Chapter 2

Bone Tissue Engineering: Past–Present–Future

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Abstract

Bone is one of the few tissues to display a true potential for regeneration. Fracture healing is an obvious example where regeneration occurs through tightly regulated sequences of molecular and cellular events which recapitulate tissue formation seen during embryogenesis. Still in some instances, bone regeneration does not occur properly (i.e. critical size lesions) and an appropriate therapeutic intervention is necessary. Successful replacement of bone by tissue engineering will likely depend on the recapitulation of this flow of events. In fact, bone regeneration requires cross-talk between microenvironmental factors and cells; for example, resident mesenchymal progenitors are recruited and properly guided by soluble and insoluble signaling molecules. Tissue engineering attempts to reproduce and to mimic this natural milieu by delivering cells capable of differentiating into osteoblasts, inducing growth factors and biomaterials to support cellular attachment, proliferation, migration, and matrix deposition. In the last two decades, a significant effort has been made by the scientific community in the development of methods and protocols to repair and regenerate tissues such as bone, cartilage, tendons, and ligaments. In this same period, great advancements have been achieved in the biology of stem cells and on the mechanisms governing "stemness". Unfortunately, after two decades, effective clinical translation does not exist, besides a few limited examples. Many years have passed since cell-based regenerative therapies were first described as "promising approaches", but this definition still engulfs the present literature. Failure to envisage translational cell therapy applications in routine medical practice evidences the existence of unresolved scientific and technical struggles, some of which still puzzle researchers in the field and are presented in this chapter.

Key words Bone, Mesenchymal stem cells, iPSC, Cell therapy, Biomaterials, Scaffolds

1 Past Cell Therapy

The standard approach proposed in the past implied the delivery of in vitro expanded cells (stem cells, progenitors, etc.) combined with biomaterials of various chemical nature and architecture.

1.1 Cells

Osteoprogenitor cells have been isolated from a variety of tissues, including periostium, bone marrow, spleen, thymus, skeletal muscle, and adipose tissue [1–8]. Osteoprogenitors have also been isolated from other tissues, such as amniotic fluid [9], chorionic villi [10], infrapatellar fat pad [11], synovium [12], and the umbilical cord [13],

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although their use in tissue engineering is not always straightforward. The most common source of stem cells remains bone marrow. Mesenchymal stem cells (MSC) can be isolated, expanded in culture, and stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat, and a variety of other connective tissues [14]. Very large numbers of MSC can be generated in culture from limited marrow samples, making it possible to engineer constructs composed of these cells together with appropriate scaffolds which can be re-introduced into the recipient. In order to obtain large numbers of osteoprogenitors for cell transplantation, culture conditions and the effects of growth factors on proliferation and differentiation of MSC are of great interest and have been investigated by several groups [15–19]. Furthermore, MSC can be transduced with various viral vectors and are, thus, interesting candidates also for somatic gene therapy in local or systemic pathologies [20–22].

One interesting source of osteoprogenitor cells is achievable in large quantities, under local anesthesia, with minimal discomfort [4, 8]. This population can be isolated from human adipose tissue harvested by suction-assisted lipectomy (liposuction) [23]. From this adipocyte-rich fraction, MSC-like cells can be isolated, maintained in vitro for extended periods with low levels of senescence. Immunofluorescence and flow cytometry show that the majority of these cells are of mesodermal or mesenchymal origin with low levels of contaminating pericytes, endothelial cells, and smooth muscle cells. Finally, they can differentiate in vitro into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors [8].

Some, if not all of the problems raised by solid tissue osteoprogenitor cells could be solved by harvesting cells with similar characteristics from peripheral blood. This of course would be the simplest source of cells to harvest and a minimally invasive approach for the donor. Few reports, starting from the historical publication by Luria and coworkers [24], suggest that it is possible to isolate a population of fibroblasts from peripheral blood [25]. These peripheral blood fibrocytes would in principle be the population of cells that reach sites of tissue injury and contribute to connective scar tissue formation. They display a distinct cell surface phenotype (CD34−/CD45−/collagen I+/β1 integrin subunit) and are an abundant source of cytokines and growth factors that function to attract and activate inflammatory and connective tissue cells. However, controversial data are often presented in the literature regarding circulating mesenchymal progenitors; this underlines the lack of incontrovertible proof of this very elusive and limited cell population and the presence of different opinions within the scientific community [26].

