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Osteogenic molecules for clinical applications: improving the BMP-collagen system

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ABSTRACT

Among the osteogenic growth factors used for bone tissue engineering, bone morphogenetic proteins (BMPs) are the most extensively studied for use in orthopaedic surgery. BMP-2 and BMP-7 have been widely investigated for developing therapeutic strategies and are the only two approved for use in several clinical applications. Due to the chemical and biological characteristics of these molecules, their authorised uses are always in combination with a carrier based on collagen type I. Although the use of these growth factors is considered safe in the short term, the very high doses needed to obtain significant osteoinduction make these treatments expensive and their long-term safety uncertain, since they are highly pleiotropic and have the capacity to induce ectopic ossification in the surrounding tissues. Therefore it is necessary to improve the currently used BMP-collagen system in terms of efficiency, biosecurity and costs. There are several strategies to increase the clinical effectiveness of these treatments. In this review we summarize the most promising results and our related work focused on this field through two different approaches: i) the development of recombinant BMPs with additional features, and ii) complementing these systems with other growth factors or molecules to enhance or accelerate osteogenesis.

Key words: BMPs, collagen, FGF-2, growth factors, osteogenesis, recombinant proteins, RGD peptide.

INTRODUCTION

Healing of bone defects and fractures caused by trauma, tumour resection or disease constitutes a significant clinical and economic problem due to the limited effectiveness of the current treatment options.

The expected time for a fracture to heal naturally is between six and twelve weeks, but there is a high rate of delayed unions, varying from 16-60% for less severe fractures to 43-100% for more severe cases. A fracture that shows motion at the bony ends and is not completely healed within 6 months is considered a non-union, whose rate has been reported to range from 4 to 10% (Garrison et al., 2007). Non-unions can not only lead to significant pain, inhibition of function and decreases in personal and professional productivity, but also enormously raise the economic implications for healthcare providers. The rate of delayed or non-unions is especially high in elderly patients, in which the titre of mesenchymal stem cells (MSCs) within the bone marrow is diminished. While one of every 10,000 bone marrow cells is estimated to be a MSC in neonates, this number decreases to one of every 2,000,000 cells in 80 year-old individuals (Caplan, 2007).

One possible option to help stabilizing fractures with a poor healing prognosis is the use of external fixation devices, although these often result in the production of unstable bone with a high probability of re-fracture (Braddock et al., 2001). For the treatment of extended bone defects following trauma, cancer resection or non-union fractures, more sophisticated treatments than the standard conservative or surgical therapies may be required. In these cases, segmental bone transport, distraction osteogenesis, bone grafting or biomaterials must be applied for reconstruction (Kneser et al., 2006).

Nevertheless, autologous bone grafts are still considered the gold standard for the treatment of non-union fractures, since they possess both important osteoconductive and osteoinductive properties. By bone grafting, the missing bone is replaced with material from the body of the same patient or with a natural substitute. When autologous bone is used, it is typically harvested from the iliac crest of the pelvis. Allografts from cadavers or living donors may also be used; these are usually sourced from a bone bank. Although bone grafting is generally successful, the limited amount of available donor tissue and the high associated morbidity, resulting in numbness or tingling at the donor site, infection or prolonged pain make the need for development of alternative therapies evident (Braddock et al., 2001).

More recently, the medical field is focused on the use of natural or synthetic biomaterials (i.e. materials which are compatible with living cells and tissues) for bone repair; the aim of these products is to mimic the osteoconductive properties of bone grafts. To confer also osteoinductive capacity to these grafts, their application in combination with osteogenic growth factors and/or biomimetic peptides is being widely studied (Lauzon et al., 2012). These signalling molecules stimulate endogenous repair mechanisms by recruiting and reprogramming the patient´s own progenitor cells; bone morphogenetic proteins (BMPs) are the most extensively studied (Reddi, 1998; Nakase et al., 2006). BMPs are a family of growth factors implicated in a variety of functions during development and in tissue regeneration (Hogan, 1996). Besides, these molecules play a key role in the development and regeneration of the skeletal system (Nakase and Yoshikawa, 2006), providing a solution for the treatment of bone fractures.

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APPLICATIONS OF BONE MORPHOGENETIC PROTEINS AND COLLAGEN TYPE I

Among the wide variety of growth factors involved in bone homeostasis, the BMPs have especially focused the attention of the researchers, because of their strong osteogenic properties and for being the only cytokines known to induce ectopic bone formation.

