

Conditionally immortalized brown preadipocytes can switch between proliferative and differentiated states

Jia Liu^{a,e,1}, Eline N. Kuipers^{b,c,1}, Hetty C.M. Sips^{b,c}, Jennifa C. Dorleijn^{b,c}, Andrea D. van Dam^d, Constantinos Christodoulides^d, Fredrik Karpe^d, Guangqian Zhou^e, Mariëtte R. Boon^{b,c}, Patrick C.N. Rensen^{b,c}, Antoine A.F. de Vries^{a,*,2}, Sander Kooijman^{b,c,d,*,2}

^a Laboratory of Experimental Cardiology, Department of Cardiology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, the Netherlands

^b Department of Medicine, Division of Endocrinology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, the Netherlands

^c Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, the Netherlands

^d Oxford Centre for Diabetes, Endocrinology and Metabolism, Radcliffe Department of Medicine, University of Oxford, Oxford OX3 7LE, United Kingdom

^e Department of Medical Cell Biology and Genetics, Guangdong Key Laboratory of Genome Stability & Disease Prevention, Shenzhen Key Laboratory for Anti-ageing and Regenerative Medicine, Health Science Center, Shenzhen University, Nanhai Ave, 3688, Shenzhen, China

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ABSTRACT

Brown adipose tissue (BAT) is a potential target to treat cardiometabolic disorders because of its capacity to combust glucose and fatty acids for thermoregulation. Its cellular and molecular investigation in humans is hampered by the limited availability of cell material and the heterogeneity of BAT between and within individuals. In this study, monoclonal lines of conditionally immortalized brown preadipocytes (iBPAs) of mouse and human origin were generated. Conditional immortalization was achieved by doxycycline-controlled expression of simian virus 40 large tumor antigen (LT) with a repressor-based Tet-On system. In the presence of doxycycline, both the murine and human cell lines showed long-term proliferation capacity with a population doubling time of ~28 h. After switching off LT expression by doxycycline removal and exposure to adipogenic differentiation medium, cells from both species acquired brown adipocyte properties. This was evidenced by the accumulation of multilocular lipid droplets, the upregulation of brown adipocyte markers including uncoupling protein 1 and an increase in lipolysis and oxygen consumption following adrenergic stimulation. Switching off LT expression before the onset of adipogenic differentiation was only critical for inducing adipogenesis in the human iBPAs, while their murine counterparts showed adipogenesis upon exposure to the adipogenic differentiation cocktail regardless of LT expression. When switched to proliferation medium, cultures of adipogenically differentiated human iBPAs de-differentiated and resumed cell division without losing their adipogenic capacity. We suggest that iBPAs represent an easy-to-use model for fundamental and applied research into BAT offering unique experimental opportunities due to their capacity to switch between proliferative and differentiated states.

Abbreviations: BAT, brown adipose tissue; BPA, brown preadipocyte; dox, doxycycline; Fabp4/FABP4, fatty acid binding protein 4; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; iBPA, conditionally immortalized monoclonal BPA; Lpl/LPL, lipoprotein lipase; LT, large tumor antigen; LTR, long terminal repeat; LUMC, Leiden University Medical Center; LV, lentiviral vector; OCR, oxygen consumption rate; PCNA, proliferating cell nuclear antigen; PD, population doubling; pH 3, phosphorylated histone H3; PP2A, protein phosphatase 2A; Pparg/PPARG, peroxisome proliferator-activated receptor γ; Ppargc1α/PPARGC1A, Pparg/PPARG coactivator 1-α; pRB, retinoblastoma protein; Prdm16/PRDM16, PR domain containing 16; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; ST, small tumor antigen; SV40, simian virus 40; T3, triiodothyronine; Ucp1/UCP1, uncoupling protein 1

* Correspondence to: A.A.F. de Vries, Department of Cardiology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, the Netherlands.

** Correspondence to: S. Kooijman, Department of Medicine, Division of Endocrinology, Leiden University Medical Center, Post Zone C7Q, P.O. Box 9600, 2300 RC Leiden, the Netherlands.

E-mail addresses: a.a.f.de_vries@lumc.nl (A.A.F. de Vries), s.kooijman@lumc.nl (S. Kooijman).

¹ Equally contributed as first authors.

² Equally contributed as last authors.

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1. Introduction

Activation of brown adipose tissue (BAT) represents a therapeutic tool to reduce cardiometabolic disease by enhancing energy expenditure with increased clearance of circulating glucose and lipids [1–3]. Cold is the physiological activator of BAT, upon which the sympathetic nerves that innervate BAT release noradrenaline that binds to β -adrenergic receptors on the cell membrane of brown adipocytes [4]. This promotes intracellular lipolysis and transport of liberated fatty acids to the mitochondria for oxidation. This process produces heat in the presence of uncoupling protein 1 (UCP1), which is uniquely expressed by brown/beige adipocytes [5–10]. In order to replenish the intracellular lipid pools, BAT takes up large amounts of triglyceride-derived fatty acids and glucose from the circulation [3].

In recent years, many (pharmacological) approaches have been identified to promote BAT activity, at least in rodents. However, the development of fundamental and applied research into human BAT has stayed behind due to the lack of high-throughput cell culture models. The use of (primary) brown adipocytes, obtained from biopsies, has important constraints: the amount of available cells is limited and there is a large heterogeneity of human BAT not only between subjects but also within one subject depending on the anatomical location from where the biopsy is taken [11]. Moreover, the (abundant) presence of intracellular lipid droplets causes mature adipocytes be very fragile and of low buoyant density. As a consequence, it is challenging to bring mature adipocytes into culture [12]. Furthermore, since mature adipocytes are in large majority post-mitotic, generation of sufficient brown adipocytes for *in vitro* experiments requires proliferation and differentiation of adipocyte precursors.

This has led to the generation of several different murine brown preadipocyte (BPA) lines such as HB2 [13], HIB 1B [14,15] and T37i [16]. HB2 cells are BPAs isolated from the interscapular BAT of p53 knockout mice, while HIB 1B and T37i cells were derived from transgenic mice expressing the simian virus 40 (SV40) large tumor antigen (LT) using constitutively active, lineage-restricted promoters to drive transgene expression. These cell lines have been helpful in increasing our understanding of adipocyte biology. However, the absence of the cellular gatekeeper protein p53 or the constitutive expression of oncoproteins limits their usefulness as model systems to study the properties of BAT. In order to study human brown adipocyte function, Shinoda et al. [17] and Xue et al. [18] generated human BPA lines by transducing the stromal vascular fraction of human supraclavicular adipose tissues with retroviral vectors encoding SV40 LT and hTERT (*i.e.* the catalytic subunit of telomerase), respectively. Following adipogenic differentiation, these cells express *UCP1* and respond to stimulation with noradrenaline and forskolin with an increase in lipolysis and mitochondrial respiration. These human BPA lines have been successfully used to identify BAT-specific genes and to investigate their function. An alternative approach to generate human brown adipocytes is by transdifferentiation of human multipotent adipose-derived stem cell-derived white adipocytes into so-called brown-in-white or “brite” adipocytes by long-term exposure to peroxisome proliferator-activated receptor γ (PPARG) agonists [19–21].

We postulated that generating conditionally immortalized monoclonal BPA (iBPA) lines is the next step towards the development of a high-throughput model for human brown adipocytes. The reasons for this were as follows: 1) Previous constitutive expression of oncoproteins such as SV40 LT to immortalize BPAs may affect their adipogenic differentiation capacity through interactions of LT with pocket proteins (in particular the retinoblastoma protein [pRB]) and with the tumor suppressor protein p53 [22,23]. 2) Proliferation and differentiation are opposing processes that generally do not occur simultaneously. In other words, continuous proliferation pressure due to permanent immortalization may reduce the adipogenic differentiation ability of BPAs. 3) The adipogenic capacity of existing BPA lines has been shown to be rapidly lost with increasing passage numbers [13].

These drawbacks prompted us to generate iBPA lines of both mouse and human origin by using a lentiviral vector (LV) expressing LT in a doxycycline (dox)-dependent manner to control cell proliferation [24,25]. The resulting monoclonal cell lines were characterized in terms of their dox-dependent LT expression level and proliferation capacity and their ability to undergo adipogenic differentiation using multilocular lipid droplet formation and expression of BAT marker genes as read-outs. In addition, the effects of adrenergic stimulation on glycerol secretion, *Ucp1/UCP1* mRNA expression and oxygen consumption of adipogenically differentiated iBPAs were studied. Finally, human iBPAs were used to demonstrate that our conditional immortalization technology allows repeated switching of the cells between proliferative and differentiated states thereby offering unique research opportunities including analysis of the molecular pathways underlying the de-differentiation and cell cycle re-entry of brown adipocytes. It may also allow dox-dependent amplification of the brown fat compartment after iBPA transplantation.

