

Toward Advanced Therapy Medicinal Products (ATMPs) Combining Bone Morphogenetic Proteins (BMP) and Cells for Bone Regeneration

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Abstract Cell-based implants with or without osteoinductive biomolecules on optimal carrier materials as an advanced therapeutic medicinal product (ATMP) are a promising strategy for poorly healing long-bone defects. This chapter will focus on ATMPs combining bone morphogenetic proteins (BMPs) and progenitor cells for the clinical treatment of large bone defects in compromised environments. We describe BMP signaling involved in the process of bone fracture healing with specific emphasis on clinically relevant BMP ligands, followed by characterization and BMP responsiveness of progenitor cells obtained from different sources. Then we explore different biomaterials and their contribution to achieve optimal BMP release and osteoinduction. Finally, we provide a perspective on the applicability of ATMPs in bone repair by reviewing the preclinical studies carried out so far in various animal models. We believe the era of regenerative medicine has just started. First-generation BMP and stem cell technologies have demonstrated that in the postnatal environment, one can successfully enhance the healing of damaged tissues by recapitulating the principles of developmental tissue formation. A second generation of products is needed that leads to successful bone healing in compromised environments.

Keywords Bone tissue regeneration • Bone morphogenetic proteins • Bone marrow stromal cells • Periosteal-derived cells • Pluripotent stem cells • Scaffold • Advanced therapy medicinal product • Animal model

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1 Introduction

Long-bone fractures are most frequently the result of trauma but can also be associated with a variety of conditions including osteoporosis, infection, tumors, and congenital diseases. Moreover, over 10 % of tibia fractures lead to delayed healing or nonunion, which greatly affects quality of life for the individual. This patient population ultimately demands an effective restoration strategy to fulfill functional requirements. Current state of the art for the reconstruction of skeletal defects involves transplantation of autologous or allogenic bone grafts, which can be harvested from sites such as the iliac crest, fibula, scapula, or radius [189]. However, the inherent drawbacks of this approach, including insufficient autologous resources, pain, and donor-site morbidity, strongly urge clinicians and researchers to explore alternative therapeutic strategies.

Several alternative strategies are emerging to treat nonhealing fractures: (1) a “smart” biomaterial device with or without growth factors, which is frequently used in non-compromised conditions, and (2) an advanced therapeutic medicinal product (ATMP) composed of cell-based implants with or without osteoinductive biomolecules on optimal carrier materials, which is typically targeted for use in compromised conditions. The combined factors in such ATMP should function synergistically with a potent regenerative effect. Hypothetically, when implanted *in vivo*, they act as a robust engine steering bone formation and integration, subsequently leading to successful healing of the defect [104]. Indeed, it is envisioned that cell-based ATMPs can overcome the limited and defective regenerative capacity of the patient. Moreover, in contrast to the single use of growth factors which seems to require high doses, the combined cell growth factor ATMP is expected to eliminate the necessity of supraphysiological doses of growth factors which could potentially induce adverse clinical complications [25]. It is anticipated that the soluble growth factors will stimulate proliferation and differentiation of progenitor cells both in carriers and defect site to form new bone tissue. Meanwhile, the implanted progenitor cells cross talk with the surrounding tissue via the secretion of signaling molecules to accelerate tissue formation, integration, and remodeling.

This chapter will focus on ATMPs combining bone morphogenetic proteins (BMPs) and stem cells for the clinical treatment of large bone defects in compromised environments. We will describe the BMP signaling that is involved in the process of bone fracture healing with specific emphasis on clinically relevant BMP ligands, followed by characterization and BMP responsiveness of stem cells obtained from different sources. Then we will explore different biomaterials and their contribution to achieve optimal BMP release and osteoinduction. Finally, we will provide a perspective on the applicability of ATMPs in bone repair by reviewing the preclinical studies carried out so far in various animal models.

2 Lessons from Biology: BMP Signaling Involved in Bone Healing

2.1 *Biological Fundamentals of Bone and Fracture Healing*

Bone formation during embryonic development involves three distinct structures that generate the skeleton. The somites give rise to the axial skeleton, the lateral plate mesoderm generates the limb skeleton and the cranial neural crest gives rise to the craniofacial cartilage and bones. Depending on the bone to be formed, two major modes of bone formation occur where both involve the transformation of a pre-existing mesenchymal tissue into bone tissue. Intramembranous ossification is a slow process that involves direct conversion of mesenchymal tissue into bone, primarily giving rise to the flat bones of the skull. The second bone-forming process, endochondral bone formation, gives rise to the long bones through a process where progenitor cells differentiate into cartilage, which subsequently is degraded, remodeled, and replaced by bone.

Throughout the life span of an individual, bones continuously undergo remodeling, leading to changes in bone size, shape, and density during growth and load-induced damage, adapting the bone to an individual's development. This remodeling process is tightly coordinated between bone-forming osteoblasts and bone-resorbing osteoclasts, the latter ones originating from hematopoietic stem cells. The interplay between these cells is regulated on both the systemic and local level by hormones, cytokines, mechanical signals, and metabolites. Imbalance, upon aging or immobilization, between bone formation and resorption, often leads to reduced bone density, osteoporosis, and fractures [68].

In healthy individuals, the skeleton acts as a scaffold by providing support and protection for the soft tissues that together make up the body. Subsequently, the bone has a complex structure and can stand high-impact and mechanical load. Fracture occurs upon severe trauma or on minor trauma in diseased bones such as osteoporosis. The majority of the fractures can heal spontaneously, due to the high regenerative potential of our skeletal system. The healing process, initiated by trauma causing the fracture, can be divided in four stages: (1) initial inflammatory response and hematoma formation, (2) callus formation, (3) remodeling of callus to immature bone, and subsequently (4) remodeling to mature lamellar bone [127]. During the initial inflammatory response, cytokines and growth factors are secreted by cells at the fracture site to recruit skeletal progenitor cells from mostly the periosteum to aid in the succeeding stages [7]. The nature of secreted stimulatory signals is partially driven by the type of fracture, hence also which healing process that will be initiated.

Fracture healing can occur through two different routes, depending on the mechanical stabilization of the fracture: intramembranous (stable fractures) or

endochondral (unstable fractures) fracture healing. In the former, osteoblasts directly produce and deposit woven bone. This process often takes place in impact or compression fractures, where the mechanical stability is high. In more mechanically unstable fractures, bone is formed through an intermediate cartilaginous tissue that can function under hypoxic conditions. The cartilage intermediate contributes to stabilization of the fracture, and upon matrix calcification, angiogenesis occurs and with new bone formation and remodeling through resorption by osteoclasts delivered through the invading blood vessels.

In clinics, over 10 % of annual tibial fractures lead to delayed or nonunions, due to the critical size of the defect, severely damaged or infected surrounding tissue, and/or genetic disorders [47]. Typically, nonunions can be characterized as hypertrophic or atrophic nonunions or a combination of both (Fig. 1) [131]. Hypertrophic nonunions are caused by excessive motion at the fracture site, causing abnormal vascularity and abundant callus formation, and these can often be successfully treated by a stabilizing fixation. Atrophic nonunions are the result of inadequate biological conditions, causing fibrous tissue to fill the fracture.



Fig. 1 Long-bone fractures. A fracture of long bones such as tibiae heals spontaneously under normal conditions (a). Under specific circumstances, the fracture can develop into an atrophic (b) or hypertrophic (c) nonunion (Radiographic images received from Professor, J. Lammens, UZ Leuven, Belgium)

2.2 *BMPs Involved in Bone Development and Fracture Healing*

Among the different ligands of the BMP family, BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-9, and GDF5 play important roles during bone development and fracture healing. During the early stages of non-compromised endochondral fracture healing, BMP-2, BMP-4, BMP-5, BMP-9, and GDF5 can be detected in activated periosteal cells and inflammatory cells in the granulation tissue [28]. As the fracture healing progresses, the expression level of these signals decreases/fluctuates. The proliferating chondrocytes express BMP-2, BMP-6, BMP-7, and BMP-9, while pre-hypertrophic chondrocytes express BMP-2, BMP-6, and BMP-7. Once cells have differentiated to hypertrophic chondrocytes, they are strongly positive for BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-9 [28, 222].

While many of the BMP ligands can exert a similar function during fracture healing as in bone development, some of them seem to play more crucial roles than others. For instance, global loss of BMP-2 leads to embryonic lethality [224]. In a limb-specific knockout of BMP-2, embryogenesis was not affected but spontaneous postnatal fractures occurred that did not heal. These data confirm that other ligands cannot compensate for the absence of BMP-2, hence ratifying its crucial role in postnatal bone development and fracture repair [196]. In similarity to BMP-2, BMP-4 and BMP-7 are present during all stages of bone development and regeneration. However, both have been reported dispensable in these processes in mice [138, 197, 198].

Nonsense mutations of the BMP-5 gene give rise to a short-ear phenotype in mice and lead to reduced plate growth and height as well as body mass [87, 133]. Upon fracture, these mice display a delayed formation and maturation of the cartilaginous fracture callus, only half the volume of healthy fracture callus [60].