The use of recombinant human BMP-2 for the treatment of open tibial fractures was investigated by the BESTT trial (the BMP-2 Evaluation in Surgery for Tibial Trauma) (Govender et al., 2002) and by a subgroup analysis (Swiontkowski et al., 2006). These studies concluded that patients treated with 1.50 mg/Kg rhBMP-2 showed fewer hardware failures, fewer infections and faster wound healing than patients in the control groups, finally leading, in July, 2002, to approval of the use of rhBMP-2 (InductOs®) by the European Medicines Agency (EMA) for the treatment of severe tibial fractures in adults. A few months later, in November, 2002, the American Food and Drug Administration (FDA) approved the use of rhBMP-2 in combination with absorbable bovine type I collagen sponges (INFUSE® Bone Graft Device) for the treatment of open fractures in long bones. rhBMP-2 was also approved in 2008 by the FDA for use in spinal fusions, in the form of a cylindrical titanium fusion cage filled with rhBMP-2/collagen sponge (InFuse® Bone Graft/LT-CAGE® Lumbar Tapered Fusion Device). This approach has been proven to be effective to achieve anterior inter-body fusion in patients with degenerative lumbar disc disease (Boden et al., 2002; Burkus et al., 2003).

Also rhBMP-7 for the treatment of tibial non-unions was investigated by a prospective, randomised clinical trial, concluding that rhBMP-7 implanted with a type I collagen carrier is a safe and effective alternative to autologous bone grafting for the treatment of tibial non-unions (Friedlaender et al., 2001). On this basis, the FDA issued a *Humanitarian Device Exemption* for the application of BMP-7 implants (OP-1® Implant) in recalcitrant long bone non-unions where autografts are unfeasible and alternative treatments had failed. Similarly, the EMA approved the use of Osigraft® for the same purposes. Since then, different clinical studies of resistant tibial non-unions treated with rhBMP-7 have been published (Pecina et al., 2001, 2003). In April, 2004, the FDA also approved the use of a combination of rhBMP-7, bovine type I collagen and carboxymethylcellulose (OP-1® Putty) for posterolateral spinal fusion after failure of alternative treatments. This decision was made based on data obtained from previous preclinical studies in dogs and clinical pilot studies (Cook, 1995; Vaccaro et al., 2002, 2004).

BMPs have also been used off-label for other clinical applications, some of them demonstrating good short-term clinical outcomes; however, their use is not completely free of associated complications (Ong et al., 2012). Clinical studies carried out with rhBMP-2 concluded that the effective osteoinductive dose of this growth factor is 1.5 mg BMP-2 / mL ACS (Valentin-Opran et al., 2002; Govender et al., 2002). Nevertheless, concentrations on the order of just hundreds of nanograms per millilitre are sufficient to induce osteoblast differentiation of mesenchymal cells *in vitro*, while in the human body, normal concentrations of BMPs are estimated at 2 ng/g of bone (Rengachary, 2002). Thus, clinical application of BMPs implies raising their local concentration more than 10^6-fold over physiological levels. However, it has been shown that after administration of rhBMP-2, the amount of growth factor that can be found in the systemic blood stream is about 0.1% of the dose used, and that these molecules have a half-life of only a few minutes (Valentin-Opran et al., 2002).

Although the use of BMP-2 and -7 is in general terms considered safe, the long-term effects of the application of such amounts of these potent, highly pleiotropic growth factors are not well known. On the other hand, the immune mechanisms triggered upon BMP implantation are not well defined, due to controversy in the literature. It appears that single applications of allogenic BMPs can promote the recruitment of macrophages, lymphocytes and plasma cells, as well as activate a moderate production of anti-BMP antibodies (Granjeiro et al., 2005). Besides these and other unknown undesired side effects, another disadvantage of the use of high doses of these growth factors is the enormous economic cost of the treatments, which is not easily assumed by many healthcare systems (Garrison et al., 2007; Alt et al., 2009).

It has been demonstrated that new bone formation can be achieved by direct application of BMPs alone (Wozney et al., 1990; Einhorn et al., 2003), but these approaches require the use of very high doses of growth factors, since they have a short half-life *in vivo* and suffer rapid systemic dispersion after injection. Application of the growth factors in combination with specific carriers can improve their osteogenic abilities (Peel et al., 2003). In these cases the aim of the carrier is to retain the growth factors at the wound site and to maintain their local concentrations, since it has been demonstrated that bone healing efficiency is correlated with the prolonged presence of BMPs at the site of implantation (Uludag et al., 2001; Woo et al., 2001). Furthermore, the carrier can act as an osteoconductive milieu, permitting its infiltration by mesenchymal cells and the ingrowth of blood vessels (Peel et al., 2003).

Keeping in mind that none of the carriers available today possess all the features to be considered ideal, despite its poor biomechanical properties collagen is the only carrier approved for clinical application of BMPs, due to its high biocompatibility, biodegradability and low immunogenicity (Hubbell, 1995; Friess, 1998). Collagen is the main protein of connective tissue in animals, and is considered the most abundant protein in mammals. Among the 28 different types of collagen, type I is the most represented in the human body and is found mainly in the dermis, tendons, endomysium, fibrocartilage, bone and scar tissue. The clinical administration of BMPs for bone regeneration is done in combination with bovine type I absorbable collagen sponges (ACS), which are soaked in the growth factor before implantation (Valentin-Opran et al., 2002). It has been shown that this form of collagen allows proper cell infiltration during new bone formation (Friess, 1998).

