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

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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], γc 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 (γc) 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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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.

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
					Phase II	1	
		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 transmembrane conductance regulator	*In vivo*	Adeno-associated virus	Intrabronchial	USA	Phase I	2	
					Phase II	2	
			Intranasal	USA	Phase I/II	3	
		Adenovirus	Intrabronchial	France	Phase I/II	1	
				USA	Phase I	3	
			Intradermal	USA	Phase I	1	
			Intranasal	Switzerland	Phase I	1	
				USA	Phase I	4	
					Phase I/II	1	
			Intranasal + intrabronchial	USA	Phase I	1	
		Naked DNA	Intrabronchial	UK	Phase I	1	
			Intranasal	UK	Phase I/II	4	
				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
				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.

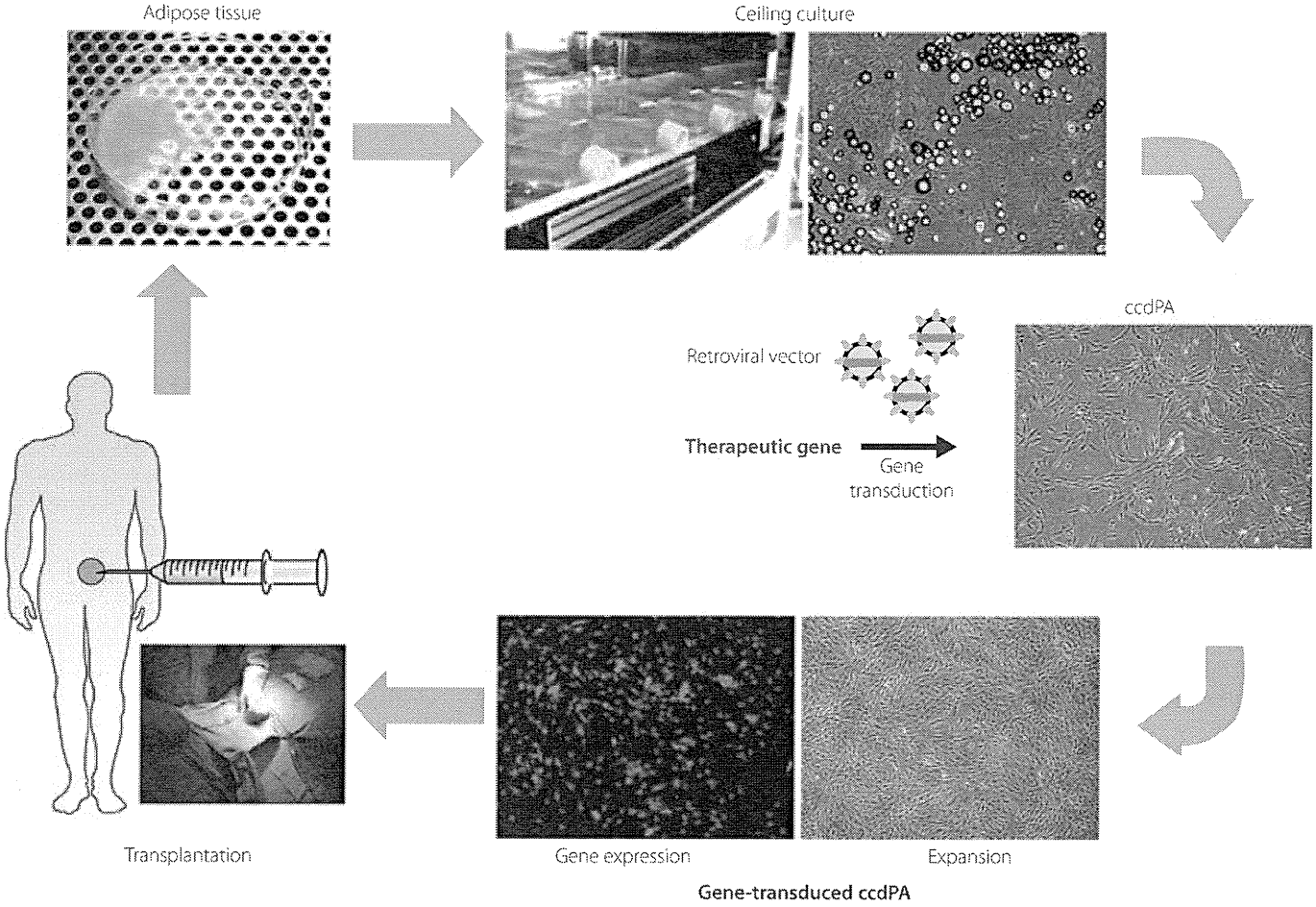

Figure 1 | Therapeutic strategy for adipocyte-based enzyme replacement therapy by *ex vivo* gene transfer. Adipose tissue is obtained by lipoaspiration from the patient. ceiling culture-derived proliferative adipocytes (ccdPA) are propagated by ceiling culture. The therapeutic gene is transduced by the 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 tissue[9] 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 culture[54]. 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 lineage[56].

