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Brown adipocytes promote epithelial mesenchymal transition of neuroblastoma cells by inducing PPAR-γ/UCP2 expression

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ABSTRACT

Neuroblastoma (NB) is an embryonic malignant tumour of the sympathetic nervous system, and current research shows that activation of brown adipose tissue accelerates cachexia in cancer patients. However, the interaction between brown adipose tissues and NB remains unclear. The study aimed to investigate the effect of brown adipocytes in the co-culture system on the proliferation and migration of NB cells. Brown adipocytes promoted the proliferation and migration of Neuro-2a, BE(2)-M17, and SH-SY5Y cells under the co-culture system, with an increase of the mRNA and protein levels of UCP2 and PPAR-γ in NB cells. The UCP2 inhibitor genipin or PPAR-γ inhibitor T0090709 inhibited the migration of NB cells induced by brown adipocytes. Genipin or siUCP2 upregulated the expression of E-cadherin, and downregulated the expression of N-cadherin and vimentin in NB cells. We suggest that under co-cultivation conditions, NB cells can activate brown adipocytes, which triggers changes in various genes and promotes the proliferation and migration of NB cells. The PPAR-γ/UCP2 pathway is involved in the migration of NB cells caused by brown adipocytes.

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Brown adipocytes; neuroblastoma; genipin; uncoupling protein; PPAR-γ

Introduction

NB is an embryonal malignancy derived from the peripheral sympathetic nervous system. Approximately 60% of primary tumours arises within the abdomen, and 30% within the adrenal medulla, and the other locations including the pelvis, head/neck, and chest. The biological heterogeneity of NB contributes to various clinical courses and prognoses of this kind of cancer [1]. NB patients are divided into very low, low, intermediate, and high-risk groups according to International Neuroblastoma Risk Group. While low and intermediate-risk NB patients display excellent outcomes, the high-risk patients show unfavourable prognoses [2]. Stage III and IV NB patients are at a high risk of undernourishment [3] and patients with Stage IV NB are frequently cachectic [4]. The tumour microenvironment (TME) consisting of endothelial cells, cancer-associated fibroblasts (CAF), immune cells, Schwann cells, and mesenchymal stromal cells (MSCs) plays an important role in the progression and metastasis of NB by multiple mechanisms [5]. CAFs in NB can differentiate into adipocytes, osteocytes, and chondrocytes [6]. Further more, many studies reported the relationship between adipocytes and tumour progression, that is, tumour-neighboured adipocytes promote cancer progression [7]. However, the precise mechanisms of adipocytes in NB development, especially that of brown adipocytes, are not clearly addressed. There is little literature on the effect of brown adipocytes on NB, and further explorations are needed.

Brown adipocytes reside in brown adipose tissue (BAT) with many large and spherical mitochondria and multilocular lipid droplets. Classic BAT inhabits the interscapular region of mice and the neck of adult humans to maintain body temperature through consuming sugar and fat to produce heat by non-shivering thermogenesis [8,9]. The process of the heat production is regulated by uncoupling protein 1 (UCP1) in the mitochondria. UCP1 and UCP2 belong to the mitochondrial anion carrier proteins family; UCP1 is mainly expressed in BAT and regarded as a marker of BAT [10], but UCP2 is mainly expressed in the pancreas, liver, kidney, brain, spleen, immune cells, and adipose tissue. They both play an important role in reducing the proton gradient in the inner mitochondrial membrane by burning the energy as heat instead of ATP

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production [11]. UCP2 overexpression has been found in many aggressive human cancers, including colon, prostate, breast, head, and skin cancers [12]. PPARs nuclear receptor superfamily includes PPARα, PPARβ/δ, and PPAR-γ, which have been identified in NB. Among these PPARs, PPAR-γ is found to be highly expressed in NB cell lines and PPAR-α is expressed at very low level, but PPAR-β/δ expression in NB cell lines is unclear [13]. PPAR-γ, as a transcription factor, may trigger tumorigenicity pathways such as metastasis, apoptosis, and proliferation [14] and its expression is related to the differentiation status of NB and outcome of patients [13].

BAT can release many molecules that transfer signals to distinct tissues and adjust systemic metabolism [15,16]. A few evidence suggest that BAT is associated with tumour progression. Activated BAT is observed in a patient with pelvic paraganglioma [17] and active cancer who have greater BAT activity and higher BAT volume than those without active cancer [18]. The total metabolic activity of BAT relates to the active neoplastic status in patients residing within the tropics, that is, a more active neoplastic status accompanies a more vigorous total metabolic activity of BAT [19]. Increased BAT volume is related to the increased likelihood of tumour-induced mortality and tumour recurrence in patients with cancer [20], especially white adipose tissue (WAT) browning in patients with cachectic gastric and colorectal cancer [21]. Cachectic tumour-bearing mice show increased thermogenesis and activation of cytokine signalling in BAT [22]. In addition, expression of brown adipose markers in tumour and host cells affects breast tumour growth in mice [23]. However, the relationship between brown fat tissues and NB has not been reported.

In the study, we aimed to determine if NB cells can activate brown adipocytes under co-cultivation conditions, which may promote proliferation and migration of NB cells. The PPAR-γ/UCP2 pathway may be associated with the migration of NB cells caused by brown adipocytes.

Results

Neuroblastoma cells and brown adipocytes show altered protein expression under co-cultured systems

To observe the relationship of brown adipocytes and NB cells, the co-culture system was applied in our experiments. Firstly, the brown adipocytes from mice were successfully separated and verified. As shown in Figure 1a-**left**, immature adipocytes were similar in morphology to fibroblasts. After induced differentiation, the extracellular matrix and cytoskeleton in immature adipocytes were transformed into those of mature cells, which gradually became round or quasi-circular, while lipid droplets in the cells were gradually accumulated and finally fused into large lipid droplets in mature adipocytes. The results from Oil red staining proved successful isolation of primary brown fat cells (Figure 1a-**right**). Secondly, the single culture or co-culture of NB cells and brown adipocytes was performed in a transwell system (pore size 0.4 μm) (Figure 1b) and was used to conduct the following experiments. The expression of the marked proteins including UCP1 and UCP2 in two kinds of cells were examined using western blotting and qRT-PCR in the co-culture system. The expression of UCP1 protein was increased in brown adipocytes co-cultured with NB cells (Figure 1c,d), which indicated that brown adipocytes could be activated under co-cultured systems. Additionally, the expression of UCP2 protein was increased in NB cells co-cultured with brown adipocytes (Figure 1e,f). In the same culture condition, results from qRT-PCR showed that UCP2 mRNA was upregulated in Neuro-2a cells (Figure 1g). However, RNA-seq results showed that UCP2 mRNA was also upregulated in NB cells (Figure 1h), which indicated that UCP2 might serve as important biomarkers in NB cells under co-culture systems.

