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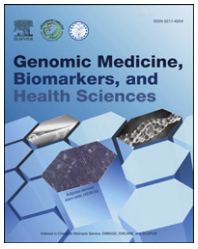

REVIEW ARTICLE

Human adipose-derived stem cells: Isolation, characterization and current application in regeneration medicine

Kuang-Hung Cheng [a,*], Tzu-Lei Kuo [a], Kung-Kai Kuo [b], Chang-Chun Hsiao [c]

[a] *Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan, ROC*
[b] *Division of Hepatobiliary Pancreatic Surgery, Department of Surgery, Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung, Taiwan, ROC*
[c] *Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Kaohsiung, Taiwan, ROC*

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Abstract Stem cell techniques and tissue engineering are the foundation of the emerging field of regenerative medicine, which requires a stable source of stem or progenitor cells. Adipose tissue has been proven to serve as an alternative, rich source of adult stem cells with multipotent ability. There has been increased interest in adipose-derived stem cells (ADSCs) for tissue engineering applications. Here, we review the current techniques for isolating, cultivating, and differentiating ADSCs, and describe them in detail. The results from many studies that we summarize in this review indicate that ADSCs and their secretory factors show great promise for use in stem cell therapy.

Introduction

Stem cells are defined as a group of undifferentiated cells that own unlimited or prolonged self-renewal ability and also have the capability to be induced into various mature somatic cell types. The individual unit of which each part of the body is composed contains many different types of somatic cell, along with stem cells (embryonic stem cells or adult somatic stem cells) that can undergo unlimited self-renewal and differentiate into different somatic cells during embryonic development. The two broad types of mammalian stem cell that have been identified so far are embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in adult tissues.

* Corresponding author. Institute of Biomedical Sciences, National Sun Yat-Sen University, 70 Lien-hai Road, Kaohsiung 80424, Taiwan.
E-mail address: khcheng@faculty.nsysu.edu.tw (K.-H. Cheng).

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In the case of somatic stem cells, some stem cells can, in certain circumstances, migrate to the injured tissue and undergo cell division to produce daughter cells that continue to differentiate into other lineages of cells which can replace dead cells and repair damaged tissue, thus maintaining the tissue's homeostasis.[1] The existence of adult stem cells helps to explain why some tissues have a strong regenerative capacity. It is well established that the adult gastrointestinal and blood system are maintained throughout life by the division and differentiation of stem cells.[2,3] In other adult tissues, notably the liver, quiescent or "facultative" stem cells are believed to become activated under conditions of stress and regeneration, but these do not participate in tissue maintenance in normal circumstances.[4]

Despite their potential importance in organ physiology and regenerative medicine, the existence and role of stem cells in most adult tissues is still controversial or ill-defined. Recently, as one advantageous technique for treating injured tissues and organs than can overcome prior problems and show a better therapeutic effect, new attention has been focused on a novel approach using cells capable of self-renewal and differentiation as a source of transplantation for damaged tissues and organs. Typical examples of such cells include tissue progenitor cells, mesenchymal stem cells, and hematopoietic stem cells.

For example, driven by a desire to find sources for new beta-cells in patients with type 1 diabetes, intense efforts have been directed towards obtaining evidence for stem cells (or at least adult cells that can give rise to beta-cells) in the adult pancreas. A variety of injury models has been used for this purpose—including partial pancreatectomy, pancreatic duct ligation, and treatment with the beta-cell toxin streptozotocin—all showing varying degrees of beta-cell neogenesis.[5–8] As is true in terms of identifying the cell of origin of pancreatic tumors, controversy exists over the origin of new beta-cells following injury. Evidence exists for beta-cell derivation from multiple sources, including pre-existing beta-cells as well as progenitor cells that reside within the pancreatic ducts, islets, and bone marrow.[9–11]

In order to address the question of whether beta-cells arise by the duplication of existing beta-cells or from non-beta-cells (whatever the source), Dor et al devised a "pulse-chase" method for labeling adult beta-cells and their progeny. These experiments demonstrated that the percentage of labeled beta-cells did not decrease with time, even following partial pancreatectomy, indicating that few if any new beta-cells were derived from an unlabeled (i.e., progenitor) cell.[12] These results do not, however, rule out the possibility that pancreatic stem cells do exist. Stem cells might have a potential that is limited to nonendocrine lineages, or they may become activated only in certain settings or upon certain forms of injury. Such settings might be recreated in cancer or with certain *in vitro* growth conditions.

Particularly noteworthy is the possibility that pancreatic cells that express the intermediate filament protein nestin, a marker of neural stem cells, could represent pancreatic stem cells.[13] Nestin-positive cells isolated from adult islets can undergo endocrine and exocrine differentiation *in vitro*.[14,15] Further, a clonal culture of cells that coexpress nestin and Pdx1, which have the capacity to undergo exocrine, endocrine, and neuronal differentiation, has been recently reported.[16] Although recent reports have suggested that nestin expression is restricted to non-epithelial cells in the pancreas,[17] it remains possible that nestin may mark a small population of somatic stem cells in many adult tissues.

