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Adipose-derived stem cell therapies for bone regeneration

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ABSTRACT

Introduction: Cell-based therapies exploit the heterogeneous and self-sufficient biological environment of stem cells to restore, maintain and improve tissue functions. Adipose-derived stem cells (ASCs) are, to this aim, promising cell types thanks to advantageous isolation procedures, growth kinetics, plasticity and trophic properties. Specifically, bone regeneration represents a suitable, though often challenging, target setting to test and apply ASC-based therapeutic strategies.

Areas covered: ASCs are extremely plastic and secrete bioactive peptides that mediate paracrine functions, mediating their trophic actions *in vivo*. Numerous preclinical studies demonstrated that ASCs improve bone healing. Clinical trials are ongoing to validate the clinical feasibility of these approaches. This review is intended to define the state-of-the-art on ASCs, encompassing the biological features that make them suitable for bone regenerative strategies, and to provide an update on existing preclinical and clinical applications.

Expert opinion: ASCs offer numerous advantages over other stem cells in terms of feasibility of clinical translation. Data obtained from *in vivo* experimentation are encouraging, and clinical trials are ongoing.

More robust validations are thus expected to be achieved during the next few years, and will likely pave the way to optimized patient-tailored treatments for bone regeneration.

KEYWORDS: Adipose tissue, Adipose-derived stem cells, ASCs, regenerative medicine, bone regeneration.

1. Introduction

Therapeutic strategies aimed at regenerating bone have undergone a significant boost during the last two decades, providing a paradigm shift in reconstructive surgery, which significantly improved clinical outcomes. Bone regeneration is needed in skeletal reconstruction of large bone defects resulting from trauma, infections, tumor resection and skeletal abnormalities, or whenever the regenerative process is compromised [1].

The best effective, clinically available, therapeutic options for skeletal reconstruction are currently restricted to autologous and allogeneic bone grafts, along with synthetic bone substitutes[2-6]. The main disadvantage of bone autografts resides in the morbidity of the donor site, where a skeletal defect is created, especially in the presence of overall poor clinical conditions, along with the limited source availability [6]. Furthermore, the complexity of autograft procedures raises other technical issues in selected skeletal sites [7].The use of allogenic bone is inherently associated with morbidities deriving from residual immune-related and infectious burden, along with reduced cost-effectiveness. Finally, synthetic bone substitutes often lack sufficient osteoinductive and osteogenic properties, while providing not always optimal osteoconduction, resorption times and biomechanical assets, especially for the treatment of large skeletal defects [8].

Indeed, the limited success of auto- and allografts in some clinical situations has stimulated the scientific research to investigate new therapeutic tools, possibly tailored to adapt to specific indications and patients' needs. On this regard, somatic stem cell-based approaches are widely considered the best effective, as they enable sustaining the physiologic osteogenic process *in vivo* and may provide effective osteoinductive stimuli [9].

Bone marrow stromal cells (BMSCs) have been widely exploited in this context, as they represent the physiological precursors for the osteoblastic cell lineage [10-12]. Nonetheless, the limited amount and stem cell yield, along with the invasive harvesting procedure, hamper a wide exploitation of bone marrow as a clinically available cell therapeutics source and have prompted the identification and characterization of additional MSC niches, located within alternative tissue sources.

To date, MSC-like multipotent stem cells have been isolated from a multitude of adult tissues, including muscle, adipose tissue, connective tissue, trabecular bone and periosteum [12], skull sutures [13], synovial fluid [14], along with perinatal tissues [15; 16]. More recently, the advent of induced pluripotent stem cells (iPSCs), obtained through genetic engineering of somatic cells, and possessing high proliferation and differentiation capabilities, has offered additional promising alternative sources for bone regeneration [17; 18]. In many cases, iPSCs have been demonstrated to exert comparable osteogenic capabilities to those displayed by MSCs [19]. Nonetheless, the possibility of reprogramming the genetic background of host

somatic cells used for iPSCs' production, may offer to unique chance to address the challenge of treating congenital skeletal disorders due to germline mutations [20;21].

In particular, tissue sources that may be collected as "waste" tissues resulting from either surgical interventions (e.g. adipose tissue from lipoaspiration or abdominoplasty),or delivery (i.e. amniotic fluid, term umbilical cord and placenta) offer a significant translational advantages, as they would allow overcoming a number of concerns related with local morbidity, safety, and ethical issues.

In particular, the placenta and related perinatal tissues represent a high-yield reservoir of mesenchymal-like multipotent stem cells, endowed with increased stemness potential, and displaying extended plasticity towards multiple lineages [16]. The osteogenic properties of these cells have been demonstrated *in vitro* [22-23]. Also, a number of preclinical studies have supported the application of these cell-based therapies for the regeneration of musculoskeletal tissues [24]. Moreover, the reproducible ability of these cells to engraft at the site of inflammation and injury, and to modulate the immune/inflammatory response in host tissues, have prompted their potential application in degenerative and immune-based conditions that may affect the musculoskeletal system [25-27].

