

Figure legends

Figure 1. The effects of platelet-rich plasma (PRP) on the 3-dimensional culture of ccdPAs.

100 μ l of fibrin gels containing 1×10^7 cells/ml of ccdPAs (FG/ccdPAs) were formed in cell culture insert and incubated in DMEM/HAM with 10% FBS for 16 hrs. The medium was replaced by DMEM/HAM in the presence or absence of different concentrations of FBS or PRP. (A) Photographs of FG/ccdPAs in the inserts were taken after 24 hr of culture. (B) The culture supernatant was collected from each well and the LDH activities expressed by the fluorescence of resorufin generated by coupled enzymatic reaction were examined. * $p < 0.05$.

Figure 2. Platelet-rich plasma (PRP) promotes the proliferation of ccdPAs.

(A) Photographs of ccdPAs were taken after 3 days in culture containing DMEM/F12-HAM in the absence of serum (a) or the presence of 2% (b) or 10% (c) FBS or 2% (d) PRP. The scale bar indicates 200 μ m. (B) Cells were seeded into 96 well plates and incubated in DMEM/F12-HAM containing 20% FBS for 24 hr. The culture medium was then replaced with DMEM/F12-HAM in the absence of serum (control) or presence of 2% FBS or 10% FBS or 2% PRP. After 3 days in CO₂ incubator, the cell number in each well was examined by DNA content in comparison to those on day 0 (open bar). The values for the serum (-), 10% FBS, and 2% PRP groups were significantly different compared with those on Day 0 ($p < 0.05$). * $p < 0.05$. (C) The cell numbers were examined in wells cultured in DMEM/F12-HAM containing various concentrations of PRP (circle), FBS (triangle), and PPP (square) after 3 days of culture similarly as Fig. 2B. The growth stimulatory effect was not significantly different between 10% and 20% FBS and PPP (data not shown).

Figure 3. PRP decreased the apoptotic cell death and caspase 3 activity induced by TNF- α and cycloheximide in ccdPAs.

(A) ccdPAs were seeded and incubated for 16 hr with medium containing 20% FBS, and the medium was then replaced with fresh medium without serum (Serum (-)) or with media containing 2% PRP, FBS, or PPP, and the cells were subsequently incubated at 37°C for 8 hr. The cells were then collected, stained by Annexin V-FITC and propidium iodide (PI), and analyzed using a Tali™ Image Based Cytometer (Life technologies). FITC-positive/PI-negative cells were considered to be apoptotic cells. * $p < 0.05$. (B) After incubation for 16 hr with medium containing 20% FBS, ccdPAs were pretreated for 2 hr with the medium containing 2% PRP or FBS. The cells were treated with TNF- α and cycloheximide for 5, 30, 60, and 120 min in the medium with 2% FBS (closed bar) or 2% PRP (open bar). Cell lysates were prepared at each time point as well as pretreatment (pre) and then were subjected to an immunoblot analysis of phosphorylated or unphosphorylated form of ERK1/2, and Actin. Results of densitometric analysis of phosphorylated form of ERK2 (pERK2) is shown below. After normalization of signals of pERK2 by Actin, each value of pERK2 was expressed as the fold increase of that at 5 min with 2% FBS. * $p < 0.05$. (C) After pretreatment for 2 hr with the medium containing 2% PRP, FBS, or PPP, the cells were incubated for 3 hr in the absence (open bars) or presence (closed bars) of TNF- α and cycloheximide, which induce apoptotic cell death. The caspase-3 activity in the lysates of collected cells were measured. * $p < 0.05$.

Figure 4. The results of the analysis of the expression of apoptosis-related genes affected by PRP.

(A) Total RNA was extracted from the cells cultured in the presence of 2% FBS or 2% PRP for 72 hr. The total RNA was subjected to cDNA synthesis and subsequent quantification of the mRNA expression for various apoptosis-related genes. The fold-changes in the quantified mRNA amounts compared to the average value of house-keeping genes were plotted for each gene (x-axis; cultured in 2% FBS, y-axis; cultured in 2% PRP).

Figure 5. Serum starvation induced the expression of the DAPK1 and BIM genes, which was prevented by cultured with 2%PRP.

The ccdPAs were seeded and incubated for 16 hr in DMEM/HAM containing 20% FBS, and the culture medium was then replaced with medium without serum (Serum(-)), or with 2% or 10% FBS, 2% PRP, or 2% PPP, followed by incubation for an additional 72 hr. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to evaluate the mRNA expression levels of DAPK1 (A) and BIM (B). The quantification of the given genes was expressed as relative mRNA level compared with a control after normalization to 18S RNA. * $p < 0.05$.

Table 1. Apoptosis-related genes affected by PRP

| Unigene | Refseq | Symbol | Description | Fold Change |
|---------------|-----------|-----------|---|-------------|
| Downregulated | | | | |
| Hs.380277 | NM_004938 | DAPK1 | Death-associated protein kinase 1 | 13.5803 ↓ |
| Hs.469658 | NM_006538 | BCL2L11 | BCL2-like 11 (apoptosis facilitator) | 5.2596 ↓ |
| Hs.5353 | NM_001230 | CASP10 | Caspase 10, apoptosis-related cysteine peptidase | 4.0726 ↓ |
| Hs.591834 | NM_003844 | TNFRSF10A | Tumor necrosis factor receptor superfamily, member 10a | 3.967 ↓ |
| Hs.513667 | NM_003946 | NOL3 | Nucleolar protein 3 (apoptosis repressor with CARD domain) | 2.8887 ↓ |
| Hs.501497 | NM_001252 | CD70 | CD70 molecule | 2.8129 ↓ |
| Hs.87247 | NM_003806 | HRK | Harakiri, BCL2 interacting protein (contains only BH3 domain) | 2.5816 ↓ |
| Hs.710305 | NM_004536 | NAIP | NLR family, apoptosis inhibitory protein | 2.3876 ↓ |
| Hs.643120 | NM_000875 | IGF1R | Insulin-like growth factor 1 receptor | 2.1732 ↓ |
| Upregulated | | | | |
| Hs.127799 | NM_001165 | BIRC3 | Baculoviral IAP repeat-containing 3 | 10.851 ↑ |
| Hs.462529 | NM_003790 | TNFRSF25 | Tumor necrosis factor receptor superfamily, member 25 | 4.0981 ↑ |
| Hs.478275 | NM_003810 | TNFSF10 | Tumor necrosis factor (ligand) superfamily, member 10 | 4.0907 ↑ |
| Hs.654459 | NM_001561 | TNFRSF9 | Tumor necrosis factor receptor superfamily, member 9 | 2.9875 ↑ |
| Hs.522506 | NM_021138 | TRAF2 | TNF receptor-associated factor 2 | 2.8331 ↑ |
| Hs.9216 | NM_001227 | CASP7 | Caspase 7, apoptosis-related cysteine peptidase | 2.2033 ↑ |
| Hs.145726 | NM_001205 | BNIP1 | BCL2/adenovirus E1B 19kDa interacting protein 1 | 2.0258 ↑ |
| Hs.194726 | NM_004874 | BAG4 | BCL2-associated athanogene 4 | 2.0241 ↑ |

Genes identified as having a change in expression > 2-fold induced by the addition of PRP in comparison to 2% FBS are shown.

