Direct Conversion of Human Myoblasts into Brown-Like Adipocytes by Engineered Super-Active PPARγ

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Objective: To determine whether super-activation of PPARγ can reprogram human myoblasts into brown-like adipocytes and to establish a new cell model for browning research.

Methods: To enhance the PPARγ signaling, M3, the transactivation domain of MyoD, was fused to PPARγ. PPARγ and M3-PPARγ-lentiviral vectors were used to convert human myoblasts into adipocytes. Brown adipocyte markers of the reprogrammed adipocytes were assessed by qPCR and protein analyses. White adipocytes differentiated from subcutaneous stromal vascular cells and perithyroid brown fat tissues were used as references.

Results: In transient transfections, M3-PPARγ had a stronger constitutive activity than PPARγ by reporter assay. Although the transduction of either PPARγ or M3-PPARγ induced adipogenesis in myoblasts, M3-PPARγ drastically induced the brown adipocyte markers of UCP1, CIDEA, and PRDM16 by 1,050-, 2.4-, and 5.0-fold, respectively, and increased mitochondria contents by 4-fold, compared to PPARγ.

Conclusions: Super-activation of PPARγ can effectively convert human myoblasts into brown-like adipocytes and provide a new approach to derive brown-like adipocytes.

Introduction

Obesity represents a major risk factor, particularly for diabetes type 2 (T2D), dyslipidemias, and cardiovascular diseases (1). Obesity develops when energy intake exceeds expenditure. The recent discovery that adult humans have functional brown adipose tissue (BAT)-mediated thermogenesis has re-energized interest in targeting BAT bioenergetics to increase energy expenditure for the treatment of obesity and associated diseases (2-4).

In mammals, two major distinctive types of adipose tissue exist, white adipose tissue and BAT; the former stores energy in the form of triglyceride whereas the latter dissipates energy as heat. Brown adipocytes, the functional component of BAT, are characterized by the presence of multilocular lipid droplets, the abundance of mitochondria, and the expression of the cell type-specific uncoupling protein 1 (UCP1). Both an increase in the number of brown adipocytes and the induction of UCP1 expression are characteristics for adipocyte “browning” (5). Great advances have been made in understanding the regulation and development of brown adipocytes or BAT in recent years (6,7) through studies on rodent models. However, because of apparent species differences in nonshivering thermogenesis, it is unclear whether the knowledge derived from the studies using rodent models can be directly extrapolated to humans. For example, systemic β-adrenergic stimulation of thermogenesis is accompanied by BAT activity in rodents (8), but not in humans (9).

Since human BAT localizes diffusely in areas of the neck, perithyroid, and supraclavicles (10,11), studies using primary brown adipocytes are scarce, probably because of the technical difficulty in obtaining such cells for research. Recently, human brown or brown-like adipocytes derived from pluripotent stem cells through overexpression of browning factors (12) and differentiation (13) have
been developed and will be an excellent cell model for differentiation studies. However, brown or brown-like adipocytes derived from somatic cells and the knowledge derived from the somatic cell reprogramming may be more applicable to human obesity treatment in the near future. To date, Activation of the PPARγ signaling is perhaps the most reproducible regimen to brown murine and human adipocytes in vitro (14-17), but there are few reports about whether clinical doses of thiazolidinediones will actually result in meaningful adipocyte browning in humans. Thus, researchers are seeking more effective means to brown human adipocytes. Recently, Hirai et al. reported (18,19) that the addition of the transactivation domain of MyoD (M3) to a transcription factor can drastically enhance its capacity to reprogram fibroblasts into induced pluripotent stem cells (iPSCs) or cardiomyocytes. Aiming to address the questions of (1) whether brown-like adipocytes can be derived from direct somatic cell conversion and (2) whether the enhancement or super-activation of PPARγ signaling can promote a deeper browning, we engineered a fusion protein of M3-PPARγ and demonstrated that it has an enhanced transcriptional activity and can effectively convert myoblasts into brown-like adipocytes.

