

World Journal of *Stem Cells*

World J Stem Cells 2021 September 26; 13(9): 1160-1359



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The *WJSC* is now indexed in Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, BIOSIS Previews, Scopus, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJSC* as 5.326; IF without journal self cites: 5.035; 5-year IF: 4.956; Journal Citation Indicator: 0.55; Ranking: 14 among 29 journals in cell and tissue engineering; Quartile category: Q2; Ranking: 72 among 195 journals in cell biology; and Quartile category: Q2. The *WJSC*'s CiteScore for 2020 is 3.1 and Scopus CiteScore rank 2020: Histology is 31/60; Genetics is 205/325; Genetics (clinical) is 64/87; Molecular Biology is 285/382; Cell Biology is 208/279.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: Yan-Xia Xing; Production Department Director: Yun-Jie Ma; Editorial Office Director: Ze-Mao Gong.

NAME OF JOURNAL

World Journal of Stem Cells

ISSN

ISSN 1948-0210 (online)

LAUNCH DATE

December 31, 2009

FREQUENCY

Monthly

EDITORS-IN-CHIEF

Shengwen Calvin Li, Tong Cao, Carlo Ventura

EDITORIAL BOARD MEMBERS

<https://www.wjnet.com/1948-0210/editorialboard.htm>

PUBLICATION DATE

September 26, 2021

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INSTRUCTIONS TO AUTHORS

<https://www.wjnet.com/bpg/gerinfo/204>

GUIDELINES FOR ETHICS DOCUMENTS

<https://www.wjnet.com/bpg/GerInfo/287>

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH

<https://www.wjnet.com/bpg/gerinfo/240>

PUBLICATION ETHICS

<https://www.wjnet.com/bpg/GerInfo/288>

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<https://www.wjnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>

Current evidence on potential of adipose derived stem cells to enhance bone regeneration and future projection

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Author contributions: Dighe AS and Cui Q contributed conception and designed the research; Le Q, Madhu V and Dighe AS contributed literature search, preparation of the first draft, tables and figures; Le Q, Hart JM and Cui Q contributed clinical trials database search and review; Dighe AS, Farber CR, Zunder ER and Cui Q contributed preparation of semifinal draft after reviewing the first draft; all authors wrote, read and approved the final manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest for this article.

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Abstract

Injuries to the postnatal skeleton are naturally repaired through successive steps involving specific cell types in a process collectively termed "bone regeneration". Although complex, bone regeneration occurs through a series of well-orchestrated stages wherein endogenous bone stem cells play a central role. In most situations, bone regeneration is successful; however, there are instances when it fails and creates non-healing injuries or fracture nonunion requiring surgical or therapeutic interventions. Transplantation of adult or mesenchymal stem cells (MSCs) defined by the International Society for Cell and Gene Therapy (ISCT) as CD105⁺-CD90⁺-CD73⁺-CD45⁻-CD34⁻-CD14⁺ or CD11b⁻-CD79⁺ or CD19⁻-HLA-DR⁻ is being investigated as an attractive therapy for bone regeneration throughout the world. MSCs isolated from adipose tissue, adipose-derived stem cells (ADSCs), are gaining increasing attention since this is the most abundant source of adult stem cells and the isolation process for ADSCs is straightforward. Currently, there is not a single Food and Drug Administration (FDA) approved ADSCs product for bone regeneration. Although the safety of ADSCs is established from their usage in numerous clinical trials, the bone-forming potential of ADSCs and MSCs, in general, is highly controversial. Growing evidence suggests that the ISCT defined phenotype may not represent bona fide osteoprogenitors. Transplantation of both ADSCs and the CD105⁺ sub-population of ADSCs has been reported to induce

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Manuscript source: Invited manuscript

Specialty type: Cell and tissue engineering

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 28, 2021

Peer-review started: March 28, 2021

First decision: May 12, 2021

Revised: May 22, 2021

Accepted: August 18, 2021

Article in press: August 18, 2021

Published online: September 26, 2021

P-Reviewer: Xu T

S-Editor: Wang LL

L-Editor: A

P-Editor: Xing YX



bone regeneration. Most notably, cells expressing other markers such as CD146, AlphaV, CD200, PDPN, CD164, CXCR4, and PDGFR α have been shown to represent osteogenic sub-population within ADSCs. Amongst other strategies to improve the bone-forming ability of ADSCs, modulation of VEGF, TGF- β 1 and BMP signaling pathways of ADSCs has shown promising results. The U.S. FDA reveals that 73% of Investigational New Drug applications for stem cell-based products rely on CD105 expression as the “positive” marker for adult stem cells. A concerted effort involving the scientific community, clinicians, industries, and regulatory bodies to redefine ADSCs using powerful selection markers and strategies to modulate signaling pathways of ADSCs will speed up the therapeutic use of ADSCs for bone regeneration.

Key Words: Mesenchymal stem cells; Adipose-derived stem cells; Endogenous stem cells; Skeletal stem cells; Bone regeneration

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Core Tip: This review systematically examined current progress and future projections of Adipose-derived Stem Cells (ADSCs) use in bone regeneration. Introduction covered the regulatory aspects of stem cell therapy and scientific concerns regarding stem cell use including ADSCs. We then analyze clinical and pre-clinical studies using ADSCs for the treatment of bone defects. We also evaluate the current understanding of ADSC's surface receptors and therapeutic subpopulations. Overall, we conclude that while mixed outcomes have been reported, a more rigorous definition of ADSCs, selection of osteogenic subpopulations, and understanding of signaling pathways will unleash ADSCs as a powerful tool in bone regeneration.

Citation: Le Q, Madhu V, Hart JM, Farber CR, Zunder ER, Dighe AS, Cui Q. Current evidence on potential of adipose derived stem cells to enhance bone regeneration and future projection. *World J Stem Cells* 2021; 13(9): 1248-1277

URL: <https://www.wjgnet.com/1948-0210/full/v13/i9/1248.htm>

DOI: <https://dx.doi.org/10.4252/wjsc.v13.i9.1248>

INTRODUCTION

Of the 7.9 million fractures sustained each year in the United States, 5% to 20% result in non-union or delayed healings[1,2]. Since these fractures do not heal naturally, they require therapeutic interventions. Transplantation of multipotent stem cells, reportedly present in practically all postnatal tissues, is an attractive therapeutic option. Mesenchymal stem cells (MSCs) isolated from bone marrow [bone marrow-derived MSCs (BMMSCs)] are thought to be true, gold-standard osteoprogenitors[3]. To streamline investigations on MSCs, the International Society for Cell and Gene Therapy (ISCT) defined MSCs in 2006 as cells satisfying the following three criteria: Plastic adherent, CD105+CD90+CD73+CD45-CD34-CD146-CD11b-CD79aorCD19-HLA-DR-, and possessing the ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*[4-6]. This remains the current definition of adult stem cells or MSCs. This school of thought suggests that MSCs exist in all adult tissues and can give rise to osteoblasts, chondrocytes, marrow stromal cells, and adipocytes. Accordingly, the U.S. Food and Drug Administration (FDA) revealed that 73% of Investigational New Drug applications for stem cell-based products rely on CD105 expression as the “positive” marker for adult stem cells[7]. The optimal utilization of MSCs has been prevented by the lack of ideal surface markers for selection and an incomplete understanding of the heterogeneity of MSCs and factors governing their bone-forming ability.

Clinical studies evaluating the exogenous addition of BMMSCs to enhance bone repair in segmental defects, nonunion of the tibia, and tibial osteotomy have shown increased healing rates[8-16]. However, several factors remain enigmatic for BMMSCs therapies, including impure cell preparations, the significant numbers of cells required to achieve satisfactory healing, supplementation of growth factors, the presence of

other cell types at a higher frequency than MSCs, and incomplete fracture healing in many patients, which suggest that more studies are required to fully understand MSCs therapy[8-10,14]. These shortcomings in addition to the invasive nature of isolating BMMSCs, their extremely low frequency in bone marrow, and the requirement of high numbers of MSCs to achieve enhancement of bone healing, diminish the enthusiasm for their therapeutic use.

In contrast, MSCs isolated from fat tissue [adipose-derived stem cells (ADSCs)] offer the following advantages over BMMSCs: ADSCs can be isolated in large numbers through a simple procedure, they possess higher proliferative capacity, their frequency is 500 times higher than BMMSCs, they are resistant to senescence, and they retain their differentiation potential for a longer period[17-25]. Given their clear clinical advantages compared to BMMSCs, ADSCs are believed by many researchers to hold great promises for implementation in regenerative medicine, specifically for the treatment of orthopedic conditions. Nonetheless, the current body of research on this topic yield confounding conclusions. The exact characterization of the osteoprogenitor population within ADSCs remains in dispute. At the same time, ADSCs utilization protocols vary greatly between different clinical and preclinical studies, which themselves are inconclusive on the nature of ADSCs' osteogenic capacity. Due to these limitations, there has been no ADSC-based orthopedic product suitable for widespread use. In this review, we attempt to capture the different aspects of current research on ADSC in the hope to highlights the importance of ADSCs for bone regeneration applications, current understanding of the subject, the obstacles facing researchers, and possible strategies to further realize ADSCs' potential as a therapeutic tool.

REGULATORY ASPECTS OF STEM CELLS THERAPY

Although there is general agreement in the scientific community that stem cell therapy holds great promise for bone repair and regenerative medicine applications, there is not much agreement on the definition of adult stem cells. Moreover, several leading experts in the field warn that the existing stem cell-based products are manufactured without vigorous testing and are not backed up by strong scientific evidence. An article titled "Clear up this stem-cell mess" published recently in *Nature* states that the confusion about MSCs is making it easier for industries to sell unproven treatments [26]. In agreement with this observation, another article in *Cell Stem Cell* comments that clinical trials using MSCs have been conducted for more than a generation, but the outcomes have fallen short of expectations[27].

A thorough understanding of the FDA guidelines is necessary for orthopedic surgeons to decide whether the stem cell-based products that they are using or being asked to use by industries are authenticated by the regulatory bodies. It is also necessary to clarify that the FDA guidelines do not establish legally enforceable responsibilities, but they describe FDA's current thinking and therefore should be viewed only as recommendations unless specific regulatory or statutory requirements are cited. This puts a greater responsibility on clinicians and scientists to make sure that the general public is aware of the effectiveness of stem cell therapy, and more importantly, the patients receiving stem cell therapy are aware of the risk to benefit ratio.

The current guidance issued by FDA is available under the docket number FDA-2017-D-6146 (<https://www.fda.gov/media/109176/download>). Adult stem cell-based products are regulated by the Center for Biologics Evaluation and Research, similar to human cells, tissues, and cellular- and tissue-based products (HCT/Ps). These regulations are provided by the FDA to HCT/P manufacturers, healthcare providers, and FDA staff, under Title 21 of the Code of Federal Regulations (CFR) Part 1271. These regulations explain the types of HCT/Ps that do not require premarket approval; and the registration, manufacturing, and reporting steps that must be taken to prevent the introduction, transmission, and spread of communicable disease by these HCT/Ps: (1) The product is minimally manipulated; (2) It is intended for homologous use and this is reflected by the labeling, advertising, and the manufacturer's objective intent; (3) The manufacture of the HCT/P does not involve the combination of the cells or tissues with another article (except for water, crystalloids, or a sterilizing, preserving, or storage agent, provided that these agents are safe); (4) The product is not dependent upon the metabolic activity of living cells for its primary function; and (5) If the product is dependent upon the metabolic activity of living cells or has a systemic effect then it must be only for autologous use

(cells isolated from the person transplanted back into the same person) or allogeneic use in a first-degree or second-degree blood relative or for reproductive use.