References

- Abuzeni PZ, Alexander RW. Enhancement of autologous fat transplantation with platelet rich plasma. *Am J Cosmet Surg* 2001;18:59-70
- Aiba-Kojima E, Tsuno NH, Inoue K, Matsumoto D, Shigeura T, Sato T, Suga H, Kato H, Nagase T, Gonda K, Koshima I, Takahashi K, Yoshimura K. Characterization of wound drainage fluids as a source of soluble factors associated with wound healing: comparison with platelet-rich plasma and potential use in cell culture. *Wound Repair Regen* 2007;15:511-20
- Andia I, Sanchez M, Maffulli N. Tendon healing and platelet-rich plasma therapies. *Expert Opin Biol Ther* 2010;10:1415-26
- Anitua E, Sanchez M, Nurdén AT, Nurdén P, Orive G, Andia I. New insights into and novel applications for platelet-rich fibrin therapies. *Trends Biotechnol* 2006;24:227-34
- Aoyagi Y, Kuroda M, Asada S, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Aso M, Saito Y. Fibrin glue increases the cell survival and the transduced gene product secretion of the ceiling culture-derived adipocytes transplanted in mice. *Exp Mol Med* 2011;43:161-7
- Aoyagi Y, Kuroda M, Asada S, Tanaka S, Konno S, Tanio M, Aso M, Okamoto Y, Nakayama T, Saito Y, Bujo H. Fibrin glue is a candidate scaffold for long-term therapeutic protein expression in spontaneously differentiated adipocytes *in vitro*. *Exp Cell Res* 2012;318:8-15
- Arvidson K, Abdallah BM, Applegate LA, Baldini N, Cenni E, Gomez-Barrena E, Granchi D, Kassem M, Konttinen YT, Mustafa K, Pioletti DP, Sillat T, Finne-Wistrand A. Bone regeneration and stem cells. *J Cell Mol Med* 2011;15:718-46
- 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* 2011;301:C181-5
- Bauer-Kreisel P, Goepferich A, Blunk T. Cell-delivery therapeutics for adipose tissue regeneration. *Adv Drug Deliv Rev* 2010;62:798-813
- Bhanot S, Alex JC. Current applications of platelet gels in facial plastic surgery. *Facial Plast Surg* 2002;18:27-33
- Billings E, Jr., May JW, Jr. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast Reconstr Surg* 1989;83:368-81
- Bir SC, Esaki J, Marui A, Yamahara K, Tsubota H, Ikeda T, Sakata R. Angiogenic properties of sustained release platelet-rich plasma: characterization *in-vitro* and in the ischemic hind limb of the mouse. *J Vasc Surg* 2009;50:870-9 e2
- Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death-apoptosis, autophagy and senescence. *FEBS J* 2010;277:2-21
- Cawthorn WP, Sethi JK. TNF- α and adipocyte biology. *FEBS Lett* 2008;582:117-31
- Cervelli V, Gentile P, Scioli MG, Grimaldi M, Casciani CU, Spagnoli LG, Orlandi A. Application of platelet-rich plasma in plastic surgery: clinical and *in vitro* evaluation. *Tissue Eng Part C Methods* 2009;15:625-34
- Chen FM, Zhang J, Zhang M, An Y, Chen F, Wu ZF. A review on endogenous regenerative technology in periodontal regenerative medicine. *Biomaterials* 2010;31:7892-927
- Chieragato K, Castegnarò S, Madeo D, Astori G, Pegoraro M, Rodeghiero F. Epidermal growth factor, basic fibroblast growth factor and platelet-derived growth factor-bb can substitute for fetal bovine serum and compete with human platelet-rich plasma in the *ex vivo* expansion of mesenchymal stromal cells derived from adipose tissue. *Cytotherapy* 2011;13:933-43
- Cho SW, Kim I, Kim SH, Rhie JW, Choi CY, Kim BS. Enhancement of adipose tissue formation by implantation of adipogenic-differentiated preadipocytes. *Biochem Biophys Res Commun* 2006;345:588-94
- Doucet C, Ernou I, Zhang Y, Lense JR, Begot L, Holy X, Lataillade JJ. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* 2005;205:228-36
- Eppley BL, Pietrzak WS, Blanton M. Platelet-rich plasma: a review of biology and applications in plastic surgery. *Plast Reconstr Surg* 2006;118:147e-59e
- Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. *Plast Reconstr Surg* 2004;114:1502-8
- Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. *Am J Sports Med* 2009;37:2259-72
- Frechette JP, Martineau I, Gagnon G. Platelet-rich plasmas: growth factor content and roles in wound healing. *J Dent Res* 2005;84:434-9
- Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res* 2007;100:1249-60
- Gozuacik D, Kimchi A. DAPK protein family and cancer. *Autophagy* 2006;2:74-9
- Jang CW, Chen CH, Chen CC, Chen JY, Su YH, Chen RH. TGF- β induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* 2002;4:51-8
- Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ, Kusumoto K. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. *Plast Reconstr Surg* 2008;122:1352-60
- Kang YH, Jeon SH, Park JY, Chung JH, Choung YH, Choung HW, Kim ES, Choung PH. Platelet-rich fibrin is a Bioscaffold and reservoir of growth factors for tissue regeneration. *Tissue Eng Part A* 2011;17:349-59
- Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell* 2009;36:487-99
- Kimura Y, Ozeki M, Inamoto T, Tabata Y. Adipose tissue engineering based on human preadipocytes combined with gelatin microspheres containing basic fibroblast growth factor. *Biomaterials* 2003;24:2513-21
- Kuramochi D, Unoki H, Bujo H, Kubota Y, Jiang M, Rikihisa N, Udagawa A, Yoshimoto S, Ichinose M, Saito Y. Matrix metalloproteinase 2 improves the transplanted adipocyte survival in mice. *Eur J Clin Invest* 2008;38:752-9
- Kurita M, Aiba-Kojima E, Shigeura T, Matsumoto D, Suga H, Inoue K, Eto H, Kato H, Aoi N, Yoshimura K. Differential effects of three preparations of human serum on expansion of various types of human cells. *Plast Reconstr Surg* 2008;122:438-48
- Kuroda M, Aoyagi Y, Asada S, Bujo H, Tanaka S, Konno S, Tanio M, Ishii I, Machida K, Matsumoto F, Satoh K, Aso M, Saito Y. Ceiling culture-derived proliferative adipocytes are a possible delivery vehicle for enzyme replacement therapy in lecithin: cholesterol acyltransferase deficiency. *Open Gene Ther J* 2011;4:1-10
- Lin Y, Hupp TR, Stevens C. Death-associated protein kinase (DAPK) and signal transduction: additional roles beyond cell death. *FEBS J* 2010;277:48-57
- Liu Y, Kalen A, Risto O, Wahlstrom O. Fibroblast proliferation due to exposure to a platelet concentrate *in vitro* is pH dependent. *Wound Repair Regen* 2002;10:336-40
- Lopez-Vidriero E, Goulding KA, Simon DA, Sanchez M, Johnson DH. The use of platelet-rich plasma in arthroscopy and sports medicine: optimizing the healing environment. *Arthroscopy* 2010;26:269-78
- Lucarelli E, Beccheroni A, Donati D, Sangiorgi L, Cenacchi A, Del Vento AM, Meotti C, Bertoja AZ, Giardino R, Fornasari PM, Mercuri M, Picci P. Platelet-derived growth factors enhance proliferation of human stromal stem cells. *Biomaterials* 2003;24:3095-100
- Man D, Plosker H, Winland-Brown JE. The use of autologous platelet-rich plasma (platelet gel) and autologous platelet-poor plasma (fibrin

glue) in cosmetic surgery. *Plast Reconstr Surg* 2001;107:229-37; discussion 238-9

Margolis DJ, Kantor J, Santanna J, Strom BL, Berlin JA. Effectiveness of platelet releasate for the treatment of diabetic neuropathic foot ulcers. *Diabetes Care* 2001;24:483-8

Martinez-Zapata MJ, Marti-Carvajal A, Sola I, Bolibar I, Angel Exposito J, Rodriguez L, Garcia J. Efficacy and safety of the use of autologous plasma rich in platelets for tissue regeneration: a systematic review. *Transfusion* 2009;49:44-56

Mebratu Y, Tesfaigzi Y. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle* 2009;8:1168-75

Muller AM, Davenport M, Verrier S, Droeser R, Alini M, Bocelli-Tyndall C, Schaefer DJ, Martin I, Scherberich A. Platelet lysate as a serum substitute for 2D static and 3D perfusion culture of stromal vascular fraction cells from human adipose tissue. *Tissue Eng Part A* 2009;15:869-75

Nakamura S, Ishihara M, Takikawa M, Murakami K, Kishimoto S, Yanagibayashi S, Kubo S, Yamamoto N, Kiyosawa T. Platelet-rich plasma (PRP) promotes survival of fat-grafts in rats. *Ann Plast Surg* 2010;65:101-6

Ning H, Liu G, Lin G, Yang R, Lue TF, Lin CS. Fibroblast growth factor 2 promotes endothelial differentiation of adipose tissue-derived stem cells. *J Sex Med* 2009;6:967-79

Oh DS, Cheon YW, Jeon YR, Lew DH. Activated platelet-rich plasma improves fat graft survival in nude mice: a pilot study. *Dermatol Surg* 2011;37:619-25

Patrick CW, Jr. Adipose tissue engineering: the future of breast and soft tissue reconstruction following tumor resection. *Semin Surg Oncol* 2000;19:302-11

Patrick CW, Jr. Tissue engineering strategies for adipose tissue repair. *Anat Rec* 2001;263:361-6