Among the adult postnatal tissue sources of MSCs, the adipose tissue (AT), given its ubiquity, the ease of retrieval, and the minimally invasive procedure required for harvesting, may be reasonably regarded as an attractive source of multipotent somatic stem cells, namely, adipose-derived stem cells (ASCs) [28]. ASCs reside in the stromal vascular fraction (SVF) of AT, from which they are easily isolated through enzymatic digestion and plastic adherence. They display BMSC-like features, including immunophenotype, trilineage potential and gene expression profile [29-31]. *In vitro* and *in vivo* models suggest that the transplantation of expanded ASCs improves bone healing through direct differentiation into mature osteoblasts and paracrine effects that facilitate migration and differentiation of resident precursors. Indeed, ASCs demonstrated relevant trophic properties that suggest the suitability for cell therapy applications: angiogenicity [30; 32-34], osteogenicity [30; 35], immunomodulation [36], and promotion of tissue remodeling [34; 37-39].

This review is, indeed, intended to focus on AT as a valuable reservoir of somatic stem cells, specifically considering the state-of-the-art on the osteogenic potential of ASCs. To this aim, we will rely on a careful and up-to-date revision of the extant scientific literature, reporting the characterization of ASCs' biological properties along with the functional validation of their capability to induce bone regeneration and healing in preclinical studies. Finally, we will report on the clinical trials that have exploited these cells for human bone regenerative applications.

2. AT as a source of somatic stem cells: adipose-derived stem cells (ASCs)

For a long time AT has been considered exclusively as an energy reservoir, hence usually discarded with surgical waste after liposuction. During the last three decades, numerous research efforts have been put forth towards recognizing AT as an endocrine organ, which controls metabolism, immunity and satiety. Thereafter, a significant breakthrough was made in 2001, when AT was originally described as an attractive new source of adult stem cells (namely, adipose-derived stem cells, ASCs) [28].

AT is a highly complex tissue comprising mature adipocytes (>90%) and a stromal vascular fraction (SVF), which includes preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, lymphocytes, and ASCs [40-42]. The density of the AT stem cell reservoir varies as a function of type of age, histotype (white or brown AT) and anatomical location (subcutaneous or visceral adipose tissue) [43-49].

Within the white fat, subcutaneous depots house a higher number of ASCs compared with visceral fat. The highest concentrations typically found in the arm region and the greatest plasticity described in cells isolated from inguinal AT [50]. Also, our research group have recently characterized the differential biological properties between the two AT layers separated by the superficial fascia in selected regions of the body, and found a higher cell viability and stemness properties in the superficial compared with the deep hypoderm [51]. Noticeably, adherent cells isolated superficial hypodermal AT showed a higher plasticity, including osteogenic potential, *in vitro* [51]. An independent study had previously reported a gender-related difference affecting the osteogenic differentiation rates in ASCs, derived from superficial-versus-deep subcutaneous AT [52].

Also, Lee and colleagues had characterized the ASCs isolated from different abdominal fat depots on the basis of the regional distribution. This study highlighted that ASCs isolated from superficial subcutaneous depots have a higher grow rate and angiogenic capability, confirming this AT layer as the most appropriate source for therapeutic fat grafting [53].

Human ASCs are usually isolated from subcutaneous AT collected through liposuction or during reconstructive surgery, through resection of tissue fragments. Standard isolation procedures imply the fractionation of AT and separation of the SVF through centrifugation, and further collagenase disruption [54]. One of the main challenges for an adequate translation of AT- and SVF-based therapeutics to the clinical setting, is the generation of a clinical grade protocol of isolation, based on minimal tissue manipulation. On this regard, a wide variety of medical devices enabling the automatic processing of AT for SVF separation and ASC isolation, are being rapidly introduced in the marketplace, sometimes without prior adequate preclinical testing and validation [55-58]

Upon SVF isolation and homogenization, ASCs are selected *in vitro* based on their plastic adherence properties, and display the typical spindle-shaped fibroblastoid morphology. They can be extensively

subcultivated in monolayer culture, and rapidly expanded, with a basal growth medium containing 10% of fetal bovine serum [31; 59; 60].

ASCs meet most of the minimal criteria set by the International Society for Cellular Therapy (ISCT) to define human mesenchymal stem cells (MSCs) [61]: plastic-adherence, *in vitro* trilineage (osteogenic, chondrogenic, and adipogenic) potential, expression of the MSC-specific antigens CD73, CD90, and CD105, and lack of hematopoietic lineage markers [61].

Nonetheless, their correct immunophenotype characterization has been long debated. Based on the hematopoietic marker CD45, the endothelial marker CD31, the perivascular marker CD146, and the stromal markers CD34, CD90, CD105 and CD117 (c-kit), four distinct populations have been defined in the SVF fraction (in uncultured conditions): putative ASCs (CD31-, CD34+/-, CD45-, CD90+, CD105-, CD117- and CD146-), endothelial-progenitor cells (CD31+, CD34+, CD45-, CD90+, CD105-, CD117+ and CD146+), vascular smooth muscle cells or pericytes (CD31-, CD34+/-, CD45-, CD90+, CD105-, CD117+ and CD146+), and hematopoietic cells (CD45+) [54; 62]. Studies on whole AT have revealed that within the stem/progenitor components, organized around small vessels, stromal multipotent cells with CD34+, CD31-, CD104-, SMA-,immunophenotype are prevalent in the supra-adventitial layer [62-64]. Pericytes and other cells defined bythe differential expression of CD34, CD31, and CD146 were sorted from the SVF of human white AT. Besides pericytes, CD34+ CD31- CD146- CD45- cells, which reside in the outmost layer of blood vessels (i.e. *tunica adventitia)*, natively express MSC markers and give rise to clonogenic multipotent progenitors, in culture, identical to BMSCs [46; 65]. Finally, studies from Cinti's group have identified a small subset of capillary endothelial cells that are plausibly capable to give rise to adipose lineage cells too [65; 66].