Unfortunately, most growth factors have little natural affinity for collagen. Pharmacokinetic studies of rhBMP-2 retention/liberation from collagen sponges *in vivo* showed a rapid initial loss followed by an exponential liberation of the growth factor (Hollinger et al., 1998). Since it has been demonstrated that a sustained release of BMPs *in vivo* may be critical for osteoinduction, most of the problems associated with the clinical application of growth factors could be palliated if these could be specifically retained at the wound site, being slowly and sustainably liberated from their carrier.

To achieve better performance of the BMP-collagen system, two main strategies have arisen: i) using recombinant DNA technology to design and produce modified BMPs with special features, and ii) complementing and/or enhancing BMP-

induced osteogenesis by including other growth factors in the system, such as angiogenic signals or other molecules as biomimetic peptides involved in bone induction.

RECOMBINANT COLLAGEN-TARGETED BMPS

As stated earlier, BMPs are involved in the regulation of numerous processes of skeletal formation and homeostasis such as migration, proliferation and differentiation of angiogenic and mesenchymal progenitor cells, matrix formation,maturation and mineralization, with their action depending on the type of BMP and their local concentrations. These growth factors are synthesized as large monomeric precursors of 400-500 amino acids consisting of an N-terminal signal peptide that directs secretion, a prodomain and a carboxy-terminal mature domain; the C-terminal mature protein is proteolytically cleaved from the prodomain at an Arg-X-X-Arg sequence by serine proteases before dimerization (Constam and Robertson, 1999). Only BMP-2 and BMP-4 possess a potential secondary cleavage site (Cui et al., 2001). The mature domain contains seven cysteines, six of which form three intracatenary disulfide bonds which will give rise to a particular quaternary structure known as the *cysteine knot*. The seventh cysteine is implicated in the dimerization process with another monomer, forming the active molecule (McDonald and Hendrickson, 1993).

In natural bone regeneration, the prolonged presence of BMPs in the local healing environment is provided by their interaction with components of the extracellular matrix. Following cleavage at the primary site, the BMPs form complexes with their prodomains; after secretion, these complexes are directly targeted to microfibrils of the extracellular matrix, where the prodomain mediates binding to fibrillins (Sengle et al., 2008). Besides, some BMPs such as BMP-2 contain another crucial binding site: a basic N-terminal domain with positive net charge that interacts with negatively charged sulfate and carboxylate groups of heparin. Although this heparin-binding site is not involved in receptor activation, it is responsible for concentrating the active growth factors by avoiding their diffusion and modulating their local action (Ruppert et al., 1996). Thus BMPs are released as soluble active forms which are capable of diffusing away from the cell of origin, or due to natural mechanisms such as the presence of ECM-binding domains or the establishment of complexes with the prodomain, being tethered and concentrated within the ECM. This anchorage of the BMPs to extracellular matrix proteins not only leads to an increase in their local concentration at the healing site, but may also allow their presentation to specific receptors of their target cells in an immobilized conformation, which could limit and/or modulate the receptor binding to initiate osteogenesis.

For use in clinical applications recombinant BMPs are combined with a carrier, not only with the aim of providing a support for bone ingrowth, but also to simulate the natural bone healing process in which BMPs are trapped in the extracellular matrix. The main problem with this approach is that the great majority of the available scaffolds do not have the ability to couple BMPs and provide a specific retention. Thus osteogenic growth factors are commonly used with simple adsorption to the carrier by soak loading, which produces an initial burst release of them with a rapid decrease of biological activity, a fact that is considered inappropriate from a physiological point of view.

One of the strategies to accomplish the specific binding of the BMPs to their delivery material is to modify the growth factors with different matrix-binding domains, allowing a controlled slow release from their carrier and protection from proteolytic degradation. Thanks to recombinant DNA technology, many proteins of therapeutic interest have been produced with modifications to target them to cells or to other proteins of the extracellular matrix (Table I) with the aim of reducing the loss of effective molecules by diffusion, uptake by cells and/or enzymatic degradation, and to maintain them at the site of application at an appropriate pharmacological level.