2. Materials and methods

2.1. LV production

LV particles were produced as detailed before [25]. The molecular structure of the shuttle plasmids used for LV production is depicted in Supplementary Fig. S1. The construction of these plasmids is described in the Supplementary Materials and Methods.

2.2. Isolation and culture of mouse and human primary BPAs

Collection of mouse tissue was in accordance with the Institute's Guide for the Care and Use of Laboratory Animals and had received approval from the Animal Experiments Committee of the Leiden University Medical Center (LUMC). Prior to the collection of human BAT, patients scheduled for surgery in the thyroid region gave written informed consent for taking biopsies and the procedure was approved by the Medical Ethical Committee of the Oxford Centre for Diabetes and performed in accordance with the principles of the revised Declaration of Helsinki.

Primary mouse BPAs were isolated from pooled interscapular BAT depots of 5 male, 5-week-old C57Bl/6J mice (Charles River Laboratories International, Wilmington, MA). Human primary BPAs were isolated from biopsies obtained from four patients (Supplementary Table 1). Biopsies of adipose tissue of brown-red color were obtained from an area close to the isthmus region of the thyroid gland. Isolated tissue was minced with scissors, washed once with DMEM/F-12, GlutaMAX supplement (Thermo Fisher Scientific; catalogue number: 10565018) and cells were separated by incubation in the same medium containing 1 mg/mL collagenase type I (Thermo Fisher Scientific, Breda, the Netherlands; catalogue number: 17018029) at 37 °C for 45 min. The resulting cell suspensions were filtered through a 200- μ m pore-sized nylon filter (Sefar, Lochem, the Netherlands; catalogue number: 03-200/39) and the cells were pelleted by low-speed centrifugation. The cell pellets were suspended in red blood cell lysis buffer (Thermo Fisher Scientific; catalogue number: 00433357) and incubated for 5 min at room temperature with occasional shaking. After pelleting, the cells were resuspended in proliferation medium [DMEM/F-12, GlutaMAX supplement containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/mL penicillin and 100 μ g/mL streptomycin (both from Thermo Fisher Scientific)] for *in vitro* culture. Proliferation medium was replaced the following day and then every other day.

2.3. Transduction of primary BPAs and generation of lines of conditionally immortalized mouse and human BPAs

Mouse primary BPAs at population doubling (PD) 2 and human

primary BPAs at approximately PD 4 were transduced with the lentiviral vectors LV.iHsEEF1A1.LT-tsA58 and LV.iHsUBC.LT-tsA58 (see Supplementary Materials and Methods), respectively. The next day, the inoculum was replaced by proliferation medium containing 100 ng/mL dox (Sigma-Aldrich, St. Louis, MO) to induce LT expression. The cells were subsequently given fresh proliferation medium with dox every other day. After 1 week of culture, the transduced cells were trypsinized and plated at a low density of approximately 10–20 cells/cm² to allow formation of single-cell clones. Two to 3 weeks later, individual cell colonies were picked and separately expanded in the presence of dox.

2.4. Culture, differentiation and adrenergic stimulation of mouse and human iBPAs

Mouse and human iBPA clones were cultured in proliferation medium supplemented with 100 ng/mL dox. Proliferation medium was replaced every other day and cells were split 1:8 after reaching ~90% confluence. For differentiation of the mouse iBPAs, confluent monolayers were established in 2% gelatin (Sigma-Aldrich; catalogue number: G9391)-coated wells. After culturing the cells for 2 days in dox-free proliferation medium, they were exposed to proliferation medium containing 5.6 nM bovine insulin (Sigma-Aldrich; catalogue number: I0305000), 126 μ M sodium ascorbate (Sigma-Aldrich; catalogue number: A7631), 1 μ M of the PPARG agonist rosiglitazone (Sigma-Aldrich; catalogue number: R2408) and 10 mM HEPES (adjusted to pH 7.40 with NaOH). This medium was replaced every other day for up to 14 days. For differentiation of the human iBPAs, cells were seeded in 2% gelatin-coated wells. After reaching confluence, the dox-containing proliferation medium was replaced by proliferation medium without dox. Two days later, differentiation was induced by exposure to proliferation medium supplemented with 150 nM human insulin (Sigma-Aldrich; catalogue number: I9278), 1 μ M dexamethasone (Sigma-Aldrich; catalogue number: D2915), 1 μ M rosiglitazone, 1 nM triiodothyronine (T3; Sigma-Aldrich; catalogue number: T6397), 500 μ M 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich; catalogue number: I7018) and 125 μ M indomethacin (Sigma-Aldrich; catalogue number: I7378), which was refreshed after 48 h and replaced on day 4 by proliferation medium supplemented with 150 nM insulin, 1 nM T3 and 1 μ M rosiglitazone. The latter medium was replaced every other day up to day 16. Exposing the murine and human iBPA clones to these differentiation cocktails will be referred to as culturing under adipogenic differentiation conditions.

The various clones were screened for their adipogenic capacity, i.e. their ability to develop multilocular lipid droplets and to express the BAT marker gene *Ucp1/UCP1*. Every other day during the differentiation process of the selected mouse and human iBPAs, pictures were taken to capture cellular morphology using the ZOE fluorescent cell imager (Bio-Rad Laboratories, Hercules, CA) and cells were collected in TriPure Isolation Reagent (Roche Life Science, Almere, the Netherlands) for RNA isolation and gene expression analysis as described below ($n = 4$ cultures per time point and experimental group).

Adipogenically differentiated iBPAs (at differentiation day 14 and 16 for the mouse and human iBPAs, respectively) were stimulated with 1 μ M noradrenaline (Sigma-Aldrich; catalogue number: A7257) or vehicle (1.7 μ M hydrochloric acid) for 8 h. Subsequently, supernatant was collected for colorimetric determination of glycerol production (Instruchemie, Delfzijl, the Netherlands) and cells were lysed in TriPure Isolation Reagent. All data was generated using cells that underwent 40–50 PDs.

2.5. Analysis of cell proliferation

To draw growth curves, primary BPAs and their conditionally immortalized counterparts were subcultured 1:4 and 1:8 on reaching confluence so that each passage corresponded to 2 and 3 PDs, respectively. To assess their proliferation rate and dependence on the activity

of SV40 LT, iBPAs were cultured at low density in medium with or without 100 ng/mL dox. At different days after culture initiation, cells were collected and counted using an Accuri flow cytometer (BD Biosciences, Breda, the Netherlands).

2.6. Western blotting

Western blotting was carried out as detailed before [26] with primary antibodies directed against LT (1:2000; Santa Cruz Biotechnology, Dallas, TX; catalogue number: sc-147), UCP1 (1:2000; Sigma-Aldrich; catalogue number: U6382), proliferating cell nuclear antigen (PCNA; 1:1000; Sigma-Aldrich; catalogue number: P8825), phosphorylated histone H3 (pH 3; 1:2000; Merck Life Science, Amsterdam Zuidoost, the Netherlands; catalogue number: 06-570) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; loading control; 1:100,000; Merck Life Science; catalogue number: MAB374) and goat anti-mouse or goat anti-rabbit IgG secondary antibodies linked to horseradish peroxidase (1:15,000; Santa Cruz Biotechnology). Data was analyzed by Quantity One software (Bio-Rad Laboratories) using the GAPDH signals for normalization purposes.

2.7. RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated using TriPure Isolation Reagent according to the manufacturer's protocol. cDNA synthesis was performed using M-MLV reverse transcriptase (Promega Benelux, Leiden, the Netherlands; catalogue number: M1705), random primers, dNTPs and RNasin ribonuclease inhibitor (all from Promega Benelux) and 1 μ g of input RNA. Real-time qPCR was carried out on a CFX96 Real-time PCR machine (Bio-Rad Laboratories) using GoTaq qPCR master mix (Promega Benelux; catalogue number: A6002). mRNA expression of the mouse iBPAs was normalized to that of the ribosomal protein-encoding reference gene *Rplp0*; mRNA expression of the human iBPAs was normalized to reference gene *LRP10*²⁷. The primer pairs used for mouse and human iBPAs are listed in Supplementary Tables 2 and 3, respectively. The forward and reverse primer of each primers pair were located in different exons except for the primers targeting murine *Pparg* transcripts.

2.8. Oxygen consumption

Oxygen consumption rate (OCR) was measured using the Seahorse XF96 analyzer (Agilent Technologies, Santa Clara, CA). Mouse and human iBPAs were cultured and differentiated in Seahorse cell culture plates as described in the section 'Culture, differentiation and adrenergic stimulation of mouse and human iBPAs'. One hour prior to the oxygen consumption measurement, the differentiation medium was replaced by Seahorse XF Base Medium (Agilent; catalogue number: 102353-100) containing 5 mM glucose (Sigma-Aldrich; catalogue number: G8769), 0.5 mM sodium pyruvate (Thermo Fisher Scientific; catalogue number: 11370-070), 1 \times GlutaMAX Supplement (Thermo Fisher Scientific; catalogue number: 35050061) and 10% FBS. Basal respiration was measured thrice followed by 6 measurements after addition of 1 μ M noradrenaline or vehicle.

