IDL in FLD patients. More cases need to be investigated to draw the definitive conclusions on the relation among *LCAT* mutation, *APOE* genotype and lipoprotein profile in FLD patients.

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Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy

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Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy

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[1]*Center for Advanced Medicine, Chiba University Hospital, Chiba University;* [2]*Department of Genome Research and Clinical Application,* [3]*Department of Plastic and Reconstructive Surgery, and* [4]*Department of Immunology, Graduate School of Medicine,* [5]*Chiba University; and* [6]*CellGenTech, Chiba, Japan*

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Asada S, Kuroda M, Aoyagi Y, Fukaya Y, Tanaka S, Konno S, Tanio M, Aso M, Satoh K, Okamoto Y, Nakayama T, Saito Y, Bujo H. Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy. *Am J Physiol Cell Physiol* 301: C181–C185, 2011. First published April 6, 2011; doi:10.1152/ajpcell.00080.2011.—Adipose tissue is expected to provide a source of proliferative cells for regenerative medicine and cell-transplantation therapies using gene transfer manipulation. We have recently identified ceiling culture-derived proliferative adipocytes (ccdPAs) from the mature adipocyte fraction as cells suitable as a therapeutic gene vehicle because of their stable proliferative capacity. In this study, we examined the capability of adipogenic differentiation of the ccdPAs compared with stromal vascular fraction (SVF)-derived progenitor cells (adipose-derived stem cells, ASCs) with regard to their multipotential ability to be converted to another lineage and therefore their potential to be used for regenerative medicine research. After in vitro passaging, the surface antigen profile and the basal levels of adipogenic marker genes of the ccdPAs were not obviously different from those of the ASCs. However, the ccdPAs showed increased lipid-droplet accumulation accompanied with higher adipogenic marker gene expression after stimulation of differentiation compared with the ASCs. The higher adipogenic potential of the ccdPAs than the ASCs from the SVF was maintained for 42 days in culture. Furthermore, the difference in the adipogenic response was enhanced after partial stimulation without indomethacin. These results indicate that the ccdPAs retain a high adipogenic potential even after in vitro passaging, thus suggesting the commitment of ccdPAs to stable mature adipocytes after autotransplantation, indicating that they may have potential for use in regenerative and gene-manipulated medicine.

gene therapy; adipose tissue-derived stem cells; adipogenesis

ADIPOSE TISSUE is now recognized as a source of proliferative cells for cell-based gene therapy (2) and for regenerative therapy (4, 5). The cells propagated from aspirated fat tissue have been shown to proliferate rapidly and differentiate into mature adipocytes both in vitro and in vivo (2, 4, 5). Although the prepared cells are highly heterogeneous with regard to differentiation and adipogenecity, two types of preparations have been methodologically reported to be sources of adipose tissue-derived proliferative cells. One is the stromal vascular fractions (SVFs), which can be obtained as a sediment by the centrifugation of collagenase-digested fat tissue (15). Numerous studies have reported that adherent cells obtained from SVFs can differentiate into not only adipocytes, but also other cell lineages, and these cells are recognized as adipose-derived stem cells (ASCs) (11). The other cell preparation is obtained from the floating mature adipocytes fraction obtained from the centrifugation, followed by a ceiling culture (13). These cells have mainly been used for the culture of mature adipocytes after proper differentiation stimulation, although their limited abilities to differentiate into other lineages have been demonstrated to be maintained in vitro (9, 10).

In the clinical application of cell-based medicine using preadipocytes to patients, it is required that the transplanted cells reside stably at the subcutaneous adipose space without unexpected proliferation or migration and that they differentiate into adipocytes to reconstruct adipose tissue. We have previously shown the transplantation of gene-transduced adipocytes to be a candidate therapy for patients lacking insulin, growth hormone, or lecithin:cholesterol acyltransferase (1, 6, 7). We have recently identified proliferative cells with a higher adipogenic differentiation potential adequate for this strategy. The proliferative adipocytes obtained immediately after a 7-day primary culture (ceiling culture-derived proliferative adipocytes, ccdPAs) have suitable gene transduction characteristics for gene therapy applications (8). The ccdPAs are expected to provide vehicle cells for protein replacement therapy using autotransplantation of exogenous gene-transduced cells. However, little is known with respect to the differences in the differentiation potential between ccdPAs and SVF-derived ASCs, and a comparison of the adipogenic status between ccdPAs and ASCs would provide insight that would be relevant for plastic and reconstructive surgery, as well as future strategies using adipose tissue-based gene therapy combined with regenerative medicine. In this study, the adipogenic potential of ccdPAs was examined compared with ASCs from SVFs as multipotential adipose tissue-derived cells.

MATERIALS AND METHODS

Cell culture and adipogenic differentiation. The study was approved by the Ethics Committee of Chiba University School of Medicine, and informed consent was obtained from the healthy volunteers. Experiments were performed with the adipose tissue specimens obtained from four different volunteers, and representative data are described in the paper. ccdPAs and ASCs were prepared according to our previous report (8). Essentially, the floating fraction and the sediment after collagenese digestion followed by centrifugation were utilized for source of ccdPAs and ASCs, respectively. The floating fraction was subjected to ceiling culture (13). The sediment was cultured by regular method to obtain adherent proliferative cells

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(ASCs). DMEM/F12-HAM (Sigma-Aldrich, St. Louis, MO) containing 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (Gentacin, Schering-Plough, Kenilworth, NJ) was used for both preparations. After 7 days primary culture, ccdPAs and ASCs were passaged twice a week with MesenPRO medium (Life Technologies, Carlsbad, CA) and used for further experiment. Bone marrow derived-mesenchymal stem cells (BM-MSC) were purchased from Lonza (Basel, Switzerland). For adipogenic induction, cells were seeded on 48-well or 6-well plates and then were incubated for 3 days to confluence. Next, growth medium was changed to adipogenic induction medium (Lonza) and cultured for 2 wk and then lipids were stained with Oil Red O.

FACS analysis. The cells cultured in MesenPRO medium for 14 days after the preparation were subjected to analysis of surface antigen as described previously (8). Fluorescein isocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies were purchased from BD Farmingen (San Diego, CA), Beckman Coulter (Fullerton, CA), or Ancell (Bayport, MN). Five thousand events were acquired for each antibody on a FACS Calibur apparatus using the CELLQuest acquisition software program (Becton Dickinson, Flanklin Lakes, NJ).

Gene expression analysis. Total RNA was prepared at each time point by RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. One microgram of total RNA was subjected to cDNA synthesis by ReverTraAce qPCR RT kit (Toyobo, Osaka, Japan). The amounts of mRNA were quantified by TaqMan methodology using ABI7500 real-time PCR apparatus. Probe and primer sets for CCAAT/enhancer binding protein δ (C/EBPδ), peroxisome proliferator-activated receptor γ2 (PPARγ2), adipocyte protein 2 (aP2), and leptin genes were purchased from Applied Biosystems (Life Technologies). A C_t value of 35 was considered as detection limit.

Statistical analysis. Data are presented as the means ± SD. Statistical comparisons were made by either Student's *t*-test or by ANOVA followed by the post hoc Dunnett test using the SPSS software program. In all cases, *P* values of <0.05 were considered to be statistically significant.

RESULTS

The ccdPAs express adipogenic markers and cell surface antigens similar to ASC cells in culture. We obtained ccdPAs after a 7-day ceiling culture as described previously (8). We first examined the expression of adipogenic markers (C/EBPδ, PPARγ2, aP2, and leptin genes) in these cells compared with the ASCs obtained from the SVF of the same fat origin after 7 days of regular plating culture in the same growth medium as the ceiling culture and also to BM-MSCs that were not related to adipocyte lineage. The messenger RNA levels of C/EBPδ in ccdPAs were significantly higher than those in ASCs at *days 1*, *4*, and *7* (Fig. 1*A*). The expression of PPARγ2 was not detected on *days 1*, *4*, *7*, or *14* in any of the three cell lines (ccdPAs, ASCs, and BM-MSCs) (data not shown). The expression of aP2 in ccdPAs and ASCs was detected on *day 1*, and the expression levels in both ccdPAs and ASCs were decreased on *day 4*. On *days 4*, *7*, and *14*, and the aP2 expression level in the ccdPAs was significantly higher than the ASCs, but it was not significantly different from the BM-MSCs, thus indicating that the aP2 expression levels on *days 4*, *7*, and *14* in ccdPAs and ASCs are not physiologically relevant to the adipose lineage (Fig. 1*B*). The expression of leptin was not detected in ASCs and BM-MSCs at any of the time points tested. However, on *days 1*, *4*, and *7*, the expression of leptin in ccdPAs was detected and became undetectable by *day 14* (Fig. 1*C*). After 14 days of preparation, the surface marker expression profiles showed no difference between ccdPAs and SVF-derived ASCs (Fig. 1*D*). Therefore, the expression levels of adipogenic genes and surface markers were not different between ccdPAs and ASCs at 14 days after preparation.

ccdPAs show a higher adopogenic response after differentiation stimulation than ASCs derived from SVF. We evaluated the adipogenesis of ccdPA during differentiation into mature adipocytes. The ccdPAs and ASCs at 14 days after preparation

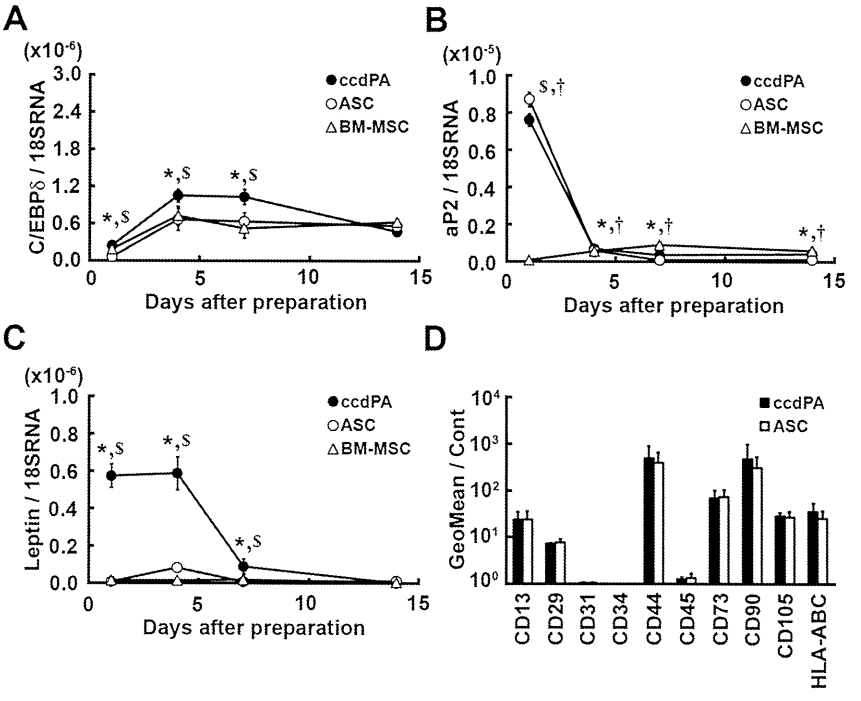

Fig. 1. Expression of adipogenic genes and cell surface markers of ceiling culture-derived proliferative adipocytes (ccdPAs) and adipose-derived stem cells (ASCs). After 7 days of primary culture with DMEM/F12-HAM supplemented with 20% fetal bovine serum (FBS), the ccdPAs and ASCs were passaged with MesenPRO medium. Bone marrow derived-mesenchymal stem cells (BM-MSCs, passage number 3 on *day 0*) were passaged in same manner. At each time point, the expression levels of mRNA for CCAAT/enhancer binding protein δ (C/EBPδ) (*A*), adipocyte protein 2 (aP2) (*B*), and leptin (*C*) were quantified by qRT-PCR. $*P < 0.05$, ccdPA vs. ASC, $\$P < 0.05$, ccdPA vs. BM-MSC, $\dagger P < 0.05$, ASC vs. BM-MSC. The expression of cell surface markers was analyzed by flow cytometry at 14 days after preparation (*D*).