The average frequency of ASCs in processed lipoaspirate is 2% of nucleated cells, and the yield is approximately 5,000 fibroblast colony-forming units (CFU-F) per gram of AT. In the bone marrow, the yield of BMSCs is approximately 100–1,000 CFU-F per milliliter [67], suggesting that AT could be a best efficient source of multipotent stromal stem cells.

Recently, the fluid portion separated by centrifugation of liposucted AT (i.e. lipoaspirate fluid, LAF) contains an ASC-like population (LAF cells) suspended in blood/saline fluid, along with tissue fractions and cell secretome (40; 68). LAF cells display the same biological features as ASCs, hence could be reasonably exploited for regenerative applications (40; 55; 69).

3. Osteoinductive properties of ASCs

The secretome of ASCs contains different pro-angiogenic and endocrine factors (adipokines) with bone inducing activity [70]. The expression of these factors can be modulated by different culture conditions, such as proliferation, differentiation and hypoxia. In particular, low oxygen levels in culture inhibit the

expression of ECM remodeling proteins, such as osteonectin, collagen type 1, collagen type 2, fibronectin 1 and TGF-β1-induced protein, while 3D culture activates the expression of several genes involved in ECM structure and related functions i.e. HGF, VEGF, KGF, b-FGF, MMP-2, and MMP-14 [44]. Interestingly, Kalinina and colleagues highlighted a specific immunophenotype (CD90+/CD73+/CD105+/CD45-/CD31-/PDGFRβ+/NG2+/CD146+(-) that induces ASCs secretion of ECM proteins (i.e. laminins, fibronectin 1, osteoblast specific factors, osteonectin, periostin, collagens and collagens interacting proteins) [71]. The effects of specific molecules both on the secretome composition and on its pro-osteogenic activity have been largely investigated [72-76]. In particular, the effect of TGF-β1 on ASCs' secretome was assessed by Rodriguez and colleagues. The Authors concluded that TGF-β1 exposure modulates the expression of several molecules in ASCs, including HGF, leptin, FGF-7 and OPN, involved in bone resorption [77]. Similarly, Overman and colleagues analyzed how osteoinductive treatments, scaffold interaction, and the cell differentiation status, affect the ASCs secretome. This study revealed that BMP2-induced osteogenic differentiation causes the increase of cytokines, such as IL-6, growth factors, such as FGF7, and adhesion molecules, such as VCAM1, in the ASCs' secretome [78]. Moreover, the vascular endothelial growth factor (VEGF), present in the secretome of both whole fresh SVF and isolated ASCs, plays a major role in the repair of fractures or bone defects. VEGF is able to activate the formation of a new network of blood capillaries, which is required during physiological bone formation and healing. In addition, VEGF plays a direct role in the recruitment of hematopoietic stem cells, involved in the formation of new bone [54].

Reasonably, the ASC secretome contribute to the composition of the acellular portion of the lipoaspirate fluid (LAF). We have previously demonstrated that this fluid is able to exert angiogenic and osteoinductive properties *in vitro* [55]. We have further investigated the proteome-peptidome composition of LAF through a top down/bottom up approach [68]. This study allowed identifying numerous bioactive proteins, peptides and paracrine factors, such as albumin and hemoglobin fragments (i.e. VV- and LVV-hemorphin-7), ubiquitin and acyl-CoA binding protein, adipogenesis regulatory factor, and perilipin-1 fragments. In addition, several molecules directly or indirectly involved in osteogenic process have been reported. In particular, the thymosin beta 4 (T.4) and beta 10 (T.10) peptides, along with their C-terminal-truncated forms, have been identified. These molecules promote angiogenesis, wound healing and tissue repair, in addition to anosteo- inductive activity. The LAF also featuredS100A6, a member of the S100 Ca^{2+}-binding protein family. S100A6 induces bone formation modulating the capability of cells to sense extracellular cations [68].The documented presence ASC-like cells and bioactive molecules in the LAF, along with its rapid and easy isolation, make this fluid attractive and suitable for regenerative medicine applications, specifically as a "minimally manipulated tissue" to be tested *in vivo*for a potentially wide range of applications.

4. Age-related changes in ASCs' biology and regenerative properties

An aging population is inevitably going to demand more in terms of regenerative strategies, including those based on fat transfer and grafting. Nonetheless, adipose tissue is not spared by the degenerative processes occurring in elderly. Aging is indeed accompanied by a loss of adipocytes' energy-expending capacity, which may contribute to the development of obesity. In this condition the accumulation of senescent cells, including perivascular stem cells and endothelial cells, along with an increase in circulating pro-inflammatory cytokines, including TNFα and IL-6, is described [79]. Aging of the adipose tissue niche leads to proliferative defects due to changes in external signals originating in the microenvironment [80]. Several studies demonstrated the effect of age on ASCs' viability and function, in both humans and animal models [79]. In particular, Rogers and colleagues demonstrated that age-related quantitative and functional loss of subcutaneous AT is associated with a selective decline in brown thermogenic adipocytes in mice [81].

