Review Article

Skeletal Regeneration: application of nanotopography and biomaterials for skeletal stem cell based bone repair

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The application of selected skeletal progenitor cells and appropriate biomimetic microenvironments and nanotopographical surfaces offer the potential for innovative approaches to bone disease treatment and bone regeneration. Skeletal stem cells, commonly referred to as mesenchymal stem cells or human bone marrow stromal stem cells are multipotent progenitor cells with the ability to generate the stromal lineages of bone, cartilage, muscle, tendon, ligament and fat. This review will examine i) the application of innovative nanotopography surfaces that provide cues for human stem cell differentiation in the absence of chemical cues, ii) unique biomimetic microenvironments for skeletal tissue repair as well as iii) data from translational studies from the laboratory through to the clinic demonstrating the potential of skeletal cell based repair using impaction bone grafting as an exemplar. The development of protocols, tools and above all multidisciplinary approaches that integrate biomimetic materials, nanotopography, angiogenic, cell and clinical techniques for skeletal tissue regeneration for *de novo* tissue formation offers an opportunity to improve the quality of life of many.

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Introduction

Skeletal tissue engineering is set to play an important role in addressing the challenges of bone regeneration in an ageing population to improve human health through prevention of disease and reparation of skeletal tissue and function. The clinical burden is significant with fractures alone costing the European economy €17 billion and the US economy $20 billion annually. Furthermore, in the US,

there are over 8 million bone fractures of which approximately 5% to 10% are associated with delayed healing or non-union. This is further compounded by recent data indicating osteoporosis affects an estimated 75 million people in Europe, USA and Japan; while it is projected that the worldwide incidence of hip fractures will increase by 310% in men and 240% in women by 2050[1]. Thus, in combination with bone loss due to trauma, tumor resection or an inability to heal due to disease or old age there is an urgent clinical need for the development of skeletal reparative strategies to address this healthcare burden. Bone tissue engineering and regenerative medicine seek to address this challenge utilizing a raft of interdisciplinary approaches including developmental biology, materials science, stem cells and bioengineering to harness the therapeutic potential of skeletal stem cells together with an appropriate scaffold, factors and an appropriate conditioned environment (bioreactor or *in vivo*). The aim being to generate a three-dimensional living tissue construct that is functionally structurally and mechanically equivalent to, if not superior to the tissue it has been designed to replace. However, a key issue in the success of bone regeneration is the source of stem cells and the absence of a definitive marker for skeletal stem cell populations; this has restricted their widespread clinical application. Similarly, scaffolds that can support bone tissue formation and modulate stem cell differentiation along appropriate lineages in combination with angiogenesis and niche development for bone will be important in delivering on cellular-based skeletal regenerative applications. Thus, an ideal scaffold for skeletal tissue regeneration would not only promote skeletal stem/ progenitor cell attachment, viability and growth, but importantly would aid differentiation of this progenitor population into a population of cells capable of bone formation.

The osteoblast, the cell responsible for bone formation, is derived from a multipotential marrow stromal cell which has been shown to support bone formation and hematopoietic marrow[2]. The term, mesenchymal stem cells (undifferentiated multipotent cells of the mesenchyme) has gained wide acceptance, although this term is nonspecific and the term skeletal stem cell (SSC) will be used throughout this review to restrict description to stem cells from bone marrow able to generate all skeletal tissues[3, 4] as, to date, the ability for regeneration or maintenance of a non-skeletal tissue compartment *in vivo* remains to be rigorously demonstrated and remains controversial. A number of studies have proposed positive selection of skeletal stem cells on the basis of an increasingly large panel of markers including CD71 (transferrin receptor), CD63, CD49a (Integrin alpha 1), CD44, the STRO-1 antigen and adhesion molecules, such as CD166 (ALCAM), CD146 (MCAM), CD106 (VCAM-1), CD54 (ICAM-1) and CD29 (Integrin beta 1)[5-7] though as yet, no single marker, or combination, defines the cell-surface profile of a demonstrably homogeneous multipotential skeletal stem cell population[8]. We routinely use the monoclonal antibody STRO-1 to immunoselect a distinct sub-population of bone marrow mononuclear cells that is enriched for multipotent clonogenic progenitor cells with bone forming capacity, as demonstrated using diffusion chambers that provide a unique closed environment[9]. This brief review will focus on the use of enriched human skeletal stem cell populations together with i) determination of innovative hydrogel regenerative microenvironments, ii) application of nanotopographical cues to modulate stem cell function in the absence of chemical cues and iii) translational and clinical developments from small animal studies through to patient studies using impaction bone grafting as an exemplar.

Hydrogel Strategies for Regenerative Microenvironments

The application of hydrogels to bone repair reflects a shift in the conceived role of biomaterials in orthopedics. Traditionally biomaterials have been applied in orthopedic contexts as bone substitute or bone filler materials for which long-term integration with existing bone and equivalent mechanical strength are fundamental design criteria. However as biomaterial research increasingly focuses on the development of biomaterials as matrices for bone regeneration that serve to deliver cells and/or growth factors to the site of damage and provide an appropriate microenvironment for bone regeneration, radically different functional properties are being specified[10, 11]. For example, while weight bearing functionality may be a useful property for an orthopedic regenerative strategy, it is arguably not a prerequisite specification as ultimately such functionality is to be provided by the regenerated tissue and in many contexts temporary support can be achieved via alternative orthopedic techniques[12]. Despite, therefore, possessing negligible potential for weight bearing functionality hydrogel technology is increasingly being applied to orthopedic problems due to the considerable potential of hydrogels

as matrices for regeneration.

