C1431T [6] or -1279G/A and His478His variants [14], the positive association of the *PPARG2 Pro12Ala* with T2D was found. In addition, some reports showed that effects of effect of the *PPARG2 Pro12Ala* polymorphism on the body weight and insulin sensitivity could be modified by dietary fatty acids or physical activity [15]. Variations in dietary intake of polyunsaturated fat versus saturated fat appeared to influence BMI and fasting insulin in Ala variant carriers [27]. Moreover, dietary intake of monounsaturated fatty acids was found to be inversely associated with insulin resistance in Ala allele carriers [28]. In the Quebec Family Study, Pro homozygous had lower BMI, waist circumference and fat mass (both subcutaneous and visceral) at baseline, but responded to an increase in dietary fat with a gradual increase in BMI and waist circumference, whereas those effects were not observed in Ala allele carriers [29]. Recently, Ruchat et al. reported that Ala allele carriers appear to be more responsive to regular endurance training in improving glucose metabolism and insulin sensitivity compared with Pro homozygous [30]. Studies in different Indian ethnicities revealed an extensive diversity of the association between the *PPARG2 Pro12Ala* and T2D, further supporting the hypothesis of gene-environment interactions. The *PPARG2 Pro12Ala* polymorphism was a strong predictor for T2D in Indian Sikhs [10] but not in South Indians from Chennai [31].

In order to clarify these above questions, the larger sample size may be necessary. Because even the previous findings claiming the association of this polymorphisms with T2D showed that homozygous for the higher risk Pro allele attributes a weak effect on T2D (only a 25% increase in diabetes risk) [3], and we revealed in the present study that the Ala-allele frequency in Vietnamese was quite low.

In conclusion, we found a low frequency of the Ala allele at the *PPARG2 Pro12Ala* and the lack of association between the *PPARG2 Pro12Ala* polymorphism and glucose intolerance in a Vietnamese population. Thus, this polymorphism is not a crucial genetic marker that predicts subjects susceptible to glucose intolerance at least in Vietnamese population with moderate BMI.

〔要　約〕

転写因子ペルオキシソーム増殖因子活性化受容体γ2（*PPARG2*）の一塩基多型である *Pro12Ala*（rs1801282）変異は，2型糖尿病のリスク軽減に関連すると多くの集団で報告されてきたが，人種によっては同様の結果が得られていない。本研究は，肥満が少ないにもかかわらず2型糖尿病が増加しているベトナム人において *PPARG2 Pro12Ala* 多型が糖代謝異常の危険因子であるかを検討することを目的とした。対象は無作為に糖代謝異常者173名とこれに性・年齢をマッチさせた310名の対照者を選んだ。*Pro12Ala* アリル頻度は，糖代謝異常者群2.9%と対照者群2.4%でともに低く，糖代謝異常と *PPARG2 Pro12Ala* 多型との有意な関連は認められなかった。すなわち，*PPARG2 Pro12Ala* 多型はベトナム人における2型糖尿病リスク予測のための確定的遺伝マーカーではない。

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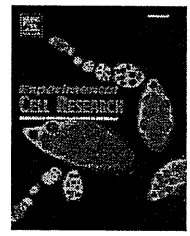

Research Article

Fibrin glue is a candidate scaffold for long-term therapeutic protein expression in spontaneously differentiated adipocytes *in vitro*

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ARTICLE INFORMATION

Article Chronology:
Received 22 March 2011
Revised version received
4 October 2011
Accepted 4 October 2011
Available online 12 October 2011

Keywords:
Adipocytes
Gene therapy
Scaffold
Fibrin glue
Adipogenesis
Lecithin:cholesterol acyltransferase

ABSTRACT

Adipose tissue is expected to provide a source of cells for protein replacement therapies via autotransplantation. However, the conditioning of the environment surrounding the transplanted adipocytes for their long-term survival and protein secretion properties has not been established. We have recently developed a preparation procedure for preadipocytes, ceiling culture-derived proliferative adipocytes (ccdPAs), as a therapeutic gene vehicle suitable for stable gene product secretion. We herein report the results of our evaluation of using fibrin glue as a scaffold for the transplanted ccdPAs for the expression of a transduced gene in a three-dimensional culture system. The ccdPAs secreted the functional protein translated from an exogenously transduced gene, as well as physiological adipocyte proteins, and the long viability of ccdPAs (up to 84 days) was dependent on the fibrinogen concentrations. The ccdPAs spontaneously accumulated lipid droplets, and their expression levels of the transduced exogenous gene with its product were maintained for at least 56 days. The fibrinogen concentration modified the adipogenic differentiation of ccdPAs and their exogenous gene expression levels, and the levels of exogenously transduced gene expression at the different fibrinogen concentrations were dependent on the extent of adipogenic differentiation in the gel. These results indicate that fibrin glue helps to maintain the high adipogenic potential of cultured adipocytes after passaging in a 3D culture system, and suggests that once they are successfully implanted at the transplantation site, the cells exhibit increased expression of the transduced gene with adipogenic differentiation.

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doi:10.1016/j.yexcr.2011.10.007

Introduction

Much attention has been paid to adipose tissue as a source of transplanted cells for cell-based gene therapy [1] and regenerative therapy [2,3]. The adipose tissue-derived preadipocytes, which can be harvested from aspirated fat tissue, proliferate rapidly and differentiate into mature adipocytes both *in vitro* and *in vivo*. Preadipocytes have been focused on as one of cell systems used to deliver therapeutic genes, since fully differentiated adipocytes are currently utilized in clinical transplantation for the correction of tissue defects in plastic and reconstructive surgery [4–6]. Therefore, we postulated that adipocyte-based protein replacement therapy via subcutaneous transplantation of gene-transduced ceiling culture-derived proliferative preadipocytes (ccdPAs) could be utilized for patients with gene deficiencies such as lecithin: cholesterol acyltransferase (LCAT) deficiency, hemophilia, lysosomal diseases, and diabetes [7–10]. The identified cells have previously been shown to differentiate into mature adipocytes in plate culture upon stimulation, and their differentiation was not affected by the exogenous gene transduction [8,11]. In the clinical application of this strategy for gene-deficient patients, it is assumed that the transplanted cells will reside stably in the subcutaneous adipose space, differentiate into adipocytes, and finally reconstruct the adipose tissue.

For successful treatment in such cell transplantation-based therapies, it is important to select suitable scaffolds for the transplanted preadipocytes, adapting the transplantation site to optimize their survival, differentiation and protein expression. In this context, fibrin glue (FG) is capable of supporting the secretion of the exogenously transduced-gene product *in vivo* [12]. In order to investigate the cell fate and protein secreting function, and also to develop alternative therapeutic applications, the establishment of an *in vitro* long-term evaluation system is required. Various synthetic and naturally-derived materials have so far been investigated as biocompatible scaffolds for adipose tissue 3D models [13–18].

