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Controlled Release Strategies for Bone, Cartilage, and Osteochondral Engineering—Part II: Challenges on the Evolution from Single to Multiple Bioactive Factor Delivery

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The development of controlled release systems for the regeneration of bone, cartilage, and osteochondral interface is one of the hot topics in the field of tissue engineering and regenerative medicine. However, the majority of the developed systems consider only the release of a single growth factor, which is a limiting step for the success of the therapy. More recent studies have been focused on the design and tailoring of appropriate combinations of bioactive factors to match the desired goals regarding tissue regeneration. In fact, considering the complexity of extracellular matrix and the diversity of growth factors and cytokines involved in each biological response, it is expected that an appropriate combination of bioactive factors could lead to more successful outcomes in tissue regeneration. In this review, the evolution on the development of dual and multiple bioactive factor release systems for bone, cartilage, and osteochondral interface is overviewed, specifically the relevance of parameters such as dosage and spatiotemporal distribution of bioactive factors. A comprehensive collection of studies focused on the delivery of bioactive factors is also presented while highlighting the increasing impact of platelet-rich plasma as an autologous source of multiple growth factors.

Introduction

In recent years, the science of tissue engineering (TE) has expanded notably for orthopedic applications, and an increasing number of TE strategies integrating controlled release systems have been further explored.[1] However, these studies face several obstacles in the development of drug delivery systems capable of matching the complexity of native tissue healing. The implementation of release systems in TE approaches using traditional drug delivery approaches and aiming for a biomimetic strategy is a tough task, as it requires the interplay between these different components to enclose the adequate degree of complexity into a TE strategy.

This review provides a comprehensive collection of studies regarding the application of delivery systems of bioactive factors for skeletal engineering. The evolution from a more simplistic approach through the use of single growth factor (GF) delivery toward dual and multiple bioactive factor presentation and the obstacles associated with this change of paradigm are assessed. The use of platelet-rich plasma (PRP), an enriched cocktail of GFs and other bioactive proteins with potential for bone, cartilage, and osteochondral engineering, is also subject of interest. The promotion of GF expression and activation through the release of genes and cells is included in the indirect GF delivery subsection. Finally, common shortcomings and challenges associated with the use of controlled release systems for skeletal engineering are discussed.

Strategies for Controlled Release of GFs

The delivery of GFs has been pursued through the application of different strategies with an overall increasing complexity. The comprehension of the tissue-healing reactions and a more effective knowledge on the function relationship of bone, cartilage, and osteochondral interface have led to the design of new carriers for controlled release of bioactive factors. This section explores the evolution from more simple and direct delivery strategies toward more current designs, in which critical parameters such as spatiotemporal and dosage control over biomolecule presentation are taken in deep consideration.

Single GF delivery

Administration of GF and other bioactive molecules to promote bone and cartilage formation and repair has

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achieved promising results. Several controlled release systems promoting the delivery of GFs are combined in strategies with cells, with the goal of acting in a synergistic manner and promoting the enhancement of new tissue formation. Co-encapsulation of GFs and cells in hydrogels[2–10] and seeding of stem cells in microparticles or scaffolds loaded with bioactive agents[11–18] are among the most common TE strategies described in literature. Typically, GFs have been included in TE strategies through three main approaches: (1) incorporation within micro- and nano-particles, which can act as supplements for *in vitro* cell cultures or can be injected into the defect sites, stimulating *in situ* tissue healing. These carriers can also be cell seeded, further enhancing the potential for inducing new tissue formation.[11,15,19–31] (2) GF-loaded microspheres can be incorporated into scaffolds or hydrogels to enhance their functionality and complexity, providing the biochemical cues to stimulate tissue regeneration.[12,32–50] (3) Bioactive factors directly dispersed, immobilized, or adsorbed into the three-dimensional construct.[3,4,6–8,51–68] The level of immobilization determines the release rate of GFs and consequently their effect on tissue formation. Several studies approach a particularly relevant immobilization method, mimicking native extracellular matrix (ECM), affinity-bound systems, through the inclusion of heparin domains on the structure, thus expecting an enhanced stability of the entrapped GF.[27,66,69–72]

Table 1 presents selected studies regarding the use of single GF release systems for bone and cartilage engineering. Current evidence based on *in vitro* and animal studies suggests that among the factors that have been investigated to date, bone morphogenetic proteins (BMPs) appear to have the highest osteoinductive potential.[73] Despite the successful reports related to the delivery of BMP for bone TE applications, when translating into clinical trials, the outcomes have not been as successful. Large doses of the GF have been required to produce an osteogenic effect.[73–79] The use of supraphysiological levels of BMP-2 might activate a negative feedback loop through BMP inhibitors such as noggin or sclerotin, which are upregulated by the presence of BMP.[80,81] Moreover, inflammation, edema, and heterotopic bone formation can occur when such high concentrations are used.[82] Complications associated to the use of recombinant human BMP-2 have been reported, including death, dysphagia, heterotopic bone formation in the spinal canal, or airway compression in cervical spine fusion. Food and Drug Administration (FDA) has been especially cautious since those reports and at this point, 75% of the spine fusions are still performed by using traditional bone grafting methods.[81] Therefore, despite its tremendous potential, BMP-2 use still presents some disadvantages regarding its bone regeneration potential. The high-cost treatment and the simultaneous stimulation of development of both osteoblasts and osteoclasts with opposite effects are additional drawbacks.[83]

Despite the complexity of angiogenic signaling pathways, extensive studies on the biology and delivery of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2, and potent GFs involved in vessel formation have been performed in both preclinical and small-scale clinical trials. Recent large-scale clinical trials[84] did not demonstrate significant therapeutic effects above placebo controls, leading to an inefficient promotion of angiogenic response. A threshold concentration of VEGF is required, above which saturation of receptors occurs and consequent down regulation of receptor expression. On the other hand, low levels of VEGF lead to insufficient expression.

Appropriate *in vitro* or *in vivo* models allowing quantitative characterization of the effects of GFs in neovascularization are crucial for the development of appropriate design criteria for therapies. *In vivo* models, including chick chorioallantoic membrane assay, corneal assay, dorsal skin chamber assay, subcutaneous implantation, and induced ischemia models, mimic certain aspects of human diseases.[84] *In vitro* models such as the embedding of endothelial cells in fibrin,[85] collagen,[86] or Matrigel[87] hydrogels can also mimic some of the *in vivo* microenvironmental features occurring during angiogenesis.

The kinetics of release strongly influences tissue regeneration. Extremes of release are in general not desirable and controlled and prolonged profile should be designed. The work of Jeon *et al.*[70] showed that prolonged delivery of BMP-2 enhanced *in vivo* the osteogenic efficacy of the protein compared to short-term delivery at equivalent dosage. Moreover, the physiological angiogenic response requires a precisely coordinated interplay between different signaling molecules and cell types. Ozawa *et al.*[88] showed that the key determinant whether VEGF-induced angiogenesis is normal or aberrant is the microenvironmental amount of GF secreted, rather than the overall dose. Other approach used to promote angiogenesis is the blocking of angiogenesis inhibitors.[89] Pro-angiogenic and anti-angiogenic factors exist in dynamic balance, and when angiogenesis inhibitors such as thrombospondin-2 are knocked out, increase in fracture vascularization can be observed.[90]

One of the critical issues in GF delivery has been the appropriate tailoring of signaling molecules dosage. There is almost no common ground in this matter and typically the use of GFs is observed within broad ranges of concentrations. For example, from our collected data in Table 2, BMP-2 and transforming growth factor (TGF)-β1 have been used *in vivo* for bone and cartilage regeneration within a range of 0.015–150 μg and 0.8 ng–1 μg per implant, respectively.

These differences cannot be explained only by animal models or discrepancies on the type of defects and ultimately emphasize the lack of knowledge regarding the therapeutic concentration of GFs. In fact, the FDA-approved BMP factors have been used in supraphysiological amounts to obtain a therapeutic effect and the required excessive amounts of proteins can be explained by an ineffective delivery system, which does not present the factor in a spatiotemporal controlled manner.

Tissue development is a highly coordinated process and it significantly benefits from strategies that mimic concomitant interactions among various factors involved in this process.[122] The presentation mode of GFs is a critical step for the generation of a specific tissue response, and several studies have focused on the evaluation of the effect of immobilization technique on cellular response.[123–131] For example, it is reported that soluble VEGF induces endothelial cell proliferation, while matrix-bound VEGF promotes vascular sprouting and branching[132] despite other studies revealing opposite responses.[86,130,133,134] Moreover, sustained activation of the Smad intracellular signaling pathway is stimulated upon culture of osteoblastic cells on immobilized BMP-2 due to prolonged phosphorylation of Smad 1/5/8 by

Table 1. Summary of Studies Performed from the Year 2000 Up to 2012 Using Single Delivery of Growth Factors to Enhance Tissue Regeneration in Bone and Cartilage Tissue-Engineering Strategy

