Adipose stem cells for bone tissue repair

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Summary

Adipose-derived stem/stromal cells (ASCs), together with adipocytes, vascular endothelial cells, and vascular smooth muscle cells, are contained in fat tissue. ASCs, like the human bone marrow stromal/stem cells (BMSCs), can differentiate into several lineages (adipose cells, fibroblast, chondrocytes, osteoblasts, neuronal cells, endothelial cells, myocytes, and cardiomyocytes). They have also been shown to be immunoprivileged, and genetically stable in long-term cultures. Nevertheless, unlike the BMSCs, ASCs can be easily harvested in large amounts with minimal invasive procedures. The combination of these properties suggests that these cells may be a useful tool in tissue engineering and regenerative medicine.

KEY WORDS: mesenchymal stem cells; adipose tissue; ASCs; osteogenic differentiation; bone tissue engineering.

Introduction

Stem cells are characterized by their ability to produce self-renewing progenitor cells that can generate one or more specialized cell types. They are divided into embryonic stem cells (ESCs) and adult stem cells (1). The use of adult stem cells has awakened huge interest. Even if they have a lower capacity of differentiating (2, 3) than ESCs (4, 5), they circumvent ethical issues and show less tumorigenicity. The first multipotent mesenchymal stem cells (MSCs) identified (6) were the bone marrow stromal/stem cells (BMSCs). Currently, these cells are most frequently used in regenerative medicine for their high differentiation potential and low morbidity during harvesting (2, 7, 8). Nevertheless, harvesting of BMSCs by bone marrow aspiration is a painful procedure, and the number of cells acquired is usually low. An alternative source of multipotent stem cells, with similar properties to those of BMSCs, is the adipose tissue (9-12). In fact, adipose stem cells (ASCs) can be easily harvested, obtaining large amount with minimal risk, and moreover they show the ability to be readily expanded, and also the capacity to undergo adipogenic, osteogenic, chondrogenic, neurogenic, and myogenic differentiation *in vitro* (3, 10-17). For these reasons ASCs are currently used for tissue regeneration or reconstruction in clinical trials. In fact, during recent years, the number of trials investigating the efficacy in treating conditions such as type I and II diabetes, liver and cardiovascular disease, limb ischemia, amyotrophic lateral sclerosis, and lipodystrophy, has increased. Moreover, ASCs appear to be more genetically stable in long-term cultures (18) compared to BMSCs (19), and show immunosuppressive properties (20, 21). Indeed, these cells are subject to study in case of immunosuppression (20-22), graft-versus-host disease (23-26), soft tissue augmentation (27, 28), multiple sclerosis (29), and bone tissue repair (30, 31). In view of all these properties, the studies of clinical and basic research are focused on ASCs, but more knowledge of the features of this cell population is needed in order to standardize the clinical strategies (32, 33). The aim of this review is to examine the differentiative capacity of ASCs and their potential role in tissue engineering.

Adipose tissue: an alternative source of stem cells

Adipose tissue, derived from the mesenchyme, is found in bone marrow, around internal organs, under the skin, and in breast tissue. In humans, it is one of the most abundant tissue types, a metabolic reservoir for packaging, storing, and releasing high-energy substrates (34), and it also has many endocrinologic properties, given that it secretes numerous polypeptides, hormones, growth factors, and cytokines (35, 36). Adipose tissue is composed of a heterogeneous set of cell populations that are defined as stromal vascular fraction (SVF) (3, 37). The SVF comprises the stromal cells, ASCs (10, 11, 38), vascular endothelial cells and their progenitors, vascular smooth muscle cells, and also cells with hematopoietic progenitor activity (39, 40). Currently, this tissue is probably considered to be one of the richest sources of adult stem cells in the human body, and therefore it holds great promise for utilization in tissue repair and regeneration.

