Chapter 5

Cartilage and Bone Regeneration – How close are we to bedside?

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LIST OF ABBREVIATIONS

ASCs Adipose-derived stem cells
ACs Articular chondrocytes
ACI Autologous Chondrocyte Implantation
AMIC Autologous matrix-induced chondrogenesis
BM Bone marrow
BMPs Bone morphogenic proteins
CRD Cartilage Repair Device
CaP Calcium phosphates
ECM Extracellular matrix
FDA Food and Drug Administration
hBMSCs Human bone marrow stromal cells
HAp Hydroxyapatite
MACI Matrix-induced Autologous Chondrocyte Implantation
MSC Mesenchymal stem cell
NCs Neuroectoderm-derived nasal chondrocytes
OA Osteoarthritis
OC Osteochondral
OCD Osteochondral Defect
OP Osteoporosis
PEG Poly ethylene glycol
PLGA Poly lactide-co-glycolide
PCL Poly ε-caprolactone
PGA Polyglycolic acid (or polyglycolide)
PLA Polylactic acid (or polylactide)

RFE Radio Frequency Energy
SF Silk fibroin
TGF-β Transforming growth factor-β
β-TCP β-tricalcium phosphate

Abstract

The treatment/regeneration of bone and cartilage diseases or defects, whether induced by rheumatism, joint dysplasia, trauma, or surgery presents great challenges that have not been fully solved by the current therapies. In the last few years, tissue engineering and regenerative medicine have been proposing advanced tools and technologies for bone and cartilage tissue regeneration, and some of which have successfully reached the market. Beyond the source of cells, the creation of superior structures for replacing defective bone and cartilage requires strong research in biomechanical signaling and synthesis of advanced biomaterials to mimic human tissues at the most varied levels. Natural and synthetic polymers, bioresorbable inorganic materials, and composites have been investigated for its potential as scaffolding materials with enhanced mechanical and biological properties. Porous scaffolds, hydrogels, and fibers are the most commonly biomimetic structures used for bone and cartilage tissue engineering. Herein, the concepts and current treatment strategies for bone and cartilage repair, as well as biomimetic strategies for bone and cartilage tissue engineering are overviewed. A global review of the ongoing clinical trials and of the scaffolds commercially available for the repair of osteochondral tissue is also presented.

Keywords: Bilayer; Tissue Engineering; Biodegradable Materials; Bone; Cartilage; Cell therapy; Ceramics; Composites; Inorganic Materials; Osteochondral; Polymers; Regenerative Medicine; Scaffold; Stem Cells.

5.1 INTRODUCTION

Osteoarthritis (OA) and Osteoporosis (OP) are among the most disabling degenerative diseases that may lead to severe complications affecting the neuromuscular system thus significantly impairing patients' quality of life (1, 2). OA is the highest-ranking disease among the musculoskeletal diseases and contributes to approximately 50% of the disease burden in this group (3). Current clinical treatments for OA and OP involve non-steroidal anti-inflammatory drug administration and surgery such as osteotomy, abrasion arthroplasty, microfracture, and autologous and allogeneic cartilage tissue grafts, and autologous chondrocytes (4). These treatments are well established and effective for reducing the patients' pain, but are not able to

completely restore the patient's mobility. Therefore, the demand for new therapeutic options for complete healing of bone, cartilage and octeochondral defects (OCDs) is significant. Bone and cartilage diseases or defects are directly related with joint degeneration. Such disorders can be caused by rheumatism, joint dysplasia and/or trauma and are particularly prevalent in countries with high life expectation. Articular cartilage damage can arise as a consequence of both acute and repetitive trauma resulting in pain, effusion and/or mechanical symptoms, affecting directly the individuals life style, as work, hobbies and daily tasks (5). Cartilage lesions in joints can have different degrees; superficial lesions, as fissures or cracks are classified as grade 1. A grade 2 abnormality is defined when cartilage is affected up to 50% of its thickness while grade 3 lesions are characterized by defects in which more than 50% of the cartilage thickness, down to the subchondral bone but without bone penetration, is affected. The final grade is the commonly termed OCD (grade 4) that results from the cartilage damage with penetration into the subchondral bone.

Although articular cartilage comprises just one type of cells, chondrocytes become less active with age and injury. Furthermore, the avascular nature of cartilage together with the declining function of chondrocytes leads to the inability of full-thickness defects to heal spontaneously. If untreated, these lesions can progress to more-serious degenerative joint conditions. Tissue engineering is a multidisciplinary field of research that employs principles of chemistry, biology, and engineering sciences towards growth, development and regeneration of damaged tissues or organs (6). It can involve the use of scaffolds combined with cells and suitable biochemical signals to design and create of-the-shelf organs and tissues substitutes. Despite the promise of tissue engineering, a better understanding of the composition, structure, and properties of bone and cartilage, can guide scientists to achieve the adequate tissue engineered grafts to ideally repair and regenerate bone and cartilage tissues. Bone and cartilage have a three-dimensional architecture with several levels of organization comprising micro- and nano-structures (Figure 5.1) (7). Cancellous bone is a porous structure, whereas cortical bone is composed of osteons that consists of a concentric series of layers (lamellae) of mineralized collagen Type I matrix. Articular cartilage presents a stratified architecture, which consists of three zones where in the superficial zone collagen Type II fibres are oriented tangentially to the articular surface, in the transitional zone have no predominant orientation and become aligned perpendicularly to the calcified cartilage, anchored in subchondral bone in the deep zone. Articular cartilage has poor intrinsic ability for healing due to its isolation from vessels and nerve supply. On the other hand, bone is a vascular and innervated tissue. While bone is mineralized, normal cartilage tissue is not mineralized and is a highly hydrated tissue.

(Insert Figure 1)

Herein, cartilage and bone physiology and disorders are discussed in terms of currently applied treatments. Their limitations and the potential solutions proposed within tissue engineering and regenerative medicine field are presented. Applied or tested materials, cells, bioactive molecules and tissue engineered techniques are briefly described. To address the question 'How close are we to bedside?', the ongoing clinical trials and the related products that have already reach the market for osteochondral (OC) tissue regeneration are also reviewed.