BMP-6 is highly expressed in the growth plate as well as during the different stages of fracture repair. However, the BMP-6 ligand is not crucial for skeletal development, maintenance, or fracture healing [59, 100]. Nevertheless, BMP-6 mutant mice displayed a reduced size of long bones, impaired growth plate function, and a delayed ossification of the developing sternum [149, 182]. GDF5, another member of the BMP family, is found throughout the growth plate of the developing long bones, and mutations in this gene have been shown to cause impaired joint morphogenesis and brachypodism in mice and man [185, 194]. During fracture repair, deletion of GDF5 does not compromise long-term fracture healing, but a delay in callus formation and remodeling suggests a role in the early phase of bone repair [30]. BMP-9 is mainly known for its regulatory role in angiogenesis, evidenced by arteriovenous malformations in BMP-9-deficient mice [155, 218]. Interestingly, recent research efforts suggest BMP-9 to be one of the most osteogenic ligands, and a first report on skeletal malformations in BMP-9-deficient mice

is currently being processed [155]. Moreover, additional support for BMP-9 as an interesting osteoinductive factor was evidenced by its role during trauma-induced heterotopic ossification [58].

The BMP signaling pathway is strictly regulated; hence, BMP antagonists are also present in fluctuating levels during fracture healing. In cartilage- and bone-forming cells as well as in granulation tissue, BMP-3, noggin, chordin, gremlin, SMAD6, and SMAD7 have been detected [222]. Moreover, BMP ligands and receptors, phosphorylated SMAD1/5/8, and BMP inhibitors are also expressed in nonunions in similarity to non-compromised fractures [92]. Interestingly, an imbalance between the level of ligands and inhibitors was reported with the most striking differences in the early cartilaginous tissue intermediates. Potentially, the disrupted balance in BMP signaling may be a mechanistic cause of the nonunion (Table 1).

2.3 Current Status of BMPs in Clinical Application

Since the discovery by Marshall Urist of BMPs and their potent bone-inducing capacity in 1965, comprehensive research efforts have led to the characterization of several ligands from the family. When it comes to bone regenerative medicine and the treatment of nonunions, BMP-7 and BMP-2 have gained most attention

Table 1 The functions of different BMPs during bone development and fracture healing

| BMP- | Knockout phenotype | Fracture healing | References |
|-------|--|---|---------------------------------|
| BMP-2 | Embryonically lethal; limb, spontaneous fractures and impaired fracture repair; chondrocyte, severe chondrodysplasia | Expressed during inflammatory, chondrogenic, and osteogenic stages | [28, 90, 91, 181, 196, 224] |
| BMP-4 | Embryonically lethal, limb: defective patterning | Expressed during chondrogenic and osteogenic stages | [28, 90, 91, 174, 210] |
| BMP-5 | Short-ear mice, reduced growth plate height, growth rate, and body mass | Expressed during mesenchymal condensation, delayed fracture callus formation and maturation | [60, 87, 133] |
| BMP-6 | Delay in sternum ossification, smaller long bones | Expressed during inflammatory, chondrogenic, and osteogenic stages | [28, 149, 182] |
| BMP-7 | Die after birth, defect in skeletal patterning, in limb: no effect | Expressed during chondrogenic and osteogenic stages | [28, 38, 90, 91, 119, 171, 198] |
| BMP-9 | Skeletal malformations, phenotype not yet published | Decreased mean levels in nonunions | [200] |
| GDF5 | Brachypodism, joint phenotype delayed callus formation, and remodeling | Expressed during chondrogenic stage | [28, 30] |

for a number of reasons including biotech-driven focus. In 2001 and 2002, FDA approved the clinical products OP-1® (BMP-7) and Infuse® (BMP-2) for the treatment of long-bone nonunion and anterior lumbar interbody fusions, respectively [2, 44]. In the following years, these approvals were extended to posterolateral fusion, posterolateral lumbar pseudarthrosis, and nonhealing tibia shaft fractures [3–5, 140].

Currently, 11 clinical trials are registered under bone morphogenetic proteins for critical bone fractures, one for BMP-7 and the remaining for BMP-2 [71] (Table 2). In the majority of these studies, the BMP ligand is delivered through the use of an adsorbable collagen sponge (ACS), a calcium phosphate matrix (CPM), or as a liquid solution in buffer. The investigated concentrations of BMP-2 are reported between 1–12 mg/ml, and the product efficacy in fracture healing was compared to autograft or allograft transplants.

Reports from these studies display that approved BMP devices function as an alternative treatment, providing similar efficacy as autologous transplants, but does not result in an superior outcome [33, 46, 81, 121]. Even though promising, a debated therapeutic outcome has been reported due to safety issues and side effects possibly related to the usage of supraphysiological doses [49, 187, 211].

2.4 BMP Signaling Pathway

2.4.1 Ligand-Receptor Binding and Oligomerization

When inducing physiological cellular responses, BMP ligands activate intracellular signaling by binding to their respective transmembrane receptors. The active receptor complex involves typically one of the type 1 receptors, activin receptor-like kinase-1 (ALK)1., ALK2, ALK3, or ALK6, and one of the type 2 receptors, BMP-receptor type 2 (BMPR2) or activin type 2 receptor (ACVR2 or ACVR2b) [178]. It has been reported that BMP-2 and BMP-4 preferentially and predominantly bind to ALK3 or ALK6, whereas BMP-6 and BMP-7 primarily bind to ALK2 [41, 193]. Moreover, BMP ligands bind to type 1 and type 2 receptors with different affinities, likely due to their structural conformation [96]. For instance, while BMP-2 and BMP-4 bind with high affinity to their type 1 receptor, BMP-7 binds with high affinity to the type 2 receptors ACVR2a or ACVR2b and less to the type 1 receptors [57, 94].

Ligand-receptor oligomerization occurs through two different mechanisms, formation of a pre-formed receptor complex (PRC) or a BMP-induced receptor complex (BRC), causing distinct downstream signaling mechanisms [139]. PRC induces signaling through the SMAD-dependent signaling pathway, while BRC-induced signaling activates the (mitogen-activated protein kinases) MAPK pathway (Fig. 2). The difference in downstream signaling, induced by the oligomerization mechanism, has been explained by two different endocytosis routes, clathrin dependent or independent [54, 69, 139].

Table 2 Clinical trials involving BMPs

| Treatment | Intervention | Control | BMP-concentration | Phase | Endpoint study | Status | References |
|--------------------------------------|---|---|---|-------|-----------------------|--------------------|-------------|
| Nonunion diaphyseal tibial fractures | BMP-7 in adjunct to fresh frozen allograft | Allograft together with DBM | OP-1® | 4 | Efficacy | Suspended | NCT00551941 |
| Nonunion diaphyseal Fractures | BMP-2 on ACS | Iliac crest autograft | InFuse® | 4 | Safety/efficacy | Withdrawn | NCT00856479 |
| Nonunion fracture | Mesenchymal stem cells, BMP-2, and collagen scaffold | ND | ND | 2 | Safety/efficacy study | Recruiting | NCT01958502 |
| Critical size tibial defects | Recombinant bone morphogenetic protein 2 | Autogenous iliac crest bone graft | InFuse® (1.5–12 mg) | 4 | Safety/efficacy | Recruiting | NCT00853489 |
| Radius fractures | BMP-2/CPM | ND | ND | 1 | Safety | Completed | NCT00161629 |
| Tibia fracture | BMP-2/CPM | Buffer/CPM | 1-2 mg/ml | 2/3 | Safety | Terminated | NCT00387686 |
| Closed fractures of the humerus | BMP-2/CPM | Buffer/CPM | 1-2 mg/ml | 2 | Safety/efficacy | Completed | NCT00384852 |
| Critical-sized fracture | Mesenchymal stem cell, HA-CaSO4, BMP-2 | ND | ND | 1 | Efficacy | Unknown | NCT01725698 |
| Osteoporosis | BMP-2/CPM injection and bisphosphonates, calcium, and vitamin D | Bisphosphonates, calcium, and vitamin D | 1-2 mg/ml unilateral intraosseous injection of 6 mL | 2 | Safety/efficacy | Completed | NCT00752557 |
| Pseudarthrosis | BMP-2 on ACS | Autologous | InFuse® | ND | Efficacy | Not yet recruiting | NCT01756144 |

2.4.2 SMAD-Dependent Signaling During Bone Formation

The SMAD-dependent signaling cascade is initiated, as the constitutively active type 2 receptor phosphorylates the (glycine-serine rich) GS domain of the type 1 receptor which subsequently phosphorylates and activates the receptor-regulated SMAD1/5/8 complex (Fig. 2) [165]. These SMADs commonly consist of a DNA-binding domain at the N-terminus and a protein-protein interaction domain at the C-terminus domain, connected through a linker domain [83]. Upon phosphorylation of the C-terminus domain by the common mediator SMAD4, the R-SMAD complex is formed and translocates to the nucleus where it regulates the expression of BMP-responsive genes [97, 118, 165, 180].

