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Historic and current strategies in bone tissue engineering: Do we have a hope in Hench?

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Abstract Professors Larry Hench and Julia Polak formed the Tissue Engineering and Regenerative Medicine Centre (TERM) at Imperial College London to foster collaborations between biologists and materials scientists. Early work at the center elucidated the biomolecular interactions between primary human osteoblasts and 45S5 Bioglass®. As research efforts expanded, the team discovered that the dissolution products of both 45S5 Bioglass® and 58S sol-gel bioactive glasses had osteoblastic stimulatory properties. To address the shortage of appropriate cells for bone tissue engineering applications, TERM scientists also demonstrated the differentiation of embryonic stem (ES) cells to osteoblasts when treated with the dissolution products of bioactive glasses. They also found that the soluble factors ascorbic acid, β-glycerophosphate, and dexamethasone preferentially differentiated ES cells to osteoblasts, and their combination with the dissolution products of bioactive glasses stimulated differentiation even further. Taken together, these results demonstrate the suitability of bioactive glasses as scaffolds for bone tissue engineering as they not only provide an osteoconductive and osteoproductive substrate, but also actively stimulate cells to express appropriate osteoblastic phenotypes. Professor Hench's vision to pioneer regenerative medicine research continues with the aim of developing novel therapeutics to treat musculoskeletal disability.

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1 History of TERM

Professor Dame Julia Polak and Professor Larry Hench, both internationally-renowned scientists in their respective fields of pathology and materials science, initiated a major collaboration in 1997 in an effort to pioneer regenerative medicine research at Imperial College London. Their first collaborative project entitled "*Cellular Mechanisms of the Observed Enhancement of Bone Growth by Bioglass*" funded a Ph.D. student and prompted a continuing partnership that resulted in the formation of a major research center. Imperial College established the Centre for Tissue Engineering and Regenerative Medicine (TERM) in July of 1999 at the Chelsea and Westminster Hospital with Professor Polak as the director and Professor Hench as the co-director. The center raised more than £5 million in research funds in its first three years and has since published over 150 research papers. While work at the center initially focused on *in vitro* interactions between bioactive glasses and osteoblasts, stem cell differentiation, and cell-based therapies to repair damaged lung epithelium, research has since expanded to include *in vitro* cultivation of cartilage and cardiac tissues as well as bioprocesses to scale up the production of engineered cellular constructs for clinical applications. The center continues to build on Professor Hench's vision to bring together internationally-renowned expertise in biomaterials, stem cell biology, and tissue engineering to usher in a new era in regenerative medicine. This review focuses on work conducted at the center to examine cell-bioactive glass interactions and stem cell differentiation to osteoblasts with the ultimate goal of creating functional and effective bone graft substitutes.

2 Bone tissue engineering

Traumatic injury as well as tumor removal often results in debilitating musculoskeletal defects. Approximately 500,000 bone grafting procedures are performed each year in the United States [1] and over 2.2 million worldwide [2]. As the population ages, musculoskeletal-related disability will become more prevalent, and will likely exceed the staggering current costs (direct and indirect) of over US$240 billion each year in the United States alone [3]. The "gold standard" for treatment of bone defects and non-healing fractures involves surgical reconstruction with an autograft, which can provide limited support, fill voids, and enhance the natural biological repair mechanisms of the bone [4]. Autologous bone, however, is often in limited supply, is not always appropriate, and has been associated with donor site morbidity [5–7]. Allogenic bone, which is also frequently utilized, is not an ideal substitute either as adverse host immune reactions [8], lack of integration [9], and disease transmission [10] have all been reported. Given the limitations of current treatment options and the need for improved clinical outcomes, the development of effective and readily available bone graft substitutes is a medical necessity. An engineered bone substitute that can mimic the native structure and replace the mechanical and biological functions of the natural tissue would be ideal to meet this need.

