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Organic cation transporter 3 (Oct3) is a distinct catecholamines clearance route in adipocytes mediating the beiging of white adipose tissue

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Abstract

Beiging of white adipose tissue (WAT) is a particularly appealing target for therapeutics in the treatment of metabolic diseases through norepinephrine (NE)-mediated signaling pathways. Although previous studies report NE clearance mechanisms via SLC6A2 on sympathetic neurons or proinflammatory macrophages in adipose tissues (ATs), the low catecholamine clearance capacity of SLC6A2 may limit the cleaning efficiency. Here, we report that mouse organic cation transporter 3 (Oct3; Slc22a3) is highly expressed in WAT and displays the greatest uptake rate of NE as a selective non-neural route of NE clearance in white adipocytes, which differs from other known routes such as adjacent neurons or macrophages. We further show that adipocytes express high levels of NE degradation enzymes Maoa, Maob, and Comt, providing the molecular basis on NE clearance by adipocytes together with its reuptake transporter Oct3. Under NE administration, ablation of Oct3 induces higher body temperature, thermogenesis, and lipolysis compared with littermate controls. After prolonged cold challenge, inguinal WAT (ingWAT) in adipose-specific Oct3-deficient mice shows much stronger browning characteristics and significantly elevated expression of thermogenic and mitochondrial biogenesis genes than in littermate controls, and this response involves enhanced β-adrenergic receptor (β-AR)/protein kinase A (PKA)/cyclic adenosine monophosphate (cAMP)-responsive element binding protein (Creb) pathway activation. Glycolytic genes are reprogrammed to significantly higher levels to compensate for the loss of ATP production in adipose-specific Oct3 knockout (KO) mice, indicating the fundamental role of glucose metabolism during beiging. Inhibition of β-AR largely abolishes the higher lipolytic and...
Abbreviations: β-AR, β-adrenergic receptor; AT, adipose tissue; ATMSC, AT-derived mesenchymal stem cell; AU, arbitrary unit; BAT, brown adipose tissue; BMR, basal metabolic rate; cAMP, cyclic adenosine monophosphate; cKO, conditional knockout; CLAMS, Comprehensive Lab Animal Monitoring System; COX2, cytochrome c oxidase subunit 2; Creb, cAMP-responsive element binding protein; dbGAP, the database of Genotypes and Phenotypes; DIO, diet-induced obesity; DNB, DNA nanoball; DOPE, discrete optimized protein energy; ETC, electron transport chain; EV, empty vector; FAO, fatty acid oxidation; FFA, free fatty acid; GO, gene ontology; goniWAT, gonadal white adipose tissue; HFD, high-fat diet; hGLUT3, human glucose transporter 3; Hsl, hormone-sensitive lipase; ingWAT, inguinal white adipose tissue; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; MAO, monoamine oxidase; MPP, methyl-4-phenylpyridinium; MSC, mesenchymal stem cell; MUSCLE, Multiple Sequence Comparison by Log-Expectation; NE, norepinephrine; NET, norepinephrine transporter; OCR, O2 consumption rate; Oct3, organic cation transporter 3; pCreb, phosphor-Creb; PKd4, pyruvate dehydrogenase kinase 4; PKA, cAMP-dependent protein kinase A; pPKA, phospho-PKA; RCR, rolling circle replication; RNA-seq, RNA sequencing; Rps18, ribosomal protein s18; RT, room temperature; SNP, single-nucleotide polymorphism; ssDNA, single-strand DNA; SVC, stromal vascular cell; SVF, stromal vascular fraction; Tβ, triiodothyronine; Tbp, TATA box-binding protein; TEM, transmission electron microscope; TF, transcription factor; Ucp, thermogenic activities in Oct3-deficient ingWAT, indicating the NE overload in the vicinity of adipocytes in Oct3KO adipocytes. Of note, reduced functional alleles in human OCT3 are also identified to be associated with increased basal metabolic rate (BMR). Collectively, our results demonstrate that Oct3 governs β-AR activity as a NE recycling transporter in white adipocytes, offering potential therapeutic applications for metabolic disorders.

Author summary

Adipose tissues (ATs) can be divided into three distinct types: white fat (or white AT [WAT]), brown fat, and beige fat. Growing evidence suggests that the development of beige fat cells in WAT, also called browning or beiging of WAT, might protect against obesity and improve systemic metabolism. Norepinephrine (NE)-induced β-adrenergic signaling is a major regulator of adaptive thermogenesis (a process of generating heat under conditions of physical activity) and leads to activation of protein kinase A (PKA) and phosphorylation of cyclic adenosine monophosphate (cAMP)-responsive element binding protein (Creb), thereby controlling the expression of thermogenic genes. In this study, we found that the catecholamine transporter, mouse organic cation transporter 3 (Oct3), is highly expressed in WAT, where it mediates NE uptake in the white fat cells in vivo and in vitro. Removing Oct3 in the fat cells leads to enhanced lipid breakdown, increased thermogenesis, and browning of WAT when stimulated by NE or cold exposure via activation of the β-adrenergic receptor (β-AR)/PKA/Creb pathway. In humans, reduced functional alleles of OCT3 are also associated with increased basal metabolic rate (BMR). Our results indicate that Oct3 is an essential regulator of NE recycling and the beiging of WAT.

Introduction

Obesity, a disease characterized by excess body fat, is a major risk factor for many human diseases, including type 2 diabetes, cardiovascular disease, and hepatic steatosis [1]. In mammals, fat is stored primarily in adipose tissue (AT), and three distinct types of ATs have been characterized: white AT (WAT), brown AT (BAT), and beige AT [2]. The morphology, function, cellular origins, and molecular features of the three types of ATs are quite distinct [3]. WAT stores nutrients as triglycerides in unilocular adipocytes, which can be used to generate free fatty acids (FFAs) by lipolysis [4]. BAT is the main tissue responsible for thermogenesis, especially when stimulated by cold [5]. After prolonged thermogenic induction, brown-like adipocytes can also be found in WAT, thus named beige or brite adipocytes [3]. The heat produced by BAT or beige AT is indispensable for survival during cold acclimatization. Also, the adaptive thermogenic beige AT may have the potential to counteract obesity and its related disorders [6, 7].

Beiging of WAT can be induced by various stimuli, including cold challenge, bile acids [8, 9], pharmacological agents, and hormones such as norepinephrine (NE) [3, 10]. NE activates β-adrenergic receptor (β-AR), which is coupled to G-proteins, resulting in increases in cyclic adenosine monophosphate (cAMP) concentration in adipocytes that enhance the activity of cAMP-dependent protein kinase A (PKA) [11]. This signal not only leads to lipolysis by phosphorylating hormone sensitive lipase (Hsl) but also by phosphorylating cAMP-responsive element binding protein (CREB), subsequently increasing the expression levels of thermogenic

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genes (UCP1, PGC1α, and DIO2) to promote nonshivering thermogenesis [11, 12]. The tightly regulated dichotomy of NE release and clearance maintains the balance between NE-induced thermogenesis and excessive NE in the vicinity of adipocytes. Thus, it is critical to elucidate the mechanism of NE clearance in AT. Although previous studies reported NE clearance mechanisms via SLC6A2 on sympathetic neurons [13] or proinflammatory macrophages in AT [14, 15], the low catecholamine clearance capacity of SLC6A2 may limit its cleaning efficiency.

Catecholamines, including NE, epinephrine, serotonin, and dopamine, act as neuromodulators in the central nervous system and as hormones in ATs and blood circulation [16]. Catecholamines are actively cleared in extracellular environment and are translocated into cells by specialized transporters that belong to two distinct transport mechanisms: neuronal transport (Uptake1, which is mediated by SLC6A2, SLC6A3, and SLC6A4) and extraneuronal transport (Uptake2, which is mediated by organic cation transporters [OCTs]) [17]. Studies have suggested a role of AT in the clearance and metabolism of catecholamines. Indeed, it has been shown that plasma concentrations of epinephrine and NE decrease after passing through AT [18, 19]. In addition, human adipocytes exhibit high expression levels and enzymatic activity of the catecholamine-degrading enzyme monoamine oxidase (MAO) [20]. Transport of catecholamines by adipocytes displays similar characteristics as the Uptake2 system and can be inhibited by the OCT3 inhibitor disprocynium 24 [20]. Ayala-Lopez et al. identified that perivascular ATs in male Sprague-Dawley rats had a NE uptake mechanism and could be reduced by norepinephrine transporter (NET) or/and OCT3 inhibitors ex vivo [21]. We reason that extraneuronal monoamine transporter OCT3 is an important route in NE clearance and adaptive thermogenesis in adipocytes complementary to sympathetic neurons or macrophages. In addition, the SLC22A3 (OCT3)-LPAL2-LPA gene cluster has been identified as a risk locus for coronary artery disease, implying its potential relationship to lipolysis [22].

