ARTICLE
Journal of Cellular Biochemistry 109:542–552 (2010)

Journal of Cellular Biochemistry

Establishment and Characteristics of Porcine Preadipocyte Cell Lines Derived From Mature Adipocytes

Hiroyuki Nobusue and Koichiro Kano*

Laboratory of Cell and Tissue Biology, College of Bioresource Sciences, Nihon University, Kanagawa, Japan

ABSTRACT

Development of established preadipocyte cell lines, such as 3T3-L1 and 3T3-F442A, greatly facilitated the study of molecular mechanisms of adipocyte differentiation under defined conditions. Most of these cell lines are derived from mouse embryos, and preadipocyte cell lines of other species have not yet been maintained in culture long enough to study differentiation under a variety of conditions. This is the first report on the establishment of porcine preadipocyte cell lines derived from mature adipocytes by a simple method, known as ceiling culture, for culturing mature adipocytes in vitro. This cell line can proliferate extensively until the cells become confluent and fully differentiated into mature adipocytes, depending on adipogenic induction. No changes in their differentiation pattern are observed during their propagation, and they have been successfully carried and differentiated for at least 37 passages. This cell line maintains a normal phenotype without transforming spontaneously, even after long-term maintenance in culture. This achievement may lead to easy establishment of porcine preadipocyte cell lines and novel model systems for studying the mechanisms of adipocyte differentiation and metabolism as a substitute for human preadipocytes. J. Cell. Biochem. 109: 542–552, 2010.

KEY WORDS: PORCINE PREADIPOCYTE CELL LINE; DEDIFFERENTIATION; DIFFERENTIATION; MATURE ADIPOCYTE; CEILING CULTURE

Adipose tissue plays a central role in the regulation of energy balance [Hwang et al., 1997; Gregoire et al., 1998]. An excess of adipose tissue, that is, obesity, is a serious problem and contributing factor to several diseases, including insulin resistance, type 2 diabetes, hypertension, cancer, and atherosclerosis [Reaven, 1988]. Identification of agents affecting adipocyte differentiation and metabolism provides us with a better understanding of ways to treat obesity and its associated disorders. The approach to the regulation of adipocyte growth and differentiation derives largely from studies with rodent preadipocyte cell lines, such as 3T3-L1 (from the immortalized Swiss 3T3 cell line) and its subclonal cell line 3T3-F442A. These cell lines have proved to be extremely useful in studying adipocyte differentiation in vitro, because they spontaneously accumulate lipids and differentiate into mature adipocytes upon growth arrest [Green and Meuth, 1974; Green and Kehinde, 1975, 1976]. The cell lines are characterized by maintenance of high proliferative capacity and stable differentiation potential during subculturing. However, these cell lines, derived from the Swiss 3T3 cell line obtained from disaggregated 17- to 19-day-old Swiss 3T3 mouse embryo in which white adipose tissue is not detected, often possess characteristics that differ from those of tissue preadipocytes [Smas and Sul, 1995; Wolins et al., 2006].

Most established stable preadipocyte cell lines are from rodents, and few preadipocyte cell lines have been established from other species. Hence, short-term primary cultures have mainly been performed [Butterwith et al., 1992; Surywan et al., 1997; Tomlinson et al., 2006]. Primary preadipocytes are derived from the stromal-vascular fraction (SVF) of adult adipose tissue; therefore, they may reflect the in vivo context and provide a stable in vitro primary culture system for identifying possible regulators [Van, 1985]. Of note, primary preadipocytes are characterized by low proliferative capacity associated with rapid decline of differentiation ability with subculturing, thereby limiting their use as cellular models. In addition, adipose precursor cells present in the SVF may fall into the following categories: (a) adipose tissue-derived stem cells that follow osteogenic, chondrogenic, and adipogenic paths [Zuk et al., 2002]; (b) cells that have undergone determination to the adipose lineage (adipoblasts); and (c) cells that differentiate into preadipocytes, a process accompanied by emergence of early or late markers. Furthermore, the SVF contains various cell types, including erythrocytes, macrophages, monocytes, endothelial cells, fibroblasts, pericytes, and preadipocytes. All these types of cells may influence adipocyte differentiation [Ramsay et al., 1992]. Because of difficulties in obtaining only preadipocytes from the SVF by

Grant sponsor: NUBS Research Grant; Grant sponsor: HAITEKU Research Grant; Grant sponsor: Nihon University Multidisciplinary Research Grant.

*Correspondence to: Dr. Koichiro Kano, Laboratory of Cell and Tissue Biology, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan. E-mail: kkano@brs.nihon-u.ac.jp

Received 5 August 2009; Accepted 22 October 2009 • DOI 10.1002/jcb.22431 • © 2009 Wiley-Liss, Inc.
Published online 9 December 2009 in Wiley InterScience (www.interscience.wiley.com).

cloning, a primary culture system based on this fraction would be unsuitable for detailed study of adipocyte differentiation. The main problem with all primary culture systems is the inability to isolate a pure population of any single cell type [Hausman et al., 1980].

Since adipocytes float in the culture medium after digestion of the adipose tissue, their isolation is easy. It has been shown that mature adipocytes isolated from fat tissue can change into fibroblast-like adipocytes and proliferate extensively by the ceiling culture method. This method exploits the buoyant property of adipocytes, which allows them to adhere to the top inner surface of a culture flask filled completely with medium [Sugihara et al., 1986, 1987] or the underside of a floating piece of glass [Zhang et al., 2000]. We recently reported the establishment of preadipocyte cell lines derived from dedifferentiated mature adipocytes of ddY mice or green fluorescent protein (GFP) transgenic mice using the ceiling culture method [Yagi et al., 2004; Nobusue et al., 2008]. These cells possess most of the characteristics of preadipocytes and retain their ability to proliferate and dedifferentiate into mature adipocytes in each subculture, even after 22 passages. We called these cells dedifferentiated fat (DFAT) cells. DFAT cells implanted in the sternum of mice can dedifferentiate into mature adipocytes in vivo and form highly vascularized fat pads that morphologically resemble normal subcutaneous adipose tissue. Therefore, DFAT cells may represent a useful model for studying the mechanism of adipocyte differentiation and adipose tissue formation in vivo and in vitro. However, rodent cells are prone to spontaneous immortalization during long-term culture [Todaro and Green, 1963; Aaronson and Todaro, 1968], and our established DFAT cell lines derived from mice have become immortalized and aneuploid [Yagi et al., 2004; Nobusue et al., 2008]. In addition, adipocyte differentiation in rodent preadipocyte cell lines does not always reflect that in other species [Tomlinson et al., 2006; Sanosaka et al., 2008], and the differentiation pattern of primary human preadipocytes and contribution of glucocorticoids to these events differ from the responses observed in rodent preadipocyte cell lines [Tomlinson et al., 2006].

Although small animal models of disease, such as rodents, often do not accurately mimic the relevant human conditions, diabetic models in pigs, which display anatomical and physiological similarities to humans, would be useful in research for developing new therapies and drugs [Larsen and Rolin, 2004; Lunney, 2007]. Thus, because of their higher lipogenic capacity and similar lipogenic patterns to human adipocytes, porcine preadipocytes are considered a better biomedical model for investigating adipocyte differentiation compared to rodent adipocytes [Pang et al., 2009]. DFAT cells may be useful porcine preadipocyte cell lines if they possess proliferation and differentiation properties identical to those of preadipocyte cell lines, maintain a normal diploid phenotype, and be effectively obtained from isolated porcine dedifferentiated mature adipocytes by ceiling culture. This could provide an excellent biomedical model for studying pharmacological and nutritional regulation of adipocyte differentiation and metabolism as a substitute for human preadipocytes.