In this study, we employed an *in vitro* long-term 3D culture system using FG as a therapeutic cell transplantation scaffold, and examined the cell survival, differentiation, and the expression of the transduced gene, of the ccdPAs cultured under these conditions.

Materials and methods

Preparation lcat gene transduced ceiling culture-derived proliferative adipocytes (ccdPA/lcat)

Subcutaneous adipose tissues were obtained from healthy donors after informed consent was obtained with approval from the ethics committee of Chiba University School of Medicine, and all studies were performed according to the guidelines of the Declaration of Helsinki. The preparation of ccdPAs, subsequent *lcat* gene transduction, and quantification of the copy number of transduced *lcat* genes were performed as described previously [8]. The cells utilized in this study had an average *lcat* gene copy number of 1.01 ± 0.03 copies/cell. LCAT secretion into the culture supernatant was examined by immunoprecipitation/western blot analysis and measuring the esterifying activity, as described previously [8].

Culture in the fibrin scaffold

Benesis (Benesis, Osaka, Japan) or Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as a clinically available material for the fibrin gel. Fibrinogen and thrombin solutions were diluted with Ringer's Solution (Fuso Pharmaceutical Industries, Osaka, Japan) containing 0.5% human serum albumin (Mitsubishi Tanabe Pharma., Tokyo, Japan). The ccdPA/*lcat* cells were suspended at 1 and 3×10^7 cells/ml in the diluted fibrinogen and thrombin solution. Because of the substantial gel shrinkage observed on Day 84 in the cultures with 3×10^7 cells/ml, the experiments were performed at a concentration of 1×10^7 cells/ml for long-term evaluation of the cultured cells. The final concentration of fibrinogen was 4, 8, or 40 mg/ml and the thrombin solution was used at 1 U/ml. To form fibrin clots, 50 μl of the cell-fibrinogen suspension was added to each cell culture insert (Falcon 3104; Becton Dickinson, Franklin Lakes, NJ), then shortly thereafter, 50 μl of the cell-thrombin suspension was added into the insert, mixed by pipetting, and incubated at room temperature for 2 h. The inserts with fibrin clots were placed in 12-well culture plates, and 1 ml and 2 ml of MesenPRO medium (Invitrogen, Carlsbad, CA), which had been shown to have the potential to significantly increase the proliferation and stability of ccdPAs and mesenchymal stem cells in comparison to conventional medium [8,19–21], were added to the inserts and wells, respectively. The plates were incubated at 37 °C in a 5% CO_2 incubator. At each time point, culture media were collected and stored at −30 °C until analysis. For collection of the culture supernatant, the culture medium was changed to fresh medium 3 days prior to the harvest.

Measurement of leptin and plasminogen activator inhibitor-1 (PAI-1)

The leptin and PAI-1secretion into the culture supernatant were examined using ELISA kits obtained from MILLIPORE (Billerica, MA) and R&D Systems, Inc. (Minneapolis, MN), respectively.

Cell viability analysis

The cell viability in the 3D gels was examined by the formation of water-soluble formazan dye using the Cell Counting Kit-8 (DOJINDO, Tokyo, Japan) according to manufacturer's instructions, with slight modifications. The FG/ccdPA/*lcat* 3D gel was prepared at a final cell concentration of 1×10^7 cells/ml with 4, 8, or 40 mg/ml of fibrinogen, and cells were subsequently cultured as described in the above section. To examine the cell viability, 100 μl or 200 μl of WST-8 solution was added to the inserts and wells, respectively, and the cells were incubated at 37 °C in a 5% CO_2 incubator for 30 min. The culture supernatants of the inserts and wells were mixed, and the absorbance was measured at 450 nm. The value without cells (negative control) at 450 nm was subtracted from each value.

Histological and immunohistochemical analyses

Photographs of the gels were taken at each time point, and the areas of each gel were calculated using the WinROOF software program (Mitani Corporation, Tokyo, Japan) and used to evaluate the degree of gel shrinkage. All samples of ccdPA/*lcat* cultured in

the fibrin scaffold were washed with PBS (Sigma-Aldrich, St. Louis, MO) and embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). These samples were stored at −80 °C until they were analyzed. Sections (5 μm thick) were fixed in 10% formaldehyde and stained with hematoxylin and eosin (HE). Adipogenic differentiation was identified by Oil Red O (Sigma-Aldrich) staining for 15 min at 37 °C, and the specimens were counterstained with hematoxylin. The cell numbers were counted, and the area positive for the Oil Red O signal was measured in four independent areas using the WinROOF software program (Mitani Corporation). We defined the lipid droplet accumulation rate (lipid area/cell number) as the lipid accumulating index.

LCAT production was investigated by immunohistochemistry using an anti-human LCAT antibody. Endogenous peroxidase was inactivated by incubating the samples in 0.3% H_2O_2 in methanol for 10–20 min. Nonspecific binding was blocked by treatment with 5% skim milk and 2% bovine serum albumin in PBS for 30 min. Specimens were then incubated with an anti-LCAT rabbit monoclonal antibody (Epitomics, Burlingame, CA) at a dilution of 1:250 in a humidified chamber at 4 °C overnight. The primary antibody reactions of LCAT were enhanced using the Envision+kit (DAKO, Glostrup, Denmark). The immunoreaction was visualized with 0.05% DAB (Sigma-Aldrich) solution for 30 s to 2 min at room temperature. After washing in distilled water, the specimens were counterstained with hematoxylin, dehydrated and mounted.

RNA extraction and quantitative real-time RT-PCR

RNA was extracted from ccdPAs on the fibrin scaffold by using the TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time RT-PCR was performed to investigate the expression level of PPARγ2, aP2, leptin, *lcat*, and 18S rRNA as an internal control. Five hundred nanograms of total RNA was used for the synthesis of cDNA, by using a ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using TaqMan Gene Expression Assays (Applied Biosystems). In all, 1–2 μl of cDNA solution corresponding to 50–100 ng of total RNA was subjected to 40 PCR cycles of 15 s at 95 °C, then 60 s at 60 °C in a 20 μl mixture containing 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems), 1 μl of TaqMan Gene Expression Assays (target gene) and 1 μl of TaqMan Gene Expression Assays (18S rRNA). The abundance of the mRNA expression of the target genes was normalized to that of 18S rRNA, and the ΔCT was produced by subtracting the mean CT of controls from the CT of each target gene.

Statistical analyses

The data are presented as the means ± S.D. Statistical comparisons were made by Student's *t*-test or by ANOVA followed by the post hoc Tukey test using the SPSS software program. The gel shrinkage, cell viability, lipid accumulating index, and mRNA levels for the *lcat*, PPARγ2, aP2, and leptin genes 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. P values <0.05 were considered to be significant.