In this study, we identified Oct3 as a novel non-neural NE clearance route in adipocytes and demonstrated its role in governing β-AR activity to mediate the beiging of WAT. Adipose-specific Oct3 knockout (KO) mice under cold acclimatization or NE administration exhibited significantly higher expression levels of genes related to thermogenesis, mitochondria biogenesis, and glycolysis in inguinal WAT (ingWAT) compared with Oct3fl/fl (Ctrl) mice. Of note, through mining of the Genome-Wide Association Studies (GWAS) Catalog, the database of Genotypes and Phenotypes (dbGAP), and UK BioBank databases, we also identified genetic variants of human OCT3 associated with higher basal metabolic rate (BMR). These findings suggest that Oct3 regulates catecholamines levels in the vicinity of the β-AR in adipocytes and therefore plays essential roles in the browning of WAT during adaptive thermogenesis. Inhibition of OCT3 may provide distinct therapeutic application by activating energy expenditure pathways.

**Results**

**Oct3 was predominantly expressed in AT and had a preferred uptake of NE**

Oct3 mRNA levels were examined in several C57BL/6J mouse tissues by real-time PCR. The highest expression levels were found in ingWAT, followed by gonadal WAT (gonWAT) (Fig 1A). In human tissues, OCT3 had the highest transcript levels in skeletal muscle and liver, followed by ATs (S1A Fig). Compared with transcript levels of other catecholamine transporters Slc6a2, Slc6a3, and Slc6a4 in murine WAT, mRNA levels of Oct3 were much higher (Fig 1B), suggesting that Oct3 is the predominant transporter for catecholamines in WAT. We further showed that adipocytes expressed high levels of NE degradation enzymes Maoa, Maob, and Comt, which was as high as the adipocytes abundant gene Adiponectin in ingWAT (Fig 1C), providing the molecular basis on NE clearance by adipocytes itself together with its transporter.
Oct3 mediating the catabolism of catecholamines in adipocytes

Oct3 was highly expressed in AT and had the greatest uptake rate of NE. (A) Tissue distribution of mouse Oct3 mRNA (8-week-old male C57BL/6 mice, n = 3). (B) mRNA expression of Oct3, Slc6a2, Slc6a3, and Slc6a4 in different mouse WATs (n = 3). (C) mRNA expression of Maoa, Maob, Comt, and adipocytes abundant gene Adiponectin in different mouse WATs (n = 3). (D) mRNA expression of Oct3 in mouse MAs and SVCs of ingWAT (n = 3). (E) Oct3 immunofluorescence staining (green) of mouse primary inguinal adipocytes. DAPI (blue), nucleus. Scale bar, 50 μm. (F) Uptake of different monoamines and MPP⁺ in HEK-293 cells stably overexpressing mouse Oct3. MPP⁺ was used as a model substrate for functional study of Oct3. n = 3 experiments. (G) Saturation kinetics of [³H]-NE transport in HEK-293 Flp-In cells stably expressing human OCT3 or mouse Oct3 (n = 3–4). (H) Immunohistochemical staining of Oct3 in ingWAT of Ctrl and adipose-specific Oct3 KO (cKO) mice. Scale bar, 50 μm. (I) [³H]-NE uptake assay in primary white and brown adipocytes from Ctrl and cKO mice. (J) Total radioactivity in ingWAT and gonWAT from Ctrl and cKO mice after [³H]-NE injection (n = 6). Data in panel B were analyzed by one-way ANOVA followed by Tukey’s test. Data in panels D, I, and J were analyzed by Student t test. The numerical data underlying this figure are included in S1 Data. AT, adipose tissue; BAT, brown adipose tissue; cKO, conditional knockout; Ctrl, control; EV, empty vector; gonWAT, gonadal white adipose tissue; HEK, human embryonic kidney; ingWAT, inguinal white adipose tissue; KO, knockout; MA, mature adipocyte; MPP, methyl-4-phenylpyridinium; NE, norepinephrine; Oct3, organic cation transporter 3; SVC, stromal vascular cell; WAT, white adipose tissue.

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Oct3. Fractionation of ingWAT showed that Oct3 was enriched in mature adipocytes rather than in stromal vascular cells (SVCs) (Fig 1D), with Perilipin used as a marker for mature
adipocytes, as its expression was limited to mature adipocytes and was not found in SVC (S1B Fig). Similar expression patterns of Oct3 in mature adipocytes and SVC were also observed in gonWAT (S1C and S1D Fig). Immunofluorescence of WAT localized Oct3 to cell membranes of adipocytes (Fig 1E).

To compare the Oct3 uptake activity against its main substrates (NE, serotonin, 1-methyl-4-phenylpyridinium (MPP⁺), epinephrine, dopamine, and tyramine), substrate uptake assays were performed in HEK-293 Flp-In cells stably expressing Oct3. NE had the highest uptake activity when cells were incubated with equivalent concentrations of catecholamines (Fig 1F). Human OCT3 and mouse Oct3-overexpressing HEK-293 cells both showed strong NE transport activities compared with empty vector (EV) in vitro, with \( K_m \) and \( V_{max} \) of 0.182 ± 0.0275 mM, 3.57 ± 0.174 nmol/mg protein/min (human) and 0.336 ± 0.0726 mM, 5.77 ± 0.448 nmol/mg protein/min (mouse), respectively (Fig 1G). Both \( K_m \) and \( V_{max} \) of OCT3 were more than 600 times greater than previously reported values of the Uptake1 transporter, SLC6A2 (0.28 ± 0.03 μM; 5.83 ± 0.49 pmol/mg protein/min) [23], consistent with OCT3 being a high-capacity, low-affinity Uptake1, transporter of NE. These data suggested an important role of OCT3 in regulating NE concentrations in peripheral tissues including AT, especially when a high concentration of NE existed, such as under NE stimulation or cold challenge.

To further examine the interactions between catecholamines and Oct3, we built a three-dimensional Oct3 homology model based on human glucose transporter 3 (GLUT3) template. Docking of structurally divergent catecholamines (NE, epinephrine, histamine, dopamine, serotonin, and tyramine) indicated that epinephrine (\( E = -45.7 \)) and NE (\( E = -43.6 \)) showed the best docking energy scores, resulting from favorable interactions formed between these two bioamines and polar residues in the primary substrate binding site of Oct3 including K215, Q242, E385, and E446 (S1E Fig).

To address the connection between Oct3, catecholamines, and adipocyte metabolism in vivo, we crossed Oct3⁰/⁰ mice with Adiponectin-Cre mice to conditionally delete Oct3 in adipocytes (S2A Fig). Disruption of Oct3 was confirmed by immunohistochemistry (Fig 1H) and western blot analysis in ATs (S2B Fig). In addition, no compensational changes were observed in expression levels of the other catecholamine transporters Slc6a2, Slc6a3, and Slc6a4 in Oct3-deficient ATs compared with controls (S2C Fig). When fed a normal chow diet, adipose-specific Oct3 KO mice were healthy and viable, with no significant differences in body weight (S2D Fig), metabolic parameters (S2E–S2H Fig), or adipose morphology (S2I Fig) compared with Ctrl mice under room temperature (RT).

To determine NE uptake activity in different adipocytes, we performed \([3]H\)-NE uptake assays in primary white and brown adipocytes. When treated with NE at a physiologically relevant concentration (0.20 μM), NE accumulation was significantly reduced (80% reduction) in Oct3 KO white adipocytes compared with controls, but not in brown adipocytes, suggesting that Oct3 is required for NE uptake in white adipocytes (Fig 1I). Moreover, in vivo NE uptake assay was performed after chemical sympathectomy, which eliminated neural NE uptake in ingWAT (S3A and S3B Fig). Oct3-null WAT showed significantly reduced total radioactivity, which reflected the accumulation of NE and its metabolites in adipocytes (73% and 65% reduction in ingWAT and gonWAT, respectively) (Fig 1J). Both the in vitro and in vivo data supported the role of Oct3 as the NE transporter in WAT.

NE-induced thermogenesis was augmented in adipose-specific Oct3 KO mice

Given that fact that NE was a preferential Oct3 substrate, we sought to investigate whether genetic deletion of Oct3 would influence NE clearance and NE-induced thermogenesis in vivo. After subcutaneous injection of low-dose NE (0.3 mg/kg), adipose-specific Oct3 KO mice...
showed significantly higher body temperature (Fig 2A and 2D) and higher maximum body temperature (Fig 2B and 2C) than that of Ctrl mice, as well as prolonged retention of increased body temperature (Fig 2A).

Adipose-specific Oct3 KO and Ctrl mice that received NE injections both demonstrated marked elevations in both whole-body O$_2$ consumption (Fig 2E and 2F) and heat production.
(Fig 2G and 2H), while these parameters remained consistent between both genotypes under non-intervention conditions (S2E–S2H Fig). The magnitude of NE-induced O$_2$ consumption (Fig 2E and 2F) and heat production (Fig 2G and 2H) in adipose-specific Oct3 KO mice was significantly higher than in the Ctrl littermates. Among the thermogenic markers tested, adipose-specific Oct3 KO mice treated with NE displayed a nearly 82-fold increase in Ucp1, a 5-fold increase in Pparc1α (Pgc1α), and a 3-fold increase in Dio2, as well as an increasing trend in Hsl and Atgl mRNA expression in ingWAT (Fig 2I). Ucp1, Pgc1α, and Dio2 were also increased in gonWAT of adipose-specific Oct3 KO mice (Fig 2J). However, the relative expression of Ucp1 in gonWAT was far lower than that in ingWAT, suggesting that ingWAT might be more responsible for whole-body thermogenesis. No significant alterations in thermogenic genes were detected in BAT of both genotypes after NE injection (S4A Fig). Collectively, these results suggested that Oct3 deficiency improved thermogenic capacity in WAT in the presence of adrenergic stimuli that resulted in an increase in core body temperature, O$_2$ consumption, and whole-body energy expenditure.

