Antibody Mediated Osseous Regeneration: A New Strategy for Bioengineering

Fernanda Coelho, Ticiana Sidorenko de Oliveira Capote, Marcell Costa de Medeiros, and Suzane Cristina Pigossi

Abstract This chapter provides a brief review of bone biology and metabolism, focusing on the regenerative potential of bone tissues. In this context, we discussed the main clinical approaches to enhance bone regeneration, concentrating on an innovative approach referred to as antibody-mediated osseous regeneration (AMOR). Bone morphogenetic proteins (BMPs) are some of the most relevant osteoinductive factors in the demineralized bone matrix. The main role of BMPs is the recruitment and differentiation of mesenchymal cells into an osteogenic lineage, resulting in new bone formation. As an alternative for the BMP-2 exogenous administration of an osteoinductive growth factor, the use of immobilized anti-BMP-2 antibodies in matrices has been proposed to capture the endogenous protein. The captured endogenous BMP-2 would be able to induce osteogenic differentiation of osteoprogenitor stem cells and improve the bone formation. In general, the association of anti-BMP-2 mAb with a scaffold has demonstrated success in new bone formation in different in vivo models with no evidence of adverse reactions.

Keywords Bone regeneration · Bone morphogenetic proteins · Antibodies · Stem cells · Osteoinductive · Osteoconductive · Osteogenic · Scaffold

Biology and Metabolism of Bone Tissue

Bone tissue has a high vascularization and its remodeling continues throughout one`s lifetime [1]. The diversity of the functions of bone is shown by its complex architecture [2]. The arrangement of the bone tissue occurs in a trabecular (cancellous bone) or compact pattern (cortical bone) [2, 3]. Interconnected trabeculae with free spaces filled by bone marrow compose the trabecular bone, and the cortical

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bone is constituted by repeating osteon units, which are composed of collagen fibers and calcium phosphate crystals [4].

Different proportions of those two architectural patterns are observed in the skeleton. Only 10% of the cortical bone is porous, being almost solid, whereas cancellous bone is more porous (50–90%) [3, 5]. According to Jimi et al. [6], the remodeling of the cancellous bone occurs at more than 30% per year and the cortical bone at approximately 3% per year. In 1 year, about 6% of all bones in the human body remodel.

Of the two components of the bone matrix: 65–70% are composed of a mineral part (hydroxyapatite), and 25–30% are made of an organic phase [3], which is composed of type I collagen (90–95%) [7].

Besides, the organic part of the bone matrix is also composed of several proteins (thrombospondin, osteonectin, osteocalcin, byglican, decorin, fibronectin, bone sialoprotein, osteopontin) with different functions [3]. The organic, non-mineralized part, presents an important role in the control of the growth and differentiation of osteocytes, osteoblasts, and osteoclasts in the bone remodeling process [2]. According to Stevens [1], over 200 different types of noncollagenous matrix proteins (sialoproteins, proteoglycans, glycoproteins, etc.) participate in a large number of signals in the immediate extracellular environment. The nanocomposite structure provides the requisite compressive strength and high fracture toughness of bone [1].

Bone tissue is composed of three cell types: osteoblasts, osteoclasts, and osteocytes. Osteoblasts are the most important cells for bone growth and metabolism; they are matrix producers [8]. They produce the extracellular matrix and regulate its mineralization [9]. Mature osteoblasts secrete and deposit most of the bone matrix proteins, besides regulating the formation of hydroxyapatite crystals in osteoids [6]. According to Jimi et al. [6], it is believed that the main functions of osteoblasts are related to the high activity of alkaline phosphatase in response to osteotropic hormones and cytokines, and the expression of a high number of extracellular matrix proteins related to bone tissue.

Osteocytes account for 90% of all cells in the adult skeleton [9]. They are derived from the osteoblasts that are included in the matrix [10]. They are mature cells found in bone lacunae, which communicate with other osteocytes through long cellular processes, and also participate in bone remodeling by sending signals resulting from mechanical stress [11]. Osteocytes may have an important role in initiating the bone cycle, perhaps by detecting microfractures or other perturbations in the bone structure and then signaling osteoclasts to those defects [7].