In the present study, we have aimed to establish a novel preadipocyte cell line derived from porcine mature adipocytes using the ceiling culture method. First, we showed that isolated porcine mature adipocytes could dedifferentiate into fibroblast-like cells and then proliferate extensively using the ceiling culture. Next, we showed that DFAT cells from pigs (DFAT-P) possess the characteristics of preadipocytes in vitro. We also identified the optimal culture conditions for adipocyte differentiation of DFAT-P cells. Furthermore, we showed that DFAT-P cells retain the ability to proliferate and differentiate into mature adipocytes and maintain a normal diploid phenotype even after long-term culturing. We propose that this cell line and the method we established here will provide useful novel model systems to study the mechanisms of adipocyte differentiation and metabolism as a substitute for human preadipocytes.

MATERIALS AND METHODS

ISOLATION OF PORCINE MATURE ADIPOCYTES

Primary adipocytes were isolated using the method of Sugihara et al. [1986, 1987] with modifications. Subcutaneous adipose tissue, obtained at a local slaughterhouse, was derived from 6-month-old male or female 3-way cross pigs. The adipose tissue was extensively washed with phosphate-buffered saline (PBS) supplemented with 80 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) and cut into small pieces in Dulbecco's modified Eagle's medium (DMEM; pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) collagenase (Type II; Sigma-Aldrich) and 2% bovine serum albumin (Fraction V; Sigma-Aldrich) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 1.8 mg/ml $NaHCO_3$, and 80 μg/ml kanamycin. After the tissue was digested by shaking the samples gently at 37°C for 1 h [Rodbell, 1964], the cell suspension was filtered through 150- and 250-μm nylon meshes (Kyoshin Rikoh, Tokyo, Japan) to remove undigested tissue debris. The filtered cells were extensively washed by centrifugation at 135*g* three times for 3 min. Floating primary adipocytes in the top layer and SVF cells at the bottom of the tube were collected separately and purified three times by centrifugation.

MICROSCOPIC ANALYSIS

The floating primary adipocytes were fixed in 4% formaldehyde (Wako, Osaka, Japan) for 10 min, washed with PBS, and stained with Hoechst 33342 (5 μg/ml; Sigma-Aldrich) in PBS for 30 min. The number of mature adipocyte nuclei was assessed by counting 2176 cells in a hemocytometer under a fluorescence microscope (Olympus IX71, Tokyo, Japan). Data from six trials were averaged.

CELL CULTURE

Isolated mature adipocytes (approximately 2×10^4 cells/cm^2) were placed in culture flasks (BD Falcon, Bedford, MA) filled with DMEM supplemented with 20% fetal calf serum (FCS; Moregate BioTech, Qld, Australia). The flasks were filled with the medium, creating an air-free environment for the cells, and then inverted and incubated at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. The cells floated up through the medium and adhered to the top inner surface (ceiling) of the flask. Approximately 1 week later, the cells were firmly attached to the ceiling and changed into fibroblast-like shapes with no visible fat droplets. The medium was changed every 4 days until confluence was reached. The SVF cells were

resuspended in DMEM containing 20% FCS in a new test tube, seeded onto 35-mm culture dishes at a density of 10^6 cells/dish, and cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air until confluence.

INDUCTION INTO ADIPOCYTE DIFFERENTIATION

After the cells reached confluence, differentiation was induced by changing the medium to DMEM supplemented with 5–40% FCS, 0.025–2 μM dexamethasone (DEX; Wako), 0.05–5 mM 3-isobutyl-1-methylxanthine (IBMX; Wako), and 0.5–50 μg/ml insulin (INS; Wako). The differentiation medium was replaced by DMEM supplemented with 20% FCS after 96 h. The cells were allowed to differentiate for 12 more days.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was isolated from cells derived from the floating adipocytes and SVF cells before and after differentiation stimuli, using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Gene expression was evaluated by RT-PCR. Total RNA (1 μg) digested with DNase I (Invitrogen) was subjected to RT-PCR analysis and reverse-transcribed using SuperScript III (Invitrogen) with a random hexamer (Invitrogen). Reverse transcripts were applied as templates for analyzing the gene expression levels using PX2 Thermal Cycler (Hybaid Ltd, Middlesex, UK) and AmpliTaq Gold (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. In general, PCR primers were designed to amplify 200–500 bp of the target sequences. Primer sequences are given in Table I. PCR conditions were as follows: 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final elongation step of 5 min at 72°C. Aliquots of PCR products were analyzed on 2% ethidium bromide-stained agarose gel.

GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPDH) ACTIVITY

GPDH activity (EC 1.1.1.8) was used as a late marker of adipocyte differentiation. Cell extracts were tested for GPDH activity according to the method described previously by Wise and Green [1979] with modification. Briefly, after removing the medium, the cells were washed twice with iced PBS. They were recovered by scraping with a rubber policeman in 25 mM Tris (pH 7.5; Sigma-Aldrich) supplemented with 1 mM ethylenediaminetetraacetate (EDTA; Nacalai Tesque Co., Kyoto, Japan) and sonicated for 10 s at 150 W. After centrifugation at 12,800*g* for 5 min at 4°C, the supernatant fraction was further centrifuged at 100,000*g* for 1 h at 4°C. The supernatant fraction thus obtained was used for subsequent assays. GPDH activity was measured according to Kozak and Jensen [1974]. The standard mixture contained 5 mM dihydroxyacetone phosphate (Sigma-Aldrich), 0.5 mM reduced nicotinamide adenine dinucleotide (Oriental Kobo Co., Osaka, Japan), and 50 mM triethanol amine (Wako) supplemented with 10 mM EDTA and 10 mM β-mercaptoethanol. The change in absorbance at 340 nm was measured using a spectrophotometer (UV-1200; Shimadzu, Kyoto, Japan). One unit of enzyme activity was defined as the quantity required to oxidize 1 nmol of NADH/min. The cytosolic protein concentrations of supernatants were measured according to the method described by Lowry et al. [1951].

LIPID STAINING

Lipid accumulation in adipocytes was observed by staining the solution with oil red O (Wako). Cells were washed three times with PBS, followed by fixation with 10% formalin in phosphate buffer for 1 h at room temperature. After fixation, the cells were washed again with PBS and stained with a filtered oil red O stock solution (0.5 g oil red O in 100 ml isopropyl alcohol) for 15 min at room temperature. The cells were then washed twice in distilled water for 15 min.

STATISTICAL ANALYSIS

Data regarding the number of nuclei per isolated mature adipocyte were analyzed by the Chi-square test with Yates' correction for continuity to determine statistical differences between each experiment. The mean cell number was examined by one-way analysis of variance. Mean GPDH activity was compared using Tukey's procedure. A probability less than 0.05 was considered significant.

RESULTS

ISOLATION OF PORCINE MATURE ADIPOCYTES AND CEILING CULTURE

Adipose tissue was digested in collagenase solution by gentle agitation to obtain uniform single-cell fractions of porcine mature adipocytes and avoid contamination by preadipocytes, fibroblasts,

TABLE I. Primer Sequence for RT-PCR

Accession number	Gene name	Primer sequence: 5′–3′
AY559454.1	Lipoprotein lipase (LPL)	Sense: AGAGAGGACTTGGAGATGTGGA Antisense: CAGGCAGAGTGAATGGGATG
AY135647.2	Adiponectin	Sense: TTGAAGGATGTGAAGGTCAGC Antisense: GGAAGCCTGTGAAGATGGAGT
AJ416020.1	Fatty acid-binding protein 4 (FABP4)	Sense: ACAGGAAAGTCAAGAGCACCAT Antisense: CGGGACAATACATCCAACAGA
AB097930.1	Peroxisome proliferator-activated receptor gamma 2 (PPARγ2)	Sense: CTTATTGACCCAGAAAGCGATG Antisense: TGAGGGAGTTGGAAGGCTCT
AF103944.1	CCAAT/enhancer-binding protein alpha (C/EBPα)	Sense: GGACAAGAACAGCAACGAGT Antisense: TCATTGTCACTGGTCAGCTC
NM_21384.1	Leptin	Sense: CATTTCTCTCTCGCTCCGCTAA Antisense: GATGGAACCCTGCTTGATGG
AK234838.1	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Sense: GGGAAGCTTGTCATCAATGG Antisense: GTTGTCATGGATGACCTTGG

and/or stromal-vascular cells [Sugihara et al., 1986]. The dissociated adipocytes were then purified by multiple rounds of centrifugation and filtration, resulting in lipid-filled unilocular adipocytes (Fig. 1A-a). In a preliminary experiment, we confirmed that porcine mature adipocytes could be obtained at levels of about 1×10^6 cells from 1 g of porcine subcutaneous adipose tissue (data not shown). To test whether the isolated mature adipocytes were mononucleate cells without tightly attached SVF cells, the nuclei of isolated mature adipocytes were stained with Hoechst 33342, and the number of mononuclear mature adipocytes was counted using a fluorescence microscope. Table II shows that 99.5% of the isolated cells were mononuclear mature adipocytes, revealing that the isolated cells were a highly homogeneous fraction consisting of mature adipocytes.

