{-/-}$ mice were housed in cold ambience for 5 days (120 h). Inguinal subcutaneous WAT (iWAT) was collected for the indicated mRNA analysis using real-time PCR. ** $P < 0.01$ and * $P < 0.05$, n = 5–6/group. RT, room temperature; LT, low temperature.
B,C. Analysing M2 macrophage markers in bone marrow-derived macrophages (BMDMs). Bone marrow cells were isolated from WT and HSPA12A knock out (*Hspa12a*$^{-/-}$) mice. After differentiation, BMDMs were incubated with IL-4 for 12 h. Cells were harvested for analysis of HSPA12A **(B)** and CD206 **(C)** levels using Immunoblotting. ** $P < 0.01$ and * $P < 0.05$, n = 3/group.
D. Overexpression of HSPA12A in Raw264.7 macrophages. Raw264.7 macrophages were infected with *Hspa12a*-adenovirus (*Hspa12a*$^{o/e}$) or empty virus (NC) for 16 h. HSPA12A protein levels were examined using immunoblotting. The blots for GAPDH were used as loading controls. ** $P < 0.01$ *vs.* NC, n = 3/group.
E. Analysing M2 macrophage markers in Raw264.7 macrophages. *Hspa12a*$^{o/e}$ and NC Raw264.7 cells were incubated with IL-4 for 12 h. Cells were harvested for analysis of the indicated mRNA levels using real-time PCR. ** $P < 0.01$, n = 6/group.

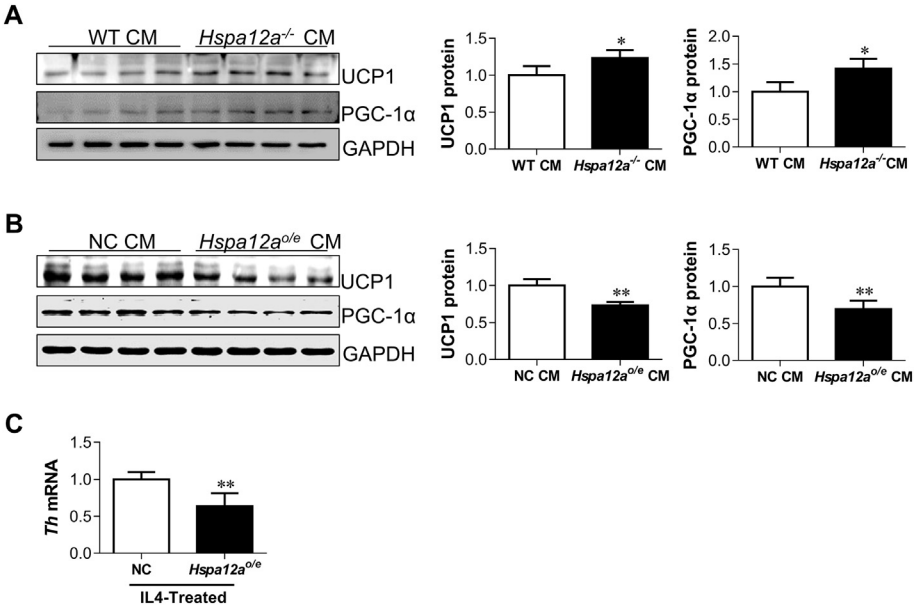

Fig. 7. Paracrine effect of macrophage HSPA12A on the browning of white adipocytes *in vitro*.

A. The conditioned medium (CM) was collected from IL-4-treated WT (WT CM) or *Hspa12a*$^{-/-}$ BMDMs (*Hspa12a*$^{-/-}$ CM). The CM was then applied to primary adipocytes. Protein examination in adipocytes was performed using immunoblotting. * $P < 0.05$ *vs.* NC CM, n = 4/group.

B. The conditioned medium (CM) were collected from Raw264.7 macrophages that with HSPA12A overexpression (*Hspa12a*$^{o/e}$ CM) or normal expression (NC CM). The CM was then applied to primary adipocytes. Protein examination in adipocytes was performed using immunoblotting. ** $P < 0.01$ *vs.* NC CM, n = 4/group.

C. Analysis of mRNA levels. ** $P < 0.01$ *vs.* NC, n = 6/group. Th, tyrosine hydroxylase.

CM or NC CM. Primary adipocytes treated with *Hspa12a*$^{o/e}$ CM showed markedly decreased UCP1 and PGC-1α expression, when compared with the primary adipocytes treated with NC CM (Fig. 7B).