TABLE I

Recombinant fusion proteins with additional binding domains to cells or extracellular matrix proteins

Protein	Modification	Reference
HGF	Collagen-binding domain of fibronectin	Kitajima et al., 2007
EGF	Cell-binding domain of fibronectin	Kawase et al., 1992
	Collagen-binding domain of C. histoliticum collagenase	Nishi et al., 1998
	Collagen-binding domain of fibronectin	Ishikawa et al., 2001
bFGF	Collagen-binding domain of C. histoliticum collagenase	Nishi et al., 1998
	Collagen-binding domain of the vWf	Andrades et al., 2001
	Fibrin-binding domain	Zhao et al., 2008
TGF-β1	Collagen-binding domain of the vWf	Tuan et al., 1996
TGF- β2	Collagen-binding domain of the vWf	Han et al., 1997
BMP-3	Collagen-binding domain of the vWf	Han et al., 2002
BMP-2	Fibrin-binding domain	Schmoekel et al., 2005
	Collagen-binding domain of the vWF	Chen et al., 2007a
	Collagen-binding domain of C. histoliticum collagenase	Chen et al., 2007b
	Collagen-binding domain of the vWF	Visser et al., 2009

Since BMPs are structurally complex proteins, large-scale *in vitro* production of these growth factors is not a simple task, especially when the coding region of the cloned gene is modified to obtain a non-native improved rhBMP. The active, mature form contains the typical cystine-knot motif that needs to be stabilized by three intracatenary and one intercatenary disulfide bonds. In addition, all BMPs have one or more putative N-glycosylation sites in their mature domains, but the presence of N-moieties is not equally important for all the members of the BMP subfamily; whereas the lack of glycosylation does not affect the activity of rhBMP-2 (Vallejo et al., 2002; Long et al., 2006; Visser et al., 2009), the binding of rhBMP-6 to its type I receptors seems to be strictly dependent on glycosylation (Saremba et al., 2008; Visser, 2009). Another important factor is the low solubility of BMPs in aqueous solutions, which makes them prone to precipitate even at relatively low concentrations. Despite these facts, some members of the BMP subfamily have been produced to date as fusion proteins with several modifications in their sequence and additional domains which confer specific affinity to several biomaterials or components of the extracellular matrix with no loss of their natural biological activity (Table I).

BMP-2 is one of the most studied members of the BMP subfamilies, not only because of being involved in nearly all stages of the bone regeneration process, but also because of the above-mentioned possibility to produce non-glycosylated active molecules in prokaryotic expression systems, applying *in vitro* folding protocols. Moreover, rhBMP-2 is one of the only two approved BMPs for clinical use in combination with absorbable bovine type I collagen sponges, whose application has shown excellent results for the treatment of open fractures in long bones and spinal fusions. In consequence, many researchers have concentrated their investigation on the modification of this protein to bind it to extracellular matrix components.

One of the proposed modifications consists of a recombinant fusion protein containing the human BMP-2 sequence and a domain which provides covalent attachment to fibrin, a natural component of the ECM also used in human surgery; a third domain was designed to allow directed cleavage for local release of the attached protein (Schmoekel et al., 2005). The aim of this construct was to modulate the activity of the protein and its retention through the enzymatic activity associated with cell invasion during the healing process. The authors showed that this modified protein combined with fibrin increased the natural healing of cranial defects in rats and rabbits compared to wild type BMP-2, in a dose-dependent manner.

Another modification consists of the addition of collagen-binding domains to the BMPs. Since collagen is not just the only carrier approved by the FDA and the EMA for bone healing applications, but also the main natural constituent of bone, collagen-targeted BMPs are of special clinical interest. Direct administration of these molecules in soluble form could increase their local concentrations by direct binding to the collagen fibers at the site of injection. On the other hand, when administered in combination with a collagenic carrier, the growth factors would be specifically retained, limiting their actions to the wound site. These approaches could reduce the doses of BMP needed to achieve bone regeneration compared to the use of native molecules, improving the safety of the treatments and reducing their costs. One of the most frequently used collagen-binding domains is the one derived from the bovine von Willebrand factor (vWF). This collagen type I-binding domain (CBD) has been identified as a decapeptide with the sequence Trp-Arg-Glu-Pro-Ser-Phe-Cys-Ala-Leu-Ser (Takagi et al., 1992), and used successfully to obtain fusion proteins with several members of the TGF-β superfamily (Table I); all these constructs showed increased collagen-binding properties without loss of their natural biological activity.

In the specific case of BMP-2, two different collagen-binding domains have been used; the one mentioned above from the von Willebrand factor (Chen et al., 2007a) and another derived from *Clostridium histoliticum* collagenase (Chen et al., 2007b). In both cases the fusion proteins contained the mature human BMP-2 sequence with the CBD fused to the N-terminus of the growth factor and an additional 6xHis purification tag. Both BMP-2-derived proteins showed specific binding to demineralized bone matrix (DBM) or collagen gels and enhanced activity *in vivo* when implanted together with DBM.

A different variant of these modifications consisted of the production of an rhBMP-2 with a modified CBD from the vWF (Fig. 1). Since the cysteine residue of the CBD can interact with any of the seven cysteines present in the mature domain of BMP-2, which may lead to wrong disulfide bond formation and the production of incorrect, inactive molecules during the *in vitro* folding process, the original Cys-7 of the decapeptide was replaced by a methionine to prevent the CBD from interfering with the correct formation of the cysteine-knot, without compromising the affinity of the CBD to collagen. An additional glycine acts as a linker between the sequences. Furthermore, this rhBMP2-CBD lacked any other additional sequences such as the commonly used 6xHis purification tag, and was purified by its natural affinity to heparin (Visser et al., 2009).