1.2 Biomaterials

The right choice of a suitable tridimensional matrix to deliver progenitor cells is of critical importance. Scaffolds are one of the most important elements required to trigger the cascade of events leading to bone repair and to mimic the extracellular matrix in a regenerating bone microenvironment. This concept implies that scaffolds do not simply deliver cells, but that they are somewhat "informative" to the cells and—thus importantly—they should be engineered as such. The primary properties of biomaterials for bone regeneration are osteoconductivity and integration with host bone tissue [27–31]. Their architecture therefore must be permissive for blood vessels to colonize even in larger structures. Finally, they should be biocompatible and resorbable. From this point of view, the new generation of bioceramics are indeed exceptional candidates [32]. Porous bioceramics (hydroxyapatite—HA and tricalcium phosphate—TCP) are osteoconductive, have a favorable bone affinity [33–35], and are free from risks of rejection or infection [31, 33, 36].

An important improvement in this field is represented by synthetic porous scaffolds. In this case in fact, the internal architecture can be intelligently designed and the density, as well as the biomechanical properties of the material, can be predetermined. The result is that the surface available for cell delivery and for consequent tissue regeneration can be maximized and may be rendered extremely wide. As outlined already, bone tissue engineering strategies attempt to provide the injured segment initially with a scaffold of poor mechanical properties, but highly permissive to new bone ingrowth and blood vessel invasion. Scaffolds will have to be eventually resorbed to allow the new bone to gradually remodel, acquiring the required mechanical properties; ideally the scaffold resorption kinetics should correspond to those of new bone deposition. HA-TCP composites have achieved these prerequisites, where HA allows a direct chemical bond with the pre-existing or with the newly deposited bone, and TCP represents the resorbable component. Interestingly, specifically designed studies have shown that neither resorption nor dissolution of TCP or Si-modified TCP would take place in the absence of new bone formation within the defective site. Indeed, orthotopic and ectopic model studies have shown that contemporary phagocytic action by macrophages and osteoclasts and deposition of new osteoid matrix are needed to generate scaffold volumes with varied densities as those seen only in cell-bearing implants. Possibly then, the precursor cells' presence on and within the scaffold, prior to implantation, may influence the ECM proteins' availability on the material surface, thus favoring attachment of the osteoclasts and their resorption activity [37].

1.3 Obstacles

Bone has been one of the most interesting models and target tissues for cell therapy. Many groups around the world have attempted to find the best approach to regenerate it. Theoretical approaches

have been applied with interesting results both in small and large animal models. Even a few clinical studies have been performed with promising results [38]. But still, at present, no routine clinical application exists. What then is the reason for this apparent gap between successful experimental models and their translation to clinical practice? First therapeutic alternatives are available basically in any medium–large sized hospital and usually they represent a consistent approach to solve the problem. However, real life situations are always more complex than any experimental setting. In other words, bone lesions (i.e. in an emergency room setting) are unpredictable in many ways (size, anatomical location, cause of the lesion, health status of the subject, etc.) and of course, they are far from being standard. Moreover, the unavailability of specific off-the-shelf scaffolds contributes to the slow adoption of cell-based tissue regenerative approaches, in spite of the fact that MSC themselves are immune-privileged. These cells, in fact, carry low levels of class 1 and no class 2 Human Leukocyte Antigens [39], properties that prompted their clinical exploitation even in allogeneic hematopoietic stem cell transplantation [40]. MSC are thus particularly advantageous in bone tissue engineering applications, since they neither induce immune nor inflammatory responses in recipient organisms [41], but cells still need time to grow, a requirement that may not match the needs of the patient or the clinical setting. Moreover, a large body of evidence indicates the loss of osteochondrogenic potential of the cells due to several factors, particularly culture conditions, passage number, length of osteogenic induction, age and health conditions of donors, cell loss after implantation and the hostile environment of the injured tissue [42, 43]. Indeed, cell pre-conditioning has been suggested to improve in vivo delivery in many experimental settings [42, 44]. Safety, legal, and ethical issues also play a role, particularly if we consider all the requirements necessary to provide a "certified safe" cell population (in terms of collateral risk-free cell availability, number of cells, and effectiveness of the cells themselves) to any patient in need of treatment [45]. In this respect, several studies are being conducted to provide adequate quantitative parameters that could predict at least the efficacy of cell-based medicinal products, particularly relating to cell viability and osteogenic potency [46]. Still no one can predict the fate of in vitro expanded stem cells a decade after they have been reintroduced in vivo.