The downstream signaling cascade of the R-SMADs can be modulated by phosphorylation of the linker region by other cellular kinases such as MAPKs and glycogen synthase kinase 3-beta (GSK-β). These compete with the receptor-mediated phosphorylation for deactivation through proteasomal-mediated degradation [50, 162]. Further fine-tuning of the signaling cascade is regulated by intracellular mediators such as small C-terminal domain phosphatases (SCP)-1 and SCP-2 and transcriptional cofactor BMP type 2 receptor-associated protein cGMP-dependent

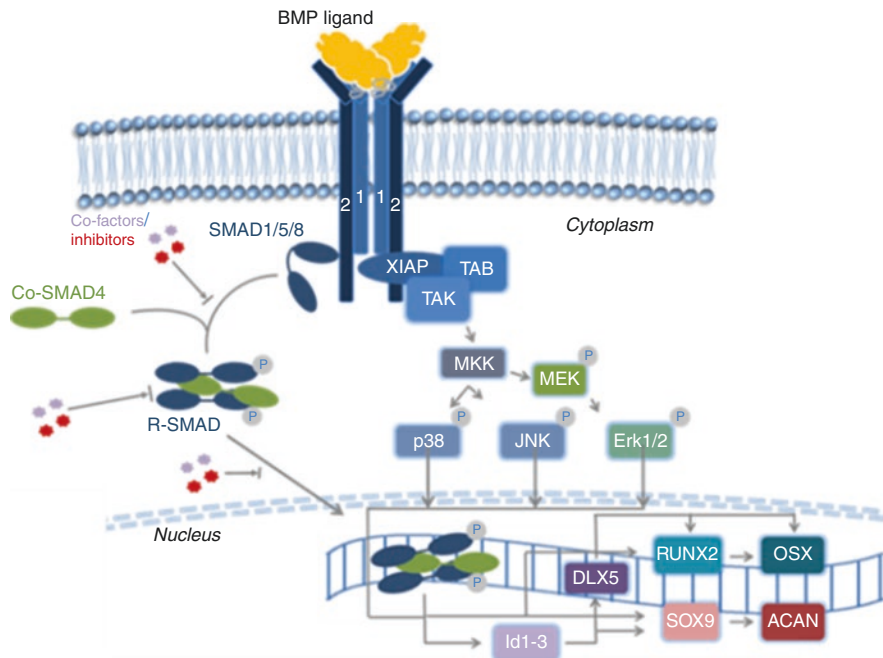


Fig. 2 Schematic view of BMP signal transduction. BMP ligands activate intracellular signaling by binding to their related transmembrane type 1 and type 2 receptors. Ligand-receptor oligomerization occurs through two different mechanisms where formation of a preformed receptor complex (PRC) mainly induces signaling through the SMAD-dependent signaling route, while BMP-induced receptor complex (BRC) preferentially activates the MAPK pathway

protein kinase 1 (cGK1) [163, 167]. Ubiquitination is another mode of regulating SMAD activity, which can lead to either proteasomal-mediated degradation causing repressed signal transduction or protein aggregate formation and regulate cellular processes as a potential protective mechanism [168]. SMAD6 and SMAD7 are also called inhibitory SMADs (I-SMADs), due to their antagonizing of the activation of R- and Co-SMADs. SMAD6 mitigates BMP signaling through competing with SMAD4 for complex formation [70]; SMAD7, on the other hand, is recruited to the receptor and induces degradation of the type 1 receptor kinase together with SMURF1 [40].

2.4.3 SMAD-Independent Signaling During Bone Formation

While the SMAD-dependent BMP signaling pathway is well investigated, less is known regarding the SMAD-independent pathways. Upon ligand binding to a pre-formed complex of the types 1 and 2 receptors, activation on gene transcription level occurs through the activation of the MAPK pathway (Fig. 2). MAPKs are evolutionary conserved enzymes that convert various extracellular stimuli into different cellular responses during biological processes such as fracture healing. The key effector enzymes p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase 1-3 (JNK) are part of a multistep cascade which is tightly regulated by phosphorylation and dephosphorylation processes [61, 80, 141]. JNK signaling is mainly known for its regulatory role in inflammation, apoptosis, and cell migration [136, 195]. The ERK-1 and ERK-2 kinases modulate cell survival, proliferation, and differentiation as well as protein synthesis in multiple cell lineages [144, 216]. Altered ERK-1/ERK-2 signaling is found in several genetic diseases with skeletal phenotypes such as neurofibromatosis type 1, suggesting a role in the regulation of skeletal development [9]. BMP-induced ERK signaling occurs through MEK1 activation, subsequently increasing Runx2 stability and transcriptional activity [82].

Among the various MAPK subfamilies, p38 kinase has attracted elevated attention in the last years and has proven essential for skeletogenesis and bone homeostasis due to its role in cell proliferation, differentiation, apoptosis, senescence, as well as cytokine production and function [56, 79, 106]. Upon BMP receptor phosphorylation, it associates with TAK1, TAB1, and XIAP, leading to activation of p38 which translocates to the nucleus [56]. Then, p38 activates transcriptional factors ATF2, c-Jun, or c-Fos to regulate BMP target genes such as RUNX2, OSX, OPN, ACAN, and ALP [103]. Each of the pathways has been proven of importance, since an effect can be seen upon inhibition, and the system is tightly controlled through fine-tuning between the activated MAPK pathways [98]. Moreover, cross talk between MAPK and SMAD signaling occurs, since it has been shown that TAK1 can modulate the duration and intensity of SMAD signaling [15, 72, 167, 177].

3 Candidate Cell Types for BMP/Cell-Based ATMPs

As aforementioned, the cells can be a driving force for tissue regeneration in cell-based ATMPs. Moreover, the necessity of (stem) cells to be included in the development of ATMPs becomes particularly important for fractures in compromised conditions, such as severely damaged surrounding tissues, elderly patients with sub-optimal conditions (e.g., diabetes and osteoporosis), or in young children with congenital disease (e.g., neurofibromatosis type 1), which all may lead to poor healing of the fracture. In such compromised conditions, the surrounding tissue may not be able to efficiently respond to the BMP stimuli. In view of this, it is a potential advantage to pre-seed the scaffold with (stem, progenitor) cells combined with a physiological dose of BMP. From a clinical point of view, it is preferable for cell-based ATMPs to have a source of human stem cells that can be derived from a small biopsy via a noninvasive initial harvest and can proliferate in large numbers and be BMP responsive including proliferate and/or differentiate into the osteochondrogenic lineage upon BMP exposure [126].

3.1 Bone Marrow Stromal Cells (BMSCs)

Bone marrow, which is composed of the hematopoietic compartment and the stroma, is the conventional source to obtain human somatic stromal cells for use in regenerative medicine. In the hematopoietic compartment, hematopoietic stem cells and committed progenitors of different specific hematopoietic lineages reside. In the stroma, stromal cells, accessory cells, extracellular matrix components, and soluble factors have been described [77]. Taking the heterogeneous population of cells into account, it is of relevance to choose a well-defined and robust methodology to isolate, characterize, and study the functionality of the expanded stromal cell [45].

3.1.1 Isolation and Expansion

BMSCs are usually isolated by cultivation of cells adherent to plastic and obtained from untreated whole bone marrow in the form of bone marrow explant or bone marrow filter washout [148]. However, this method may lead to low yield of isolation because a large proportion of erythrocytes reside in the untreated bone marrow and their presence may interfere with the initial attachment of BMSCs [6]. An alternative method to isolate BMSCs is through an initial isolation of mononuclear cells by a Ficoll-Hypaque gradient before further cultivation [45]. By removing the unwanted high-density blood cells, this method is helpful to increase the number of colony-forming unit (CFU) in the primary BMSC culture [6].

The isolated BMSCs are usually cultured for expansion in basal medium supplemented with irradiated fetal bovine serum (FBS) [105]. FBS batches may differ from one to another, which could deeply affect the proliferation rate, reproducibility, and consistency of the production process [23]. Furthermore, FBS raises a general concern regarding immunological issues due to potential transfer of xenogeneic proteins as well as communicable disease such as prion-transmitted bovine spongiform encephalopathy, hence, posing potentially a long-term health risk [122]. In consequence, the regulatory authorities encourage replacing the FBS with a non-xenogeneic alternative, albeit GMP-compliant FBS batches are available and used in clinical-grade manufacturing [23].

As an alternative, human platelet lysate (hPL), a blood-derived product prepared as a clinical-grade reagent, has drawn attention for BMSC expansion, since it is a rich source of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF)[19]. Previous studies revealed that hPL-expanded MSCs have comparable characteristics with those cultured in the presence of FBS [16]. Furthermore, hPL increases proliferation capacity of BMSCs, hence providing more efficient expansion [22]. However, also hPL shows important variability in its growth factor content, and a clinical-grade preparation poses still concern.

3.1.2 Characterization and BMP Responsiveness

In vitro, BMSCs represent a phenotypically heterogeneous population of cells. Fernandez Vallone et al. comprehensively reviewed the current progress on the phenotypic characterization of BMSCs using the fluorescence activated-cell sorter (FACS) and magnetic separation techniques [45]. Also our results demonstrated that primary cultures of human BMSCs are positive for the following markers: Strol-1, CD73, CD49, CD105, CD90, CD146, CD147, and lack of expression of CD14, CD20, CD34, and CD45. However, the aforementioned marker expression decreases during in vitro passaging, in association with the disappearance of multipotency of BMSCs [137]. When subjected to appropriate culture conditions, BMSCs readily differentiate into the osteoblastic and chondrogenic lineages, which is particularly of interest for bone regeneration. Research showed that BMSCs even possess greater osteogenic potential than either chondrogenic or adipogenic potential [137]. Moreover, their osteogenic potential appeared to be one of the last lineage commitment phenotypes to be lost [137, 188].

The age and skeletal site of harvest of BMSCs can affect their responses to BMP exposure. Osyczka et al. [142] assessed BMP-2 responsiveness (100 ng/ml supplemented in serum-containing and serum-free medium) of BMSCs harvested from adult maxilla, mandible, and iliac crest BMSCs from the same individuals and pediatric iliac crest. Their results showed that adult orofacial BMSCs were more BMP-2 responsive than iliac crest BMSCs based on higher gene transcripts of alkaline phosphatase, osteopontin, and osteogenic transcription factors *MSX-2* and *Osterix* in serum-free insulin-containing medium. Pediatric iliac crest BMSCs were more

responsive to recombinant human BMP-2 (rhBMP-2) than adult iliac crest BMSCs based on higher expression of alkaline phosphatase and osteopontin in serum-containing medium [142].