**Methods**

**Molecular cloning**

The M3 DNA fragment was obtained by PCR amplification on the template of M3O (fusion protein of M3 with Oct4) (20), and cloned into a Gateway pEntr vector (Invitrogen, Carlsbad, CA) to generate pEntr-M3. pEntr M3-PPARγ was constructed by cloning of human PPARγ1 into pEntr-M3 at the 3′-end in frame by Infusion (Clontech, Mountain View, CA). To make the lentiviral destination vector pSMPUW-CMV-DEST, a fragment of the CMV promoter-ccdB was cloned into the universal lentiviral vector pSMPUW (Cell BioLabs, San Diego, CA). Standard Gateway LR cloning protocol was utilized to generate pLenti-M3-PPARγ, PPARγ and GFP by using LR Clonase II reaction (Invitrogen). cDNA inserts of all clones were verified without mutation by restriction enzyme digestion and DNA sequence analysis.

**Reporter assays**

Human HEK293 cells were cultured in 6-well plates in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Luciferase reporter assays were performed by transfecting 1 μg of PPRE3x-tk-Luc reporter construct, 1 μg of pSMPUW-M3-PPARγ, pSMPUW-GFP control, or 2 ng of pCMV- Renilla (Promega) into HEK293 cells using LipoD293 (SigmaGen Laboratories, Rockville, MD). The cells were cultured in the presence or absence of rosiglitazone (1 μM) for 48 h after transfection and lysed for luciferase and renilla (for correction) activity assay by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

**Production of lentivirus**

HEK293T cells were used for lentivirus production. To produce lentiviruses, 1.2 μg of the transfer vector (lenti-M3-PPARγ, lenti-PPARγ, or lenti-GFP), 1.2 μg of pCD/α-BH*DD (Addgene plasmid 17531) and 0.2 μg of pVSVG (Cell Biolabs) were co-transfected into HEK293T cells using LipoD293 reagent (SigmaGen) in a 6-well plate. LipoD293/DNA complex-containing medium was removed and replaced with fresh medium (DMEM/F12 supplemented with 10% FBS and 100 U/ml penicillin-streptomycin) 16 h post-transfection. Cell medium containing the viral particles was collected twice at 24 and 48 h after the medium change, passed through a 0.22 μm filter, and either used fresh or stored at 4°C for up to one week for cell infection.

**Human studies**

The human study protocols were approved by the institutional review board of the University of Maryland. All subjects provided informed consent. To isolate human myoblasts, a percutaneous muscle biopsy was obtained from the lateral portion of the vastus lateralis. The tissue samples were cut into small pieces (~10 mg), washed in Hanks buffer without calcium or magnesium and digested with a mixture of collagenase IV (1 mg/mL, Sigma-Aldrich, St Louis, MO) and trypsin (Gibco, 0.025%) in PBS for 30 min at 37°C. Tissue pieces and cells were centrifuged for 5 min at 170 x g, resuspended in complete SkBM medium with Singlequots supplements containing rhEGF, fetuin, BSA GA-1000, insulin and hydrocortisone (Lonzka, Walkersville, MD), 100 U/mL penicillin-streptomycin and 10% FBS and plated on collagen-coated plates. Myoblasts were allowed to grow out of the tissue pieces and were passaged in the complete SkBM medium minus insulin and hydrocortisone. Human adipose stromal vascular cells (AdSVCs) were isolated as described before (21,22). Human BAT were obtained from dissection of the perithyroid adipose tissues during surgery for obesity-related diseases.

