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Regenerative medicine and tissue engineering in orthopaedic surgery

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1. ABSTRACT

Orthopedic surgery is going through a serious paradigm shift; instead of simply replacing damaged tissues with prosthetic or allograft material, the aim is to regenerate them. This endeavor has generated the field of regenerative orthopaedics, an increasingly expanding area of research with hopes of providing new and better treatments for diseases and injuries affecting the musculoskeletal system. As part of this process, we are witnessing a substantial accumulation of new cellular and molecular insights into connective tissue function, coupled with emerging new concepts in stem cell biology and scaffolding technologies. Indeed, any successful strategy to regenerate musculoskeletal tissues can be portrayed as an intricate interplay between the three main constituents of the regenerative system: cells, environment and scaffolds. This review is not meant to be exhaustive and comprehensive, but aims to highlight concepts and key advances in the field of regenerative orthopaedics and tissue engineering, as well as to present current possibilities for clinical translation.

2. INTRODUCTION

Regenerative medicine is a transdisciplinary field that combines advances in biology, chemistry, clinical medicine, engineering, and material sciences. The ultimate goal is to recreate and reestablish natural healing processes which eventually lead to regeneration of damaged tissues and organs. Orthopaedics has emerged as one of the most attractive fields for implementation of regenerative strategies. Trauma and diseases involving musculoskeletal tissues result in severe pain and disability for hundreds of millions of people worldwide and represent major challenges for the orthopedic surgeons. Moreover, several orthopedically relevant tissues respond very well to regenerative stimuli.

Any successful strategy that attempts to regenerate musculoskeletal tissues can be portrayed as an intricate interplay between three main constituents: cells, environment and scaffolds (Figure 1).

This review addresses current achievements in the field of regenerative orthopaedics, particularly focusing

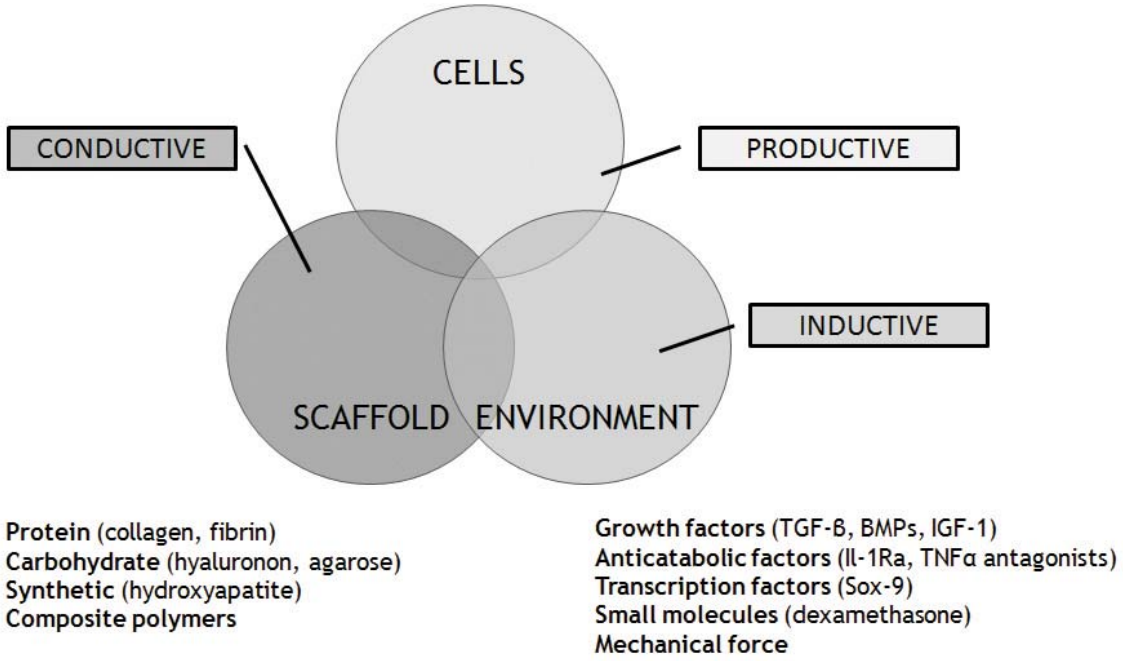

Figure 1. Schematic representation of interplay between the basic components of regenerative orthopaedics paradigm ("regenerative triad").

on bone and cartilage repair and regeneration. We describe different types and sources of cells, the multiple bioactive signals implicated in bone and cartilage repair and regeneration, and discuss matrices and scaffolds. We also provide a brief description of potential clinical applications.

3. CELLS

3.1. Basic concepts

Tissues may be restored with differentiated cells of the type normally found in the target tissue, or with progenitor cells, including stem cells, that can differentiate into the mature cells of the tissue. Each type of cell has its advantages and disadvantages. In principle, using differentiated cells removes the need to coax cells into following certain prescribed lineages and reduces the likelihood of aberrant differentiation into unwanted cell types. However, this dichotomy is blurred because many types of differentiated cells undergo phenotypic modulation ("de-differentiate") when passaged. Nevertheless, differentiated cells are thought to be less likely to undergo malignant transformation and are therefore considered to be safer. Use of differentiated cells usually requires sacrifice of uninjured tissue to provide cells for repair of the injured site, whereas progenitor cells can often be harvested without such collateral damage. Moreover, progenitor cells are usually available in much higher numbers.

Regardless of the source and type of cell used for regenerative purposes, the degree to which the cells are passaged is an important variable. If sufficient numbers of the right type of cell can be recovered, it is sometimes possible to use them directly to promote tissue repair without the need for cell culture or other major processing. This provides a big advantage because, at least in the United States, such cells are considered "minimally manipulated" and their regulatory route to the clinic is greatly facilitated. In most cases, however, the cells will need to be manipulated by exposing them to morphogenetic stimuli, purifying them, combining them with scaffolds or passaging. Any manipulation of cells outside the operating room requires a Good Manufacturing Process (GMP) facility, which greatly increases cost and complexity. These complications could be minimized if allogeneic cells could be used and an universal donor established for all patients. As discussed below, there is interest in using allografted mesenchymal stem cells (MSCs) in this fashion.

3.2. Differentiated Cells

Differentiated cells have been used in orthopaedic surgery mainly to treat articular cartilage defects. Autologous chondrocyte implantation (ACI) is a groundbreaking method that was introduced into the clinic by Mats Brittberg and Lars Peterson back in 1994, and marked the beginning of tissue engineering era in human orthopedic medicine (1). In the original method cartilage is biopsied from non-weight bearing part of the knee joint articular surface, enzymatically digested, expanded in monolayer culture, and then reinjected under an autologous periosteal flap, sutured onto the cartilage defect. The initial outcomes of the ACI procedure for this first group of patients indicated good to excellent results for 92% of isolated lesions and for 67% of multiple lesions. Despite the initial enthusiasm and promising clinical results, limitations of the classical ACI procedure have become apparent. These are related to the complexity of the surgical procedure, the biological response of the periosteum, chondrocyte culturing conditions and the graft fixation

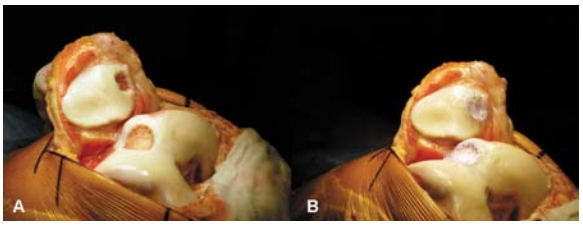

Figure 2. Second generation of autologous chondrocyte implantation. (a) Debrided lesions on patella and trochlea. (a) Chondrocytes implanted under the sutured collagen membrane.

method (2). Human articular cartilage chondrocytes have only limited proliferation potential, and when grown as a monolayer they tend to dedifferentiate with passage, losing both their chondrogenic phenotype, and redifferentiation potential.