The adult mammalian liver has been shown to have a high regenerative capacity; in response to chronic liver diseases, liver injury, or hepatectomy, repopulation occurs by the proliferation of progenitor cells located in the liver, known as oval cells, when hepatocyte replication is impaired or delayed.[18] Detailed mechanisms of the stages leading to liver progenitor cell activation have been studied using animal models of hepatocarcinogenesis and liver regeneration after toxic chemical injury. Under these conditions, small oval cells with a large nuclear-to-cytoplasm ratio containing an oval-shaped nucleus were specially expanded to increase the number of cells present. The histoimmunochemical analysis suggested that epithelial oval cells residing within the canal of Hering could be resident liver progenitor cells, which are high expressors of the hematopoietic stem cell markers CD34, c-kit (CD117), and Thy-1 (CD90). Such cell populations may become central players in the future treatment of liver diseases requiring clinically relevant liver regeneration.[19,20]

To date, the best characterized adult stem cell population considered to possess multipotent capacity is that of bone marrow mesenchymal stem cells, often referred to as multipotent stromal cells (MSCs). These are a heterogeneous population of fibroblast-like cells that are found in most adult organs; MSCs, the archetypal multipotent progenitor cells derived from cultures of developed organs, are of unknown identity and tissue wide distribution.[21] For more than a decade, advances have been made in isolating and expanding MSCs from human bone marrow, umbilical cord blood, and fat.[22,23] Multipotent MSCs maintain stem/progenitor cell activity and can differentiate into lineages of many mesodermal tissues including bone, myocardiocyte, cartilage, skeletal muscle, and neuron, with different growth conditions produced by combining them with specific hormones or growth factors.

Fat tissue consists of mature adipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, monocytes, and lymphocytes. Using collagenase digestion followed by centrifugation, the pellet called referred to as the stromal-vascular cell fraction (SVF) has come increasingly into focus in adipose stem cell research, since this compartment provides a rich source of pluripotent adipose tissue-derived stem cells (ADSCs).[24] There is a confusing inconsistency in the literature when using terms describing multipotent precursor cells derived from adipose tissue stroma, such as processed lipoaspirate cells, adipose tissue-derived stromal cells, preadipocytes, adipose stroma vascular cell fraction, and others. The term SVF corresponds to enriched ADSCs and describes ADSCs obtained immediately after collagenase enzyme digestion. The critical point is the requirement for a detailed molecular and cellular characterization of multipotent stem cells within the adipose stroma.

Recently, Rodriguez et al described the isolation and culture of adipose tissue-derived stem cells with multipotent differentiation capacity at the single-cell level.[25] These cells can maintain their stem cell characteristics

with long-term passaging and develop the unique features of human adipocytes. Meanwhile, subcutaneous adipose tissue is abundant and easy to obtain, and repeatable access to subcutaneous adipose tissue provides a clear advantage over the isolation of MSCs from bone marrow. Liposuction is a common surgical procedure that is safe, and a large number of cells can be obtained with minimal risk.

ADSCs have been reported to have the potential for endothelial and macrophage differentiation.[26] In addition, an initial effort has been made regarding the differentiation of ADSCs across the germ layer-specific tissues ("cross-differentiation") into nonmesenchymal tissues such as neurons or endocrine pancreatic cells.[27] Here, we decided to use the term ADSCs in our review as a compromise, and then only for cells that we were able to passage many times and that showed multipotential differentiation capacity, and/or could be molecularly characterized using a multipanel of mesenchymal differentiation markers (Table 1 and Fig. 1).

This review will focus on isolation and purification procedures for ADSCs, their molecular characterization, and their capacity for differentiation that could be employed in regenerative medicine. Considering future prospects, we will also discuss the mechanisms and future role of ADSCs in tissue engineering and in the repair and regeneration of injured tissue.

Isolation and characterization of adipose-derived mesenchymal stem cells

ADSCs are morphologically similar to MSCs obtained from bone marrow or other tissue after placing it in growth medium *in vitro*. ADSCs possess the progenitor activity to differentiate into cells of mesenchymal origin, such as adipocytes, myocytes, chondrocytes, and osteocytes. Previous studies adjusting the conditions of the culture media used to grow ADSCs showed that most of the successful media contained high concentrations of Ca^{2+} ions, which may induce or enhance the differentiation of ADSCs.

Here we followed the protocol established by Dr. Chang,[28] which employs the growth medium K-NAC and grows human ADSCs in K-NAC medium with a lower calcium level (0.09 mM), as the low-calcium K-NAC medium may prevent differentiation and prolong the lifespan of the ADSCs.[28] Another major feature of the K-NAC medium is its supplementation with the antioxidants NAC and Asc-2P; NAC is a cysteine prodrug that enhances the production of glutathionine. The extra supplementation with these two chemicals may change the cellular redox conditions and enhance the progenitor potential and lifespan of the stem or precursor cells. Indeed, culturing the ADSCs in K-NAC medium gave rise to significantly better proliferation capacity, and cultures could be maintained for as long as 20 or more passages without losing their mesenchymal stem cell markers.

Other factors such as donor age, fat tissue type (white or brown adipose tissue), and localization (subcutaneous or visceral adipose tissue) of the adipose tissue, type of surgical procedure, culturing conditions, plating density, and media formulations are also able to affect the growth rate and differentiation capacity of ADSCs. However, neither the type of surgical procedure nor the anatomical site of the adipose tissue affects the total number of viable cells that can be obtained from the SVF fraction. Discarded adipose tissue was collected during surgery with the informed consent of the patients, and approval by the hospital. Further, the Institutional Review Board regulates all research activities involving human subjects.

The protocols below describe in detail how we isolated the ADSCs in our laboratory (Fig. 2). In brief, immediately after the biopsy, adipose tissue (0.1—2 mg) was transported from the hospital to the laboratory in 15 mL ice-cold growth medium (K-NAC medium with 10% fetal calf serum). In the laboratory, using a sterile scalpel and forceps, we cut the biopsy into small pieces and maintained these in a digestion buffer in a culture dish under a laminar flow hood. Tissue without fat (white, soft and easy to shred) was discarded (some biopsies contained a majority of nonadipose tissue).