In culture flasks completely filled with medium, mature adipocytes floated and adhered well to the top inner surface of

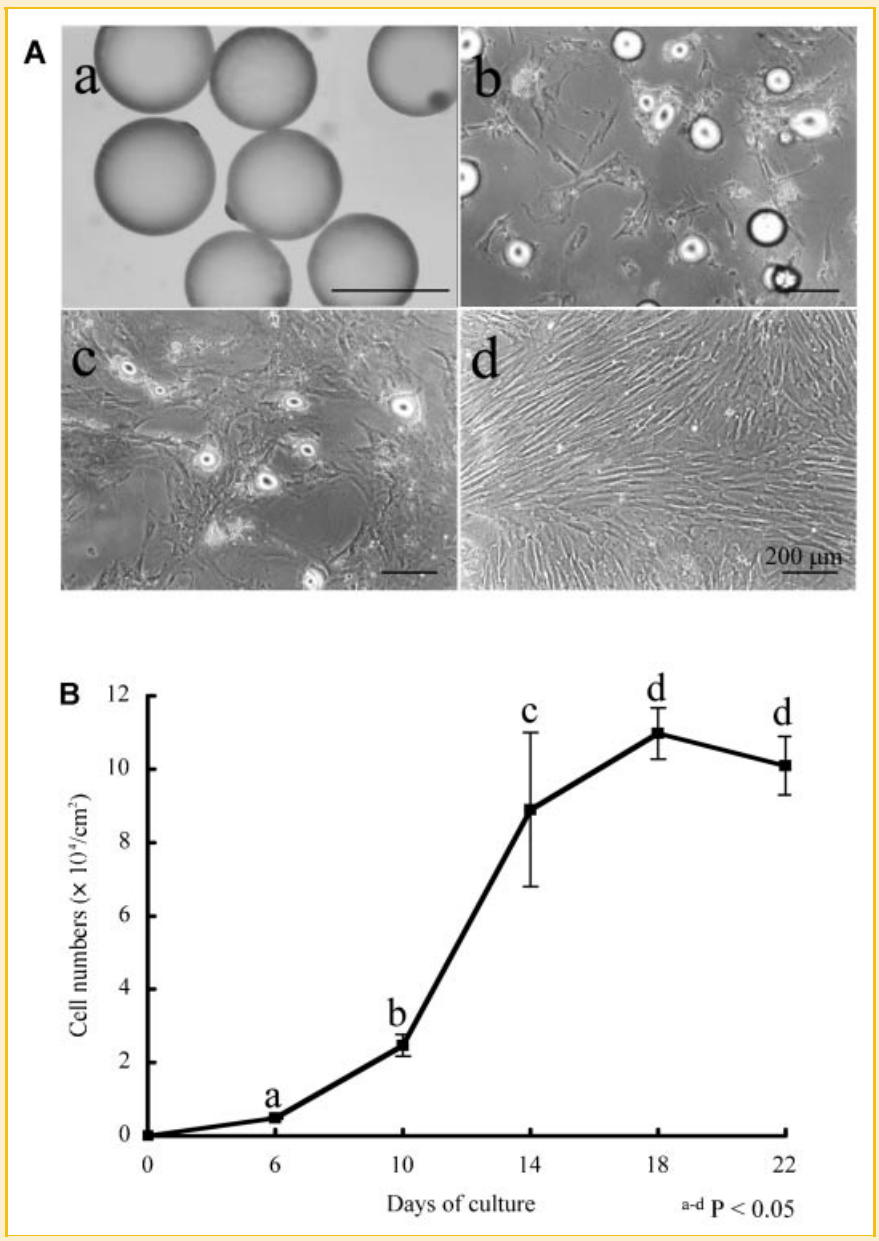

Fig. 1. Morphological changes in dedifferentiating cells from porcine mature adipocytes by the ceiling culture method. A: Morphology of isolated mature adipocytes. a: These cells are lipid-filled unilocular adipocytes without attached stromal-vascular cells. Morphology of adipocytes on day 6 of culture. b: Porcine mature adipocytes changed into multilocular adipocytes, followed by generation of fibroblast-like cells (DFAT-P cells). Morphology of adipocytes on day 10 of culture. c: DFAT-P cells actively proliferated, whereas multilocular adipocytes markedly decreased. Morphology of adipocytes on day 14 of culture. d: Multilocular adipocytes disappeared completely, and DFAT-P cells became confluent. Bars, 200 μm. B: Proliferation activity of DFAT-P cells obtained after ceiling culture. DFAT-P cells were collected by trypsinization and centrifugation after 6 days of ceiling culture or later, and the number of cells per flask was determined. Bars indicate mean ± standard deviation (SD) of five independent experiments performed on the least triplicate dishes. $^{a-d}P < 0.05$.

TABLE II. Number of Nuclei in Adipocytes Stained by Hoechst 33342

Total adipocytes[a]	No. of cells with 1 nucleus/cell	No. of cells with 2 or more nuclei/cell
382	382 (100%)[b]	0 (0%)[c]
364	361 (99.2%)[b]	3 (0.8%)[c]
333	333 (100%)[b]	0 (0%)[c]
368	365 (99.2%)[b]	3 (0.8%)[c]
352	349 (99.1%)[b]	3 (0.9%)[c]
377	375 (99.5%)[b]	2 (0.5%)[c]
2167	2165 (99.5%)[b]	11 (0.5%)[c]

Mature adipocytes were isolated from porcine adipose tissue by enzymic digestion and multiple cycles of centrifugation and filtration. Data from six trials were averaged.
[a]Number of nuclei in adipocytes was assessed by counting 2,176 cells.
[b]Values in the same row with different superscripts are different ($P < 0.05$).
[c]Values in the same row with different superscripts are different ($P < 0.05$).

the flask. Porcine mature adipocytes changed phenotypically to become multilocular and then fibroblast-like in appearance during ceiling culture, in accordance with our previous reports [Yagi et al., 2004; Nobusue et al., 2008]. After 6 days in ceiling culture, multilocular adipocytes and fibroblast-like cells were present in the flasks (Fig. 1A-b). After culturing for 10 days, multilocular adipocytes markedly decreased and fibroblast-like cells whose cytoplasm did not contain lipid droplets proliferated actively (Fig. 1A-c). After 14 days, the multilocular adipocytes disappeared completely and the fibroblast-like cells became confluent (Fig. 1A-d). We refer to these fibroblast-like cells as DFAT-P cells in this article. DFAT-P cells were collected by trypsinization, and the floating unilocular/multilocular adipocytes in the top layer and the fibroblast-like cells with no lipid droplets at the bottom of the tube could be separated by centrifugation after 6 days of ceiling culture. The proliferation activity of the DFAT-P cells obtained was then examined (Fig. 1B). The number of DFAT-P cells was approximately 0.5×10^4 cells/cm^2 after 6 days of ceiling culture. The number of DFAT-P cells rapidly increased from 2.5×10^4 cells/cm^2 to 11×10^4 cells/cm^2 between 10 and 18 days and then became confluent. These results indicate that DFAT-P cells can be effectively obtained from porcine mature adipocytes by the ceiling culture method.

