HHS Public Access

Author manuscript

Stem Cell Res. Author manuscript; available in PMC 2020 July 23.

Published in final edited form as:

Stem Cell Res. 2018 July ; 30: 12–21. doi:10.1016/j.scr.2018.04.007.

Derivation and characterization of a UCP1 reporter human ES cell line

Suranjit Mukherjee[a,b], **Tuo Zhang**[c], **Lauretta A. Lacko**[a], **Lei Tan**[a], **Jenny Zhaoying Xiang**[c], **Jason M. Butler**[a,d,e], **Shuibing Chen**[a,*]

[a]Department of Surgery, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA

[b]Program of Pharmacology, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA

[c]Genomic Core, Weill Cornell Medical College, New York, NY, USA

[d]Department of Medicine, Weill Cornell Medical College, New York, NY, USA

[e]Ansary Stem Cell Institute, Weill Cornell Medical College, New York, NY, USA

Abstract

Interest in human brown fat as a novel therapeutic target to tackle the growing obesity and diabetes epidemic has increased dramatically in recent years. While much insight into brown fat biology has been gained from murine cell lines and models, few resources are available to study human brown fat in vitro, which makes the need for new ways to derive and study human brown adipocytes imperative. Human ES cell based reporter systems present an excellent tool to identify, mark, and purify cell populations of choice. In this study, we detail the derivation and characterization of a novel human ES UCP1 reporter cell line that marks UCP1 positive adipocytes in vitro. We targeted a mCherry reporter to the UCP1 stop codon via CRISPR-Cas9 based gene targeting. The brown adipocytes derived from reporter cells express UCP1, display high mitochondrial content, multi-locular lipid morphology, and exhibit functional properties such as lipolysis. The mCherry positive cells purified after cell sorting show elevated expression of brown fat marker genes and a high similarity to isolated human brown fat via RNA-seq analysis. Finally, we demonstrate the utility of this reporter to real time monitor UCP1 expression upon stimulation. This reporter cell line thus presents new opportunities to study human brown fat biology by enabling future work to understand early human brown fat development, perform disease modeling, and facilitate drug screening.

*Corresponding author. shc2034@med.cornell.edu (S. Chen).

Author's contributions

S.M. and S.C. designed the project. L.L. helped with fluorescence imaging. T.Z. and J.Z.X. performed bioinformatics analysis. L.T. helped with molecular cloning. J.M.B helped with oxygen consumption assessment. S.M. and S.C. prepared the manuscript.

Disclosure of potential conflicts of interest

None.

1. Introduction

The growing obesity and diabetes epidemic around the world has made it clear that gaining deeper insights into human adipocyte biology is imperative. One area of interest in the field is the exploitation of the metabolic properties of brown fat, a recently re-identified type of adipose tissue that is present in adult humans (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). Several studies have gained insights into the development, thermogenic activation, and metabolically favorable properties of brown fat using murine in vitro and in vivo models (Boström et al., 2012; Cao et al., 2004; Seale et al., 2011; Seale et al., 2008; Tseng et al., 2008). Unfortunately, few resources exist to study human brown fat in vitro. Recently, clonal isolation and immortalization of pre-adipocytes from supraclavicular human neck fat has revealed new human brown fat selective genes important to thermogenic function, as well as new cell surface markers indicative of thermogenic potential (Shinoda et al., 2015; Xue et al., 2015). However, these isolated cells are already committed to the pre-adipocyte state, and limit any efforts to study earlier commitment steps in human brown fat specification. In addition, few applications have been reported regarding the use of these pre-adipocytes for gene targeting.

Human embryonic stem (ES) cells present a consistent and reproducible source from which to derive tissue specific cell types that can be used to gain early developmental insights, model human diseases, and perform high throughput drug screening. Although protocols have been published to differentiate human ES cells to brown adipocytes (Ahfeldt et al., 2012; Guénantin et ah, 2017; Mohsen-Kanson et al, 2013; Nishio et al, 2012), these approaches suffer from a variety of reasons, such as the use of exogenous transcription factor expression, purity, and cell yield. Overall, these technical limitations reduce the variety of biological questions and applications that can be realized using human ES derived brown adipocytes.

We hypothesized that the development of a reporter cell line that marks UCP1 positive cells would be beneficial for the study of human brown adipocytes by providing a resource to overcome many of limitations mentioned. Human ES reporter systems have previously been developed and are useful in their ability to identify and quantify cell populations of interest, perform lineage tracing, and enable the purification of cell types of choice (Bu et al., 2009; Schwach et al., 2017; Sluch et al., 2015; Wu et al., 2016; Xia et al., 2017). We chose UCP1 as the reporter gene given that the presence of the UCP1 mitochondrial protein is the distinguishing feature between brown/beige and white adipocytes (Cannon and Nedergaard, 2004). In this technical report, we detail the derivation and characterization of a *UCP1: mCherry* human ES reporter line and highlight the opportunities that will now be present with the use and application of such a reporter system.

2. Materials and methods

2.1. sgRNA design and Cas9 vector assembly

To target the human UCP1 stop codon, Cas9 sites were identified using the online CRISPR design tool (crispr.mit.edu). A 90 bp region surrounding the UCP1 stop codon (40 bp before the stop codon, 50 bp after stop codon) was provided as the template. 3 pairs of sgRNAs

were then selected and cloned into a Px330 vector (Addgene #42230) using appropriate overhangs as described previously (Ran et al., 2013).

2.2. Surveyor assay for Cas9 constructs

The assembled Cas9 vectors targeting the UCP1 stop codon were transfected into 293 T cells for indel analysis. Briefly, 1×10^6 293 T cells were plated into a well of 6-well plate on day 1. The following day, 4 μg of Cas9 construct were transfected. 72 h after transfection, DNA was harvested and amplified by PCR, followed by DNA hybridization for heteroduplex formation. 10 μL of hybridized DNA was then subjected to digest by 0.2 μL of T7 endonuclease (NEB cat #M0302) and ran on a 2.5% agarose gel to identify the presence of expected bands. The guide sequence with the highest cutting efficiency and closest proximity to the UCP1 stop codon was chosen for targeting (5′ ttttcttgaagctgattatg 3′).

2.3. Generation of reporter construct

The UCP1 reporter plasmid was generated in a step-wise manner using traditional restriction enzyme/cloning methodology. Left and right homology arms flanking the UCP1 stop codon were PCR amplified using the following primers:

Left arm forward: 5′ agatctccacattgggaagtgaagagaaac 3′.

Left arm reverse: 5′ gaattcttatgtggcacagtccatagtctg 3′.

Right arm forward: 5′ ggtacctcagcttcaagaaaatgatgtaac 3′.

Right arm reverse: 5′ tctagatgaatatattaactgacaactgtgg 3′.