Pires Fraga MF, Nishio RT, Ishikawa RS, Perin LF, Helene A, Jr., Malheiros CA. Increased survival of free fat grafts with platelet-rich plasma in rabbits. *J Plast Reconstr Aesthet Surg* 2010;63:e818-22

Ramesh S, Wildey GM, Howe PH. Transforming growth factor β (TGF β)-induced apoptosis: the rise & fall of Bim. *Cell Cycle* 2009;8:11-7

Ramjaun AR, Tomlinson S, Eddaoudi A, Downward J. Upregulation of two BH3-only proteins, Bmf and Bim, during TGF beta-induced apoptosis. *Oncogene* 2007;26:970-81

Redler LH, Thompson SA, Hsu SH, Ahmad CS, Levine WN. Platelet-rich plasma therapy: a systematic literature review and evidence for clinical use. *Phys Sportsmed* 2011;39:42-51

Ren D, Tu HC, Kim H, Wang GX, Bean GR, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science* 2010;330:1390-3

Sadati KS, Corrado AC, Alexander RW. Platelet-rich plasma (PRP) utilized to promote greater graft volume retention in autologous fat grafting. *Am J Cosmet Surg* 2006;23:203-211

Sclafani AP. Applications of platelet-rich fibrin matrix in facial plastic surgery. *Facial Plast Surg* 2009;25:270-6

Stashower M, Smith K, Williams J, Skelton H. Stromal progenitor cells present within liposuction and reduction abdominoplasty fat for autologous transfer to aged skin. *Dermatol Surg* 1999;25:945-9

Sterodimas A, de Faria J, Nicaretta B, Pitanguy I. Tissue engineering with adipose-derived stem cells (ADSCs): current and future applications. *J Plast Reconstr Aesthet Surg* 2010;63:1886-92

Tobita M, Uysal AC, Ogawa R, Hyakusoku H, Mizuno H. Periodontal tissue regeneration with adipose-derived stem cells. *Tissue Eng Part A* 2008;14:945-53

Torio-Padron N, Borges J, Momeni A, Mueller MC, Tegtmeier FT, Stark GB. Implantation of VEGF transfected preadipocytes improves vascularization of fibrin implants on the cylinder chorioallantoic membrane (CAM) model. *Minim Invasive Ther Allied Technol* 2007;16:155-62

Vogel JP, Szalay K, Geiger F, Kramer M, Richter W, Kasten P. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. *Platelets* 2006;17:462-9

Warne JP. Tumour necrosis factor α : a key regulator of adipose tissue mass. *J Endocrinol* 2003;177:351-5

Welsh WJ. Autologous platelet gel: clinical function and usage in plastic surgery. *Cosmet Dermatol* 2000;13:13-18

Wildey GM, Patil S, Howe PH. Smad3 potentiates transforming growth factor β (TGF β)-induced apoptosis and expression of the BH3-only protein Bim in WEHI 231 B lymphocytes. *J Biol Chem* 2003;278:18069-77

Wu W, Zhang J, Dong Q, Liu Y, Mao T, Chen F. Platelet-rich plasma - A promising cell carrier for micro-invasive articular cartilage repair. *Med Hypotheses* 2009;72:455-7

Yamada Y, Nakamura S, Ito K, Sugito T, Yoshimi R, Nagasaka T, Ueda M. A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology. *Tissue Eng Part A* 2010;16:1891-900

Yamaguchi M, Matsumoto F, Bujo H, Shibasaki M, Takahashi K, Yoshimoto S, Ichinose M, Saito Y. Revascularization determines volume retention and gene expression by fat grafts in mice. *Exp Biol Med (Maywood)* 2005;230:742-8

Yoshimura K, Suga H, Eto H. Adipose-derived stem/progenitor cells: roles in adipose tissue remodeling and potential use for soft tissue augmentation. *Regen Med* 2009;4:265-73

Yu J, Zhang L, Chen A, Xiang G, Wang Y, Wu J, Mitchelson K, Cheng J, Zhou Y. Identification of the gene transcription and apoptosis mediated by TGF- β -Smad2/3-Smad4 signaling. *J Cell Physiol* 2008;215:422-33

Yu W, Wang J, Yin J. Platelet-rich plasma: a promising product for treatment of peripheral nerve regeneration after nerve injury. *Int J Neurosci* 2011;121:176-80

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;7:211-28

Figure 1. Fukaya *et al.*

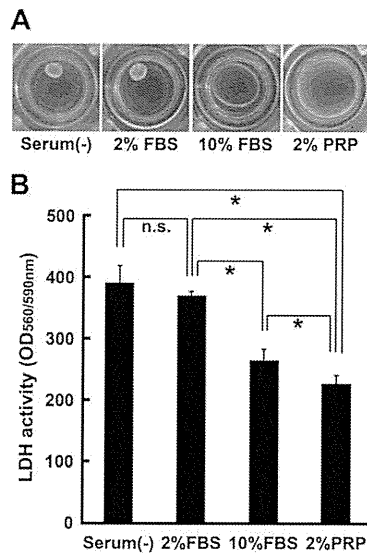


Figure 2. Fukaya *et al.*

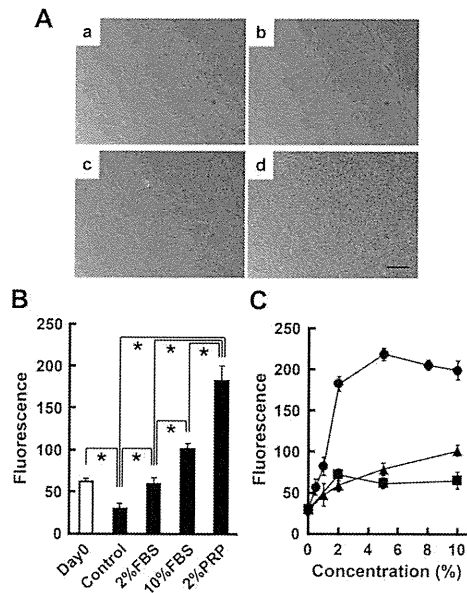


Figure 3. Fukaya *et al.*

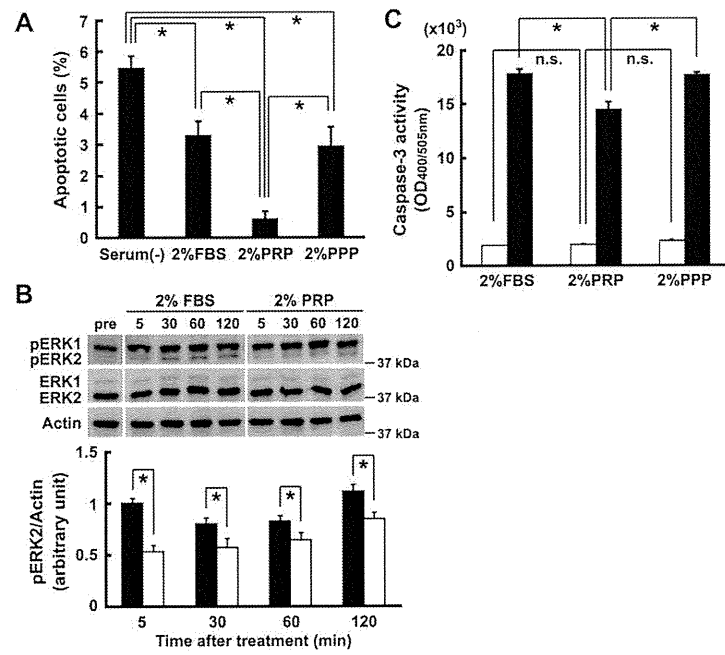


Figure 4. Fukaya *et al.*

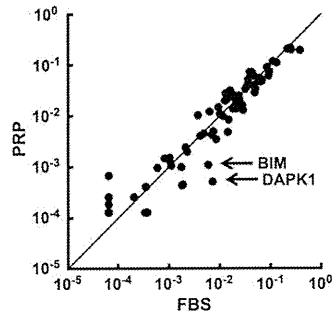
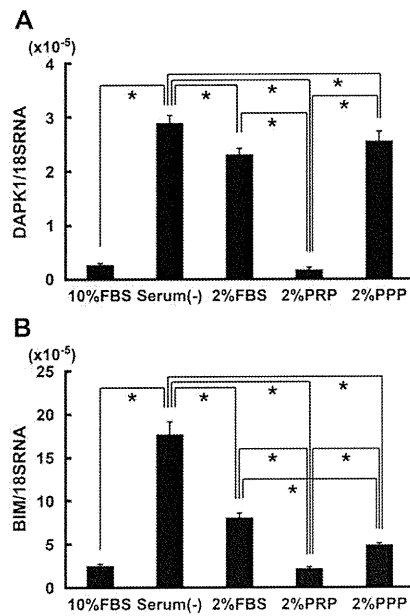


Figure 5. Fukaya *et al.*

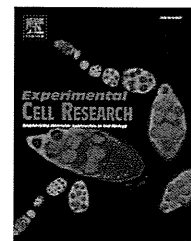




ELSEVIER

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

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

Yasuyuki Aoyagi^{a,b}, Masayuki Kuroda^{a,b,*}, Sakiyo Asada^{a,b}, Shigeaki Tanaka^c,
Shunichi Konno^c, Masami Tani^c, Masayuki Aso^c, Yoshitaka Okamoto^a,
Toshinori Nakayama^d, Yasushi Saito^e, Hideaki Bujo^b

^aCenter for Advanced Medicine, Chiba University Hospital, Chiba University, Chiba, Japan

^bDepartment of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan

^cCellGenTech, Inc., Chiba, Japan

^dDepartment of Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan

^eChiba University, Chiba, Japan

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 auto-transplantation. 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.