Adverse clinical outcomes of the ACI procedure have been also analyzed, and based on 7,500 procedures reported to the U.S. Food and Drug Administration from 1996 to 2003, the most common adverse event was graft failure, accounting for 24.8% of all adverse events followed by delamination, accounting for 22.1% and tissue hypertrophy, accounting for 17.7% (3). To overcome these limitations, modifications to the original method have been introduced. The issue of chondrocyte dedifferentiation has been recently addressed by Saris and coworkers where they used pre-screened chondrocytes to treat symptomatic joint surface defects on the femoral condyles of the knee (4). Second generation ACI includes the use of a bi-layer collagen membrane instead of periosteal flap (Figure 2). The use of collagen membranes simplifies and shortens the operative procedure, and eliminates the hypertrophy found when using the periosteal flap (5-6). Further development of the ACI has brought third generation procedures which combine three-dimensional, biodegradable scaffolds with cultured chondrocytes (7). Many materials, such as collagen-gel, hyaluronan polymer or polylactin have been used for the third generation ACI, but the search for improved scaffolds continues (8). Since most of these techniques require only fibrin glue to fix the construct, the procedure can be done through a small incision or even arthroscopically (9). Fourth generation ACI is already on the horizon and the focus has shifted to include the use of stem cells, gene therapy and other advanced technologies (10).

3.3. Stem Cells

Stem cells are self-renewing, unspecialized cells capable of differentiating into multiple different cell types (11). These properties provide many advantages for the development of novel tissue regeneration strategies, and stem cells continue to be of unprecedented public, scientific and clinical interest.

Embryonic stem cells (ESCs). ESCs were originally isolated from mouse embryos by Nobel Prize winner Martin Evans and his coworkers almost thirty years ago (12). Isolation of ESCs from human embryos followed in the late 1990s (13). These cells are considered to be truly pluripotent, meaning that they can differentiate into all cells that arise from the three germ lines (14). Examples include the differentiation of ESCs into hematopoietic cells, neurons, osteoblasts, chondrocytes and hepatocytes (15-19). Theoretically ESCs offer many advantages for regenerative medicine, but several ethical and practical questions limited their use. For instance, certain countries prohibit the isolation of ESCs from human embryos. More biological limitations include possible tumor formation and immunological incompatibility. The recent discovery that human fibroblasts may be reprogrammed to become pluripotent by transfer of cDNAs expressing four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc), might obviate the ethical issues surrounding the use of ES cells from human embryos (20, 21). Discovery of these so called induced pluripotent stem (iPS) cells opens exciting new avenues for basic research and regenerative medicine (22).

Adult stem cells (ASCs). Although stem cells derived from post-natal individuals are much more limited in their differentiation capacity than those derived from embryos, they bring many advantages to regenerative medicine. In theory, one could harvest ASCs, stimulate the appropriate differentiation pathways, seed them onto three-dimensional scaffolds and transplant them back into the patient. Since the cells are autologous there are no immunological obstacles, and all the ethical and practical issues surrounding the use of ESCs are also circumvented. Moreover, ASCs are thought to present a much lower risk of malignant transformation. ASCs are known to be present in many tissues including central nervous system, heart and intestine (23-25). However, the research focus of the orthopedic scientists and clinicians has traditionally been directed towards the use of MSCs which can differentiate into cells of a variety of different connective tissues, including osteoblasts (bone), chondrocytes (cartilage), adipocytes (fat), tenocytes (tendon), and myoblasts (muscle) (26-30).

What we now call MSCs were originally described by Friedenstein and coworkers (31) as fibroblastoid cells isolated from rodent bone marrow explants on the basis of their ability to adhere to plastic, which distinguishes them from non-adherent hematopoetic stem cells (HSCs) also present in the bone marrow. If grown *in vitro* they rapidly proliferate and single cells give rise to distinct colonies (colony-forming unit fibroblasts – CFU-F). By modifying their biological environment it is possible to induce their differentiation along several different pathways, including osteogenic, adipogenic and chondrogenic lineages (26,32). Because defining criteria for MSCs do not exist, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSCs (33). First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR surface molecules. Third, MSCs must be able to differentiate into osteoblasts, adipocytes and chondrocytes

in vitro. Although MSCs have been isolated from almost every tissue in the body including bone marrow, fat, muscle, synovium, peripheral blood, brain, liver, and placenta, recent data suggest that a sub-set of these cells resides near the vasculature and are referred to as pericytes (34).

When injury occurs, MSCs are activated and they secrete bioactive signals which are both immunomodulatory and trophic (35). Immunosupression is particularly exhibited against T-cells which are responsible for antigen recognition and presentation; this property has been used to combat steroid resistant graft-versus-host-disease (GvHD) (36). Simple infusion of culture-expanded, allogeneic human MSCs effectively suppresses GvHD and induces remission without any adverse events. Bases on these remarkable results, similar trials have been initiated for the treatment of inflammatory bowel disease (Crohn's disease), type I diabetes and chronic obstructive pulmonary disease (COPD) (34). Because MSCs have immunosuppressive properties, it may be possible to allograft them for the purposes of tissue regeneration. If successful, this would obviate the need for treating each patient with autologous cells, thereby simplifying and streamlining the entire process. Such an advance would provide an enormous boost to regenerative orthopaedics. The trophic activities of MSCs include acceleration of angiogenesis, mitotic stimulation of local progenitors, anti-apoptosis and anti-scarring effects (37). The ability of MSCs to secrete such factors is increasingly recognized as an important component of their ability to promote tissue regeneration. Indeed, according to an emerging school of thought, the regenerative properties of MSCs do not reside so much in their ability to differentiate into replacement, differentiated cells, as in their ability to stimulate local healing through the secretion of trophic factors.

4. ENVIRONMENT

Bone and cartilage, the two tissues highlighted in this review, represent opposite ends of the regenerative spectrum. Bone is one of the few organs in the adult human with the ability to heal spontaneously without scarring. Cartilage, on the other hand, has almost no endogenous repair capacity.

When it occurs naturally, repair comprises a complex cascade of different, overlapping events controlled by local stimuli that provide signals at sites of injury. These recruit mesenchymal progenitors that undergo lineage commitment in a spatiotemporally controlled manner and mature through a series of differentiation steps. In response to appropriate signals the progenitor cells first proliferate and then secrete extracellular matrix as a prelude to reconstituting the original tissue (38). To the extent that regenerative orthopaedics seeks to emulate the natural healing process, one of its main goals is to control the environment by providing specific inductive signals.

4.1. Inductive signals for cartilage regeneration

4.1.1. General principles

Despite their common progenitors, bone and cartilage are very different tissues. Cartilage is avascular, aneural and alymphatic tissue produced by chondrocytes that are embedded at low density within the extracellular matrix. For these reasons, cartilage is the tissue with modest reparative and regenerative capabilities.

There are two fundamental approaches to the tissue engineering of cartilage. One begins with progenitor cells, such as MSCs, which differentiate into chondrocytes and form new cartilage. The other uses chondrocytes to form new cartilage. The first approach does not require the sacrifice of existing cartilage to recover autologous chondrocytes, but introduces complexities associated with inducing the chondrogenic differentiation of progenitors. The latter approach sacrifices existing cartilage as a source of autologous chondrocytes with which to from additional, new, repair cartilage. This avoids the need to promote the chondrogenic differentiation of precursors, although expanded chondrocytes undergo phenotypic drift that may require attention. The use of articular chondrocytes also reduces the likelihood that progenitor cell differentiation will continue to the hypertrophic phase of maturation, leading the endochondral ossification and the deposition of bone instead of cartilage at the defect site.

It should also be pointed out that there is a large difference between repairing cartilage that has become damaged as a result of trauma to an otherwise normal joint, and repairing the loss of cartilage resulting from a disease such as arthritis. In the latter case, cartilage repair is complicated by, among other things, the concomitant presence of a disease process.

4.1.2. Growth factors and hormones

Numerous morphogens are able to stimulate the chondrogenesis of progenitor cells, the deposition of matrix by chondrocytes, or both. Examples include TGF-beta, several BMPs, IGFs, FGFs, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Several of these have shown potential in regulating the growth, repair and regeneration of cartilage (39-42).