Left and right homology arms were then cloned into the pGEM-T vector plasmids (Promega A1360) and sequenced for integrity following transformation. The right homology arm was then cloned into a gene targeting compatible vector (Addgene #22733) using sequential BglII and KPN1 digests (NEB) together with a short 51 bp linker containing a Mlu*I* restriction site to facilitate the cloning of the loxp-pgk-puro-loxp selection cassette.

Following sequential digests with BglII and KPN1 of the backbone vector containing the linker and right homology arm, the left homology arm was double digested with BglII and ECOR1 and ligated together in a single step with an IRES-puro-p2A-mCherry reporter cassette that was developed in-house. This construct was then transformed and validated with bi-directional sequencing to confirm integrity of the construct.

To incorporate the loxp-pgk-puro-loxp selection cassette, primers were designed with Mlu*I* overhangs to amplify the selection cassette from Addgene vector #31938. The primer sequences used to amplify the cassette were: Forward (5′ acgcgtgtaagaattccgatcatatt 3′) Reverse (5′ acgcgtgaacctcttcgagggaccta 3). After T/A cloning and transformation of the PCR product, the plasmid and donor backbone were digested with MluI (NEB) and ligated together followed by bidirectional sequencing to confirm the presence of the selection cassette.

2.4. Generation of reporter human ES cell clones

Knock-in of the UCP1 reporter construct was performed on H1 hES cells cultured on matrigel (Corning, #47743–715) in mTESRl media (Stemcell Technologies). Briefly, 90% confluent cells were digested into single cells with accutase. 1 million cells were then centrifuged and resuspended with 4 μg of the CRISPR construct and 2 μg of reporter plasmid in nucleofection media per manufacturer's instructions (Lonza cat # VPH-5022). Cells were then electroporated using an Amaxa Nucleofector II device using program A-23. Cells were then plated onto matrigel coated plates in mTESRl media with 10 μM ROCK inhibitor. 48 h after electroporation, cells were changed to mTESRl media containing 0.5 μg/mL puromycin for 48 h with daily media changes. Cells were then split to single cell clones on matrigel coated 96 well plates in 10% C02. After 48 h, single cell clones were switched to 0.5 μg/mL puromycin in mTESRl media for 48 h after which point genotyping of clones was performed. 50 individual colonies were picked and screened for reporter integration using the following primer combination: Forward: *5′*aggggctggaggtaggaaaa 3' Reverse: *5′*catgtaaagcatgtgcaccg 3′. This internal/external primer pair amplifies a 1.58 kb PCR product 200 bp upstream of the left homology arm up to the IRES element of the reporter construct, thus indicating a possible integration.

Excision of the loxp-pgk-puro-loxp cassette was facilitated via a second round of electroporation as described above using 2 μg of plasmid vector expressing Cre recombinase (Addgene #pOG231).

2.5. Copy number assay

A custom copy number probe targeting the mCherry sequence of the reporter was generated using the Custom TaqMan Copy Number Assay (ThermoFisher, #4400294). The custom copy number assay was used together with a TaqMan copy number reference assay Rnase P (ThermoFisher, #4403326) and run on a Roche Lightcycler 480 qRT-PCR instrument according to manufacturer's instructions. Data was quantified using the Applied Biosystems Copy Caller software.

2.6. Differentiation of UCP1: mCherry H1 hES cells to brown adipocytes

UCP1: *mCherry* H1 hES cells were differentiated according to a published protocol (Ahfeldt et al., 2012). Briefly, reporter cells were placed into suspension culture to form embryoid bodies for 7 days, after which embryoid bodies were plated onto 0.1% gelatin coated dishes with DMEM supplemented with 10% FBS and 10 μg/mL bFGF to promote mesenchymal cell outgrowth. After reaching 90% confluency, cells were trypsinized and passaged for 4–5 rounds prior to plating for infection with doxycycline inducible PPARγ, CEBPβ, and PRDM16 lentiviral constructs. These cells were placed into adipogenic medium containing Knockout Serum Replacement, dexamethasone, insulin, and rosiglitazone, along with 700ng/mL doxycycline for 14 days. Finally, doxycycline was removed and the cells were allowed to mature for another 6 days or longer as experiments required.

2.7. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min, followed by incubation with blocking buffer (0.3% triton X-100, 5% horse serum in PBS) for 1 h. Primary antibodies were applied at the following dilutions in blocking buffer at 4 degree C overnight: anti-UCPl antibody 1:200 (Sigma, #U6382), anti-mCherry antibody 1:500 (ThermoFisher, #M11217). Alexa-Fluor 488 and 594 secondary antibodies (ThermoFisher) were incubated 1:500 at room temperature. Nuclei were stained with DAPI. BODIPY (Invitrogen, #D3922) was used at 10 μg/mL and incubated for 15 min in PBS. Mitotracker (ThermoFisher #M22426) was used at 100 nM and incubated for 30 min in PBS prior to fixation.

2.8. Oxygen consumption analysis

Oxygen consumption assessment was determined using a Seahorse XFp instrument. *UCP1: mCherry* H1 hES cells were plated onto gelatin coated microplates and differentiated to brown adipocytes. Cells were stimulated with 1.25 μm oligomycin, 0.5 FCCP pm, and 1.5 μm anti-mycin/rotenone. Assay was carried out and quantified according to manufacturer's instructions.

2.9. Glycerol release assay

To perform the glycerol release assay, brown adipocytes derived from *UCP1: mCherry* H1 hES cells were serum starved for 1 h in 1% FBS/DMEM. Cells were incubated in Hank's balanced salt solution with 20 μm isoproterenol or 10 pm forskolin for 1 h. The supernatant was collected and measured for glycerol content using fluorescence detection kit (Abcam, #65337). Glycerol content was normalized to protein concentration measured using a protein assay kit (ThermoFisher, #23227).

2.10. Cell sorting

For isolation of mCherry positive cells for RNA-seq, differentiated cells were digested with 0.25% trypsin for 15 min until cells detached from plates. Cells were then digested with Liberase TM (Sigma, #5401119001) at 0.04 μg/mL in 2% BSA/DPBS on a rocking shaker at 37 degrees C for 1 h. Cell suspension was then filtered through a 70 pm mesh strainer and stained with DAPI prior to cell sorting. Cells were sorted on a BD Influx cell sorter equipped with 488 nm laser as the FSC/ SSC source, a 355 nm laser for excitation of DAPI, and a 561 nm laser for excitation of mCherry. Non-targeted H1 hES cells differentiated into brown adipocytes using the same protocol were used to set up the gate for mCherry negative cells. The rest of the live cells were defined as mCherry positive cells.

2.11. RNA isolation

For the assessment of gene expression at differing days of differentiation, RNA was isolated with the Qiagen RNeasy Kit (#74136) according to manufacturer instructions.

To purify RNA from the sorted cells, cells were sorted directly into 750 μL lysis buffer using the Agilent Absolutely RNA Microprep kit (#400805) and RNA isolated according to kit instructions.

