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TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Concise Review: Cell-Based Strategies in Bone Tissue Engineering and Regenerative Medicine

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ABSTRACT

Cellular strategies play an important role in bone tissue engineering and regenerative medicine (BTE/RM). Variability in cell culture procedures (e.g., cell types, cell isolation and expansion, cell seeding methods, and preculture conditions before in vivo implantation) may influence experimental outcome. Meanwhile, outcomes from initial clinical trials are far behind those of animal studies, which is suggested to be related to insufficient nutrient and oxygen supply inside the BTE/RM constructs as some complex clinical implementations require bone regeneration in too large a quantity. Coculture strategies, in which angiogenic cells are introduced into osteogenic cell cultures, might provide a solution for improving vascularization and hence increasing bone formation for cell-based constructs. So far, preclinical studies have demonstrated that cell-based tissue-engineered constructs generally induce more bone formation compared with acellular constructs. Further, cocultures have been shown to enhance vascularization and bone formation compared with monocultures. However, translational efficacy from animal studies to clinical use requires improvement, and the role implanted cells play in clinical bone regeneration needs to be further elucidated. In view of this, the present review provides an overview of the critical procedures during in vitro and in vivo phases for cell-based strategies (both monoculture and coculture) in BTE/RM to achieve more standardized culture conditions for future studies, and hence enhance bone formation. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:98–107*

INTRODUCTION

Bone is one of the most transplanted tissues, with more than 2.2 million bone graft procedures being performed annually worldwide [1]. Bone tissue engineering/regenerative medicine (BTE/RM) approaches, with the triad principle of applying combinations of the three building blocks: supporting scaffolds, growth factors, and functionally active cells to (re)generate biologically functional tissues, have been suggested as promising strategies to regenerate bone [2].

The potential of BTE/RM constructs becomes especially challenging under compromised conditions, such as in elderly patients with suboptimal medical conditions (e.g., osteoporosis, diabetes, and cancer), or in cases in which the bone defect dimensions are (far) beyond those that can spontaneously heal. Consensus on the difficulty of healing bone defects under such conditions illuminates that the bone regenerative capacity arising from only a scaffold material is often insufficient, and that additional BTE/RM approaches should arise from preseeding the scaffold with cells or incorporating growth factors within the scaffolds. Small successes have been reported for in vitro experiments and even animal studies with cell-laden scaffolds, but translation of these results to the clinic for bone regenerative applications has been insignificant so far [3]. Several issues can be attributed to the lack of this clinical success. First, the quality and quantity of the used cells and the preculture conditions after cell seeding onto the scaffolds are variable and limited, and tiny variations within these procedures may substantially influence the outcome. Second, cells within a construct are subjected to inflammatory conditions and limited nutrient supply on implantation because surgical intervention generates tissue damage and the diffusion of nutrients and oxygen from the nearest capillary is limited to only 150 μm to 200 μm [4]. Researchers have pointed out that rapid vascularization into cell-based BTE/RM-constructs is pivotal to clinical success [3]. From a cellular point of view, the solution for insufficient vascularization is either coculture of osteogenic cells with angiogenic cells [5] or changing the priming differentiation pathway of stem cells (SCs) from osteogenic to chondrogenic, because cartilage is an avascular tissue with less susceptibility to limited vascularization [6].

The aim of this review is to summarize the current state-of-the-art in cell-based BTE/RM in terms of critical procedures and efficacy of monoculture (osteogenic) and coculture approaches.

Although the authors are aware of the major importance of scaffold properties and the potential of growth factor incorporation and release, the intention is to focus on the cellular component in BTE/RM approaches, and hence critically review the experimental, preclinical, and clinical efforts on this topic.

Critical Procedures

To restore bone defects in clinical applications, some critical issues that are inherently related to the cell quality and quantity (e.g., cell types/sources, cell isolation, and yield), cell seeding efficiency and preculture conditions must be considered, and finally in vivo conditions should be taken into consideration.

Stem Cell Sources

A source of human cells that can be derived in large numbers from a small and easy initial harvest and can differentiate into bone-forming cells is preferable for cell-based BTE/RM constructs [7]. Various cell types have been explored for BTE/RM, each with its own potential and premise.

Nonadult SCs

Nonadult SCs contain two categories: embryonic stem cells (ESCs) and SCs isolated from perinatal tissue, such as aborted fetal tissue and discarded tissue at birth (e.g., umbilical cord and placenta). ESCs are pluripotent, but consistency on bone formation capacity by ESCs progeny has not been achieved [8, 9] and ethical issues exist. The latter category, positioned between embryonic and adult SCs, is multipotent SCs. These cells have similar bone formation capacity compared with adult mesenchymal SCs (MSCs) [10]. Nevertheless, that they can form tumors [11] on in vivo implantation makes the use of these cell types controversial.

Adult SCs

Adult MSCs play a predominant role in the field of BTE/RM. The most common sources are bone marrow (BM), adipose tissue (AT), and dental pulp (DP).

