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Bioactive Scaffolds for the Controlled Formation of Complex Skeletal Tissues

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1. Introduction

Most skeletal tissues have the innate capacity to regenerate, at least to a certain extent, without therapeutic intervention. Bone for example has the capacity to repair fractures up to a critical size with the help of local or recruited cells of the patient's body, and the defects heal by forming new bone that is indistinguishable from uninjured tissue (Deschaseaux et al., 2009; Place et al., 2009). However, traumatic fractures of the lower extremities are one group of orthopedic injuries that often require prolonged rehabilitation, or multiple procedures to achieve maximal functional recovery, and comprise the bulk of nonunion or delayed-union fractures (Deschaseaux et al., 2009). In contrast, articular cartilage, ligaments and tendons are known to have a limited capacity for self-repair and methods to augment their natural healing response are still highly investigated.

Biomaterials used to restore the structure and function of lost tissues and organs have evolved greatly due to the large knowledge accumulated on material-protein and material-cell interactions. Current commercially available biomaterials are engineered to be non-toxic, non-immunogenic, and hemocompatible. In contrast to elements of the extracellular matrix of living systems, however, these scaffolds are very poor in information, which makes them suboptimal for many tissue engineering applications. These passive biomaterials are unlikely to guide cell migration and differentiation or controlled matrix deposition, a problem that becomes even more evident in complex tissues with more than one cell type. Furthermore, they also cannot induce tissue neoformation while preventing other undesirable tissue repair processes such as scarring, and they are unable –with the notable exception of some materials in bone regeneration- to promote functional tissue integration in the host, including vascular and/or nervous connectivity. Finally, these passive scaffolds largely lack the capacity to induce cell differentiation, which becomes a major limitation for their use together with current stem cell-based therapies (Huebsch & Mooney, 2009). A promising strategy to overcome these limitations is to design bioactive scaffolds capable of recruiting tissue-forming cells from the body in such a way that they promote cell adhesion and tissue formation within their matrix. Moreover, materials that can recruit endogenous cells of the patient into scaffolds, avoid the expense and difficulties

associated with culture, storage and distribution of cells, not to mention other immunological considerations (Discher et al., 2009; Place, ES et al., 2009).

Bioactivity is defined as having the capacity to interact with a living tissue or system. In terms of scaffold design, we define a bioactive scaffold as one in which biological functionality has been integrated to provide an information-rich support material for tissue engineering. Bioactive scaffolds are designed to control cell- and tissue responses, and to provide a more efficient integration with the host. In this manuscript we will cover the most important scaffolds presenting a biomimetic composition (Section 2), the main strategies to introduce biomimetic features in scaffolds (Section 3), their expected applications in skeletal tissue engineering and the main challenges remaining for the successful application of this concept (Section 4). We will highlight methods of how scaffold materials can be modified to be bioactive, how a scaffold can promote the body's innate capabilities to heal and recruit cells to the site of injury. Bioactive scaffolds have already found their way into clinics and hold great promise to serve as a manageable alternative to tissue engineered implants that face a vast array of regulatory and logistical issues. In the end, products on the market need to show convincing clinical performance as well as cost-effectiveness. We will therefore not cover the traditional tissue engineering approach to seed and culture scaffolds ex vivo with cells before implantation. However, many of the methods presented here have been discovered from in vitro cell cultures and might as well be of high importance in these strategies.

2. Scaffolds with a biomimetic composition

Three dimensional biopolymer networks are major players in physiological wound healing. It is assumed that wound healing progresses through three stages: (i) blood clotting and inflammation; (ii) granulation tissue; (iii) tissue remodeling. Each of these stages can be associated to the prevalence of a particular biopolymer network, i.e. fibrin (stage i), unstructured collagen/glycosaminoglycan (GAG, for an acronyms list refer to Section 6) extracellular matrix (stage ii), structured collagen/GAG extracellular matrix (stage iii) (Braiman-Wiksman et al., 2007). The delicate choreography of deposition and remodeling of these biopolymer networks is coupled to the activation of relevant signaling cascades, directing immune cell invasion, inflammatory processes, progenitor cell attraction, differentiation, extracellular matrix (ECM) deposition, ECM remodeling and revascularization of the newly formed tissue graft (Gillitzer & Goebeler, 2001). These critical processes in wound healing underline the importance of fibrin, collagens and GAGs in tissue formation and fundament their recognition as bioactive polymers for tissue engineering. Besides, this biomimetic approach has also suggested the potential of reprocessed tissues for tissue/organ engineering. Reprocessed tissues are complex, structured scaffolds prepared by performing different extraction treatments (i.e. demineralization, decellularization) to biological tissues.

2.1 Fibrin

Fibrin is a biopolymer derived from the crosslinking of fibrinogen, the major protein involved in blood coagulation. As a medical product, fibrin gel is a blood derivative, typically presented as a two syringe-system with a fibrinogen solution and a thrombin solution having a common port. Once casted, fibrinogen is cleaved by thrombin, resulting in

the formation of a gel in a few seconds. In Europe, fibrin glue has been available as tissue adhesive, sealant and hemostat from the 1980's. Its approval by the FDA was delayed until 1998 due to security standards regarding blood derivative processing. Fibrin is now regularly used in surgery around the world for a broad number of indications (Spotnitz, 2010).

Fostered by its success in surgery, fibrin has gained increased attention in tissue engineering strategies. Fibrin has the great advantage that it can be delivered by minimally-invasive methods to the tissue defect, a property that is only shared with a few materials capable of gelling in situ (Kretlow et al., 2007). Due to this controlled gelling capacity, fibrin glue can be casted in defined shapes and be applied to fill wound beds. For cell delivery, fibrin glue excels in many ways, as cells can be injected with fibrinogen solution and are then retained at the wound site as the fibrin gels. As fibrin has been designed by nature as the emergency "physiological scaffold" used in wound healing, fibrin gels are well structured to accommodate cells (T.A.E. Ahmed et al., 2008). As a cell delivery matrix, fibrin glue has been used with mesenchymal stem cells (MSCs) for bone (Dozza et al., 2011; Liao et al., 2011), cartilage (T.A. Ahmed et al., 2011; J.S. Park et al., 2011), ligament and tendon regeneration (Lim et al., 2004; Soon et al., 2007). It has also been used with chondrocytes in cartilage regeneration (Eyrich et al., 2007; S.J. Lee et al., 2011; Peretti et al., 2006; Singh et al., 2011), with osteoblasts for bone regeneration (S.J Kim et al., 2007), and fibroblasts in tendon regeneration (Chun et al., 2003).

Fibrin glue can also be used as a drug delivery device for growth factors (GFs), either in combination with cell transplantation or not. Several studies have shown that fibrin glue can provide sustained release of GFs during a few days to one week (Spicer & Mikos, 2010). Longer GF retention times can be achieved by modifications in the GF (Schmoekel et al., 2004), by integrating high GF-affinity moieties in the fibrin chains (Merritt et al., 2010), or by covalent linking of the GFs (Drinnan et al., 2010; Schmoekel et al., 2005). Examples of GFs that have been integrated in fibrin gels in skeletal tissue engineering applications include BMP-2 (Kang et al., 2011; Schmoekel et al., 2004; Schmoekel et al., 2005), TGF-β1 and TGF-β3 (Drinnan et al., 2010; J.S. Park et al., 2011; W. Wang et al., 2010b). A similar concept to GF-loaded fibrin is platelet-rich fibrin, a new blood-derivative formed by platelet concentration. Platelet-rich fibrin also comprises fibrinogen for its polymerization in situ, although handling is different as compared to fibrin glue (Dohan et al., 2006). Due to this different processing, platelet-rich fibrin contains a concentrate of GFs that are present in platelets (i.e. PDGF, TGF, IGF, VEGF, EGF), transforming this product in a truly blood-clot biomimetic (Eppley et al., 2004). Platelet-rich fibrin, however, presents some disadvantages compared to fibrin glue encapsulating GFs. These are mainly related to potential variability in GF-content between batches and undefined GF doses.

Besides those indicated for GF delivery, fibrin glue has some important shortcomings. Firstly, fibrin is known to shrink as gelation occurs, and overall, fibrin lacks adequate mechanical properties (Sierra, 1993). Those limitations are particularly critical to skeletal tissue engineering, as many tissues to be regenerated are load bearing. Moreover, fibrin gels are known to degrade in a few days in vivo, due to the activation of plasminogen by the cells, which leads to formation of the serine protease plasmin and to subsequent fibrinolysis (Ye et al., 2000). Again, this short biodegradation time is probably inadequate for most applications in skeletal tissue engineering, as regeneration of these tissues usually takes several weeks or months. To address these limitations, fibrin glue scaffolds can be optimized

by using a solid porous scaffold for reinforcement (Schagemann et al., 2010; Z.H. Wang et al., 2010a; W. Wang et al., 2010b), or by the use of enzymatic inhibitors (Fussenegger et al., 2003). Eyrich et al. reported some optimized conditions that lead to fibrin gels that were stable for up to three weeks in vitro (Eyrich et al., 2007).