3.8.3. Deficiency of HSPA12A in adipocytes did not affect the paracrine effect of macrophage HSPA12A on the browning of white adipocytes

The primary preadipocytes were isolated from WT and *Hspa12a*$^{-/-}$ mice. After differentiation, the adipocytes were incubated with *Hspa12a*$^{-/-}$ CM or *Hspa12a*$^{o/e}$ CM, respectively. The protein levels of UCP1 and PGC-1α in *Hspa12a*$^{-/-}$ primary adipocytes were comparable with that in WT primary adipocytes following treatment with either *Hspa12a*$^{-/-}$ CM or *Hspa12a*$^{o/e}$ CM (Fig. S6A and B).

3.8.4. Overexpression of HSPA12A in adipocytes did not affect the paracrine effect of macrophage HSPA12A on the browning of white adipocytes

The primary preadipocytes were isolated from WT mice and overexpressed with HSPA12A by infection with HSPA12A-lentivirus. After differentiation, the primary adipocytes were incubated with *Hspa12a*$^{-/-}$ CM or *Hspa12a*$^{o/e}$ CM, respectively. Immunoblotting showed no difference of UCP1 and PGC-1α protein levels between NC and *Hspa12a*$^{o/e}$ adipocytes following treatment with either *Hspa12a*$^{-/-}$ CM or *Hspa12a*$^{o/e}$ CM (Fig. S7A and B).

3.8.5. Overexpression of HSPA12A in macrophages decreased tyrosine hydroxylase (TH) expression

Evidence demonstrates that M2 macrophages express tyrosine hydroxylase (TH) to induce WAT browning [26]. RNA analysis demonstrated that HSPA12A overexpression decreased *Th* mRNA expression in IL-4-treated Raw264.7 macrophages (Fig. 7C).

4. Discussion

The main finding of this study is that deficiency of HSPA12A promoted cold-induced WAT browning and thermogenic adaptation. This action was mediated, at least in part, through the paracrine effects of macrophage HSPA12A on white adipocyte browning. Targeting HSPA12A strategies might be developed as a potential therapeutic intervention in the browning-associated physical and pathophysiological events.

Heat shock proteins (HSPs) compose a family that exerts multiple chaperone functions such as preventing protein aggregation and facilitating protein folding [27]. Not only in protecting from harmful stimuli [18,28,29], HSPs are associated with adipose homeostasis. Indeed, obese humans display increased expression of HSP60, HSC70, HSP72, HSP90 and GRP94 and decreased expression of DNAJB3/HSP40 in adipose tissues, and physical exercise restores HSPs expression to normal levels with attenuated inflammatory response [30]. Moreover, HSP60 mediates the obesity-associated metabolic disorders and adipose tissue inflammation, whereas HSP70 shows a protective role against obesity-induced insulin resistance [31,32]. Of particular interest to this study, genetic disruption of HSP20 increases WAT browning and energy expenditure in mice [33]. In this study, we observed that adipose tissues exhibited a relative high expression of HSPA12A, and its expression and nuclear translocation was increased in iWAT during cold-induced browning. More importantly, deficiency of HSPA12A enhanced the cold-induced upregulation of UCP1, the surrogate of browning, in iWAT of mice. Our data suggest that HSPA12A is a novel player for WAT browning.

Warm-blood animals require extra heat production to protect them from cold stress. Non-shivering thermogenesis is a cold-induced increase in heat production not associated with the muscle activity of shivering [34]. The classical BAT was considered as the main non-shivering thermogenic depot where UCP1 diminishes the proton gradient by uncoupling cellular respiration, which dissipates electrochemical energy into heat [3]. Recently, a new subset of brown-like adipocytes has been identified in WAT, which is characterized by the high expression of UCP1 and induced through the process known as browning. Browning of WAT not only fights against low ambient temperature, but also promotes energy expenditure which prevent individuals from obesity and metabolic abnormality [8,9]. In the present study, we observed that HSPA12A deficiency significantly palliates the cold-induced decrease in the body temperature. Concomitantly, iWAT of *Hspa12a*$^{-/-}$ mice showed increased expression of thermogenic genes compared to WT controls after cold exposure. Altogether, the data suggest a regulatory effect of HSPA12A in the adaptive thermogenesis by modulating WAT browning after cold exposure.

Browning of WAT is induced upon certain stimuli, for instance chronic cold exposure, and characterized by the inducible upregulation of UCP1. Transcriptional regulation of UCP1 counts on its 5′ non-coding regulatory region which interacts with transcription factors and transcription co-factors, such as PGC-1α [35]. PGC-1α acts as a stimuli sensor and is strongly induced by cold exposure and/or β-adrenergic signaling and ultimately leads to the upregulation of UCP1 [36].