Osteoclasts, the tissue-resorbing and macrophage-like cells, degrade the bone structure through a combination of localized acidification, removing the mineral, and breaking down the matrix by protease secretion [11]. Osteoclasts are active at the beginning of the bone remodeling cycle and have the function of resorbing the existing bone. They attach their fenestrated membrane to the mineralized matrix on the surface of the bone, isolating a microenvironment, which will be a local site of bone resorption. There is a decrease in pH, and potent enzymes (β-glycerophosphatases, acid phosphatase, β-glucuronidases, aryl-sulfatases, cysteine-proteinases, metalloproteinases, etc.) are released. There is the formation of a depression in the bone due to the erosion, termed as lacunae [7].

Osteogenesis occurs not only during embryogenesis. It occurs throughout life and is related to the bone remodeling process in adults. It is a tightly regulated, active process initiated by stem cells for the purpose of forming a normal vascularized bone structure. The formation of bone tissue depends on the association of factors such as the expression of soluble molecules (hormones, growth factors, vitamins, cytokines, ions, etc.), specific cell types (osteoclasts and mesenchymal stem cells), scaffolds (extracellular matrix molecules, hydroxyapatite), and mechanical stimuli [12]. The bone tissue may be formed by intramembranous and/or endochondral ossification. Mesenchymal condensation nuclei are formed, where cells can differentiate directly into osteoblasts (intramembranous ossification), or into chondrocytes (endochondral ossification) [12].

Bone remodeling occurs by the balanced activity between osteoclasts and osteoblasts [10]. The beginning of the bone remodeling occurs in the quiescent phase. After the osteoclasts are attracted to the new site, they promote erosion of the bone matrix, forming lacunae with sizes of approximately a 50 μm depth and 100 μm in diameter. This process requires about 10 days. Resorption is discontinued and osteoblasts are attracted to bone remodeling site. Osteoblasts secrete an osteoid matrix composed primarily of type 1 collagen, filling the lacunae. This process requires about 80 days. The newly formed matrix is mineralized with hydroxyapatite. The remodeled area goes into the quiescent phase to complete the bone cycle of 60–120 days [7]. The process of bone remodeling is exemplified in Fig. 1.

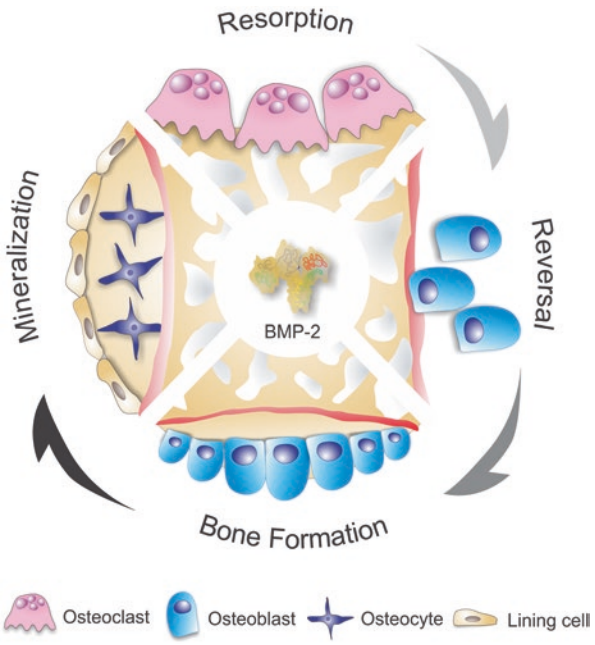

Fig. 1 The process of bone remodeling. (Source: Prepared by the authors)

Bone Regeneration

Unlike other tissues, bone can regenerate and repair itself in a process called bone regeneration. This process is a very efficient and is a rigorously regulated process where all components of bone tissue are involved to optimize the repair and restore skeletal function. It is characterized by a sequence of biological events of bone induction and bone conduction, which involves several types of cells, extracellular and intracellular molecular signaling pathways [11, 13].