Despite being a material with a long history of application, fibrin has been studied recently in some advanced tissue engineering strategies for bone regeneration. Dozza et al. showed that MSCs seeded in fibrin scaffolds resulted in better bone formation around an uncemented hip prosthesis and better bone-prosthesis contact compared to the control (Dozza et al., 2011). Liao et al. showed that MSCs seeded in either platelet-rich fibrin or a Medpor® sheet filled with platelet-rich fibrin resulted in approximately 4-fold higher bone formation as compared to empty defects in a mandibular reconstruction model (Liao et al., 2011). Leong et al. confirmed the osteogenic differentiation of MSCs in fibrin glue filled polycaprolactone/tricalcium phosphate scaffolds (Leong et al., 2008).

Fibrin-based devices have also been recently studied for cartilage and ligament/tendon regeneration. MSCs seeded in fibrin glue containing TGF-β3 showed the capacity to regenerate articular cartilage (J.S. Park et al., 2011). Eyrich et al. demonstrated the capacity to form cartilage with chondrocyte-seeded stable fibrin gels (Eyrich et al., 2007). Wegener et al. used polyester scaffolds filled with a fibrin gel (with or without chondrocytes) to repair cartilage defects in the femoral condyle in sheep (Wegener et al., 2010). This study confirmed cartilage formation and improved O'Driscoll scores in defects filled with these scaffolds. Lee et al. formed a cartilage layer by seeding chondrocytes in fibrin gels on top of auricular implants as a strategy to minimize morbidity associated with implant dislodgement (S.J. Lee et al., 2011). Wang et al. used polyester/fibrin gel constructs loaded with MSCs and TGF-β1 to promote restoration of full-thickness cartilage defects (W. Wang et al., 2010b). Also recently, Scotti et al. used fibrin gels to promote cell adhesion to devitalized spongiosa cylinders used for engineering osteochondral grafts (Scotti et al., 2010). In two studies by the laboratory of Lee, MSCs embedded in fibrin glue were able to promote better osseointegration of tendon grafts (Lim et al., 2004; Soon et al., 2007).

A clinical study on mandibular degree II furcation defects has shown that patients treated with platelet-rich fibrin glue and an open flap debridement presented statistical improvements in all clinical parameters as compared to patients treated with just open flap debridement (Pradeep & Sharma, 2011). In another clinical study, MSCs in platelet-rich fibrin glue were implanted in patients with condyle defects for cartilage regeneration (Haleem et al., 2010). The study showed improvement of patient's symptoms in all subjects over the follow-up period of 12 months, achieving nearly normal arthroscopic scores after the follow-up and partial to total cartilage surface regeneration.

2.2 Collagen

Collagen is a family of proteins that are the main components of connective tissue, and the most abundant proteins in mammals (>90% of the ECM) (Van der Rest & Garrone, 1991). Collagen type-I is a major component of scar tissue, while this and other types of collagens are present in physiological tissue (and regenerated tissue if correctly remodeled) (Fratzl 2008). Specifically in skeletal tissues, collagen type-I is preeminent in bone, tendons and muscle. Collagens in cartilage change with specific tissue types: collagen type-II is preeminent in hyaline cartilage, collagens type-I and –II are present in fibrocartilage and collagen type-X is a marker of hyperthrophic cartilage. Collagen type-I is a long, stiff, triple-

stranded helical structure comprising two identical chains (i.e. one $\alpha 1$(I)-chain and one $\alpha 2$(I)-chain). This $\alpha 2$(I)-chain contains the sequence repeat (G-X-Y)n, X being frequently proline and Y hydroxyproline, that allow the formation of a triple helix (Ricard-Blum & Ruggiero, 2005). In physiological tissues, collagens show very defined structures. For instance, collagen type-I forms parallel, longitudinal fibers in tendons and ligaments and long bones. In lamellar bone, collagen type-I fibrils run in parallel to other fibrils in the same layer, but are aligned in the opposite direction in alternating layers (Fratzl 2008). In cartilage, collagen type-II is crosslinked to proteoglycans by the action of collagen type-IX that acts as a biological "spacer" (Fratzl 2008; Lodish, H et al., 2002).

As materials for tissue scaffolds, collagens have been thoroughly investigated due to their biomimetic nature, their biocompatibility and abundance. Indeed, collagen type-I has shown to be degradable, and highly biocompatible, particularly after proteolytic removal of telopeptides (Glowacki & S. Mizuno, 2008). Currently, collagen is FDA approved for implantation. Collagen scaffolds can be prepared in two forms: as hydrogels or as fibers in a mesh like structure (also named as "collagen sponges" or "collagen foams"). Collagen fibers can be reinforced by crosslinking, a modification that can be performed with either physical or chemical methods (Glowacki & S. Mizuno, 2008). Some chemical crosslinkers such as glutaraldehyde have been related to cytotoxicity (Middelkoop et al., 1995), although others have shown that this limitation can be overcome by protocol optimization (Nimni et al., 1987). Today, other more biocompatible crosslinkers can be selected (Kubow et al., 2009; Mekhail et al., 2010). Collagen fibers manufactured by optimized protocols present an adequate porous microstructure and promote cell adhesion (Glowacki & S. Mizuno, 2008). In skeletal tissue engineering, collagen scaffolds have been used for seeding osteoblasts (Laflamme & Rouabhia, 2008), periosteal cells (Ryu et al., 2010), MSCs (Brady et al., 2010; Dozza et al., 2011; Jäger et al., 2008), chondrocytes (Oliveira et al., 2010; Zhou et al., 2011) and fibroblasts (George et al., 2008; Mekhail et al., 2010). Collagen type-I has shown the capacity to specifically upregulate osteoblastic function and bone formation (Lynch et al., 1995), and this property has been linked to the interaction of this collagen with integrin α2β1 (M. Mizuno et al., 2000). On the other hand, collagen type-II scaffolds have shown favorable interactions for cartilage tissue engineering and cartilage regeneration, particularly in combination with GAGs (Chang et al., 2007; Wu et al., 2010). These results highlight, once again, the importance of providing the cells with scaffold compositions that mimic their natural environment.

To enhance their regenerative properties even further, collagen scaffolds can be loaded with GFs such as BMPs (Laflamme & Rouabhia, 2008), TGF (Pabbruwe et al., 2010) and FGF (Pang et al., 2010). Collagen-based sponge constructs be impregnated in BMP-2 are already available as a FDA-approved commercial product for spinal reconstruction (Infuse™, Medtronic, Inc.). Nevertheless, collagen has rather limited controlled release capacity, with most occurring in the first days (Maeda et al., 1999). Release properties can be improved to a limited extent by crosslinking (Fujioka et al., 1998) or drastically, by embedding in the scaffold a suitable drug delivery device (Fujioka et al., 1998; J.E. Lee et al., 2004).

Apart from its limited capacity to control drug release, the main limitations of collagen scaffolds are their poor mechanical properties. Indeed, collagen scaffolds show no rigidity and shrink due to the cohesive forces that appear as ECM is deposited. As with fibrin, this shrinking is observed already in vitro, and clearly show the intrinsic limitations of this material to reconstruct tissue constructs with defined shapes: ultimately, one of the main objectives of scaffold implantation. Besides, this lack of mechanical competence might

compromise the integrity of the scaffold upon implantation resulting in implant failure, non-union or even ectopic tissue formation. Again, it needs to be considered that for most skeletal tissue engineering applications, scaffolds will be exposed to considerable mechanical stress. The mechanical properties of collagen scaffolds can be improved by the integration of a reinforcing substructure (Zhou et al., 2011) or using composites of collagen with mechanically competent materials (Lee et al., 2004). Apart from this limitation, collagen scaffolds might lead to some minor immunological problems upon implantation if the telopeptides are not correctly removed from the materials by proteolysis (S. Mizuno & Glowacki, 1996).

Due to their many intrinsic advantages, collagen scaffolds have been applied to the regeneration of all skeletal tissues. Zhang et al. studied chitosan/collagen scaffolds bioactivated with an adenovirus carrying a BMP-7 transgene and seeded with periodontal ligament cells in dental implant defects (Y. Zhang et al., 2007). Histomorphological and qRT-PCR analysis were consistent with more bone formation and higher expression of bone-matrix markers (alkaline phosphatase, osteopontin, bone sialoprotein). Xu et al. studied collagen type-I scaffolds integrating bone sialoprotein for their capacity to induce osteoblast differentiation, and found that this treatment resulted in early mineral deposition by proliferating repair-cells in calvarial defects. Pabburke et al. implanted collagen-based constructs seeded with MSCs in sheep meniscal disks, and confirmed by histomorphometric analysis that they improve integration compared to the scaffolds alone and the control (Pabbruwe et al., 2009; Pabbruwe et al., 2010). Zhou et al. showed that substructure-reinforced collagen matrixes seeded with chondrocytes supported neocartilage formation (Zhou et al., 2011). Chen et al. tested the potential of genipin-crosslinked collagen type-II scaffolds seeded with MSCs for cartilage repair in an osteochondral defect and observed that after 2 months, there were clear signs of cartilage formation in the defect (W.C. Chen et al., 2011). After 24 weeks, cartilage in the defect presented the same structure as native cartilage. A recent study has confirmed the efficacy of knitted silk-collagen sponge scaffolds as support for embryonic stem cell-derived MSCs in tendon regeneration (J.L. Chen et al., 2010).