1)Hydrogel structure and delivery

In their broadest definition hydrogels are highly hydrated, three dimensional networks of large organic molecules or small inorganic particles formed by physical or chemical interaction[13]. The high water content (>90%) of hydrogels facilitates diffusion of oxygen and nutrients and contributes to the biocompatibility of the material suggesting hydrogels as excellent candidates for tissue regeneration matrices[14, 15]. Hydrogels can be categorized according to their microstructure as per the distinctions proposed by Flory[16] between; 1) covalently bonded polymer networks; 2) polymer networks formed through physical aggregation of polymer chains; and 3) ordered lamellar structures (as in the mesophases of inorganic clays). Considerable attention has been paid to the mode of gelation of hydrogels, with a major goal being the development of injectable hydrogels[17, 18]. As well as allowing for minimally invasive delivery of cells and molecules, injectability and gel-formation *in situ* allows for regenerative constructs to effectively fill spaces, and perfuse porous structures, such as bone graft material, without requiring elaborate prefabrication procedures.

The different microstructures of physically networked and covalently networked polymer hydrogels are analogous to their mode of gelation and typically give rise to altered mechanical properties. Both these characteristics are of relevance to tissue engineering. The networks of physical polymer gels are formed of various reversible links including molecular entanglements, ionic interactions, hydrogen bonds, hydrophobic associations and Van der Waals forces[14, 15, 17]. These associations are non-permanent or reversible as they can be formed or disrupted by physical changes such as pH, temperature and ionic strength[19]. The reversible nature of these networks facilitates minimally invasive delivery through the mixing and injection of gel/cell/factors prior to gelation *in situ*[19, 20]. Thermoresponsive gelation is a widely studied approach to the formation of physical hydrogels where a sol-gel phase transition is engineered to occur as body temperature is approached[21]. For example, Triblock copolymers, using various combinations of synthetic molecules such as poly(l-lactic acid) (PLLA), poly(lactic-co-glycolic acid), (PLGA) and poly (ethylene glycol) (PEG) (e.g. PLGA-PEG-PLGA or PEG-PLLA-PEG), have been widely applied in tissue regeneration approaches. Phase transition to a macroscopic gel is achieved through the sequential assembly, bridging and packing of micelles in response to an increasing temperature[17, 21].

The ability of physical hydrogels for biocompatible *in situ* gelation is a significant advantage, however the physical and ionic cross-linking mechanisms, particularly in naturally derived molecules such as collagen or fibrin, are difficult to control and can result in gel inhomogeneities complicating the regenerative outcome[17]. In contrast, while the process of chemical cross-linking and the toxicity of certain cross-linking agents creates challenges for in situ gelation, chemically cross-linked hydrogels enable considerably more control over the micro-structure of the gel allowing for mechanical properties which can be tailored according to the number of crosslinks in the network and, depending on the nature of the crosslinks, longer degradation times[22]. Synthetic polymers such as poly(ethylene glycol) (PEG), poly(propylene fumurate) (PPF), and poly(N-isopropylacrylamide) (PNIPAAM), in particular provide versatile platforms, and have been extensively developed for regenerative medicine applications[23]. Thus various cross-linking approaches compatible with good cell viability have been developed. Photoinitiated polymerisation of, particularly PEG macromolecular monomers, are particularly well studied, though the reliance on a photo-source for activation of free-radicals may not be suitable for deep-tissue applications[17, 23]. Other approaches include Micheal-type conjugate addition reactions, Schiff-based reactions and the use of the cross-linker genipin, all of which allow for non-cytotoxic gel-network formation[17].

2)Hydrogels for regeneration

In addition to providing a route for the delivery of stem-cells, the tissue regeneration matrix serves to provide a regenerative micro-environment, or niche, directing cell behavior[24]. Critical to this function is the ability to control the presentation, in space and time, of bioactive molecules that direct the growth and differentiation of progenitor populations. In addition to concerns to enhance efficacy, systemic toxicity is a risk due to the multiple bio-efficacy of many growth factors and so controlled local delivery is also important in relation to safety[25]. This however constitutes a considerable challenge, as the open polymer networks that characterize many hydrogels typically result in the rapid release of incorporated soluble molecules. For example