In a cautionary observation, Skovrlj *et al*[28] reported that all five commercially available cellular bone matrices for spine fusion, Osteocel Plus (NuVasive, San Diego, CA, United States), Trinity Evolution (Orthofix, Lewisville, TX, United States), Cellentra Viable Cell Bone Matrix (Biomet, Warsaw, IN, United States), AlloStem (AlloSource, Centennial, CO, United States), and Ovation (Osiris Therapeutics, Columbia, MD, United States), contain live, allogeneic MSCs but claim to meet the FDA criteria under Section 361, 21 CFR Part 1271, and have not undergone FDA premarket review. All of these products are composed of MSCs derived from freshly procured cadaveric bone marrow, cadaveric adipose tissue, or chorion layer of the placenta.

It is important to take notice of the fact that there is no stem cells-based product currently approved by the FDA that can be used for bone tissue engineering purposes or for the treatment of bone diseases. The list of all cell and gene therapy products approved by the FDA can be found on FDA's website: <https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products>. Thus, detailed investigation on bone-forming potential of stem cells *in vitro* and *in vivo* followed by non-industry sponsored clinical studies evaluating the efficacy of stem cells are required. Since ADSCs can be isolated in a non-invasive procedure, in abundant numbers, for autologous use, they offer a promising option for stem cell-based bone repair therapies.

THE CLINICAL TRIALS USING ADSCS

To elucidate the possible clinical benefits of using ADSCs, many clinical trials have been initiated. The clinical trials that will be used in our analysis were acquired from Clinicaltrials.gov in December 2020 using the combination of keywords "Orthopedic Disorder (Condition) - Adipose Stem Cells (Other terms)" and "Bone (Condition) - Adipose-Derived Stem Cells (Other terms)". The first combination of keywords returned 74 registered trials while the second combination returned 17 trials. Following content review, we eliminated any overlapping trials, trials that did not directly involve orthopedic conditions, trials that did not include human patients, and trials that did not explicitly state the use of ADSCs. This resulted in 70 trials being included in this analysis. We found only four trials that addressed bone healing or bone regeneration using ADSCs: NCT02140528, NCT04340284, NCT03678467, and NCT03678467. While NCT03678467 is an ongoing trial, the results of the other three are not published. We, therefore, searched PUBMED using the keywords "adipose stem cells", "orthopedic", and "clinical" with the filter "Clinical Study" and found 10 relevant articles[29-38]. The general distribution of the 80 included clinical trials can be seen in Figure 1. The outcomes of clinical trials on bone regeneration are summarized in Table 1.

From the number of trials, it is clear that there is tremendous interest in ADSCs as a therapeutic tool for a variety of orthopedic disorders. The earliest trials were started in 2008. However, the number of initiated trials has been on an upward trend since this time. Moreover, only 37% of trials indicated as completed (total = 29). We will be seeing a large number of trials ending in 2021 (total = 17), which will have important implications for the field. The majority of the trials are in Phase 1 or 2, evaluating the safety and initial efficacy of treatment with ADSC. Only 6 trials (8%) are in phase 3 and one is in phase 4. Of the 29 completed trials, 19 corresponding publications could be found on PubMed using the National Clinical Trial registration number. Of these, we will review in detail 10 publications directly investigating bone regeneration using ADSCs.

SAFETY OF ADSCS ESTABLISHED IN CLINICAL TRIALS

In 2013, Pak *et al*[29] published the outcomes of long term follow up of 91 patients undergoing injections of autologous ADSCs with platelet-rich plasma in various joints to evaluate the safety of this treatment modality. Participants were observed for an average of 16 mo. During this time, magnetic resonance imaging (MRI) evaluation showed no evidence of neoplasm. Common adverse events included swelling of injected joints, tenosynovitis, and tendonitis, all of which were either successfully

Table 1 Summary of the clinical trials involving treatment of the bone defects using adipose-derived stem cells

Bone defect treated	Study duration and length of follow up	n	Intervention	ADSCs source	ADSCs number	Outcome	Ref.
Avascular necrosis of hip, osteoarthritis of hip/knee/ankle, spinal disc herniation	2009-2012, 30 mo	91	Intraarticular injection of SVF with PRP	Autologous SVF from abdominal tumescent liposuction	10 mL of SVF	No evidence of neoplasm, no serious adverse events, common adverse events (swelling of injected joints, tenosynovitis, and tendonitis) were either successfully managed or self-limited, established safety of ADSCs	Pak <i>et al</i> [29]
Upper arm fracture in elderly patients (62-84 yr)	2012-2014, 6 mo	8	SVF seeded porous silicated-hydroxyapatite microgranules with fibrin hydrogel implant	Autologous SVF from abdominal tumescent liposuction	800 microliters of SVF	Evidence of osteogenesis at graft site; circumstantial evidence for direct contribution of SVF cells to fracture healing	Saxer <i>et al</i> [30]
Large cranial defect	2008-2010, 12 mo	4	ADSCs-seeded β -tricalcium phosphate implant	Autologous ADSC from abdominal subcutaneous liposuction	15×10^6 cells	Noted equivalence between newly generated tissue and native bone	Thesleff <i>et al</i> [31]
Large cranial defect	2008-2016, approximately 7 yr	5	ADSCs-seeded β -tricalcium phosphate implant	Autologous ADSC from abdominal subcutaneous liposuction	15×10^6 cells	This study was long term follow up of Thesleff <i>et al</i> [31]; unsatisfactory long-term outcome with significant resorption	Thesleff <i>et al</i> [32]
Cranio-maxillofacial hard-tissue defects	2012-2014, up to 52 mo	13	ADSCs-seeded bioactive glass or β -tricalcium phosphate scaffolds, at times with recombinant hBMP-2	Autologous ADSC from anterior abdominal wall liposuction	Up to 160×10^6 cells	Majority of patients achieved satisfactory clinical and radiographic results; three experienced significant resorptions of the ADSCs graft	Sándor <i>et al</i> [33]
Long bone nonunion from bone tumor resection or pseudoarthrosis	2012-2014, 39 mo	6	ADSCs seeded decellularized bone matrix	Subcutaneous autologous ADSCs	Up to 200×10^6 cells	50% of the patients achieved bone regeneration and union	Dufrane <i>et al</i> [34]
Maxillary sinus floor elevation	2009-2015, 36 mo	10	SVF seeded β - tricalcium phosphate implant	Autologous SVF from abdominal tumescent liposuction	20×10^6 cells	Experimental group exhibited significantly more bone healing compared to control	Prins <i>et al</i> [35]
Alveolar cleft osteoplasty	2015-2016, 6 mo	10	Lateral ramus cortical bone plate with ADSCs-mounted natural bovine bone mineral	Autologous ADSCs from buccal fat pad	1.0×10^6	No significant different in bone regeneration found between experimental group and controls	Khojasteh <i>et al</i> [36]
Mandibular fracture	2010-2015, 12 wk	20	Direct application of ADSCs	Autologous ADSCs	Unreported	Significantly more osteogenesis in ADSCs-treated group compared to control	Castillo-Cardiel <i>et al</i> [37]
Nonunion following subtalar arthrodesis	2010-2016, 24 mo	140	ADSC-seeded partially demineralized bone matrix	Allograft ADSCs	Unreported	Inferior bone union rate in ADSCs treated group compared to autograft; equivalent clinical evaluations	Myerson <i>et al</i> [38]

ADSCs: Adipose-derived stem cells; SVF: Stromal Vascular Fraction; PRP: Platelet-rich plasma.

managed or self-limited. Evaluation of pain using VAS suggested that most patients experienced a significant reduction in pain three months post-operation.

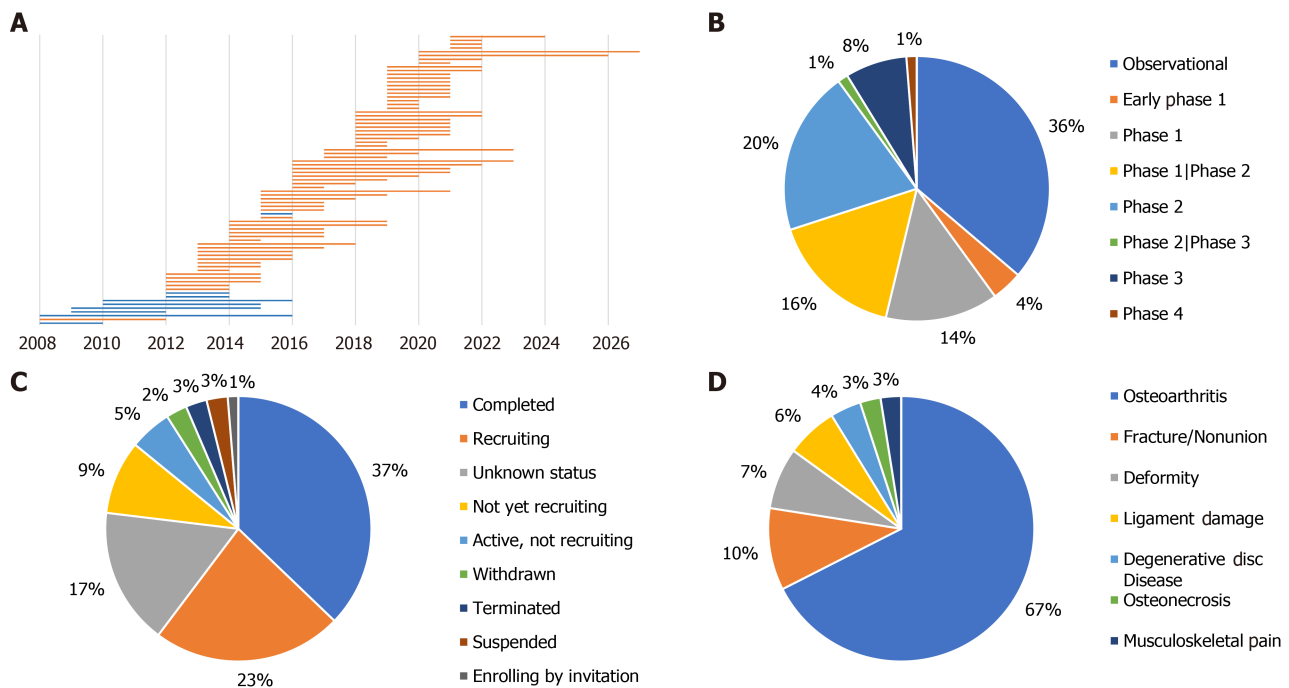


Figure 1 The clinical trials utilizing adipose-derived stem cells. The data were retrieved from clinical trials databases Clinicaltrials.gov and PUBMED. A: Distribution of adipose-derived stem cells (ADSCs) clinical trials' start and end dates. Clinical trials on bone regeneration with both identified start and end date are represented in blue. All others are represented in orange; B: Distribution of ADSCs clinical trials' status; C: Distribution of ADSCs clinical trials' phase; D: Distribution of ADSCs clinical trials' targeted condition.

OUTCOMES OF CLINICAL TRIALS USING ADSCS FOR BONE REGENERATION

Saxer *et al*[30] published in 2016 the results of a study investigating the safety and feasibility of a stromal vascular fraction (SVF) (construct in the treatment of proximal humeral fractures in elderly patients. The construct was made from unexpanded and undifferentiated human SVF derived from abdominal adipose tissue seeded onto a silicated-hydroxyapatite and fibrin hydrogel scaffold. The construct was tested first on male nude rats' 6 mm critical-sized femoral defects. Compared to cell-free control, the SVF-seeded construct was associated with significantly better mineralization and bone volume. Histological staining also confirmed *de novo* osteogenesis and angiogenesis in SVF-treated rats. The construct was subsequently tested on 8 patients aged 62-84 with displaced, low-energy, proximal humerus fractures who were followed prospectively for up to 12 mo post-surgery. The study confirmed that it was feasible for liposuction, SVF isolation, graft manufacturing, and implantation to all be completed intraoperatively. The implant was deposited into the void space created following open reduction and internal fixation. Over the follow-up period, the authors reported no adverse reaction that could be linked to the graft. Pain evaluation *via* VAS showed no lasting donor site pain and generally diminished operation site pain. Within one year of the surgery, five out of eight patients had their plates removed, which provided the opportunity for biopsy. The other three patients achieved subjective therapeutic goals and declined plate removal. Histological and micro-CT analysis confirmed osteogenesis at the graft site, either directly connected to or separated from the pre-existing bone. Bone ossicles were also found in scaffold pores. The authors considered these findings as circumstantial evidence for the direct contribution of SVF cells to fracture healing.

