Generation of osteoinductive grafts by three-dimensional perfusion culture of human bone marrow cells into porous ceramic scaffolds

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INTRODUCTION

CHAPTER 1

BONE REPAIR

1.1 Bone tissue: biology, structure, and function

Bone is a dynamic, highly vascular, and mineralized connective tissue, characterized by its hardness, resilience, growth mechanisms, and its capability to remodel itself throughout the life-time of an individual.

Bone performs several key functions within the body: it not only provides structural support and protection to bodily organs, but is also responsible for maintaining mineral homeostasis, and is the primary site for the synthesis of blood cells. Furthermore, it is capable of maintaining an optimal shape and structure throughout life, via a continuous process of renewal and remodelling, through which it's able to respond to changes in its mechanical environment, in order to meet different loading demands, thus maintaining an optimal balance between form and function [1].

Simply, bone is a dense multi-phase composite, made up of cells embedded in a very well-organized matrix, which is composed of both organic and inorganic elements; however, both structure and proportion of its components widely differ with age, site and history, resulting in many different classifications of bone that exhibit various mechanical and functional characteristics.

Histologically, mature bone is classified in two different types of tissue, one of which is relatively dense, known as cortical bone, while the other consists of a network of struts or trabeculae surrounding interconnected spaces, known as trabecular or cancellous bone (Fig.1). Bone surfaces consist of cortical bone, and the thickness of this protective layer increases in mechanically demanding regions, such as the shafts of long bones, while cancellous bone is found in the interior of bones, such as within the femoral head, and vertebra.

Bone as an organ is composed of three main elements: (i) bone matrix, providing mechanical strength and acting as the body's mineral store, (ii) bone cells, responsible for maintaining the structure of the matrix, regulating its oxygen and nutrient supply, and storing or releasing minerals as required, and (iii) bone marrow with its associated vascular network, providing the source of stem cells and representing the main means of communication and interaction with the rest of the body.

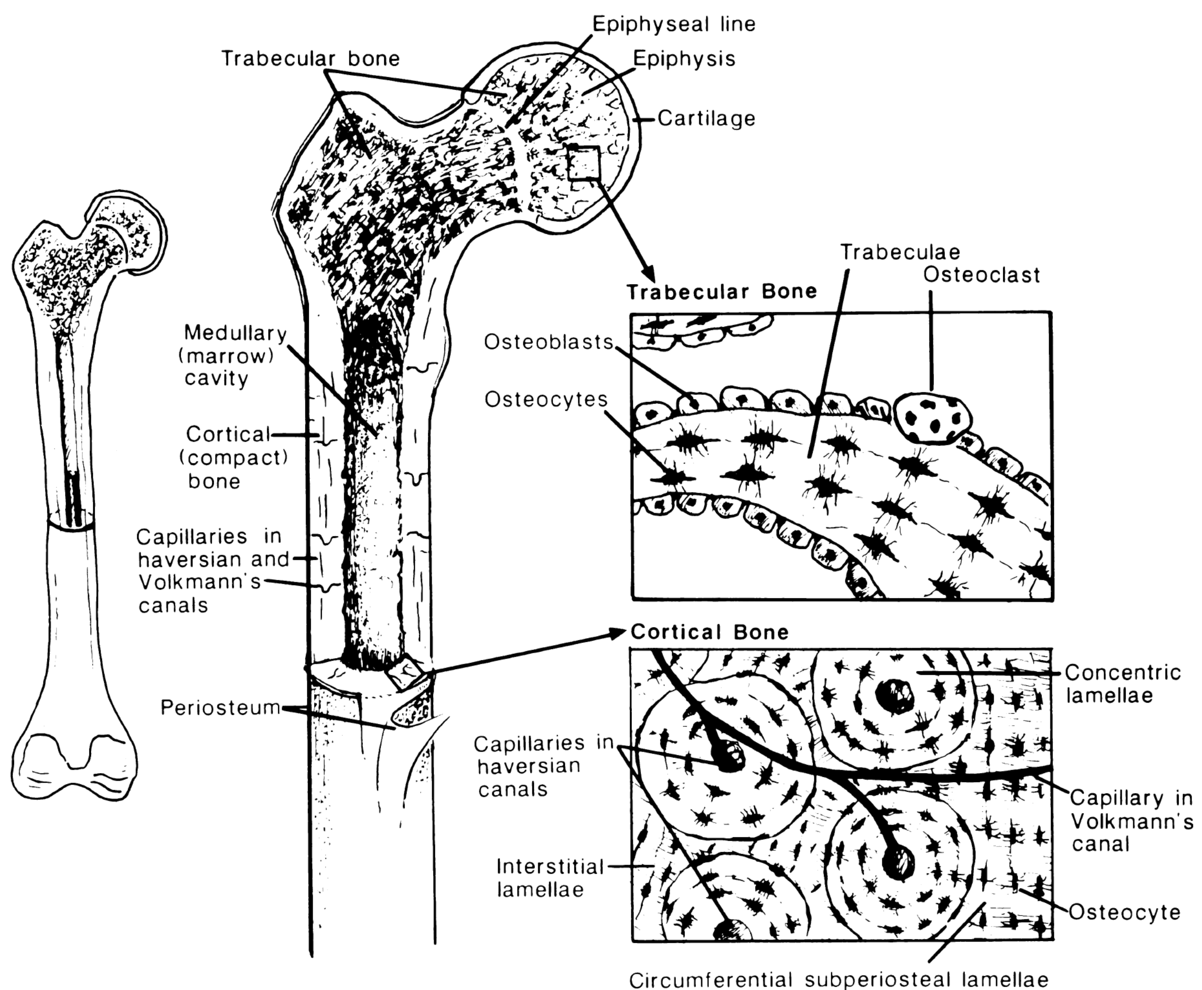

Fig.1. Schematic diagram of cortical and trabecular bone, showing the different microstructures (Reproduced from Hayes WC: Biomechanics of cortical and trabecular bone: Implications for assessment of fracture risk).

Bone extracellular matrix has two main components: the organic collagen fibres and the inorganic bone mineral crystals. Together they make up approximately 95% of the dry weight of bone,

the remainder being composed of other organic molecules, collectively known as the non-collagenous proteins.

Collagen accounts for 70-90% of the non-mineralized components of the bone matrix; it consists of carefully arranged arrays of tropocollagen molecules, which are long rigid molecules composed of three left-handed helices of peptides, known as α-chains, which are bound together in a right-handed triple helix. Bone contains mostly type-I collagen, which is composed of tropocollagen molecules containing two identical and one dissimilar α-chains ($\alpha 1(I)_2\, \alpha 2$).

The main inorganic phase within the bone matrix is usually incorrectly referred to as hydroxyapatite (HA), a hydrated calcium phosphate ceramic, with a similar crystallographic structure to natural bone mineral, which has a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$; however, bone-apatite is characterized by calcium, phosphate and hydroxyl deficiency, internal crystal disorder, and ionic substitutions, thus resulting in the presence of significant levels of additional trace elements within bone mineral: it is not a direct analogue of HA, but more closely a carbonate-substituted apatite. All these factors contribute to an apatite that is insoluble enough for stability, yet sufficiently reactive to allow the in vivo crystallites to be constantly resorbed and reformed as required by the body.

The most important non-collagenous organic constituents of bone matrix are four proteins: osteocalcin (OC), bone sialoprotein (BSP), osteopontin (OP) and osteonectin (ON). They are produced by bone cells and their relative composition within the bone matrix appears to be self-regulating through a feedback effect on their expression by osteoblasts. They all appear to be multi-functional, and are all involved in regulating bone mineralization and remodelling.

Bone matrix also contains a great number of growth factors, including fibroblast growth factors (FGFs), insuline-like growth factors (IGFs), plateled-derived growth factors (PDGF), transforming growth factor-beta (TGFβ) superfamily, and bone morphogenic proteins (BMPs): they play several critical roles in regulating cell proliferation and differentiation, inducing the complete sequence of endochondral bone formation, when cartilage forms first and is subsequently replaced by bone.

The major types of bone cells are osteoblasts, osteocytes and osteoclasts, respectively responsible for production, maintenance, and resorption of bone; they are highly specialized

differentiated cells, and they generally don't proliferate. Less differentiated cells of the same lineage are required for the control of bone cell populations, and, as demands are made on or by the bone, these cells proliferate and differentiate as required: such cells are generally known as stem cells, and in the case of bone formation are often referred to as osteogenic cells.

The osteogenic bone-forming cells originate from the mesenchymal bone marrow stromal cell line and exist in the endosteum and periosteum [2]. Biochemical signalling molecules stimulated during remodelling and fracture healing, result in a local increase of this cell population. However, the local environment also determines the route of differentiation undertaken by osteogenic cells, resulting in the evolution of either osteoblasts or chondroblasts: if the environment surrounding a differentiating osteogenic cell has a high vascular content, as in healthy bone, the cell will differentiate into an osteoblast which will produce bone; once the osteoblast has been surrounded by bone, it differentiates into an osteocyte, and becomes involved in the nutrition and maintenance of the local bone. In contrast, if the environment surrounding a differentiating osteogenic cell has little or no vascular content, as in a recent fracture site, the cell will differentiate into a chondroblast and cartilage will be produced; once the chondroblast is surrounded by cartilage, it then differentiates into a chondrocyte, which maintains the surrounding collagenous matrix until it's replaced by bone during endochondral ossification.

In contrast osteoclasts are derived from monocytes, thus they originate from the haemopoietic stem cell lineage: under the influence of specific signalling proteins or cytokines, mononuclear monocytes migrate to the resorption site and fuse with either other monocytes or a multi-nucleated macrophage, before differentiating into the specialized osteoclast, an aggressive cell responsible for bone resorption [3].

1.2 Bone formation: development, healing, and repair

Bone is unique among all the vertebrate tissues in its ability to heal via formation of new bone: most of the other tissues, such as heart, muscle and brain heal by replacement with connective tissue rather than original tissue. Furthermore, in a mature animal, the molecular and cellular patterns of bone repair after injury are similar to bone formation in an embryo, suggesting analogous mechanisms for the control of bone formation in adult and embryonic skeletons [4]. In an embryo, a condensation of primitive mesenchymal cells can transform into bone via either intramembranous or endochondral ossification: intramembranous ossification occurs when the mesenchymal cells are transformed into osteoprogenitor cells and then directly into osteoblasts, resulting in the direct formation of bone; endochondral ossification occurs via a two-step process where mesenchymal cells transform into chondroblasts which lay down a collagenous template, subsequently ossified by invading osteoblasts. The final mature bone formed by both processes is virtually indistinguishable, and the mechanisms dictating which route is taken are poorly understood.

Fractured bone heals through endochondral ossification: a haematoma is formed, resulting from injury to the periosteum and local soft tissue; as a consequence of this disruption in the blood supply, osteocytes nearest to the fracture die, resulting in local necrosis of the bone around the fracture; simultaneously, there is a demand for the repair of the bone, the stabilization of the damaged area and the removal of the dead tissue; in response to this, macrophages and fibroblasts are recruited to the site to remove tissue debris, and to express extracellular matrix, respectively. In response to growth factors and cytokines released by these inflammatory cells, mesenchymal stem cells recruited from the bone marrow and periosteum, proliferate and differentiate into osteoprogenitor cells. This leads to an apparent thickening of the periosteum and the production of collars of external fracture callus around the fracture site. Those osteoprogenitor cells that lie close to undamaged bone, differentiate into bone osteoblasts and form an osteoid, which is rapidly calcified into bone, while those farther away become chondroblasts and form cartilage; concurrent angiogenesis is induced, and, as soon as cartilage has formed and the fracture site stabilized, it is replaced by cancellous bone via endochondral ossification, in which osteoclasts and osteoprogenitor cells invade the cartilaginous

callus preceded by capillary formation. The uncalcified material is then resorbed, and new bone is deposited on the remaining spinicules of calcified cartilage. Woven bone is finally remodelled into lamellar bone, bone marrow is restored within cancellous regions, and successive layers of bone gradually fill the spaces between trabeculae of cortical bone. Load-bearing capabilities and a new vascular network are thus restored.

Although the vast majority of bone defects spontaneously heal with minimal treatment, among the 6 millions fractures occurring every year in the United States, 5-10% require further treatment for compromised healing because of either interposition of soft tissue, improper fracture fixation, loss of bone, metabolic diseases, impairment of blood supply or infection. Furthermore, in certain clinical settings, large pieces of bone must be resected to treat benign and malignant tumours, osteomyelitis, as well as bone deficiences, and abnormal loss in the maxillo-facial area; in addition, bone is typically subject to progressive degeneration as a result of age and disease (i.e. osteoporosis).

Considering all these challenging situations, bone function can often be restored only by surgical reconstruction: bone grafting, the procedure of replacing missing bone with material from either the patient's own body (autografting) or that of a donor (allografting) is used in the surgical procedures since many years. Autologous bone harvested from donor sites such as the iliac crest, is the preferred treatment [5]: grafts of this kind are osteoconductive (they provide a scaffold on which bone cells can proliferate), osteoinductive (they induce proliferation of undifferentiated cells and their differentiation into osteoblasts), and osteogenic (they provide a reservoir of skeletal stem and progenitor cells that can form new bone); however, the amount of bone that can be safely harvested is limited, while the additional surgical procedure may be complicated by donor-site pain and morbidity. Modern allografting using material stored within bone banks overcomes these difficulties; however, the demand exceeds the supply, there is no assurance of freedom from disease, and healing can be inconsistent [6].

As an alternative to these two types of bone grafts, a wide variety of synthetic substrates have been developed and are actually in clinical use, with mixed success and surgical acceptance: such materials in fact are generally biocompatible and osteoconductive, thus supporting adhesion, proliferation, and differentiation of osteogenic cells from surrounding tissues, and ultimately leading

to bone formation; however, these materials are not osteoinductive, providing only the scaffold which has to be invaded by bone-forming bioactive cells [7, 8]: reasoning that they typically give good results only when implanted in small defects, where interactions between material's surface and local cells and proteins are sufficient to repair the bone defect. In addition, metals, although providing immediate mechanical support at the site of the defect, exhibit poor overall integration with the tissue at the implantation site, and can fail because of infection or fatigue loading; on the other hand, ceramics have very low tensile strength and are brittle, thus they cannot be used in locations of significant torsion, bending, or shear stress [9].

Thus it's clearly seen that repair of bone defects is actually still a big challenge for the orthopaedic, reconstructive, and maxillo-facial surgeons: it's in this scenario that a promising field of science called Tissue Engineering is emerging since the last few years.

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CHAPTER 2

CELL-BASED ENGINEERING OF BONE TISSUE

2.1 General concepts

As defined by Langer and Vacanti [1], tissue engineering is an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function. In contrast to classical biomaterials approaches, it's based on the understanding of tissue formation and regeneration, and aims at inducing new functional tissue, rather than implanting new spare parts: researchers foresee to reach this goal by combining knowledge from physics, chemistry, engineering, materials science, biology and medicine in an integrated manner.

Engineering of osteoinductive grafts can be achieved by loading 3D scaffolds with either bone morphogenetic proteins, or osteogenic cells: regarding the first approach, the growth factor can be incorporated within a polymer scaffold, which, by degradation, will release the factor with defined kinetics, or, alternatively, injected directly at the site, together with an osteoconductive material, aiming at recruitment and differentiation of mesenchymal progenitor cells localized in the neighboring original bone tissue. The second approach does require osteogenic cells, which can be obtained from biopsies of different tissues (i.e. bone marrow, periosteum, adipose tissue...): typically, osteogenic cells are obtained from the bone marrow, where they represent a very small percentage (approximately 0.01%) of the total number of nucleated cells. Therefore, to obtain a sufficient number of cells for bone tissue engineering applications, BMSC are typically first selected and expanded in vitro by sequential passages in monolayer (2D) prior to loading into three-dimensional (3D) porous scaffolds, which prime cell differentiation towards the osteogenic lineage and provide the template for the in vivo bone-like tissue formation [2, 3].

The growth factor-based approach, since it doesn't require ex vivo cell processing, appears more simple, but, on the other hand, it opens biological questions, such as how the overdose of one single molecule could recapitulate the complex set of molecular events physiologically involved in the safe and stable formation of bone tissue. The cell-based approach, although it's apparently less convenient in terms of logistics and costs, is actually becoming more and more appealing because based on more physiologic signals, possibly inducing mechanisms of regeneration which are closer to those naturally occurring in normal functional living organisms. However, 2D-expanded BMSC have a dramatically reduced differentiation capacity in comparison with those found in fresh bone marrow [4, 5], placing potential limits on their clinical utility.

Required components for cell-based bone tissue engineering are cells, extracellular matrix, intercellular communications, cell-matrix interactions, and growth factors; in addition, since bone has a three-dimensional (3D) configuration, a 3D structure, a scaffold, is typically used, in order to provide the template for tissue development in a 3D manner.

The in vitro culture of 3D cell-scaffold constructs under conditions that support efficient nutrition of cells, possibly combined with the application of mechanical forces to direct cellular activity and phenotype, is another important step towards the development of functional grafts for the treatment of lost or damaged tissues [6].