2 Present Challenges

2.1 Informative Substrates

An emerging philosophy aims to circumvent the traditional approach of recreating the complexity of living tissues ex vivo. In this context, the most ambitious strategy attempts to develop synthetic materials that establish key interactions with cells in ways that

unlock the body's innate powers of organization and self-repair. The complex cell–biomaterial interaction moves on multiple spatial and temporal scales. Therefore, in order to effectively influence cell behavior, scaffold materials must bear complex information, coded in their *physical* and *chemical* structures. In particular, bio-scaffolds must be properly designed to allow the spatial organization of stem cells and provide the basis for recreating a microenvironment mimicking their physiological *niche*. Stem cell niche is defined as a dynamic microenvironment that balances stem cell ability to maintain tissue homeostasis and repair throughout the lifetime of an organism [47]. In principle, stem cells in their niche make decisions to remain in a quiescent state, undergo self-renewal, or exit the niche upon exposure to local or systemic stimuli. These signals are actively coordinated and presented in a temporally and spatially regulated manner. Proper microenvironmental cues given by the biomaterial may be "informative" for cells, stimulating specific cellular responses.

2.2 Scaffold Physical Properties

Regardless of the chemistry or topography of the scaffolds, and prior to its implantation at the injured site, the primary function that a scaffold provides to the seeded cells is a physical support for adhesion. This implies close contact between cellular (endogenous) or secreted (exogenous) proteins and the scaffold itself. A few macromolecular classes encompass almost all the main extracellular matrix constituents, including collagens, elastin, proteoglycans, hyaluronic acid, and adhesion glycoproteins such as fibrinogen, fibronectin, tenascins, and thrombospondins. Independently of the scaffold, the mechanisms of cell adhesion rely on the deposition of extracellular matrix (ECM) components secreted by the seeded cells [48]. The ECM secretion pattern and the initial sensed resistance of the substrata are coupled to cytoskeletal alterations by a feed-back loop, through the concerted action of selectins, cadherins, and integrins [49, 50]. These mechanosensors and adhesion proteins, in turn, may direct cell differentiation toward a specific lineage. Indeed cells of mesenchymal origin adhere and contract on a variety of different substrates, for example uncoated or collagen-coated acrylamide gels and glass. Such a wide range of recognized surfaces parallels a wide variation in matrix stiffness sensing [51]. The resistance that a cell feels when it deforms the ECM can be measured, and ranges from 0.1 kPa (in soft tissues) to 1.0–20.0 kPa (muscle) to >25.0 kPa for bone. By varying matrix elasticity, Engler and collaborators were able to demonstrate that matrix stiffness can specify the MSC lineage differentiation, regardless of the culturing conditions or nutrients used [52]. Local sensing of force is then actively transduced into biochemical signals that regulate cell shape, growth, differentiation, and even death [53]. Interestingly, nuclear deformations also take place in response to cytoskeletal modifications, cell cycle and division: the nucleus is

quite stiff and resists distortion for brief periods, whereas it undergoes deformation for longer periods, granting the continuous timescale spectrum for varied genome expression kinetics [54] and hence a physiological base for differentiation as a consequence of the adhesion substrate characteristics.

2.3 Topographical Surface Modifications of Scaffolds

Progenitor cell fate is also affected by topographic cues of the scaffolds (i.e. *topological conditioning*). Recently, it has been reported that cells are able to decode the topographic signals of the scaffold and respond to the shape of the microenvironment by priming a specific cell differentiation commitment [55]. Thus, nanostructured biomaterials such as nanoparticles, nanofibers, nanosurfaces, and nanocomposites have gained increasing interest in regenerative medicine, since they offer a temporary ECM for regenerative cells [56–58]. Topography may also be relevant for hydrophilicity and for specific protein adsorption, as shown by the selective take-up of proteins relevant for cell attachment, such as fibronectin and vitronectin, on fibrous meshes with nanoscale fiber diameters [59]. Indeed, the interaction of cells with the surrounding milieu is in the nanometer scale and for prosthetic applications in orthopedics, cell attachment to grooved materials [60] and to nanocrystalline coatings [61] has long been documented. Thus nanoscaled topography of synthetic materials has been tailored to resemble the original surrounding tissue and mammalian cells have demonstrated a response to topographical surface variations [62, 63]. For mesenchymal cells, specific nano-patterning(s) may be compliant with the peculiar distribution(s) of adhesion molecules, mimicking the one that cells would adopt in a specific stiffness/elasticity context of an underlying contact surface. The patterning would "anticipate" the cell response to a specific substratum, thus forcing the consequences of cell adhesion, as in the case of neuronal differentiation of MSC toward neuronal lineages when cultured onto gratings of 350 nm line width [64].