In 2011, Thesleff *et al*[31] presented the results of treatment of 4 patients with critical-size calvarial defects that underwent cranioplasty using grafts of ADSCs seeded on beta-tricalcium phosphate granules. ADSCs were obtained autologously from participants' subcutaneous abdominal fat, isolated, and expanded over three weeks. Participants were evaluated over a one-year follow-up period with computed tomography (CT) scans, which showed ossification. Hounsfield Unit measurements with CT scans showed approximate equivalence between normal bone and regenerated tissue. No serious adverse event was reported. In 2017, the same research group released the results of a 6-year follow-up on the same cohort of patients plus

one more[32]. Unfortunately, the authors determined that the long-term outcomes of ADSCs beta-TCP grafts for cranioplasty remained unsatisfactory. Among the five patients who underwent the procedure, four needed revision surgeries at 0.9, 2.0, 2.2, and 7.3 years following the original operation. Indications for re-operation included infect, partial resorption of graft, complete resorption, and meningioma recurrence. The grafts were then either removed and replaced with titanium, strengthened with titanium mesh, or the patient underwent craniotomy in the case of meningioma. Only one patient retained the original graft at the time of publication, but her skull X-ray did show some level of graft resorption.

Sándor *et al*[33] in 2014 reported a case series involving 13 patients with craniomaxillofacial bone defects, three of the frontal sinuses, five of the cranial bones, three of the mandibles, and two of the nasal septa. ADSCs from participants were harvested from abdominal subcutaneous fat, expanded, and seeded on either bioactive glass or beta-tricalcium phosphate scaffolds. In the three mandible cases, rhBMP-2 was also added. Follow-up periods ranged between 12 and 52 mo and showed satisfactory clinical and radiographic results for patients with mandibular, frontal sinus, nasal septum defects. Of the participants with cranial defects, two achieved clinically and radiographically satisfactory ossifications, while the other three experienced significant resorptions of the ADSC graft. One of the nasal septum defect patients resumed habitual nasal picking during follow-up and needed graft removal.

Dufrane *et al*[34] published a study in 2015 describing the treatment of 6 patients with long bone nonunion resulting from either bone malignancy or pseudoarthrosis. These patients were treated using 3D bone grafts produced from subcutaneous ADSCs, incubated in osteogenic media, and delivered using demineralized bone matrix (DBM) without scaffolding. During the follow-up period of 47 mo, no acute adverse events or tumorigenicity were reported, but there were two instances of infection. Three out of six patients achieved bone regeneration and union.

Prins *et al*[35] published a study in 2016 evaluating the use of autologous SVF, rich in ADSCs, seeded in calcium phosphate ceramics for maxillary sinus floor elevation. SVF was obtained from the participants' abdominal wall. A total of 10 participants received either bilateral implants, with one side being SVF with ceramics and one side being ceramics control or a unilateral implant of just SVF with ceramics. Follow-up over three years showed no serious adverse event. Follow-up biopsy and micro-CT showed active bone formation in the study arm with statistical differences in bone volume over control, most notably in SVF with β -tricalcium phosphate ceramics group.

In 2017, Khojasteh *et al*[36] published a phase I clinical trial following 7 patients with alveolar clefts treated with autogenous bone osteoplasty in combination with buccal fat pad derived ADSCs. Patients were divided into three treatment arms: Anterior iliac crest (AIC) spongy bone with a collagen membrane, lateral ramus cortical bone plate with ADSCs, and AIC spongy bone with ADSCs and collagen membrane. Results indicated bone generation in all three experimental arms, weakest in the AIC only group and strongest in the AIC with ADSCs group. However, the differences were not statistically significant. No serious adverse event was reported.

Castillo-Cardiel *et al*[37] published in 2017 the results of a single-blind, randomized, clinical trial involving 20 patients with mandibular angle fractures. Participants were separated into two groups, a control group receiving fracture reduction only and a stem cell treatment group receiving fracture reduction with application of ADSCs as well. ADSCs were obtained from abdominal fat 24 h prior to the mandibular procedure. Evaluation of bone regeneration over 12 wk showed statistically significant improvement in ossification in the ADSC group compared to control.

In 2019, Myerson *et al*[38] published a multicenter, randomized controlled study to compare safety and efficacy of ADSCs in subtalar arthrodesis (bone fusion of the subtalar joint involving ankle bone and heel bone) with classic bone autograft. This study included 140 patients enrolled in two study arms receiving either autologous bone grafts or ADSCs. Autologous bone grafts were obtained from either the iliac crest or the distal tibia. ADSCs were obtained autologously and deposited on partially demineralized cancellous bone. Patients were followed up for over two years using clinical scores such as AOFAS, SF-12, and FFI-R as well as radiographic evaluation for the fusion of the subtalar joint. Imaging showed a lower rate of fusion in the ADSCs group compared to autograft control. Nonetheless, both groups showed equivalent clinical evaluations.

There are three clinical trials registered at Clinicaltrials.gov with no published outcomes. NCT02140528 sought to evaluate the safety and efficacy of the injection of allogeneic ADSCs on the healing of tibial fractures in 40 patients. Patients were separated into two groups receiving either ADSCs injections or placebo. NCT04340284

is a retrospective report on the outcomes of 11 patients receiving fluoroscopic guided percutaneous injections of SVF to the site of long bone nonunion. Healing was evaluated over 12 mo using SF-12 and radiographic imaging. ADSCs were also considered for Spinal Cord Injury, which was investigated in trial NCT02981576. This study enrolled 14 participants separated into two arms receiving three intrathecal injections of either autologous ADSCs or autologous bone marrow-derived MSCs. Follow-up was done over 12 mo using ASIA impairment score as well as MRI imaging.

Finally, clinical trial NCT03678467 is an open-label trial using an autologous, anatomically shaped bone graft made from patients' own ADSCs specifically for patients' mandible injury or deformity. The main goal of the study is to assess the safety of the treatment. Six patients will be followed over 12 mo on the number of adverse events, quality of life, and bone regeneration with CT scans.

In summary, a total of ten different studies conducted on total of 307 patients suggest that the use of ADSCs is safe, but there is limited evidence that ADSCs can significantly enhance bone regeneration.

RESERVATIONS ABOUT USING ADSCS FOR BONE REGENERATION

Considering the abundant availability of ADSCs and ease of their isolation, several investigators have attempted to use ADSCs to enhance bone regeneration. These studies were conducted using conventional preparations of ADSCs satisfying the ISCT definition of adult stem cells or MSCs as CD105+CD90+CD73+CD45-CD34-CD14orCD11b-CD79 α orCD19-HLA-DR- cells. These studies suggested a limited ability of ADSCs to induce bone formation or to enhance bone repair and raised serious doubts about their therapeutic utility. The outcomes of the investigations are summarized in this section.

Primary ADSCs failed to enhance bone healing, in defects created in rat calvaria and sheep tibia[39,40]. In a canine maxillary alveolar cleft model, autografts induced significantly higher bone formation than ADSCs-seeded on hydroxyapatite/beta-tricalcium phosphate scaffolds[41]. Godoy Zanicotti *et al*[42] used titanium surface as the scaffold for delivery of ovine ADSCs to repair sheep femur epicondyle defects. Histology and histomorphometry were used to evaluate the implants one month after surgery. Using PKH26 cell-tracking dye, the authors were able to confirm the persistence of ADSCs in the defect area at one month. Unfortunately, based on histomorphometry results, no significant difference in regenerated bone tissue was found among all experimental and control groups.

When human ADSCs (hADSCs) were implanted in immunodeficient animals, they failed to induce any ectopic bone formation in 8 wk[43-46]. Spheroids of human bone marrow-derived MSCs, but not hADSCs, could consistently induce ectopic bone formation in immunodeficient mice[47]. Surprisingly, hADSCs did not survive in the calvarial defects of nude mice after two weeks, although the recipient mice lacked T cells[48]. While the theory of paracrine factors released by hADSCs in these two weeks being sufficient for bone regeneration awaits more investigation, these data raise questions about the usefulness of ADSCs for bone regeneration in healthy (immunocompetent) recipients. Corroborating this notion, ADSCs could not enhance calvarial defect healing in immunocompetent rats[39].

Attempts by other investigators to improve the bone-forming ability of hADSCs, by the addition of BMP-2 have also failed in a femoral defect model in T-cell deficient nude rats[49]. This was proposed to be the consequence of the failure of hADSCs to respond to BMP-2 *in vitro*[50]. In agreement with this finding Runyan *et al*[51] found that recombinant human BMP-2 formed more bone than autologous ADSCs and recombinant human BMP-2 in combination in a porcine model of the periosteal envelope. Keibl *et al*[52] tested a fibrin scaffold embedded with ADSCs and BMP-2 in the treatment of a non-critical size rat femur defect model. At two- and four weeks post-treatment, the authors found no major difference among the groups indicating no effect of BMP-2 on ADSCs potential and ADSCs alone could not induce any bone repair. This questions the ability of ADSCs to induce bone formation and also their ability to respond to BMP-2. Interestingly, this problem could be overcome by overexpressing BMP-2 and BMP-7 both in ADSCs. Qing *et al*[53] reported that only the BMP-2/BMP-7 transduced ADSCs, but not non-transduced ADSCs, BMP-2 only ADSCs, and BMP-7 only ADSCs, showed complete filling of the defect area in rat femur defects. However, the combination of growth factors present in non-activated platelet-rich plasma (nPRP), such as PDGF, TGF- β , bFGF, and VEGF, did not show any

beneficial effect on ADSCs during rabbit calvarial defect healing[54]. There was little difference between the nPRP-ADSCs group, ADSCs alone, and PRP alone in terms of newly formed bone surface or volume.

Mazzoni *et al*[55] evaluated the osteogenic capacity of ADSCs on a hydroxyapatite-collagen hybrid scaffold in 50 patients undergoing malar augmentation. The authors reported the follow-up over three years which showed implant stability and osteointegration but histological samples from patients revealed osteogenesis and mature bone only in 70% of specimens.

Testing human stem cells in T-cell deficient animal models has been a regular practice but that may not be the ideal way to test the potential of ADSCs. Recent advances in the bone regeneration field suggest that certain T-cell subsets, CD4+CD25+FoxP3+ Treg cells being a prominent one, are required for stem cells to initiate the bone formation process. We believe that this could explain, at least partially, the inability of ADSCs to induce bone formation in T-cell lacking mice and rats.

PRECLINICAL STUDIES

Publications on preclinical studies utilizing ADSCs were obtained from OVID Medline using the search keywords: “ADSCs”, “Stem Cells”, “Animals”, “Mesenchymal Stem Cells”, “Tissue Engineering” and “bone regeneration”, which returned 90 studies. An additional 14 studies were included from past collections by the researchers. After the elimination of studies that either lacked *in vivo* experiments, lacked a focus on ADSCs, or overlapped with other sections, 52 pre-clinical studies, investigating the bone-forming ability of ADSCs using various animal models, are summarized in this section and in Table 2.

COMPARISON OF ADSCS WITH SVF AND MSCS

Kang *et al*[56] compared canine MSCs from adipose tissue, bone marrow, umbilical cord blood, and Wharton’s jelly in terms of their osteogenic potential *in vitro* and *in vivo*. ADSCs showed the highest proliferation capacity at all passages *in vitro*. Measured levels of ALP activity were highest in ADSC and umbilical cord blood-derived MSCs. When stem cells were mixed with β -TCP and implanted into the canine segmental defects created in the radial diaphysis, comparable bone healing was observed in all stem cells groups which were significantly higher than the scaffold control group as determined by radiographic union, histology analysis, and the ratio between newly formed bone over total defect size.