CHARACTERISTICS OF ADIPOCYTE DIFFERENTIATION IN DFAT-P CELLS

After the DFAT-P cells were stimulated by an adipogenic cocktail, we characterized their adipocyte differentiation. Figure 2A shows the gene expression pattern of adipogenic markers during adipocyte differentiation. The density of the amplified cDNA band for some adipogenic markers was normalized with respect to the density of the corresponding band for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). From the start of the growth phase, the expression of fatty acid-binding protein 4 (FABP4) mRNA was detected. The expression of lipoprotein lipase (LPL), adiponectin, peroxisome proliferator-activated receptor gamma 2 (PPARγ2), and CCAAT/enhancer-binding protein alpha (C/EBPα) mRNA was enhanced after adipogenic induction (4 days). On day 8, the expression of leptin mRNA, a marker of functionally mature adipocytes, was detected at a low level in DFAT-P cells.

Before adipogenic induction, DFAT-P cells had a fibroblast-like appearance and did not contain lipid droplets (Fig. 2B-a). Following adipogenic induction, the cellular morphology of DFAT-P cells dramatically changed to a stellar appearance and accumulated small lipid droplets in the cytoplasm (Fig. 2B-b). The number and size of lipid droplets increased at day 12 (Fig. 2B-c).

Next, we measured GPDH activity, a late marker of adipocyte differentiation, in DFAT-P and SVF cells as a control. As shown in Figure 2C, the GPDH activity of DFAT-P cells slightly increased for the first 4 days after adipogenic induction, then rapidly increased, and reached the maximum activity of approximately 160 U/mg protein at day 12. The GPDH activity of SVF cells was approximately 2.6-fold lower at day 12 (60 U/mg protein). Increase in GPDH activity was not detected in the cells not treated with adipogenic cocktail.

Figure 2D shows oil red staining after adipogenic induction. DFAT-P cells cultured in the presence of the adipogenic cocktail showed a remarkable increase in lipid droplets in contrast to the negative control (Fig. 2D-a,c). The oil red-positive areas were greater in DFAT-P cells than SVF cells (Fig. 2D-a,b). These results show that DFAT-P cells possess most of the characteristics of preadipocytes and that the adipogenic ability of DFAT-P cells was higher than that of SVF cells.

EFFECT OF FCS, DEX, IBMX, AND INS TREATMENT ON ADIPOCYTE DIFFERENTIATION OF DFAT-P CELLS

To investigate the optimal concentration of FCS, DEX, IBMX, and INS for maximal adipogenesis of DFAT-P cells, confluent cells were transferred to adipogenic medium containing various concentrations of FSC and three hormones (Fig. 3A–D). For FCS, the GPDH activity of DFAT-P cells increased in a dose-dependent manner, the optimum concentration being 20% (Fig. 3A). Consequently, a medium supplemented with 20% FCS was used in further experiments. The GPDH activity of DFAT-P cells exposed to DEX, IBMX, and INS dose-dependently increased 12 days after adipogenic induction. However, treatments with more than 0.25 μM DEX, 0.5 mM IBMX, or 5 μg/ml INS did not significantly increase the GPDH activity (Fig. 3B–D). Thus, the optimal concentrations of DEX, IBMX, and INS were 0.25 μM, 0.5 mM, and 5 μg/mL, respectively.

Finally, the effect of each combination of DEX, IBMX, and INS on adipocyte differentiation of DFAT-P cells was examined (Fig. 3E). Although treatment with DEX or IBMX alone increased GPDH activity, INS treatment did not result in a significant increase compared to the control. The GPDH activity of cells stimulated by combinations of two hormones was higher than that of cells exposed to single hormones. In the combination of three hormones together, the GPDH activity was highest among each hormonal combination, the value of that was increased over 50-fold compared with the control (no induction).

EFFECT OF LONG-TERM SUBCULTURE ON PROLIFERATION AND DIFFERENTIATION OF DFAT-P CELLS

Cell lines are typically characterized by maintenance of high proliferative capacity and stable differentiation potential during