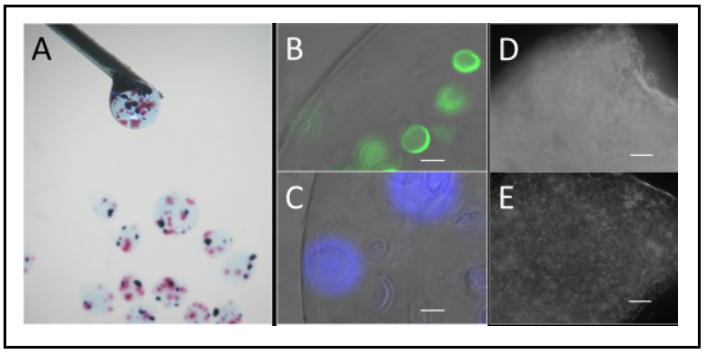


Fig.1 Clay base hydrogels for tissue regeneration
Injectable suspensions of clay nano-particles self organize into gels via electrostatic interactions allowing encapsulation of cells and proteins (A). Sub encapsulation of microcapsules containing FITC labeled lysozyme (B) and DAPI labeled dsDNA (C) after 5 days indicated alternate protein distributions related to the different electrostatic properties of the encapsulated molecules. Co-addition of the matrix molecule fibronectin enhanced the matrix secretion of encapsulated bone marrow stromal cells (D) compared with controls (E).

one study calculated the diffusivity of the relatively large molecule, bovine serum albumin, encapsulated in a hyaluronic acid/PEG hydrogel to be little under 10% of it's diffusivity in water[26, 27].

Thus various approaches have been developed to modify hydrogels to control the release of encapsulated molecules. Such modifications have included, increased cross-link density[26, 28], incorporation of charged or lipophilic sections and functional groups[29-31] or co-encapsulation of charged or lipophilic micro-particles[32, 33].

We have recently investigated the potential of gels formed by the electrostatic interactions of clay nano-particles to localize biological molecules[34]. Clay-protein interactions, via cation exchange, hydrophobic and interlamellar mechanisms, have long been harnessed, typically in tablet form, to delay or localize the action of therapeutic molecules[35, 36]. Using low concentration (1.5-3%) hydro-dispersions of laponite, which self-organize in response to an ionic media, we observed minimal release of encapsulated protein over 72 hours, and conversely a rapid uptake of protein from the surrounding media. This high sorptive potential allowed the co-localization of the adhesion molecule fibronectin, and the angiogenic factor vascular endothelial growth factor 165 ($VEGF_{165}$) to induce an angiogenic response *in vitro* and in a murine defect model[34]. The facility for *in situ* self-assembly in response to physiological saline together the capacity for protein localization without the need for complex chemical/physical approaches offers a simple yet powerful means to develop and deliver microenvironments for tissue regeneration (Fig.1).

In addition to encapsulation, direct covalent immobilization of growth factors is also an important means of achieving growth factor localization[37, 38]. As well as minimizing non-target effects, immobilization of the growth factors can, in fact, enhance local performance. Bentz et al.[37] demonstrated an enhanced fibroblast response (resulting in new collagenous connective tissue deposition) when transforming growth factor beta 2 (TGF-β2) was conjugated to collagen via a PEG based chain as compared to admixed formulations of collagen and TGF-β2. Furthermore, such an approach, provided for the incorporation of enzymatically-degradable elements in the design of hydrogels thus allowing for cell mediated scaffold degradation and growth-factor release[39]. One such approach utilized a matrix metalloproteinase (MMP) degradable PEG scaffold to control VEGF delivery and allow invading local endothelial cell-mediated release of the growth factor[40].

In vivo, the extracellular matrix, not only mediates the diffusion of chemical and biological signals, but is further associated with directing cell growth and differentiation via direct interaction with cell surface receptors. Thus, for example, Type I collagen, the major organic component of bone extracellular matrix, is chemotactic to fibroblasts possessing high affinity cell binding domains and type I collagen-specific binding has been found to mediate the osteogenic response of human bone marrow stromal cells[3, 41, 42]. Due to their hydrophilic nature hydrogels do not readily absorb the biological molecules that direct cell behavior. While presenting a challenge for the manufacture of biological environments, this also constitutes an opportunity for the bottom-up construction and assessment of biological environments with minimal biological interference from the hydrogel scaffold[43]. Common approaches have involved covalently incorporating into the polymer network proteins or peptide sequences. Matrix and adhesion molecules such as fibronectin[44, 45] and the RGD peptide (Arginine — Glycine — Aspartic acid), ubiquitous in extracellular matrix and promoting integrin-receptor type binding to most cell types[46-48], have been extensively studied in this respect. Recent approaches have combined pep-

tide sequences that self-organize into hydrogels with an RGD-based peptide and a peptide sequence mimicking the molecule VEGF allowing a hydrogel that is able to self-organize in situ and stimulate angiogenesis[49, 50].

The use of two-photon chemistry that can allow complex protein binding patterns under the control of a multiphoton confocal laser, has provided a basis to control the concentration of biological molecules over the range of 10-20 μm in three dimensions[51]. This approach, in which chemical binding sites are patterned by photo-chemistry and then subsequently flushed to bind the biomolecule of interest, was used to create a 3D gradient of immobilized $VEGF_{165}$ to induce a chemotactic response in endothelial cells[52]. This approach was further developed by utilizing different orthogonal physical binding pairs to allow the simultaneous spatial control of two different growth-factors involved in retinal precursor cell differentiation, sonic hedgehog and ciliary neurotrophic factor[53]. The use of photo-chemistry has also been developed to enable control over the temporal presentation of biological molecules to encapsulated cells *in situ*. A recent study has demonstrated the ability to utilize two different photo-initiated reactions, responsive to different wave-lengths, as above in a tightly spatially controlled manner, to control the alternate binding and release of an RGD peptide to allow single cell-level control of adhesion events[54, 55].