Adipose stem cells

Recently, ASCs have been isolated from adipose tissue (2, 11). These cells, like other MSCs (i.e. BMSCs), show a spindle or stellate shape, the capacity to adhere to plastic to form fibroblast-like colonies (Figure 1), an extensive proliferative capacity, and the ability to differentiate into several multilineage cells such as osteoblasts, adipocytes, chondroblasts, myocytes, tendocytes, and ligament cells (3, 10-17, 41). In addition to their potential to differentiate, ASCs have also been shown to possess some level of plasticity, transdifferentiating *in vitro* into epithelial cells, hepatocytes, and neural

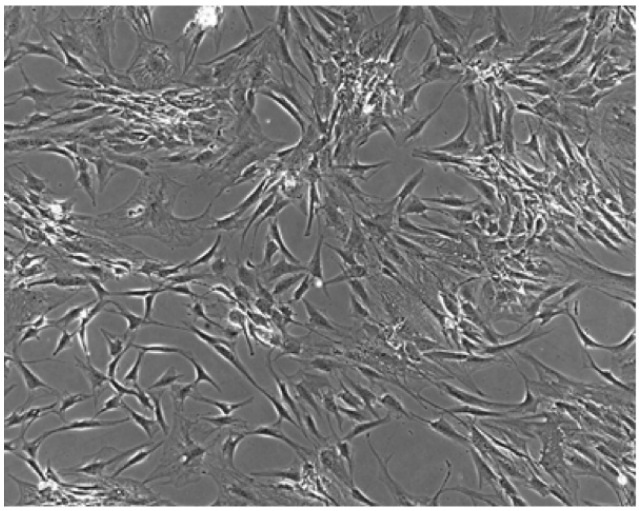

Figure 1 - ASCs undifferentiated. Observation with phase contrast microscope: ASCs with shaped form.

cells (42-44). In culture, these cells express stem cell-surface markers such as: CD13, CD29, CD44, CD73, CD90, CD105, CD133 and CD166 (45-51). Therefore, ASCs match the four criteria for the identification of human mesenchymal stem cells proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (52): they have to be plastic-adherent when maintained under standard culture conditions; they must have the ability to osteogenic, adipogenic, and chondrogenic differentiation; they must express CD73, CD90, CD105; they must lack the expression of hematopoietic linage markers (CD14, CD11b, CD34, CD45, CD19, CD79). Moreover, ASCs can be easily harvested in great amounts from fat tissue; i.e., after cosmetic liposuctions, once adipose tissue is digested with collagenase (63), approximately ~5000 cell/ml fat can be obtained (64). For the reasons mentioned above, ASCs may represent a valuable and alternative tool in tissue engineering. Some studies have also reported that ASCs possess the ability to suppress a mixed lymphocyte reaction in a dose-dependent and time-dependent manner (53, 54), that they are immunoprivileged (20, 21), due to lack of major histocompatibility complex class II expression (45, 55), and they have the capacity to suppress the proliferation of activated allogeneic lymphocytes (54-56). This suggests that ASCs can have potential as immunoprivileged universal donor cells with the capacity to be used in the allogeneic setting and to reduce graft-versus-host disease (20, 54). Nevertheless, recent studies have shown that the immunosuppressive effects of ASCs may induce tumorigenesis (57, 58). Conversely, other studies show that these cells possess tumorsuppressive capacity (59-61). The debate is still open at this time.

Many names have been utilized to describe these cells: processed lipoaspirate cells (PLA), preadipocytes, adipose-derived adult stem (ADAS) cells, adipose-derived stromal cells (ADSCs), adipose stromal cells (ASCs), multipotent adipose-derived stem cells (MADS), and adipose mesenchymal stem cells (AdMSCs) (37). To solve the problem, the International Fat Applied Technology Society has proposed a standardized nomenclature by adopting the term adipose derived stem/stromal cells (ASCs) to identify the isolated, plastic-adherent, multipotent cell population (39, 62).