Brown adipocytes increases the proliferation and migration of NB cells in co-culture system

Co-culture of NB cell lines and brown adipocytes was performed in a transwell system (pore size 0.4 μm) (Figure 2a), and the migration of NB cells affected by brown adipocytes was also tested in a transwell system (pore size 8 μm) (Figure 2b).

In the co-culture system, the proliferation and migration of NB cells were determined with SRB or crystal violet staining. Data showed that the cell viability of Neuro-2a, BE(2)-M17, or SH-SY5Y cells was increased after the cells were co-cultured with brown adipocytes (Figure 2c,d), but there was no change in the cell viability of Neuro-2a, BE(2)-M17, and SH-SY5Y cells treated with BCA-CCM (Figure 1e). The protein expression of cyclin D1 (Figure 2f,g) and CDK4 (Figure 2f,h) in Neuro-2a, BE(2)-M17, or SH-SY5Y cells was obviously increased after the cells were co-cultured with brown adipocytes, which indicated that brown adipocytes might promote the proliferation of Neuro-2a, BE(2)-M17, or SH-SY5Y cells by upregulating the expression of cyclin D1 and CDK4. Therefore, it was suggested that brown fat cells in a co-culture system were able to promote tumour cell

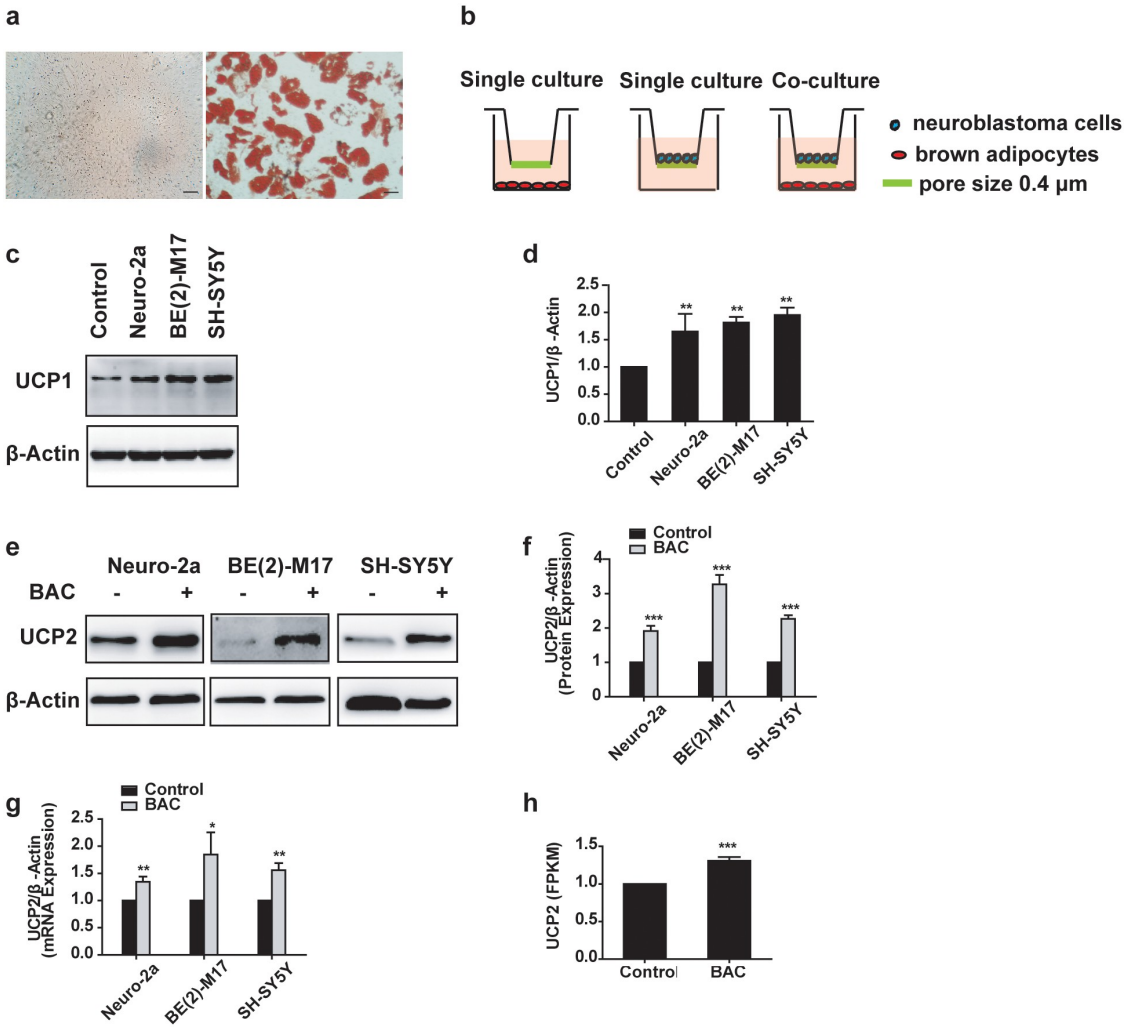

Figure 1. Neuroblastoma cells and brown adipocytes show alteration of protein levels under co-culture systems. (a) Brown adipocytes are detected by Oil Red O. Brown preadipocytes (left, scale bar: 200 μm) and differentiated brown adipocytes after the induction stained with Oil Red O (right, scale bar: 100 μm). (b) Schematic diagram of single-culture or co-culture of neuroblastoma cell lines and brown adipocytes. (c) Western blotting is used to detect the expression of UCP1 in brown adipocytes after co-culture with Neuro-2a, BE(2)-M17, and SH-SY5Y cells for 72 h. (d) The expression of UCP1, as analysed by grey-scale scanning. (e) The expression of UCP2 protein in Neuro-2a, BE(2)-M17 and SH-SY5Y cells after co-culture with brown adipocytes for 72 h, as detected by western blotting. (f) The expression of UCP2 protein, as analysed by grey-scale scanning. (g) The level of UCP2 mRNA in Neuro-2a, BE(2)-M17, and SH-SY5Y cells after co-culture with brown adipocytes for 72 h, as detected by qRT-PCR. (h) The level of UCP2 mRNA in Neuro-2a cells after co-culture with brown adipocytes for 72 h, as detected by RNA seq. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$ vs. Control, *Mean* ± *SD*, n = 3.

proliferation, while brown fat cells cultured alone do not exert the enhanced effect. In the migration system, after the NB cells in the upper chamber were stained with crystal violet and dissolved in acetic acid, the values of absorbance showed that cell migration of Neuro-2a, BE (2)-M17, or SH-SY5Y was increased after cells were co-cultured with brown adipocytes (Figure 2i,j). The expression of vimentin (Figure 2k,l) and N-Cadherin (Figure 2k, m) protein was clearly increased, but the expression of E-cadherin (Figure 2k,n) protein was significantly decreased in NB cells co-cultured with brown adipocytes. Thus, it was shown that brown adipocytes promoted the migration of Neuro-2a, BE(2)-M17, or SH-SY5Y by regulating epithelial–mesenchymal transition (EMT).

UCP2 is involved in cell migration of NB cells co-cultured with brown adipocytes

From the above results, brown adipocytes affected the proliferation and migration of NB cells in co-culture system. In addition, UCP2 expression in NB cells was