The TGF-beta family. All three isoforms of TGF-beta, TGF-beta1, TGF-beta2, TGF-beta3, and several BMPs have potent chondrogenic properties (43-45). Not only do these molecules induce the differentiation of MSCs into chondrocytes, but they also increase matrix synthesis by existing chondrocytes. Moreover, there is evidence that the TGFs maintain the phenotypes of articular chondrocytes and prevent their continuation into the hypertrophic phase. TGF-beta1 is found in cartilage in the proliferative, upper hypertrophic zones and induces early stage of chondrogenesis as well as production of aggrecan and collagen type II (46). TGF-beta3 has been detected in all zones of cartilage and plays the role in chondrogenic maturation (47). It has been reported that scaffolds loaded with TGF-beta1 could recruit MSCs and induce the process of cartilage formation when implanted in ectopic site (48). One concern with using TGF-beta in cartilage repair is the dramatic synovial fibrotic response it triggers. Any use of TGF-beta would need technologies that ensure it remains restricted strictly to cartilage.

BMPs. BMP-2 and -7 have shown efficacy in animal models of cartilage repair. BMP-7 has also shown efficacy as an agent that restores and protects articular

cartilage in osteoarthritis. Studies on their receptors have shown that BMPR1A and BMPR1B have distinct roles during chondrogenesis (49-50). The transcription factor Sox9 expression might be under direct control of TGFbeta/BMP activity, and factors downstream of TGFbeta/BMP pathways, such as Smads and TAK1 (TGF-beta activated kinase), control chondrogenesis-specific enhancers of the Sox9 gene, listed below together with description of Sox9 (47).

IGFs. IGFs are important for both cartilage and bone repair and have been described in detail in the section concerning bone regeneration, earlier in this review. IGF-1 is expressed in developing and mature cartilage. However, it cannot induce the chondrogenic differentiation of MSCs and its major effects in terms of cartilage repair appear are mediated via chondrocytes. IGF-1 is considered a candidate for articular cartilage repair because it stimulates both cell proliferation as well as the synthesis of key matrix constituents like aggrecan and type II collagen by chondrocytes (51-52). Moreover, IGF-1 is a survival factor for chondrocytes, protecting them from cell death. The addition of IGF-1 chondrocyte grafts enhanced chondrogenesis in cartilage defects, including the incorporation into surrounding cartilage, in large full-thickness repair models. IGF-2 plays a similar role to IGF-1, but shows less effect.

FGF-2. There are 22 members of FGF family in humans, but FGF-2 has been largely investigated as a potent stimulator of chondrocyte proliferation (53-54). In cartilage tissue, FGF-2 strongly binds to cell surface heparin molecules and stimulates chondrocyte division. Moreover, FGF-2 inhibits terminal differentiation of chondrocytes and calcification. It was reported that FGF-1 and -18 also stimulate chondrocyte proliferation (55). However, the role of FGF-2 in chondrogenesis is still not clearly understood with respect to extracellular matrix regulation. The literature contains conflicting data suggesting that FGF-2 can act to promote cartilage repair, as well as to promote cartilage destruction. Ellman *et al.* (56) observed that FGF-2 exerted catabolic and anti-anabolic effects on human articular cartilage. It is well established that FGF-2 interacts with FGFR1 and FGFR3 in cartilage, and these two receptors play critical, yet opposite roles in growth plate cartilage biology, promoting proliferation and differentiation, respectively. Binding of FGF-2 to FGFR1 receptor results in activation of following downstream signaling cascades: PKCdelta, NFkappaB, Ras-Raf-MAPK (including three subgroups ERK, JNK, and p38) and PI3K/Akt (57-59). Activation of those pathways results in elevated expression of matrix metalloprotease 13 (MMP-13) and suppresses proteoglycan synthesis. Binding of FGF-18 to FGFR3 suppresses cellular proliferation and promotes mesenchymal cell differentiation towards the chondrocyte lineage but the signaling cascades responsible for this are largely unknown. The anabolic effect of FGF-18 in cartilage suggests that this growth factor could be useful in promoting repair of damaged cartilage, especially if signaling via FGF-2 and FGFR1 is suppressed.

4.1.3. Transcription factors

Sox. Mesenchymal condensation is one of the first steps in chondrogenesis. It is first mediated by paracrine factors and subsequently by Sox2, a transcription factor that belongs to the SRY (sex-determining region on Y chromosome) family and contains the high mobility group box (HMG-box) DNA binding domain. The expression of Sox9 is regulated by members of FGF, TGF-beta, BMP and Wnt families (60). Sox9 is responsible for the expression of some key genes in chondrogenesis: Sox5, Sox6, collagen II alpha1, collagen XI alpha1 and cartilage–derived retinoic acid-sensitive protein (CD-RAP). Sox5 and Sox6 are required for the expression of collagen IX alpha1 and aggrecan (61). The so-called Sox trio (Sox 5, 6 and 9) regulates many important events throughout chondrogenesis thanks to the different spatiotemporal expression of transcriptional cofactors CBP, p300, TRAP230, PGC-1alpha and TCF.

4.1.4. Anticatabolic factors

Molecular destruction of the cartilage matrix is mainly driven by an increased activity of MMPs (-2, -3. -13) and aggrecanases (ADAMTS-4 and -5). These enzymes are induced by catabolic cytokines such as interleukin-1 (IL-1) or tumor necrosis factor-alpha (TNF-alpha), but also by matrix degradation products and other non-physiologic stimuli (62-63). IL-1 and TNF-alpha downregulate synthesis of the most abundant cartilage matrix components collagen type II and aggrecan and induce the expression of MMP-1 (collagenase 1), MMP-3 (stromelysis 1), MMP-13 (collagenase 3), and ADAMTS-4 (aggrecanase 1) (64). IL-1 acts through the three pathways of MAPK signaling, ERK, JNK and p38, as well as through NFkappaB (65).

Because breakdown of the cartilaginous matrix opposes repair and regeneration, the combination of an anti-catabolic agent, such as the interleukin-1 receptor antagonist (IL-1Ra), in combination with other previously described inductive molecules may be particularly effective in restoring cartilage, especially in an inflammatory environment or in joints with osteoarthritis. Other anti-inflammatory, immunomodulatory and anti-erosive mediators, such as, soluble receptors for TNF (TNFsR) or IL-1 (sIL-1R), IL-4 or IL-10, and inhibitors of MMPs, may be administered in this context (66, 67).

4.1.5. Mechanical forces

Biomechanical signals are perceived by cartilage in magnitude-, frequency-, and time-dependent manners. Static and dynamic biomechanical forces of high magnitudes induce proinflammatory genes and inhibit matrix synthesis. Contrarily, dynamic biomechanical signals of low/physiologic magnitudes are potent antiinflammatory signals that inhibit catabolic cytokines such IL-1 or TNF-alpha −induced inhibition of matrix synthesis. Recent studies have identified NF-kappaB transcription factors as key regulators of biomechanical signal−mediated proinflammatory and antiinflammatory actions (68). The application of hydrostatic pressure, scaffold compression, and stretching also stimulated the expression of the early chondrogenic marker Sox9 (69-71). All three types of the mechanical load enhanced transcriptional activity of chondrogenic markers and long term glycosaminoglycan (GAG) deposition (72). It can be concluded that mechanical stimulation represents an

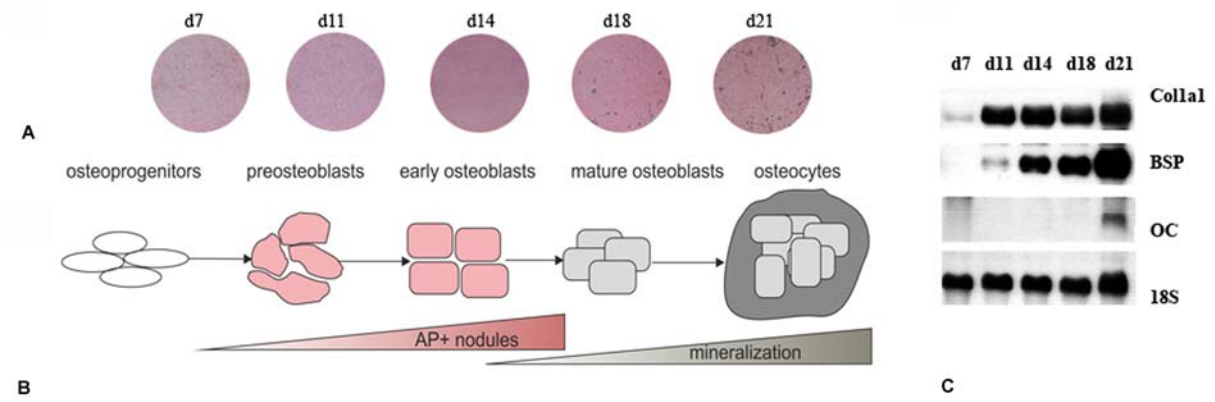

Figure 3. Osteogenic differentiation of bone marrow – derived mesenchymal progenitors in culture. Alkaline phosphatase and Von Kossa staining (a) observed at days 7,11, 14, 18 and 21 of the culture, present developing nodules with alkaline phosphatase activity and mineralization as a visual markers of osteoblast lineage differentiation (a). Northern blot analysis (c) presents gradual elevation of oseoblast mRNA markers Col1a1, BSP and OC as differentiation progresses.

additional and an efficient tool to improve MSCs differentiation and that providing the appropriate mechanical environment will lead to greater success of engineered tissues in the clinic.