were plated and grown for 3 days to confluency and then stimulated for adipogenic differentiation with medium containing insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and indomethacin (IND), and the appearance and adipogenic gene expression were analyzed for 14 days. A histological analysis suggested that the lipid droplet formation had increased in the ccdPAs compared with the ASCs (Fig. 2*A*). An adipogenesis-related gene analysis showed that the expression of PPARγ2 was detectable on *day 1* in both ccdPAs and ASCs and was gradually increased until *day 8* and then declined in both cell lines (Fig. 2*B*). The PPARγ2 expression in ccdPAs was higher than that of ASCs at all time points of stimulation (Fig. 2*B*). The aP2 expression was maximal on *day 8* or *10* (Fig. 2*C*), and its expression was also higher in ccdPAs than in ASCs at all time points (Fig. 2*C*). Therefore, ccdPAs show a higher adipogenic response during differentiation in vitro.

ccdPAs retain higher adipogenic potential than ASCs during in vitro passaging. We next examined the capability of adipogenic differentiation during passaging. Cells freshly harvested after 7 days of primary culture (designated as *day 0* in this text) and the cells that were further cultured until *day 7*, *14*, and *42* were subjected to adipogenic differentiation. During the passage period, the doubling time of ccdPAs and ASCs was not significantly different (1.60 ± 0.34 days vs. 1.57 ± 0.32 days) when they were grown in MesenPRO medium. The histological observations (Fig. 3*A*) showed that both cell lines gradually lost their capabilities for adipogenic differentiation during in vitro passage. At 14 days after stimulation, there was a clear difference in the numbers of differentiated lipid droplet-containing cells. A gene expression analysis showed that the ccdPAs expressed significantly increased levels of aP2 mRNA compared with the SVF-derived ASCs when the cells that were passaged for 0, 7, 14, and 42 days after preparation were subjected to adipogenic stimulation (Fig. 3*B*). These results show that the ccdPAs retain a higher adipogenic potential than the ASCs during in vitro passaging.

ccdPAs exhibit an increased response to the partial adipogenic stimulation compared with ASCs. To further characterize the adipogenic status of the ccdPAs in terms of lineage, we employed different combinations of DEX, IBMX, and IND. After 14 days of stimulation, fine lipid-containing cells were observed in the presence of DEX alone in both the ccdPA and ASC cultures (Fig. 4*A*) but not in the presence of IBMX or IND alone (data not shown). We next omitted each reagent from the full cocktail with DEX, IBMX, and IND. Notably, ccdPAs formed relatively large lipid droplets when IBMX was omitted, whereas the ASCs formed only fine droplets (Fig. 4*A*). Moreover, it was difficult to observe any lipid droplet in the ASCs cultured without IND, whereas the ccdPAs formed lipid droplets. We therefore compared the mRNA levels of the PPARγ2 and aP2 genes in ccdPAs and ASCs (Fig. 4*B*). The ccdPAs expressed both adipogenic genes at levels approximately twofold of those in ASCs on *day 14* after incubation with the full stimulatory cocktail (Fig. 4*B*). The difference in the PPARγ2 mRNA levels of ccdPAs and ASCs was increased to 14-fold when the cells were cultured without IND (Fig. 4*C*). The difference in the aP2 mRNA levels of ccdPAs and ASCs were also obviously increased by ~90-fold under the conditions without IND (Fig. 4*D*). Therefore, the ccdPAs clearly have an increased adipogenic differentiation potential during the partial stimulation in the presence of DEX.

DISCUSSION

We have shown that gene-transduced adipocytes can supply insulin (6) and growth hormone (7) at levels sufficient to

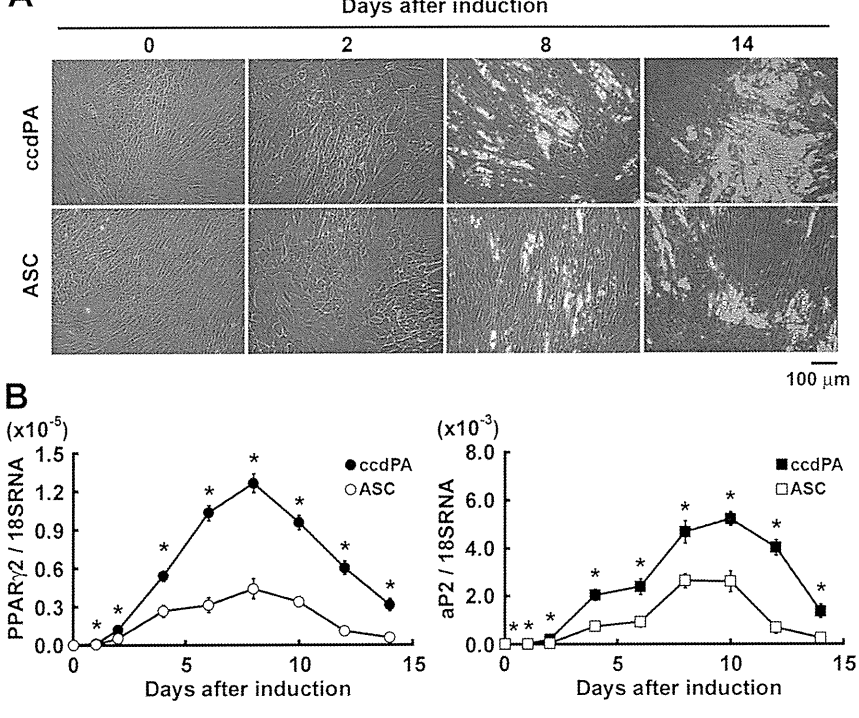

Fig. 2. Comparison of the expression of adipogenic markers in ccdPAs and ASCs during the induction of adipogenesis. *A*: adipogenic induction was performed using ccdPAs (*top*) and ASCs (*bottom*) cultured for 14 days in MesenPRO medium following 7 days of primary culture. The appearance of cells at each time point is shown. *B*: levels of peroxisome proliferator-activated receptor γ2 (PPARγ2) and aP2 gene expression were examined at each time point by qRT-PCR. $^*P < 0.05$.

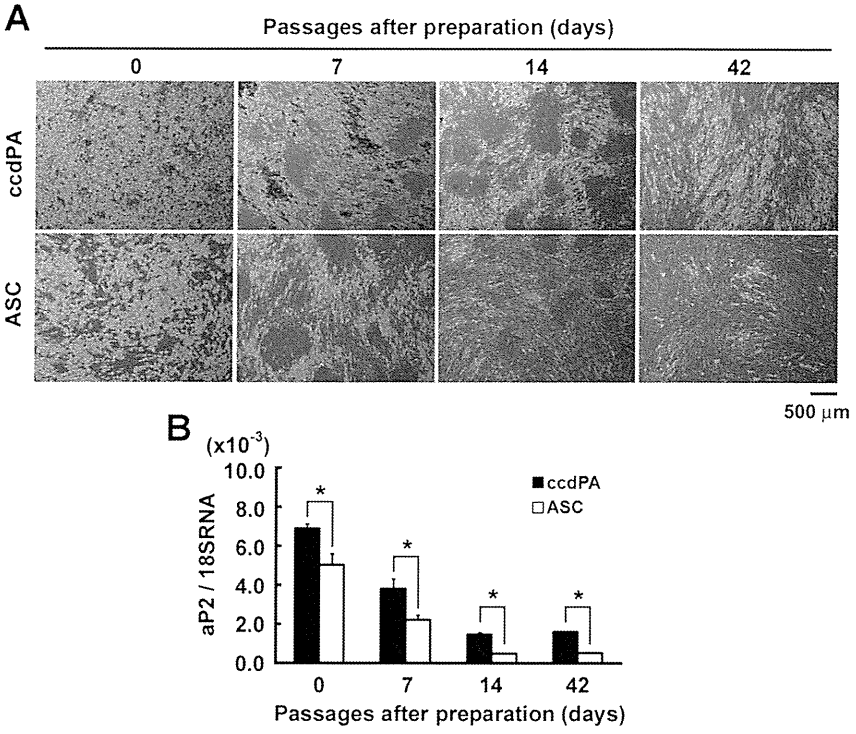

Fig. 3. The effects of consecutive in vitro passaging on the adipogenic potential of ccdPAs and ASCs. The ccdPA and stromal vascular fraction (SVF)-derived cells were obtained after a 7-day ceiling culture and were further cultured in MesenPRO medium for 7, 14, or 42 days. Cells were seeded and incubated for 3 days to confluency, and the medium was replaced by adipogenic induction medium. On *day 14*, the differentiation of the cells was evaluated by the appearance of lipid droplet formation (*A*) and by the expression of the aP2 gene as determined by qRT-PCR (*B*). $*P < 0.05$.

provide improvement of systemic disturbances in animal models. During the development of adipocyte-based protein replacement therapy, the transplanted cells are required to exhibit stable and controllable characteristics of gene transduction efficiency, maintenance of the transduced gene, proliferation, and survival after transplantation, in addition to posing a minimal risk for unexpected phenotypic changes. Considering the successful outcomes for these applications, the properties required for the transplanted adipocytes are different from those for typical regenerative medicine, i.e., homogeneity to