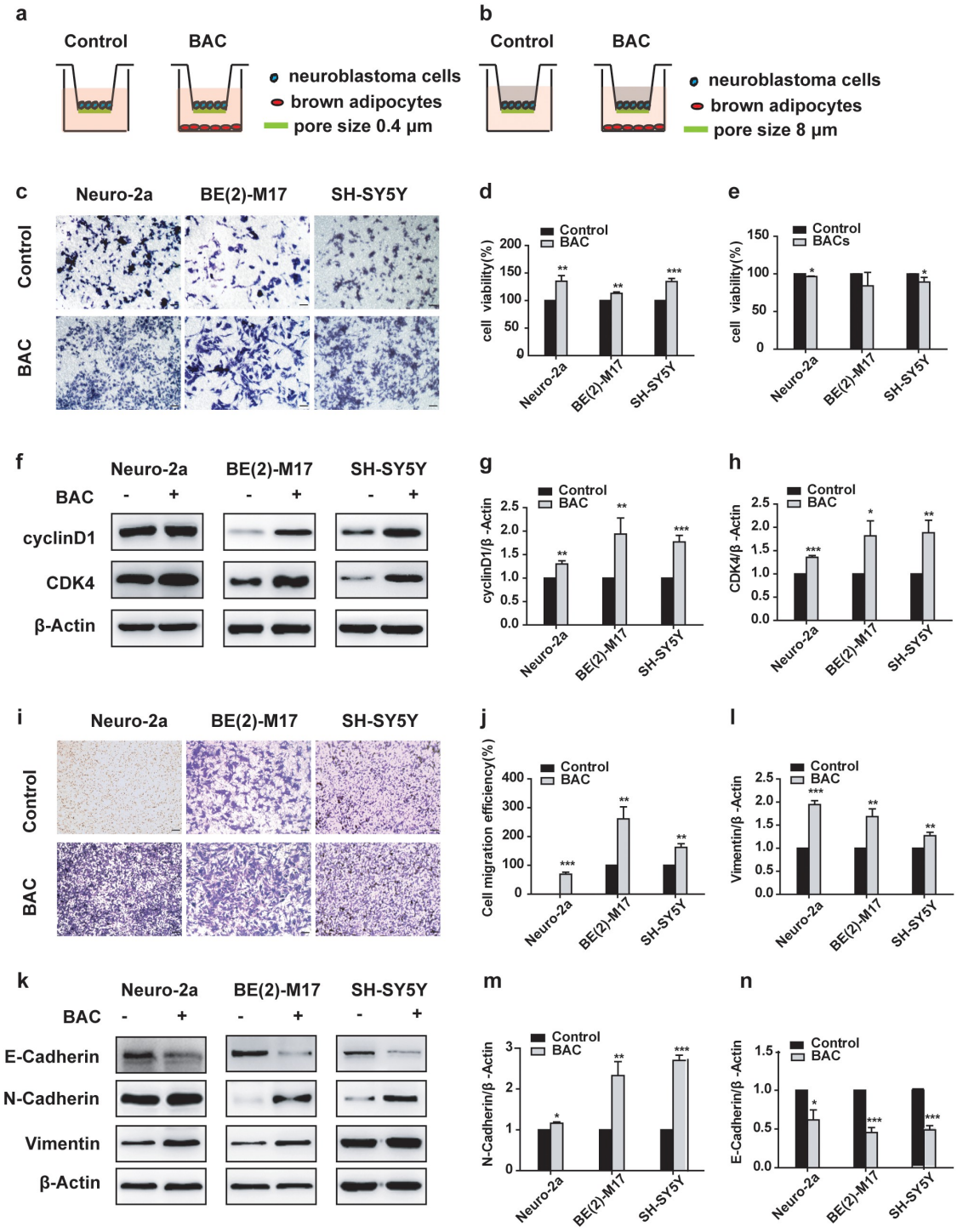

Figure 2. Neuroblastoma cells co-cultured with brown adipocytes show the increase of growth and migration capacities. (a) Schematic diagram of single-culture or co-culture of neuroblastoma cell lines and brown adipocytes. (b) Schematic diagram of the migration of neuroblastoma cell lines affected by brown adipocytes. (c) The growth of Neuro-2a, BE(2)-M17, and SH-SY5Y cells after co-culture with brown adipocytes for 72 h, as observed with crystal violet staining. Scale bar: 100 μm. (d) Statistical analysis histogram for c. (e) The viability of Neuro-2a, BE(2)-M17, and SH-SY5Y cells after co-culture with CCM of BACs for 72 h, as detected by SRB assay. (f) The expression of cyclin D1 and CDK4 protein in Neuro-2a, BE(2)-M17, and SH-SY5Y cells after co-culture with brown adipocytes for 72 h, as detected by western blotting. (g-h) The expression of cyclin D1 and CDK4 protein, as analysed by grey-scale scanning. (i) The migration of Neuro-2a, BE(2)-M17, and SH-SY5Y cells after being induced by brown adipocytes for 48 h, seen with crystal violet staining. Scale bar: 100 μm. (j) Statistical analysis histogram for i. (k) The expression of E-Cadherin, N-Cadherin, and Vimentin protein in Neuro-2a, BE(2)-M17, and SH-SY5Y cells after co-culture with brown adipocytes for 72 h, as detected by western blotting. (l-n) The expression of Vimentin, N-Cadherin, and E-Cadherin protein, as analysed by grey-scale scanning. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$ vs Control, *Mean* ± *SD*, n = 3.

increased, which required further investigation, leading to the use of UCP2 inhibitors genipin and siRNA in the following experiments. At first, we tested the effect of genipin on the proliferation of NB cells. Genipin at 0–30 μM had little effect on the cell survival of Neura-2a cells (Figure 3a) and at 0–60 μM had the same effect on BE(2)-M17 (Figure 3b); therefore, 30 μM and 60 μM genipin were chosen for the next experiments. As

