obtained human ccdPA regarding the differentiation, cell surface marker expression, transgene stability and cell growth, in comparison to the un-transduced cells.

Circulating LCAT supplementation by the implantation of lcat-gene transduced ccdPA in mice

The capacity of human ccdPA as a vehicle for *lcat*-gene product delivery was assessed in mice. A cell suspension containing 1.5 x 10^6 cells was transplanted into the fat tissue of immuno-deficient mice, and LCAT protein secreted into serum was determined by the IP-Western technique. Human LCAT was clearly detected in sera of all transplanted mice at day1 (**Figure 5a**), and detectable after a month in mice (**Figure 5b**). A densitometric analysis revealed that the concentration of human LCAT was estimated to be 0.26 ± 0.19 μg/ml at day1. Real-time PCR quantification showed 42.9 ± 27.1% (Day1), 1.0 ± 1.0% (1 month), and 1.2 ± 0.7% (3 months) of the *lcat* gene of that of the transplanted cells at Day0 to exist in the adipose tissue transplanted with *lcat*-gene transduced ccdPA. These results suggested that about 1% of the *lcat*-gene transduced ccdPA survived for 3 months after the transplantation of cells into fat tissue of mice.

DISCUSSION

This study evaluated human ccdPA as a vehicle for therapeutic genes. Adipose-derived stem cells (ASC) differentiate into multilineage cells upon induction and are expected to be an ideal resource for regenerative cell therapy.[18] The current study utilized autologous ccdPA prepared from the subcutaneous fat of patients as a vehicle for therapeutic protein replacement therapy. Adipose tissue contains two major sources of proliferative cell populations; namely, floating and pellet fractions after centrifugation of collagenase-digested fat tissue. ASC is derived from the pellet fraction, SVF.[16,17] The ceiling culture of the SVF-removed floating fraction can presumably further enrich the cells derived (or dedifferentiated) from mature adipocytes by the buoyant property of adipocytes during the ceiling culture periods. This cell-based therapy was developed from the mature adipocyte cultures since SVF consists of a heterogeneous cell population, including blood cells, fibroblasts, and endothelial cells[14,15] and has some risk yielding a cell population with abnormal phenotype in long-term *in vitro* culture.[26-28]. Although the adipocytes-derived cell population obtained via the ceiling culture still have the potential for chondrogenic or osteogenic differentiation *in vitro*[29,30], the adipogenic potential observed in these cells in comparison to SVF-derived cells[29] was consistent with the current study purposes.

MesenPRO medium, a medium optimized for mesenchymal stem cells, provided

some advantage in the preparation of ccdPA through higher expansion capacity in comparison to DMEM/FBS (**Figure 1**). On the other hand, the MesenPRO medium was less effective for the propagation of human ccdPA in the ceiling culture than DMEM/FBS. Considering the fact that lipid-droplet containing adipocytes produce progeny cells in ceiling culture[19,20,30-32], MesenPRO medium seems unsuitable for the proliferation of mature adipocytes in ceiling culture. The FACS analysis showed that the obtained ccdPA shared a similar profile of the surface markers with that of the previously reported adipose-derived cells[29,30,33,34] (**Figure 2**). The certain population of the ccdPA retained a mature adipocyte maker (CD36) at the early stage and lost it eventually. These adipogenic properties would be suitable for optimized the stable survival of the cells to survive in the fat tissue and to keep producing therapeutic protein for a long period.

Simple exposure of the virus vector supernatant resulted in 40-50% transduction efficiency (**Figure 3a,b**), thus suggesting that human ccdPA serve as an excellent recipient of retrovirus vector-based therapeutic applications in contrast to cell populations in which efficient transduction requires drug selection[4,35] or multiple rounds of transduction.[36-38] The *lcat*-expressing retrovirus vector was constructed using pDON-AI originated by Kim et al.[39,40] as a backbone. The risk of replication-competent retrovirus (RCR) occurrence was minimized by eliminating all the unnecessary structural genes from the MoMLV genome in this vector. In fact, no RCR was detected in the vector preparations (data not shown). A single exposure with 2.0×10^9 RNA copies/ml of CGT_hLCATRV was selected to keep minimize the copy number of the transgene of each cell. The transduction efficiency was correlated with the integrated copy number (**Figure 3a,b**), the integration sites seemed to be randomly distributed, no clonal expansion was detected by a Southern blot analysis of transgene after expansion culture (**Figure 4e**), and no increase in the integrated copy number was observed in the preparations (**Figure 4d**). No evidence of transformation was observed in soft agar assay, either at the time of implantation (after three weeks from fat tissue removal) or after a long-term extended culture (data not shown).