Results

ccdPAs secrete the functional protein translated from an exogenously-transduced gene, as well as physiological adipocyte proteins, in the 3D fibrin gel culture system

The suitability of fibrin gel as a scaffold in the 3D culture system for the secretion of protein produced in stable exogenous gene-expressing preadipocytes was studied using ccdPA/*lcat*, the ceiling culture-derived proliferative adipocytes transduced with the human *lcat* gene [8]. The FG/ccdPA/*lcat* 3D gel was prepared at a final cell concentration of 1×10^7 cells/ml, and cultured in MesenPRO medium (see Materials and methods). The secreted LCAT protein, the exogenously transduced-gene product, was detected by immunoprecipitation/western blot analysis in the culture supernatant (Fig. 1A). The supernatant of the ccdPA/*lcat* in 3D gel showed significantly increased cholesterol esterifying activity in comparison to that of ccdPA cultures (without *lcat* gene transduction) in the gel (Fig. 1B). The amount of leptin and PAI-1, an active molecule important for the regulation of lipid and glucose metabolism, and a regulator in plasminogen activator/plasmin system [22–24], respectively, were increased in the course of 3D culture (Fig. 1C). Thus, the functional transduced gene product was secreted, in addition to the physiologically produced adipocyte-derived proteins, in the media of the FG/ccdPA/*lcat* 3D gels.

The ccdPAs survive for at least 84 days in 3D gels in a fibrinogen concentration-dependent manner

We analyzed the viability of preadipocytes cultured in the FG/3D gel for longer periods, up to 12 weeks. Obvious gel shrinkage was observed time-dependently in the wells cultured with the 4 or 8 mg/ml concentrations of fibrinogen at a cell concentration of 1×10^7 cells/ml (Fig. 2A). The WST-8 assay showed that the cell viabilities in the gels cultured with 4 or 8 mg/ml fibrinogen were significantly lower than that of the cells treated with 40 mg/ml, throughout the culture period (Fig. 2B). The cell viabilities were significantly and positively correlated with the extents of gel shrinkage among the analyzed points with the three different concentrations of fibrinogen ($p<0.05$, $r=0.934$) (Fig. 2C). Thus, the long-term viability of ccdPAs (for up to 84 days) was regulated by the fibrinogen concentrations in the 3D gel culture system.

The ccdPAs accumulate lipid droplets spontaneously and in a fibrinogen concentration-dependent manner in 3D gels

We next analyzed the lipid accumulation in ccdPAs to determine their ability to differentiate into mature adipocytes in the 3D gel culture system. Oil red O staining of embedded cells in sections suggested that the cells spontaneously started to accumulate lipid droplets around Day 28, and then the droplets became larger and more prevalent during the reamining period (Fig. 3A, at 4 mg/ml fibrinogen). The lipid accumulation analysis clearly showed that the cells cultured with 4 or 8 mg/ml of fibrinogen started to accumulate intracellular lipids after 56 days, and the cells cultured in the 40 mg/ml concentration started the accumulation after 84 days in culture (Fig. 3B). The ratios of lipid accumulation/cell number on Days 56 and 84 were significantly higher in the cells cultured with 4 mg/ml of fibrinogen than those cultured

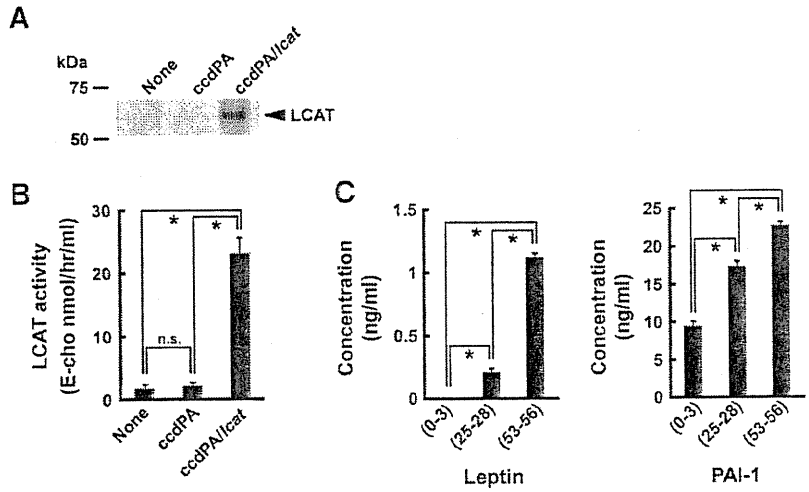

Fig. 1 – 3D fibrin gel culture of *lcat*-expressing ccdPAs. 3D fibrin gels were prepared without cells (None), or with ccdPA with or without *lcat* gene transduction, and were subsequently cultured for up to 84 days. The LCAT secretion was examined by immunoprecipitation/western blot analysis (A) and by measuring the cholesterol-esterifying activity (B) in the culture supernatant from Day 4 to Day 7. *$p<0.05$. (C) Leptin and PAI-1 were quantified by an ELISA in the three day culture supernatant of the FG/ccdPAs/*lcat* culture collected from Day 0 to Day 3 (0–3), from Day 25 to Day 28 (25–28), and from Day 53 to Day 56 (53–56). *$p<0.05$. Leptin in the supernatant from Day 0 to Day 3 was below detection limit.

in 8 or 40 mg/ml of fibrinogen, and were also higher in those cultured with 8 mg/ml of fibrinogen than in those cultured with 40 mg/ml of fibrinogen (Fig. 3C), suggesting that the lower concentrations of fibrinogen induce lipid accumulation in ccdPAs earlier and to a greater extent in the gel. The observation that the cell numbers in a single field apparently increased during the culture period in Fig. 3A, together with the identified gel shrinking (see Fig. 2), prompted us to analyze the cell density in the gel as a regulator of lipid accumulation at different fibrinogen concentrations. The quantitation of cell numbers in sections showed that cell densities were gradually increased during the culture from 7 days to 84 days, and reached numbers on Day 84 that were about 3 to 4-fold those on Day 7 days at all of the above concentrations of fibrinogen (Fig. 3D). This suggests that the lipid accumulation starts at a cell density of 200/field in the gel. Therefore, ccdPAs spontaneously accumulate lipid droplets, and the accumulation can be regulated by the fibrinogen concentration, possibly through the effects of the cell density in the 3D gel.