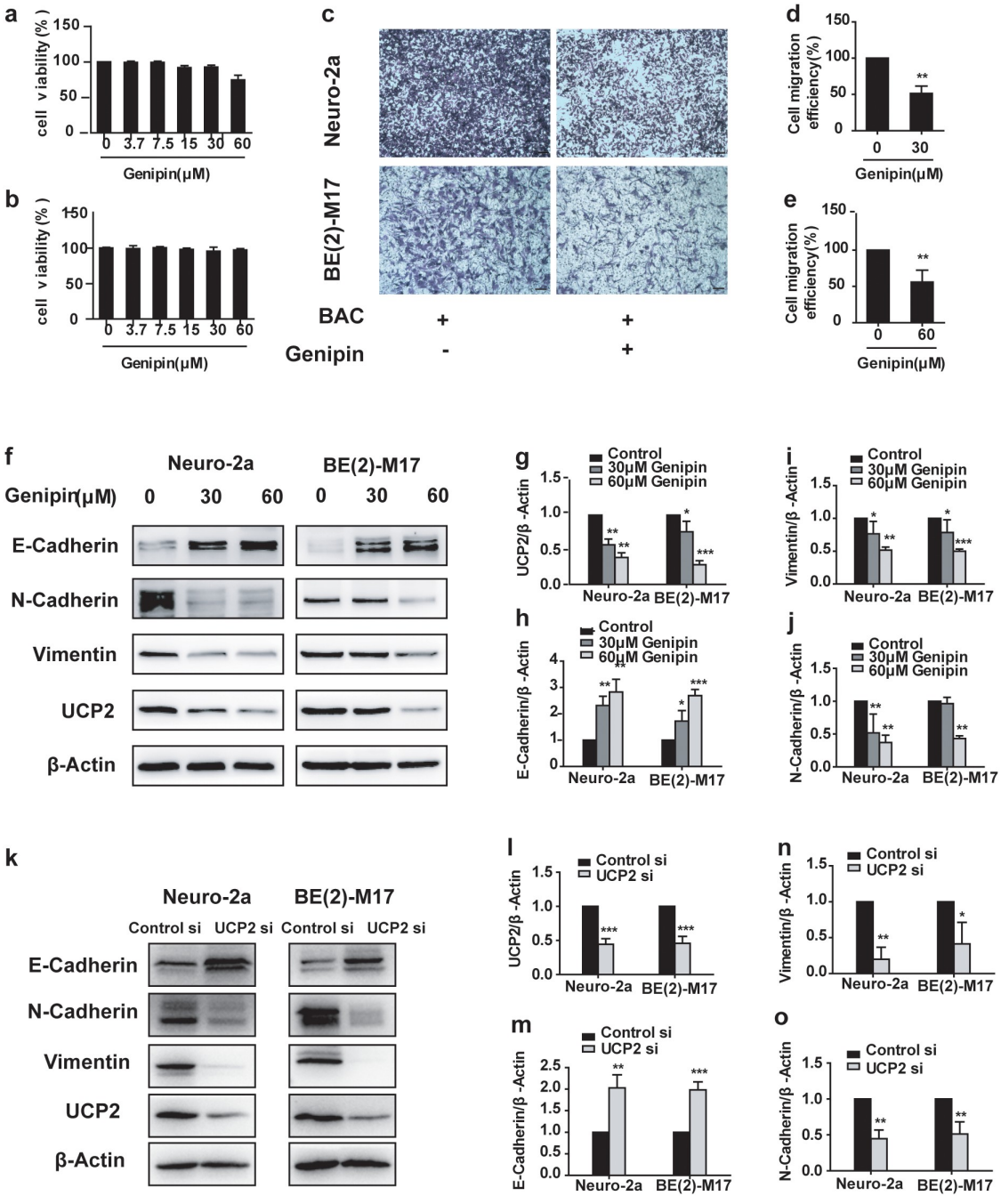

Figure 3. UCP2 modulates the migration of neuroblastoma cells induced by brown adipocytes. (a-b) The viability of Neuro-2a, and BE(2)-M17 cells after treatment with genipin for 48 h, as detected by SRB assay. (c) The effect of genipin on the migration of Neuro-2a (30 μM) and BE(2)-M17 (60 μM) cells induced by brown adipocytes for 48 h, as detected by crystal violet staining. Scale bar: 100 μm. (d-e) Statistical analysis histogram for Neuro-2a and BE(2)-M17 cells. (f) Western blotting is performed to examine the expression of UCP2, E-Cadherin, N-Cadherin, Vimentin protein in Neuro-2a and BE (2)-M17 cells after culture with 30 μM or 60 μM genipin for 48 h. (g-j) The expression of UCP2, E-Cadherin, Vimentin, and N-Cadherin protein, as analysed by grey-scale scanning. (k) Western blotting is used to detect the expression of UCP2, E-Cadherin, N-Cadherin, and Vimentin protein in Neuro-2a or BE(2)-M17 cells after culture with 50 nM siUCP2 for 48 h. (l-o) The expression of UCP2, E-Cadherin, Vimentin, and N-Cadherin protein, as analysed by grey-scale scanning. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$ vs Control, *Mean* ± *SD*, n = 3.

presented in Figure 3, 30 μM and 60 μM genipin suppressed the migration of Neura-2a (Figure 3c,d) and BE(2)-M17 (Figure 3c,e) induced by brown adipocytes, respectively. Second, the migration of the NB cells was examined using a transwell assay. Finally, EMT-associated proteins in NB cells were tested after genipin or siUCP2 treatment using western blotting. Genipin inhibited the expression of UCP2 protein (Figure 3f,g), upregulated the expression of E-cadherin (Figure 3f,h), and downregulated the expression of vimentin (Figure 3f,i) and N-cadherin (Figure 3f,j) in Neuro-2a and BE(2)-M17 cells. Similarly, siUCP2 inhibited the expression of UCP2 protein (Figure 3k, l), upregulated the expression of E-cadherin (Figure 3k, m), and downregulated the expression of vimentin (Figure 3k,n) and N-cadherin (Figure 3k,o) in Neuro-2a and BE(2)-M17 cells. From this, genipin inhibited the migration of NB cells induced by brown adipocytes via EMT regulated by UCP2. Moreover, inhibition of UCP2 caused changes in the expression of E-Cadherin, N-Cadherin, and vimentin protein in NB cells.

PPAR-γ regulates the migration of neuroblastoma cells induced by brown adipocytes