Four components are related to the bone regeneration process: (a) morphogenetic signals, (b) response to the signal by the host cells, (c) a suitable carrier for the growth of the host cells, and (d) a well-vascularized and viable tissue of the host [4, 14]. Inflammatory cells, vascular cells, osteoclasts, and osteochondral progenitor cells are cells that are present in the repair process [15]. Increased expression of pro-inflammatory, osteogenic, and angiogenic growth factors released at the site of the bone lesion induces signaling cascades for tissue repair [16].

Several growth factors are present in the bone regeneration process, including: bone morphogenetic proteins (BMPs); fibroblast growth factor (FGF), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF); transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) [17].

According to Schindeler et al. [15], bone healing occurs in a four-stage process: inflammation (formation of hematoma, infiltration of the hematoma by inflammatory cells, secretion of cytokines and growth factors, invasion of mesenchymal stem cells, formation of granulation tissue); formation of soft callus (formation of a cartilaginous callus and fibrocartilaginous tissue); formation of hard callus (removal of soft callus, revascularization, osteoblasts in high activity, formation of a mineralized bone matrix); and bone remodeling (the newly formed bone is remodeled into cortical and/or trabecular bone).

Physiological and pharmacological components influence the fracture healing, such as the location and extent of the lesion, infection, biomechanical forces, diseases, nutrition, and genetics [15].

Clinical Approaches to Enhance Bone Regeneration

As previously discussed, bone is a mineralized conjunctive tissue, with a special healing capacity which ensures that the bone injuries and fractures heal without scar formation [11]. However, the successful bone regeneration becomes more complicated depending on the size of the defect since large defects present greater difficulty of repair [18]. In this cases bone substitutes (autogenous, homogenous, heterogeneous, or synthetic) have been used to replace the missing bones.

The ideal bone substitute must present osteoconductive, osteogenic, and osteoinductive properties: osteoconductive bone substitute is able to stimulate osteogenic cells attachment, survival, and migration; osteoinductive bone substitutes induces stem cells differentiation toward an osteoblastic lineage by physical and biochemical

factors, and osteogenic bone substitutes contains stem cells able of differentiating into osteogenic cells in the bone defect [4]. Based on that, the autogenous bone graft is therefore the gold standard since it has the osteoinductive factors and osteogenic cells required for bone regeneration [19]. Autogenous bone graft is removed from another part of the patient's body (tibia, iliac crest, mandible, or skullcap), which restricts its applications essentially due to the limited quantity of the autograft that can be achieved [20]. Moreover, the operating time required for harvesting autografts is expensive with substantial donor site injury often related with pain, infection, and hematoma [21].

Therefore, viable alternatives including allograft (from human donors/cadavers) and xenograft (from a nonhuman) bone are also regularly used for bone defect regeneration when autologous bone graft is not available [22]. However, although the biomechanical stability and elasticity are similar to autologous bone, the absence of osteogenicity associated with the lower rate of graft incorporation represents limitations to those bone grafts [2]. Furthermore, host rejection, disease transmission, and infection risk (even infrequent) have limited their uses [16].

Against all these limitations, synthetic bone substitutes have been developed as a safer, less expensive, and less invasive alternative compared to these bone implants. Those bone substitutes can be produced from biomaterials as hydroxyapatite (HA), bioactive glass, tricalcium phosphate (TCP), polymers, and ceramics [23]. The isolated use of those materials presents only an osteoconductive role, limiting their use in bone reconstruction [18]. Consequently, researchers have been proposed the association of diverse compounds as growth factors, hormones, and drugs to the synthetic bone substitutes to ensure osteoinductive properties and to improve the regenerative potential of those materials [18].