2.12. Gene expression analysis by QPCR

Total RNA was reverse transcribed using the PrimeScript 1st strand cDNA Synthesis Kit (Clontech, #6110A). QPCR was performed in triplicate using Light Cycler 480 SYBR Green Master Mix (Roche, #04887352001). Primers used for analysis are listed below.

Target name	Forward (5′ → 3′)	Reverse (5′ → 3′)
UCP1	tctacgacacggtccagg	gtctgactttcacgacctctg
PGClα	gccaaaccaacaactttatctcttc	cacacttaaggtgcgttcaatagtc
FABP4	tcatgaaaggcgtcacttcc	gcttgctaaatcagggaaaaca
CYC1	cttcgcggggtagtgttgg	ggccagacttcgacgacaa
ELOVL3	atgtagttctgccccacagc	aaggacatgaggccctttt
CIDEA	ggcaggttcacgtgtggata	gaaacacagtgtttggctcaaga
KCNK3	ctacgagcactggaccttctt	cgtaaggatgtagacgaagctga

Expression data was first normalized to GAPDH then normalized to day 0 cells in Fig. 2c and mCherry negative cells in Fig. 3b. Statistical significance was determined using $n = 3$ biological replicates, Student's *t*-test, $p < 0.05$ S.E.M.

2.13. RNA-SEQ

cDNA libraries were generated using TruSeq RNA Sample Preparation (Illumina). Each library was sequenced using single reads in a HiSeq2000/1000 (Illumina).

2.14. Bioinformatics analysis of mCherry positive and negative cells

Gene expression data of cells differentiated from clonally isolated pre-adipocytes from primary human brown and white fat was retrieved from an online database (E-MTAB-2602). Gene expression data of cells differentiated from isolated pre-adipocytes from primary interscapular fat and inguinal fat were retrieved from NCBI (GEO: GSE66686). The expression data was normalized for each gene across different samples by subtracting their mean and then dividing by their s.d. To remove batch effect introduced by experiments, expression data was normalized separately for human brown and white fat samples, and for mCherry positive and negative samples. The top 942 differentially expressed genes between human brown and white fat samples were identified by using DESeq2 with adjusted p-value < 0.05. 836 of those genes mapped to the mouse genome. The heat-map plot was generated using R pheatmap package, red color indicates higher expression, and blue color indicates lower expression. A hierarchical clustering was performed using R hclust with Euclidean distance.

Pathway enrichment analysis on mCherry positive and negative cells was performed using the DAVID function-annotation tool on genes 5 fold enriched in mCherry positive cells versus negative cells.

The Gene Expression Omnibus (GEO) accession number for RNA-seq data reported in this study is GSE109163.

2.15. UCP1 upreguladon in white adipocytes

UCP1: *mCherry* H1 hES cells were directed towards white adipocyte differentiation as detailed (Ahfeldt et al., 2012). White adipocyte cells were incubated with 10 μM forskolin and imaged using a Nikon Biostation CT live imaging system for 36 h. Fluorescence intensity was quantified using ImageJ.

3. Results

3.1. Creation of a UCP1: mCherry H1 hES cell line

To label and isolate UCP1 positive brown adipocytes in vitro, we designed a reporter construct to be knocked-in to the UCP1 genomic locus using CRISPR-Cas9 technology. The donor construct contained an 840 bp left and 460 bp right homology arm, an IRES driven puromycin-P2A-mCherry selection cassette, and a PGK-puromycin selection cassette flanked by loxp sites to assist in drug based selection of ES clones with correctly integrated donor constructs (Fig. 1A). We chose to incorporate both puromycin based selection and mCherry expression elements into the reporter given that mature, differentiated adipocytes are quite difficult to purify and isolate. We hypothesized that one, or both selection elements could assist in the process of isolation and characterization of UCP1 positive adipocytes. To facilitate knock-in of the reporter construct, three pairs of sgRNAs were designed to target near the UCP1 stop codon. Surveyor assays were performed to determine cutting efficiency. sgRNA #3, which provided the highest cutting efficiency, was chosen for gene-targeting experiments (Fig. IB).

The CRISPR Cas9 construct and donor vector were introduced into H1 hES cells via nucleofection. After subsequent puromycin selection and sub-cloning, 50 colonies were selected for further characterization, and 2 clones were identified to have successful integration of the donor vector (Fig. 1C). One clone was chosen for further validation. Using primers external and internal to the donor vector, the clone demonstrated correct and error-free integration of the donor vector into the targeted region of the *UCP1* genomic locus (Fig. 1D). This was further corroborated by sequencing of the entire integrated construct and flanking areas 5′ and 3′ of the donor vector's homology arms (data not shown). To excise the PGK-puro cassette, the hES cell clone was transiently transfected with Cre recombinase. Eight clones demonstrated excision of the cassette. Sub-clone 5, which showed the most robust cell growth, was chosen for further characterization, hereafter referred to as *UCP1: mCherry* H1 hES cell line (Fig. 1E). To determine the homozygous or heterozygous status of *UCP1: mCherry* H1 hES cell line, PCR was performed using *5′* and 3′ primers external to the region of integrated reporter in *UCP1* gene. The lack of the wild type UCP1 band in *UCP1: mCherry* H1 hES cell line indicated that both alleles were targeted (Fig. 1F, left panel). The homozygosity of the reporter line was further validated using a copy number assay (Fig. 1F, right panel).

3.2. Directed differentiation of UCP1: mCherry H1 hES cells

To determine the ability of *UCP1: mCherry* H1 hES cells to label brown adipocytes in culture, the reporter line was differentiated into brown adipocytes using a published protocol (Ahfeldt et al., 2012). Briefly, *UCP1: mCherry* H1 hES cells were differentiated

using embryoid body suspension culture, followed by monolayer culture for expansion of mesenchymal progenitor cells (MPCs). After infection with doxycy-cline inducible PPARγ, PRDM16, and CEBPβ lentiviral constructs, MPCs were maintained in adipogenic medium containing doxycycline for 14 days, followed by an additional 6 days culture in the absence of doxycycline (Fig. 2A). During the induced differentiation, mCherry positive adipocytes were detected around day 6, and increased dramatically between days 14 and 20 (Fig. 2B). qRT-PCR analysis was used to monitor the expression of brown adipocyte marker genes, *UCP1* and *PGCla,* along with the general adipocyte marker, *FABP4* (Fig. 2C). The expression levels of *FABP4* peaked around day 6, correlating with the presence of increased lipid droplets in the immature adipocytes (Fig. 2B). More importantly, levels of *PGCla* and *UCP1* peaked at day 20, corroborating the observation of increased mCherry positive adipocytes from day 20 onwards.