Thus, for a successful cell-based engineering of osteoinductive grafts, the following issues should be carefully addressed and combined: (i) identification of a reliable cell source, (ii) selection of the right scaffold material and architecture, (iii) development of the adequate 3D cell-scaffold culture system, and (iv) use of the appropriate culture media supplements.

2.2 Cell sources

Cell sourcing is the first issue to deal with for development of engineered bone grafts. The characteristics of an ideal cell source include: no immunorejection, graft-versus-host disease or

tumorigenicity, availability in pertinent quantities, controlled cell proliferation rate, consistent osteogenic potential, as well as controlled integration in the surrounding tissues.

The first and most obvious choice for non-immunogenicity is the use of autologous osteoblasts harvested from a biopsy of the patient himself; unfortunately, relatively few cells are available for harvesting, potentially resulting in some degree of donor-site morbidity, and their expansion rate is relatively low, limiting the number of cells available to be seeded on the scaffolds, even following expansion in vitro [7].

An alternative is the use of xenogenic cells (obtained from non-human donors): this methodology would solve the issue of the low cell number, but potentially it would introduce serious problems, such as the immunogenic response and the transmission of infectious agents [8].

It is in this context that stem cell biology appears as the most valid and promising solution. Stem cells are defined as undifferentiated cells with the capacity for self- renew, and multilineage differentiation [9]. However, stem cells have different degrees of differentiation potential, ranging from the totipotency (ability to form the embryo and the trophoblast of the placenta) of the zygote, to the pluripotency (ability to differentiate into almost all cells that arise from the three germ lines) of embryonic stem cells, and lastly to the multipotentiality (capability of producing a limited range of differentiated progeny, related to the embryonic origin of the tissue where they are found) of adult stem cells, which are present in the fully differentiated tissues [9].

In the field of bone tissue engineering, there has been a special interest in the stem cells located in the bone marrow, known as Mesenchymal Stem Cells (MSC). The idea that bone marrow contained some kind of osteogenic precursor cells started in 1963, when it was shown that by implanting pieces of bone marrow under the renal capsule, it was possible to obtain an osseous tissue [10]. After this, some in vivo studies by Friedenstein revealed the possible existence of osteogenic stem cells in the bone marrow [11], [12]: to better understand the nature and origin of these cells, he then developed a method to isolate fibroblast-like cells from the marrow, basing on their ability to adhere to cell culture plastic [13]; later he coined the term colony-forming units fibroblastic (CFU-f) to describe the fibroblastic, non-phagocytic and clonogenic nature of these cells [14]. Almost twenty years later, Caplan gave these cells the name they have today, Mesenchymal Stem Cells [15], and he

showed that these cells, when placed in the adequate conditions, can differentiate into different cells of mesenchymal origin, capable of giving origin to bone, cartilage, fat, tendon, as well as all the other tissues of mesenchymal origin [16].

Although their high differentiation potential makes them a very appealing candidate as a cell source for bone tissue engineering, there are several key issues that still need to be addressed: (i) the percentage of MSC present in the bone marrow is very low (1 in every 10^5 cells) [16], thus their expansion is highly time consuming; (ii) with an increased number of passages, MSC progressively loose their differentiation potential [17]; (iii) although several stem cell surface markers for the isolation and characterization of MSC were described, the high heterogeneity of MSC cultures actually makes very difficult the establishment of universal markers to identify the MSC with multilineage potential within the whole bone marrow cell population [18].

For these reasons, attempts have been recently made to isolate MSC from alternative tissues rather than the bone marrow, e.g. fat [19] or periosteum [20]; however, in spite of the rather invasive procedure of bone marrow harvest, and the limited and highly variable amount of cells that can be isolated from this tissue [21], MSCs from bone marrow currently represent the most reliable and widely used cell source for the experimental induction of bone tissue formation.

Based on these considerations, the experimental system we developed for generating osteoinductive constructs was based on MSC from the bone marrow, alternatively called bone marrow stromal cells (BMSC), or mesenchymal progenitor cells (MPC).

2.3 Scaffolds

Bone matrix in vivo is a 3D scaffold for bone cells, providing them with a tissue-specific environment and architecture, and serving as a reservoir of water, nutrients, cytokines, and growth factors: in this sense, and in order to restore function or regenerate bone tissue, one needs a template, a scaffold, that will act as a temporary matrix for cell proliferation and extracellular matrix deposition, with consequent bone in-growth until the new bone tissue is totally restored or regenerated, as well as

a template for the vascularization of the neo-tissue. This means that an appropriate 3D scaffold is an essential component for a successful tissue engineering strategy.

A wide number of biodegradable and bioresorbable materials, as well as scaffold designs, have been experimentally and clinically studied. Ideally, a scaffold should have the following characteristics: (i) three-dimensional and highly porous interconnected structure, with a large surface-to-volume ratios, for cell growth and flow transport of nutrients and metabolic waste; (ii) biocompatible and bioresorbable composition, with a controllable degradation and resorption rate to match cell/tissue growth; (iii) suitable surface chemistry for cell attachment, proliferation, and differentiation; (iv) mechanical properties to match those of the tissue at the site of implantation, and (v) easy structure to manufacture, sterilize and handle in the surgery room [22].

Regarding the selection of the adequate material for bone tissue engineering applications, up to now several possibilities have been proposed, such as metals, ceramics, and polymers; metals however, as well as some ceramics, are not biodegradable, which reduces the choice of an appropriate material to: (i) biodegradable ceramics, both from natural (e.g. coralline hydroxyhapatite), or synthetic origin (e.g. synthetic hydroxyhapatite, β-tricalcium phosphate); and (ii) synthetic polymers (e.g. collagen, fibrinogen, hyaluronic acid, polycarbonates, poly-α-hydroxyacids, polyanhydrides) [23].

Ceramics are well known to support the osteogenic phenotype of osteoblasts [24], and to prime the differentiation of MSC towards the formation of bone tissue [3]. Even though it seems possible to design a standardized hydroxyapatite ceramic scaffold with the help of rapid prototyping techniques [25], the scaffold architecture (i.e. size and interconnectivity of the pores), as well as its mechanical properties, are better controlled using synthetic polymers [26]; on the other hand, the ability of synthetic polymers to induce osteogenic differentiation is generally much lower than that of ceramics, unless growth factors are incorporated and released in a controlled fashion.

Based on these considerations, our experimental system was based on porous ceramic scaffolds (8 mm diameter, 4 mm thickness) made of 100% hydroxyapatite, and with a porosity of 83% (Fin-ceramica Faenza, Faenza, Italy).

2.4 3D-culture systems

Bone is a highly structured and mechanically active 3D tissue: the biological environment is derived from a dynamic interaction between active cells experiencing mechanical forces, and a continuously changing 3D matrix architecture [27]: in order to develop engineered bone tissue in vitro, it is thus needed to establish adequate cell/scaffold culture systems mimicking the dynamics of the in vivo environment.

The first step in establishing the 3D culture is the cell seeding on 3D scaffolds, that is the dissemination of cells within a scaffold; there are evidences that the cell seeding phase might play a crucial role in determining the progression of tissue formation [28]: the initial distribution of cells within the scaffold in fact, has been related to the distribution of tissue subsequently formed within the final engineered construct [29], suggesting that uniform cell-seeding could establish the basis for uniform tissue generation. Although static loading of cells onto scaffolds is by far the most commonly used seeding method, several studies reported low seeding efficiencies [30, 31] and non-uniform cell distributions within the scaffolds [32], owing, in part, to the manual and operator-dependent nature of the process.

In addition, if the obtained 3D cell/scaffold constructs are cultured in static conditions, although a three-dimensional structure is provided to the cells during their expansion, not only the dynamics of the in vivo environment are far from being reproduced [33], but even the needed supply of oxygen and soluble nutrients within the constructs represents a challenge: in this regard, it has been shown that in static culture conditions, due to mass-transport limitations, viable osteogenic cells can be supported into 3D scaffolds for only short distances from the scaffold surface [34].

Therefore, the use of bioreactors, both for cell seeding on the 3D scaffolds, and for the subsequent culture of the obtained cell-scaffold constructs, likely appears to be a promising solution to overcome the above mentioned limitations of the “static approach”.

Up to now, four main representative models of bioreactor systems for cell seeding and/or culture have been proposed: (i) spinner flasks [35], where scaffolds are attached to the needles hanging from the lid of the flask, and connective forces, generated by a magnetic stirrer bar, allow continuous

mixing of the medium surrounding the scaffolds, thus enhancing external mass-transfer; (ii) rotating wall vessels reactors [36], where scaffolds are maintained floating on an horizontal axis by a dynamic laminar flow; (iii) bioreactors applying computer-controlled mechanical forces, such as dynamic compression [37, 38], where constructs can be engineered under physiological loading conditions; (iv) bioreactors based on flow perfusion [27], where cell suspensions are perfused through the scaffold, and a very efficient and homogeneous cell distribution and nourishment throughout the scaffold can be achieved [32], thus increasing the mineralized matrix deposition [27].

In our study we aimed at obtaining a uniformly seeded construct, where cell growth and differentiation could be further sustained by efficient and widespread nourishment throughout the entire construct: considering how beneficial is perfusion flow for achieving these results, a perfusion bioreactor system was used in this work both for cell seeding and subsequent culture of BMSC on 3D porous ceramic scaffolds.

2.5 Culture media supplements

As a common basis of the many different approaches currently considered for cell-based engineering of bone tissue, there is the worldwide accepted concept that during the culture of osteogenic cells, it's appropriate to apply specific growth factors, in order to enhance cell differentiation and proliferation, thus obtaining sufficient numbers of osteogenic cells which, combined with an osteoconductive scaffold, can then be implanted as an osteoinductive graft.

Growth factors are cell secreted cytokines which, by binding to specific receptors, initiate intracellular signalling pathways, leading to different events such as promotion or prevention of cell adhesion, proliferation, migration, and differentiation, by up- or down-regulating the synthesis of several proteins, growth factors and receptors. In the field of bone regeneration, Urist first popularized the concept of a bone-generating protein in 1965, when he made the discovery of bone morphogenetic proteins (BMP) [39], which are including the most popular molecules used for bone tissue engineering.

Among the several different cocktails of growth factors currently proposed as the most appropriate for inducing osteogenic differentiation of MSC, the most typically used is containing dexamethasone, β-glycerophosphate, and ascorbic acid [40]. On the other hand, given the limited amount of available MSC, their proliferation is another key issue to keep into account in culturing them: it has been shown that, among different growth factors, fibroblast growth factor-2 (FGF-2) is the most effective in (i) promoting MSCs expansion in vitro, and (ii) maintaining them in a more immature state [3]. Interestingly, the combination of FGF-2 and dexamethasone, results not only in a high proliferation rate, but also in a final cell population with a high osteogenic commitment and bone forming capacity [41]. Nevertheless, it's still controversial among the researchers whether it's worth aiming at obtaining a cell population highly differentiated, or if it's better to maintain it in a more immature state.

In order to increase both MSC proliferation and their osteogenic differentiation capacity, in our experimental system culture medium containing fatal bovine serum was supplemented with FGF-2, dexamethasone, and ascorbic acid [3, 41, 42].

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CHAPTER 3

A NOVEL APPROACH FOR CELL-BASED ENGINEERING OF BONE TISSUE

3.1 Rationale

One of the major challenges to be faced for the routine clinical use of engineered bone-tissues is related to their manufacturing process, which, at present, is costly, impractical, and not enough standardized.

In this context, we consider that likely in the future, specific tissues could be engineered within closed bioreactor units, with advanced control systems, that would facilitate streamlining and automating the numerous labour-intensive steps. Starting from a patient's tissue biopsy, a bioreactor system could isolate, expand, seed and differentiate specific cell types on a scaffold, thereby performing the different processing phases within a single closed and automated system (Fig. 2), [1].

Such a bioreactor would (i) minimize operator handling, (ii) eliminate the need for large and expensive GMP (good manufacturing practice) facilities, and (iii) enable competent hospitals and clinics to carry out autologous bone-tissue engineering for their own patients, thus eliminating logistical issues of transferring specimens between locations. This would result in reducing the costs for engineering osteoinductive substitutes, which would not remain confined within the context of academic studies or restricted to elite social classes, but would become easily accessible for the health system and the community.

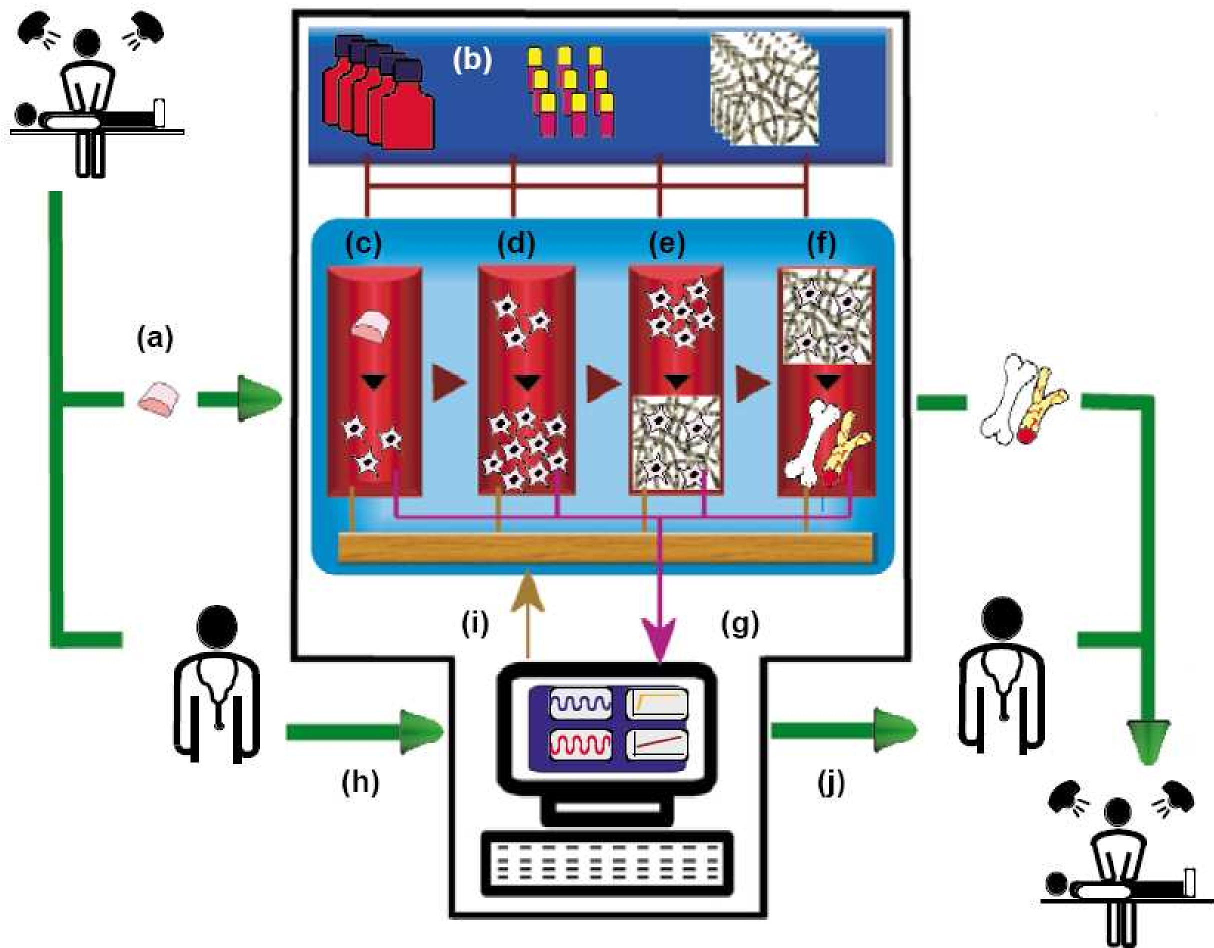

Fig. 2. Vision for a closed-system bioreactor for the automated production of tissue-engineered grafts. (a) The surgeon would take a biopsy from the patient and introduce it into the bioreactor located on-site at the hospital. (b) All reagents (e.g. culture medium, medium supplements, and scaffolds) would be stored in compartments under appropriate conditions (i.e. temperature, humidity). The bioreactor system could then (c) automatically isolate the cells, (d) expand the cells, (e) seed the cells onto a scaffold, and (f) culture the construct until a suitably developed graft is produced. (g) Environmental culture parameters and tissue development would be monitored and inputs fed into a microprocessor unit for analysis. In conjunction with data derived from clinical records of the patient (h), the inputs would be used to control culture parameters at pre-defined optimum levels automatically (i) and provide the surgical team with data on the development of the tissue, enabling timely planning of the implantation (j). Figure generated by M. Moretti.(Trends Biotechnol. 2004;22:80-6)

3.2 Goals and experimental system

The main aims of this thesis were (i) to identify and develop a system that could be reproducibly used to streamline manufacture of osteoinductive grafts based on human bone marrow stromal cells (BMSC) in the context of regenerative medicine (Chapter 4), (ii) to characterize the developed system in order to identify the key elements responsible for its reproducible and efficient performance (Chapter 5), and (iii) to extend its use to a sheep cell source (Chapter 6), thus opening the way to test the osteoinductivity of orthotopic implants in a large animal model, as a first step towards the potential extension of its use to clinical applications.