Many researchers have described the "holy grail" of bone substitutes as an engineered tissue composed of a mechanically sound, biocompatible, bioresorbable scaffold that incorporates a sufficient number of appropriate, functional cells. Upon implantation the engineered graft would ideally resorb and serve as a guide to regenerate the native tissue [11–13]. The search for this ideal scaffolding material and the source and means to provide the constituent cells, however, remains elusive. Researchers have explored a number of biologically-derived scaffold materials for bone applications including collagen [14–16], fibrin [17], hyaluronic acid [18], chitosan [19, 20], alginate [16, 21, 22], hydroxyapatite [23], and agarose [16], among others. While these biologically-derived materials tend to be biocompatible and biodegradable, concerns regarding mechanical strength, degradation rates, and batch-to-batch variability have called their use into question. As an alternative, synthetic polymers have also been extensively explored. The FDA-approved copolymer poly(lactic-co-glycolic acid) (PLGA) [24–30], as well as other poly(α-hydroxy) esters [31, 32], have been widely studied as scaffolds for bone tissue engineering. Other synthetic polymers such as poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHVB) [33], poly(ε-caprolactone) (PCL) [34], and poly(ethyl methacrylate) (PEMA) [35] have also been examined. Many synthetic polymers, however, require potentially cytotoxic organic solvents in manufacturing, have poor surface attachment properties, create acidic byproducts upon degradation, and may not be suitable for bone applications leaving the field without an ideal alternative.

Tissue engineering strategies, however, require more than simply an osteoconductive scaffold. Therefore, much research has likewise been devoted to exploring cell sources for bone applications. Marrow-[36–39] and adipose-[40–42] derived and embryonic [38, 43] stem cells have been examined by a number of research groups. Embryonic stem cells can likely proliferate indefinitely and have the potential to differentiate into cell types from all three germ layers [44]. Current derivation protocols, however, can only produce allogenic lines which may elicit an immune response upon implantation. Autologous stem cells, on the other hand, are inherently non-immunogenic but are difficult to isolate and likely lack the pluripotency of stem cells derived from embryos. Current differentiation protocols are not ideal in either case as they fail to yield pure populations of osteoblasts. Adult cells, such as primary cultures of osteoblasts have also been explored [31, 35, 45], but it is difficult to obtain sufficient quantities of such cells for therapeutic use.

While many of the scaffolding materials currently being explored for bone tissue engineering provide a structural support and biocompatible surface for cell adhesion and proliferation, they often lack a means to guide constituent cells towards an appropriate phenotype and functionality. That is, they lack chemical, mechanical, or electrical stimuli that may guide tissue-specific cellular responses and differentiation. Even polymers specifically designed to deliver bone-inducing molecules to constituent cells may still suffer from pharmokinetic loss of protein as the scaffold is biologically and physically degraded. Bioactive glasses, on the other hand, form a hydroxyapatite layer when exposed to body fluids and bond to living bone *in vivo* [46]. Since their discovery in the late 1960's, bioactive glasses have been known to be osteoconductive [46] and osteoproductive [47]. However, they have also been found to be osteoinductive [48], and do not induce local or systemic toxicity, inflammation, or a foreign body response [49]. In short, bioactive glasses possess many of the qualities associated with an ideal scaffolding material for a bone graft substitute. Under the direction of Professor Hench, researchers at TERM have examined the interactions between bioactive glasses and osteogenic cells in an effort to elucidate the role of their interactions with one another and take the necessary steps to create effective, functional bone graft substitutes.

3 Research conducted at TERM

3.1 45S5 Bioglass®

As an effective engineered bone substitute will provide an environment for appropriate cell proliferation and