LCAT is synthesized in the liver tissue and secreted into blood stream where it converts cholesterol to cholesterol ester in association with other accessory proteins on HDL and LDL.[21] LCAT protein produced by the human *lcat*-gene transduced ccdPA was a glycosylated protein (data not shown) with a molecular weight and *in vitro* enzymatic activity equivalent to that of human serum LCAT. An animal study indicated that the human LCAT protein secreted from the implanted transduced human ccdPA was detected in the blood samples (**Figure 5**). The serum of familial LCAT deficiency

patients exhibits less than 10% LCAT activity of the healthy subjects.[21] Patients with partially inactive LCAT enzyme (8.3-15% activity of normal) have no renal complications.[24,41] Plasma infusion in patients, which restores 17.3% LCAT activity, results in a significant improvement of lipoprotein profiles.[42] These observations suggest that LCAT activity seems necessary to be more than 10-20% to prevent the development of the symptoms. The circulating LCAT protein concentration is approximately 6 μg/ml.[43,44] A densitometric analysis showed that transplantation of 1.5 x 10^6 of *lcat*-expressing human ccdPA nearly achieved 5% of the healthy control level on day1 in mice, implicating that 10^9-10^{10} cells would yield a therapeutic effect in patients. The fact that the *lcat*-gene transduced human ccdPA could be expanded to nearly 10^{10} cells within two weeks after gene transduction from 1 g of fat tissue suggested the potential of human *lcat*-gene transduced ccdPA to rescue LCAT deficient patients. Considering the differences in the lipoprotein metabolism between mice and human, the future strategy to investigate the efficacy of human LCAT replacement therapy may be to establish an *in vitro* evaluation system employing serum obtained from familial LCAT deficiency patients.

In summary, this study has established a procedure to prepare *lcat*-gene transduced human ccdPA for cell based gene therapy. These cells have ability to differentiate to mature adipocytes and secret functional human LCAT protein. Animal studies showed that the implanted cells successfully supplied a therapeutic level of LCAT into the serum, suggesting the feasibility of the ccdPA-mediated gene therapy. Considering the fact that only 1% of the transduced gene was retained in transplanted adipose tissue at one month or later, future studies must be focused on the improvement of the cell survival rate and more long-lasting production of the transgene product. In this context, numerous studies have been performed to develop and assess the biomaterials for a scaffold and cytokines for induction of angiogenesis to increase cell survival.[10,45,46] A clinical trial of *ex vivo* gene therapy for 22 months has shown the implantation of autologous fibroblasts genetically modified to express human nerve growth factor into the forebrain improved the rate of cognitive decline in the subjects with Alzheimer disease,[47] thus indicating that the local delivery of therapeutic protein using autologous fibroblasts as a cell vehicle is therefore clinically relevant. The establishment of clinically applicable procedures for such transplantation using biomaterials with implanted cells, gene-transduced human ccdPA would be useful to obtain further applicable autologous cells for *ex vivo* gene therapy in patients with serum protein deficiencies who require long-term therapeutic protein supplements.