OCR values were adjusted for cell input using the CyQUANT Cell Proliferation Assay Kit (Thermo Fischer Scientific; catalogue number: C7026) according to the manufacturer's instruction. The last measurement point before compound addition was used for quantification.

2.9. Statistical analysis

Data are represented as means \pm standard error of the mean (SEM). The effects of adrenergic stimulation on iBPAs were analyzed by Student's *t*-test. GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA) was used for all calculations. Differences at *P* values < 0.05

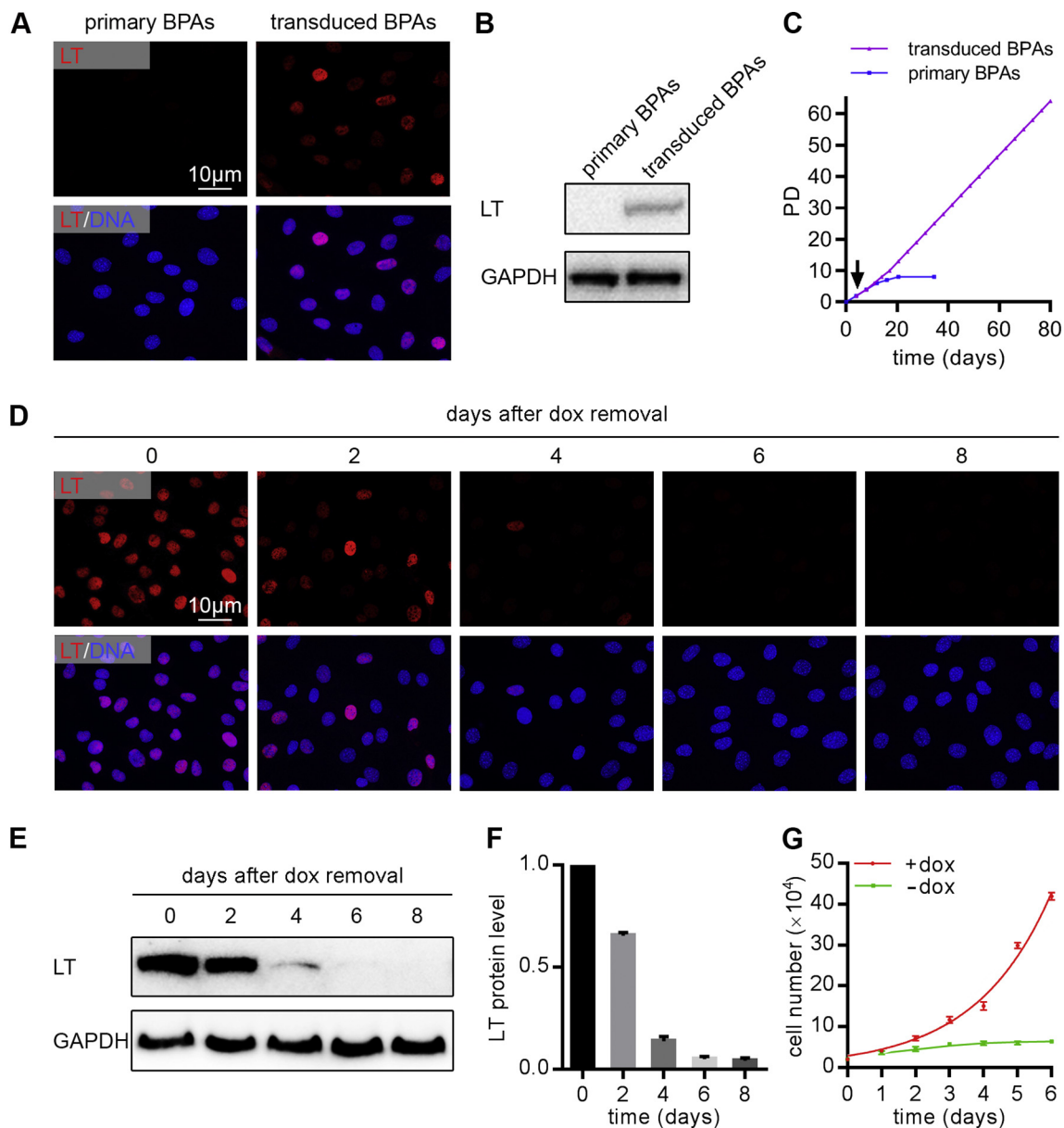


Fig. 1. Conditional immortalization of mouse BPAs. (A, B) Confirmation of LT expression in dox-exposed LV.iHsEEF1A1.LT-tsA58-transduced BPAs (8 PDs) by immunocytochemical staining (A) and Western blotting (B). In both cases, dox-treated primary BPAs that underwent 8 PDs served as negative controls. Cell nuclei were visualized by Hoechst 33342 (blue) and stained for LT (red). (C) Growth curves of primary BPAs and LT-tsA58-transduced BPAs. Since the iBPAs were transduced at PD 2 (arrow) this was the starting point for the analysis of their proliferation rate. Cells were cultured in the presence of dox. (D-F) Analysis by immunocytochemistry (D) and Western blotting ((E, F) of LT expression in clone #6 iBPAs before (day 0) and at 2, 4, 6 and 8 days after dox removal. (G) Quantification of cell numbers in cultures of clone #6 iBPAs exposed for the indicated days to medium with or without dox. BPAs, brown preadipocytes; dox, doxycycline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iBPAs, conditionally immortalized brown preadipocytes; LT, large T; PD, passage doubling.

were considered statistically significant.

3. Results

3.1. Generation of conditionally immortalized mouse BPAs

Mouse primary BPAs were isolated from the pooled interscapular BAT depots of 5 mice and transduced with an LV conferring dox-dependent expression of the SV40 LT (Supplementary Fig. S1). Immunocytochemical staining (Fig. 1A) and Western blotting (Fig. 1B) showed LT expression in the transduced BPAs but not in primary BPAs following the exposure of both cell types to dox. Primary BPAs gradually stopped proliferating after about 8 PDs (Fig. 1C), whereas the transduced LT-expressing BPAs kept proliferating for at least 60 PDs

without showing any noticeable decrease in their proliferation rate (Fig. 1C).

Low-density cultures of the LT-expressing murine BPAs were used for isolating single-cell clones, which were picked and numbered in order of appearance. From over 50 rapidly growing cell clones, clone #6 was selected to further study the long-term dox-dependent proliferation capacity of iBPAs since it was one of the most adipogenic clones (see below). LT expression of clone #6 cells before (day 0) and 2, 4, 6 and 8 days after dox removal was analyzed by immunocytochemical staining and Western blotting. Clone #6 displayed dox-inducible LT expression; LT was highly expressed in the presence of dox, and LT levels gradually declined in its absence, being no longer detectable 6 days after dox removal (Fig. 1D, E and F). Consistent with these findings, iBPAs showed dox-inducible proliferation capacity as