In this work we used a previously developed bioreactor system [2] for perfusing three-dimensional (3D) porous ceramic scaffolds first with cells (seeding phase), and subsequently with culture medium (expansion phase) within a single and closed environment: as shown in Fig. 3, 3D scaffolds were placed within chambers (one scaffold per chamber) which were positioned at the bottom of two vertical Teflon tubes, and connected each other at their base through a U-shaped tubing, whereas the top of the tubes were connected with a computer-controlled syringe pump (Fig. 4A).

To avoid any risk of mechanically induced cell damage from a pumphead, the flow path didn't recirculate the cell suspension through the scaffold and the pump: based on the bioreactor design previously developed for efficient and uniform cell seeding [2], the flow pathway was designed to pump the headspace above the cell suspension back and forth from one Teflon tube to the other, thereby generating an alternating flow of the cell suspension through the scaffolds. Cell settling and cell attachment to bioreactor components were minimized by its vertical orientation, component material properties, and by minimizing the surface area of horizontal surfaces where cells would tend to accumulate (Fig. 4B).

3D scaffolds were lightly press-fit and clamped within the scaffold chamber, such that fluid flow couldn't deviate around the scaffold, but had to flow through its pores. The chamber was manufactured from polycarbonate and polished until translucent, thus permitting the detection of possible air bubbles (Fig. 4C). Teflon FEP tubes (6 mm i.d.; Cole Parmer) were connected to

disposable three-way stopcocks (Hi-Flow™; Medex GmbH) via polypropylene luer adaptors (EM-Technik GmbH), and stopcocks were then connected to the scaffold chamber via its luer connections.

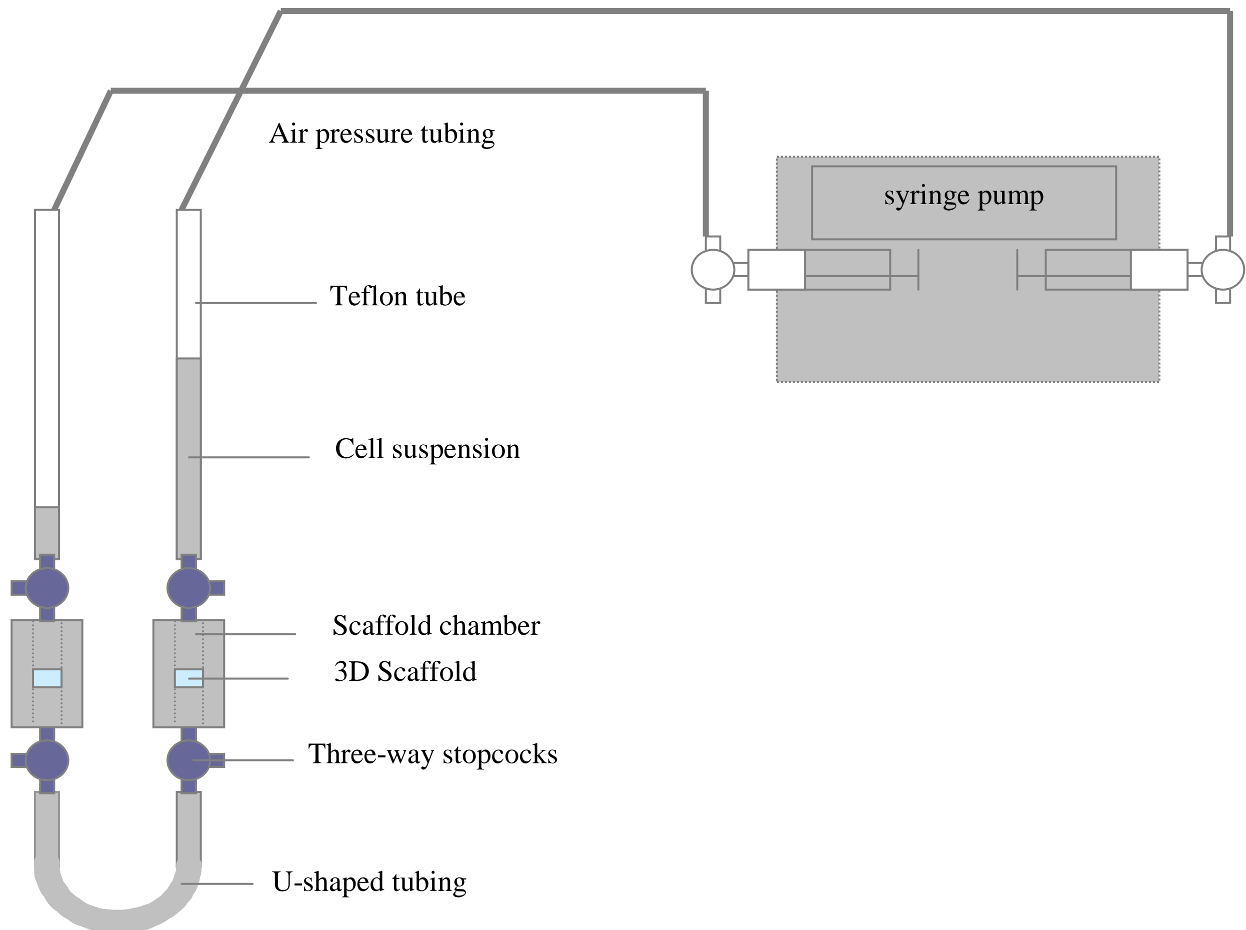

Fig.3. Schematic representation of the developed perfusion bioreactor system for uniform seeding and culture of BMSC on 3D porous scaffolds.

After isolating the nucleated cells from human bone marrow aspirates, they were resuspended in medium containing fibroblast growth factor-2 (FGF-2), dexamethasone, and ascorbic acid, and cell suspension were introduced within each tube: starting from a status of equilibrium in the level of liquid present in the two "twin" tubes connected by the U-shaped tubing, flow of the cell suspension was induced by the use of the computer-controlled syringe pump, at the flow rate inserted by the user (400 µm/sec); the direction of the flow was then reversed when the selected volume (V_1) of cell suspension had been perfused: in the following perfusion sequence, a double volume (V_1 x 2) was thus

A. Computer-controlled syringe pump

C. Scaffold chamber

B. Perfusion bioreactor

Fig. 4. Main components of the perfusion bioreactor system.

perfused in the opposite direction. This pattern was then repeating itself until all the cells were attached to the scaffold (5 days).

Because scaffolds were press-fit into the chamber, the cell suspension could not deviate around the scaffold, and was therefore forced to flow through its pores. At the end of the cell seeding phase, stopcocks were simply rotated to divert flow through Interlink® injection sites (Becton Dickinson), which were connected to empty syringes, thus collecting the old medium without removing the system from the incubator; old medium was then replaced by fresh medium through an other syringe placed on the other side of the system, similarly connected to the stopcock of the twin-tube via Interlink® injection sites. Medium was then perfused through the constructs at a lower velocity (100 µm/sec) for additional 14 days (cell expansion phase), with two media changes per week.

Fig. 5. Complete bioreactor system placed in the incubator.

Following the total 19 days of perfusion within the bioreactor system (Fig. 5), constructs were either removed from the system and ectopically implanted in nude mice in order to assess their capability of bone formation, or further perfused with collagenase and trypsin solutions, respectively for 60 and 20 minutes, in order to extract the expanded cells from the generated constructs, and thus characterize them through several in vitro assays (cell number, colony-forming units efficiency [CFU-f], Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction [Real-Time PCR], Fluorescence-Activated Cell Sorting Analysis [FACS]), as described in detail in the following three Chapters.

Using the defined experimental system, we first investigated whether human BMSC can be seeded, expanded and differentiated in 3D ceramic scaffolds by perfusing the nucleated cells of marrow aspirates through the scaffold pores, bypassing the conventional process of monolayer expansion. We then compared the osteoinductivity of the resulting 3D constructs with that obtained using monolayer-expanded BMSC (Chapter 4, [3]).

In order to validate the possibility of extending the use of the developed 3D-culture system for generating osteoinductive grafts of clinically relevant size, we then investigated whether a minimum cell density is required for the reproducibile bone tissue formation (Chapter 5).

We finally investigated whether the use of the developed 3D-culture system could be extended to engineer osteoinductive constructs based on ovine BMSC (Chapter 6, [4]): this would allow us to test the osteoinductivity of orthotopic implants in a sheep model.

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METHODS
AND RESULTS

CHAPTER 4

THREE-DIMENSIONAL PERFUSION CULTURE OF HUMAN BONE MARROW CELLS AND GENERATION OF OSTEOINDUCTIVE GRAFTS

Enclosed is the pdf-file of the Paper published in Stem Cells 2005;23:1066–1072.

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Three-Dimensional Perfusion Culture of Human Bone Marrow Cells and Generation of Osteoinductive Grafts

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Key Words. Bone marrow stromal cells • Bone marrow cells • Colony formation • Expansion
Hematopoiesis • Mesenchymal stem cells • Osteoprogenitor • Tissue regeneration

ABSTRACT

Three-dimensional (3D) culture systems are critical to investigate cell physiology and to engineer tissue grafts. In this study, we describe a simple yet innovative bioreactor-based approach to seed, expand, and differentiate bone marrow stromal cells (BMSCs) directly in a 3D environment, bypassing the conventional process of monolayer (two-dimensional [2D]) expansion. The system, based on the perfusion of bone marrow–nucleated cells through porous 3D scaffolds, supported the formation of stromal-like tissues, where BMSCs could be cocultured with hematopoietic progenitor cells in proportions dependent on the specific medium supplements. The resulting engineered constructs, when implanted ectopically in nude mice, generated bone tissue more reproducibly, uniformly, and extensively than scaffolds loaded with 2D-expanded BMSCs. The developed system may thus be used as a 3D in vitro model of bone marrow to study interactions between BMSCs and hematopoietic cells as well as to streamline manufacture of osteoinductive grafts in the context of regenerative medicine. STEM CELLS *2005;23:1066–1072*

INTRODUCTION

Bone marrow stromal cells (BMSCs) have received increasing experimental and clinical interest, owing to their surprising degree of plasticity [1–3] and their potential use for treatment of genetic [4] or immunologic [5] pathologies. In the field of regenerative medicine, BMSCs have been most extensively used for bone repair because their default pathway seems to be osteogenic [6]. This has led to encouraging findings in heterotopic models [7, 8], in orthotopic implants [9, 10], and in a few clinical cases [11]. Given their low frequency among bone marrow–nucleated cells (approximately 0.01%), BMSCs are typically selected and expanded by sequential passages in monolayer (two-dimensional [2D]) cultures. However, 2D-expanded BMSCs have a dramatically reduced differentiation capacity compared with those found in fresh bone marrow [12, 13], which limits their potential use for therapeutic purposes [6, 14].

Reasoning that a three-dimensional (3D) culture system may represent a more physiological environment than a Petri dish for a variety of cells [15, 16] and that fluid flow is an important component for seeding and culturing BMSCs in 3D environments [17, 18], we aimed in this work at developing an innovative procedure to seed and expand BMSCs directly into porous 3D scaffolds under perfusion. We demonstrated that perfusion of bone marrow–nucleated cells through the pores of 3D ceramic scaffolds resulted in the efficient expansion of clonogenic BMSCs and in the generation of highly osteoinductive grafts. Moreover, the developed system allowed us to coculture BMSCs with hematopoietic cells and to support hematopoiesis.

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Materials and Methods

Bone Marrow Cell Culture

Bone Marrow Aspirates

Bone marrow aspirates (20- to 40-ml volumes) were obtained from eight healthy donors (36–54 years old) during routine orthopedic surgical procedures in accordance with the local ethical committee (University Hospital Basel) and after informed consent. Nucleated cells were isolated from aspirates by Ficoll density-gradient centrifugation. The initial number of BMSCs, defined as the number of fibroblast colony-forming units (CFU-F) in the fresh marrow aspirates, averaged 21 ± 7 per 10^5 nucleated cells.

Culture Medium

Unless otherwise stated, medium (α-modified Eagle's medium) containing 10% fetal bovine serum was supplemented with 5 ng/ml fibroblast growth factor-2, 10 nM dexamethasone, and 0.1 mM L-ascorbic acid-2-phosphate to increase BMSC proliferation and osteogenic commitment [8, 19]. In some experiments, medium was alternatively supplemented with 2 ng/ml interleukin-3, 10 ng/ml stem cell factor, and 20 ng/ml platelet-derived growth factor-bb to support maintenance of hematopoietic cells in culture [20] (hematopoietic medium).

3D Culture

Using a perfusion bioreactor system we previously developed for cell seeding of 3D scaffolds [18], an average of 18.4 ± 6.6 million freshly isolated bone marrow–nucleated cells were perfused through 8-mm-diameter, 4-mm-thick disks of porous (total porosity, $83\% \pm 3\%$; pore size distribution: 22%, <100 μm; 32%, 100–200 μm; 40%, 200–500 μm; 6%, >500 μm) hydroxyapatite ceramic (Engipore; Fin-Ceramica Faenza, Faenza, Italy, http://www.finceramicafaenza.com) at a superficial velocity of 400 μm per second (previously determined to result in efficient and uniform cell seeding). Based on CFU-F assays of five marrow aspirates, an estimated average of $4.8 \pm 2.6 \times 10^3$ BMSCs was perfused through each disk, corresponding to 4 BMSCs per cm^2 of ceramic surface area. Such clonogenic BMSC seeding density was previously described to prolong BMSC lifespan and differentiation potential [14]. After 5 days (cell seeding phase), harvested medium was plated in tissue culture dishes to quantify the fraction of CFU-F not seeded. Fresh medium was then added to the system, and the cell-ceramic constructs were perfused for an additional 14 days (cell expansion phase) at a velocity of 100 μm per second (previously determined to support cell viability throughout the scaffold thickness), with medium changes twice a week. As a control, bone marrow–nucleated cells from each donor were plated on tissue-culture dishes (2D expansion) using the same initial cell number/surface area as in the 3D ceramic disks and cultured for 19 days without passaging, with the same schedule of medium changes as for the 3D culture.

Bone Formation Assays

Construct Implantation

Constructs from four independent experiments, after the cell seeding or cell expansion phases of 3D culture, were implanted ectopically in recipient nude mice (CD-1 nu/nu, 1 month old; Charles River Laboratories, Sulzfeld, Germany, http://www.criver.com/index.html) in accordance with institutional guidelines. As a control, we implanted ceramics seeded with 2D-expanded BMSCs at the same density as measured in the corresponding 3D cultured constructs after the cell expansion phase. Seeding of 2D-expanded BMSCs was performed by static loading of a cell suspension. We previously reported that the fraction of cells retained in the scaffolds after seeding by static loading was similar to that obtained using the described perfusion device, although cells seeded statically were less uniformly distributed [18].

Quantitative Assessment of Bone Tissue Formation

Eight weeks after implantation, constructs were fixed in 4% formalin, decalcified (Osteodec; Bio-Optica, Milan, Italy, http://www.bio-optica.it), paraffin embedded, and sectioned at six different levels (5-μm-thick sections at 600-μm intervals). For each cross-section, stained by hematoxilin/eosin, six images (covering most of the total cross-sectional area) were used to quantify the amount of bone tissue normalized to the total available pore space, as previously described [21]. The uniformity of bone tissue formation was quantitatively determined from the average ($\bar{x}$) and standard deviation (s) of the bone amounts measured in each cross-section [18] as follows:

$$\%\ uniformity = 100 \times \left(1 - \left(s/\bar{x}\right)\right)$$

Cell Characterization

Scanning Electron Microscopy

Constructs cultured in the 3D system after the cell expansion phase were fixed in 4% formalin, dehydrated, critical point dried, and coated with 20 nm of Au. Scanning electron microscopy observation was performed with an ESEM XL 30 (Philips, Amsterdam, The Netherlands, http://www.philips.com) with 10-kV acceleration.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

mRNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), treated with DNAse, and retrotranscribed into cDNA, as previously described [19]. Polymerase chain reaction was performed and monitored with the ABI Prism 7700 Sequence Detection System (PerkinElmer/Applied Biosystems, Rotkreuz, Switzerland, http://www.perkinelmer.com), and

expression levels of genes of interest (bone sialoprotein [BSP], collagen type I [CI], and osteopontin [OP]) were normalized to the 18S rRNA. Previously determined levels of expression of the genes of interest in human osteoblast cultures, also normalized to 18S rRNA [19], were used as reference.

Cell Extraction

After the cell expansion phase in the 3D culture system, cells were extracted from the ceramic pores by perfusing a solution of 0.3% collagenase and 0.05% trypsin/0.53 mM EDTA at 400 μm per second. Extracted cells were assessed for the ability to form fibroblastic and hematopoietic colonies and characterized by flow cytometry, as described below.

CFU-F Assay

CFU-F assays of expanded cells were performed by plating four cells per cm^2 in tissue culture dishes. After 10 days of culture, cells were fixed in 4% formalin and stained with 1% methylene blue, and the number of colonies was counted.