To investigate the molecular mechanism of UCP2 regulation in NB cells co-cultured with adipocytes, transcriptome analysis was applied in the co-culture system. The results displayed that top 20 DEGs were obtained in Neura-2a co-cultured with brown adipocytes by screening genes with Q value ≤ 0.05 and log2Ratio ≥ 1 (Figure 4a). The top five KEGG pathways that DEG participated in were ranked as follows: cytosolic DNA-sensing pathway, NOD-like receptor signalling pathway, alanine, aspartate, and glutamate metabolism pathway, amino sugar and nucleotide sugar metabolism pathway, and PPAR signalling pathway (Figure 4b). The results suggested that metabolism-related pathways and PPAR signalling pathways might be involved in the mechanism of brown fat affecting NB. The results were verified using western blotting and qRT-PCR, which demonstrated that brown adipocytes upregulated the protein (Figure 4c,d) and mRNA (Figure 4e) expression of PPAR-γ in Neuro-2a and BE(2)-M17 cells co-cultured with brown adipocytes. The PPAR-γ inhibitor T0090709 was used in the following experiment. At first, we examined the effect of T0090709 on the proliferation of Neura-2a and BE(2)-M17. As shown in Figure 4, T090709 at 0–10 μM had no effect on the proliferation of Neura-2a (Figure 4f) and BE(2)-M17 (Figure 4g), thus the concentrations were used for the migration experiments in the co-culture system. T0090709 at 10 μM decreased the migration of the indicated NB cells induced by co-cultured brown adipocytes (Figure 4h,i), and T0090709 at 10 and 20 μM inhibited the protein expression of UCP2 (Figure 4j,k) and PPAR-γ (Figure 4j,l) in Neuro-2a and BE(2)-M17 cells, which suggested that inhibition of PPAR-γ reduced UCP2 protein expression in NB cells. Therefore, PPAR-γ was related to the increased migration of NB cells induced by brown adipocytes and may be related to the inhibition of UCP2 expression.

Discussion

It is reported that brown fat tissues are associated with the progression of cancer. Activated brown fat exists in some cachectic patients, causing energy wasting in cancer cachexia [24]. However, the underlying relationship between brown fat and NB still needs to be addressed. Previous data demonstrate that the expression of UCP1 from brown fat tissues is increased in cancer cachexia. Cachectic mice show a change from WAT to BAT with increased UCP1 expression, which contributes to enhanced lipid mobilization and energy wasting [25]. Lewis Lung Carcinoma cell-conditioned medium induces the expression of UCP1 in primary white adipose cells. Adipose tissue browning and cachexia were discovered in Lewis Lung Carcinoma tumour-bearing mice, which can be inhibited by PTHrP neutralization [26]. Upregulation of UCP1 protein and mRNA level is detected in WAT of both late-stage cancer cachexia mice and patients [21]. Our results showed that NB cells induced the increase of UCP1 expression in brown adipocytes from the co-culture system. Thus, brown adipocytes co-cultured with NB cells showed the same characteristics as brown fat in cancer cachexia.