Ablation of Oct3 enhanced catecholamine-stimulated lipolysis and β-adrenergic signaling in WAT

Activation of β-AR signaling has been found to promote lipolysis via the PKA-mediated phosphorylation of Hsl. To investigate the role of Oct3 in catecholamine signaling in lipolysis, we tested in vivo lipolysis by measuring serum concentration of FFAs. There was no difference in basal lipolysis, while NE injection enhanced lipolysis more significantly in adipose-specific Oct3 KO mice than in Ctrl (Fig 3A). Epinephrine had a similar effect on lipolysis in vivo (S4B Fig). Increased levels of Hsl phosphorylation at two PKA-responsive serine residues (pHsl-S563 and pHsl-S660) were observed in ingWAT (Fig 3B) and gonWAT (Fig 3C) of adipose-specific Oct3 KO mice 4 hours after NE stimulation, which is necessary for Hsl translocation into lipid droplets and NE-induced lipolysis in rodents [24]. No noticeable difference was observed in Hsl phosphorylation 4 hours after injection in BAT between the two genotypes (S4C Fig). One possible explanation for these results was that deletion of Oct3 led to impaired NE uptake into adipocytes and extracellular NE accumulation. Therefore, excess NE would activate β$_3$-AR, increase cAMP concentration, and phosphorylate PKA, which would promote lipolytic activity via phosphorylating Hsl at S563 and S660 [25].

Next, we compared basal and NE-stimulated glycerol release from WAT explants derived from adipose-specific Oct3 KO mice and Ctrl mice. The rates of glycerol release were similar under basal conditions in both genotypes, but were enhanced more in Oct3 KO AT when treated with NE (Fig 3D). Ablation of Oct3 in primary adipocytes led to an elevated response to adrenergic stimulation, as demonstrated by an increased lipolytic response to NE (Fig 3E) and intracellular signaling molecule activation (pHsl-S563 and pHsl-S660) (Fig 3F).

To compare NE uptake capacity in Ctrl and Oct3 KO adipocytes, we assayed intracellular NE levels using immunofluorescence. Primary adipocytes were treated with catecholamine-degradation enzyme inhibitors decynium-22 in the presence or absence of NE and were then incubated with anti-NE antibodies. Staining patterns were similar between unstimulated adipocytes; however, Oct3 KO adipocytes had lower signals compared with Ctrl under NE stimulation, indicating that ablation of Oct3 in adipocytes impaired NE uptake (Fig 3G).

We next investigated the role of Oct3 on lipolysis in vitro by using a gain-of-function approach. Preadipocytes were transfected to produce Oct3-overexpressing 3T3-L1 cells (Oct3-OE). Although Oct3 overexpression did not significantly affect adipocyte differentiation based on Oil Red O staining (S4D Fig) or measurement of basal lipolysis (Fig 3H), Oct3-OE cells displayed attenuated NE-stimulated lipolysis compared with EV cells (Fig 3H), likely a result of enhanced NE transport to adipocytes accompanied by reduction of extracellular NE. In
Fig 3. Genetic ablation of Oct3 augmented NE-stimulated lipolysis in WAT. (A) Basal and NE-stimulated lipolysis by measuring serum FFAs in Ctrl and cKO mice (n = 6). (B, C) Protein levels of Hsl phosphorylated at Ser563 and Ser660 (pHsl-S563 and pHsl-S660, respectively) and total Hsl in ingWAT (panel B) and gonWAT (panel C) of Ctrl and cKO mice with NE (“NE”) or without NE injection (“Ctrl”). (D) Ex vivo glycerol release from AT explants in the absence (“basal”) or presence of NE (“NE”) (n = 4). (E) In vitro glycerol release from differentiated SVF-derived adipocytes from Ctrl and cKO mice in the absence or presence of NE (n = 4). (F) Protein levels of pHsl-S563, pHsl-S660, and total Hsl in differentiated SVF-derived adipocytes from Ctrl and cKO mice in the absence or presence of NE. (G) Immunofluorescence analysis of intracellular NE (green) in differentiated SVF-derived adipocytes from Ctrl and cKO mice. Adipocytes were either untreated or treated with NE (100 nM) for 10 minutes. DAPI (blue), nucleus. Scale bar, 50 μm. (H) Basal and NE-stimulated lipolysis, as measured by glycerol release from differentiated 3T3-L1 adipocytes stably transfected with EVs or Oct3 (Oct3-OE) (n = 3). Data in panels A, D, E, and H were analyzed by Student t test. The numerical data underlying this figure are included in S1 Data. cKO, conditional knockout; Ctrl, control; EV, empty vector; FFA, free fatty acid; gonWAT, gonadal white adipose tissue; Hsl, hormone-sensitive lipase; ingWAT, inguinal white adipose tissue; KO, knockout; NE, norepinephrine; Oct3, organic cation transporter 3; OE, over-expression; pHsl, phosphorylated hormone-sensitive lipase; SVF, stromal vascular fraction; WAT, white adipose tissue.
addition, Oct3-OE and EV cells were treated with the adenylyl cyclase agonist forskolin (10 μM), and similar lipolytic capacities were observed (S4E Fig), indicating that cAMP signaling (blocked by forskolin) and downstream lipolytic capacity were not primary causes of the difference in NE-stimulated lipolysis in our model.

In light of the enhanced lipolysis in Oct3 KO AT after NE stimulation, we examined the effect of adipose-specific KO of Oct3 on the liver. Fatty liver was not observed (S4F Fig), and there was no significant difference in liver triglyceride levels between genotypes 4 hours after NE injection (S4G Fig). One possible reason was that single NE injection represented only a short-term effect on lipolysis and FFA flux in blood, and another was that the 4-hour duration time might be insufficient to generate significant differences in fat accumulation in the liver.

**Oct3 deficiency induced white to brown fat transition and enhanced thermogenesis**

To study the role of Oct3 in physiologically stimulated thermogenesis, adipose-specific Oct3 KO and Ctrl mice were exposed to a 4°C ambient environment for 1 month. Both groups had similar rectal temperatures under RT (Fig 4A). However, adipose-specific Oct3 KO mice showed significantly higher rectal temperatures (by 1.7°C) than those of Ctrl littermates after prolonged cold acclimation (Fig 4A and 4B). As seen in S5A Fig, body weight of adipose-specific Oct3 KO mice had a slight but significant decrease compared with that of Ctrl mice after sustained cold exposure. We also observed a more reddish appearance of ingWAT in adipose-specific Oct3 KO mice compared with Ctrl (Fig 4C). Notably, adipose-specific Oct3 KO ingWAT contained more clusters of uncoupling protein 1 (Ucp1)-positive multilocular adipocytes than Ctrl after cold exposure (Fig 4D). Whole-body energy expenditure of mice after cold challenge was measured by Comprehensive Lab Animal Monitoring System (CLAMS). Adipose-specific Oct3 KO mice showed markedly higher O2 consumption rates (OCR) (Fig 4E), CO2 production rates (Fig 4F), and heat production (Fig 4G) than Ctrl, indicating a higher BMR. In addition, higher NE levels were observed in Oct3 KO ingWAT (S5B Fig), probably due to accumulation of extracellular NE derived from sympathetic nerve endings during cold challenge. Meanwhile, NE concentration in serum was also slightly increased, probably due to a leakage of extracellular NE in WAT into blood circulation (S5C Fig). These results showed the role of Oct3 in altering NE distribution between adipocytes and blood circulation.