Nevertheless, it is noted that BMPs are relatively inefficient in inducing human BMSC to undergo osteogenesis, albeit they are strong inducers for rat and mouse BMSCs [143]. It is shown that mouse-derived BMSCs respond to BMP-2, BMP-4, BMP-6, BMP-7, and GDF5 and further undergo chondrogenic differentiation [24, 43, 172, 173]. However, human BMSCs respond in a different way to distinct BMPs. Continuous stimulation of BMP-2, -4, or -7 upregulated the osteochondrogenic gene expression (e.g., NOGGIN, BMP-2, osteopontin) in human BMSCs [36]. However, they failed to enhance alkaline phosphatase activity, an indicator of osteogenic differentiation [36, 37]. In addition, continuous stimulation of BMP-2 with relatively high dosage (100 ng/ml) significantly increased human BMSC proliferation [36]. In contrast, short-term BMP-2 stimulation at lower doses (10–20 ng/ml) is more effective to induce *in vitro* osteogenic differentiation, evidenced by significantly increased gene expression of RUNX2, COLI, ALP, and OCN, as well as protein levels of COLI and ALP [36]. It was hypothesized that the impaired BMP response of human BMSCs is correlated with the absence of ALK6 expression [143]. However, the overexpression of ALK6 in human BMSCs had no effect on alkaline phosphatase mRNA transcripts, suggesting that the precise relationship between BMP receptor ALK6 and osteoblast-related genes remains to be defined [143]. There is limited research focusing on systematic *in vivo* evaluation of cell-based implants combining BMSCs and BMP. Wang et al. [207] reported a moderate increase of bone formation when loading BMSCs and BMP on calcium phosphate cements subcutaneously implanted in nude rats after 8 weeks, and such improved bone formation can be further enhanced by additional low dosage of bFGF (50 ng/ml).

3.2 *Periosteum-Derived Cells (PDCs)*

Anatomically, the periosteum is a thin vascular membrane that covers the external surface of the bone except for the articular joint surfaces of the long bones. It serves as an attachment site for tendons, ligaments, and muscles and is a rich source of blood vessels that deliver 70–80 % of the blood supply to the bone cortex [26]. Microscopically, the periosteum is composed of an outer fibrous layer and an inner cambium layer. The fibrous layer contains fibroblasts, collagen, and elastin along with a nerve and microvascular network [8], while the inner cambium layer consists of progenitor cells with the capacity to differentiate into osteoblasts and chondrocytes [64, 183].

The osteogenic potential of the periosteum was revealed early in the eighteenth century, when the integrity of the periosteum was found crucial to achieve successful fracture healing [39, 102]. Upon fracture, progenitor cells in the periosteum adjacent to the fracture undergo extensive expansion and differentiation to form a

cartilaginous fracture callus [31]. The cartilaginous callus progressively bridges the fractured bone fragments, followed by replacement by the bone, resulting in the formation of a hard callus which eventually is remodeled to the original cortical and trabecular bone configuration by osteoclasts.

3.2.1 Isolation and Expansion

To isolate periosteal tissue from the patient, a periosteum elevator, shaped like a curved chisel, is typically used to cut off the Sharpey's fibers that anchor the periosteum to the bone, hence maintaining the integrity of the periosteum [27]. Periosteum-derived cells (PDCs) are then harvested by enzymatic digestion of the tissue or by spontaneous cell egression from the biopsy onto plastic cell culture flasks [156].

In culture, PDCs exhibit a fibroblast-like morphology, which is stably maintained over several passages [156]. During in vitro expansion, PDCs do not express osteogenic and chondrogenic properties; however, they can be induced to differentiate into the osteogenic, chondrogenic, and adipogenic lineage by exposing them to specific differentiation medium [34, 156, 202], confirming their multi-lineage potential at the single-cell level.

3.2.2 Characterization and BMP Responsiveness

During expansion, over 90 % of human PDCs express CD73, CD90, and CD105 [156, 202], while lacking the presence of hematopoietic markers such as CD14, CD20, CD34, and CD45 (Ji et al. submitted). In addition, it has been reported that PDCs express perivascular cell markers, including α SMA [130], CD146 [156], and PDGF receptor beta [202], most likely due to their perivascular location [132, 159]. This concept is further underscored by our recent report that PDC enhanced vasculogenesis by adapting a pericyte-like phenotype when they were implanted in vivo [202].

Our data show that continuous in vitro stimulation of BMP-2, BMP-4, BMP-6, and BMP-9 (100 ng/ml) significantly enhanced the osteochondrogenic differentiation of human PDCs, evidenced by the upregulation of *SOX9*, *ACAN*, *RUNX2*, *OSX*, *DLX5*, and *IDI1*. Through mRNA transcript analysis, the BMP-induced differentiation could be correlated to the expression of BMP type 1 and type 2 receptors Bolander et al., Eur Cell Mater. 2016 Jan 5;31:11-25. PMID: 26728496.

Upon coating onto calcium phosphate (CaP) carriers followed by hPDC seeding and 5-week in vivo implantation in nude mice, only BMP-2- and BMP-6-containing constructs gave rise to ossicle formation, including cartilage intermediates, trabeculae-like structures embedded in bone marrow with a surrounding cortex-like bone structure. In these ossicles, the implanted human PDCs contributed to 20 % of de novo bone (Bolander et al. submitted). Such enhanced in vivo bone formation might be correlated with the activation of SMAD-dependent pathway and MAPK pathway within hPDCs induced by BMP and Ca²⁺ exposure (Bolander et al. submitted).

3.3 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defined properties of embryonic stem cells [124]. Since iPSCs can be derived directly from adult tissues, they not only bypass the need for embryos, but can be made in a patient-matched manner, which means that each individual could have their own pluripotent stem cell line, revealing a potential in personalized medicine.

3.3.1 Generation of iPSCs

In 2006, Yamanaka et al. first reported the generation of mouse iPSCs using retroviral transduction with 24 transcription factors highly expressed in embryonic stem (ES) cells [89]. This cluster of genes was gradually reduced to four key genes that encode the transcription factors OCT4, SOX2, KLF4, and c-Myc [191]. Shortly after the initial reprogramming success in the mouse, Yamanaka et al. [190] reported the generation of iPS cells from adult human dermal fibroblasts using a retroviral system with the same four factors: *OCT3/4*, *SOX2*, *KLF4*, and *c-Myc*. Concurrently, Yu et al. [219] reported the generation of human iPSCs from human somatic cells with lentivirus using a slightly different combination of genes including OCT4, SOX2, NANOG, and LIN28. Notably, the conversion from human somatic fibroblast to iPSCs is very low, with reported transduction rate from 0.001 to 1 %, depending on different vectors and gene combinations [89]. In 2012, Zhou et al. [229] reported a detailed protocol for generating human iPSCs from exfoliated renal epithelial cells present in urine, which allow a less-invasive and cost-effective sample harvest procedure and up to 4 % retroviral transduction efficiency.

3.3.2 Characterizations and BMP Responsiveness

Human iPS cells are similar to human ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity, with capacity to further differentiate into cell types of the three germ layers including teratoma formation. Based on the guideline from the International Stem Cell Banking Initiative (ISCBI), Marti et al. [128] published a detailed characterization of iPSCs. In summary, human iPSCs demonstrate the following characteristics: (i) pluripotency – human iPSCs positively express human ES cell markers, such as pluripotent markers placental alkaline phosphatase (hPLAP); nuclear transcription factors OCT4, NANOG, and SOX2; the keratin sulfate antigens Tra-1-60 and Tra-1-81; and the glycolipid antigens SSEA3 and SSEA4. (ii) Differentiation – In vitro, human iPSCs colonies can form large aggregates called embryoid bodies (EBs), which should differentiate

spontaneously to different cell types derived from the three germ layers (spontaneous differentiation) or can be cultured in different substrates with different media to favoring differentiation toward a specific lineage (guided differentiation). Furthermore, the iPSCs will proliferate and differentiate *in vivo* in the tissue where they are injected and ultimately form a teratoma that contains multiple tissues from the three primordial germinal layers characterized by specific markers [11] (Table 3).

Recently, we reported in collaboration with Tsumaki labs the reprogramming of human dermal fibroblast into induced chondrogenic cells (iChon cells) using lentivirus system for Klf4, c-Myc, and Sox9 [192]. The iChon cells demonstrated a highly hypertrophic differentiation capacity *in vitro* and direct or indirect contribution to cartilage and bone formation *in vivo* [192], which highlights the promise of cellular reprogramming for the creation of functional skeletal cells that can be used for novel bone healing strategies.

The generation of iPSCs is regulated by multiple types of signaling cascades, including those mediated by BMPs. A recent study demonstrated that BMP signaling during the early stage of iPSC induction can induce a set of miRNAs associated with the mesenchymal-to-epithelial transition (MET), which can accelerate the generation of iPSCs [161]. Such enhancement might be mediated by a receptor complex consisting of ALK3 and BMPR2, since suppression of ALK3 and BMPR2 inhibited the generation of iPSCs [161]. Hamasaki et al. [66] recently showed that constitutive activation of ALK2 affected both the upregulation of pluripotent markers and the downregulation of fibroblastic markers during the early phase of iPSC generation, thus resulting in incomplete reprogramming. The role of ALK3 and ALK6 in the generation of iPSCs in cellular reprogramming still remains unknown.