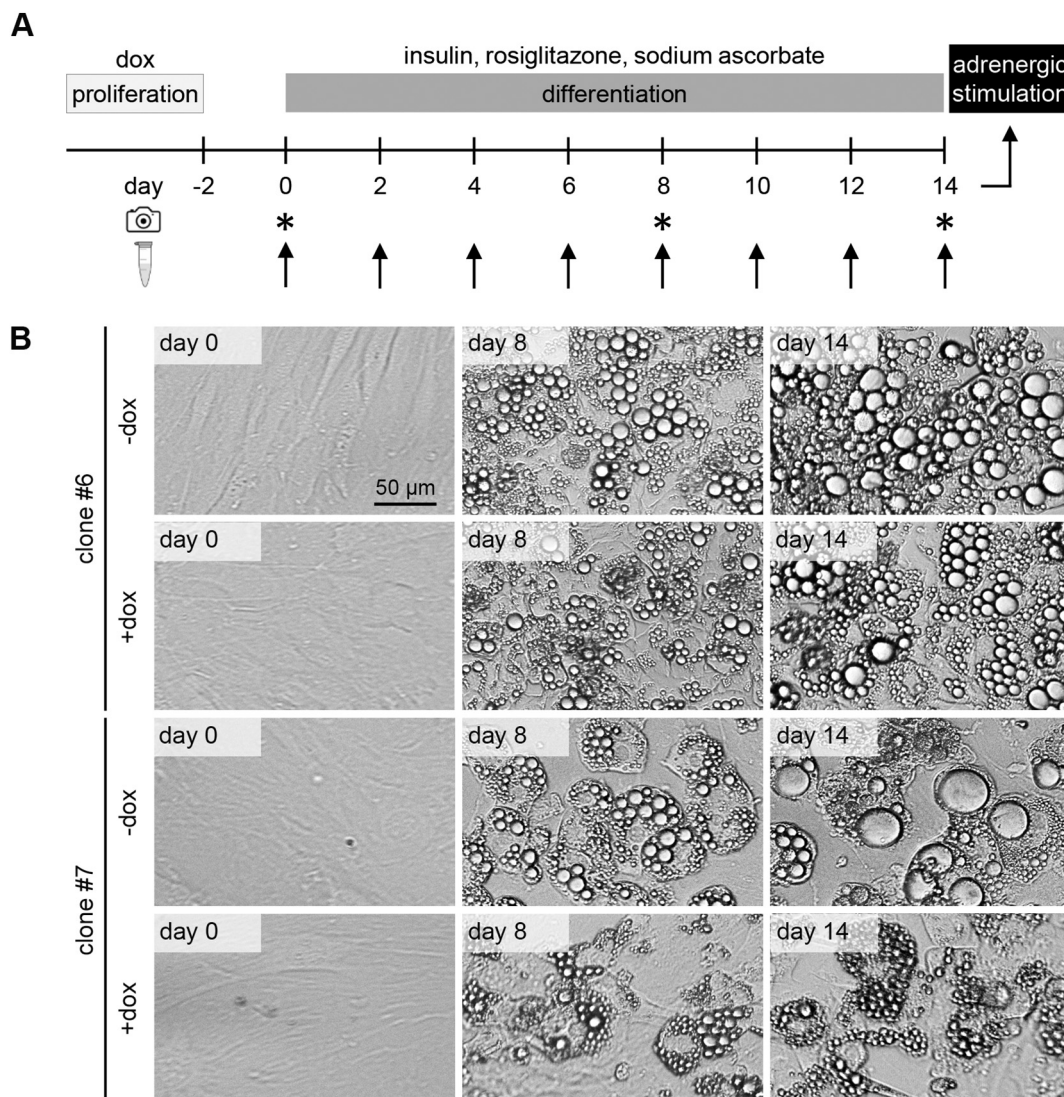


Fig. 2. Adipogenic capacity of mouse iBPAs. (A) Protocol used to differentiate mouse iBPAs into brown adipocytes. Images were taken (*), and RNA and protein samples were collected (†) at the indicated time points. (B) Phase-contrast images showing the morphological changes associated with the adipogenic differentiation of clone #6 and #7 iBPAs in the presence or absence of dox. dox, doxycycline; iBPAs, conditionally immortalized brown preadipocytes.

demonstrated by immunostaining for the cellular proliferation marker Ki-67, which was no longer observed 6 days after dox withdrawal (Supplementary Fig. S2). In the presence of dox, cells proliferated with an average doubling time of ~28 h, whereas in the absence of dox, the cell number slightly increased up to day 4 and then remained constant (Fig. 1G). The small increase in the number of iBPAs during the first 4 days after dox removal is likely due to the slow decline of the LT expression level following transgene silencing. Taken together, the expression of LT enabled BPAs to continuously proliferate and bypass senescence.

3.2. Mouse iBPAs possess adipogenic capacity

In order to assess their adipogenic capacity, cells from each clone were cultured in proliferation medium and when these clonal preadipocyte cell lines reached 100% confluence (day -2) the dox-containing proliferation medium was replaced by dox-free proliferation medium. Two days later (i.e. on day 0) the cells were induced to differentiate to brown adipocytes under adipogenic differentiation conditions for 14 days (Fig. 2A). Cells from clones #6 and #7 exhibited the highest differentiation capacity based on the extent of lipid droplet formation and *Ucp1* expression level (Supplementary Fig. S3). To better

characterize the adipogenic potential of these clones, microscopic images of cell cultures subjected to adipogenic differentiation conditions were taken. The cells of both clones gradually accumulated small lipid droplets acquiring the multilocular phenotype typical for brown adipocytes and after 14 days of adipogenic differentiation most cells were lipid-laden (Fig. 2B and Supplementary Fig. S4). Interestingly, dox did not block the adipogenesis of mouse iBPAs since even in its presence, the cells stopped proliferating and formed lipid droplets when exposed to adipogenic differentiation conditions (Fig. 2B).

Mouse iBPAs were further analyzed by RT-qPCR to assess the expression of brown adipocyte markers during adipogenesis without proliferation pressure (i.e. in absence of dox). Expression of the unique BAT marker *Ucp1* showed a robust induction starting at day 4 of adipogenic differentiation for both clones (Fig. 3A). The *Ucp1* mRNA level peaked at differentiation day 12 for clone #6 and at differentiation day 10 for clone #7 (Fig. 3A). Consistently, the UCP1 protein in clone #6 iBPAs was first detected at day 6 of differentiation and gradually increased afterwards until differentiation day 12 (Supplementary Fig. S5). The genes encoding transcriptional coactivators PR domain containing 16 (*Prdm16*), PPARG coactivator 1- α (*Ppargc1a*), PPARG (*Pparg*), fatty acid binding protein 4 (*Fabp4*) and lipoprotein lipase (*Lpl*) followed similar patterns of expression, i.e. expression of all these markers was

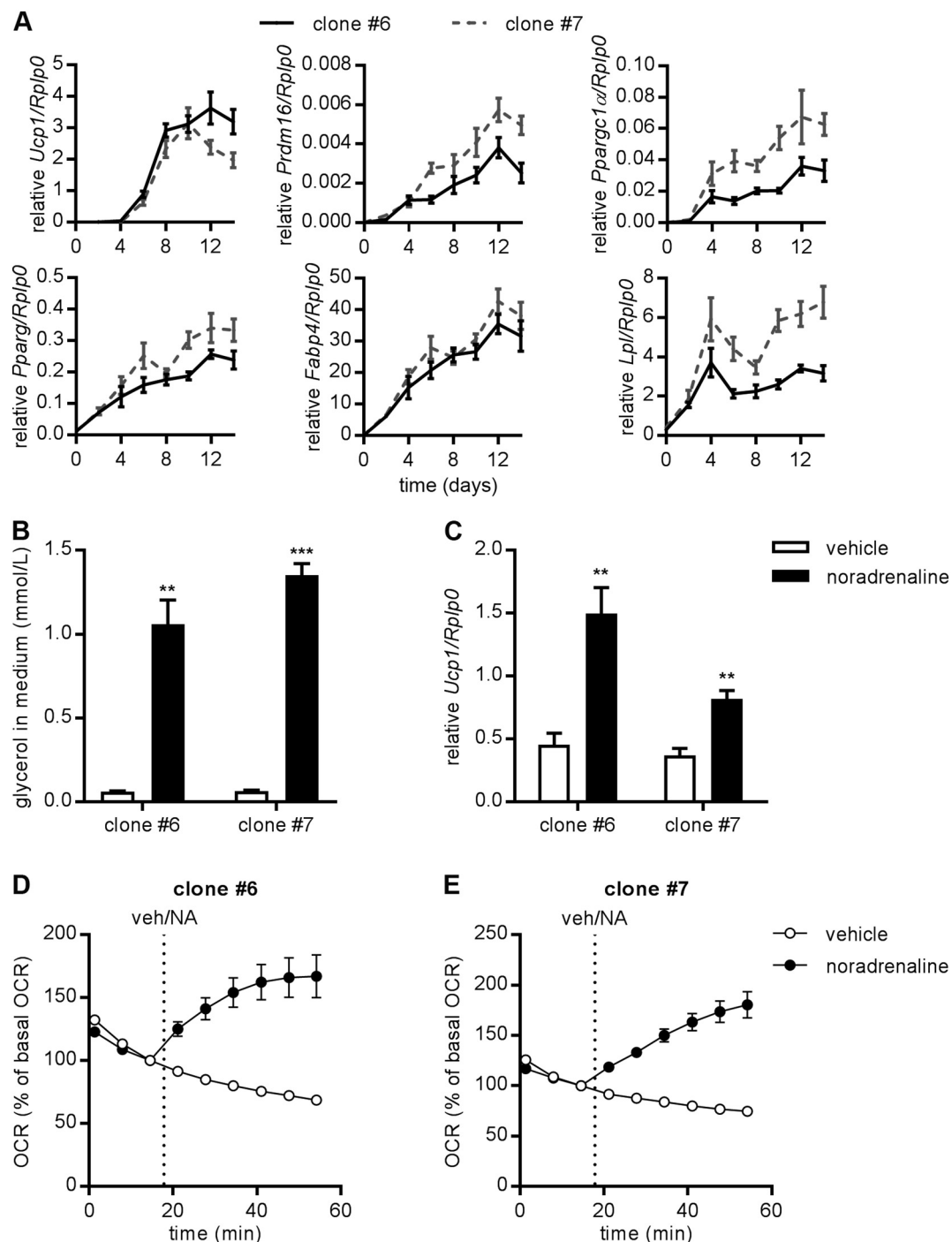


Fig. 3. Genetic profiles and functional properties of adipogenically differentiated mouse iBPAs. (A) RT-qPCR analysis of *Ucp1*, *Prdm16*, *Pparg1α*, *Pparg*, *Fabp4* and *Lpl* gene expression in clone #6 and #7 iBPAs on the indicated days of adipogenic differentiation normalized to *Rplp0* expression. (B–E) Effect of adrenergic stimulation on clone #6 and #7 iBPAs. Both clones were stimulated with 1 μ M noradrenaline for 8 h, at differentiation day 12, after which glycerol concentrations in the culture media (B) and *Ucp1* mRNA levels in the cells (C) were measured. (D, E) OCR in adipogenically differentiated mouse iBPAs after treatment with vehicle or noradrenaline using Seahorse respirometry. Data are means \pm SEM ($n = 4$ in A, B, C, D; $n = 7–12$ in D, E). ** $P < 0.01$; *** $P < 0.001$. iBPAs, conditionally immortalized brown preadipocytes; NA, noradrenaline; OCR, oxygen consumption rate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; veh, vehicle.

induced from the start of adipogenic differentiation and, peaked at differentiation day 12, except for the *Lpl* gene, which showed bimodal expression (Fig. 3A). Based on these data, we conclude that conditionally immortalized murine BPAs possess adipogenic capacity and that optimal differentiation of iBPAs is reached at 12–14 days after initiation of adipogenesis.