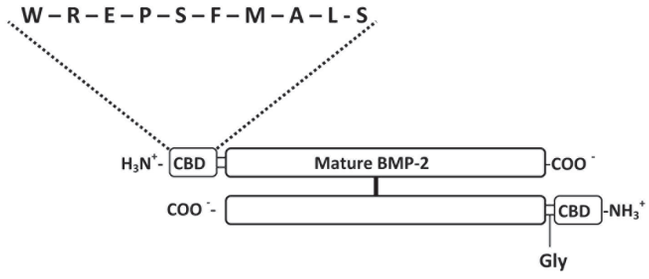

Figure 1. Schematic representation of the genetically engineered rhBMP2-CBD fusion protein, produced by Visser et al., 2009.

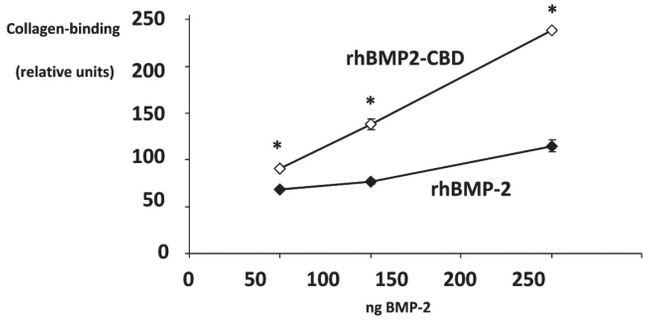

Figure 2. rhBMP2-CBD affinity to absorbable collagen sponges. Protein remaining in ACSs after 7 days of washing with PBST. (*p<0.001). Modified from Visser et al., 2009.

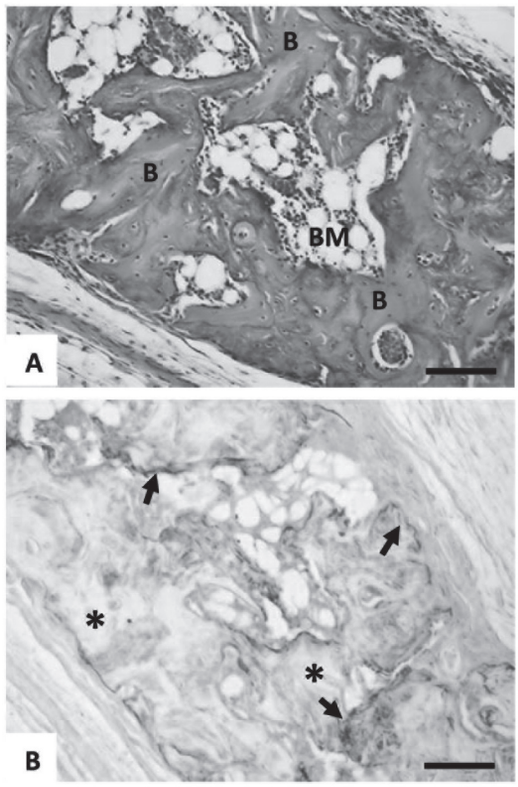

Figure 3. *In vivo* osteogenic activity of rhBMP2-CBD. Ectopic bone formation when implanted in combination with ACS. **(A)** Hematoxylin-eosin staining of implants, showing the formation of a mature trabecular bone with medullar cavities. **(B)** Immunostaining with an anti-osteopontin antibody. **Arrows:** osteoblasts expressing osteopontin; **asterisks:** osteocytes expressing osteopontin; B: mature bone trabecular; **BM:** bone marrow. Scale bars =100 μm

The resulting protein construct exhibited an enhanced specific affinity to absorbable bovine type I collagen sponges in a dose-dependent manner; this binding was demonstrated to be stable over time (Fig. 2). In addition, when implanted *in vivo* together with ACSs which, unlike DBM are free of any other endogenous growth factors, low concentrations of this rhBMP2-CBD were able to induce new bone formation in rats (Fig. 3). These and other studies suggest that the design and production of recombinant modified BMPs might be useful to improve the current results obtained with the clinical application of these growth factors.

IMPROVING THE BMP-COLLAGEN SYSTEM

The formation of new bone where there was no bone before is a complex, multifactorial process that involves neoangiogenesis and the recruitment and differentiation of osteoprogenitor cells. Although BMPs alone are able to trigger this entire process, even in non-osseous environments, many studies have demonstrated that co-administration of BMPs with other growth factors can enhance or accelerate osteogenesis compared to BMPs alone.