In order to examine global gene expression changes in ingWAT, RNA from ingWAT of both genotypes were subjected to RNA sequencing (RNA-seq) analysis. Ablation of Oct3 led to significantly differential expression of 2,521 genes in ingWAT (adjusted p < 0.05) (S5D Fig and S1 Table). Widespread up-regulation of BAT-selective and beige genes were identified in Oct3 KO ingWAT (Fig 4H), while WAT-selective genes showed a down-regulation tendency (Fig 4I). Furthermore, expression of genes involved in fatty acid oxidation (FAO) also showed substantial up-regulation (Fig 4J). Based on the RNA-seq data analysis, we conducted real-time PCR analysis on genes involved in thermogenesis and adipose physiology. Oct3 KO ingWAT from cold-exposed mice had significantly higher mRNA expression levels of thermogenic genes Ucp1, Dio2, Pgc1α, Cidea, Ppara, Pgc1β, Tmem26, and Slc27a2—as well as genes that showed an up-regulation trend, including Tbx1 (Fig 4H)—whereas WAT-selective genes Gsn, Sncg, and Rarres2 were significantly decreased (Fig 4I). Several key genes of FAO were significantly up-regulated in Oct3 KO ingWAT, including Cpt1a, Hadha, Hadhb, Acdvl1, and Acsl5 (Fig 4J). Moreover, a negative regulator of FAO, Twist1, was significantly reduced in Oct3 KO ingWAT, by 80% (Fig 4J). No significant changes in morphology of BAT and gonWAT from both genotypes were observed (S5E Fig), consistent with unchanged mRNA levels of Pgc1α, Dio2, and other thermogenesis genes, as well as representative FAO genes (S5F and S5G Fig).
Given the close relationships between Oct3, NE, and thermogenesis, we next focused on the NE/β-AR/cAMP pathway. Western blot analysis showed that phospho-PKA (pPKA) substrate (Fig 4K), phospho-Creb (pCreb), and downstream Ucp1 and Pgc1α levels (Fig 4L) were significantly elevated in ingWAT from adipose-specific Oct3 KO mice compared with Ctrl. Protein levels of pCreb, Ucp1, and Pgc1α remained unchanged in BAT (S5H Fig) and gonWAT (S5I Fig). Moreover, genes encoding components of the cAMP/PKA pathway (Adcy3 and Adcy10) were also up-regulated in ingWAT after cold exposure (S5J Fig). Based on the data above, we hypothesized that the enhanced browning effect in adipose-specific Oct3 KO mice during cold challenge may be due to higher metabolic rate and elevated thermogenic gene expression.

**Ablation of Oct3 promoted mitochondria biogenesis and a remodeling of energetic metabolism in ingWAT during cold challenge**

We next characterized the effect of Oct3 deletion on mitochondria in ingWAT after cold exposure. Electron microscopy showed more accumulated mitochondria and enlarged mitochondrial area (Fig 5A), a larger number of mitochondria per cell area (Fig 5B), and increased mitochondrial DNA content (Fig 5C) in Oct3 KO adipocytes from ingWAT compared with Ctrl. Larger surface area of mitochondria was also observed in Oct3 KO ingWAT stained with Mitotracker (Fig 5D and 5E). Consistently, the gene ontology (GO) analysis of RNA-seq data revealed that differential genes associated with mitochondrial component were enriched (Fig 5F and S1 Table). Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlighted specific changes in genes associated with oxidative phosphorylation, glycolysis and fatty acid metabolism, and citric acid cycle (Fig 5G and S1 Table).

In ingWAT of adipose-specific Oct3 KO mice, the increase in cold-induced thermogenesis was coupled with broad up-regulation of electron transport chain (ETC) genes (Fig 5H). Real-time PCR analysis showed that expression of mitochondrial respiratory chain complexes genes (Cox7b, Uqcrb, Uqcrh, and Cytochrome c) were remarkably increased in Oct3 KO ingWAT compared with Ctrl under cold stimulation (Fig 5I). In line with these changes, western blot analysis showed that protein levels of cytochrome c oxidase subunit 7b (Cox7b), Uqcrb, Uqcrh, and Cytochrome c were significantly elevated in Oct3 KO ingWAT from mice under cold exposure (Fig 5J). We found no difference in the expression of ETC genes in BAT (S5K Fig) and gonWAT (S5L Fig) between genotypes.

The profound differences of glycolytic genes in Oct3 KO ingWAT suggested that Oct3 deletion resulted in a metabolic rewiring during cold exposure (Fig 5K). Real-time PCR analysis of ingWAT showed up-regulation of glycolytic genes (Hk2, Pdk4, Pfkl, Eno1, Gapdh, Pfkm, and Pkm1) in adipose-specific Oct3 KO versus Ctrl mice (Fig 5L). The protein levels of a key enzyme of glycolysis, Hk2, and the amount of lactate (the product of glycolysis) were up-regulated in ingWAT from adipose-specific Oct3 KO mice (Fig 5M and 5N). These alterations...
were selective in ingWAT, as no significant difference between groups was observed in BAT (S5K and S5M Fig) or gonWAT (S5L and S5M Fig).

Collectively, our results suggested that the elevated browning effect in adipose-specific Oct3 KO mice may be owing to increased energy expenditure, mitochondrial biogenesis, and enhanced glycolysis in ingWAT.

Enhanced thermogenesis and lipolysis resulted from overactivating the NE/β-AR/PKA signaling pathway in adipose-specific Oct3 KO mice

To further investigate the relationship between Oct3-depletion–induced NE-stimulated thermogenesis and β-AR signaling, we firstly cotreated mice with NE and propranolol, a pharmacological β-AR inhibitor. An elevated thermogenic response in adipose-specific Oct3 KO mice was observed, including a more significant increase in core body temperature (Fig 6A), O$_2$ consumption (Fig 6B), and heat production (Fig 6C) following single NE treatment, as described before (Fig 2A–2H). However, propranolol pretreatment abolished the elevation of thermogenic response (Fig 6A–6C), indicating that the increased energy expenditure in adipose-specific Oct3 KO mice was mediated by the β-AR signaling pathway.

To estimate the functional relevance of Oct3 for thermogenic capacity of adipocytes, we performed plate-based respirometry on primary adipocytes from preadipocytes isolated from SVC of ingWAT, which maintained expression of beige-related genes. Although the OCR curves were nearly identical in primary adipocytes of both genotypes in the absence of NE (S6A Fig), Oct3 KO beige adipocytes displayed a higher OCR than that of Ctrl cells after NE stimulation (Fig 6D). Additionally, pharmacological inhibition of β-AR by propranolol significantly blunted the NE-stimulated OCR increase to a similar extent in both groups (Fig 6D).

Primary adipocytes derived from ingWAT were treated with NE and triiodothyronine (T$_3$) to induce Ucp1 expression [26]. An approximate 1.9-fold increase in Ucp1 mRNA expression in Oct3 KO beige adipocytes was found even in the absence of NE compared with Ctrl, and another 3.7-fold increase occurred in response to NE treatment (Fig 6E). Meanwhile, acute propranolol-induced inhibition of β-AR similarly blunted the NE-induced increase in Ucp1 transcripts in the beige both groups of adipocytes (Fig 6E). As a control, no noticeable difference was observed in mRNA expression of the NE-insensitive gene Ap2 between groups (S6B Fig). Additionally, Ucp1 transcripts and Creb phosphorylation showed no response to treatment of other Oct3 substrates (epinephrine, dopamine, and serotonin) (S6C and S6D Fig).

As NE was known to activate β-AR and trigger PKA-Creb signaling, we examined whether the enhanced thermogenesis in Oct3 KO beige adipocytes was regulated by the PKA/Creb pathway. NE-stimulated Oct3 KO adipocytes showed robust phosphorylation of PKA substrates and Creb, without changes in total Creb (Fig 6F). Meanwhile, these effects were largely
**Fig 6. Oct3 deficiency induced a thermogenesis and lipolysis program through the NE/β-AR/PKA signaling pathway.** (A–C) Maximal body temperature change (panel A), maximal $O_2$ consumption change ($ΔO_2$) (panel B), and maximal heat production change (ΔHeat) (panel C) of Ctrl and cKO mice ($n = 6$ in body temperature measurement; $n = 4$ for oxygen consumption and heat production measurement). NE was injected into mice with propranolol (“NE + prop”) or without propranolol (5 mg/kg, s.c.) pretreatment (“NE + vehicle”). (D) The OCR in Oct3 KO beige adipocytes and Ctrl. Cells were acutely stimulated with NE in the absence or presence of propranolol at the indicated time point (arrow). Ctrl or Oct3 KO adipocytes with NE ($n = 7$ for both); Ctrl or Oct3 KO adipocytes with NE and propranolol ($n = 8$ for both). (E) $Ucp1$ mRNA expression in differentiated primary inguinal adipocytes from Ctrl and cKO mice after stimulation with NE and T$_3$. (F) Western blotting of pPKA substrate, pCreb, and total Creb in primary inguinal adipocytes from Ctrl and cKO mice stimulated with NE in the presence or absence of propranolol ($n = 3$). (G) In vitro glycerol release from differentiated SVF-derived adipocytes in the presence or absence of NE and propranolol ($n = 3$). (H) Western blotting of pHsl-S563, pHsl-S660, and total Hsl in differentiated SVF-derived adipocytes from Ctrl and cKO mice in the presence or absence of NE and propranolol. (I) Schematic diagram of Oct3-regulating thermogenesis and lipolysis in ingWAT. Data in A–E and G were analyzed by one-way ANOVA followed by Tukey’s test. The numerical data underlying this figure are included in S1 Data. β-AR, β-adrenergic receptor; AC, adenyyl cyclase; cAMP, cyclic adenosine monophosphate; Creb, cAMP-responsive element binding protein; Ctrl, control; FFA, free fatty acid; Gs, Gs alpha subunit; Hsl, hormone-sensitive lipase; ingWAT, inguinal white adipose tissue; KO, knock out; NE, norepinephrine; OCR, $O_2$ consumption rate; Oct3, organic cation transporter 3; pCreb, phosphorylated cAMP-responsive element binding protein; pHsl, phosphorylated hormone-sensitive lipase; PKA, protein kinase A; pPKA, phospho-PKA; prop, propranolol; s.c., subcutaneous; SVF, stromal vascular fraction; T$_3$, triiodothyronine; TF, transcription factor; TG, triglyceride; VO$_2$, oxygen consumption.