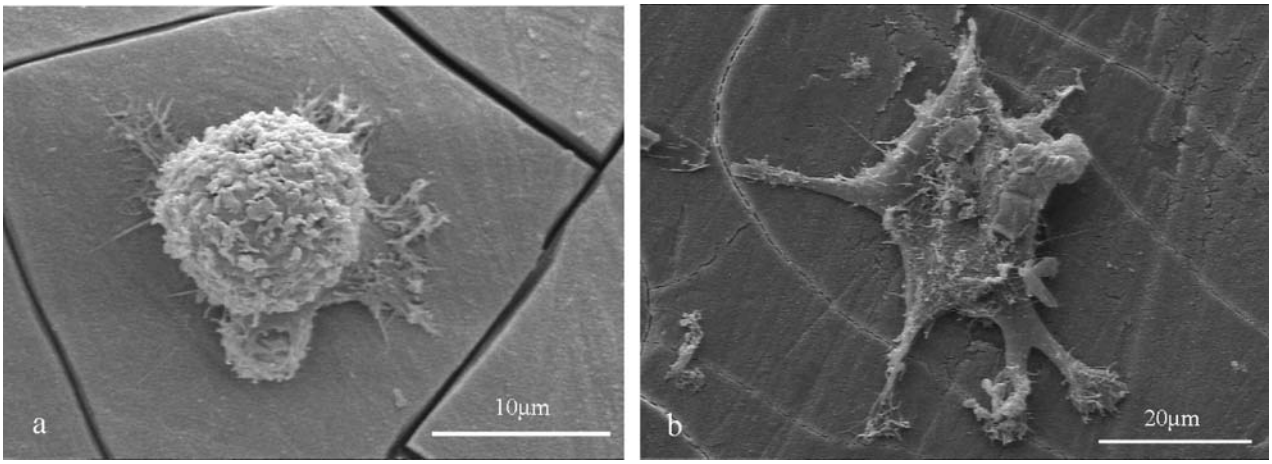

Fig. 1 (a) Scanning electron micrograph of human primary osteoblast cultured on a 45S5 Bioglass® disc for 2 days. (b) Osteoblast in contact with 45S5 Bioglass® disc after 6 days in culture. The osteoblast has assumed a more flattened morphology and is anchored to the substrate by multiple lamelliodia. With kind permission of Springer Science and Business Media [50]

differentiation, early work at TERM focused on evaluating the use of Bioglass® as a scaffold to induce bone formation *in vitro*. One of the first TERM collaborative efforts with Professor Hench examined human primary osteoblasts cultured for up to 12 days on 45S5 Bioglass® discs (Fig. 1). Xynos *et al.* demonstrated that the human osteoblasts cultured on Bioglass® had an enhanced proliferation potential when compared to osteoblasts grown on tissue culture plastic. Their results were confirmed by flow cytometric analyses, which showed increased cell populations in both the S and G2/M phases of the cell cycle [50]. Similarly, osteoblasts cultured on Bioglass® demonstrated an augmented commitment to the osteoblastic phenotype and a striking ability to form mineralized collagen nodules. That is, the study correlated osteogenesis on a bioactive substrate with a sequence of biological events involving cell proliferation, differentiation, and morphology.

Although the mechanism by which Bioglass® affects cells is uncertain, it has been hypothesized that its osteoinductive capabilities arise from a combination of both cell-substrate contact as well as through the release of soluble ions. After finding that culturing human primary osteoblasts on Bioglass® scaffolds caused increased cell proliferation, osteoblastic commitment, and the formation of bone nodules, the research team at TERM explored the effects of the dissolution products of Bioglass® on human primary osteoblasts. By culturing primary human osteoblasts in Bioglass®-conditioned medium, Xynos and colleagues were able to demonstrate that the dissolution products alone stimulated cell proliferation and the upregulation of a number of genes, including the bone mitogenic growth factor, IGF-II [51]. Based on these results the authors proposed that the increased cell proliferation seen in osteoblast cultures was likely a result of IGF-II activation mediated by the ionic dissolution products of 45S5 Bioglass®.

Given these results, Xynos and colleagues expanded their previous efforts to explore the effects of the dissolution products of 45S5 Bioglass® on human primary osteoblast cultures using cDNA microarray techniques [52]. They discovered that the dissolution products of Bioglass®—Ca, P, and Si—had a direct effect on the gene-expression profile of the cultured osteoblasts. Most notably, genes known to be involved in osteoblast metabolism and bone homeostasis were upregulated. Extracellular matrix regulators such as metalloproteinases-2 and-4, as well as cell surface receptors CD44 and integrin $\beta 1$, were also upregulated in addition to genes that affect the cell cycle such as *RCL* and *G1/S specific cyclin D*. These results, reviewed by Professor Hench in 2004 [53], offered new insights into the mode of action of bioactive glasses in the induction of bone formation *in vitro* and possibly *in vivo*.