Cell proliferation is the result of cell growth and division, which is essential for tumorigenesis, but its dysfunction is the key characteristic of all tumours [27]. The proliferation of NB cells can be altered by the tumour microenvironment substantially [5]. Our results showed that the growth of Neuro-2a, BE(2)-M17, or SH-SY5Y was not affected by brown adipocyte conditioned medium, but the survival rate and expression of cyclin D1 and CDK4 protein were increased in Neuro-2a, BE(2)-M17, or SH-SY5Y cells co-cultured with brown adipocytes. RNA-seq results demonstrated that the transcription levels of multiple genes were changed in Neuro-2a cells co-cultured with brown adipocytes. Thus, brown adipocytes co-cultured with NB cells might be different from the cells cultured alone with conditioned medium. Additionally, cyclin D1 and CDK4 were involved in the increased growth of NB cells co-cultured with brown adipocytes. EMT refers to a process of changes from epithelial to mesenchymal

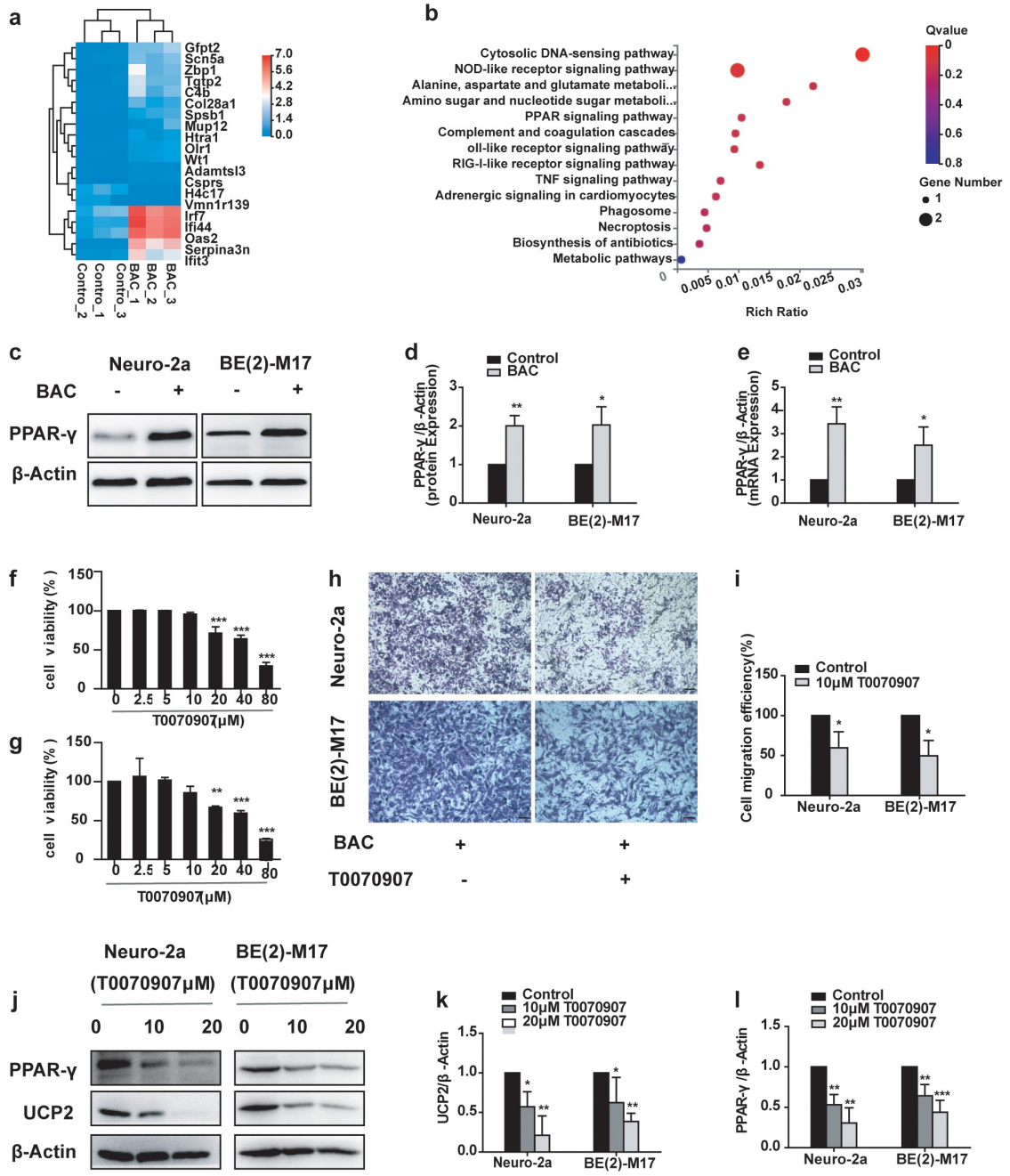

Figure 4. PPAR-γ regulates the migration of neuroblastoma cells induced by brown adipocytes. (a) Cluster of TOP20 DEGs obtained in Neuro-2a cells co-cultured with brown adipocytes for 72 h. The horizontal axis was the log2 of the sample (expression value +1), and the vertical axis was the gene. (b) Enriched KEGG pathways for TOP20 DEGs obtained in Neuro-2a cells co-cultured with brown adipocytes for 72 h. The total DEGs are subjected to KEGG pathway enriched analysis. X-axis shows Rich Factor, and Y-axis shows KEGG pathways. (c) Western blotting is used to detect the expression of PPAR-γ protein in Neuro-2a or BE(2)-M17 cells after co-culture with brown adipocytes for 72 h. (d) The expression of PPAR-γ protein, as analysed by grey-scale scanning. (e) The mRNA level of PPAR-γ in Neuro-2a after co-culture with brown adipocytes, as detected by qRT-PCR. (f-g) The viability of Neuro-2a and BE(2)-M17 cells after being treated with T0070907 for 48 h, as detected by SRB assay. (h) Crystal violet staining is performed to detect the effect of 10 μM T0070907 on the migration of Neuro-2a and BE(2)-M17 cells induced by brown adipocytes for 48 h. Scale bar: 100 μm. (i) Statistical analysis histogram for h. (j) Western blotting is used to detect the expression of UCP2 and PPAR-γ protein in Neuro-2a and BE(2)-M17 cells after being cultured with 10 μM or 20 μM T0070907 for 48 h. (k-l) The expression of UCP2 and PPAR-γ protein, as analysed by grey-scale scanning. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$ vs Control, *Mean* ± *SD*, n = 3.

phenotype with a feature of EMT accompanying the reduction of E-cadherin to increase cell motility and decrease adhesion, increased expression of N-cadherin to equilibrate the reduction of E-cadherin, and upregulation of vimentin

to enable cell motility [28]. Our results demonstrated that brown adipocytes promoted the migration of NB cells and changed the expression of EMT-associated proteins under the co-culture system. Thus, the migration of NB cells may be regulated by EMT under the co-culture system.

