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Methods and Procedures in Adipose Stem Cells: State of the Art and Perspective for Translation Medicine

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Stem cells have potential in the retrieval and repair of injured tissue and renovation of organ function. To date, several studies have been carried out to elucidate how differentiation of stem cells can be used in regenerative medicine applications. Adipose tissue is an abundant and accessible source of stem cell, useful for regenerative therapeutic use. Adipose stem cells (ASCs) are favorable for future translational research and can be applied in many clinical settings. Adipose tissue repair has been recently adopted in clinical trials to prove that ASCs can be successfully used in patients. Variability in cell culture procedures (isolation, characterization, and differentiation) may have an influence on the experimental outcome. In this report, we consider the selection mechanisms of ASCs using flow cytometry, cell culture, freezing/thawing, cell cycle evaluation, histochemistry/immunofluorescence, and differentiation of ASCs. Both researchers and regulatory institutions should consider a new policy for GMP procedures and protocols, paying special attention to stem cell bio-physiology, to facilitate more clinically oriented studies. ASCs show angiogenic properties, with prospects of repairing tissue damaged by radiotherapy, as well as possessing the ability to heal chronic wounds. They can also be useful in surgical practice. We focus on the potential clinical application of ASCs that are currently available regarding translational medicine and the methods and procedures for their isolation, differentiation, and characterization.

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Stem cells are designated to preserve the structural and functional integrity of tissues, replacing mature injury cells. Mesenchymal stem cells are multipotent adult stem cells (MSC) present in all mature human tissues and are likely to reside in a tissue-specific region remaining inactive until re-activated by disease or injury. "A stem cell is self-renewable and capable of differentiating into at least two distinctive cell types (Parker et al., 2004; Mao et al., 2006; Kolios and Moodley, 2013)." "These two properties must both be satisfied for a cell to be defined as a stem cell. Self-renewal denotes that undifferentiated daughter cells are a precise replica and can further replicate many generations without losing their original characteristics (Tanabe 2014; Zheng and Cheung, 2010; Mao et al., 2006)." Cells of an immortalized cell line can abundantly reproduce generations but are inadequate in multi-lineage differentiation. Multilineage differentiation considers the ability of a single stem cell to differentiate into different cell types. For example, a single population of MSCs can differentiate into both adipocytes and osteocytes. Pre-adipoblasts can differentiate into adipoblasts, but cannot differentiate into other mesenchymal lineages, such as chondrocytes or osteocytes, unless they revert back to MSCs through de-differentiation.

In the adult, MSCs maintain sufficient physiological tissue turnover and, upon injury or disease, differentiate to initiate tissue regeneration. MSCs have been experimentally differentiated into all mesenchymal or connective tissue lineages (Pittenger et al., 1999) and, in many cases, have been used for tissue engineering. Regeneration of tissue "structures from stem cells" is an "insurmountable effort until advances from several seemingly unrelated disciplines—such as cell and molecular biology, polymer chemistry, molecular genetics, materials science, and mechanical engineering—converged into the self-assembling field of tissue engineering (Nerem, 1992; Langer and Vacanti, 1993; Mao et al., 2006)." "To engineer a functional biological structure, cells must" receive indications to differentiate and instructions on positional cues (Mao et al., 2006). They need to produce, by synthesis, adequate extracellular matrix molecules and retain the shape and dimension of the injured tissues or organs. Cell growth and differentiation in previously unfamiliar or unknown environments may be assisted by biomimetic scaffolds. Niches of human stem cells have been extensively studied and different sources have been used to repair and regenerate tissue including mesenchymal tissue, adipose tissue, dental pulp, and other tissues (Laino et al., 2006; Kaewsuwan et al., 2012; Bluguermann et al., 2013). For example, adipose tissue stem cells (ASCs) have been engineered for adipose and connective tissue building and repair. These cells successfully differentiate and proliferate into pre-adipoblasts, which leads to a production of adipose tissue in vitro. Moreover, these cells are then transplanted into immunosuppressed rats, which subsequently give rise to a complete vascularised adipose and loose connective tissue (D'Andrea et al., 2008; Ferraro et al., 2013). These findings are important in adipose tissue repair due to their origin, their differentiative abilities, proliferation rate, and long lifespan (Casadei et al., 2012). In a broad context, adult stem cells may be used in tissue engineering and regenerative

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medicine as an effective therapeutic mechanism in demolition pathologies.