ASCs: from ward to bench

ASCs can be isolated from adipose tissue coming from plastic surgery or biopsies. When the starting material is obtained from liposuction procedures, the isolation is faster as a result of finely minced tissue fragments (65). Using whole tissue pieces as starting material, the tissue is minced manually (35). In our lab, after having obtained informed consent in accordance with the Institutional Review Board protocol, we have utilized the following practice: the adipose tissue biopsies are immediately placed in McCOY'S 5A medium supplemented with 22mM HEPES and 100 IU/ml penicillin, 100 µg/ml streptomycin, pH 7.4, and transported to the laboratory, with processing within 30 min of excision. In the lab the samples were minced into small pieces (0.2-0.5mm) and the fragments were washed with McCOY'S 5A medium, centrifuged at 200 g for 10 min, resuspended in Ham's F12 Coon's modification medium supplemented with 20% FBS and 3 mg/ml collagenase type I, digested for 3 h at 37°C, mechanically dispersed and passed through a sterile 230-mm stainless steel tissue sieve. The undigested tissue trapped in the sieve was discarded, while the infranatant containing the preadipocyte fraction was collected and the cells were sedimented by centrifugation at 300 g for 5 min. The pellet was incubated with an erythrocyte lysis buffer for 2 min at room temperature, and the remaining cells were cultured in 100 mm culture plates in growth medium: Ham's F12 Coon's modification medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1 ng/ml basic fibroblast growth factor, and incubated at 37°C in humid atmosphere with 5% CO2. The medium was refreshed twice a week (66).

ASCs: differentiation potential

ASCs can differentiate into several different cell types (Table 1) (10, 11, 15, 67-74) and the lineage-specific differentiation is directly related to the expression of specific phenotypic markers and mature tissue genes. However, the mechanisms that drive these cells into the specialized lineage are not yet clear.

Table 1 - Differentiation potential of ASCs.

Lineage	References
Adipose	75,77,78,80
Bone	35,77,85,86,88,89
Cartilage	17,35,68,101,103
Skeletal muscle	69,113,114
Cardiac muscle	70, 115-117
Endothelium	124,125,128
Pancreas	16,139,140
Hepatic	15,135,141,142
Erithelial	113,136
Nervous	137,138,143,144

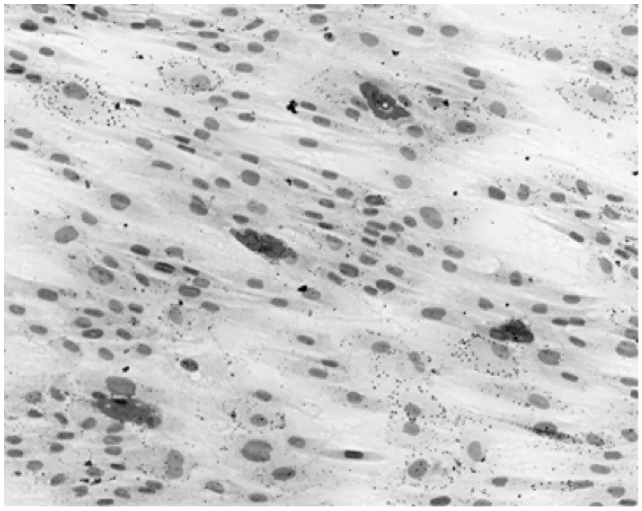

Figure 2 - Adipogenic differentiation of ASCs. Oil Red O staining: adipocytes in red, nuclei in blue-violet.

Adipogenic differentiation

ASCs have a notable capacity to differentiate into adipocytes (75-77), which is very important in developing techniques to repair soft tissue defects, especially after oncological surgery (78). The adipogenic induction medium contains insulin or IGF-1, triiodothyronine, transferrin, isobutylmethylxanthine, hydrocortisone or dexamethasone, indomethacin or thiazolidinedione, pantothenate, and serum (10, 11, 79, 80). A week after induction, lipid containing vacuoles accumulate in ASCs, which can be detected by Oil Red O or Nile red staining (Figure 2) (11, 81), and express several genes and proteins involved in lipid biosynthesis, metabolism and accumulation, including peroxisome-proliferating activated receptor γ, adipocyte fatty-acid binding protein, and leptin can be detected (10, 11, 82-84).