A further recent development in the application of hydrogel technologies to bone repair, is the incorporation of inorganic components into the hydrogel to provide nucleation sites for mineralization[56]. Bone matrix itself incorporates within a continuous collagenous organic phase a dispersed calcium phosphate inorganic phase in the form of hydroxyapatite (HA). As well as imparting mechanical strength, HA provides an important mode of localizing osteogenic signaling molecules in bone matrix. The incorporation of an inorganic phase into hydrogel matrices is therefore likely to constitute an important step towards the development of a regenerative microenvironment for bone repair.

The micro-environment that fosters stem cell mediated tissue regeneration consists of the structural proteins of the extra-cellular matrix, and the tightly regulated soluble signals that perfuse it. The unique facility of these hydrogel strategies for self-assembly, cell delivery and retention of the vital extracellular components provides considerable potential for the bottom-up assembly and *in vivo* application of such skeletal regenerative microenvironments.

Nanotopography for stem cell research and regenerative medicine

The importance of the physical environment including topography[57, 58], stiffness[59] and chemistry[60-62] in the regulation of stem cell fate has become widely recognized. However, the notion that topography can influence cell fate *in vitro* is not a new concept. In the 1940s, Weiss[63] reported on the orientation of cultured cell axons. Later, Curtis and Varde attributed alignment to a cellular response to topographical cues[64]. Approaches using surface topography, in particular nanoscale topography, to direct the differentiation of adult skeletal stem cells and embryonic stem cells are largely informed by the *in vivo* environment. For example, 2-50nm mineral grain dimensions of woven and lamellar bone have been reported at sites of bone turnover[65]. Bone apatite and collagen composite provide a rich topographical environment on the bone surface. In addition, the extracellular matrix, rich in nanoscale features, provides a scaffold for cell adherence, proliferation, stem cell self-renewal and specific differentiation within the niche environment[66]. Thus, current approaches to improve *in vitro* expansion and differentiation of skeletal stem cells and to improve success and longevity of orthopedic implants *in vivo* are applying topographical strategies and materials[67].

Synthetic surface topography, for experimental use, range from disordered, rough surfaces with millimeter dimensions to highly ordered, nanometer patterned surfaces. In particular, biocompatible nanomaterials with topographical features of 1-100nm, in at least one dimension, are produced by electron beam lithography (EBL) with 10nm precision over cm^2 areas[68]. Such nanotopographical surfaces may prove useful in overcoming some of the challenges faced by regenerative medicine, in particular in the field of orthopedics. For example, osteoblasts were reported to have enhanced adhesion to nanoscaled alumina, titania, HA, titanium alloy (Ti6Al4V), and cobalt-chromium-molybdenum alloy compared to adhesion to micron scaled ceramic materials[69, 70]. In contrast, and of significant therapeutic benefit, adhesion of fibroblasts to these nanomaterials was reduced, potentially overcoming fibrous encapsulation of implants leading to poor osseointegration[69].

1) Manipulation of adult skeletal stem cells using nanotopography

The use of nanotopography to influence skeletal stem cell fate avoids the use of chemical differentiation inducing

factors which are widely used in current differentiation protocols limiting translation to the clinic. Current research utilizes nanoscale topographical surfaces of diverse geometries to influence stem cell fate.

In contrast to flat titanium surfaces, 80nm diameter nanotubular surfaces induced higher levels of adhesion and proliferation plus enhanced alkaline phosphatase activity of rat marrow stromal cells[71]. Using human bone marrow derived cells, we have previously reported that a random arrangement 120nm diameter nanopits produced a high cell density of adhered skeletal stem cells. In contrast, a regular hexagonal arrangement of nanopits of the same dimensions resulted in low cell density attributed to low adhesion on this geometry[57]. Geometry can also be used to manipulate skeletal stem cell fate. A near square arrangement of nanopits (displaced by 50nm in x and y axis) induced the osteogenic differentiation of unsorted human bone marrow and STRO$^+$ human skeletal stem cells in the absence of soluble, chemical osteogenic factors[57]. Expression of osteopontin and osteocalcin was observed in cell types cultured on near square nanotopographical surfaces. In comparison, a planar flat control surface of the same material failed to induce differentiation[57] indicating that nanotopography alone is sufficient to induce differentiation.

Adherence of adult skeletal stem cells was reported to be enhanced on 30nm diameter titanium oxide nanotubes in comparison to 100nm diameter nanotubes. In contrast, 70nm and 100nm diameter nanotubes induced the elongation of cells promoting osteogenic differentiation[72]. However, this differential effect on directed differentiation may also be attributed to cell density; whereby reduced seeding densities promote osteogenic differentiation and high seeding densities promote adipogenic differentiation[60, 73].

Culture on nanoislands in the presence of osteogenic factors, was reported to enhance alkaline phosphatase activity and mineralization of human mesenchymal stem cells compared to a flat control when nanoislands were 12 and 21nm in height but this effect was not observed with 45nm nanoislands[74]. Nevertheless, defining nanoscale thresholds at which cells switch behaviour from that of proliferation to lineage specification and differentiation offers insight for the development of these surfaces for clinical use.