5.2 CONCEPTS AND TREATMENT STRATEGIES

In order to understand what sort of bone and cartilage tissue engineering strategies could be best for repair/reconstruct defects, it is important first to recognize the structure, concepts and current therapeutic approaches targeting the different bone and cartilage lesions.

5.2.1 Bone

Bone is a complex, highly organized and specialized connective tissue with many functions. All bones have a mechanical function providing attachment to various muscle groups. In addition, in some parts of the body, bones provide a protective function to vital structures - skull (brain), ribs (lungs, heart) and pelvis (bladder, pelvic viscera). Some bones retain their hematopoietic function in adults - vertebrae, iliac crests, proximal parts of femur and humerus (3, 4). All bones serve as a reservoir of calcium and actively participate in the calcium homeostasis in the body.

Bone is composed of cortical (compact) (80%) and trabecular (cancellous or spongy) (20%) tissues. Cortical bone tissue forms the outer shell, or cortex, of the bone and has a dense structure with a porosity of about 5-10% (3-5). It is the primary component of the long bones of the arm and leg and other bones, where its greater strength and rigidity are needed. Trabecular bone tissue typically occupies the interior region of bones and is composed of thin plates, or trabeculae, in a loose mesh structure with porosity of 50-90%. It is highly vascularized and frequently encloses the bone marrow (BM) with high proportions of mesenchymal and hematopoietic stem cells. Trabecular bone tissue has a higher surface area but is less dense and stiff, and weaker than cortical bone.

Bone has the ability to remodel, by altering its size, shape and structure to meet the mechanical demands placed on it. Bone remodeling is a dynamic, lifelong process in which resorption is followed by formation, respectively involving the activity of osteoclasts and osteoblasts (6). During bone formation, also called osteogenesis, pre-osteogenic cells are stimulated to migrate through a provisional matrix, which in a therapeutic/regenerative approach could be represented by bone-graft substitutes or a blood clot (7). The migrating cells then start a differentiation process that results in the secretion of the new bone matrix.

Bone defects are often associated to a disease state (e.g. OA, OP, osteomyelitis, and osteogenesis imperfect) and trauma related injuries resulting from primary tumor resection and orthopedic

surgeries (e.g. total joint arthroplasty and implant fixation). In addition, spinal fractures, called vertebral compression fractures, are the most common fracture in patients with OP, affecting nearly 700,000 people each year, typically postmenopausal women. However, others fractures like fractures of the hip, wrist, and proximal humerus are commonly observed in patients with OP (8).

Treatments used by orthopedic surgeons for the reconstruction and repair of bone defects and fractures are mainly internal fixation and bone allografts and autografts (9). Allografting involves the transplant of tissue from one individual to another with a different genotype but of the same species, carrying the risk of immunomediated rejection or transmission of infectious diseases. By its turn, autograft is a piece of tissue that is transplanted from one part of the body to another in the same individual. This procedure ensures the long-term survival of graft and subsequent successful reconstruction due to intrinsic features such as osteoconductivity and, histocompatibity. Autografting is thus considered the clinical gold standard method (8, 9). However, a number of complications including infection, vascular injuries, chronic donor site pain and morbidity have been reported with the using autografts (10).

Other approaches, such as vascularised fibula autograft, Ilizarov bone transfer technique, and Masquelet technique, where autologous bone grafting alone is not recommended due to the risk of resorption, have been used particularly for long bone defects reconstruction (11-13). Nevertheless, these techniques are mainly limited to cancer patients which have OP and suffer from impaired wound healing.

5.2.2 Cartilage

Articular (hyaline) cartilage regeneration is a priority of orthopedic care because the clinical need is expanding with the aging population (mainly in developed countries). Articular cartilage enables the joints to tolerate shearing forces and absorb shock and loads up to 20 times the body's weight. As health care is evolving, people live longer and population ages. Moreover societies are increasingly more dynamic, competitive and physically more demanding. With time articular cartilage increasingly bears more prolonged and cumulative skeletal stresses and shearing forces, increasing the potential to the development of degenerative diseases of cartilage as OA. Worldwide estimates indicate that 9.6% of men and 18% of women $\geq$ 60 years have symptomatic OA last decade (2). Joint surface defects are ubiquitous, with reported prevalence of arthritis of about 31% in knees, 17% in hip and 7% in hands (10). In respect to prevalence of joint pain, 38% is incident in knees, 18% in the shoulders, 14% in hands and hip and 16% in lower back.

While long-term research goals for cartilage regeneration focus on harnessing stem cell therapies alone or in combination with biodegradable materials, in the near term orthopedists choose from multiple treatment strategies to manage cartilage injuries. When injuries occur fracturing or damaging the tibia or knee, and the patient wants to continue to practice their normal lifestyle (or his physical activity in the case of sportsmen for example) is usually indicated surgical

intervention (11). That type of surgery can be performed by arthroscopy, and at least from the standpoint of controlling pain, arthroscopic surgery has as main advantage a significant reduction of postoperative pain (12). Literature describes that the minor the peripheral tissue damage, the lower the nociceptive stimulus at the surgery site, which will be crucial for the patient to have less pain after surgery (13). Procedures such as the suprapatellar approach for nail insertion are seen as options to avoid late postoperative knee pain (14). This minimally invasive approach uses an easy entry point, promoting lesions only in the Hoffa's body, which is usually removed during arthroscopy because it can be inflamed or damaged and to better visualize the knee also (15).

Currently the methods for the treatment of cartilage defects (e.g. OA) include the insertion/transplantation of OC tissue, cells, scaffolds or growth factors (GFs), alone or in combination, or even the use of radio frequency energy (RFE) methods (16). Procedures that are normally used for treatment of small articular cartilage defects include also RFE, chondrocyte implantation and BM stimulation techniques as drilling, debridement or microfracture. In the case of large defects, scaffolds and mosaicplasty are commonly used in the treatment (Figure 5.2) (17). Although current methods for articular cartilage defects are promising, no treatment has resulted in complete regeneration of the hyaline cartilage and the subchondral bone.