4.2. Inductive signals for bone regeneration

4.2.1. Growth factors and hormones

Bone has high regenerative potential and self-healing capabilities. Bone may be formed in one of two ways, both of which involve the differentiation of uncommitted mesenchymal precursors. Endochondral bone formation requires the differentiation of MSCs into chondrocytes which lay down cartilage, later resorbed and replaced by bone. Chondrogenesis is discussed in the next section. Intramembranous bone formation requires the differentiation of MSCs directly into osteoblasts. As summarized in Figure 3, this is a multi-step process involving the sequential induction of various genes culminating in the deposition of a mineralized extracellular matrix.

Despite bone's remarkable ability to heal, certain types of trauma and many medical conditions impair its repair. A number of osteogenic stimuli hold regenerative potential under these conditions. Here we describe several key inducers of bone differentiation that are currently being used or investigated for bone tissue engineering purposes.

Bone morphogenetic proteins (BMPs). BMPs are members of TGF-beta superfamily of growth factors and are the most widely utilized factors in bone tissue engineering applications (73-74). BMPs are key regulators of cellular growth and differentiation and regulate tissue formation in both developing and mature organisms (75). Twenty unique BMP ligands have been identified and categorized into subclasses based on amino-acid sequence similarity (76-77). BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9 have been reported to have strong efficacy in inducing bone formation (78-83). BMP-3, however, inhibits osteogenesis. BMP-7 and BMP-2 are well studied members of this family of growth factors and are now being used clinically as the active ingredients of OP-1 (Stryker, Kalamazoo, MI, USA) and Infuse (Medtronic, Minneapolis, MN, USA) respectively, to induce new bone formation in spine fusions and long bone nonunion fracture (84-85). BMP-2 and BMP-7 belong to two closely related BMP subclasses, namely the BMP-2/4 subclass and the BMP-5/6/7 subclass (41). BMP activities are mediated by serine/threonine kinase receptors (86). Three type I receptors (BMPR1A, BMPR1B, ACVR1A) and three type II receptors (BMPR2, ACVR2A, ACVR2B) have been identified (87). BMP activated receptors phosphorylate the intracellular signaling proteins Smad-1, -5 and -8, which form complexes with the common mediator Smad-4 (88). Activated Smad complexes translocate to the nucleus and act as transcription factors to induce expression of target genes. Other BMP signaling pathways, including p38/MAPK and PI3K/Akt pathways are also involved in BMP osteoinductive signaling (89-91).

IGF-1. IGFs are polypeptides with high sequence similarity to proinsulin. They allow cells to communicate with their environment through complex regulatory systems composed of two cell-surface receptors (IGF1R and IGF2R), two ligands (IGF-1 and IGF-2), a family of six high-afinity IGF binding proteins (IGFBP 1-6), and IGFBP degrading proteases. IGF-1 functions as an inductive molecule mediating the effects of hormones, growth factors, cytokines and morphogens during the process of fracture healing as well as in the control of tooth development (92). IGF-1 exerts its function by binding to the IGF-1 receptor inducing autophosphorylation of the intracellular kinase domain of the receptor. Upon receptor activation, a number of protein substrates, including insulin-receptor substrate 1 (IRS-1) and Src and collagen protein (SHC), are activated and transduce multiple signaling pathways, including PI3K/PDK-1/Akt and Ras/Raf-1/MAPK. IGF-1 is critical for both linear growth of the bone and bone remodeling (93).

Vascular endothelial growth factor (VEGF). Since bone is highly vascularized tissue, angiogenesis plays a major role in skeletal development and fracture repair. VEGF stimulates the proliferation and migration of

endothelial cells mediating vasculogenesis, angiogenesis and the formation of the lymphatic vascular system (94). Although VEGF is a critical regulator in physiological angiogenesis it also plays a significant role in skeletal growth. Strong evidence also implicates VEGF in the recruitment, survival and activity of osteoblasts (95). It acts as a mediator of various osteoinductive factors such as TGF-beta1, IGF, FGF-2, which in turn regulate the expression pattern of VEGF. VEGF-mediated enhancement of endothelial cell migration into the extracellular matrix to develop vascular beds will be critical for the survival of implanted bone constructs. Therefore, there is growing appreciation that the endochondral route of osteogenesis generates its own vasculature and is therefore less dependent on the application of exogenous angiogenic factors (96).

Wnts. Wnts are secreted glycoproteins with 19 members. There are currently 4 different signaling pathways through which any given Wnt protein can act. The best studied of these pathways is a canonical or Wnt/beta-catenin signaling pathway. The three other pathways are planar cell polarity (PCP) pathway, the Wnt/Ca^{2+}, and a Protein Kinase A pathway involving CREB (97). The Wnt/beta-catenin pathway is now recognized as an important regulator of bone mass and bone cell functions. This pathway is important in osteoblasts for differentiation, proliferation and the synthesis of bone matrix, whereas osteocytes use the Wnt/beta-catenin pathway to transmit signals of mechanical loading to cells on the bone surface. The Wnt/beta-catenin pathway is activated by binding appropriate Wnt to co-receptor complex involving Lrp5 or Lrp6 and one of the frizzled family members. That leads to the activation of Dishevelled (Dsh) and downstream phosphorylation of glycogen synthase kinase-3beta (GSK-3beta). GSK-3beta is a key component of a degradation complex controlling free intracellular levels of beta-catenin (98). When beta-catenin accumulates in the cytoplasm it translocates to the nucleus, interacts with Tcf/Lef family of transcription factors regulating expression of genes important for differentiation, proliferation and apoptosis of bone cells.

4.2.2. Small molecules

Dexamethasone (DEX). DEX is a synthetic steroid that has been traditionally used experimentally to differentiate MSCs into osteoblasts in cell culture; it has been reported that human MSCs readily undergo osteoblastic differentiation and deposit mineral when exposed to 10^{-7} M DEX, ascorbic acid and beta-glycerophosphate for 21 days *in vitro* (99). Although effective, this approach is not practical for clinical application since it requires prolonged exposure to DEX. It has been shown that short-term *in vitro* exposure to higher doses of DEX is sufficient to commit MSCs irreversibly to osteoblastic differentiation and promising, preliminary, clinical results were achieved using autologous bone matter and freshly aspirated MSCs as graft, with DEX as the signaling molecule (100). Therefore, DEX may be an attractive option to be used intraoperatively during a single surgical procedure *in situ*ations where bone restoration is needed. DEX induces osteoblast differentiation through two related molecules IGF-2 and IGFBP-2, as well as integrin alpha 5 (ITGA5). All three molecules are strongly upregulated in dexamethasone-treated MSCs. Furthermore, the increased expression of these genes is associated with increased expression of the osteoblast phenotypic genes Runx2, ALP and COl1A1 (101).

HDAC inhibitors. Histone deacetylases (HDACs) and histone acetyltransferases participate in chromatin remodeling and the regulation of gene expression. The addition of acetyl groups to histones leads to the recruitment of coactivatiors and the relaxation conformation that is necessary for transcriptional activation. Removal of acetyl groups by HDACs results in condensed chromatin structure that is restrictive to transcription. Mammalian HDACs are organized into four classes – I, II, III and IV (102). HDACs inhibitors (HDIs) affect activities of class I, II and IV by blocking a channel that leads to the active site and a catalytic zinc ion (103). HDIs are divided into several classes including hydroxamic acids such as trichostatin A (TSA), short chain fatty acids such as valproic acid (VPA) and sodium butyrate (NaB), and benzamides such as MS-275 (104). Treatment of MSCs with HDIs increased expression of genes associated with osteoblast differentiation and enhanced mineralization (105). Recent studies showed that transcription factor Runx2 interacts with HDACs and that HDAC/Runx2 interactions are important in regualtion of osteoblast differentiation (106-107).