Small pieces of tissue were then digested with 5 mL collagenase A solution. Collagenase digestion was performed at 37 °C in a water bath. The tube was shaken vigorously every 5 minutes until dissociation of the tissue pieces occurred. This took between 20 and 30 minutes depending on the size of the adipose tissue fragments. Collagenase digestion was stopped after 30 minutes even if a few fragments were not totally dissolved. The collagenase digestion reaction was stopped by adding 10 mL DMEM + 10% fetal calf serum medium.

The crude SVF was separated from the adipocyte fraction by centrifugation at 800 × *g* for 10 minutes at room temperature. The fat and oil fractions (the upper fraction) were discarded, and cells from the SVF were seeded onto one (or two if starting with 2 g of tissue) 100-mm culture

Table 1 Molecular phenotype of adipose tissue-derived stem cells (ADSCs).

ADSC-positive cellular markers and genes	ADSC-negative cellular markers and genes
CD13	CD 31
CD29	**CD 34**
CD44	**CD45**
CD49	**CD 117**
CD90	CD 13 3
CD105	**HLA-DR**
CD106	STRO-1
CD166	Lin
Fibronectin	HLA II
αSMA	
Vimentin	
Collagen-1	

All the gene and surface marker expression profiles apply to in vitro-expanded cells, not primary cells.
As a minimal prerequisite, mesenchymal stem cells must express CD105 and CD90 and lake the hematopoietic lineage markers c-kit$_P$CD34$_P$CD45 and H LA-DR (printed in boldface).
αSMA = smooth muscle cell-specific alpha actin; ADSC = adipose tissue-derived mesenchymal stem cell; HLA = Human leukocyte antigen.

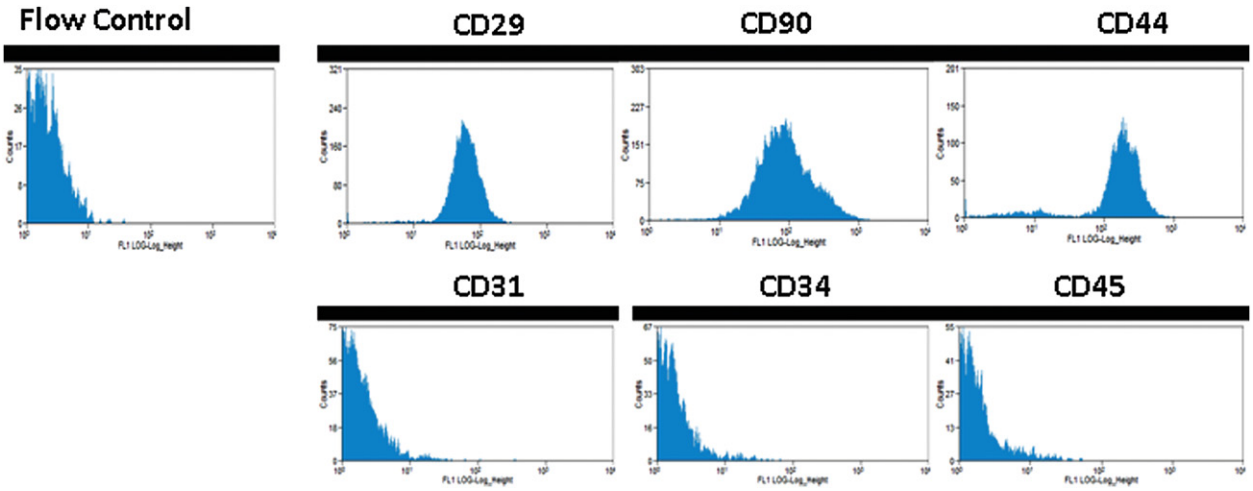

Figure 1 Flow analysis of human adipose-derived stem cells (ADSCs). Cells of the human ADSC stable cell line PLA6 were harvested and specific cell surface antigens were detected. Cells stained with a fluorescein isothiocyanate (FITC)-conjugated nonspecific IgG were examined as a control. Flow cytometric analysis shows that human ADSC-PLA6 cells do not express CD31, CD34, and CD45, but do express CD29, CD90, and CD44.