Hematopoietic Colony-Forming Unit Assay

Hematopoietic colony-forming unit assays were performed as previously described [22] to quantify the following types of hematopoietic clonogenic cells: neutrophils, macrophages, burst-forming-unit-erythroid, and granulocyte-erythroblast-macrophage-megakariocyte. Briefly, 2.5×10^5 cells per ml were cultured in medium containing 1.75 U/ml erythropoietin, 2.625 ng/ml granulocyte-colony stimulating factor, 40 U/ml granulocyte macrophage colony stimulating factor, 40 U/ml interleukin-3, and 62.5 ng/ml stem cell factor. After 14 days, the colonies were classified and counted.

Fluorescence-Activated Cell Sorting Analysis

Cell suspensions were incubated with antibodies against CD105 (Serotec), STRO-1, BSP, CI, OP (all from Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), nerve growth factor receptor (NGFR), or CD45 (both from Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and analyzed using a FACSCalibur flow cytometer (Becton, Dickinson and Company). Reactions with anti-BSP, -OP, or -CI were proceeded by membrane permeabilization with BD Cytofix/Cytoperm Plus Kit (Becton, Dickinson and Company). Positive expression was defined as the level of fluorescence greater than 95% of corresponding isotype-matched control antibodies.

Results and Discussion

BMSC Expansion Under 3D Perfusion

Using a bioreactor system recently developed for efficient and uniform seeding of anchorage-dependent cells into 3D scaffolds [18], we perfused the nucleated cells of human bone marrow aspirates in alternate directions through the pores of disk-shaped ceramic scaffolds, and we hypothesized that BMSCs would attach to the ceramic substrate and proliferate. The number of BMSCs perfused through each scaffold, estimated by CFU-F assays, averaged $4.8 \pm 2.6 \times 10^3$ cells. Medium was first changed after 5 days (cell seeding phase), which resulted in the elimination of the non-attached cell population, containing negligible numbers of CFU-F (<1% of those seeded in the scaffolds). Fresh medium was further perfused for an additional 14 days (cell expansion phase), during which time the total number of cells, monitored by Alamar blue, was found to increase at a nearly exponential rate (Fig. 1). At 19 days, the number of BMSCs found within the ceramic pores, calculated as the $CD105^+$ fraction of the extracted cells, averaged $9 \pm 3 \times 10^5$ cells for each scaffold. These data demonstrate that BMSCs can be seeded and extensively expanded (average of 8.2 ± 0.9 doublings in 19 days) by perfusion of bone marrow cell suspensions through 3D porous scaffolds, thereby avoiding typical 2D expansion.

Bone Formation by Expanded BMSCs

The osteoinductivity of the constructs resulting from BMSC seeding and expansion in the porous ceramic under perfusion (total of 19 days culture) was verified by ectopic implantation in nude mice. Reproducible, extensive, and markedly uniform bone formation was found in implanted constructs from four out of four independent experiments, performed using aspirates from different donors. Mature lamellar bone, organized in typical bone/marrow ossicles [23], filled an average of 52.1% ± 7.7% of the total available pore space and was distributed throughout the scaffold volume with high uniformity (Fig. 2). In contrast,

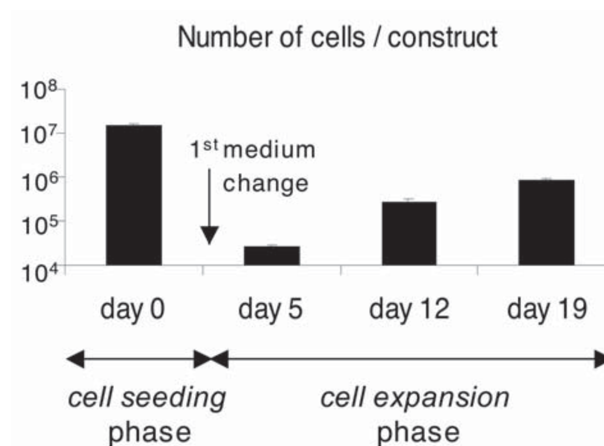

Figure 1. Total number of cells per construct detected in the three-dimensional (3D) system by Alamar blue assays. At day 0, the number of cells corresponds to the total number of cells added to the 3D system. At day 5, after removing the non-adherent cells with the first medium change, the total number of cells corresponds to the cells attached to the scaffold.

when 2D-expanded BMSCs from the same donors were loaded into ceramic scaffolds at the same density as measured in the corresponding 3D cultured constructs, bone tissue was formed in only one of the four experiments. Moreover, in those constructs positive for bone formation, bone tissue filled only 9.6% ± 2.7% of the total available pore space and was localized to scattered peripheral regions (Fig. 2). The increased osteoinductivity of constructs generated using the developed system may have been supported by the ceramic substrate used for BMSC expansion [24], the 3D cell–cell interactions during culture [25], the regimen of fluid flow applied [17, 26], or combinations of these variables that remain to be further elucidated. Interestingly, constructs implanted immediately after the cell seeding phase, in which BMSCs were attached to the ceramic but had not significantly expanded, were never osteoinductive. This suggests that a critical density of osteoprogenitor cells is necessary to initiate bone formation and points out the limit of approaches based on direct implantation of scaffolds mixed with bone marrow aspirates, especially considering the known variability in the number of BMSCs per aspirate volume [27].

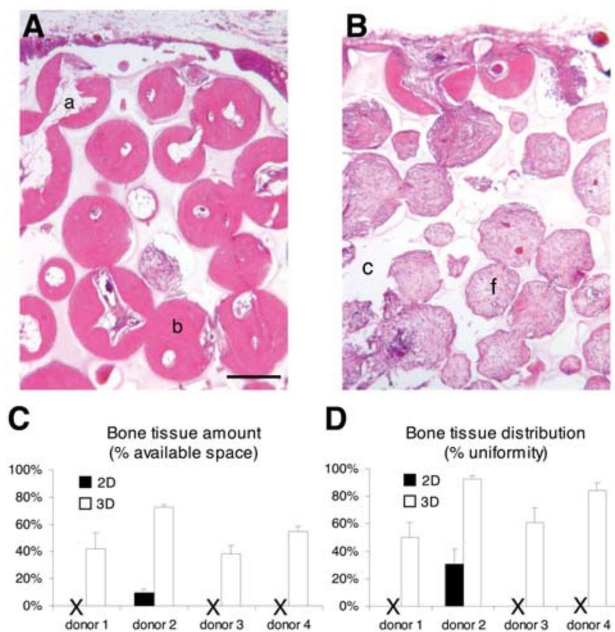

Figure 2. Bone tissue formation by bone marrow stromal cells (BMSCs) expanded in monolayers (two-dimensional [2D]) or under three-dimensional (3D) perfusion. **(A, B):** Representative hematoxilin/eosin-stained cross-sections of BMSC-ceramic constructs implanted ectopically in nude mice and harvested after 8 weeks. BMSCs expanded directly in the ceramic scaffolds in the 3D system yielded massive and uniformly distributed bone tissue **(A)**, in contrast to BMSCs loaded in the ceramic after traditional 2D culture **(B)**. White spaces correspond to the decalcified ceramic (c), whereas scaffold pores are filled with fibrous (f), adipose (a), or bone (b) tissue. Bar = 400 μm. **(C, D):** Quantitative image analysis of constructs generated using bone marrow aspirates from four independent donors further highlighted the increased reproducibility, amount **(C)**, and uniformity **(D)** of bone tissue formation after BMSC expansion under 3D compared with 2D. Values are presented as mean and SE of the percentages calculated for each cross-section. The crosses indicate no bone formation in any of the implanted constructs.

BMSC Characterization

We then preliminarily characterized the morphology, phenotype, and clonogenicity of cells seeded and expanded within the developed 3D system. Scanning electron microscopy indicated the formation of a stromal-like tissue within the ceramic pores, consisting of a 3D network of spheroidal cells in contact with heterogeneously shaped fibroblastic cells (Fig. 3A). The mRNA expression levels of genes encoding for the osteoblast-related proteins BSP, CI, and OP averaged, respectively, 3.6%, 35.3%, and 48.0% of those previously quantified in human osteoblast cultures [19] (Fig. 3B). Levels were similar to those measured in 2D-expanded BMSCs and lower than those measured in BMSCs after osteogenic differentiation [19]. Fluorescence-activated cell sorting analyses indicated that 68% ± 18% of the cells extracted from the ceramic scaffolds were positive for CD105, a surface marker typically expressed by cells of the mesenchymal lineage (Fig. 3C). These CD105$^+$ cells expressed low levels of STRO-1

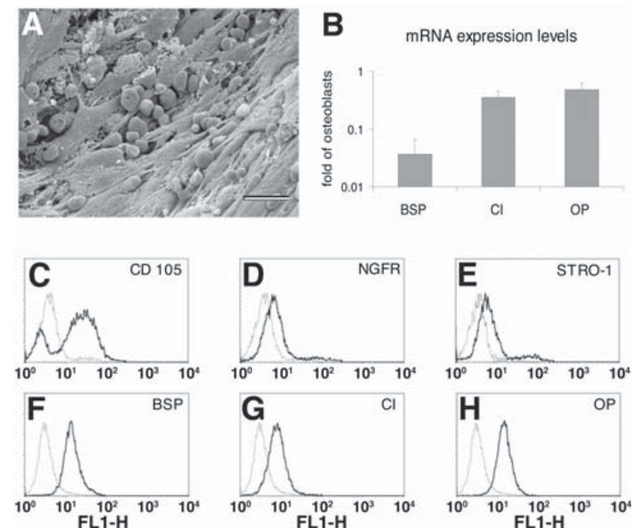

Figure 3. Morphology and phenotype of cells expanded under three-dimensional (3D) perfusion. **(A):** Scanning electron microscopy images of the constructs generated by perfusion of bone marrow–nucleated cells through the pores of ceramic scaffolds for 19 days. The ceramic pores were filled with a stromal-like tissue, consisting of a 3D network of heterogeneously shaped cells and extracellular matrix. Bar = 10 μm. **(B):** mRNA expression levels of bone sialoprotein (BSP), collagen type I (CI), and osteopontin (OP) in the cells. Values are presented as mean and SE of three independent experiments. **(C–H):** Surface markers expressed by cells extracted from the ceramic scaffolds after 19 days culture. Cells positive for **(C)** CD105 expressed low levels of **(D)** nerve growth factor receptor (NGFR) and **(E)** STRO-I and high levels of **(F)** BSP, **(G)** CI, and **(H)** OP. Light line, isotype control; dark line, specific antibody.

(proposed as a marker of early mesenchymal progenitors [28]) and NGFR (proposed as a marker of multipotent BMSCs [29, 30]) and high levels of BSP, OP, and CI (Figs. 3D–3H). The percentage of CD105$^+$ cells capable of forming a fibroblastic colony (CFU-F) was markedly higher after expansion in the 3D than in typical 2D cultures (29.4% vs. 10.7%, respectively). Taken together, these data suggest that BMSCs generated in the developed 3D system were neither early undifferentiated mesenchymal precursors nor fully differentiated osteoblast-like cells but comprised a large population of clonogenic osteoprogenitor cells. Future studies should address whether changes in the substrate used (e.g., scaffold composition or architecture), flow rate, and culture medium composition will regulate the phenotype, proliferation, and multilineage differentiation capacity of the expanded BMSCs.

Hematopoietic Cell Characterization

The finding that a substantial fraction of the cells cultured in the developed 3D system was not of the mesenchymal lineage, as suggested by the rounded morphology and demonstrated by the lack of expression of CD105, induced us to investigate whether both hematopoietic and mesenchymal cells were cocultured within the ceramic pores. Indeed, in the engineered constructs we found cells positive for CD45, a surface marker of hematopoietic cells, at percentages (30% ± 15%) equivalent to those of cells negative for CD105 (Figs. 4A–4I). It is likely that cocultured hematopoietic cells, possibly including CD14-positive adherent macrophages, regulated the phenotype of BMSCs [31] and played a critical role in determining the osteoinductivity of the constructs, possibly by maintaining a higher fraction of clonogenic BMSCs. It has been described that upon transplantation into a host animal, BMSCs form an ectopic ossicle in which bone cells, myelosupportive stroma, and adipocytes are of donor origin whereas hematopoiesis and the vasculature are of recipient origin [23]. Considering that in our 3D system human hematopoietic cells were coimplanted with BMSCs, future studies should aim at determining whether human cells contributed to hematopoiesis in this model.

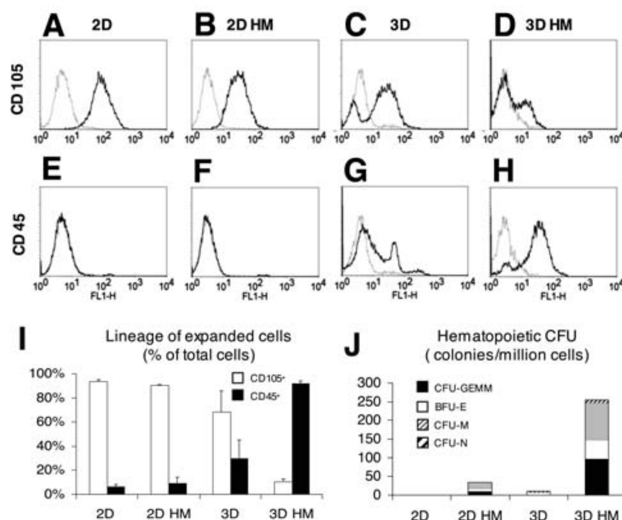

Figure 4. Fraction and clonogenicity of hematopoietic cells. **(A–H):** Representative profiles of cells labeled for CD105 or CD45 after two-dimensional (2D) or three-dimensional (3D) culture in standard or hematopoietic medium (HM). Light line, isotype control; dark line, specific antibody. **(I):** Percentages of CD105$^+$ and CD45$^+$ cells in the above conditions. Values are presented as mean and SE of four independent experiments. **(J):** Quantification of the following types of hematopoietic colony-forming units present within the populations generated in the above conditions: neutrophils (CFU-N), macrophages (CFU-M), burst-forming-unit-erythroid (BFU-E), and granulocyte-erythroblast-macrophage-megakariocyte (CFU-GEMM).

We next hypothesized that, through the addition of specific medium supplements, the developed 3D culture model allows the regulation of the relative proportions of hematopoietic and mesenchymal cells. Using supplements typically used for culture of hematopoietic cells (i.e., interleukin-3, stem cell factor, and platelet-derived growth factor-bb, hematopoietic medium) [20], the fraction of CD45$^+$ cells found after 19 days of 3D culture was increased to more than 90% (Fig. 4I) whereas BMSC proliferation capacity was still sustained (average of 4.5 ± 0.7 doublings in 19 days). Interestingly, the use of this culture medium further increased the percentage of CFU-F within CD105$^+$ cells from 29.4%–38.8% and generated relevant fractions of hematopoietic CFUs, including those with a mixed phenotype, indicative of early multilineage progenitor populations (Fig. 4J). Remarkably, the use of the same medium supplements in 2D cultures was not able to modulate the fractions of hematopoietic/mesenchymal cells nor their clonogenicity, possibly due to the fact that most of the non-adherent cells were not entrapped within the 3D niches of the ceramic or newly formed stromal-like tissue and were thus discarded during medium changes. This evidence further highlights the potential of the developed culture system, in which the 3D configuration under perfusion flow provides an extension of the concept of stromal feeder layer for the support and development of hematopoietic cells [23, 32] and thus modifies standard paradigms for culture of bone marrow cells.

Conclusions

Our study validates the simple but innovative concept that BMSCs can be seeded and expanded by perfusion culture through the pores of 3D scaffolds starting from minimally processed bone marrow aspirates and avoiding 2D culture expansion. The developed approach was used for the reproducible, spatially uniform, highly efficient, and simplified manufacture of osteoinductive grafts. Incorporating in the system features like automated medium change, monitoring and control of pH, gases, and metabolites are likely to lead to the development of a closed system for the automated and controlled production of autologous BMSC-based bone substitutes. Compared with previously proposed perfusion systems [17, 33], the elimination of the 2D culture would allow for

a one-phase, streamlined procedure that could thus generate engineered bone grafts at reduced costs and make them commercially viable against alternative off-the-shelf osteoinductive materials (e.g., based on the delivery of growth factors). In this context, however, scaling up of the procedure to clinically relevant sizes will have to address the challenge of maintaining cell viability in larger constructs, both during in vitro culture and upon grafting.

Beyond the relevance in the field of bone tissue engineering, our results validate the developed process as a first step toward ex vivo tissue engineering of bone marrow as a model to investigate proliferation, differentiation, and interactions among different types of bone marrow cells in a more physiological environment than previously established systems (e.g., Petri dishes or spinner flasks [20]). The developed culture system may be further explored for the expansion under perfusion of $CD34^+$ hematopoietic stem cells from bone marrow or cord blood within an engineered 3D stromal network. Finally, the same paradigm of bypassing 2D expansion by direct 3D perfusion culture may be used for the engineering of other 3D tissues and organs.