4.2.3. Transcription factors

Transcription factors are key targets for various hormonal, local and external regulatory molecules that bind to promoters regulating genes that define the cell phenotype. The osteoblast commitment, differentiation, and function are governed by many transcription factors, resulting in the expression of genes responsible for the plasticity of the osteoblastic phenotype (108). Since transcription factors are intracellular proteins they need to be delivered in the cell by gene transfer in order to induce bone differentiation.

The principal transcriptional regulator of osteoblast differentiation is *Runx2* (CBFA1, AML3), a member of the Runt familiy of transcription factors. Molecular studies of Runx2 indicated that the expression of Runx2 is both necessary and sufficient for MSC differentiation towards the osteoblast lineage (109-110). Runx2 binds to the Runx consensus sequence named osteoblast-specific element (OSE2) that can be found in the promoter of all major genes that define osteoblast phenotype including type I collagen alpha1 chain, osteopontin (OP), bone sialoprotein (BSP) and osteocalcin (OC). The most described factors in literature that actively enhance function of Runx2 are Rb, TAZ, HoxA10, BAPX-1, Smad 1 and 5, C/EBPbeta and δ, and Menin (111). Runx2 degradation can be accelerated by Smurf1, but factors such as YAP, TAZ, and WWP1-Schnurri may counteract the loss (112).

Osterix (Osx) is zinc finger transcription factor specifically expressed by osteoblasts. Osx transcription is

positively governed by Runx2 and acts by directing pre-osteoblasts to immature osteoblasts (113). Osx was found to interact with nuclear factor of activated T cells (NFAT) resulting in expression of COL1A1 (114). This coactivation stimulates Wnt/beta-catenin pathway, an important pathway controlling bone formation and bone mass described in previous section.

ATF4 or CREB2 (cAMP response element binding protein 2) interacts with Runx2 to regulate transcriptional activity of osteocalcin (115). ATF4 is phosphorylated by the kinase Rsk2 controlling the amino-acid transportation in osteoblasts, an important step in bone formation.

AP1, a transcription factor composed of the Fos and Jun families is also an important regulator of bone formation (116).

Homeobox proteins including Dlx3, Dlx5 and Dlx6 as well as Msx1 and Msx2 have an important role in osteoblastogenesis acting like repressors or activators of transcription of Runx2 and other bone markers like bone sialoprotein (BSP), OC, OP and ALP (117-122).

Helix-loop-helix (HLH) proteins including Id and Twist are expressed during proliferating stage of osteoblasts and are important negative regulators of osteoblast maturation (123).

PPARγ2 (proliferation-activated receptor γ2) is transcription factor that plays an important role in lineage determination. Increased expression of PPARγ induces adipocyte differentiation and reduces osteoblast differentiation of mesenchymal cells. PPARγ2 binds to Runx2 inhibiting its transcriptional activity (124).

C/EBPs (CCAAT/enhancer –binding proteins) are transcription factors that also regulate genes critical for adipocytic/osteoblastic differentiation. Both C/EBPbeta and C/EBPδ activate osteocalcin gene transcription and synergize with Runx2 to regulate bone-specific expression.

4.2.4. Mechanical forces

An appreciation of the biomechanical attributes of bone is critical to understanding of both, pathogenesis of metabolic bone disease and the emerging possibility of controlling the bone mass and structure through mechanical stimuli. Mechanical load applied to bone is perceived mostly by osteocytes, although induced fluid flow through the lacunar-canalicular system reaching osteoblasts is also a critical component. Perception of load triggers a number of intracellular responses including the release of PGE_2 into lacunar-canalicular fluid acting in the autocrine and/or paracrine fashion. Binding of PGE_2 to its receptors leads to the activation of Wnt/beta-catenin pathway, reduction of sclerostin and Dkk1, binding of Wnt proteins to Lrp5-Fz and amplification of load signal (125). Other signaling pathways that are important in response mechanical loading may also crosstalk with the Wnt/beta–catenin signaling pathway. For example activation of integrins leading to stimulation of integrin-linked kinase, which can phosphorylate GSK- 3beta is another intersection that needs to be explored and potentially any pathway that activates Akt could crosstalk with the Wnt/beta-catenin pathway (126). Many types of mechanical stimulation have been used in tissue engineering in order to improve growth of the MSCs. The application of flow perfusion, fluid flow and scaffold streching stimulate the gene expression levels of Osx and Runx2 and therefore the process of osteoblast differentiation (127-130).

5. SCAFFOLDS

Most current research is focused on resorbable scaffolds whose main function is to provide temporary, three-dimensional templates to which cells can adhere and synthesize extracellular matrix (ECM). As the scaffold resorbs, it is progressively replaced by newly formed, functional tissue (131). An ideal scaffold for tissue engineering in orthopaedics should posses certain characteristics. It should be biocompatible, meaning that the scaffold and its products of degradation must not be toxic to surrounding tissues, and should not induce immunological response.

It should possess mechanical stability allowing containment and orientation of the seeded cells, but also be able to undergo controllable biodegradation allowing eventual replacement by matrix components synthesized by the implanted cells. Another crucial requirement is the porosity of the scaffold which should enable diffusion of nutrients and bioactive signals, migration, proliferation, and adherence of the cells. Porosity also facilitates uniform distribution of the cells throughout the carrier. From the practical standpoint, the scaffold would be easy to manufacture, store, and handle, and would be versatile in terms of application for different clinical settings (132). Although many different materials have been used in orthopedic science to manufacture scaffolds, it is convenient to divide them in three major groups: natural, synthetic and combined scaffolds (Table 1.) (133).

Natural scaffolds normally have excellent biological compatibility, but often lack mechanical stability and there are issues with their sourcing, processing and possible disease transmission. On the other hand, synthetic scaffolds can be designed to be mechanically superior, with different macro- and microstructural properties. Their main disadvantages include inferior biocompatibility, mainly related to their potential to lower pH by the release of acidic products, and the elicitation of inflammatory responses within local tissues (8).

Because musculoskeletal tissues are so diverse, it is impossible to design an universal scaffold that would be suitable for all strategies, and different clinical applications require different scaffolds. For example, cartilage is avascular, aneural and alymphatic, with a disperse chondrocyte population, and even the smallest chondral defects have limited potential to heal. Bone, however, is highly vascularized with tremendous regeneration potential. Only in certain clinical situations (e.g. segmental defects due to trauma or tumor resection) does it not heal naturally.

Table 1. Natural and synthetic materials used in scaffold manufacturing

Natural materials	Protein-based	Collagen
		Fibrin
		Gelatin
	Carbohydrate-based	Hyaluronan
		Agarose
		Alginate
		Chitosan
Synthetic materials		Carbon fiber
		Polylactic / polyglycolic acids (PLLA, PLGA)
		Teflon
		Dacron
		Polybutyric acid
		Bioactive glass
		Hydroxyapatite

5.1. Scaffolds for cartilage repair and regeneration

Natural scaffolds. Various natural materials have been used for cartilage repair and regeneration, including collagen, gelatin, hyaluronic acid (hyaluronan), fibrin, alginate and chitosan. Collagen-based scaffolds, particularly matrix-associated chondrocyte implantation (MACI), are the most widely used for cartilage repair (134). Collagen is a major component of many connective tissues, giving them flexibility and tensile strength. Essentially, chondrocytes are seeded between two collagen layers in the operating room, implanted directly into the defect, and contained within the defect with fibrin glue. Gelatin is the denatured form of collagen and is also an interesting option for cartilage engineering (135). Reports indicate that it can be used as delivery carrier for growth factors such as TGF-beta and FGF-2 (136-137). Another strategy is to combine gelatin with a nonwoven PLLA scaffold, enabling uniform cell seeding into the 3D scaffold (138). Hyaluronan-based scaffolds are also good option for cartilage repair, and recent studies showed that use of this scaffold decreases expression of collagen type I, but increases production of markers of the authentic chondrogenic phenotype - collagen type II and aggrecan (139). Fibrin-based scaffolds are being extensively used for cartilage tissue engineering, as they exhibit excellent biocompatibility and various bioactive molecules can be easily mixed with them (140-141). Alginate is a natural anionic polysaccharide present in the cell walls of brown algae, that solidifies in the presence of multivalent cations such as Ca^{2+}. This reversible gelation process offers the possibility to encapsulate cells or growth factors within the hydrogel. Reports indicate that alginate beads stimulate chondrogenesis while preserving the original carrier shape. Alginate can be also prepared in an injectable form that could be used as a minimally invasive implant material (142-143). The biocompatibility of chitosan and its similarity to glycosaminoglycans make it attractive as a scaffold for cartilage engineering. It is formed from chitin, an abundant natural polysaccharide, primarily obtained as a by-product of shellfish, such as crabs and shrimps (144). When seeded on chitosan scaffolds, MSCs maintain viability above 90%,; chondrogenesis is improved by altering the 3-D structure of the scaffold (i.e. decreasing the fiber diameter) (145).