(Insert Figure 2)

Drilling procedure aims to pierce the underlying subchondral bone, thereby inducing bleeding at the defect site and allowing the formation of a blood clot which contains BM mesenchymal stem cells (MSCs) that will differentiate helping in cartilage regeneration (18). Drilling was described by Pridie and Gordon (19) and it is known to cause thermal necrosis of the subchondral bone, as well as to result in an uneven repair surface and for these reasons is not a favored method of treatment (18, 20). Debridement is the simple excision of the damaged cartilage and has been shown to improve symptoms for five years or more (21). Opinion is divided as to whether arthroscopic debridement has any place in the treatment of established degenerative disorders, as OA, although this debate does not apply to the treatment of localized symptomatic chondral defects.

Microfracture, which is based on marrow-stimulation that creates fibrocartilage (as drilling and debridement) at the site of the procedure, with varied amounts of collagen Types I, II and III (20), was introduced by Steadman et al (22) 20 years ago, and is one of the most used methods for cartilage repair. This type of cartilage is less durable, less resilient and less able to withstand shearing forces than native articular cartilage, composed mainly by collagen Type II (Table 5.1). While this approach can have good results in smaller lesions, clinical studies reflect the lack of durability over a long-term follow-up. By its turn, treatment of OCD in the foot has also been

considered a great challenge due to the different biomechanical features as interestingly reported elsewhere (23).

TABLE 5.1 Types of collagen and associated genes and cartilages phenotypes outcomes			
Type	**Outcome**	**Gene(s)**	**Disorders**
I	Fibrocartilage	COL1A1, COL1A2	Osteogenesis imperfecta
II	Hyaline cartilage, makes up 50% of all cartilage protein	COL2A1	Collagenopathy, Types II and XI
X	Hypertrophic and mineralizing cartilage	COL10A1	Schmid metaphyseal dysplasia
XI	Cartilage	COL11A1, COL11A2	Collagenopathy, Types II and XI

Mosaicplasty, or OC cylinder transplantation, was first described in 1993 (24). In this procedure, OC plugs are taken with a cylindrical cutting device and used to fill an articular cartilage defect. Advantages of this technique are on one hand the immediate filling of the deffects with mature, hyaline articular cartilage and on the other the simultaneous treatment of both chondral and OCDs. However, donor site morbidity is a concern and Hangody and Fules (25) recommend the limiting of the area to be treated to 1 to 4 cm^2. There are also technical difficulties in restoring the surfaces of both cartilage and bone to produce a smooth, convex joint surface. The thickness of the donor cartilage may differ from that of the area to be treated and the reconstitution of the important subchondral layer may not occur. In addition, lateral integration rarely occurs (26) raising the concern that synovial fluid may penetrate through the subchondral layer possibly causing cyst formation.

Autologous chondrocyte implantation (ACI) can result in more hyaline-like cartilage within the treated defect. The technique of ACI was first performed by Peterson et al (27) in Gothenburg in 1987 and it was the first application of cell engineering in orthopedic surgery. In ACI, healthy cartilage cells are harvested, cultured and then re-implanted into the defect under a patch, in a second-stage surgery. Brittberg et al (28) presented the results of 23 patients with a mean follow-up of 39 months. Good or excellent clinical results were reported in 70% of cases (88% of femoral condylar defects). Of the biopsies from treated femoral condylar lesions, 11 of 15 had a

hyaline-like appearance. A more recent publication from the same group showed durable results up to 11 years following the treatment of OC lesions (29).

Current drawbacks to this procedure are hypertrophy of the patch that can lead to further surgery and unreliable biological potential of the re-implanted cartilage cells. Furthermore, histological analysis apparently show that ACI is capable of producing hyaline-like tissue in some specimens, however the best repaired tissue is not morphologically or histochemically identical to normal hyaline cartilage, and fibrocartilage can be found frequently.

A variation of the ACI technique using culture-expanded BM stromal cells has the advantage of not requiring an additional arthroscopic procedure in order to harvest articular cartilage (30).

All of these techniques encounter limited success due to issues which include fibrocartilage formation, chondrocyte de-differentiation, and lack of tissue integration and mechanical support.

5.3 BIOMATERIALS FOR BONE AND CARTILAGE REGENERATION

The rationale of using biomaterials as scaffolds in tissue regeneration is to obtain a temporary three-dimensional structure for the *in vitro* growth of living cells and its subsequent implantation into the lesion area followed by its biodegradation as newly tissue is being formed. Several natural and synthetic polymers, bioactive inorganic materials, and their combinations have been employed for bone and cartilage tissue engineering and regeneration. Polymers have great stiffness and mechanical strength, and natural polymers add advantages such as their resemblance with the extracellular matrix (ECM), specific degradation rates due to the susceptibility to the action of enzymes, and improved recognition by the living body. Bioactive inorganic materials, such as calcium phosphates (CaP) and bioactive glasses, have good biocompatibility, osteoconductivity, and bioresorbability. Despite, they present poor mechanical properties that hinder its use in load-bearing applications. The combination of these different types of materials result in composite structures with significantly enhanced mechanical and biological properties for bone tissue engineering. In opposition, cartilage tissue is engineered using natural/synthetic polymers.

The most promising polymers, inorganic materials and composites and their properties are briefly described as follows.

4.3.1 Polymers

Natural polymers, also known as biopolymers, have been extensively used owing their ability to interact with cells and to be susceptible to enzymatic degradation providing space for tissue ingrowth (31). Naturally occurring polymers most widely explores for bone and cartilage repair/regeneration are: i) proteins (e.g. silk fibroin (SF), collagen and gelatin); ii) polysaccharides (e.g. chitosan, alginate, gellan gum and derivatives); and iii) glycosaminoglycans (e.g. hyaluronic acid) (31).

In comparison to biopolymers, synthetic polymers have several advantages; excellent processing characteristics, excellent mechanical and physical properties (e.g. elastic modulus, strength, and degradation rates), and bioresorbability (32). However, many of these polymers present several disadvantages, such as the possibility of causing persistent inflammatory reactions and not being capable to integrate with host tissues (33). The most used polymers are polyglycolic acid (or polyglycolide - PGA), polylactic acid (or polylactide - PLA), poly lactide-co-glycolide (PLGA), poly (D,L- lactic acid) (PDLLA), poly ethylene glycol (PEG), and poly ε-caprolactone (PCL). These polymers have received special attention since they can be self-reinforced to gain better strength properties (34).