Immunofluorescence staining using antibodies against brown adipocyte markers was performed to further characterize the cellular identity of mCherry positive cells. First, staining using UCP1 antibody demonstrated robust co-expression with mCherry and UCP1, confirming proper activity of the mCherry reporter system (Fig. 2D). Mi-totracker staining demonstrated high mitochondrial content in the differentiated cells compared to MPCs, further confirming brown adipocyte identity (Fig. 2E). In addition, BODIPY staining exhibited lipid droplet presence throughout the culture of hES cell-derived brown adipocytes, but not in undifferentiated MPCs. More importantly, multiple small lipid droplets were found in each mCherry positive cell, which is a morphological feature characteristic of differentiated brown adipocytes (Fig. 2F).

Functionally, brown adipocytes contain a dense mitochondrial content to support thermogenic activity. Oxygen consumption assessment demonstrated elevated maximal respiration compared to baseline, indicative of enhanced mitochondrial capacity (Fig. 2G). Additionally, brown adipocytes catabolize lipids to fuel the thermogenic pathway (Townsend and Tseng, 2014). To determine the ability of *UCP1: mCherry* H1 hES cell derived brown adipocytes to break down lipids, we performed a glycerol release assay. After a 1 h stimulation with 20 μM isoproterenol, a p-adrenergic agonist, or 10 μM forskolin, a cAMP activator, a significantly higher level of glycerol release was observed in differentiated brown adipocytes versus unstimulated control cells (Fig. 2H).

3.3. Purified mCherry positive cells express brown adipocyte transcript signature

Reporter systems are advantageous in that they can be used to isolate cell populations of interest without contamination of other cell types in heterogeneous cell cultures. To investigate the gene expression profile of mCherry positive cells, differentiated cells were separated into mCherry positive (35.3%) and negative cells (64.7%) using FACS (Fig. 3A). qRT-PCR analysis confirmed the significant upregulation of key brown adipocyte genes in the mCherry positive cells, including *UCP1, PGCla, CIDEA, CYC1,* and *ELOVL3* (Fig. 3B). In addition, *KCNK3,* a gene encoding a potassium channel protein that recently identified as a marker highly enriched in UCP1 positive human adipocytes (Shinoda et al., 2015), was highly expressed in mCherry positive cells.

RNA-seq analysis was performed to analyze the global gene expression profile of mCherry positive cells. Previously reported RNA-seq data from human brown and white fat (ArrayExpress E-MTAB-26032) and mouse interscapular and inguinal white fat (GEO GSE66686) were used as references. Clustering analysis validated that mCherry positive cells closely resemble human and mouse brown fat, while mCherry negative cells resemble human and mouse white fat (Fig. 3C). A heat map using 836 differentially expressed genes ($p < .05$) between human brown and white fat also demonstrated that a majority of genes upregulated in human brown fat are highly expressed in mCherry positive cells, while most of the genes that are upregulated in human white fat are highly expressed in mCherry negative cells (Fig. 3D). Additionally, mCherry negative cells preferentially express white preadipocyte and adipocyte selective genes such as *TCF21, SPHK1, DPT, Serpina3,* and *EDNRA* (Kajimura et al., 2008; Sharp et al., 2012; Timmons et al., 2007) (Fig. 3E).

In order to better understand the function behind the differentially expressed gene profiles between mCherry positive and negative cells, we performed pathway analysis of genes that were five-fold enriched in mCherry positive cells. Gene ontology analysis revealed that genes enriched in mCherry positive cells were related in mitochondrial activity, glucose responsiveness, cAMP response, and lipid metabolism. KEGG analysis also revealed enrichment of pathways involved in adi-pocytokine signaling, fatty acid metabolism, regulation of lipolysis, and PPAR signaling in mCherry positive cells (Fig. 3F). Together, these results suggest that the *UCP1: mCherry* H1 hES cell line is capable of identifying cells that closely resemble human brown fat and are enriched for biological processes characteristic of brown adipocytes.

3.4. Real time monitoring of UCP1 upregulation in white adipocytes using the UCP1: mCherry H1 hES cell line

The development of a platform to identify compounds that promote UCP1 upregulation and browning in human white adipocytes would greatly benefit the drug discovery process. One application of our reporter line is the real-time monitoring of *UCP1* expression. As a proof of concept, MPCs derived from *UCP1: mCherry* H1 hES cells were converted to white adipocytes by infecting with dox-inducible PPARγ and differentiating for 14 days in adipogenic medium with doxycycline, followed by an additional 6 days in culture with doxycycline-free adipogenic medium (Ahfeldt et al., 2012). The derived white adipocytes were monitored for their response to cAMP agonist stimulation. As shown in Fig. 4, the addition of 10 μM forskolin upregulates *UCP1* in white adipocytes as indicated by the increased mCherry expression over the course of 36 h, with a plateau in expression by 24 h. The increased detection of mCherry signal with forskolin addition validates the ability of *UCP1: mCherry* H1 hES cells to real-time monitor the upregulation of UCP1, which can be applied in cell screening studies to identify further compounds that promote UCP1 upregulation.

4. Discussion

The growing interest in human brown fat biology has made apparent the need for better tools and cell lines to study the genetic pathways and factors that govern the thermogenic process

in human brown adipocytes. The development of reporter cell lines using human ES cells provides not only a tool to track and monitor expression changes in a gene of interest, but to do it in a relevant human cell type. In this report, we have detailed the derivation and characterization of the first human ES UCP1 reporter cell line.

As demonstrated, the *UCP1: mCherry* H1 hES cell line faithfully tracks changes in UCP1 expression throughout the course of the brown adipocyte differentiation period. The derived mCherry positive cells expressed morphological and functional features characteristic of brown adipocytes and importantly, demonstrated co-expression with UCP1.

An additional advantage with the reporter system is the ability to purify and isolate mature, lipid-filled adipocytes. The isolation and purification of mature fat cells is notoriously challenging. However, with the presence of *UCP1: mCherry* reporter, it is now possible to isolate pure populations of UCP1 positive brown adipocytes from heterogeneous cultures. The purified mCherry positive cells exhibited elevated expression of brown fat markers, such as *PGC1 α, CIDEA,* and *ELOVL3.* RNA-seq further demonstrated that mCherry positive cells more closely resemble isolated human brown fat. However, we observed some overlap in gene expression pattern between white adipocytes and our mCherry positive cells. This could possibly be due to the slightly stochastic nature of the lentiviral mediated differentiation protocol, where a fraction of MPC cells could receive a greater ratio of PPARγ vector over CEPBβ and/or PRDM16 during the infection process, leading to a more white adipocyte-like gene signature. A similar rationale could also explain why we observed mCherry negative cells exhibiting some similarity to brown adipocyte gene expression, despite clustering more closely with white adipocytes. Overall, these results emphasize the need for better, step-wise differentiation protocols that utilize small molecules and/or growth factors to differentiate brown adipocytes from human ES cells.