These studies demonstrate that nanoscale materials with directed differentiation properties have regenerative medical applications in the *in vitro* differentiation of skeletal stem cells to produce osteogenic cell types for research purposes or for transplantation to assist in the repair or replacement of lost or damaged bone. Furthermore, application of suitable nanotopographical patterns to implant surfaces may enhance osseointegration where the implant is in contact with the bone marrow skeletal stem cell population. In comparison to the near square arrangement of nanopits, a regular ordered arrangement of nanopits did not induce differentiation in the absence of chemical osteogenic factors[57]. In fact, this geometric pattern (Fig.2A) maintained the skeletal stem cell state over multiple passages[58] a phenomenon not observed with passage on tissue culture plastic

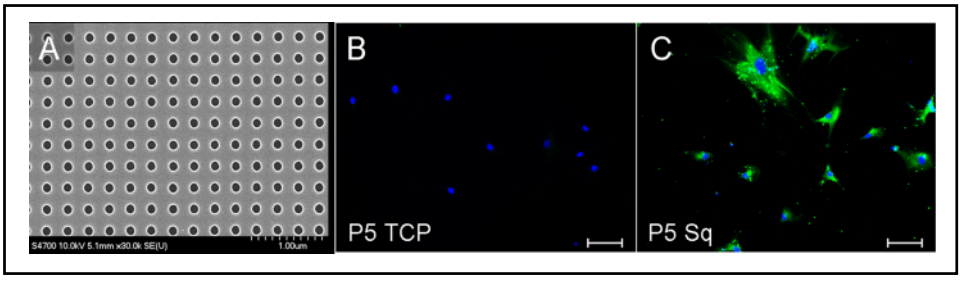


Fig.2 A square arrangement of nanopits maintains STRO the skeletal stem cell state over multiple passages

An SEM displaying a square arrangement of nanopits (A). The STRO$^+$ populations of adult bone marrow cells were seeded directly onto tissue culture plastic (TCP) (B) or nanotopographical substrates (Sq — square arrangement of nanopits) (C). Cells were incubated on these surfaces in basal media (a-MEM plus 10% FCS and Penicillin-Streptomycin). Cell density was maintained below 80% confluence by passage every 3-5 days. Cells were fixed with 4% paraformaldehyde the day after the fifth passage. Immunofluorescent staining was conducted using STRO antibody hybridoma supernatant and Alexafluor 488 secondary antibody (green) and nuclei counterstained using DAPI (blue).

or planar control surfaces. The expression of STRO-1 was undetectable after 5 passages on tissue culture plastic (Fig.2B), yet still detectable after passage 5 on the square nanotopographical pattern (Fig.2C). These observations indicate that the square arrangement of nanopits may be suitable for *in vitro* expansion of skeletal stem cells for research purposes or prior to directed differentiation for regenerative therapies.

2)Nanotopographical cues for ESC differentiation

While all stem cells have the properties of self-renewal and potency, embryonic stem cells (ESCs) are truly pluripotent, providing, potentially, a useful resource for regenerative medical applications. Typical culture methods for ESCs involve expansion on a mitotically inactivated feeder layer of cells; traditionally murine embryonic fibroblasts (MEFs)[75] and more recently human derived feeder cells such as fetal fibroblasts[76], foreskin fibroblasts[77, 78] or adult bone marrow cells[79]. Thus ESC maintenance incorporates a rich environment of soluble secreted factors and physical topographical cues provided by the feeder layer of cells. In feeder-free culture systems, chemical cues are provided by MEF conditioned media[80] or the addition of basic fibroblast growth factor (bFGF)[81, 82] in order to maintain ESC self-renewal. However, tissue culture plastic surfaces must have a surface coating of, for example, Matrigel[80], a soluble basement membrane extract of Engelbreth-Holm-Swarm mouse sarcoma, in order to provide a topographical environment for ESC self-renewal maintenance. The implementation of topography to maintain ESC self-renewal or to direct differentiation may overcome a number of challenges and risks associated with the use of animal derived surface coatings and supplementary factors. Initially, approaches to manipulate ESCs with topography focused on ridge and groove patterns, nanotubes or nanofibrils. ESCs were reported to align and elongate in the direction of these patterns, forming morphologically elongated cells with neural cell marker expression[83-86]. However, neural differentiation is reported to be the default lineage of differentiation in the absence of chemical cues[87, 88].

We hypothesized that the near square arrangement of nanopits, which induced the differentiation of adult skeletal stem cells in the absence of osteogenic factors, could direct the differentiation of human ESCs. Utilizing near square nanotopography surfaces, hESCs seeded in a basal medium lacking differentiation inducing factors (withdrawal of FGF and conditioned medium) were observed to differentiate towards a mesodermal lineage without detectable expression of neural markers[89, 90]. Furthermore, markers of skeletal stem cells (STRO1 and CD44) were detected in cell types resulting from differentiation of hESCs on planar or near square surfaces. Interestingly, following further incubation, skeletal stem cell markers were reduced in cells on near square surfaces indicating further differentiation. Consistent with this, later markers of primitive human stromal cell differentiation were detected with an enhancement in CD63, ALCAM, collagen I and RUNX2 observed. Interestingly, the adipogenic marker *PPARγ* was not detectable. Given the limited availability of adult skeletal stem cells, directed differentiation of hESCs to skeletal stem cell types for research purposes offers a renewable source of cells for research purposes. In addition, nanotopography directed hESC differentiation avoids the use of complex chemicals in culture media which may interfere with downstream applications.