© 2011 Elsevier Inc. All rights reserved.

* Corresponding author at: Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Japan. Fax: +81 43 226 8130.

E-mail address: kurodam@faculty.chiba-u.jp (M. Kuroda).

0014-4827/\$ – see front matter © 2011 Elsevier Inc. All rights reserved.

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 μ l of the cell-fibrinogen suspension was added to each cell culture insert (Falcon 3104; Becton Dickinson, Franklin Lakes, NJ), then shortly thereafter, 50 μ l 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% CO₂ 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-1 secretion 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 μ l or 200 μ l of WST-8 solution was added to the inserts and wells, respectively, and the cells were incubated at 37 °C in a 5% CO₂ 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 ($5\ \mu\text{m}$ 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 PPAR γ 2, 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 \pm 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*, PPAR γ 2, 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 remaining 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

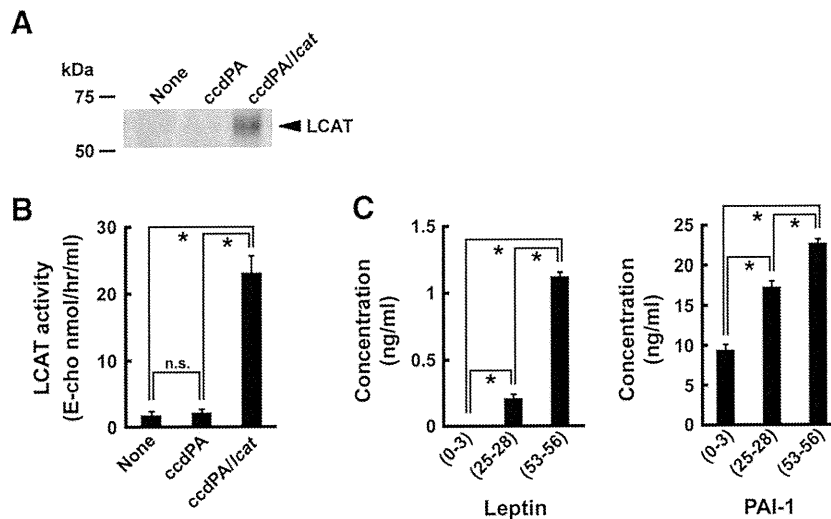


Fig. 1 – 3D fibrin gel culture of *lcat*-expressing ccdPAs. 3D fibrin gels were prepared without cells (None), or with ccdPA with or without *lcat* gene transduction, and were subsequently cultured for up to 84 days. The LCAT secretion was examined by immunoprecipitation/western blot analysis (A) and 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

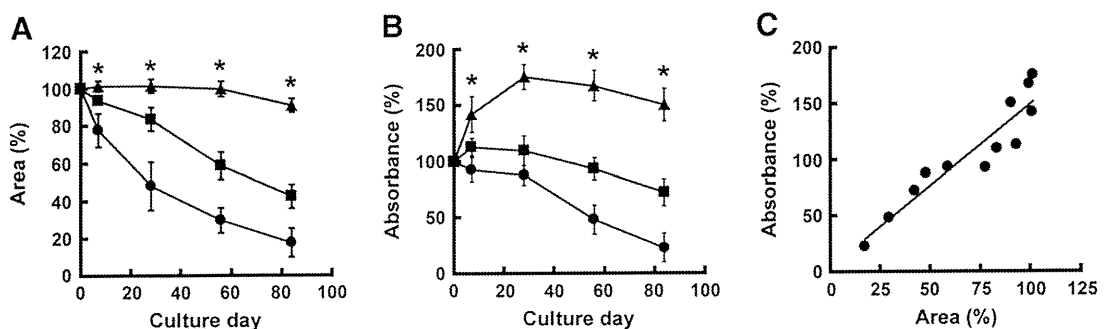


Fig. 2 – The status of 3D gel shrinkage and the survival of ccdPAs/*lcat* in the gel. The 3D fibrin gels were prepared with ccdPAs/*lcat* using 4 (closed circle), 8 (closed square), or 40 mg/ml (closed triangle) of fibrinogen, and were subsequently cultured for up to 84 days. (A) The status of gel shrinkage 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 \pm 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 ($p < 0.05$).

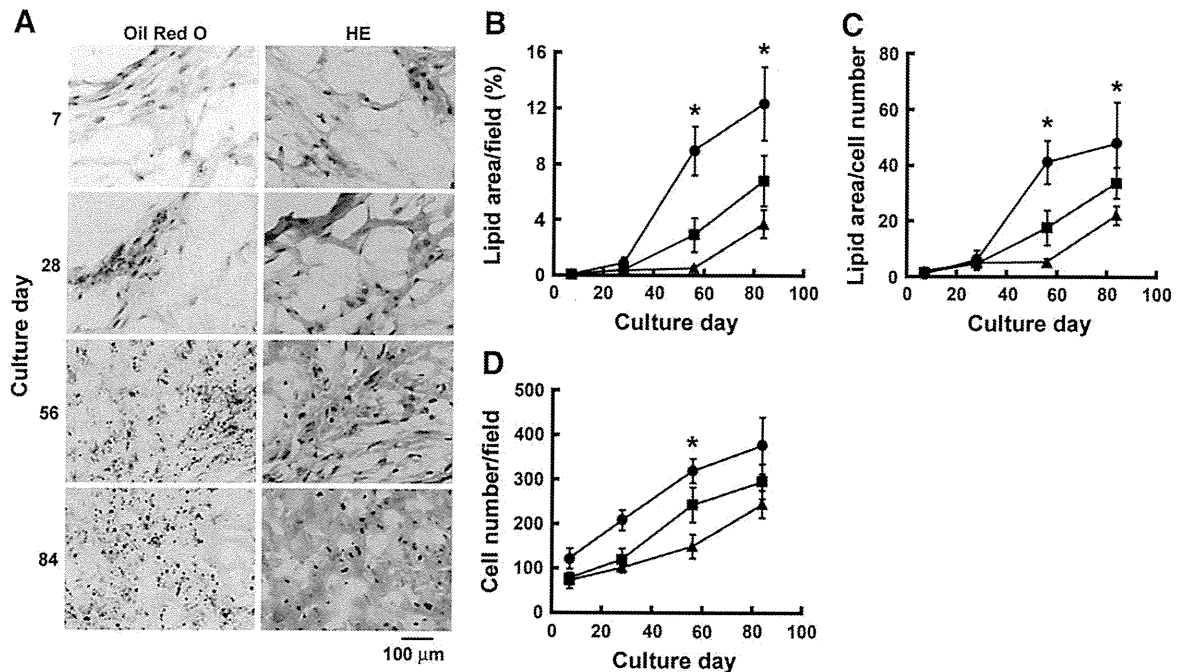


Fig. 3 – The effects of the fibrinogen concentration on the lipid accumulation of ccdPAs cultured on fibrin scaffolds. (A) Representative data from the histological analyses of cross-sections with 4 mg/ml fibrinogen are shown. Lipid accumulation was observed by Oil Red O staining (left panel). The sections 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 \pm 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 PPAR γ 2, 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 PPAR γ 2 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