Lastly, the *UCP1: mCherry* H1 cells open the doors for future screening applications that seek to find novel small molecules that promote the browning of human white fat. The proof-of-principle study using the known UCP1 activator, forskolin, has demonstrated the capacity of the mCherry reporter to track, in real-time, UCP1 expression based on the mCherry signal. With this reporter platform, we anticipate further screening efforts using mCherry expression as a readout to identify small molecules that promote UCP1 upregulation, with further cellular characterization and mechanistic analysis of hit compounds to determine whether a stable remodeling of white adipocytes to brown adipocytes has taken place. Furthermore, with the ability to monitor UCP1 expression in real time, additional insight can be gained regarding a compound's potential activity, helping to discern whether an increase in UCP1 expression is transitory, or if a more permanent remodeling to a brown fat state has taken place in a white adipocyte.

5. Conclusions

In summary, we have demonstrated the derivation and characterization of a UCP1: mCherry H1 hES cell reporter line that marks human brown adipocytes in vitro. The ability of the mCherry reporter to track UCP1 expression lends this cell line to future applications in the study of human brown fat biology, such as improving differentiation protocols, gaining

greater understanding of early human brown fat development, and performing disease modeling for diabetes and obesity.

Acknowledgements

We would like to thank Chad Cowan (Harvard Stem Cell Institute) for providing lentiviral constructs for brown adipocyte differentiation. We'd also like to thank Dr. Michael Poulos for his helpful insights on RNA isolation from sorted cells.

Funding

This study was funded through the following grants: 1 DP3DK111907–01 (NIH), 1R01NS099270 – 01 (NIH), C029156 (NYSTEM). Research reported in this publication was also supported by the Office of the Director of the National Institutes of Health under Award Number S10OD019986 to Hospital for Special Surgery.

References

Ahfeldt T, Schinzel RT, Lee Y-K, Hendrickson D, Kaplan A, Lum DH, Camahort R, Xia F, Shay J, Rhee EP, Clish CB, Deo RC, Shen T, Lau FH, Cowley A, Mowrer G, Al-Siddiqi H, Nahrendorf M, Musunuru K, Gerszten RE, Rinn JL, Cowan C. a, 2012. Programming human pluripotent stem cells into white and brown adipocytes. Nat. Cell Biol 14, 209–219. 10.1038/ncb2411. [PubMed: 22246346]

Boström P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach K. a, Bostrom EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Hpjlund K, Gygi SP, Spiegelman BM, 2012. A PGCl-a-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481, 463–468. 10.1038/naturel0777. [PubMed: 22237023]

Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, Roberts DJ, Huang PL, Domian IJ, Chien KR, 2009. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460, 113–117. 10.1038/nature08191. [PubMed: 19571884]

Cannon B, Nedergaard J, 2004. Brown adipose tissue: function and physiological significance. Physiol. Rev 84, 277–359. 10.1152/physrev.00015. 2003 [PubMed: 14715917]

Cao W, Daniel KW, Robidoux J, Medvedev AV, Bai X, Lisa M, Spiegelman BM, Collins S, Puigserver P, Floering LM, 2004. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol. Cell. Biol 24, 3057–3067. [PubMed: 15024092]

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng Y-H, Doria A, Kolodny GM, Kahn CR, 2009. Identification and importance of brown adipose tissue in adult humans. N. Engl. J. Med 360, 1509–1517. 10.1056/NEJMoa0810780. [PubMed: 19357406]

Guénantin A-C, Briand N, Capel E, Dumont F, Morichon R, Provost C, Stillitano F, Jeziorowska D, Siffroi J-P, Hajjar RJ, Feve B, Hulot J-S, Collas P, Capeau J, Vigouroux C, 2017. Functional human beige adipocytes from induced pluripotent stem cells. Diabetes 66, dbl61107. 10.2337/dbl6-1107.

Kajimura S, Seale P, Tomaru T, Erdjument-Bromage H, Cooper MP, Ruas JL, Chin S, Tempst P, Lazar MA, Spiegelman BM, 2008. Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. Genes Dev. 22, 1397–1409. 10.1101/gad.1666108. [PubMed: 18483224]

van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts J.M. a F.L., Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJJ, 2009. Cold-activated brown adipose tissue in healthy men. N. Engl. J. Med 360,1500–1508. [PubMed: 19357405]

Mohsen-Kanson T, Hafner A-L, Wdziekonski B, Takashima Y, Villageois P, Carriere A, Svensson M, Bagnis C, Chignon-Sicard B, Svensson P-A, Casteilla L, Smith A, Dani C, 2013. Differentiation of human induced pluripotent stem cells into brown and white adipocytes: role of Pax3. Stem Cells 6, 1459–1467. 10.1002/stem.1607.

Nishio M, Yoneshiro T, Nakahara M, Suzuki S, Saeki KK, Hasegawa M, Kawai Y, Akutsu H, Umezawa A, Yasuda K, Tobe K, Yuo A, Kubota K, Saito M, 2012. Production of functional classical brown adipocytes from human pluripotent stem cells using specific hemopoietin cocktail

without gene transfer. Cell Metab. 16, 394–406. http://dx.doi.Org/10.1016/j.cmet.2012.08.001. [PubMed: 22958922]

Ran FA, Hsu PD, Wright J, Agarwala V, Scott D. a, Zhang F, 2013. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc 8, 2281–2308. 10.1038/nprot.2013.143. [PubMed: 24157548]

Schwach V, Verkerk AO, Mol M, Monshouwer-Kloots JJ, Devalla HD, Orlova VV, Anastassiadis K, Mummery CL, Davis RP, Passier R, 2017. A COUP-TFII human embryonic stem cell reporter line to identify and select atrial cardiomyocytes. Stem Cell Rep. 9, 1–15. http://dx.doi.Org/10.1016/j.stemcr.2017.10.024.

Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki M. a, Beier DR, Spiegelman BM, 2008. PRDM16 controls a brown fat/skeletal muscle switch. Nature 454, 961–967. 10.1038/nature07182. [PubMed: 18719582]

Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, Cohen P, Cinti S, Spiegelman BM, 2011. Prdml6 determines the thermogenic program of subcutaneous white adipose tissue in mice. J. Clin. Invest 121, 53–56. http://dx.doi.org/ 10.1172/JCI44271.96.

Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, Hu H, Wang L, Pavlova Z, Gilsanz V, Kajimura S, 2012. Human BAT possesses molecular signatures that resemble beige/brite cells. PLoS One 7 (e49452). [PubMed: 23166672]

Shinoda K, Luijten IHN, Hasegawa Y, Hong H, Sonne SB, Kim M, Xue R, Chondronikola M, Cypess AM, Tseng Y-H, Nedergaard J, Sidossis LS, Kajimura S, 2015. Genetic and functional characterization of clonally derived adult human brown adipocytes. Nat. Med 1–8. 10.1038/nm.3819. [PubMed: 25569538]

Sluch VM, Davis CO, Ranganathan V, Kerr JM, Krick K, Martin R, Berlinicke CA, Marsh-Armstrong N, Diamond JS, Mao H-Q, Zack DJ, 2015. Differentiation of human ESCs to retinal ganglion cells using a CRISPR engineered reporter cell line. Sci. Rep 5, 16595. 10.1038/srepl6595. [PubMed: 26563826]

Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, Nedergaard J, Cannon B, 2007. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proc. Natl. Acad. Sci 104, 4401–4406. 10.1073/pnas.0610615104. [PubMed: 17360536]

Townsend KL, Tseng Y-H, 2014. Brown fat fuel utilization and thermogenesis. Trends Endocrinol. Metab 1–10. http://dx.doi.Org/10.1016/j.tem.2013.12.004.