The stem population, isolated and cultured in vitro, is arranged in different cell types with different stages of differentiation. There is extensive use in laboratories of detection and isolation of stem cells (SC) techniques such as tissue surgical removal under sterile conditions, digestion in collagenase/dispase, detection, and selection by selective and specific markers. For example, SC isolated from adipose tissue express CD34 and CD90 antigens such as mesenchymal stem cells (De Francesco et al., 2009). The SC isolation technique used in most laboratories is based on the use of flow-cytometers with cell sorter or FACS (Fluorescent Activated Cell Sorter). This procedure is effective in providing high purity and successful population separation using multiple parameters of fluorescent intensity, limited cell death and examination of a large number of cells. On the other hand, flow cytometry presents some drawbacks, such as semisterility of samples and the considerable expenses in reagents and equipment maintenance as well as lengthy sample preparation and stem cell sorting processes. New methods and protocols may be proposed to select and isolate stem cells without fluorescent marker staining or use of magnetic beads. Research on new methods may consider the biophysical differences among the cell populations to identify the peculiarities for use in biomedical and clinical laboratories. It may be important to strive for simplicity, to reduce the time needed for sample preparation, to obtain maximum sterility stem cells, to work towards disposability potential with subsequent decrease in costs, maintenance of cell vitality and integrity with real time response, which are fundamental in biomedical, clinical, and surgical contexts. Based on our previous experimental findings, we, hereby report on the main procedures used in the selection of ASCs by flow cytometry, cell culture, freezing/thawing, and differentiation of ASCs. We also underline the importance of new methods/procedures for subsequent applications in biomedical/clinical/surgical fields. Moreover, this review examines the current research possibilities in adipose stem cells and summarizes data from in vitro studies, pre-clinical animal models, and recent clinical trials concerning the use of ASCs in regenerative medicine.

Materials For Adipose Stem Cells Research

Reagents and solutions for cell cultures, immunofluorescence, immunohistochemistry, and electronic microscopy

Chlorexidin gel (Forhans, NY), stored at room temperature until exposure date; Type I Collagenase (GIBCO, Invitrogen, San Giuliano Milanese, Milan, Italy), stored at 4°C until exposure date; Dispase (GIBCO), stored at 4°C until exposure date; Gentamicin solution (Sigma, Milan, Italy), stored at room temperature until exposure date; PBS w/o Ca++, Mg++, (Lonza, Milan, Italy), stored at 4°C until exposure date; 70 μm Falcon strainers (BD Pharmingen, Buccinasco, Milan, Italy), stored at room temperature; DMEM culture medium (Sigma), stored at 4°C until exposure date; alpha-MEM culture medium (Sigma) stored at 4°C until exposure date; high glucose DMEM culture medium (Sigma), stored at 4°C until exposure date; Fetal Bovine Serum, South American Origin, (GIBCO), stored at −20°C until exposure date; Horse Serum (Sigma) stored at −20°C until exposure date; L-Ascorbic acid (Sigma), stored at room temperature until exposure date; L-glutamine (Sigma), stored at −20°C until exposure date; Penicillin and streptomycin, Pen/Strep, (Lonza), stored at −20°C until exposure date; EDTA (Sigma), stored at room temperature until exposure date; Bovine Serum Albumin (BSA) fraction V (Sigma), stored at room temperature until exposure date; Toluidine blue (Sigma), stored at room temperature; Paraformaldehyde (Sigma), stored at room temperature; Trypsin-EDTA solution (Lonza), stored at 4°C until exposure date; Dexamethasone (Sigma), stored at +4°C until exposure date; Recombinant human insulin (Sigma), stored at +4°C until exposure date; Indomethacin (Sigma), stored at room temperature until exposure date; 3-isobutyl-1-methyl-xantina, IBMX, (Sigma), stored at −20°C until exposure date; methylcellulose (Sigma), stored at room temperature until exposure date; mercaptoethanol (Sigma), stored at room temperature until exposure date; beta-glycerophosphate (Sigma), stored at +4°C until exposure date; ITS-Premix (Becton-Dickinson, Buccinasco, Milan, Italy) stored at +4°C until exposure date; sodium pyruvate (GIBCO) stored at +4°C until exposure date; TGF-β1 (Peprotech, Hamburg, Deutschland) stored at +4°C until exposure date; hydrocortisone (Sigma) stored at −20°C until exposure date; Oil red-O (Sigma), stored at room temperature until exposure date; Alizarin Red (Sigma), stored at room temperature until exposure date; Alcian Blue (Sigma), stored at room temperature until exposure date; Calcein-AM (Dojindo Molecular Technologies, Gaithersburg, MD), stored at −20°C until exposure date; Dimethyl sulfoxide, DMSO, (Sigma), stored at room temperature, until exposure date; Threalose (Sigma) stored at room temperature, until exposure date.