	Growth factor	In vitro (*dose range*)	In vivo (*dose range*)	In vitro/in vivo *models*	*References*
Bone (osteogenesis)	BMP-2	0.1–2.5 μg per carrier	100 ng/mL 0.015–150 μg/implant	*In vitro* osteogenic differentiation. Subcutaneous implantation and intramuscular and critical-size bone defects.	6, 7, 9, 15, 26, 43, 46, 58, 59, 61, 62, 65, 66, 68, 69, 91–101
	FGF-2	—	20 μg–2.4 mg per implant	*In vivo* critical-size defects.	102–106
	IGF-I	500 ng/mL	30 μg–5 mg per implant 500 ng/mL	Bone critical-size defects such as periodontal and segmental tibia defects. *In vivo* subcutaneous implantation.	24, 25, 107
	TGF-β1	5 ng per mg of carrier 5–40 ng per carrier	2 μg per implant	*In vitro* osteogenic differentiation. *In vivo* ectopic and orthotopic models.	16, 67, 108
	BMP-7	—	3.3–3.5 mg per implant 5 μg/mg carrier polymer	*In vivo* critical-size defects and subcutaneous implantation.	50, 64, 109
	PDGF	100–400 ng per carrier	22–75 μg per implant 0.3–1 mg/mL per implant	*In vivo* critical-size defects. *In vitro* osteogenic cultures.	110–112
Angiogenesis	VEGF	0–70 ng/mL	250 ng–3 μg/construct	*In vivo* subcutaneous and ischemic hindlimb implantation. *In vitro* cultures with endothelial cells.	87, 113–116
	FGF-2	—	4–50 μg/construct	*In vivo* subcutaneous and ischemic hindlimb implantation.	47, 117, 118
Cartilage (chondrogenesis)	TGF-β1	3–600 ng/scaffold 100–600 ng/mL 10 ng per construct	0.8 ng–1 μg/implant	*In vitro* cultures in a basal and chondrogenic medium Full-thickness cartilage defects. Assembled MSC sheets with microspheres.	2, 8, 19, 20, 32–35, 38, 39, 52, 56, 67, 119, 120
	TGF-β3	25–100 ng/scaffold 600 ng/mL scaffold	100 ng–1 μg/scaffold 100 ng/mL	Subcutaneous and intramuscular implantation in mice. *In vitro* cultures.	21, 23, 36, 51, 53, 55, 121
	IGF-I	25–100 μg/scaffold	3–30 μg/construct	*In vitro* assays. Subcutaneous and articular cartilage defects implantation.	5, 41, 54, 55
	BMP-2	500 ng/system	3 μg/construct	*In vitro* chondrogenic differentiation. *In vivo* articular cartilage defect implantation.	22, 57

BMP, bone morphogenetic protein; FGF, fibroblast growth factor; IGF, insulin growth factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

Table 2. Dual or Multiple Bioactive Factor-Controlled Delivery Systems Aiming for Bone, Cartilage, and Osteochondral Regeneration

Bioactive factor 1	*Bioactive factor 2*	*Material/ carrier*	*Incorporation method*	*Release mechanism and quantification*	*Kinetics*	In vitro/ In vivo	*Biological effect*	*Application Reference (Year)*
$VEGF_{165}$ 2 μg per implant	PDGF-BB 3 μg per implant	Foaming of PDGF-BB pre-encapsulated in PLG microspheres + VEGF dispersed in freeze-dried alginate	Encapsulation	*In vitro* radiolabeling tracing	Cumulative release after 1 month: ~25 pmol of VEGF and 5–70 pmol of PDGF-BB	*In vivo* (rat subcutaneous implantation and hindlimbs of NOD mice)	Elevated vessel density and maturity.	Angiogenesis 44 (2001)
FGF-2 1 μg per mg of scaffold	VEGF 0.7 μg per mg of scaffold	Freeze-dried collagen sponge modified or not with heparin	Impregnation	Not performed	—	*In vivo* (rat subcutaneous implantation)	Dual-delivery formulation scored the highest blood vessel density and maturity.	Angiogenesis 71 (2007)
VEGF 25 and 100 ng per implant	KGF or Ang-1 or PDGF 25 and 100 ng per implant	Hyaluronic acid-based hydrogels and heparin-modified HA gels	Encapsulation	Not performed	—	*In vivo* (mouse ear pocket)	Largest vascularization responses were consistently produced by gels delivering the GF combination VEGF + KGF.	Angiogenesis 147, 148 (2006/2010)
VEGF 1.5 μg layer 1 and 3 μg layer 2	PDGF 3 μg layer 1	PDGF-loaded PLG microspheres assembled with VEGF-dispersed alginate	Encapsulation	*In vitro* radiolabeling	75% and 50% cumulative release of VEGF and PDGF after 1 month, respectively	*In vivo* (mouse ischemic hindlimbs)	Layer with sequential release of GFs led to smaller density, but enhanced size and maturity of blood vessels.	Angiogenesis 149 (2007)
VEGF (0.6 molar ratio)	PDGF-BB (1 molar ratio) TGF-β1 (1 molar ratio)	Alginate scaffolds (triple GF combinations: molar ratio: 0.6:1:1)	Impregnation (total loading GFs 100 ng/scaffold)	*In vitro* ELISA assay	Burst release in alginate scaffolds, but sustained profile for alginate-sulfate scaffolds.	*In vivo* (rat subcutaneous implantation)	Higher blood vessel density and percentage of mature vessels for the triple GF system when compared with the FGF-2 control scaffold	Angiogenesis 150 (2009)
VEGF 3 μg per implant	PDGF 3 μg per implant	PDGF-loaded PLG microspheres in VEGF-loaded alginate hydrogel	Encapsulation	*In vitro* radiolabeling	50%/10% and 80%/70% cumulative release of VEGF and PDGF after 2 and 30 days, respectively.	*In vivo* (rat unilateral hindlimb ischemia)	GF combination established a more functional and stable vascular network.	Angiogenesis 151 (2010)
FGF-2 1 or 5 μg/mL	VEGF 1 or 5 μg/mL	PEG–heparin hydrogels	Immobilization	*In vitro* ELISA assays and radiolabeling	10% cumulative release after 4 days.	*In vivo* (CAM model for angiogenesis)	Dual delivery induced HUVEC migration *in vitro* and enhanced vessel formation *in vivo*.	Angiogenesis 152, 153 (2010/2011)
VEGF 20 ng per mg dry gel	SDF-1 IGF-1 20 ng per mg dry gel	Photocrosslinkable dextran/ PEGDA hydrogels	Encapsulation	*In vitro* ELISA assay	20%–60% cumulative release in 4 days	*In vivo* (rat subcutaneous implantation)	VEGF delivery is needed for functional neovascularization and enhanced by additional angiogenic GFs.	Angiogenesis 154 (2011)
FGF-2 250 ng per membrane	VEGF 250 ng per membrane	Self-assembled hyaluronic acid/heparin–peptide amphiphile membrane	Incorporation with heparin-affinity domains	*In vitro*, ELISA assay	Dependent on heparin concentration. 100% cumulative release after 14 days.	*In vivo* (CAM model for angiogenesis)	Enhanced angiogenic response with higher vessel density.	Angiogenesis 155 (2011)
VEGF 6.4 μg per scaffold	PDGF-BB 6.4 μg per scaffold	Codeposited electrospun PCL/ Col fiber and electrospraying GF-loaded HA gel	Encapsulation	*In vitro* ELISA assay	40% and 25% cumulative release of VEGF and PDGF-BB, respectively	*In vitro* (coculture of HUVEC/fibroblasts)	Instigation of capillary networking on the surface and interior of the scaffolds.	Angiogenesis 156 (2011)
FGF-2 2 μg per implant (renew dose from day 1 to day 3)	PDGF 5 μg per implant (renew dose from day 3 to day 7)	Cellulose acetate hollow fibers injected in a sequential manner with GF solutions (days 0–3 with FGF-2 and days 3–7 with PDGF)	Incorporation	*In vitro* ELISA assay	Fast and strong release during each 24-h delivery cycles.	*In vivo* (mouse subcutaneous implantation)	Increased endothelial cell migration and formation of red blood cell-filled neovessels, suggesting integration with existing vasculature	Angiogenesis 157 (2011)
BMP-2 30 μg per implant	VEGF 20 μg per implant	Silk hydrogels	Encapsulation	*In vitro* ELISA assay	Highly controlled and linear release profiles	*In vivo* (rabbit maxillary sinus floor augmentation)	Synergistic effect on bone formation and angiogenesis.	Bone + Angiogenesis 158 2011
SDF-1 5 μg per implant	BMP-2 3 μg per implant	Gelatin hydrogels	Adsorption/ impregnation	*In vivo* radiolabeling	80% and 45% burst release of SDF-1 and BMP-2, respectively	*In vivo* (rat ulna critical-size defect)	Enhanced recruitment of osteogenic cells, angiogenesis, and osteogenic differentiation of host cells.	Bone + angiogenesis 159 (2011)
PDGF 250 ng TGF-β1 100 ng	VEGF 350 ng	VEGF pre-encapsulated in PLGA microspheres dispersed in GF-loaded brushite cements	Encapsulation	*In vivo* radiolabeling	90% cumulative release after 21 days for PDGF and TGF-β1 and after 56 days for VEGF.	*In vivo* (rabbit femur defect)	Combination of VEGF and PDGF increases blood vessel density and surface area. TGF and VEGF did not act synergistically.	Bone + angiogenesis 160 (2011)

(*continued*)

Table 2. (Continued)