Tseng Y-H, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, Tran TT, Suzuki R, Espinoza DO, Yamamoto Y, Ahrens MJ, Dudley AT, Norris AW, Kulkarni RN, Kahn CR, 2008. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Nature 454, 1000–1004. http://dx.doi. org/10.1038/na ture07221.

Virtanen KA, Taittonen M, Ph D, Laine J, Savisto N, 2009. Functional brown adipose tissue in healthy adults. N. Engl. J. Med 360, 1518–1525. [PubMed: 19357407]

Wu J, Hunt SD, Xue H, Liu Y, Darabi R, 2016. Generation and validation of PAX7 reporter lines from human iPS cells using CRISPR/Cas9 technology. Stem Cell Res. 16, 220–228. http://dx.doi.Org/10.1016/j.scr.2016.01.003. [PubMed: 26826926]

Xia N, Fang F, Zhang P, Cui J, Tep-Cullison C, Hamerley T, Lee HJ, Palmer T, Bothner B, Lee JH, Pera RR, 2017. A Knockin reporter allows purification and characterization of mDA neurons from heterogeneous populations. Cell Rep. 18, 2533–2546. http://dx.doi.Org/10.1016/j.celrep.2017.02.023. [PubMed: 28273465]

Xue R, Lynes MD, Dreyfuss JM, Shamsi F, Schulz TJ, Zhang H, Huang TL, Townsend KL, Li Y, Takahashi H, Weiner LS, White AP, Lynes MS, Rubin LL, Goodyear LJ, Cypess AM, Tseng Y-H, 2015. Clonal analyses and gene profiling identify genetic biomarkers of the thermogenic potential of human brown and white preadipocytes. Nat. Med 21, 760–768. 10.1038/nm.3881. [PubMed: 26076036]

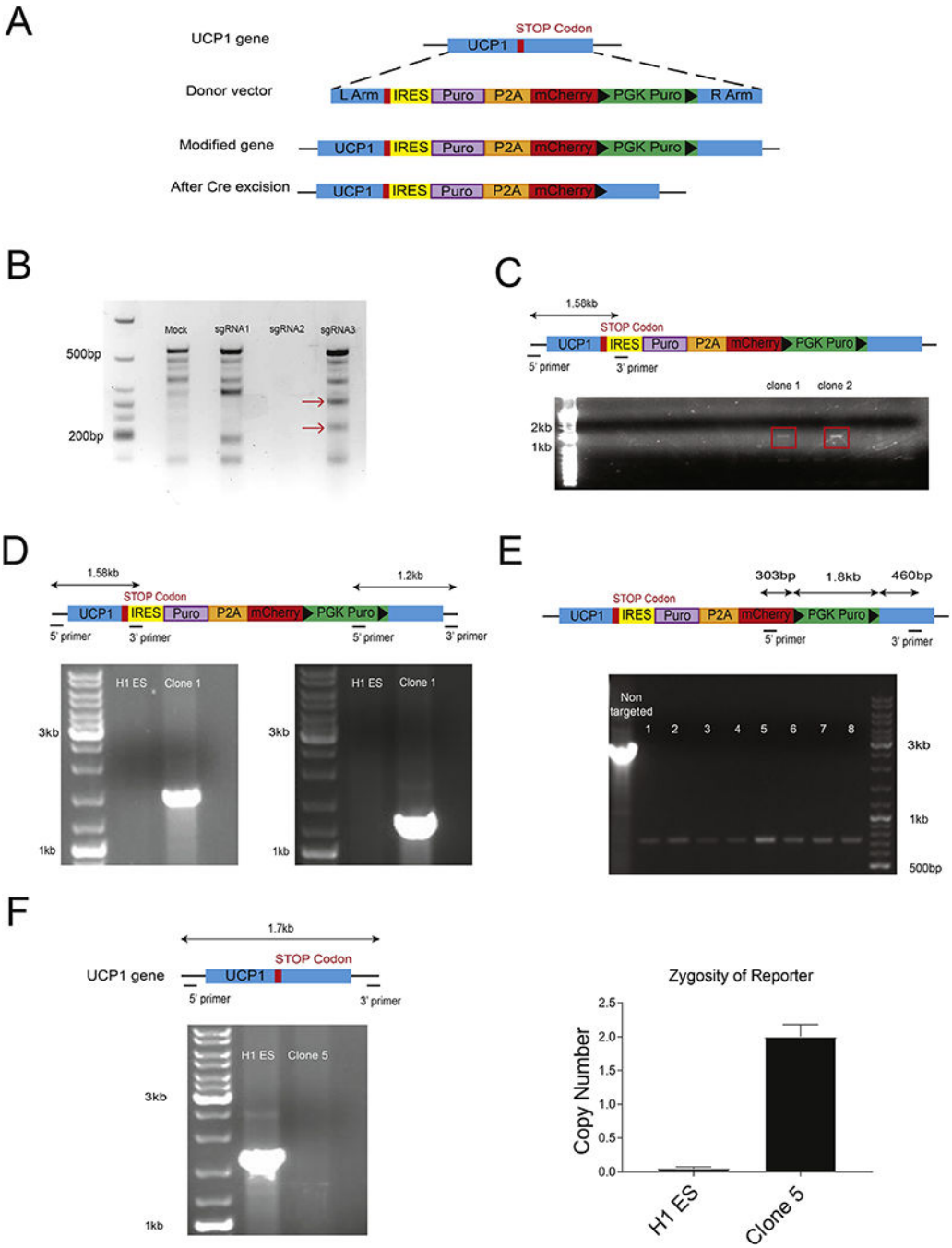

Fig. 1.

UCP1: mCherry H1 hES cell line design and integration confirmation.

(A) Gene targeting strategy and reporter design.

(B) Surveyor nuclease assay of three guide RNAs targeting the *UCP1* stop codon. Red arrows indicate expected bands.

(C) Identification of targeted ES clones after puromycin selection. PCR primers external and internal to the reporter respectively were used to detect integration. 2 clones were identified, with clone 1 selected for further study.