GENE-TRANSDUCED ADIPOCYTES AS VEHICLE CELLS

MoMLV-mediated gene transduction in human ccdPA resulted in a high gene transduction efficiency[55]. 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 model[35]. 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 failure[57]. LCAT deficiency is caused by mutations in the *lcat* gene, and more than 40 different mutations have been identified to date[58]. 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 genomes[55]. 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 strategy[59]. 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 patients[41].

The potential safety issues related to the ccdPA have been carefully addressed[55]. 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 10^{12} cells from 1 g of fat tissue within 1 month after fat tissue preparation[55]. 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.

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

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

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

gene therapy; adipose tissue-derived stem cells; adipogenesis

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

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

MATERIALS AND METHODS

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

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

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

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

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

RESULTS

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

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

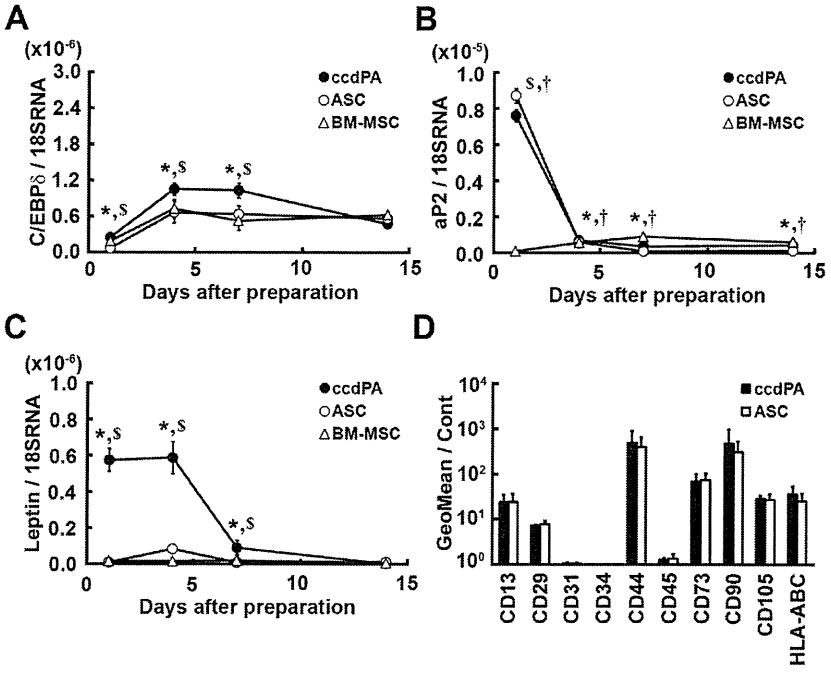

Fig. 1. Expression of adipogenic genes and cell surface markers of ceiling culture-derived proliferative adipocytes (ccdPAs) and adipose-derived stem cells (ASCs). After 7 days of primary culture with DMEM/F12-HAM supplemented with 20% fetal bovine serum (FBS), the ccdPAs and ASCs were passaged with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