3.2 58S sol-gel bioactive glass

Despite the plethora of favorable data indicating its effectiveness as a potential scaffold for bone graft substitutes, 45S5 Bioglass® has been criticized for a number of its limitations such as its high processing temperatures, narrow range of bioactive compositions, and limited textural features [54–57]. Therefore, other bioactive glass materials with enhanced properties have been proposed as alternatives. Professor Hench, in collaboration with researchers at TERM, expanded the group's original focus and began examining bioactive glasses created with the sol-gel processing technique (see review [58]). When compared to the 45S5 system, sol-gel bioactive glasses have higher rates of apatite-layer formation, exhibit more rapid bone bonding, show improved homogeneity and purity, and have excellent degradation/resorption properties [59–61]. Some of the first work

examined the effect of the dissolution products of 58S sol-gel bioactive glass on human osteoblasts derived from fetal long bones [62]. Preliminary studies of osteoblastic marker gene expression at the mRNA level indicated that specific ranges of concentrations may enhance the osteoblastic phenotype. A more thorough analysis with gene microarrays demonstrated that a single 24-hour treatment of human fetal osteoblasts with the dissolution products of 58S sol-gel bioactive glass resulted in over 10,000 transcripts expressed by the cells [63]. These transcripts included osteoblast-related genes that code for growth factors and their associated receptors, protein components of the extracellular matrix (ECM), enzymes involved in ECM degradation, as well as transcription factors and other osteoblast-associated markers.

Given these results, the team continued to examine 58S sol-gel bioactive glasses. Bielby *et al.* cultured primary human and murine osteoblasts in 58S-conditioned medium that contained known concentrations of Si, Ca, P, and Na [64]. The authors noted increased cell proliferation and increased bone nodule formation in both species. Apoptosis, however, was stimulated in murine cultures treated with glass-conditioned medium, but inhibited in human osteoblast cultures. The authors proposed that in murine cultures, the increased apoptosis may have correlated with temporal changes in cell populations normally associated with the formation of fracture callus. In the human cultures, they hypothesized that the lack of bioactive glass-induced apoptosis may, in fact, help maintain mitogenically active osteoblast populations, even in elderly patients. Perhaps more importantly, however, the authors demonstrated that the 58S sol-gel bioactive glass system was at least as suitable as the melt-derived 45S5 system, but without its drawbacks, as a potent material for inducing osteoblastic differentiation.

While primary cultures of osteoblasts are appropriate for examining the suitability of a particular biomaterial for clinical applications, their use in engineering a bone graft has been called into question. Autologous cells, while potentially appropriate, are difficult to obtain in sufficient quantities for therapeutic use. Allogenic cells, on the other hand, may present an immune reaction and procuring enough cells is likely still prohibitively difficult. Embryonic stem (ES) cells derived from the inner cell mass of a pre-implantation blastocyst, however, have remarkable proliferative potential and the ability to reproducibly differentiate into various cell lineages [44, 65]. Therefore, the TERM research group, under the direction of Professor Hench, next examined the differentiation of murine ES cells exposed to the dissolution products of 58S sol-gel bioactive glass [66]. Bielby *et al.* reported that addition of the extracts from the dissolution of the bioactive glass was as effective in stimulating osteogenic differentiation as the addition of the glucocorticoid dexamethasone, a known factor used in osteogenic differentiation of ES cells [67, 68]. Analyses of mineralized nodule formation, alkaline phosphatase activity, and gene expression further demonstrated that the combination of sol-gel bioactive glass dissolution products with dexamethasone enhanced the differentiation of the ES cells to the osteoblastic lineage even further. These results marked a significant step forward in research efforts into the therapeutic uses of bioactive glasses. That is, they demonstrated that bioactive glasses were not only valuable as a three-dimensional matrix for cell attachment, growth, and differentiation, but also as a guide to assist in the differentiation and derivation of specific cell types—an important step in the development of an effective bone graft substitute.