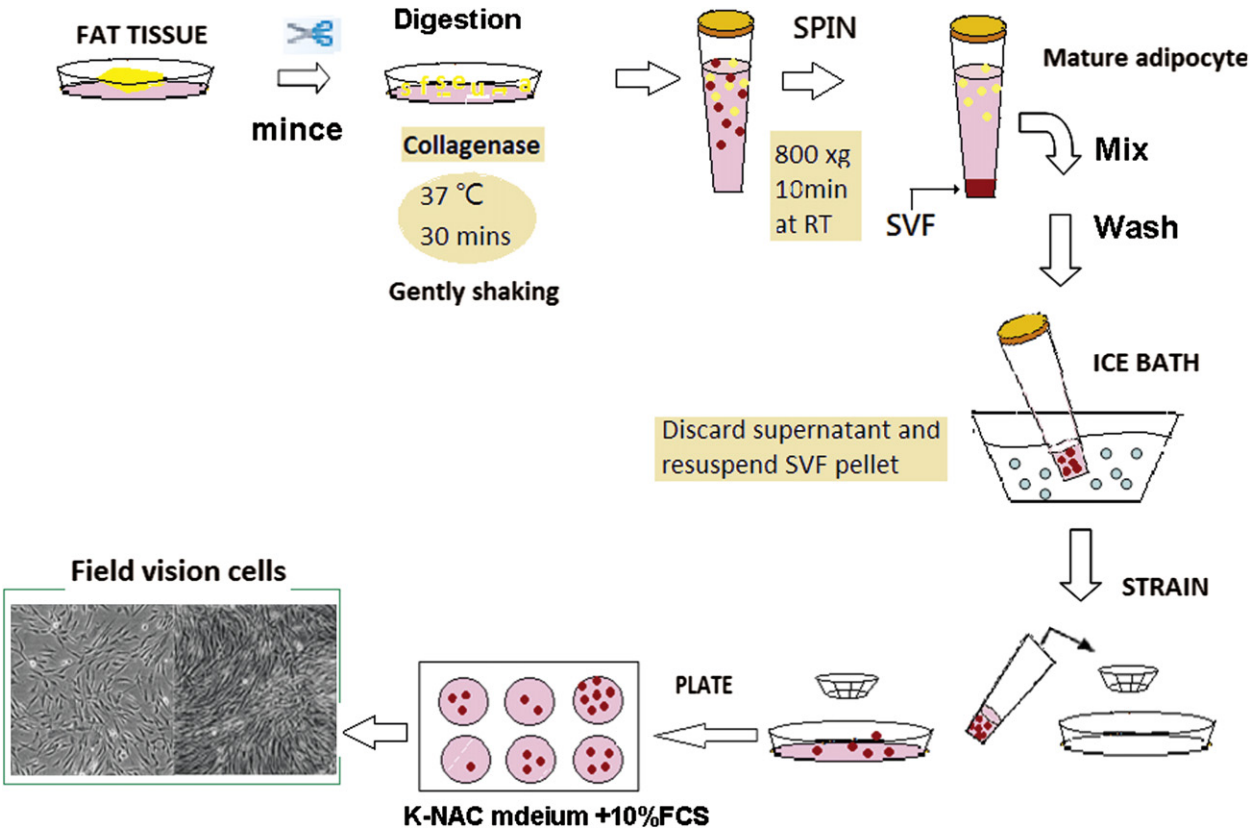

Figure 2 Diagram showing the procedure for acquiring adipose tissue and isolating adipose-derived stem cells (ADSCs). In brief, adipose tissue was collected by needle biopsy or liposuction aspiration. ADSCs were isolated from adipose tissue by washing the fat tissue several times with 1 × phosphate-buffered saline containing 5% penicillin/streptomycin before mincing. After removal of the debris, the sample was placed in a sterile tissue culture plate with 0.05% collagenase digestion buffer for tissue digestion. The adipose sample was then pipeted up and down several times to further disintegrate any adipose tissue aggregates. After inactivation of collagenase with K-NAC medium with 10% fetal calf serum (FCS), the supernatant was aspirated, and the cell pellet was resuspended in a maximum of 5 mL K-NAC medium supplemented with 10% FCS. After centrifugation, the cell suspension was remixed and filtered through a 100-μm cell strainer. Finally, cell pellets were plated onto a tissue culture plate and incubated at 37 °C in 5% carbon dioxide in an incubator.

dish in 10 mL growth medium. After overnight incubation, the culture medium was replaced with fresh medium, and the adherent cells were maintained for expansion. Adherent cells were dissociated when they reached 60–70% confluence. ADSCs were shown to be able to proliferate in culture for more than 10 passages without reaching senescence.

Subsequently, for the transfer, culture and subculture of ADSCs, the culture medium was aspirated off, and the cells were washed with 1 × phosphate-buffered saline. The phosphate-buffered saline was then aspirated off and 1 mL trypsin solution added, ensuring that the trypsin covered the cell monolayer. The culture was then incubated at 37 °C in 5% carbon dioxide for 2–3 minutes. After checking under an inverted microscope that the cells had detached from the bottom of the dish, 10 mL growth medium was added to inactivate the trypsin, and the cells were resuspended by pipeting several times. The cells were then transferred to a sterile tube and centrifuged at 800 × *g* for 5 minutes at room temperature. The medium was aspirated off, and the cell pellet was resuspended in 5 mL growth medium by pipeting up and down two or three times.

When the cells had been counted, 2.5×10^5 cells were added to 10 mL prewarmed growth medium and then transferred to a 100-mm dish, the medium being changed every other day. Three to four days later, the cultures were trypsinized, as for the centrifugation step above. Cultures were subcultured before the cells had reached 70% confluence. The cells were harvested, pipeted into freezing tubes and stored in liquid nitrogen.

The name adipose derived stem cells has been utilized as these cells display additional and specific characteristics. Human MADS are isolated from human adipose tissue, and human MADS cell lines can be established using the protocol above. Cells exhibit characteristics similar to those of mesenchymal stem cells, i.e., they have the capacity for self-renewal, as cells can be expanded *in vitro* for more than 20 population doublings (i.e., around 30 passages in our hands so far) while maintaining a normal diploid karyotype and the potential to undergo differentiation into adipocytes, chondrocytes/osteoblasts, hepatocyte, islet β-cells, and gial-like cells (Table 2). In *in vitro* cultivation, these cells enter the adipose lineage at a high rate, the differentiation yield being estimated to be more than 80%, and they differentiate into cells displaying a unique combination of properties similar to those of native adipocytes. Therefore, ADSCs are an appropriate model cell line with which to study human fat cell metabolism and to uncover the genes involved in the early steps of adipogenesis.