Toplu *et al*[57] created the bone defects on the bilateral zygomatic arches of 20 rats. On one side, the defect was left for secondary healing and on the other side, SVF was injected into the defect site. After 20 wk, Micro-CT analysis and histology confirmed a significantly larger volume of newly formed bone in the SVF-injected side[57].

GROUP 1: PRE-DIFFERENTIATED ADSCS

Kim *et al*[58] treated rabbit 20 mm mid-diaphyseal ulna bone defects using SVF on a PLGA scaffold. Animals were treated with scaffold alone, PLGA containing undifferentiated SVF cells, and PLGA with osteogenically induced SVF cells. Since the PLGA-osteogenic SVF group showed significantly higher bone volume, the authors concluded that osteogenic differentiation was necessary for optimal bone regeneration by SVF. Osteogenically induced ADSCs-seeded coral scaffold showed statistically significant more healing of the canine bilateral full-thickness parietal defect model in comparison with control scaffold[59].

Investigators have also explored pre-differentiating ADSCs into endothelial lineage. Shah *et al*[60] compared osteogenesis induced by ADSCs differentiated into osteogenic lineage with those differentiated into endothelial lineage. Undifferentiated control ADSCs and differentiated ADSCs were used to treat rats’ calvarial defects. The authors were not able to find any statistically significant difference in osteogenesis and angiogenesis among these groups. Sahar *et al*[61] also compared ADSCs differentiated into endothelial lineage with ADSCs differentiated into osteogenic lineage when implanted in a critical size rat calvarial defect model. The results showed that undiffer-

Table 2 Summary of the preclinical studies involving bone regeneration induced by transplantation of adipose-derived stem cells

Animal model	Scaffold used	ADSCs per implant	Time frame	Defect healing outcomes	Ref.
-Beagle Dogs; -Unilateral radial segmental defect-10 mm	β -TCP/poly l-lactide-co-glycolide-co- ϵ -caprolactone composite scaffold	1×10^6 canine ADSCs	20 wk	33.90 ± 4.31	Kang <i>et al</i> [56]
-Wistar albino rats; -Middle zygomatic arch defect; -3 mm wide	No scaffold	Rat inguinal fat pad derived SVF	20 wk	The average new bone growth in the experimental group was 1.1 mm, significantly higher than control	Toplu <i>et al</i> [57]
Group 1: Pre-differentiated ADSCs					
-New Zealand white rabbits; -Mid-diaphysis of left ulna; -20 mm long	Porous polylactic glycolic acid scaffold	1×10^6 rabbit SVF cells	8 wk	Approximately 55%	Kim <i>et al</i> [58]
-Beagle dogs; -Parietal bone; -20 mm \times 20 mm full-thickness defect	Coral scaffold	60×10^6 of canine ADSCs	24 wk	84.19 ± 6.45	Cui <i>et al</i> [59]
-Lewis rats; -Calvarial defect -8 mm wide	Polylactic scaffold	0.1×10^6 rat ADSCs	8 wk	Coculture of endothelial- and osteoblast-induced ADSC showed no significant improvement over undifferentiated cells	Shah <i>et al</i> [60]
-Lewis rats; -Calvarial defect; -8 mm wide	Poly (D,L-Lactide) scaffold	0.1×10^6 rat ADSCs	8 wk	Osteogenic-induced ADSC generated $0.91 \pm 0.65 \text{ mm}^3$ new bone, significantly higher than endothelial-induced ADSC	Sahar <i>et al</i> [61]
Group 2: FGF, VEGF, PDGF, and ADSCs					
-Osterix \square mCherry reporter mice; - Closed transverse diaphysis fractures of the right femur	No scaffold	0.3×10^6 wild-type mice ADSCs	35 d	The experimental group induced significantly larger mineralized surface and bone callus compared to cell-free and non-transduced controls.	Zhang <i>et al</i> [62]
-Balb/c nude mice; -Parietal bone defect; -4 mm wide	Whitlockite \square reinforced gelatin/heparin cryogels	1×10^6 human ADSCs	8 wk	> 16%	Kim <i>et al</i> [63]
-CD1 nude mice; -Parietal bone defect; -4 mm wide	Coral scaffold	1.5×10^6 human ADSCs	8 wk	95.40%	Behr <i>et al</i> [64]
-Sprague Dawley rats; -Distal femoral cancellous bone -3.5 mm wide and 5 mm deep defect	Trimodal mesoporous bioactive glass scaffold	20×10^6 cell/mL until saturation; rat ADSCs	8 wk	14.25 ± 3.57	Du <i>et al</i> [65]
-Nu/Nu J mice; -Parietal bone; -4 mm wide	Polycaprolactone - fibrin scaffold containing heparin-conjugated decellularized bone	0.2×10^6 human ADSCs	12 wk	The experimental group induced a significantly larger new bone volume compared to the control without PDGF	Rindone <i>et al</i> [66]
Group 3: BMP and ADSCs					
-Sprague Dawley rats; -Full-thickness parietal bone defect -5 mm wide	Polylactic glycolic acid scaffold	0.0025×10^6 human ADSCs	8 wk	33.3 ± 29.0	Park <i>et al</i> [67]
-Chinese white rabbits; -Full-thickness calvarial defects; -8 mm	Fibrin gel matrix	3×10^6 rabbit ADSCs	12 wk	Approximately 48	Lin <i>et al</i> [68]
-Japanese white rabbits; -Segmental radial defect; -15 mm	Nano-hydroxyapatite/recombinant human-like collagen/poly (lactic acid) scaffold	2×10^6 cells/ml; rabbit ADSCs	12 wk	97.25 ± 2.06	Hao <i>et al</i> [69]

-Taiwan Lee-Sung minipigs; -Mid-shaft left femur defect; -30 mm long	Apatite coated poly (L-lactide-co-glycolide) scaffolds	100×10^6 cells/animal; minipig ADSCs	12 wk	Experimental group's new bone formation showed equivalent density and volume compared to native bone and is significantly better than non-transduced control	Lin <i>et al</i> [70]
-CD-1 nude mice; -Full-thickness parietal bone defect -3 mm wide	Porous poly(lactic-co- glycolic acid) scaffold	3×10^6 cells/mL; ADSC from C57BL/6 mouse	6 wk	77%	Fan <i>et al</i> [71]
-Nude mice; -Parietal bone defect; -4 mm wide	Polylactic glycolic acid scaffold	5×10^5 human ADSCs	12 wk	83%	Li <i>et al</i> [72]
-Nude mice; -Subcutaneous implantation	Porous poly(lactic-co- glycolic acid) scaffold	0.01×10^6 rat ADSCs	4 wk	Transduced ADSC construct induced more bone and vessel formation compared to cell-free and non-transduced control	Weimin <i>et al</i> [73]
-CD \square 1 nude mice; -Right parietal bone defect; -4 mm wide	Polylactic glycolic acid scaffold	0.15×10^6 human ADSCs	6 wk	Up to 100%	Levi <i>et al</i> [74]
-Athymic nude rat; -Mandible defect; -5 \times 5 mm	Chitosan/ chondroitin sulfate scaffold	0.25×10^6 ADSCs from C57BL/6 mouse	8 wk	Approximately 43%	Fan <i>et al</i> [75]
Group 4: Genetically manipulated ADSCs					
-BALB/c nude mice; -Subcutaneous implantation	β -tricalcium phosphate scaffold	2×10^6 human ADSCs	8 wk	Approximately 30%	Wang <i>et al</i> [76]
-Sprague Dawley rats; -Calvarial defect; -8 mm wide and 1 mm thick	Poly (sebacoyl diglyceride) scaffold	Rat ADSCs	8 wk	50.53 ± 4.45	Xie <i>et al</i> [77]
Group 5: Engineered scaffolds and ADSCs					
-C57BL6/J mice; -Mid femur defect; -2 mm	Strontium-substituted hydroxyapatite poly (γ -benzyl-L-glutamate) scaffold	5×10^6 C57BL6/J mice ADSCs	8 wk	Approximately 38%	Gao <i>et al</i> [78]
-Sprague Dawley rats; -Full-thickness femur defect; -4 mm wide	NaB/polylactic glycolic acid scaffold	1×10^6 rat ADSCs	4 wk	ADSC-seeded poly lactic glycolic acid scaffold with 0.05% NaB induced the highest bone density, compared to cell-free control and other concentration of NaB	Doğan <i>et al</i> [79]
-Balb/c nude mice; -Cranium defect; -4 mm wide	SiRNA lipidoid nanoparticle immobilized on polydopamine coated PLGA scaffold	1.0×10^6 human ADSCs	8 wk	Approximately 75%	Shin <i>et al</i> [80]
-Sprague Dawley rats; -Calvarial defect; -5 mm wide	Collagen-resveratrol scaffold	0.05×10^6 human ADSCs	2 wk	Undifferentiated ADSC-seeded construct exhibited better osteogenesis compared to controls and osteoinduced ADSC seeded scaffold	Wang <i>et al</i> [81]
-Athymic nu/nu mice; -Subcutaneous implantation	Alginate microspheres	0.5×10^6 rabbit ADSC	12 wk	Approximately 41%	Man <i>et al</i> [82]
Group 6: Manipulation of recipient host and ADSCs					
-Sprague-Dawley rats; -Calvarial defect; -7 mm wide	Polylactic glycolic acid scaffold	1×10^6 human ADSCs	12 wk	Approximately 60%	Wang <i>et al</i> [83]
-C57 black/DBA mice; -Supracondylar right femur defect -0.9 mm wide	Hydrogel	0.3×10^6 mice ADSC	8 wk	Approximately 50%	Deng <i>et al</i> [84]
-Osteoporotic Sprague-Dawley female rats; -Distal epiphysis left femur defect; -3 mm wide	Gelatin	2×10^6 rat ADSCs	5 wk	Approximately 23%	Li <i>et al</i> [85]