Antibodies

CD29 CYCROME (BD Pharmingen, Buccinasco, Milan, Italy); CD31 FITC (PECAM-1) (BD Pharmingen); CD34 PE (Milteniy Biotec, Calderara di Reno, Bologna, Italy); CD44 FITC (BD Pharmingen); CD45 CYCROME (BD Pharmingen); CD54 CYCROME, (BD Pharmingen); CD90 FITC (BD Pharmingen); CD105 FITC (Santa Cruz, CA); CD117 PE (Becton and Dickinson, Buccinasco, Milan, Italy); CD133 PE (Milteniy Biotec, Calderara di Reno, Bologna, Italy); Flk-1 (Santa Cruz); Von-Willebrand (domain 1 and 2) (Santa Cruz); Angiotensin-converting-enzyme, ACE, (Santa Cruz); PPAR-gamma (Abcam, Cambridge, UK); Adiponectin (Abcam), GLUT-4 (Abcam); Osteocalcin (Santa Cruz); Collagen Type II (Santa Cruz); Aggregan (Santa Cruz); MyoD (Abcam).

All antibodies are stored at 4°C in the dark until exposure date as indicated from manufacturer.

Reagents for cell cycle analyses

Iodide propide solution (Sigma), stored at 4°C in the dark until exposure date; Sodium citrate (Sigma), stored at room temperature until exposure; RNAse A (Sigma), stored at −20°C in the dark until exposure date.

Equipments

BD FACSAria II Near UV Upgrade kit for three laser system (Becton Dickinson, Franklin Lakes, NJ); Fluorescence Microscope (Nikon TE 2000-S, Milan, Italy).

The procedures that are preferably and mainly used in the laboratories in order to detect, isolate, proliferate, and differentiate ASCs include the following steps.

Methods For Adipose Stem Cells Research

Adipose tissue extraction and digestion

Adipose tissue are extracted from abdomen, mammary regions, troncanteric regions of healthy adults. Prior to extraction, each subject was checked for systemic infection or diseases. Only disease-free subjects must be selected. Before extraction, the skin is covered with 0.3% chlorexidin gel (Forhans, NY) for 2 min and then remove the adipose tissue. Once removed, the adipose tissue is immersed in a digestive

solution (3 mg/ml I type collagenase (GIBCO) plus 4 mg/ml dispase (GIBCO) and 5 μl/ml gentamicin (40 μg/ml) (Sigma) for 1 h at 37°C. The solution was then filtered with 70 μm Falcon strainers (BD Pharmingen) to remove the tissue pieces not digested (Fig. 1).

Cell culture

After filtration, cells are immersed in DMEM culture medium (Sigma) supplemented with 10% FBS (GIBCO), 100 μM 2P-ascorbic acid (Sigma), 2 mM L-glutamine (Lonza, Milan, Italy), 100 U/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza) and placed in 75 cm two flasks with filtered valves. Flasks were incubated at 37°C and 5% CO_2 and the medium changed twice a week. Just before cells become confluent, they can be subdivided into new flasks. The number of passages must be reduced to the minimum and performed avoiding cell stress and senescence. This is because the interactions between cells are of paramount importance to obtain the deposition of the extracellular matrix. Stem cells are sorted (see below) only when their number reaches at least 1,000,000 per flask. This number is achieved around day 14, when they are still undifferentiated.