4.3.2 Bioactive inorganic materials

Inorganic materials often used for bone repair and regeneration are CaP, namely hydroxyapatite ($Ca_5(PO_4)_3OH$, HAp) and β-tricalcium phosphate ($Ca_3(PO_4)_2$, β-TCP), bioactive glasses, and glass-ceramics owing their bioactivity, biocompatibility and osteoconductivity (35, 36).

HAp is crystalline and is the most stable and least soluble CaP in an aqueous solution down to a pH of 4.2 (36). Aqueous precipitation (37, 38), hydrothermal synthesis (39), solid-state reaction using calcium oxide, calcium hydroxide or calcium carbonate, and hydrolysis of other CaP, have been methods used to prepare HAp. The detailed information on HAp synthesis and preparation is well established (40). β-TCP is a high temperature phase of CaP, which can only be obtained by its thermal decomposition at temperatures above 800ºC. β-TCP is biodegradable and has been extensively used as bone substitute, either as granules or blocks, or even in CaP bone cements (41). The resorption capability of HAp and β-TCP is different though their similarity in terms of chemical composition. It is believed that HAp has a slow resorption rate (1 to 2% per year) and may be integrated into the regenerated bone tissue, while β-TCP is completely reabsorbed (42, 43). Therefore, clinical applications have been performed by combining HAp with β-TCP, which forms the biphasic CaP, improving the bioresorbability and strength of the bone substitutes (35, 40, 44). Nevertheless, these materials are limited to non-load bearing applications due to their poor mechanical properties.

Bioactive glasses and glass-ceramics have been used in bone regeneration due to their capability to react with physiological fluids thus bonding with bone through the formation of HAp layers at the implant interface thus stimulating bone growth (45, 46). These type of materials are osteogenetic and osteoconductive, while CaP exhibit only osteoconductive properties (41). It has also been found that reactions on bioactive glass surfaces release concentrations of Si, Ca, P and Na ions, thus inducing intracellular and extracellular responses (47). They are also able to improve osteoblast adhesion, vascularization *in vivo*, enzymatic activity, and differentiation of MSCs (48). In addition, glasses have shown great potential as reinforcing materials since they fully degrade in aqueous media (49, 50). Bioactive glasses are brittle materials; this limitation can be solved by the development of glass-ceramics or by the combination with an additional phase as a polymer, forming a composite (51, 52). There are different compositions of bioactive

glasses, based on silicate, phosphate, and borate, which can be obtained through melt-quenching (53) and sol-gel process (54). The most widely investigated bioactive glass for biomedical applications is the silicate-based glass designated 45S5, also known by its commercial name Bioglass® (55). These types of glasses have higher chemical durability and durability limits as compared to others bioactive glasses (56). By its turn, phosphate-based glasses have unique dissolution properties in aqueous fluids, while borate-based bioactive glasses have faster degradation rates and are able to completely convert into apatite (57, 58).

Bioactive inorganic materials can be doped with trace elements (e.g. strontium, zinc, magnesium, manganese, silicon), which can influence bone health and enhance biocompatibility, while strengthening the mechanical properties of the implants (59). Besides, minerals and traces of metal elements may provide physicochemical modifications in the produced materials, which can accelerate bone formation and resorption *in vivo* (60, 61).

4.3.3 Composites

Composite materials embracing a natural/synthetic polymeric matrix and bioactive inorganic materials, as fillers, appeared as a strategy to mimic the human bone, which is a three-dimensional composite composed of organic, inorganic and cellular phases, strictly assembled to form the natural bone tissue. Composites are the combination of two or more materials, with different compositions and properties, resulting in a single structure with significantly improved mechanical and biological properties. Special interest has been attributed to nanocomposites for bone tissue engineering and regeneration due to the nanosized features of the fillers which can intensely improve the tissue bonding capacity of the polymeric matrices, that the individual materials cannot attain thus allowing the production of better biomaterials (7). The nanoparticles have large surface area when compared to the conventional micro-sized fillers, thus offering improved mechanical properties, while maintaining the osteoconductivity and biocompatibility of the fillers, as well as, cell adhesion and differentiation (62).

Many combinations of polymers and inorganic materials have been proposed for the production of nanocomposites which final properties will be dependent. As aforementioned, the most common polymers used are of natural origin (collagen, gelatin, silk, chitosan, alginate, hyaluronic acid, and gellan gum) (63-69). By its turn, some synthetic polymers (e.g. PEG, PLA, PGA, PLGA, and PCL) have been also used and applied in the clinics. On the other side, nanosized fillers include nanoparticles of CaPs and bioactive glasses, carbon nanotubes, nanofibers, and nanoplatelets. These nanoparticles have been prepared through different processes, namely wet chemical precipitation (38), sol-gel synthesis (70), hydrothermal synthesis (71), mechanochemical synthesis (72), microwave processing (73), spray drying methods (74), and electrospinning (75), while nanocomposites have been prepared by simple mechanical mixing or co-precipitation.

Further details on nanomaterials processing techniques and applications in bone tissue regeneration can be found elsewhere (76).

4.4 BONE AND CARTILAGE TISSUE ENGINEERING

Biomimetic strategies to develop new bone and cartilage tissue engineered constructs rely on bioactive structures able to mimic the natural tissue ECM in order to promote cell adhesion, migration, growth and matrix deposition forming a tissue-like substitute. These structures embrace three-dimensional porous and fibrous scaffolds, and hydrogels, with specific design, controlled degradation rate, mechanical properties, and porosity for efficient gases, nutrients, and regulatory factors transport.

4.4.1 Bone tissue engineering

Bone tissue engineering focuses on alternative treatment strategies to reduce the shortcomings of the current clinical treatments (i.e. infection, vascular injuries, immune rejection, chronic donor site pain and morbidity) by using the combination of materials science, engineering principles, and cell biology. Hence, the fabrication of composite constructs hierarchically structured from nano- to macro-size ranges inspired by the nature of bone, has been followed (77).

Conventional technologies, such as foam replica method (78), solvent casting and particulate-leaching (79), freeze-drying (80), phase separation (81), and gas foaming (82), often inexpensive, simple and flexible to optimize physico-chemical properties, have been used to fabricate scaffolds. Rapid prototyping (83) and electrospinning (84) are sophisticated techniques for the production of respectively, 3D structures and fibers, that allow the possibility of incorporating pharmaceutical agents. Molecular self-assembly is another strategy available for the production of nanofibers by creating supramolecular architectures (85).