Zhu and collaborators demonstrated that ASCs isolated from human liposucted subcutaneous AT display a reduced plasticity (in terms of osteogenic potential), in older compared with younger female donors, regardless of the cellular yield [82]. The gene expression profiles of human senescent subcutaneous AT specimens, also pointed to a significant a decreased ASCs' yield, growth kinetics and differentiation capacities in older donors [83; 84]. In addition, ASCs from older donors display increased oxidative stress markers, coupled with a reduced detoxification capability [85], possibly explaining their impaired proliferation and plasticity [86].

Ye and collaborators compared ASCs isolated from orbital AT of old-versus-young donors, and found fewer progenitor cells, reduced proliferative rates, increased senescent features and decreased trilineage potential, despite no significant differences in overall cellular yield and immunophenotype [87]. Finally, a significant decrease in ASCs' yield and angiogenic capacity, has been demonstrated also in visceral fat depots of elder individuals [88]. Taken together, these data strongly highlight the dramatic effect of aging on ASCs' properties that must be taken into account in the design and development of autologous AT-based regenerative treatments in the elderly.

5. OSTEOGENIC PROPERTIES: Preclinical studies

In vitro assays aimed at demonstrating ASCs' multipotency have been widely utilized as part of the standard characterization protocols. These involve the induction with culture medium supplemented appropriate differentiation *stimuli*, followed by lineage-specific stainings and gene expression profiling [66]. Nonetheless, in order to achieve robust and sound scientific evidence of the functionally effective cell plasticity, *in vivo* transplantation assays are mandatory [89].

A plethora of studies have been conducted attempting to obtain valid *in vivo* data demonstrating the osteogenic potential of ASCs and to adequately translate *in vitro* findings to a clinical level [90]. Several animal models have been designed, employing either allogenic or xenogenic cells transplantation. When employing human ASCs, nude or athymic animals embody a reliable model for studying osteogenic

processes, as injured bone repair requires the participation of both the immune and hematopoietic niches. Although nude animals demonstrate a blunted inflammatory response, they can still mount an inflammatory B-cells and NK-cells response and possess the surrounding osteogenic precursor cells from the periosteum [90; 91].

The *in vivo* osteogenic potential of experimental design can be evaluated in an elementary model, relying on local intramuscular injection inducing ectopic bone formation [30; 92-102]. Calvarial defects offer the benefit of studying bone healing in animal models, allowing an easy quantification of the amount of newly formed bone within a bidimensional defect [92; 103-135]. Several models, mostly rodent, have been described to assess calvarial defects and it has been reported that a 4mm mouse parietal bone defect is sufficient to offer a reliable and easily repeatable prototype [91]. Long bone skeletal defect models have been widely employed, as they are able to mimic the clinical condition of bone fracture or injury under load bearing stress [117; 136-153]. In particular, the femur offers special benefits, due to its larger shaft, tolerating a wider defect and allowing the placement of external fixator devices or distracters [136-142; 151; 152].

The study of ASCs for bone regeneration has largely involved the insertion of biomaterials in rat and nude mouse models. Furthermore, to demonstrate the application and optimization of ASC therapies, these defect models have been also experimented in other different species, achieving successful results. To this aim, either undifferentiated ASC (i.e. in the absence of any prior *ex vivo* osteogenic induction) or uncultured SVF [92; 100; 111] have been exploited, paving the way to an easier translation of preclinical evidence to the clinical setting.

Taken together, the numerous studies published so far on this topic, have reported a huge amount of data demonstrating the efficacy of ASC-based approaches for inducing bone regeneration/healing *in vivo*. Nonetheless, based on the heterogeneity of experimental designs, a direct comparison and systematic account of all studies would be ineffective. Instead, Table 1 reports a tabular view of the most relevant studies designed on this topic, categorizing publications according to the experimental model and species employed, the use of scaffolds or additional treatments, and the origin of graft [22; 23; 30; 92-159].

Growth factors, which are naturally expressed within the healthy bone matrix or during fracture healing, have been explored as promoter of the direct development of the structures of osteogenic tissue and the differentiation of bone cells [160]. The osteoinductive potential of recombinant BMPs has been broadly demonstrated in animal models and clinical studies [90-92; 161]. Since the Food and Drug Administration (FDA) approved the use of recombinant human BMP-2 for spine fusion and granted a device exemption for the use of BMP-7 to treat recalcitrant nonunions, the interest in BMPs increased rapidly as long as the number of published studies [142]. However, the results of both animal and clinical studies have been

somewhat disappointing and recent evidence has suggested that BMPs, even in combination with ASCs, should not be considered the best viable strategy for inducing bone healing [142]. Furthermore, although high doses of recombinant BMPs induce bone formation, recombinant proteins are expensive, and there are concerns about potential oncogenic effects, considering their pleiotropic functions.

New transcription factors involved in the osteogenic process have been, also, reported, including runt-related transcription factor 2 (RUNX2), vascular endothelial growth factor (VEGF), LIM mineralization protein (LMP), Sonic Hedgehog (SHH) and Nell-1. Several studies, reviewed by Romagnoli and colleagues [2], demonstrated that the over-expression of these genes significantly increases the osteogenic potential of ASCs.