Group 7: Allogeneic ADSCs						
-New Zealand white rabbits; -Ulna defect; -15 mm	Demineralized bone matrix	60 × 10 ⁶ rabbit ADSCs	12 wk	Both allogeneic and autologous ADSC seeded construct induced almost complete defect repair while cell-free control remained unrepaired	Gu <i>et al</i> [86]	
-Sprague Dawley rats; -Ulna defect; -8 mm long	Demineralized bone matrix	60 × 10 ⁶ rat ADSCs	24 wk	Radiographs and histology confirmed superior bone healing in the experimental group compared to cell-free control	Wen <i>et al</i> [87]	
-Beagle Dogs; -Parietal bone defect; -20 × 20 mm	Coral scaffold	60 × 10 ⁶ of canine ADSC	24 wk	Approximately 70%	Liu <i>et al</i> [88]	
-Wistar rats; -Left radius defect; -4 mm long	Heterogeneous deproteinized bone	0.1 × 10 ⁶ rat ADSCs	8 wk	Radiographs and histology confirmed improved healing in osteoinduced ADSC/scaffold group compared to undifferentiated ADSC, cell-free, and blank controls	Liu <i>et al</i> [89]	
Group 8: Non-manipulated or unaltered ADSCs						
Decellularized matrices						
-CD1 nude mice; -Distal femur defect - 3 mm	Human cancellous bone scaffold	0.5 × 10 ⁶ human ADSCs	8 wk	hADSCs-seeded scaffold induced significantly superior defect healing compared to cell-free scaffold	Wagner <i>et al</i> [90]	
-C57BL/6 mice; -Calvarial defect; -4 mm wide	Extracellular matrix deposited on porcine small intestinal submucosa	0.0025 × 10 ⁶ of human ADSCs	4 wk	21.77 ± 6.99	Zhang <i>et al</i> [91]	
-Institute of Cancer Research mice; - Full-thickness parietal defect; -4 mm wide	Decellularized tendon	1.0 × 10 ⁶ human ADSCs	8 wk	86%	Ko <i>et al</i> [92]	
-Sprague Dawley rats; -Two-wall periodontal intrabony defect; -2.6 × 2.0 × 2.0 mm	Amniotic membrane	0.3 × 10 ⁶ human ADSCs	3 wk	ADSC-seeded scaffold resulted in a significantly smaller defect size than the control	Wu <i>et al</i> [93]	
Ceramics						
-Sheep; -Tibia; -3.2 cm long defect	Hydroxyapatite-based particle in a semi-solid milieu	56 × 10 ⁶ human ADSCs	12 wk	The experimental group showed bridging and significantly better healing compared to control	Ben-David <i>et al</i> [94]	
-New Zealand White rabbits; -Full-thickness proximal medial tibia defect; -8 mm wide	Hydroxyapatite	0.2 × 10 ⁶ rabbit ADSCs	8 wk	The new bone area was equivalent between seeded and unseeded scaffold; however, ADSC seeded construct represented preferable histological characteristics	Arrigoni <i>et al</i> [95]	
-New Zealand White rabbits; -Full-thickness proximal medial tibia; -8 mm in diameter	Hydroxyapatite	1.5 × 10 ⁶ rabbit ADSCs	8 wk	ADSC-seeded scaffold exhibited better scaffold resorption than cell-free scaffold and superior histological characteristics compared to all controls	De Girolamo <i>et al</i> [96]	
-Fisher 344 rats; -Calvarial defect; -5 mm wide	Hydroxyapatite	0.4 × 10 ⁵ rat ADSCs	8 wk	16.88 ± 1.52	Xia <i>et al</i> [97]	
-T and B cell-deficient NOD SCID mice; -Subcutaneous implantation	Type I collagen (30%) and magnesium-enriched hydroxyapatite	1 × 10 ⁶ human ADSCs	8 wk	hADSC-seeded presented improved osteogenesis and angiogenesis compared to cell-free scaffold control	Calabrese <i>et al</i> [98]	
-Miniature Pigs; -Mandibular defect -3 cm × 1 cm × 2 cm	Tri-calcium phosphate- poly (D,L-lactide-co-glycolide) scaffolds	5 × 10 ⁶ porcine ADSCs	12 wk	34.8 ± 4.80	Probst <i>et al</i> [99]	

Bioactive glass						
-Wistar rats; -Full-thickness calvarial defect; -8 mm wide	Bioactive glass	0.5×10^6 rat ADSCs	12 wk	ADSC-seeded scaffold group exhibited significantly more bone repair and higher bone density compared to blank control. ADSC construct's result was equivalent to that of autologous bone graft		Saçak <i>et al</i> [100]
-Sprague Dawley rats; -Parietal bone defect; -8 mm wide	Icariin doped bioactive glass	0.5×10^6 rat ADSCs	12 wk	The experimental group saw the complete repair of the defect while all controls showed various degrees of incomplete healing; repair in the experimental group is characterized by mature bone and complete scaffold resorption		Jing <i>et al</i> [101]
Polymers						
-Wistar rats; -Calvarial defect; -5 mm wide	Polycaprolactone scaffold	0.05×10^6 human ADSCs	8 wk	Both undifferentiated and osteo-induced ADSC-seeded scaffold resulted in preferable histological features and higher expression of osteogenesis and angiogenesis markers		Caetano <i>et al</i> [102]
Platelet-rich plasma as carrier material						
-Beagle dogs; -Tibial defects; -10 mm wide	Activated platelet-rich plasma	1.0×10^6 human ADSCs	6 wk	68.97 ± 0.91		Cruz <i>et al</i> [103]
-F344 rat; -Calvarial defect; -5 mm wide	Activated platelet-rich plasma	0.2×10^6 rat ADSCs	8 wk	95.60		Tajima <i>et al</i> [104]
Hybrid materials						
-New Zealand white rabbits; -Calvarial defect; -10 mm wide	Hyaluronic acid-g-chitosan-g-poly (N-isopropylacrylamide) embedded with biphasic calcium phosphate microparticles and PRP	0.1×10^6 rabbit ADSCs	16 wk	The experimental group induced obvious significant bone formation and defect bridging. Cell-free scaffold control showed negligible defect repair		Liao <i>et al</i> [105]
-Sprague Dawley rats; -Parietal defect; -5 mm wide	Multi-layered stacking of electrospun polycaprolactone/gelatin membranes	0.006×10^6 rat ADSCs	12 wk	Up to 90%		Wan <i>et al</i> [106]
-Balb/c nude mice; -Calvarial defect; -4 mm wide	1H,1H,2H,2H-per- fluorodecyl acrylate (97%) and glycidyl methacrylate coated paper scaffold	1.0×10^6 cells/paper human ADSCs	8 wk	92%		Park <i>et al</i> [107]

ADSCs: Adipose-derived stem cells; SVF: Stromal Vascular Fraction; PRP: Platelet-rich plasma.

entiated ADSC or osteogenic ADSC induced a significantly higher amount of bone tissue than endothelial ADSCs group which was equivalent to acellular control.

GROUP 2: FGF, VEGF, PDGF, AND ADSCS

Modulation of the bone-forming ability of ADSCs by expressing genes of FGF, VEGF, PDGF or by tethering these proteins to scaffolds has been reported. Zhang *et al* [62] created bone defects in mouse femurs and injected control ADSCs or ADSCs transduced with bFGF intramuscularly adjacent to the fracture site. While no significant improvement was observed in the ADSCs group, improved bone healing

was observed in the ADSCs-bFGF group. Interestingly, using GFP-assisted observation, the authors identified that at day 21, only a very small fraction of the originally implanted ADSCs remained in the healing callus. This supported the idea that ADSCs' role in bone healing is more reliant on its paracrine function rather than direct cell replacement[62].

Kim *et al*[63] studied VEGF-transduced ADSCs for the treatment of mouse calvarial bone defects. At week 8, microCT and histology revealed that groups with ADSCs exhibited faster bone regeneration. In that, the VEGF overexpression group was found to have significantly more bone healing. hADSCs pre-treated with VEGF also showed beneficial effects. Behr *et al*[64] implanted hADSCs that were pre-treated with 2 µg VEGF into critical-size calvarial defects of nude mice using coral scaffold. Quantification of defect filling at week 8 by microCT showed that the VEGFA treated ADSCs group yielded significantly better bone regeneration than all other groups including untreated ADSCs. VEGF is likely to have a direct effect on the differentiation of ADSCs and it might also increase the bone healing rates indirectly by improving angiogenesis during bone regeneration. Du *et al*[65] have reported that the bioactive glass implant pre-vascularized *in vitro* for 7 d using endothelial cells and then seeded with ADSCs was associated with enhanced angiogenesis and significantly more bone regeneration in rat femur critical size defect compared to acellular scaffold and non-vascularized ADSC-seeded implant.

Similar to FGF and VEGF modulation, PDGF has been reported to improve the osteogenic ability of ADSCs. Rindone *et al*[66] designed a scaffold in which PDGF-BB was anchored using heparin-conjugation and simple electrostatic force. When implanted in murine calvarial defects, the experimental group containing ADSCs and PDGF-BB showed significantly higher bone formation compared to controls without PDGF-BB.

GROUP 3: BMP

BMP-2 is FDA-approved for the treatment of bone injuries and is currently being widely used to treat bone defects. It is known to govern osteogenic differentiation of stem cells. Naturally, it has been the focus of investigation for improving the bone-forming potential of ADSCs.

Park *et al*[67] investigated the ability of BMP-2 transduced ADSCs for the treatment of full-thickness parietal bone defects in rats. Similar to control receiving no implant, scaffold only group and scaffold with osteogenically induced ADSCs group showed either no or incomplete filling. However, mice receiving BMP-2 transduced ADSCs showed complete healing at week 8. Lin *et al*[68] compared bone formation induced by BMSCs and ADSCs in rabbit calvarial defect model. BMSCs and ADSCs were transduced with a BMP4-carrying-adenovirus vector and seeded on a fibrin gel scaffold. Both transduced BMSC and ADSC groups showed a significantly higher amount of newly regenerated bone tissue compared to their respective non-transduced control. No difference was identified between transduced BMSC and transduced ADSC groups. Hao *et al*[69] investigated the potential of BMP-2 overexpressing ADSCs in a rabbit critical size radial segmental defect. The authors reported that animals treated with transfected ADSCs-seeded scaffolds demonstrated recanalization of the radial medulla, bone contour modeling, and scaffold degradation. No significant defect repair was found in either scaffold only or scaffold with non-transfected ADSCs groups.

Lin *et al*[70] overexpressed BMP-2 and VEGF genes in ADSCs and the resulting cells were seeded on a PLGA scaffold which was implanted in a minipig massive segmental left femoral defect model. Bone regeneration in the experimental group was observed as early as two weeks post-procedure and progressively increased to complete union at 12 wk. PET evaluation also revealed improved angiogenesis in the experimental group compared to the control.

Strategies that promote BMP signaling in ADSCs have also been used successfully. Fan *et al*[71] coated PLGA scaffolds with Phenamil and BMP-2 and then seeded the scaffold with ADSCs. Phenamil is a derivative of the diuretic Amiloride, that acts as a powerful stimulator of BMP-2 signaling. The authors hypothesized that using Phenamil would allow optimal osteogenesis while reducing the needed BMP-2 dose to avoid adverse effects. The construct was tested on a mouse calvarial bone defect model. The authors reported that ADSCs-loaded scaffold treated with both Phenamil and BMP-2 induced significantly improved bone regeneration compared to ADSCs-loaded scaffold with BMP-2 alone group as measured by micro-CT. Li *et al*[72]

transduced ADSCs to upregulate expression of BMP-2 and miR-148b using a Cre/LoxP-based baculovirus hybrid before seeding onto gelatin-coated PLGA scaffold. miR-148b is a miRNA identified for its osteogenic property when acting with BMP-2. When this construct was used to treat critical-sized calvarial bone defects in nude mice, the authors found that at 12 wk post-procedure, the experimental group showed significantly improved bone healing compared to controls with either no transduction, transduction with only miR-148b, or only BMP-2. Weimin *et al*[73] expressed LIM mineralization protein 1 (LMP-1) and hypoxia-inducible factor 1 (HIF-1 α) genes in ADSCs to promote osteogenesis. LMP-1 was recognized as a positive intracellular regulator of osteogenesis, upstream of BMP-2, while HIF-1 α initiated angiogenesis[41]. After lentiviral expression of genes in ADSCs, the resulting cells were seeded onto a PLGA scaffold and tested on the dorsal subcutaneous pockets of nude mice. Based on histological analysis, the authors claimed that there was more neo-osteogenesis found in LMP-1 and HIF-1 α expressing ADSCs than found in controls.

Lentiviral delivery of shRNA to inhibit expression of Noggin, an inhibitor of BMP-2 signaling, in ADSCs has been shown to improve their osteogenic potential[74]. The transduced ADSCs induced more rapid and complete healing of the calvaria defect in nude mice in comparison with non-transduced ADSCs. BMP-2 addition and Noggin inhibition together are known to further improve bone healing outcomes. Fan *et al*[75] transfected ADSCs with lentivirus silencing the expression of noggin and loaded them onto a chitosan and chondroitin sulfate scaffold, coated with apatite to ensure BMP-2 attachment and controlled release. The construct induced significantly more bone repair in a rat mandibular defect model in comparison with blank scaffold, scaffold with BMP-2, or scaffold with ADSCs (Nog-/-) at 8 wk.

GROUP 4: GENETICALLY MANIPULATED ADSCS

Wang *et al*[76] found that Prostaglandin G/H synthase 1 (PTGS1) is expressed in ADSCs in response to TNF- α in inflammatory conditions and that PTGS1 knockout ADSCs showed higher osteogenic potential. When PTGS1 knockout ADSCs were mixed with Synthograft (Bicon), a commercial beta-tricalcium phosphate product, and were transplanted into the dorsal subcutaneous tissue of mice, they induced significantly more bone formation at week 8 compared to control[76]. Xie *et al*[77] used lentivirus to transduce ADSCs to upregulate the expression of miR-135, a microRNA recognized for its role in regulating osteogenesis. Transduced ADSCs were implanted in rats' critical-sized calvarial bone defect model. The authors observed almost complete sealing of defect area when treated with miR-135 transduced ADSCs. All other groups showed from nonexistent to incomplete filling of the defect.