Acknowledgments

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CHAPTER 5

THE OSTEOINDUCTIVITY OF ENGINEERED BONE CONSTRUCTS IS RELATED TO THE DENSITY OF CLONOGENIC BONE MARROW STROMAL CELLS IMPLANTED

Enclosed is the Paper currently in preparation.

The osteoinductivity of engineered bone constructs

is related to the density of clonogenic bone marrow stromal cells implanted

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Abstract

Reproducibility in bone formation is a key issue for generating bone substitutes based on bone marrow stromal cells (BMSC) and three-dimensional (3D) scaffolds for clinical use. The concept that BMSC seeding density on 3D scaffolds may influence the reproducibility of subsequent bone formation may seem intuitive, but limited data are currently available. In this study we hypothesized that presence or absence of bone in ectopically implanted constructs is related not to the number of total BMSC, but to the number of clonogenic BMSC (colony forming unit-fibroblast, CFU-f) present in the constructs at the time of implantation.

Human BMSC were seeded and expanded on 3D porous ceramic scaffolds by perfusing the nucleated cell fraction of marrow aspirates, and the generated constructs were assessed for cell number, cell clonogenicity, and for their osteoinductivity following ectopic implantation in nude mice.

The number of clonogenic BMSC, but not the number of total BMSC, was positively correlated to the initial cell seeding density. The number of total BMSC was similar in osteoinductive and not osteoinductive constructs, whereas that of clonogenic BMSC was significantly different: an apparent threshold (at around 3.0E+05 CFU-f) could be observed, discriminating between osteoinductive and not osteoinductive constructs.

These results indicate that CFU-f play a fundamental role in determining the capability of the constructs to form bone. The identification of specific markers for clonogenic BMSC after expansion will be necessary to establish protocols for predicting bone formation and/or to enrich CFU-f populations within expanded BMSC.

Key words: Bone marrow stromal cells, clonogenicity, CFU-f, bone tissue engineering, osteoinductivity.

Introduction

One of the most typical approaches for engineering osteoinductive tissues is based on seeding and possibly culturing bone marrow stromal cells (BMSC) into three-dimensional (3D) porous ceramic scaffolds, which prime cell differentiation towards the osteogenic lineage and provide the template for in vivo bone tissue formation [1, 2]. BMSC, which are typically defined by their capacity to adhere on plastic [3] and form a fibroblastic colony (CFU-f) [4], represent a very low fraction (approximately 0.01%) [5] among the nucleated cells of the bone marrow. In order to overcome such a low frequency, prior to statically loading them into ceramic scaffolds, BMSC are typically expanded in monolayer (2D). Culture expansion in 2D causes BMSC to progressively lose their early progenitor properties [6] and differentiation potential [7], and to decrease their capability to form colonies [8] and to induce bone tissue formation upon ectopic implantation in nude mice [6].

We recently demonstrated that human BMSC can be extensively expanded in 3D ceramic scaffolds by directly perfusing the nucleated cells of marrow aspirates through the scaffold pores, thus bypassing the conventional process of 2D-expansion [9]. BMSC expanded in the 3D perfusion system were found to be more clonogenic than those expanded in 2D, possibly due to a variety of reasons, including the maintenance of hematopoietic cells in culture. When the ceramic constructs containing the 3D-expanded BMSC were ectopically implanted in nude mice, bone tissue formation was more reproducible, abundant and uniform as compared to scaffolds loaded with the same number of 2D-expanded BMSC [9]. In order to validate the possibility of using the developed perfusion-based approach for the generation of osteoinductive grafts in amounts sufficient for clinical use, it is mandatory to identify a lower limit of cell density allowing for reproducible bone tissue formation across different donors. The concept that BMSC seeding density may influence the reproducibility of bone formation in tissue-engineered constructs may seem intuitive, but limited data are currently available to support this conclusion. Recent studies showed that when hydroxyapatite scaffolds seeded with different BMSC densities were ectopically implanted in nude mice, constructs with higher seeding densities appeared to contain significantly more bone [10]. However, it is difficult to establish a reproducible relation between cell seeding density and bone forming capacity of engineered

constructs if cells are not uniformly distributed throughout the entire scaffold, as it typically occurs if cells are statically loaded into porous scaffolds [11].

In this study, we aimed at investigating the relation between cell density and in vivo bone forming capacity of constructs generated in the developed 3D-system and then implanted in nude mice. The perfusion system used for generating the constructs allowed uniform cell distribution throughout the scaffolds, and the following ectopic implantation of the generated constructs allowed to determine their intrinsic osteoinductive properties in a supporting but not inducing environment.

Considering the previously established association between the higher clonogenicity of BMSC expanded in the 3D-system and the more reproducible and extensive osteoinductivity of the resulting constructs, as compared to those based on 2D-expanded BMSC, we now hypothesized that the presence or absence of bone in the constructs following ectopic implantation is related not to the total number of implanted BMSC, but to the number of CFU-f present in the construct at the time of implantation.

Materials and Methods

Cell isolation and culture

Bone marrow aspirates. Bone marrow aspirates (20-40 ml volumes) were obtained from 9 healthy donors (36-54 years old) during routine orthopedic surgical procedures, in accordance with the local ethical committee (University Hospital Basel) and after informed consent. Nucleated cells were isolated from aspirates by Ficoll density gradient centrifugation.

CFU-f assay. The initial number of bone marrow stromal cells (BMSC), defined as the number of colony-forming units fibroblastic (CFU-f) in the fresh marrow aspirates, was identified by plating 3.5E+03 nucleated cells/ cm^2 in tissue-culture dishes. Following 10 days of culture, cells were fixed in 4% formalin, stained with 1% methylene blue, and the number of colonies counted. Clonogenicity of marrow aspirates was expressed as the percentage of the initial nucleated cells which were able to form a fibroblastic colony.

Culture medium. Medium (α-Modified Eagle's Medium) containing 10% fetal bovine serum was supplemented with 5 ng/ml fibroblast growth factor-2, 10 nM dexamethasone, and 0.1 mM L-ascorbic acid-2-phosphate, in order to increase BMSC proliferation and osteogenic differentiation capacity [12, 13].

Cell culture. Using a perfusion bioreactor system we previously developed for seeding and culture of BMSC into the pores of 3D scaffolds [9, 11], freshly isolated bone marrow nucleated cells were perfused through disks (8 mm diameter, 4 mm thick) of porous (total porosity: 83 ± 3%; total surface area: 1260 cm^2; total volume: 200 cm^3) hydroxyapatite ceramic (Engipore, Fin-ceramica Faenza, Faenza, Italy) at a superficial velocity of 400 µm/sec (previously determined to result in efficient and uniform cell seeding), at concentrations ranging from 7.50E+05 to 2.25E+07. At the first medium change, after 5 days, unseeded cells were reintroduced into the system with fresh medium; the cell-ceramic constructs were then perfused for an additional 14 days at a velocity of 100 µm/sec (previously determined to support cell viability throughout the scaffold thickness), with medium changes twice a week. The presence of unseeded CFU-f in the medium following the first medium change was negligible.

Expanded cells characterization

Cell extraction. Following 19 days of culture under perfusion, cells were extracted from the ceramic pores by perfusing a solution of 0.3% collagenase and of 0.05% trypsin/0.53 EDTA at 1000 µm/sec, respectively for 60 and 20 minutes. Efficiency of cell extraction, evaluated by assessing the amount of DNA in the cell-extracted scaffolds as previously described [14], averaged 85±5%. Extracted cells were assessed by cytofluorimeter for mesenchymal or hemopoietic surface markers expression and their ability to form fibroblastic colonies as described below.

Cytofluorimetric analysis. Suspensions of extracted cells were labeled with APC-conjugated antibody against CD45 and FITC-conjugated antibodies against CD14, CD44, CD90, CD105, or PE-conjugated antibodies against CD34, CD29, CD73, or CD166 (all from Becton Dickinson and Company, Franklin Lakes, NJ). Positive expression was defined at a level of fluorescence greater than 97% of the corresponding isotype-matched control antibodies. Positive expression of CD45 was used to identify

cells of the hemopoietic lineage. Negative expression of CD14 and CD34, and positive expression of CD105, CD29, CD44, CD73, CD90, and CD166 (typical mesenchymal markers) were used to identify the mesenchymal lineage of CD45 negative cells, hereafter referred to as BMSC.

Cell proliferation. The number of BMSC doublings was calculated from the initial number of CFU-f seeded and the total number of CD45- cells extracted from the constructs after 19 days culture, assuming that all seeded CFU-f attached to the scaffolds [9].

CFU-f assay. CFU-f assays of the expanded cells were performed by plating 35 cells/cm^2 in tissue-culture dishes. Following 10 days of culture, cells were fixed in 4% formalin, stained with 1% methylene blue, and the number of colonies counted. Clonogenicity was expressed as the percentage of expanded BMSC, and was used to calculate the final number of CFU-f in the ceramic scaffolds.

Bone formation assay

Following 19 days of culture under perfusion, 32 constructs from 16 experimental groups generated in 9 independent experiments, i.e. with cells from 9 different donors, were harvested from the bioreactor system and ectopically implanted in recipient nude mice (CD-1 nu/nu, female, 1 month old; from Charles River, Germany), in accordance with institutional guidelines. Two constructs from the same experimental group were implanted in separate animals. Eight weeks after implantation, constructs were fixed in 4% formalin, decalcified (Osteodec, Bio-Optica, Italy), embedded in paraffin, cross-sectioned at 3 different levels (5 µm thick sections, at 500 µm intervals), and stained by Hematoxilin/Eosin.

Statistical analysis

Values are presented as mean ± standard deviation. Differences among experimental groups were assessed by two-tailed Student's *t* test or the Welch modified *t* test, in accordance with Levene's test for homogeneity of variances, after assessment of normality by skewness and kurtosis. A value of $p=0.05$ was selected as the threshold of statistical significance. Correlations were assessed using Pearson's test (two-tailed), and considered statistically significant with $p< 0.05$.

Results

Initial clonogenicity and cell seeding densities

Based on the clonogenicity of the marrow aspirates, which averaged 0.013% ± 0.005% of the initial nucleated cells, an estimated 60 to 4500 BMSC were initially perfused through the porous ceramics (Table 1).

Donor number	Initial number of nucleated cells per scaffold	CFU-f in the marrow aspirate (% of nucleated cells)	Initial number of BMSC per scaffold
1	9.00 E+06	0.010	900
2	4.20 E+06	0.016	670
3	8.40 E+06	0.010	840
4	1.00 E+07	0.010	1000
5	7.50 E+06	0.010	770
6	9.00 E+06	0.022	2000
7	2.50 E+06	0.014	350
7	7.50 E+06	0.014	1100
7	2.25 E+07	0.014	3200
8	7.50 E+05	0.008	60
8	7.50 E+06	0.008	600
8	2.25 E+07	0.008	1800
9	7.50 E+05	0.020	150
9	2.50 E+06	0.020	500
9	7.50 E+06	0.020	1500
9	2.25 E+07	0.020	4500

Table 1. Initial cell densities.

Characterization of extracted cells

Cell lineages. Cells extracted from ceramics were analyzed for the expression of CD45 by cytofluorimetry to quantify the relative fractions of CD45+/- cells and to further characterize the non-hemopoietic (i.e., CD45-) population. The CD45- fraction was confirmed to be of the mesenchymal lineage by the expression of adhesion molecules (CD44 and CD166), integrin CD29, and additional mesenchymal markers (CD73, CD90, CD105) [7], and by the lack of expression of CD34 and CD14 (Fig. 1).

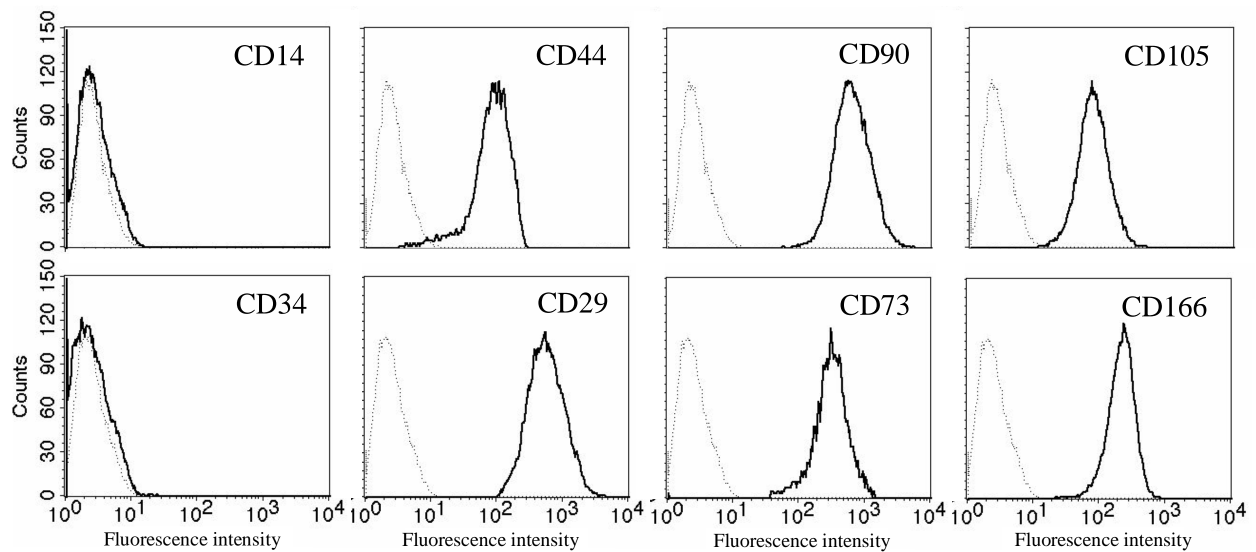
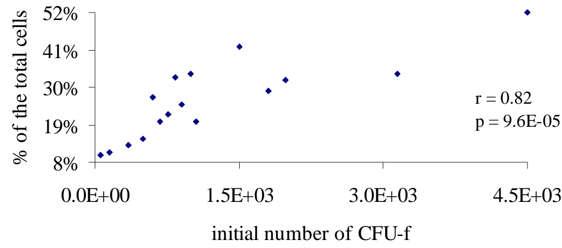
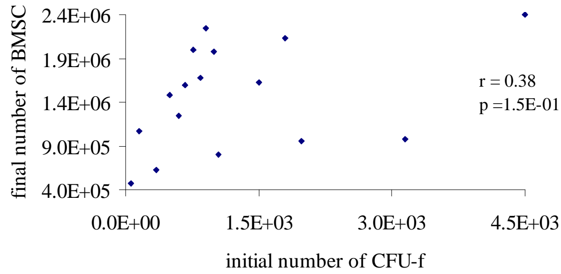


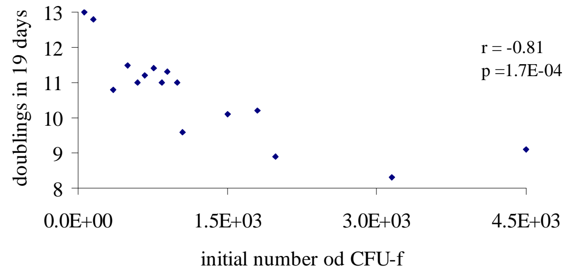
Fig. 1. Phenotype of expanded BMSC. Expression levels of FITC-conjugated anribodies against CD14, CD44, CD90, CD105, and PE-conjugated antibodies against CD34, CD29, CD73, CD166, by expanded CD45 negative cells.

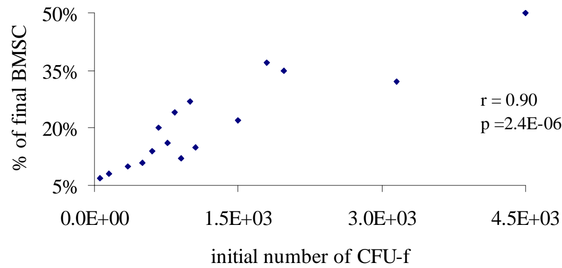
Cell proliferation. As shown in Fig. 2a, as the initial number of CFU-f in constructs increased, a higher fraction of the final cell population consisted of hemopoietic cells (from 10%-52%). The complementary mesenchymal fraction of the final population was constituted by variable amounts of BMSC (Fig. 2b), ranging from 4.8E+05 to 2.4E+06 per construct, with no correlation to the number of initial CFU-f. The proliferation rate of BMSC decreased significantly with increasing initial CFU-f, such that the total number of doublings throughout 19 days of culture ranged from a maximum of 13 BMSC doublings to a minimum of only 8 (Fig. 2c).

Clonogenicity. In all experimental conditions, the final numbers of CFU-f following 19 days of culture were higher than the initial number seeded. Final numbers of CFU-f in the constructs were positively correlated to the initial numbers of CFU-f loaded in the ceramic scffolds (Fig 2e). Interestingly, increasing initial CFU-f densities gave rise to more clonogenic final BMSC populations, with percentages of CFU-f reaching 50% of the final BMSC population (Fig. 2d).