Bioactive factor 1	*Bioactive factor 2*	*Material/ carrier*	*Incorporation method*	*Release mechanism and quantification*	*Kinetics*	In vitro/ In vivo	*Biological effect*	*Application Reference (Year)*
BMP-2 0–3.75 μg per construct	IGF-I 0–1 μg per construct	Gelatin coatings with three different release kinetics	Encapsulation	*In vitro* ELISA assays	Three distinct profiles	*In vitro* osteogenic differentiation (SaOS-2 and BMSCs)	Recreation of different stages of bone healing. IGF-1 stimulated cell proliferation.	Bone 161 (2004)
BMP-2 1.25 and 2.5 μg per construct	IGF-I 50 and 100 ng per construct	Gelatin coatings	Impregnation	*In vitro* ELISA assay	Early release of BMP-2, followed by release of BMP-2 and IGF-1 after 5 days	*In vitro* osteogenic differentiation (C3H cells and BMSCs)	Sequential delivery promoted increased ALP activity, while simultaneous delivery of both GFs did not promote higher ALP and calcium deposition.	Bone 162 (2004)
BMP-2 200 ng per implant	TGF-β3 20 ng per implant	RGD-immobilized alginate hydrogels	Encapsulation	Not performed	—	*In vivo* (ectopic implantation in SMID mice)	Coencapsulation with 1 million BMSCs; dual release promoted significant bone formation, whereas single delivery of GFs failed.	Bone 163 (2004)
BMP-2 200 ng per implant	TGF-β3 20 ng per implant	PLGA scaffold filled with RGD–alginate containing the GFs	Encapsulation	Not performed	—	*In vivo* (rat critical-size femoral defect implantation)	Greater mineralized matrix production, but lacked consistent bone union.	Bone 164 (2007)
BMP-2 2 μg per implant	VEGF 12 μg per implant	GF-loaded gelatin microparticles incorporated in PPF scaffold	Adsorption/swelling	Done in previous studies	—	*In vivo* (rat cranial critical-size defects)	Dual delivery favors early bone formation. After 12 weeks, results are similar to the BMP-2-only group.	Bone 45 (2008)
BMP-2 40 ng per scaffold	BMP-7 40 ng per scaffold	GF-loaded PLGA and PHBV nanocapsules incorporated in chitosan-based scaffolds	Encapsulation	*In vitro* BCA assay of model protein	BMP-2 early delivery followed by BMP-7 in sequential mode	*In vitro* osteogenic differentiation (50.000 BMSCs per scaffold)	Sequential release enhanced ALP activity, while simultaneous delivery failed to give high ALP values.	Bone 12 (2009)
BMP-2 50 ng per mg of carrier	IGF-I 50 ng per mg of carrier	GMA microparticle–gelatin composite scaffolds	Encapsulation	*In vitro* ELISA assay	90% cumulative release after 1 month	*In vitro* osteogenic differentiation (0.5 million PDLFs per scaffold)	IGF-1 enhanced biological effect of BMP-2 and osteogenic markers.	Bone 17 (2009)
BMP-2 9.2 μg per implant	VEGF 2 μg per implant	BMP-2-loaded PLGA microspheres entrapped in a PPF scaffold and combined with a VEGF-loaded gel	Encapsulation	*In vivo* radiolabeling tracing	VEGF-burst release within the first 3 days and sustained delivery of BMP2 for 56 days.	*In vivo* (rat subcutaneous and critical-size femoral defects)	Dual release enhanced ectopic bone formation in comparison with the BMP-2-only group. The same was not observed in the orthotopic model.	Bone 165 (2009)
VEGF 320 ng per implant	PDGF 250 ng per implant	VEGF-loaded alginate microspheres dispersed in chitosan sponges and PDGF-loaded brushite paste	Encapsulated/ dispersed	*In vitro* and *in vivo* radiolabeling	70% and 80% cumulative release of VEGF and PDGF in 3 weeks, respectively	*In vivo* (rabbit femurs critical-size defect implantation)	PDGF alone increased bone formation, but the dual system significantly augmented new bone formation.	Bone 166 (2010)
BMP-2 20 μg per implant	VEGF Immersion in a 20 μg/mL solution	PDLLA foam-entrapping BMP-2 and VEGF-loaded alginate fibers	Impregnation and entrapment	*In vitro* ELISA assays	Linear profile for VEGF while BMP-2 release starts at the 2nd week	*In vivo* (mouse segmental femur-defect model)	Seeded with 0.2 million hBMSCs. Seeded constructs showed significantly higher bone formation.	Bone 18 (2010)
BMP-7 125 μg per implant	TGF-β3 25 μg per implant	Calcium carbonate/ hydroxyapatite constructs	Impregnation/ swelling	Not performed	—	*In vivo* (intramuscular implantation)	The dual-delivery system promoted significantly higher bone formation.	Bone 60 (2010)
SDF-1 10 μg per implant	BMP-2 5 μg per implant	Collagen pellets	Impregnation	Not performed	—	*In vivo* (rat subcutaneous implantation)	Enhanced homing of MOPCs to the implant and ectopic bone formation induced by a suboptimal BMP-2 dose.	Bone 63 (2011)
BMP-2 40 μg/mL solutions; 6 μg per implant	VEGF 40 μg/mL solutions; 4 μg per implant	Polyelectrolyte multilayer nanofilms on PCL/β-TCP scaffolds	Adsorption	*In vitro* ELISA assays	GF loading and release are dependent on the number of layers	*In vitro* and *in vivo* (rat intramuscular implantation)	Recapitulation of a more complete bone tissue architecture precipitated by PEM-mediated release of dual GF from the scaffold.	Bone 167 (2011)
BMP-2 ~100 μg per implant	Wnt1 ~20 μg per implant	Gelatin sponges incorporating β-TCP granules	Impregnation/ adsorption	*In vitro* radiolabeling	10% and 40% burst release of BMP-2 and Wnt1, respectively.	*In vivo* (middle-aged rat subcutaneous implantation)	Increased osteoid formation in rats with decreased potential for bone formation.	Bone 168 (2011)
BMP-2 2.5 μg per implant	TGF-β1 25 ng per Implant	Collagen sponges	Encapsulation	Not performed	—	*In vivo* (intramuscular and mouse calvarial defect)	TGF-β1 strongly enhances osteoinductive activity of BMP-2 by regulating osteoblast and osteoclast generation.	Bone 169 (2011)

(*continued*)

Table 2. (Continued)

Bioactive factor 1	*Bioactive factor 2*	*Material/ carrier*	*Incorporation method*	*Release mechanism and quantification*	*Kinetics*	In vitro/ In vivo	*Biological effect*	*Application Reference (Year)*
BMP-2 12 μg and 120 μg per implant (ectopic and orthotopic, respectively)	VEGF 0.4 μg and 4 μg per implant (ectopic and orthotopic, respectively)	Biphasic calcium phosphate scaffolds loaded with PLGA microparticles and gelatin hydrogels	Encapsulation	Not performed	—	*In vivo* (intramuscular and ulna defects in Beagles)	Timing of BMP-2 release largely determines speed and amount of ectopic bone formation independent of VEGF release	Bone 170 (2012)
TGF-β1 200 ng per gram of gel	IGF-I 100 ng per gram of gel	Loaded microspheres incorporated within OPF hydrogels	Impregnation/ swelling	*In vitro* ELISA assay	45% and 32% cumulative release of TGF-β1 and IGF-I, respectively	*In vitro* (chondrogenic differentiation)	Enhancement of collagen type II and aggrecan expression; Hydrogels coencapsulated with 20 million MSCs/mL	Cartilage 37, 171 (2005/2009)
TGF-β1 200 ng per gram of gel	IGF-I 200 ng per gram of gel	Loaded microspheres incorporated within OPF hydrogels	Impregnation/ swelling	*In vitro* ELISA assay	80% and 60% cumulative release, respectively	*In vivo* (osteochondral defect in rabbits)	IGF-I positive effects were not maintained in the dual-delivery system.	Cartilage 172 (2007)
TGF-β3 100 ng/mL	Ascorbate 80μM	Encapsulation in the PNIPAM hydrogel	Encapsulation	*In vivo* bioimaging	Almost complete release after 4 weeks	*In vivo* (subcutaneous implantation in rabbits)	Coencapsulation with 1 million chondrocytes. Enhanced accumulation of the ECM.	Cartilage 3 (2007)
TGF-β3 100 ng/mL	Dex 100 nM	Encapsulation in PNIPAM–hyaluronate composite hydrogels	Encapsulation	*In vivo* bioimaging (FITC and Cy5.5-conjugation)	After 17 days, the implanted are presented significantly less biomolecules.	*In vivo* (subcutaneous implantation in nude mice)	Coencapsulation with 5 million chondrocytes. Enhanced proliferation and maintenance of chondrocyte phenotype.	Cartilage 4 (2007)
TGF-β3 100 ng/mL	Dex dosage not clear	Heparin-bound TGF-coated PLGA microspheres containing Dex	Encapsulation and immobilization	*In vitro* *In vivo* bioimaging (FITC and Cy5.5-conjugation)	80% and 85% cumulative release after 4 weeks, respectively	*In vivo* (subcutaneous implantation in nude mice)	Rabbit MSCs seeded on the microspheres. Accumulation of the ECM throughout the construct.	Cartilage 11 (2009)
TGF-β3 100 ng per disk	PTHr-P 50 ng per disk	Loaded microspheres incorporated within hyaluronic acid hydrogels	Encapsulation	*In vitro* ELISA assay	100% TGF-β3 released after 5 days; PTHrP not quantified	*In vivo* (subcutaneous implantation in nude mice)	Codelivery induced modest decrease of hypertrophy in newly differentiated chondrocytes; Hydrogels coencapsulated with 20 million MSCs/mL.	Cartilage 36 (2011)
BMP-2 3.125–9.375 μg per mg of material	IGF-I 3.125–9.375 μg per mg of material	Gradients of PLGA and silk microspheres within alginate hydrogel	Encapsulation	*In vitro* ELISA assay	Linear GF gradients along the scaffold	*In vitro* hMSC cultures	The osteogenic and chondrogenic differentiation of seeded hMSCs corresponded to the gradient distribution of BMP-2 and reverse distribution of BMP-2/IGF-I.	Osteochondral 173 (2009)
BMP-2 1.5 μg per scaffold	TGF-β1 0.75 μg per scaffold	Sintering PLGA microspheres disposed in GF concentration gradients	Encapsulation	Performed in previous studies (*In vitro* ELISA assay)	6% and 10% cumulative release after 21 days	*In vivo* (rabbit condyle implantation)	Complete bone ingrowth with an overlying cartilage layer well integrated with surrounding cartilage.	Osteochondral 174, 175 (2010/2011)
BMP-6 500 ng per construct	TGF-β3 250 ng per construct	Loaded microspheres incorporated within glycol chitosan hydrogels	Encapsulation	*In vitro* BCA assay	95% and 80% cumulative release within 70 days	*In vitro* formulation design	No work with cells.	40 (2011)
BMP-4 200 and 300 ng per layer for *in vitro* and *in vivo* assays, respectively	TGF-β1 200 and 300 ng per layer for *in vitro* and *in vivo* assays, respectively	GF-affinity-bound alginate hydrogels	Immobilization	Not performed	—	*In vivo* (subchondral rabbit defects)	Stem cells migrating into the defect are able to sense the biological cues spatially presented in the hydrogel and respond by differentiation into the appropriate cell lineage.	Osteochondral 176 (2012)