Similar to ES cells, pluripotency and differentiation of iPSCs are also regulated by BMPs. However, many studies have highlighted differences between mouse and human ES cells regarding the response to extrinsic signals. For instance, Ying et al. [217] reported that BMP-4 sustains self-renewal of mouse ES cells by inducing the expression of ID genes. In contrast, in human ES cells, BMP-4 has been shown to

Table 3 Characteristic markers expressed in human iPSCs

| | | |
|------------------------|----------|---|
| Pluripotency | | hPLAP, Oct4, Nanog, Sox2 Tra-1-60, Tra-1-81; SSEA3, SSEA4, SSEA1 (mouse) |
| Differentiation | Endoderm | α -1-Fetoprotein FoxA2 |
| | Mesoderm | Brachyury (nuclear) (Muscle-like tissue) α -smooth muscle actin and α -sarcomeric actin (Cartilage-like tissue) Sox9, fibronectin, chondroitin sulfate |
| | Ectoderm | Pax6, Sox1, Tuj1 |

induce specification into the trophoblastic lineage [212], as well as germ cell lineage differentiation [209]. Consistently, Hamasaki et al. [66] showed that the BMP-4 or BMP-7 reduced the colony-forming capacity of iPSCs and directed iPSCs into both mesodermal and endodermal lineage. Therefore, we should be very careful to interpret the data obtained from mouse iPSCs and to extrapolate the results for studies using human cells.

4 Scaffolding Material for BMP Cell-Based ATMPs for Bone Regeneration

4.1 Clinical Perspectives of Desired Scaffold Properties

Effective clinical repair of bone defects is highly dependent on mechanical stability in the defect site and requires osteogenic cells and osteoinductive growth factors in combination with a proper delivery system, conceptualized as the “diamond concept” that provides the optimum mechano-biological conditions for bone regeneration [53]. The standard clinical practice for fracture immobilization is by using internal or external fixators to prevent micro-motion that will lead to scar tissue or cartilage formation. This technique is necessary especially when non-load-bearing biomaterial is used as the BMP delivery system/scaffold within an ATMP. Alternatively, metallic scaffolds with high mechanical strength could play a role in alleviating the adverse effects arising from mechanical instability. Although metallic scaffolds provide temporary supports to patients to regain immediate mobility, the non-biodegradability of the metals has limited its clinical applications. Nevertheless, significant research efforts on developing biodegradable metallic scaffolds with high mechanical strength are being carried out in order to overcome this limitation [223].

In addition to the mechanical stability aspect, the biodegradation kinetic of a biomaterial needs to match the bone formation process, to precisely control the release of BMPs, to guide cell differentiation and bone tissue formation, and to timely provide free space for blood vessel ingrowth and bone tissue formation. It is being suggested that an ideal biomaterial for bone defect repair should be partially degraded by 7 weeks and fully degraded around 14 weeks post-implantation, slightly depending on the defect nature including defect site, size, and patient profile. Moreover, the degradation by-products should not or minimally interfere with the activation of BMP signaling, if possible rather enhance the molecular and cellular cascade of bone healing. Therefore, the pharmacokinetic profile of a BMP-based ATMP should preferably be sustained over an appropriate period of time that matches the bone healing process in accordance to the cell proliferation and differentiation and mineralization effects elicited by BMP, instead of long but low concentration of BMP release or initial burst release. In fact, a delicate balance in concentration of BMP loading onto scaffolds is required. Furthermore, the pharmacokinetic profile should be specific to BMP ligands (due to different amino acid sequences of the BMP subtypes), the type of fracture or the application, host species

(different optimal release profiles are required), and implantation site. These factors would determine the form of the BMP delivery system conformations (from injection, micro-/nanoparticles to 3D porous scaffold), formulation (single or composite materials), and the type and amount of BMP in use.

Host environment is another crucial factor that needs to be considered thoroughly for designing an effective BMP-based ATMP therapy for bone defects, including the suitable BMP dosing and the concentration of BMP at the graft site. However, findings from animal studies are not easy to be translated into a clinical protocol as the BMP concentration used in animal studies appears to be lower than the dose required in patients. Moreover, the host environment is rich in a variety of organic and inorganic molecules that potentially influence the interaction between the biomaterials and BMP as well as the BMP bioactivity, such as in vivo temperature and bodily fluid pH and osmolarity. Other clinical implications of BMP treatment that require careful considerations include the route of administration and BMP antibody formation (i.e., 38 % of treated patients in some trials).

Ideally, a BMP carrier should (1) be biodegradable or present adequate porosity to allow the formation of an interface with the surrounding biological tissues for cell infiltration, vascularization, and new bone formation, (2) possess full biodegradability for complete integration of healed bone tissues, (3) provoke some mild inflammatory responses to activate the healing process, and (4) protect BMPs from deactivation while releasing the protein in a time- and space-controlled way to promote bone regeneration. On top of the requirements from the biomaterial's point of view, other stringent criteria for clinical usage include adaptability to the wound site, surgical malleability, as well as patient specificity or customization in respect to the treatment duration, anatomical geometry of the defect, and vascularity [65]. Lastly, the ATMPs need to be sterile without either loss of material integrity or deactivation of BMPs. Therefore, the respective manufacturing pipelines require special production and handling processes that would give rise to conveniently sterilizable, surgeon-friendly implants, stable over time with well-defined storage procedures (long shelf life). By combining manufacturing technologies, minimal manual intervention in the production pipeline is highly preferable for efficient commercial scale-up manufacturing of the respective BMP-based ATMPs, an additional criterion that would facilitate approval by regulatory agencies.

4.2 Injectable Materials for BMP- and Cell-Based ATMPs

Due to its water solubility, albeit rather poorly soluble, BMPs can be dissolved in water-based buffer solutions (e.g., physiological saline) and delivered in vivo simply via injection. New generation of BMPs is being developed that improve the solubility. Local injection is a potential minimally invasive delivery technique for treatment of delayed and nonunions, spinal fusion, and acceleration of healing of closed fractures. However, injection of BMPs in solution results in burst release of

BMP molecules, hence, a rapid clearance from the defect into surrounding tissues which reduces the differentiation effect and potentially causes toxicity and heterotopic bone formation.

To overcome these potential adverse effects, BMPs are often added into a protein carrier for precise injection into the defect to ensure sustainable BMP release to enhance long duration of local-acting differentiation effects. Besides maintaining the local BMP concentration, the carriers also provide protection to BMPs from deactivation by harmful conditions such as endogenous enzyme digestion and protein denaturation due to pH shifts. For this, collagen is often used as BMP delivery vehicle because it is easy to prepare in an injectable hydrogel form and can be obtained as purified recombinant human collagens that are free of animal components from reliable and chemically defined sources. Moreover, the binding affinity of BMPs to collagen can be modulated by changing the pH or isoelectric point of the two proteins in order to obtain specific BMP-release profiles to enhance bone formation [52]. Alternatively, gelatin is a cost-effective collagen-derived protein carrier that could provide controlled BMP release, by changing the electrical nature of gelatin via acidic or alkaline preparation process of collagen. In fact, delivery of BMP using collagen or gelatin as carriers showed increased retention ranging from 15 to 55 % as compared to less than 5 % of BMP dose remaining at the application site when no carrier was used [73].

Furthermore, the BMP release and bioactivity can be modulated by varying the extent [213] or employing site-specific enzymatic cross-linking of BMP onto gelatin [101]. Hyaluronic acid, a natural extracellular matrix (ECM), has been used as an effective injectable control release system to augment bone formation due to its specific chemical structures that allow chemical modification to ease cross-linking and for covalent binding of BMPs [129]. Self-assembly silk fibroin is another interesting injectable BMP hydrogel due to its processing flexibility, biodegradability, biocompatibility, and high mechanical toughness. The BMP release can be tailored by adjusting the enzymatic degradability of silk fibroin via the control of the crystalline state, molecular weight, and secondary structure [150, 157, 226]. Other natural-origin biopolymers that are used as injectable BMP hydrogel include alginate [18, 76], fibrin [145, 214], chitosan/chitin [184], and heparin [107]. Several studies showed that the composites of the above mentioned biopolymers either simply by mixing two biopolymers [147] or by conjugation [e.g., heparin-conjugated fibrin [215] provided more sustainable BMP release and improved in vivo bone regeneration as compared to using collagen alone as a carrier.

Synthetic polymers offer an advantage over the natural-origin biopolymers of being free from the risk of disease transmission. These polymer carriers are biodegradable, and thus allow for a controlled release of BMPs by fine-tuning the material degradation kinetics to match in vivo bone healing processes. Poly(lactic acid) (PLA) was an initial carrier to be used for BMP delivery, but it was ineffective due to the release of acidic degradation by-products that deactivate BMP. Subsequently, poly(lactic-co-glycolic acid) (PLGA) received particular attention because it combines the absorptive stability of PLA with mechanical strength of polyglycolic acid (PGA) and offers tunable biodegradability by varying the proportion of the two

components. Poly(ethylene glycol) (PEG) is a bio-inert hydrophilic polymer that is versatile for hydrogel formation or for conjugating with biomolecules including growth factors, cell adhesion peptides, and enzymes for controlling matrix degradation (e.g., matrix metalloproteinase)[179, 230]. Because of its unique chemical structure (i.e., two hydroxyl end groups), PEG can be converted into other functional groups to obtain a tunable physical state. This tunable state renders the PEG injectable and in situ cross-linkable either via a temperature-dependent liquid-semisolid transition [called thermosensitive polymers, such as polypropylene fumarate-co-ethylene glycol [14], poly-D,L-lactic acid-polyethylene glycol (PLA-PEG) block copolymers [85, 160]] or via in situ polymerization through chemical [e.g., poly(ethylene glycol) fumarate[179]] or photo-cross-linking mechanisms [e.g., poly(ethylene glycol) diacrylate [35, 228]]. Furthermore, synthetic polymers also provide higher mechanical properties (such as torsional strength) than the biopolymers which are crucial for healing large bone defects. However, additional materials for intervention may hinder BMP release from the bulk or alter BMP molecular integrity and thus compromise its bioactivity. Nonetheless, these materials are often bio-inert and lacking bone-inducing effects. This has led to the development of injectable, in situ setting ceramic cements as BMP delivery carriers.