3.3. Noradrenaline activates adipogenically differentiated mouse iBPAs

To investigate the response of adipogenically differentiated iBPAs to adrenergic stimulation, the cells were exposed to the non-selective adrenergic agonist noradrenaline [27] upon which glycerol release was quantified and *Ucp1* expression was determined. As expected, the glycerol concentration in the culture medium significantly increased after

8 h of stimulation with noradrenaline (17.6-fold, $P < 0.001$ for clone #6; 21.3-fold, $P < 0.001$ for clone #7) pointing to increased intracellular lipolysis (Fig. 3B). Consistent with the increased lipolysis, *Ucp1* expression also showed a significant increase after noradrenaline stimulation (3.3-fold, $P < 0.001$ for clone #6; 2.2-fold, $P < 0.05$ for clone #7, Fig. 3C). Furthermore, oxygen consumption was measured using Seahorse respirometry before and after the addition of noradrenaline. Clone #6 and #7 appeared to have similar basal OCRs (Supplementary Fig. S6A) and both clones acutely increased their OCR in response to noradrenaline (up to 67 and 78% above the basal respiration rate for clone #6 and clone #7, respectively, Fig. 3D and E). Together, these data demonstrate that adipogenically differentiated mouse iBPAs are responsive to adrenergic stimulation.

3.4. Human iBPAs possess adipogenic capacity only after shutdown of LT expression

Human primary BPAs were isolated from a biopsy taken from the supraclavicular fossa of four patients and monoclonal cell lines of human BPAs were generated in a similar fashion as for the mouse iBPAs (Supplementary Fig. S1). Similar to the results obtained with the mouse iBPAs, LT-tsA58 strongly increased the proliferative capacity of human BPAs, i.e. while human primary BPAs stopped dividing at PD 8, human iBPAs kept on proliferating at the same rate for at least 60 PDs (Supplementary Fig. S7). From patient 1, clones #2 and #3 were selected for further characterization as they performed best in terms of proliferative capacity, lipid droplet formation and *UCP1* gene expression. Confluent monolayers of these clones were exposed to adipogenic differentiation conditions in the absence or presence of dox (Fig. 4A). Consecutive microscopic images taken 2 days apart revealed that cells from both clones gradually accumulated small lipid droplets only in the absence of dox (Fig. 4B). After 16 days of differentiation in the absence of dox, most cells were lipid-laden, while cells cultured in the presence of dox did not show any signs of lipid droplet formation (Fig. 4B).

Since dox negatively impacted on the formation of lipid droplets by the human iBPAs as determined by microscopy, we next analyzed the expression patterns of brown adipocyte markers by RT-qPCR during adipogenic differentiation in the absence and presence of dox (Fig. 4C). The expression of *UCP1* increased starting at day 4 of adipogenic differentiation for both clones cultured without dox, whereas the *UCP1* expression of cells cultured in the presence of dox remained low. *UCP1* expression of clone #3 peaked at day 14 of adipogenic differentiation and at this time point was > 4 times higher than the *UCP1* expression of clone #2. *PRDM16* mRNA levels took an unexpected course being lower at differentiation day 16 than at the start of adipogenic differentiation) except for clone #3. In this clone, *PRDM16* mRNA levels peaked at differentiation day 8 and were very similar at day 1 and day 16 of adipogenic differentiation without dox. When the cells were cultured in the absence of dox, *PPARGC1A*, *FABP4* and *LPL* expression robustly increased between differentiation day 4 and 8, after which *PPARGC1A* expression stabilized at a somewhat lower level. *FABP4* and *LPL* expression further increased after differentiation day 8, peaking around differentiation day 10–12 and day 10–14, respectively. When the cells were cultured in the presence of dox, expression of these three markers did not significantly change/increase during the entire differentiation period. Lastly, *PPARG* expression showed a similar induction during the first 4 days of adipogenic differentiation under all culture conditions. Subsequently, *PPARG* expression of clone #3 cultured without dox further increased and peaked at differentiation day 8. In contrast, *PPARG* expression of clone #2 cultured without dox remained fairly stable while in both clones cultured in the presence of dox *PPARG* expression declined again. Based on these data, we conclude that conditionally immortalized human BPAs are able to adopt a multilocular phenotype and a gene expression pattern compatible with brown adipocytes only in the absence of dox and that optimal differentiation is reached at 14–16 days after initiation of adipogenesis.

3.5. Noradrenaline activates adipogenically differentiated human iBPAs

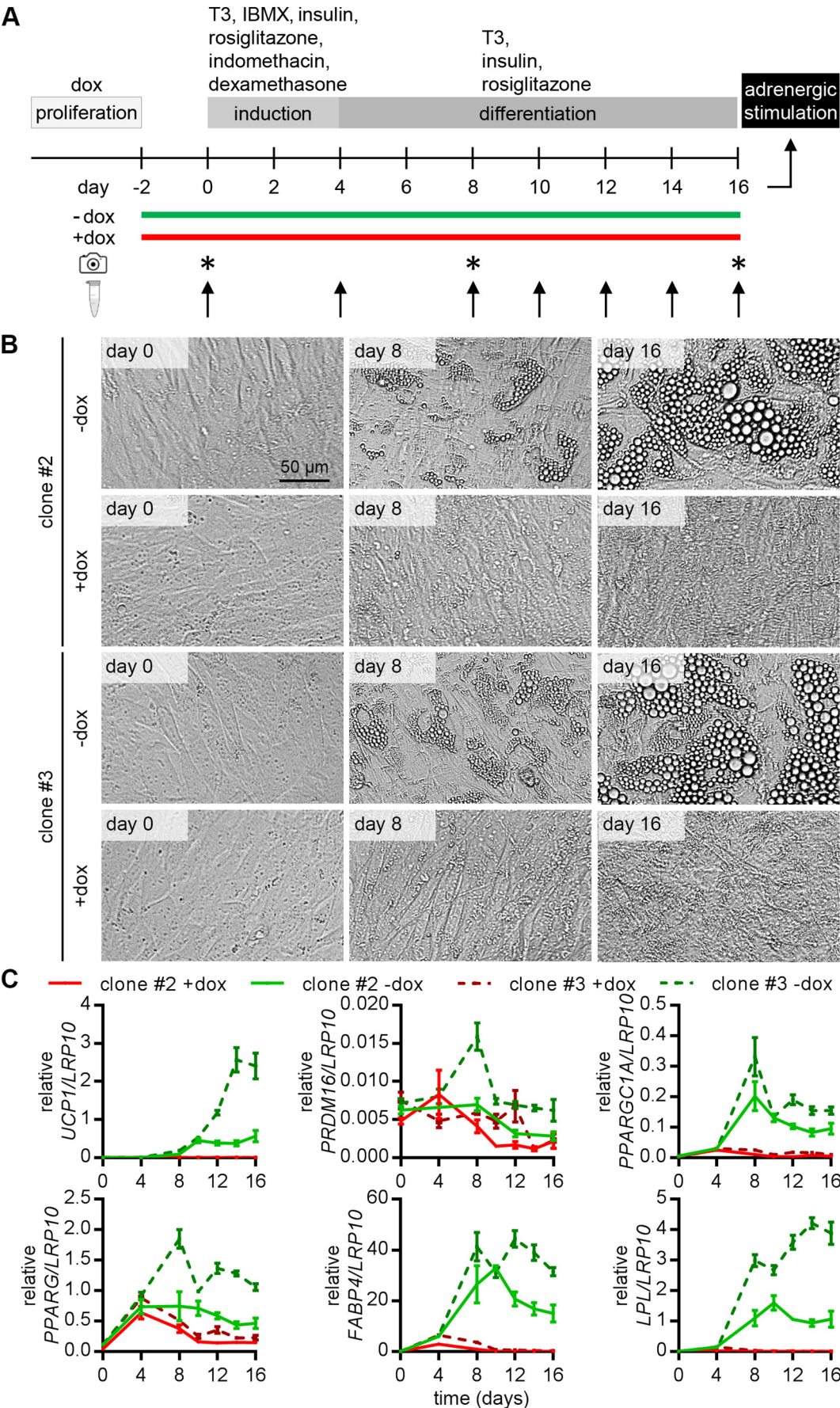
Similar to the mouse iBPAs, we also investigated the response of adipogenically differentiated human iBPAs to adrenergic stimulation. Stimulation of adipogenically differentiated iBPAs with noradrenaline increased the glycerol concentration in the culture medium with 43% ($P < 0.01$, Fig. 5A) and 200% ($P < 0.001$, Fig. 5A) over basal values for clone #2 and clone #3, respectively. Although *UCP1* expression levels were lower in clone #2 compared to clone #3, noradrenaline stimulation increased *UCP1* expression in both clones (clone #2: +292%, $P < 0.05$ and clone #3: +198%, $P < 0.05$, Fig. 5B). Also, basal OCRs were similar (Supplementary Fig. S6B) and OCRs of both clones increased in response to noradrenaline stimulation (up to 106 and 93% above the basal respiration rate, for clone #2 and clone #3, respectively, Fig. 5C–D).