Osteogenic differentiation

In vitro osteogenic differentiation of ASCs can be obtained using medium supplemented with ascorbic acid, b-glycerophosphate, dexamethasone and/or 1,25 vitamin D_3 (3, 85, 86). Another factor that can strongly induce the osteogenic differentiation is bone morphogenic protein 2 (BMP2), belonging to the transforming growth factor-β (TGF-β) superfamily. Indeed, some studies show *in vitro* and *in vivo* differentiation of ASCs in osteoblast-like cells when transfected with *BMP2* gene (87, 88). ASCs cultured in the presence of these factors express genes and proteins associated with the osteoblast phenotype, including alkaline phosphatase (Figure 3A), type I collagen, osteopontin, osteonectin, Runx2, BMP-2, BMP-4, and BMP receptors I and II (11, 35, 76, 77, 85, 89, 90). Moreover, when cultured between 14 and 28 days *in vitro* in osteogenic medium, ASCs start to produce calcium phosphate mineral within their extracellular matrix, which can be detected by Alizarin Red or von Kossa staining (Figure 3B) (10, 11). Importantly, ASCs undergoing osteogenic induction are able to adhere to scaffolds, migrate, proliferate, and differentiate when transplanted in bone tissue *in vivo* (91-93). This type of construct can be effective to regenerate damaged bone tissue (92, 94-97). Finally, since it is demonstrated that while the adipogenic potential is unchanged compared to age, the osteogenic potential decreases with an increase of the same (98). All these conditions should be evaluated for designing clinical treatment for patients.

Chondrogenic differentiation

Many studies have demonstrated the ability of ASCs to undergo chondrocytic differentiation (10, 17, 68, 99) using medium supplemented with insulin growth factor, ascorbate-2-phosphate, dexamethasone, L-proline, BMP6 and 7, and TGF-β (100-106). Routinely, for chondrogenic differentiation, ASCs are cultured in micro mass culture or pellet culture systems (8, 107). These practices mimic the embryonic de-