GROUP 5: ENGINEERED SCAFFOLDS

Gao *et al*[78] developed a microcarrier from Strontium (Sr)-substituted hydroxyapatite, which was found to release Sr ions, known activators of the Wnt/b-catenin pathway, consistently at the right concentration. When these microcarriers were seeded with ADSCs and implanted into mouse femur nonunion defect, they were found to stimulate significantly more bone formation than control at 8 wk. Doğan *et al*[79] incorporated boron into PLGA scaffold (NaB/PLGA) and seeded ADSCs onto it to test this implant in a rat femur defect model. One month after implantation of ADSC-NaB/PLGA, the ADSC-NaB/PLGA group showed the highest Hounsfield units which represented superior bone regeneration compared to all other groups.

Shin *et al*[80] constructed a system in which siRNA lipidoid nanoparticles, designed to target and silence the osteogenesis inhibitor guanine nucleotide-binding protein alpha-stimulating activity polypeptide (GNAS), were immobilized on PLGA scaffolds, and hADSCs were seeded onto this PLGA scaffold for treating mouse critical-sized calvarial defect. The authors hypothesized that using this system, hADSCs could undergo genetic modification and osteogenic induction after being seeded onto the scaffold, eliminating the need for activation using culture-based protocols. At eight weeks post-procedure, the experimental group showed significantly more bone regeneration in comparison with no treatment control, construct without siRNA, and construct with scrambled siRNA.

Wang *et al*[81] combined collagen scaffold with Resveratrol (RSV), an antioxidant with anti-inflammatory and osteogenic properties, and seeded ADSCs on the construct. The authors reported that hADSCs-seeded collagen-RSV scaffold was the most effective in bone regeneration in a rat calvaria defect model when compared with other groups: collagen scaffold only, collagen scaffold with resveratrol, ADSCs seeded collagen scaffold, and ADSCs seeded collagen scaffold with resveratrol, based on their micro-CT results at 12 wk.

Man *et al*[82] evaluated the effect of PRP on the osteogenic potential of ADSCs encapsulated in alginate microspheres. The microspheres were combined with 5% PRP, 10% PRP or 15% PRP and injected subcutaneously in athymic nude mice. Only groups receiving ADSC-Alginate with 10% and 15% PRP showed mineralization at 1 and 3 mo with the 15% PRP group showing a dose-dependent increase.

GROUP 6: MANIPULATION OF RECIPIENT HOST

Wang *et al*[83] used the hADSC-seeded PLGA constructs for the treatment of rat critical-size calvarial defect and also evaluated the impact of locally injected Alendronate (Aln), a bisphosphonate often used for the treatment of osteoporosis. At 12 wk the acellular groups (control and PLGA-Aln) showed limited bone formation while both PLGA-ADSCs and PLGA-ADSC-Aln showed abundant mature neo-osteogenesis. Complete bridging of the defect was observed only in the PLGA-ADSC-Aln group.

Deng *et al*[84] found that exendin-4 enhanced the ability of ADSC to induce bone regeneration in a mouse femur metaphyseal defect model. Exendin-4 is a glucagon-like peptide 1 receptor agonist previously recognized for its role in glycemic control, control of bone resorption, and increased bone mass[84]. After creating the femur metaphyseal defect, the authors planted hydrogels with ADSCs into the defect site followed by daily intraperitoneal exendin-4. This experimental group was compared with wild-type non-defective bone, defect bone without treatment, and defect bone treated with ADSC only. The results of bone regeneration after 8 wk showed that this experimental group exhibited significantly more repair than ADSCs only group as well as the controls.

Li *et al*[85] reported that miR-214 targeted the Wnt pathway to favor adipogenesis in ADSCs isolated from osteoporotic ovariectomized rats and this microRNA was found at a high level in aged osteoporotic patients as well. Implantation of ADSCs genetically manipulated to silence miR-214, but not unaltered ADSCs isolated from osteoporotic rats, could lead to complete healing of critical size femoral metaphyseal defects in ovariectomized rats when delivered using a gelatin scaffold.

GROUP 7: ALLOGENEIC ADSCS

The use of allogeneic stem cells is currently prohibited by FDA. However, there is limited data available that allogeneic ADSCs can be as effective as autologous ADSCs in rabbit, canine, and rat bone defect models.

Gu *et al*[86] investigated the osteogenic capacity of ADSCs-seeded DBM to treat critical-sized ulna defects in a rabbit model. Micro-CT was used to compare three experimental groups: Allogenic ADSC-seeded DBM, Autologous ADSC-seeded DBM, and DBM only. The authors reported that both Allogenic and Autologous ADSC groups showed bone formation that bridged the defect gap. DBM alone group, on the other hand, did not show bridging of the defect but only loose fibrous tissue. Wen *et al*[87] also used a DBM as a scaffold for allogeneic rat ADSCs to promote bone healing in rat critical-sized ulnar bone defect model. At 24 wk, superior osteogenesis in defects treated with ADSCs-DBM was recognized grossly and radiographically. ADSCs-DBM treatment was also associated with significantly higher ulnar bone strength than those treated with DBM only. Allogeneic ADSCs were shown to be as effective as autologous ADSCs for the treatment of cranial critical-sized defects in canine models as well[88]. There was no significant systemic immune reaction as measured by the ratio of CD4/CD8 as well as serum levels of IL-2, IL-4, IL-10, IFN- γ , and TGF- β 1. MicroCT evaluation showed equivalent bone regeneration between allogeneic and autologous groups with both groups inducing significantly better healing than the scaffold-only group. When GFP-positive ADSCs were implanted, they could still be detected in osteocyte lacunae and bone matrix at 24 wk, pointing to their direct role in osteogenesis.

Liu *et al*[88] found that allogeneic ADSCs require pre-differentiation to be effective. Undifferentiated allogeneic ADSCs failed to induce bone formation. They seeded allogeneic ADSCs on heterogeneous deproteinized bone (HDB) and delivered the construct for the treatment of critical-sized bone defects in a rat radius model[89]. The authors investigated four groups: no implant, HDB implant only, non-induced ADSCs seeded on HDB or osteogenically induced ADSCs on HDB scaffold. It was found that at 8 wk, the group treated with osteogenic ADSCs on HDB showed evident bridging with new bone completely filling the defect area. All other controls, on the other hand, did not improve healing at 8 wk.

GROUP 8: SCAFFOLD TYPES USED FOR NON-MANIPULATED OR UNALTERED ADSCS

To this end, many different materials have been experimented with *in vitro* and *in vivo* in combination with ADSCs. Commonly used materials include decellularized tissues used as matrix, ceramics, polymers, as well as hybrid materials.

Decellularized matrices

Various natural matrices such as human cancellous bone, porcine small intestinal submucosa, bovine tendon, human amniotic membrane, have been used successfully, after their decellularization, for delivery of unaltered ADSCs and this approach has attained reasonable success in enhancing bone regeneration.

Wagner *et al*[90] investigated the combination of hADSCs and freeze-dried human cancellous bone for treatment of femur critical-sized defect in rats. They optimized the seeding density of ADSCs and found that a cell number of 250000 cells (84600 cells/100mm³) was optimal. At 4 wk, the authors observed a significant elevation of bone regeneration in the ADSCs group compared to unseeded control. Zhang *et al*[91] explored a scaffold made from the extracellular matrix (ECM) deposited on porcine small intestinal submucosa (SIS). This porcine SIS construct was cultured with osteoblasts to induce deposition of osteogenic ECM, followed by decellularization and ADSCs seeding. The ADSCs-seeded ECM-SIS scaffold induced significantly more healing of mouse critical-sized calvarial defects than SIS only, ECM-SIS, ADSC-SIS groups. Ko *et al*[92] evaluated decellularized, bovine Achilles and neck tendons as the scaffold for osteogenically induced hADSCs to evaluate bone regeneration in a mouse calvarial bone defect model. The implant was placed in two layers into the calvarial bone defects and its capacity for bone regeneration was evaluated. Results at 4 and 8 wk showed significantly better filling of the defect in the experimental group compared to all controls. Wu *et al*[93] obtained amniotic membranes (AM) during cesarian delivery, decellularized them, and co-cultured with ADSCs to initiate seeding. This construct was used to treat two-wall periodontal bone defects in rats. CT imaging of the defect 29 d after implantation showed a smaller defect volume in the ADSCs-AM group compared to no treatment control, AM only, and ADSCs only.

Ceramics

Hydroxyapatite and β -tricalcium phosphate are the two most widely used matrices in this group and have shown successful outcomes in supporting ADSCs-mediated bone regeneration. ADSCs seeded and grown on hydroxyapatite-based mineral particles could successfully treat full cortex segmental tibial defect in sheep[94]. Following implantation of the ADSCs-seeded particles, statistically higher newly formed bone volume was observed in the treatment group compared to the control. Arrigoni *et al* [95] compared bone regeneration in a rabbit critical-sized tibial defect model mediated by hydroxyapatite alone and ADSCs-seeded hydroxyapatite implant. The authors reported that the ADSCs-seeded group displayed superior performance. de Girolamo *et al*[96] also reported somewhat similar findings in the rabbit model when they used autologous ADSCs seeded hydroxyapatite scaffold to treat full-thickness defects in New Zealand rabbit's proximal tibial epiphysis. At 8 wk, the authors reported that there were no significant differences in defect filling and bone mineral density, but the ADSCs-scaffold group induced the most mature bone that was quite similar to native tissue. The structure of hydroxyapatite is reported to play an important role. Based on *in vitro* results and data from the healing of the rats' bilateral calvarial defects, the micro-nano-hybrid structure, which is a hybrid of nanorod and microrod, was found to be the most effective surface topography for the delivery of ADSCs[97]. Calabrese *et al*[98] evaluated the ectopic bone formation induced by hADSC seeded on a collagen-

hydroxyapatite scaffold through subcutaneous implantation in mice. The scaffolds seeded with ADSCs exhibited faster hydroxyapatite formation and increased vascular generation, both statistically significant in comparison with scaffold control.

Probst *et al*[99] examined the efficacy of pig ADSCs with tri-calcium phosphate poly (lactic-co-glycolic) acid scaffold for regeneration of critical-sized mandibular bone defects in minipigs. ADSCs were induced with an osteogenic medium prior to seeding. MicroCT showed a significantly higher ratio of bone volume to total volume in the ADSCs group in comparison with control but even in the test group, the regenerated bone volume was only about one-third of the defect size.

Bioactive glass

Saçak *et al*[100] investigated bone regeneration in calvarial bone defect of mice using ADSCs seeded bioactive glass. The authors divided the animals into four groups either untreated, treated with autologous bone graft, treated with bioactive glass, or treated with ADSC-seeded bioactive glass. Bone regeneration in the ADSC-seeded bioactive glass group and autologous bone graft treatments were equivalent.

Jing *et al*[101] doped 45S5 Bioglass with Icariin, a flavonoid glucoside isolated from the plant *Herba Epimedii* and then seeded the implant with ADSCs. Implantation of the Icariin-doped, ADSC-seeded scaffold resulted in the complete repair of the rat calvarial defect in 12 wk. Groups receiving no scaffold, Bioglass only, and ADSC-seeded Bioglass without Icariin only exhibited partial repair. The authors reported that when cultured with Icariin, ADSCs upregulate their expression of VEGF, thus promoting angiogenesis which was the mechanism behind their enhanced osteogenic potential.