Being one of the first and most widely used materials studied for tissue engineering, collagen has already been investigated in several clinical studies (Rodkey et al., 1999; Stone et al., 1997) that have ultimately resulted in collagen regulatory approval. Intense clinical research continues, as new indications for collagen-based scaffold technologies are sought and long-term clinical outcomes are reevaluated. In a recent study, Crawford et al. studied the safety and healing potential of commercial cartilage matrix seeded with autologous chondrocytes (NeoCart) in the human knee (Crawford et al., 2009). The study revealed significantly reduced pain in the treatment group, with neocartilage formation that was well integrated. Welsch et al. compared cartilage repair in the femoral condyle after autologous chondrocyte transplantation in a hyaluronan-based scaffold or a collagen-based scaffold (Welsch et al., 2010). Results based on morphological scores and T2-mapping showed that most parameters were similar for both scaffolds, however, collagen showed significantly better constitution of the surface and higher T2-relaxation times.

2.3 Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear polysaccharides consisting of repeating hexuronic acid bonded to a hexosamine (Iozzo, 2000). GAGs are usually sulfated and linked to a protein domain (a structure that is named proteoglycans). Hyaluronan is exceptional in this regard as it is the only kind of GAG that is neither modified by sulfonation nor attached

covalently to proteins. Nevertheless, hyaluronan does attach by non-covalent interactions to other molecules of the ECM and to cells. Other important GAGs are dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate and keratan sulfate. GAGs have important physiological functions as "space fillers", in shock absorption, in preventing coagulation, and in GF binding. Studies have also shown the importance of GAGs in embryogenesis and tissue regeneration (Fedarko et al., 1992; Toole, 2001). However, the complete functionality of GAGs is still a topic requiring further investigation.

Unmodified GAGs tend to form polymer solutions or gels that dissolve slowly due to the high molecular weight of the components. This dissolution of the matrices together with their poor mechanical properties are unsuitable for tissue engineering. Therefore, GAGs are most often not used by themselves to form scaffolds, but rather as composites (Garcia-Fuentes et al., 2008; Min et al., 2010; Moss et al., 2010; S.J. Park et al., 2009; Schagemann et al., 2010). This role is even more prominent for heparin and heparan sulfate, which are typically added in very small proportions as GF-complexing agents in drug delivery formulations (Biondi et al., 2008; N.X. Wang & von Recum, 2011). Alternatively, GAGs can be chemically modified as a more insoluble compound (e.g. Hyaff®, Fidia Farmaceutici s.p.a) or as crosslinked networks (Crescenzi et al., 2003; Zheng Shu et al., 2004). In skeletal tissue engineering studies, GAG-containing scaffolds have been seeded with osteoblast-like cells (Wagner et al., 2007), chondrocytes (Nuernberger et al., 2011; Schagemann et al., 2010), MSCs (J.L Chen et al., 2010; Min et al., 2010; Schwartz et al., 2011), and fibroblasts (Irie et al., 2011; Sawaguchi et al., 2010). Hyaluronan interacts specifically with cells through CD44, RHAMM and ICAM-1 (Tammi et al., 2002; Toole, 2004). These interactions trigger signaling cascades that could result in the observed capacity of hyaluronan to instruct tissue neo-formation and regeneration. For illustration, hyaluronan has shown pro-proliferative properties (Zou et al., 2004), capacity to enhance unspecific ECM deposition (Garcia-Fuentes et al., 2009), to induce cartilage (Allemann et al., 2001; Williams et al., 2003; Yamane et al., 2005) and ligament (Cristino et al., 2005) formation, capacity to maintain embryonic stem cells in undifferentiated state (Gerecht et al., 2007), and capacity to modulate inflammatory and catabolic markers (Grigolo et al., 2005; Homandberg et al., 2004). Hyaluronan biological properties, though, seem to be very dependent on its molecular weight, and the presence of hyaluronan oligosaccharides have been connected to chondrocyte-driven chondrolysis (Knudson et al., 2000).

GAG-based or GAG-modified scaffolds have also been applied with GFs for sustained delivery. Again, this mimics a physiological process, as sulfated GAGs are GF reservoirs in the ECM. For most GFs, GAG binding occurs through specific non-covalent interactions between a so-called heparin binding domain in the polypeptide and the sulfated GAG (Iozzo, 2000). Modification of drug delivery systems with sulfated GAGs for specific binding of GFs is a usual technological strategy, commonly practiced as a surface modification (i.e. "surface heparinization") (Biondi et al., 2008; N.X. Wang & von Recum, 2011). An alternative but still rather unexplored strategy is the design of delivery systems based on crosslinked sulfated GAGs for GF-delivery applications. Drug delivery devices modified with sulfated GAGs have been applied to the delivery of TGF-β1 (Chou et al., 2006; Lee et al., 2004) and FGF-2 (Mi et al., 2006) among others. Heparin and other sulfated GAGs known to interact with GFs can enhance markedly the affinity of these molecules for the drug delivery system and change the release profile of the device, as reviewed before (N.X. Wang & von Recum, 2011). In general terms, when sulfated GAGs are integrated in the composition of a

hydrogel, they show high affinity for the GF and result in sustained release profiles (Benoit & Anseth, 2005b). In the work of Cai et al. (Cai et al., 2005), FGF-2 was released from several crosslinked GAG-based hydrogels in a sustained way for over a month. Moreover, it was found that hydrogels presenting immobilized heparin further enhanced the retention of FGF-2. Interestingly, the bioactivity of the released GF was confirmed both in the study of Benoit et al. (Benoit & Anseth, 2005b) and in that from Cai et al. (Cai et al., 2005).

GAG-based materials, particularly hyaluronan, have been extensively investigated for skeletal tissue regeneration. Patterson et al. prepared hyaluronan hydrogels with different degradation rates by photo-crosslinking to control the release of BMP-2 in a rat calvarial defect model (Patterson et al., 2010). They showed that tissue regeneration could be achieved with any of the BMP-2 loaded hydrogels tested. However, the fastest and the slowest degrading hydrogels resulted in more organized bone structures. Kang et al. investigated the possibility of coating porous polyester scaffolds with a fibrin/hyaluronan matrix loaded with BMP-2, and the effect of such constructs in MSCs (Kang et al., 2011). The results showed that MSCs were differentiated more efficiently and resulted in better bone formation when BMP-2 was sustainably released from the fibrin/hyaluronan constructs as compared to BMP-2 in solution. Chen et al. showed that polyester scaffolds coated with hyaluronan/methylated collagen were able to enhance bone formation by MSCs as compared to non-coated polyester systems (M. Chen, et al., 2010). In the study published by Irie et al., chitosan/hyaluronan fibers seeded with fibroblasts were tested for rabbit medial collateral ligament reconstruction (Irie et al., 2011). The in vivo test indicated that ligaments treated with cell-seeded scaffolds showed enhanced collagen type-I deposition and mechanical strength as compared to non cell-seeded ones.

Cartilage and osteochondral structures have been the main focus of attention for GAG-based scaffolds, as GAGs are particularly important components of cartilage ECM. Tan et al. showed the potential of new oxidized hyaluronan/N-succinyl chitosan systems that are able to gel in situ for the delivery of chondrocytes (Tan et al., 2009). Eggelet et al. tested a cell-free polyester/hyaluronan scaffold to cover microfractured full-thickness articular cartilage defects in sheep, and achieved significant improvements in histological structure, and in collagen type-II content as detected by immunohistochemistry (Erggelet et al., 2009). Im et al. designed a hyaluronan-atelocollagen/hydroxyapatite-tricalcium phosphate composite scaffold for testing in osteochondral defects in minipigs (Im et al., 2010). Clinical scoring, histological and mechanical results showed comparable results for this composite scaffold with or without seeded chondrocytes and for defects refilled with the removed osteochondral pieces. Moreover, all these groups showed significantly higher performance than the negative control (empty defects), but comparable in many aspects to native cartilage. In the studies of Moss et al. and Huang et al., GAG-based scaffolds were used for reconstitution of the nucleus pulposus of the intervertebral disc (Huang et al., 2011; Moss et al., 2010). The results of these studies sustained the possibility to maintain disc height and induce intervertebral disc regeneration upon implantation of these scaffolds. Interestingly, the study of Moss et al. compared hyaluronan scaffolds with a composite of hyaluronan and an elastin-based peptide (Moss et al., 2010). Although the composites presented better mechanical properties, this improvement did not translate in enhanced regeneration.

Nehrer et al. and Welsch et al. have published clinical evaluations of hyaluronan scaffolds seeded with chonodrocytes for cartilage regeneration (Nehrer et al., 2009; Welsch et al., 2010). The study by Nehrer supported the interest of Hyalograft C for cartilage regeneration in healthy young patients with isolated cartilage defects, but not for salvage procedures

(Nehrer et al., 2009). Similarly positive results were obtained by Welsch et al. for condyle regeneration, although this study showed some slight advantages of collagen-based scaffolds compared to the hyaluronan-based ones.