ALP, alkaline phosphatase; Ang, angiopoietin; BCA, bicinchoninic acid; BMSCs, bone marrow mesenchymal stem cells; CAM, chorioallantoic membrane; Col, collagen; Dex, dexamethasone; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GF, Growth factor; GMA - glycidyl methacrylate; HA, hyaluronic acid; HUVEC, human umbilical vein endothelial cells; KGF, Keratinocyte growth factor; MOPCs, circulating bone marrow-derived osteoprogenitor cells; NOD, nonobese diabetic; OPF, oligo(poly(ethylene glycol) fumarate); PCL, polycaprolactone; PDLLA, poly(D,L-lactic acid); PEG, polyethylene glycol; PEGDA, poly(ethylene glycol) diacrylate; PEM, polyelectrolyte multilayer; PHBV, polyhydroxybutyrate–valerate; PLG, PLGA, poly(lactic-co-glycolic acid); PNIPAM, poly(*N*-isopropylacrylamide); PTHrP, parathyroid hormone; RGD, arginine–glycine–aspartic acid; SCID, severe combined immunodeficiency; SDF, stromal-derived growth factor; TCP, tricalcium phosphate; Wnt1, Wingless family.

cellular exposure to immobilized BMP-2 relative to treatment with soluble BMP-2.[135]

Several studies[127,136–138] have shown that the culture of osteoblast precursor cells on substrates modified with immobilized BMP-2 can significantly increase the expression of osteogenic differentiation markers. These immobilization techniques can even be combined with patterning approaches.[139] In particular, immobilization with affinity-bound ligands has been demonstrated to promote a strong attachment of GFs to the matrix and to enhance their potency. For example, the osteoinductive effects of recombinant human BMP-2 in combination with a complex of heparin and chitosan in a gel formulation were shown to be superior when compared to recombinant human BMP-2 implanted with type I collagen in a rat model.[140] Jeon *et al.*[70] also observed that the heparin-conjugated scaffolds allowed a long-term delivery of BMP-2, which ultimately resulted in the enhancement of the *in vivo* osteogenic efficacy of the protein.

Re'em *et al.*[141] also showed that controlled release of TGF-β1 affinity-bound alginate scaffolds enhanced human mesenchymal stem cells (MSCs) chondrogenic differentiation and *in vivo* deposition of cartilaginous ECM in an ectopic model in nude mice. Reyes *et al.*[142] analyzed osteochondral regeneration postimplantation of a bilayered scaffold loaded with BMP-2 or TGF-β1 and observed that the higher concentration of BMP-2 gave rise to higher quality cartilage with improved surface regularity 2 weeks postimplantation.

Despite the partial success of simple GF therapy, it is clear that the field is not taking full advantage of the potency of these signaling molecules. The complexity of native tissue-healing cascades, which involves several GFs and chemokines, communicating with each other through positive and negative feedback mechanisms, is obviously lacking in current TE and regenerative strategies incorporating the release of a single GF. Even if the carrier and release kinetics are appropriately designed, a single signaling molecule will not be able to promote bone and cartilage regeneration by itself. Therefore, the development of TE strategies incorporating delivery systems for multiple GFs has emerged to overcome the hurdles previously found and to increase the functionality of the constructs following a biomimetic approach.

Multiple GF delivery

Appropriate delivery of multiple GFs and other bioactive agents might reduce the dosage of factors required to achieve the desired effect, in essence increasing the potency of the molecules. Precise control over temporal sequence of release and presentation of GFs is critical because coexistence of destabilizing and stabilizing factors may cancel each other's effects.[84] The hallmark study of the use of dual and multiple GF release for TE was the one performed by Richardson and his team.[44] Angiogenesis is one of the most relevant mechanisms involved in bone healing, which is characterized by complex cascades of GFs. VEGF in particular has shown its potency to promote therapeutic angiogenesis. However, the vessels induced by single delivery of VEGF frequently display morphological and functional abnormalities, such as leaky vessels and unusually large irregular lumens.[88] Richardson *et al.*[44] successfully designed a VEGF and platelet-derived growth factor (PDGF)–BB dual release system with distinct delivery profiles, promoting a rapid generation of mature vascular network. Moreover, they showed the thin line between the therapeutic effect of a successful combination of GFs, delivered at the appropriate time and dosage, and the antagonist effect of this combination. In this case, high levels of PDGF before sufficient pericyte recruitment result in destabilized vessel network and subsequent regression. Upon *in vivo* implantation, the mechanism of dual delivery allows the formation of larger and more mature blood vessels as opposed to smaller, incomplete vessels formed using a single deliver technique.[143] Moreover, formation of truly functional vasculature will likely require control over the location and the magnitude of the angiogenic region. Tight spatial regulation often results from the combined action of stimulatory and inhibitory factors.[144] Yuen *et al.*[144] employed a dual-release system based on a poly(lactic-co-glycolic acid) (PLG) scaffold incorporating layers loaded with angiogenic stimulatory factor and inhibitory anti-VEGF. This led to a spatially sharp angiogenic region, sustained over 3 weeks.

Table 2 presents a detailed description of some of the most relevant studies performed regarding the application of dual or multiple bioactive factor delivery systems for bone, cartilage, and osteochondral regeneration.

The choice of the appropriate combinations of GFs is one of the critical hurdles for bioactive factor delivery in TE.[82] As an example, Vonau *et al.*[145] designed a dual-delivery system composed by recombinant human BMP-2 and FGF-2 in a collagen sponge, which ultimately resulted in decreased bone formation in a rabbit model of tibial fracture.

The modulation of the adequate delivery kinetics is another major design requirement that can significantly affect the outcome of the strategy. Setting and combining fast and slow-release profiles can enhance tissue formation by closely mimicking native interactions in ECM. For example, Jaklenec *et al.*[146] designed modular scaffolds resultant from the fusion of PLGA microparticles, tailored for different delivery rates of GFs (delayed and burst release), allowing sequential delivery of insulin growth factor (IGF)-I and TGF-β1.

On a first analysis of Table 2, the diversity of combinations and dosages of GFs is clear. Furthermore, the use of different materials processed in distinct architectures and the specific drug-loading procedures do not contribute for the homogenization of the outcomes. One obvious limitation of several studies that assessed their drug delivery system *in vivo* is the *in vitro* quantification of the release profile of the bioactive factors and consequent assumption that the kinetics would follow a similar pattern *in vivo*. These observations rise obstacles for the establishment of a correlation between the delivery kinetics and the measured outcomes and decreases significantly the efficiency of predictability of a newly proposed drug delivery system.

Regarding the selection of bioactive factors, since the lack of bone formation is often due to the limited ability of the surrounding tissue to induce a vascular supply at the regenerating location, one of the most common combinations for bone regeneration is the dual release of osteogenic and angiogenic GFs, since those two processes are interconnected during bone healing. Vessel formation is the earliest process in bone regeneration to promote the recruitment of progenitor cells to start the osteogenic differentiation process.[177]

Dual immobilization has also been performed to further improve angiogenesis. VEGF has been coimmobilized with

angiopoietin-1 (Ang-1), a GF known for its support for vessel stability and maturation, and enhanced endothelial cell infiltration was achieved.[126]

The combined release of BMP-2 and VEGF is commonly approached with mixed results, and control over amount and timing of GF delivery is critical. VEGF dosage must be tightly controlled as excessive amounts of this GF can inhibit osteogenesis and cause severe leakage and hypotension.[167] Combined results of Kempen *et al.*[165] and Patel *et al.*[45] suggest that the enhanced effect of VEGF and BMP-2 combination is both time- and location-dependent, which is not surprising due to the complexity of the pathways involved in bone healing. While Kempen *et al.*[165] displayed an extremely high VEGF burst release of around 80%, De la Riva *et al.*[166] reduced this stage to 20% and obtained considerable enhanced bone formation in the dual-release system they proposed (combination of VEGF and PDGF). Shah *et al.*[167] showed that dual release of VEGF and BMP-2 from polyelectrolyte multilayers induced a more complete bone architecture than the single dose of BMP-2, promoting a greater initial concurrent vascularization process and consequent introduction of more cells in the interior on the scaffolds. However, a recent report Geuze *et al.*[170] demonstrated that the timing of BMP-2 release largely determined the rate and amount of bone formation independently of VEGF release kinetics. Moreover, at orthotopic location, no significant effect on bone formation was found from a timed release of GFs, suggesting that time-release effects are location dependent.

Other combinations have also been explored to promote bone regeneration. Luong *et al.*[178] developed an interesting study on the effects of the coprecipitation of different amounts and combinations of FGF-2 and BMP-2 into an apatite coating on the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). These studies revealed that FGF-2 increased cell proliferation, but high concentrations inhibited osteogenic differentiation. On the other hand, a threshold concentration of BMP-2 was required to induce significant osteogenesis. The sequence of delivery of BMP-2 (300 ng/mL) and FGF-2 (2.5 ng/mL) also provided relevant data, and it was observed that the release of FGF-2 followed by BMP-2 or the delivery of BMP-2 followed by the simultaneous delivery of FGF-2 and BMP-2 enhanced osteogenic differentiation more significantly than the simultaneous delivery of both factors.[178]

Strobel *et al.*[179] applied a multiple and sequential delivery coating, promoting the release of gentamicin, IGF-1, and BMP-2. The earlier action of IGF-1 promoted cell proliferation, while BMP-2 stimulated alkaline phosphatase (ALP) activity. Kohara and Tabata[168] also explored an interesting route by developing a dual-release system based on gelatin hydrogels loaded with calcium phosphates and with BMP-2- and Wnt1-inducible signaling pathway protein 1. The Wnt-signaling pathway is predicted to control bone mass, and the dual-release system was evaluated in middle-aged mice with decreased bone formation potential. The simultaneous delivery of both factors increased osteoid formation.

One interesting finding of Ripamonti *et al.*[60] was the apparent role of osteoclast activity on the implanted macroporous constructs. In their study, bisphosphonate zoledronate was loaded onto the surface of the scaffolds to inhibit osteoclastic activity after intramuscular implantation. Osteoclasts surfacing and modifying the surface of the implanted constructs might initiate bone formation by carving topographical modifications and releasing calcium ions. The inhibited scaffolds showed lack of new bone formation, indicating the critical role of osteoclasts in the spontaneous induction of new bone formation.