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eliminated by blockade of β-AR signaling (Fig 6F), indicating that β-AR signaling was required for enhanced thermogenic activity under Oct3 deficiency.

We next aimed to determine the requirement of NE/β-AR pathway in lipolysis induced by Oct3 ablation. Propranolol at 10 μM partially but significantly inhibited the enhancing effect of NE on lipolysis and Hsl phosphorylation, while 100 μM propranolol showed complete inhibition in Oct3 KO adipocytes (Fig 6G and 6H), indicating that this effect relied on β-AR function.

Based on these collective results, we propose the following model (Fig 6I). Oct3 locates to cell membranes of adipocytes and uptakes NE, which thereby decreases NE concentration in extracellular medium, and enhancing the NE concentration and leading to enhanced lipolysis, glycolysis, thermogenesis, and WAT browning via β-AR/cAMP/PKA pathway activation.

**Adipose-specific Oct3 KO mice fed with high-fat diet showed enhanced thermogenesis, lipolysis, and improved insulin sensitivity under cold exposure**

Next, we explored the effect of adipose-specific Oct3 KO on diet-induced obesity (DIO). There was no difference in body weight between adipose-specific Oct3 KO and littermate Ctrl mice fed with a high-fat diet (HFD) and housed at thermoneutrality (30°C) (S7A Fig) or at RT (S7B Fig) for 12 weeks. We then subjected HFD-fed mice of both genotypes (housed at thermoneutrality for 12 weeks) to 4°C cold exposure. Higher body temperature was evident in adipose-specific Oct3 KO mice after the first 4 hours (S7C Fig). After 1-week cold acclimation, the body weights of adipose-specific Oct3 KO mice decreased more (S7D Fig) despite no difference in food intake (S7E Fig), serum glucose (S7F Fig), triglyceride (S7G Fig), cholesterol (S7H Fig), insulin (S7I Fig), and adiponectin (S7J Fig). Meanwhile, serum FFAs increased about 21% during cold stimulation in adipose-specific Oct3 KO mice (S7K Fig), implying higher lipolysis owing to lower NE clearance under Oct3 deficiency. Furthermore, glucose tolerance was improved in adipose-specific Oct3 KO mice treated with Ctrl mice (S7L Fig). These data showed that thermogenesis and lipolysis under cold stimuli were still enhanced in adipose-specific Oct3 KO mice treated with HFD, and insulin sensitivity was also improved.

**Human OCT3 polymorphisms were associated with BMR**

To determine the relevance of the studies in mice to human, BMR with polymorphisms within the human OCT3 locus was explored through mining of GWAS Catalog, dbGAP, and UK Biobank databases. Among the 11 single-nucleotide polymorphisms (SNPs) in the OCT3 locus,
four independent SNPs were associated with BMR with $p < 5 \times 10^{-8}$ (S8A Fig and S3 Table). From the GTEx Portal, we noted that 10 of the 11 SNPs are significantly associated with human OCT3 expression levels in ATs (subcutaneous and/or visceral omentum) (S8B and S8C Fig). Each of the alleles, which was associated with lower expression levels of OCT3 in AT, was correlated with higher BMR in the UK Biobank participants.

To further confirm the relevance to humans of the studies in mice, we performed experiments on human AT and primary human AT-derived mesenchymal stem cells (ATMSCs) that were induced to differentiate into adipocytes. Both human AT and differentiated ATMSCs showed high expression levels of OCT3 and NE-degrading enzymes (S8D–S8F Fig). These results were consistent with studies on human adipocytes showing that NE uptake existed and was inhibited by OCT3 inhibitors [20]. Furthermore, inhibition of OCT3 led to an increased lipolytic response to NE in differentiated ATMSCs (S8G Fig), similar to the observations in mouse stromal vascular fraction (SVF)-derived adipocyte model (Fig 4E).

Although human functional genomic data and cell studies provided cues for metabolic roles of OCT3, further investigations are needed to define the subtle roles of human OCT3 in AT.

**Discussion**

Brown and beige AT are two key drivers for the dissipation of chemical energy to stimulate thermogenesis [27]. The current study demonstrates that genetic deletion of Oct3 enhances ingWAT browning and up-regulates NE-induced thermogenesis, lipolysis, and mitochondrial biogenesis by attenuating NE clearance. These findings support the non-neural role of Oct3 as a catecholamine scavenger for beige AT microenvironments in adaptive thermogenesis.

Recent studies have proposed a neural mechanism for NE uptake by Slc6a2 in special macrophages in AT [14, 15]. However, a proinflammatory state seems necessary to enhance their NE uptake in macrophages [14]. In addition, considering the high-affinity, low-capacity nature of Slc6a2 ($K_m$: 0.28 ± 0.03 μM; $V_{max}$: 5.83 ± 0.49 pmol/mg protein/min) [23], a NE transport system with greater transport capacity may be needed to meet increased metabolic demand during long-term cold challenge. With high expression in adipocytes and high capacity for catecholamines, Oct3 ($K_m$: 0.183 ± 0.0275 mM; $V_{max}$: 3.57 ± 0.174 nmol/mg protein/min) may function in part to clear catecholamines in white AT microenvironments. However, the quantitative contribution and exact conditions of extraneuronal NE uptake through Oct3 versus neuronal NE clearance remains to be investigated.

Cold challenge induces stronger browning and NE/β-AR/PKA signaling in ingWAT of adipose-specific Oct3 KO mice. Consistent with sustained action of catecholamines, protein levels of pPKA substrate, pCreb, and downstream Ucp1 and Pgc1α are all up-regulated. Pgc1α is the crucial transcriptional co-activator that is highly induced after cold exposure [28]. In white adipocytes, ectopic expression of Pgc1α induces Ucp1 expression and essential enzymes in mitochondria ETC and also increases mitochondrial DNA content [28, 29]. Mitochondria biogenesis and expression of ETC is significantly increased in ingWAT of adipose-specific Oct3 KO mice after cold challenge, which may result from elevated Pgc1α protein expression. Moreover, genes encoding fatty acid β-oxidation were exclusively up-regulated in ingWAT of Oct3-deficient mice after cold exposure. Consequently, more FFAs may be utilized as the primary fuel source for thermogenesis through Ucp1. These chain reactions are likely driven by more NE available due to Oct3 deletion.

During browning of ingWAT in adipose-specific Oct3 KO mice, metabolic rewiring of glucose metabolism to glycolysis is another significant phenomenon observed. Upon cold exposure, key enzymes in glycolysis and pyruvate dehydrogenase kinase 4 (Pdk4, the key regulatory enzyme linking glycolysis to the citric acid cycle) are significantly up-regulated in ingWAT of
adipose-specific Oct3 KO mice. Higher expression of Pdk4 restricts the conversion of glucose to acetyl-CoA and reprograms it towards a higher glycolytic metabolism [30]. Along with other key glycolytic enzymes, the enhanced glycolysis in adipose-specific Oct3 KO mice may compensate for the loss of mitochondrial ATP production due to heat-generating mitochondrial uncoupling [31]. Recent reports have demonstrated that expression of glycolytic genes and Pdk4 are induced by β-AR agonists in BAT [32]. The enhanced glycolytic activity in ingWAT of cold-exposed adipose-specific Oct3 KO mice most likely results from stronger adrenergic stimulation through blunted NE catabolism by Oct3 deficiency. To date, there is little information available regarding the role of glucose metabolism in WAT. RNA-seq data also showed that these glycolytic genes are more highly expressed in ingWAT in adipose-specific Oct3 KO mice after cold exposure (S1 Table). These findings revealed that Oct3-mediated glucose metabolism is important to WAT energy homeostasis.

The differential responsiveness of WAT and BAT in adipose-specific Oct3 KO mice may result from several factors. First, Oct3 has a much lower expression in BAT compared with WAT. Second, current studies suggest that BAT is more densely innervated compared with WAT [33], and neuronal Slc6a2 may be largely responsible for the clearance of catecholamines released in BAT after NE injection or cold exposure [34]. Although browning of WAT can lead to metabolic improvements, the marked increase in energy expenditure (Fig 4E–4G) may also come from other sources such as skeletal muscle, cardiac and respiratory work, and liver, stimulated by enhanced circulating NE level [30–32]. Under HFD treatment at RT or thermoneutrality, similar body weights between two genotypes are observed. It is probably due to drastically reduced density of sympathetic nerves in ingWAT under HFD [35]. On the other hand, the Uptake1 system might be sufficient to compensate for the KO effect of Oct3. The overall proposed mechanism for Oct3 effects on WAT browning is summarized in Fig 6I.

Exploration of the Gene Atlas database for genetic associations of human BMR with variants in OCT3 shows consistency with some of the results in the adipose-specific Oct3 KO mouse model. In particular, several reduced expression variants of OCT3 are positively correlated with BMR in human. Though a greater baseline BMR is not observed in the adipose-specific Oct3 KO mice, metabolic rate is greater after either NE administration or cold challenge. Our study suggests an important clinical implication as β3-AR plays a central role in regulating nonshivering thermogenesis, which enhances energy expenditure and has potential to treat obesity [36]. Inhibition of OCT3 might block NE transport into adipocytes and mimic β3-AR agonists, resulting in browning of WAT and enhanced lipolysis. In summary, our results demonstrate that Oct3 is essential in catecholamine clearance in adipocytes of WAT and blunts intracellular responses upon sustained catecholamine stimulation by regulating the activity of β-AR. Development of OCT3-specific inhibitors may shed light on improving metabolic disorders.