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

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

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

DISCUSSION

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

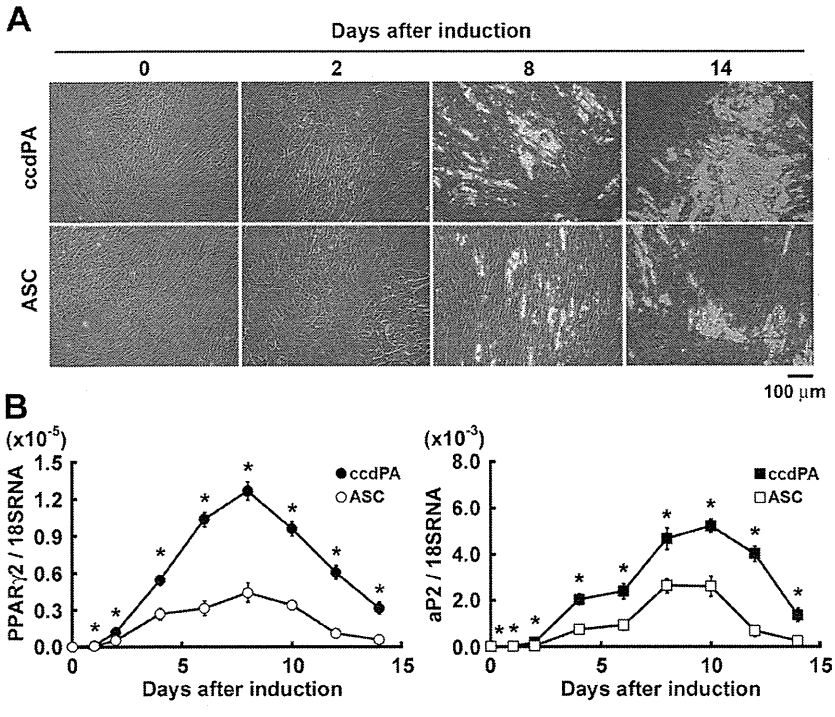

Fig. 2. Comparison of the expression of adipogenic markers in ccdPAs and ASCs during the induction of adipogenesis. *A*: adipogenic induction was performed using ccdPAs (*top*) and ASCs (*bottom*) cultured for 14 days in MesenPRO medium following 7 days of primary culture. The appearance of cells at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

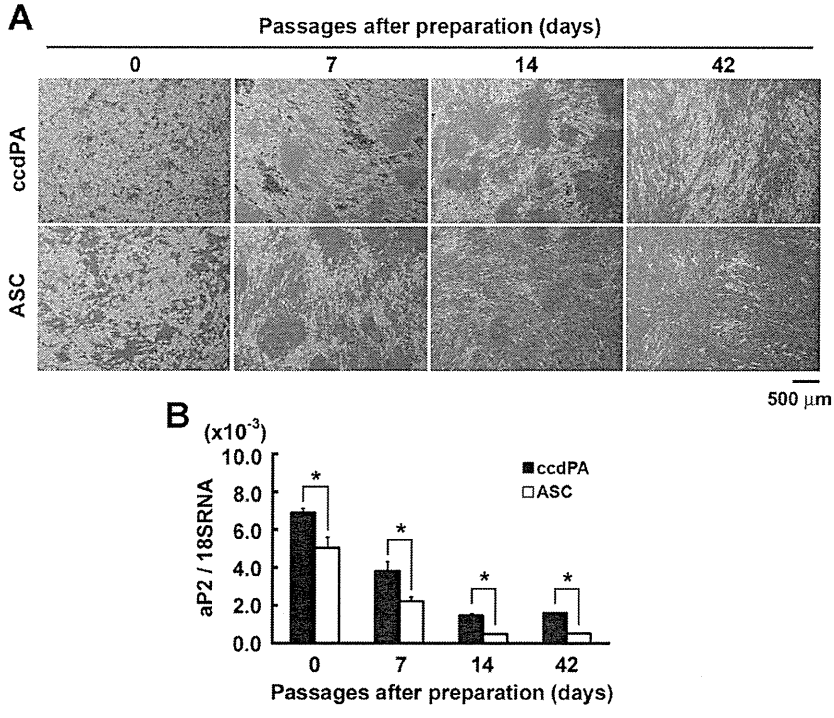

Fig. 3. The effects of consecutive in vitro passaging on the adipogenic potential of ccdPAs and ASCs. The ccdPA and stromal vascular fraction (SVF)-derived cells were obtained after a 7-day ceiling culture and were further cultured in MesenPRO medium for 7, 14, or 42 days. Cells were seeded and incubated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