Synthetic Tissue Scaffolds for Bone Regeneration

Three-dimensional scaffolds are normally prepared with porous biodegradable materials which have the mechanical support and preserve the space necessary for cell growth and matrix production during new bone formation [4]. Moreover, the scaffold structure is able to transport diverse compounds in the target space in a high local concentration with the smallest side effects [18]. The ideal synthetic tissue scaffolds should be: (a) biocompatible without eliciting an immune response; (b) osteoconductive, osteoinductive, and osteogenic, promoting bone ingrowth; (c) absorbable in a predictable manner, with biocompatible components, and at the same time of bone growth; (d) easily adaptable to an irregular wound site; and (e) sterilizable without property modification. Additionally, the scaffolds need to present pore sizes with approximately 200–400 μm, correct mechanical and physical properties and not stimulate soft tissue growth at bone/implant interface [14].

The choice of the material to produce a scaffold is an essential stage since its properties will determine the mechanical and physical properties [3]. Several materials including biodegradable ceramics and polymers have been proposed. Ceramics

can be from natural (e.g., coralline HA) or synthetic origin (β-TCP or synthetic HA) [24]. Although being osteoconductive and osteoinductive, those materials have some major drawbacks including small mechanical stability, which limits their applications for large bone defects regeneration. Likewise, their degradation/dissolution rates are difficult to estimate [3]. As an alternative, the natural (animal or vegetal source) and synthetic biodegradable polymers have been used. Those materials showed little immunogenic potential, bioactive behavior, chemical versatility, and capability of host's tissue interaction [3].

The synthetic tissue scaffolds also act as carriers to deliver different compounds to the bone defect area improving local protein retention and sustain a slow release, increasing the osteoinductive potential of the material [16]. According to Vo et al. [16] "the strategies for protein absorption into scaffolds involve either non-covalent (surface adsorption, physical entrapment, affinity binding, ionic complexation) or covalent immobilization on or into the delivery system (chemical conjugation)". The choice of the type of absorption is based on the material's physicochemical properties and interactions between the protein, defect type, and carrier [25]. Beyond the several hormones, growth factors and drugs, the antibody incorporation was proposed in an approach referred to as "antibody-mediated osseous regeneration (AMOR)" [26].

AMOR: Antibody Mediated Osseous Regeneration

Several therapies involving antibodies have been studied in genomic research based on its high degree of affinity and specificity to the antigen or target molecule that guarantee a high level of efficiency with fewer adverse events. The mechanism of action of the antibodies and the ability of targeting several molecules allows them to be applied to a wide range of therapeutic targets [27]. Currently, the number of monoclonal antibodies (mAbs) approved in clinical research is surprisingly growing in different therapeutic areas, including cancer treatment, organ transplantation, inflammatory disease, respiratory disease, cardiovascular disease, ophthalmologic disease, and infection [27, 28]. Based on these antibody therapies advantages, Freire et al. [26] proposed a strategy using BMP-2 specific immobilized antibodies (BMP-2 Abs) to promote bone regeneration. In this strategy, the BMP-2 specific immobilized antibodies can sequester endogenous BMP-2 and induce new bone formation. This new approach is referred to as AMOR.

Osteoblasts are responsible for the synthesis and secretion of BMPs. BMPs induce the differentiation of mesenchymal stem cells (MSCs), which stimulate the process of osteogenesis, allowing for healing and bone remodeling [29, 30]. Traditionally, pathways leading to differentiation of MSCs into osteoblasts are activated by BMPs binding to a membrane-specific ligand receptor named of BMP type 1 (BMPR1) and BMP type 2 receptors (BMPR2). Receptor binding stimulates signal transduction through the phosphorylation of various homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis

elegans proteins (Sma) (SMAD) proteins and their nuclear translocation. The SMADs also function as transcription factors, controlling the expression of essential osteogenic genes involved in osteoblast proliferation (*Msx2*), matrix synthesis (*RUNX2*, osteopontin-*SPP1*, alkaline phosphatase-*ALPL*) and inhibition of osteoclast differentiation (*TNFRSF11B*- osteoprotegerin) [31]. According to [32]: "In addition to BMP/SMAD signaling, Mitogen Activated Protein Kinases (MAPK) cascades represent an alternative, non-canonical pathway for BMP-2 signal transduction. BMP-2 activates the signaling pathways p38, extracellular-signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK1/2) to induce the activation and expression of a specific transcription factor related to *RUNX2*. *RUNX2* plays an essential role in the osteoblastic differentiation of MSCs and directly stimulates the transcription of important downstream target genes, including those encoding osteocalcin (*Bglap*), collagen type 1 (*Col1A1*) and osteopontin (*Spp1*)". The mechanism of action of BMP-2 on the expression of osteogenic markers can be verified through the signaling cascade in Fig. 2.

Based on this high osteogenic activity, the use of recombinant human bone morphogenetic protein-2 (rhBMP-2) as a viable alternative to bone grafts has been approved by the US Food and Drug Administration (FDA) for clinical use and has been investigated in different bone cicatrization applications. Preclinical and clinical researches have demonstrated that an absorbable collagen sponge combined with rhBMP-2 can induce new bone formation with clinical and radiographic results equivalent to autogenous grafting [33–35]. However, according to [36]: "The clinical application of rhBMP-2 is associated with a number of biological and logistic drawbacks, including (1) requirement for administration of rhBMP-2 at superphysiological doses, (2) inability to sustain growth factor concentration over extended periods of time, (3) lower biological activity of rhBMP-2 relative to endogenous counterparts and (4) high costs of rhBMP-2". Therefore, the use of BMP-2 specific immobilized antibodies has been investigated to replace the exogenous pathway and avoid the adverse effects associated with rhBMP-2 use [26, 37].

This approach aims to capture endogenous BMP-2 using specific Abs immobilized in a solid scaffold. The binding scheme of the anti-BMP-2 antibodies in scaffolds for the capture of endogenous BMP-2 can be visualized in Fig. 3.

According to Freire et al. [26]: "To participate in AMOR, an Abs molecule must have the following properties: (1) high affinity for binding to endogenous BMP-2; (2) binding of BMP-2 epitopes remotely from the BMP-2 receptor-binding domains; (3) involve the BMP-2 cellular receptor on osteoprogenitor cells by the Ab-BMP-2 immune complex; (4) intracellular signal transduction by the Ab-BMP-2 immune complex; (5) absence of an adverse local or systemic immunological response in the host; and (6) mediation of osteogenic differentiation by Ab-BMP-2 immune complexes".

Freire et al. [26] performed in vitro and in vivo studies to evaluate the ability of the immobilized anti-BMP-2 Abs to capture the endogenous BMP-2 and mediate the formation of a new bone tissue. In these studies, antibodies were obtained from the immobilization of rhBMP-2 (Infuse®; Medtronic) in mice. Many clones were formed using the ClonaCell-HY hybridoma cloning kit (StemCell Technologies).

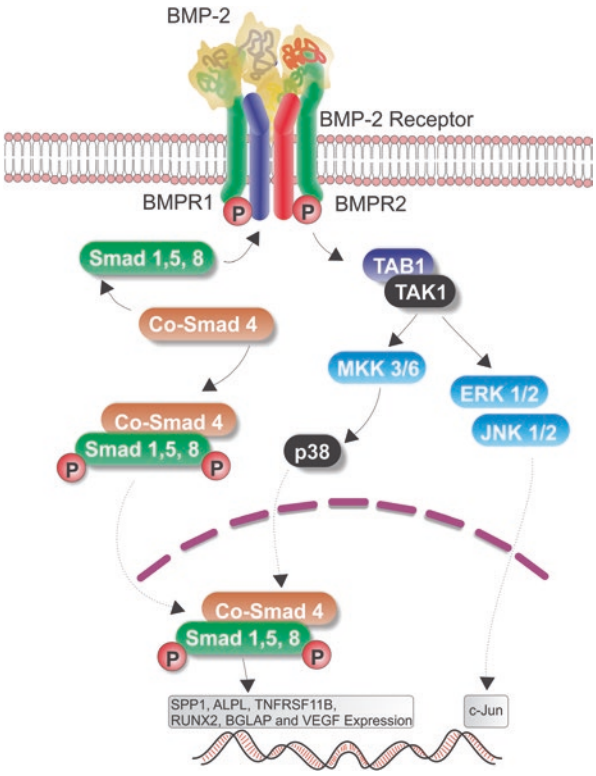

Fig. 2 BMP-2 signaling markers in the expression of osteogenic markers. (Source: Prepared by the authors)