Bone regeneration: the clinical need

Skeletal disorders requiring the regeneration or *de novo* production of bone as a consequence of significant bone loss present the orthopaedic surgeon with a considerable reconstructive challenge. These include traumatic bone loss from high velocity injuries, fracture non-union due to the biological failure of normal bone healing, surgical excision of bone for infection or tumour, joint arthrodesis and revision arthroplasty surgery. Autologous bone is widely considered the "gold standard" for restoring lost bone stock because of its biological and mechanical properties, but it is of limited supply and results in significant donor site morbidity. Allograft is a good alternative, overcoming some of these issues, but concerns over allograft immunogenicity, risk of disease transmission and cost, have led to the need for alternative grafts and the subsequent development of scaffolds to act as bone graft substitutes. The "Diamond Concept" outlines the principles advocated in the development of a scaffold to optimize bone graft incorporation[91]. Naturally occurring biomaterials (demineralized bone matrix, collagen, hydrogels)[92-100], bioresorbable synthetic polymers[101-105], ceramics (HA, beta-tricalcium phosphate)[106-113], silicon-based compounds (bioactive glasses, glass ionomers)[114-120] and trabecular metal (tantalum, titanium)[121-127] have all been developed for use as bone graft substitutes. However, while their osteoconductive and me-

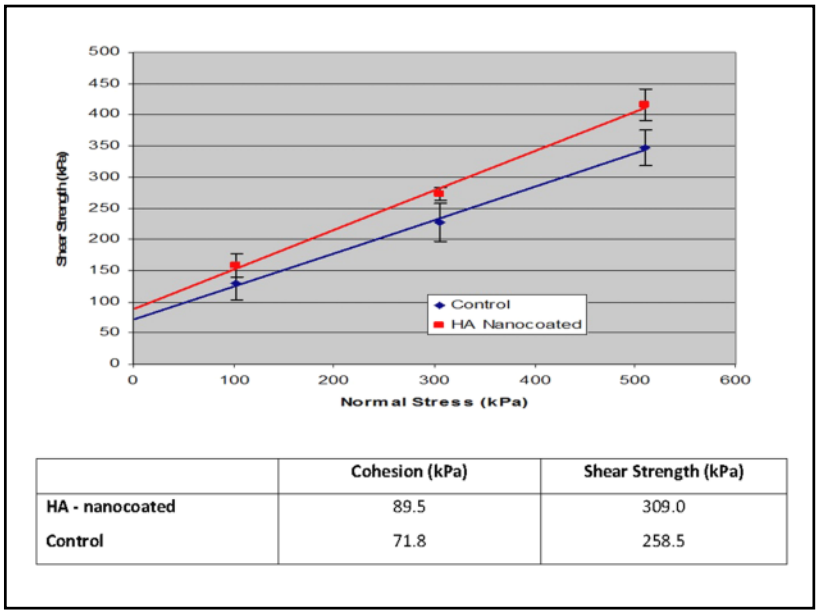
Table 1 Summary of tissue engineering strategies successfully translated into clinical practice

Scaffold/TE strategy	Cell type/preparation	Anatomical location	Clinical Situation	Refs
HA	Autologous periosteum-derived cells	Thumb - distal phalanx	Trauma	129)
HA	Autologous marrow-derived cells	Long bones	Defects following osteotomy for lengthening/trauma	128,139)
HA & titanium mesh cage	Autologous bone marrow	Mandible	Oral neoplasia	130,137)
Alumina-ceramic prosthesis	Culture-expanded skeletal stem cells	Ankle	Osteoarthritis	132)
HA	Autologous stem cells and platelet-rich plasma	Maxilla	Reduced alveolar bone crestal height	133)
Allograft	Autologous bone marrow aspirate	Femoral head	Cyst/osteonecrosis	136)
HA	Culture-expanded autologous skeletal stem cells	Femur/tibia	Benign bone tumors	135)
Titanium mesh plate	Culture-expanded skeletal stem cells and platelet-rich plasma	Alveolar cleft	Congenital cleft lip and alveolus	134)
No scaffold - local application	Skeletal stem cells and platelet-rich plasma	Femur/tibia	Achondroplasia/ hypochondroplasia	138)
No scaffold - local application	Platelet-rich plasma	Spine/ mandible/ maxilla	Degenerative/ congenital	140, 141, 143)
No scaffold - percutaneous injection	Autologous concentrated bone marrow aspirate	Tibia	Non-union following trauma	131)
No scaffold - direct injection during surgical procedure	Concentrated autologous bone marrow aspirate	Femoral head	Osteonecrosis	142)

chanical properties auger well for their application, in isolation bone graft substitutes often lack the necessary osteogenic and osteoinductive properties. The last decade has seen a significant expansion in the application of tissue engineering strategies to address this problem and recent advancements in techniques have led to the successful clinical translation of some of these strategies (Table 1)[128-143]. The technique of impaction bone grafting and its role in revision arthroplasty surgery will be explored further to illustrate some of these concepts.