Core-shell microcapsules using PLGA and alginate were also developed for dual bioactive factor-release system. The core and shell domains presented different release patterns and when incorporating BMP-2 and dexamethasone and cocultured with BMSCs in a hydrogel construct resulted in enhanced expression of osteogenic markers.[180] It has also been reported that the coadministration of VEGF, IGF-1, stromal-derived growth factor (SDF)-1, and Ang-1 led to a dramatic increase in angiogenic response, specifically the size and number of arterioles when compared to the single delivery of these individual factors.[154]

Another interesting group of studies involve the use of chemoattractant chemokines selected to promote host cell migration. For example, SDF-1 chemotactic gradients have been shown to affect migration patterns of both injected and host MSCs.[181–183] Therefore, the targeted delivery of SDF-1 can be used as a new approach to create an artificial signaling center without implantation of exogenous MSCs. Despite the potential of inflammation modulation, the critical situation is to distinguish between regenerative and damaging inflammation processes in bone. Aberrant inflammation has been implicated as a significant factor in bone injuries that fail to heal; thus, tight control over this process must be tailored.[184]

An obvious interesting approach for a multiple delivery system is the regeneration of orthopedic interfaces, such as ligament-to-bone,[185,186] tendon-to-bone,[187] and cartilage-to-bone.[188] Many approaches have been used to fabricate scaffolds for osteochondral application by changing material composition, mechanical properties, and architecture between the chondrogenic and osteogenic layers.[172–174,188–193] Hierarchical scaffolds loaded with inductive factors disposed in two phases or even in gradients, capable of stimulating each layer toward the maturation of the specific tissue, are being increasingly explored. By using gradients of multiple bioactive factors, multiple tissue regeneration can be addressed via a single-cell source; that is, stem cells can differentiate along different lineages within the same constructs.[194] Current design challenges for engineering biomimetic gradients are caused mostly by a question of scale, as it is not an easy task to mimic the micro- and nanoscale gradients reported at tissue-to-tissue interfaces, such as the osteochondral one.[195]

The application of dual-delivery systems for cartilage regeneration has also been under intensive investigation. Bian *et al.*[36] successfully implanted subcutaneously in nude mouse constructs containing TGF-β3-loaded microspheres, resulting in superior cartilage matrix formation when compared to groups without the GF or with the protein added directly to the gel. However, calcification was observed, hence suggesting hypertrophy of chondrocytes. To counter this aberrant hypertrophy, parathyroid hormone-related protein (PTHrP) has been employed to inhibit hypertrophy of chondrocytes or MSCs during chondrogenesis. To prevent this, the authors implanted a dual-delivery system releasing both TGF-β3 and PTHrP, resulting only in partially reduced calcification and failing to prevent extensive mineralization. These results might be explained by the quick release of the

hormone during the first week of implantation, and the system should be optimized to delay the release of PTHrP until the second week of implantation, when MSCs begin to express hypertrophic markers.

The combination of TGF-β1 and IGF-1 has been intensively explored by Mikos and coworkers.[171,172] Codelivery of both GFs resulted in enhanced expression of chondrogenic markers in some of the studies; however, *in vivo* data also showed that the dual-delivery system did not maintain the positive effect of IGF-1 as a single-bioactive-factor delivery system. These observations emphasize that even if the sequence of GFs may be appropriate, dosage and release kinetic optimization are required.

The application of dual-release systems for osteochondral regeneration has also been increasingly reported. One of the most interesting findings of the study of Holland *et al.*[172] was the apparent lack of TGF-β1 and IGF-1 synergy when simultaneously delivered to treat rabbit osteochondral defects. The GFs were delivered at different rates, with IGF-1 released throughout the first weeks of healing, while TGF-β1 was expected to be released within the first few days. The individual release of IGF-1 consistently produced better regeneration outcomes, therefore showing the need to optimize the proper combination, dosage, and release kinetics of different GFs to achieve the desired biological effect. Mohan *et al.*[174] also observed that sintering BMP-2- and TGF-β1-loaded microspheres disposed in oppositely oriented gradients could lead to appropriate scaffolds aiming for osteochondral regeneration, as demonstrated *in vivo* through implantation in rabbit condyle defects.

Wang *et al.*[173] investigated the release of BMP-2 and IGF-I inside PLGA and silk fibroin microspheres, suspended in a gradient pattern inside alginate gels and observed a direct effect over osteogenic and chondrogenic differentiation of seeded hMSCs. Dormer *et al.*[175] designed PLGA microsphere-sintered constructs incorporated with gradients of BMP-2 and TGF-β1, and despite obtaining promising data, acknowledged the difficulty in sustaining the gradient patterns for long term *in vitro* due to the quick diffusion of the molecules. Re'em *et al.*[176] designed a bilayered system, spatially presenting the chondroinductive TGF-β1 in one layer and the osteoinductive BMP-4 in a second layer via affinity binding to the matrix. When implanted in subchondral defects in rabbits, the constructs were able to induce the migration of host stem cells that sensed the biological cues spatially presented in the hydrogel and responded by differentiating into the appropriate cell lineage.

Another rather important conclusion taken from the compilation of studies focusing on the engineering of drug delivery systems for bone, cartilage, and osteochondral applications is the lack of *in vivo* proper tracing and quantification of the bioactive factor being released in the host. Most of the reports that assessed the *in vivo* release profiles used radioactivity as a tracing agent to identify the implanted molecules of interest.[160,166,168]

Indirect GF delivery

The development of systems capable of promoting the delivery of inductive molecules has seen a tremendous improvement over the last decades. A key challenge in the application of GFs is their eventual inefficient delivery. In this review, we classify indirect growth delivery strategies as the ones leading to the production of these bioactive signals without incorporating them directly in the system. In that sense, gene therapy and cocultures can be included in this approach. An extensive overview of these approaches is out of the scope of this review. One possible route to overcome this issue is through the use of genetically engineered cell therapy, which has become a cutting-edge approach for tissue regeneration, and it has been under intensive experimental evaluation.[196,197] Cell-based gene delivery approaches to induce bone formation through the transfection of cells with BMP-2, 4, 6, 7, and 9 genes have led to superior bone formation in several animal models.[198–203] Gene therapy approaches for cartilage regeneration have also been explored with transfection of cells with BMP-2, BMP-7, TGF-β1, IGF-1, among others, acting individually or in synergy.[204–208] Blocking angiogenic genes to prevent osteocalcification by using antiangiogenic factors or competitive inhibitors have also shown to promote cartilage regeneration.[209] Menendez *et al.*[210] also assessed osteochondral repair after injection with adenoviral vectors of BMP-2 and BMP-6. Cartilage and subchondral bone regeneration was supported; however, it was insufficient to provide long-term quality osteochondral repair.

Meinel *et al.*[211] compared adenovirus gene transfer and protein delivery of BMP-2 on osteogenesis of human MSCs cultured on silk biomaterials. The transfected cells produced BMP-2 within a range of 0–40 ng/mL, and control cultures were supplemented with the same amount of exogenous GF. Results demonstrate that transfection resulted in higher levels of expression of osteogenic marker genes. However, it should be noted that the exogenous BMPs were supplemented in a culture medium, and in our opinion, despite the interesting data obtained with this study, the spatiotemporal control over the release kinetics of the protein should be regarded to obtain a fair comparison between both strategies.

The synergistic effect of combinations of genes encoding for specific GFs and transcription factors on bone and cartilage regeneration has been intensively explored. For bone formation, studies regarding gene combinations of BMP-2 and VEGF, BMP-2,[212] VEGF, and Ang-1[213,214]; BMP-4 and VEGF[122,215]; BMP-7 and PDGF-BB[216]; Runx 2 and Osterix[217]; and FGF-2 and sonic hedgehog[218] have been published.

For cartilage regeneration, some examples of combinations that have been used to enhance the formation of new tissue include BMP-2 and IGF-1,[219] IGF-1 and IL-1,[220] FGF-2 and IGF-1,[221,222] Sox5, 6 and 9 genes,[223,224] and TGF-β3 and collagen I silencing short hairpin RNA.[225] Osteochondral defects have also received particular interest by the combination of BMP-2 and TGF-β1.[226] Phillips *et al.*[185] have also observed graded mineral deposition through the creation of a gradient of Runx2/Cbfa1 oriented along the length of collagen scaffold aiming for orthopedic interfacial TE.

Gene therapy can be applied through two main mechanisms, either the direct delivery of genes into cells and tissues or through transfection of transplanted cells and further seeding onto the construct and/or implantation to the defect site. Both strategies share the vision of cells as factories for bioactive factors.[89] The biggest hurdle for the translation of stem cell transplantation into clinical practice has been the *in vitro* expansion conditions to achieve the required amounts of cells for a successful therapeutic outcome.[227]

The importance of cell-to-cell interactions in the context of TE is also a critical issue.[228] Cells can stimulate the production of chemoattractant and trophic factors, stimulating neighboring cell populations. During endochondral bone formation, a cascade of signaling occurs between chondrocytes and osteoprogenitor cells that ultimately lead to bone formation via a cartilage intermediate. Chondrocytes can provide morphogenic signals that induce osteogenic differentiation of MSCs.[229]

In development, vascularization precedes osteogenesis, and it is suggested that microvessels accelerate bone formation even before flow has been established.[230] For example, the coculture of target tissue cells together with endothelial cells is a promising approach to promote vascularization. In the case of bone, considering the intricate connection between angiogenesis and osteogenesis, it is not surprising that the interactions between osteoblasts and endothelial cells happen to be so relevant. Endothelial cells are osteoinductive, as they drive MSCs toward the osteoblastic phenotype. Cocultures of endothelial cells with bone marrow stem cells, osteoblast-like cells, and osteoblast progenitor cells have shown pronounced mineralized matrix production, enhanced microvascular network formation, and increases bone regeneration.[230]

In cartilage, mature chondrocytes are demonstrated to secrete TGF-2, BMP-2, and IGF-1 to direct and enhance MSC chondrogenesis with substantial increase in tissue volume, mass, and ECM production.[231]

The special case of PRP

Another promising strategy based on the use of GFs for stimulation of bone, cartilage, and osteochondral healing is through the use of PRP. The implementation of an autologous technology represents a new translational procedure acting as an alternative to the limitations observed with the current TE and regenerative medicine cell-based approaches.[232] In the past two decades, the increasing knowledge on the physiological roles of platelets in wound healing and tissue injury suggests the potential of using platelets as therapeutic tools.[233] Platelets are anucleated cytoplasmatic fragments that form an intracellular storage pool of proteins vital to wound healing. When activated, they release a group of biologically active proteins and other molecules that bind to transmembrane receptors of target cells, leading to the expression of gene sequences that ultimately promote the recruitment, growth, and morphogenesis of cells.[234,235] Most of the GF content is stored in the alpha-granules of the platelets. Platelets activation can be initiated by a number of methods, such as shear forces caused by fluid flow, contact with a variety of materials, including collagen and basement membranes of cells, and thrombin.[236,237] Upon activation of the platelets with thrombin, calcium, or temperature cycles, proteins are released.