Finally, the limited success of auto- and allo-bone grafts in some clinical situations has stimulated the investigation of a wide variety of biomaterials to design osteoconductive scaffolds for clinical applications. Well characterized biomaterials, such as hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), biphasic calcium phosphate (BCP), implemented or not with bioactive glasses, have been widely explored in preclinical studies and are currently used in different clinical applications [162; 163]. Taken together, these results demonstrated the potential of bioactive scaffold in bone remodeling, providing an additional effective strategy for treating bone defects. Indeed, in the last years, several *in vitro* and *in vivo* studies highlighted the osteo-inductive role of biomimetic scaffolds on ASCs, showing how the use of the biopolimers as substrate to growth could embody a useful trigger for the differentiation of the ASCs toward the osteoblastic phenotype[54; 164-170].

6. Clinical use of ASCs for bone regeneration/reconstruction

Despite the numerous successful preclinical applications of ASCs-based therapy for bone regeneration, few clinical trials have been reported, and completed to date. The official international clinical trial database (https://clinicaltrials.gov/, keywords: adipose derived stem cells AND bone) counts 19 studies, 7 of which focusing on ASCs and bone regeneration/reconstruction, excluding those with “unknown status”. These include two completed trials and one terminated. Four studies investigated the use of ASCs in bone or composite graft applied to different bone defect models. Two trials were designed to determine whether ASCs injection was effective in severe osteoarthritis. One trial aimed on studying ASCs effect on avascular necrosis of femoral head. However, no result or relevant data have been reported, to date.

Lendeckel and colleagues described the use of autologous ASCs combined with bone graft and fibrin glue to treat a large pediatric post-traumatic calvarial defect in a case report [171]. Three-month follow-up CT scan showed almost complete calvarial healing with a stable osteo-integrated graft. Mesimäki and colleagues

described a novel method to reconstruct a major maxillary defect in an adult patient using autologous ASCs combined with recombinant human BMP-2 and β-TCP granules. The patient's healing was clinically uneventful, obtaining new, mature, vital and vascularized bone eight months after surgery, with good osteointegration and stability [172]. Thesleff and colleagues employed ASCs for calvarial reconstruction, testing alternative biomaterials (βTCP and resorbable mesh bilaminate scaffold) and obtaining successful results in adult patients [173]. Sandor and colleagues reported the successful reconstruction of large anterior mandibular bone defects using ASC seeded on a βTCP pre-molded scaffold, custom based on patient's' CT scans [174]. The same Authors reviewed a 13 cases series of cranio-maxillofacial hard-tissue defects reconstructed with either bioactive glass or βTCP scaffolds seeded with ASCs, reporting successful integration and bone regeneration in 10 cases [175]. Pak and colleagues described the complete resolution of avascular necrosis of the femoral head treated with ASCs and PRP injection [176]. In a clinical trial, Castillo-Cardier and colleagues investigated autologous ASCs application in mandibular angle fractures as an alternative to conventional reduction treatment, evaluating healing time and ossification rate; at 12 weeks follow-up CT scan revealed higher percentage of ossification in the experimental group [177]. Dufrane and colleagues proved the feasibility of a scaffold-free three-dimensional ASCs graft, designed for facing reconstruction of long bone defect in the context of congenital pseudarthrosis or tumor resection [178]. Prins and colleagues evaluated the potential effect of freshly isolated SVF seeded on either βTCP or biphasic calcium phosphate carriers in patients undergoing maxillary sinus floor elevation, using a one-step surgical procedure, proving the feasibility, safety and efficacy of the technique, irrespective of the bone substitute [179].

Despite ASCs are being proven to be suitable candidates for tissue reconstruction in several surgical applications, they are still far from being an "off-the-shelf" product, based on the current regulatory issues. The national regulatory agencies (i.e. the Food and Drug Administration in US and the European Medicines Agency in EU) provide the official rules and guidelines that guarantee safe and controlled procedures, requiring Good Manufacturing Practice (GMP) to be fulfilled during cell therapy production and applications [180]. Accordingly, whenever cell culture expansion is required to produce a cell-based treatment to be used in a clinical setting, this is labeled as an "advanced cell therapy". GMP-proof facilities (i.e. cell factories) are mandatorily needed for the entire cell processing procedure, in this case. It is also recommended to use approved GMP-manufactured, or anyway appropriately validated clinical grade reagents. Therefore, given that most applications described in the scientific literature imply extensive *ex vivo* processing of AT and SVF for ASCs isolation and expansion, these rules should be satisfied to move forward into the clinical setting. Conversely, the procedures involved in the isolation and use of fresh SVF or LAF (not requiring cell culture stages) are classified as minimal tissue processing. This setting does not require classified environments, hence would be closer to clinical translation, based on the existing regulatory issues.

7. ASCs-based bone regeneration in geriatric applications

Nowadays, stem cell therapies should inevitably address the increased medical challenges deriving from the progressive aging of the population (at least in western countries), associated with an inherently increased demand for regenerative applications to treat the structural frailty of older patients. Hence, the design and translation of AT-based experimental regenerative strategies are expected to cope with these issues [181]. In particular, decreased bone mass and mineral density, along with degenerative joint disease, scarcopenia and muscle weakness, are indeed part of the frail phenotype affecting the entire musculoskeletal system in elder people. To date, few, although encouraging, results have been achieved in clinical studies exploiting ASCs for the treatment of typical geriatric conditions, specifically affecting the skeletal system. Pak and colleagues reported the safety and feasibility of percutaneous intraarticular injections of uncultured ASC-containing SVF (associated with platelet-rich plasma) in patients suffering from chronic or degenerative joint disease [182].