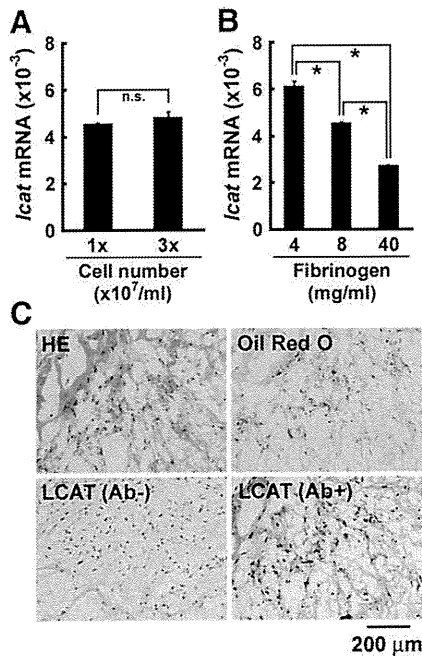


Fig. 4 – The expression of the exogenously-introduced *lcat* gene. The expression of the introduced *lcat* mRNA was compared between cultures originally generated using two different cell densities (A) and for the cultures using the three different fibrinogen densities (B) at Day 56 by quantitative RT-PCR.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

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 heterogeneous 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

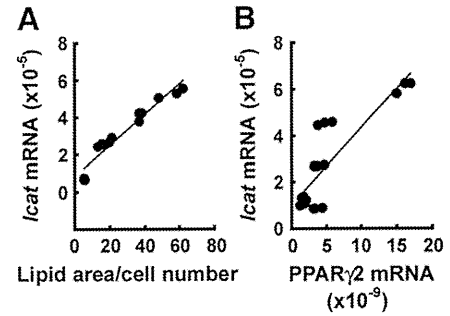


Fig. 6 – The correlations between the *lcat* expression level and lipid accumulation/cell number and PPAR γ 2 expression. The correlation of the expression of *lcat* with lipid accumulation/cell number (A) or PPAR γ 2 expression (B) was evaluated in ccdPAs cultured with different concentrations of fibrinogen in the 3D gel. * $p < 0.05$.

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

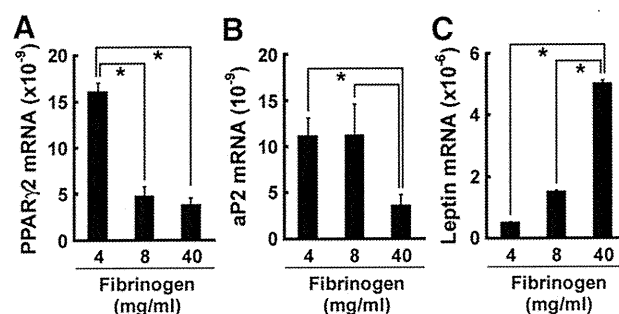


Fig. 5 – The effects of the fibrinogen concentration on adipocyte differentiation. The gene expression of adipose-specific PPAR γ 2 (A), aP2 (B), and leptin (C), on Day 56 were compared among the cultures grown with different fibrinogen concentrations by quantitative RT-PCR. The data are presented as the means \pm 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 mature 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.).

REFERENCES

- [1] L. Casteilla, B. Cousin, V. Planat-Benard, P. Laharrague, M. Carmona, L. Penicaud, Virus-based gene transfer approaches and adipose tissue biology, *Curr. Gene Ther.* 8 (2008) 79–87.
- [2] J.K. Fraser, I. Wulur, Z. Alfonso, M.H. Hedrick, Fat tissue: an underappreciated source of stem cells for biotechnology, *Trends Biotechnol.* 24 (2006) 150–154.
- [3] J.M. Gimble, A.J. Katz, B.A. Bunnell, Adipose-derived stem cells for regenerative medicine, *Circ. Res.* 100 (2007) 1249–1260.
- [4] E. Billings Jr., J.W. May Jr., Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery, *Plast. Reconstr. Surg.* 83 (1989) 368–381.
- [5] C.W. Patrick Jr., Adipose tissue engineering: the future of breast and soft tissue reconstruction following tumor resection, *Semin. Surg. Oncol.* 19 (2000) 302–311.
- [6] C.W. Patrick Jr., Tissue engineering strategies for adipose tissue repair, *Anat. Rec.* 263 (2001) 361–366.
- [7] S. Asada, M. Kuroda, Y. Aoyagi, H. Bujo, S. Tanaka, S. Konno, M. Tanio, I. Ishii, M. Aso, Y. Saito, Disturbed apolipoprotein A-I-containing lipoproteins in fish-eye disease are improved by the lecithin:cholesterol acyltransferase produced by gene-transduced adipocytes *in vitro*, *Mol. Genet. Metab.* 102 (2011) 229–231.
- [8] M. Kuroda, Y. Aoyagi, S. Asada, H. Bujo, S. Tanaka, S. Konno, M. Tanio, I. Ishii, K. Machida, F. Matsumoto, K. Satoh, M. Aso, Y. Saito, Ceiling culture-derived proliferative adipocytes are a possible delivery vehicle for enzyme replacement therapy in lecithin: cholesterol acyltransferase deficiency, *Open Gene Ther. J.* 4 (2011) 1–10.
- [9] M. Ito, H. Bujo, K. Takahashi, T. Arai, I. Tanaka, Y. Saito, Implantation of primary cultured adipocytes that secrete insulin modifies blood glucose levels in diabetic mice, *Diabetologia* 48 (2005) 1614–1620.
- [10] M. Kuroda, H. Bujo, M. Aso, Y. Saito, Adipocytes as a vehicle for ex vivo gene therapy: novel replacement therapy for diabetes and other metabolic diseases, *J. Diabetes Invest.* 2 (2011) 333–340.
- [11] S. Asada, M. Kuroda, Y. Aoyagi, Y. Fukaya, S. Tanaka, S. Konno, M. Tanio, M. Aso, K. Satoh, Y. Okamoto, T. Nakayama, Y. Saito, H. Bujo, 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 (2011) C181–C185.