2.4 Micro-environment

The optimization of the interactions between a scaffold matrix and cellular counterparts of the constructs can also be pursued by a contemporary specific biomimetic functionalization and/or nanostructuration of the interface. Clearly, once a cell has somewhat "decoded" its substrate and has ignited a new gene expression program in response to exogenous/endogenous stimuli, the secreted extracellular matrix proteins will modify the microenvironment and further drive the cell along a specific differentiation pathway. Experimental settings, in which passive adsorption of two matrix proteins, vitronectin (VN) and type collagen I (Col I), was tested on polymeric substrates showed that treated substrates mediated MSC adhesion and differently induced activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signal transduction pathways [65]. Hence, the de novo synthesis

and deposition of ECM proteins by MSC alters the chemical identity of the polymeric substrate, altering the integrin expression profiles by a feed-back loop mechanism. These changes, in turn, cause modifications in the MAPK and PI3K signaling pathways, ultimately influencing the osteogenic differentiation of the seeded cells. Larger amounts of fibronectin and Col I and lower levels of VN were in fact deposited on poly(lactic) glycolic acid scaffolds over a 28-day period. Accordingly, cells also provided higher levels for α5β1 and α2β1 integrins (receptors for fibronectin and Col 1, respectively), and reduced levels for αVβ3 integrin (VN receptor). Relevant to the osteogenic differentiation of the cells, adhesion to Col I and fibronectin has been shown to induce the MAPK cascade, in particular the activation of the ERK1/2 system, which is critical for the activation of the osteogenic transcriptional factor Runx2 [66, 67]. Specific integrins then seem to be preferred or even required for the osteogenic differentiation of MSC; however a bio-functionalization of a scaffold surface should not focus on the presentation of a uniform coating to engage a single receptor, but rather identify the properties that control the presentation of integrin-specific epitopes within the coatings [68].

Clearly several chemical–physical modifications can be attempted and performed on almost any specific substrata even in a multiple fashion, provided that the proper chemistry is used. Indeed, many different strategies are currently being tested [69], including simple coatings [70], the contemporary use of genetic engineering and structural approaches [71, 72] and combinations of matrix-mimicking ligands and engineered structured nanomatrices [73]. The same natural extracellular matrix is per se able to induce specific cell commitment [74]. It is not surprising then, that the combination of topographical and chemical cues may result in a synergistic effect, in some cases informative enough to directly address adult MSC stem cells to non-canonical differentiation pathways, such as the neuronal one. Interestingly, the effects of a nano-patterned surface were even stronger than single biochemical induction on controls grown on un-patterned surfaces [64]. The cells are, therefore, major players in tissue regeneration approaches and the successful reconstruction of normal tissue depends on the properly simulated activity of the available progenitors.

3 Will Tissue Engineering and Cell Therapy Still Be Valuable?

In a number of studies, autologous marrow samples have been harvested and osteoprogenitors were isolated and expanded in culture [75]. A critical size segmental defect was surgically created in a long bone. The surgical lesion was filled with biomaterials carrying autologous in vitro expanded osteogenic progenitors. Radiographic and histological analysis of the retrieved specimens

revealed excellent integration of the host bone/implants and an amount of neo-formed bone significantly higher in the scaffolds loaded with osteoprogenitors than in acellular control grafts. The results of these studies were in good agreement suggesting an important advantage in bone formation and therefore, in the healing of the segmental defect when marrow-derived osteoprogenitors were delivered together with a proper biomaterial scaffold. It is surprising that, after initial enthusiasm over very encouraging large animal study results, only two pilot clinical studies have been performed [76, 77]. Although material science technology has resulted in clear improvements in the field of regenerative medicine, no ideal bone substitute has been developed yet and hence large bone defects still represent a major challenge for orthopedic and reconstructive surgeons. A number of bone substitute biomaterials are readily available. The intended clinical use defines the desired properties of engineered bone substitutes. Anatomical defects in load bearing long bones, for instance, require devices with high mechanical stability whereas for craniofacial applications, initially injectable or moldable constructs are favorable. Therefore, the most intriguing concept is the priming of the natural processes of bone regeneration driven by cells, through the use of materials able to mimic a specific pre-existing microenvironment.