Materials and methods

Ethics statement

All experiments were performed in accordance with guidelines of the Institute for Laboratory Animal Research of Tsinghua University. The experimental procedures were approved by the Administrative Committee of Experimental Animal Care and Use of Tsinghua University, licensed by the Science and Technology Commission of Beijing Municipality (SYXK-2014-0024), and they conformed to the National Institutes of Health guidelines on the ethical use of animals.

Animals

Adiponectin-Cre mice were obtained from the Jackson Laboratory (stock NO.010803). Oct3<sup>fl/fl</sup> mice were generated in the Ying Xu lab (Soochow University), backcrossed with C57BL/6J.
mice for at least 8 generations, and subsequently intercrossed with Adiponectin-Cre mice.
Mice were housed under a 12-hour light/dark cycle (light period 7:00–19:00) at constant tem-
perature (22˚C RT). Food and water were available ad libitum. Male mice were 8 to 10 weeks
of age when used for experiments.

**Uptake assays**
HEK-293 Flp-In cells (Invitrogen, Carlsbad, CA) stably overexpressing human or mouse
OCT3 were generated, maintained, and used for uptake assays as previously described [37].
For comparison between human OCT3 and mouse Oct3 NE uptake kinetics (Fig 2E), uptake
time was 2 minutes at 37˚C. $K_m$ and $V_{max}$ were calculated by fitting the data to Michaelis-Men-
ten equations using GraphPad Prism software (La Jolla, CA). For [3H]-NE uptake in primary
adipocytes, the same procedures were performed as described above.

**Cold challenge and indirect calorimetry**
For cold challenge experiments, male mice were placed at 16˚C for 1 week, and then 3 weeks
at 4˚C. Metabolic measurements were measured using CLAMS (Columbus Instruments,
Columbus, OH). Mice were individually housed in cages. Food and water were provided ad
libitum.

**Mouse Oct3 three-dimensional–structure model and catecholamine
 docking**
The amino acid sequence of the mouse Oct3 was retrieved from the UNIPROT database
(Q9WTW5) [38]. Homology models of Oct3 was generated via four steps. First, the primary
sequence of Oct3 was submitted to the RCSB Protein Data Bank [39] to search for suitable
template structures using PSI-BLAST [40]. Human glucose transporter 3 (hGLUT3) having its
crystal structure determined in complex with D-glucose at 1.5 Å resolution in an outward-
occluded conformation (PDB ID: 4ZW9) [41] is the closest structurally characterized protein
to Oct3, sharing 24% sequence identity. Second, a sequence alignment between Oct3 and
hGLUT3 was computed by Multiple Sequence Comparison by Log-Expectation (MUSCLE)
[42]. Third, a total of 500 homology models were generated for Oct3 with the standard “auto-
model” class in MODELLER [43] on the basis of hGLUT3 template. Finally, these 500 models
were evaluated by the discrete optimized protein energy (DOPE) score [44], and the best rank-
ing model was selected.

Molecular docking screens against the selected Oct3 homology model was performed with
a semi-automatic docking procedure. All docking calculations were performed with DOCK
3.6 [45]. The docking database contains seven compounds, dextroamphetamine, NE, epineph-
rine, dopamine, serotonin, histamine, and tyramine. The docked compounds were ranked by
the docking energy that is the sum of van der Waals, Poisson-Boltzmann electrostatic, and
ligand desolvation penalty terms.

**Histology and immunofluorescence analysis**
Fresh tissue samples were fixed in 4% paraformaldehyde, dehydrated in serial alcohol, embed-
ded in paraffin, cut into 5-μm–thick sections, and stained with hematoxylin–eosin (HE). Ucp1
immunohistochemical staining was performed as previously described [46]. Specimens were
prepared and observed by transmission electron microscope (TEM) (H-7650; Hitachi, Japan)
as previously described [47]. Mitochondria numbers were counted in more than 10 images for
each specimen.
Mouse ingWAT frozen sections were then blocked for 30 minutes in PBS containing 10% normal goat serum at RT. For Oct3 staining, sections were incubated with a rabbit anti-mouse Oct antibody (1:200; OriGene Technologies, Rockville, MD) followed by staining with a Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:300; Invitrogen, Carlsbad, CA). Then, sections were stained with DAPI for 10 minutes. Images were obtained with a Nikon A1 confocal microscope (Nikon Corp., Japan).

For mitochondria staining, deparaffinized slices of ingWAT from Ctrl and cKO mice were incubated with 250 nM MitoTracker (Molecular Probes, OR, USA) for 1 hour at RT. Slides were washed with PBS and had coverslips mounted. Images were obtained with a Nikon A1 confocal microscope (Nikon Corp., Japan). Mitochondria area fraction percentage was calculated as previously described [48] using NIS-Elements software (Nikon Corp., Japan). More than 20 fields were scored per group.

For detection of intracellular NE by immunofluorescence, primary adipocytes were incubated with medium containing NE (100 ng/ml) for 20 minutes at 37°C. After blocking, the adipocytes were next incubated with anti-noradrenaline (ab8887, 1:50, Abcam, Cambridge, MA) overnight at 4°C. The following procedures of immunofluorescence were described earlier.

**Differentiation of primary adipocytes and 3T3-L1 cells**

Isolation of stromal-vascular cells from ingWAT and BAT was performed as described previously [49, 50]. Cells were cultured in growth medium DMEM/F-12 containing 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO₂. For induction to differentiate, cells were incubated with induction medium containing 5 mg/ml insulin, 1 mM dexamethasone, 1 μM rosiglitazone, 0.5 mM 3-isobutyl-1-methylxanthine, 1 nM T₃, and 125 nM indomethacin for 2 days. Cells were then maintained with 1 μM rosiglitazone and 1 nM T₃ until maturation. Where indicated, cells were treated with T₃ (10 μM), norepinephrine (100 nM) for 4 hours to induce Ucp1 expression.

3T3-L1 preadipocytes were incubated with an induction medium DMEM containing 1 μg/ml insulin, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 2 μM rosiglitazone for 2 days, and then with a differentiation medium DMEM containing 1 μg/ml insulin for another 2 days. Fresh DMEM was supplied every 2 days until maturation. For Oil Red staining, fully differentiated 3T3-L1 cells were fixed with 4% paraformaldehyde, followed by Oil Red O incubation for 2 hours.

**Determination of NE-induced thermogenesis**

Ctrl and cKO mice (males, 8 weeks, chow diet) were intraperitoneally injected with pentobarbital (75 mg/kg), which does not inhibit nonshivering thermogenesis [51, 52]. For NE treatment, mice were subcutaneously injected with 0.3 mg/kg NE under anesthesia. For β-AR inhibition assay, propranolol (5 mg/kg) was given 20 minutes before NE. The core body temperature was recorded with a rectal probe connected to a digital thermometer (Yellow Spring Instruments). Infrared thermal images were obtained using a FLIR E60 compact infrared thermal imaging camera and were analyzed using FLIR Tools software (FLIR, North Billerica, MA).

**Determination of NE-induced lipolysis**

For in vivo lipolysis, 0.3 mg/kg NE or saline was injected intraperitoneally into Ctrl and cKO mice. Tail blood was taken from mice 20 minutes after injection of NE or saline. Serum was separated for the determination of FFA using NEFA C kits (Wako, Oxoid SA, France).

For ex vivo lipolysis, about 50 mg WAT samples from overnight fasted mice were incubated in KRB buffer (12 mM HEPES, 121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, and 0.33 mM...
CaCl$_2$) containing 2% FA-free BSA and 2.5 mM glucose, and then stimulated with 1 μM NE. Glycerol release was measured by using free glycerol reagent (Sigma-Aldrich, USA).

For in vitro lipolysis, glycerol release from differentiated SVF-derived or 3T3-L1 adipocytes was measured as previously described [53]. Lipolysis was stimulated by incubation of NE (1 μM, Sigma-Aldrich, USA) or forskolin (10 μM, Sigma-Aldrich, USA).

**In vivo uptake**

Chemical sympathectomy was performed by unilateral denervation in ingWAT on one side and sham-operation on the collateral side to eliminate SNS NE release and uptake, as described previously [54]. The effectiveness of sympathectomy was confirmed by immunofluorescence of tyrosine hydroxylase (Th).

After 3 days of recovery from surgery, mice were given intraperitoneal injection of 0.5 mg/kg of NE spiked with $[^3H]$-NE (PerkinElmer, CT, USA). After 30 minutes, mice were transcardially perfused with ice-cold PBS and washed for 4 to 5 minutes. IngWAT was immediately isolated with the major blood vessels removed and homogenized in Solvable (PerkinElmer CT, USA) overnight. Total radioactivity in ingWAT homogenates was determined by liquid scintillation counting. Protein content of homogenates was measured by BCA assay (Thermo Scientific, USA).