BM-MSCs

BM is the predominant source for adult MSCs. BM-derived MSCs (BM-MSCs) have multipotential differentiation capacity, including osteogenic potential, and can perform pericyte-like functions by secreting both angiogenic and stabilization factors in the process of vessel formation [12]. Although BM-MSCs are widely used in laboratory and preclinical studies, they have several disadvantages, including donor site morbidity and side effects, limited proliferation capacity, and inferior differentiation potential in aged individuals [13]. Thus, alternatives to BM-MSCs have been explored.

AT-MSCs

AT is a popular alternative source for MSCs because of easy and less invasive harvest procedures and larger yield compared with BM [14]. AT-derived MSCs (AT-MSCs) are similar to BM-MSCs regarding gene expression and osteogenic capacity [15] and can also exert pericyte-like functions [16]. Nevertheless, the bone-forming capacity of AT-MSCs needs further confirmation, and whether AT-MSCs exhibit bone-forming capacity similar to that of BM-MSCs is still controversial. Moreover, preclinical safety and efficacy as well as long-term in vivo studies are required before AT-MSCs can be evaluated clinically.

DP-MSCs

The noninvasive manner of obtaining DP from deciduous/extracted teeth or even nonextracted crown fractured teeth makes it an ideal source for MSCs. DP-derived MSCs (DP-MSCs) have similar gene expression, a faster proliferation rate, and a higher percentage of SCs in the harvested population compared with BM-MSCs [17] and express pericyte markers [18]. Some researchers have reported that DP-MSCs have at least equal bone-forming capacity compared with BM-MSCs [19, 20], and others showed only the formation of connective tissue [21] or dentin pulp-like complex formation [22] from DP-MSCs, suggesting the requirement for in-depth studies on the mechanism of bone-forming capacity by DP-MSCs.

Isolation and Expansion

High-quality cells in relevant quantities are often crucial to satisfy clinical demand (i.e., the successful restoration of a bone defect). To achieve this, effective cell isolation and expansion of harvested cells are of utmost importance. Inadequate isolation methods can lead to polluted isolates and hence inconsistency in cell marker expression [23]. Further, long-term expansion has demonstrated decreased stem cell proliferation and differentiation capacity [24]. Nevertheless, a broadly accepted protocol has not been established for isolation and large-scale expansion of human MSCs, which also makes the comparison among reported results from publicly available databanks difficult.

Isolation of BM-MSCs, AT-MSCs, and DP-MSCs

The isolation of MSCs is a general procedure, meaning tissue harvesting and treatment (mincing and/or enzymatic digestion), washing, filtering and centrifugation, and plating. BM-MSCs can be obtained from either bone chips (cortical or trabecular bone) or BM. The SCs isolated from these two sources have been shown to be identical in terms of phenotype and multilineage differentiation capacity [25]. AT-MSCs can be isolated from a resected adipose block or lipoaspirate. The latter method is easier because lipoaspirate consists of finely minced fat fragments with less volume and a more homogeneous population of cells. Isolation of MSCs from DP basically relies on two methods. The first one is the outgrowth method (i.e., cell migration out of the pulp fragments and adherence to cell culture plates), which is rarely applied because it takes substantially longer to obtain similar cell numbers compared with enzymatic digestion. This latter method is comparable to the earlier mentioned digestion method for BM-MSCs (Fig. 1). Bone chips, resected AT, and DP tissue require mincing, whereas BM and lipoaspirate proceed directly to a washing procedure. After washing, enzymatic digestion is performed for all the tissues except BM aspirates followed by filtering and gradient density centrifugation. The isolated cells are plated for adhesion-dependent selection. To increase the purity of the isolated cells, cell separation methods such as cell sorting using cell surface markers (e.g., CD73, CD105, and Stro-1) are frequently used (Fig. 1).

Expansion of BM-MSCs, AT-MSCs, and DP-MSCs

For expansion, essential culture parameters comprise the type of basic culture medium and the nutritional source (e.g., serum), cell passaging density, and doubling numbers. α-minimum essential

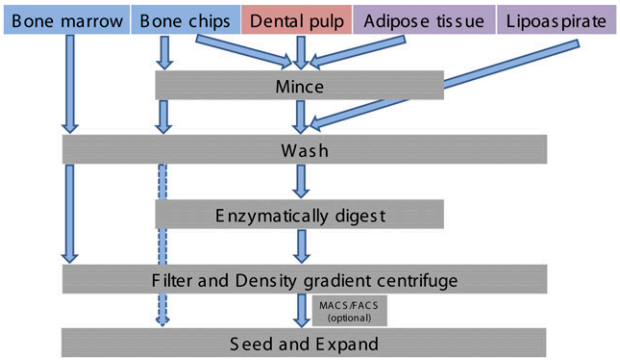

Figure 1. Scheme for isolation of mesenchymal stem cells derived from bone, adipose tissue, and dental pulp. Abbreviations: FACS, fluorescence activated cell sorting; MACS, magnetic activated cell sorting.