- [12] Y. Aoyagi, M. Kuroda, S. Asada, H. Bujo, S. Tanaka, S. Konno, M. Tanio, I. Ishii, M. Aso, Y. Saito, Fibrin glue increases the cell survival and the transduced gene product secretion of the ceiling culture-derived adipocytes transplanted in mice, *Exp. Mol. Med.* 43 (2011) 161–167.
- [13] C. Fischbach, J. Seufert, H. Staiger, M. Hacker, M. Neubauer, A. Gopferich, T. Blunk, Three-dimensional *in vitro* model of adipogenesis: comparison of culture conditions, *Tissue Eng.* 10 (2004) 215–229.
- [14] X. Kang, Y. Xie, D.A. Kniss, Adipose tissue model using three-dimensional cultivation of preadipocytes seeded onto fibrous polymer scaffolds, *Tissue Eng.* 11 (2005) 458–468.
- [15] W.J. Li, R. Tuli, X. Huang, P. Laquerriere, R.S. Tuan, Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold, *Biomaterials* 26 (2005) 5158–5166.
- [16] J.R. Mauney, T. Nguyen, K. Gillen, C. Kirker-Head, J.M. Gimble, D.L. Kaplan, Engineering adipose-like tissue *in vitro* and *in vivo* utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds, *Biomaterials* 28 (2007) 5280–5290.
- [17] R. Nieto-Aguilar, D. Serrato, I. Garzon, A. Campos, M. Alaminos, Pluripotential differentiation capability of human adipose-derived stem cells in a novel fibrin-agarose scaffold, *J. Biomater. Appl.* 25 (2011) 743–768.
- [18] J.H. Kang, J.M. Gimble, D.L. Kaplan, *In vitro* 3D model for human vascularized adipose tissue, *Tissue Eng. Part A* 15 (2009) 2227–2236.
- [19] G. Eibes, F. dos Santos, P.Z. Andrade, J.S. Boura, M.M. Abecasis, C.L. da Silva, J.M. Cabral, Maximizing the ex vivo expansion of human mesenchymal stem cells using a microcarrier-based stirred culture system, *J. Biotechnol.* 146 (2010) 194–197.
- [20] F.D. Santos, P.Z. Andrade, M.M. Abecasis, J.M. Gimble, L.G. Chase, A.M. Campbell, S. Boucher, M.C. Vemuri, C.L. Silva, J.M. Cabral, Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions, *Tissue Eng. Part C Methods* (2011), doi: 10.1089/ten.tec.2011.0255 [Electronic publication ahead of print].
- [21] T. Jiang, W. Liu, X. Lv, H. Sun, L. Zhang, Y. Liu, W.J. Zhang, Y. Cao, G. Zhou, Potent *in vitro* chondrogenesis of CD105 enriched human adipose-derived stem cells, *Biomaterials* 31 (2010) 3564–3571.
- [22] J.K. Sethi, A.J. Vidal-Puig, Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation, *J. Lipid Res.* 48 (2007) 1253–1262.
- [23] S.P. Poulos, D.B. Hausman, G.J. Hausman, The development and endocrine functions of adipose tissue, *Mol. Cell. Endocrinol.* 323 (2010) 20–34.
- [24] P. Wang, E. Mariman, J. Renes, J. Keijer, The secretory function of adipocytes in the physiology of white adipose tissue, *J. Cell. Physiol.* 216 (2008) 3–13.
- [25] C.P. Barnes, S.A. Sell, E.D. Boland, D.G. Simpson, G.L. Bowlin, Nanofiber technology: designing the next generation of tissue engineering scaffolds, *Adv. Drug Deliv. Rev.* 59 (2007) 1413–1433.
- [26] E. Dawson, G. Mapili, K. Erickson, S. Taqvi, K. Roy, Biomaterials for stem cell differentiation, *Adv. Drug Deliv. Rev.* 60 (2008) 215–228.
- [27] K. Sun, C.M. Kusminski, P.E. Scherer, Adipose tissue remodeling and obesity, *J. Clin. Invest.* 121 (2011) 2094–2101.
- [28] Y. Kimura, M. Ozeki, T. Inamoto, Y. Tabata, Adipose tissue engineering based on human preadipocytes combined with gelatin microspheres containing basic fibroblast growth factor, *Biomaterials* 24 (2003) 2513–2521.
- [29] D. Kuramochi, H. Unoki, H. Bujo, Y. Kubota, M. Jiang, N. Rikihisa, A. Udagawa, S. Yoshimoto, M. Ichinose, Y. Saito, Matrix metalloproteinase 2 improves the transplanted adipocyte survival in mice, *Eur. J. Clin. Invest.* 38 (2008) 752–759.
- [30] H. Ning, G. Liu, G. Lin, R. Yang, T.F. Lue, C.S. Lin, Fibroblast growth factor 2 promotes endothelial differentiation of adipose tissue-derived stem cells, *J. Sex. Med.* 6 (2009) 967–979.
- [31] N. Torio-Padron, J. Borges, A. Momeni, M.C. Mueller, F.T. Tegtmeier, G.B. Stark, Implantation of VEGF transfected preadipocytes improves vascularization of fibrin implants on the cylinder chorioallantoic membrane (CAM) model, *Minim. Invasive Ther. Allied Technol.* 16 (2007) 155–162.
- [32] A. Bouloumie, C. Sengenès, G. Portolan, J. Galitzky, M. Lafontan, Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation, *Diabetes* 50 (2001) 2080–2086.
- [33] G. Croissandeau, M. Chretien, M. Mbikay, Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes, *Biochem. J.* 364 (2002) 739–746.
- [34] H.R. Lijnen, E. Maquoi, L.B. Hansen, B. Van Hoef, L. Frederix, D. Collen, Matrix metalloproteinase inhibition impairs adipose tissue development in mice, *Arterioscler. Thromb. Vasc. Biol.* 22 (2002) 374–379.
- [35] H.S. Moon, H.G. Lee, J.H. Seo, C.S. Chung, D.D. Guo, T.G. Kim, Y.J. Choi, C.S. Cho, Leptin-induced matrix metalloproteinase-2 secretion is suppressed by trans-10, cis-12 conjugated linoleic acid, *Biochem. Biophys. Res. Commun.* 356 (2007) 955–960.
- [36] Y. Wu, C.M. Smas, Wdnm1-like, a new adipokine with a role in MMP-2 activation, *Am. J. Physiol. Endocrinol. Metab.* 295 (2008) E205–E215.

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

Masayuki Kuroda^{1*}, Hideaki Bujo^{2*}, Masayuki Aso³, Yasushi Saito⁴

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 1990¹, 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 gene¹². Clonal expansion was also reported in X-CGD gene therapy trials¹¹ 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 cells¹⁴, although the precise mechanisms underlying the development of leukemia in such patients are not completely understood¹⁵.

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

¹Center for Advanced Medicine, Chiba University Hospital, ²Department of Genome Research and Clinical Application, Graduate School of Medicine, ³Chiba University, and ⁴CellGenTech, Inc., Chiba, Japan

*Corresponding authors. Masayuki Kuroda Tel: +81-43-222-7171 or +81-43-441-4121

Fax: +81-43-226-8130 E-mail address: kurodam@faculty.chiba-u.jp

Hideaki Bujo Tel: +81-43-222-7171 Fax: +81-43-226-8130

E-mail address: hbujo@faculty.chiba-u.jp

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 steps¹⁸, and the development of the treatment has been shifted to more efficient in vivo transduction methodologies¹⁹. 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 vector²⁰, 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 treatment²¹.

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 disease²⁵. 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 improvement²⁶. 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 lifespan³⁸. 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 cells³⁹. 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 effects³⁵. 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 vivo⁴². 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.

Table 1 | Clinical gene therapy trials for metabolic diseases

| Diseases and transgene | Gene delivery | Vector | Administration route | Trial country | Phase | Number | References |
|--|-----------------------|------------------------|-----------------------------|---------------|------------|--------|------------|
| Alpha-1 antitrypsin deficiency | | | | | | | |
| Alpha-1 antitrypsin | In vivo | Adeno-associated virus | Intramuscular | USA | Phase I | 2 | 60–62 |
| | | Naked DNA | Intranasal | USA | Phase I | 1 | |
| Cystic fibrosis | | | | | | | |
| Alpha-1 antitrypsin | In vivo | Naked DNA | Intranasal | USA | Phase I | 1 | 63–66 |
| Cystic fibrosis | In vivo | Adeno-associated virus | Intrabronchial | USA | Phase I | 2 | |
| transmembrane conductance regulator | | | Intranasal | USA | Phase II | 2 | |
| | | Adenovirus | Intrabronchial | USA | Phase I/II | 3 | |
| | | | Intradermal | France | Phase I/II | 1 | |
| | | | Intranasal | USA | Phase I | 3 | |
| | | | Intranasal | USA | Phase I | 1 | |
| | | | Intranasal | Switzerland | Phase I | 1 | |
| | | | Intranasal | USA | Phase I | 4 | |
| | | | Intranasal | USA | Phase I/II | 1 | |
| | | Naked DNA | Intranasal + intrabronchial | USA | Phase I | 1 | |
| | | | Intrabronchial | UK | Phase I | 1 | |
| | | | Intranasal | UK | Phase I/II | 4 | |
| | | | Intranasal | USA | Phase I | 5 | |
| | | | Intranasal + intrabronchial | UK | Phase I | 1 | |
| Familial hypercholesterolemia | | | | | | | |
| Low-density lipoprotein receptor | Ex vivo (Hepatocytes) | Retrovirus | Intrahepatic | USA | Phase I | 1 | 16–18 |
| Gaucher's disease | | | | | | | |
| Glucocerebrosidase | Ex vivo (CD34 + PBC) | Retrovirus | Bone marrow transplantation | USA | Phase I | 1 | 67,68 |
| | | | Intravenous | USA | Phase I/II | 1 | |
| | | | Intravenous | USA | Phase I | 1 | |
| Huntington's disease | | | | | | | |
| Ciliary neurotrophic factor (CNTF) | Ex vivo (BHK) | Naked DNA | Intracerebral | Switzerland | Phase I | 1 | 69,70 |
| | | | | France | Phase I | 1 | |
| Lipoprotein lipase deficiency | | | | | | | |
| Lipoprotein lipase (LPL) | In vivo | Adeno-associated virus | Intramuscular | Netherlands | Phase I/II | 1 | 19,71,72 |
| | | | | Canada | Phase I | 1 | |
| Mucopolysaccharidosis type I (Hurlers syndrome) | | | | | | | |
| Alpha-L-iduronidase | Ex vivo (BMC) | Retrovirus | Bone marrow transplantation | UK | Phase I/II | 1 | 73,74 |
| | Ex vivo (Fibroblasts) | | Intraperitoneal | France | Phase I | 1 | |
| Mucopolysaccharidosis type II (Hunter disease) | | | | | | | |
| Iduronate-2-sulfatase | Ex vivo (PBC) | Retrovirus | Intravenous | USA | Phase I | 1 | 75 |
| Mucopolysaccharidosis type VII | | | | | | | |
| Beta-glucuronidase | Ex vivo (CD34+PBC) | Lentivirus | Intravenous | USA | Phase I | 1 | 76–78 |
| Ornithine transcarbamylase deficiency | | | | | | | |
| Ornithine transcarbamylase | In vivo | Adenovirus | Intrahepatic | USA | Phase I | 1 | 20,79 |
| Pompe disease | | | | | | | |
| Acid-alpha glycosidase | In vivo | Adeno-associated virus | Intramuscular | USA | Phase I/II | 1 | 80–82 |
| Familial lecithin-cholesterol acyltransferase deficiency | | | | | | | |
| Lecithin-cholesterol acyltransferase | Ex vivo (Adipocytes) | Retrovirus | Subcutaneous | Japan | Phase I | 1 | 42,55,59 |

Summarized according to the Clinical Trials Database provided by the Journal of Gene Medicine (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>). Protocol of clinical trial for lecithin-cholesterol acyltransferase deficiency by our group is now under review by Ministry of Health, Labour and Welfare. BHK, baby hamster kidney cells; BMC, bone marrow cells; PBC, peripheral blood cells.