PRP can be easily obtained through centrifugation cycles of blood samples, and after activation of the platelet concentrates and consequent liberation of their protein content, the enriched GF cocktail can also be called platelet lysates (PLs).[234] The standard protocol for the preparation of PRP from autologous blood is based on a two-step centrifugation process to separate blood components into different layers: the separation and concentration steps. For the separation step, blood is centrifuged to separate red blood cells from platelets and plasma. For the concentration step, the supernatant composed of platelets and plasma is collected and centrifuged again to isolate the platelets.[238,239] However, in the literature, several isolation procedures can be found, and they can be divided in three main groups according to the differing parameters in the platelet isolation: (1) final concentration of platelets in PRP; (2) protocol for the activation of PRP; (3) other variations from the standard protocol.[240] There are several classifications to categorize platelet concentrates based on relative concentrations of platelets, leukocytes, and fibrin, but herein the general abbreviation is going to be used.

An attractive approach for the addition of GFs to increase bone, cartilage, and osteochondral regeneration is the addition of PRP to the defect site.[241] PRP in the liquid state can be used to disperse and encapsulate cells upon activation of the platelet concentrate, which clots, forms a hydrogel, and creates a 3D environmental support for cells.[242] Moreover, these hydrogels are particularly attractive, because they can be used as minimally invasive injectable systems, with *in situ* fast gelling abilities. This way, PRP can both act as a GF source and also as a cell carrier for TE applications. Even in strategies where the main role of PRP is the supply of GFs, the presence of the protein concentrate in the structure typically enhances the stability of the scaffold/material and might even work as a glue, as, for example, in constructs build up on the assembly of particles.

The mechanism by which PRP may work has not been fully explored.[243] However, it is known that the GFs present in the platelet concentrate promote healing by attracting undifferentiated cells into the newly formed matrix and by triggering cell proliferation.[244] Moreover, PRP plays a significant role on the regulation of inflammation, as it may interact with macrophages and limit the degree of inflammation,[245] and on vascularization, as it promotes new capillary growth.[246]

There are conflicting reports in the literature with several studies concluding a positive effect of the concentrates in either bone and/or soft tissue healing, while other do not report a significant benefit from the use of the enriched protein suspension for the regeneration of tissues.[247–251] One of the reasons for this disparity is the difference in platelet density used for the different published studies. Platelet count may vary according to the preparation technique, ranging from two- to several fold above the physiological levels.[238,252] To confirm the efficacy of this cocktail of GFs, the isolation procedure should be standardized.[253] PRP effects change according to its preparation, activation, concentration, protocol for administration, and the material used for the combination.[247] Table 3 summarizes some studies regarding the use of PRP for bone, cartilage, and osteochondral applications. It is clear from the list that there is a pronounced variability in the experimental conditions, specifically on the preparation and isolation of PRP by the suggestion of different centrifugation cycles in time and force.[254,255] Centrifugation force in particular might be a critical step in PRP preparation, as the applied mechanical forces may damage the platelets leading to GF loss.[256]

Despite the common extreme variability and donor dependency in the amount of GFs present in the platelet concentrate, typically, the proliferation enhancement is not

Table 3. Summary of Studies Regarding the Application of PRP for Controlled Delivery Systems Aiming for Bone, Cartilage, and Osteochondral Regeneration

Source	Isolation	Mean/range platelets	Activation protocol	GFs concentration (ng/mL)	GF source/ structure	Materials	Application	Cell source	Comments	Reference (Year)
Dogs	(1) 1100 rpm, 5 min (2) 2500 rpm, 5 min	0.935–1.84 million	Thrombin and calcium	Not quantified	Structure	PRP gels with cells	Bone regeneration mandible defects	Dog BMSCs	Bone regeneration comparable to autogenous bone graft.	262 (2004)
Rabbits	(1) 2400 rpm,10 min (2) 3600 rpm,10 min	1.2 million per μL	Thrombin	PDGF-BB: 225–240; TGF-β1: 65–100	GF source	PRP-loaded gelatin hydrogels	Bone regeneration. Rabbit radius defect	No cells	PRP-loaded hydrogels stimulated bone regeneration. Gelatin activates PRP.	253 (2005)
Humans	(1) 840 *g*, 10 min (2) 1310 *g*, 10 min	0.95 million per μL	Not mentioned	Not quantified	GF source	Collagen type I scaffold	Bone regeneration. Sheep tibia defects	No cells	No effect of PRP on bone regeneration.	263 (2006)
Rats	(1) 2400 rpm, 10 min (2) 3600 rpm, 15 min	0.6 million per μL	Thrombin and calcium	Not quantified	GF source	PCL-TCP scaffold	Bone regeneration. Rat femur defect	No cells	Accelerated early vascular ingrowth and improved long-term functional integration.	264 (2007)
Rats	(1) 147 *g*, 15 min	Three-fold increase	Thrombin and calcium	Not quantified	Structure	HA-TCP particles + PRP gel	Bone regeneration. Rat cranial defect	No cells	PRP implantation showed no effect on bone formation and neovascularization.	265 (2007)
Rabbit	(1) 1800 rpm,10 min (2) 3600 rpm, 10 min	Not quantified	Thrombin and calcium	Not quantified	Structure	PRP gel	Cartilage regeneration. Rabbit subcutaneous implantation	Rabbit chondrocytes	Combination with cells produced cartilage unlike PRP alone.	266 (2007)
Rabbit	(1) 800 rpm, 15 min (2) 2000 rpm, 10 min	1 million per μL	One thermal cycle	PDGF-BB: 3.23; TGF-β1: 78.41; VEGF: 138.79	GF source	PRP-loaded gelatin hydrogels	Meniscus regeneration. Rabbit meniscus defect	No cells	Enhanced meniscal repair in the group containing PRP.	267 (2007)
Dogs	(1) 3000 rpm, 8 min	0.6 million per μL	Calcium	Not quantified	GF source	PRP-loaded bioactive glass foam	Bone regeneration. Mandible defects	No cells	Thicker area and more mature bone formation.	268 (2008)
Humans	(1) 200 *g*, 15 min (2) 200 *g*, 10 min	0.5–1 million per μL	Calcium	Release quantification	GF source/ structure	Alginate–PRP capsules	Bone regeneration. *In vitro* cultures with osteoblastic cells	SaOS-2	Enhanced cell proliferation and ALP activity.	269 (2008)
Mice	(1) 2400 rpm, 10 min (2) 3600 rpm, 10 min	5.17 million	Gelatin contact	PDGF-BB: 49; VEGF: 0.064; IGF-1: 7.58; SDF-1α: 10.1	GF source	Gelatin hydrogel	Therapeutic angiogenesis. Mouse ischemic hindlimb model	Endothelial cells (*in vitro*)	Restoration of blood perfusion by restoring angiogenesis.	270 (2009)
Goats	(1) 800 rpm, 15 min (2) 2000 rpm, 15 min	1.2 million per μL	Thrombin and calcium	PDGF-BB: 25.74; TGF-β1: 128.88; FGF-2: 81.15; VEGF: 161.53	GF source	TCP–chitosan composite scaffold	Bone regeneration. Goat tibia defects	BMSCs (*in vitro*). No cells (*in vivo*)	Complete repair of the defect within 16 weeks.	271 (2010)
Mini pigs	(1) 3200 rpm, 15 min	1 million per mm^3	Thrombin and calcium	PDGF-BB: 15; TGF-β1: 40	GF source	Bone graft	Bone regeneration. Pig tibial defect	No cells	Complete bridging of the defect area.	259 (2010)
Humans	(1) 150 *g*, 20 min; (2) 2200 *g*, 15 min	1 million per μL	Thermal cycles	PDGF-AB:185.67; TGF-β1: 97.11	GF source	Mineralized collagen	Bone regeneration. Sheep tibia defects	Ovine BMSCs and ovine ASCs	PRP compensated the inferior osteogenic potential of ASCs.	272 (2010)
Humans	SmartPReP2 (Harvest Technologies Corp)	0.95 million per μL	Thermal cycles	VEGF: 0.3–1.6; FGF-2: 0–0.8	GF source	Silk-, PGA-, and PCL-electrospun scaffolds	General tissue engineering applications	Adipose stem cells and macrophages	Enhanced bioactivity of the scaffold	273 (2011)
Rats	Labofuge 400R	4.21 million per μL	Thrombin and calcium	TGF-β1: 423.87	GF source	Collagenous bovine matrix	Bone regeneration. Rat intramuscular implantation	Rat BMSCs	Autogenous GFs had no effect on the capacity of BMSCs to form new bone.	274 (2011)
Humans	(1) 3000 rpm, 6 min	Not quantified	Thrombin	TGF-β1: 0.1–2	GF source	Collagen-coated plates	Cartilage regeneration. Inflammation-induced model	Cytokine-induced osteoarthritic chondrocytes	Restoration of collagen II and proteoglycan production postinduced inhibition.	275 (2011)
Rabbits	(1) 2400 rpm,10 min (2) 3600 rpm, 15 min	Not quantified	Not specified	Not quantified	GF source	TCP granules	Bone regeneration. Rabbit calvaria defect	No cells	Apparent formation of more mature bone upon use of double-dose PRP (one at day 0 and another at day 15).	276 (2012)
Humans	Platelets obtained from blood bank	1 million per μL	Thermal cycles	TGF-β1: 200 pg; PDGF-BB: 200 pg; cumulative release after 1 month	GF source	PDLLA scaffolds entrapping PL-loaded CH/CS NPs	Osteogenic differentiation	Human ASCs	Earlier osteogenic differentiation of ASCs with enhanced osteocalcin expression	291 (2012)
Rabbit	(1) 1500 rpm, 10 min (2) 3000 rpm, 10 min 2)	Not specified	Not specified	Not quantified	GF source	GPT hydrogels	Chondrogenic differentiation	Joint chondrocytes	Formation of cartilage and perichondrium in the PRP-enriched hydrogels	277 (2012)
Rabbit	(1) 250 *g*, 10 min (2) 1000 *g*, 10 min	1.6 million per μL	Calcium	Not quantified	Structure/GF source	PRP hydrogels	Chondrogenic differentiation	Rabbit BMSCs and ASCs	Contribution of implanted cells was significant, and BMSCs achieved stronger levels of chondrogenic induction	278 (2012)