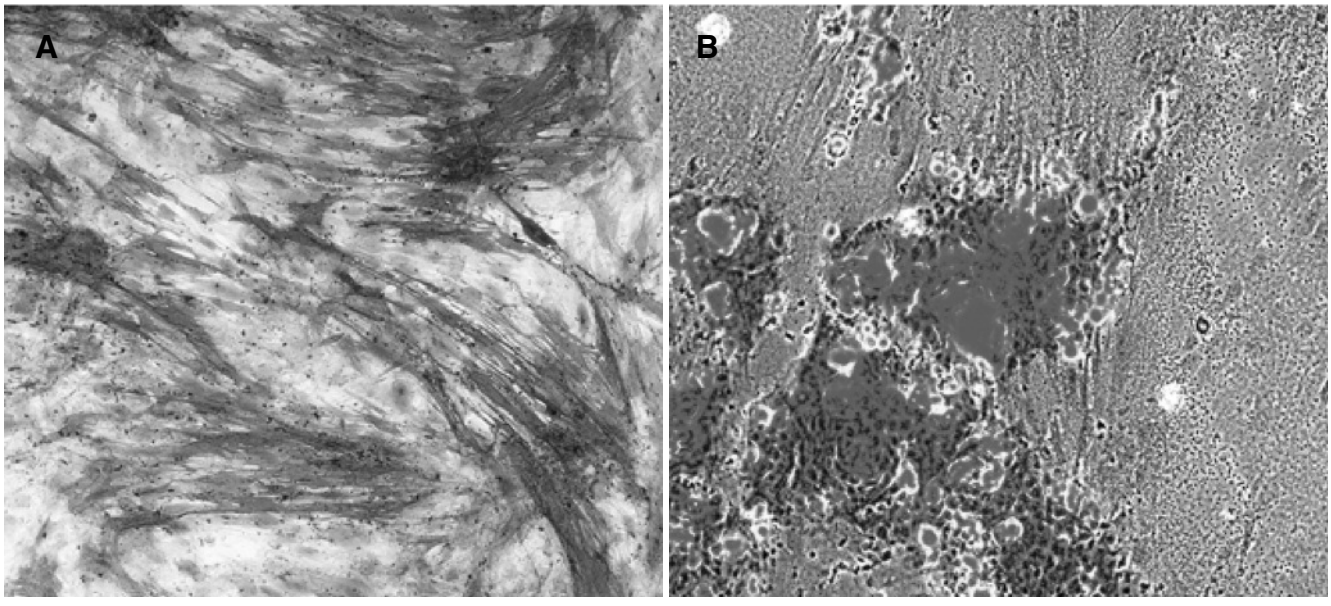

Figure 3 - Osteogenic differentiation of ASCs. A. ALP activity (in red) in osteogenic differenziate ASCs; B. Hydroxyapatite deposits: calcium deposits in red, ASCs in gray-blue.

velopment conditions of cartilage tissue, increase the cell-to-cell interaction, and lead to the production of a cartilage-like matrix (108). Alternatively, ASCs can be seeded into polyglycolic acid scaffolds that mimic the composition of the native cartilage (74, 109). Moreover, it is demonstrated that ASCs on elastin-like polypeptide material can grow and express the chondrogenic phenotype without the chondrogenic medium (110). So, when ASCs are maintained *in vitro* in an appropriate 3D environment, with or without induction medium, they will start to synthesize the cartilage extracellular matrix proteins, including COL II, COL VI, and aggrecan (68, 109, 111). The potential of ASCs in cartilage tissue engineering is also demonstrated in *in vivo* studies in animal models (112).

Myogenic and cardiomyogenic differentiation

Using specific medium that contains induction factors such as dexamethasone and hydrocortisone, it is possible to obtain ASCs myogenic differentiation (10, 113). *In vitro*, after undergoing these culture conditions, ASCs express muscle-specific marker including a heavy chain of myosin, myogenic determination factor 1 and myogenin (10, 11, 69), and they adopt elongated, multinucleated structures that closely resemble myotubes (10, 11, 69). *In vivo* studies showed that the implantation of ASCs in muscular dystrophy mice restores the dystrophin expression in the muscles (114). These results are particularly promising for genetic diseases characterized by progressive muscle degeneration.

ASCs also have the capacity to differentiate in cardiomyocytes, when seeded in medium supplemented with interleukin 3 and 6 (70, 115-117). Nevertheless, few studies are reported, and in most of them a low percentage of differentiation has been reported (118-120). *In vitro* expanded ASCs have been shown to improve cardiac function when administered in animal model with myocardial infarction (120).

Endothelial differentiation

It has been reported that ASCs have the potential for endothelial differentiation (33, 121-125) and can participate in blood vessel formation, as they are able to secrete a number of proangiogenic factors, such as vascular endothelial growth factor and platelet-derived growth factor (13, 126-130). These characteristics are very important, because the regenerated tissues need to contain vascular systems to allow both the tissue and the differentiated cells to survive. Studies performed on animal models have allowed the development of an osteogenic and vasculogenic construct using human adipose stromal-vascular cell fractions (125, 131). These studies prove that human ASCs under perfusion flow in a three-dimensional environment are able to form bone tissue and blood vessels after implantation in nude mice. What is more, the blood vessels formed by human ASCs were functionally connected to the mouse vascular network and contained mouse erythrocytes (125, 131).

ASCs: transdifferentiation potential

It has long been believed that tissue-specific progenitor cells can only differentiate into native tissue cell types. Recent studies have challenged this view. Many experiments have revealed that adult stem cells may retain the potential to transdifferentiate from one phenotype to another, either *in vitro* or after transplantation *in vivo* (132, 133). ASCs can differentiate, upon specific stimulus, into cells of ectodermal and endodermal origin such as pancreatic cells (16, 134), hepatocytes (135), epithelial cells (113, 136) and neuronal cells (11, 137, 138), although the mechanisms are not yet clear.