The most studied combinations are those that combine a BMP with an angiogenic factor. This idea is mainly based on two premises: i) new blood vessels are necessary to supply nutrients, other growth factors and cells to the new forming tissue, and ii) blood vessels might themselves be the source of osteoprogenitor cells. This latter hypothesis is based on recent studies that have shown that MSCs express perivascular cell markers and that isolated pericytes can give rise to cells from the myogenic, chondrogenic, osteogenic and adipogenic lineages (da Silva Meirelles et al., 2008; Crisan et al., 2008; Nombela-Arrieta et al., 2011; Feng et al., 2011). Whether pericytes are the real MSCs or not, it seems clear that angiogenic factors can act synergically with BMPs to enhance osteogenesis, despite some controversy in the literature.

One of these angiogenic molecules is vascular endothelial growth factor (VEGF), which has been shown to modulate proliferation, migration and tube formation by endothelial cells. Although numerous studies have reported that VEGF plays an important role during bone formation and healing (Street et al., 2002) and that its co-administration with BMP-2 can enhance bone formation under certain circumstances (Kempen et al., 2009), other authors have described only synergic effects in some cases, or even a total lack of effect of VEGF on osteogenic variables (Young et al., 2009). This is not surprising, however, considering that BMP-2 and VEGF are contradictory signals for a single cell, and that to achieve bone formation *in vivo* both factors must act sequentially and on different cell populations. Thus a proper delivery system for both growth factors simultaneously should be able to deliver them not only at the correct relative and absolute doses, but also following a convenient temporal pattern. Since blood vessels need to supply nutrients and cells to the target area, an initial but finite VEGF-stimulus would be desirable, followed by prolonged liberation of the differentiating factor. Taking this into account, some authors already have proposed some approaches in this line, such as the development of biphasic composites to deliver VEGF and BMP-2 with different liberation rates (Kempen et al., 2009).

The importance of the dose of the angiogenic factor becomes especially clear when fibroblast growth factor-2 (FGF-2 or bFGF) is used. This growth factor is not only a stronger inducer of blood vessel formation than VEGF, but also a potent mitogen for a wide variety of cells such as fibroblasts, myocytes, osteoblasts and chondrocytes. Therefore, during the early stages of natural bone healing FGF-2 plays a critical role in angiogenesis and mesenchymal cell proliferation, which has made this growth factor a promising candidate to be used in combination with BMPs for skeletal regenerative medicine purposes. In fact, many authors have investigated the effect of FGF-2, alone or together with BMP-2, on the osteogenic cells themselves and on *in vivo* bone formation. Although the results of these studies may seem sometimes contradictory, as they range from inhibition by FGF-2 of the expression of osteogenic markers in cultured cells (Rodan et al., 1989; Kato and Iwamoto, 1990; Hurley et al., 1993; Iwamoto et al., 1995; Delany and Canalis, 1998) or of bone formation *in vivo* (Bland et al., 1995; Andreshak et al., 1997; Sakano et al., 2002) to enhanced osteogenesis (Nagai et al., 1995; Lu and Rabie, 2002; Power et al., 2004), most probably these observations are just reflecting the complexity of signalling by FGF-2.

However, much evidence suggests that FGF-2 acts synergically with BMP-2 to enhance *in vitro* differentiation of osteoprogenitor cells or bone formation *in vivo* only when used at low doses, while suppressing osteogenesis when

larger amounts are used. This type of biphasic dose-dependent response is not rare in growth factor biology and has been reported by many authors for FGF-2, which enhanced bone formation in bone grafts (Wang and Aspenberg, 1996) and in a mandibular defect model (Zellin and Linde, 2000) at low doses, while inducing fibrous tissue formation at higher doses. *In vitro* studies on MC3T3-E1 mouse preosteoblasts showed how a low dose (2 ng/mL) of FGF-2 mainly induced proliferation, as shown by the expression of early markers of cell growth, and inhibited the expression of classical osteogenic markers (collagen type I, alkaline phosphatase, osteocalcin), while BMP-2 induced mineralization (Hughes-Fulford and Li, 2011).

Although the fact that BMP-2 is approved by the FDA and the EMA for its use in orthopaedic surgery has forced most research groups to focus on this specific BMP, FGF-2 can also respond synergically with other members of the BMP subfamily such as BMP-6, which has been shown to be a more potent inducer of osteogenesis than both BMP-2 and BMP-7 (Visser et al., 2012: Vukicevik and Grgurevic, 2009). In fact, the combination of a low dose of FGF-2 with a suboptimal dose of BMP-6 *in vivo* enhances the osteogenic activity of BMP-6, resulting in faster and greater bone formation (Fig. 4). Furthermore, a low dose of FGF-2 also affects positively the osteogenic differentiation of MSCs induced by BMP-6 *in vitro* (Fig. 5), since under these conditions an equilibrium between growth and differentiation seems to be reached in the cell population, resulting in an increased overall expression of the osteogenic marker alkaline phosphatase (Visser et al., 2012).