ASCs, adipose-derived stem cells; CH/CS, chitosan/chondroitin sulfate; GPT, gelatin-poly(ethylene glycol)-tyramine; HA-TCP, hydroxyl apatite and tricalcium phosphate; NPs, nanoparticles; PGA, polyglycolic acid; PRP, platelet-rich plasma.

impaired.[240] However, it has also been reported before that the stimulation effect of the lysates on cell proliferation and differentiation is dose dependent.[257] Previous studies suggest that range of 2-fold to 8-fold increase in platelet concentration above physiological levels of blood samples is required to obtain positive results from PRP. Lower concentrations have suboptimal effects, whereas higher concentrations might have a paradoxically inhibitory effect.[238,258,259] A platelet count of 1 million platelets per μL has also become the benchmark of therapeutic PRP.[260,261]

Clearly, PRP also needs to be properly activated to achieve full degranulation of platelets.[238] The addition of calcium chloride activates PRP, because it replaces the calcium bound by the anticoagulant agent, used to avoid coagulation of the collected whole blood. This calcium allows the conversion of prothrombin to thrombin, thus activating the coagulation cascade. Exogenous thrombin can also be provided, and it has been commonly used as a PRP activator; however, the use of animal-derived thrombin has raised some concerns regarding the potential significant immunogenic and bleeding side effects, including high rates of thrombosis.[243,258] Moreover, some studies reported the loss of osteogenic and chondrogenic potential of platelet concentrate upon activation with thrombin.[279]

Studies have also shown that using thrombin as an activator can result in the bolus release of GFs, with nearly 100% of the protein content released within 1 h. Clearly, this method fails to maximize the potential of PRP, as GFs are cleared before they can even promote healing.[273] Moreover, the need for platelet activation with exogenous thrombin before injection in unclear, since upon PRP injection into connective tissues, it comes in contact with tissue factor, which can activate platelets and initiate the formation of the fibrin 3D matrix.[237,280] To overcome this limitation, other stimulus has been increasingly pursued, especially mechanical destruction through thermic shock.[240,253,281,282]

Table 3 summarizes some studies promoting the delivery of GFs present in PRP for stimulation of bone, cartilage, and osteochondral regeneration. Kim *et al.*[283] evaluated how different activation protocols would affect the concentrations of GFs in the lysates. Curiously, the four activation methods (calcium chloride, a nonionic surfactant, thrombin and calcium chloride, thrombin and calcium chloride with preactivation with shear stress, and collagen) promoted completely different effects on the release of PDGF, TGF-β, FBF-2, and VEGF, and there was no clear pattern on which one of them was more effective. However, the activation method that promoted a higher release of VEGF also stimulated higher bone mineral density and content in critical-size rat craniotomy defects after implantation of β-TCP and the different groups of PRP.

The large list of biological mediators stored in platelets includes the proteins fibrinogen, fibronectin, and vitronectin, which are known to act as cell adhesion molecules.[257] Platelets store essential GF, including PDGF, TGF-β, IGF-1, and EGF.[238,239,253,284] PRP may act as an exogenous source of TGF-β for bone healing, directing BMSCs to resorption sites.[285] Furthermore, platelets contain different angiogenic factors, such as VEGF, Ang-2, FGF-2, and antiangiogenic proteins, including endostatin and thrombospondin-1, regulating the formation of new blood vessels.[232,239,284]

Localized angiogenic factor delivery has proven beneficial for bone regeneration in various animal models by promoting neovascularization, bone turnover, osteoblast migration, and mineralization. Considering that PRP can release factors involved in angiogenesis, platelet concentrates have been used aiming for that goal. Hu *et al.*[286] reported that PRP possibly starts the angiogenic process by recruiting endothelial cells that line blood vessels and initiates bone regeneration. The angiogenic role of PRP has been reported by Kajikawa *et al.*[287] and Lyras *et al.*,[288] which, respectively, observed the role of PRP as an activator of circulation-derived cells in the early phase of tendon healing and on early neovascularization enhancement in full-thickness defects of patellar tendon, respectively.

When activated, PLs can even form 3D structures such as hydrogels and scaffolds based on the production of a fibrin network from the fibrinogen released from the platelets and converted to fibrin through the action of thrombin. However, most of the studies report a significant and pronounced volume shrinkage in these structures.[289] It has been stated that the positive role of PRP on bone healing is more related to the fibrin-supporting matrix than for the GFs content.[274] Moreover, the matrix prolongs the exposure of cells to those GFs. On the other hand, it has been stated that PRP enhances osteoprogenitor cell number in the defect area, thus stimulating tissue regeneration.[261]

Marx *et al.*[290] proposed the use of PRP to enhance the initial stages of bone wound healing. The GFs and chemokines present in the platelets play critical roles on the chemotaxis, cell proliferation and differentiation, angiogenesis, vascular modeling, and bone formation.[234,252] Bertoldi *et al.*[247] evaluated the effect of PRP on different stages of bone formation to optimize the administration protocol of the platelet concentrate. They observed that an initial and single dose of PRP was not as effective as the frequent addition of PRP for a long period, which ultimately led to enhanced ALP production and mineralization in osteoblast cultures. These studies support the need for the development of controlled release systems for PRP to enhance tissue regeneration. As an example, Dutra *et al.*[268] observed superior maturity of bone formation when PRP was associated with bioactive glass foams when compared with nonloaded sponges.

Present research shows that the enrichment with PRP can influence the early stage of bone healing, gradually decreasing the exerted effect with time.[259] It is believed that TGF-β and PDGF promote the healing of soft and bone tissues through stimulation of collagen production to improve wound-healing formation and the initiation of callus formation.[253] Santo *et al.*[291,292] also showed that *in vitro* controlled release of PLs led to a faster osteogenic differentiation of human adipose-derived stem cells, with a stronger contribution during earlier stages in culture.

Several studies reported the effective response of PLs in the repair and regeneration of a variety of tissues other than bone, including cartilage.[293,294] The application of PRP in cartilage repair is relatively new, and there are limited *in vivo* studies regarding its use for that specific application. However, it has been shown that PRP stimulates chondrocyte and MSC proliferation and cartilage ECM synthesis of proteoglycans and collagen type II.[260] Besides the significant role of TGF-β on bone formation, TGF-β is also one of the most important GFs involved in the process of cartilage regeneration.[295] Wu *et al.*[266] documented new cartilage tissue in rabbits injected with chondrocytes/PRP mixtures and production of large amounts

of proteoglycans and collagen fibrils, whereas Akeda *et al.*[296] showed enhanced proliferation and proteoglycan and collagen synthesis on porcine chondrocytes. Direct injection of PRP into patient's knee has been increasingly investigated, and preliminary results are promising.[295,297,298]

Clinical studies comparing the role of approved BMPs and PRP on bone healing have been done.[299] Calori *et al.*[299] reported a clinical report regarding the positive role of both BMP-7 and PRP on treatment of long-bone nonunions, with BMP-7 promoting a slightly better healing response in the treated patients. Preclinical evidence has demonstrated that PRP enhances osteoprogenitor cell proliferation, promotes angiogenesis, and enhanced fracture healing and bone regeneration. In a randomized tibial osteotomy trial in 33 patients,[300] the authors reported a superior radiographic and histological healing in defects treated with PRP when compared with the controls, although clinical and functional outcomes did not show significant differences. Considerable effort has also been done to evaluate the role of PRP and PLs as replacement or supplements for *in vitro* cultures.[243,295,301–305] The use of animal-derived serum raises concern regarding its immunological response and possible prion and virus transmission and the application of an easily isolated and autologous source of proteins for *in vitro* cell expansion and differentiation. To translate the culture of MSCs into clinical practice, PLs have also been used to replace fetal bovine serum, thus avoiding the use of animal-derived proteins, showing to accelerate cell expansion, and thus reducing the duration of *ex vivo* manipulation.[240,282,306]

Despite the impressive amount of research with PLs and PRP, a limited number of human clinical trials investigating the use of this concentrate were carried out so far.[252] There is still room for improvement on the use of PRP as a therapeutic agent for skeletal regeneration. The data obtained from studies with humans are mostly obtained from case reports with small sample sizes and in majority from maxillofacial area, with little data regarding the effect of PRP in critical defects in the axial skeleton.[241]

Moreover, PRP can be used as a useful tool to study the mechanisms underlying the action of several GFs and cytokines. Its widespread availability conjugated with its rich composition provides a highly competent source of signaling molecules. The level of complexity limits the comprehension of the biological phenomenon promoted by PLs; however, there is a potential to maximize this degree of complexity by developing mechanisms of isolating specific GFs from the whole cocktail. Following this approach, PLs could be used not only as a bioactive factor source for tissue regeneration but also as a high-throughput analysis tool for assessing the role of specific molecules released by the platelets.