**Cell culture and differentiation**

Human myoblasts were grown in the complete SkBM medium minus insulin and hydrocortisone until 90–100% confluence in 12-well plates and were infected by exposure to lentiviral supernatant (~4 MOI) and polybrene (8 μg/mL, Millipore, Billerica, MA) daily for two days. After the second lentiviral infection, human myoblasts or AdSVCs underwent adipogenesis according to the nonmodified adipogenesis protocol (23). Briefly, the cells were cultured in the adipocyte induction medium DMEM/F12 (Gibco) containing d-Biotin (33 nM, Sigma), human insulin (70 nM, Sigma I2778), dexamethasone (100 nM, APP pharmaceuticals, Schauburg, IL), pantothenate (4 μg/mL, Sigma), human transferrin (10 μg/mL, Calbiochem), 3,3′, 5-triiodo-l-thyronine sodium salt (2 nM, Sigma), rosiglitazone (1 μM, Enzo Lifesciences, Farmingdale, NY), isobutyl-methylxanthine (IBMX, 0.5 mM, Sigma), 10% FBS, 100μ/mL penicillin-streptomycin. After 72 h, cells were switched to the adipocyte differentiation medium (induction medium without rosiglitazone and IBMX) for additional 8 days or until collection.

**RNA extraction, cDNA synthesis, and RT-qPCR**

Total RNAs were extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) with on-column DNase digestion (Qiagen). cDNAs were synthesized using AMV Reverse Transcriptase kit (Promega) from 1μg of total RNA. Quantitative PCR was performed on Light Cycler 480 (Roche, Indianapolis, IN) using primers listed in Supporting Information Table S1. β-actin was used as a reference gene.

**Western blot**

Cultured cells were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 6.8) and 2% SDS. The protein concentration was
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MitoTracker staining and quantification
Cells were incubated with 100 nM MitoTracker Red CMXRos (Invitrogen), which contains a mildly thiol-reactive chloromethyl moiety for labeling mitochondria, for 30 min at 37°C at about 100% confluency and then washed twice with PBS before imaging. Confocal imaging was performed using a Zeiss LSM510 microscope (Carl Zeiss MicroImaging). Ten cells of each group were randomly picked for quantification of the red fluorescence signal by ImageJ software.

Statistics
qPCR and Western analyses were conducted in cells from three to four subjects. Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA). Data are expressed as mean ± SEM. Group comparisons were determined using ANOVA or two-tailed Student’s t test, and P < 0.05 was considered significant.

Results
Fusion of M3 to PPARγ enhances PPARγ signaling
Fusion of the M3 transacting domain to transcription factors Oct4 and Mef2 enhances their cellular reprogramming activities (19,20). We speculated that M3 may enhance the transcriptional activity of PPARγ, and engineered a synthetic protein of M3-PPARγ by fusing M3 to the N-terminus of PPARγ (Figure 1a). To evaluate the transcriptional activity of M3-PPARγ, we examined the luciferase activities of a promoter reporter (PPRE 3x-Luc) which contains three peroxisomal proliferator responsive elements (PPRE) by transient transfection assays in HEK293 cells. As shown in Figure 1b, basal promoter activity with the control vector GFP was low in the cells but increased by 17-fold after transfection of PPARγ, which was further augmented by about 1-fold in the presence of the PPARγ ligand rosiglitazone. Impressively, M3-PPARγ increased the promoter activity by 82.6- and 4.8-fold, respectively, when compared to GFP control and PPARγ. The M3-PPARγ remained responsive to rosiglitazone by increasing the luciferase activity by 177%. These results indicate that M3-PPARγ is constitutively super-active in activating the PPRE reporter and remains responsive to the PPARγ ligand.

M3-PPARγ directly converts myoblasts into brown-like adipocytes
We then generated lentiviruses expressing M3-PPARγ, PPARγ, and GFP. The myoblasts transduction rate was nearly 100% after two rounds of the viral infection at an MOI of 4. We confirmed the protein expression of PPARγ and M3-PPARγ at the expected size (Figure 2a) in myoblasts collected 48 h post-infection. Next, we tested human primary myoblasts derived from five individual muscle biopsies for adipogenesis in response to the adipogenic cocktail containing insulin, dexamethasone, and IBMX. Myoblasts transduced with GFP remained fibroblast-like during the entire adipogenic process. In contrast, cells transduced with PPARγ and M3-PPARγ in the presence or absence of rosiglitazone (1 μM), and assayed for luciferase activities. Data are expressed as mean ± SEM (n = 3). ** P < 0.01.