(D) DNA gel demonstrating integration of reporter at *UCP1* stop codon in clone 1. External and internal primers were designed for 5' and 3' ends of reporter. Left gel indicates integration of 5' side, with the right gel indicating integration on the 3' end. Non-targeted HI hES cells were used as a negative control.
(E) Cre mediated excision of loxp-pgk-puro cassette from clone 1. 8 sub-clones were identified indicating removal of selection cassette. Sub-clone 5 was selected for further study.
(F) Sub-clone 5 is homozygous for the reporter on both alleles. *(Left panel)* Gel demonstrates lack of the wild type band in sub-clone 5 vs wild type H1 hES cells using primers flanking the UCP1 stop codon. *(Right panel)* Copy number assay confirms homozygous status of sub-clone 5 vs wild type H1 hES cells.

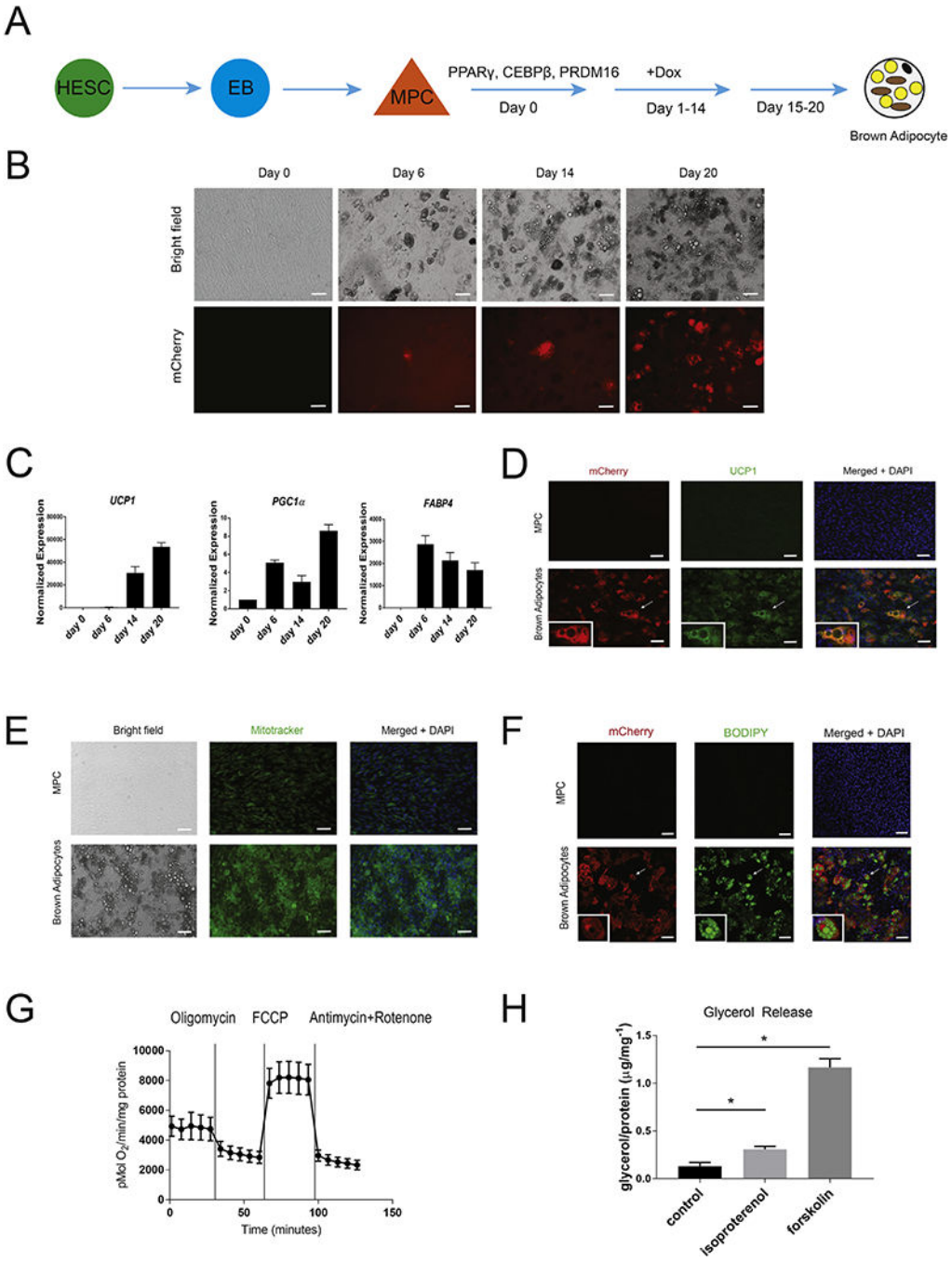

Fig. 2.

Differentiation protocol and characterization of mCherry positive cells.

(A) Scheme of the stepwise differentiation strategy to differentiate *UCP1: mCherry* H1 hES cells to brown adipocytes.

(B) Fluorescence imaging of the differentiation of *UCP1: mCherry* H1 hES cells at days 0, 6, 14 and 20 of differentiation. Scale bar = 50 μm.

(C) qRT-PCR analysis of differentiating reporter line cultures. Expression was normalized to GAPDH (n = 3 biological replicates, Student's t-test, *p < 0.05, data represented as mean ± S.E.M).

(D) Immunostaining of *UCP1: mCherry* H1 hES cell-derived cells using antibodies against mCherry (red) and UCP1 (green). Differentiated mCherry positive cells show co-expression of UCP1. Arrow refers to cell in inset image. Scale bar = 50 μm.

(E) Mitotracker staining (green) of *UCP1: mCherry* H1 hES cell-derived cells. Scale bar = 50 μm.

(F) Immunostaining of *UCP1: mCherry* H1 hES cell-derived cells using antibody against mCherry (red) and BODIPY against lipid droplets (green). Arrow refers to cell in inset image. Scale bar = 100 μm.

(G) Oxygen consumption assay on reporter line derived brown adipocytes.

(H) Glycerol release assay of *UCP1: mCherry* H1 hES cell-derived cells. Cells were incubated with either 20 pm isoproterenol or 10 pm forskolin for 1 h (n = 3 biological replicates, Student's t-test, *p < 0.05, data represented as mean ± S.E.M).