Endodermal tissue-derived differentiation

Pancreatogenic differentiation

Some studies report that ASCs can be differentiated into cells with a pancreatic endocrine phenotype expressing insulin, glucagon, and somatostatin (16). Indeed, if transplanted into diabetic mice, they can regulate blood glucose levels by releasing human insulin (139), or if transplanted in pancreatectomized rats, to induce C-peptide-positive cells from ASCs by observing expression of genes related to early pancreatic differentiation (140).

Hepatogenic differentiation

The differentiation of ASCs into hepatocyte-like cells has also been investigated (15, 141, 142). When hepatogenically induced, ASCs express albumin and α-fetoprotein, and show LDL uptake and production of urea. When these cells are predifferentiated *in vitro,* and after a transplantation into a SCID mouse model, they express albumin and promote the hepatic integration *in vivo* (135).

Epithelial differentiation

Currently, there are studies which show that, under optimized conditions, ASCs express epithelial marker protein and complete their epithelialization after implantation (113, 136), i.e. a tissue-engineered airway construct with a 3D structure of fibrin and ASCs was marked as a prototype vocal fold replacement.

Ectodermal tissue-derived differentiation

Neurogenic differentiation

Many studies have demonstrated the neurogenic potential of ASCs (11, 137, 138). When seeded in medium containing factors such as valproic acid, insuline, hydroxyanisole, epidermal growth factor, fibroblast growth factor, and indomethacin (11), ASCs express neuronal and/or oligodendrocytic markers (14, 143, 144) such as neuron specific enolase, neuron specific nuclear protein, nestin, intermediate filament M, and glial fibrillary acidic protein. Moreover, they adopt a neuronal morphology with a small cell body and multiple neurite-like projections (11). However, chemical signal transduction properties of ASCs in neurogenesis need to be characterized further to use these cells in the regeneration of the central or peripheral nervous system following traumatic injury.

Possible bio-applications of the ASCs

ASCs in immunologic disorders

Graft-versus-host disease (GvHD)

GvHD is a disorder that can be manifested after tissue or organ allogenic transplant. These cases are characterized by massive activity of the donor's immunologic system cells versus host. Some *in vivo* studies have reported good results in

humans, using ASCs for treating severe and acute GvHD (23-26).

Diabetes

One study, effected in insulinopenic diabetic patients treated using insulin-producing ASCs transfused, reported 30-50% decreased insulin requirements in all patients with a 4- to 26-fold increase in serum c-peptide levels during a follow-up period of 2.9 months on average (145).

Immunosuppression by ASCs

The use of ASCs for immunologic diseases, such as autoimmune-induced rheumatoid arthritis (RA) or inflammatory bowel disease, is currently under investigation (20-22, 146). Results show that the systemic infusion of ASCs in human patients is an important regulator of immune tolerance, with the ability to suppress T-cell and inflammatory responses, and inducing the activation of antigen-specific regulatory T-cells.

ASCs in tissue engineering

Tissue engineering is a field of research that uses biomaterials, growth factors, and stem cells to repair, replace or regenerate tissues and organs damaged by diseases or injuries (147, 148). ASCs are ideal candidates for utilization in tissue engineering practices, especially if the receiver is at the same time the donor (autologous transplant), since, as demonstrated above, they are able to self-renew, to commit to multiply cell lineages (2, 12), and easily harvested in large amounts with minimal invasive procedures (40).

Soft-tissue augmentation

The extensive *in vitro* proliferative capacity of ASCs is very important for the volume return of tissue in oncological patients after surgery, especially in breast cancer cases. Also, soft-tissue augmentation is a practice often used in plastic surgery. These techniques consist in the utilization of hyaluronic acid-based spongy scaffolds seeded with ASCs and then implanted subcutaneously (149).