medium (α-MEM) is the optimal bare culture medium for isolation and expansion of human BM-MSCs [26], AT-MSCs [27, 28], and DP-MSCs [29, 30]. Human serum [31] or human platelet lysate (PL) [32–34] has been explored as a replacement for fetal bovine serum (FBS) because FBS carries potential hazards related to bovine pathogens and immunological issues. No consensus regarding optimal cell-seeding density for expansion exists, although low-density cell seeding has been recommended (e.g., for BM-MSCs, 50~100, or 1,000 cells per cm^2 for expansion; for AT-MSCs, 100~200 cells per cm^2, and for DP-MSCs, 800~1,000 cells per cm^2) because this is associated with higher proliferation rates [26, 35, 36].

Small variations within each step of an isolation procedure, including enzyme type and enzymatic digestion time, centrifugation speed and time, or washing liquid (either phosphate-buffered saline or sodium saline), may change the quality and quantity of the isolated SCs and hence influence the experimental outcomes. Additionally, the site of tissue harvest can influence the phenotype of the isolated SCs [37], the percentage of SCs in the whole population [38], and their osteogenic differentiation capacity [39]. However, this review did not reach any significant conclusion about standardized and optimized SC isolation and expansion procedures, which sheds light on the need for relative studies.

Cell-Seeding Techniques and Preculture on Scaffolds

Cell seeding and preculture on scaffolds are essential procedures before in vivo implantation. Seeding requirements for cell-based BTE/RM constructs for potential clinical use should allow maximized utilization of donor cells, minimal time for anchorage-dependent and shear-sensitive cells (e.g., osteoblasts) in suspension, and spatially uniform distribution of attached cells [40].

Cell-Seeding Efficiency

Cell-seeding efficiency, which might further correlate with bone-forming capacity, can be increased by either selecting proper scaffolds and modifying the scaffold surface or by optimizing cell-seeding methods. For the former method, the criteria for three-dimensional scaffolds are explored in tandem with properties such as porosity, interconnectivity, biodegradability, and mechanical integrity. As such, diverse forms of scaffolds (e.g., bulk vs. hydrogels; fibrous vs. foam) with different components (e.g., polymers, ceramics, and metal) have been explored. Various scaffold properties affect cell-seeding efficiency; for instance, scaffolds that have more regular and homogeneous pores and are more accessible for the cell suspension during drop seeding have higher cell-seeding efficiency (e.g., a fiber deposited vs. a foam scaffold) because they avoid cell aggregate entrapment in the small and irregular internal pores [41]. Furthermore, scaffold surfaces can be modified through pretreatment with adhesive proteins (e.g., preimmersion in serum or fibronectin). For the latter method, cell-seeding volume and cell-seeding time affect cell-seeding efficacy as well as cell viability significantly [41]. Moreover, the combination of low pressure (vacuum) with vibration can help remove potential air bubbles around scaffolds and allow more cells to penetrate deeply into the scaffolds to enhance bone formation, especially for porous scaffolds, in comparison with the commonly used static seeding methods [42]. Additionally, dynamic cell seeding (e.g., perfusion bioreactor) has shown to yield higher cell-seeding efficiencies and more homogenous cell distribution compared with static cell seeding [43].

Cell-Seeding Density and Medium Perfusion

Cell proliferation after cell seeding is mainly regulated by contact-inhibition between adjacent cells, which is determined by cell seeding density [44] and nutrient transfer efficiency as a result of the medium perfusion rate, which also determines cell viability [45].

Initial seeding density can alter the expression of osteogenic genes by controlling the distance of paracrine signals among cells. Although no systematic studies on the optimal cell-seeding density are available because of the variety of scaffold properties (Table 1), it has been agreed that a certain threshold of cell density is essential to achieve successful bone regeneration in vivo. Low seeding densities may compromise cellular contact and hence influence bone formation, and high seeding densities do not necessarily benefit cell behavior because the overloaded cells may result in limited nutrient transport and insufficient waste removal from the internal structures [46]. In general, below a certain threshold (i.e., the optimal density), osteogenic marker expression and extracellular matrix production (i.e., mineralization) capacities are enhanced with increasing cell-seeding density. In contrast, when the cell-seeding density exceeds this optimal density, a further increase in cell-seeding density can reduce the bone regenerative capacity of cells [46].

Medium perfusion is increased in dynamic cultures using bioreactor systems, leading to an improved nutrient delivery and mechanical stimulation to the cells as well as enhanced osteogenic differentiation compared with static cultures [47]. Three classes of bioreactor systems have been widely used in BTE: spinner flasks, rotating wall, and perfusion systems. Each of these has proven effective to culture cells for BTE. Perfusion systems have been shown to perfuse media effectively throughout the scaffold rather than creating only a homogenous media solution on the exterior of a scaffold [48].