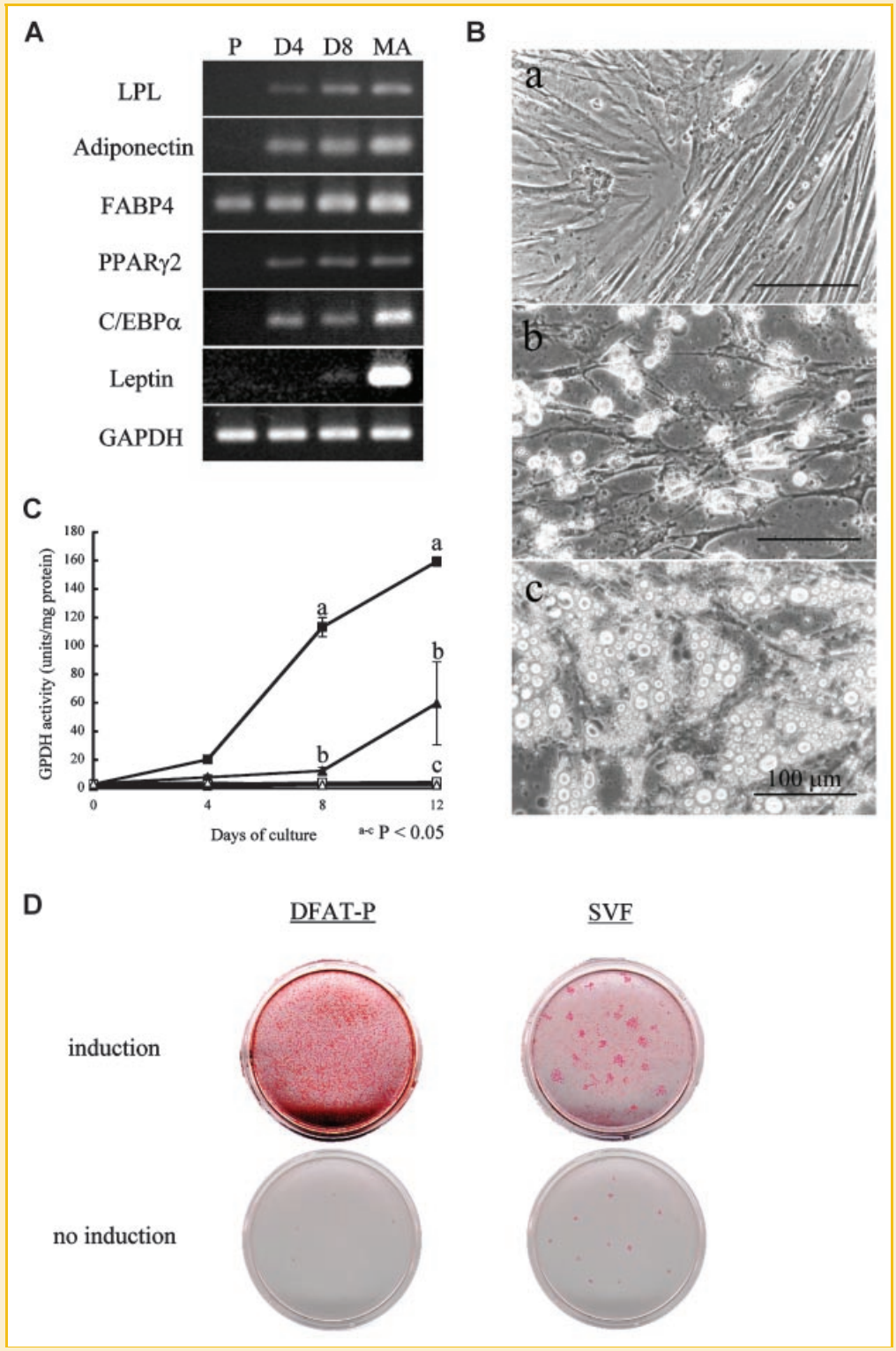

Fig. 2. Characteristics of adipocyte differentiation in DFAT-P cells. A: Gene expression pattern of adipogenic markers during adipocyte differentiation of DFAT-P cells (P sample was obtained 2 days before adipogenic induction when the cells were in the exponential growth phase; D4 and D8 samples were obtained 4 and 8 days after induction, respectively; and MA sample was obtained from isolated porcine mature adipocytes as a positive control). The density of each amplified cDNA band for some adipogenic markers was normalized to the density of the corresponding band for GAPDH. B: Morphological changes in DFAT-P cells after adipogenic induction. (a) Before adipogenic induction, DFAT-P cells had a fibroblast-like appearance and did not contain lipid droplets. (b) At 4 days after adipogenic induction, the morphology of DFAT-P cells dramatically changed into a stellar appearance with accumulation of small lipid droplets in the cytoplasm. (c) At day 12, the number and size of the lipid droplets were greater. Bars, 100 μm. C: Comparison of GPDH activity during adipocyte differentiation. ■, DFAT-P cells (differentiation); ▲, SVF cells (differentiation); □, DFAT-P cells (no differentiation); and △, SVF cells (no differentiation). Bars indicate mean ± SD of four independent experiments performed on the least triplicate dishes. $^{a-c}P < 0.05$. D: Oil red O staining 12 days after adipogenic induction. DFAT-P and SVF cells cultured in the presence of the adipogenic cocktail (induction) showed remarkable increase in lipid droplets in contrast to the negative control (no induction). Oil red-positive areas were greater in DFAT-P cells than SVF cells.

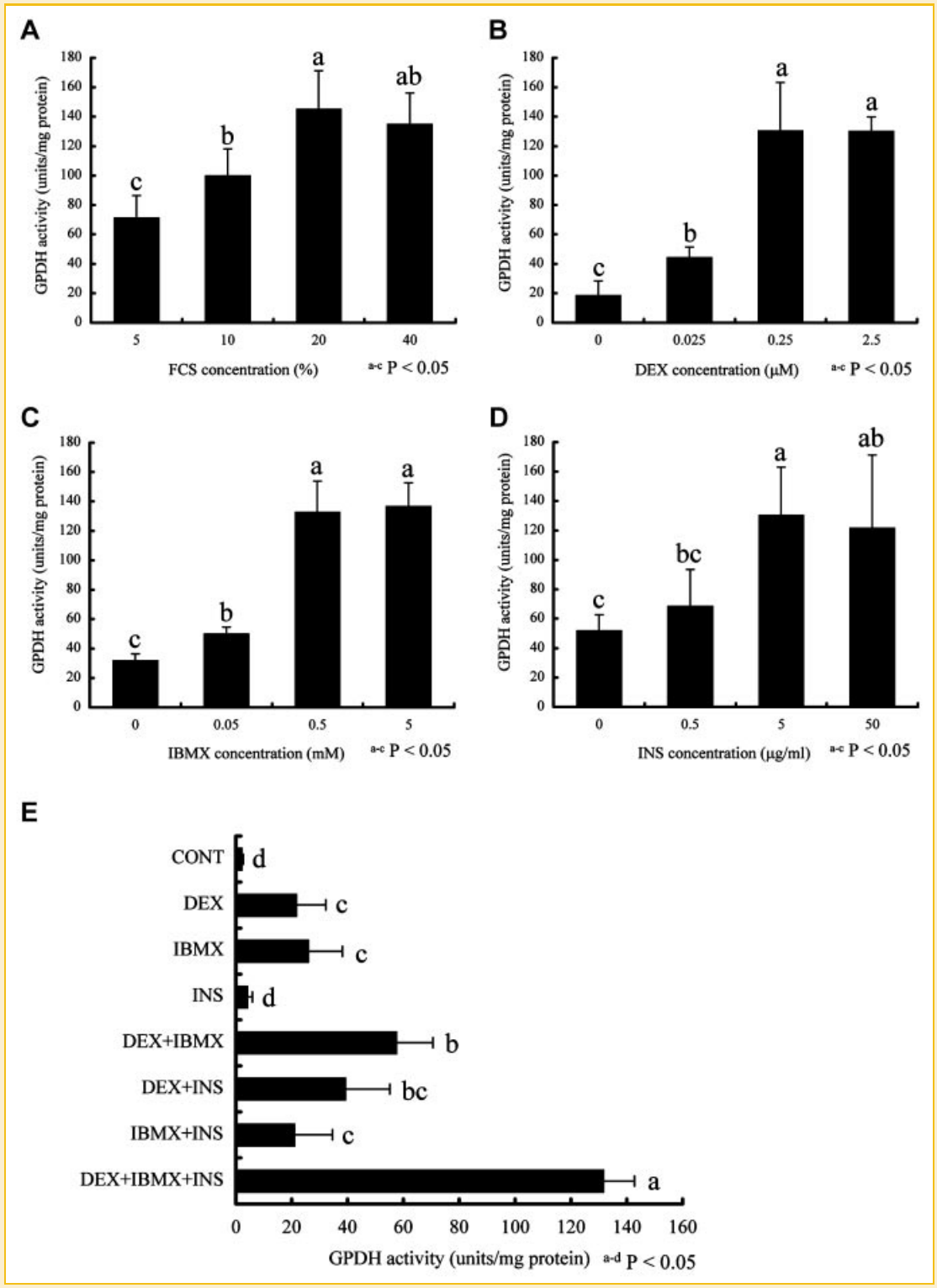

Fig. 3. Effect of different FCS, DEX, IBMX, and INS concentrations on adipocyte differentiation of DFAT-P cells. A: FCS, the confluent cells were cultured in DMEM containing 0.25 μM DEX, 5 μg/ml INS, 0.5 mM IBMX supplemented with 5%, 10%, 20%, and 40% FCS, respectively. B: DEX, The confluent cells were cultured in DMEM containing 20% FCS, 5 μg/ml INS, 0.5 mM IBMX supplemented with 0, 0.025, 0.25, and 2.5 μM DEX, respectively. C: IBMX, The confluent cells were cultured in DMEM containing 20% FCS, 0.25 μM DEX, 5 μg/ml INS supplemented with 0, 0.05, 0.5, and 5 mM IBMX, respectively. D: INS, the confluent cells were cultured in DMEM containing 20% FCS, 0.25 μM DEX, 0.5 mM IBMX supplemented with 0, 0.5, 5, 50 μg/ml INS, respectively. E: Effect of each combination of DEX, IBMX, and IBMX on adipocyte differentiation of DFAT-P cells. The confluent cells were cultured for 12 days in various combinations. CONT, growth medium; DEX, 0.25 μM DEX; IBMX, 0.5 mM IBMX; INS, 5 μg/ml INS. The GPDH activity was determined 12 days after adipogenic induction. Values are mean ± SD of five independent experiments performed on the least triplicate dishes. $^{a-c}P < 0.05$.

subculturing. They are useful for a consistent and reproducible culture system. To test whether DFAT-P cells could be used as a cell line, DFAT-P cells established from five individual pigs were examined in terms of proliferation and differentiation in each subculture (Fig. 4). The number of DFAT-P cells derived from four pigs briefly decreased at 6 and 7 passages; however, the proliferation ability of DFAT-GFP cells derived from three individual pigs was maintained until 37 passages (Fig. 4A). Figure 4B shows the GPDH activity of DFAT-P cells 10 days after adipogenic induction in each subculture. There was no difference in GPDH activity among subcultures in three of five clones. However, the GPDH activity of the other two clones rapidly decreased until 22 passages, equivalent to the negative control (no induction). These results indicated that DFAT-P cells could retain the ability to proliferate and differentiate into mature adipocytes in each subculture until at least 37 passages, and therefore, possessed the characteristics suitable for a cell line.