Bone tissue repair

Many studies have been carried out to evaluate the ASCs or scaffold-ASCs construct capacity in bone tissue repair. At this time, three of these studies report success. In the first case, in the animal model, ASCs are plated on an apatite-coated resorbable scaffold. This construct has been used for the treatment of a murine calvarial defect. The results demonstrated a range of 70-90% closure of the defect within 12 weeks (96). In the second case, with a patient affected by keratocyst, maxillary reconstruction, has been done utilizing synthetic bone combined with autologous ASCs, previously expanded *ex vivo*. The results show mature bone structures developed within the construct (31). In the third case, the patient affected by severe calvarial fracture was treated with autologous ASCs applied in combination with autologous bone. The ASCs were supported in place using autologous fibrin glue, and mechanical fixation was achieved with resorbable macroporous sheets. The postoperative course was uneventful and new bone formation was observed three months after the reconstruction (30). However, further studies are required to assess and verify the safe outcome of the clinical procedure using *in vitro* expanded stem cells. In our laboratory, we have studied the growing and osteogenic differentiation ability of ASCs on hard biocompatible biomaterials, usually used in orthopedic-prothesic replacement (66). ASCs were seeded on titanium alloy (Ti6Al4V) and, at different times, were evaluated for cell growth and adhesion, and osteogenic differentiation. The results show good cell expansion and adhesion on the Ti6Al4V alloy surface. Moreover, when seeded in the presence of osteogenic medium, ASCs express the osteoblastic phenotype, which is characterized by an increase, time-dependent, of alkaline phosphatase activity, by deposits of osteopontin, osteonectin, and hydroxyapatite (Figure 4), and by the expression of a pattern of genes characteristic of the same phenotype as *ALP*, *RUNX2*, *SMAD1*, *OCN* and *OPN.*

Clinical trials

In the view the results described above, and to improve the safety and efficacy of ASCs for tissue regeneration or reconstruction, 18 clinical trials have been initiated (Table 2), and only 2 have been completed so far. The clinical trials using ASCs can be consulted on www.clinicaltrials.gov.

Future perspectives

It is well know that adipose tissue can be harvested in large quantities with minimal invasive procedures, obtaining large amounts of ASCs with high plasticity (134, 150-153). Moreover these ASCs exhibit immunomodulatory and angiogenetic properties (20, 21). These properties can have opposite effects. Reports have shown that the immunosuppressive capacity of the ASCs may in some cases favour the growth of tumour cells (57, 58), even if other studies report contrary

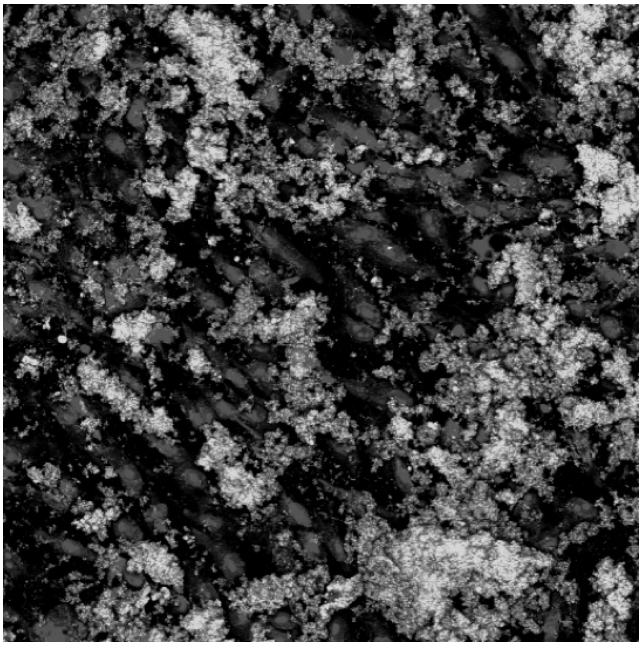

Figure 4 - ASCs seeded on titanium alloy (Ti6Al4V). LSM confocal microscopy: nucleuses in red, calcium deposits in green.