MATERIALS AND METHODS

Construction of pCGThLCAT, retrovirus vector plasmid encoding human lcat gene. pDON-AI vector (TaKaRa Bio Inc., Shiga, Japan) was used as a recipient for human *lcat* cDNA. *lcat* cDNA was derived from total RNA prepared from HepG2 cells. Resulting cDNA was amplified by PCR using primer pair as follows; 5'-ATCGGATCCAGGGCTGGAAATGGGGCCGCCC-3' (forward primer) and 5'-ATCGGATCCGTCGACGGAAGGTCTTTATTCAGGAGGCGGGGG-3' (reverse primer). Forward primer contained *Bam*HI site (underlined) and Kozak sequence, and reverse primer contained *Sal*I site (underlined). Reverse primer also eliminated polyA signal from original *lcat* cDNA. Amplified PCR product was digested by *Bam*HI and *Sal*I and cloned into corresponding sites of pDON-AI. Then, neomycin resistant gene was removed by *Sal*I and *Xho*I digestion and subsequent self-ligation, producing pCGThLCAT.

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

Isolation of ccdPA from human fat tissue. Ceiling culture technique was employed to isolate human ccdPA with some modifications. Subcutaneous adipose tissues were obtained from 16 healthy volunteers with ages ranging from 19 to 42 years after informed consent was obtained with approval and guidelines of the ethical committee at Chiba University School of Medicine according to the Declaration of Helsinki. Fat tissue was weighed and each 1.0 g was transferred into 50 ml conical tubes containing 1.5 ml of Hank's balanced salt solution (HBSS) with 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ). Same volume of HBSS containing 4 mg/ml collagenase (Collagenase NB 6 GMP Grade, SERVA, Heidelberg, Germany) was added into each tube and digested for 1 hr at 37°C with gentle shaking. Then, the solution was diluted with 10 ml of DMEM/F12-HAM containing 20% fetal bovine serum (FBS, SAFC

Biosciences, Lenexa, KS) and 40 μg/ml gentamicin, mixed, and centrifuged at 400g for 1 min. Steps of dilution to removal of pellet were repeated 4 times to collect floating cell fraction. The floating fraction was filtered with 500 μm mesh (Netwell™ Insert, Corning Inc., Corning, NY) for ceiling culture. Pellet was collected as stromal-vascular fraction (SVF) for another purpose by aspiration. Floating fraction was seeded into flasks, completely filled with DMEM/F12-HAM containing 20% FBS and 40 μg/ml gentamicin. Before incubation the medium was equilibrated with 5% CO_2 at 37°C overnight. After 7 days ceiling culture, cells that grew at the ceiling and the bottom surfaces were detached separately with TrypZean (Sigma) treatment and seeded into flasks for next step.

Cell culture. Dulbecco's modified Eagle's medium [DMEM]/F12-HAM was purchased from Sigma-Aldrich (St. Louis, MO). MesenPRO medium was from Invitrogen (Carlsbad, CA). Cells were detached by TrypZean treatment, harvested by centrifugation at 300g at 4°C, re-suspended, seeded in fresh growth medium. Expansion culture was performed in CellSTACK-1, 2, 5, and 10 Chamber (Corning Inc.) flasks. Passage was performed essentially twice a week.

Gene transduction. In preliminary experiments, the acceptability of MoMLV vector for human ccdPA propagated in the course of ceiling culture showed that longer culture resulted in more resistance to retrovirus vector transduction (data not shown). Thus, the cells obtained by 7 day-ceiling culture were evaluated as a recipient of retrovirus vector mediated gene transduction. After 7 days ceiling culture, human ccdPA were seeded and incubated in DMEM/F12-HAM supplemented with 20%FBS and 40 μg/ml gentamicin at 37°C for 24 hrs. To examine the gene transduction conditions, 2.5×10^5 cells were seeded into T25 flasks. PS (Novo-Protamine Sulfate, 100 mg for I.V. Injection, Mochida Pharm. Co. Tokyo, Japan) was used to optimize transduction conditions (0.5-16 μg/ml). For gene transduction, vector stock solution was thawed just before use. Gene transduction was performed in the presence of 20% FBS and 8 μg/ml PS at 37°C for 24 hrs. Virus vector concentration used for transduction was 2.0×10^9 RNA copies/ml, unless otherwise mentioned. After transduction, the medium was changed to growth medium. In this study, we also utilized *ZsGreen*-gene expressing retrovirus vector to evaluate the human ccdPA as a recipient for gene transduction.