Several studies have been reporting the development of porous structures for bone tissue engineering using diverse materials and techniques (65, 78, 86-88). For example, Oliveira et al (78) developed macroporous HAp scaffolds with controlled morphology using the sponge replica method. The structures showed a porosity of ~70%, and highly interconnected macropores with a diameter in the range of 50–600 µm (Figure 5.3). Later, Barbani et al (86) produced a gelatin/HAp nanocomposite scaffold with elastic modulus similar to natural bone, using freeze-drying technique. It was shown that HAp scaffolds supported the adhesion and proliferation of human MSCs onto the scaffolds.

(Insert Figure 3)

Yan et al (87) prepared a composite scaffold of SF and nanosized CaP combining solvent casting and freeze-drying methods that allowed the formation of a homogeneous macroporosity and porosity distribution (Figure 5.4). The scaffolds are also characterized by the good mechanical properties and stability, and self-mineralization capability which represent a major feature for bone tissue engineering.

(Insert Figure 4)

Eftekhari et al (89) developed a novel porous scaffold composed of cotton-sourced cellulose microcrystals, HAp nanoparticles and PLLA with enhanced mechanical strength for bone tissue regeneration. A different preparation method, by applying cryogelation method as an alternative to freeze-drying, was used to prepared collagen/nano-HAp scaffolds for bone regeneration (90). The scaffolds showed improved mechanical properties and allowed high cells proliferation.

Hydrogels have been also explored in the context of bone tissue engineering due to their structural and compositional similarities with the ECM that allow efficient mass transfer as well as the encapsulation of cells and biomolecules (91). These structures comprise a hydrophilic porous network that can be controlled by solvent casting and particulate leaching, phase separation, gas foaming, solvent evaporation, freeze-drying, and blending with non-cross-linkable linear polymers (92). Hydrogel networks can be engineered, into different sizes and shapes, as thin films, sheets, spheres, rods, hollow tubes, and bellows, due to their unique physical properties (93). Excellent reviews regarding a deep description of hydrogels properties were recently reported (94).

Hydrogels have been produced combining synthetic or/and biopolymers and inorganic biomaterials, with desired physical properties, reproducibility, and biological activity for use in bone tissue engineering. For example, Gaharwar et al (95) developed hydrogels incorporating PEG and HAp presenting highly porous structures and interconnected porous structure with pore sizes of 100−300 nm (Figure 5.5). The results also showed osteoblast cell adhesion and bioactive attachment sites for the osteoblastic cells. An injectable and thermo-sensitive PEG-PCL-PEG copolymer, collagen, and nanosized HAp hydrogel for guided bone regeneration was developed by Fu et al (96). The results revealed good biocompatibility, biodegradability and new bone tissue formation after implanting the structures in rats. Gantar et al (97) developed bioactive glass-reinforced gellan-gum hydrogels with an open and well-interconnected porosity of about 80% and a pore size of ~100-200 μm.

(Insert Figure 5)

Fiber-based scaffolds for bone tissue engineering are another good option to mimic the fibrous structure of ECM. Likewise, nanoscale fibrous scaffolds with similarities with the network of collagen fibrils of native ECM have received particular interest to enhance cell adhesion, proliferation and differentiation (98). Electrospun fibers have been explored as scaffolds similar to natural ECM to engineer and repair the bone tissue. Rajzer et al (99) prepared composite fibrous scaffolds with electrospun PCL and gelatin/CaP fibers with diameter in the range of 2-6.5 µm and porosities of $74.3 \pm 7.0\%$ and $86.7 \pm 2.3\%$, respectively for the gelatin side and for the PCL side of composite scaffold (Figure 5.6). *In vitro* tests proved the bioactivity of the scaffolds by the higher activity of ALP. Chae et al (84) fabricated alginate/HAp fibrous scaffolds via electrospinning, composed of random nanofibers holding homogeneously distributed HAp nanocrystals.

(Insert Figure 6)

5.4.2 Cartilage tissue engineering

The limited ability of articular cartilage to regenerate has prompted the development of cell-based tissue engineering techniques, such as ACI. However, the complexity of ACI and contraindications in wider clinical applications has driven the development of matrix-assisted chondrocyte implantation, which uses scaffolds to provide mechanical stability and to support chondrogenesis. Laboratory and clinical studies have examined the management of larger lesions using tissue-engineered cartilage (100). To improve neotissue formation, cells can be cultured in vitro in 3D matrices with exogenous stimuli, such as GFs, to promote graft maturation and biomechanical integrity.

In order to outperform the currently used methods for treatment of grade 4 defects (penetration of the subchondral bone), novel tissue engineering approaches propose addressing OC regeneration by means of using bilayered scaffolds in combination with stem cells. That approach has in account the use of structures with two layers with different physical properties, usually a bioactive layer with a ceramic phase for the bony part, and a non-bioactive layer, composed by polysaccharides or proteins, or even biodegradable synthetic polymers for the cartilage-like layer (33, 101).

OC tissue is mainly composed by osteoblasts and chondrocytes. The two neighboring, but different cell lines have extremely different in vivo physiologic conditions, which have to be understood and replicated in vitro to obtain improvements in OC tissue regeneration.

Osteoblasts and chondrocytes are mononucleated cells that are derived from MSCs by GFs, via different signaling transcription pathways. Those factors can exhibit different and often opposite effects in the modulation of cells metabolism depending on their maturation stage and phenotype. Many adult tissues contain cell niches that in response to injury, for example, provide

stem cells that are able to differentiate into multiple cell linages, including chondrocytes. The adult stem cells have gained significant attention over the past decade and became frontline management for cartilage defects in the very recent past. BM is one of the main cell niches used to this ends, presenting good potential and results (102-104). However there are more MSCs niches with potential to be used for cartilage and bone differentiation (105), as umbilical cord- and adipose-derived stem cells (ASCs). In the case of ASCs, Hoffa's body is recently being explored as an interesting autologous source of cells to regenerate cartilage in knee-associated disorders (106, 107). Hoffa's body, which is a fat pad of ASCs, has to be removed during an arthroscopy to facilitate the visualization of the knee and surgery handling, and also to avoid tissue inflammation as was explained before. This way, this tissue can be considered too as a promising source of ASCs with great potential to differentiate into chondrocytes and osteoblasts (108). Recently, also strategies using endothelial cells in co-culture with osteoblasts or stem cells are being used to promote vascularization in bony part (109, 110).