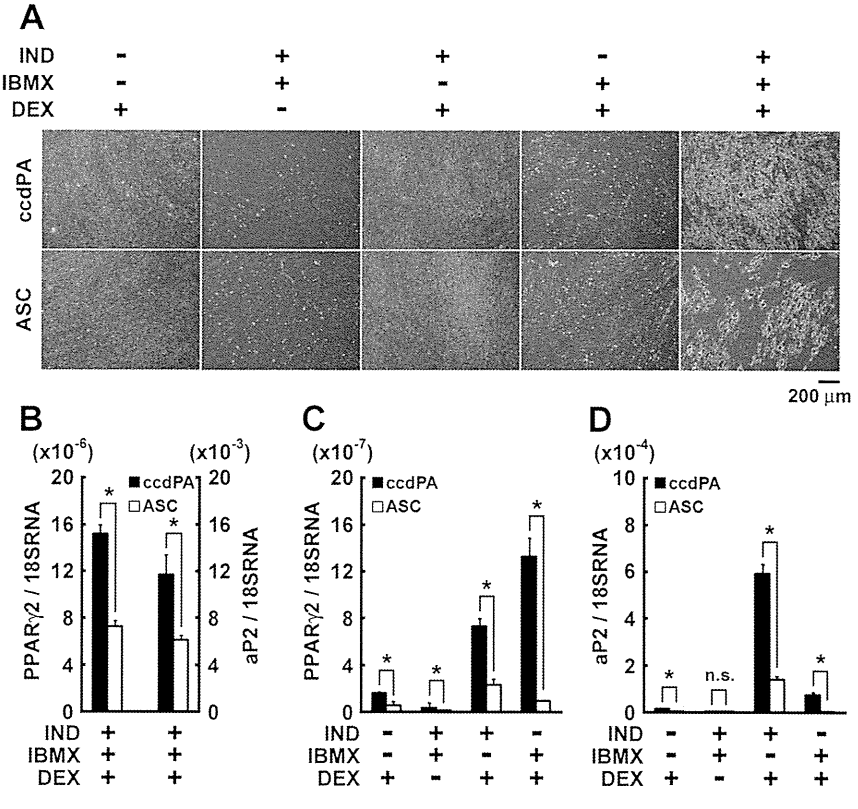

Fig. 4. Effects of differentiation-inducing agents on the adipogenecity and the gene expression levels in ccdPAs. *A*: cells were cultured for 2 wk in growth medium before induction. The appearances of the ccdPAs and ASCs on *day 14* after adipogenic induction with medium containing combinations of the indicated agents are shown. Insulin was included in all medium for the adipogenic induction. The accumulated lipids were stained with Oil Red O. The expression levels of the PPARγ2 and aP2 genes in the cells induced by the full cocktail (*B*) and different combinations (*C*, *D*) of the reagents were quantified on *day 14*. IND, indomethacin; IBMX, 3-isobutyl-1-methylxanthine; DEX, dexamethasone. $*P < 0.05$.

maintain cell stability, but not heterogeneity to keep the multipotentiality.

We have previously utilized the ceiling culture technique to obtain proliferative cells for retrovirus-mediated gene transduction and designated these cells as ccdPAs (8). We identified the optimal primary culture period to be 7 days for high transduction efficiency with minimal integrated copies of therapeutic gene per cell. The obtained gene-transduced ccdPAs stably maintain the exogenously introduced gene during their subsequent culture in vitro. In the present study, we further addressed their adipogenic potential to clarify the suitability of ccdPAs as transplantation cells for use in long-term protein replacement therapy.

The ccdPAs showed increased expression levels of mRNA for the aP2 and leptin genes on *day 1* after 7 days of ceiling culture (see Fig. 1, *C* and *D*). These expression levels of late genes for adipogenic markers had declined to baseline within 7 days of the following culture. At 14 days after preparation, these cells showed no significant difference in their morphological appearance and surface antigen profiles compared with ASCs. However, they exhibited clearly different responsiveness to adipogenic stimuli (see Fig. 2). Even after consecutive in vitro passages, the ccdPAs still had a higher adipogenic potential than the ASCs (see Fig. 3). This higher adipogenic potential was reflected by the observation that ccdPAs expressed increased levels of PPARγ2 and aP2 mRNAs compared with the SVF-derived ASCs (see Figs. 2 and 3). The differences between ccdPAs and ASCs in terms of the mRNA levels for the PPARγ2 and aP2 genes were even more pronounced when the cells were cultured without IND (see Fig. 4). These results suggest that ccdPAs can be easily differentiated into mature adipocytes and/or that ccdPAs are highly homogeneous preadipocytes, most of which retain an adipogenic potential higher than that of ASCs. On the other hand, these results imply that the ccdPAs are less suitable for applications as regenerative medicine in which the cells are intended to differentiate into other cell lineages. In the present study, we used MesenPRO medium as the regular culture medium for ccdPAs, since the medium has greater advantages for expansion capability (8) and the chromosomal stability. It is possible that different culture conditions may be required to be developed for these regenerative medicine purposes. The implication of these findings for the therapeutic strategies based on adipocyte engineered protein delivery includes many metabolic diseases in addition to congenital circulating enzyme deficiencies. The high adipogenic potential of ccdPAs suggests the possible use of ccdPA for improving the cosmetic and metabolic abnormalities observed in lipodystrophy (3, 12, 14). The expandability of the transplanted ccdPA with the secretion properties of leptin and other cytokines should therefore be further studied in future studies.

In summary, ccdPAs retain their capability for adipogenic differentiation longer than ASCs, although the basal levels of the adipogenic differentiation markers examined are undistinguishable between the two cell lines. More precise investigations of ccdPAs using SVF-derived ASCs as reference cells will be helpful not only to distinguish ccdPAs from ASCs but also to provide a better understanding of the mechanism of adipogenesis and the physiology of adipose tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Fibrin glue increases the cell survival and the transduced gene product secretion of the ceiling culture-derived adipocytes transplanted in mice

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Abbreviations: ccdPA, ceiling culture-derived proliferative adipocytes; LCAT, phosphatidylcholine-sterol O-acyltransferase; PPAR, peroxisome proliferator-activated receptor

Abstract

The development of clinically applicable scaffolds is important for the application of cell transplantation in various human diseases. The aims of this study are to evaluate fibrin glue in a novel protein replacement therapy using proliferative adipocytes and to develop a mouse model system to monitor the delivery of the transgene product into the blood and the fate of the transduced cells after transplantation. Proliferative adipocytes from mouse adipose tissue were transduced by a retroviral vector harboring the human lecithin-cholesterol acyltransferase (*lcat*) gene, and were subcutaneously transplanted into mice combined with fibrin glue. The *lcat* gene transduction efficiency and the subsequent secretion of the product in mouse adipocytes were enhanced using a protamine concentration of 500 μg/ml. Adipogenesis induction did not significantly affect the *lcat* gene-transduced cell survival after transplantation. Immunohistochemistry showed the ectopic enzyme production to persist for 28 days in the subcutaneously transplanted gene-transduced adipocytes. The increased viability of transplanted cells with fibrin glue was accompanied with the decrease in apoptotic cell death. The immunodetectable serum LCAT levels in mice implanted with the fibrin glue were comparable with those observed in mice implanted with Matrigel, indicating that the transplanted *lcat* gene-transduced adipocytes survived and functioned in the transplanted spaces with fibrin glue as well as with Matrigel for 28 days. Thus, this *in vivo* system using fibrin is expected to serve as a good model to further improve the transplanted cell/scaffold conditions for the stable and durable cell-based replacement of defective proteins in patients with LCAT deficiency.

Keywords: adipocytes; enzyme replacement therapy; fibrin tissue adhesive; phosphatidylcholine-sterol O-acyltransferase; tissue scaffolds; transplantation

Introduction

Aspirated fat is a common source of autologous tissue transplantation for the correction of tissue defects in plastic and reconstructive surgery (Billings and May, 1989; Patrick, 2000, 2001). Recent studies have shown that the preadipocytes in aspirated fat are multipotential and implicated in the source of cell-based therapies (Stashower *et al.*, 1999; Zuk *et al.*, 2001; Gimble *et al.*, 2007). One such potential is the high capability for exogenous gene transduction and the secretion of transgene products (Ito *et al.*, 2005). We have recently identified human ceiling culture-derived proliferative adipocytes (h-ccdPA) in subcutaneous adipose tissue, and proposed the application of gene-transduced h-ccdPA to the long-lasting replacement therapy for a variety of inherited or acquired gene-defective diseases (Asada *et al.*, 2011; Kuroda *et al.*, 2011).

A key factor in the protein delivery system via the autotransplantation of various types of gene-transduced cells is the regulation of the survival and the secretory function of these cells at the transplanted space. We have shown that the nutri-

tional condition of the recipient is one of the important factors for the survival and the gene expression of adipocytes in the fat graft after subcutaneous transplantation in mice (Matsumoto *et al.*, 2002). In addition, the secretion of vascular endothelial growth factor (a bioactive molecule secreted from the vascular system) around the transplanted graft in recipients is also important for long-term cell survival (Yamaguchi *et al.*, 2005). Particularly, recent studies have highlighted the importance of various cytokines for the regulation of cell function and the surrounding matrix conditions (Kimura *et al.*, 2003; Cho *et al.*, 2006; Torio-Padron *et al.*, 2007b; Kuramochi *et al.*, 2008; Ning *et al.*, 2009). Together with the consideration of cytokine delivery for the transplanted cells, the development of scaffolds for transplantation contributes to the early construction of the surrounding matrix around the transplanted site. Insulin gene-transduced cells transplanted with Matrigel as a scaffold have been shown to survive as insulin-secreting adipocytes for three months after transplantation (Ito *et al.*, 2005). It is therefore critical to set up an appropriate clinically applicable scaffold for the adipocyte transplantation into patients, which allows not only a longer survival of the implanted cells but also guarantees a longer-lasting secretion of the therapeutic gene product into the blood stream.

In this study, we have optimized the gene transduction conditions for the most effective retroviral vector-mediated gene transduction using ceiling culture-derived proliferative adipocytes from mouse adipose tissue (m-ccdPA). We established a mouse model for the transplantation with the expanded human enzyme gene-transduced m-ccdPA for the evaluation of protein delivery in the serum of the mice. Using an *in vivo* model, we analyzed the effect of fibrin glue (Malafaya *et al.*, 2007; Mano *et al.*, 2007; Neuss *et al.*, 2008) as a clinically applicable scaffold on the efficacy of the circulating enzyme delivery.