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

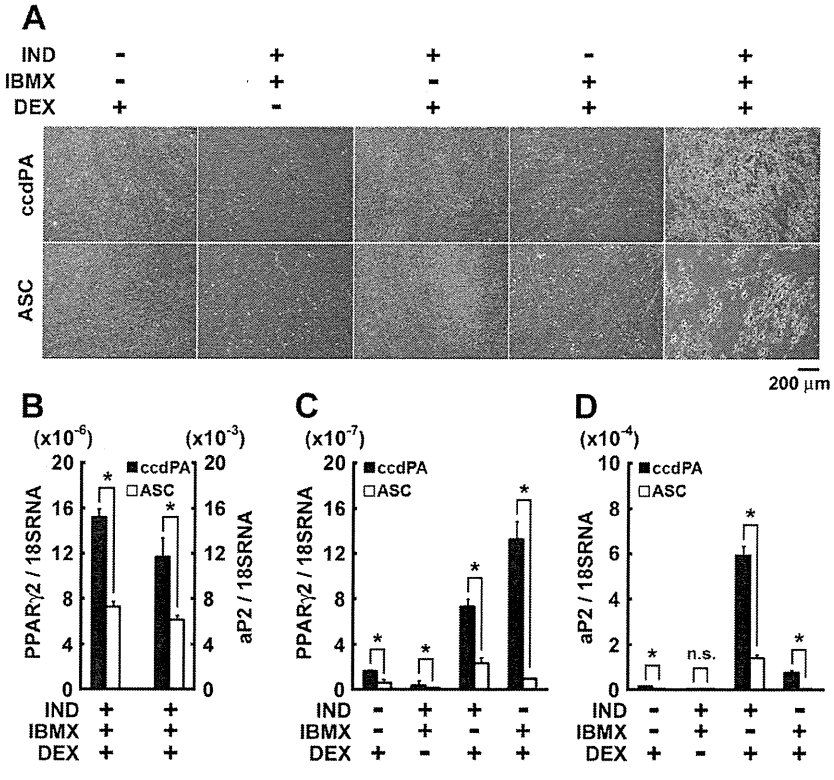

Fig. 4. Effects of differentiation-inducing agents on the adipogenecity and the gene expression levels in ccdPAs. *A*: cells were cultured for 2 wk in growth medium before induction. The appearances of the ccdPAs and ASCs on *day 14* after adipogenic induction with medium containing combinations of the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

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

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

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

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

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DISCLOSURES

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

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ORIGINAL ARTICLE

Faster decline of physical performance in older adults with higher levels of baseline locomotive function

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Aim: The purpose of this longitudinal study was to determine whether the rate of decline in community-dwelling older adults varies according to baseline locomotive function levels.

Methods: This longitudinal study was conducted in community-dwelling older adults in Kyoto, Japan. In addition to information about falls, physical performance was assessed using a series of tests, including 10-m walking time, timed up and go (TUG) test, functional reach, one-leg stand test, and five chair stand test. The outcomes for each patient were measured once in 2009 and then followed up 1 year later. The change in physical performance was then determined. We divided the participants into tertiles (T1, T2, and T3) according to timed up and go test results, and the differences among the three groups were compared.

Results: Of the 252 individuals who were enrolled in the study, 231 (91.6%) completed the 12-month follow-up: 77 in the T1 group; 78 in the T2 group; and 76 in the T3 group. The T1 group showed a significantly larger decrease than the T2 and T3 groups in the 10-m walking time and TUG tests ($P < 0.05$). However, there were no significant differences in functional reach, one-leg standing test, or five chair stand test among the three groups. In the T1 group, the number of falls and elderly who had developed fear of falling increased during the study period.

Conclusions: This study demonstrated that elderly with the highest baseline performances were more likely to show a greater decline in locomotive performance than the other groups. Further study is required to elucidate the mechanism of faster physical functional decline in robust elderly. **Geriatr Gerontol Int 2011; ••: ••–••.**

Keywords: level of frailty, locomotive function, longitudinal study, robust elderly.

Introduction

Maintenance of physical performance in later life is an important component of healthy aging.[1] Walking speed has been identified as one of the most influential physical performances associated with deterioration in activity of daily living among older adults.[2] The timed up and go test (TUG) is a simple tool developed to screen basal mobility performance, which has been shown to be significantly associated with activity of daily living in frail older adults.[3] Thus, evaluating walking speed and TUG is important for predicting the risk of functional decline.

Several cross-sectional studies have shown that a gradual decline in physical performance is significantly

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associated with age.[4,5] Several longitudinal studies have also found a time-dependent decline in the physical performance of community-dwelling older adults.[6,7] However, few studies have addressed the factors involved in longitudinal change in physical performance. Therefore, we conducted several studies to demonstrate that the differential factors are related to daily activities and depend on community-dwelling older adults' level of frailty.[8] Our data suggests that a resistance training program is effective for improving physical performance in frail elderly, but not in non-frail elderly,[9] indicating a difference in the effect of physical training on elderly persons with varying levels of physical fitness. Therefore, it is important to examine longitudinal changes in the physical performance of elderly persons with varying levels of physical fitness.