The effects of cell density and the fibrinogen concentration on the expression of the exogenously introduced gene in ccdPAs

An exogenously transduced gene has previously been shown to be stably expressed for 3 months in plate culture without any modification of the proliferative activity of ccdPAs [8]. We herein examined the transduced gene expression in cells cultured in 3D-fibrin gel (3D/FG). The *lcat* expression levels were not significantly different between the cells that were seeded at a density

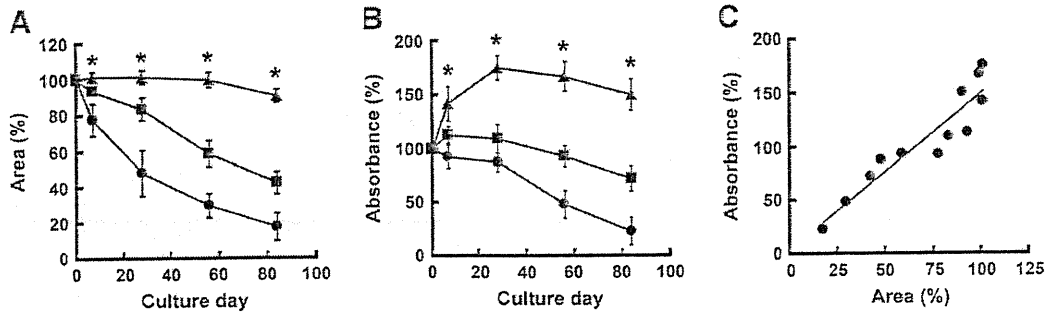

Fig. 2 – The status of 3D gel shrinkage and the survival of ccdPAs/*lcat* in the gel. The 3D fibrin gels were prepared with ccdPAs/*lcat* using 4 (closed circle), 8 (closed square), or 40 mg/ml (closed triangle) of fibrinogen, and were subsequently cultured for up to 84 days. (A) The status of gel shrinkage was expressed by the area (%) using the area of the original gel as 100%. (B) The viability of the cells in the gels was also examined, and expressed using the absorbance values with original gels as 100%. The data are presented as the means ± SD ($n=4$). Asterisks (*) depict that the differences were significant among the three concentrations of fibrinogen ($p<0.05$). (C) The correlation of the cell viability with the degree of gel shrinkage was evaluated in ccdPAs cultured with the three different concentrations of fibrinogen in the 3D gel ($p<0.05$).

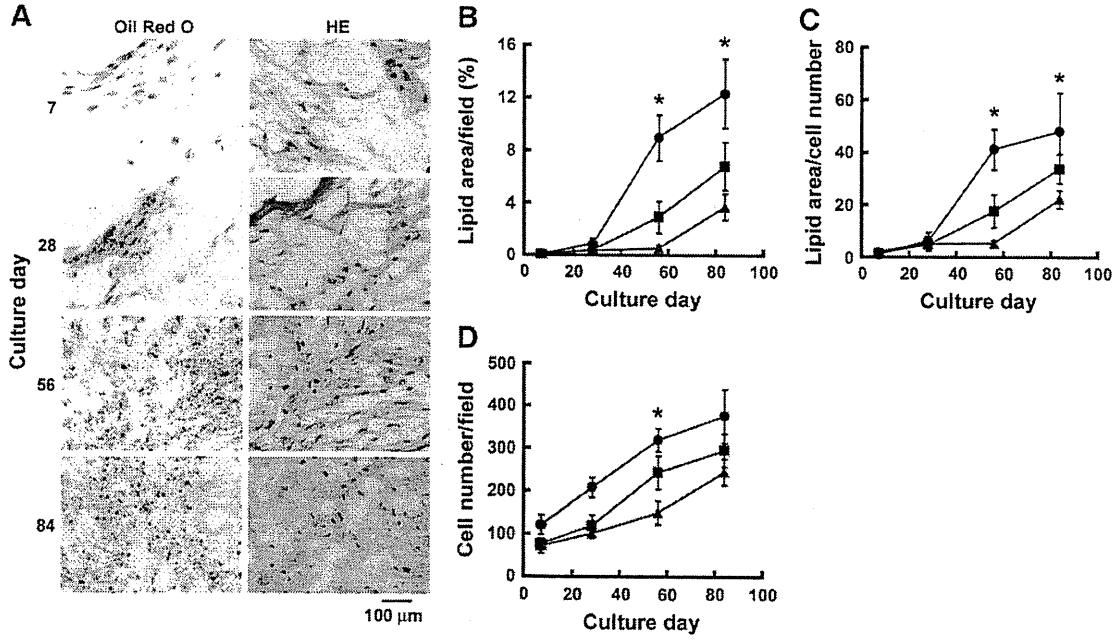

Fig. 3 – The effects of the fibrinogen concentration on the lipid accumulation of ccdPAs cultured on fibrin scaffolds.
(A) Representative data from the histological analyses of cross-sections with 4 mg/ml fibrinogen are shown. Lipid accumulation was observed by Oil Red O staining (left panel). The sections were examined by staining with hematoxylin and eosin (HE) (right panel). (B–D) The cross-sections of the 3D fibrin gels with 4 (closed circle), 8 (closed square), and 40 mg/ml (closed triangle) fibrinogen were examined. (B) Lipid droplets were quantified by evaluating the red colored area using Oil Red O stained specimens. (C) The lipid accumulation rates were evaluated based on the lipid accumulating index. (D) The cell number in the fibrin gels was evaluated by counting the number of nuclei. The data are presented as the means ± SD ($n=4$). Asterisks (*) depict that the differences were significant among the three concentrations of fibrinogen ($p<0.05$).

of 1×10^7 cells/ml or 3×10^7 cells/ml after 56 days in culture (Fig. 4A). We observed that the *lcat* gene expression levels in the cells after 56 days of culture were dose-dependently decreased by the fibrinogen concentration (Fig. 4B). When the fibrinogen concentration was 40 mg/ml, there was a 55% decrease in *lcat* expression in comparison to that at 4 mg/ml. Immunostaining showed that the translated *lcat* gene product could be clearly observed in ccdPA cultures on Day 56 (Fig. 4C). Furthermore, the immunodetected signal was intensely detected in the lipid accumulation area, rather than the area without lipid accumulation. Therefore, the expression levels of the transduced exogenous gene with its product in ccdPAs were maintained at 56 days in 3D/FG, and affected by the fibrinogen concentration.

The effects of the fibrinogen concentration on the adipogenic differentiation of ccdPAs in 3D gel

The above results suggested that the fibrinogen concentration caused decreased exogenous gene expression in ccdPAs, possibly by inhibiting the differentiation of adipocytes in the 3D/FG. Therefore, we analyzed the effect of the fibrinogen concentration in the gel on the expression levels of genes important for adipocyte differentiation in the cultured ccdPAs. The increased fibrinogen concentration resulted in a decrease in the mRNA level of PPARγ2, a differentiation-related transcription factor (Fig. 5A). The expression of aP2, another adipogenesis-related gene, was also significantly decreased by the 40 mg/ml fibrinogen concentration (Fig. 5B). In contrast, the expression of leptin, an adipocyte-secreting hormone, increased due to the increased fibrinogen concentration (Fig. 5C). These results indicated that the fibrinogen concentration modifies the adipogenic differentiation of ccdPAs and their exogenous gene expression levels, in the 3D gels.