To promote stem cell differentiation, GFs can be introduced in scaffolds in order to induce a faster host tissue response to the implanted matrix of material. In vitro osteogenic differentiation of ASCs could be induced by dexamethasone, L-ascorbic acid-2-phosphate, β-glycerophosphate, bone morphogenic proteins (BMPs), fibroblast growth factor (FGF), platelet-derived GF and transforming growth factor-β (TGF-β) (111, 112). Chondrogenic differentiation of ASCs requires GFs such as members of the TGF-β and BMPs families.

Steinwachs et al (113) reported the technique of autologous matrix-induced chondrogenesis (AMIC). AMIC involves the joint use of the Chondro-Gide (Geistlich Biomaterials, Wolhausen, Germany) collagen Type I / III membrane as a scaffold over a defect treated by microfracture. Short-term results were encouraging, however, long-term follow-up data is needed to substantiate preliminary findings (114). A novel approach to enhance cartilage repair with AMIC is to deliver GFs that selectively recruit and stimulate MSCs from the subchondral BM to invade cell-free scaffolds. Such GFs can be tailored also to activate chondrocytes in the surrounding healthy tissue to help filling the cartilage defect remodeling the tissue.

Matrix-assisted chondrocyte implantation (MACI) (Genzyme, Oxford, United Kingdom) is a later surgical technique of cartilage repair and is a third generation variant of conventional ACI. Instead of injecting cultured chondrocytes underneath a periosteal or collagen Type I / III cover, the cells are pre-loaded onto a commercially-produced porcine collagen patch. At the second stage of the operation, the patch is manually cut to cover the dimensions of the cartilage defect and held in place with tissue glue and, where necessary, sutures. Currently there is a limited data on the mid- to long-term follow-up success of such a technique.

Recently a new promising cell niche for articular cartilage regeneration was investigated by Pelttari et al (115). The authors showed that adult human neuroectoderm-derived nasal chondrocytes (NCs) can be constitutively distinguished from mesoderm-derived articular chondrocytes (ACs) by lack of expression of specific HOX genes, including HOXC4 and HOXD8. In contrast to ACs, serially cloned NCs could be continuously reverted from differentiated to dedifferentiated states, conserving the ability to form cartilage tissue in vitro and

in vivo. NCs could also be reprogrammed to stably express HOX genes, typical of ACs, upon implantation into goat articular cartilage defects, directly contributing to cartilage regeneration.

The effect of the scaffolds pore size was studied by Zhang et al (116) using porcine Type I collagen scaffolds applied for cartilage regeneration. The results obtained could help to establish the ideal conditions for future strategies for one of the most promising targets of regenerative medicine, the OC regeneration. The collagen porous scaffolds were prepared by means of using pre-prepared ice particulates that had diameters of 150–250, 250–355, 355–425 and 425–500 μm. The collagen porous scaffolds prepared with ice particulates 150–250 μm in size best promoted the expression and production of Type II collagen and aggrecan (116).

Adachi et al (117) evaluated the implantation of tissue engineered cartilage-like tissue composed by autologous chondrocytes cultured in atelocollagen gel for the treatment for full-thickness cartilage defects of the knee. Arthroscopic analysis was performed 2 years after implantation. According to the International Cartilage Repair Society scale, in 64 of 73 knees (87.7%) the implanted constructs were graded normal or nearly normal. The authors concluded that the procedure can be suggested for repairing full-thickness cartilage defect of the knee (117).

Lu et al (118) used a baculovirus system that exploited FLPo/Frt-mediated transgene recombination and episomal mini-circle formation to genetically engineer rabbit ASCs. The baculovirus system conferred prolonged and robust TGF-β3/BMP-6 expression in rASCs cultured in porous scaffolds, which critically augmented rASCs chondrogenesis and suppressed osteogenesis/hypertrophy. Twelve weeks after implantation into full-thickness articular cartilage defects in rabbits, these engineered constructs displayed cartilage-specific zonal structures without signs of hypertrophy and degeneration, and eventually integrated with host cartilage (118). Wang et al (119) investigated the repair of articular cartilage defects with tissue-engineered cartilage constructed by acellular cartilage matrices from the rabbit ear and seeded with ASCs. After in vitro chondrogenic differentiation for 2 weeks, the constructs were implanted in 4mm cartilage defects in rabbits. Articular cartilage defects of the rabbits implanted with tissue engineered constructs were filled with chondrocyte-like tissue with smooth surface, while in the group implanted with acellular scaffolds, the defect was filled with fibrous tissue (119). Forming a stable interface between the subchondral bone and tissue engineered cartilage components remains a major challenge. Dua et al (120) investigated the utility of HAp nanoparticles to promote controlled bone-growth across the bone-cartilage interface in an in vitro engineered tissue model system using BM derived stromal cells. Samples incorporated with HAp demonstrated significantly higher interfacial shear strength (at the junction between engineered cartilage and engineered bone) compared with the constructs without HAp, after 28 days of culture.

5.5 CLINICAL TRIALS

Significant strategies for regenerative medicine appeal to the use of cell therapy or tissue engineering, the first apply the effect of cell signaling for clinical application, the last approach

combine scaffolds with bioactive signaling molecules and cells for tissue repair and regeneration. Among bioactive molecules, recombinant bone morphogenetic proteins (BMPs) growth factors are the most common used for bone growth and healing due to their osteoinduction ability. Nevertheless, some complications relating to the off-label use of BMP-2 in spinal and trauma surgery have been reported to result in the formation of ectopic epidural bone associated with severe neurological impairment in anterior interbody fusion surgery (121, 122).