Synthetic scaffolds. Synthetic scaffolds provide interesting options for tissue engineering, because their structural and mechanical properties can be designed according to the particular clinical application. Scaffolds with a relatively high content of water and porous, viscoelastic properties are very much like cartilage, supporting the chondrogenic differentiation of progenitor cells and deposition of a cartilaginous ECM.

The most common materials used for cartilage repair and regeneration are poly(alpha-hydroxy esters), such as polylactic acid (PLA), polyglycolic acid (PLG) or copolymers of these two (PLGA) (146). Recent reports suggest that is feasible to use chondrogenically induced ASCs and a PGA/PLA scaffold to repair full-thickness cartilage defects in a porcine model (147). Another interesting approach combines allogeneic synovium-derived stem cells (SDSCs) seeded into non-woven PGA mesh and incubated in rotating bioreactor systems before implantation into defects (148). Recently, many investigators explored possibilities to combine certain bioactive cues into the scaffolds in order to facilitate the chondrogenic process. For example, BMP-6 in the presence of TGF-beta1 was effective in improving GAG and total collagen production when the cells were pre-treated with FGF-2 prior seeding into PGA scaffolds (149). Other interesting materials are poly(ethylene fumarate) or PPF and poly(ethylene glycol) or PEG. A novel modification of PPF termed oligo(poly(ethylene glycol) fumarate) or OPF has been recently developed. The presence of double bonds in the main chain enables it to crosslink into a hydrogel, and the hydrolysis of ester linkages results in degradation of the crosslinked hydrogel. In recent study by Guo *et al.*(150) used rabbit MSCs that were encapsulated in OPF hydrogels, combined them with TGF-beta1-loaded gelatin microparticles and successfully transplanted into rabbit osteochondral defects. Photopolymerized PEG hydrogels are attractive carriers for cartilage tissue engineering because of their ability to mimic the aqueous environment and mechanical properties of native cartilage. By simple changes in the gel structure or addition of bioactive cues to these synthetic systems, it is possible to affect chondrocyte gene expression and ECM evolution (151-152).

5.2. Scaffolds for bone regeneration

Natural scaffolds. Natural materials used for bone tissue engineering are similar to those used for cartilage and include fibrin, collagen, silk, alginate, coral and chitosan (153-154). Most of them have excellent biocompatibility and are readily degraded under physiological conditions. Drawbacks include inferior biomechanical properties, potential disease transmission and difficulties in processing. They can be used alone or in combination with other natural and synthetic materials. Additional improvements of these delivery systems include incorporation of molecular signals that control cellular functions or addition of genetically engineered stem cells that express osteogenic genes (155-156). Since collagen (especially type I) is the main component of the ECM, and it can be readily isolated from animal species, it has been extensively used in different bone regeneration strategies.

A recent study showed that it is feasible to engineer autologous bone grafts for maxillary sinus

augmentation (157). Cells derived from the mandibular periosteum were cultured *in vitro* for 2 weeks with autologous serum, transferred onto a collagen matrix and then transplanted into the sinus. A recent study by Glatt *et al.* (158) showed that a collagen sponge containing rhBMP-2, used in conjunction with locally applied tobramycin, enhances bone formation within critical size defects created in the rat femur.

Fibrin is a natural material derived from blood clots, and it can be enzymatically crosslinked to form glue, which can be applied in an injectable form. A proof-of-principle study reported by Muller *et al.* (159) showed that it is possible to seed freshly isolated adipose tissue cells containing mesenchymal and endothelial progenitors onto a fibrin hydrogel, and wrap it around bone substitute materials based on beta-tri calcium phosphate (beta-TCP), hydroxyapatite (Hap), or an acellular xenograft. The resulting construct (generated in only 3 hours) was able to induce production of osteocalcin and bone sialoprotein when implanted into nude mice. Silk is a natural material produced by silkworm (*Bombyx mori*), and has been used as a suture material for centuries. Recent advances in genetic modification and cloning techniques, along with versatile processing and structural modifications have resulted in the production of highly purified natural polymer (160). The unique mechanical properties, excellent biocompatibility and slow degradation of these fibers provide important design options for different applications in skeletal tissue engineering (161). Premineralized silk fibroin protein scaffolds combined with MSCs modified to over-express BMP-2 successfully repaired mandibular bony defects in a rat model (162). A similar study indicated that silk scaffolds and rhBMP-2 can be used as composite osteoinductive implants, and induce new bone formation in critical size defect in a nude rat model (163). Alginate and gelatin hydrogels, with or without addition of growth factors and progenitor cells, have been also successfully used in various experimental settings to induce bone formation (164-165).

Synthetic scaffolds. Apart from polymers of alpha-hydroxy esters such as PLA or PGA that are most commonly used materials for manufacturing carriers for skeletal tissues engineering, certain inorganic materials such as calcium phosphate cement (CPC), bioactive glass, HAp and beta-TCP have proven to be useful alternative to promote bone formation (166). CPC is particularly interesting due to its self-setting ability *in vivo*, injectability, biocompatibility and degradability. Essentially, CPC forms HAp through a cement reaction at body temperature and in a physiological environment. Upon mixing with a liquid phase, it forms a paste which is able to set and harden after being implanted within the body, creating a perfect fit for irregular bone defects (167). It is highly versatile and can be combined with other materials, growth factors and progenitor cells to obtain a construct which will promote bone regeneration *in situ* (168). Examples from literature include CPC-silk constructs enhanced by addition of an osteogenic cocktail (beta-glycerophosphate, ascorbic acid, and dexamethasone) and MSCs, and CPC-chitosan constructs enhanced by addition of MSCs (169-170). A preclinical study with adult male dogs showed that a HAp/beta-TCP/rhTGF-beta2 construct promoted local bone regeneration in 3 mm defects, in a dose dependent manner (171). It is also possible to combine organic and inorganic polymers to create constructs that have superior mechanical properties and storage qualities. Interconnected-porous calcium hydroxyapatite ceramics (IP-CHA), and the synthetic biodegradable block co-polymer (PLA-PEG) have been mixed with rhBMP-2 and implanted into a rabbit radius model. At 8 weeks after implantation, all bone defects in groups treated with rhBMP-2 were completely repaired with sufficient strength (172). In a similar study CPC was reinforced with chitosan fibers and implanted into bone defects in nine dogs (173). After 20 weeks, new callus from the healthy tissue of the defect entirely integrated with the CPC-fiber implant and new bone was formed as the implant degraded.

6. POTENTIAL FOR CLINICAL TRANSLATION

Advances in surgical techniques along with discovery of new implant materials have revolutionized the field of orthopaedic surgery in the last few decades. Total joint replacements and technologically advanced osteosynthetic implants are the most obvious examples. Although there is no doubt that these achievements improved and saved many lives, they have certain limitations. Artificial medical devices cannot completely substitute biological function, nor can they recreate the natural environment within the damaged tissues. Along with that, the clinical need to treat bone and cartilage defects effectively is expected to increase as the ageing population continues to grow. Tremendous advances in basic science of regenerative medicine and tissue engineering present opportunities to address many of the current clinical challenges. In order to do that, it is of utmost importance to move innovation from bench to bedside and validate it in a scientific manner.