The relationship between adipogenic differentiation and exogenous gene expression of ccdPAs in 3D gel

Finally, we analyzed the relationship between the exogenous gene expression and the adipogenic differentiation in 3D/FG with different concentrations of fibrinogen. The *lcat* gene expression levels significantly and positively correlated with the amount of lipid accumulation in the ccdPAs ($p<0.05$, $r=0.967$) (Fig. 6A). The *lcat* gene expression level was also significantly correlated with the PPARγ2 mRNA expression in the ccdPAs ($p<0.05$, $r=0.852$) (Fig. 6B). Therefore, the levels of exogenously transduced gene expression in the cultures with different fibrinogen concentrations were dependent on the extent of adipogenic differentiation in the 3D gels.

Discussion

For long-lasting enzyme replacement therapy, the transplanted cells need to stably and functionally survive at the transplanted site. To achieve this general requirement, the candidate cell types

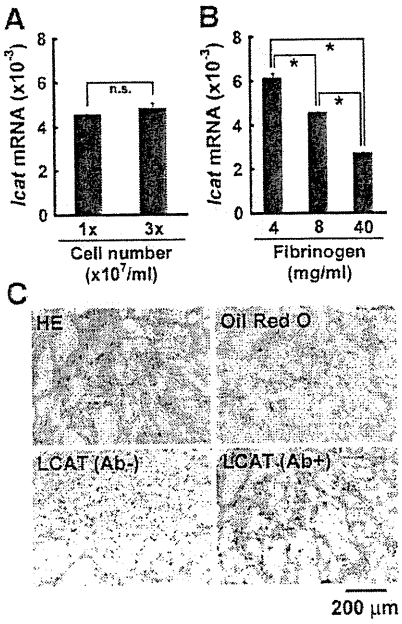

Fig. 4 – The expression of the exogenously-introduced *lcat* gene. The expression of the introduced *lcat* mRNA was compared between cultures originally generated using two different cell densities (A) and for the cultures using the three different fibrinogen densities (B) at Day 56 by quantitative RT-PCR. The mRNA expression of the treated group relative to the control was calculated using the ΔCT method. All PCR experiments were performed in triplicate. The data are presented as the means ± SD (n = 3). (C) The histological and immunostaining analyses of ccdPA/*lcat* HE in cross sections of 3D/FG on day 56 (*p<0.05).

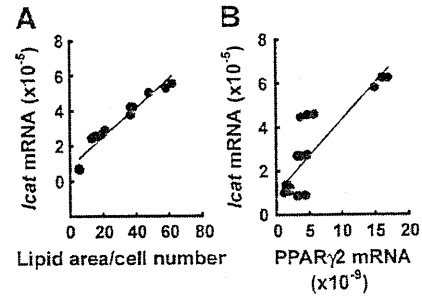

Fig. 6 – The correlations between the *lcat* expression level and lipid accumulation/cell number and PPARγ2 expression. The correlation of the expression of *lcat* with lipid accumulation/cell number (A) or PPARγ2 expression (B) was evaluated in ccdPAs cultured with different concentrations of fibrinogen in the 3D gel. *p<0.05.

are adipocytes and their progenitor cells (preadipocytes). In fact, aspirated adipocytes are widely available and currently used for cell transplantation in plastic surgery. ccdPAs are homogeneous cells identified from heterogenous preadipocytes with high adipogenic potential in long-term plate culture [11]. The homogeneity of the ccdPAs seems to be suitable for stable gene transduction in comparison to using the conventional ASCs (adipose tissue-derived stem cells) as a gene transfer vehicle. The exogenously transduced gene expression did not affect the adipogenic differentiation in a plate culture system [8]. Based on these previous findings, we evaluated the transduced gene expression characteristics of the ccdPAs using an *in vitro* 3D culture system in order to determine whether the homogenous preadipocytes could be applied for long-term protein supplementation of the exogenously transduced gene products. The ccdPAs differentiated into adipocytes which were characterized by lipid droplet accumulation and the expression of adipogenesis-specific genes in fibrin gel 3D culture, without the need for any artificial stimulation. The expression levels of the exogenously transduced gene were associated with the lipid accumulation properties of the preadipocytes in the gel. Importantly, the adipogenic potential was modified by the fibrinogen concentrations in the 3D gel, possibly as a result of the regulation of gel shrinking, and therefore the cell density, throughout the long-term culture. Thus, we propose that the 3D culture system is a candidate system suitable for evaluating gene-transduced preadipocytes prior to their subsequent physiological application.

The ccdPAs secreted the functional protein produced by the exogenously transduced gene, as well as endogenous gene products, in 3D culture (see Fig. 1). Using the exogenously transduced product-secreting preadipocytes, we investigated the cell properties using 1×10^7 cells/ml with three different concentrations of fibrinogen for 84 days in culture. The histological observations clearly showed that the fibrinogen concentration regulated the

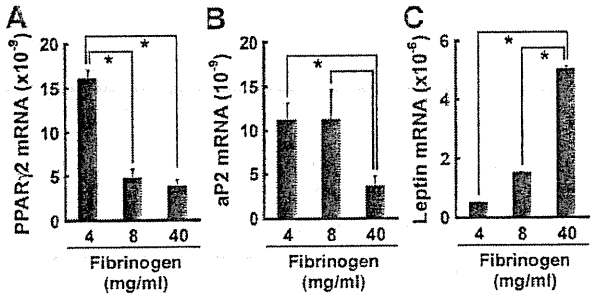

Fig. 5 – The effects of the fibrinogen concentration on adipocyte differentiation. The gene expression of adipose-specific PPARγ2 (A), aP2 (B), and leptin (C), on Day 56 were compared among the cultures grown with different fibrinogen concentrations by quantitative RT-PCR. The data are presented as the means ± SD (n = 3). *p<0.05.

gel shrinkage, as well as the survival of the cultured cells in the gel (see Fig. 2). The resulting changes in cell density in the shrunken gel regulated the lipid accumulation levels of the ccdPAs (see Fig. 3). Although the conditions are not applicable for the culture of other kinds of cells with adipogenic potential, the importance of the fibrinogen concentrations was clearly indicated as a regulator of the transduced gene expression in 3D culture (see Fig. 4). The gene expression levels of the ccdPAs were highly dependent on the fibrinogen concentration for at least 84 days, possibly because of the effects of fibrinogen on the regulation of cell adiposity and lipid accumulation (see Figs. 5 and 6). Although the precise mechanisms underlying the relationship between exogenous gene expression and adipogenic differentiation need to be elucidated in future, the induction of adipogenic differentiation of ccdPAs could complement the loss of transplanted cells after transplantation for stable protein replacement therapy. Therefore, the exogenously transduced gene expression was altered with the fibrinogen in the gel system, and therefore, the determination of the optimal fibrinogen concentration is important for the appropriate modification of the adipogenic status of the ccdPAs in 3D gel culture to ensure the therapeutic effect.