The purpose of this longitudinal study was to determine whether the rate of decline in older adults differs according to baseline locomotive function levels.

Methods

Participants

Study participants were recruited through ads in the local press requesting healthy community-dwelling volunteers. A total of 252 Japanese participants, 65 years and older living in Kyoto city, were included in the baseline survey in October 2009. One year later in October 2010, the second survey was conducted. We screened 332 people, and 252 who agreed to participate were enrolled. Of the 252 individuals, 231 (91.7%) completed the 12-month follow-up (Fig. 1).

The screening process was used to exclude participants based on the following criteria: severe cardiac, pulmonary, or musculoskeletal disorders; comorbidities associated with an increased risk of falling such as Parkinson's disease and stroke; and use of psychotropic drugs. Written informed consent was obtained from each participant for the trial in accordance with the guidelines approved by the Kyoto University Graduate School of Medicine and the Declaration of Helsinki, 1996.

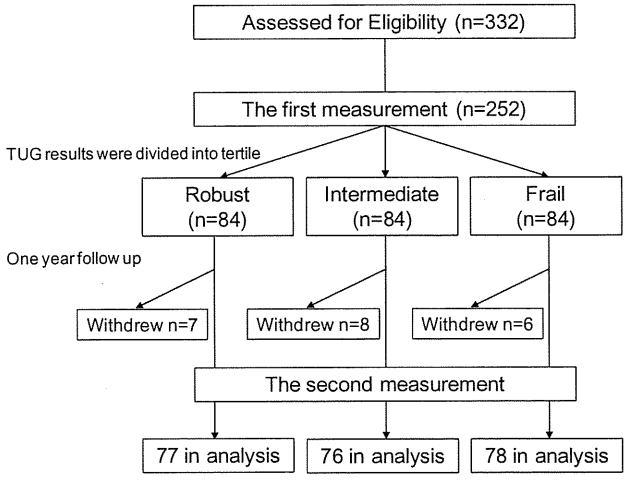

Figure 1 A flow chart showing the distribution of participants.

Outcome measures

All participants underwent five tests for measurements: 10-m walking time,[10] TUG test,[3] functional reach (FR),[11] one-leg standing (OLS) test,[12] and five chair stand (5CS) test.[13] Outcome measures were conducted in October 2009 and October 2010. No exercise program was prescribed to participants during the interim period. Before the study started, all researchers were trained by one of the authors (MY) on correct protocols for administering the assessment measures. If a participant normally used a walking aid, this aid was used during the 10-m walking time and TUG tests.

In the 10-m walking time test, participants walked 15 m at a comfortable pace, as determined by the individual. A stopwatch was used to record the time required to reach the 10-m point that was marked in the middle of the path. The test-retest reliability using the intertrial correlation coefficient (ICC; 1.1) was 0.943. The better performance of the two trials was used as the walking time score in the analysis. In the TUG test, participants were asked to stand up from a standard chair with a seat height of 40 cm, walk a distance of 3 m at a maximum pace, turn, walk back to the chair, and sit down. The test-retest reliability using the ICC (1.1) was 0.929. The TUG score was defined as the better performance of the two trials. In the FR test, each participant was positioned next to a wall with one arm raised at 90° and fingers extended. A meterstick was mounted on the wall at shoulder height. The distance that a participant could reach while extending forward from an initial upright posture to the maximal anterior leaning posture, without moving or lifting the feet, was measured in centimeters according to the position of the tip of the third finger against the mounted meterstick. The distances measured in the two trials were averaged to obtain the FR score. The test-retest reliability using the ICC (1.1) was 0.915. In the OLS test, participants were instructed to start from a standing position with a comfortable base as support with their eyes open and arms at their sides. They were then instructed to stand unassisted on either leg. OLS was measured in seconds from the time one foot was lifted from the floor to when it touched the ground or the standing leg. The test-retest reliability using the ICC (1.1) was 0.905. The participants stopped the OLS if the time exceeded 60 s. In 5CS, participants were asked to stand up and sit down five times as quickly as possible. They were timed from the initial sitting position to the final standing position

at the end of the fifth stand. The test-retest reliability using the ICC (1.1) was 0.954. The 5CS score was defined as the better performance of the two trials. The percent change for physical performance was calculated as follows:

Percent change (%) = 100 × (2010 measurement − 2009 measurement)/2009 measurement

Falls and the fear of falling

Participants were interviewed about falling during the past year and their fear of falling in 2009 and 2010. Falls were defined as all situations in which a participant suddenly and involuntarily came to rest upon the ground or a surface lower than their original station.[14] Falls resulting from extraordinary environmental factors (e.g. traffic accidents or falls while riding a bicycle) were excluded.