Combinatory strategies

The latest trends on the application of biomimetic TE approaches require the combination of several biochemical cues, presented through different mechanisms. Therefore, it is expectable that to mimic the complexity of the native ECM, indirect and direct delivery of GFs should be included in the strategy to produce formation of new functional tissue.

The codelivery of PRP with other GFs has also shown high potential. Considering the ability of PRP to stimulate undifferentiated tissue healing mainly through cell proliferation, the combination of this cocktail of GFs with a potent inducing signaling molecule, such as BMP-2 for bone, could lead to a strong enhancement of tissue formation and more importantly to a more directed regeneration pathway toward a specific lineage. The osteogenic potential of BMP-2 and PRP has already been demonstrated.[307,308] It has been demonstrated that PRP reduces the osteoclast-mediated bone collagen degradation, suggesting the inhibition of osteoclast activation.[309] Since BMP-2 stimulates the generation of osteoblasts, but also osteoclasts, the combination of both agents could lead to a more favorable strategy. The combination of gene delivery and direct GF delivery has also been explored. Luo *et al.*[310] evaluated the potential of codelivering VEGF and the gene encoding for BMP-2.

To the best of our knowledge, there are no reports of studies regarding the combination of direct and indirect GF delivery for cartilage and osteochondral interface regeneration.

Common Shortcomes and Challenges

The limited success of the current approaches using GFs as therapeutics indicates that substantial challenges need to be addressed. The optimization of biomaterial design and site-specific pharmacological actions of GFs remain challenging in translational bone and cartilage regeneration studies.[165] It is difficult to determine through *in vitro* experiments the effect of a specific GF, because it is highly dependent on the state of cellular differentiation, growth conditions, and the presence/absence of other GFs.[32] The varying effects of strategies to deliver GFs are often related to the delivery vehicle used, the concentrations and combinations of bioactive molecules delivered, and the reliance on host cells for new tissue formation.[163] Furthermore, it is impossible to know the exact concentrations of GFs present within each specific tissue to provide an exogenous dosage of bioactive factors. Upon injury, tissues upregulate GFs and chemokines in a dose-dependent manner that is influenced by the degree of injury and by the particular clinical background of each host. The infinite number of factors influencing this secretion creates this unpredictable scenario, making it difficult for a TE strategy to provide the appropriate amount of biological cues.[311] Moreover, scale-up is one of the parameters impairing translation of general GF delivery into clinical practice.

The establishment of a correlation between effective drug delivery strategies *in vitro* and *in vivo* is not an easy task, and translating an *in vitro* successfully generated construct to an *in vivo* setting is a limiting step. Typically, dosages required to promote an efficient response on cell behavior are typically lower for *in vitro* studies. While timing of the protein release is important, dynamic nature of the healing zone makes it difficult to assess the state of the defect. At tight control, over dosage of GFs is required, since there is a tenuous line separating therapeutic from pathological effects.[312]

There is not a standardized procedure to evaluate the release systems, and the regeneration profiles are highly dependent on species, age, tissue, and health report. GFs may be degraded more quickly in humans than in animals; the biology of the receptor-ligand complex may differ, and the pharmokinetics of the delivery system might be less favorable in humans than in the animal models.[73] The assessment

of a treatment effect is typically performed in a homogeneous group of animals, oppositely to a strong heterogeneity naturally found in a group of patients. The negligent monitoring and underestimation of physiological parameters can also lead to erroneous conclusions. Hence, the standardization of protocols, animal models, and characterization assays to assess the dosages, targeting, and efficiency of GF delivery vehicles is of uttermost relevance, suggesting an increasing need of accurately designed clinical trials. The definition of an appropriate blinding, sample size calculation, and timing of outcome assessment is critical.[313,314] Therefore, reassessing measurable and reproducible outcomes is required to ensure a proper analysis. Furthermore, the concept of reverse translation can be adapted, that is, fully understand the mechanisms behind the pathologies in humans to properly design new efficient animal models. The establishment of systematic review and meta-analysis of animal models and the application of mathematical modeling may aid in the selection of the most promising treatment strategies for clinical trials.

Another common problem faced in some of the work published in the literature is the lack of clear information regarding the actual dosage of GFs delivered by the construct *in vivo* in the defect.[15,315] Improved noninvasive assessment tools are needed to monitor the extent of tissue healing and to trace the location of drugs and drug delivery systems after implantation. There are many imaging modalities that can be used in clinical practice, and their choice is determined by the specific diagnostic, availability, sensitivity, specificity, resolution, and cost–effectiveness.

The ability to combine both targeted imaging and therapeutic agents within the same carrier, allowing the visualization of targeted drug delivery sites and to deliver therapeutics simultaneously, is an exciting advance in this field.[316,317] Some examples of imaging modalities include magnetic nanoparticles for cell labeling and observation by magnetic resonance imaging and quantum dots, carbon nanotubes, or radionuclides for *in vivo* observation of cells by positron-emission tomography (PET) or single-photon-emission computed tomography.[318–320]

Although PET provides great sensitivity, it lacks spatial resolution. On the other hand, optical imaging has great sensitivity and temporal resolution, but lacks spatial resolution and sufficient penetration depth. The combination of molecular and anatomical imaging allows the simultaneous assessment of spatial and temporal aspects of tissue healing.[321] Further improvements for the development of theranostic systems are critical to provide a live and noninvasive monitoring of drug delivery and to assess in real-time instantaneous host tissue responses.[322–324]

The therapeutic outcome of GF treatment also depends on its quantity, concentration, administration route, time of application, and the experimental sites chosen for the study. Relevant details such as the differences in surgical procedures between ectopic and orthotopic sites are critical and commonly ignored. Orthotopic procedures typically lead to larger hematomas, which are a source of endogenous chemotactic, angiogenic, and mitogenic GFs.[165] Moreover, different anatomical sites require therapeutic doses depending on degree of vascularization, defect size, and number of resident cells.[325] Therefore, appropriate carrier systems are critical for the delivery, retention, and release of GFs at the implanted site to achieve the desired effect.[68,326] Despite the differences in the use of scaffold carriers, GFs dosages and combinations, animal models, and consequently the tissue regeneration outcomes, the potential of GF therapy are undeniable. This can lead to the development of more cost–effective and adverse effect-free GF treatment.

In case of the highly promising PRP/PLs, the preparation and delivery of the lysates are likely to be critical, but the biological significance of different preparations of PRP remains unclear, and no standardized method has been developed, and there are only few studies that consider the factors involved in platelet activation and conditions at the PRP-delivered site.[283] The enriched composition of PRP makes it hard to realize which component is mainly responsible for the observed cellular responses. An important step toward determining an optimal PRP preparation is adoption of a standardized nomenclature for PRP products to accurately reflect platelet concentrations.

In addition to efficacy and safety, simplicity is an important consideration for any regenerative strategy, because the combination of multiple bioactive components may not have a realistic chance of clinical translation due to the cost or regulatory approval barriers.[327] Regarding cell therapy, an increasing number of clinical trials assessing the potential of stem cells for bone and cartilage regeneration are undergoing. The demanding logistics, the lack of FDA-approved and off-the-shelf devices incorporating human cells, time-consuming procedures, safety issues, and insufficient amount of cells to achieve the desired goal have been impairing translation of the widely investigated cell therapy toward clinical practice.[328] In that sense, the application of bioactive cues such as cytokines and GFs capable of promoting stem cell homing induction is seen as safer and more practical procedures. However, the above-listed limitations have also limited their success, as the majority of drugs that enter clinical trials after extensive animal testing fail to achieve FDA approval due to lack of safety or efficacy in humans.

While much effort has been dedicated to identifying which biochemical cues are most critical and fabricating appropriate material delivery systems, opportunities and challenges exist for developing advanced drug delivery strategies to accelerate differentiation processes toward committed pathways.[329] The authors believe that the answer for the definition of an appropriate delivery system relies on the development of dynamic systems, capable of responding *in situ* to the encountered *in vivo* conditions. The design of such smart strategies requires the understanding of the mechanisms underlying the activation and production of native bioactive factors to further incorporate those concepts into the formulation of the new sophisticated systems. Therefore, it is our belief that the development of nanotechnology approaches might provide the scientific community with more information regarding the characterization of native tissues and to produce more sophisticated materials with enhanced control over specific properties, structure, architecture, and functionality. It is anticipated that the current developments in nanotechnology would significantly improve the current understanding of the structure–function relationships at native tissues. Spatial patterning of biological cues, vital for tissue healing, and the ability to more closely mimic the native transition in composition and function properties are among the critical contributions from nanotechnology

advances. The application of a nanotechnology-based platform through high-throughput screening is a powerful tool, as it allows the miniaturization of the assays, and consequently promotes the quick analysis of numerous parameters influencing the biological processes.

The miniaturization of the systems, in particular drug delivery systems for bone, cartilage, and osteochondral interface, allowed a range of new opportunities in the design of more elegant strategies for the stimulation of tissue healing. Cell engineering is a powerful tool to manipulate cell fate and differentiation, and one of the most effective mechanisms is through cell internalization, which allows an intracellular target and deliver of drugs.[318] The use of nanoparticles also enables their permeabilization across biological membranes and overall shows an enhanced targeting efficiency of the delivery system. Due to their greater surface area-to-volume ratios, nanoparticles also present higher drug loadings and drug bioavailability. The size similarity of native nanoscale components to engineered drug-loaded nanomaterials also enables their use as building blocks for bottom-up colloidal systems such as injectable gels for bone and cartilage regeneration.

Patterning,[330,331] surface immobilization,[332,333] creation of gradients,[334,335] self-assembly,[336,337] and layer-by-layer deposition[338,339] are among the techniques that are currently being further improved and explored due to advances in nanotechnology. This significant list of contributions highlights the decisive role of nanotechnology on the development of multifunctional biomaterials for bone, cartilage, and osteochondral engineering.

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Disclosure Statement

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