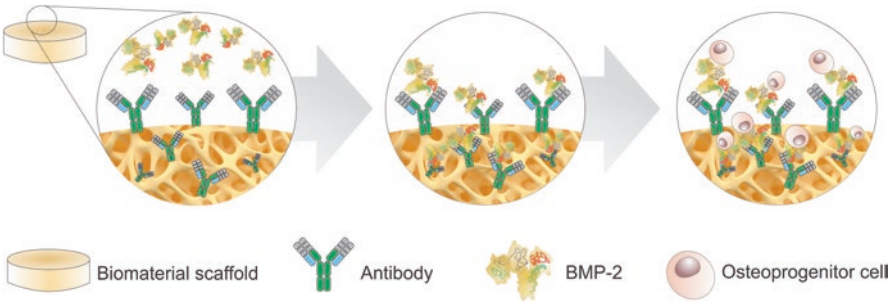

Fig. 3 Anti-BMP-2 immobilization scheme on membranes. Demonstration of capture of endogenous BMP-2 and osteoprogenitor stem cells. (1) Anti-BMP-2 mAb is withheld on membranes; (2) mAb binds endogenous BMP-2; and (3) specific receptor osteoprogenitor cells are attracted by endogenous BMP-2 and promote bone differentiation. (Source: Prepared by the authors)

Thereafter, the competence of the anti-BMP-2 Abs to link to BMP-2 and to permit BMP-2 in the immune complex to link to the BMP cell receptor was assessed by a flow cytometry assay. Through this assay it was observed that some mAbs could link to BMP-2 and permit BMP-2 to bind to cells, but most prevent the binding of BMP-2 to its cellular receptors. In the in vivo analysis, the BMP-2 antibody was immobilized on an absorbable collagen sponge and surgically placed in a rat calvarial defect. Micro-CT analysis demonstrated that bone regeneration was promoted by only a few anti-BMP-2 Abs clones immobilized on absorbable collagen sponge (ACS). The in situ expression of BMP-2 and osteocalcin was evaluated by immunohistochemistry. The analysis revealed higher expression of these markers in sites with greater bone regeneration. These studies demonstrated the competence of anti-BMP-2 Abs to link to endogenous BMP-2 and mediate the formation of a new bone in vivo, presenting a strategy for improved tissue engineering [26].

Investigation of the AMOR Approach in Animal Models

The AMOR approach may be used in clinics, and it is interesting to demonstrate its effectiveness in animal models. Besides the study [26], a variety of in vivo experimental approaches have been reported in the literature.

Ansari et al. [37] compared the effectiveness of a murine anti-BMP-2 mAb immobilization technique using diverse matrices including titanium microspheres, alginate hydrogel, and ACS. Those matrices were surgically grafted on rat critical-sized calvarial defects. After 8 weeks, the bone regeneration process was most effectively evidenced in the three types of scaffolds with immobilized anti-BMP-2 mAb compared to the isotype control mAb. The titanium scaffold presented greater bone formation followed by ACS. However, the titanium scaffold is not biodegradable, which limits its applications. In all the scaffolds used, the presence of the BMP-2, -4, and -7 antigens was identified through the immobilized anti-BMP-2 mAb, reinforcing the efficiency of the AMOR strategy for bone regeneration.