2.4 Reprocessed tissue scaffolds

From the point of view of biomimetism, reprocessed tissue scaffolds (e.g. demineralized bone matrix and decellularized tissues) represent probably the most advanced scaffolds available. These natural scaffolds recapitulate not only the complex composition, but also the structure of the native tissues (Danti et al., 2007; Traphagen & Yelick, 2009). This cannot be achieved with the scaffolds prepared from extracted ECM compounds such as those presented before, because the processing steps used to extract the biopolymers completely erases the morphology, histological topology, the precise interactions between different components, the molecular alignment, and other subtle cues from the materials. While both demineralized bone matrix (DBM) and decellularized tissue can be considered similar concepts ("reprocessed tissue scaffolds"), their processing methods and characteristics differ and are discussed separately herein.

Demineralized bone matrix is prepared by acid extraction of allograft bone (Urist, 1965). It results in the elimination of the major part of the mineral phase and the immunogenic components of bone, but retains collagen that provides a structured osteoconductive scaffold and a soluble protein fraction comprising several GFs, BMPs among them (Dinopoulos & Giannoudis, 2006; Reddi, 1998). It is also usual to retain a very low fraction of mineral bone phase after the demineralization step (2% approx.). The exact composition of DBM -including the GF content- will depend heavily on the exact protocol followed for mineral extraction, and thus, DBM from different commercial sources might show considerable variability. Extensive studies have shown that DBM is osteoinductive and osteoconductive (Dahners & Jacobs, 1985; Martin Jr et al., 1999). Osteoinduction mechanism by DBM seems to be very dependent on the environment. DBM implanted in submuscular/subcutaneous tissue undergoes ossification through a process recapitulating several steps from the endochondral bone formation process. DBM implanted in calvarial defects direct MSC differentiation to osteoblasts, similarly to the process followed in intramembranous ossification (J. Wang & Glimcher, 1999a, 1999b; J. Wang et al., 2000). DBM by itself is presented in the form of a powder, but is usually mixed with other suitable materials (e.g. hyaluronan, poloxamer) to form a paste that can be easily casted at the bone defect site (Dinopoulos & Giannoudis, 2006; Juang Ming Yee et al., 2003). Mixing DBM with other materials, however, changes the product from a regulatory perspective.

DBM can be supplemented with suitable cell types as a strategy to promote tissue regeneration. For instance, some articles report on seeding chondroblasts in DBM as a potential alternative in cartilage tissue engineering (Jin et al., 2006; Z.H. Wang et al., 2010a). A large number of studies have also investigated MSC-seeded DBM (e.g. see Gurevitch et al., 2003; Liu et al., 2010a). DBM can also be supplemented with additional GFs in an attempt to boost their regenerative capacity. DBM has been mainly loaded with BMPs (Lammens et al., 2009) –and particularly, with BMP-2- for bone tissue engineering (Lammens et al., 2009; H. Lin et al., 2008). DBM has also been loaded with other GFs such as TGF-β1 (Moxham et al., 2009). Overall, DBM presents very little capacity to bind additional GFs, and therefore is not an ideal choice as a sustained delivery system. Modification, for instance by heparin addition, can increase GF binding affinity. First DBMs were introduced

to the clinics during the 1990's, and there are currently more than 20 commercial products available based on this technology. Therefore, there is currently large clinical evidence supporting their efficacy for several orthopedic conditions (Irinakis, 2011; Y.K. Kim et al., 2010; Topuz et al., 2009).

Decellularized tissue scaffolds are prepared by cell lysis, induced by physical and/or chemical treatments, followed by cell component removal treatments. Mostly, chemical methods are used for this last step (Gilbert et al., 2006). Similarly to DBM, decellularized tissues present the biomimetic topology, tissue-conductive capacity and regeneration-inductive properties arising from the presence of physiological amounts of GFs in the ECM. However, these beneficial characteristics are heavily dependent on the processing protocol followed for decellularization (Gilbert et al., 2006; Ingram et al., 2007; Woods & Gratzer, 2005). Moreover, this protocol might also influence other critical parameters such as the decellularization efficacy and the complete removal of chemical agents from the natural scaffold. Although decellularized tissues and organs are being investigated in several tissue engineering applications, they have not been so popular for skeletal tissue engineering, as scientist have focused on these technologies for engineering high-complexity, localized organs such as the heart (Ott et al., 2008). That is except for DBM, which can be considered also a decellularized tissue scaffold. Some articles have supported the concept of decellularized cartilage as a chondroconductive matrix in cartilage tissue engineering (Elder et al., 2010; Gong et al., 2011; Hou et al., 2011; Secretan et al., 2010; Stabile et al., 2010). Indeed, in vitro tests have shown the possibility to form cartilage tissue in vitro by chondrocyte seeding on a decellularized tissue scaffold (Gong et al., 2011). This ability to support cartilage formation translates to advanced in vivo models such as a for larynx reconstruction in a rabbit model (Hou et al., 2011), or for meniscus reconstruction in sheep (Stabile et al., 2010). First proof-of-concept of the therapeutic interest of this concept in humans is available through the successful transplantation of an MSC-seeded decellularized airway in a 30-years old patient with bronchomalacia (Macchiarini et al., 2008). Decellularized tissues have also been applied to ligament and tendon tissue engineering in vitro, with successful results (Abousleiman et al., 2009; Deeken et al., 2011; Ingram et al., 2007; MacLean & Gratzer, 2011; Woods & Gratzer, 2005). Further animal experiments and clinical studies will be necessary to validate the potential of this strategy. Similarly, other studies have indicated the possibility to use decellularized tissue scaffolds also for skeletal muscle repair and adipose-tissue engineering (L. Flynn et al., 2007; L.E. Flynn, 2010; Gillies et al., 2010; Merritt et al., 2010).

3. Strategies for scaffold bioactivation

As compared to nature's derived biopolymers, synthetic materials for tissue engineering offer important advantages such as more reproducible manufacture, tailored mechanical and biodegradation properties, and diverse processing options. With a few exceptions (e.g. titanium) synthetic materials do not interact favorably with the body, rendering them incapable of actively promoting tissue regeneration. Despite this limitation, synthetic biomaterials can be engineered to promote their capacity to cross-talk with relevant cell populations. Indeed, bioactive scaffolds can also be prepared from synthetic materials by physical adsorption or chemical immobilization of biomolecules or oligopeptides on the scaffold surface, or by physical entrapment of bioactive molecules alone or incorporated in a

drug delivery system into the scaffold. These strategies can also be applied to enhance the bioactivity of scaffolds made from ECM-native materials such as those covered in the previous section.

3.1 Scaffolds as drug delivery systems

Engineered tissues need not only to fill a defect and to integrate into a host tissue, but they also need to meet the demands of a constantly changing - through growth or adaptation - tissue. It was hypothesized that those tissues capable of growing with time could be engineered by supplying growth stimulus signals to cells from the biomaterial used for cell transplantation (Alsberg et al., 2002). Smart drug delivery systems might not only be able to transmit one single signal to the cells, but multiple signals in concert in a timely controlled release pattern. This release may be controlled through properties of the drug delivery system itself: examples of those are biodegradation-controlled release devices or stimuli-sensitive systems (polymeric networks that react to changes in pH, temperature or to the presence of enzymes). In a further degree of sophistication, drug release can also be triggered through external physical stimuli such as ultrasounds or electrical fields (Deckers et al., 2008; Sirivisoot et al., 2011). Another method of remote drug delivery relies on magnetic nanoparticles and thermally sensitive vesicles (de Cogan et al., 2011; Mart et al., 2009a; Mart et al., 2009b). In these devices, heat originating from magnetic stimulation of nanoparticles induces swelling of the themosensitive polymer and the subsequent drug release.

In tissue engineering, polymeric matrices used as scaffolds can double as drug delivery systems, a strategy that has been mainly used for soluble signalling molecules such as GFs (e.g. BMPs or TGF-β). GFs and cytokines have shown to be potent inducers of cell migration, proliferation, differentiation and new tissue formation. Many of them are also important morphogens in embryogenesis and natural wound healing through their action on stem cells. Therefore, there are large expectations set on GF therapeutic efficacy, particularly in combination with stem-cell-based therapies. Besides, many researchers believe that through the chemoattractant properties of these molecules, drug delivery devices will be able to mobilize the resident stem cell pool from the injured host, making reiterative any strategy based on stem cell supplementation. Cell recruitment and migration to the site of injury may be promoted through various signaling molecules. Many of these factors, e.g. TGF-βs, BMPs and IGF-1, are not only involved in cell attraction but also affect cell proliferation and differentiation (Lieberman et al., 2002; Reddi, 2001a; Reddi, 2001b; Sundelacruz & Kaplan, 2009).