Predifferentiation of Cells

Predifferentiation of the cells into osteogenic [49, 50] or chondrogenic lineage [6, 51] before in vivo implantation has been assumed essential for enhancing osteogenesis in vitro and in vivo in comparison with nonpredifferentiated (i.e., osteogenic or chondrogenic) cells. However, the optimal preculture time for bone regeneration is either controversial (for osteogenic differentiation) or lacking (for chondrogenic differentiation). Short osteogenic induction is probably insufficient for inducing cell

Table 1. In vivo preclinical studies involving human mesenchymal stem cells transplantation (monocultures) in BTE/RM

Cell sources	Scaffold	Seeding density	In vitro preculture time	In vivo time (max)	Species and repair sites	Evaluation method	Efficacy of bone formation (Fold change, cell seeded/cell-free)	Reference
BM-MSCs	Mineralized collagen	$20x10^6$/construct (5 cm^3)	1 day PM	24 weeks	Sheep tibia	Histology	Bone percentage (%): 18.0/13.0 = 1.4	[77]
BM-MSCs	Chitosan	$1x10^6$/construct (5 mm Ø, 1 mm H)	2 weeks OM	8 weeks	Mouse calvarium	Micro-CT	More bone formation in cell-seeded constructs: (No quantitative analysis)	[78]
BM-MSCs	PCL	$3x10^6$/construct (5 mm Ø, 9 mm H)	1 day PM	12 weeks	Rat femur	Micro-CT	Bone volume (mm^3): 25.0/10.0 = 2.5	[10]
BM-MSCs	PCL/β-TCP	$1x10^6$/construct (4 mm Ø, 8 mm H)	1 day PM	3 weeks	Rat femur	Micro-CT	Bone volume (mm^3): 1.0/0.3 = 3.3	[79]
BM-MSCs	Hyaluronic acid	$1x10^6$/construct	1 day PM	4 weeks	Rat calvarium	Histology	Bone percentage (%): 61.0/16.0 = 3.8	[14]
BM-MSCs	PLGA	$0.5x10^6$/construct	9 days in PM +14 days in OM	20 weeks	Rat calvarium	Histology	Bone percentage (%): 54.0/20.0 = 2.7	[80]
BM-MSCs; AT-MSCs	Collagen	Not clear	Not clear	8 weeks	Rat calvarium	X-ray	Bone mineral density percentage (%): 100.0/60.0 = 1.7 (for both BM-MSCs and AT-MSCs)	[81]
AT-MSCs	Collagen; PLGA; HA	$1x10^6$/construct (size is unclear)	8-12 hours PM	18 weeks	Rat calvarium	Histology	Bone mineral density (g/cm^2): 4.0 (collagen); 1.6 (PLGA); 2.0 (HA)	[82]
AT-MSCs	PLGA	$0.5x10^6$/construct (8 mm Ø, 0.8 mm H)	2 weeks OM	12 weeks	Rat calvarium	Histology	Bone percentage (%): 72.0/38.0 = 1.9	[54]
AT-MSCs	Ti	$1x10^6$/construct (5 mm Ø)	1 week OM	8 weeks	Rat calvarium	Histology	Bone percentage (%): 151.0/57.0 = 2.6	[60]
AT-MSCs	PLGA	$0.15x10^6$/construct (size is unclear)	1 day PM	8 weeks	Mouse calvarium	Histology	Bone percentage (%): 100.0/42.0 = 2.4	[83]
AT-MSCs	BCP	$75x10^6$/ml suspension	1 day PM	16 weeks	Rat femur	Histology	Bone percentage (%): 89.0/40.0 = 2.2	[84]
DP-MSCs	Collagen	$1x10^6$/construct (13 mm Ø, 1.5 mm H)	10 days OM	6 weeks	Rat parietal bone	Histology	Bone percentage (%): 69.0/38.0 = 1.8	[85]

Abbreviations: AT, adipose tissue; β-TCP, β-tricalcium phosphate; BCP, biphasic calcium phosphate; BM, bone marrow; BTE, bone tissue engineering; CT, computed tomography; DP, dental pulp; H, height; HA, hydroxyapatite; Ø, diameter; OM, osteogenic medium; PCL, polycaprolactone; PLGA, poly(lactic-co-glycolic acid); PM, proliferation medium; RM, regenerative medicine; Ti, titanium.

Search conditions for Table 1:

For human BM-MSCs:

(i) (human bone marrow mesenchymal stem cell* OR human bone marrow derived mesenchymal stem cell* OR human bone marrow mesenchymal stromal cell* OR human multipotential stromal cell*) AND (bone tissue engineering OR bone regenerat* OR bone form*) AND (spinal OR cranial OR cavarial OR femur OR femoral OR radial OR jaw OR mandible OR mandibular OR maxilla OR tibia), and

(ii) (human bone marrow mesenchymal stem cell* OR human bone marrow derived mesenchymal stem cell* OR human bone marrow mesenchymal stromal cell* OR human multipotential stromal cell*) AND (bone tissue engineering OR bone regenerat* OR bone form*) AND (animal* OR in vivo).