Polymers

Caetano *et al*[102] evaluated the use of polycaprolactone, a semi-crystalline biodegradable polymer, as a scaffold for human ADSCs to treat critical-size calvarial bone defects in rats. They compared undifferentiated hADSCs with hADSCs cultured in osteogenic conditions. The authors found osteoid tissue larger in size and more organized in groups treated with both types of ADSCs. Immunohistochemical staining revealed that the undifferentiated ADSCs group showed the highest percentage of cells with BMP-2 expression. The two groups with ADSCs showed equivalent angiogenesis, assessed *via* CD31 staining, which was significantly higher than no ADSCs groups.

Platelet-rich plasma as carrier material

Cruz *et al*[103] evaluated the use of platelet-rich plasma activated with calcium chloride as the carrier for ADSCs to treat 10 mm wide, beagle dog tibial bone defects. Four defects were introduced in each animal. The defects were treated with clot, PRP only, autogenous bone graft, or ADSCs-seeded PRP. Histological analysis showed that the PRP-ADSCs group induced significantly more bone formation when compared to control, autogenous bone graft, and PRP only.

Tajima *et al*[104] similarly explored activated PRP as the scaffold for delivering ADSCs to rat calvarial defects. Based on micro-CT results at 4 and 8 wk following the surgery, the authors found that ADSCs-seeded PRP demonstrated significantly larger regenerated bone area and volume compared to treatment with ADSCs-seeded type 1 collagen, PRP only, type 1 collagen only, and PBS control. The authors also confirmed that ADSCs transplanted by this manner differentiated into osteoblasts, by creating a construct using GFP expressing ADSCs-seeded PRP and observing cells staining positive for both GFP as well as osteocalcin.

Hybrid materials

Liao *et al*[105] used hyaluronic acid-g-chitosan-g-poly(N-isopropylacrylamide) (HA-CPN) embedded with biphasic calcium phosphate microparticles and PRP as the organic matrix for delivery of rabbit ADSCs to critical-size cranial bone defects in rabbits. This experimental implant induced significant bone formation, almost completely covering the defect area while the control showed only negligible bone formation at 16 wk.

Wan *et al*[106] designed a construct involving multi-layer stacking of three ADSCs-seeded polycaprolactone/gelatin electro-spun membranes. The construct was implanted into a model of calvarial defects in rats on bilateral parietal bones. The authors reported that the ADSCs seeded multilayer membrane group showed significantly more bone regeneration at higher density than those found in control and scaffold-only groups.

Park *et al*[107] evaluated a paper-based multi-layer scaffold for delivery of ADSCs to a mouse calvarial defect model. Based on their *in vitro* results, the authors determined that a scaffold of commercial weighing paper coated with 1H,1H,2H,2H-perfluorodecyl acrylate (97%) and glycidyl methacrylate was most suitable. The authors compared two stacks of scaffolds only, two stacks of ADSCs-seeded scaffolds, three stacks of alternating ADSCs-seeded scaffolds (A) and HUVEC seeded scaffolds (H), and finally five stacks of alternating A-H-A-H-A scaffolds. All ADSCs seeded scaffolds increased bone healing after 8 wk compared to the blank control and scaffold-only groups. HUVEC-seeding did not show any statistically significant difference but there was a trend of increased bone healing.

SURFACE RECEPTORS EXPRESSION-BASED SELECTION OF SUB-POPULATIONS OF ADSCS AS A RELIABLE STRATEGY TO IMPROVE THE THERAPEUTIC POTENTIAL OF ADSCS

Since investigations utilizing ADSCs in their un-purified and unaltered form have given mixed results, strategies to create ADSCs-based formulation that can enhance bone healing, unambiguously and reliably are necessary. Therefore, the search for the osteogenic sub-population of ADSCs has been initiated worldwide. Since a common molecular marker for all osteogenic progenitors has not been discovered and the precise identity of true skeletal stem cells, required for homeostasis and repair of the postnatal skeleton, remains elusive, investigators have used various surface markers for the selection of osteogenic sub-populations of ADSCs. The results of this investigation are summarized in this section and in Table 3.

CULTURED CELLS VS UNCULTURED SVF

Culture-expanded, horse ADSCs showed superior osteogenic ability when implanted in nude rats than that induced by the uncultured SVF[108].

CD146

Interestingly, a mixture of two distinct FACS-purified hADSCs populations (CD146⁺CD34⁻ and CD146⁺CD34⁺) induced ectopic bone formation and also healed 60% of calvarial defect created in immunodeficient mice[109-111]. When FACS-purified CD146⁺CD34⁻ cells were compared with unpurified SVF for their bone-forming ability using the ectopic bone formation assay and in the calvarial defect model, cells showed greater bone formation. Moreover, BMP2 treated cells showed more bone formation but with a massive adipogenic response. Usage of Nel-1 in place of BMP2 avoided adipogenesis to selectively promote only bone formation[110]. A study has shown that both CD146⁺CD34⁻ and CD146⁺CD34⁺ subpopulations from hADSCs undergo tri-lineage differentiation and express adult stem cell markers CD105, CD90, CD73. CD34⁺ cells pre-cultured in an osteogenic medium for 3 d could induce bone formation in calvarial defects[112].

CD90

FACS-purified CD90⁺ hADSCs, but not unpurified hADSCs, induced bone healing in calvarial defects of nude mice[113]. However, when CD90⁺CD34⁺ hADSCs were implanted in nude mice using a collagen sponge, they generated only adipose tissue after 4 wk[114]. This indicates the relevance of CD34 expression. In another study, mADSCs were FACS-purified into CD90⁺CD105⁻, CD90⁺CD105⁺, CD90⁻CD105⁻, and CD90⁻CD105⁺ populations. Marker expression of cells in basal medium, in osteogenic medium, and BMP2 transfected cells were determined. BMP2 transfection and culturing in an osteogenic medium were found to decrease the expression of CD105 [115]. CD105^{low} and CD90⁺ subpopulations were purified from hADSCs and compared with each other for their osteogenic potential. CD90⁺ cells were found to be more osteogenic compared to CD105^{low} cells *in vitro* as measured by ALP, Alizarin Red staining, and mRNA expression of Runx2, Ocn, Opn. When sorted cells were

Table 3 Specific markers used for selection of sub-populations of adipose derived stem cells showing superior bone forming ability

Ref.	Marker	Study outcome and salient findings
	CD146	
James <i>et al</i> [110]	CD146 ⁺ CD34 ⁺ CD45 ⁻ (Pericytes) + CD146 ⁺ CD34 ⁺ CD45 ⁻ (Adventitial cells)	Intramuscular ectopic bone formation in SCID mice; FACS purified, human, pericytes + adventitial cells produced significantly more ectopic bone formation than SVF; BMP2 enhanced osteogenic as well as adipogenic differentiation, whereas Nel-1 promoted only bone formation when tested in ectopic bone formation assay; 250000 cells were implanted intramuscularly in SCID mice for 4 wk using collagen sponge or DBX+ β -TCP + 3.5 -11.25 μ g of BMP2 or 350 μ g Nel-1
James <i>et al</i> [109]	CD146 ⁺ CD34 ⁺ CD45 ⁻ (Pericytes) + CD146 ⁺ CD34 ⁺ CD45 ⁻ (Adventitial cells)	Human pericytes + adventitial cells together make up around 40% of SVF from human lipoaspirate (60 patients tested) both types representing around 20% and these numbers do not change with age, gender, or body mass index; FACS purified, human, pericytes + adventitial cells induce significantly more healing in mouse calvarial defect than SVF; 250000 cells were implanted to critical size (3 mm) calvarial defect in SCID mice for 8 wk using PLGA
Meyers <i>et al</i> [112]	CD146 ⁺ CD34 ⁺ CD45 ⁻ (Pericytes) + CD146 ⁺ CD34 ⁺ CD45 ⁻ (Adventitial cells)	It was feasible to purify human pericytes + adventitial cells using a multi-column approach of magnetic beads; Purified pericytes + adventitial cells could enhance critical size (4 mm) calvarial defect created in SCID mice; 250000 cells were implanted to critical size (4 mm) calvarial defect in SCID mice for 8 wk using PLGA
	CD90	
Chung <i>et al</i> [115]	CD90 ⁺	CD90 ⁺ cells induced almost complete healing of critical size (4 mm) calvarial defect in nude mice compared to CD105 ^{low} (approximately 75%), CD105 ^{high} (approximately 65%), and CD90 ⁻ (40%) by micro-CT; Taken together CD90 ⁺ cells are more osteogenic compared to CD105 ^{low} cells; 150000 cells were implanted to critical size (4 mm) calvarial defect in SCID mice for 8 wk using PLGA
Ferraro <i>et al</i> [113]	CD90 ⁺ CD34 ⁺	Implantation of human CD90 ⁺ CD34 ⁺ ADSCs in nude mice resulted in the formation of only fat tissue surrounded by loose connective tissue; 250000 cells were implanted subcutaneously in nude mice for 4 wk using a collagen sponge
	CD105	
Levi <i>et al</i> [120]	CD105 ^{low}	FACS-sorted, human, CD105 ^{low} sub-population of ADSCs significantly enhanced bone regeneration (> 95%) in critical size (4 mm) calvarial defect in CD1-nude mice compared to CD105 ^{high} (approximately 40%) and unsorted ADSCs (50%-60%); Knockdown of CD105 in ADSCs (shCD105) resulted in improving their ability to induce bone formation (> 60%) compared to ADSCs transfected with control shRNA (30%); 150000 cells were implanted to critical size (4 mm) calvarial defect in nude mice for 8 wk using PLGA-HA
Madhu <i>et al</i> [123]	CD105 ⁺ CD34 ⁻ ; CD105 ⁺ CD34 ⁺ ; CD105 ⁺ CD34 ⁺ ; CD105 ⁺ CD34 ⁻	FACS-purified, mouse, CD105 ⁺ CD34 ⁻ ADSCs that responded maximally to BMPs <i>in vitro</i> failed to induce ectopic bone formation upon their subcutaneous implantation immunocompetent syngeneic mice; FACS-purified CD105 ⁺ CD34 ⁻ ADSCs responded the least to BMPs <i>in vitro</i> . A bone marrow-derived, clonal, osteoprogenitor population showing the similar phenotype of CD105-CD34- induced robust bone formation; OM preconditioned 1×10^6 cells were implanted subcutaneously in Balb/c mice for 4 wk using Matrigel
Chan <i>et al</i> [128]	AlphaV ⁺ CD200 ⁺ CD105 ⁻ D90 ⁻	Mouse skeletal stem cells that give rise to bone were identified as AlphaV ⁺ CD200 ⁺ CD105 ⁻ D90 ⁻ cells and were present in the femoral growth plate; They were not present in adipose tissue; however, when a collagen sponge loaded with BMP-2 was implanted in adipose tissue, the authors reported de novo formation of AlphaV ⁺ CD200 ⁺ CD105 ⁻ D90 ⁻ cells in the adipose tissue; Subcutaneous implantation of 10 μ g BMP2+ Collagen Sponge in nude mice for 4 wk
Chan <i>et al</i> [131]	PDPN ⁺ CD164 ⁺ CD73 ⁺ CD146 ⁻	The human counterpart of mSSC was discovered and was found to be of phenotype PDPN ⁺ CD164 ⁺ CD73 ⁺ CD146 ⁻ ; Human adipose stroma did not naturally contain these cells but when it was mixed with BMP-2 and injected subcutaneously it led to skeletal reprogramming and induced formation of PDPN ⁺ CD164 ⁺ CD73 ⁺ CD146 ⁻ human skeletal stem cells; 10×10^6 cells with 10 μ g BMP2 + Matrigel were subcutaneously implanted in nude mice for 4 wk
	CXCR4	
Xu <i>et al</i> [133]	CXCR4 ⁺	CD146 ⁺ CD34 ⁺ CD45 ⁻ cells were FACS-purified from hard (human periosteum) and soft (adipose and dermal tissue). Cells isolated from hard tissue but not the soft tissues showed a strikingly high tendency for skeletogenesis; This corresponded to high CXCR4 signaling in periosteal cells; Inhibition of CXCR4 signaling abrogated bone-forming potential of CD146 ⁺ CD34 ⁺ CD45 ⁻ periosteal cells; CXCR4 ⁺ cells from soft tissue (adipose) derived CD146 ⁺ CD34 ⁺ CD45 ⁻ cells represented osteoblastic/non-adipocytic precursor cells; 1×10^6 cells were implanted intramuscularly in nude mice for 4 wk using DBM putty
	PDGFR α	
Wang <i>et al</i> [134]	PDGFR α ⁺	Lineage tracing using PDGFR α reporter mice showed that PDGFR α expression marks different sub-populations in the adipose tissue; PDGFR α ⁺ and PDGFR α ⁻ fractions both are multipotent progenitor cells, however, PDGFR α ⁺ ADSCs-derived ectopic implants ossify to a greater degree than PDGFR α ⁻ cell fractions; 1×10^6 PDGFR α ⁺ or PDGFR α ⁻ cells were implanted intramuscularly in nude mice for 8 wk using HA- β -TCP; Or Subcutaneous implantation of 2.5 μ g BMP2 + Matrigel into the inguinal fat pad of PDGFR α ⁺ -CreER for 8 wk

ADSC: Adipose-derived stem cells; FACS: Fluorescence-activated cell sorting; SCID: Severe combined immunodeficiency; BMP: Bone morphogenetic protein; TCP: Tricalcium phosphate; PLGA: Polylactic glycolic acid; HA: Hydroxyapatite; DBM: Demineralized bone matrix.

implanted into calvarial defects of nude mice, CD90⁺ cells showed more bone formation[113].