An intriguing future alternative, given the advancing knowledge on the biology of stem cells, is going to be recruiting and properly addressing resident stem cells toward a regeneration pathway more than toward a reparation process. This in theory should be possible using appropriate soluble signals, able to deviate cells from a path and redirect them in a desired direction. Alternatively, more recent research has prompted the use of inducible pluripotent stem cells (iPSCs) for disease modeling, drug effectiveness evaluation, and therapeutic applications. The enormous potential for the generation of patient-specific stem cells able to differentiate into any lineage has boosted attempts to resolve their limitations in tissue engineering and regenerative medicine applications: random genomic integration of the transgenes, tumorigenic risk associated with the use of *c-myc*, the potential immunogenicity of autologous-derived iPSCs due to insufficient reprogramming and genetic instability. These severe risk factors have sparked a debate on the use of iPSCs for regenerative medicine applications. However, in order to circumvent these aspects, iPSCs could complete an in vitro differentiation into the needed cell type before transplantation, as suggested by the work of Araki et al. [78]. Nonetheless, epigenetic aberration patterns can be generated following directed differentiation. Therefore, in spite of the low immunogenicity of differentiated cells derived from iPSCs of a syngeneic source, immunogenicity must be thoroughly evaluated for each single protocol intended for clinical translation [79]. Screening and reliable protocols to assess the tumorigenic potential of individual iPSC

lines are also needed. Induced mesenchymal stem cells have already been generated and they maintain the potential to differentiate into osteoblasts, chondrocytes, or adipocytes starting from cord blood CD34+ cells [80]. Interestingly iPSC cells transduced according to the traditional Yamanaka protocol [81] were sensitive to nanotopographical patterning of the culture substrate, linking the previously described effects of topographical-induced differentiation pathways to an epigenetic status of the cell [82].

4 Conclusions

As a whole, a scaffold properly designed for tissue engineering applications must bear a structure planned on different spatial scales, in order to mimic the complex MSC niche [83]. Not all aspects of the niche will be needed to enhance stem cell self-renewal, but the simultaneous presence of many of these, such as chemical and multi-scale architectural cues, will be required to prompt specific cell differentiation and tissue ingrowth. Pre-commitment of MSC grown on a specific matrix cannot be overcome by the presence of soluble factors in the growth medium: indeed proper surface sensing has evidenced the existence of new requirements for progenitor cell lineage differentiation. For example, the osteogenic differentiation of MSC seeded onto electrospun poly(ε-caprolactone)/ECM scaffolds is maintained even if the cell culture medium is devoid of dexamethasone, a molecule normally required in standard osteogenic induction of plastic-adherent MSC cultures [84]. This observation as well as the many others in the field are of paramount relevance for MSC tissue engineering applications, particularly for bone reconstruction applications, where several rounds of ex-vivo cell duplications are needed and are normally performed on standard disposable culture plasticware. In this respect, recent lines of research have evidenced that the sensitivity of stem cells to the mechanical microenvironment is indeed a new parameter that must be considered when addressing induction strategies and the physical in vivo and ex vivo microenvironments for tissue engineering applications. All these approaches and specific aspects (scaffold stiffness compliance, surface topography and tri-dimensionality, scaffold chemistry) will have to be integrated into scaffold engineering to properly foster tissue regeneration. Whether this is feasible remains to be seen, given the high level of complexity of the dynamic interactions among the different components. These aspects, however, have become even more relevant, particularly if the same pluripotent progenitor cells are used for multiple tissue repairs within tissue engineered composites, such as in the case of osteochondral defects. Significantly, recent findings have also raised the possibility that an injured microenvironment may lose compliance, due to insufficient sensitivity and remodeling

options of stem cells once in a non-inducing environment, such as a fibrotic scar [85]. Given the influence of the microenvironment on repair outcomes, then, an additional challenge will also need to be addressed: to provide the proper cell "pre-commitment" in vitro to partially overcome an inappropriate pathological microenvironment in vivo, at the lesion site.

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