Ceramic cements, such as calcium phosphate or hydroxyapatite, have been shown to have high binding affinity for BMP molecules [108, 206], thus making them suitable carriers for effective delivery of BMP in addition to their well-known osteoconductive and osteoinductive effects. The osteoinductive effect of calcium phosphate is beneficial as BMP devices as currently formulated must be used at very high concentrations to be effective [55]. In fact, ceramic pastes incorporated with rhBMP-2 showed to accelerate healing of critical-sized bone defects in pre-clinical large animals, such as canine [42] and nonhuman primate [170]. Bioactive glass is another promising bone-inducing biomaterial and delivery vehicle for BMPs due to its unique ability to bond to living bone and promote bone regeneration [220]. It has been reported that BMPs can be covalently immobilized onto bioactive glass effectively via surface functionalization techniques such as silanization [205] or physical absorption onto apatite coating formed on bioactive glass [123]. The benefits of injectable synthetic polymer and ceramic carriers for BMP delivery gave rise to the development of injectable composite carriers that were found to enhance bone formation and were linearly dependent on the amount of additional calcium phosphate powder in respect to the rhBMP-2/calcium phosphate ratio [84]. Nevertheless, lack of open-pore structures or low porosity of the hardened paste appears to be the major drawback of this delivery method, which may interrupt BMP release kinetics and prevent ingrowth of surrounding tissues and the formation of neo-tissues, thereby compromising or delaying bone formation. For this, injectable micro- or nanocarriers that are encapsulated or chemically immobilized with BMPs are developed to circumvent these drawbacks by providing a higher specific area for BMP release and interparticle open spaces for tissue growth. For instances, these injectable micro- or nanocarriers have been reported to be successfully made from PLGA [152], chitosan [125], silk fibroin [17], polycaprolactone [12], and calcium phosphate [208].

Recently, carbon nanotube (CNT) was reported to be a promising biomaterial for bone tissue engineering [1]. In addition to the high mechanical strength, surface functionalizing the nanotube surface with BMPs was shown to be feasible and gave rise to controlled release of BMPs and accelerated chondrogenic and osteogenic differentiation of progenitor cells and in vivo bone formation [112]. Interestingly, an inhibitory effect of CNT was found on carboxylated CNT that showed to inhibit proliferation and differentiation of precursor cells which may be modulated via a SMAD-dependent BMP signaling pathway [113]. This indicates that further investigation is necessary to gain more insights into the biomedical applicability of CNT as BMP delivery system, in addition to the potential cytotoxicity effects due to intracellular accumulation of CNTs [62] or generation of reactive oxygen species [151].

4.3 Solid Porous Scaffolds for BMP- and Cell-Based ATMPs

Three-dimensional (3D) scaffolds play an important role in tissue regeneration by providing attachment sites, void spaces, as well as bioactive signals for cells to grow and differentiate into specific lineages. Specifically, it aims to provide a precise microenvironment for optimal expansion and control of differentiation of precursor cells that subsequently lead to 3D functional organ formation. Conventional techniques are employed to produce 3D porous scaffolds in solid (e.g., salt leaching, porogen sacrifice, and gas foaming), fibrous (e.g., electrospinning), and microspheres (e.g., water-to-oil emulsion and droplet generation). These scaffolds could act as efficient drug delivery systems, delivering BMP homogeneously in a three-dimensional manner which is an important criterion to elicit bone formation in all or a targeted direction.

It is known that the clinical efficacy of recombinant human BMPs (rhBMPs) will depend on the carrier system used to ensure an effective delivery of adequate protein concentrations to the defect site [134]. Various modes of BMPs incorporation into the scaffolds have been developed and showed promising bone formation outcomes [20]. The most convenient method is by physical absorption onto porous scaffold, whereby BMPs are randomly impregnated within the delivery matrix without chemical bonding. However, physical absorption will lead to an initial burst release of BMPs. BMPs can also be incorporated into the porous scaffolds by entrapment within a hydrophobic polymeric matrix during scaffold production in order to obtain an extended period of BMP release. The risk of BMP protein denaturation and loss of bioactivity could arise due to temperature changes during the production process or pH shift due to material degradation. Hydrogel scaffolds made from extracellular matrix (e.g., hyaluronic acid, heparin sulfate, heparin proteoglycans) or charged polymers (e.g., chitosan, alginate, or synthetic polyelectrolytes) are interesting biomaterials for BMP delivery, attributed to the strong affinity of BMPs or via ion complexation binding of BMPs with the biomaterials. Modification of surface chemistries of the porous scaffolds for immobilization of BMPs via covalent binding showed to be more promising than any nonspecific immobilization methods. This immobilization can be achieved

by either modifying the chemical backbone structures of the biomaterials or grafting functional groups that are specific for BMP molecules onto the surface of scaffolds. Alternatively, BMP protein with a domain of specific binding to the scaffolds can be produced due to the great versatility of the recombinant technology nowadays. Therefore, chemical immobilization of BMPs has provided feasibility to develop “smart” BMP-releasing scaffolds which guaranteed precise dosing and control over BMP release such as via cell-mediated activity [114], light [93], temperature [115], and pH changes [51]. Incorporation of other essential biological cues to enhance cell adhesion and growth on the porous scaffolds is an attractive approach to enhance the biological functions of the porous scaffolds. For instance, hyaluronic acid scaffold was reported to be superior over collagen gel as carrier for a gradual and sustainable release of functional rhBMP-2 [86], and covalent grafting of fibronectin fragments within the hyaluronic acid structures enhanced cell attachment and spreading, as well as improved quality of ectopic bone formation [88].

Besides biochemical signals between cells, physical parameters of the scaffolds are shown to exhibit significant effects on tissue formation starting at the single-cell level. Indeed, the behavior of stem cells or osteochondro-progenitors is strongly influenced by the geometrical features of scaffold pores. It is reported that small pore sizes (<500 μm in diameter) gave rise to lower scaffold permeability (than the bigger pore size; >500 μm in diameter), thus, resulting in significantly higher *in vitro* cell seeding efficiency but faster occlusion of the pores that blocked further cell growth [201]. *In vivo*, subcutaneous implantation of porous hydroxyapatite scaffolds (in combination with BMP-2) with pore sizes of 300–400 μm resulted in highest bone formation, whereas pore size of 90–120 μm gave rise to cartilage tissues, a phenomenon that was dependent on the vascular invasion [99, 199]. Additionally, the pore curvature imposed active mechanical forces that influenced the speed of cell growth, which resulted in a curvature-driven cell growth pattern that was associated with distinct patterns of actin organization and alignment [63, 95]. Interestingly, a study using sheep critical-sized bone defects showed that the scaffold architecture directed bone tissue organization through structural guidance and load transfer, while BMP stimulation accelerated bone formation without altering the bone tissue microstructure at different length scales [29]. These findings indicated important implications toward the understanding of natural processes of bone defect healing and bone remodeling, as well as important clues for designing optimum 3D porous scaffolds [158].

Advances in 3D additive manufacturing (e.g., selective laser melting/sintering, fused deposition modeling, and solid free-form fabrication) have opened up the feasibility to fabricate synthetic 3D microenvironments that mimic the regulatory characteristics of natural extracellular matrices (ECMs) and ECM-bound growth factors in addition to the indispensable biological and physical criteria required on the scaffolds to warrant success during *in vitro* 3D culture and *in vivo* tissue formation [10]. Since BMPs are delicate proteins that are vulnerable to temperature and pH, it is of utmost importance that the employed 3D printing technology must not compromise the bioactivity of the incorporated BMPs; otherwise incorporation of BMPs is to be carried out on the surface of scaffolds after the production process via the aforementioned methods. Examples of 3D-printed porous scaffolds for BMP delivery include

polymer-based scaffolds [e.g., polycaprolactone [225]], hydrogels [e.g., PEG [164, 175]], ceramics [e.g., biphasic calcium phosphate [186]], and metallic [e.g., titanium alloys [227]]. This technology has potential to fulfill the needs for engineering an efficient upscale production of ATMPs with quality attributes of high controllability and reproducibility. Table 4 shows a summary of different types of biomaterials and their advantages and disadvantages as potential BMP-related ATMPs.