Impaction bone grafting: tissue engineering and regeneration

The number of primary hip and knee arthroplasty procedures performed in England and Wales between 2006 and 2011, increased from 120,000 to 163,000 per annum, while the number of revision procedures, accounting for approximately 10% of cases, almost doubled to 15,000 per annum[144]. In the United States alone, revision hip and knee arthroplasty procedures are projected to increase by 137% and 601% respectively between 2005 and 2030, with the greatest requirement in those under the age of 65[145, 146]. These figures are only set to rise given the demographics of an aging population together with an increase in patient functional expectation and demand. Revision arthroplasty can be complicated by significant bone loss as a consequence of osteolysis, stress shielding, implant removal, fracture and/or infection. Impaction bone grafting (IBG) is a recognized technique for restoring bone stock. First introduced by Slooff in the Netherlands in the early 1980's using autograft for acetabular reconstruction[147], the technique was later modified by the Exeter Hip Group in the United Kingdom for femoral reconstruction[148]. The technique, using fresh frozen morcellised allograft, forms the basis of modern day impaction grafting and remains the "gold standard" in femoral and acetabular reconstruction with extensive bone loss. IBG studies have demonstrated 99% survivorship of the acetabular component and 89% survivorship of the femoral component at 10 and 20 years respectively[148, 149], although these encouraging results have



	Cohesion (kPa)	Shear Strength (kPa)
HA - nanocoated	89.5	309.0
Control	71.8	258.5

Fig.3 The effects of biologically activating allograft

Superior shear strength and interparticulate cohesion of HA-nanocoated allograft biocomposite was observed compared to the uncoated allograft control.

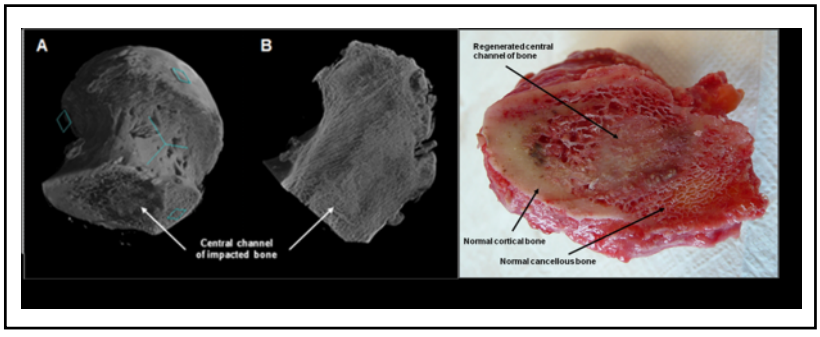


Fig.4 Successful graft incorporation into the femoral head following IBG for avascular necrosis

The central channel of impacted bone is seen to have osseointegrated well on 3D reconstructed views following μCT analysis (A, B) and macroscopically (C) in a retrieval analysis specimen two 2 years post-surgery.

not been replicated outside the major centers[150, 151]. The success of IBG is thought to be dependent on both mechanical and biological factors, with implant failure arising from a lack of bone graft incorporation, poor biological fixation at the interface and an inability to resist shear forces. We have shown that a graded particulate mix of morsellized allograft can improve the shear strength properties of the graft bed[152] and that resistance to shear forces can be increased by extensive washing of the graft prior to impaction[153]. Furthermore, we have demonstrated the mechanical properties, on the femoral and acetabular side, can be enhanced by local fluid drainage and the application of a vibrating tamp during the impaction process[154-156], resulting in reduced peak loads and hoop strains transmitted to the femoral cortex, and improved resistance to stem subsidence[154]. These techniques enhance prosthetic stability (particularly around the proximal and middle femoral regions) and, critically, reduce the potentially damaging impaction loads and associated fracture risk.

Morsellized impacted allograft provides a mechanical scaffold with excellent osteoconductive properties but displays negligible osteoinductive potential in isolation. In 1985, Burwell reported the beneficial effects of adding bone marrow to allograft on new bone formation and graft incorporation in an *in vivo* animal model[157]. We have demonstrated the efficacy of human bone marrow stromal cells in combination with IBG and allograft in both *in vitro* and *in vivo* models, with proliferation and differentiation of the stromal cells following impaction resulting in increased interparticulate cohesion and shear strength, and conferring a mechanical advantage over allograft alone[158]. Studies have also shown that the ability of a living composite of human bone marrow stromal cell-allograft construct to resist shear forces can be significantly enhanced by increasing the initial seeding density, with a $2x10^5$ cells/cm^2 seeding density giving a 16 per cent increase in shear strength