For human AT-MSCs:

(i) (human fat derived mesenchymal stem cell* OR human fat tissue derived mesenchymal stem cell* OR human adipose tissue derived mesenchymal stem cell* OR human lipoaspiration) AND (spinal OR cranial OR cavarial OR femur OR femoral OR radial OR jaw OR mandible OR mandibular OR maxilla OR tibia), and

(ii) (human fat derived mesenchymal stem cell* OR human fat tissue derived mesenchymal stem cell* OR human adipose tissue derived mesenchymal stem cell* OR human lipoaspiration) AND (animal* OR in vivo).

For human DP-MSCs:

(i) (human dental pulp mesenchymal stem cell* OR human dental pulp derived mesenchymal stem cell* OR human dental pulp stem cell*) AND (spinal OR cranial OR cavarial OR femur OR femoral OR radial OR jaw OR mandible OR mandibular OR maxilla OR tibia), and

(ii) (human dental pulp mesenchymal stem cell* OR human dental pulp derived mesenchymal stem cell* OR human dental pulp stem cell*) AND (animal* OR in vivo).

differentiation, whereas long duration osteogenic induction leads to an apoptotic process [52]. Longer preculture time (2 weeks) has been shown to induce more bone formation compared with shorter time (1 week) for both human BM-MSCs [53] and human AT-MSCs [54]. From a clinical point of view, one-step surgical techniques, in which freshly isolated cells (without expansion) are used directly within the operation theater, are recommended for clinical applications of cell-based bone regenerative strategies [55]. This suggests that a balance is needed between the bone regeneration efficacy caused by the relatively longer preculture time and the benefits that patients gain from the one-step surgery.

The aforementioned seeding techniques and preculture conditions (for monocultures) are also used for cocultures. Additional parameters should be considered for cocultures, including choice of cell origin and cell type, culture medium, and cell ratio. The effects of origin of either MSCs (e.g., BM-MSCs or AT-MSCs) or angiogenic cells (e.g., ECs, endothelial progenitor cells [EPCs]) need further investigation. Furthermore, it should be noted that cellular communication in cocultures is cell-type specific. For instance, when ECs were replaced by primary chondrocytes or fibroblasts in osteoblasts/ECs coculture, no increasing effects on alkaline phosphate activity were observed [56]. The culture medium used in cocultures is different depending on the research aims (i.e., angiogenesis or osteogenesis). EC culture medium (EM) has been shown to be more suitable for vessel formation and stabilization, whereas osteogenic medium (OM) has been shown to favor osteogenic differentiation of MSCs [57]. It was reported recently that more bone formation was observed for cells cocultured continuously in OM compared with sequential culture medium variations (e.g., first culturing cells in EM and subsequently in OM) before in vivo implantation [58]. Further, it was recently shown that BM-MSCs/ECs cultured in OM achieved higher mineralization while maintaining angiogenic capacity in vitro [5]. This indicates that OM may be a more optimal culture medium for cocultures for future research on bone regeneration capacity of such cell-based constructs. A cell ratio of 1:1 is often chosen in cocultures, which was also demonstrated recently as optimal for both BM-MSC/EC [5] and AT-MSC/EC [59] cocultures.

One of the crucial aspects of BTE/RM is the evaluation/prediction of bone healing capacity for the tissue-engineered constructs. To further test such bone-forming efficacy, in vivo studies including both preclinical research and clinical trials are necessary.

Preclinical Studies

Monocultures

Because MSCs from humans (rather than other animal cell source) are the cell type essential for future clinical application, and orthotopic implantation (i.e., bony defects) is the preferred model to mimic the clinical situation compared with ectopic implantation sites, a literature review on preclinical studies using BM-MSCs, AT-MSCs, and DP-MSCs of human origin for repair of bony defects was performed in PubMed. A total of 13 such preclinical studies were available, among which 7 included BM-MSCs, 6 included AT-MSCs (including one paper studying both BM-MSCs and AT-MSCs), and 1 included DP-MSCs (Table 1). For human BM-MSCs, three studies used rat cranial defects, one used mouse cranial defects, one used sheep tibial defects, and two used rat femoral defects. For human AT-MSCs, four studies used rat cranial defects, one used mouse cranial defects, and one used rat femoral defects. For human DP-MSCs, the only available study used rat parietal bone defects. Cell-based constructs demonstrated more efficacy in restoring bone defects in comparison with cell-free constructs, with a fold range of 1.4 to 3.8, 1.6 to 4.0, and 1.8 compared with cell-free control constructs for BM-MSCs, AT-MSCs, and DP-MSCs, respectively.