Besides the limited data available from clinical trials, several preclinical studies have tested the feasibility and efficacy of ASC-based strategies for the treatment of age-related bone disorders, particularly osteoporosis. To this aim, animal models of ovariectomy-induced osteoporosis have been widely exploited, accounting for a multitude of preclinical reports. A recent metanalysis of preclinical studies in different animal models of osteoporosis attempted to combine this huge amount of data [183] Focusing on ASCs, the results consistently indicate that ASCs-based treatments were able to improve bone mineral density and reduce bone loss in different osteoporotic animal models [183-185] . In addition, local injection of ASCs infected with lentiviral vectors expressing human alpha-1 antitrypsin protein improved bone-morphometric parameters and succeeded in partially reversing the ovariectomy-induced bone loss [186].

Furthermore as regenerative stem-cell therapies are almost entirely based on an autologous approaches, it is reasonable to consider whether osteoporosis could influence the biological properties of ASCs, when harvested from osteopenic patients. Indeed, the cell regenerative capacity and osteogenic potential of ASCs, isolated from the inguinal subcutaneous AT were found impaired in osteoporotic mice [187]. Similarly, ASCs from ovariectomized rats exhibited a comparable proliferation capacity compared with controls, but showed relatively lower osteogenic potential in a critical-size calvarial defect model [188].
Another study demonstrated that the recovery of osteoporosis achieved by ASCs transplantation, tended to decrease with donor age in osteoporotic mice [185].
While the aforementioned studies reported results of local ASCs transplantations, anew regenerative therapy for the prevention of bone loss, employing the systemic administration of aspirin and allogeneic ASCs, was tested in an ovariectomized mice model and demonstrated temporary recovery of bone loss [189] .

These data collectively provide promising clues towards the design and development of advanced ASC-based stem cell therapies for the treatment of age-associated bone frailty and osteoporosis. Nonetheless, further testing in the clinical setting need to be implemented, and patient-tailored approaches should be defined in order to cope with the several co-morbidities affecting an aged patient.

8. Concluding remarks

During the last decades the scientific literature agreed to indicate ASCs as a new promising tool to be exploited in bone regenerative applications. Current regulatory issues in matter of "bioprocess engineering products" encouraged the development of closed devices for the isolation of ASCs/SVF, enabling minimal tissue manipulation. Preliminary outcomes of clinical studies are confirming the results obtained in animal models, suggesting that either native or cultured ASCs, alone or in combination with biomimetic scaffolds and/or treatments, are able to improve bone healing. Taken together, these data highlight the growing translational relevance of the use of ASCs for bone repair.

9. Expert opinion

Since the original description, 15 years ago, of adipose-derived stem cells (ASCs), research on these cells has become a cutting-edge topic in the field of regenerative medicine, with over 8500 papers published in PubMed, to date. Our research group, interested in bone biology and genetics, started focusing on ASCs with the aim of identifying a suitable cell source, alternative to BMSCs, to be exploited in experimental biological therapies aimed at regenerating bone.

For the reasons detailed in this review, ASCs potentially offer a number of advantages over adult stem cells from other sources (such as bone marrow, amniotic membrane, etc), in terms of feasibility of clinical translation. In particular, not only adipose tissue is found basically in all individuals, with extended availability and relatively easy harvesting, but it also yields high amounts of viable stem cells, compared with other tissue sources. The recently achieved standardization of nomenclature and isolation protocols, along with the improved characterization of stem cell niches, is rapidly leading to the development of safer and more targeted autologous transplantation protocols, with optimized patient-tailored prioritization of harvesting sites.

Besides their multipotency and trophic features, ASCs display immune-modulatory and paracrine effects exerted through their secretome, similarly to mesenchymal stromal cells isolated from other tissue sources. These features enlarge significantly the ground of actual and potential applications of ASC-based therapies.

Despite the limited comparability of the outcomes obtained through *in vivo* transplantation assays, the exploitation of ASCs in bone regenerative strategies has evolved at the same pace as their *in vitro* and *ex vivo* biological characterization. Even though the adipogenic niche is distinct from the skeletogenic niche, by proper definition, a number of biological similarities suggest a reciprocal ex/interchange between these two systems. Indeed, adipose tissue is found in spatial proximity with both angiogenic and osteogenic precursors within a bone segment, and represents a closely interacting domain, with bi-directional plasticity. ASCs indeed indirectly contribute to the homeostasis of bone development, remodeling and healing, *in vivo*, mostly due to the secretion of bioactive molecules exerting paracrine effects on the osteogenic lineage. Several preclinical studies, and selected clinical trials, to date, have confirmed that ASCs represent suitable cell therapies for regenerating large bone defects, whenever endogenous BMSCs are not sufficient, and/or transplanted BMSC are not feasible, to sustain osteogenesis. In most cases, in order to achieve successful bone healing, the designed strategies involve genetic engineering and/or ex vivo osteoinductive priming of ASCs, prior to *in vivo* implantation (see details on preclinical studies and clinical trials in this review). Nonetheless, distinct studies reported the capability of undifferentiated native ASCs to drive the osteogenic process *in vivo*. This capability is reasonably the result of a fruitful combination of their demonstrated plasticity and their bioactive peptide-enriched secretome, enabling the delivery of trophic paracrine effects at the site where new bone synthesis is required.