Fluorescence activated cell analysis and sorting

Cells are detached using 0.02% EDTA solution in PBS and collected (10 min at 1,000 rpm). Cells are washed in 0.1% BSA in PBS, then incubated in a solution of antibody (1 μg/μl). After incubation, cells are then washed in the same solution (see above) and are ready for observation. Antibodies used for sorting are the following mouse anti-human antibodies: CD90 FITC (BD Pharmingen), CD117 PE (BD Pharmingen), CD34 PE (Milteniy Biotec), flk-1 (Santa Cruz) and CD45 CYCROME (BD Pharmingen). The sorting is performed using both morphological (high side scatter and low forward scatter) and antigenic criteria (first with CD90 and CD34). Only cells that expressed all these markers are selected, in order to obtain a homogeneous population (Fig. 2).

Colony efficiency assays and proliferation potential

To evaluate colony efficiency and proliferation potential of sorted stem cells, single cells obtained by limiting dilutions are plated. Numbers of clones and cells are evaluated. After three weeks of culture, cells are stained with 0.1% (v/v) toluidine blue (Sigma) in 1% paraformaldehyde (Sigma). The number of clones (>50 cells) are counted as described in d'Aquino et al. (2007).

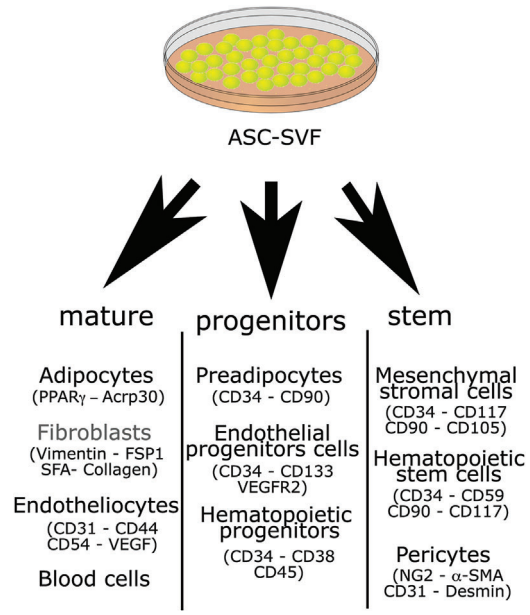

Fig. 2. Adipose stem cells characterization. Cells are detached, washed and incubated in a solution of antibody. Cells are ready for observation by Flow Cytometer. We observed mature cells (adipocytes, fibroblasts, endotheliocytes, and blood cells), progenitor cells (preadipocytes, endothelial progenitor cells, hematopoietic progenitors) and stem cells (mesenchymal stromal cells, hematopoietic stem cells, and pericytes). The sorting is performed using both morphological and antigenic criteria.

Differentiation

Sorted cells can be challenged to assess their multipotency (Fig. 3). For adipocyte differentiation, the culture medium must be supplemented with 10% FBS and 1 μM dexamethasone, 10 μM recombinant human insulin, 200 μM indomethacin and 0,5 mM 3-isobutyl-1-methyl-xantina (IBMX) (all purchased from Sigma) twice a week for two weeks. Oil red-O (Sigma) staining is used to identify lipid-laden fat cells.

For endothelial differentiation, the cells were plated in 24-well plates in a semisolid growth medium that consist of 0.9% methylcellulose in DMEM 30%, FBS 1%, bovine serum albumin (BSA), 10–4 mol/L mercaptoethanol and 2 mmol/L

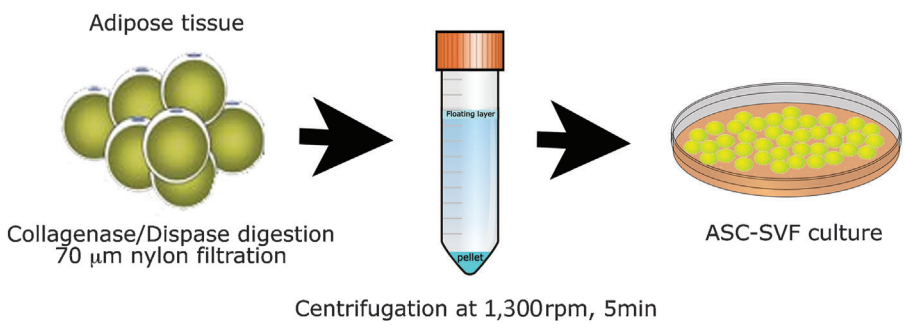

Fig. 1. Adipose stem cells isolation. Adipose tissue are extracted from abdomen, mammary regions, trochanteric regions of healthy adults. The adipose tissue is immersed in a digestive solution for 1 h at 37°C. the solution was filtered and immersed in DMEM culture medium. Flasks were incubated at 37°C and 5% CO_2 and the medium changed twice a week.