Numerous studies have been conducted to search for a suitable scaffold for cell-based therapies to characterize and enhance the differentiation efficiency of progenitor or multi-potential cells. These materials must fulfill several requirements, including mechanical support and the ability to guide tissue reconstruction, as well as biocompatibility, biodegradability, and easy handling [25,26]. In order to ensure that successful outcomes can be obtained from subcutaneous adipocyte transplantation, the prevention of apoptosis of transplanted cells and facilitation of remodeling in the transplanted region by transplanted cells through communications with surrounding tissues/cells are important. We have shown that transplanted murine ccdPAs with fibrin gel could be clearly identified in the transplanted sites of recipient mice 14 days after transplantation and the fibrin gel decreased the apoptosis of the transplanted ccdPAs [12]. Several matrix metalloproteinases (MMP) and angiogenic growth factors were previously shown to be involved in the remodeling of adipose tissue [27]. Previous reports by our group and other investigators have shown the importance of various cytokines and MMPs for the successful transplantation of adipocytes [28–31] as well as the development of adipose tissue [32–36]. These combinations with our fibrin gel condition could facilitate the development of adipocyte-based gene therapies.

In this study, higher concentrations of fibrinogen were effective for decreasing the gel shrinkage throughout the culture period, probably ensuring cell viability in the gel. On the other hand, a higher concentration of fibrinogen seemed unsuitable for preadipocytes to maturate into adipocytes soon after transplantation, leading them to reside stably at the transplantation site. Considering the application of fibrinogen/3D gel for clinical transplantation, the concentration of fibrinogen may be a determinant required to ensure the survival of the preadipocytes and to maintain the stable long-term therapeutic effects. Further analyses of the behavior of preadipocytes in fibrin gel are expected to enable us to optimize the clinical transplantation conditions in the future.

In summary, we have herein demonstrated that ccdPAs differentiate into adipocytes without artificial stimulation, and that their exogenously transduced gene expression level was modified by the fibrinogen concentration in the 3D gel. The concentration effects may have been caused by the adipogenic status, in association with the cell density of the cultured cells in the gel. The 3D culture system therefore serves as useful evaluation system for long-lasting protein replacement therapy using the cells with adipogenic potential for the development of an effective gene expression system using transplanted cells.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This study was supported by Health and Labour Sciences Research Grants for Translational Research, Japan (H. B.), and by the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), and MEXT (Japan) (Y.O., Y.N., and H.B.).

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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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Am J Physiol Cell Physiol 301:C181-C185, 2011. First published 6 April 2011;
doi:10.1152/ajpcell.00080.2011

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Am J Physiol Cell Physiol 301: C181–C185, 2011.
First published April 6, 2011; doi:10.1152/ajpcell.00080.2011.

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

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Submitted 18 March 2011; accepted in final form 4 April 2011

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 CELL-Quest 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

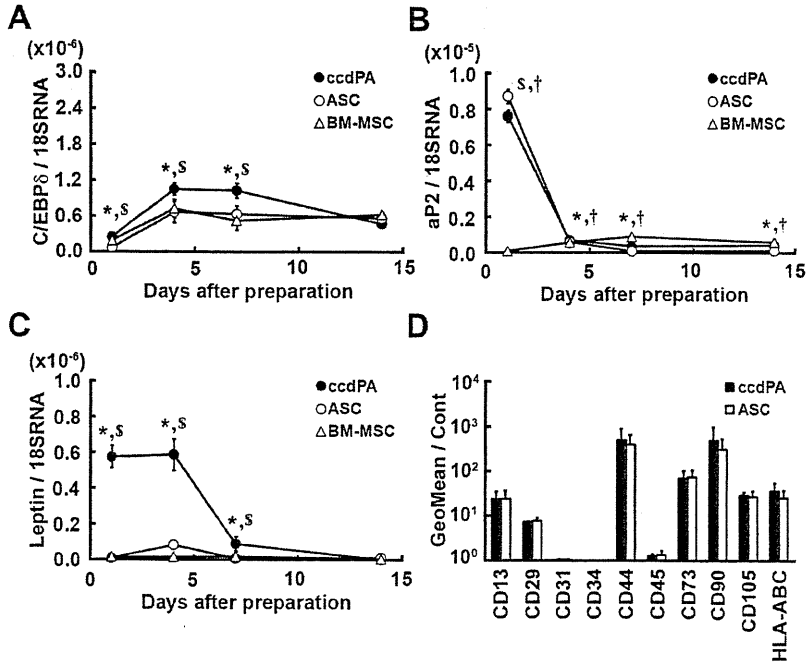

Fig. 1. Expression of adipogenic genes and cell surface markers of ceiling culture-derived proliferative adipocytes (ccdPAs) and adipose-derived stem cells (ASCs). After 7 days of primary culture with DMEM/F12-HAM supplemented with 20% fetal bovine serum (FBS), the ccdPAs and ASCs were passaged with 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$, ccdPA vs. BM-MSC, †$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

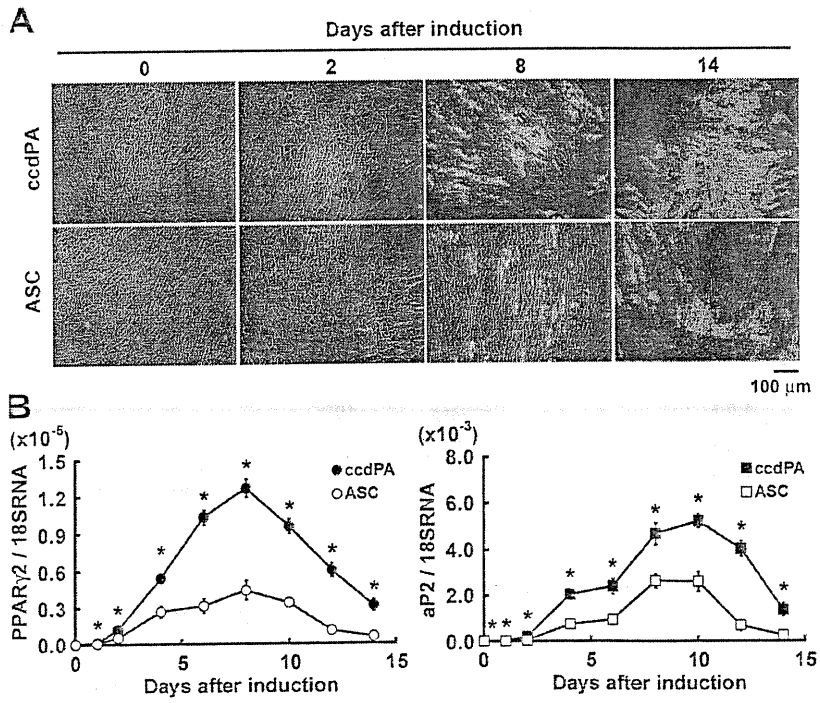

Fig. 2. Comparison of the expression of adipogenic markers in ccdPAs and ASCs during the induction of adipogenesis. *A*: adipogenic induction was performed using ccdPAs (*top*) and ASCs (*bottom*) cultured for 14 days in MesenPRO medium following 7 days of primary culture. The appearance of cells at 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. $^*P < 0.05$.