Growth factors and cytokines – although very potent – usually present short half-lives, especially in physiological media. Also, their potential is highly dependent on their spatial and temporal action pattern, and a change in local concentration may have a huge effect (Uebersax et al., 2009). It follows that systemic administration of GFs is inherently inefficient, since it results in fast GF elimination and indiscriminate biodistribution. Ultimately, the therapeutic concentration of the GF may never be reached at the site of injury. To overcome this, drug delivery strategies are designed to (i) provide a platform for the localized delivery of the GF at the site of implantation creating a favorable concentration gradient, (ii) to protect the bioactivity of the molecule and (iii) to provide a controlled release pattern of the drug over a desired time frame. To add a further layer complexity, in developmental pathways, different factors become active at different times, and GF release

profiles that recapitulate these dynamics are likely to provide more leverage over cell behavior than those that apply these signals indiscriminately (Place et al., 2009). This is a central dilemma in tissue regeneration: on one hand, biomimetic platforms integrating complex release behavior are required for an optimal therapeutic outcome, but on the other hand, engineering needs to be kept to a reasonable level to provide technologically-feasible medical solutions (see section 4.2).

Two approaches have been mainly used for scaffold bioactivation: GFs can be encapsulated in a selected drug delivery system such as a microsphere or nanoparticle formulation, and these can be incorporated into the scaffolds. Alternatively, GFs might be incorporated directly into the scaffold itself (Holland & Mikos, 2006; Holland et al., 2007; Liu et al., 2010b; Luginbuehl et al., 2004; Shi et al., 2011; Uebersax et al., 2009). For example, IGF-1 has been incorporated into biodegradable poly(lactide-co-glycolide) microspheres and used as a treatment for 10-mm segmental tibial defects in sheep (Meinel et al., 2003). The application of 100 µg IGF-1 per defect resulted in the downregulation of inflammatory marker genes at the site of injury and the induction of new bone formation that bridged the defect within 8 weeks. Uebersax et al. explored the alternative approach, where IGF-1 was directly incorporated into porous 3D silk fibroin scaffolds (Uebersax et al., 2008). Silk scaffolds incorporating IGF-1 were able to preserve GF bioactivity, and prompted chondrogenic stimuli to seeded MSCs in vitro. By definition, implantation of GF-loaded scaffolds results in the localized delivery of the signaling molecule. Still, a certain fraction of the incorporated drug can reach the lymphatics or the circulation and distribute to non-target tissues. Therefore, even for these localized therapies, potential adverse effects of GF need to be carefully monitored. For example IGF-1 has been shown to be involved in diabetes or in the development of colorectal and breast cancers (Dunger et al., 2005; Putney & Burke, 1998; Sandhu et al., 2002).

Controlled or sustained release patterns may also be achieved through non-covalent association with matrix components, for example, with GAGs (section 2.3) (L. Zhang et al., 2006). In vivo, GAGs have critical roles in the regulation of GF activity. This includes sequestering GFs from the tissue liquid phase, acting as GF reservoir, preventing their degradation and presenting them to cell-surface receptors (Place et al., 2009). Regiospecific sulfation patterns that enable specific interactions between GFs and GAGs have been introduced in other biomaterials that are not natively from the ECM. This modification resulted in specific GF-scaffold bindings. For example, this has been done in alginate hydrogels and in silk fibroin scaffolds that have been decorated with sulfonated moieties capable of influencing the release of GFs such as FGF-2 (Freeman et al., 2008; Wenk et al., 2010).

Release upon cell demand is mainly based on protease-sensitive polymer modifications. For this strategy, peptide sequences that can be cleaved by proteases are introduced to hydrogel crosslinking groups, and the GF is loaded in this hydrogel. In the absence of proteases, the small mesh size of the hydrogels prevents the GF from being released. In their presence, proteolytic activity cleaves the crosslinks, increases the mesh size of the hydrogels resulting in the GF diffusion out of the polymeric mesh. This is again a biomimetic concept, since physiologically, the ECM not only provides structural and biochemical cues for cells in contact, but can also promote cell invasion and remodeling upon cell-triggered proteolysis, a process that ultimately results in tissue regeneration. Lutolf et al. used synthetic poly(ethylene glycol) (PEG) hydrogels containing crosslinking matrix metalloproteinase substrates that can undergo cell-mediated proteolytic degradation (Lutolf et al., 2003a). Upon degradation of the

matrix, entrapped BMP-2 was released and bone regeneration was achieved within 5 weeks in critical size cranial defects in rats (Lutolf et al., 2003b). This effect was highly reduced when BMP-2 was entrapped into matrix metalloproteinase-insensitive hydrogels.

All drug release strategies mentioned above may also be combined resulting in even more complex strategies. For example Haberstroh et al. showed that human intervertebral disc-derived nucleus pulposus cell migration can be promoted through a serum fraction gradient in a concentration dependent manner and that both TGF-β3 and hyaluronan were able to promote cell differentiation and matrix generation (Haberstroh et al., 2009). They concluded that a bioactive scaffold containing serum and TGF-β3 or hyaluronan might be an excellent candidate for cell-free biological treatment strategies in preventive and curative approaches for degenerative disc disease.

There are other complex drug delivery strategies for tissue engineering that have not been covered in this manuscript. For example cells can be used as drug delivery systems. Especially immunocytes that are mobile and can migrate across impermeable barriers can be exploited as trojan horses for drug delivery (Batrakova et al., 2011). Applied cells may also be genetically modified in order to support immunomodulating, homing or paracrine activities at the implant (Myers et al., 2010). Besides, scaffolds can also be used for gene delivery, together with gene nanocarriers or by themselves. This last concept is usually referred as "gene activated matrices" and has attracted some attention for their use for skeletal tissue regeneration (Geiger et al., 2005; Guo et al., 2006). Besides this alternative methodologies based on genetically engineered cells and gene therapy, it is important to bear in mind that not only physiological factors can influence cell differentiation and tissue regeneration. Synthetic small molecular drugs such as dexamethasone, β-glycerophosphate or ions such as strontium released from bioactive glasses have also been shown to influence cell proliferation and differentiation (Chung et al., 1992; Isaac et al., 2011). Indeed, the potential of small molecular weight drugs for tissue engineering has been dramatically demonstrated by their capacity to markedly enhance cell-reprogramming efficiency, and even substitute many of the transgenes used in these processes (Huangfu et al., 2008).

3.2 Scaffolds bound to biological functionalities

In section 2, we highlighted the capacity of purified ECM compound scaffolds to provide sites for cell attachment and cell-substrate crosstalk (Fig.1). These scaffolds exert bioactivity because they bear complex information, coded in their physical and chemical structures. Unfortunately, scaffolds fully made out of natural ECM molecules present several limitations related to their difficult purification and processing and their suboptimal mechanical properties (Place et al., 2009). These limitations have broadened the range of scaffold materials investigated towards synthetic scaffolds that are functionalized with bioactive moieties.

Most cell receptors do not require interacting with the whole ligand for their activation. Often, a relatively short peptide fragment of about 40 amino acids is sufficient to provide the desired signal to the cells. Indeed, it has been shown that small peptides can activate specific cellular pathways: binding their respective receptors, triggering signal transduction and leading to appropriate cells responses (Chung & T.G. Park, 2007; Place et al., 2009). The required peptide sequences can either be obtained from biological sources or can be chemically synthesized (Sreejalekshmi & Nair, 2011). As these small fragments are easier to produce synthetically and usually show quick refolding under physiological conditions,

they might represent a more cost-effective, easier to manipulate, alternative to GFs or other signaling proteins. Moreover, they have been shown to have a higher stability against conformational change, easy controllability of surface density and orientation, and more favorable ligand-receptor interaction (Chung & T.G. Park, 2007; Hersel et al., 2003; Lutolf et al., 2003b; Ruoslahti, 1996). Sreejalekshmi et al. has recently reviewed peptide-modified scaffolds, a manuscript that includes a decision-tree-type flow chart indicating probable cellular outcomes resulting from a given modification (Sreejalekshmi & Nair, 2011).