Cocultures

Although animal studies confirmed the capability of cell-based constructs to form bone and integrate with the host tissues, the repair efficacy could be more promising once the key hurdle of insufficient vascularization can be overcome, which would require clinically sized, fully viable bone grafts to be engineered and implanted. A 50% loss of viable cells in monoculture cell-based constructs, as shown by the presence of apoptotic cells, was observed within two days after implantation in vivo [60], most likely because cells encountered an ischemic and inflammatory environment [3]. In view of this, coculture approaches have shown advantages in both vascularization and bone formation compared with monocultures (Table 2). Currently, six preclinical studies have been reported on orthotopic implantation using tissue-engineered constructs seeded with cocultures using human MSCs (i.e., four for BM-MSCs, two for AT-MSCs, and none for DP-MSCs) (Table 2). For human BM-MSCs, one study used rat cranial defects, two used rat femoral defects, and one used rabbit ulna defects. These studies demonstrated a range fold of bone-forming efficacy of 1.2 to 1.4 (cocultures compared with MSCs monocultures) and vessel-forming efficacy of 1.0 to 10.0 (compared with MSCs monocultures) or 2.5 to 2.7 (compared with ECs/EPCs monocultures). For human AT-MSCs, the studies demonstrated a range fold of bone-forming efficacy of 0.3 to 1.3 (cocultures compared with MSCs monocultures). Although cocultures of human DP-MSCs with ECs have been shown to enhance osteogenic and angiogenic potential in vitro [61], in vivo studies using DP-MSCs in coculture approaches are still lacking.

Fate of the Implanted Cells

After in vivo implantation, the fate/contribution of the seeded cells (both MSCs and ECs/EPCs) and the possible mechanisms of their bone-forming (for MSCs) or vessel-forming (for ECs/EPCs) capacity have been explored. The seeded MSCs can proliferate and differentiate into osteogenic lineages to contribute directly to bone formation [62, 63] and have the potential to recruit cells from the host, which can be MSCs [64] or ECs/EPCs [64, 65], to induce/enhance bone formation indirectly. Similarly, implanted EPCs may also act in this way on bone formation by either forming stable vasculature through coculturing with MSCs [66] or by secreting chemotactic factors (e.g., vascular endothelial growth factor) to recruit ECs/EPCs from the host and thereby stimulate vascularization in bone defects [67]. Several studies have shown the synergistic contribution to bone formation [68] as well as vessel formation [69] of both donor and host cells. Most of the studies demonstrated that bone [14, 70] and blood vessel formation [69, 71] were mainly from the implanted donor cells.

Clinical Studies

MSCs have been used in clinical trials in various fields of RM such as treatment of graft versus host disease, diabetes mellitus, amyotrophic lateral sclerosis, and liver failure. However, to our knowledge, human clinical trials addressing cell-based constructs for bone repair are limited to case reports (Table 3), which

Table 2. In vivo preclinical studies involving human MSC transplantation (cocultures) in BTE/RM

Cell sources (cell ratio and medium)	Scaffold	Seeding density	In vitro preculture time	In vivo time (max)	Species and repair sites	Evaluation method	Efficacy of bone and vessel formation (fold change, coculture/monoculture)
BM-MSCs/ECs (not clear, PM)	PLGA	1×10^6 /construct (8.5 mm Ø, 2 mm H)	1 hr	12 wk	Rat calvarium	Histology	Bone percentage (%): 33.0/23.0 (MSCs) = 1.4; donor-derived vessels percentage (%): 2.0/0.2 (MSCs) = 10.0 [86]
BM-MSCs/EPCs (1:1, PM)	β-TCP	0.5×10^6/construct (granule, 0.7–1.4 mm)	0 d	8 wk	Rat femur	Histology	Bone percentage (%): 1.2/1.0 (MSCs) = 1.2; vessel density (/mm^2): 0.4/0.4 (MSCs) = 1.0 0.4/0.15 (EPCs) = 2.7 [87]
BM-MSCs/EPCs (1:1, PM)	β-TCP	0.5 x 10^6/construct (granule, 0.7–1.4 mm)	0 d	1 wk	Rat femur	Histology	New bone mass: 7.2/5.4 (MSCs) = 1.3; vessel density (/mm^2): 3.2/0.45 (MSCs) = 7.1 3.2/1.3 (EPCs) = 2.5 [88]
BM-MSCs/ECs (1:1, OM)	β-TCP	1.0 x 10^6/construct (3.5 x 2 x 15mm)	2 wk	16 wk	Rabbit ulna	Histology	Bone percentage (%): 37.0/29.0 (MSCs) = 1.3; vessel percentage (%): 5.9/4.9 (MSCs) = 1.2 [89]
AT-MSCs/ECs (2:1, OM)	PCL/PLGA/ β-TCP	0.3 x 10^6/construct (8 mm Ø, 2 mm H)	2 d	12 wk	Rat calvarium	Micro-CT	Bone percentage (%): 74.0/58.0 (MSCs) = 1.3 [90]
AT-MSCs/ECs (1:1, OM)	Ti	1 x 10^6/construct (5 mm Ø)	1 wk	8 wk	Rat calvarium	Histology	Bone percentage (%): 44.0/151.0 (MSCs) = 0.3 [60]