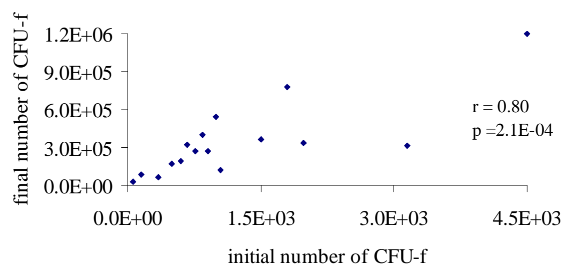


Fig. 2. Positive correlations between the initial number of seeded CFU-f and the final fraction of hemopoietic cells (A), the final fraction of CFU-f (D), and the final number of CFU-f (E). Negative correlation between the initial number of seeded CFU-f and the number of doublings of BMSC in 19 days (C). No correlation between the initial number of seeded CFU-f and the final number of BMSC (B).

Bone formation

A total of 32 specimens from 9 independent experiments were ectopically implanted in nude mice. Following 8 weeks in vivo, extensive and uniform bone formation was found in 16 implanted constructs, whereas only fibrous tissue was observed in the other 16 implants. The presence or absence of bone formation was then related to the number of CFU-f and/or BMSC within the ceramics at the time of implantation. There was no significant difference ($p = 0.164$) between the final number of BMSC in constructs that contained either bone or only fibrous tissue (Fig. 3a). In fact, constructs with BMSC densities ranging from the low through high end of our range resulted in both osteoinductive and non-osteoinductive grafts. In contrast, there was a significant difference ($p = 0.010$) between the final number of CFU-f in constructs that generated bone or no bone (Fig. 3b). An apparent threshold in the number of implanted CFU-f could be observed, discriminating between ostoinductive (containing> 3.0E+05 CFU-f) and not osteoinductive (containing < 3.0E+05 CFU-f) constructs (Fig. 3c).

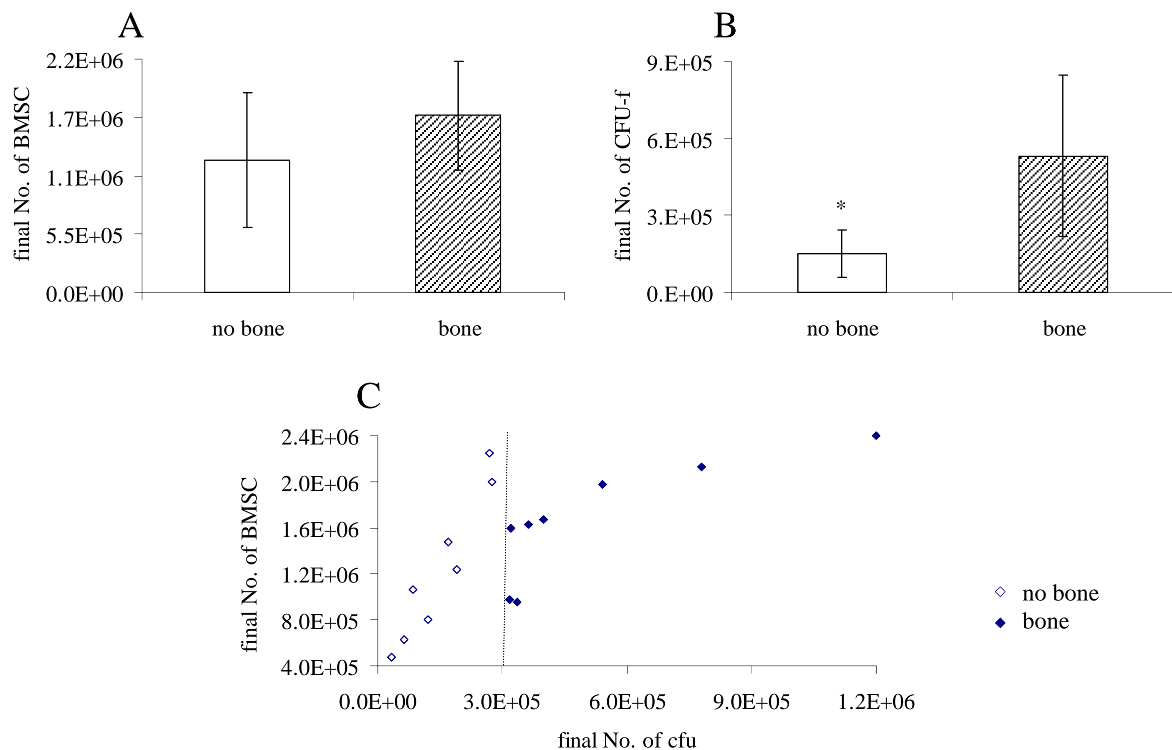


Fig. 3. Relation between the presence (shaded) or absence (white) of bone in the generated constructs and the number of implanted BMSC (A) or CFU-f (B): osteoinductive and not osteoinductive constructs contained similar numbers ($p = 0.164$) of BMSC (A) but statistically different numbers ($p = 0.010$) of CFU-f (B). In C, each dot represents the numbers of implanted BMSC and CFU-f in each experimental group (2 constructs per group) which formed bone (filled dots) or fibrous tissue (unfilled dots). The dashed line indicates the threshold in the number of CFU-f (3.0E+05) discriminating between osteoinductive and not osteoinductive constructs.

Discussion

The aim of this study was to identify a relation between cell density and in vivo bone forming capacity of constructs based on bone marrow stromal cells (BMSC) and porous ceramic scaffolds ectopically implanted in nude mice.

By using a recently developed 3D-system for the uniform seeding and culture of BMSC on 3D porous ceramic scaffolds, we demonstrated that bone forming capacity of the generated constructs is related to the number of implanted colony-forming units fibroblastic (CFU-f). We identified in 3.0E+05 CFU-f the apparent threshold discriminating between osteoinductive and not osteoinductive constructs. These results indicate that constructs are osteoinductive if containing a minimum number of CFU-f, and suggested that the presence of high amounts of CFU-f plays a fundamental role in determining the capability of the constructs to form bone.

Several factors could promote the production of constructs containing high numbers of CFU-f. The most obvious parameter influencing the final number of CFU-f is their initial number: in fact we observed that increasing initial densities of CFU-f resulted in a final BMSC population which was much more clonogenic than that obtained seeding low numbers of CFU-f. In addition we observed that increasing initial numbers of CFU-f resulted in a final population containing higher fractions not only of clonogenic BMSC, but also of hemopoietic cells: basing on recent studies indicating the existence of a crosstalk between hemopoietic and mesenchymal bone marrow cells enhancing the number of CFU-f [15], the high clonogenicity obtained in the osteoinductive constructs could have been determined by the co-culture of high fractions of hemopoietic cells. Further studies should investigate the correlation between the maintenance of high fractions of hemopoietic cells and the osteoinductivity of the generated constructs.

On the other hand we observed that the total number of final BMSC is related neither to the number of CFU-f initially seeded nor to the bone forming capacity of the generated constructs: these results suggest that that final number of BMSC cannot be considered as a parameter to predict the osteoinductive capacity of the constructs.

The phenotypic characterization of BMSC expanded in the developed 3D-system showed a uniform expression of mesenchymal markers: this indicates that the markers monitored in this study

could not discriminate between clonogenic and not clonogenic BMSC. There has been an increasing interest in the last decade in the identification of specific markers for the osteoprogenitor cells contained in the bone marrow. Simmons demonstrated [16] the use of STRO-1 antibody for enrichment of mesenchymal precursors: this antibody was shown to bind to all the cells associated with CFU-f activity of human bone marrow [17] and was used to identify CFU-f of the bone marrow with osteoprogenitor properties [18]. Other studies [19-21] suggested several other markers (CD73, CD49a, CD63/HOP-26, CD166/SB-10) identifying bone marrow subsets containing all the CFU-f. The identification of specific markers for clonogenic BMSC following in vitro expansion, will allow to prospectively isolate CFU-f-enriched populations prior to their implantation.

Considered that (i) constructs were osteoinductive if containing at least 3.0E+05 CFU-f, (ii) this final number of CFU-f was achieved by seeding at least 1.5E+03 CFU-f, and (iii) the typical clonogenicity of human bone marrow aspirates is 1.0E+02 CFU-f per million of nucleated cells, we can derive that by seeding at least 1.5 E+07 nucleated cells per scaffold, the resulting construct should be predictably osteoinductive. Therefore, in order to scale-up the generation of osteoinductive constructs based on 100% hydroxyapatite scaffolds for producing implants of clinical-relevant size (e.g. scaffolds of 5 cm^3), at least 3.75 E+08 nucleated cells per 5 cm^3 scaffold should be seeded. However, preliminary studies should demonstrate that the increment of the scaffold size would not negatively effect (i) the uniform cell distribution and nourishment within the scaffold during in vitro culture, and (ii) the in vivo survival of the cells throughout the entire construct, which were obtained in this study. In addition, such a large number of nucleated cells cannot be easily obtained from the typical amount of harvested bone marrow (20-40 ml). Combining protocols for the selection of purified populations of CFU-f from the bone marrow [22] and/or culture conditions for the selective survival of subset of cells enriched in CFU-f [8], it will be likely possible in the future to obtain from limited bone marrow volumes highly clonogenic cell populations, i.e. containing sufficient numbers of CFU-f for generating reproducibly osteoinductive constructs of clinically-relevant size.

Acknowledgments

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CHAPTER 6

ENGINEERING OF OSTEOINDUCTIVE GRAFTS
BY ISOLATION AND EXPANSION OF OVINE BONE MARROW STROMAL CELLS DIRECTLY ON 3D CERAMIC SCAFFOLDS

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Engineering of Osteoinductive Grafts by Isolation and Expansion of Ovine Bone Marrow Stromal Cells Directly on 3D Ceramic Scaffolds

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Abstract: In this work, we investigated whether osteoinductive constructs can be generated by isolation and expansion of sheep bone marrow stromal cells (BMSC) directly within three-dimensional (3D) ceramic scaffolds, bypassing the typical phase of monolayer (2D) expansion prior to scaffold loading. Nucleated cells from sheep bone marrow aspirate were seeded into 3D ceramic scaffolds either by static loading or under perfusion flow and maintained in culture for up to 14 days. The resulting constructs were exposed to enzymatic treatment to assess the number and lineage of extracted cells, or implanted subcutaneously in nude mice to test their capacity to induce bone formation. As a control, BMSC expanded in monolayer for 14 days were also seeded into the scaffolds and implanted. BMSC could be isolated and expanded directly in the 3D ceramic scaffolds, although they proliferated slower than in 2D. Upon ectopic implantation, the resulting constructs formed a higher amount of bone tissue than constructs loaded with the same number of 2D-expanded cells. Constructs cultivated for 14 days generated significantly more bone tissue than those cultured for 3 days. No differences in bone formation were found between samples seeded by static loading or under perfusion. In conclusion, the culture of bone marrow nucleated cells directly on 3D ceramic scaffolds represents a promising approach to expand BMSC and streamline the engineering of osteoinductive grafts.
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Keywords: mesenchymal stem cells; perfusion; 3D culture; bone formation; bone marrow cells; tissue engineering

INTRODUCTION

Bone marrow stromal cells (BMSC) represent a phenotypically and functionally heterogeneous population of mesenchymal precursors providing support for hematopoieses and contributing to the physiological regeneration of bone, cartilage, adipose, muscle, and other connective tissues. For bone tissue engineering applications, BMSC are typically first expanded in monolayer (2D), in order to overcome their very low fraction among bone marrow cells. The expanded cells are then statically loaded into three-dimensional (3D) porous ceramic-based scaffolds, which prime cell differentiation towards the osteogenic lineage and provide the template for bone tissue formation (Haynesworth et al., 1992; Martin et al., 1997). The resulting constructs have been demonstrated to be osteoinductive in ectopic models (Cancedda et al., 2003; Muraglia et al., 1998), and to support repair of large segmental defects in sheep (Kon et al., 2000; Petite et al., 2000) and in humans in a few clinical cases (Quarto et al., 2001). In some studies, it was further proposed that in vitro culture of 2D-expanded BMSC in 3D scaffolds under appropriate conditions could accelerate and enhance in vivo bone tissue formation (Kruyt et al., 2004; Mendes et al., 2002; Sikavitsas et al., 2003b; Wang et al., 2003).

In all these approaches, the phase of BMSC expansion in 2D is associated not only with biological concerns, such as the loss of cell differentiation capacity with serial passaging (Banfi et al., 2000), but also with a non-standardized and labor-intensive production of the osteoinductive grafts (e.g., for serial passaging or seeding of the expanded cells into a 3D scaffold). In addition, the static loading of the cells into 3D scaffolds may result in non-uniform distributions, with higher cellular density at the surface layers (Wendt et al., 2003).

Based on these considerations, in this study we aimed at generating osteoinductive constructs exclusively within a 3D culture environment. In particular, we tested the hypothesis that ovine BMSC can be isolated and expanded within 3D ceramic scaffolds by direct loading and culture of bone marrow nucleated cells into the scaffold pores. The ectopic

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osteoinductivity of the resulting constructs was compared to that of ceramics loaded with 2D-expanded BMSC or with bone marrow nucleated cells, implanted without 3D pre-culture. Moreover, based on the demonstrated efficacy of 3D perfusion systems to improve the efficiency and uniformity of cell seeding within porous 3D scaffolds (Bancroft et al., 2002; Sikavitsas et al., 2003; Wendt et al., 2003), we investigated whether dynamic perfusion seeding of bone marrow nucleated cells within 3D ceramics could improve the amount and uniformity of bone formed in vivo as compared to static cell loading.

MATERIALS AND METHODS

Generation and Implantation of Constructs

Isolation of Bone Marrow Nucleated Cells

Bone marrow aspirates (10 mL volume) were obtained from the iliac crest of two adult sheep (average age: 3 years) under total anesthesia and diluted 1:3 with phosphate buffered saline (PBS). Bone marrow nucleated cells were isolated using a density gradient solution (Ficoll, Histopaque®, Sigma Chemical, Buchs, CH) and counted with a standard nuclear stain (methylene blue, Fluka Chemie GmbH, Buchs, CH).

Culture Medium

Cells were cultured in alpha-modified Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES buffer, 1 mM sodium pyruvate, and 1 ng/mL fibroblast growth factor-2 (FGF-2). FGF-2 was used in order to enhance BMSC proliferation and maintenance of the post expansion bone formation capacity (Martin et al., 1997). All cultures were performed within 37°C/5% CO_2 humidified incubators.

Scaffolds

Ceramic scaffolds (Engipore, Finceramica Faenza, Italy) were porous discs (8 mm diameter, 4 mm thick) with $80\% \pm 3\%$ macro-porosity. The scaffolds were made of 100% hydroxyapatite, with a 1.67 Ca/P ratio, 0.63 g/cm^3 density, and 0.93 m^2/g specific surface, resulting in about 1,200 cm^2/scaffold.

Bone Marrow Cell Culture in 3D

Freshly isolated bone marrow nucleated cells were seeded in the pores of the ceramic scaffolds (1.1E + 07 nucleated cells/scaffold) either by static loading or by direct perfusion. For static loading, scaffolds were pre-wet in culture medium, blotted dry on a sterile gauze, and transferred into 12-well plates previously coated with a thin layer of 1% agarose, in order to prevent cell adhesion. Cells were resuspended at a density of 2.8E + 08 cells/mL, and 40 μL aliquots of the suspension were slowly dispersed over the top surface of the scaffolds with a micropipette. After 1 h, 4 mL of culture medium were gently added along the side of the wells. Constructs were maintained in culture for up to 14 days, with medium changes twice a week.

For perfusion seeding, 1.1E + 07 bone marrow nucleated cells were resuspended in 4 mL of medium and perfused in alternate directions at a flow rate of 1.2 mL/min through the pores of each scaffold, using a previously developed bioreactor system (Wendt et al., 2003). After 3 days, constructs were transferred into 12-well plates in fresh medium, and maintained in static culture for up to an additional 11 days (total = 14 days from the beginning of cell seeding), with medium changes twice a week.

Bone Marrow Cell Culture in 2D

Freshly isolated bone marrow nucleated cells were plated in plastic dishes (approximately 1E + 05 cells/cm^2), with medium changes twice a week. After approximately 10 days, when dishes were subconfluent, attached cells were harvested with 0.05% trypsin/0.53 mM EDTA and replated at 3E + 03 cells/cm^2. Following 14 days of 2D expansion, cells were statically loaded into ceramics using a number equivalent to, or 10-fold higher than the number of BMSC measured in the 3D cultured constructs.

Construct Implantation

The osteoinductive capacity of generated constructs was assessed by ectopic implantation in nude mice (CD-1 nu/nu, Charles River, Sulzfeld, Germany) for 8 weeks. Constructs based on 3D culture of bone marrow cells were implanted 3 or 14 days after the beginning of the seeding. Ceramics seeded with 2D-expanded BMSC were directly implanted, without 3D pre-culture. Three constructs for each experiment and experimental group were implanted in separate mice.

Analytical Methods

Fibroblastic Colony Forming Unit (CFU-f) Assays

In order to determine the fraction of clonogenic fibroblastic cells in the bone marrow aspirate, 2E + 05 nucleated cells were plated in 28 cm^2 tissue culture dishes; medium was changed after 3 days and twice a week thereafter. When colonies were clearly visible but not yet overlapping (after approximately 2 weeks), they were fixed in 4% buffered formalin, stained with 1% methylene blue and counted.