Flow cytometry. Cells were detached by TrypZean treatment and harvested, re-suspended with medium, and counted with a NucleoCounter (ChemoMetec, Allerød,

Denmark). Cells were harvested, washed twice with FACS buffer (phosphate buffered saline, PBS, containing 2% FBS). Fluorescein isocyanate (FITC) or phycoerythrin (PE)-conjugated antibody were purchased from BD Farmingen (San Diego, CA) (Anti-CD10, 13, 29, 31, 44, 59, 90, 106, 146, and HLA-ABC) or Beckman Coulter (Fullerton, CA) (Anti-CD9, 34, 36, 45, 49d, 54, 65, and 117). Anti-CD105 (FITC) was from Ancell Corporation (Bayport, MN). Aliquots of cell suspension (4.5 x 10^4 cells) were mixed with primary antibody in the total volume of 90 μl and incubated for 30 min at RT. The cell suspension was washed twice with FACS buffer, and the cells were fixed in 200 μl of FACS buffer containing 1% paraformaldehyde. Five thousand events were acquired for each antibody on FACS Calibur apparatus using CELLQuest acquisition software (Becton, Dickinson and Company, Flanklin Lakes, NJ). *ZsGreen* expression in human ccdPA was also examined similarly. Un-transduced cells were cultured in parallel, and used as a negative control.

Quantification of transduced gene. According to preliminary experiments, the integrated copy number became stable at least 1 week after transduction. Therefore, we have taken the cell samples 11 or 12 days after gene transduction for determination of the integrated copy number. Cells were harvested, re-suspended in PBS, and frozen in -20°C. Genomic DNA was extracted with DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany). Integrated vector copy number was quantified by SYBR® *Premix Ex Taq*™ (Perfect Real Time) kit (TaKaRa Bio Inc.). Known amount of pCGThLCAT DNA was used as a standard. Primer pair used was from Retrovirus Titer Set (TaKaRa Bio Inc.). This assay system was customized by TaKaRa Bio Inc. Value of DNA content of human normal cell (6 pg/cell)[48] was used for calculation for averaged integrated copy number. Genomic DNA from mice adipose sections was extracted by Gentra Puregene kit (QIAGEN). Existence of transduced gene in transplanted adipose tissue was quantified by TaqMan Gene Expression Master Mix (Applied Biosystems) using a *lcat* cDNA specific primers and probe designed by Probe Finder Software (Roche Diagnostics, Mannheim, Germany). Both assays were performed using ABI7500 Real-time PCR system (Applied Biosystems, Foster City, CA).

Detection of LCAT protein. For detection of human LCAT in culture medium and mice sera, test samples 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 gentle rotation. Twenty microliters 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 five times with 1 ml of PBS-NP40 buffer, and eluted by boiling in 10 μl of 2 x Laemmli's sample buffer. Immunoprecipitated samples and standards (purified human LCAT (Roar Biomedical, Inc., New York, NY) or human plasma HDL (Calbiochem, Merck, Darmstadt, Germany)) were separated by 10% acrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked for 1 hour in SuperBlock T20 (TBS) (Thermo Fisher Scientific Inc., Rockford, IL) and incubated overnight at 4°C with the anti-LCAT rabbit polyclonal antibody (Novus Biologicals, Littleton, CO). After washing the membrane filter with Tris-buffered saline containing 0.1% Tween 20 was incubated with TrueBlot anti-Rabbit IgG HRP (1:5000) (eBioscience) for 1 hour at room temprature. The signals were detected by SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) with LAS1000 apparatus (FUJI film, Tokyo, Japan).