3.3 *In vitro* differentiation of stem cells

As researchers at TERM realized the importance of generating large quantities of functional osteoblasts for engineering bone graft substitutes, they continued their focus on the directed differentiation of ES cells. In their 2001 publication, Buttery and colleagues detailed a consistent and reliable method to differentiate murine ES cells to mineralizing osteoblasts [68]. They discovered that the addition of ascorbic acid, β-glycerophosphate, and dexamethasone resulted in the formation of discrete mineralized bone nodules surrounded by an extracellular matrix marked by the presence of osteocalcin and collagen type 1. Interestingly, they also found that co-culture of murine ES cells with primary calvarial fetal osteoblasts likewise stimulated osteogenic differentiation. A later study examining gene expression with cDNA microarray techniques and RT-PCR also determined that the addition of these particular soluble factors resulted in the upregulation of *osteopontin, HSP-47,* and *IGF-II,* genes known to be involved in osteoblast differentiation [69]. They also reported the downregulation of genes involved in ES cell differentiation to other phenotypes. Most notably, *Stra-13* or hematopoietic specific protein E3, a gene known to be associated with neuronal differentiation was substantially downregulated. Moreover, by labelling the cells with the antibody cadherin-11, the authors were able to magnetically sort the cells and obtain a purified population of cells with specific osteoblastic characteristics. These results detailed a straightforward method to create a potentially unlimited supply of functional osteoblasts with the potential for therapeutic use.

When Bielby and colleagues, also in TERM, applied these same methods to human ES cells, they were able to obtain mineralizing osteoblasts as determined by alizarin red S staining and immunostaining with osteocalcin, as shown in Fig. 2 [67]. When seeded on poly(D, L-lactide) (PDLLA) scaffolds and implanted subcutaneously in SCID mice, the differentiated cells gave rise to mineralized tissue that stained positively for human osteocalcin. The transfer of these protocols from mouse ES cells to human was an important step in establishing the feasibility of using ES cells for bone graft