We assessed participants' fear of falling by asking a single yes-or-no question with a high test-retest reliability, "Are you afraid of falling?"[15] This question was asked during the interviews in 2009 and 2010. The test-retest reliability using the kappa coefficient was 0.960.

Statistical analysis

We divided the participants into tertiles (T1, T2, and T3) according to TUG test results. TUG was chosen for several reasons. First, it is a simple measure of physical function that involves lower extremity strength, dynamic balance, gait, and agility. Second, TUG has been shown to identify physical function limitations in geriatric patients in a clinical setting.[16,17]

We analyzed the outcome measurements using a two-way ANOVA. Tukey tests were used for post-hoc analysis. Differences in the physical variables between elderly who had or had not fallen and between those with or without a fear of falling were analyzed by two-way ANOVA. Data were analyzed using SPSS v. 18.0 for Windows (Chicago, IL, USA). A *P*-value of <0.05 was considered statistically significant for all analyses.

Results

Of the 252 individuals, 231 (91.7%) completed the 12-month follow-up: 77 in T1 group (91.7%), 78 in T2 group (92.9%) and 76 in T3 group (90.5%) (Fig. 1). There were no significant differences in all performance measurements and age between men and women.

Baseline characteristics

There were significant differences in age (T1, 73.9 ± 6.6; T2, 79.1 ± 7.0; T3, 82.0 ± 6.9; F = 25.2, $P < 0.001$), walking time (T1, 7.4 ± 1.4 sec; T2, 9.7 ± 2.8 sec; T3, 12.7 ± 2.6 sec; $P < 0.05$), TUG (T1, 6.9 ± 0.9 sec; T2, 9.2 ± 0.9 sec; T3, 12.7 ± 1.3 sec; $P < 0.05$), FR (T1, 29.0 ± 7.0 cm; T2, 26.5 ± 6.7 cm; T3 21.3 ± 7.1 cm; $P < 0.05$), OLS (T1, 19.5 ± 13.6 sec; T2, 10.0 ± 10.7 sec; T3, 5.4 ± 5.5 sec; $P < 0.05$), and 5CS (T1, 8.5 ± 2.4 sec; T2, 10.4 ± 2.1 sec; T3, 13.5 ± 3.8 sec; F = 28.0, $P < 0.001$). There were no significant differences in height or weight (Table 1).

Follow-up measures

There were significant differences in walking time (T1, 8.0 ± 1.9 sec; T2, 9.3 ± 2.0 sec; T3, 12.3 ± 2.7 sec; $P < 0.001$), TUG (T1, 7.5 ± 1.5 sec; T2, 9.3 ± 1.8 sec; T3, 13.0 ± 3.2 sec; $P < 0.001$), FR (T1, 30.2 ± 8.8 cm; T2, 27.6 ± 8.4 cm; T3, 21.0 ± 6.5 cm; $P < 0.001$), OLS (T1, 19.0 ± 12.8 sec; T2, 8.7 ± 9.4 sec; T3, 4.3 ± 3.8 sec; $P < 0.001$), and 5CS (T1, 7.4 ± 2.0 sec; T2, 9.5 ± 3.2 sec; T3, 13.6 ± 5.5 sec; $P < 0.001$) (Table 1, Fig. 2).

Group-time interactions are summarized in Table 1. A statistically significant group-time interaction was observed for walking time and TUG ($P < 0.05$).

Falls and fear of falling

In the T1 group, the number of falls and elderly who had developed a fear of falling increased between baseline and follow-up (falls, 19.5% to 27.2%; fear of falling, 13.0% to 26.0%). There were no significant differences in FR, OLS, or 5CS. In T2 and T3 groups, the number of falls and elderly who had developed fear of falling did not change between baseline and follow-up (Table 1).