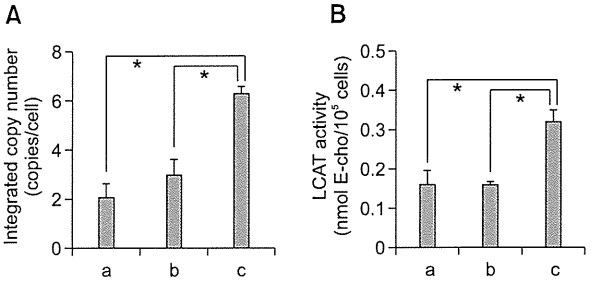

Figure 1. Enhanced gene transduction efficiency in m-ccdPA. Integrated copy numbers (A) and LCAT activity in the culture medium (B) after retroviral vector-mediated human *lcat* gene transduction were analyzed. Single round (b) and two rounds (a, c) of exposure to CGT_hLCATRV in the presence of 8 (a) or 500 μg/ml (b, c) of PS. Transductions with 8 μg/ml (a) and 500 μg/ml (b, c) of PS were performed overnight and one hour, respectively. Data are presented as the mean ± SD (n=3). *P < 0.05.

Results

Establishment of human *lcat* gene transduced m-ccdPA (m-ccdPA/*lcat*)

We have recently established h-ccdPA, which secretes functionally active hLCAT, a key circulating enzyme for serum cholesterol esterification, and proposed a novel cell-based gene therapy by the subcutaneous transplantation of the cells for the long-lasting replacement of the protein in the patients with LCAT deficiency (Kuroda *et al.*, 2011). In order to establish the most suitable mouse model for the evaluation of the effect of the scaffold on the survival and function of the transplanted adipocytes, we first prepared m-ccdPA for the *lcat* gene transduction as donor cells for the recipient mice. The biochemical characterization showed that the prepared m-ccdPA have morphological features and surface antigen expression patterns similar to those of h-ccdPA (unpublished data). Our preliminary experiments showed that the transduced m-ccdPA secreted a much lower amount of hLCAT than the h-ccdPA when the average copy number

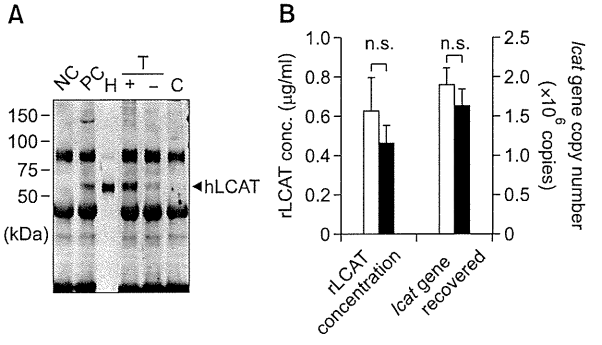

Figure 2. Detection of hLCAT and survival of human *lcat* gene after transplantation of *lcat* gene-expressing m-ccdPA. Human *lcat* gene-transduced mouse ccdPA (5×10^6 cells) were subcutaneously transplanted in nude mouse with fibrin glue as a scaffold. (A) Existence of hLCAT protein in mice sera was detected by IP-Western experiments. 15 μg human high density lipoprotein (HDL) was loaded for quantification of signals (H). Mouse serum with (PC) or without (NC) 15 μg HDL were subjected to IP-Western. The gene-transduced (T) m-ccdPA were transplanted with (+) or without (-) fibrin glue. Sera (100 μl) from the mice and mice transplanted with un-transduced (C) m-ccdPA were subjected to IP-Western analysis. (B) Human *lcat* gene-transduced mouse ccdPA (5×10^6 cells) were transplanted after three days of culture with (open bars) or without (closed bars) adipogenic differentiation medium. The serum concentrations of the hlcat protein were quantified by densitometric analysis (left), and the human *lcat* gene was quantified in excised implants (right).

of human *lcat* cDNA/cell was equivalent. Two rounds of one hour exposures with CGT_hLCATRV in the presence of 500 μg/ml of PS significantly improved the transduction efficiency compared with two rounds of overnight exposures in the presence of 8 μg/ml of PS, a concentration which was originally used for the gene transduction of h-ccdPA (Figure 1A: Kuroda *et al.*, 2011). The LCAT activity in the culture medium significantly increased in the cells with the same transduction conditions (Figure 1B).

Transplantation of m-ccdPA/*lcat* in nude mice

We transplanted the above established m-ccdPA/*lcat* subcutaneously into nude mice to examine the effect of fibrin glue as a scaffold on the secretion of hLCAT from the surviving cells without immunoreactive conditions. Blood samples collected from the mice transplanted with or without the fibrin glue were subjected to immunoprecipitation/Western (IP-Western) procedures 7 days after transplantation (Figure 2A). hLCAT was immunologically-detected clearly in the m-ccdPA/*lcat* transplanted mice, and not in the vehicle-transplanted mice (Figure 2A). The serum from the mice transplanted with the fibrin glue showed apparently increased signal intensity in comparison to those from the mice without fibrin glue (Figure 2A), indicating that the fibrin glue is effective for the cell survival after transplantation. The signal intensity analysis suggested that the concentration of the circulating hLCAT protein is over or equivalent to those of the 15 μg of human HDL, which is a major distribution site of LCAT (Fielding and Fielding, 1995).

Several reports have shown that mouse (Mizuno *et al.*, 2008) and human (Cho *et al.*, 2006) pre-adipocytes after adipogenic induction were superior in survival potential when implanted into nude mice. We therefore examined whether adipogenic differentiation affects hLCAT delivery and the survival of m-ccdPA/*lcat* after implantation with fibrin glue. RT-PCR analysis showed that the PPARγ2 expression level was significantly induced in cells cultured in adipogenesis-inducing medium for three days prior to transplantation (data not shown). The serum hLCAT concentration was not significantly different between mice transplanted with differentiation-induced cells and those transplanted with uninduced cells (Figure 2B). The *lcat* gene recovery analysis suggested that the adipogenesis-inducing pretreatment did not affect the cell survival rate (Figure 2B). These results indicate that the transplanted m-ccdPA/*lcat* implanted with fibrin glue survive at least 14 days after transplantation in immunosuppressive conditions.

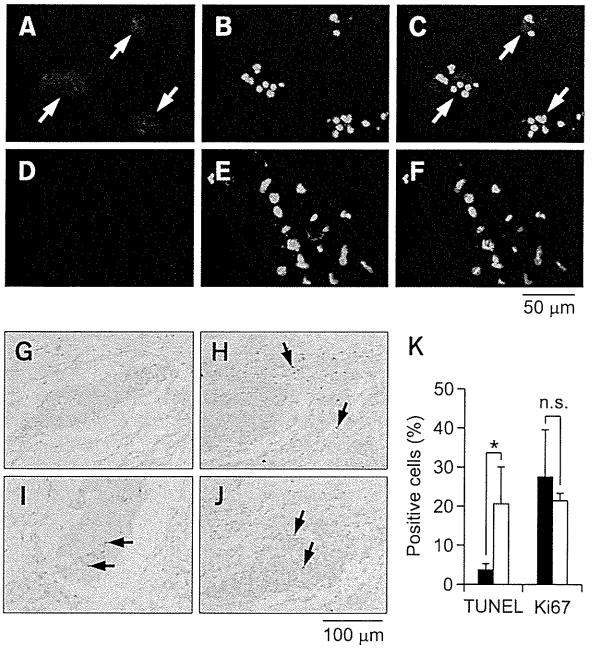

Figure 3. Immunohistochemical analysis of transplanted *lcat* gene-expressing m-ccdPA. Sections of implants from the cells with transduced (A, B, and C) or un-transduced (D, E, and F) by retroviral vector at day 28 were prepared and LCAT-immunostaining was performed. Implants were taken upon observation of PKH26 fluorescence. Immunohistochemical staining of hLCAT in fixed implants was done using rabbit anti-hLCAT monoclonal antibody as a primary antibody. Alexa Fluor 488 goat anti-rabbit IgG was used as a secondary antibody. The slides were counterstained with DAPI. Photographs of hLCAT staining (A and D), DAPI staining (B and E), and merged images (C and F) were shown. TUNEL (G and H) and Ki67 (I and J) staining of explants on 15 days after transplantation with (G and I) or without (H and J) fibrin glue were performed. Cells with positive signal were counted in four independent areas (K). $^*P < 0.05$.

Effect of fibrin glue on hLCAT delivery in m-ccdPA/*lcat*-transplanted B6 mice

We and others have already shown that exogenous gene-transduced adipocytes survive more than 28 days when subcutaneously transplanted with Matrigel, which is used as an experimental scaffold in many studies (Kitagawa and Kawaguchi, 1999; Ito *et al.*, 2005; Piasecki *et al.*, 2008). In order to consider the possibility of fibrin glue as a clinical scaffold, we analyzed the effect of fibrin glue on hLCAT delivery in comparison to Matrigel in B6 mice. The m-ccdPA/*lcat* was subcutaneously transplanted into B6 mice with fibrin glue. hLCAT immunostaining revealed that the m-ccdPA survive 14 days after transplantation and express hLCAT protein in B6 mice (Figures 3A-3F). The hLCAT expression was still detectable in the transplanted m-ccdPA 28 days after transplantation (data not shown). The TUNEL staining of transplanted sections excised on 15 days after transplantation showed that the

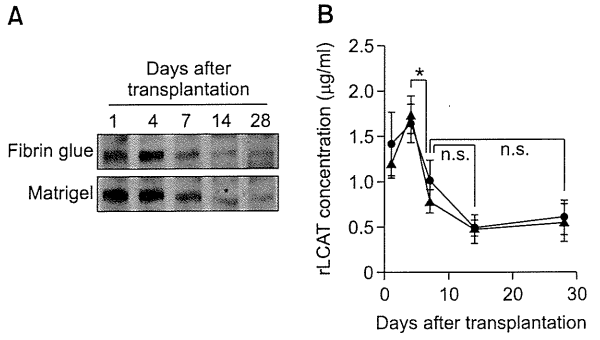

Figure 4. Effect of fibrin glue on hLCAT protein delivery. Human *lcat* gene-transduced m-ccdPA were subcutaneously transplanted in C57BL/6J mice using fibrin glue or Matrigel as scaffolds. Representative data of the experiments were shown (A), in which hLCAT delivery was monitored in a single mouse. Concentrations of hLCAT protein in cell-transplanted mice sera with Matrigel (closed circle) or fibrin glue (closed triangle) were quantified by densitometric analysis after IcP-Western experiments (B). $^*P < 0.05$.

apoptotic cell death in the cells with fibrin glue was significantly decreased in comparison to those without fibrin glue (Figures 3G, 3H and 3K). On the other hand, there was no significant difference in Ki67 staining between the cells with and without fibrin glue (Figures 3I, 3J and 3K). The IP-Western analysis showed that the hLCAT protein was detected at least up to 28 days after transplantation in the serum of mice. The collected mouse sera were analyzed for hLCAT protein by IP-Western blotting and hLCAT protein was detected up to 28 days after transplantation (Figure 4A). Densitometric analysis (Figure 4B) showed that from day 4 to day 7, the density became significantly decreased, and from day 7 the concentration of hLCAT protein became relatively constant. The hLCAT levels in the serum from m-ccdPA/*lcat* transplanted with fibrin glue were comparable with those from m-ccdPA/*lcat* transplanted with the Matrigel reagent. These results showed that fibrin glue, a common clinically available material, worked as a scaffold for the *in vivo* delivery of the hLCAT protein.