Table 4 Types of biomaterials as potential BMP-related ATMPs: advantages and disadvantages

| Types of biomaterials | Advantages | Disadvantages |
|--|--|---|
| A. Non-ceramic based | | |
| 1. <i>Natural-origin</i> (e.g., collagen, gelatin, fibrin, chitin/chitosan, alginate, hyaluronic acid, and agarose) | Biocompatible and biodegradable Allows clinical malleability according to defect geometry and application (injectable, moldable putty like, sponges, hydrogels, 3D-printed porous scaffolds) Simple incorporation of BMP into the biomaterials | Risk of disease transmission and immunogenic Limited sources and impurities contamination Low mechanical properties |
| 2. <i>Synthetic polymers</i> (e.g., polylactic acid, polylactic-glycolic acid, polycaprolactone, polyethylene oxide, polyethylene glycol, polypropylene, polyvinyl alcohol) | Biocompatible, biodegradable, and free of risk of disease transmission Available by mass production via chemical synthesis Tunable chemical and material properties for specific BMP release and material degradation profiles to match bone healing Allows cells encapsulation and chemical immobilization of biomolecules to enhance biological activity No or low intervention with BMP bioactivity Presence in hydrogel form or 3D-printed porous scaffolds to allow patient-customary implant design | Potential deactivation of BMP and immunogenic due to acidic degradation products Insufficient mechanical strength for load-bearing applications No bone-inducing property thus required high BMP dosage to achieve the desired therapeutic effect |
| B. Ceramic-based biomaterials | | |
| 1. <i>An organic animals' bone granules</i> | Confers superior osteoinductivity due to high similarity of chemical composition and structure to native bone Biocompatible, biodegradable, and non-immunogeni Ideal delivery vehicle for BMPs due to high binding affinity and material degradation Possess physiological calcium and phosphate ions release kinetics for stimulating bone formation | Risk of zoonosis transmission and limited sources Limited sources and impurities contamination and toxicity Inconsistent bone formation outcomes due to the variation in animals and production factors |

(continued)

Table 4 (continued)

| Types of biomaterials | Advantages | Disadvantages |
|---|--|---|
| 2. <i>Calcium carbonate</i> (e.g., corals, egg shells) | Alternative resources and cost effective Can be synthesized into calcium phosphate-based apatites | Risk of zoonosis transmission and impurities contamination |
| 3. <i>Calcium phosphate and bioactive glasses</i> (e.g., hydroxyapatite, tricalcium phosphate, biphasic CaP, octacalcium phosphate, calcium pyrophosphate, dicalcium phosphate) | Widely used as synthetic bone substitutes due to its excellent osteoconductivity and osteoinductivity Possess higher biomechanical strength than polymer- or hydrogel-based biomaterials Allows fine-tuning of the material degradation and BMP release kinetics Synthetic and thus free of risk of disease transmission and impurities contamination High affinity for BMPs binding, and unlimited availability Can be formed into paste-like or 3D-printed porous scaffolds | Rigid, brittle, and requires fixators in load-bearing application May induce adverse inflammatory responses and osteoclastic resorption May interfere BMP signaling activation |
| C. Metals (e.g., titanium-based, cobalt-chromium, zirconium, stainless steel, tantalum, magnesium alloys) | Biocompatible and offers excellent mechanical strength Can be produced into implants with desired defect geometry and 3D-printed porous scaffolds Allows surface immobilization of BMPs for controlled delivery applications Bio-inert and thus not interfering with BMP effects Provide immediate mechanical support and mobility to patients | Nonbiodegradable and requires surgical intervention due to implant wear off Risk of metal toxicity or chronic inflammatory responses |
| D. Composites made from the above biomaterials (e.g. Composites of CaP with collagen, hydrogels or polymers; Hydrogel or CaP-coated of collagen, polymer or metallic sponges or scaffolds) | Improved mechanical strength, osteoconductivity and osteoinductivity Higher versatility and flexibility in fine-tuning the material properties and bioactivities Offers higher technological flexibility for different clinical implications and demands Improved BMPs delivery as compared to single material delivery | Lack of technological tools as well as knowledge on the BMP-material-host interactions for developing an ideal biomaterial that optimally elicits bone regeneration based on BMP technology |

5 Toward ATMP Combining BMP and Cells

Since powerful “raw materials” are now available in clinical grade such as BMPs, CE-approved smart biomaterials, and GMP-manufactured cell suspensions such as BMSCs or periosteal-derived cell populations, we have set out to produce combinations of these that exceed the biological potency of the single products such as BMPs or biomaterials only. These combination products are envisioned to be of use for large bone defects in compromised environments, with sick tissues, lack of stem or progenitor cells close by, and where the implant needs to drive semiautonomously the process of tissue formation and integration despite an unfavorable environment. This may be in genetic diseases such as NF1, where the periosteum compartment is simply ineffective or an aging patient with diabetes and osteoporosis or osteomalacia.

The search for these optimal combination products is quite challenging and should be based on the principles of developmental engineering as a concept of “in vitro biomimetics of in vivo tissue development” [109, 110]. In short, the design of cell-based products should integrate the concepts of developmental biology, so that the behavior of networks of genes, proteins, or cells that govern the unfolding of developmental processes could be related to the design parameters. In addition, it is necessary to involve new methodologies such as design of experiment (DoE) approach to determine the optimal setup for each design parameters. We recently conducted a full-factor DoE analysis of bone formation capacity induced by ATMPs with different calcium phosphate scaffolds, BMP loading dosage, and cell seeding dosage (Ji and Kerkhofs et al. in preparation). Our data indicates that indeed the proper dosage combinations of BMPs and cells seeded on specific scaffolds can generate skeletal tissue intermediates with higher bone-forming potency, improved bone quality, and more active contribution from donor cells, exceeding these of smart biomaterials only with growth factors or cells.

To turn this into robust manufacturing processes, new enabling technologies such as perfusion bioreactors in combination with biosensors are required. Such setup provides several advantages for a manufacturing pipeline, including (1) direct cell-cell and cell-extracellular matrix interaction, (2) direct control over shear stress development, and (3) accurate sensor readouts at the outlet of the bioreactors. It also helps to develop structurally defined and functionally effective complex 3D-engineered constructs at the patient scale using scale-out strategies [146]. In addition, noninvasive imaging will be necessary to further tailor the quality characteristics of specific stem cell culture as well as for more complex 3D TE construct culture [146]. Furthermore, regulatory requirements are evolving for these novel 3D products and their manufacturing processes. Therefore, the effective bioreactor systems with incorporation of multiple sensors would provide information-rich processes for the manufacturing of TE products that could meet regulatory demands [146].

6 Preclinical Evaluation of ATMPs Combining BMP and Cells

BMP, stem cells, and biomaterials can be considered as “raw materials” in the development of ATMPs. Although recent progress has been achieved in BMP production, (stem) cell culture and expansion as well as new biomaterials fabrication, respectively, the translation of knowledge from in vitro model systems to in vivo and upscaling to the clinical setting is still challenging. Therefore, it is necessary to use sequential animal model systems to fully understand the biological performance of these devices in a living organism before translation into the clinics can be made. The following section will focus on animal models suitable for preclinical evaluation of BMP-/cell-based ATMPs.

6.1 Ectopic Model

Particularly for bone regeneration, the ectopic model provides a relatively controlled and clean system to evaluate the in vivo de novo bone formation capacity of human cell-based ATMPs. Therefore, this is suitable as a first-line screening model to identify the biocompatibility, toxicity, and bioactivity of ATMPs. The three most commonly used ectopic models are subcutaneous, intramuscular thigh, and under-the-kidney capsule implantation [169]. Despite the advantages of the ectopic model, the differences in the inflammatory, immunological, biochemical, and mechanical environment between ectopic and orthotopic locations are distinct, which greatly affects the bone-forming process induced by the ATMP. For instance, Levi et al. [111] showed that adipose-derived stem cells successfully ossified a critical size defect. However, the same implants did not result in significant bone formation in the ectopic model.

Another concern comes from the different tissue responses between immune-deficient and immune-competent animals upon ATMP implantation. Liu et al. [116] showed that hydroxyapatite tricalcium phosphate (HA-TCP) scaffolds combined with mouse BMSCs were much less osteoinductive in syngeneic immune-competent mice than immune-deficient mice when implanted ectopically. Furthermore, recipient T lymphocytes were found to inhibit bone formation in immune-competent mice via inflammatory factors such as IFN gamma and TNF alpha. In a different study, gene expression profiles of the implants showed that T lymphocyte differentiation and activation gene markers were upregulated in immune-competent mice in comparison to immune-deficient mice [21]. Our recent data confirmed that BMP-6-mouse PDCs combined implants induced bone formation in the ectopic model in immune-deficient mice, but failed to do so when tested in immune-competent mice (Fig. 3).

On the other hand, it is well known that a proper inflammatory response is an essential part of the natural bone healing process [135]. Consequently, modulation of inflammation in ATMP implantation is of utmost importance. More recent

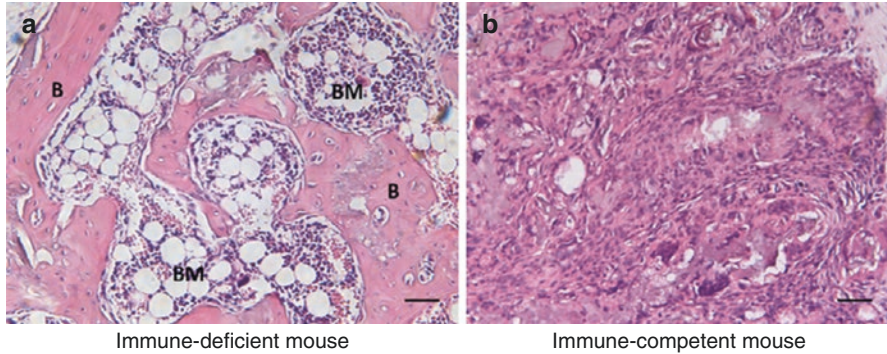


Fig. 3 Mouse PDC-mediated bone formation in immune-deficient and immune-competent mouse. HE staining of tissue explants from immune-deficient mouse (a) and immune-competent mouse (b) 6 weeks after subcutaneous implantation of BMP-6-coated scaffolds with syngeneic mouse PDCs (scale bar = 100 μ m, B bone, BM bone marrow)

emphasis has been given to the modulation of the inflammatory reaction toward improved bone regeneration. New strategies based on surface modifications of biomaterials, coupling of anti-inflammatory drugs to biomaterials, delivery of growth factors, and infusion of MSCs have been explored [48, 117, 153, 176]. For instance, it was reported that Nel-like molecule-1 (NELL-1), a protein first identified for its osteoinductive properties in craniosynostosis patients, could suppress the BMP-2-induced inflammatory reaction in vivo [176]. Furthermore, systemic infusion of MSCs had a positive effect on reducing IFN gamma and TNF alpha and promoted bone formation when scaffolds combined with MSCs were implanted ectopically in immune-competent mice [117]. Nevertheless, there are no methods that generate the same amount of bone in immune-competent mice compared to immune-deficient mice without concerns regarding its clinical safety. Therefore, further studies are required to fully understand the interaction between the immune system and bone tissue formation, providing new insights for successful application of bone tissue engineering strategies.