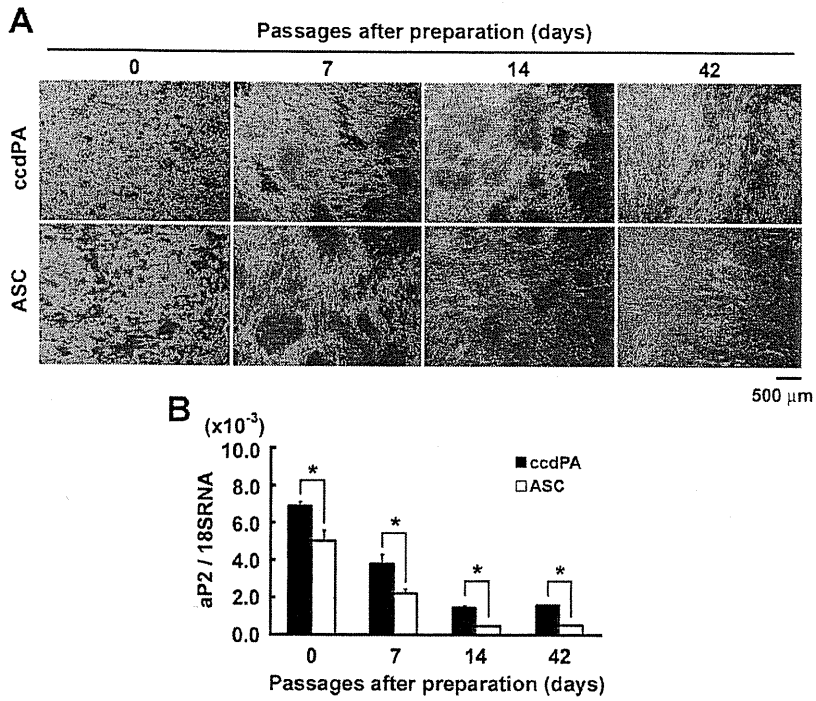

Fig. 3. The effects of consecutive in vitro passaging on the adipogenic potential of ccdPAs and ASCs. The ccdPA and stromal vascular fraction (SVF)-derived cells were obtained after a 7-day ceiling culture and were further cultured in MesenPRO medium for 7, 14, or 42 days. Cells were seeded and incubated 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

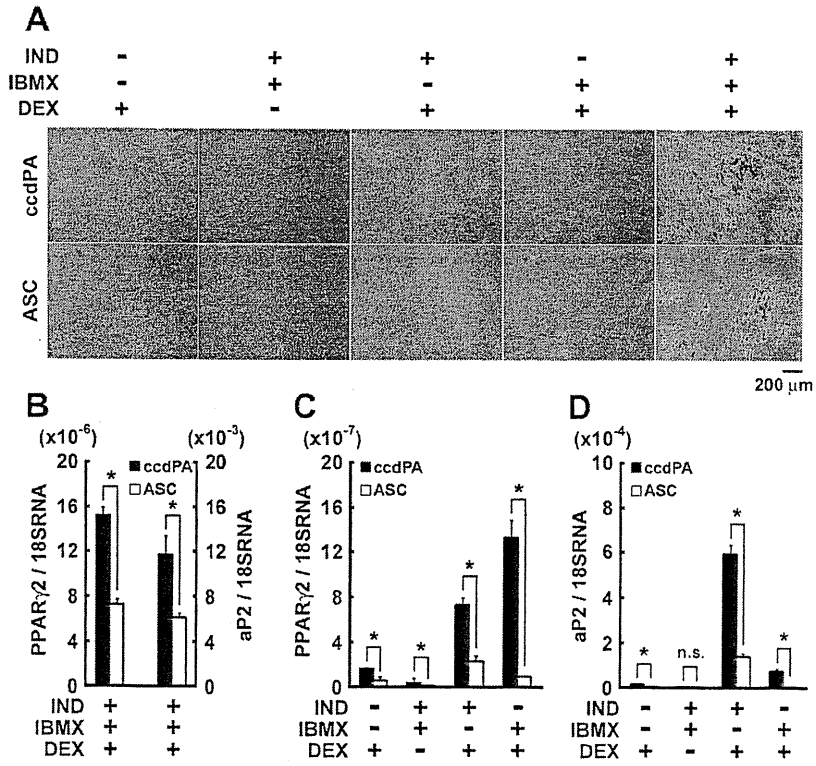

Fig. 4. Effects of differentiation-inducing agents on the adipogenecity and the gene expression levels in ccdPAs. *A*: cells were cultured for 2 wk in growth medium before induction. The appearances of the ccdPAs and ASCs on *day 14* after adipogenic induction with medium containing combinations of the 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.

GRANTS

This study was supported by Health and Labour Sciences Research Grants for Translational Research, Japan (to H. Bujo), and in part by the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT, Japan (to Y. Okamoto, T. Nakayama, and H. Bujo).

DISCLOSURES

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

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Adipocytes as a vehicle for ex vivo gene therapy: Novel replacement therapy for diabetes and other metabolic diseases

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ABSTRACT

Because of its availability and recent advances in cell biology, adipose tissue is now considered an ideal target site for the preparation of recipient cells and for the transplantation of gene-transduced cells for supplementation of therapeutic proteins. Inherited or acquired serum protein deficiencies are the ideal targets for gene therapy. However, to develop an effective ex vivo gene therapy-based protein replacement treatment, the requirements for the recipient cells are different from those for standard gene therapy that is intended to correct the function of the recipient cells themselves. To meet the requirements for such a therapeutic strategy, recent in vitro and animal model studies have developed new methods for the preparation, culture, expansion and manipulation of adipose cells using advanced gene transduction methods and transplantation scaffolds. In this short review, we introduce the progress made in novel adipose tissue-based therapeutic strategies for the treatment of protein deficiencies by our group and other investigators, and describe their future applications for diabetes and other metabolic diseases. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00133.x, 2011)

KEY WORDS: Adipocyte, Gene therapy, Metabolic disease

INTRODUCTION

Since the first gene therapy trial against advanced melanoma using gene-transduced lymphocytes was published in 1990[1], numerous therapeutic clinical trials have been carried out, and inherited monogenic disorders represent approximately 8% of the diseases targeted by gene therapy applications (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Recent studies on the biology of pluripotent stem or progenitor cells have suggested the sustained production of therapeutic proteins to be a potential treatment strategy for patients with a variety of genetic disorders[2–5]. The ability of cells to self-renew at a high proliferation rate has led to the expectations that these cells might be ideal targets for retroviral vector-mediated transgene delivery for permanent correction of the defect, not only for immunodeficiencies, but also for a variety of inherited or acquired metabolic diseases, including diabetes mellitus.