According to our characterization, ADSCs must express CD44, CD90, and CD105 (Table 1 and Fig. 1), and must lack expression of the endothelial lineage and hematopoietic lineage markers CD31, CD34, and CD45 (Table 1 and Fig. 1).[29–31] The possible known ADSC expression profile of surface markers and genes is summarized in Table 1 according to data derived from our analysis and other published literature, and these expression data prove that polypotent ADSCs are adult progenitor cells. Meanwhile, the lack of HLA-DR expression and the immunosuppressive properties of ADSCs make these cells suitable for *in vivo* allogenic transplantation procedures as they are free of the risk of tissue rejection. ADSCs do not provoke *in vitro* alloreactivity of incompatible lymphocytes, and they can suppress mixed lymphocyte reactions and lymphocyte proliferative responses to mitogens. These findings support the idea that ADSCs share immunosuppressive properties with bone marrow MSCs and therefore might represent an alternative source for stem cell therapy.

Table 2 Experimentally used chemicals and growth factors that trigger the differentiation of adipose tissue-derived stem cells (ADSCs).

Type of differentiation	Differentiation factors
Adipogenic	Insulin, IBMX, dexamethasone, rosiglitazone, indomethacin,
Chondrogenic	5-Aza BMP-6, 5-Aza BMP-7, GFG-2, TGF-β_1, TGF-β_2, TGF-β_3, dexamethasone, IGF-1
Osteogenic	1,25$(OH)_2D_3$, β-glycerophosphate, ascorbic acid, BMP-2, dexamethasone, valproic acid.
Cardiomyogenic differentiation	TSA, 5-Aza
Hepatic	HGF,OSM,DMSO
Neurogenic	EGF, FGF, 25uM fluvastatin, B27/ Neurobasal medium (B27/N medium), 5-azacytidine
Pancreatic/ endocrine	Activin-A exendin-4, pentagastrin, HGF, nicotinamide, high glucose concentration

l,2B$(OH)_2D_3$ = 1,25-dihydroxys-cholecalciferol; BMP = bone morphogenetic; DMSO = dimethyl sulfoxide; EGF = epidermal growth factor; FGF = fibroblast growth factor; HGF, hepatocyte growth factor; IBMX = 3-isobutyl-1-meyhylxanthine; IGF = insulin-like growth Factor; IL = interleukin; OSM = oncostain M; TGF = transforming growth factor; TSA = trichostatin A: 5-Aza, 5-azacytidine.

Mechanisms of potential therapeutic utility of ADSCs: Lineage-specific differentiation potential

Differentiation in osteogenesis

Several studies have focused on the utilization of ADSCs or MSCs as an alternative to the use of autologous chondrocytes to repair damage to the articular cartilage in the knee.[32] Many studies have shown that ADSCs can be differentiated in culture into osteoblasts and chondrocytes.[33,34] In our analysis, osteogenesis could be induced using culture medium supplemented with 1 nM dexamethasone, 2 mM β-glycerolphosphate, and 50 μM ascorbate-2-phosphate. The ADSCs were maintained in this medium for approximately 2 weeks, and the osteogenic medium was replaced every 2–3 days. *In vitro* osteoblast differentiation was commonly monitored using the marker osteocalcin, which was sequentially expressed during the differentiation process. Mineralization was assessed by staining the cells with 40 mM Alizarin Red (pH 4.1) after fixation in 10% formalin.

In *in vivo* studies, Hattori et al. in 2004 compared the osteogenic and bone-forming capacities of ADSCs in porous β-tricalcium phosphate as a scaffold on which to implant the ADSCs for bone regeneration.[35] The availability of a cell source replacing human bone would be strongly beneficial for cell therapy, bone tissue engineering, and the development of new therapeutic options to enhance the regenerative capacity of human bone. Further, the *in vivo* formation of bone was investigated in nude mice using β-tricalcium phosphate scaffolds seeded with ADSCs.[36]

In addition, Lendeckel et al used adipose-derived stem cells mixed with autologous fibrin glue to keep the stem cells in place and to augment the limited amount of bone available for calvarial reconstruction, with milled cancellous bone serving as an osteoconductive scaffold.[37] Mechanical fixation was achieved by two large, resorbable macroporous sheets that also acted as a soft tissue barrier. The postoperative course was uneventful, and computed tomography scans showed new bone formation and near-complete calvarial continuity 3 months after transplantation.

Taken together, these two *in vivo* studies utilizing these ADSCs are very attractive for cartilage and bone tissue engineering since subcutaneous fat is abundant in the human body and the liposuction procedure is minimally invasive for the patient.

Differentiation into hepatocytes

In recent years, various reports have indicated that the growth of ADSCs in a medium under specific conditions encourages their differentiation toward the endoderm hepatocyte lineage. These cells have acquired the capability for hepatocyte-specific synthetic functions, albumin production, low-density lipoprotein uptake, ammonia detoxification, drug metabolism, and ammonia clearance. Moreover, transplantation of these cells into different animal models of liver failure has demonstrated that these cells can recover liver function and improve the markers of liver injury.