6.1. Cartilage repair and regeneration

The restoration of damaged articular cartilage remains one of the biggest challenges in modern clinical orthopaedics. There is no pharmacological treatment that promotes the repair of the cartilage, and non-operative treatment inevitably leads to the development of premature osteoarthritis (174). Current treatment modalities include microfracture, transplantation of ostechondral grafts and ACI, each having its own benefits and shortcomings. Original versions of these modalities utilize only a part of the "regenerative triad" (cells, environment and scaffold), and it is necessary to introduce improvements to existing methods, and also to develop new and innovative approaches.

Microfracture and other bone marrow stimulation techniques involve penetration of the subchondral plate in order to recruit MSCs into the chondral defect. The formation of a stable clot that fills the lesion is of paramount importance to achieve a successful outcome. The technique is safe, easy and cheap, with excellent short-term results when used in small cartilage defects (175).

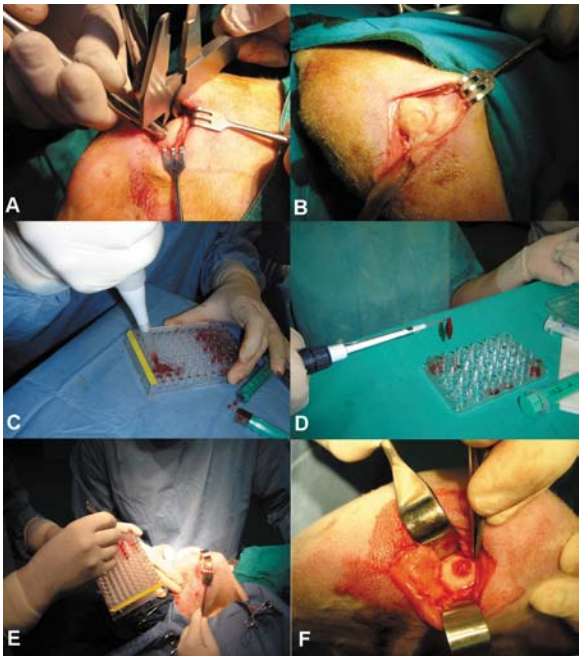

Figure 4. Implantation of a gene plug. (a) An adaptation of standardized mosaicplasty instrumentation was used to create a chondral defect on the weight-bearing surface of the medial condyle in sheep. (b) Care was taken not to penetrate the subchondral plate. The defect measured 6.2 mm in diameter. (c) Aspirated bone marrow was immediately mixed with adenoviral vector suspension. (d) Genetically modified bone marrow formed a clot - gene plug. (e) Pressfit implantation of the gene plug into the defect. (f) The plug was stable and well-placed within the defect. The joint was rinsed with saline prior to closing.

However, the resulting repair tissue is primarily fibrocartilage, with structural and functional properties that are inferior to natural hyaline cartilage, making it susceptible to clinical deterioration starting as early as 18 months postoperatively (176). One possible solution is to introduce scaffolds which posses better biological and mechanical properties then blood clot. Erggelet *et al.* (177) showed in an animal model that covering the microfractured defect with cell-free, freeze-dried implants made of PGA and hyaluronan, significantly improved repair tissue formation. In similar study, Hoemann *et al.* (178) used chitosan-glycerol phosphate/autologous whole blood implant to cover the defect, resulting in improved cartilage repair compared with microfracture alone. Further improvements can be obtained by introducing chondrogenic cues or eliminating catabolic cues, especially inflammation, from the local environment. Rabbit studies by Kuo *et al.* (179) reported that when compared to each treatment alone, the combination of microfracture and collagen sponges loaded with BMP-7, increase both the quality and quantity of repair tissue. In a horse model, Morisset *et al.* (180) showed a synergistic effect of microfracture and local gene therapy with IL-1Ra and IGF-1.

As previously mentioned, the original ACI method has been, and still is being, modified and improved in order to obtain tissue that is equal in structure and function to natural hyaline cartilage. Three-dimensional scaffolds are used to carry and orient cells within the defect, providing a temporary matrix, which is subsequently replaced by ECM produced by the cells. Furthermore, it has been shown that chondrocytes tend to lose their chondrogenic phenotype when expanded in monolayer cultures, but regain it when seeded in three-dimensional systems using scaffolds (181). A recently published, short-term pilot study showed it is safe and feasible to use alginate beads containing human mature allogenic chondrocytes to treat symptomatic knee cartilage defects (182). The study included 21 patients, and results indicate that the proposed technique provides clinical and histologic outcomes that are similar to those of other cartilage repair techniques. Another interesting approach uses a hyaluronan-based scaffold seeded with autologous chondrocytes. The results of a prospective clinical case series with 2 to 7 years' follow-up, indicated that this technique provides clinical improvement in healthy young patients with single cartilage defects (183). MSCs are an attractive cell source alternative for the original ACI method, because of their inherent capacity to differentiate into chondrocytes. The first clinical results for the transplantation of MSCs seeded in a collagen type I hydrogel was reported in 2004 by Wakitani and coworkers (184). They reported on two patients with patellar defects treated with collagen gel/MSCs construct and covered with a periosteal flap. Subsequently the procedure was performed on 41 patients, and neither tumors nor infections were observed between 5 and 137 (mean 75) months of follow-up. Chondrogenesis of MSCs is precisely orchestrated process which involves many growth factors and signaling molecules (185). By modifying the local cellular environment, it is possible to enhance formation of more natural cartilage tissue within the defect. Recent studies by Pascher *et al.* (186) and Ivkovic *et al.* (187) proposed a novel, abbreviated, *ex vivo* protocol utilizing vector-laden, coagulated bone marrow aspirates for gene delivery to cartilage defects (Figure 4). In this approach freshly aspirated bone marrow is transduced with an adenoviral vector carrying gcDNA encoding TGF-beta1. The marrow is allowed to clot creating a so called gene plug, and then immediately transplanted into the defect. A similar approach by Guo *et al.* (188) uses autologous MSCs modified with the TGF-beta1 gene. These are seeded onto chitosan scaffolds to form gene-modified constructs, and implanted into full-thickness articular cartilage defects in rabbits' knees. Rather than using single therapeutic genes, it might be more effective to combine several genes. Indeed, a recent study by Steinert *et al.* (189) showed that co-expression of IGF-1, TGF-beta1 and BMP-2 results in improved chondrogenesis of MSCs. However, the use of multiple genes greatly complicates the regulatory approval process and thereby slows clinical translation.

6.2. Bone regeneration

Bone and liver are the only two tissues that can spontaneously heal and restore function without significant scarring. Adult bone is a highly vascularized tissue which undergoes constant remodeling, enabling it to regenerate after injury. However, in certain clinical situations where

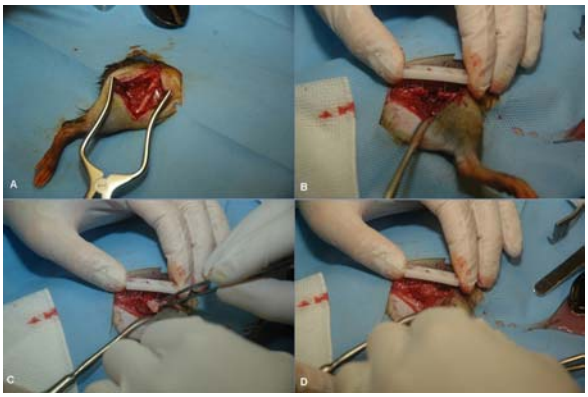

Figure 5. Bone regeneration using fresh bone marrow-derived MSCs, gelatin scaffold and rhBMP-2. (a) Critical-sized defect in a nude rat model. (b) Stabilization of the defect with external fixation. (c) Implantation of a construct. (d) Wound before closure.