Discussion

For the development of long-lasting protein replacement therapy by gene-transduced ccdPA, the use of a clinically applicable scaffold is one plausible approach for the improvement of survival and/or secretion function of transplanted cells in patients. Various types of materials have been proposed (Malafaya *et al.*, 2007; Mano *et al.*, 2007; Neuss *et al.*, 2008) as scaffolds for cell transplantation. In this study, we have chosen fibrin glue because it is already commonly used in clinics and an easy-to-use kit is commercially available. In order to evaluate the effect of fibrin glue as a scaffold in the survival and function of transplanted adipocytes, we established an autologous mouse model system using m-ccdPA/*lcat*. The results using the mice showed that fibrin glue supported human enzyme delivery from the transplanted m-ccdPA/*lcat* at a level equivalent to Matrigel, which is known as an efficient scaffold in experimental models. Thus, fibrin glue could be a candidate as a scaffold in the clinical transplantation of h-ccdPA/*lcat* in LCAT-deficient patients to prevent the development of renal insufficiency and/or corneal opacity.

Preliminary experiments showed that hLCAT protein was secreted by m-ccdPA/*lcat in vitro*, however, hLCAT was barely detectable in the transplanted mouse serum probably because of the lower capability of secretion. The integrated copy number and the LCAT activity in the culture medium could be elevated approximately three fold with the conditions suitable for m-ccdPA (Figure 1). As a result, IP-Western analysis was sensitive enough for the quantification of the serum hLCAT protein in the mice, and the analysis indicated that the delivered protein is equivalent to that of 15 μg of HDL (Figure 2A). These optimizations enabled us to establish an *in vivo* mouse model to monitor the effect of fibrin glue as a scaffold for the transplanted m-ccdPA. Adipogenic differentiation did not significantly affect the hLCAT delivery and the cell survival in this model using fibrin glue as a scaffold with m-ccdPA (Figure 2B). In this context, our results may suggest that the transplanted cells with fibrin glue were differentiated into adipocytes without adipogenic pretreatment (Cho *et al.*, 2006; Torio-Padron *et al.*, 2007a). The immunohistochemical observation did not clearly show that ccdPA would undergo adipogenic differentiation after transplantation, but the transplanted ccdPA were clearly identified as hLCAT-delivery cells in the transplanted sites of the recipient mice (Figures 3A-3F). Immunohistochemical analysis of transplanted sections suggested that action of fibrin glue was prevention of apoptotic cell death rather than proliferation stimulation of the transplanted cells after transplantation (Figures 3G-3K), and thus, caused the increase in the hLCAT-delivery into circulation after transplantation in mice. The analysis of the m-ccdPA/*lcat* with fibrin glue revealed that the serum hLCAT concentration decreased to one half in a week, and became relatively stable at 7-14 days after transplantation (Figure 4). We could therefore discern that the hLCAT-positive cells survived and functioned for at least one month

using the m-ccdPA/*lcat* transplanted mouse model.

The current study showed that the implanted cells successfully supplied a therapeutic level of hLCAT into the serum, and suggested the feasibility of ccdPA-mediated gene therapy using the ccdPA. However, there are several remaining issues to be resolved before the clinical application of this therapy if we anticipate extending this cell implantation technique to various diseases other than LCAT deficiency. First, the survival period of ccdPA needs to be assessed after transplantation into the recipient. The previous model using insulin-secreting adipocytes showed that the blood glucose-reducing activity was stably observed for two months (Ito *et al.*, 2005). The stability of the ccdPA needs to be evaluated for longer periods using the mice established in this study. Second, the protein delivery by the transplanted ccdPA into the serum is unstable at the initial phase to 7 days after subcutaneous transplantation, although the delivery became constant after the 7-day phase up to a month. The characterization of the transplanted ccdPA including the interaction between the differentiation and secretion functions is in progress using this model. Before obtaining the knowledge of the multi-phase cell conditions in the recipients, the application of this cell therapy would be restricted to the enzyme deficiency in recipients without the overdose toxicity in the enzyme-mediated metabolism. In order to resolve the above remaining problems for wide clinical applications, the established autologous cell transplantation model enables us to evaluate the effects of the environmental conditions of the transplanted ccdPA on the survival and/or function of cells in detail, which is critical for successful cell-based gene therapies in humans.

Methods

Cell culture

Dulbecco's modified Eagle's medium/F12-HAM (Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ) was used as culture media except for the adipogenic induction in which PGM-2 Bullet kit (Lonza, Basel, Switzerland) was used. The m-ccdPA were prepared from 7-8 weeks male C57BL/6J mice as described (Kuroda *et al.*, 2011).

Optimization of gene transduction

Human *lcat* gene-expressing amphotropic retrovirus vector, CGT_hLCATRV (Kuroda *et al.*, 2011), was used for gene transduction at the concentration of 2.0×10^9 RNA copies/ml. Based on the report of Landazuri *et al.* (2007), we examined 100-500 μg/ml of protamine sulfate (PS, Novo-Protamine Sulfate, 100 mg for I.V. Injection, Mochida Pharm. Co. Tokyo, Japan) to enhance transduction efficiency in comparison to 8 μg/ml. Gene transduction was performed at 37°C in the presence of 20% FBS and PS. Subsequently, LCAT activities secreted in culture medium were measured to examine the effect of transduction conditions using artificial liposome substrate as described (Kuroda *et al.*, 2011).

Real-time PCR and RT-PCR

Genomic DNA extractions from cultured cells and mice transplants, and quantification of transduced human *lcat* gene were performed as described (Kuroda *et al.*, 2011). Total RNA was prepared by RNeasy Plus Mini kit (QIAGEN). Single-stranded cDNA was synthesized with ReverTra Ace-α-™ kit (TOYOBO, Osaka, Japan). PPARγ2 expression was examined by RT-PCR using primers as follows; PPARγ2-F (5'-GGTGAAACTCTGGGAGATTC-3') and PPARγ2-R (5'-CAACCATTGGGTCAGCTCTTG-3'). The amplification was performed with TITANIUM Taq DNA polymerase (TaKaRa Bio Inc.) under the following condition: 94°C for 5 min/94°C for 30 s, 58°C for 30 s, and 72°C for 90 s (28 cycles)/72°C for 7 min. The amplified products were subjected to 2% agarose gel electrophoresis and visualized with staining with GelStar® Nucleic Acid Stain reagent.

Detection of LCAT protein

Mice sera were diluted up to 500 μl with ice-cold phosphate buffered saline containing 0.2% Nonidet P-40 (PBS-NP40) and incubated with 2.5 μl of anti-LCAT rabbit monoclonal antibody (EPITOMICS, Burlingame, CA) overnight at 4°C with rotation. Twenty microliters of TrueBlot™ anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) was added and incubated with rotation for 2 h at 4°C. Beads/proteins complex was washed with PBS-NP40, and treated by boiling in 10 μl of 2 × Laemmli's sample buffer. Samples and standards (recombinant human LCAT (Roar Biomedical, Inc., New York, NY) or human plasma HDL (Calbiochem, Merck, Darmstadt, Germany)) were subjected to western blotting using anti-LCAT rabbit polyclonal antibody (Novus Biologicals, Littleton, CO) and TrueBlot anti-Rabbit IgG HRP (1:5000) (eBioscience) as primary and secondary antibody, respectively. The signals were detected by SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) with LAS1000 apparatus (FUJI film, Tokyo, Japan). Preliminary experiments demonstrated that the efficiency of recovery of input human LCAT (hLCAT) as HDL into mice serum was 101.0 ± 9.5%.

In vivo experiment

Animal experiments were carried out according to the Guidelines for Animal Research of Chiba University or

ORIENTAL YEAST Co., Ltd.. Male nude and C57BL/6J mice (Charles River Japan) were used as recipients. The cells were stained using PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling (Sigma-Aldrich) to identify the transplanted cells *in vivo*.

Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as clinically available fibrin glue. Fibrinogen solution and thrombin solution were diluted four and two times using DMEM-HAM/F12 (Sigma) respectively before use. Cells were suspended at 1×10^7 cells/ml by diluted thrombin solution, and injected subcutaneously into the mouse with same volume of diluted fibrinogen solution using injection apparatus included in Bolheal kit. We also transplanted the cells suspended in Matrigel (BD Biosciences, Bedford, MA) at 5×10^6 cells/ml. In both cases, 5×10^6 cells were transplanted.

All mice were allowed free access to regular chow and water. Three animals were sacrificed to take serum samples at day 1, 14, 28. In C57BL/6J mice experiments, blood samples were taken from tail without sacrifice to monitor the hLCAT delivery in same animal at day 1, 4, 7, 14 and 28. Transplanted region was taken under fluorescent microscopic observation by SZX16 reflected fluorescence system (OLYMPUS corp. Tokyo, Japan).

Histological staining

The explanted tissues were fixed in 4% paraformaldehyde following replaced 30% gum-saccarose and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan). Immunohistochemical staining was performed using anti-LCAT rabbit monoclonal antibody (250:1; EPITOMICS) and Alexa Fluor 488 goat anti-rabbit IgG (1000:1; Invitrogen) as primary and secondary antibody, respectively. The slides were counterstained with DAPI using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). TUNEL staining of the explanted tissues were performed using *In situ* Apoptosis Detection Kit (TaKaRa Bio Inc., Shiga, Japan). Ki67 imuunostaining was performed using anti-mouse Ki67 Rabbit polyclonal antibody (Abcam plc., Cambridge, UK), followed by biotin-conjugated anti-Rabbit Ig/HRP-conjugated streptavidin reaction. Signals were visualized by HRP reaction with DAB and the slides were counterstained with hematoxylin for TUNEL and Ki67 staining.

Statistical analysis

Data are presented as means ± S.D. Statistical comparison were made by Student's *t*-test or by ANOVA followed by the post hoc Tukey test to compare using SPSS software. In all cases, *P* values of less than 0.05 were considered as significant.