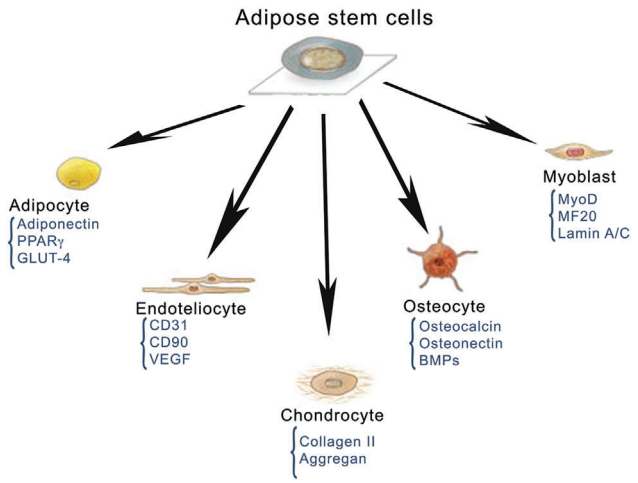

Fig. 3. Adipose stem cells differentiation. Sorted cells can be challenged to assess their multipotency in differentiative medium (adipogenic, endothelial, chondrogenic, osteogenic, and myogenic differentiation).

L-glutammine (all purchased from Sigma), with or without Vascular endothelial growth factor (VEGF, 50 ng/ml).

For osteogenic differentiation, the medium contained alpha-MEM (Sigma), 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen), 100 nM dexamethasone (Sigma), 50 uM magnisum L-ascorbic acid-phosphate (Sigma) and 4 mM beta-glycerophosphate (Sigma). Mineral deposition was detected by staining with Alizarin Red (Sigma).

For chondrogenic differentiation, the protocol provides a standard pellet culture method. The culture medium contained with high glucose DMEM (Sigma), 1% ITS + Premix (Becton--Dickinson), 100 mM sodium pyruvate (Invitrogen), 10 ng/ml TGF-β1 (Peprotech), 100 nM dexamethasone (Sigma), 120 uM magnesium L-ascorbic acid-phosphate (Sigma) and 100 U/ml penicillin and 100 ug/ml streptomycin (Sigma). The pellets were formed in 5 ml polypropylene tubes (Becton-Dickinson) by centrifugation of 200,000 cells at 600 $g \times 5$ min. Alcian blue (Sigma) staining is used to identify chondrocytes.

For myogenic differentiation, tiny longitudinal multinucleated cells were observed when the cells are grow in DMEM (Sigma), 10% FBS, 5% Horse Serum (Sigma), 50 uM hydrocortisone (Sigma), 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen).

Cell cycle analysis

Cell cycle is analyzed by flow cytometry. Cells are harvested in phosphate-buffered saline (PBS) containing 2 mM EDTA (Sigma), They are washed once with PBS and stained with 50 ug/ml iodide propide (Sigma) in sodium citrate (Sigma) buffer 0,1% plus 1 mg/ml RNAse A (Sigma) for 2 h at room temperature in the dark. Stained nuclei are analyzed with a fluorescence-activated cell sorter (FACS) ARIAII (Becton-Dickinson) and the data analyzed using a Mod-Fit cell cycle analysis program version 3.1 (Becton-Dickinson). Analyses are performed in four's for each sample and time from day 15 up to the 45th day of culture as described in d'Aquino et al. (2007).