Characteristics of elderly with or without falls

Group-time interactions are summarized in Tables 2, 3, and 4. In T1 group, a statistically significant group-time interaction was observed for TUG and 5CS ($P < 0.05$). However, we did not find any significant differences in T2 and T3 groups (Tables 2, 3 and 4).

Characteristics of elderly with or without fear of falling

Group-time interactions are summarized in Tables 2, 3, and 4. In T1 group, a statistically significant group-time interaction was observed for TUG ($P < 0.05$) (Table 2). In T2 group, a statistically significant group- time interaction was observed for TUG and 5CS ($P < 0.05$) (Table 3). In T3 group, there were no significant differences (Table 4).

Discussion

In the current study, we have shown that elderly with the highest baseline performances are more likely to show a decline in locomotive performance than the

Table 1 Comparison of outcome measurements among the three groups

	T1 (≤8.2) (n = 77)	T2 (8.3–10.9) (n = 76)	T3 (≥11.0) (n = 78)	F-value	*P*-value	*Post-hoc*
Age	73.9 ± 6.6	79.1 ± 7.0	82.0 ± 6.9	25.2	<0.001	†‡§
Height (cm)	157.1 ± 9.0	155.0 ± 8.1	155.8 ± 10.9	0.5	0.620	–
Weight (kg)	57.7 ± 9.8	56.5 ± 8.3	54.5 ± 10.1	0.7	0.492	–
Gender, female	57 (74.0)	60 (78.9)	60 (76.9)			–
Falls, n (%)						
2009	15 (19.5)	20 (26.3)	26 (33.3)			
2010	21 (27.2)	22 (28.9)	28 (35.9)			
Fear of falling, n (%)						
2009	10 (13.0)	29 (38.2)	36 (46.2)			
2010	20 (26.0)	30 (39.5)	37 (47.4)			
Walking time (sec)						
2009	7.4 ± 1.4	9.7 ± 2.8	12.7 ± 2.6	9.227	<0.001	†‡§
2010	8.0 ± 1.9	9.3 ± 2.0	12.3 ± 2.7			†‡§
Change (%)	5.3 ± 17.6	–5.1 ± 25.2	–3.4 ± 20.1			†‡
Timed up and go (sec)						
2009	6.9 ± 0.9	9.2 ± 0.9	12.7 ± 1.3	3.361	0.037	†‡§
2010	7.5 ± 1.5	9.3 ± 1.8	13.0 ± 3.2			†‡§
Change (%)	5.8 ± 14.1	2.4 ± 16.2	2.6 ± 22.6			†‡
Functional reach (cm)						
2009	29.0 ± 7.0	26.5 ± 6.7	21.3 ± 7.1	1.254	0.291	
2010	30.2 ± 8.8	27.6 ± 8.4	21.0 ± 6.5			
Change (%)	5.5 ± 28.0	5.8 ± 28.1	–3.3 ± 37.9			
One-leg standing (sec)						
2009	19.5 ± 13.6	10.0 ± 10.7	5.4 ± 5.5	0.906	0.439	
2010	19.0 ± 12.8	8.7 ± 9.4	4.3 ± 3.8			
Change (%)	–5.3 ± 41.4	–2.9 ± 31.2	–6.7 ± 32.8			
Five chair stand (sec)						
2009	8.5 ± 2.4	10.4 ± 2.1	13.5 ± 3.8	0.217	0.885	
2010	7.4 ± 2.0	9.5 ± 3.2	13.6 ± 5.5			
Change (%)	–10.0 ± 24.5	–6.4 ± 33.3	1.0 ± 31.7			

†T1 *versus* T2. ‡T1 *versus* T3. §T2 *versus* T3.