Subsequently, monoclonal cell lines from BAT biopsies of three other human adult individuals were generated by conditional immortalization as described above. One clone was selected from each individual based on its adipogenic differentiation capacity (i.e. *UCP1* expression level) to investigate their response to adrenergic stimulation. When cultured under adipogenic differentiation conditions, clones from all three patients showed an increase in *UCP1* expression from differentiation day 4 onwards with the clone from patient 4 reaching the highest level of *UCP1* mRNA (Fig. 5E). Moreover, all three clones also showed increased glycerol release (Fig. 5F) and induction of *UCP1* expression (Fig. 5G) 8 h following noradrenaline stimulation. Together, these data demonstrate that adipogenically differentiated human iBPAs derived from different patients are responsive to adrenergic stimulation.

3.6. Cultures of iBPAs can switch between proliferative and differentiated states

To investigate whether adipogenically differentiated human iBPAs can resume cell division, we replaced the differentiation medium with proliferation medium containing dox at day 10 of differentiation (Fig. 6A). At this particular time point the majority of cells contained visible lipid droplets (Fig. 6B, day 10). During the next 6 days, in some areas the percentage of cells with visible lipid droplets decreased while in other areas adipogenesis continued resulting in foci of lipid-laden cells containing larger lipid droplets than at day 10 of differentiation (Fig. 6B, day 16). Subsequently, the percentage of cells with visible lipid droplets rapidly declined in the entire culture, which was accompanied by an increase in total cell number (Fig. 6B, day 20). After 10 days of exposure to proliferation medium, the cultures had become so crowded that the cells had to be passaged. During passaging, these cells exhibited a mesenchymal morphology (Fig. 6B, days 21, 27 and 30 and Supplementary Fig. S8A), were Ki-67-positive (Supplementary Fig. S8B) and were actively dividing with a PD time similar to that of human iBPAs that had not been adipogenically differentiated.

After 3 passages at a split ratio of 1:8, i.e. 30 days after the start of the first round of adipogenesis, the cells were once again exposed to adipogenic differentiation conditions (Fig. 6A). This instigated a second round of adipogenesis, during which the cells gradually accumulated lipid droplets resulting in the multilocular phenotype typical for brown adipocytes (Fig. 6B, days 38, 42 and 46). The molecular signature of these cells was further assessed by RT-qPCR and Western blot analysis of *UCP1*/*UCP1* expression. The brown adipocytes from the first and second round of adipogenic differentiation showed a similarly strong increase in *UCP1* mRNA and *UCP1* protein levels between the start of the differentiation process, when *UCP1*/*UCP1* expression is hardly detectable, and differentiation day 16 (Fig. 6C). Expression of the late G1/S/early G2 phase marker PCNA and the late G2/M phase marker pH3 both showed the opposite trend, i.e. a large decrease in protein level between day 0 and day 16 of adipogenic differentiation (Supplementary Fig. S8C). Overall, these results demonstrate that human iBPA cultures



(caption on next page)

Fig. 4. Adipogenic capacity of human iBPAs. (A) Protocol used to differentiate human iBPAs into brown adipocytes. Images were taken (*), and RNA samples were collected (†) at the indicated time points. (B) Phase-contrast images showing the morphological changes induced by adipogenic stimulation of clone #2 and #3 iBPAs cultured with (+dox) and without (–dox) continuous proliferation pressure. (C) RT-qPCR analysis of *UCP1*, *PRDM16*, *PPARGC1A*, *PPARG*, *FABP4* and *LPL* gene expression in clone #2 and #3 iBPAs on the indicated days of culturing under adipogenic differentiation conditions with and without dox. Expression levels were normalized to *LRP10* expression. Data are means \pm SEM ($n = 4$). dox, doxycycline; IBMX, 3-isobutyl-1-methylxanthine; iBPAs, conditionally immortalized brown preadipocytes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; T3, triiodothyronine.