Cell attraction
e.g. concentration dependent

Cell attachment
+ migration

Cell differentiation
+ ECM production

cell adhesion molecules
ECM binding sites
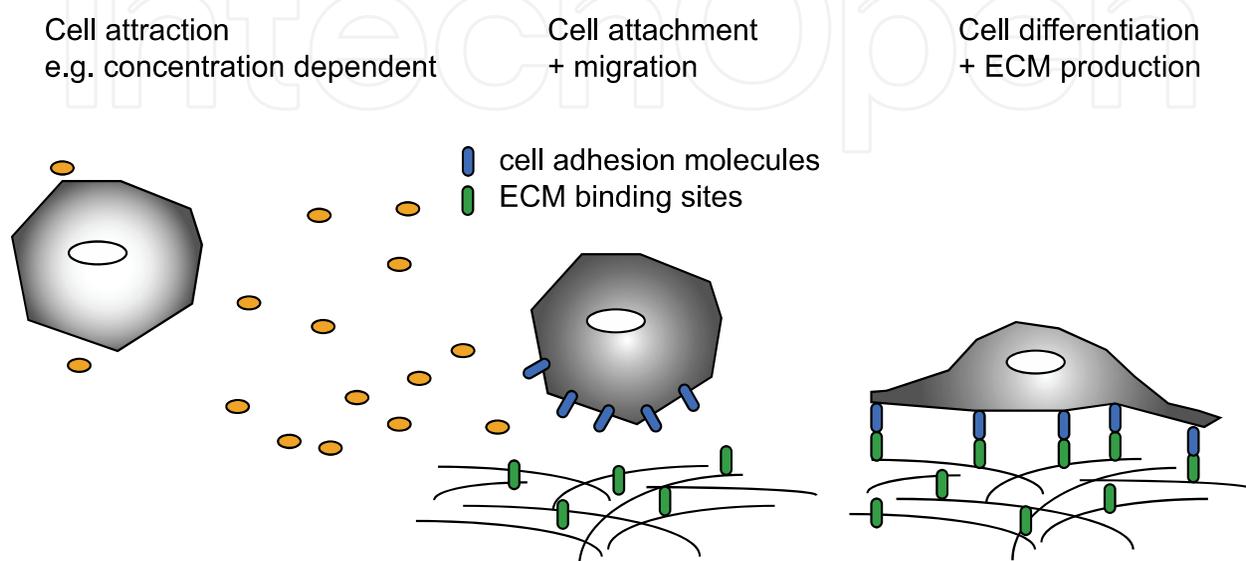
Fig. 1. Scaffolds as devices capable to activate the host stem cell pool. Growth factors activate the stem cell pool and induce their migration to the scaffold. Stem cells adhere to the scaffold by integrin binding and undergo GF/scaffold-directed differentiation

3.2.1 Fibronectin and small cell adhesion motifs

Cell adhesion is a prerequisite to the success of scaffold-based tissue engineering strategies. In native tissues, fibronectin is one essential component of the ECM that mediates cell-matrix interaction. Cells can bind to fibronectin through transmembrane receptor proteins of the integrin family, which mechanically interlink the actin cytoskeleton to the ECM through an elaborate adhesion complex. The binding process between ECM and the dimeric integrin receptors on the cells surface is then followed by a cascade of signaling events leading to the up- or downregulation of the expression of several genes. It is important to note that the binding of integrins happens through dimerization of different alpha and beta subunits, and therefore, association with diverse ligands can lead to different effects in the cell. Some specific functions of biopolymers can be attributed to small functional domains, and these may be incorporated in synthetic analogs in place of the full protein (Dunehoo et al., 2006; Schense et al., 2000; Silva et al., 2004). Probably the best-known sequence binding to integrins is arginine-glycine-aspartic acid (RGD). It is found in many ECM proteins, including fibronectin, laminin, collagen type-IV, tenascin and thrombospondin (Benoit & Anseth, 2005a; Comisar et al., 2007; Underwood et al., 1995). However, the RGD motif and its derivatives are not the only integrin-binding sequence used for scaffold modification. For example, polyethylene terephthalate surfaces modified with the cell adhesion motif

GRGDSPC showed improved differentiation of pre-osteoblastic cells as compared to non-grafted surfaces (Zouani et al., 2010). Alginate hydrogels modified with G_4RGDY were able to support the formation of growth-plate-like structures in a co-culture of mouse osteoblasts and chondrocytes (Alsberg et al., 2002). In order to promote cell attachment, an adhesion motif can be simply mixed with the scaffold material or coated on the scaffold surface. Syndecan-binding peptides such as AG73 (RKRLQVQLSIRT) have shown enhanced integrin-mediated biological activities in scaffolds made out of collagen, laminin-11 and fibronectin (Yamada et al., 2011). Morphological analysis indicated that these effects were, at least partially, mediated by enhanced cellular attachment and cell spreading. Attia et al. have shown that polyurethane scaffolds containing an anionic dihydroxy oligomer coated with fibronectin promoted not just cell attachment, but also cell alignment parallel to the scaffold fibers (Attia et al., 2010). This featured scaffold topology was able to increase collagen production after seeding with annulus fibrosus cells. Genetically engineered biopolymers provide another method for the integration of bioactive sequences. Girotti et al. engineered an ECM-analogue of a recombinant multi(bio)functional elastin-like protein polymer with integrated REDV cell adhesion sequences for tissue engineering purposes (Girotti et al., 2004). Cells are known to be very sensitive to the mechanical properties of the substrate (Engler et al., 2006), but also to directional mechanical forces transduced through the ECM (Kurpinski et al., 2006). In skeletal tissues, where mechanical stresses are so important and even necessary for cell stimulation, the integration of adhesion motifs capable of transducing mechanical forces into the right cell signals is of critical importance (Fig. 2).
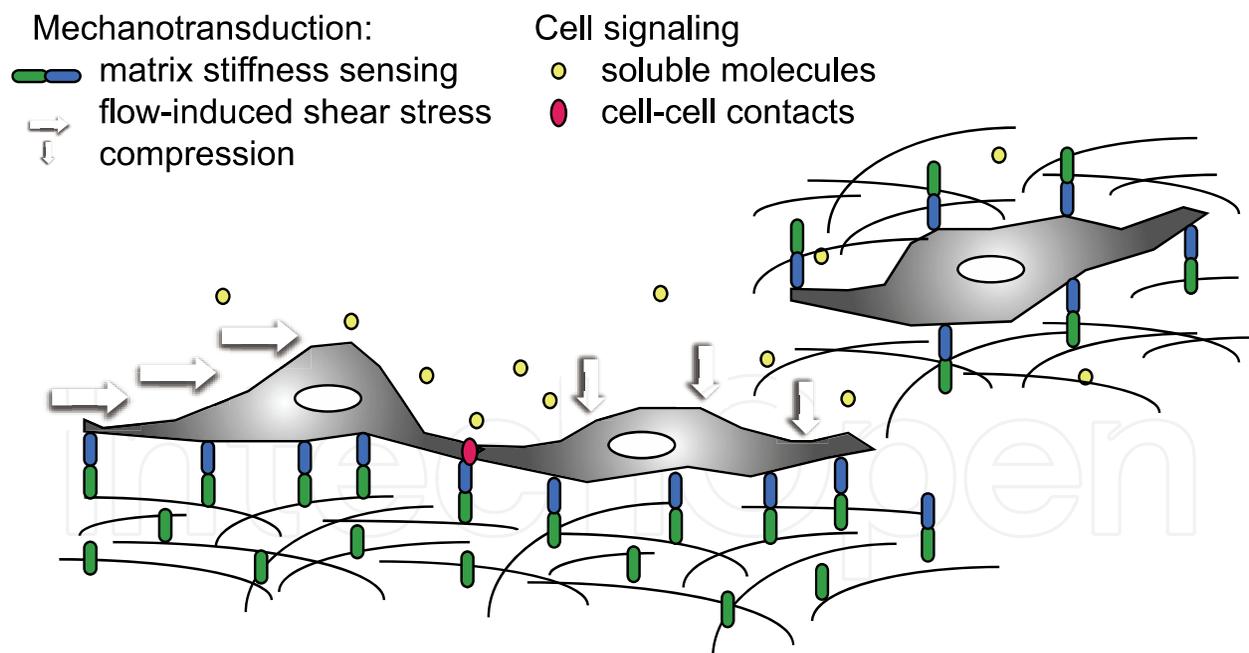

Fig. 2. Cell differentiation and extracellular matrix deposition can be affected by many different signals, e.g. mechanotransduction or cell signaling

3.2.2 Growth factors
Scaffolds may not only be modified with bioactive sequences, whole GFs have been shown to retain their bioactivity upon conjugation to scaffold materials. Through covalent

immobilization on the scaffold surface in either random or specific orientation many disadvantages of controlled GF delivery can be circumvented. However, in order to transmit the desired signal to the cells, the moiety must be accessible. This requires the GF to be exposed on the scaffold surface and not incorporated in the polymer core. The application of polymeric spacers such as PEG can provide higher biological activity due to reduced interaction between the GF and the scaffold. For example, collagen matrices were covalently modified with horseradish peroxidase as a model protein with or without PEG as a linker. Introduction of PEG resulted in higher long-term stability of the enzyme (Chen et al., 2002). Recombinant TGF-β2 was either covalently bound through a PEG spacer or admixed to injectable bovine dermal fibrillar collagen (Bentz et al., 1998). PEG-TGF-β2 showed a significantly higher in vitro and in vivo bioactivity than admixed TGF-β2. The authors concluded that covalent binding of TGF-β2 by their method allowed to preserve the GF's biological activity in vitro, and even potentiated their response in vivo. Karageorgiou et al. have shown that immobilization of BMP-2 on a silk fibroin surface was more efficient in terms of osteogenic differentiation of MSCs in vitro than delivery of the GFs in solution (Karageorgiou et al., 2004). In a follow-up study, they could show that with the same protocol, porous 3D scaffolds modified with BMP-2 implanted into rat critical size femoral defects improved bone formation similarly to the same scaffolds seeded with either undifferentiated MSCs or with MSCs predifferentiated for 4 weeks in vitro before implantation (Kirker-Head et al., 2007). Clearly, the protocol used for biofunctionalization is a critical step for these technologies. De Mel et al. have recently published a review on biofunctionalization of biomaterials for accelerated in situ endothelialization (de Mel et al., 2008).