But not only angiogenic factors may be useful to enhance the osteogenic activity of BMPs, since the stimulation of pathways other than the BMP-mediated smad signalling pathway can trigger osteogenic responses. Cell adhesion to the extracellular matrix is important for osteoblast proliferation and differentiation as well as for matrix mineralization; this process is primarily mediated by a family of transmembrane receptors called integrins. When the integrins expressed by a cell recognize specific motifs present in many extracellular matrix proteins, they cluster together to form focal adhesions associated with the actin cytoskeleton. These focal adhesions not only mediate cell adhesion and migration, but can also trigger signalling pathways involved in mesenchymal cell commitment and osteoblast differentiation (Garcia and Reyes, 2005). Among the different integrin-recognition motifs identified in extracellular matrix proteins, the arginine-glycine-aspartic acid (RGD) triplet present in fibronectin, bone sialoprotein and osteopontin is one of the most widely studied. Several groups have reported increased osseointegration of titanium implants when these were previously functionalized with integrin-directed biomimetic peptides (Germanier et al., 2006; Petrie et al., 2008). Also, the combination of RGD-based biomimetic peptides with BMP-2 or BMP-2-derived peptides has shown some promising results, such as enhancing the proliferation and expression of osteogenic markers of human MSCs (Moore et al., 2011) and osteogenesis *in vivo* (Park et al., 2010). To exert their effect, RGD-biomimetic peptides have to be anchored to the biomaterial of election; a broad range of approaches have been used to achieve this for many different implantable materials. For the functionalization of titanium implants with RGD-peptides strategies such as simple adsorption (Song et al., 2010) or chemical linking through gold-thiol chemistry (Ferris et al., 1999) or electrodeposited PEG (Oya et al., 2009) have been carried out. In the case of collagen type I, functionalization with this type of biomimetic peptide has been done through chemical procedures such as using thiol or phosphonate anchors (Roessler et al., 2001), periodate activation (Zhang et al., 2005) or hetero-bifunctional coupling agents (Monteiro et al., 2011). To avoid these chemical modifications to functionalize ACSs with biomimetic peptides, one approach has been the design of an RGD peptide with the CBD from the vWF (Fig. 1). The resulting collagen-targeted

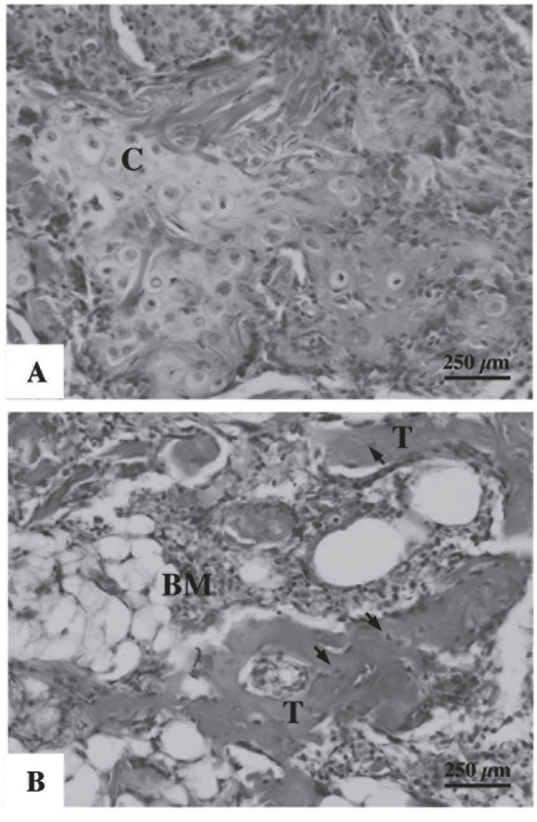

Figure 4. Ectopic bone formation *in vivo*. ACSs loaded with 300 ng BMP-6 **(A)** or 300 ng BMP-6 + 20 ng bFGF **(B)** 14 days after intramuscular implantation, stained with hematoxylin-eosin. **C:** cartilage in transition to bone; **BM:** bone marrow-like tissue; **T:** bone trabecular; **arrows:** osteocytes.

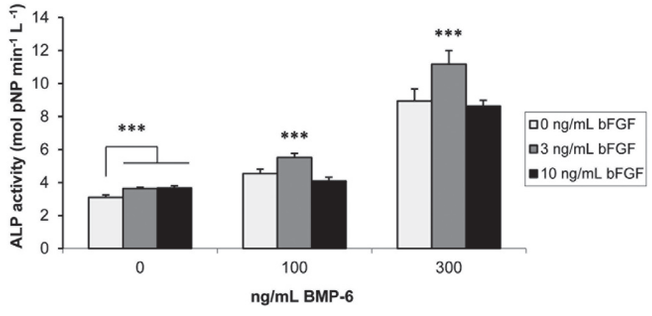

Figure 5. Alkaline phosphatase activity of rat MSCs cultured with BMP-6 and/or bFGF for 7 days. Comparisons are between different bFGF concentrations for every BMP-6 concentration. *** $p < 0.001$. Modified from Visser et al., 2012.