We next conducted qPCR to selectively measure the expression of representational adipocyte and myocyte genes at differentiation day 11 in comparison to the in vitro differentiated white adipocytes and BAT. Fatty acid binding protein 4 (FABP4), an adiposity marker, was barely expressed in GFP-expressing myoblasts, but was induced by ~30,000-fold and 128,000-fold in PPARγ- and M3-PPARγ-reprogrammed cells, respectively. The expression pattern of another adiposity gene CIDEC or FSP27 was similar to that of FABP4.
Leptin was induced by 1.5- and 18.6-fold in PPARγ and M3-PPARγ-transduced cells, respectively, versus the GFP control (Figure 4a). Interestingly, the adipogenic transcription factor C/EBPβ was induced by 4- and 9-fold in PPARγ and M3-PPARγ cells whereas PPARγ transcripts were less induced in M3-PPARγ (132-fold) than PPARγ-transduced cells (387-fold). For selective brown adipocyte genes (24), compared to the GFP control, PPARγ-overexpression induced brown fat-selective genes including CIDEA (46-fold), PRDM16 (2.8-fold) and UCP1 (47-fold). Remarkably, further induction was observed in M3-PPARγ cells for genes of CIDEA (2.4-fold), PRDM16 (5-fold) and UCP1 (1,057-fold) over PPARγ cells (Figure 4b). Finally, we measured myocyte-specific genes Myosin (25) and MyoD (26) and found their expressions were significantly reduced in M3-PPARγ cells by ~0.6-fold ($P < 0.05$ for both), but
were not significantly reduced in PPARγ cells (Figure 4c), compared to the GFP control. In reference to the white adipocytes differentiated from AdSVCs [i.e., induced white adipocytes (iWA)] and the perithyroid BAT, the expressions of most white adipocyte and adiposity markers in PPARγ and M3-PPARγ-induced adipocytes were higher than iWA except leptin. On the other hand, the expression of brown adipocyte markers UCP1, PRDM16 and CIDEA in the M3-PPARγ-reprogrammed adipocytes was significantly higher than that in iWA and PPARγ-induced adipocytes. As for UCP1 and PRDM16, their expression levels were close to the perithyroid BAT. These data showed that the M3-PPARγ-reprogrammed adipocytes were expressing high levels of brown adipocyte genes.
UCP1 is considered to be both the molecular and functional marker of browning. We then conducted Western blot analysis to determine UCP1 protein expression. As shown in Figure 5, UCP1 protein was not detectable in GFP cells, but the protein band became visible in the PPARγ-expressing cells. Strikingly, the protein was increased by 47-fold in M3-PPARγ cells versus PPARγ cells (P < 0.01).

Brown adipocytes are rich in mitochondria and the increase in mitochondria content is considered a typical sign of adipocyte browning. We then conducted MitoTracker fluorescence analysis by confocal microscopy in the cells transduced with GFP, PPARγ, or M3-PPARγ. As depicted in Figure 6, the GFP myoblasts remained a myoblast morphology and stained strongly for mitochondria, whereas the staining in the PPARγ-induced adipocytes was reduced by ~71%. The fluorescence intensity was increased by 3-fold in the M3-PPARγ group than the PPARγ group (P < 0.01). This result indicated that M3-PPARγ induced mitobiogenesis.

Discussion
In this study, we show that human myoblasts can be converted into brown-like adipocytes through cellular reprogramming by super-activation of PPARγ signaling. Since the functionalities of the reprogrammed adipocytes have yet to be characterized in more detail, we call our reprogramming-derived, high UCP1-expressing cells brown-like adipocytes. Although M3-PPARγ appeared to induce brown adipocyte gene expression, especially UCP1 to a great extent, it induced white adipose genes, such as leptin, as well. Whether M3-PPARγ selectively promotes browning is not definite at this stage, so we define the M3-PPARγ-mediated, general enhanced PPARγ signaling above the degree of activation by ligand on wild-type PPARγ as “super-activation.” Although brown or brown-like adipocytes have been obtained from human pluripotent stem cells through over-expression of browning factors (12) and differentiation (13), this is the first report, to our knowledge, demonstrating that human brown-like adipocytes can be derived through cellular reprogramming of nonpluripotent somatic cells.