Scanning Electron Microscopy

Following 14 days of 3D culture, constructs seeded by static loading or perfusion were assessed for cell morphology by scanning electron microscopy. Scaffolds were washed in cacodylate buffer at 4°C for 10 min, fixed in 2.5% buffered

glutaraldehyde for 30 min at 4°C, and washed three times in cacodylate buffer at 4°C. Scaffolds were then dehydrated and imaged (Leo Stereo Scan 440 S, link-GEM, Oxford, UK).

Stain for Living Cells

The spatial distribution of living cells within the scaffold after the seeding phase was qualitatively assessed by staining with MTT (Sigma, St. Louis, MO). Constructs seeded by static loading or by perfusion were bisected, rinsed in PBS, placed in 12-well plates, and incubated at 37°C for 1.5 h with 3 mL of 0.12 mM MTT. Insoluble purple salts formed in the presence of living cells.

Cell Proliferation

Following 14 days of 3D culture, cells were extracted from the pores of the scaffolds by sequentially perfusing the constructs with 0.3% collagenase for 30 min followed by 0.05% trypsin/0.53 mM EDTA for 10 min (Braccini et al., 2005). To evaluate the efficiency of cell extraction, the amount of DNA in the cell-extracted scaffolds was then assessed using the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), following overnight digestion with 1 mL protease K solution, using amounts of known cell numbers as standards (Wendt et al., 2003). Cells expanded in 2D were also detached after 14 days, using the same reagents. Cell proliferation was defined as the number of doublings during the expansion phase, based on the initial number of CFU-f seeded.

Cytofluorimetric Analysis

Analysis with collagen type I labeling was performed to identify the fraction of mesenchymal cells in the different experimental groups. Cells expanded in 2D or extracted from 3D scaffolds were permeabilized with 250 μl Cytofix/Cytoperm Plus™ (Becton Dickinson, San Diego, CA), incubated with an antibody against collagen type I (SP1D8, Developmental Studies Hybridoma Bank, Iowa), rinsed, stained with FITC-conjugated goat anti-mouse IgG, and assessed cytofluorimetrically using a FACSCalibur flow cytometer (Becton Dickinson).

Quantification of Bone Formation

Explants were fixed in 4% buffered formalin for 24 h, decalcified with Osteodec (Bio Optica, Milan, Italy) for 5 h, paraffin embedded, cross-sectioned (5 μm thick) at six different levels and stained with hematoxylin–eosin. All sections were quantitatively analyzed by computerized bone histomorphometry following acquisition of microscopical images both in transmitted and fluorescent light (excitation wavelength 546 nm, emission wavelength 590 nm), as previously described (Martin et al., 2002). Briefly, the amount of bone tissue was quantified in each section as the area of fluorescent tissue, and the area available for tissue ingrowth was determined by subtracting the area of undegraded scaffold, quantified in the transmitted light images, from the total cross-sectional area. For each section, the amount of bone tissue was then calculated as a percentage of the total space available for tissue ingrowth. Each construct was assessed in two cross-sections at each of the six levels of depth. For each construct, the amount of bone formation was determined as the average of the percentages measured in the 12 sections. The percent uniformity of bone tissue formation was determined as $100\% \times (1 - Cv)$, where Cv is the coefficient of variation (i.e., the standard deviation divided by the average) of the percentages measured in the 12 sections.

Statistical Analysis

Two experiments were independently performed using cells from the different sheep. Data on bone tissue formation are presented as mean ± standard deviation of values determined for each construct in the two independent experiments ($N = 3$ constructs per experiment). Differences were assessed using Mann–Whitney U-tests and considered statistically significant with $P < 0.05$.

RESULTS

Cell Morphology and Lineage

Seeding and culture of bone marrow nucleated cells in the ceramic scaffolds resulted after 14 days in the presence of cells adhered to the scaffold surface (Fig. 1). The morphology of the cells did not appear different following seeding by perfusion (Fig. 1A) or by static loading (Fig. 1B).

Enzymatic extraction of cells from the ceramic scaffolds under perfusion was highly efficient, with less than 15% of the total cells remaining in the scaffolds. Essentially all cells extracted from the constructs after 14 days of 3D culture, independent of the seeding modality, were positive for the expression of collagen type I, similarly to cells expanded in 2D (Fig. 2). This result indicates that cells isolated and expanded in the 3D system, starting from the diverse population of bone marrow nucleated cells, were of mesenchymal and not hematopoietic lineage. Consistent with the typical findings in 2D cultures, these mesenchymal cells are thus the progeny of the initial CFU-f, and will be referred to as BMSC.

Cell Proliferation

The average number of CFU-f in the fresh bone marrow aspirates was $0.011\% \pm 0.005\%$ of the total number of nucleated cells. Assuming that all CFU-f attached to the ceramic scaffolds, approximately $1.2E+03$ CFU-f were initially seeded in each scaffold. After 14 days of 3D culture, the average number of extracted cells was increased to $5E+05$ per scaffold. The fact that cells were of the

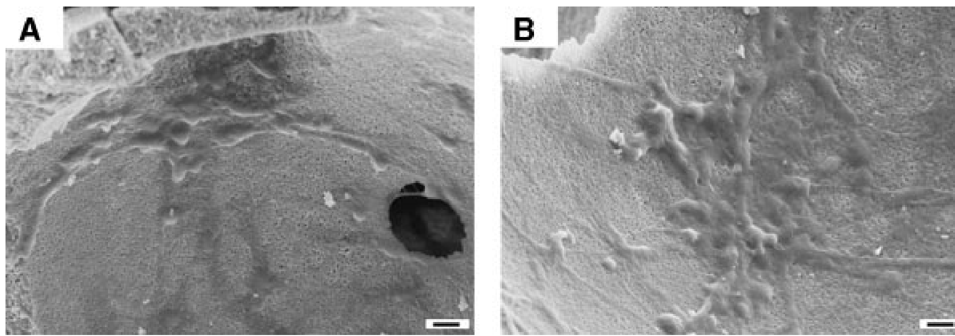

Figure 1. Scanning electronic microscopy of three-dimensional (3D) constructs. Representative images of ceramic 3D scaffolds cultured for 14 days following seeding of bone marrow nucleated cells by perfusion (A) or static loading (B). Cells were adhered to the ceramic scaffolds and displayed a similar morphology in the two conditions. Scale bar = 10 μm.

mesenchymal lineage, based on the expression of collagen type I, indicates that a large progeny of BMSC was generated within the ceramic scaffolds by extensive proliferation of the seeded CFU-f (Fig. 3). The number of doublings was similar if cells were seeded by static loading or by perfusion, but significantly higher if cells were expanded in 2D for the same time period.

In Vivo Bone Formation

Ceramic scaffolds seeded with bone marrow nucleated cells, either by static loading or by perfusion, with or without additional 3D culture, were all able to form bone tissue upon ectopic implantation in nude mice for 8 weeks, as assessed by hematoxylin–eosin staining. In all groups, the pattern of bone formation was typical of the so called ''ossicles,'' with bone tissue starting from the ceramic surface, gradually filling the pore cavities and leaving space for marrow elements (Bianco et al., 1998) (Fig. 4A). The extent of bone tissue ingrowth within the ceramic pores qualitatively appeared more progressed in constructs implanted after 14 days of 3D culture (Fig. 4C) than in those implanted immediately after the 3 days required for cell seeding (Fig. 4B).

The amount of bone formed in the different experimental conditions, expressed as a percentage of the area available for tissue ingrowth, was quantified using computerized image analysis (Fig. 5A). After 8 weeks of implantation, constructs generated by 3D culture of bone marrow cells formed approximately twice the amount of bone than those directly implanted following seeding. The modality of the cell seeding (i.e., by static loading or by perfusion) did not influence the amount of formed bone. Scaffolds loaded with 2D expanded cells formed bone tissue at amounts comparable to those of 3D cultured constructs only if they were seeded with 10-fold the number of cells measured in the 3D constructs. The percentages of bone formation measured in this study were overall comparable to those reported in previous works using goat (Kruyt et al., 2004) or human cells (Martin et al., 2002).

The uniformity of bone tissue formation appeared to be higher in constructs cultured for 14 days than in those implanted directly after cell loading, independent of the seeding modality (Fig. 5B). However, due to the relatively large variabilities, no statistically significant differences between experimental groups were found.

DISCUSSION

In this study, we demonstrated that osteoinductive grafts can be generated using exclusively a 3D culture system, by direct isolation and expansion of ovine BMSC within 3D porous ceramic scaffolds. Upon ectopic implantation, the resulting constructs formed more bone tissue than those implanted without any 3D pre-culture or those based on the same

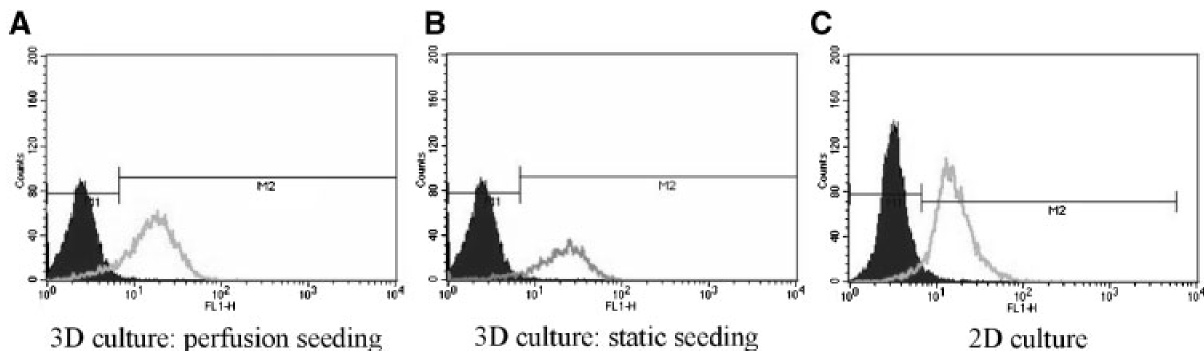

Figure 2. Expression of collagen type I. Representative cytofluorimetric analysis of collagen type I expression by bone marrow cells seeded on 3D scaffolds by perfusion (A) or by static loading (B), as compared to cells expanded in 2D (C). In each group, essentially all cells were positive for collagen type I and thus appeared to belong to the mesenchymal lineage.

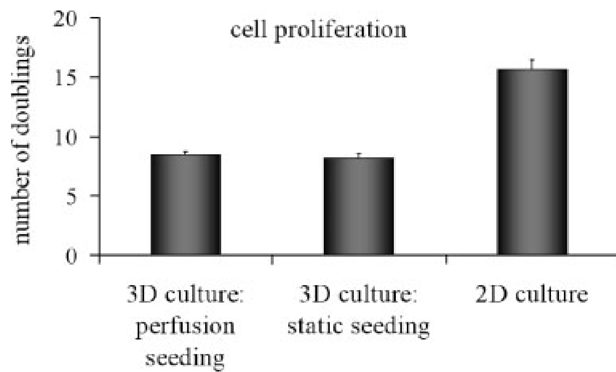

Figure 3. Cell proliferation in 2D or 3D. Cell proliferation was measured after 14 days of culture either in monolayer (2D) or within 3D ceramic scaffolds, seeded by perfusion or by static loading of a cell suspension. Cells proliferated extensively in all experimental groups, although the number of doublings was significantly higher by 2D expansion.

number of 2D-expanded cells. Moreover, the amount of bone formed was similar if bone marrow cells were seeded by static loading or by perfusion.

BMSC isolation and expansion within 3D scaffolds was achieved by seeding the whole population of bone marrow nucleated cells, containing only a minor fraction of clonogenic mesenchymal precursors (CFU-f). During culture, BMSC were selectively expanded due to their capacity to attach to the ceramic surface, whereas cells of the hematopoietic lineage were progressively eliminated from the system, likely due to the lack of specific medium supplements and/or to the removal of non-adherent cells at each medium change. The possibility of selectively expanding BMSC directly within 3D scaffolds opens new opportunities to the simplified and streamlined production of osteoinductive grafts, bypassing the typical phases of 2D cell passaging and 3D scaffold seeding/culture. Interestingly, the density of CFU-f initially seeded, calculated from the surface area of the scaffold and the fraction of CFU-f in the marrow aspirate, was as low as 1 cell/cm^2. While it is remarkable that a large number of BMSC could be generated within the scaffolds starting from such a small density of CFU-f, it would be important for scale-up purposes to identify a minimal threshold in the CFU-f density, which is sufficient for the generation of osteoinductive grafts in our system.

The finding that BMSC directly expanded in 3D scaffolds were able to induce more efficient formation of bone than cells expanded in 2D for the same time could be explained by a number of factors, including (i) cell interactions with a ceramic substrate (Ducheyne and Qiu, 1999; Ohgushi et al., 1996), (ii) the lower number of cell doublings, known to be associated with a progressive decrease in the osteogenic capacity (Banfi et al., 2000; Derubeis and Cancedda, 2004; Sugiura et al., 2004), and/or (iii) the likely deposition of pericellular and extracellular matrix during 3D culture (Bancroft et al., 2002; Mendes et al., 2002; Sikavitsas et al., 2003). It is noteworthy that as compared to cells expanded in 3D scaffolds, BMSC expanded in 2D could generate similar amounts of bone tissue if seeded at a 10-fold higher number.

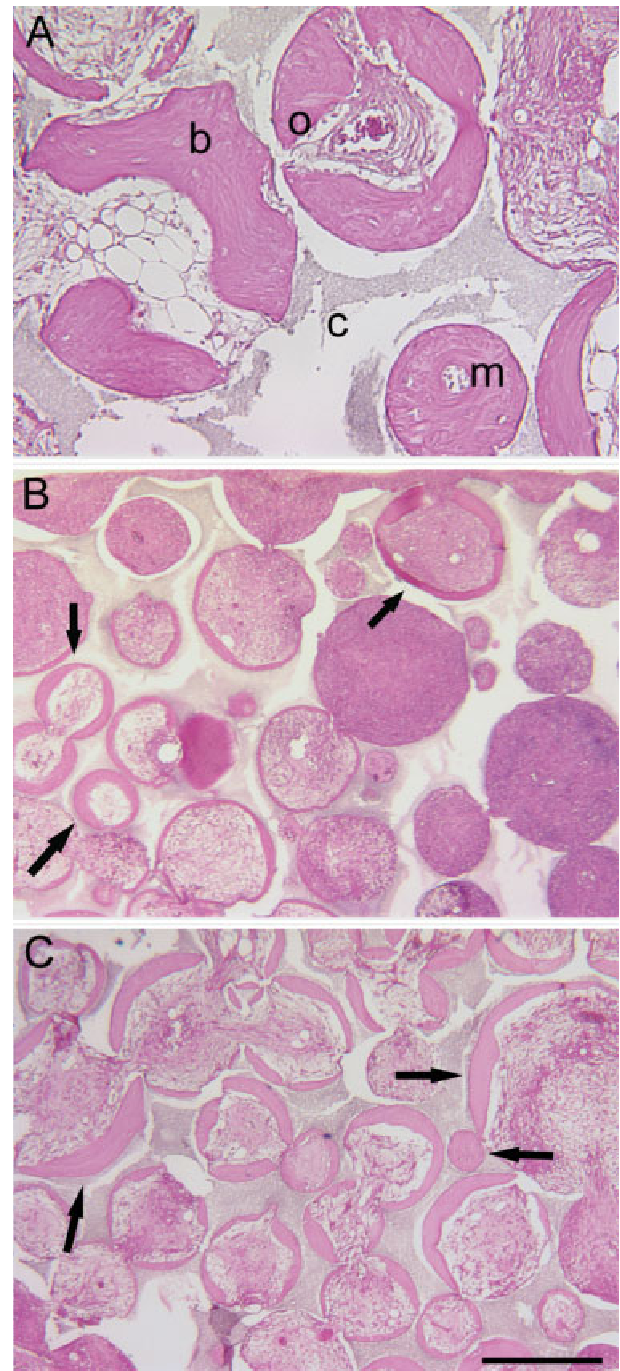

Figure 4. Histological sections of explants. Representative hematoxilin–eosin stained cross-sections of constructs implanted ectopically in nude mice and harvested after 8 weeks. A: In all groups, bone tissue formation displayed the typical pattern of an "ossicle," with lining osteoblasts (o) depositing bone matrix (b) starting from the ceramic (c) surface, gradually filling the pore cavities and leaving space for marrow elements (m). Constructs were implanted after 3 days (B) or 14 days (C) of 3D culture, following static loading of bone marrow cells. Similar results were achieved following perfusion seeding of bone marrow cells. Arrows in B and C indicate deposited bone matrix. Scale bar = 100 µm (A) or 300 µm (B, C). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

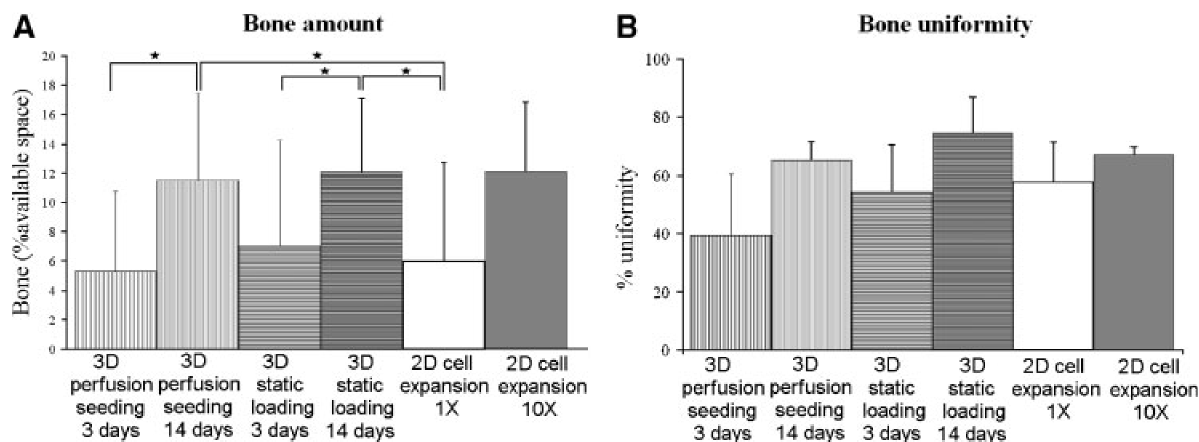

Figure 5. Bone tissue quantification. The amount (A) and uniformity (B) of bone tissue formation were quantified by computer-based image analysis in constructs implanted ectopically in nude mice and harvested after 8 weeks. Constructs were implanted after 3 or 14 days of 3D culture, following bone marrow cell seeding by perfusion or by static loading. Constructs were also seeded with 2D-expanded bone marrow stromal cells (BMSC), statically loaded at the same (1×) or 10-fold higher (10×) density of cells measured following 3D culture, and immediately implanted. Statistically significant differences are indicated with asterisks.