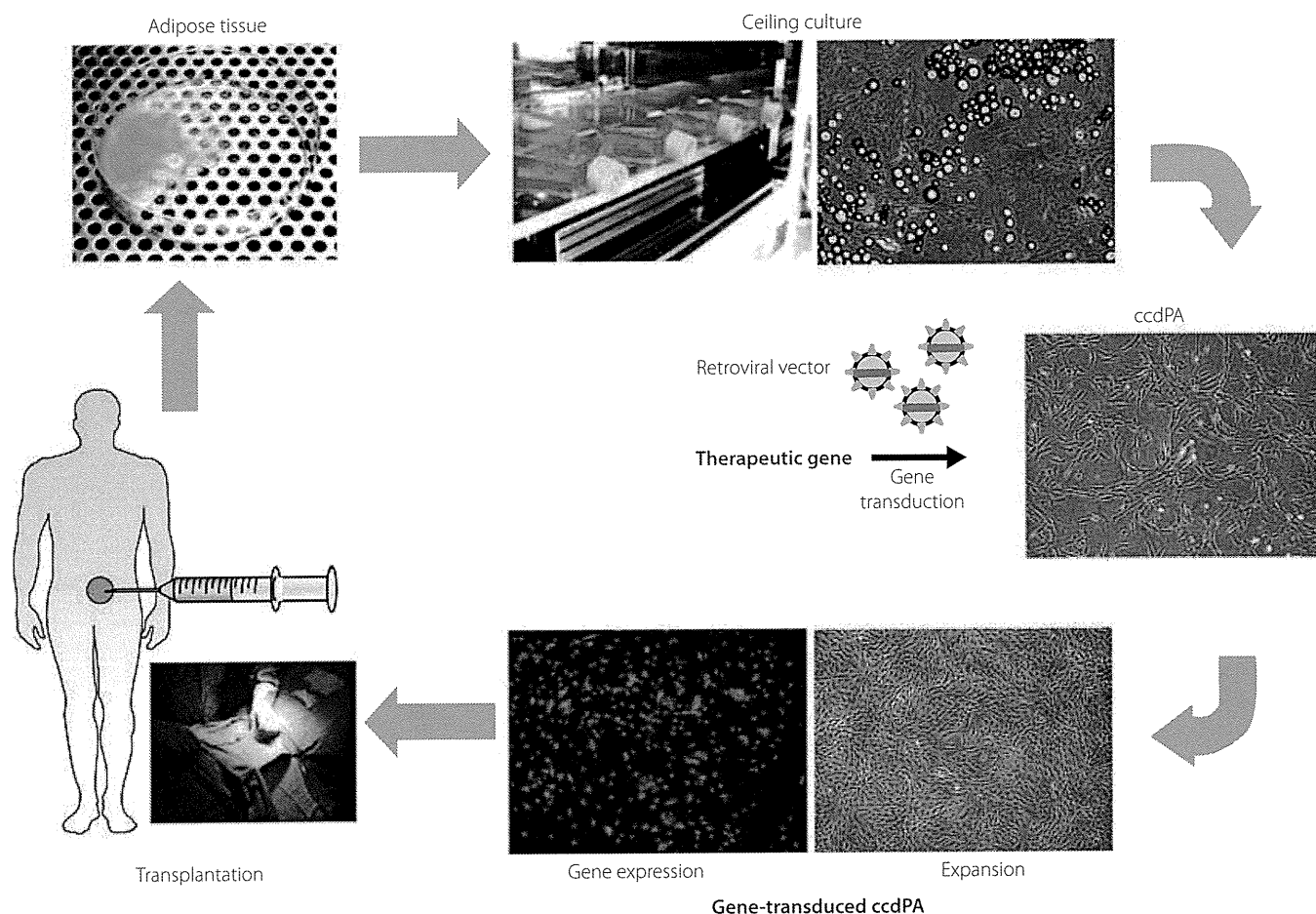


Figure 1 | Therapeutic strategy for adipocyte-based enzyme replacement therapy by ex vivo gene transfer. Adipose tissue is obtained by lipoaspiration from the patient. ceiling culture-derived proliferative adipocytes (ccdPA) are propagated by ceiling culture. The therapeutic gene is transduced by the retroviral vector. ccdPA stably secreting the therapeutic protein are expanded and harvested. Harvested cells are subcutaneously transplanted with the appropriate scaffold.

PREADIPOCYTES WITH HIGH ADIPOGENIC POTENTIAL

Recently, adipose tissue has been demonstrated to be a source of proliferative cells for cell-based therapies, such as regenerative medicine and gene transfer applications. Two types of preparation have been reported to be sources of adipose tissue-derived proliferative cells. One is stromal vascular fractions (SVF), which can be obtained as a sediment by the centrifugation of collagenase-digested fat tissue⁹ and is the most commonly used technique. The adherent cells obtained from SVF are now recognized as adipose tissue-derived stem cells (ASC), which are pluripotent and can differentiate to yield various cell types, including cardiomyocytes, chondrocytes and osteoblasts, in addition to adipocytes, thus providing a relatively heterogeneous cell population appropriate for regenerative therapy^{51–53}. However, these data show that SVF are heterogeneous, and therefore imply that SVF might not

result in a stable therapeutic gene vehicle for gene therapy purposes.

The other cell preparation is obtained from the floating mature fat cell fraction obtained after the centrifugation, followed by a ceiling culture⁵⁴. Because the cells are propagated using the buoyant properties of mature adipocytes in this preparation, the progeny cells are more homogeneous than ASC. Proliferative adipocytes were propagated by the ceiling culture technique from the mature adipocyte fraction, and the cells were designated as ceiling culture-derived proliferative adipocytes (ccdPA)⁵⁵. The ccdPA are nearly homogeneous and show only a trace of mature adipocytes by analysis of surface antigen profiles. On stimulation to induce differentiation, the ccdPA showed increased lipid droplet accumulation accompanied with higher adipogenic marker gene expression compared with the ASC, even after in vitro passaging, suggesting the commitment of ccdPA to the mature adipocyte lineage⁵⁶.

GENE-TRANSDUCED ADIPOCYTES AS VEHICLE CELLS
 MoMLV-mediated gene transduction in human ccdPA resulted in a high gene transduction efficiency⁵⁵. In search of optimal transplantation conditions, the 3-D long-term culture system using fibrin gel, a tissue sealant utilized in the clinic, was established. The gene-transduced ccdPA spontaneously accumulate lipid droplets without any artificial stimulation in 3-D culture using the fibrin glue (Aoyagi Y, Kuroda M, Asada S, Tanaka S, Konno S, Tanio M, Aso M, Okamoto Y, Nakayama T, Saito Y, Bujo H, unpublished observations, 2010). Interestingly, the fibrinogen concentration was shown to affect the lipid accumulation in the cells. The expression of the transduced gene was correlated with cell differentiation (Aoyagi Y, Kuroda M, Asada S, Tanaka S, Konno S, Tanio M, Aso M, Okamoto Y, Nakayama T, Saito Y, Bujo H, unpublished observations, 2011).

In one study, the insulin gene-transduced cells were propagated, and the efficacy of these cells was evaluated in a diabetic mouse model³⁵. The transplantation of the cells improved hyperglycemia and blood HbA_{1c} concentrations in a manner that was dependent on the cell number, without causing hypoglycemia. The plasma insulin concentration was dependent on the implanted cell number, and the systemic effect of the circulating insulin was confirmed by a marked improvement in bodyweight reduction and liver glycogen content. Thus, the autotransplantation of gene-transduced ccdPA could serve as a novel clinical application for a variety of systemic metabolic disorders.