Measurement of LCAT activity. To assess LCAT activities in culture medium, we used liposomes containing lecithin-cholesterol as substrate. Procedure of Ishii et al.[49] was modified to optimize. Two hundred micro liter of [^{3}H]-cholesterol (American Radiolabeled Chemicals, Inc., St. Louis, MO) was transferred into glass tube, and evaporated to dryness by flushing N_2 gas, and 5 ml of substrate mixture of Anasolv® LCAT kit (SEKISUI MEDICAL Co. Tokyo, Japan) was added. The solution was vortexed for 1 min and sonicated by Digital Sonifier® Model 250 (BRANSON, Danbury, CT) with amplitude of 40%, and 0.5 second pulse on-off cycles for 1 min in an ice bath. The sonication was repeated six times. Sonicated mixture was centrifuged at 3,000 rpm and stored at 4°C until use. Reaction mixture contained 100 μl of labeled substrate, 4.5 mM of β-mercaptoethanol, 36 μg of apolipoprotein A1 (Athens Research & Technology, Athens, GA), and 100 μl of culture medium in total volume of 220 μl. Reaction was performed at 37°C for 1 hr, and the reaction was terminated by addition of 1.6 ml of chloroform/methanol (2:1). One hundred micro liter of water was added, and organic phase was obtained by centrifugation. [^{3}H]-cholesteryl esters were separated from unesterified labeled cholesterol by thin layer chromatography (TLC). Fifty micro liter of organic phase was spotted on to Whatman flexible 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). Developed TLC plates were air-dried and stained with Iodine (Wako Pure Chemicals, Osaka, Japan). Spot of cholesteryl ester was cut and the radioactivity was determined by liquid scintillation spectrometry.

Adipogenic differentiation assay. The ability to differentiate to adipocyte was examined. 3.5 x 10^4 cells of human ccdPA were seeded into BioCoat™ Collagen I 48-well Multiwell Plates (BD Biosciences) and grown to confluency (for 3 days). Differentiation was induced with PGM Bullet Kit (Lonza, Basel, Switzerland) and the cells were incubated for 2 weeks. Culture medium was removed and the cells were fixed in 4% paraformaldehyde, washed with PBS twice, 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 triglyceride was examined to confirm the adipogenic differentiation using Triglyceride E-test™ kit (Wako Pure Chemicals). Briefly, cells were lysed in 50 μl of lysis solution containing 50 mM Tris-HCl, 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.01% Tween20, and then sonicated. 30 μl of lysate was used for the assay. Protein content of the lysate was also determined with Quick Start Bradford Dye Reagent (Bio-Rad Laboratories Inc.).

Clonality analysis by Southern blotting. Abnormal amplification of specific cell clones resulted by integration of retrovirus vector genome sequence was examined by Southern blotting procedure essentially according to DIG (Digoxigenin) application manuals (Roche Diagnostics). Cells were harvested, suspended in PBS, and kept at -20°C until use. Genomic DNA was extracted using Gentra Pure Gene kit (QIAGEN) and digested with *Hin*dIII (Roche Diagnostics). Digested DNA was concentrated by ethanol precipitation and 6 μg of the DNA was subjected to agarose gel electrophoresis, followed by capillary transfer to positive-charged nylon membrane (Roche Diagnostics). Human *lcat* cDNA in pCGThLCAT was used as a template to synthesize probe by PCR DIG Probe Synthesis kit (Roche Diagnostics). Hybridization was performed at 50°C overnight. Membrane was washed and reacted with Anti-digoxigenin-AP, Fab fragments (Roche Diagnostics). Membrane was washed and the signals were detected by using CDP-Star with LAS1000 apparatus (FUJI film). As positive control, 293 (European Collection of Cell Cultures) cells were transduced by neomycin-resistant gene containing version of *lcat*-expressing retrovirus vector and typical single copy-integrated clones were selected by quantification of the integrated copy number as mentioned above.

Colony formation assay by soft-agar containing medium. An anchorage-independent colony formation was examined by soft-agar assay using CytoSelect™ 96-well Cell Transformation Assay kit (Cell Biolabs, Inc., San Diego, CA) according to

manufacturer's instructions. Ten thousands of gene-transduced human ccdPA were seeded into 96-wells plates in at least triplicates, along with 100, 1,000, and 10,000 cells of HeLa cells (European Collection of Cell Cultures) as positive control. DNA contents were also quantified using Gemini XPS fluorescent micro-plate reader (Molecular Devices, Sunnyvale, CA).