The wide range of applications of induced pluripotent stem cells (iPSCs) stimulated the curiosity of Wu et al. [38]. The iPSCs are somatic cells collected from patients, which could be transformed into pluripotent stem cells, using a suitable sequence of signaling proteins and growth factors, exhibiting the same pluripotency of embryonic stem cells (ESCs). In this context, Wu et al. [38] described an approach using BMP-2 Abs to guide osteogenic differentiation of iPSCs (derived from mesenchymal stromal cells or iMSCs) in an in vivo ectopic bone formation model. For this, subcutaneous injection of alginate microbeads with iMSCs and encapsulated anti-BMP-2 Abs were perfomed in 12 eight-week-old male mice. The presence of the anti-BMP-2 antibody was able to involve the BMP-2 recalls in the iMSCs. A subcutaneous implantation locus loaded with the iMSCs and anti-BMP-2 showed increased bone formation and vascularization in mice compared to exogenous BMP-2. The exogenous BMP-2 exhibited significantly lower dystrophic calcification and vascularization, which revealed that the anti-BMP-2 Ab/BMP2 immune

complex was able to dictate the acquirement of the osteogenic phenotype of iMSCs and subsequent mineralization.

Guo et al. [39] investigated the AMOR approach for a nonunion tibia defect repair in a nonhuman primate model. Six animals of the species *Macaca fascicularis* were operated on for a 20 mm segmental osteotomy in their tibias. The isotype matched control mAb and the investigated material (absorbable collagen sponge incorporated with anti-BMP-2 mAb) were introduced into the defects created. After a surgical period of 12 weeks, histological, histomorphometric, and cone beam computed tomography (CBCT) analyses were performed. Quantitative 3D volumetric analysis by CBCT demonstrated the formation of a larger volume of mineralized tissue at sites that were implanted with an absorbable collagen sponge incorporated with anti-BMP-2 mAb compared to sites implanted with an isotype-matched control mAb; and the histological and histomorphometric analysis indicated that sites introduced with anti-BMP-2 showed new bone formation with a higher percentage of bone volume compared to the isotype matched control mAb.

Xie et al. [40] investigated the application of the AMOR approach in a mandibular continuity defect repair in nonhuman primates. Critical-sized mandibular continuity defects were formed in six adult male *Macaca fascicularis.* Collagen sponges (CS) incorporated with anti-BMP-2 mAbs were locally implanted. Three animals were designated to experimental (AMOR) and three to control (isotype-matched mAb) groups. 2D and 3D analysis of CBCT and histological examination demonstrated an increased bone density and volume in regions treated with anti-BMP-2 ACS-mAb compared to control in 6 and 12 weeks, postoperatively.

Khojasteh et al. [41] evaluated the AMOR strategy on the repair of a canine segmental mandibular continuity defect model. Consequently, a 15 mm unilateral segmental defect was created in the mandible and fixed with a titanium plate. Inorganic bovine bone mineral with 10% collagen (ABBM-C) was incorporated with anti-BMP-2 mAb or isotype-matched mAb. The rhBMP-2 served as a positive control. Morphometric analyses were observed by CBCT and histological images. Bone densities within healed defect sites at 12 weeks after surgery were 1360.81 ± 10.52 Hounsfield Unit (HU), 1044.27 ± 141.16 HU, and 839.45 ± 179.41 HU, in sites with implanted anti-BMP-2 mAb, rhBMP-2, and isotype mAb groups, respectively. Osteoid bone formation in anti-BMP-2 mAb (42.99% ± 8.67) and rhBMP-2 (48.97% ± 2.96) groups was not significantly different but was higher than in sites with an isotype control mAb (26.8% ± 5.35). In this way, the results of this study confirmed the feasibility of AMOR in a large clinically relevant animal model.

Conclusion

The efficiency of the AMOR approach in bone tissue engineering has been demonstrated in several in vivo studies. In general, the association of anti-BMP-2 mAb with a scaffold has demonstrated success in the new bone formation in different

in vivo models with no evidence of adverse reaction. The effectiveness of this approach suggests that this strategy could be introduced into clinical use of tissue engineering with promising results.

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