Immunohistochemistry and Immunofluorescence analyses

For adipogenic, endothelial, osteogenic, chondrogenic, and myogenic differentiation staining, cells in P6 well plates were washed in PBS and fixed with 4% PFA for 30 min at 4°C, then washed three times in PBS for 10 min and incubated in PBS/5% FBS for 60 min at 4°C. After a double washing in PBS for 10 min at room temperature, cells were incubated overnight at 4°C with monoclonal anti-human antibodies (diluted 1:100 in PBS). Wells were washed in PBS three times for 10 min at room temperature and incubated for 90 min at 4°C with the secondary FITC- or PE-conjugated antibody (diluted 1:200 in PBS 1×) (Santa Cruz). Moreover, cells were stained with DAPI (49, 6-diamidino-2-phenylindole) (Invitrogen) diluted 1:10,000 (5 mg/ml) in PBS for 7 min at room temperature. Cells incubated for 90 min at 4°C only with conjugated secondary antibodies were used as negative control. Cells were then observed under a fluorescence microscope (Nikon Instruments Italia, Calenzano, Firenze, Italy). Primary antibodies for adipogenic differentiation staining included: anti-PPARγ, anti-adiponectin, and anti-GLUT4 (all puchased from Abcam); for endothelial differentiation staining: anti-CD31, anti-CD90, anti-VEGF (all purchased from Santa Cruz); for osteogenic differentiation staining: anti-osteocalcin (Santa Cruz); for chondrogenic differentiation staining: anti-Collagen Type II and anti-Aggregan (all purchased from Santa Cruz); for myogenic differentiation staining: anti-MyoD (Abcam). Immunohistochemical analyses were performed with a DAKO CYTOMATION kit (En Vision + System-HRP-AEC, Dako Italia, Milan, Italy) according to the manufacturer's protocol.

For Calcein staining, a solution of 50 μM calcein-AM in PBS (Dojindo Molecular Technologies), is added to culture medium (1/10 v/v) for 30 min, at 37°C with 5% CO_2. After incubation, cells are washed twice with PBS. The observation is performed using a fluorescence microscope (Nikon TE 2000-S) with 490 nm excitation and 512 nm emission filters.

Cryopreservation

If a long-term storage is needed during the study, it is possible to safely cryopreserve adipose tissue stem cells using the following method. Cells (stocks of 500,000 for stem cells), are frozen in 4% dimethyl sulfoxide (DMSO) (Sigma), 6% threalose (Sigma) in DMEM at 90% FBS in a cryotube (Lonza). They are immersed in liquid nitrogen. Storage can be prolonged for up to 1–2 years. At the end of the freezing period, cells can be quickly thawed by the addition of 1 ml of medium at 10% FBS at 37°C and then added to 10 ml of the same medium. Cells are pelletted at 1,500 rpm for 10 min. The supernatant is removed. Fresh medium is added to the tube. Cells are then placed in flasks and cultured at 37°C in a 5% CO_2 atmosphere. They are vital and the amount of apoptotic cells is less than 10%.

New Procedures

There are standard techniques for cell harvest, culture, expansion, and enrichment of stem cells. Many laboratories use the same techniques based on surgical removal of tissue under sterile conditions, transported to a laboratory in a physiological solution, digestion in collagenase/dispase, centrifugation and, then, use of selective markers using fluorescence activated cell sorters (FACS) or magnetic beads. The traditional techniques of selection and enrichment of adipose stem cells are based essentially on the use of flow-cytometry with cell sorter (Tucker and Bunnell, 2011). Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single cells, which flow in a fluid stream through a beam of light (Wang et al., 2008). The characteristics analyzed include a cell's relative size, granularity or internal complexity, and relative fluorescence intensity. These new developments in detection and isolation of cells using specific marker expression are mainly due to flow cytometers with sorting instrumentation. Cell sorting has been