Acknowledgements

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Ceiling Culture-Derived Proliferative Adipocytes are A Possible Delivery Vehicle for Enzyme Replacement Therapy in Lecithin: Cholesterol Acyltransferase Deficiency

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Abstract: Human proliferative adipocytes propagated *via* ceiling culture technique from subcutaneous fat tissue (designated as ccdPA) were herein evaluated for their potential as a recipient for retroviral vector-mediated gene transduction of a therapeutic protein delivery. Exposure to the ZsGreen-expressing vector supernatant using a cell preparation generated by a 7-day ceiling culture induced a 40-50% transduction efficiency, with less than two integrated copies of viral genome per cell on average. The *lcat* gene-transduced human ccdPA secreted functional LCAT protein, correlating with the integrated copy number of vector genome. The gene-transduced cells could be expanded up to nearly 10^{12} cells from 1 g of fat tissue within one month after fat tissue preparation. The cells also maintained the potential to differentiate into adipocytes *in vitro*. The presence of human LCAT protein in serum was immunologically identified upon transplantation of *lcat*-expressing ccdPA into the adipose tissue of immune-deficient mice. These results indicated that human ccdPA has a novel therapeutic potential for LCAT-deficient patients. The clinical application in combination with cell transplantation shed a light on a development of a life-long protein replacement therapy for LCAT-deficient patients.

Keywords: Protein replacement therapy, lecithin:cholesterol acyltransferase, adipocyte, ceiling culture, gene therapy.

INTRODUCTION

The intriguing biology of pluripotent stem or progenitor cells has suggested the sustained production of therapeutic proteins to be a treatment for patients with serum protein deficiencies [1, 2]. The ability of cells to self-renew at a high proliferation rate has led to the expectations that these cells are ideal targets for retroviral vector-mediated transgene delivery. Studies examining this concept have described the treatment of various diseases in animal models [3-10].

Lecithin:cholesterol acyltransferase (LCAT) is a plasma protein responsible for the conversion of plasma unesterified cholesterol into cholesteryl ester, and plays a central role in the formation and maturation of high-density lipoproteins (HDL), which are involved in reverse cholesterol transport. Genetic LCAT deficiencies have been identified, and more than forty different mutations have been identified to date (refer to HGMD: http://www.hgmd.cf.ac.uk/ac/index.php). Plasma LCAT is either absent or exhibits no catalytic activity in patients with a familial LCAT deficiency. Cholesteryl ester levels are markedly reduced in lipoproteins, abnormal cholesterol deposition is observed in the tissues of these patients, and patients often develop corneal opacity, anemia, proteinuria, and renal failure [11]. The efficacy of LCAT replacement therapy was shown by infusion of normal plasma [12, 13], but the effects were transient. In addition, replacement therapy with recombinant LCAT protein has not been established mainly because this is a rare condition, and due to the associated expenses for production of the recombinant protein. Therefore, life-long treatment with autologous cell-based therapy may contribute to the continuous replacement of enzymes.

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Recently, much attention has been paid to adipose tissue as a source of proliferative cells for cell-based gene therapy [14] and for regenerative therapy [15, 16]. Two types of preparations have been reported to be sources of adipose-tissue derived proliferative cells. One is stromal-vascular fractions (SVFs), which can be obtained as sediment by the centrifugation of collagenase-digested fat tissue [17, 18]. The obtained cells are pluripotent and can differentiate to yield various cell types, including cardiomyocytes, chondrocytes, and osteoblasts, in addition to adipocytes [19]. The other cell preparation is obtained from the floating mature fat cell fraction of the centrifugation, followed by a ceiling culture [20]. The cultured cells maintain the ability to differentiate into mature adipocytes at a high frequency [10, 20, 21], and are presumably more committed to the adipocytes lineage.

In the present study, a target cell population was prepared from adipose tissue using the ceiling culture technique to develop a cell-based gene therapy of LCAT-deficient patients, and we designated the target cells as ceiling-culture derived proliferative adipocytes (ccdPA). The current study established this production procedure, and optimized the gene transduction conditions of human ccdPAs as therapeutic gene recipient cells. In addition, we assessed the capability and the safety of the *lcat* gene-transduced ccdPA as a LCAT-secreting device for protein replacement therapy. Therefore, we developed stable protein-producing human ccdPAs with self-renewing and high expansion capacities.

MATERIALS AND METHODS

Construction of pCGThLCAT, A Retroviral Vector Plasmid Encoding the Human *Lcat* Gene

The pDON-AI, Moloney Murine Leukemia virus (MoMLV) vector plasmid (TaKaRa Bio Inc., Shiga, Japan) was used as a recipient for the human *lcat* cDNA. The *lcat* cDNA was derived from total RNA prepared from HepG2 cells. The resulting cDNA was amplified by PCR using the following primer pair: 5'-ATCGGATCCAGGGCTGGAAA TGGGGCCGCCC-3' (forward) and 5'-ATCGGATCC GTCGACGGAAGGTCTTTATTCAGGAGGCGGGGG-3' (reverse). The forward primer contained a *Bam*HI restriction site (underlined) and a Kozak sequence, and the reverse primer contained a *Sal*I restriction site (underlined). The reverse primer also eliminated the polyA signal from the original *lcat* cDNA. The amplified PCR products were digested by *Bam*HI and *Sal*I and cloned into the corresponding sites of the pDON-AI plasmid. Thereafter, the neomycin resistant gene was removed by *Sal*I and *Xho*I digestion and subsequent self-ligation, yielding the pCGThLCAT plasmid.

Production of the Amphotropic Retroviral Vector

The GMP grade retroviral vector CGT_hLCATRV was produced by TaKaRa Bio Inc. In brief, the pCGThLCAT vector was transfected into the ecotropic packaging cell line GP+E86 (ATCC#: CRL-9642), and the supernatant was collected. The supernatant was used to infect the amphotropic packaging cell line GP+envAM-12 (ATCC#: CRL-9641) to produce a master cell bank (MCB) for vector production. CGT_hLCATRV was prepared from culture supernatant of the MCB. The vector solution was aliquoted and stored at -80 °C until use. The vector titer was quantified by TaKaRa Bio Inc. using the One Step SYBR PrimeScript RT-PCR Kit with primer pairs from Retrovirus Titer Set (TaKaRa Bio Inc.). The *ZsGreen*-gene expressing retrovirus vector was similarly propagated.

Cell Culture and Medium

Dulbecco's modified Eagle's medium [DMEM]/F12-HAM (Sigma-Aldrich, St. Louis, MO) and MesenPRO medium (Invitrogen, Carlsbad, CA) were used to maintain cultured cell lines. Fetal bovine serum (FBS) was purchased from SAFC Biosciences (Lenexa, KS). Cell passaging was performed twice a week.

Isolation of ccdPAs from Human Fat Tissue

Subcutaneous adipose tissues were obtained from 16 healthy volunteers (C001-C016) with ages ranging from 19 to 42 years after informed consent was obtained with the approval and guidelines of the ethical committee at Chiba University School of Medicine, according to the Declaration of Helsinki. Ceiling culture techniques [20] were employed and optimized using C001-C012 fat tissues to isolate human ccdPAs as follows. Fat tissue was weighed, and each 1.0 g was digested with gentle agitation for 1 hr at 37 °C in 3 ml of Hank's balanced salt solution (HBSS) containing 2 mg/ml collagenase (Collagenase NB 6 GMP Grade, SERVA, Heidelberg, Germany) and 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ). Thereafter, the solution was diluted with 10 ml of DMEM/F12-HAM containing 20% FBS and 40 μg/ml gentamicin (DMEM/FBS), mixed, and centrifuged at 400 x g for 1 min. The pellet was removed as an SVF. The dilution steps were repeated 4 times to collect the floating cell fraction. The floating fraction was filtered with a 500-μm mesh (Netwell Insert, Corning Inc., Corning, NY) and seeded into flasks, which were filled with DMEM/FBS. After 7 days ceiling culture, cells that grew at the ceiling surfaces were harvested and seeded into flasks for the subsequent steps.

Gene Transduction

In preliminary experiments, the acceptability of the MoMLV vector for human ccdPA propagated in the course of ceiling culture revealed that longer culture times resulted in a higher resistance to retroviral vector transduction (data not shown). Therefore, the cells obtained by 7 day-ceiling culture were evaluated as a potential recipient for retroviral vector-mediated gene transduction. Human ccdPAs were seeded and incubated in DMEM/FBS at 37 °C for 24 hrs. Protamine sulfate (PS, Novo-Protamine Sulfate, 100 mg for I.V. Injection, Mochida Pharm. Co. Tokyo, Japan) was used to optimize the transduction conditions (0.5-16 μg/ml). Gene transduction was performed in the presence of 20% FBS and 8 μg/ml PS at 37 °C for 24 hrs. The viral vector concentration used for transduction was 2.0 x 10^9 RNA copies/ml, unless otherwise specified. After transduction, the medium was replaced with growth medium.

Flow Cytometry

Cells were suspended in phosphate buffered saline containing 2% FBS (PBS/FBS). Fluorescein isocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies were purchased

from BD Farmingen (San Diego, CA) or Beckman Coulter (Fullerton, CA), or Ancell Corporation (Bayport, MN). Aliquots of cell suspensions (4.5 x 10^4 cells) were mixed with primary antibody in a total volume of 90 μl and were incubated for 30 min at RT. The cell suspension was washed twice with PBS/FBS, and the cells were fixed in 200 μl of PBS/FBS containing 1% paraformaldehyde. Five thousand events were acquired for each antibody on a FACS Calibur apparatus using the CELLQuest acquisition software program (Becton, Dickinson and Company, Flanklin Lakes, NJ). *ZsGreen* expression was also examined in human ccdPAs. Non-transduced cells were used as a negative control.

Quantification of Transduced Gene

Genomic DNA was extracted from cultured cells and mouse adipose sections with the DNeasy Blood & Tissue kit and the Gentra Puregene kit (QIAGEN, Hilden, Germany), respectively. The integrated vector copy number was quantified with the SYBR *Premix Ex Taq* (Perfect Real Time) kit (TaKaRa Bio Inc.). A known amount of pCGThLCAT DNA was used as a standard. The primer pairs were from the Retrovirus Titer Set (TaKaRa Bio Inc.). The DNA content in a human normal cell (6 pg/cell) [22] was used for calculating the average integrated copy number. Existence of transduced gene in transplanted adipose tissue was quantified with a TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) using *lcat*-cDNA specific primers and probes designed by the Probe Finder Software program (Roche Diagnostics, Mannheim, Germany). All the real-time PCR reactions were performed using the ABI7500 Real-time PCR system (Applied Biosystems).