Higher levels of UCP2 protein are significantly expressed in the human head and neck, pancreatic, prostate, and prostate tumour tissues [29]. UCP2 catalyzes the transport of aspartate and facilitates tumour growth. The cell growth of kRASmut human pancreatic ductal adenocarcinoma is inhibited by UCP2 silencing *in vitro* and *in vivo* [30]. Genipin treatment or UCP2 knockdown promotes ROS production, decreases glucose uptake, and inhibits cell survival in lung cancer cells with high UCP2 expression [31]. Overexpression of UCP2 is detected in glioma and correlated with poor prognosis in glioma patients, but glioma cells with silenced UCP2 exhibit the inhibitory state of proliferation and migration [32]. The radiosensitivity of cervical cancer cells is augmented by UCP2 inhibition [33]. Metastatic tumour cells contain high expression of UCP2 [34]. High expression of UCP2 is discovered in gallbladder cancer tissues, which is relevant to poor outcomes in gallbladder cancer patients [35]. Glutamine deficiency causes the reversible and rapid decrease of UCP2 expression, and reduction of viability and metabolic activity of NB cells [36]. In our experiments, the results showed that mRNA and protein expression of UCP2 were increased in NB cells co-cultured with brown fat cells. Genipin, a potent UCP2 inhibitor [37], inhibited the migration of NB cells caused by brown adipocytes. Genipin or siUCP2 increased the expression of E-cadherin and decreased the expression of N-cadherin and vimentin. E-cadherin, N-cadherin, and vimentin are the marked proteins of EMT. Therefore, the results further indicate that UCP2 participates in the migration of NB cells induced by co-cultured brown adipocytes through the regulation of EMT.

PPAR-γ is found to be highly expressed in NB cell lines and its expression is related to the differentiation status of NB and outcome of patients [13]. The results from RNA-seq illustrated that the PPAR pathway was changed in Neuro-2a cells co-cultured with brown adipocytes. Increased levels of PPAR-γ protein and mRNA were detected in Neuro-2a and BE(2)-M17 cells. The function of PPAR-γ on tumour progression is disputed, some studies indicate that PPAR-γ agonists can inhibit the growth of NB cells [38]. However, it has been shown that PPAR-γ agonists suppressed tumour development in a PPAR-γ-independent pathway [39,40]. PPAR-γ plays a role in promoting tumour formation in neuroblastoma [41], which are consistent with our results. It has been reported that UCP2 protein expression is upregulated in islets with overexpressed PPAR-γ [42] and activated PPAR-γ increases mitochondrial UCP2 expression in Sprague–Dawley rats [43]. In addition, UCP2 expression is modulated by activated PPAR-γ in BAT of rats and mice [44]. From these evidence, it was supposed that PPAR-γ would modulate the expression of UCP2 in our experimental system, so the PPARγ inhibitor T0070907 was used to verify this hypothesis. As the results indicated, T0070907 inhibited the protein expression of UCP2 and the migration of Neuro-2a and BE(2)-M17 cells caused by brown adipocytes. Therefore, it is speculated that the PPARγ/UCP2 pathway is involved in modulating the migration of NB cells.

Han et al. reported that recombinant murine IL-6 significantly increases UCP1 mRNA levels in a model of pre-brown adipocytes for 3T3-L1 pre-white adipocytes transfected with Cebp/b [21]. In our co-culture system, the primary adipose adipocytes will be also affected by the NB cells and then secrete BATokines that influences the tumour cells. IL-6 is reported to serve as one of BATokines [15,16]. To study the major component in the supernatant of the co-culture system that exerts the effect on NB cells, the transcriptome sequencing and analysis were used in the co-culture system. The results showed that the IL-6 mRNA was increased in the Neuro-2 cells (data not shown). The value of log2 fold change in Neuro-2 in the co-culture system was 2.22 as compared to the control. In fact, IL-6 is expressed in many malignant tumours including myeloma, melanoma, renal carcinoma, and prostate cancer to stimulate cancer cell proliferation as an autocrine loop and modulate the progression of the tumours. In advanced refractory prostate cancer patients, the content of serum IL-6 is increased in an androgen-independent manner as a sign of poor prognosis. IL-6 could upregulate PPAR-γ expression in PC-3 cells, a prostate cancer cell line [45]. We speculated that IL-6 may be involved in regulating the functions of NB cells and brown adipocytes in our co-culture system. Also, the mechanism of the cytokine will be further explored in the future.

In summary, brown adipocytes can be activated by NB cells under co-culture conditions, and promote the growth and migration of NB cells, accompanied by the changes of cell cycle, EMT and metabolism-related protein, and at the transcription level of multiple genes. The PPAR-γ/UCP2 pathway is involved in the migration of NB cells caused by brown adipocytes.

Material and methods

Cell culture and reagents

Mouse NB cell line Neuro-2a, human NB cell line BE(2)-M17, and SH-SY5Y were purchased from the Cell Center of the Chinese Academy of Medical Science. Neuro-2a, BE

(2)-M17, and SH-SY5Y were cultured in MEM, DMEM, and DME/F12 (HyClone, Logan, UT, USA) medium containing 10% FBS (Gibco Grand Island, NY, USA), respectively, and grown in a 37°C incubator with 5% CO_2. Genipin or T0090709 was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Anti-UCP1, anti-N-Cadherin, anti-Vimentin, anti- E-Cadherin, anti-UCP2, anti-CDK4, anti-cyclin D1, and anti-PPARγ antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA), and secondary antibody and anti-β-actin antibodies were purchased from (Proteintech, Wuhan, China). Other chemical reagents were provided by Sigma (Sigma-Aldrich, St. Louis, MO, USA).