6.2 Orthotopic Model

Orthotopic models refer to studies in which the bone is formed in its correct and relevant anatomical location. These can be applied in different species to fulfill specific research questions, which can be categorized as (1) understanding of mechanism of action and (2) clinical upscaling, feasibility, safety, and efficacy prediction. For instance, to understand the mechanism underlying bone formation, small rodents such as mice and rats are preferred due to availability of immune-deficient animals for xenograft-based experiment [169]. For clinical translation, the defect should be upscaled in a clinically relevant setting with critical size, which is “above

Table 5 Orthotopic models in different animals with critical size

| Animal | Advantages | Disadvantages | Calvarial defect | Segmental long-bone defect | References |
|------------------------|---|---|---|--|--------------------|
| Rodents (mice and rat) | Easy to perform surgery Availability of immunodeficient animals Availability of specific disease-target gene knockout animal | Relatively thicker and fewer trabeculae than humans Mice and rat do not have Haversian system Periosteum in rats and is well vascularized, hence improving bone healing | Mice: 5 mm diameter Rat: 8 mm diameter | Mice: 0.4 cm in the radius, 0.5 cm in the femur Rat: 1 cm in the radius, 0.4–0.5 cm in the tibia, 0.5–1 cm in the femur | [13, 32, 203, 204] |
| Dog | Tractable nature Similar bone mineral density to humans | Higher rate of solid bony fusion when compared to humans Low nonunion rates Ethical issues and negative public perception Significant inter-animal variations due to breed diversity | N/A | 0.3–2.5 cm in the radius 2–2.5 cm in the ulna 2.1–7 cm in the femur | [74, 154] |
| Sheep | Docile animals with easy outdoor housing Similar body weight to humans Hind limb anatomy similar to humans Dimension of long bones suitable for human implants | Higher trabecular bone density than humans Late skeletal maturity, with Haversian remodeling at 7–9 years of age | N/A | 3–3.5 cm in the tibia 2.5 cm in the femur | [74, 203, 221] |
| Pig | Better social acceptance Bone mineral density and healing similar to humans | Hind limb anatomy is different to humans Rapid growth rate Difficult handling | N/A | 2.5–3 cm in the radius | [74, 203, 221] |

the threshold size intraosseous defect dimensions that will not heal spontaneously during the lifetime of the animal” [166]. Therefore, large animals are more appropriate. Table 5 summarizes the advantages and disadvantages when applying orthotopic models in different animal species.

From a surgical point of view, orthotopic models can be categorized as (1) calvarial defect and (2) segmental long-bone defect, which has different critical sizes

depending on location, age, and animal species (Table 5). The calvarial defect model provides a good non-loading-bearing bone-healing environment with relative biological inertness due to poor blood supply and limited access of bone marrow, which is thought to resemble the atrophic mandibular bone in humans. Furthermore, it provides a good simultaneous environment to study the intramembranous ossification and allows the establishment of a uniform, reproducible, and standardized defect. The standard rodent calvarial bone defect is typically created by using a trephine drill that makes a circular defect in the cranial skeleton on the midline [189]. It is suggested that the sagittal suture and the dura mater underlying the defect have to be carefully protected during the surgery which is important for the cranial skeleton healing. Furthermore, the filling materials should be strong and sufficiently resistant to avoid the dilation of brain tissue beneath the defect [78]. The rodent models are the first-choice models for *in vivo* testing of regenerative and/or therapeutic approaches but are not suited to the establishment of long-term studies and immediately translation to a clinical setting.

Segmental long-bone defects allow researchers to test and understand the tissue formation destined for long-bone healing with mechanical loading and in upscaled treatment modalities for clinical application. The creation of segmental long-bone defects is usually done in an osteotomy approach, which utilizes a drill or saw to surgically remove the required length of the bone from a predetermined site, producing a consistent defect in all animal species. After filling the defect, it can be internally fixed with either bone plates or intramedullary rods [74] or by external fixation such as the Ilizarov fixation technique. In addition, we recently developed a sheep segmental tibial defect bone model, which provides additional insights on the handling, safety, feasibility, and upscaling possibilities of different regenerative treatments. However, also in this large-animal bone defect model, discussions still remain in defining a critical size defect being the one that does not achieve spontaneous healing during the lifetime of the animal. Therefore, the design of a large-animal model has to be stringent, where factors such as the age of the animal, the defect size, and the fixation material used will have a significant impact. Moreover, this phenomenon of spontaneous bone regeneration, which can occur in a large-animal bone model and thus can interfere with a regenerative treatment applied in the defect, can be seen as “background noise” and can therefore lead to over-enthusiastic conclusions about the actual effect of a regenerative treatment (Fig. 4).

7 Cell-Based Combination Products: Challenges and Perspectives

Bone fracture healing is essential for the quality of life and even survival. Therefore, a natural tightly regulated cascade of cellular and molecular events has evolved in evolution leading to a successful healing process allowing the individual to survive and resume normal function within 6–10 weeks. However, the bone-healing process gets delayed and leads to a nonunion or nonhealing fracture when the defects are too

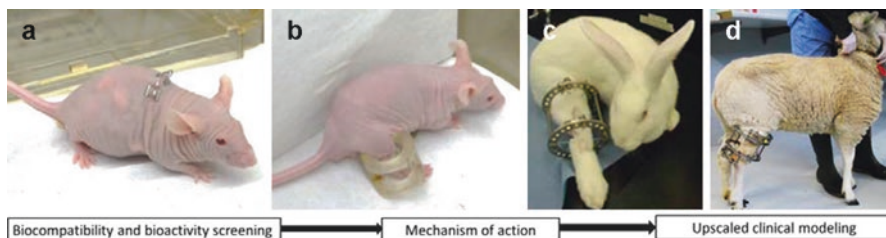


Fig. 4 Animal models for preclinical evaluation of regenerative treatment possibilities for bone regeneration. (a) Ectopic model in rodents is mainly used for biocompatibility and bioactivity screening. Orthotopic defect with Ilizarov fixation technique in mouse (b) and rabbits (c) is usually used to study the mechanism of action underlying the tissue formation. The upscaled orthotopic defect in sheep (d) is useful for clinical translation

large or comprising conditions such as infection and diseased bones arise. In the animal world, a nonunion or nonhealing fracture results inevitably to death. In humans we have the challenge to try to obtain healing by other means in an attempt to restore function and thus independence of the patient.

Novel solutions have been developed in the past decades, including the discovery of antibiotics to fight infection, new surgical techniques and instrumentation to obtain full immobilization, and bone distraction osteogenesis as developed by Ilizarov [67, 75]. In addition, impressive progress in our knowledge on the cell and developmental biology of bone as well as fracture healing has triggered the discovery of new growth and differentiation factors such as BMPs and the development of smart biomaterials. This in turn has led to an unprecedented number of opportunities and strategies to enhance bone healing.

Despite all these stellar developments, there are still quite some clinical challenges, and growing in number, also due to the aging population. These include large bone defects in compromised environments in the patient with comorbidities such as cardiovascular disease, diabetes, osteoporosis, and osteomalacia. In addition, large bone defects as a result of revisions of joint prostheses are becoming a real challenge in daily clinical practice.

In view of this, we need to turn to more sophisticated strategies, combining and improving all the powerful tools and insights that nature has provided us. Opportunities include the use of (stem) cell technologies, the development of more sophisticated growth factor formulations, and the optimization of biologically relevant scaffolds that are enhancing the biological processes and not just sitting there as an inert material. Ultimately, the dream is to combine all these to create living tissue intermediates or provisional tissues that upon implantation steer the healing process in the right direction, also called developmental engineering [109, 110]. Growing knowledge and insights on both materials engineering and cell biology is crucial to implement the essential natural temporal and spatial complexity within the synthetic microenvironment that recapitulates developmental and healing processes of cell proliferation, differentiation, and tissue morphogenesis [120].

To produce these living tissues “of the shelf,” we have serious manufacturing challenges. In combination with robust *in vitro* culture technology that mimics closely the *in vivo* “biological chamber,” upscaled tissue engineering constructs or ATMPs could be engineered into sufficiently pre-differentiated tissue intermediates that are directly recognized by the microenvironment and readily initiate the cascade of bone regeneration. In this perspective, bioreactors with sophisticated online monitoring systems tracking all relevant cellular metabolic profiles and culture environment readouts become critical assets. Novel enabling technologies such as biosensors will be instrumental for industrial manufacturing modular processes for cell-based combination products.

In conclusion, we believe the era of regenerative medicine has just started. First-generation BMP and stem cell technologies have demonstrated that in the postnatal environment, one can successfully enhance the healing of damaged tissues by recapitulating the principles of developmental tissue formation. The stage is set; it is up to us to take on the challenge for the second-generation products that lead to the creation of living replacement body parts.

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