Despite the already existing evidence, it is likely and highly desirable that, in the next years, uncontroversial information would derive from improved and reproducible preclinical studies, from the successful completion of clinical trials, and from the implementation of optimal osteoconductive scaffolds. Altogether, these scientific facts will enable confirming that ASCs-based treatments could regenerate a fully functional bone tissue, with a correct structural architecture and efficient integration, at least in selected skeletal sites. Lastly, new challenges are being offered to the biomedical research community, by the progressive aging of the Western world population, along with the rapid development of genomic technologies that allow defining patient-specific backgrounds. These factors are delineating a rapidly changing medical scenario, in which targeted cell-based therapies will need to be as precise as possible, and personalized to cope with specific demands of wellbeing and improved performance, from the aging population.

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ARTICLE HIGHLIGHTS BOX:

- Adipose tissue houses multipotent somatic stem cells (aka adipose-derived stem cells, ASCs) residing in perivascular niches.

- Significant advancements in ASCs' research have been achieved in the last decades, leading to improved knowledge of their biology and potentialities.

- ASCs exert osteoinductive properties, by secreting bioactive molecules and growth factors that mediate their paracrine trophic effects.

- ASCs are extremely plastic and proved to be capable of inducing bone regeneration in distinct animals models.

- Strict regulatory issues are to be met in order to achieve a safe and efficient clinical translation of the numerous experimental data on ASC-based therapies.

- Clinical trials are ongoing to provide the final confirmation of the correct and feasible exploitation of ASCs for the treatment of bone defects and of disorders characterized by impaired endogenous osteogenesis and bone remodeling.

Table 1. Preclinical studies on ASC osteoregenerative potential.

Experimental model	Species	Scaffold / administration	Additional*ex vivo*/*in vivo* treatment	Grafttype	Reference
Calvarialdefect	Rat	PLGA	Alendronate	Xenogeneic	103
Calvarialdefect	Rabbit	HA-PLGA, collagensponge	BV-BMP2/ TGF β3	Allogeneic	104
Calvarialdefect	Mouse	PLGA	Dura mater	Xenogeneic	105
Calvarialdefect	Rat	β -TCP	Lenti-miR-31	Allogeneic	106
Calvarialdefect	Mouse	Custom scaffold	NOGGIN shRNA-Knockout	Xenogeneic	30
Calvarialdefect	Dog	HA-PLGA	None	Xenogeneic	108
Calvarialdefect	Mouse	Systemicinjection	None	Allo/xenogeneic	109
Calvarialdefect	Mouse	Local injection	None	Xenogeneic	110
Calvarialdefect*	Rat	DBM, PLA	None	Xenogeneic	111
Calvarialdefect	Rat	MAP-coated PCL/PLGA	None	Xenogeneic	112
Calvarialdefect	Rat	HA- β-TCP	None	Xenogeneic	113
Calvarialdefect	Rat	PLGA	None / ostegenic medium	Xenogeneic	114
Calvarialdefect	Dog	Coral	Osteogenicinduction	Autologous	115
Calvarialdefect	Dog	Coral	Osteogenicinduction	Allogeneic	95
Calvarialdefect	Pig	Collagen sponge	Osteogenic induction	Autologous	116
Calvarialdefect*	Rat	DBX	Osteogenic induction	Allogeneic	117
Calvarialdefect	Rat	PCL-PLGA- β-TCP	Osteogenic induction + HUVEC	Xenogeneic	118
Calvarialdefect	Mouse	pDA-PLGA	rhBMP-2	Xenogeneic	119
Calvarialdefect	Rabbit	Collagensponge	rhBMP-2	Allogeneic	120
Calvarialdefect	Mouse	HA-PLGA	Sonic hedgehog signaling Induction	Xenogeneic	121
Calvarialdefect	Rat	Local injection	VEGFa	Xenogeneic	122
Calvarialdefect	Rat	Local injection	PRP	Allogenic	123
Calvarialdefect	Rabbit	Fibronectin-treated PLA/PLA	None / Osteogenic induction	Allogenic	124
Calvarialdefect	Rat	PLA	None/ Osteogenic induction / endothelial induction/ coculture	Allogenic	125
Calvarialdefect	Rabbit	TCP/BAG	BMP-2/ BMP-7/ VEGF	Autologous	126
Calvarialdefect	Rat	Bio-Oss + Collagen type I	None	Xenogeneic	127
Calvarialdefect	Dog	PCL+β-TCP/ASCs-sheet+ PCL+β-TCP	None / Osteogenic induction	Autologous	128
Calvarialdefect	Rabbit	BAG/ TCP	Iron-labeling	Autologous	129
Cavarialdefect	Rabbit	Polyamide/ PLGA/DAM	Osteogenic induction	Autologous	130
Calvarialdefect	Mouse	PLGA	BMP-2/miR-148b baculovirus vectors	Xenogenic	131
Calvarialdefect	Rat	CH+HA	17β-Estradiol	Allogenic	132
Calvarialdefect	Mouse	Decellularizedtendon	Osteogenic induction	Xenogenic	133
Calvarialdefect	Mouse	PLGA	None	Autologous	133
Calvarialdefect	Rat	BAG	None	Autologous	134
Calvarialdefect	Mouse	SPCL	None	Autologous	135
Ectopic bone formation/ Calvarial defect*	Mouse/Rat	HC	None	Xenogenic	92
Ectopic bone formation	Mouse	PLGA	BMP2/RUNX2 bicistronic vector	Xenogeneic	93
Ectopic bone formation	Mouse	PRP + alginate microsphere	None	Allogeneic	94