First, Kim et al intravenously injected ADSCs into a mouse model with partial hepatectomy and showed the integration of ADSCs into the liver,[38] which suggested that mesenchymal stem cells derived from adipose tissues could be utilized for cell therapy in the liver. Afterward, other groups used various cytokine mixtures to induce ADSCs to differentiate into functional hepatocyte-like cells. In 2005, Seo et al were the first to show that human ADSCs differentiated into hepatocyte-like cells upon treatment with hepatocyte growth factor, oncostatin M, and dimethyl sulfoxide.[39]

Banas et al further sorted $CD105^+$ cells from ADSCs and treated them with fibroblast growth factor and hepatocyte growth factor for 3 weeks. During the step of hepatic differentiation, the cells showed a remarkable transition from a bipolar fibroblast-like morphology to a round epithelium-like shape. When treated with dexamethasone to induce cell maturation, the cells became quite dense and round with clear or double nuclei after they had reached the late stage of differentiation.[40] The hepatogenic potentiality of ADSCs was confirmed by the detection of hepatic-specific markers and biochemical functions. Albumin, the major protein produced by the hepatocytes, was synthesized and secreted into the medium at days 30 and 50. The expression and activity of the microsome cytochrome P enzymes involved in drug and xenobiotic metabolism, as well as sterol and bile acid synthesis, indicates hepatocyte specificity. The authors then implanted the ADSCs-derived hepatocytes into CCl4-injured nude mice and observed direct incorporation into the liver. In those mice, some liver functions, such as ammonia concentration level, were improved, as was the level of glutamic—pyruvic aminotransferase level in the peripheral blood, which is a marker of the damaged liver.

Recently, Dr. Yamamoto's group analyzed the gene expression profiles of ADSC-derived hepatocytes using several microarray methods.[41] This report provides evidence that the transcriptome and signal pathways of ADSC-derived hepatocytes are similar to those of human primary hepatocytes. A decrease in Twist and Snail expression, as well as upregulated E-cadherin and α-catenin, indicated that epithelial mesenchymal transition (EMT) occurred in the differentiation of ADSCs into hepatocytes.

Liang et al found that the mitogen-activated protein kinase pathway is involved in the hepatogenic induction process of ADSCs *in vitro*, and the extracellular signal-regulated kinase pathway seems to play a more important role in this step than do the c-Jun N-terminal kinase and p38 pathways[42]. Several groups have shown that culturing ADSCs in a cocktail of growth factors can differentiate them into cells that take on hepatocyte properties. Analysis of these cells on both transcriptional and translational levels has demonstrated the activation and expression of several hepatocyte-specific pathways after differentiation. The efficiency of differentiation may not, however, be adequate for therapeutic application.

More recently, in 2010, the latest study carried out by Lue et al identified five critical transcriptional factors important in hepatocyte development that were missing in these growth factor-driven hepatocyte-like cells, and speculated that a deficiency of these factors was contributing to incomplete transdifferentiation. In summary, they transfected FOXA1, FOXA2, SOX17, GATA4 and HNF4a factors to ADSCs using lentiviral vectors.[43] The data implied that transducing ADSCs with a combination of endodermal transcriptional factors could indeed result in an enhancement of albumin gene expression when compared with untransduced ADSCs after 10 days of culture. Thus, the direct transdifferentiation of MSCs into endoderm-like cells rather than growth factor-driven hepatocyte-like cells may be another potential route for the establishment of a therapy for the injured liver.

Differentiation to islet beta cells

Adipose tissue is a noteworthy organ that regulates the body's fat mass and nutrient homeostasis. It secretes a large number of adipokines that modulate homeostasis, blood pressure, and lipid and glucose metabolism. Adipokines such as leptin, adiponectin, and visfatin are known insulin-sensitizers and play a major role in glucose homeostasis.

In 2003, Kojima et al showed that extrapancreatic insulin-producing cells could be detected in multiple

organs, including the liver, spleen, bone marrow, and adipose tissue of diabetic mice.[44] In the same year, Dr. Roy found adipocytes from a carp that could express the insulin gene and secrete immunoreactive and biologically active insulin.[45] This evidence directly supports the possibility that ADSCs can differentiate into insulin-producing cells.

Notably, Timper et al showed that ADSCs cultured in high glucose (25 mM), hepatocyte growth factor (5 ng/mL), and several other factors (exendin-4, pentagastrin, activin-A, betacellulin, and nicotinamide) could differentiate into insulin-producing cells.[46] Initially, these cells expressed not only the stem cell markers nestin, ABCG2, SCF, and Thy-1, but also the pancreatic transcription factor Isl-1. After 3 days' treatment followed by upregulation of Isl-1, pdx-1, and Ngn-3 expression in mRNA, C-peptide-positive cells could be found among the differentiated ADSCs. In addition, these cells also released somatostatin into the culture medium. Another group, that of Lee et al, used regenerating pancreas extract to induce ADSCs for pancreatic differentiation.[47] Pancreas extract from regenerating pancreas after partial pancreatectomy is known to contain factors that induce islet neogenesis in rats with streptozotocin-induced diabetes. All these features make ADSCs the pre-eminent candidate to differentiate into the pancreatic endocrine lineage for use in cell-based therapies for diabetes.

Chandra et al used a similar method of culture with liver extracts to transform mesodermal ADSCs into definitive endoderm lineage, then into pancreatic endoderm, and finally into islet-like cell aggregates.[48] Their differentiation protocol was divided into three stages. First, definitive endoderm differentiation was achieved with insulin—transferrin—selectin, 4 nM activin A, 0.5 mM sodium butyrate, and 50 nM 2-mercaptoethanol. Pancreatic endoderm was induced in the next step with 0.3 mM taurine, and finally pancreatic hormone-expressing islet-like cell aggregates were induced with GLP-1, niacin, and a supraphysiological level of taurine (3 mM) for 5 days. *In vivo*, transplantation of mature islet-like cell aggregates by implanted capsules could restore normoglycemia within 2 weeks in streptozotocin-induced diabetic mice.