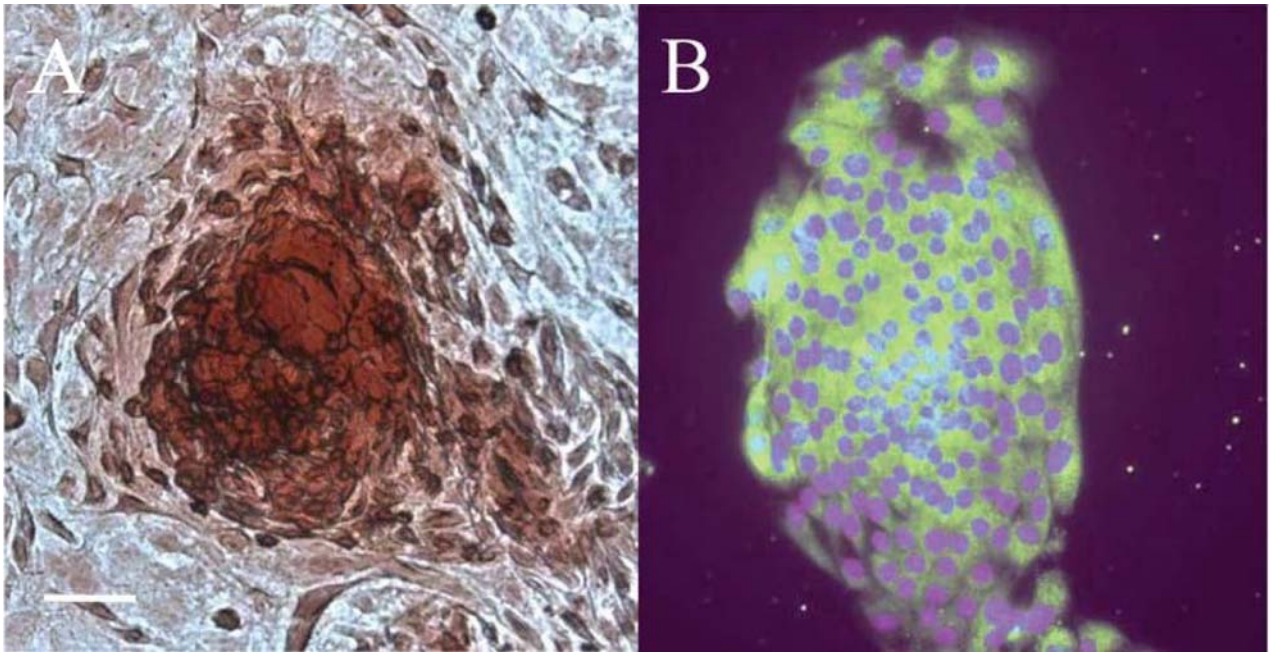

Fig. 2 (A) Mineralized bone nodule formed by differentiated human ES cells after 21 days in culture. Alizarin red S stain. Scale bar: 40 μm. (B) Immunostaining of bone nodule formed by human ES cells maintained in culture for 21 days. The human isoform of osteocalcin appear green while the DAPI nuclear counterstain is blue. Confocal laser scanning micrograph. Original magnification: 100×. Reproduced with kind permission of Mary Ann Liebert, Inc., Publishers [67]

substitutes—risks associated with xenogenic transplants may be insurmountable in human therapeutic use of stem cells. Furthermore, the demonstration that human ES cells mineralize in three-dimensional scaffolds *in vivo* marked an important step in demonstrating the feasibility of constructing bone graft substitutes *in vitro* prior to *in vivo* implantation.

The major drawback of manipulating cell culture conditions to differentiate cells towards a particular phenotype, however, is that the proportion of cells generated with such protocols can be relatively low. Therefore, research at TERM also considered genetic manipulation of murine ES cells in order to make them overexpress osterix, a transcription factor specific to bone. Tai *et al.* found that osteoblastic-specific gene markers were upregulated and bone nodule formation was significantly increased in cultures that overexpressed osterix [70]. Interestingly, the authors also found that *Sox-9* and *PPAR-*γ, genes associated with chondrocytic and adipocytic differentiation, respectively, were also upregulated early on, but then downregulated after 7 days in culture. These results suggest that the differentiation of murine ES cells proceeds through a mesodermal progenitor population before entirely committing to the osteoblast lineage. To explore this angle further, Tai and colleagues transfected various ES cells lines (human and murine) with an Osx-GFP fusion protein reporter system and definitively demonstrated that osterix regulates and commits precursor cells to the osteoblast lineage and prevents them adopting other mesodermal phenotypes [71].

4 Future directions

Current research at TERM continues to examine ES cell differentiation to osteoblasts and novel methods to create sufficient numbers of cells for therapeutic applications. The TERM research team also continues to explore the effects of the dissolution products of bioactive glasses on stem cells. Ongoing projects are attempting to evaluate the effects of specific dissolution products on murine ES cells in an effort to elucidate the specific the means by which bioactive glasses induce osteogenesis *in vitro*.

While the past six years have witnessed massive leaps in the field of bone tissue engineering, the goal of creating a functional, implantable, engineered bone substitute still remains elusive. Bioglass® and other bioactive glasses would seem an ideal substrate for enhancing bone growth and directing cell differentiation *in vivo* and *in vitro*, however, the mechanical properties of scaffolds created with these materials are insufficient for load-bearing applications because the ceramic is inherently too brittle. Composite materials that combine synthetic polymers with bioactive glasses may be an alternative as they show improved mechanical properties and allow for mineralization at their surface [72, 73]. Their surface properties and cellular interactions, however, still need to be evaluated further, and they may still lack sufficient bending and compressive properties to repair fractures in long bones.

The provision of an adequate number of functional cells remains an obstacle as well. While both adult and embryonic stem cell populations can be cultured and produce bone *in vitro*, using current methods to produce such cells in sufficient numbers to create a bone graft for a single patient would require an exorbitant amount of labor, supplies, and time. Better methods to bioprocess clinically useful numbers of appropriate cells are obviously required. Finally, as in all areas of tissue engineering, size remains a problem. Current engineered tissues are inevitably limited in size by diffusion. That is, constructs more than a few millimeters in any dimension suffer from necrosis because of limited nutrient transport. Successful tissue substitutes will have to overcome this limitation to achieve a therapeutic benefit.

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