Different stem cell sources as, human MSCs, human BM stromal cells (hBMSCs), and human endometrial stem cells have been proposed for bone regeneration. It was reported a clinical trial in which autologous BMSCs were seeded in macroporous HAp scaffolds showing a promising outcome of functional bone recovery, with good implant integration and host bone formation during 6 to 7 years post-surgery (123). Other ongoing and complete clinical trials using several tissue engineering strategies for bone and cartilage repair/regeneration are well reported (124-126). There is a variety of bone scaffolding products currently available in the market for clinical utility. On the contrary, few scaffolds are being commercialized for OC regeneration. This fact reflects the great challenge when addressing the simultaneous regeneration of two distinct tissues such as bone and cartilage. Considering all bone and cartilage tissue engineering and regenerative medicine strategies for clinical application, ongoing and completed clinical trials (with no reported results yet) for OC repair/regeneration using scaffolds or cell therapies, or even scaffolds combined with cells pre-cultures *in vitro* are summarized in Table 5.2.

TABLE 5.2 Overview of ongoing and complete clinical trials using strategies for OC regeneration. Information obtained from https://clinicaltrials.gov/.

NCT number	Date and phase	Name of the clinical trial	Patients age	Follow-up	Procedure
NCT00891501	2006-2014 Phase 2 and 3	The Use of Autologous BM MSCs in the Treatment of Articular Cartilage Defects	15-55 yrs	n.d.	BM MSCs aspiration and implantation
NCT00560664	2007-2013 Phase 3	Comparison of ACI Versus Mosaicoplasty	18-50 yrs	24 mths	Autologous chondrocytes transplantation and mosaicoplasty
NCT00945399	2008-2011	Comparison of Microfracture Treatment and CARTIPATCH® Chondrocyte Graft Treatment in Femoral	18-45 yrs	18 mths	ACI and microfracture

	Phase 3	Condyle Lesions			
NCT00793104	2008-2012 Phase 3	Evaluation of the CR Plug (Allograft) for the Treatment of a Cartilage Injury in the Knee	≥ 18 yrs	24 mths	Placement of allograft CR Plug in primary injury site
NCT00821873	2008-2012 Phase 3	Evaluation of the CR Plug for Repair of Defects Created at the Harvest Site From an Autograft in the Knee	18-55 yrs	24 mths	CR Plug implantation in the harvest site
NCT01409447	2009-2011	Repair of Articular OCD	18-60 yrs	12 mths	Biphasic OC composite implantation
NCT00984594	2009-2012 Phase 3	Evaluation of a Composite Cancellous and Demineralized Bone Plug (CR-Plug) for Repair of Knee OCDs	18-55 yrs	24 mths	Autograft implantation in the primary defect site; CR-Plug implantation in the harvest site
NCT01183637	2010-2014 Phase 2	Evaluation of an Acellular OC Graft for Cartilage Lesions	≥ 21 yrs	24 mths	Microfacture
NCT01159899	2010-2014 Phase 0	Transplantation of BM Stem Cells Stimulated by Proteins Scaffold to Heal Defects Articular Cartilage of the Knee	30-75 yrs	12 mths	Transplantation of BM stem cells activated in knee arthrosis
NCT01209390	2010-2016	A Prospective, Post-marketing Registry on the Use of ChondroMimetic for the Repair of OCDs	18-65 Yrs	36 mths	Chondromimetic
NCT01473199	2011	BioPoly RS Knee Registry Study for Cartilage Defect	≥ 21 yrs	5 yrs	BioPoly RS partial resurfacing knee

		Replacement			implantation
NCT01290991	2011-2012	A Study to Evaluate the Safety of Augment™ Bone Graft	18-40 yrs	12 mths	Augment Bone Graft
NCT01410136	2011-2014	Chondrofix OC Allograft Prospective Study	18-70 yrs	24 mths	Allogeneic OC grafting
NCT01477008	2011-2014 Phase 3	BiPhasic Cartilage Repair Implant	Up to 54 yrs	12 mths	Marrow Stimulation
NCT01282034	2011-2015 Phase 4	Study for the Treatment of Knee Chondral andOC Lesions	18-60 yrs	24 mths	Marrow stimulation - Drilling or Microfractures
NCT01471236	2011-2017 Phase 4	Evaluation of the Agili-C Biphasic Implant in the Knee Joint	18-55 yrs	24 mths	Agili-C Bi-phasic implantation and mini-arthrotomy or arthroscopy
NCT01347892	2011-2019	DeNovo NT Ankle LDC Study	≥ 18 yrs	5 yrs	DeNovo NT Natural Tissue Grafting
NCT01747681	2012-2013	Results at 10 to 14 Years After Microfracture in the Knee	18-80 yrs	10 yrs	Microfracture
NCT01554878	2012-2014	Observational Study on the Treatment of Knee OC Lesions of Grade III-IV	30-60 yrs	12 mths	Knee surgery
NCT01920373 (cancelled)	2013 Phase 1	Platelet-Rich Plasma vs Corticosteroid Injection as Treatment for Degenerative Pathology of the Temporomandibular Joint	n.d.	6 mths	Corticosteroid and platelet rich plasma injection

NCT01799876	2013-2015	Use of Cell Therapy to Enhance Arthroscopic Knee Cartilage Surgery	18-68 yrs	12 mths	Autologous cell and standard microfracture arthroscopic surgery
NCT02005861	2013-2016 Phase 3	"One-step" BM Mononuclear Cell Transplantation in Talar OC Lesions	15-50 yrs	24 mths	BM derived cells transplantation on collagen scaffold
NCT02011295	2013-2017 Phase 4	BM Aspirate Concentrate Supplementation for OC Lesions	18- 95 yrs	24 mths	Ankle arthroscopy with debridement and microfracture
n.d.: not defined					

5.6 COMMERCIAL PRODUCTS

The process of commercialization of the scaffolds for implantation involves multiple stages of R&D replications before reaching the final approval from the governing bodies. R&D stages ensure safety and efficacy of the implants, which involve the production of medical grade scaffolds followed by animal testing under regulatory approved conditions. Scaffolds for bone tissue engineering are classified as biomedical devices under Class II- Medium Risk (127). For bone regeneration there are no tissue engineered approaches fully approved for clinical application. Instead, just engineered materials/scaffolds already regulatory approved are arriving in the clinic as bone grafts (without the combination of cells), such as Infuse® Bone Graft (Medtronic Sofamor Danek) used for fusion of spinal cage, Osigraft (Stryker Biotech) for long bone non-unions applications, and Grafton® Orthoblend (OsteoTech) as a bone void filler for small and large defects, have been successfully reported. Despite their efficacy in bone regeneration, clinical translation of scaffold-based bone therapies is limited to small defects due to insufficient mechanical integrity.