A further research group used human eyelid adipose-derived stem cells that could also normalize hyperglycemia in diabetic mice. For nuclear reprogramming methods, exogenously induced expression of the *Pdx1* gene, a key regulator of pancreatic bud development and beta-cell differentiation, induces the differentiation of both embryonic stem cells and bone-marrow-derived mesenchymal stem cells into insulin-producing cells.[49] Kajiyama et al showed that transfer of the *Pdx1* gene into ADSCs could differentiate them into insulin-producing cells *in vivo*, significantly decreasing blood glucose levels and increasing survival rates in mice with streptozotocin-induced diabetes.[50] These pieces of research may be a useful reference when considering the clinical application of ADSCs in developing a cell-based therapy for diabetes mellitus.

Neuronal differentiation

The central nervous system, made up of the brain and spinal cord, is an amazing nerve impulse conduction machine. Peripheral nerves link the brain and spinal cord to the other parts of the body, such as muscles and skin. The nervous system consists of two types of cell: nerve cells (neurons) and their associated supporting cells, glial (neuroglial) cells, which occupy the spaces between neurons and release neuronal growth factors, neuroprotective molecules, and neuronal transmitters that are essential for maintaining the survival and function of neurons.

The nervous system possesses some very fragile structures that can be damaged by pressure, and key mediators in the regeneration of such injured nervous tissue are glial cells.[51] The three types of supporting cell in the central nervous system are the astrocytes, oligodendrocytes, and microglia. The supporting cells of the peripheral nervous system are known as Schwann cells. The therapeutic approach usually uses direct end-to-end surgical repair of the injured nerves for minor damage, whereas autologous nerve grafts are required for larger defects. Despite tremendous surgical advances in recent years, functional recovery is still poor, and neuronal cell therapy and tissue engineering techniques enhancing the beneficial endogenous responses to nerve injury could provide a novel therapeutic strategy.

Recent studies have shown that ADSCs can be induced to differentiate into Schwann-like cells and have compared this differentiation potential with that of bone marrow stromal cells (BMSCs);[52] we will now primarily discuss the use of ADSCs for neuronal therapy after brain injury, traumatic injuries, and demyelinating lesions. Many recent reports have indicated that the ADSCs can also be induced into neurospheres and neuronal-like cells *in vitro*, and that an intracerebral transplantation of ADSCs can improve the neurological deficits seen after cerebral ischemia in mice or rats.[27,53—55] Thus, ADSCs could be an ideal alternative cell source for neuronally related cells. Several studies have indicated that ADSCs could be converted into neurospheres, and that these neurospheres could be induced to become neuron-like cells. Some of those neuron-like cells can even form myelin structures around neuronal neurites.

Briefly, ADSC were taken through three to five passages to grow them detached, and were then replated in serum-free DMEM/F12 medium supplemented with epidermal growth factor and basic fibroblast growth factor. Few cells adhered to the surface of the flasks. A large number of small spheres of floating cells appeared after 2—4 days in the conversion culture, and these spheres were able to proliferate *in vitro* for up to 2 months. Neurospheres could be passaged every 7—10 days, with an estimated doubling time of 3 days. More than 80% of the ADSCs converted into neurospheres, and about 70% of neurosphere cells were able to express nestin protein, whereas a small proportion (less than 10%) of the neurosphere cells expressed β-tubulin III, the glial marker GFAP, S-100, and p75 in neurosphere cells. As soon as the neurospheres were plated in poly-L-lysine-coated slides cultured in B27/Neurobasal medium (B27/N medium), they began to grow attached, and finally gained a neuronal morphology that was positive for neuronal marker β-tubulin III and GFAP.

A recent study has employed a different induction method. ADSCs were first exposed to 5-azacytidine, a demethylating agent capable of changing gene expression

through demethylation.[56] Further differentiation was achieved by maintaining the cells in Neurobasal medium containing B27 supplement to induce neural differentiation.[56]

In our studies, we have also been able to demonstrate the ability of ADSCs to differentiate into neuron-like cells by simply maintaining ADSCs in K-NAC medium containing 25 μM fluvastatin for 3 days to obtain neuron-like cells that were positive for two neural markers, GFAP and MAP. The success and progress of the *in vitro* studies of ADSCs has no doubt led us to the fundamental question of whether these cells are capable of survival and are able to function after transplantation into the central or peripheral nervous system.

One recent study has described the survival and migration ability of ADSCs that had been transplanted into rats. This group cultured ADSCs in Neurobasal medium with B27 supplement, 5-azacytidine, and growth factors.[57] After implantation into the lateral ventricle of the rat brain, the ADSCs were able to survive and migrated to multiple areas of the brain. When ADSCs were transplanted into animals with focal ischemia resulting from middle cerebral artery occlusion, they migrated into the injured cortex, suggesting that ischemia-induced factors facilitate donor cell migration; in addition, physical behavioral testing demonstrated that ADSCs improved functional recovery after cerebral artery occlusion.[58] The report implied that the future clinical application of ADSCs for tissue engineering would help the development of cell-based therapeutics for diseases of the nervous system. Moreover, these ADSCs can secrete many growth factors, such as pigment epithelium-derived factor, and can promote survival and neurite outgrowth in neurons. Neuron-like cells derived from ADSCs may also be useful in the near future for treating diseases of the peripheral (e.g., nerve injuries) and central (e.g., multiple sclerosis) nervous systems.