CD105, TGF- β 1 SIGNALING AND BMP-RESPONSIVENESS

While ADSCs have been conventionally characterized by positive expression of CD105 [39,40,59,116,117], many groups have also observed considerable amounts of phenotypic variability within ADSCs during *in vitro* expansion [118,119]. Our group and other laboratories are actively investigating the role of CD105 in determining the osteogenic potential of ADSCs. CD105 is the co-receptor of the TGF- β 1 signaling pathway and is known to enhance signaling of the main receptors Alk1 and Alk5 through phosphorylation of the downstream mediators - Smads2/3.

It has been shown that FACS-purified CD105^{low} hADSCs possess more osteogenic differentiation potential than CD105^{high} and unsorted cells *in vitro*, and also show decreased TGF- β 1 and Smad2 phosphorylation. Treatment with TGF- β 1 significantly reduces the osteogenic differentiation of CD105^{low} ADSCs *in vitro*. In contrast, treatment with the Alk5 inhibitor enhances osteogenic differentiation. Moreover, CD105 knockdown promoted the bone-forming potential of ADSCs in immunodeficient animals *in vivo* [120-122].

We FACS-purified 4 different sub-populations of mADSCs; CD105⁺CD34⁺, CD105⁺CD34⁻, CD105⁻CD34⁺ and CD105⁻CD34⁻ and tested their BMP-responsiveness *in vitro*. Only CD105⁺CD34⁻ cells, showing the classical MSCs phenotype, responded to BMPs while others did not show significant response. We hypothesized that the ADSCs population maximally responding to BMPs *in vitro* would possess the ability to induce bone formation, and therefore investigated the bone-forming potential of CD105⁺CD34⁻ ADSCs in immunocompetent mice. Our hypothesis was clearly refuted and CD105⁺CD34⁻ ADSCs could not induce any bone formation [123]. Although we did not test the bone-forming ability of other three FACS-purified populations in that study, we found that bone marrow-derived D1 osteoprogenitor cells isolated from the same Balb/c mouse strain, did not express CD105 and did not respond to BMPs *in vitro*, but showed robust ability to induce bone formation [123,124]. Data from our group and others suggest that CD105⁻ population represents true osteoprogenitors and inhibition of TGF- β 1 signaling can improve the bone-forming ability of ADSCs. However, the bone-forming ability of CD105⁻ ADSCs is not yet established in immunocompetent hosts. FACS purified CD105⁻ human bone marrow-derived MSCs showed superior osteogenic efficacy when compared to CD105⁺ cells *in vitro*. In critical-size defects created in the tibia of canine, CD105⁻ MSCs implantation led to superior bone healing with complete bone remodeling, while CD105⁺ MSCs implants failed to remodel resulting in the defect site filled with fibrocartilaginous tissue [125]. In sum, these studies showed that CD105⁻ cells have more osteogenic potential *in vitro* as well as *in vivo*.

We have shown that simultaneously inhibiting TGF and BMP signaling pathways by using small chemical inhibitors induces neuronal differentiation of hADSCs *in vitro* and neurite outgrowth *in vivo* [126]. Previously this was demonstrated in ESCs and iPSCs, but not in adult ADSCs. It is well established that activin/nodal signaling contributes to the maintenance of pluripotency of hESCs. Activin/nodal/TGF- β and BMP pathways naturally antagonize each other because they compete for a common signal transducer Smad4. Inhibition of activin/nodal/ TGF- β signaling results in trophoblast differentiation, similar to induction of trophoblast differentiation by BMP-4 [127]. These findings reveal the crucial roles of TGF- β and BMP signaling in deciding the fate of ADSCs.

In a recent discovery, the phenotype of mouse skeletal cells (mSSC) has been described as the CD45⁻Tier119⁻Tie2⁻AlphaV⁺Thy6C3⁺CD105⁻CD200⁺ cells which were isolated from femoral growth plates of the mice [128]. These CD105⁻ cells were able to form bone *in vivo* when implanted beneath the kidney capsule of T-cell deficient mice. Surprisingly, these cells were not efficiently engrafted, suggesting their requirement for a supportive niche. When these cells were transplanted with unsorted cells, they could form both bone and cartilage. Blocking VEGF signaling promoted chondrogenesis. Subcutaneous implantation of BMP2 in a collagen sponge in mouse inguinal pad formed ectopic bone; however, it did not originate from circulating SSCs recruited to implanted sites but SSCs formation was induced in the adipose tissue. It is not clear whether the CD105⁺ or CD105⁻ population of adipose tissue contributed to SSCs formation and this ectopic bone formation. Co-delivery of BMP2 with VEGF inhibitor into adipose tissue favored cartilage formation over bone [128]. We have shown that the crosstalk between BMP and VEGF signaling pathways enhances osteogenic differentiation of hADSCs through the p38 signaling pathway. Mineralization was abrogated when the p38 signaling pathway was inhibited [129]. We also found that VEGF could crosstalk with a downstream signal mediator of BMP, LIM mineralization protein 1 (LMP1) to enhance cell mineralization and ectopic bone formation mediated

by osteoprogenitors[130]. Similar to mSSCs, human skeletal stem cells (hSSCs) formation has also been reported, by the discoverers of mSSCs, in BMP2 treated adipose tissue. hSSCs displayed the phenotype PDPN⁺CD73⁺CD146⁺CD146[131].

CD271

CD34⁺CD271⁺ hADSCs showed increased osteogenic differentiation compared to CD34⁺CD271⁻ and SVF whereas adipogenic and chondrogenic differentiation were similar[132].

CXCR4

FACS purified CD146⁺CD31⁻CD45⁻ hADSCs isolated from different origins such as the periosteum, adipose, and dermal tissue display different degrees of osteogenic capabilities. Periosteal cells also express standard adult stem cell markers (CD105, CD90, CD73), Gli1, PDGFR α , and CXCR4; and are known to be more osteogenic *in vitro* as well as *in vivo* unlike soft tissue-derived CD146⁺CD31⁻CD45⁻ ADSCs. Inhibition of CXCR4 expression abolishes the ability of these ADSCs to induce ectopic bone formation. Unsorted ADSCs as well as CD146⁺ ADSCs further selected for CXCR4⁺ show enhanced osteogenic potential *in vitro* and *in vivo*[133].

PDGFRA

PDGFR α ⁺CD34⁺, PDGFR α ⁺CD34⁻, PDGFR α ⁻CD34⁺, and PDGFR α ⁻CD34⁻ were sorted from SVF of mouse adipose tissue from PDGFR α ⁺CreER and PDGFR α ⁻CreER mice. The authors found that PDGFR α ⁺CD34⁺ ADSCs displayed more osteogenic potential *in vitro*. They also found that subcutaneously implantation of PDGFR α ⁺ cells and subcutaneous implantation of BMP2 into inguinal fat pads of PDGFR α ⁻CreER mice formed more bone as compared to controls[134].

CD105 AND SSEA3 EXPRESSING MUSE CELLS

Multilineage-differentiating stress-enduring (Muse) cells were first identified from bone marrow, which are of interest. These cells are positive for mesenchymal and embryonic stem cell markers CD105 and SSEA3. Muse cells comprise a small population of MSCs in BM-MSCs (1%-2%) and ADSCs (5%). 250000-500000 cells can be obtained from one gram of lipoaspirate. Adipose-derived Muse cells spontaneously differentiate into all three germ layers: mesodermal, endodermal, and ectodermal cell lineages and have non-tumorigenic and immunomodulatory properties. Muse cells have been successfully used for regeneration of skin, muscle, liver, kidney in different animal disease models however it has not been tested for its osteogenic differentiation potential[135].

Thus, the selection of subpopulations of ADSCs can harness abundantly available ADSCs for applications in bone regeneration.

CONCLUSION

The safety of ADSCs is reasonably established since they have been tested in 79 clinical trials including 580 patients total and there have been no serious adverse events reported. However, the clinical trials, as well as the pre-clinical studies investigating the potential of ADSCs in enhancing bone regeneration, have given confounding outcomes. In some cases, they were reported to enhance bone healing whereas, in others, they have failed to do so. It is also difficult to compare outcomes of different studies as investigators have used different animal models, delivery methods, and genetic manipulation of ADSCs. In many of the pre-clinical studies, T cell-deficient hosts were used. This transplant scenario is unlikely to provide a realistic picture of the osteogenic potential of ADSCs since T cells are likely to modulate bone regeneration induced by exogenously added adult stem cells. After careful review of

all the published reports, it is safe to conclude that ADSCs in their unaltered and unpurified form cannot be considered as reliable therapy for bone repair yet. Two major steps can be taken to solve this problem - first is to develop potency assays for each batch of ADSCs used in clinical and pre-clinical studies to allow comparison of outcomes of different studies and second is to search for a unique and reliable set of surface markers to define ADSCs. The current definition of adult stem cells can no longer be applied to ADSCs since both CD105⁻ as well as CD105⁺ fractions of ADSCs have been shown to possess bone forming potential. Surface markers such as CD146, AlphaV, CD200, PDPN, CD164, CXCR4, and PDGFR α will play an important role in defining osteogenic population within ADSCs in coming years. Areas such as the role of endogenous bone-progenitors in bone regeneration induced by exogenously added ADSCs and BMP-responsiveness of ADSCs also need immediate attention. Most of the studies published so far have not evaluated the survival and differentiation of transplanted ADSCs as well as recruitment of endogenous bone-progenitors to investigate whether the regenerated bone is donor stem cells-derived or originates from endogenous precursors. While BMPs are thought to promote differentiation of stem cells into the osteogenic lineage and BMP-overexpression has increased bone-forming potential of ADSCs in certain animal models, some investigators have also reported that ADSCs do not respond to BMPs. This observation and recent findings that implantation of BMP in adipose stroma leads to skeletal reprogramming and de novo formation of skeletal stem cells in adipose tissue, together, demand urgent attention of the scientific community to signaling pathways of ADSCs during osteogenic differentiation and after BMP stimulation. VEGF, BMP and TGF- β signaling pathways are the most important ones in this regard. Although the current clinically tested ADSC therapies do not yet appear to induce bone repair reliably, the ADSC optimizations described in this manuscript, based on cell subset purification and stimulus/activation, show great promise, and could potentially dominate stem cell-based therapies such as bone regeneration in the future.

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