Monitoring human LCAT secretion in mouse model. To examine the ability to secrete LCAT protein *in vivo*, animal experiment was performed in Central Institute for Experimental Animals (CIEA, Kanagawa, Japan). The protocols of the study was reviewed beforehand and approved by the Animal Ethics Committee of CIEA and performed according to the Ethical Guidelines for Animal Experimentation from CIEA. To identify the transplanted cells *in vivo*, the cells were stained using PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling (Sigma-Aldrich) one passage before transplantation with slight modification of manufacturer's instructions. After expansion, cells were detached by TrypZean treatment and harvested, washed with Ringer solution containing 0.5% human serum albumin (Benesis Corp. Osaka, Japan) four times, re-suspended to the cell concentration of 3×10^7 cells/ml. 50 μl of cell suspension was injected into the adipose tissue between the shoulder-blades of NOD/Shi-*scid* IL-2Rγ^{null} (NOG) mouse.[50] Buffer alone was injected as control. All mice were bred in vinyl-isolator and six animals were sacrificed to take serum samples at each time point (Day1, 1, 3, and 6 months). Transplanted region was taken under fluorescent microscopic observation by SZX16 reflected fluorescence system (OLYMPUS corp. Tokyo, Japan) and sections were frozen at -80°C until use. Detection of LCAT in mice serum and quantification of the transplanted *lcat* gene in adipose tissues were performed as described in previous sections.

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 to compare using SPSS software. Integrated copy number and positive rate and LCAT activity were analyzed to determine whether there was a linear correlation between them. For this analysis, we calculated a linear correlation coefficient (Pearson r value) and a corresponding P value (two tailed) based on these assumptions. In all cases, P values of less than 0.05 were considered as significant.

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FIGURE LEGENDS

Figure 1 Comparison of DMEM/FBS and MesenPRO media for preparation of human ccdPA. (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) Gene transduction was performed for 2.5 x 10^5 cells (at day0) in DMEM/FBS (closed triangle) or MesenPRO medium (closed circle). Transduced cells were passaged, and cell samples at each time point were subjected to integrated copy number quantification. Data are presented as the mean ± SD (n=3). *p<0.05 vs. MesenPRO medium at twelve days after transduction. (c) The cell numbers were counted during the proliferation for 35 days in DMEM/FBS (closed triangle) or MesenPRO medium (closed circle) after gene transduction with DMEM/FBS. Cell numbers are presented from 1 g fat tissue . Data are presented as the mean ± SD (n=3). *p<0.05 vs. MesenPRO medium at each day after seeding.

Figure 2 Cell surface antigen profiles of isolated human ccdPA by ceiling culture. (a) The cells were harvested at 7 days after ceiling culture, and immuno-stained with the corresponding antibodies (solid line) or isotype control (dotted line), and subjected to FACS analysis. Histograms for each antibody are presented. (b) CD36-positive cells was examined in the cells harvested from ceiling culture (CF7(7)), and 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.

Figure 3 *In vitro* evaluation of human ccdPA as a recipient of MoMLV-based retrovirus vector mediated gene transduction and a vehicle for 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) upon single-round exposure of 2.0 x 10^9 RNA copies/ml of virus vector are shown. The cells (C010 and C011) were exposed to transduction mixture one day (Day1) or two days (Day2) 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 x 10^5 cells. After a densitometric analysis of immuno-detected signals for human LCAT protein (60-65kDa), integrated copy number and LCAT amount (arbitrary units, AU) were plotted (Pearson r value of linear coefficient, 0.953, p<0.05). (d) Culture medium incubated with 1 x 10^5 cells for 3 days were subjected to assay of LCAT activities. The

activity was presented by esterified cholesterol production from the cholesterol by the medium of human ccdPA (Pearson r value of linear coefficient, 0.954, $p<0.05$).