Considering that BMSC proliferation was considerably faster in 2D than in 3D (i.e., approximately eight more doublings in 14 days), it follows that under the described conditions the typical 2D expansion of BMSC would support the generation of larger osteoinductive constructs as compared to expansion in 3D scaffolds. Thus, while the demonstrated paradigm of expanding BMSC directly in 3D is highly relevant to overcome practical issues of 2D–3D cell manipulation in the generation of osteoinductive grafts, future studies will have to address the development of improved protocols for BMSC expansion in 3D, which might also lead to enhanced cell proliferation.

Scaffolds loaded with bone marrow cells and pre-cultured for 14 days generated higher amounts of bone than those implanted only 3 days after seeding. A prolonged cultivation time may have enhanced the osteoinductivity of the engineered grafts by allowing (i) more efficient colonization of the scaffold pores by an increased number of cells, (ii) BMSC differentiation towards a more committed osteoblastic phenotype, and/or (iii) increased deposition of a bone-like matrix within the scaffold. The deposition of extracellular matrix has been previously postulated as the main reason why 3D pre-culture of 2D-expanded BMSC within 3D scaffolds enhances the osteoinductivity of the resulting implant (Kruyt et al., 2004; Mendes et al., 2002). It is worth noting that in our system the deposition of extracellular matrix would not require an additional culture phase, but would be accomplished during cell expansion within the porous scaffold.

Using a recently developed perfusion bioreactor system (Wendt et al., 2003), we demonstrated that ovine BMSC can also be seeded within ceramic scaffolds by perfusing bone marrow nucleated cells in alternate directions through the scaffold pores. Although this seeding technique did not result in superior uniformity or amount of bone tissue formation as compared to static cell loading, the method would be more suitable for standardization in closed bioreactor systems. In addition, considering the importance of perfusion flow for enhancing mass transport and application of shear forces during the culture of osteogenic cells in a 3D environment (Bancroft et al., 2002; Botchwey et al., 2003; Cartmell et al., 2003; Gomes et al., 2003; Martin et al., 2004; Mauney et al., 2004; Meinel et al., 2004; Sikavitsas et al., 2003a,b; Wang et al., 2003; Wendt et al., 2003), it will be interesting to address whether prolonged perfusion after the phase of cell seeding would further enhance the osteoinductivity of the grafts engineered in our system as compared to a static 3D culture.

In conclusion, in this work we have demonstrated the possibility, starting from ovine bone marrow, to generate osteoinductive grafts using exclusively a 3D culture system, bypassing the conventional phase of BMSC expansion in 2D. Ongoing studies are aimed at using the same principle to generate constructs of size and shape relevant for orthotopic implantation in experimental bone defects. These experiments, together with the recent finding that the described paradigm is also applicable to a human cell source (Braccini et al., 2005), will open the way to the streamlined engineering of osteoinductive grafts for use in defined clinical applications.

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SUMMARY AND CONCLUSIONS

CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1 Summary: aims and results of this work

The main aims of this thesis were (i) to identify and develop a system that could be reproducibly used to streamline manufacture of osteoinductive grafts based on human bone marrow stromal cells (BMSC) in the context of regenerative medicine, (ii) to characterize the developed system in order to identify key elements responsible for its reproducible and efficient performance, and (iii) to extend its use to a sheep cell source, thus opening the way to test the osteoinductivity of orthotopic implants in a large animal model.

Bone Marrow Stromal Cells (BMSC), which are typically defined by their capacity to adhere on plastic [1] and form a fibroblastic colony (CFU-f) [2], represent a very low fraction (approximately 0.01%) among the nucleated cells of the bone marrow. Therefore, to obtain a sufficient number of cells for bone tissue engineering applications, BMSC are typically first selected and expanded in monolayer (2D) prior to loading into 3D scaffolds. However, 2D-expansion causes BMSC to progressively lose their early progenitor properties and differentiation potential [3-5], and to decrease their capability to form colonies and to induce bone tissue formation upon ectopic implantation [3], placing several potential limits on their clinical utility. To bypass the process of 2D-expansion and its associated limitations, we used an innovative bioreactor-based approach to seed, expand, and differentiate BMSC directly in a 3D ceramic scaffold [6]. Nucleated cells, freshly isolated from a bone marrow aspirate, were introduced into the bioreactor system and perfused through the pores of 3D ceramics for five days, then further cultured under perfusion for an additional two weeks. Using the developed procedure, BMSC could be seeded and extensively expanded within the 3D environment of the ceramic pores. Interestingly, we found that the 3D-generated constructs contained both

hemopoietic cells and BMSC, whose relative fractions could be modulated by appropriate media supplements, and that a consistent fraction of expanded BMSC was clonogenic. In contrast, following the typical 2D-expansion, cells of the hemopoietic lineage could not be maintained, and, consistently with previous studies, only a minor fraction of expanded BMSC was still clonogenic. When constructs were ectopically implanted in nude mice, those engineered in the bioreactor reproducibly generated bone tissue that was uniformly distributed throughout the scaffold volume and filled up to 60% of the ceramic pores. In marked contrast, when similar numbers of 2D-expanded BMSC were loaded into ceramic scaffolds and implanted, bone was infrequently generated, and even in the most osteoinductive constructs, it was localized to peripheral regions, filling only 10% of the ceramic pore volume [6].

Considering the need of reproducibility or at least of predictability in the osteoinductive ability of the constructs for their standardized clinical use, in order to validate the possibility of extending the use of the developed bioreactor-based approach for generating osteoinductive grafts of clinically relevant size, we then investigated whether a minimum cell density was required for the reproducible bone tissue formation. Based on the established association between the higher clonogenicity of BMSC expanded in the 3D-system and the more reproducible and extensive osteoinductivity of the resulting constructs, as compared to those based on 2D-expanded BMSC, we demonstrated that presence or absence of bone in the constructs following ectopic implantation is related not to the total number of implanted BMSC, but to the number of CFU-f present in the construct at the time of implantation. In particular, we identified an apparent threshold in the amount of CFU-f discriminating between osteoinductive and not osteoinductive constructs.

The developed bioreactor-based approach has been validated in a heterotopic model. Before envisioning a clinical trial in human, a study in a large animal model is needed to validate the safety and the surgical feasibility of the overall procedure. Thus, in the perspective of testing our novel approach for repairing experimental bone defects in a sheep model, it was first necessary to validate our system using ovine BMSC. We demonstrated that osteoinductive constructs can be generated by perfusing 3D ceramic scaffolds with the nucleated cell fraction of ovine bone marrow aspirates [7]. Ongoing studies in the context of an EU-funded Project are aimed at testing the capability of the

generated constructs to repair large bone defects in sheep (i.e. defects around titanium implants inserted into trabecular bone of the proximal humerus, and postero-lateral spinal fusion in lumbar-region).

7.2 Relevance of the achieved results and future perspectives

Possible uses of the developed system

This study has significant implications for clinical applications of osteoinductive grafts considering that the elimination of the 2D-expansion phase would facilitate the development of a more streamlined, effective, reproducible, and economical manufacturing process of autologous BMSC-based bone grafts [8].

The developed system represents also a promising approach towards establishing a 3D in vitro model system of bone marrow, which could be used to investigate interactions between different populations of bone marrow cells in a more physiological environment than previously established systems [9].

The same paradigm of bypassing 2D-expansion by direct 3D culture under perfusion of BMSC on 3D scaffolds may be used for generating other tissues and organs of mesodermic origin (e.g. cartilage [10, 11], tendons and ligaments [12], heart [13]).

The innovative 3D-culture system used in this work for generating osteoinductive grafts gave the interesting result of yielding a final BMSC population which was more clonogenic than following the typical 2D-culture. Thus, the developed system could be used to extensively expand cells for all those therapeutic purposes where large amounts of mesenchymal progenitors are needed [14, 15].

Finally, the characterization by microarrays of differential gene expression in 3D- and 2D-expanded BMSC, could open the way to identify gene sets involved in the maintenance of progenitor cells features.

Up scaling of bone tissue engineering

One of the most obvious relevance of the present work is represented by the possibility to extend the innovative bioreactor-based approach to the generation of osteoinductive grafts of clinically-relevant size. In this context, several issues should be addressed.

First of all scaling up the procedure to clinically relevant sizes will have to face the challenges of uniformly seeding the cells throughout larger constructs, and maintaining their viability both during in vitro culture and upon grafting. Even following successful seeding and culture, cell viability in large constructs cannot be maintained after in vivo transfer, unless early blood supply is achieved. However, invasion of host blood vessels requires several days to weeks, depending on the size and porosity of the construct. Therefore, cells in the center of the construct which are not supplied by diffusion would die before they can be reached by growing vessels. One of the possible solutions to the mentioned issue is constituted by co-culturing BMSC with endothelial progenitor cells, which could potentially generate a new vascular network after implantation by anastomosing to the host vessels directly and/or by releasing angiogenic factors [16].

In this work we established a relation between the clonogenicity and the osteoinductivity of BMSC. In order to predictably generate osteoinductive grafts of clinically relevant size, based on our results, a very large amount of CFU-f should be initially seeded. However, relatively limited numbers of CFU-f can be easily obtained from the typical amount of harvested bone marrow. Recent studies proposed several markers identifying CFU-f in the fresh bone marrow with osteoprogenitor properties [17-20]. Further studies should identify specific markers for clonogenic BMSC after expansion, thus allowing the establishment of protocols for enriching CFU-f populations within expanded BMSC.

Role of hemopoietic cells

The high clonogenicity of BMSC cultured in the 3D bioreactor system could be due to a variety of factors, including the maintenance of substantial fractions of hemopoietic cells in culture, which was observed in our system. Interestingly, when high numbers of cells were initially seeded, we observed an up-regulation of both fractions of hemopoietic cells and CFU-f.

Recent studies indicate the existence of a crosstalk between hemopoietic and mesenchymal bone marrow cells enhancing the growth of CFU-f [21]. Based on the established association of the high clonogenicity of BMSC both with the co-culture of hemopoietic cells and with a reproducible osteoinductive capacity of the resulting constructs, further studies should investigate whether hemopoietic cells enhance the osteoinductivity of the constructs not only indirectly (e.g., by maintaining BMSC more clonogenic) but also directly (e.g., by releasing specific cytokines in vivo).

In our study we demonstrated that fractions of hemopoietic and mesenchymal cells can be modulated by using specific culture conditions: it would be interesting to test whether by maintaining/expanding an increased number of hemopoietic cells, it could be possible to enhance the osteoinductivity of the generated constructs.

Possible improvements of the developed system

The approach used in this work for cell-based engineering of osteoinductive grafts was based on (i) bone marrow nucleated cells as a source of BMSC, (ii) 100% Hydroxyapatite (HA) ceramics as 3D scaffolds, (iii) 3D perfusion flow as a culture system, and (iv) culture medium containing fetal bovine serum, and supplemented with FGF-2, dexamethasone, and ascorbic acid. The combination of these parameters gave rise to the reproducible, uniform, and extensive bone formation upon ectopic implantation of the generated constructs in nude mice. Results obtained in this study were very encouraging, but alternatives for the mentioned elements could further improve the developed system.

Cell source. The ideal source of autologous mesenchymal progenitor cells should provide large number of cells from living individuals without using invasive procedure, meaning without any patient morbidity: such a cell source could be represented by human blood. Very recent studies indicate the presence of human circulating CD14+ monocytes exhibiting mesenchymal differentiation potential [22], and of circulating osteoblast-lineage cells expressing bone-related markers [23]. Likely in the future, the development of methods to harvest large amounts of these cells from peripheral blood might lead to generating bone substitutes with minimal patient morbidity.

Scaffolds. The ideal 3D-scaffold for engineering bone should provide an initial support for osteoprogenitor cells to deposit mineralized bone matrix; then it should be slowly resorbed at the same

time newly formed bone tissue grows inside the scaffold. A high porosity and a high degree of interconnection among the pores are an absolute requirement for the vascularization of the implant and the new bone formation; chemical composition plays a major role in the resorbability of the biomaterial. Scaffolds used in this study (100% HA) have high osteoconductive properties and high porosity (83%), but low resorption rate. Composite scaffolds based on tricalcium phosphate/HA (TCP/HA) have been recently proposed as a valid alternative to the 100% HA ceramics for their faster resorption upon orthotopic implantation in large animal models [24]. Ongoing studies are aimed at determining the feasibility of using our perfusion bioreactor system to generate osteoinductive grafts based on several types of biphasic bioceramic scaffolds (e.g. TCP/HA, carbonated/HA, collagen/HA). In addition, adsorption of extracellular matrix molecules such as fibronectin [25] or collagen [26] onto the HA surface has been shown to significantly increase cell adhesion and proliferation, and thus represents a further potential improvement of our system.

3D-culture system. Concerning the perfusion bioreactor system used in this study for producing osteoinductive constructs, several features should be included in the developed design in order to make it clinically usable at large scale. An automated, reproducible, controlled, GMP-compatible production unit for the generation of engineered bone should include (i) on-line monitoring and control of the chemico-physical parameters of the cell-culture (e.g. pH, gases, metabolites) in order to monitor cell growth, predict the effective number of cells present in the graft and determine the appropriate time for implantation, (ii) automated medium change, thus excluding manual intervention and reducing costs of manufacture and risk of contamination, and (iii) reduced size, in order to allow easy location in dedicated areas, possibly within hospitals.

Culture media supplements. In our system BMSC were cultured in medium containing fetal bovine serum (FBS) and supplemented with several growth factors. However, for widespread clinical applications, contact with FBS must be minimized since it is putative source of prion or virus transmission, and the use of exogenous recombinant growth factors should be avoided for their unknown long-term effects in human cells. Platelet lysis releases a wide variety of growth factors including FGF-2, and platelet lysate has been recently described as a powerful and safe substitute for fetal bovine serum, promoting BMSC proliferation without loss of their intrinsic differentiation

properties [27]. Likely in the future, the platelet lysate, being itself a source of growth factors, could be considered not only as a substitute of FBS, but also as the only culture medium supplement needed during culture of BMSC to be used in clinical applications. Based on recent studies in large animal models, BMSC combined with platelet-rich plasma are proven to be potent angiogenic inducers [28], thus representing a promising solution not only to overcome problems related to the use of serum and growth factors during in vitro culture, but also to possibly improve the in vivo bone graft vascularization and integration.

7.3 Schematic summary

The most relevant results generated in this work can be summarized as follow:

- Human or ovine BMSC can be seeded and extensively expanded on 3D ceramic scaffolds by perfusing directly the nucleated cell fraction of marrow aspirates through the scaffold pores, thus bypassing the conventional 2D-expansion phase.
- 3D-generated constructs are more reproducibly, extensively and uniformly osteoinductive than those based on 2D-expanded cells.
- The used bioreactor-system allows co-culture of hemopoietic cells and BMSC.
- The relative fractions of hemopoietic cells and BMSC can be modulated by using appropriate media supplements.
- 3D-expanded BMSC are more clonogenic than following 2D-expansion.
- Bone formation occurs if a sufficient density of CFU-f is implanted.
- Bone formation is not related to the number of implanted BMSC.
- High initial cell densities generate constructs containing high fractions of both hemopoietic cells and of CFU-f.

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International Patent Applications:

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