Not only complete GFs can be attached to scaffold surfaces, but also oligopeptides such as those derived from BMP-2 or BMP-7 that have been immobilized on alginate hydrogels and glass, respectively (Kirkwood et al., 2003; Saito et al., 2003; Suzuki et al., 2000). These in vitro studies, performed with a murine multipotent MSC culture or with primary rat calvarial osteoblastic cell populations showed that immobilized oligopeptides may have the capacity to induce osteoblastic differentiation and mineralization in a more predictable manner than the entire GF. This might be attributed to the lower complexity of the molecule and therefore increased stability. Implantation of a BMP-2 derived oligopeptide covalently coupled to alginate into the calf muscle of rats resulted in ectopic bone formation (Suzuki et al., 2000). Synthetic peptide mimics have also been applied for the promotion of angiogenic responses (X. Lin et al., 2006): F2A4-K-NS is a synthetic mimic of FGF-2 that has been shown to trigger signal transduction as monitored by the stimulation of ERK1/2 phosphorylation in human umbilical cord endothelial cells. In cell-based assays, it increased cell migration, cell proliferation and gelatinase secretion. When used in similar quantities, F2A4-K-NS achieved comparable response endpoints to those associated with FGF-2 stimulation.

As mentioned before, wound healing and tissue repair might be stimulated by GF release, but optimal effects are only achieved when several chemical signals are provided in the necessary order and at precise times during the regeneration process. This is probably one of the main disadvantages of GF conjugation to scaffolds, as there is no easy way to provide a time-dependent switch to activate/deactivate the provided signaling.

3.2.3 Other binding moieties: cleavage sequences and oligosaccharide domains

Incorporation of cleavage sequences is an interesting concept to provide on-demand GF delivery (see 3.1), and also to control cell invasion (Place et al., 2009). In this concept,

cleavage sequences engineered in multidomain peptides are integrated in the polymeric network of the scaffold. This function mimics the multilayered bioactivity of the ECM, where enzymatic remodeling can liberate "cryptic sites" contained within the ECM proteins. A nice example was provided by Lutolf et al., who rendered a synthetic PEG hydrogel network as amenable to both proteolytic degradation and cell invasion through the integration of integrin binding sites and substrates for matrix metalloproteases (MMP) (Lutolf et al., 2003a). Primary human fibroblasts were demonstrated to proteolytically invade these networks, a process that depended on MMP substrate activity, adhesion ligand concentration and network crosslinking density.

While most ligands that have been tested for tissue engineering so far were proteins, many scientists are turning to other biomolecules of high biological functionality: polysaccharides. Indeed, small oligosaccharide domains are involved in many cell-cell and cell-substrate interactions (Brown et al., 2008; Scaglione et al., 2010). Recent developments in understanding the role of natural polysaccharides and improved chemical synthesis of defined oligosaccharides will potentially result in the discovery several new targets of interest in skeletal tissue engineering.

4. Key challenges

4.1 Engineering large tissue constructs – mechanistic issues

Since the first works showing the possibility to instruct cells to aggregate and form 3D-tissue constructs, tissue engineers have debated between forming tissues and organs de novo or just boosting the body's intrinsic capacity for self-repair. Although in the long run the first of these concepts will probably result in technologies and therapeutic approaches of high medical interests, nowadays the second approach has gathered all the big success stories from a clinical perspective (Place et al., 2009). Devices that enhance tissue regeneration take advantage of environmental cues present at the wound site (i.e. progenitor cells, GFs and tissue ECM present at the wound borders) and accelerate the repair process by establishing a conductive matrix and by providing supplementary simulative factors. These factors can coordinate cell chemokinesis, cell differentiation and induction of tissue-specific ECM formation by committed cells. Many of these technologies in the form of bioactivated scaffolds and drug delivery devices are already a reality (Infuse™, Medtronic, Inc.) and others will become part of the orthopaedist's arsenal in the next few years. A second generation of these technologies could integrate stem cells as a potential opportunity to promote further regeneration. However, it is still unclear whether additional stem cell transplantation results in enhanced regeneration in biomimetic scaffolds (Im et al., 2010). Devices driven by this concept of enhanced regeneration will most likely not be applicable to wounds above a certain size. In those cases, it is easy to argue that regeneration will be limited by nutrient and oxygen diffusion, as well as by the diffusion/migration of pro-regenerative factors (i.e. GFs, cells) (Muschler et al., 2004).

Tissue engineers study in vitro tissue formation as a potential way to generate tissues that could be later implanted in larger defects. While this concept presents several practical disadvantages compared to the most direct approach described above, it is possible that it could result in well-structured tissues that could ultimately be combined in the form of larger constructs. Still, this larger constructs will be deprived of oxygen once formed and implanted, making it critical to induce the quick vascularization of the implanted tissue. In this approach, enhanced tissue construct survival could be achieved by co-implantation of

drug delivery devices capable of releasing proangiogenic GFs (Richardson et al., 2001) or by transplanting cells genetically modified to enhance their survival under hypoxic conditions (R.P. Ahmed et al., 2010). Still, conventional in vitro tissue engineering strategies will be unsuitable for very large tissue construct or for regenerating complex tissues/organs.

Some very recent work indicated that through the use of relevant progenitor cells and sophisticated bioreactors, complex and even vascularized tissues might be formed in vitro (Tsigkou et al., 2010). Additionally, other studies have shown that even small organs can be engineered in vitro by culturing cells in reprocessed tissue scaffolds (i.e. decellularized tissues) (Ott et al., 2008). While these advances are really impressive, we must bear in mind that the mechanisms used for tissue/organ neogenesis in vitro are far closer to those involved in tissue regeneration than in physiological organogenesis. In vitro tissue formation occurs by the penetration of committed cell populations into a scaffold, the formation of cell-cell and cell-scaffold interactions and the formation of new tissue. That would also be, simplified, the sequence of events leading to tissue remodeling after cell colonization of bloods clots during wound regeneration. On the other hand, organ morphogenesis is a highly orchestrated process occurring during embryogenesis where the tissues are formed by a sequence of very defined events in a growing construct. In morphogenesis, cells undergo sequential differentiation steps under a very defined spatial and temporal regulation. This regulation is usually defined by gradients of morphogens, but also by cell polarity occurring from integrin-mediated cell-cell interactions (Krasnow, 1997). Further studies will define if in vitro strategies based on tissue regeneration mechanisms such as those tested by Ott et al. and Tsigkou et al. can result in functional, clinically useful tissues and organs.

In any case, knowing which tissue formation mechanism you are trying to recapitulate is important when designing a tissue-engineering strategy. For example, this is pertinent to bone engineering, where most strategies try to induce MSC differentiation to osteoblasts despite of the fact that this process is only physiological in intramembranous bone regeneration. For long bones, the physiological growth and regeneration process is through endochondral ossification. Endochondral ossification is a multistep process where cartilage is initially formed at the defect site, the cartilage becomes hyperthropic, and then it is remodeled into bone by osteoclasts and osteoblasts. Some steps of endochondral bone formation are recapitulated in DBM implanted subcutaneously (J. Wang & Glimcher, 1999a), and this process has also been studied as an advanced tissue engineering strategy in some recent works (Oliveira et al., 2010).

4.2 Engineering a complex environment

Tissue regeneration and tissue neoformation are very complex phenomena. Indeed, the cascade of events after a lesion is formed comprises blood clot formation, inflammation, formation of scar tissue and tissue remodeling. Every one of these processes requires a finely orchestrated cascade of cellular and molecular events. As an illustration, Gerstenfeld et al. have investigated the levels of GFs, other pro-inflammatory cytokines and MMPs occurring during bone regeneration after a traumatic injury (Gerstenfeld et al., 2003), and their studies show the involvement of a myriad of different molecules, present only at specific stages in the process. This complexity is not as surprising: GFs are known to have synergistic effect for many applications as they trigger coordinated effects in their effector cells. Moreover, it is known that all GF effects are very context-specific and concentration-dependent, and therefore, changes in the target cells induced by one GF could drastically change the effect of

a second GF (Massagué, 2000). This results in the necessity to design a very complex environment if we want to recreate all this cues in biomimetic scaffolds.

But is this complexity compatible with realistic engineering solutions for tissue regeneration? If something we have learnt from the decline and rise of the tissue engineering field it is the necessity to look for realistic objectives and to avoid overengineered technologies (Place et al., 2009). In short, technologies that are now a success are those that have kept concepts simple. Dealing with the problem of engineering acceptably simple technologies to manipulate a complex process requires a practical approach. First, it is necessary to study how sophisticated your system needs to be for each specific application. For many applications, just the addition of a good tissue-conductive material might suffice. For other applications, this material could be bioactivated with an inducer or seeded with relevant cell populations. Optimization of these strategies could probably rely on high-throughput analysis and systems biology to identify the main signals required for inducing tissue regeneration (Anderson et al., 2004; Anderson et al., 2005; Langer & Tirrell, 2004). For more complex challenges, engineers could turn to reprocessed tissue scaffolds that might be capable of producing some complex tissue structures. Addressing the challenges with technologies that integrate the level of complexity needed to meet the demands of the medical problem addresses complexity from a management perspective. From a technical perspective, many research groups are working in technical solutions that allow implementing complex signaling in the scaffolds with relatively simple chemistries. Examples highlighted in this chapter on the use of small synthetic peptides (Sreejalekshmi & Nair, 2011) or molecules (Ding et al., 2003) and on the formation of material composites including biomimetic compounds might be just some of these simple technologies.