Figure 4 Characterization of *lcat*-gene transduced ccdPA in culture. The *lcat*-gene transduced (a, b, c) and un-transduced (d, e, f) cells of C013 were incubated for two weeks with (b, c, e, f) or without (a, d) differentiation stimulation. The appearance of cells was observed with (c, f) or without (a, b, d, e) Oil Red O staining (magnification bar, 100 μm). (g) The cells of C013 were gene transduced and the resulted cells were passaged. The cell numbers were counted during the proliferation for 35 days. The cells were transduced by the conditions of 1.3×10^9 RNA copies/ml on Day2 (closed circle), 1.3×10^9 RNA copies/ml on Day1 (closed triangle), 2.0×10^9 RNA copies/ml on Day1 (closed rhombus), or 3.1×10^9 RNA copies/ml on Day1 (closed square). Doubling times were 32.2 ± 5.8 (closed circle), 31.5 ± 4.0 (closed triangle), 31.6 ± 3.9 (closed rhombus), and 31.3 ± 4.4 hrs (closed square), respectively. The doubling time of control (un-transduced) cells (open circle) was 31.5 ± 4.7 hrs. Data are presented as the mean ± SD (n=3). No significant difference was observed in comparison to control cells. (h) The *lcat*-gene transduced cells (closed bars) and un-transduced cells (open bars) were expanded in MesenPRO medium for two weeks after gene transduction. Values of Geo/mean for 19 different surface antigens were examined by a flow cytometry analysis. Data are presented as the mean ± SD (n=3). (i) Integrated copy number of *lcat*-gene transduced ccdPA was followed during *in vitro* culture. Symbols are same as shown in Figure 4g. Data are presented as the mean ± SD (n=3). (j) A clonal analysis was performed by Southern blotting in C013 samples. Genomic DNA samples in C013 were prepared from the cells 18 days after gene transduction. Lanes 1 and 2, *lcat*-gene transduced clones obtained by transduction of 293 cells, lanes 3, 4, and 5, *lcat*-gene transduced human ccdPA with different integrated copy number (lane 3; 0.90 ± 0.20, lane 4; 1.65 ± 0.12, and lane 5; 1.79 ± 0.23 copies/cell), lane 6, un-transduced (control) cells. A smeared faint signal was observed in *lcat*-gene transduced ccdPA (shown by arrow).

Figure 5 Circulating human LCAT in NOG mice transplanted with *lcat*-gene transduced human ccdPA. The cell suspension containing 1.5×10^6 cells of *lcat*-expressing human ccdPA (C014, Transplanted) and Ringer's solution containing 0.5% HSA (Control) were injected into fat tissue of NOG mice. After one day (a, b) or one month (b) the mice were sacrificed and serum samples were collected at each time point. D1; next day of injection, M1; 1 month after injection, H; 15 μg of HDL (control). At 1 month after transplantation, LCAT was detected in serum of two mice from six mice. At

3 months or later, LCAT was hardly detected in serum (data not shown).

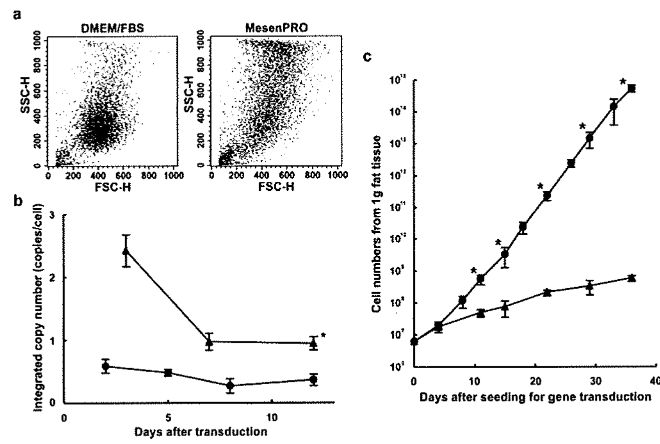


Figure 1.