Detection of LCAT Protein

Culture medium and mice sera were diluted to a volume of 500 μl with ice-cold phosphate buffered saline containing 0.2% Nonidet P-40 (PBS-NP40) and were incubated with 2.5 μl of anti-LCAT rabbit monoclonal antibody (EPITOMICS, Burlingame, CA) for 18 hrs at 4 °C with gentle rotation. Twenty micro-liters of TrueBlot anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) was added and incubated with rotation for 2 hrs at 4 °C. Bound proteins were pelleted by centrifugation, washed with PBS-NP40, and eluted by boiling in 10 μl of 2X Laemmli's sample buffer. Immunoprecipitated samples were subjected to immunoblotting. Purified human LCAT (Roar Biomedical, Inc., New York, NY) or human plasma HDL (Calbiochem, Merck, Darmstadt, Germany) was used as a standard. An anti-LCAT rabbit polyclonal antibody (Novus Biologicals, Littleton, CO) and TrueBlot anti-Rabbit IgG HRP (1:5000) (eBioscience) were used as primary and secondary antibodies, respectively. The signals were detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) and the LAS1000 apparatus (FUJI film, Tokyo, Japan).

Measurement of LCAT Activity

The procedure described by Ishii *et al.* [23] was modified to prepare the liposome substrate for the LCAT analyses. Two hundred microliters of [^{3}H]-cholesterol (American Radiolabeled Chemicals, Inc., St. Louis, MO) were evaporated to dryness by flushing N_2 gas, and 5 ml of the substrate mixture of Anasolv LCAT kit (SEKISUI MEDICAL Co. Tokyo, Japan) was added. The solution was sonicated with a Digital Sonifier Model 250 (BRANSON, Danbury, CT) at an amplitude of 40% and 0.5 second pulse cycles for 1 min a total of six times in an ice bath. The sonicated mixture was centrifuged at 3,000 rpm and stored at 4 °C until use. The reaction mixture contained 100 μl of labeled substrate, 4.5 mM β-mercaptoethanol, 36 μg of apolipoprotein A1 (Athens Research & Technology, Athens, GA), and 100 μl of culture medium in a total volume of 220 μl. The reaction was performed at 37 °C for 1 hr, and was terminated by the addition of 1.6 ml of chloroform/methanol (2:1). One hundred microliters of water was added, and the organic phase was obtained by centrifugation. Fifty microliters of the organic phase was spotted onto Whatman flexible thin layer chromatography (TLC) plates (Whatman plc, Kent, UK). Sample-spotted plates were developed with standards of cholesterol and cholesterol oleate in a glass tank using a solvent mixture of hexane/ethyl ether/acetic acid (146:50:4) by TLC. Developed TLC plates were air-dried and stained with iodine (Wako Pure Chemicals, Osaka, Japan). Cholesteryl ester spots were excised, and the radioactivity was determined by liquid scintillation spectrometry.

Adipogenic Differentiation Assay

Human ccdPA (3.5 x 10^4 cells) were seeded into BioCoat Collagen I 48-well Multiwell Plates (BD Biosciences) and grown to confluency over 3 days. Differentiation was induced with the PGM Bullet Kit (Lonza, Basel, Switzerland), and the cells were incubated for 2 weeks. The cells were fixed in 4% paraformaldehyde, washed twice with PBS, incubated with 60% isopropanol for 1 min, and stained with Oil Red O solution (Chemicon International, Inc. Temecula, CA) for 20 min. The accumulation of triglycerides was examined to confirm adipogenic differentiation using the Triglyceride E-test kit (Wako Pure Chemicals) according to the manufacturer's instructions. The protein content of the lysate was also determined with the Quick Start Bradford Dye Reagent (Bio-Rad Laboratories Inc.).

Clonality Analysis by Southern Blotting

Abnormal amplification of specific cell clones resulting from the integration of the retroviral vector genomic sequence was examined by Southern blotting according to the DIG (Digoxigenin) protocol (Roche Diagnostics). Genomic DNA extracted with the Gentra Pure Gene kit (QIAGEN) was digested with *Hin*dIII (Roche Diagnostics). Digested DNA (6 μg) was subjected to agarose gel electrophoresis, followed by capillary transfer to a positively-charged nylon membrane (Roche Diagnostics). Human *lcat* cDNA was used as a template to synthesize probes by the PCR DIG Probe Synthesis kit (Roche Diagnostics). Hybridization was performed at 50 °C overnight. The membrane was washed and reacted with Anti-digoxigenin-AP, Fab fragments (Roche Diagnostics). The signals were detected using CDP-Star with the LAS1000 apparatus (FUJI film). As positive control, 293 (European Collection of Cell Cultures) cells were transduced with a neomycin-resistant gene-containing version of the *lcat*-expressing retroviral vector, and typical single copy-integrated clones were selected.

Colony Formation Assay by Soft-Agar Containing Medium

Anchorage-independent colony formation was examined by the soft-agar assay using the CytoSelect 96-well Cell Transformation Assay kit (Cell Biolabs, Inc., San Diego, CA). Ten thousand gene-transduced human ccdPAs were seeded into 96-well plates in triplicates along with 100, 1000, or 10,000 HeLa cells (European Collection of Cell Cultures) as a positive control.

Monitoring Human LCAT Secretion in Mouse Model

Animal experiments were performed in the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan) according to the Ethical Guidelines for Animal Experimentation from CIEA to examine the delivery of LCAT protein *in vivo*. To identify the transplanted cells, cells were stained using the PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling (Sigma-Aldrich) one passage prior to transplantation. Expanded cells were harvested, washed with Ringer solution containing 0.5% human serum albumin (HSA, Benesis Corp. Osaka, Japan) four times, and re-suspended to a final cell concentration of 3×10^7 cells/ml. The cell suspension (50 μl) was injected into the adipose tissue between the shoulder-blades of NOD/Shi-*scid* IL-2Rγ^{null} (NOG) mice [24]. Buffer alone was injected as a control. All mice were bred in a vinyl-isolator and six animals were sacrificed to collect serum samples at each time point (Day 1, and at 1, 3, and 6 months). Six and three animals were used for the transplanted and control groups, respectively, for each time point. The transplanted region was taken using fluorescent microscopy on a SZX16 reflected fluorescence system (OLYMPUS corp. Tokyo, Japan), and sections were frozen at -80 °C until use.

Statistical Analysis

Data are presented as means ± S.D. Statistical comparison were made by Student's *t*-test or by ANOVA followed by the post hoc Dunnett or Tukey test using the SPSS software program. The integrated copy number, positive rate, and LCAT activity were analyzed to determine whether there was a linear correlation between these variables. For this analysis, we calculated a linear correlation coefficient (Pearson r value) and the corresponding P-value (two tailed) based on these assumptions. In all cases, P-values of less than 0.05 were considered to be statistically significant.

RESULTS

Preparation of Gene-Transduced Human ccdPAs

The optimization of cell-processing steps was carried out with fat tissues obtained from 16 healthy volunteers (C001-C016). Adipose tissue-derived proliferative cells were assessed for their suitability in ceiling culture, gene transduction, and cell expansion, using two culture media, DMEM/F12-HAM supplemented with 20% FBS (DMEM/FBS) and MesenPRO medium, respectively. The ceiling culture was performed in DMEM/FBS in comparison to MesenPRO medium. The cell yield of C012 after the ceiling culture from 1 g adipose tissue was $7.1 \times 10^5 \pm 1.0 \times 10^5$ and $2.1 \times 10^5 \pm 0.2 \times 10^5$ cells in DMEM/FBS and MesenPRO medium, respectively, showing that a higher cell yield was obtained in DMEM/FBS than in MesenPRO medium ($p<0.05$). The flow cytometric analyses showed that cells in DMEM/FBS tended to be homogeneous in shape and size, in comparison to those grown in MesenPRO medium (Fig. **1a**). The gene transduction of the cells after the ceiling culture was next assessed using the two medium types. The above cells which were frozen after ceiling culture (C010) in DMEM/FBS were recovered, incubated for 4 days, and seeded for gene transduction in MesenPRO medium or DMEM/FBS medium. After transduction with the *lcat*-expressing retroviral vector, the cells were passaged several times in the respective medium, and cell samples were subjected to copy number quantification 12 days after transduction. DMEM/FBS was more effective than MesenPRO medium for the gene transduction of human ccdPAs when a retroviral vector was employed under the appropriate conditions (0.94 ± 0.10 copies/cell vs. 0.36 ± 0.09 copies/cell, $p<0.05$). Finally, the effects of the

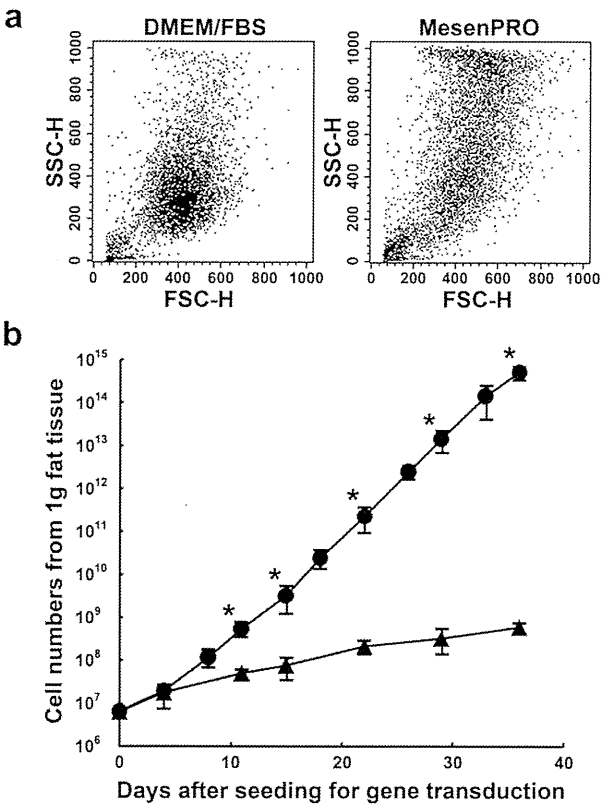

Fig. (1). Comparison of DMEM/FBS and MesenPRO media for the preparation of human ccdPAs. (**a**) The cells (C012) prepared by ceiling culture in DMEM/FBS (left panel) or MesenPRO medium (right panel) were subjected to a FACS analysis. The dot-plot (forward-scattered vs. side-scattered) of both cell populations are shown. A representative plot is shown for each medium. (**b**) The cells derived from C013 were used for expansion. Cell numbers were counted during proliferation for 35 days in DMEM/FBS (closed triangle) or MesenPRO medium (closed circle) after gene transduction in DMEM/FBS. Cell numbers are presented from 1 g of fat tissue. Data are presented as the mean ± SD (n=3). *$p<0.05$ vs. MesenPRO medium at each day after seeding.

incubation media on the gene-transduced cell expansion were examined in C013 cells. The doubling times of the cells in the MesenPRO medium were significantly shorter than those in DMEM/FBS (31.7 ± 4.8 hours vs. 119.4 ± 29.6 hours, $p<0.05$). The transduced cell number expanded to more than 3 x 10^4 fold of the original number in a month when grown in MesenPRO medium (Fig. **1b**). Therefore, DMEM/FBS was chosen for the ceiling culture and gene transduction, and the MesenPRO medium for cell expansion of ccdPA, respectively, in subsequent experiments.