Table 2 - Clinical trials utilizing ASCs.

Trial	Condition	Design
Study of Autologous Fat Enhanced w/ Regenerative Cells Transplanted to Reconstruct Breast Deformities After Lumpectomy	Breast Neoplasms Carcinoma, Ductal, Breast Mammoplasty Mastectomy, Segmental, Lumpectomy, Breast Reconstruction	Phase IV
Safety and Efficacy of Autologous Cultured Adipocytes in Patient with Depressed Scar	Depressed Scar	Phase II/III, Completed
Efficacy and Safety of Adipose Stem Cells to Treat Complex Perianal Fistulas Not Associated to Crohn's Disease (FATT1)	Complex Perianal Fistulas	Phase III, Completed
Safety and Efficacy of Autologous Adipose-Derived Stem Cell Transplantation in Type 2 Diabetics	Type 2 Diabetes Mellitus	Phase I/II
Intraarterial Infusion of Autologous Mesenchymal Stem Cells From Adipose Tissue in Diabetic Patients With Chronic Critical Limb Ischemia	Chronic Critical Limb Ischemia	Phase I/II
Safety and Efficacy of Autologous Adipose-Derived Stem Cell Transplantation in Patients With Type 1 Diabetes	Type 1 Diabetes Mellitus	Phase I/II
Safety and Efficacy Study of Autologous Cultured Adipose -Derived Stem Cells for the Crohn's Fistula	Crohn's Fistula	Phase I
Safety Study of Autologous Cultured Adipose -Derived Stem Cells for the Fecal Incontinence	Fecal Incontinence	Phase I
Autologous Mesenchymal Stem Cells From Adipose Tissue in Patients With Secondary Progressive Multiple Sclerosis	Secondary Progressive Multiple Sclerosis	Phase I/II
Allogenic Stem Cells Derived From Lipoaspirates for the Treatment of Recto-Vaginal Fistulas Associated to Crohn`s Disease (ALOREVA)	Rectovaginal Fistula Crohn Disease	Phase I/II
Randomized Clinical Trial of Adipose Derived Stem Cells in the Treatment of Pts With STElevation Myocardial Infarction	Myocardial Infarction Coronary Arteriosclerosis Cardiovascular Disease Coronary Disease	Phase I
A Randomized Clinical Trial of Adipose-Derived Stem Cells in Treatment of Non Revascularizable Ischemic Myocardium	Ischemic Heart Disease Coronary Arteriosclerosis Cardiovascular Disease Coronary Disease Coronary Artery Disease	Phase I
Autologous Adipose-Derived Stem Cell Transplantation in Patients With Lipodystrophy	Lipodystrophy	Phase I
Liver Regeneration Therapy by Intrahepatic Arterial Administration of Autologous Adipose Tissue Derived Stromal Cells	Liver Cirrhosis	Phase I
Autologous Stem Cells Derived From Lipoaspirates for the Non-Surgical Treatment of Complex Perianal Fistula	Anal Fistula	Phase II
Long-term Safety and Efficacy of Adipose-derived Stem Cells to Treat Complex Perianal Fistulas in Phase II Patients Participating in the FATT-1 Randomized Controlled Trial	Complex Perianal Fistula	Phase II
Liver Regeneration Therapy Using Autologous Adipose Tissue Derived Stromal Cells	Liver Cirrhosis	Recruiting
Tissue Partitioning in Early Childhood	Changes in Bone Mineral Content Changes in Bone Marrow Adipose Tissue	No yet recruiting

results (59-61). In addition, although ASCs appear to be fairly stable genetically in long-term cultures, some chromosomal aberrations also arise transiently, at least when cells are cultured for approximately one month (18, 154). However, it is demonstrated that ASCs can be cultured safely during a standard *ex vivo* expansion period of approximately 6-8 weeks (155). Consequently, it is very important to improve methods to assess the safety of ASCs, not only *in vitro*, but also *in vivo* and clinically, to prevent anaphylactic reactions and tumorigenicity in the recipient.

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