synthetic peptide demonstrated stable binding to ACSs without performing any chemical linking, and induced the differentiation of MC3T3-E1 mouse preosteoblasts and rat bone marrow-derived MSCs (Fig. 6). Furthermore, *in vivo* experiments showed that ACSs functionalized with CBD-RGD and loaded with a sub-functional dose of BMP-2 formed ectopic bone in rats, while non-functionalized sponges loaded with the same amount of BMP-2 did not (Fig. 7). These results indicate that the combination of this biomimetic peptide with the currently used collagen-BMP system might be a promising approach to improve osteogenesis and to reduce the doses of BMPs needed in clinical orthopaedics (Visser et al., 2013).

Besides RGD, other motifs such as FHRRIKA (consensus heparin-binding motif), PHSRN (from fibronectin) or GFOGER (from collagen type I) are also involved in integrin-mediated adhesion and signalling and might be useful for designing BMP-delivery systems with higher osseointegrative properties (Garcia AJ and Reyes CD, 2005).

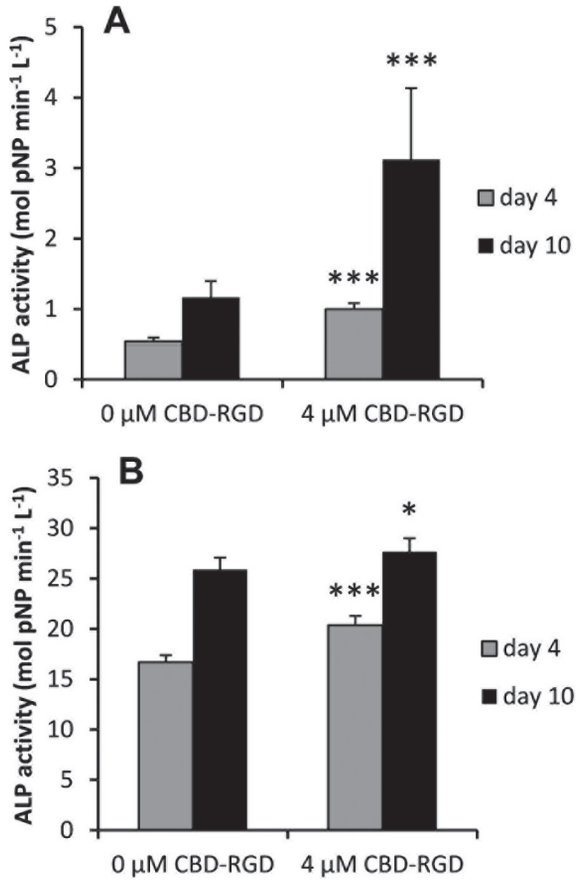

Figure 6. Alkaline phosphatase activity of MC3T3-E1 mouse preosteoblasts **(A)** and rat bone marrow derived MSCs **(B)** cultured in medium supplemented with or without 4 µM soluble CBD-RGD for 4 or 10 days. * $p < 0.05$; *** $p < 0.001$. Modified from Visser et al., 2013.

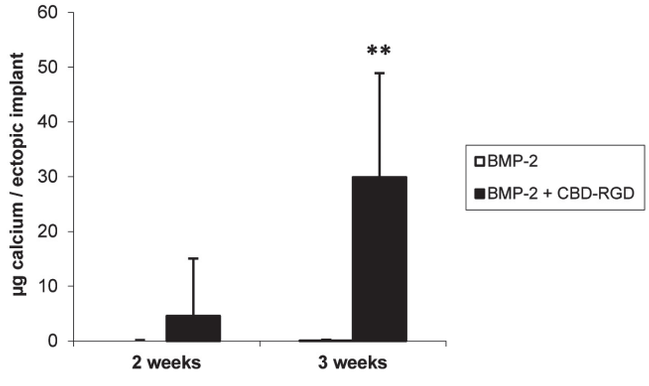

Figure 7. Calcium content of ectopic bone formed in ACSs loaded with 300 ng BMP-2 or 300 ng BMP-2 + 5 µg CBD-RGD, two and three weeks after intramuscular implantation. ** $p < 0.01$. Note that the value of the bars that correspond to BMP-2 alone is zero at both time-points. Modified from Visser et al., 2013.

CONCLUSION

In conclusion, although BMPs alone have proven to be potent and useful tools in orthopaedic surgery and it has been demonstrated that bone healing efficiency is correlated with the prolonged presence of BMPs at the site of implantation and is a complex multifactorial process, its modification with different ECM-binding domains and/or their complementation with other growth factors or osteoinductive molecules might yield more effective or even tailor-designed systems in the future for those cases in which BMPs alone are not sufficient, being a better and safer alternative for bone repair.

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