**RNA-seq analysis**

For RNA-seq analysis of ingWAT, total RNA was extracted using the RNeasy Lipid Mini Kit (Qiagen, Hilden, Germany) based on the manufacturer’s instructions. Quality of RNA was analyzed using the Agilent 2100 (Agilent Technologies, Palo Alto, CA). The poly-A-containing mRNA was purified by using poly-T oligo-attached magnetic beads. Purified mRNA was fragmented into small pieces using divalent cations under elevated temperature. RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers, followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. A single “A” base was added to cDNA fragments, and the adapter was subsequently ligated. The products were then purified and enriched with PCR amplification. PCR yield was quantified by Qubit, and samples were pooled together to make a single-strand DNA circle (ssDNA circle), which gave the final library.

DNA nanoballs (DNBs) were generated with the ssDNA circle by rolling circle replication (RCR) to enlarge the fluorescent signals at the sequencing process. The DNBs were loaded into the patterned nanoarrays and had 50 bp of single end read through the BGISEQ-500 platform. The DNB-based nanoarrays and stepwise sequencing were combined using Combinational Probe-Anchor Synthesis Sequencing Method and were analyzed.

Heatmap for differential genes was produced using pheatmap package of R. GO enrichment analysis and KEGG analysis for differential genes were performed in clusterProfiler package [55] version 2.2.4 of R using default settings.

**Western blotting**

Tissues and cells were lysed with RIPA buffer containing Halt Protease/Phosphatase inhibitors (Thermo Fisher Scientifics, Pittsburgh, PA). The primary antibodies used in this study were as follows: anti-phospho (Ser563)-HSL (#4139), anti-phospho (Ser660)-HSL (#4126), anti-HSL (#4107), anti-pPKA Substrate (#9621), and anti-phospho (Ser133)-CREB (#9198) (all from Cell Signaling Technology); anti-SLC22A3 antibody (#ab191446), anti-ATP5A (#ab176569), anti-COX7B (#ab140629), anti-UQCRH (#ab134949), anti-UQCRB (#ab190360), and anti-beta Actin (ab8226) (all from Abcam); anti-UCP1 (#GTX112784) (Genetex); and anti-PGC1α (#20658) (Proteintech Group Inc).
Real-time PCR analysis

Total RNA was extracted by RNeasy Mini Lipid Tissue Kit (QIAGEN, Hilden, Germany). Reverse transcription (Tiangen, Beijing, China) and SYBR green quantitative PCR (Transgen, Beijing, China) were performed according to the manufacturer’s instructions. Primers for target sequences were as shown in S2 Table. Relative gene expression level was normalized by TATA box-binding protein (Tbp) expression levels, unless otherwise indicated, since TBP expression was stable across ATs [56].

For mitochondrial DNA analysis, mouse tissues were homogenized, and genomic DNA was extracted by TIANamp genomic DNA kit (Tiangen, Beijing, China). SYBR green quantitative PCR (Transgen, Beijing, China) was performed in duplicate using mitochondrial DNA specific primers for mitochondrial Cox2 and normalized by amplification of the nuclear gene ribosomal protein s18 (Rps18).

Lactate concentration

Lactate concentration in the ATs was measured by using a Lactate Assay Kit (Biovision, USA) following the instructions of the manufacturer.

Seahorse experiments

Cellular OCRs were determined using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse Biosciences, Chicopee, MA). Primary adipocytes from ingWAT were seeded at 20,000 cells/well. Differentiation was induced as described above, and the cells were analyzed on day 6. Before the measurements, the cells were washed twice with assay medium (XF DMEM + 25 mM glucose + 2 mM pyruvate + 4 mM glutamine) and incubated in 175 μL of assay medium for 1 hour in an incubator without CO₂ at 37˚C. Port injection solutions were prepared as follows: NE (1 μM final concentration) or NE with propranolol (50 μM final concentration), oligomycin (2 μM final concentration), FCCP (1 μM final concentration), and a cocktail containing rotenone (1 μM) and antimycin A (1 μM). Each cycle consisted of mix 5 minutes, wait 0 minutes, and measure 5 minutes.

Human genetic association studies for BMR

Publicly available resources were surveyed to identify human genetic studies associated with BMR. The resources included GWAS Catalog (https://www.ebi.ac.uk/gwas/home), dbGAP (https://www.ncbi.nlm.nih.gov/gap), and UK BioBank resource (http://geneatlas.roslin.ed.ac.uk/, https://www.biorxiv.org/content/early/2017/08/18/176834).

In Gene Atlas, which housed the results of all the genetic association studies analyses of the UK Biobank cohort (N = 408,455), BMR was available for over 7,000 participants. HaploReg version 4.1 [57] and GTEx Portal [58] were used to determine the location, consequence of the variants, and the effect of the variants on transcript levels of the gene. Information about the methods used for determining BMR of the participants were available online (http://biobank.ctsu.ox.ac.uk/crystal/docs/Anthropometry.pdf).

HFD treatment

Six-week-old adipose-specific Oct3 KO and Ctrl mice were treated with HFD for 12 weeks under RT or thermoneutrality (30˚C). For the groups under thermoneutrality, mice were then cold-stimulated at 4˚C for 7 days. Body temperatures of mice were monitored at the first 8 hours under cold exposure. Body weights were recorded before and after cold exposure. Food intake was recorded daily during cold exposure. Blood was collected into EDTA tubes, and
Plasma was separated by centrifugation. Plasma triglyceride and total cholesterol levels were determined by respective assay kits (Nanjing Jiancheng Biotechnology Institute, Nanjing, China). Commercially available ELISA kits were used to measure plasma adiponectin (Abcam), insulin (ALPCO, Salem, NH), serum and tissue NE (Rocky Mountain Diagnostics, Colorado Springs, CO) levels following instructions from the manufacturers. Plasma FFAs was measured NEFA C kits (Wako, Oxoid SA, France). Liver triglyceride content was measured using Triglyceride Quantification Assay Kit (Abcam).

**Oral glucose tolerance test**

Oral glucose tolerance test was performed on mice fed with 12-week HFD under thermoneutrality and then acclimated cold exposure for 7 days by oral gavage of glucose (2 g/kg) after 12-hour overnight fasting.

**ATMSCs**

Human MSCs derived from human AT, a kind gift from Dr. Yanan Du (Tsinghua University), were cultured in human MSC growth medium (Wuhan Viraltherapy Technologies Co. Ltd, Beijing) containing 10% calf serum and penicillin G. Adipogenic differentiation of human MSCs were performed as previously described [59].

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.0 (http://www.graphpad.com/scientific-software/prism). ANCOVA was conducted for in vivo metabolic data. Other analysis was conducted by Student t test (for comparison of two experimental conditions) or one-way ANOVA followed by Tukey’s test (for comparison of three or more experimental conditions). Data are represented as mean ± standard deviation. Statistical significance was calculated and indicated (*p < 0.05, **p < 0.01, ***p < 0.001).

**Supporting information**

S1 Fig. Oct3 was highly expressed in AT. (A) Tissue distribution of human OCT3 mRNA (n = 3). (B) mRNA expression of Perilipin in mouse MA and SVCs of ingWAT (n = 3). (C) mRNA expression of Oct3 in mouse MA and SVC of gonWAT (n = 3). (D) mRNA expression of Perilipin in mouse MA and SVC of gonWAT (n = 3). (E) Three-dimensional–structure modeling of Oct3 and molecular docking of different monoamines. The three-dimensional Oct3 homology model was based on human GLUT3 template in the outward-facing-occluded (“occluded”) conformation in complex with D-glucose. The predicted homology models contained the whole target sequence including the 12 transmembrane helices and the primary substrate binding site. After overall structural quality evaluation with DOPE scores, the best-scored model was further assessed based on its ability to discriminate between catecholamines (NE, epinephrine, histamine, dopamine, serotonin, and tyramine) and d-AMPH, which was not a substrate for Oct3 and only exerted weak inhibitory effects on Oct3-mediated uptake. The numerical data underlying this figure are included in S1 Data. AT, adipose tissue; d-AMPH, dextroamphetamine; DOPE, discrete optimized protein energy; GLUT3, glucose transporter 3; gonWAT, gonadal white adipose tissue; ingWAT, inguinal white adipose tissue; MA, mature adipocyte; NE, norepinephrine; Oct3, organic cation transporter 3; SVC, stromal vascular cell.