crucial in the isolation of stem cells, starting from heterogeneous cell populations. "There are a number of methods reported in literature for isolating the adipose cells from the lipoaspirate (Gronthos et al., 2001; Zuk et al., 2001; Aust et al., 2004; Boquest et al., 2006; Locke et al., 2009)." The lipoaspirate is thoroughly washed and collagenase is used to digest floating adipose tissue to break down the extracellular matrix that proceeds to filtration and centrifugation. Following centrifugation, the end product pellet is referred to as SVF, which has a heterogeneous cell population with circulating blood cells, fibroblasts, perycites, endothelial cells as well as "preadipocytes" or adipocyte progenitors. The technique of liposuction has greatly simplified the mincing of human tissue, which was previously done manually. The procedures generate finely minced tissue fragments, without altering the viability of isolated SVF cells (Moore et al., 1995; Lalikos et al., 1997; Oedayrajsingh-Varma et al., 2006; Bunnell et al., 2008). "The recovery of ASCs can be improved further by manipulating the centrifugation speed (Yoshimura et al., 2006; Bunnell et al., 2008; Ferraro et al., 2011)." To facilitate this process, several groups have fabricated devices to automate the cell isolation (Hicok and Hedrick, 2011; Guven et al., 2012). However, the available "ready to use" prototypes have not always produced satisfactory clinical and scientific results. Nevertheless, some investigators have devised a new clinical process of tissue regeneration, using a mechanical disaggregation. This innovative approach allows "mechanical disaggregation of the tissue without manipulation of the matrix (Brunelli et al., 2013)." The mechanical isolating procedure is "easier, safer, cheaper, and faster than traditional currently performed enzymatic procedures (Raposio et al., 2014)." Some case reports were conducted to report and analyze the clinical outcomes of autologous micro-graft obtained with a new protocol so called "Rigenera" (Brunelli et al., 2013; Graziano et al., 2013). This system encourages stem cells to be generated during surgical procedures. The subcutaneous adipose tissue provides a clear advantage due to the ease with which it can be accessed as well as to the ease of isolating the residing stem cells. However ASCs localize in the stromal-vascular portion can be isolated using enzymatic digestion. Carelli et al. (2014) report that micro fragmented human lipoaspirated adipose tissue results as a better stem cell source. The ASCs derived from micro fragmented lipoaspirate tissue have been characterized and are multi-potent with a good differentiation potential. Thus, "Rigenera" protocol, which is primarily utilized for other types of connective tissues such as dental pulp, could be used for the adipose tissue. Actually, this system was not specifically designed for management of adipose cell application but it may be fundamental in therapeutic fat grafting and be proposed as an alternative to current approaches.

"Identification of the ASC surface immunophenotype has provided a mechanism to enrich or purify the stem cell population directly from the heterogeneous SVF cells (Hutley et al., 2001; Miranville et al., 2004; Sengenes et al., 2005; Gimble et al., 2007, 2011)." Flow cytometry and immunomagnetic beads have been used by researchers to make positive and negative selections within the SVF of cell subpopulation. For example, purification by magnetic bead coupling has been performed by some researchers (De Francesco et al., 2009) to remove $CD45^+$ and $CD31^+$ cells prior to differentiation experiments. Likewise, positive selection has been similarly performed using CD34 and other antigens.

Current Pre-Clinical and Clinical Findings

Numerous studies show the multipotency of ASCs. ASCs are of mesenchymal origin, potential lineages include the adipogenic (Wosnitza et al., 2007), osteogenic (Arrigoni et al., 2009), chondrogenic (Roux et al., 2013), endothelial (Wosnitza et al., 2007; De Francesco et al., 2009) and myogenic lineages (Stern-Straeter et al., 2014) (Fig. 3). However, ASCs have also been shown to possess the potential to differentiate into non-mesenchymal lineages including myocardial cells (Bai and Alt, 2010), neuron-like cells (Zavan et al., 2010) and epithelial lineage (including hepatocytes and pancreatic cells) (Baer, 2011). Although an in vitro culture system is useful to investigate ASCs differentiation, we may consider in vivo investigations to study the potential of adipose stem for cell tissue engineering. Therefore, experiments on animal models have been used to regenerate and repair tissue with or without appropriate scaffolds. The materials currently used in ASCs tissue regeneration include collagen (Ferraro et al., 2013), hyaluronic acid (Pandis et al., 2009), polylactic-co-glycolic acid (Kang et al., 2008), and other "smart" materials (Bressan et al., 2013). ASCs have been used in preclinical models to treat various diseases effecting a range of tissues and organs as summarized in Table 1. Most preclinical studies using ASCs have been carried out on rodents considering them as ideal models for their size, cost, availability, and access. Approval of clinical trials has been applied in many countries (Table 2) following the accumulated data. Current clinical trials are