To examine whether DFAT-P cells spontaneously transformed during long-term subculture, we determined the ploidy of these cells at passage 35 by metaphase analysis. Seventy-four percent of the cells were diploid (38 chromosomes), 11% contained 1 additional chromosome, 8% had less than 38 chromosomes because of random loss, 6% showed structural abnormalities, and 1% had more than 58 chromosomes. These data suggest that even after long-term maintenance in culture, most cells maintain a normal phenotype.

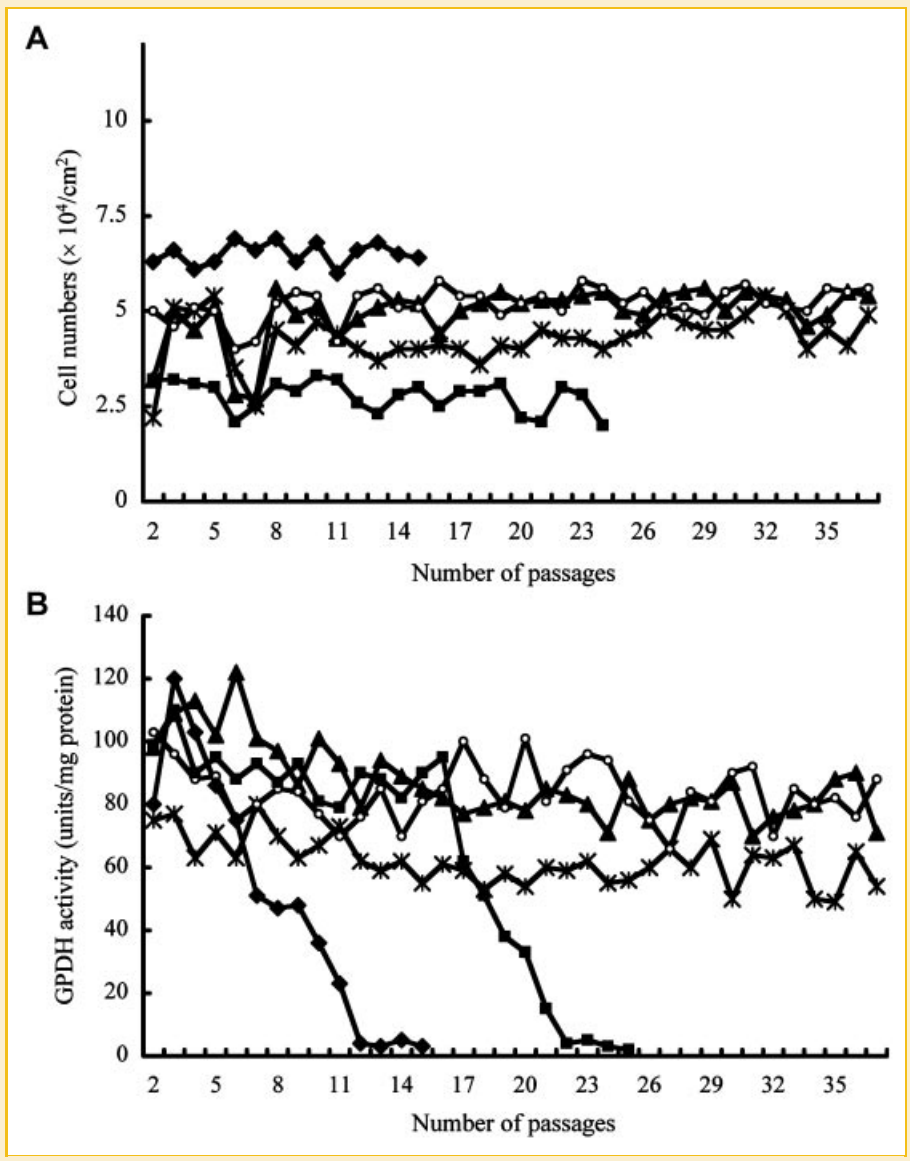

Fig. 4. Effect of long-term subculture on the ability of DFAT-P cells to proliferate and differentiate. A: Proliferation activity of DFAT-P cells in each subculture. DFAT-P cells established from five individual pigs were placed into culture dishes at an initial density of 10^3 cells/cm^2 and cultured in DMEM supplemented with 20% FCS. At 10 days, the cells were harvested by trypsinization, and the number of cells per dish was determined. B: GPDH activity of DFAT-P cells in each subculture. The confluent DFAT-P cells established from five individual pigs were cultured in DMEM containing 20% FCS, 0.25 μM DEX, 0.5 mM IBMX, and 5 μg/ml INS. GPDH activity was determined at 10 days after adipogenic induction. ▲, Clone 1; *, clone 2; ○, clone 3; ■, clone 4; ◆, clone 5.

DISCUSSION

In this study, we describe the establishment of novel porcine preadipocyte cell lines, DFAT-P, derived from subcutaneous fat tissue. DFAT-P cells originate from a homogeneous cell population derived from a single fraction of mature adipocytes. These cells proliferate extensively until they become confluent and differentiate into mature adipocytes upon treatment with DEX, IBMX, and INS. These cells proliferate more effectively compared to SVF cells derived from porcine adipose tissue. The growth rate of DFAT-P cells is stable with increasing passage number, and the cells were successfully maintained and differentiated until at least passage 37. DFAT-P cells maintain a normal diploid phenotype even after long-term maintenance in culture. We thus propose that DFAT-P cells and the method that we have described here provide a novel model system for studying the mechanisms of adipocyte differentiation and metabolism, as a substitute for human preadipocytes.

The possible contamination of the mature adipocyte population with stromal cells, such as preadipocytes and adipose tissue-derived stem cells, was a concern in our study. Several researchers have reported that collagenase processing, including multiple cycles of centrifugation and filtration, is critical for obtaining uniform single-cell suspensions of mature adipocytes [Sugihara et al., 1986, 1987; Shigematsu et al., 1999; Zhang et al., 2000; Justesen et al., 2004; Tholpady et al., 2005]. In our previous experiments, microscopic and fluorescence-activated cell sorting analysis of floating primary cells derived from GFP transgenic mice stained for neutral lipid with Nile red and analysis of nuclei with 4′6-diamidino-2-phenylindole revealed that 98.8% of cells within the floating cell fraction are mononuclear mature adipocytes with no stromal-vascular cells [Nobusue et al., 2008]. In the present study, we counted the number of nuclei per porcine mature adipocyte stained by Hoechst 33342 to determine the number of mononuclear mature adipocytes, without contaminating preadipocytes and adipose tissue-derived stem cells attached to the mature adipocytes. The analysis indicated that 99.5% of the isolated porcine mature adipocytes have a single nucleus (Table II). This result is consistent with those of previous reports [Shigematsu et al., 1999; Justesen et al., 2004; Tholpady et al., 2005; Nobusue et al., 2008]. Considering past and present results, our findings indicate that a homogeneous mature adipocyte population can be easily isolated from porcine subcutaneous adipose tissue by enzymic digestion and multiple rounds of centrifugation, and filtration.

To obtain primary preadipocytes, several passages should eliminate contamination by other cell types by cloning using a limiting dilution method, because primary preadipocytes are derived from the SVF of adult adipose tissue. However, because primary preadipocytes are characterized by low proliferation, it is difficult to obtain sufficient numbers for in vitro experiments [Darimont and Mace, 2003]. DFAT-P cells can be obtained from small amounts of adipose tissue. We reproducibly isolated 1×10^6 mature adipocytes from approximately 1 g of porcine subcutaneous adipose tissue. We found that 2.4×10^4 cells/cm^2 mature adipocytes were needed to obtain sufficient numbers of DFAT-P cells (11×10^4 cells/cm^2 in primary culture). Furthermore, the growth ability of DFAT-P cells is stable with increasing passage number. These findings indicate that DFAT-P cells may be applicable to various in vitro experiments because adequate quantities of these cells can be prepared from small amounts of adipose tissue.