Pharmacologically upregulating PGC-1α has been reported to hold the therapeutic potential to conquer metabolic disorders by facilitating browning of WAT [23]. In this study, we observed that the cold-induced upregulation of PGC-1α and UCP1expression was enhanced in WAT of *Hspa12a*$^{-/-}$ mice following cold exposure. The finding indicates an involvement of PGC-1α in the regulation of HSPA12A in WAT browning.

We then sought answers for how HSPA12A regulated browning. WAT Browning could be regulated by either an adipocyte-autonomous or an inter-organic pathway. Unexpectedly, our *in vitro* experiments using primary adipocyte cultures showed no effect of adipocyte HSPA12A on their browning, suggest that the browning was not affected by adipocyte HSPA12A autonomously. Recent studies have revealed that the immune cells play important roles in WAT browning [37,38]. Macrophages are resident innate immune cells in WAT and are a highly heterogeneous, plastic population that undergoes pleiotropic coordinated responses to immunological environment of activation, known as “pro-inflammatory” M1 or “immunoregulatory” M2 [39–41]. In WAT, the M2 macrophages secrete catecholamines to induce browning [24,25,42]. Indeed, we observed that HSPA12A deficiency promoted M2 macrophage polarization in mice during cold exposure. Inversely, overexpression of HSPA12A in macrophages impeded IL4-induced M2 macrophage polarization and reduced the expression of TH. Most importantly, the conditioned medium collected from *Hspa12a*$^{o/e}$ macrophages significantly reduced the expression of UCP1 and PGC-1α in the browning-treated primary white adipocytes. Collectively, the findings indicate that the white adipocyte browning was modulated by macrophage HSPA12A in a paracrine-dependent manner.

Upregulation and translocation are two major features of most inducible heat shock proteins. Evidence suggests that heat shock transcription factor 1 (HSF1) is the major heat shock transcriptional factor that binds heat shock element in the promoter of heat shock proteins and controls rapid induction of heat shock protein, such as HSP25, HSP22 and HSP70, in the cells subjected to various environmental stresses [43,44]. However, it unknown that whether the cold-induced HSPA12A upregulation in WAT is relied on HSF1. Translocation of HSPs is beneficial for cells to increase tolerance against stresses by exerting chaperone functions such as protection proteins from denature, stabilization of cytoskeleton and et al. [45]. Moreover, nuclear accumulation of HSP90 is observed in cancer cells and shows association with poor survival in patients with non-small Cell Lung Cancer [46]. Of particular interest, our recent study has demonstrated that nuclear HSPA12A promotes hepatic macrophage (Kupffer cell) M1 polarization through modulating the conformation of pyruvate kinase M2 [47]. Indeed, we observed a nuclear accumulation of HSPA12A during WAT browning, while knockout of HSPA12A enhanced WAT browning and macrophage M2 polarization, suggesting a possibility that HSPA12A nuclear accumulation may be related to the gene expression for controlling macrophage polarization. Following housing in cold ambience, HSPA12A showed upregulation and nuclear accumulation, and body temperature showed quickly drop. When taken account that knockout of HSPA12A attenuated the cold-induced drop of body temperature in mice, it is possible that HSPA12A plays an important role in the thermogenic adaptation. However, at this stage, we cannot conclude whether there is a feedback inhibition HSPA12A to control the thermogenic impact. It is worthwhile to elucidate the detail role of HSPA12A in thermogenic controlling in further studies.

In summary, this study demonstrates that deficiency of HSPA12A showed beneficial effects on WAT browning, M2 macrophage polarization and thermogenic adaption in mice following cold challenge. The mechanism involves paracrine interactions between macrophages and adipocytes. Further studies should provide direct *in vivo* evidence for macrophage HSPA12A in WAT browning using HSPA12A tissue-specific targeted animals. Our data suggest that targeting HSPA12A might provide meaningful advances for the management of WAT browning and the related physiological events such hypothermia adaptation and pathological disorders such as obesity and cancer-related cachexia.

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Author contributions

L.L. and Z.D. conceived the project and designed the experiments; H.C., T.Q., X.Z., Q.K., X.M., Q.M., and X.C. performed the animal study procedures and the *in vitro* experiments; H.C. collected and analysed the data; H.C., L.L., and Z.D. interpreted the data and prepared the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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