TABLE 1. Preclinical animal studies using ASCs

Tissue	Defect	Animal model	References
Adipose/soft tissue	Burn	Rabbit	Piccinno et al., 2013
	Radiation trauma	Rat	Huang et al., 2013
	Skeletal muscle trauma	Mice	Lee et al., 2014
Bone	Craniofacial defect	Canine	Liu et al., 2013
Liver	Acute failure	Rat	Koellensperger et al., 2013
Inflammation	Endotoxemia	Rat	Shin et al., 2013
Skin	Wounds	Murine	Huang et al., 2012
Vascular	Hind limb ischemia	Murine	Harada et al., 2013

TABLE 2. Clinical trials studies using ASCs

Indication	Study type	Number of patients/follow-up	References
Soft tissue			
Breast reconstruction	Comparative	10/12 months	Gentile et al., 2012
Irradiation	Case report	20/31 months	Rigotti et al., 2007
Orthopedic			
Craniofacial	Case report	2/12 months	Mesimaki et al., 2009
Osteoarthritis	Phase I and II	18/6 months	Jo et al., 2014
Immune			
Pulmonary fibrosis	Phase Ib	14/12 months	Tzouvelekis et al., 2013
Crohn's disease	Phase II	43/12 months	Yong et al., 2013
Cardiovascular			
Myocardial ischemia	Study design		Qayyum et al., 2012
Hind limb ischemia	Phase I	7/2 weeks	Bura et al., 2014

enrolling a number of patients for regeneration of soft tissue, craniofacial tissue, cardiovascular tissue, and immune disease (Tobita et al., 2011). There is evidence that ASCs have a potential clinical impact on many acute and chronic diseases.

Conclusion

The aim of this paper is to provide primary assistance regarding the basic characteristics of adipose tissue stem cells. We, hereby, propose to simplify the advancement of international standards contingent on reproducible parameters, guaranteeing timely delivery of safe and effective ASC-based tools to the medical community. In conclusion: (1) adipose tissue is a remarkable site of stem cells; (2) collecting stem cells from adipose tissue is a non-invasive procedure that can be performed in adulthood and childhood after common plastic surgery; (3) there is less probability of sacrificing tissue when collecting adipose tissue stem cells; (4) the multipotency of the adipose tissue stem cells enables development of numerous cytotypes; (5) transplantation of new-formed adipose and connective tissues resulting from adipose tissue stem cells will promote vascularized adult tissue and permit the graft to be integrated with the surrounding host blood supply; (6) adipose tissue stem cells can be cryopreserved and stored for long periods; (7) adipose tissue stem cell is ideal for tissue engineering and for clinical use in several pathologies in which essential substance for growth and repair is lacking; (8) new improved methods are necessary for their isolation, expansion, and use in therapy. We may consider the importance of testing the secretion of a defined factor to determine a specific set of genes or microRNA. Moreover, we need to refine and standardize assays and methods to estimate quantities and reproducibility of adipose tissue stem cells.

Concluding Remarks

Not withstanding the enormous progress in stem cell research to date, their implementation, and success rate in clinical trials is yet to be ascertained. There has been a lot of convincing research done on the basic biology of stem cells, but there is still much to learn regarding events in clinical settings. In the last decade, significant expertise has been acquired in isolation, in the study of morphological features, molecular biology, and the differentiation potential of stem cells in vitro. Moreover, abundant studies have been performed on animal models for the assessment of stem cells. Few clinical trials have been conducted to date in humans with a three-year follow-up (Mesimaki et al., 2009; Qayyum et al., 2012; Tzouvelekis et al., 2013; Yong et al., 2013; Bura et al., 2014; Jo et al., 2014). Despite the optimal results reported, no other human studies have been conducted yet. The situation for other stem cell types, excluding hematopoietic stem cells for leukemia treatment, is similar. In order to fully develop the clinical application potential of research done in this field, both researchers and clinicians need to find new strategies and to work towards an improvement in GMP procedures. The GMP procedures that must be followed by researchers/clinicians for the engraftment of autologous cells, are not necessarily indicative of research on humans. These procedures are considered sufficiently arduous, lengthy, and expensive to discourage researchers and clinicians from engaging in clinical trials.

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