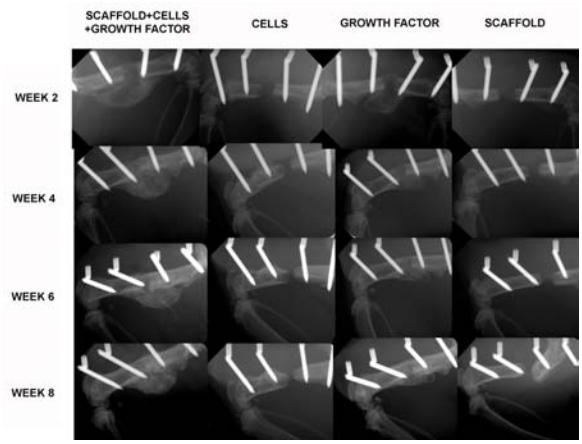

Figure 6. Radiographs of different treatment groups indicate that combination of a cell-loaded scaffold and growth factor show superior bone regeneration after 8 weeks.

extensive injury, disease or malformation cause large defects, it is necessary to resort to tissue grafting and reconstruction. It is estimated that half a million bone grafting procedures are performed annually in the United States (190). Autologous bone grafting is the gold standard in orthopaedic surgery, as it provides all necessary components of the regeneration triad (osteoblasts, growth factors and bone-supporting matrix), and is completely immunocompatible with the host. However, several constraints remain such as limited availability, donor site morbidity and the requirement for secondary procedure. Allogeneic and xenogeneic bone transplantations are viable alternative treatments, but they are not without limitations such as tissue matching, possible disease transmission, and inability to integrate with surrounding tissues. Regenerative medicine and tissue engineering concepts emerged as potential alternative approaches to deal with difficult clinical situations caused by severe bone loss. Current models of bone regeneration are exploiting the paradigm that cellular differentiation can be modulated by the same factors known to be involved in the process of embryonic bone development (191). To do this, it is necessary to expose cells embedded in a suitable three-dimensional scaffold to an environment containing the appropriate biochemical and biophysical signals from embryology (Figure 5 and 6).

Adult MSCs are capable of differentiation into osteoblasts and are of obvious utility for bone tissue engineering. Bone marrow-derived MSCs (BMSCs) have been most commonly used for this purpose.[192] Quarto and coworkers (193) reported the use of cultured BMSCs seeded on hydroxyapatite to treat long bone defects in three patients. Callus formation and integration was observed as early as 2 moths postoperatively, but the hydroxyapatite resorbed only slowly and was still present several years of surgery. A similar approach was used by Shayesteh *et al.* (194) for maxillary sinus augmentation. A novel, intraoperative procedure that utilizes the reamer-irrigator-aspirator (RIA) (Synthes Inc., Paoli, PA, USA) system to recover autologous bone along with progenitor cells from bone marrow has been recently described. In a preliminary clinical study, the materials recovered by RIA were loaded on gelatin scaffold, briefly treated with a high concentration of dexamethasone, and then implanted into the fracture site. A total of 13 patients were treated in this study, and preliminary clinical results confirmed that the proposed method is a safe and promising approach to the treatment of segmental bone defects and non-unions (100). Recently a group from Columbia University successfully engineered fully viable, clinically sized, and precisely shaped temporomandibular joint grafts by culturing BMSCs in a bioreactor (195).

Since the initial number of MSCs obtained from bone marrow is rather low (approximately 0.01% of the nucleated marrow cells), alternative sources of these progenitors must be considered. Adipose-derived MSCs (AMSCs) are an attractive source due to their accessibility and abundance within the adipose tissue (approximately 1 to 5% of isolated nucleated cells) (196). When cultured on porous scaffolds such as PLA/TCP, these cells are capable of producing bone-like constructs (197). Addition of various osteoinductive molecules to the cells/scaffold construct can further enhance bone formation *in vivo*. BMPs, TGF-beta and IGF-1 have been shown to poses osteoinductive properties which make them suitable for incorporation into different carrier systems (198). Examples include combined use of BMP-2 and TGF-beta2 loaded on porous-coated titanium scaffold in a dog humerus study, and rhBMP-2/PLA-DX-PEG/beta-TCP construct studied in a critical-sized rabbit bone defect model (199-200). In order to survive and effectively integrate into surrounding tissue, these constructs must become attached to the host vasculature. Therefore, vascular endothelial growth factor (VEGF) has attracted the attention of the bone researchers. Although primarily known for its angiogenic properties, VEGF is also implicated in recruitment, survival and function of osteblasts (201). Indeed, recent studies confirmed the synergistic effect of an angiogenic (VEGF) and an

osteogenic (e.g. BMP-2) growth factor for bone regeneration (202).

7. OUTLOOK

New discoveries and innovations in regenerative orthopaedics and tissue engineering continue to emerge, and have the potential to overcome the shortage of suitable autografts and allografts for enhanced bone and cartilage healing.

Elucidating the optimal source and harvest method of the cells still remains a challenge. Each of the mentioned sources – primary cells, MSCs, ESCs and iPS cells has certain advantages and disadvantages. Rather than finding a "universal" cell source, it is more likely that different clinical applications will require different cell sources. Stem cell-based approaches have distinct advantages over other treatments, and three major areas have emerged as the most promising ones, namely, the mesenchymal differentiation of ESCs, the therapeutic use of MSCs, and the identification of new tissue specific stem cells (203). Bone and cartilage regeneration are highly complex processes, precisely orchestrated by the sequential, spatiotemporal expression of different growth factors and cytokines. For example, bone regeneration starts with inflammatory phase and ends in mineralization of ECM. Recent evidence suggests that the combination of angiogenic and osteoinductive signals is a promising strategy. On the contrary, inflammation is detrimental to the formation of new cartilage, and should be prevented. Ideally the local environment for cartilage regeneration should be free from inflammatory cytokines, and rich in chondrogenic factors and biological cues that will diminish hypertrophy of newly formed cartilage. Therefore, the delivery systems for bone and cartilage regeneration should not be limited to a single morphogenetic factor, but should be optimized for the delivery of multiple signals in specific spatiotemporal patterns.

Another important component of musculoskeletal tissue engineering is the development of effective bioreactors. These devices are dynamic culture systems used to control and maintain the cell microenvironment in order to permit or induce desired biological and chemical processes (204). Bioreactors allow cells and scaffold interactions in more natural 3D settings, producing a clinically relevant construct that is ready for application. This is termed bioprocessing and refers to a translation of laboratory-based practices to clinical practice, with special attention devoted to automation, quality assurance and regulation (205).

The design and innovation of carrier systems and scaffolds will continue to be one the most dynamic fields within the field of regenerative orthopaedics. Emphasis will be placed on the development of complex structures that posses multiple layers within a single unit in which each layer has distinct porosity or chemical structure. Differences in mechanical properties, such as stiffness or elasticity, have been shown to be very important in terms of cell differentiation and phenotypic behavior. For example, a recent study indicated that carrier rigidity profoundly affects cell morphology, focal adhesions, cytoskeletal contractility and stem cell differentiation (206). Incorporation of various bioactive molecules, improved control of their release kinetics, and discovery of new biomimetic materials are also expected to impact greatly the field regenerative orthopaedics and tissue engineering. Emerging bioprinting methodologies can help us create tissue engineered constructs with precisely defined three-dimensional organization (207). This technology is based on the use of simple ink-jet printers that can fabricate persistent biomimetic patterns in order to improve tissue regeneration.

Finally, it is of utmost importance to stress the need for translation from laboratory to operating room. At present, there is a huge disproportion between preclinical and clinical work in regenerative orthopaedics and tissue engineering arena. The most likely reasons include the demanding logistics of harvesting, expanding and re-transplanting autologous cells, expensive production of biomimetic scaffolds, numerous regulatory hurdles and costly clinical trials. These are exciting times for orthopaedic surgery, and closer cooperation between basic scientist, clinicians and regulatory agencies is necessary in order to overcome mentioned obstacles, and move these new technologies from bench to bedside.

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Abbreviations: MSCs: mesenchymal stem cells, ACI: autologous chondrocyte implantation, ESCs: embryonic stem cells, ASCs: adult stem cells, iPS: induced pluripotent stem cells, HSCs: haematopoetic stem cells, CFU-F: colony forming unit – fibroblast, CD: cluster of differentiation, HLA: human leukocyte antigen, TGF: transforming growth factor, BMP: bone morphogenetic protein, IGF: insulin-like growth factor, FGF: Fibroblast growth factor, PDGF: platelet-derived growth factor, ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs, TNF: tumor necrosis factor, DEX: dexamethasone, Dkk1: Dickkopf-related protein 1, ECM: extracellular matrix

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