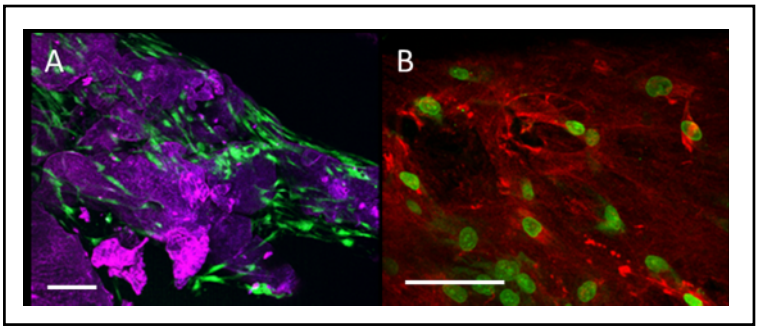


Fig.5 Cell survival and differentiation on PLA-HA + HBMSCs scaffold
A milled mix of PLA-HA composite were seeded with cells at $5x10^5$ cells/ml and incubated in osteogenic media (a-MEM, 10% FCS, Penicillin-Streptomycin, ascorbate-2-phosphate and dexamethasone) (A) live-dead stain confirming cell survival on the scaffold at 2/52 incubation (B) COL 1 (red) against DAPI nuclear (green) stains illustrating osteogenic activity. Scale bars = 100 μm.

(p<0.001)[159]. Other studies have looked at the effects of precoating allograft with type 1 collagen, fibronectin or nano-HA particles, prior to human bone marrow stromal cell (HBMSC) seeding and impaction[159] (Fig.3). The addition of vaterite (calcium carbonate) microspheres to the impacted allograft/HBMSC construct has also been shown to augment bone formation in an *in vivo* murine model[160]. These tissue engineering strategies, in combination with IBG, have been successfully translated to the clinical setting in a series of patients with early stage avascular necrosis of the femoral head. Allograft seeded with autologous HBMSCs from an iliac crest aspirate was impacted into a canal drilled into the avascular segment of bone from the lateral femoral cortex. Parallel *in vitro* analysis of these impacted samples has confirmed that autologous HBMSCs seeded onto the scaffold not only remain viable but exhibit an osteogenic phenotype[136]. Interestingly, retrieval analysis of the femoral head sample, from a patient that subsequently had a hip arthroplasty procedure as a consequence of disease progression, demonstrated excellent graft incorporation into the hosts own bone[161](Fig.4).

The concerns and limitations surrounding the use of autograft and allograft have necessitated the development and fabrication of alternative bone graft substitutes for use in IBG. *In vitro* studies have demonstrated that poly (DL-lactic acid) (PLA), when augmented with HBMSCs, can support osteogenic differentiation and improve the mechanical properties of the scaffold, compared to PLA alone[159]. These observations have been replicated *in vivo* in a subcutaneous murine model, with an increased angiogenic response in the living composite[159]. Further studies, using an array of high and low molecular weight polymers as allograft substitutes, have found that the milled, high molecular weight forms of both PLA and poly (DL-lactic-co-glycolic acid) (PLGA), possess the mechanical shear strength and HBMSCs compatibility characteristics desirable for clinical use[163]. The production of a porous version of these polymers using a supercritical CO_2 foaming technique, with pore sizes between 50 and 200 μm was found to maintain the mechanical strength of the polymer/HBMSC construct by improving resistance to shear forces and enhancing cellular compatibility and cohesion between the polymer particles[164]. The addition of HA particles to the porous matrix has been found to further enhance the osteoinductivity of the scaffold both *in vitro* and in a murine *in vivo* model (Fig.5). IBG provides a useful strategy for replacing bone stock in contained defects, however such an approach is limited if the bone loss is too extensive or the defect is uncontained. To address such conditions, porous trabecular metal has been used, and we have shown *in vitro* the ability of tantalum trabecular metal to support the growth and osteogenic differentiation of HBMSCs[165].

As arthroplasty becomes increasingly more common in younger people and as life expectancy increases, the number of people with substantial bone loss requiring surgery will inevitably increase. The challenge will be to develop biologically active constructs, with optimal mechanical properties, capable of promoting osseointegration. Despite ongoing research efforts and recent clinical success of tissue engineering strategies, the widespread uptake of this technology has yet to be fully realized.

Conclusions

Skeletal tissue regeneration using skeletal stem cells offers the prospect of new alternative therapies for bone and cartilage regeneration. Critical in this process of tissue repair is the cell source and bone marrow derived skeletal stem cells offer an exciting possibility in attaining clinical efficacy. Other approaches using human embryonic and induced pluripotent stem cells provide a clear challenge to

generate reproducible homogenous skeletal populations and yet offer exciting vistas for future skeletal reparative approaches. Crucial in the development of a cell based strategy together with enhanced understanding of skeletal stem and progenitor biology, cell fate and function are new approaches that provide cues for differentiation and function and appropriate niches for tissue development (including analysis of the inflammatory milieu). Nanotopography templates provide a powerful tool for skeletal stem cell modulation of function and stemness whilst biomimetic environments that provide stem cell niche and angiogenic cues will undoubtedly inform and enhance skeletal tissue repair. These are exciting times in bone tissue regeneration and the challenge will be to harness developmental biology, biomaterial science, bioengineering, translational biomedicine and stem cell science to deliver simple, safe and reproducible skeletal cell based strategies for bone augmentation for an ageing population.

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