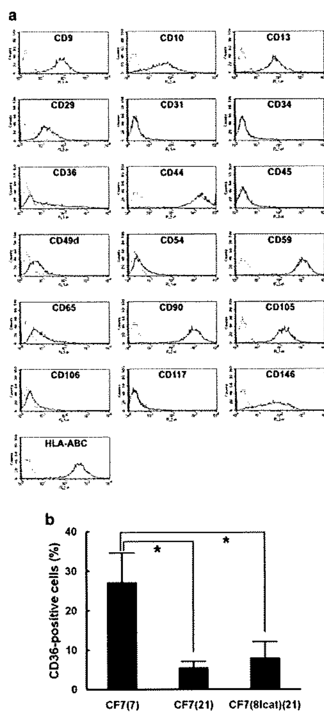
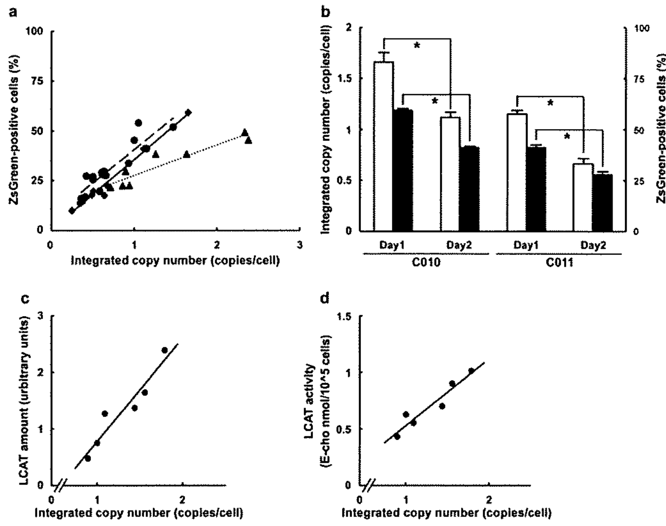


Figure 2.



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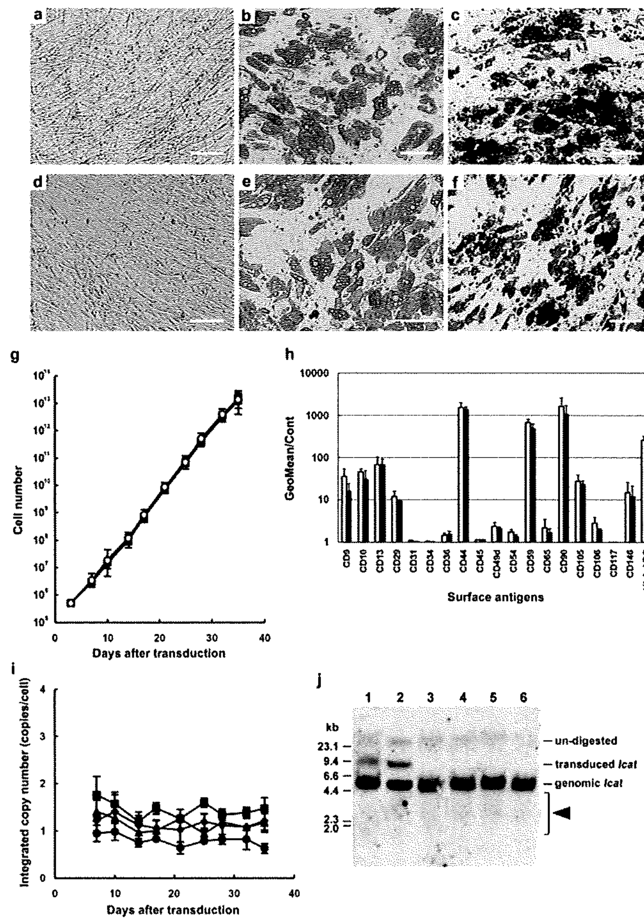

Figure 4.

Figure 5, Kuroda et al.

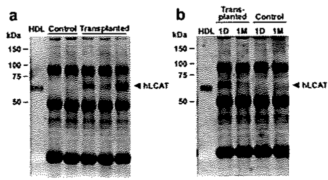

Figure 5.

Disturbed apolipoproteinA-I containing lipoproteins in fish eye disease is improved by lecithin: cholesterol acyltransferase produced by the gene-transduced adipocytes

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