4.3 Controlling stem cell fate and individual variability

New tissue engineering strategies can benefit enormously from the concomitant use of stem cells. However, to fulfill the potential of these technologies, improved control over stem cell fate needs to be exerted, particularly in some applications. For instance, some scaffolds have been designed to maintain stem cells in an undifferentiated state for several passages, thus enhancing their capacity for renovation and cell population growth. Differentiation of stem cells can be achieved by their exposition to defined media. This approach is very efficient for some skeletal tissues (e.g. bone), but leads to suboptimal results for others: hyaline cartilage, skeletal muscles and tendon (Brent et al., 2005; Buckwalter & Brown, 2004). When designing tissue engineering strategies for these applications it is important to consider whether environmental cues from the host tissue might help to improve the differentiation process. Indeed, some experiments have shown tissue repair upon implantation of stem cells despite the questionable ability of these cells to differentiate to these tissues. In cases with modest stem cell contribution to tissue regeneration, bioactive scaffold materials can help to direct stem cell differentiation by introducing important environmental cues: nanotopology (Yim & Leong, 2005), mechanical properties of the supporting scaffold (Engler et al., 2006), or chemical moieties (Anderson et al., 2004). In extremely difficult situations, directed cell differentiation might be addressed by gene therapy approaches (Selvaraj et al., 2010). Controlling stem cell differentiation is critical not only to engineer tissues matching the structure, composition and properties characteristics of native ones, but also to prevent undesirable processes such as orthotopic tissue formation, scar formation and even, potential tumor induction (Amariglio et al., 2009).

Reliance on stem cell therapies raises also the topic of individual variability (Odorico et al., 2001). Stem cells collected from different donors are known to respond very differently to inductive stimuli, to present different capacities for GF secretion and ultimately, to have extremely different regenerative capacities. When designing tissue engineering devices based on stem cells, it would be critical to have quick tests ensuring a minimum of activity, similarly to the approach taken with conventional drugs. However, this concern does not only apply to tissue engineering products containing stem cells, but also to scaffolds and drug delivery devices intended for conduction and induction of a stem cell-mediated responses. A case in point would be the elder or diseased population, where resident stem cell pools might be partially depleted and their full pro-regenerative capacity compromised. The efficacy of tissue engineering devices will need to be tested in these populations, and specific recommendations be issued to take into account this potential variability.

Tissue engineered products based on in vitro cultured stem cells face another important limitation related to their necessary culture time. In vitro, tissues need to be cultured for several weeks and in many cases, this time is not an option for a therapeutic intervention. Even in those cases where this time is available, the postponed surgery will result in an uncomfortable situation for the patient, in increased medical and social costs and in potential worsening of the medical condition. Medical technologies that can be applied immediately out-of-the-box are therefore preferred, and bioengineers should consider this important limitation and the competing therapeutic options when deciding for these technologies.

4.4 Technical challenges

Although encapsulation and controlled release of macromolecules has now been studied for several decades, they are difficult technologies to get right due to the easy denaturation of the drug, to the difficulties to predict release in vivo, and even to potential patient-specific issues that could change the release kinetics of the system. This problem translates to GF-release devices, where potential denaturation of the drug combines with the added concern of their very high manufacturing costs. Growth factor denaturation can occur at three steps during their integration in a drug delivery device: (i) during encapsulation, (ii) upon storage and (iii) upon implantation in the release phase. In practical terms degradation during storage (ii) is infrequent and only applies to formulations in aqueous phases, or with materials that could cross-react with the GFs. For most solid formulations where the matrix is stable for long periods of time, this process should not be a concern. GF degradation during encapsulation (i) is usually a concern, and in most cases, can only be minimized and not completely avoided (Sah, 1999). To prevent GF degradation, exposure to high temperatures should be avoided, and high shearing forces and solvent/water interfaces minimized as possible. In any case, it is possible to enhance GF stability by adding other proteins and protecting groups that block the solvent/water interface. This works particularly well with GFs because of the low loading required that could lead to a high disproportion between the protecting protein/compound and the GF. Growth factor denaturation during the release phase (iii) is a concern for many polymeric systems whose degradation byproducts might react with the proteins. This is best illustrated by polyesters, probably the most used materials for sustained release of GFs. When implanted in the body, polyesters are known to hydrate and start their decomposition, which can occur at the surface but also at the inner matrix (Rezwan et al., 2006; Zhu et al., 2000). As the polyester

discompose in the core of the matrix forming acidic oligomers, these remain trapped in the polymeric network and induce an acidic microclimate that can degrade the GF. This general mechanism applies similarly to polymers where the degradation products can react with or just degrade the GFs. For GF delivery, it is probably best to directly select materials that do not result in reactive degradation products, or that undergo only surface-erosion (e.g. polyanhydrides) (Tabata et al., 1993). However, further protection of GFs in polyesters can be achieved by incorporating buffering agents (Zhu et al., 2000). Interestingly, some polyester composites have shown capacity to protect macromolecules both during encapsulation and release (Csaba et al., 2005), and these technologies have already been applied for GF-delivery (d'Angelo et al., 2010). In any case, designing devices capable of providing GF release for several months and in a bioactive form continues to be a challenging objective.

Another strategy to achieve sustained exposure of cells to GFs or other signaling molecules is covalent tethering to the scaffold. Covalent tethering of proteins presents the difficulty of unselective binding of the protein to the polymer. Indeed, although some more selective reactions have been designed (i.e. cysteine based linking) (Backer et al., 2006), most protocols use reactions where protein can be bound through several aminoacids of the peptidic chain. This lack of specificity could result in a potential bioactivity loss if a critical aminoacid region is modified with the polymer. However, in general terms good bioactivity has been observed with immobilized GFs (Bentz et al., 1998). On the other hand, lack of specificity when linking biomolecules to synthetic materials raises another concern, linked to a difficult regulatory pathway with materials where no clear molecular structure can be defined.

5. Concluding remarks

We have gone a long way in the field of tissue engineering from the first discoveries pointing to the conductive properties of tissue scaffolds. The experience from reprocessed tissues (DBM, for example) and other ECM-containing products soon pointed to the importance of a biomimetic approach in scaffold design. In the last years, biopolymer-derived and bioactivated synthetic scaffolds have been thoroughly studied and GF delivery integrated in many devices. With a good share of hype and bumps on the way, we have now several tissue engineering products available on the market, most for therapeutic indications related to skeletal tissue engineering, and some having been transformed in great commercial successes (e.g. Infuse™ from Medtronic, Inc.). Tissue engineering potential, however, does not need to be limited to regeneration in relatively simple injury models. Recent studies showed the possibility to engineer body parts integrating several tissues (e.g. ostochondral grafts, vascularized bone) or even small organs. Whether these studies could translate into products of clinical interest is still uncertain, but worth exploring. Probably further optimization of the biomimetic scaffolds together with improved bioreactors will be necessary to realize these challenging goals. Another technology to be expected commercially in the next few years are biomaterials integrating stem cells. For those applications, scaffolds integrating functionalities capable of instructing stem cell proliferation, differentiation and tissue formation will be required, and this concept should be a top priority in tissue engineering, bioregenerative medicine and in the government's scientific agendas.

6. Acronyms used

BMP: bone morphogenic protein
DBM: demineralized bone matrix
ECM: extracellular matrix
EGF: epidermal growth factor
FGF: fibroblast growth factor
GF: growth factor
IGF: insulin-like growth factor
MMP: matrix metalloprotease
MSC: mesenchymal stem cell
PDGF: platelet-derived growth factor
PEG: poly(ethylene glycol)
qRT-PCR: quantitative reverse transcription polymerase chain reaction
TGF: transforming growth factor
VEGF: vascular endothelial growth factor

7. Acknoledgments

8. References

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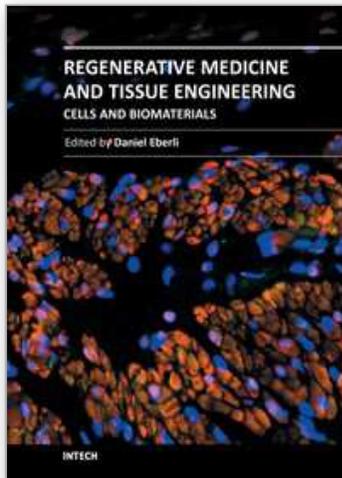
Regenerative Medicine and Tissue Engineering - Cells and Biomaterials

Edited by Prof. Daniel Eberli

ISBN 978-953-307-663-8

Hard cover, 588 pages

Publisher InTech

Published online 29, August, 2011

Published in print edition August, 2011

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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