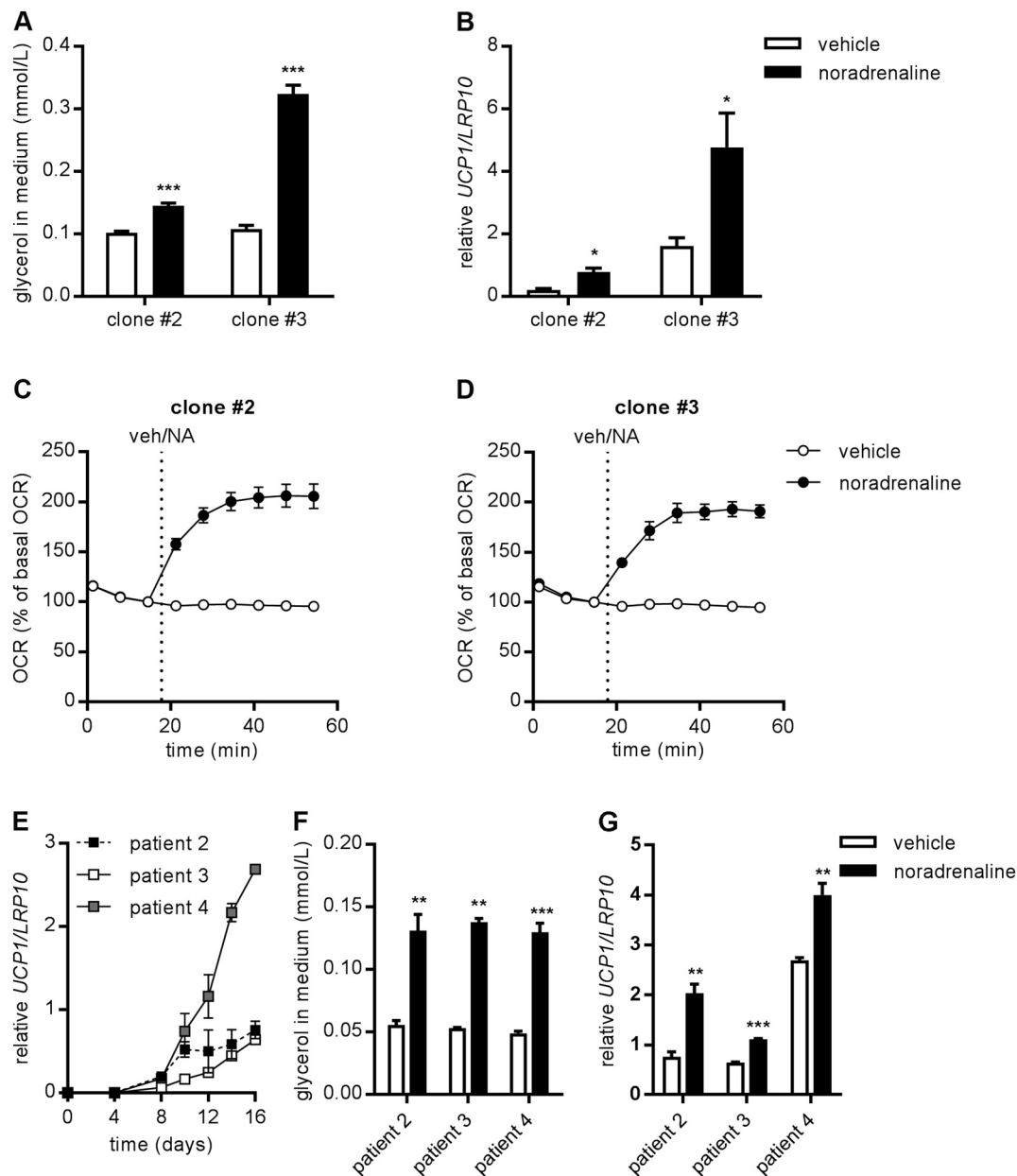


Fig. 5. Functional properties of adipogenically differentiated human iBPAs. (A–D) Effect of adrenergic stimulation on clone #2 and #3 iBPAs. Both clones were stimulated with 1 μ M noradrenaline for 8 h, at differentiation day 16, after which glycerol in the culture medium (A) and *UCP1* mRNA levels in the cells (B) were measured. (C, D) OCR in adipogenically differentiated human iBPAs upon treatment with vehicle or noradrenaline using Seahorse respirometry. (E) RT-qPCR analysis of *UCP1* gene expression during adipogenic differentiation in human iBPA clones derived from 3 additional patients (i.e. patients 2–4). (F, G) Effect of adrenergic stimulation on human iBPA clones from patients 2, 3 and 4. These clones were stimulated with 1 μ M noradrenaline for 8 h, at differentiation day 16, and glycerol concentrations in the culture media (F) and *UCP1* mRNA levels in the cells (G) were measured. Data are means \pm SEM ($n = 4$ in A, B; $n = 10$ –12 in C, D; $n = 3$ in E, F, G). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. iBPAs, conditionally immortalized brown preadipocytes; NA, noradrenaline; OCR, oxygen consumption rate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; veh, vehicle.

can undergo multiple rounds of proliferation and brown adipogenesis (Fig. 6D).

4. Discussion

In this study, robust and tightly controlled expansion of monoclonal populations of mouse and human BPAs was accomplished by providing the cells with a dox-regulated SV40 LT expression unit via lentiviral

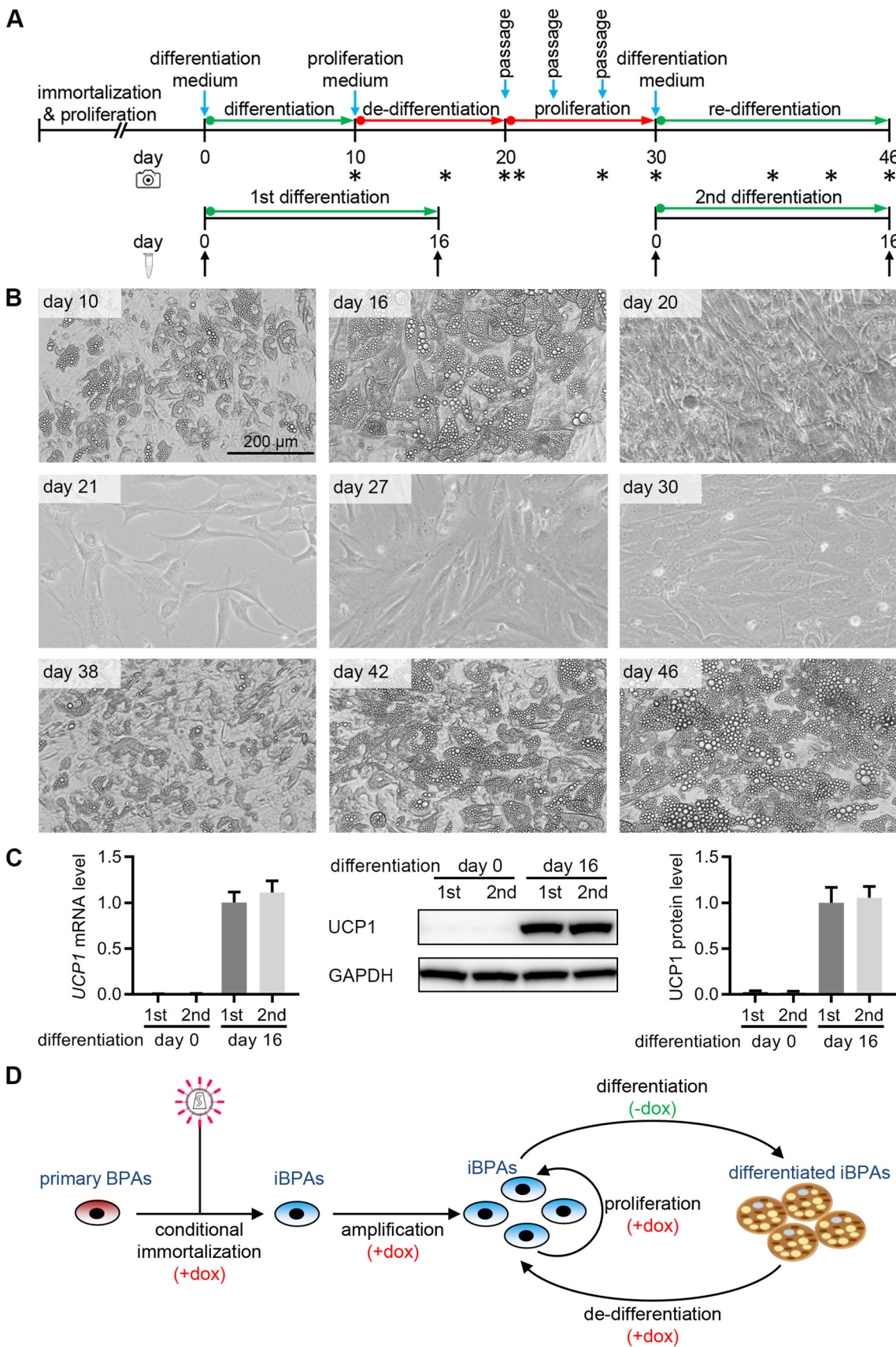


Fig. 6. Switching of human iBPAs between proliferative and differentiated states. (A) Experimental design: At day 0, human iBPAs were cultured under adipogenic differentiation conditions for 10 or 16 days to induce brown adipogenesis. The cells that had been exposed to adipogenic differentiation conditions for 10 days were subsequently cultured in proliferation medium for 20 days (18 days with dox and 2 days without dox) and passaged whenever the cell cultures reached 90% confluency. Thereafter, the cells were re-exposed to adipogenic differentiation conditions for 16 days. Images were taken (*), and RNA and protein samples were collected (†) at the indicated time points. (B) Phase-contrast images showing the morphological changes induced in human iBPAs following two consecutive shifts from proliferation to adipogenic differentiation conditions. (C) RT-qPCR and Western blot analysis of *UCP1*/UCP1 expression in human iBPAs at the start and the end of the first and second round of brown adipogenesis. *UCP1* mRNA and UCP1 protein expression levels were normalized to *LRP10* and GAPDH expression, respectively. Data are means \pm SEM ($n = 4$ for RT-qPCR and $n = 3$ for Western blot). (D) Schematic representation of BPA immortalization, amplification and differentiation and one or more subsequent cycles of de-differentiation, proliferation and re-differentiation. BPAs, brown preadipocytes; dox, doxycycline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iBPAs, conditionally immortalized brown preadipocytes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean.

transduction. The presence of dox in the culture medium induced LT expression and caused rapid iBPA expansion, while after dox removal, LT expression faded and the cells stopped dividing. In contrast to murine iBPAs, which could undergo brown adipogenesis both in the absence and presence of dox, human iBPAs could only differentiate into brown adipocytes when LT expression was switched off. In addition, when exposed to dox-containing proliferation medium, cultures of adipogenically differentiated human iBPAs lost their multilocular appearance as well as other properties characteristic for brown adipocytes (e.g. *UCP1*/UCP1 expression) and became proliferative again. After re-exposure of these proliferating cultures to adipogenic differentiation conditions, the cells underwent a second round of brown adipogenesis. Importantly, adrenergic stimulation of adipogenically differentiated iBPAs of both murine and human origin strongly increased their lipolytic activity and oxygen consumption.

Permanently immortalized murine BPAs have proven to be useful tools for studying brown adipocyte biology and responses to pharmacological treatments [13–16,28]. However, these cells generally display a rapid loss of adipogenic capacity upon repeated cell divisions [13], limiting their applicability in high-throughput assays. Also, several important differences have been observed between murine and human BAT [29], which may limit the usefulness of immortalized murine BPAs as a model system for human brown adipocytes. Hence, the immortalization of human BPAs was pursued, which yielded cell lines that were either polyclonal [30] or monoclonal with a slow proliferation rate and/or with an unknown relationship between proliferation and adipogenic differentiation ability [17,18]. In favourable contrast, our monoclonal mouse and human iBPA lines retained full adipogenic capacity for > 60 PDs, thereby providing abundant cell sources for high-throughput studies. Another limitation of non-conditionally immortalized BPAs is the potential influence of permanent expression of immortalization genes on the adipogenic differentiation ability of BPAs or on the phenotype of the brown adipocytes derived from the immortalized cells. Conditionally immortalized mouse BPAs have been generated using the temperature-sensitive LT mutant *tsA58* [31]. However, even at the non-permissive temperature of 39 °C, the binding of LT-*tsA58* to p53 is only partially inhibited whereas its ability to repress pRB remains unaltered [32]. Lastly, immortalization of cells without clonal selection might result in unstable cell lines, since faster proliferating cells will overgrow the slower proliferating ones and thereby change the composition of the lines over serial passaging. Similar issues do not apply to our monoclonal iBPA lines, which due to the dox dependency of LT expression, can be amplified as required and adipogenically differentiated in a synchronous manner and in the absence of immortalization gene expression. Altogether, these assets provide a clear advantage in the assessment of differentiation stage-dependent gene expression profiles over existing BAT models.