Search conditions for Table 2:
Based on the search conditions from Table 1, additional search condition of (coculture* OR co-culture* OR co-seed* OR coseed* OR cotransplant* OR co-transplant* OR co-implant*) OR (endothelial cell* OR endothelial progenitor cell*) was added.
Abbreviations: AT, adipose tissue; BM, bone marrow; BTE, bone tissue engineering; ECs, endothelial cells; EPCs, endothelial progenitor cells; H, height; MSC, mesenchymal stem cells; Ø, diameter; OM, osteogenic medium; PCL, polycaprolactone; PLGA, poly(lactic-co-glycolic acid); PM, proliferation medium; RM, regenerative medicine; TCP, tricalcium phosphate; Ti, titanium.

emphasizes the need for randomized control trials and systematic clinical studies. The available case reports cover long bone defects, jaw defects, alveolar cleft regeneration, and sinus augmentation. A search on clinicaltrials.gov using a combination of search terms revealed 24 relevant studies. Eight studies included the terms "bone marrow derived mesenchymal stem cells" AND "bone." Two studies included the terms "adipose tissue derived mesenchymal stem cells" AND "bone." No studies included the terms "dental pulp stem cells" AND "bone." Five studies included the terms "mesenchymal stromal cells" AND "bone." Two studies included the terms "mesenchymal stromal cells" AND "bone regeneration," and seven studies included the terms "stem cells" AND "bone regeneration."

These studies are dedicated to the treatment of bone defects and diseases, such as bone cysts, osteoarthritis, osteonecrosis, spinal fusion, and atrophic nonunion fractures using human MSC monocultures. Some of these studies are still recruiting patients. To date, BM-MSCs are still the main source for clinical trials as well as case reports, and reports on the use of coculture-based constructs for clinical bone regeneration are lacking. Clinical studies conducted so far have demonstrated that it is safe to use human MSCs for bone regeneration, but the outcomes are substantially less promising compared with those of animal studies. In two patients, only 34.5% and 25.6% of bone regeneration (of the original defect volume) was found after four months when BM-MSC-loaded demineralized bone matrix was used to restore human alveolar cleft defects [72]. Meijer et al. observed bone regeneration in 50% (3/6) of patients, and bone formation was induced in only one patient by the tissue-engineered construct when BM-MSCs seeded β-tricalcium phosphate granules were implanted in jaw defects. A remarkable finding of this study was that despite the low number of construct contributions to clinical bone formation, parallel ectopic implantations with the constructs in nude mice showed bone formation in all specimens [73]. The discrepancy between preclinical results and clinical outcomes is likely explained by several causes. As mentioned, because of the unstandardized cell preparation protocol, the cell populations used are often heterogeneous, which makes comparison among outcomes from different studies difficult. In addition, the long-term survival and proliferation of implanted cells and to what extent these cells can contribute to bone formation remain largely unknown, especially in critical sized bone defects. In view of this, cell tracing such as luciferase labeling enables real time in vivo evaluation of the cells, which is particularly interesting to determine the contribution and distribution of the implanted cells. Finally, the comparison of bone regeneration mechanisms between animals and human beings at a molecular level is necessary to understand the underlying reasons further.

Conclusion

Bone regeneration in clinics is compromised in some cases, for example, in patients with typical risk factors like smoking or diabetes, and the incidence of these risk factors is increasing from 5% to 10% (for a healthy population) up to 40% [74]. Cell-based BTE/RM has emerged as an attractive approach for bone regeneration in preclinical studies, although no definite answer about its use has been given for clinical studies. A standardized method

Table 3. Clinical studies using human MSC-based tissue-engineered constructs

Cell sources and treatment	Scaffold	Number of patients	Repair sites	Evaluation method(s)	Reported outcomes
BM-MSCs	HA	3	Long bone defects (1 tibia, 1 ulna, 1 humerus)	X-rays and micro-CT scans	Abundant callus formation along the implants and good integration at the interfaces with the host bones 2 months after surgery [91]
BM-MSCs	HA	4	Long bone (limb)	X-rays and micro-CT scans	No major complications occurred; complete integration between implants and host bone 5–7 months and maintained 6–7 years after surgery [92]
BM-MSCs (with PRP)	Ti	1	Alveolar cleft	Micro-CT scans	Regenerated bone bridged the cleft after 6 months [93]
BM-MSCs	DBM/Calcium sulphate	2	Alveolar cleft	Micro-CT scans	There is 34.5% and 25.6% integrity of the bone defects for the two patients [73]
BM-MSCs (with fibrin glue)	Ti	1	Mandible	Dental CT scans	Successful bone regeneration in large segmental defects of the jaw [94]
BM-MSCs	BCP	7	Sinus augmentation	X-rays and biopsy	The mean percentage of the newly formed bone was 41%; bone height was 11 mm after 1 year compared with the initial height of 2 mm, with a successful rate of 93% (28/30) for dental implants [95]
BM-MSCs	HA (particles)	6	Jaw	X-rays and biopsy	Bone regeneration was observed in 50% (3/6) of the patients, and bone formation was induced by the tissue-engineered construct in only 1 patient [96]
BM-MSCs	Allogenic mandible	3	Mandible	Micro-CT scans	Bone healing was observed in 2 of the 3 patients [97]
AT-MSCs (with BMP-2)	β-TCP	1	Maxilla	X-rays	Regenerated bone resembled mature maxillary bone 2 months after surgery [98]
DP-MSCs	Collagen	17	Mandible	X-rays and biopsy	Bone regeneration in the defects was complete and stable 1 year after surgery [99]