GPDH activity, a late marker of adipocyte differentiation in vitro, in DFAT-P cells was approximately 2.6-fold higher than that in SVF cells 12 days after adipogenic induction (Fig. 2C). The oil red-positive areas were greater in DFAT-P cells than in SVF cells (Fig. 2D). SVF cells contains various cell types besides preadipocytes, including erythrocytes, macrophages, monocytes, endothelial cells, fibroblasts, and pericytes. Therefore, it is suggested that only a few SVF cells can respond to the adipogenic cocktail. However, DFAT-P cells constitute a homogeneous cell population derived from a single fraction of porcine mature adipocytes. Consequently, we strongly suggest that all DFAT-P cells uniformly possess the characteristics of preadipocytes; therefore, a homogeneous preadipocyte population can be obtained from porcine mature adipocytes by ceiling culture without cloning by a limiting dilution method.

It is generally known that 3T3-L1 and 3T3-F442A spontaneously accumulate lipids and differentiate into mature adipocytes upon growth arrest [Green and Meuth, 1974; Green and Kehinde, 1975, 1976]. In this study, DFAT-P cells cultured in the presence of the adipogenic cocktail showed an increase in GPDH activity and accumulation of lipid droplets, whereas this was absent in cells cultured in medium without the adipogenic cocktail (Fig. 2C,D). Furthermore, spontaneous adipogenesis is not observed in primary human preadipocytes after they become confluent [Koellensperger et al., 2006], and this property is consistent with that of DFAT-P cells. Therefore, the tight control of differentiation in DFAT-P cells could be used advantageously in screening and identifying molecules that are important in inducing adipocyte differentiation.

It is known that members of the C/EBP family and PPARγ are involved in terminal differentiation of the adipogenesis process by subsequent transactivation of adipocyte-specific genes [Gregoire et al., 1998; Rangwala and Lazar, 2000]. Notably, PPARγ2 and C/EBPα are together identified as an adipocyte-specific master switch for terminal differentiation [Rosen and MacDougald, 2006]. DFAT-P cells expressed these master genes only after adipogenic induction (Fig. 2A). This finding indicates that DFAT-P cells are dedifferentiated cells until the preadipocyte phase, and can dedifferentiate into mature adipocytes upon adipogenic induction. FABP4 is an important regulator of systemic insulin sensitivity as well as lipid and glucose metabolism. It is most widely used as a marker of terminally differentiated adipocytes [Bernlohr et al., 1985; Amri et al., 1991; MacDougald and Lane, 1995]. In this study, FABP4 mRNA expression was clearly detected even before adipogenic induction in DFAT-P cells (Fig. 2A). Though the reasons for this are unclear, we assume that DFAT-P cells partly retain the characteristics as adipocytes because these cells are derived from dedifferentiated mature adipocytes.

FCS contains high levels of the adipogenic factor acting directly on adipocyte differentiation, except growth and other various factors [Kuri-Harcuch and Green, 1978]. In addition, it has been previously reported that adipogenesis was induced in 3T3-F442A cells by exposure to FCS in a dose-dependent manner. In this study, the GPDH activity of DFAT-P cells after adipogenic induction significantly increased when supplemented with about 20% FCS in a

dose-dependent manner (Fig. 3A). Consequently, it is suggested that adipogenic factors included in serum promote adipocyte differentiation in DFAT-P cells. Previous studies have reported a significant dosage effect of hydrocortisone and insulin on GPDH activity, achieving maximum effect at a concentration of 100 ng/ml (0.25 μM) and 100 nM (5 μg/ml) in stromal-vascular cells from porcine adipose tissue [Ramsay et al., 1989; Surywan et al., 1997]. These hormonal concentrations are generally used for culturing the preadipocyte cell lines 3T3-L1, 3T3-F442A, and RMB-AD [Rubin et al., 1978; Marko et al., 1995]; they correspond with optimal concentrations of INS and DEX in DFAT-P cells (Fig. 3A,B). It has been reported that 0.5 mM IBMX is very effective in promoting adipocyte conversion of 3T3-L1 cells [Russell and Ho, 1976], and this dose is widely used for several preadipocyte cell lines [Rubin et al., 1978; Marko et al., 1995]. The optimal concentration of IBMX in DFAT-P cells is 0.5 mM, in accordance with previous studies (Fig. 3C).

3T3-L1, 3T3-F442A, and RMB-AD preadipocyte cell lines undergo differentiation upon treatment with DEX, INS, and IBMX [Green and Kehinde, 1975; MacDougald and Lane, 1995; Marko et al., 1995]. When DFAT-P cells were treated with DEX, INS, and IBMX, the GPDH activity was higher than other hormonal combinations (Fig. 3E). Therefore, the adipocyte differentiation cocktail provides the best culture conditions for in vitro studies of adipocyte differentiation in DFAT-P cells. Previous studies reported that INS has a positive effect on adipocyte differentiation in mammals [Ramsay et al., 1992; Surywan et al., 1997]. In contrast, we found that INS alone did not have a direct effect of adipocyte differentiation in DFAT-P cells compared to DEX or IBMX alone (Fig. 3E). Steinberg and Brownstein [1982] have suggested that INS is not an inducer of adipocyte differentiation but merely enhances lipid synthesis in cells committed to differentiation. DEX, a synthetic glucocorticoid agonist, strongly stimulates differentiation at early stages in cultured preadipocytes. Thus, DEX is recognized as an essential inducer of adipocyte differentiation for in vitro studies of cell lines and primary culture in most mammals [Wolf, 1999; Hausman, 2003]. Russell and Ho [1976] have reported that IBMX, a synthetic compound, triggers the differentiation process by rapidly and irreversibly programming the fibroblasts to differentiate into adipocytes. In our study, addition of DEX and INS or IBMX and INS enhanced the rate of adipocyte differentiation, although INS alone was ineffective (Fig. 3E). Taken together, these findings suggest that INS does not function as a molecular inducer of adipocyte differentiation in DFAT-P cells. Instead, it leads to an increase in lipid deposition of adipocytes already committed to differentiation by DEX or IBMX.

Our collaborative research group has clarified that the in vitro developmental rate of the nuclear transfer embryo reconstructed with DFAT-P cells was comparable to that of the control fetal fibroblast-derived embryos and that transfer of 1,004 nuclear transfer embryos with DFAT-P cells to five recipients led to five cloned piglets [Tomii et al., 2005]. These findings suggest that the nuclear condition of DFAT-P cells is highly stable even after subculture. Moreover, donor DFAT-P cells labeled with the fluorescent cell-tracer stain were successfully transplanted into the cloned pigs without immune rejection, and they differentiated into mature adipocytes in vivo 3 weeks after transplantation [Shimada et al., 2006]. Thus, we propose that DFAT-P cells are available for studying adipose tissue formation and development as well as pharmacological and nutritional regulation of adipocyte growth and differentiation in vivo.

In summary, we have established novel porcine preadipocyte cell lines, DFAT-P, derived from dedifferentiated mature adipocytes by the ceiling culture method for in vitro mature adipocytes. DFAT-P cells can proliferate extensively until they became confluent. These cells fully differentiate into mature adipocytes upon treatment with DEX, INS, and IBMX. No changes in their ability to proliferate and differentiate were observed during propagation of these cells, and they have been successfully carried and differentiated until at least 37 passages. Furthermore, DFAT-P cells maintain a normal diploid phenotype even after long-term maintenance in culture. This achievement may lead to easy establishment of porcine preadipocyte cell lines in a reproducible fashion as a substitute for human preadipocytes and novel model systems for studying the mechanisms of adipocyte differentiation and metabolism in vitro and in vivo.

ACKNOWLEDGMENTS

We thank to Masashi Hashimoto (Kobe University) for technical assistance. This work was supported by NUBS Research Grant and HAITEKU Research Grant to Nihon University. This work was also supported by a Nihon University Multidisciplinary Research Grant.

REFERENCES

Aaronson SA, Todaro GJ. 1968. Development of 3T3-like from Balb-c mouse embryo cultures: Transformation susceptibility to SV40. J Cell Physiol 72: 141–148.