Differentiation to cardiomyocytes

A heart attack (also known as a myocardial infarction) commonly causes the death of cardiomyocytes, which are replaced by scar tissue. Cardiomyocytes are terminated, differentiated cells that cannot regenerate when they are damaged by cardiac infarction or cardiomyopathy. ADSCs, on the other hand, are pluripotent cells with the potential to differentiate into other lineage cells under appropriate stimulation.

In the first study of 2003, Rangappa et al treated ADSCs with various concentrations of 5-azacytidine and incubated them for different lengths of time.[59] After treatment with 5-azacytidine, the adult ADSCs were transformed into cardiomyocytes. Some cells presented binucleation and extended cytoplasmic processes to adjacent cells after 1 week's treatment. Further, 20–30% of the cells increased in size and formed a ball-like appearance after 2 weeks' treatment. At 3 weeks, some cells began to beat spontaneously in culture when observed under a phase-contrast microscope. These differentiated cardiomyocyte-like cells stained positive for myosin heavy chain, α-actinin, and troponin-I in cytochemistry analysis. Moreover, those differentiated cells maintained this phenotype and had not dedifferentiated up to 2 months after treatment with 5-azacytidine.

In 2008, Zhang et al applied the same method of cultivation of ADSCs *in vitro* in order to investigate the differences between ADSCs and MSCs grown *in vitro* and their differentiation into cardiomyocytes.[60] After treatment with a consistent dose of 5-azacytidine for several weeks, they obtained the same cardiomyocyte-like appearance after 3 weeks of treatment. They also claimed that the percentage of ASCs that differentiated into cardiomyocytes after treatment with 5-azacytidine was significantly higher than that of MSCs. This report indicated that ADSCs have advantages over MSCs in terms of tissue content, homology, growth, and differentiation rate, suggesting that ADSCs are a more suitable resource for the differentiation of cellular cardiomyoplasty than MSCs.

In 2010, Choi et al used 5-azacytidine, a modified cardiomyogenic medium, and the histone deacetylase inhibitor trichostatin A in a co-culture of neonatal rat cardiomyocytes.[61] In this study, they examined and monitored cardiomyogenic differentiation protocols step by step using 5-azacytidine treatment that reduced both cardiac actin and troponin T mRNA expression. Incubation in modified cardiomyogenic medium only slightly increased gene expression (1.5-fold), with 27.2% of cells coexpressing NKX2.5/sarcomeric α-actin. Most importantly, many of those differentiated cells showed spontaneous contractions accompanied by calcium transients in culture. ADSCs showed Ca^{2+} transients and contractions synchronous with surrounding rat cardiomyocytes (at approximately 100 beats/minute). Gap junctions also formed between the cells, as observed by dye transfer. This method used co-culture with contracting cardiomyocytes to increase the differentiation efficiency of ADSCs into cardiomyocytes.[62] Together, these observations confirm that ADSCs isolated from fatty tissue can be chemically transformed into cardiomyocytes and provide a potential cardiac differentiation system using autologous cells for myocardial repair or tissue engineering.

Current clinical applications

In the first clinical case, ADSCs were used for the regenerative treatment of traumatic calvarial bone defects.[37] A 7- year-old girl with post-traumatic calvarial defects was treated with autologous cancellous iliac bone combined with ADSCs, fibrin glue, and a biodegradable scaffold. Postoperative computed tomography scanning showed new bone formation, and almost complete calvarial continuity was obtained.

Autologous ADSC therapy was also used to treat fistulas in patients with Crohn's disease. In a pilot study of five patients with Crohn's disease, the external opening in six out of eight fistulas could be closed by inoculating the fistulas with autologous ADSCs.[63] Administration of expanded ADSCs (20–60 million cells) in combination with fibrin glue is an effective and safe treatment for complex perianal fistula and appears to achieve higher rates of healing than fibrin glue alone.[64]

Since this report was published, ADSCs have also been used to repair tracheomediastinal fistulas caused by cancer

ablation. In another acute clinical trial, ADSCs were administered intravenously to patients with acute steroid-refractory graft-versus-host disease.[65] The graft-versus-host disease resolved completely in five out of six patients, four of whom were alive, without side effects, after a median follow-up period of 40 months.

Clinical trials of ADSCs for the treatment of acute myocardial infarction and chronic heart failure have begun in Europe. Although the results of these studies have not been published, ADSCs as well as bone marrow-derived MSCs are a promising source of cell-based therapies for the treatment of cardiovascular diseases.

Conclusion and future perspective

In our laboratory, we were recently able to isolate and expand the ADSC culture system from human adipose tissue. ADSCs share similar characteristics with MSCs, but the former system has some advantages, including ease of use, simplicity, and a less invasive process for isolation and purification.

However, many critical issues regarding the application of ADSCs for regenerative medicine and tissue engineering remain unclear. There are several key questions that still need to be answered, and these must be explored before clinical trials are conducted: Which factors control the stem/progenitor activity of ADSCs? How can the processes of proliferation and differentiation of ADSCs be controlled *in vitro* and *in vivo*? Which signal pathways control these processes? Will ADSCs cause tumor formation after transplantation?

Meanwhile, adipose tissues preparation, techniques to expand and maintain cell culture *in vitro*, the degree of purity needed for clinical application, methods for monitoring and ensuring the quality of the ADSCs, and techniques to evaluate cell differentiation potential before implantation should also be identified and standardized. Although the latest results are very encouraging, more detailed and confirmatory research is necessary before future clinical protocols can be constructed. We believe, however, that ADSCs will be a good autologous resource for regenerative medicine.

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