Search conditions for Table 3:
(human mesenchymal stem cell* OR human stem cell* OR human mesenchymal stromal cell* OR human marrow stromal cell*) AND (clinic* OR case report* OR clinical trial*) AND (bone tissue engineering OR bone regenerat* OR bone form*).
Abbreviations: AT, adipose tissue; BCP, biphasic calcium phosphate; BM, bone marrow; BMP, bone morphogenetic protein; CT, computed tomography; DBM, demineralized bone matrix; DP, dental pulp; HA, hydroxyapatite; MSC, mesenchymal stem cells; PRP, platelet-rich-plasma; TCP, tricalcium phosphate; Ti, titanium.

Table 4. Opportunities and threats for cell-based constructs in clinical application for BTE/RM

Opportunities	Threats
Various cell sources available with osteogenic capacity	Limited cell number for clinical cases in which there are large defects, or for one-step surgery
Through expansion, sufficient cell numbers can be obtained	Longer expansion may lead to cell deformation and decreased differentiation capacity
Efficient cell seeding on scaffolds can be achieved before in vivo implantation	Cell survival is compromised after in vivo implantation
Successful bone regeneration using cell-based constructs has been demonstrated in preclinical studies	There have been less promising outcomes of bone regeneration using cell-based constructs in clinics
In some clinical case reports using cell-based strategies for BTE/RM, bone regeneration was observed	Because of the lack of a control group in studies and case reports, the contribution of implanted cells cannot be ascertained

Abbreviations: BTE, bone tissue engineering; RM, regenerative medicine.

of using cell-based tissue engineering constructs in clinical work contains a protocolized cell isolation and culture method; valid characterization of the isolated cells, and efficient cell seeding with an optimized cell source, scaffold type, and cell seeding parameters (e.g., density, time, and volume). A proper predifferentiation method and time are also needed.

Based on the promising outcomes from preclinical studies, in which cell-based constructs (irrespective of monoculture and cocultures) enhanced bone formation compared with acellular constructs, and cocultures showed more pronounced vessel and bone formation than monocultures, it is logical and straightforward to proceed the translational step from bench to bedside. Because of the limited number of systematic clinical studies and the lack of proven examination methods (e.g., biopsy), very few studies were able to demonstrate the efficacy of cell-based strategies for BTE/RM in a clinical setting. Therefore, a number of areas of ongoing active research are directly relevant to the translation of cell-based constructs for bone regeneration into clinics, including development of bioreactor systems for standardized and scalable cell expansion methods; scale up to clinically sized (large) bone

constructs with potential for further integration with host tissues; evaluation of engineered bone grafts in large animal models; rapid establishment of vascularization through the implanted bone graft; and marketing of off-the-shelf products. For the benefit of patients, intraoperative graft manufacture, also called one-step surgery, in which the graft is assembled during the surgical procedure, may be of interest. A prerequisite for this method is obtaining sufficient cell numbers for cell seeding and bone regeneration without expansion. Moreover, by using cocultures, the needed number of MSCs can be reduced while maintaining equal vascularization and bone formation.

As an overview, the opportunities and threats for cell-based tissue engineered constructs for clinical application in BTE/RM are listed in Table 4. For clinical studies, several aspects should be considered. There is a need for a reduction of the time lapse from cell isolation to in vivo implantation to avoid long waiting times and patient discomfort and ensure the multipotential differentiation capacity of MSCs, which can be lost during (long-term) expansion. Because of the immunomodulatory properties of MSCs [75], standardized off-the-shelf products containing allogeneic MSCs seem feasible. The contribution of implanted MSCs to the regenerative process is still unclear because of the combination of osteoinductive scaffolds and/or growth factors and the lack of control groups (e.g., bare scaffolds) in clinical trials. To determine the regeneration efficacy of cell-based constructs, quantitative measurements should be performed, whereas the current assessment for bone formation is mostly radiological examination (e.g., x-ray and micro-computed tomography). This makes it difficult to distinguish between newly formed bone and the implanted scaffolds, especially when radiopaque scaffolds are used. It is necessary to identify donors with appropriate osteogenic capacity before in vivo implantation. There is no certain correlation between osteogenic capacity in vitro and bone formation in vivo [76], although researchers are seeking indices to help predict the bone formation capacity. Longer follow-up periods are recommended to evaluate the long-term safety and efficacy of human MSC-based tissue-engineered constructs in bone regeneration.

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Author Contributions

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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