FDA approved scaffolds for craniofacial applications, such as Osteoplug and Osteomesh, are so far produced by Osteopore™. These scaffolds are composed of filaments of three-dimensional inter-woven PCL polymer. They have higher mechanical strength and its architecture minimizes potential injuries to the exposed brain through the burr holes. Long-term clinical trials revealed significant bone regeneration with adequate resorption rate and no adverse reactions (128).

Concerning cartilage tissue engineering, MACI is the better established technique for cartilage repair and the only FDA approved cell-based regenerative approach. Named CARTICEL, uses patient own cartilage cells (chondrocytes) to treat and repair the articular cartilage damage in the knee (28). However, nowadays there are some acellular approaches entering into the market. In fact, some of the products present in table 5.2 are already being commercialized.

Bilayered or multilayered scaffolds, consisting of bone- and cartilage-like layers, seem to be the most promising strategy to achieve OC lesion regeneration (129). Some studies have revealed promising results conjugating multilayer structures for OC regeneration and some acellular products are already being commercialized (101, 130). Among these few structures already in clinic, only three were reported in literature (Figure 5.7) one is a bilayer PLGA-calcium-sulphate co-polymer porous structure (Figure 5.7 a) (131). The second OC scaffold is a nanostructured biomimetic HA-collagen scaffold with a porous 2D trilayer composite structure, mimicking the whole OC anatomy (Figure 5.7 b) (130). The Kensey Nash Cartilage Repair Device (CRD) is a biphasic (Figure 5.7 c), bioresorbable scaffold intended to be implanted at the site of a focal articular cartilage lesion or OCD in the knee (132). This CRD technology utilizes a biphasic design that contains two discrete layers. The chondral phase consists of a unique bovine collagen type I matrix. The subchondral phase consists of β-TCP mineral suspended within a porous bioresorbable synthetic polymer scaffold (132). Other OC scaffolds are still under preclinical investigation (130).

(Insert Figure 7)

5.7 CONCLUSIONS AND FUTURE DIRECTIONS

It is known that tissue engineered technologies can take up to 20 years for reaching the market, and despite progress in many fields, this timeframe has yet to be shorten. Accordingly, tissue engineering, which has officially given its first steps during the late eighties, has not supplied many products to the bedside. Cell therapy strategies, as well as its first allogeneic stem cell therapy products, have been successfully applied for only few applications. Therefore, it is clear that, in spite of recent advances, tissue engineering has much to deliver in respect to combined products comprising biomaterials, growth factors/bioactive molecules and cells (differentiated or undifferentiated). Innovative strategies, such as the ones aforementioned, present out of the box solutions for some of the present challenges in the field, and may constitute major breakthroughs in future in order to finally catalyze the translation of tissue engineered products from bench to bedside.

Using a product comprising a biomaterial seeded with cells from the own patient is appealing. Despite this, the culture conditions, mechanical stimuli, GFs cocktails and environment conditions (as oxygen tension and tonicity) have to be better understood, correlated and optimized for the ideal regenerative product to be obtained.

In a static cell culture system the environment is constantly changing due to accumulation of metabolites, nutrient consumption in the culture medium and the consequent change in pH. This situation is clearly not representative of the *in vivo* state, in which cells are maintained in equilibrium between the constant supply of nutrients and removal of products through the cellular secretory mechanism. Moreover the metabolic requirements of a complex 3D environment are substantially larger than the necessary to maintain cell monolayers. To overcome these difficulties several different bioreactor systems have been developed. A bioreactor can be described as a device or dynamic system for culturing cells or tissues under controlled conditions either biochemical or mechanically. Different bioreactors have been reported in the literature, including mixed flasks, rotating vessels, perfused cartridges, and bioreactors with different mechanical stimulation. For cartilage or bone tissue engineering separately there are some more options to improve the tissue maturation, as bioreactors with pneumatic compression, hypoxia chambers and flow perfusion bioreactors. The main limitation of the current bioreactors for OC tissue engineering is that the newly formed tissue(s) is not homogeneously distributed within the constructs. Furthermore, there are just one bioreactor adapted for bilayered scaffolds (OC-related applications) that support different culture medium for each layer of the bilayered constructs, allowing inducing rotatory stimulus, compression and vertical movement to avoid cell sedimentation and undesired tissue malformation, at the same time. Therefore, the developments in OC tissue regeneration and OC products in particular will be greatly dictated by the future developments in bioreactors and dynamic culture techniques/systems.

ACKNOWLEDGMENTS

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FIGURE CAPTIONS

FIGURE 5.1   Bone and cartilage micro- and nano-sized cylinder formation. *Reprinted from Ref. (7), with permission.*

FIGURE 5.2   Representative images as summary of cartilage regeneration techniques: (a) full-thickness focal chondral lesion; (b) debridement; (c) microfracture; (d) ACI; and (e) MACI. *Reprinted from Ref. (17), with permission.*

FIGURE 5.3   HAp scaffolds: a) macroscopic image; b) and c) microstructure. *Reprinted from Ref. (78), with permission.*

FIGURE 5.4   SF/nano-HAp scaffolds: a) macroscopic image (scale bar: 3 mm); b1), b2) and b3) microstructure (scale bar: 500 nm). *Reprinted from Ref. (87), with permission.*

FIGURE 5.5   Microstructure of the hydrogel with 15% of nanosized HAp concentration. Arrow indicates the polymer-nanoparticle aggregates (scale bar 2 µm). *Adapted from Ref. (95), with permission.*

FIGURE 5.6   Microstructures of the composite fibrous scaffold Gel/CaP/PCL: a) gelatin side and b) PCL side. Scale bar 10 um. *Adapted from Ref. (99), with permission.*

FIGURE 5.7 Scaffolds commercialized for OC regeneration: (a) TruFit CB® implants in increasing diameters (7, 9 and 11 mm) (*Reprinted from Ref. (133) with permission); (*b) Maioregen® scaffold presenting three different gradient layers *(Reprinted from Ref. (132) with permission)*; and (c) CRD technology utilizes a biphasic design that contains two discrete layers (*Reprinted from Ref. (134), with permission).*