Ectopic bone formation	Mouse	β-TCP	None	Xenogeneic	95
Ectopic bone formation	Rat	HA	None	Xenogeneic	96
Ectopic bone formation	Rat	Matrigel	Osteogenicinduction	Xenogeneic	97
Ectopic bone formation	Rat	DBM	Osteogenicinduction	Xenogeneic	30
Ectopic bone formation	Mouse	Carbon nanotubes	rhBMP2	Xenogeneic	99
Ectopic bone formation	Rat	PLDA	rhBMP2	Xenogeneic	100
Ectopic bone formation*	Mouse	BMM+PRP	None	Allegenic	101
Ectopic bone formation	Mouse	β-TCP	Chondrogenicinduction / None	Xenogenic	102
Ectopic bone formation	Mouse	polyurethane + spheroids	None / Osteogenic induction	Allogenic	103
Femurdefect	Mouse	Systemicinjection	None	Allogeneic	136
Femurdefect	Rat	Fibrinmatrix	rhBMP2	Allogeneic	137
Femurdefect	Rat	β-TCP	Lenti-BMP2/7	Allogeneic	138
Femur defect	Rat	Collagen gel	None	Xenogeneic	139
Femurdefect	Rabbit	PLGA	BMP2 and VEGF baculovirus vectors	Autologous	140
Femurdefect	Sheep	Titanium	Osteogenic induction / serum deprivation	Autologous	141
Femurdefect	Rat	Collagen-ceramic	BMP-2-carrying adenovirus	Xenogenic	142
Femurdefect + distractor	Rat	Type I collagen gel	None	Allogenic	143
Ulna defect	Rabbit	PLGA	None / osteogenic medium	Xenogeneic	117
Ulna defect	Rabbit	DBM	None / Osteogenic induction	Allogenic	124
Radialdefect	Dog	β -TCP	None	Allogeneic	144
Radialdefect	Rabbit	PLA/PCL + vascularizedperiosteum	Ad-Cbfa1	Allogeneic	136
Radial defect	Rabbits	HA-PLA-COL	Ad-hBMP2	Allogeneic	145
Tibia defect	Rabbit	HA	None	Autologous	146
Tibia defect + distractor	Rabbit	Local injection	None	Autologous	147
Tibia defect	Rabbit	HA	None	Autologous	148
Tibia defect	Dog	PRP	None	Xenogeneic	149
Tibia defect	Mouse	Injection	None	Autologous	150
Femurosteochondraldefect	Rabbit	Local injection	Bovine BMP	Allogeneic	151
Femurosteochondraldefect	Rabbit	Ceramics, biphasicmaterials	none	allogeneic	152
Spinal fusion	Mouse	Local injection	rhBMP6 nucleofection	Xenogeneic	170
Spinal fusion	Rat	Lyophilized human cancellous bone	Gal-KO + osteogenicinduction	Xenogeneic	169
Spinal fusion/ Femurdefect	Pig	3D-DBM	DBM + Osteogenic induction	Autologous	129
Vertebraldefect	Rat	Fibrin gel	rhBMP6 nucleofection	Xenogeneic	171
Mandibledefect	PIg	Local-systemicinjection	None	Allogeneic	174
Mandibledefect	Rat	HA/COL	None	Xenogeneic	173
Mandibledefect	Rabbit	CH+CS	BMP2 + NOGGIN shRNA-Knckout	Xenogenic	133
Mandibledefect	Pig	DBM	3D- Osteogenic induction	Autologous	147
Alveolardefect	Rat	PLGA	None	Allogenic	146

HA: hydroxyapatite; PLGA: poly(lactic-co-glycolic acid); PLA/PCL: polylactic acid/polycaprolacton; Ad-Cbfa1: adenoviral expression vector carrying the Cbfa1 gene; DBM: demineralized bone matrix; β -TCP : beta-tricalcium phosphate; Lenti-miR-31: lentivirus expression vector carrying the microRNA-31; p-DA: polydopamine; PRP: platelet-rich plasma; Lenti-BMP2/7: lentivirus expression vector carrying either the BMP2 or the BMP7 gene, MAP: mussel adhesive proteins, NOGGIN shRNA : short hairpin ribonucleic acid to knockdown NOGGIN gene, COL: collagen; BAG: bioactive glass; DAM decellularized amniotic membrane; BV-BMP2/ TGF β3: baculovirus expression vector carrying either the BMP2 or the TGF β3 gene; BMP-2/miR-148b: baculovirus vectors

carrying the microRNA-148b and BMP2 genes; CH: Chitosan; CS: chondroitin sulfate; Gal-KP: galactosyl-knock-out; a-CaP: amorphous calcium phosphate; 3D-DBM: three-dimentional demineralized bone matrix. BMM: bone mineral matrix. SPCL: starch-polycaprolactone. HC: Engineered and devitalized hypertrophic cartilage. *: these studies were based on uncultured SVF instead of culture-amplified ASCs.

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