Gene expression profiles of mouse and human iBPAs during differentiation into brown adipocytes showed several similarities with rising mRNA expression levels of the genes coding for UCP1, PPARGC1A, FABP4 and LPL. Interestingly, while in murine iBPAs, expression of the gene encoding PRDM16 was induced during adipogenic differentiation, this was not the case for the human iBPAs. PRDM16 is a known transcriptional coregulator of BAT development and is suggested to repress

white adipocyte gene expression and to promote the expression of beige/brown adipocyte genes [33,34]. Using mouse adipocytes, Ohno et al. [35] have shown that PRDM16 is required for PPARG agonist-induced brown adipogenesis. In line with this finding and considering the fact that *UCP1* is a target gene of PRDM16, it might be expected that the mRNA expression patterns of *UCP1* and *PRDM16* would follow a similar trend. Indeed, others have reported a strong correlation between *PRDM16* and *UCP1* expression in human brown adipocytes from the perirenal depot [36]. Nonetheless, even though our human iBPAs do not show a clear adipogenic differentiation-dependent increase in *PRDM16* expression, they efficiently differentiate into multilocular brown adipocytes that are responsive to adrenergic stimulation. Perhaps, PRDM16 activity during the differentiation of our human iBPAs is post-transcriptionally regulated. However, since to date most studies on the regulation of BAT formation have been performed in mice and given the differences between murine and human BAT, additional studies are required to determine the precise role of PRDM16 and other factors in human brown adipogenesis.

We found that continuous LT expression, as induced by dox, inhibited the adipogenic differentiation of the human iBPAs, whereas the presence of dox in the culture medium did not inhibit adipogenesis of the mouse iBPAs. Previously, dox has been shown to negatively impact mitochondrial function [37,38], which could theoretically impact adipogenic differentiation capacity. However, the concentration of dox used in our iBPA cultures (i.e. 100 ng/mL) was well below the concentration shown to disturb mammalian mitochondrial function [38] and mouse iBPAs readily differentiated into brown adipocytes at this dox concentration. Thus, it is more likely that another mechanism is underlying the discrepancy between mouse and human cells. Since the preadipocytes were isolated from young mice *versus* from middle-aged humans, perhaps the age of the preadipocyte donor affects the capacity to differentiate under continuous proliferation pressure. Moreover, different promoters were used to drive LT expression in murine and human BPAs and this may have resulted in different expression levels of LT, thereby contributing to differences in the adipogenic differentiation potential between dox-exposed murine and human iBPAs. The inter-species difference may also be caused by less pronounced effects of the pRB pathway on human compared to mouse cells with respect to adipogenesis. Several studies have shown that inactivation of the pRB pathway inhibits white adipogenesis but facilitates brown adipogenesis in mice [23,39,40], whereas no correlation was found between *RB1*, the gene encoding pRB, and *UCP1* gene expression in human adipose tissue [41]. Even though dox did not block the adipogenesis of mouse iBPAs, there are some subtle differences between mouse iBPAs adipogenically differentiated with or without dox. At late stages of adipogenic differentiation, cells cultured with dox on average appear to contain somewhat more and smaller lipid droplets than mouse iBPAs differentiated without dox. Furthermore, the kinetics of lipid droplet accumulation seems slightly slower for murine iBPAs maintained in differentiation medium with dox. At present, we don't know whether differentiation of mouse iBPAs into brown adipocytes in the presence of dox (i) merely proceeds somewhat slower, (ii) is impaired and thus results in less fat accumulation or, oppositely, (iii) yields a browner phenotype reflected by the generation of smaller lipid droplets.

Whereas continual LT expression blocked the subsequent adipogenic differentiation of our human iBPAs, 10.8% of the clonal LT-transduced human BPA lines generated by Shinoda et al.¹⁷ displayed high adipogenic capacity. To immortalize human BPAs, Shinoda and colleagues used a gammaretroviral vector containing an intron-less wild-type LT gene, expression of which was driven by the virus' long terminal repeat (LTR), while our human iBPAs were generated using a lentiviral vector that contains a human *UBC* promoter-driven temperature-sensitive LT gene with intron. Pre-mRNA of the LT gene with intron, via alternative splicing, gives rise to LT, 17kT and small tumor antigen (ST) [42,43]. The lack of the intron abolishes ST expression [42]. ST contains a cellular protein phosphatase 2A (PP2A) binding region which inhibits the pro-apoptotic activity of PP2A, thereby increasing the viability of cells [44,45]. Although the use of different versions of the LT gene could potentially explain the apparent discrepancy between our results and theirs, Shinoda et al. did not study LT expression in their cells. Hence, loss of LT expression prior to or during adipogenic differentiation of Shinoda's cells cannot be ruled out. Another factor that might have contributed to the different behavior of our cells and those of Shinoda et al. is the use of different conditions to induced brown adipogenesis. The same holds true for the human BPA clones made by Xue et al.¹⁸. Adipogenic differentiation of these clones, which were generated with a gammaretroviral vector containing an LTR-driven human TERT expression unit, also involved the use of a treatment regimen different from ours. A clear advantage of our iBPAs is their high proliferation speed and capacity. Both the murine and human iBPAs have a doubling time of ~28 h and can be massively expanded. In contrast, the fastest proliferating cell line of Xue et al.¹⁸ (i.e. Sub4 hBAT-SVF) divides approximately once every 6 days and the proliferation speed and capacity of the LT-immortalized human BPAs made by Shinoda et al.¹⁷ have not been reported. Another important finding of our study is that the selected murine and human iBPA clones can differentiate into brown adipocytes until at least PD 60 while it is unclear until which PD the immortalized BPAs of Xue et al. and Shinoda et al. retain their adipogenic differentiation capacity. Finally, since Xue et al. did not perform single-cell cloning, the cellular composition and therefore properties of their TERT-immortalized BPA cultures may undergo substantial changes during repeated passaging due to clonal selection of the fastest-growing cells.

Our iBPA cell lines provide many opportunities for future research. Mouse iBPA clone #6 and human iBPA clone #3 retain full adipogenic capacity for > 60 PDs, implying that in each case > 10¹⁸ brown adipocytes could be generated from a single preadipocyte. This provides an abundant cell source for fundamental and applied research [46]. The tightly controlled LT expression/proliferation used in this study provides a safeguard against tumor development and creates an opportunity for iBPAs to become a transplantable cell source. The recent finding that mature brown adipocytes of mice retain proliferative capacity [47,48] and that mature white adipocytes in murine mammary glands can undergo pregnancy-related cycles of de-differentiation, proliferation and re-differentiation [49] indicates that adipocytes possess remarkable phenotypic plasticity. However, the molecular mechanisms underlying this plasticity remain unclear. Our iBPAs might provide a good model system to gain more insight into the pathways involved in adipocyte plasticity and, after transplantation, allow dox-dependent regulation of the size of the BAT compartment *in vivo*. Besides, a better understanding of the species-specific role of LT and pRB in adipogenesis will help to further understand the mechanisms of brown adipogenesis as well as the conversion of white into brown adipocytes and *vice versa*. iBPAs are unique in their ability to vary the timing of LT expression and therefore vary the pocket protein activity in relation to the moment of adipogenic stimulation. This property makes iBPAs particularly attractive for studying the role of cell cycle regulators including those directly targeted by LT in the adipogenic differentiation of BPAs.

In conclusion, we established lines of conditionally immortalized mouse and human BPAs with long-term proliferation ability and

adipogenic capacity. Following adipogenesis, these cells display a multilocular lipid droplet appearance as well as a gene expression signature and metabolic capacity akin to brown adipocytes. The generated cell lines have many potential applications and can, for example, be used to investigate BPA proliferation and differentiation, brown (pre) adipocyte metabolism and regulation of *Ucp1/UCP1* activity and thermogenesis, for brown (pre)adipocyte-based disease modelling, BPA transplantation studies and drug development.

Author contributions

A.A.F.V. and S.K. designed the experiments. J.L., E.N.K., H.C.M.S., J.C.D. and A.D.D. performed experiments and analyzed data. C.C. and F.K. provided human BAT biopsies. J.L. and E.N.K. wrote the original draft. G.Z., M.R.B., P.C.N.R., A.A.F.V. and S.K. supervised research and reviewed the manuscript. All authors have read and approved the final version of the manuscript.

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Transparency document

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbalip.2019.08.007>.

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