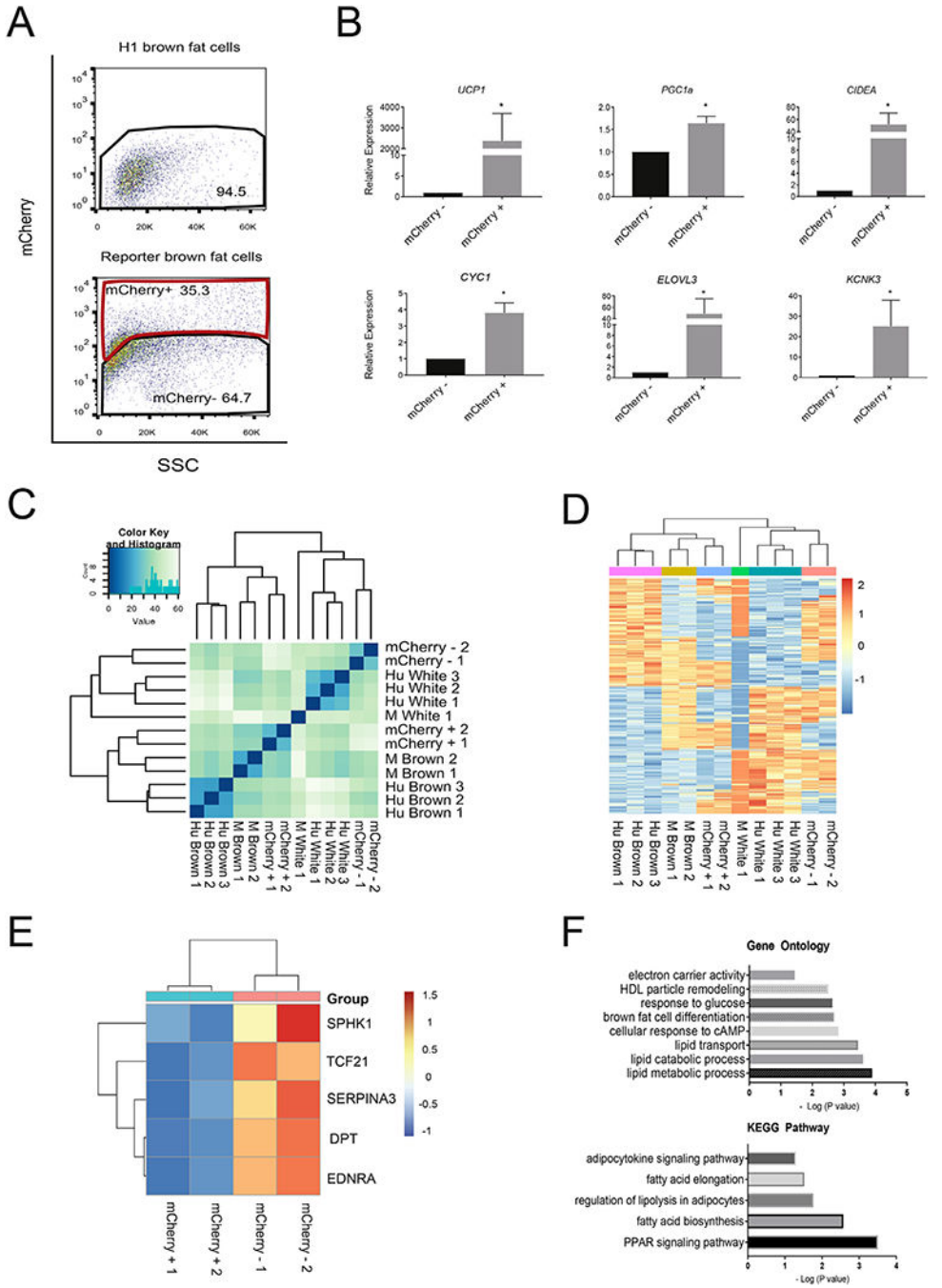

Fig. 3.

Gene expression profiling of purified mCherry positive cells.

(A) Representative flow plots for FACS analysis of *UCP1: mCherry* H1 hES cell derived brown adipocytes. Wild-type hES cell derived brown adipocytes were used as the negative control to set up the gate.

(B) qRT-PCR of brown adipocyte marker genes *UCP1, PGCla., CIDEA, CYC1, ELOVL3,* and *KCNK3* in mCherry positive versus negative cells, (n = 3 biological replicates, Student's *t*-test, *p < 0.05, data represented as mean ± S.E.M).

(C) Heat map showing sample-to-sample distances. Distance between two samples was calculated by comparing the normalized expression data with Euclidean distances.
(D) Heat map showing differentially expressed genes of human brown and white fat. Selected genes included all differentially expressed protein coding genes with adjusted p-value < 0.05.
(E) Heat map showing expression levels of white adipocyte-selective genes in mCherry positive versus negative cells.
(F) Gene Ontology and KEGG pathway analysis of genes 5-fold upregulated in mCherry positive versus negative cells.

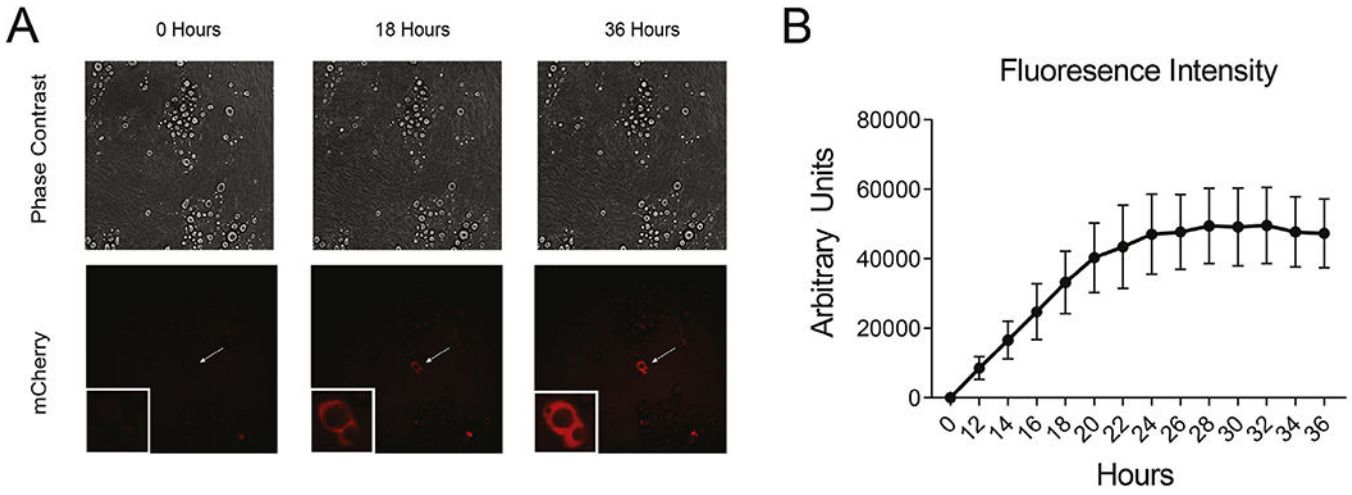

Fig. 4.

Real-time monitoring of UCP1 upregulation in *UCP1: mCherry* H1 hES cell-derived white adipocytes.

(A) Representative phase contrast and fluorescence images of *UCP1: mCherry* H1 hES cell-derived white adipocytes after stimulation with 10 μM forskolin. Time lapse photos at 0, 18, and 36 h after forskolin addition. Arrow refers to cell in inset image.

(B) Quantification of fluorescence intensity of mCherry positive cells after 10 μm forskolin stimulation over 36 h.