Since the breakthrough discovery that adult skin fibroblasts can be reprogrammed into iPSCs (27), the great plasticity of cell conversion from one type to another has been well recognized. Moreover, cell lineage conversion occurs amenably between developmentally related types of cells because of a lower epigenetic barrier (28-30). Since brown adipocytes share common precursor cells with skeletal myocytes (31-33), we reasoned that human myoblasts would be a type of somatic cells well-suited for the reprogramming study to derive brown-like adipocytes. Although many transcription factors, hormones, and growth factors (34-37) are reported to induce browning in murine model systems, only a few have shown browning activities in human cells. Exceptionally, activation of PPARγ

**Figure 6** Induction of mitobiogenesis by M3-PPARγ. (A) Representative image of MitoTracker (red) staining of human myoblasts transduced with GFP, PPARγ, and M3-PPARγ 11 days after treatment with adipose differentiation cocktail. (B) Quantitative fluorescence signal of MitoTracker staining. Data are presented as mean ± SEM; ** P < 0.01 versus PPARγ (n = 10). Scale bar: 10 μm for all images. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
signaling reproducibly induces UCP1 expression in human adipocytes. We thus speculated that super-activation of PPARγ would promote human adipocyte browning and engineered a M3-PPARγ fusion protein, which indeed activated the reporter gene activity by 3-fold more than the wild-type PPARγ (Figure 1). Although both PPARγ and M3-PPARγ induced adipogenesis, the super-active PPARγ drastically stimulated UCP1 gene expression by ~1,050-fold and protein expression by ~47-fold versus the PPARγ group, and induced significant mitobiogenesis. Moreover, using in vitro differentiated white adipocytes and perithyroid BAT as references, we showed that the M3-PPARγ-reprogrammed cells express brown adipocyte markers at levels much closer to BAT than the white adipocytes do. Collectively, we demonstrate the browning characteristics of the M3-PPARγ-reprogrammed adipocytes at both the molecular and cellular levels.

Although the browning approach taken in the study is artificial, our success in reprogramming human myoblasts into brown-like adipocytes has several implications. Firstly, we demonstrate the feasibility of deriving brown-like cells through cellular reprogramming of somatic cells, providing a new cell model for browning research. Secondly, our study shows that PPARγ super-activation can significantly promote browning, revealing that human adipocytes have a great capacity for browning. Although PPARγ activation by thiazolidinediones can cause browning of adipocytes in vitro, significant adipocyte browning at clinical doses of the medication has not been noticed in human subjects. Thus, a super-activation of PPARγ may be needed for browning in humans. Whether an enhancement of the PPARγ signaling pathway would promote further browning was unknown before. Fusion of VP16, a viral transacting domain, to PPARγ (38) can enhance PPARγ’s transcriptional activity; however, whether it would induce adipocyte browning has not been studied. In this study, we chose the M3 domain based on the following considerations: (1) the M3 domain would more likely maintain its transacting activity in myoblasts where MyoD is expressed natively and (2) the M3 domain appears to possess stronger transacting activity than VP16 for cellular reprogramming (19). The mechanism by which M3-fusion increases PPARγ signaling is not clear. Whether the addition of M3 domain nonspecifically increases PPARγ signaling or preferentially activates browning genes is an important question with regard to the PPARγ-mediated browning. Presumably, the addition of M3 to the N-terminus of PPARγ may enable PPARγ to recruit more or new co-activators to target genes or to facilitate the chromatin accessibility to co-transcription factors (19). Further investigation may lead to new strategies to selectively activate browning genes.

In summary, we have derived human brown-like adipocytes through cellular reprogramming of somatic cells, providing a new cell model to study adipocyte browning and opening up a new avenue towards the derivation of brown adipocytes.

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