Amri EZ, Betrand B, Ailhaud G, Grimaldi P. 1991. Regulation of adipose cell differentiation. I. Fatty acids are inducers of the aP2 gene expression. J Lipid Res 32:1449–1456.

Bernlohr DA, Doering TL, Kelly TJ, Jr., Lane MD. 1985. Tissue specific expression of p422 protein, a putative lipid carrier, in mouse adipocytes. Biochem Biophys Res Commun 132:850–855.

Butterwith SC, Peddie CD, Goddard C. 1992. Effect of transforming growth factor-alpha on chicken adipocyte precursor cells in vitro. J Endocrinol 134: 163–168.

Darimont C, Mace K. 2003. Immortalization of human preadipocytes. Biochimie 85:1231–1233.

Green H, Meuth M. 1974. An established pre-adipose cell line and its differentiation in culture. Cell 3:127–133.

Green H, Kehinde O. 1975. An established pre-adipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5:19–27.

Green H, Kehinde O. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7:105–113.

Gregoire FM, Smas CM, Sul HS. 1998. Understanding adipocyte differentiation. Physiol Rev 78:783–809.

Hausman GJ, Campion DR, Martin RJ. 1980. Search for the adipocyte precursor cell and factors that promote its differentiation. J Lipid Res 21: 657–670.

Hausman GJ. 2003. Dexamethasone induced preadipocyte recruitment and expression of CCAAT/enhancing binding protein alpha and peroxisome

proliferator activated receptor-gamma proteins in porcine stromal-vascular (S-V) cell cultures obtained before and after the onset of fetal adipogenesis. Gen Comp Endocrinol 133:61–70.

Hwang CS, Loftus TM, Mandrup S, Lane MD. 1997. Adipocyte differentiation and leptin expression. Annu Rev Cell Dev Biol 13:231–259.

Justesen J, Pedersen SB, Stenderup K, Kassem M. 2004. Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. Tissue Eng 10:381–391.

Koellensperger E, von Heimburg D, Markowicz M, Pallua N. 2006. Human serum from platelet-poor plasma for the culture of primary human pre-adipocytes. Stem Cell 24:1218–1225.

Kozak LP, Jensen JT. 1974. Genetic and developmental control of multiple forms of L-glycerol 3-phosphate dehydrogenase. J Biol Chem 249:7775–7781.

Kuri-Harcuch W, Green H. 1978. Adipose conversion of 3T3 cells depends on a serum factor. Proc Natl Acad Sci USA 75:6107–6109.

Larsen MO, Rolin B. 2004. Use of the Gottingen minipig as a model of diabetes, with special focus on type I diabetes research. ILAR J 45:303–313.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurements with the folin phenol regent. J Biol Chem 193:265–275.

Lunney JK. 2007. Advances in swine biomedical model genomics. Int J Biol Sci 3:179–184.

MacDougald OA, Lane MD. 1995. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 64:345–373.

Marko O, Cascieri MA, Ayad N, Strader CD, Candelore MR. 1995. Isolation of a preadipocyte cell line from rat bone marrow and differentiation to adipocytes. Endocrinology 136:4582–4588.

Nobusue H, Endo T, Kano K. 2008. Establishment of a preadipocyte cell line derived from mature adipocytes of GFP transgenic mice and formation of adipose tissue. Cell Tissue Res 332:435–446.

Pang WJ, Yu TY, Bai L, Yang YJ, Yang GS. 2009. Tissue expression of porcine FoxO1 and its negative regulation during primary preadipocyte differentiation. Mol Biol Rep 36:165–176.

Ramsay TG, White ME, Wolverton CK. 1989. Glucocorticoids and the differentiation of porcine preadipocytes. J Anim Sci 67:2222–2229.

Ramsay TG, Rao SV, Wolverton CK. 1992. In vitro systems for the analysis of the adipose tissue in domestic animals. J Nutr 122:806–817.

Rangwala SM, Lazar MA. 2000. Transcriptional control of adipogenesis. Annu Rev Nutr 20:535–559.

Reaven GM. 1988. Role of insulin resistance in human disease. Diabetes 37:1595–1607.

Rodbell M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lypolysis. J Biol Chem 239:375–380.

Rosen ED, MacDougald OA. 2006. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 7:885–896.

Rubin CS, Hirsch A, Fung C, Rosen OM. 1978. Development of hormone receptors and hormonal responsiveness in vitro. J Biol Chem 253:7570–7578.

Russell TR, Ho R. 1976. Conversion of 3T3 fibroblasts into adipose cells: Triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutylxanthine. Proc Natl Acad Sci USA 73:4516–4520.

Sanosaka M, Minashima T, Suzuki K, Watanabe K, Ohwada S, Hagino A, Rose MT, Yamaguchi T, Aso H. 2008. A combination of octanoate and oleate promotes in vitro differentiation of porcine intramuscular adipocytes. Comp Biochem Physiol B 149:285–292.

Shigematsu M, Watanabe H, Sugihara H. 1999. Proliferation and differentiation of unilocular fat cells in the bone marrow. Cell Struct Funct 24:89–100.

Shimada A, Tomii R, Kano K, Nagashima H. 2006. Transplantation and differentiation of donor cells in the cloned pigs. Biochem Biophys Res Commun 344:455–462.

Smas CM, Sul HS. 1995. Control of adipocyte differentiation. Biochem J 309:697–710.

Steinberg MM, Brownstein BL. 1982. A clonal analysis of the differentiation of 3T3-L1 preadipocyte cells: Role of insulin. J Cell Physiol 113:359–364.

Sugihara H, Yonemitsu N, Miyabara S, Yun K. 1986. Primary cultures of unilocular fat cells: Characteristics of growth in vitro and changes in differentiation properties. Differentiation 31:42–49.

Sugihara H, Yonemitsu N, Miyabara S, Toda S. 1987. Proliferation of unilocular fat cells in the primary culture. J Lipid Res 28:1038–1045.

Surywan A, Swason LV, Hu CY. 1997. Insulin and hydrocortisone, but not triiodothyronine, are required for the differentiation of pig preadipocyte in primary culture. J Anim Sci 75:105–111.

Tholpady SS, Aojanepong C, Llull R, Jeong JH, Mason AC, Futrell JW, Ogle RC, Katz AJ. 2005. The cellular plasticity of human adipocytes. Ann Plast Surg 54:651–656.

Todaro GJ, Green H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17:299–313.

Tomii R, Kurome M, Ochiai T, Wako N, Ueda H, Hirakawa K, Kano K, Nagashima H. 2005. Production of cloned pigs by nuclear transfer of preadipocytes established from adult mature adipocytes. Cloning Stem Cells 7:279–288.

Tomlinson JJ, Boudreau A, Wu D, Atlas E, Hache RJ. 2006. Modulation of early human preadipocyte differentiation by glucocorticoids. Endocrinology 147: 5284–5293.

Van RLR. 1985. The adipocyte precursor cell. In: Cryer A, Van RLR, (Eds.) New perspectives in adipose tissue: Structure, function and development. Butterworths. London, UK: pp 353–382.

Wise LS, Green H. 1979. Participation of one isozyme of glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. J Biol Chem 254:273–275.

Wolf G. 1999. The molecular mechanism of the stimulation of adipocyte differentiation by a glucocorticoid. Nutr Rev 57:324–326.

Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Park C, Choi K, Bickel PE. 2006. OP9 mouse stromal cells rapidly differentiate into adipocytes: Characterization of a useful new model of adipogenesis. J Lipid Res 47:450–460.

Yagi K, Kondo D, Okazaki Y, Kano K. 2004. A novel preadipocyte cell line established from mouse adult mature adipocytes. Biochem Biophys Res Commun 321:967–974.

Zhang HH, Kumar S, Barnett AH, Eggo MC. 2000. Ceiling culture of mature human adipocytes: Use in studies of adipocyte function. J Endocrinol 164:119–128.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.