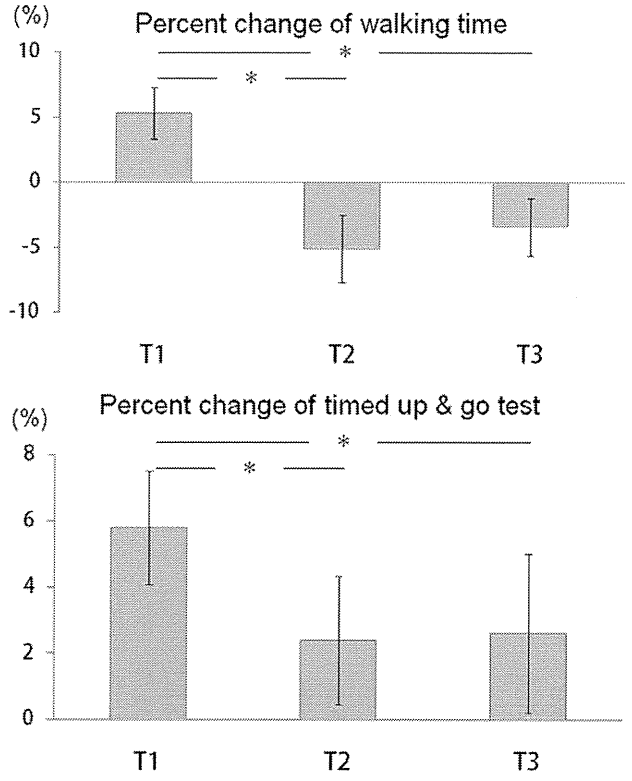

Figure 2 Percent change in the walking time and timed up and go tests among the three groups. The means ± SEM in T1, T2, and T3 groups are shown. *$P < 0.05$.

others. These results are quite unexpected. However, Koster *et al.* compared the leg strength of octogenarians and septuagenarians, and found more rapid decline in leg strength in septuagenarians.[18] Moreover, it has been reported that active community-dwelling elderly men and women respectively lose 0.8% and 0.7% of lean leg mass per year on average.[18] These characteristics of the aging process might account for why the T1 group showed a greater decrease in locomotive functions. Yet, it is possible that a floor effect may account for this longitudinal maintenance of physical performance in the T2 and T3 groups. Previous cross-sectional studies have shown an age-dependent gradual decline in locomotive function,[4,5] while longitudinal studies have shown a time-dependent decline in locomotive function in community-dwelling older adults.[6,7] The subjects of these studies were community-dwelling older adults with a relatively high level of performance, presumably equivalent to our study's T1 or T2 groups. However, participants in our study had a wider range of physical performance levels. For example, the reference values for TUG were 8.1 s for persons aged 60 to 69 years old, 9.2 s for persons aged70 to 79 years, and 11.3 s for persons aged 80 to 99 years.[19] The reference values for the T1 group was 6.9 s, 9.2 s for the T2 group, and 12.7 s for the T3 group. Thus, the level of physical performance may have affected our results.

The T1 group showed a significantly larger decrease in 10-m walking time and TUG than the other groups. An earlier longitudinal study indicated that the age-related decline was accelerated in lower extremity performance.[7] Walking speed, in particular, is a good predictor for the onset of functional dependence in a Japanese community population.[2] In the same manner, TUG has been shown to be significantly associated with activities of daily living function in frail older adults.[3] Thus, physical training, such as resistance training, maintains level of activity of daily living in healthy elderly is very important.

The T1 group showed an increase in falls (19.5% to 27.2%) and fear of falling (13.0% to 26.0%) in 2010. In general, at least one-third of people aged 65 and older fall at least once annually.[20,21] In addition, the major risk factor for fear of falling is shown to be history of at least one fall.[22] In the T1 group, elderly with a fear of falling were more likely to show a decline in locomotive performance than elderly without a fear of falling. Several studies have indicated that people who are afraid of falling appear to enter a debilitating spiral of loss of confidence, restricted physical activities, physical frailty, lack of social participation, falls, and loss of independence.[23–28] Therefore, it is possible that the increased fear of falling is associated with decreased locomotive function in T1 group.

There were several limitations of this study that warrant mention. First, although we used TUG to define frailty, TUG may not be sufficient to define frailty. The Edmonton Frail Scale adopts eight other domains such as cognition, general health status, functional independence, social support, medication use, nutrition, mood, and continence other than TUG.[29] Further study is required to test the levels of these domains in this cohort. Second, the standard deviations for the percent change values are quite large, which shows major individual differences. These factors may have affected the current results. Third, the results of men and women were combined in this research because of the relatively small sample size. A larger sample size is required to analyze in each gender. Finally, participants were probably more motivated and showed greater interest in health than the general population of older adults.

This was a longitudinal study to demonstrate that the participants with the highest baseline levels of performance were more likely to show a greater decline in locomotive performance than the other groups. Further study is needed to explore the mechanism of a faster decline in physical performance in the robust elderly. Future work should also be done to determine whether the effects of a training program on physical performance differ according to the level of physical well-being.