EX VIVO GENE THERAPY FOR IMMUNODEFICIENCIES

The most impressive outcomes of ex vivo gene therapy trials have been reported in subjects with immunodeficiencies as a result of monogenic disorders, including adenosine deaminase deficiency (ADA-SCID)[6,7], cc chain deficiency (X-SCID)[8,9] or X-linked chronic granulomatous disease (X-CGD)[10,11], where the treatments were combined with the infusion of ex vivo gene-corrected hematopoietic cells. Among these trials, the treatment for X-SCID caused the oncogenesis of gene-transduced cells through the clonal expansion of the cells with the activation of cellular oncogenes as a result of insertion of the MLV LTR sequence into the promoter region of the LMO2 gene[12]. Clonal expansion was also reported in X-CGD gene therapy trials[11] and myelodysplasia with monosomy 7 was caused by the insertional activation of ecotropic viral integration site 1 (EVI1)[13].

To correct the immune disorder in these patients, it is necessary for the infused gene-corrected cells to grow, differentiate into multiple hematopoietic lineages and reconstruct the immune system. In the case of X-SCID, the introduced gene (cc) is essential for the maturation of T cells, hence, only the gene-transduced cells grow and mature into functional lymphocytes, causing in vivo selection of the gene-corrected cells[14], although the precise mechanisms underlying the development of leukemia in such patients are not completely understood[15].

EX VIVO GENE THERAPY FOR FAMILIAL HYPERCHOLESTEROLEMIA

The liver is one of the primary sites of metabolic activity, and is thus the target organ of the pathogenesis for many metabolic disorders. Hepatocytes are the major cell type in the liver and have the ability to proliferate after injury, making them seem

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Received 3 April 2011; accepted 6 April 2011

like an ideal target for ex vivo gene therapy purposes. Using essentially the same technique, in which a partial hepatectomy followed by MoMLV-mediated gene transduction and reinfusion of the cells was carried out, a total of five familial hypercholesterolemia patients were treated[16,17]. However, levels of serum cholesterol reduction in these patients were moderate, and metabolic responses after gene transfer varied substantially among the five recipients. Thus, the strategy has not been carried out again to date, as a result of the invasiveness of the procedure and ineffective cell engraftment in addition to difficulties in cell preparation steps[18], and the development of the treatment has been shifted to more efficient in vivo transduction methodologies[19]. The various gene therapy trials carried out for the treatment of various metabolic deficiencies are summarized in Table 1.

CURRENT PROGRESS IN OTHER DISEASES

Genetic and acquired disorders causing secreted serum enzyme deficiencies have also been postulated to be ideal targets for gene therapy applications. In these diseases, the deficient protein functions systemically, and its defect causes severe complications in target organs. Therapeutic genes expressed by a viral vector are directly infused into the target tissues (in vivo gene therapy), or therapeutic gene-transduced cells are transplanted (ex vivo gene therapy) and, subsequently, functional proteins are produced systemically to improve the symptoms through protein replacement therapy.

In the former strategy, the gene transduction efficiency might vary depending on the tissue and cell types, and unexpected ectopic gene transduction is not completely prevented. Acute toxicity has been observed after the clinical use of an adenoviral vector[20], leading to limited further use. The efficacy of the currently available AAV vectors was shown to be hampered by the pre-existing host immune system, resulting in limitations of their applications to a clinical trial for hemophilia B treatment[21].

In the latter strategy, these side-effects can be minimized by preparing the recipient cells in vitro, and gene transduction efficiency is controllable and checked before transplantation, although cell preparation steps are required. In addition, transplanted cells are required to reside and/or survive in the patient rather than replicate, in order to continue providing a therapeutic level of protein secretion. Hemophilia has been indicated to be one of most obvious candidates for protein replacement therapy. Although considerable efforts have been expended to apply ex vivo gene therapy to treat these patients, no obvious clinical benefits were observed[22–24]. However, transplantation of genetically-modified fibroblasts into the forebrain was shown to be effective in clinical gene therapy trials of Alzheimer's disease[25]. Another approach using encapsulated-cell biodelivery technology to provide nerve growth factor (NGF) release (the product name is NsG0202) is currently being studied in a clinical trial. In this strategy, cells are enclosed by an immunoprotective, semi-permeable, hollow fiber membrane, enabling the influx of nutrients and outflow of NGF, and preventing the direct contact of the cells with the host tissue and immune system. Preliminary results have shown good safety and tolerability with no serious adverse events, and an increase in the expression of cortical nicotinic receptors, and three patients have shown cognitive improvement[26]. However, these strategies were designed for local supplementation of NGF. There is thus an absolute necessity for a novel approach to systemic delivery of therapeutic proteins. Therefore, long-lasting protein replacement therapy using gene-transduced cells is needed to provide a sufficient therapeutic strategy for systemic metabolic diseases.

ADIPOSE TISSUE AS A TARGET TISSUE FOR EX VIVO GENE THERAPY

To develop life-long protein replacement therapy through transplantation of gene-transduced cells, adipose tissue has been explored as a suitable target for several reasons. First, aspirated fat is a common source of autologous tissue transplantation for the correction of tissue defects in plastic and reconstructive surgery[27–29]. Adipose tissue is well-vascularized, and now is recognized as an important endocrine and secretory organ[30–33], and thus could enable the systemic delivery of the therapeutic protein in cell-based gene therapy applications[34–37]. Fat cells have been shown to have a relatively long lifespan[38]. With regard to safety concerns, lipoaspiration or resection of adipose tissue and fat grafting are routinely carried out in the plastic and reconstructive surgery field with minimal risk. Adipocyte-based therapeutic strategy for enzyme replacement therapy is shown in Figure 1.

Recently, adipogenic potential has been shown to suppress the tumorigenic activity of ink4a knockout mesenchymal stem cells[39]. Furthermore, if the gene-transduced cells show an abnormal phenotype, the transplanted cells residing in the transplantation space could be easily excised. In fact, it has already been shown that the transplanted cells can be excised on occurrence of unexpected or abnormal effects[35]. These findings should encourage researchers to develop an adipose tissue-based life-long and risk-manageable treatment for patients with serum protein deficiencies.

SCAFFOLD DEVELOPMENT FOR CELL TRANSPLANTATION

For the successful treatment of such cell transplantation-based therapies, it is important to select suitable scaffolds for the transplanted preadipocytes, adapting the transplantation site to optimize their survival, differentiation and protein expression. These materials must fulfill several requirements, including mechanical support and the ability to guide tissue reconstruction, as well as biocompatibility, biodegradability and easy handling[40,41]. In this context, fibrin glue is capable of supporting the secretion of the exogenously transduced gene product from preadipocytes in vivo[42]. Considering the previous reports showing the importance of various cytokines for the regulation of cell function and the surrounding matrix conditions[43–50], these combinations with our fibrin gel condition could improve the outcomes of adipocyte-based gene therapies.