AN EX VIVO GENE THERAPY TRIAL USING EXOGENOUS GENE-TRANSDUCED ADIPOCYTES

Lecithin-cholesterol acyltransferase (LCAT) deficiency has been identified as a genetic metabolic disorder. Cholesteryl ester levels are markedly reduced in lipoproteins, and abnormal cholesterol deposition is observed in the tissues of these patients, who often develop severe complications including corneal opacity, anemia, proteinuria and renal failure⁵⁷. LCAT deficiency is caused by mutations in the *lcat* gene, and more than 40 different mutations have been identified to date⁵⁸. Protein replacement treatment was suggested to be effective; however, no approach for the permanent correction of the symptoms has been reported.

However, in a previous study, the human *lcat* gene was transduced into human ccdPA by a retroviral vector. The transduced cells secreted functional LCAT protein *in vitro*, correlating with the integrated copy number of vector genomes⁵⁵. The secreted LCAT protein clearly ameliorated the disturbed high-density lipoprotein subpopulation profile caused by impaired LCAT function in patients' serum by the *in vitro* incubation assay, strongly suggesting the feasibility of our strategy⁵⁹. An application of this *in vitro* assay system to evaluate the responsiveness of patients is now under investigation. The LCAT delivery achieved in the mouse model with the clinically available fibrin scaffold was enough to suggest the efficacy of the ex vivo gene therapy strategy to prevent a poor prognosis in those patients⁴¹.

The potential safety issues related to the ccdPA have been carefully addressed⁵⁵. Gene transduction did not affect the cell

growth, adipogenic differentiation or surface antigen profiles of the cells. The averaged integrated copy number was stable during the *in vitro* expansion process, and clonal expansion was not observed, indicating no predominant growth of gene-transduced cells. The transplantation experiments showed no signs for side-effects.

CONCLUSION

There are high hopes that a successful gene therapy approach can be developed in the future to treat rare genetic defects. Numerous studies have been carried out to develop such treatment strategies, both on the basic level and in the clinic. Although hematopoietic cells are proven target cells for ex vivo gene therapies, especially for immune-related diseases in which those cell functions are primarily affected by the gene defects, they might not be suitable targets for the many metabolic diseases that result in impairment of multiple organs. The physiological functions and applicability of adipose tissue would enable researchers to develop a novel therapeutic strategy to deliver therapeutic proteins systemically.

Mature adipocytes have been explored as a source of target cells for ex vivo gene therapy. Propagated ccdPA would provide an excellent platform for a novel adipocyte-based protein replacement therapy for patients with serum protein deficiencies who require long-term therapeutic protein supplements. A good manufacturing practice production procedure has been established, and the gene-transduced cells can be expanded up to nearly 10¹² cells from 1 g of fat tissue within 1 month after fat tissue preparation⁵⁵. To further expand the adipocyte-based therapeutic strategy for the supplementation of other proteins, it will be necessary to evaluate the characteristics of ccdPA from various kinds of fat diseases, such as those from subjects with metabolic syndrome, which might affect the secretion function of adipose tissues, and to develop an allogeneic transplantation method for patients with lethal conditions in childhood, as well as to establish the necessary transplantation procedure. After the careful consideration of the safety in combination of efficacy, the novel transplantation therapy developed using adipocytes might be applicable not only for genetic deficiencies, but also for lifestyle-related diseases, including diabetes mellitus.

ACKNOWLEDGMENTS

We thank Dr Itsuko Ishii, Dr Fumiaki Matsumoto, Dr Masaharu Ichinose and Dr Kaneshige Satoh for helpful and valuable suggestions for our research. We also thank Sakiyo Asada, Yasuyuki Aoyagi, Yoshitaka Fukaya, Shunichi Konno and Shigeaki Tanaka for their assistance in preparing this manuscript. This study was supported in part by Health and Labour Sciences Research Grants for Translational Research, Japan (HB).

REFERENCES

1. Rosenberg SA, Aebbersold P, Cornetta K, et al. Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified

- by retroviral gene transduction. *N Engl J Med* 1990; 323: 570–578.
2. Kumar S, Chanda D, Ponnazhagan S. Therapeutic potential of genetically modified mesenchymal stem cells. *Gene Ther* 2008; 15: 711–715.
 3. Nienhuis AW. Development of gene therapy for blood disorders. *Blood* 2008; 111: 4431–4444.
 4. Qasim W, Gaspar HB, Thrasher AJ. Progress and prospects: gene therapy for inherited immunodeficiencies. *Gene Ther* 2009; 16: 1285–1291.
 5. Reiser J, Zhang XY, Hemenway CS, et al. Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases. *Expert Opin Biol Ther* 2005; 5: 1571–1584.
 6. Aiuti A, Cattaneo F, Galimberti S, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* 2009; 360: 447–458.
 7. Onodera M, Ariga T, Kawamura N, et al. Successful peripheral T-lymphocyte-directed gene transfer for a patient with severe combined immune deficiency caused by adenosine deaminase deficiency. *Blood* 1998; 91: 30–36.
 8. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 2000; 288: 669–672.
 9. Hacein-Bey-Abina S, Hauer J, Lim A, et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2010; 363: 355–364.
 10. Kang EM, Choi U, Theobald N, et al. Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long-term correction of oxidase activity in peripheral blood neutrophils. *Blood* 2010; 115: 783–791.
 11. Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* 2006; 12: 401–409.
 12. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003; 302: 415–419.
 13. Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med* 2010; 16: 198–204.
 14. Cavazzana-Calvo M, Fischer A. Gene therapy for severe combined immunodeficiency: are we there yet? *J Clin Invest* 2007; 117: 1456–1465.
 15. Pike-Overzet K, van der Burg M, Wagemaker G, et al. New insights and unresolved issues regarding insertional mutagenesis in X-linked SCID gene therapy. *Mol Ther* 2007; 15: 1910–1916.
 16. Grossman M, Rader DJ, Muller DW, et al. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. *Nat Med* 1995; 1: 1148–1154.
 17. Grossman M, Raper SE, Kozarsky K, et al. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat Genet* 1994; 6: 335–341.
 18. Nguyen TH, Mainot S, Lainas P, et al. Ex vivo liver-directed gene therapy for the treatment of metabolic diseases: advances in hepatocyte transplantation and retroviral vectors. *Curr Gene Ther* 2009; 9: 136–149.
 19. Vaessen SF, Twisk J, Kastelein JJ, et al. Gene therapy in disorders of lipoprotein metabolism. *Curr Gene Ther* 2007; 7: 35–47.
 20. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003; 80: 148–158.
 21. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006; 12: 342–347.
 22. Chuah MK, Collen D, Vandendriessche T. Preclinical and clinical gene therapy for haemophilia. *Haemophilia* 2004; 10(Suppl. 4): 119–125.
 23. Murphy SL, High KA. Gene therapy for haemophilia. *Br J Haematol* 2008; 140: 479–487.
 24. Petrus I, Chuah M, Vandendriessche T. Gene therapy strategies for hemophilia: benefits versus risks. *J Gene Med* 2010; 12: 797–809.
 25. Tuszynski MH, Thal L, Pay M, et al. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med* 2005; 11: 551–555.
 26. Mangialasche F, Solomon A, Winblad B, et al. Alzheimer's disease: clinical trials and drug development. *Lancet Neurol* 2010; 9: 702–716.
 27. Billings E Jr, May JW Jr. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast Reconstr Surg* 1989; 83: 368–381.
 28. Patrick CW Jr. Adipose tissue engineering: the future of breast and soft tissue reconstruction following tumor resection. *Semin Surg Oncol* 2000; 19: 302–311.
 29. Patrick CW Jr. Tissue engineering strategies for adipose tissue repair. *Anat Rec* 2001; 263: 361–366.
 30. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004; 89: 2548–2556.
 31. Ronti T, Lupattelli G, Mannarino E. The endocrine function of adipose tissue: an update. *Clin Endocrinol (Oxf)* 2006; 64: 355–365.
 32. Trayhurn P, Beattie JH. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 2001; 60: 329–339.
 33. Wozniak SE, Gee LL, Wachtel MS, et al. Adipose tissue: the new endocrine organ? A review article. *Dig Dis Sci* 2009; 54: 1847–1856.
 34. Casteilla L, Cousin B, Planat-Benard V, et al. Virus-based gene transfer approaches and adipose tissue biology. *Curr Gene Ther* 2008; 8: 79–87.