Characterization of Human ccdPAs

The cell surface antigen profile was analyzed by FACS for human ccdPAs (Fig. **2a**). The populations of $CD31^-$/$CD45^-$ cells were significantly increased in the ccdPA preparation, in comparison to SVF-derived cells (99.1 ± 0.3% vs. 95.6 ± 0.1%, $p<0.05$), indicating that ceiling culture technique excludes CD31-positive and/or CD45-positive cell populations in comparison with cells prepared from SVF. The ccdPAs were positive for CD9, CD10, CD13, CD29, CD44, CD59, CD90, CD105, CD146, and HLA-ABC, and

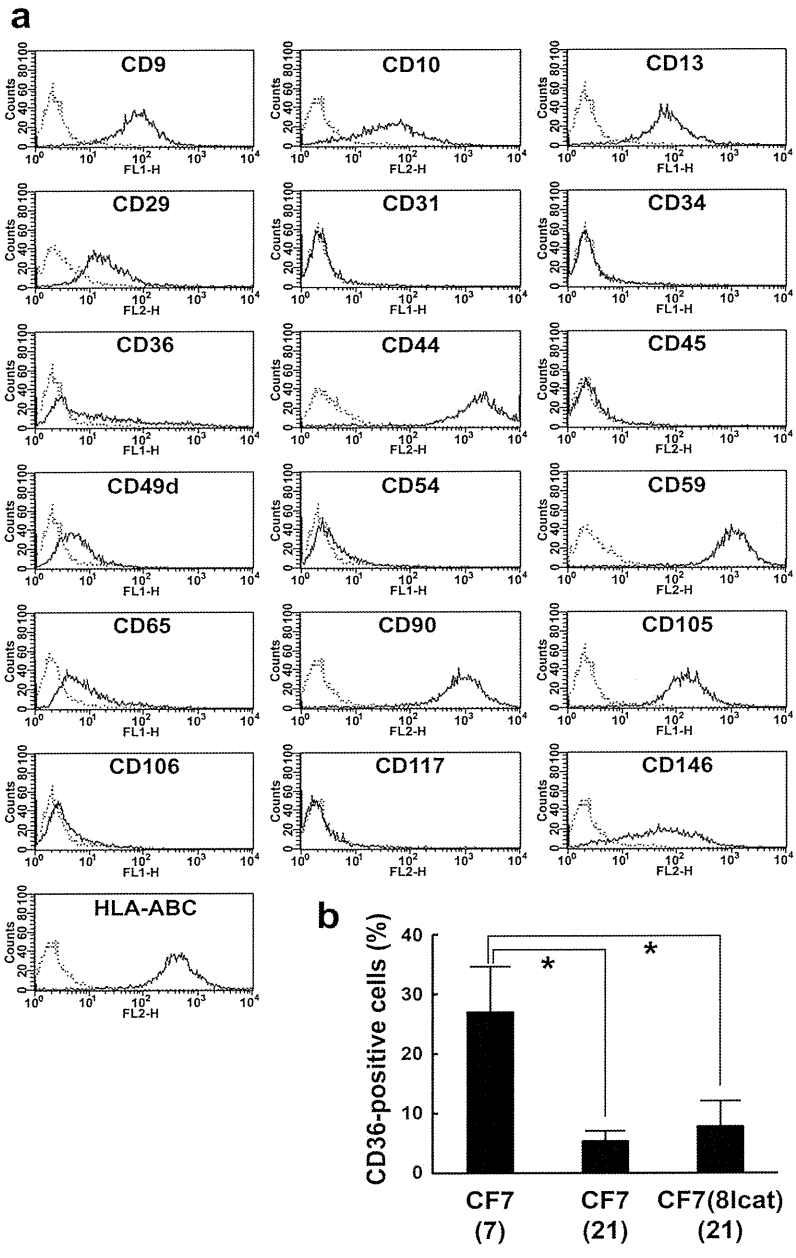

Fig. (2). Cell surface antigen profiles of isolated human ccdPAs by ceiling culture. (**a**) The cells were harvested at 7 days after ceiling culture, and were immuno-stained with the corresponding antibodies (solid line) or an isotype control (dotted line), and were subjected to a FACS analysis. Histograms for each antibody are presented. (**b**) CD36-positive cells was examined in the cells harvested from the ceiling culture (CF7(7)), the cells expanded after *lcat*-gene transduction (CF7(8lcat)(21)), and the cells expanded without gene transduction (CF7(21)). The ratio of CD36-positive cells in the prepared cells is presented as the positive cell rate (%). Data are presented as the mean ± SD (n=3). *$p<0.05$.

negative for CD31, CD34, CD45, CD54, and CD106. They were moderately positive for CD49d and CD65, and a substantial number of cells were positive for CD36, a marker for adipocytes [25]. The populations of CD36-positive cells after a 14-day *in vitro* culture of ccdPAs were significantly lower than those at 7 days ($p<0.05$, Fig. **2b**).

Retroviral Vector-Mediated Gene Transduction and Transduced Gene-Derived Protein Secretion in Human ccdPA

Human ccdPAs were evaluated as a recipient of MoMLV-based gene transduction using various concentrations of the vector and PS with single round of transduction using a ZsGreen-expressing vector. Two types of cells were analyzed, one cell type just after harvesting from the ceiling culture (CF7(7)), while another type was further cultured in the normal manner for an additional week (CF7(14)) in DMEM/FBS. The integrated copy number could be increased to approximately 1.7 and 2.5 copies/cell in CF7(7) and CF7(14) cells, respectively, and a good linear correlation was observed between the integrated copy number and the transduction efficiency (percentage of ZsGreen-positive cells) (Fig. **3a**). The transduction efficiency and the integrated copy number were significantly different between the cells of same batch at Days 1 and 2 of gene transduction (Fig. **3b**). These results showed that the cells with a higher transduction efficiency of the transduced gene and a lower integrated copy number were obtained by transduction for cells which were seeded and incubated overnight following a 7-day ceiling culture (CF7(8)). The CF7(8) cells were examined as a potential recipient for the human *lcat* gene. The transduction analyses using the *ZsGreen* vector showed that a vector concentration of 2.0×10^9 RNA copies/ml resulted in a good correlation between the integrated copy number and ZsGreen-positive cells in two different cell batches (Fig. **3a**). The use of the maximum achievable concentration (3.1×10^9 RNA copies/ml) of CGT_hLCATRV was compared with that using a concentration of 2.0×10^9 RNA copies/ml. Transduction of CF7(8) cells with 3.1×10^9 or 2.0×10^9 RNA copies/ml of the vector resulted in no difference in the integrated copy number (1.65 ± 0.12 vs. 1.56 ± 0.23 copies/cell). The LCAT protein produced by the *lcat* gene-

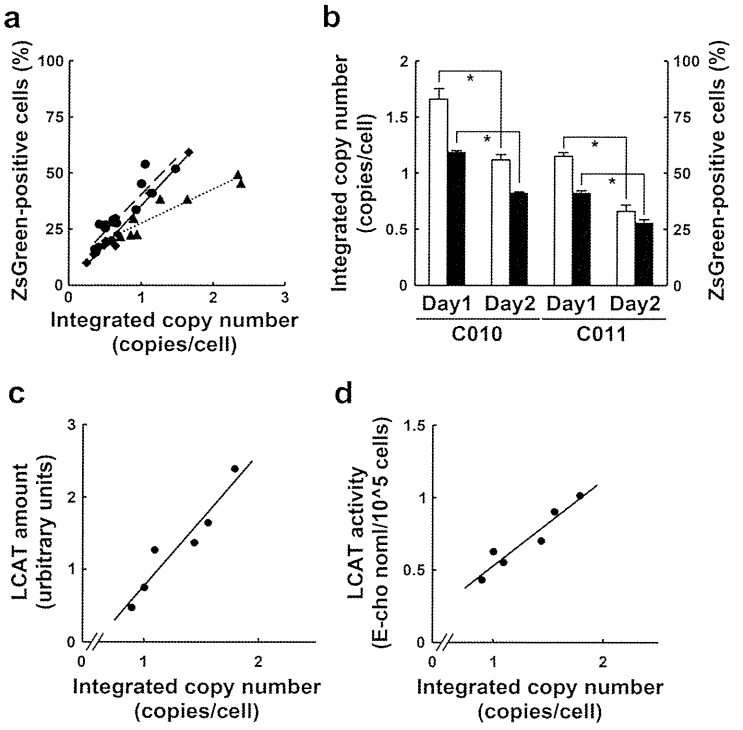

Fig. (3). *In vitro* evaluation of human ccdPAs as recipients of MoMLV-based retroviral vector-mediated gene transduction and a vehicle for the secretion of functional LCAT protein. (**a**) Integrated copy number (copies/cell) and ZsGreen-positive cells (%) were plotted for C010 CF7(7) (closed rhombus), C011 CF7(7) (closed circle), and C011 CF7(14) (closed triangle). Lines are drawn with Pearson r-values of 0.991, 0.908, and 0.937 for C010 CF7(7) (solid line), C011 CF7(7) (broken line), and C011 CF7(14) (dotted line), respectively ($p<0.05$). (**b**) Integrated copy numbers (copies/cell, open bars) and ZsGreen-positive cells (%, closed bars) after a single round of exposure of 2.0×10^9 RNA copies/ml of virus vector are shown. The cells (C010 and C011) were exposed to the transduction mixture one day (Day 1) or two days (Day 2) after seeding. Data are presented as the mean ± SD (n=3). *$p<0.05$. (**c**) Secreted LCAT protein was detected by immunoprecipitation/immunoblotting in culture medium incubated for 3 days with 1×10^5 cells (C013). After a densitometric analysis of immunodetected signals for human LCAT protein (60-65kDa), the integrated copy number and LCAT level (arbitrary units) were plotted (Pearson r-value of linear coefficient, 0.953, $p<0.05$). (**d**) Culture medium incubated with 1×10^5 cells (C013) for 3 days were subjected to assay of LCAT activities. The activity was presented by esterified cholesterol production from the cholesterol in the medium of human ccdPAs (Pearson r value of linear coefficient, 0.954, $p<0.05$).