Isolation and identification of primary brown adipocytes

All animal experiments were approved by the Ethical Committee Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College. Newborn female BALB/c mice were purchased from SIPEIFU (Beijing, China) and treated according to the requirements of animal ethics. BAT was slowly taken out from the neck of the BALB/c mouse in the ultra-clean bench. The tissue was digested with collagenase (Sigma-Aldrich, St. Louis, MO, USA) solution for 30 min. The cell digestion solution was filtered with a cell sieve, and then the cell suspension was centrifuged at 1000 rpm for 5 min. Finally, the cell pellet was resuspended in DME/F12 medium containing 15% FBS and grown in a 37°C incubator with 5% CO_2. After the cell confluence reaches 100%, insulin (20 nM), rosiglitazone (1 μM), T3 (1 nM), IBMX (0.5 mM), dexamethasone (1 μM) were added to the proliferation medium. The oil drops were stained with Oil Red O staining solution, and pictures were taken under a microscope.

Cell Conditioned Medium (CCM) Preparation

When brown adipocytes grew to 80% confluence, the complete medium was replaced with serum-free DMEM/F12 and the cells were cultured for another 24 h. The serum-free medium of brown adipocytes was collected and centrifuged at 1000 rpm for 5 min to remove cell debris, and the supernatant was collected.

Co-culture system

Co-culture of NB cell lines and brown adipocytes was performed in a transwell system, 1×10^5 Neuro-2a, BE(2)-M17, or SH-SY5Y cells were planted in transwell inserts (pore size 0.4 μm, Corning, New York, NY, USA), respectively. Additionally, 2×10^5 primary brown adipocytes were cultivated on the bottom of six-well cell culture receiver plate. NB cells or brown adipocytes were cultured (either on six-well culture plates or in transwell inserts) alone served as control. The cells were co-cultured for 72 h, then analysed using western blotting or cell proliferation studies.

Cell viability assay

Cells were seeded in 96-well plates at a density of 3×10^4–5×10^4 cells/mL for 200 μl per well. After the cells adhered to the plates, each culture well was replaced with culture medium with or without CCM, and then Genipin or T0090709 and each treatment group was set up in triplicates. After 48 h of culture, the cells were treated with 10% trichloroacetic acid for 1 h, and then stained with sulforhodamine B. The protein-bound dye was dissolved by Trisbase and absorbance was measured at 570 nm by a microplate reader (Thermo Fisher, Waltham, MA, USA).

Migration assays

In the cell migration system, a total of 0.5×10^5 NB Neuro-2a, BE(2)-M17, and SH-SY5Y cells were seeded in transwell inserts (pore size 8 μm, Corning, New York, NY, USA), respectively, in serum-free medium. Primary brown adipocytes (1×10^5 cells) were cultured on the bottom of cell culture receiver plate, and NB cells were seeded in transwell inserts alone served as control. The cells were then cultured for 48 h. The cells on the opposite side of membrane were stained with crystal violet solution after removing all the cells on the upper side of the membrane with a cotton swab, and pictures were taken under an inverted microscope. The nested membrane was dissolved with 33% acetic acid, and the absorbance at 570 nm was read with a microplate reader (Thermo Fisher, Waltham, MA, USA).

Western blotting

After the cells were washed twice with PBS, the total protein was extracted with RIPA lysis solution, and the concentration was determined by the BCA method (Thermo Fisher Scientific, Waltham, MA, USA). The protein was denatured with loading buffer and heating, and 20 μg protein sample was taken for SDS-PAGE electrophoresis separation. The protein was then transferred to the PVDF membrane (Millipore, Billerica, MA, USA), blocked with 5% milk at room temperature for 1 h, incubated with the primary antibody overnight

and the secondary antibody for 1 h, and then reacted with the chemiluminescent solution (Thermo Fisher Scientific, Waltham, MA, USA). The pictures were collected by the imaging system (Proteinsimple, Santa Cara, CA, USA) and quantitated by image J.

RNA extraction and quantitative PCR

The total cell RNA was extracted according to RNAfast200 kit (Fastagen Biotech, Shanghai, China), and the RNA was reverse transcribed using TOYOBO's ReverTra Ace® qRT-PCR Master Mix with gDNA Remover kit (TOYOBO). Applied Biosystems 7500 PCR system was used for TB Green fluorescence PCR amplification.

siRNA transfections

A total of 1×10^5 NB cell lines Neuro-2a or BE(2)-M17 cells were planted in six-well plates. After 24 h, the riboFECT CP Transfection Kit (Ribobio, Guang Zhou, China) was used to carry out the transfections with 50 nM siRNA for 48 h (Mouse UCP2 siRNA sense: GGAAAGGGACUUCUCCCAATT, antisense: UUGG GAGAAGUCCCUUUCCTT; human UCP2 siRNA sense: GCUAAAGUCCGGUUACAGATT, antisense: UCUGUAACCGGACUUUAGCTT).

Transcriptome sequencing and analysis

Co-culture of Neuro-2a cell lines and brown adipocytes was performed in a transwell system. A total of 1×10^5 Neuro-2a cells were planted in transwell inserts (transwell, pore size 0.4 μm, Corning, New York, NY, USA). Primary brown adipocytes (2×10^5) were cultivated on the bottom of six-well cell culture Receiver Plate. The Neuro-2a cells were cultured in transwell inserts alone served as control. The cells were co-cultured for 72 h, and then Trizol Reagent was used to extract total RNA from Neuro-2a cells. Agilent 2100 (Agilent Technologies, Norwood, MA, USA) was used to measure the purity and quality of total RNA, three biological replicates were included in each treatment. BGISEQ-500 was applied to sequence the six samples. Transcriptome data was calculated by Genomics Institute (Shenzhen, China).

Statistical analysis

Graph Pad Prism software was used for statistical analysis. Three independent experiments were performed for groups of data that were presented as mean ± SD. Comparisons between two groups were done using Student's t-test. One-way ANOVA were used for multiple comparisons. Statistical significance was set at $P < 0.05$.

Author contributions

CSZ designed, wrote, and edited the paper. GZJ wrote the paper, analysed the data, and conducted the experiments. SY performed some cell cultures. WDW made some experiments for reviewers' comments. YJG participated in the design of the project.

Availability of data and materials

Data sharing not applicable – no new data generated.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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