(TIF)

S2 Fig. Characterization of adipose-specific Oct3 KO (cKO) mice. (A) Schematic diagram of the construction of cKO mice. Oct3<sup>−/−</sup> mice were generated by locating LoxP sites in cis...
flanking in exon 2 of the Oct3 allele. (B) Western blot analysis of Oct3 in multiple ATs from Ctrl and cKO mice. (C) Analysis of gene expression by real-time PCR in ingWAT and gon-WAT from Ctrl and cKO mice (n = 6). (D) Body weight of Ctrl and cKO mice. (E–H) Metabolic parameters of Ctrl and cKO mice under RT (n = 4). (E) O2 consumption; (F) CO2 production; (G) heat production; (H) RER. (I) Representative HE staining in BAT, ingWAT and gonWAT (n = 3–4). Scale bar, 100 μm. Data in C–D were analyzed by Student t test. Data in E–H were analyzed by ANCOVA analysis. The numerical data underlying this figure are included in S1 Data. AT, adipose tissue; cKO, conditional knockout; Ctrl, control; gonWAT, gonadal white adipose tissue; HE, hematoxylin–eosin; ingWAT, inguinal white adipose tissue; RER, respiratory exchange ratio; RT, room temperature; Oct3, organic cation transporter 3. (TIF)

S3 Fig. In vivo NE uptake and analysis of CLAMS by ANCOVA. (A) Experimental scheme of in vivo NE uptake assay in AT from Ctrl and cKO mice. (B) Representative figures of ingWAT immunolabeled by anti-tyrosine hydroxylase (Th) to verify successful denervation (when ingWAT was successfully denervated, Th, a sympathetic nerve marker, would be significantly decreased). (C) Body composition of Ctrl and cKO mice. The total mass of mice consists of lean mass and fat mass (n = 8). (D, E) Multiple linear regression model and ANCOVA analysis for coefficient estimates of oxygen consumption (panel D) and energy expenditure (panel E) to lean mass and fat mass in Ctrl and cKO mice (n = 8). (F, G) The relationship of oxygen consumption (panel F) and energy expenditure (panel G) to body weight (n = 8). Data in S3D–S3G Fig were analyzed by ANCOVA to determine statistical differences. The numerical data underlying this figure are included in S1 Data. AT, adipose tissue; cKO, conditional knockout; CLAMS, Comprehensive Lab Animal Monitoring System; Ctrl, control; ingWAT, inguinal white adipose tissue; NE, norepinephrine. (TIF)

S4 Fig. Thermogenic and lipolytic response in BAT of Ctrl and cKO mice after NE stimulation. (A) mRNA expression of thermogenic and lipolytic genes in BAT of Ctrl and cKO mice after NE injection (n = 3–4). (B) Basal and epinephrine-stimulated serum FFAs in Ctrl and cKO mice (n = 6). (C) Protein levels of pHsl-S563, pHsl-S660, and total Hsl in BAT of Ctrl and cKO mice with NE (“NE”) or without NE injection (Ctrl). (D) Oil red O staining of 3T3-L1 cells stably transfected with EVs or Oct3 (Oct3-OE) induced by differentiation medium. (E) Basal and forskolin-stimulated lipolysis, as measured by glycerol release from differentiated 3T3-L1 adipocytes stably transfected with EVs or Oct3 (Oct3-OE) (n = 3). (F) Representative micrographs of livers of Ctrl and cKO mice with NE (“NE”) and without NE injection (Saline), stained with HE. (G) Hepatic triglyceride levels of Ctrl and cKO mice with NE (“NE”) and without NE injection (Saline) (n = 6). Data in panels A, B, E, and G were analyzed by Student t test. The numerical data underlying this figure are included in S1 Data. BAT, brown adipose tissue; cKO, conditional knockout; Ctrl, control; EV, empty vector; FFA, free fatty acid; NE, norepinephrine; Oct3, organic cation transporter 3; pHsl, phosphorylated hormone-sensitive lipase. (TIF)

S5 Fig. Thermogenic response in BAT and gonWAT after cold challenge. Ctrl and cKO mice were housed at 4˚C for 1 month. (A) Body weights of Ctrl and cKO mice (n = 6). (B, C) NE content in ingWAT (panel B) and serum (panel C) from Ctrl and cKO mice after cold exposure (n = 6). (D) Heat maps of significantly changed gene expressions in ingWAT of Ctrl and cKO mice. (E) Representative HE staining in BAT and gonWAT. Scale bar, 100 μm. (F) mRNA expression of thermogenic genes, catecholamine degradation enzymes, and FAO genes
in BAT \((n = 4)\). (G) mRNA expression of thermogenic, WAT-selective and FAO genes in gon-WAT \((n = 4)\). (H, I) Protein levels of pCreb, total Creb, Ucp1, and Pgc1α in BAT (panel H) and gonWAT (panel I). (J) mRNA expression of cAMP-PKA pathway component genes in ingWAT \((n = 4)\). (K, L) mRNA expression of ETC and glycolytic genes in BAT (panel K) and gonWAT (panel L) \((n = 4)\). (M) The amount of lactate in BAT and gonWAT. Data in A–C, F–G, and J–M were analyzed by Student \(t\) test. The numerical data underlying this figure are included in S1 Data. BAT, brown adipose tissue; cKO, conditional knockout; Ctrl, control; ETC, electron transport chain; FAO, fatty acid oxidation; gonWAT, gonadal white adipose tissue; ingWAT, inguinal white adipose tissue; NE, norepinephrine; PKA, protein kinase A.

S6 Fig. Characterization of Oct3 knockout primary inguinal adipocytes. (A) OCR measured by Seahorse in differentiated primary inguinal adipocytes from Ctrl and cKO mice \((n = 6)\). (B) Ap2 mRNA expression in differentiated primary inguinal adipocytes from Ctrl and cKO mice after stimulation with NE and T3 in the presence or absence of propranolol \((n = 3)\). (C) Ucp1 mRNA expression in differentiated primary inguinal adipocytes from Ctrl and cKO mice after stimulation with epinephrine, dopamine, and serotonin in the presence of T3 \((n = 3)\). (D) Western blotting of pCreb and total Creb in primary inguinal adipocytes from Ctrl and cKO stimulated with epinephrine, dopamine, and serotonin in the presence of T3. Data in A–C were analyzed by Student \(t\) test. The numerical data underlying this figure are included in S1 Data. cKO, conditional knockout; Ctrl, control; NE, norepinephrine; OCR, \(O_2\) consumption rate; Oct3, organic cation transporter 3; pCreb, phosphorylated cAMP-responsive element binding protein; T3, triiodothyronine.

S7 Fig. Effect of Oct3 deficiency on HFD-fed mice under cold exposure. (A) Body weight curves of Ctrl and cKO mice fed HFD for 12 weeks at thermoneutrality (30˚C) (panel A) or RT (panel B) (thermoneutrality, \(n = 8\); RT, \(n = 6\)). (C–L) Ctrl and cKO mice in panel A were then subjected to a cold challenge (4˚C) for 1 week \((n = 8)\). (C) Body temperature. (D) Body weights. (E) Food intake. (F) Fed blood glucose. (G) Plasma triglyceride. (H) Plasma cholesterol. (I) Plasma insulin. (J) Plasma adiponectin. (K) Plasma FFAs. (L) Glucose tolerance test. All data were analyzed by Student \(t\) test. The numerical data underlying this figure are included in S1 Data. cKO, conditional knockout; Ctrl, control; FFA, free fatty acid; HFD, high-fat diet; Oct3, organic cation transporter 3; RT, room temperature.

S8 Fig. Polymorphisms in human SLC22A3 and their associations with BMR and transcript levels. (A) A regional plot of SLC22A3 locus. SNPs were plotted by chromosome 6 against association with BMR in UK Biobank participants \((N \sim 7,000)\). An SNP, rs555754 (purple circle), and its proxies are the top signals in SLC22A3 locus. Estimated recombination rates \((\text{cM/Mb})\) were plotted in blue to reflect the local LD structure. The SNPs surrounding the significant SNP, rs555754, were color coded to reflect their LD with this SNP. This LD was taken from pairwise \(r^2\) values from the 1000 Genomes Nov 2014 EUR (hg19). Genes, the position of exons, and the direction of the transcription from the UCSC Genome Browser were noted. This plot was created using LocusZoom (http://locuszoom.org/genform.php?type=yourdata). The associations for each variant in this plot (effect size of the reference allele and \(p\)-value) were shown in S3 Table. (B, C) SLC22A3 transcript levels in adipose subcutaneous (panel B) and adipose visceral omentum (panel C) were significantly associated with the top two SNPs in the locus zoom plot in panel A. The figure and data were available in GTEx portal (gtexpertal.org). The x-axis showed the genotype for the SNP and the y-axis showed the expression...
levels of SLC22A3 as quantified by RNA-seq method. A full list of all eQTL for each SNPs were available in S3 Table. (D) mRNA expression level of catecholamine transporters and catecholamine degradation enzymes in human AT (n = 3). (E) Oil Red O staining of undifferentiated and adipogenically differentiated ATMSC. (F) mRNA expression level of catecholamine transporters and catecholamine degradation enzymes in differentiated ATMSC (n = 3). (G) In vitro glycerol release from differentiated ATMSC untreated or NE-treated to stimulate lipolysis, incubated with vehicle or OCT3 inhibitor decynium-22 (10 μM) (n = 3). Data in panel G were analyzed by Student t test. The numerical data underlying this figure are included in S1 Data.

AT, adipose tissue; ATMSC, AT-derived mesenchymal stem cell; BMR, basal metabolic rate; eQTL, expression quantitative trait locus; LD, linkage disequilibrium; NE, norepinephrine; Oct3, organic cation transporter 3; SNP, single-nucleotide polymorphism.

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