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Segmental bone defects: from cellular and molecular pathways to the development of novel biological treatments

Spyros G. Pneumaticos [a], Georgios K. Triantafyllopoulos [a], Efthimia K. Basdra [b], Athanasios G. Papavassiliou [c, *]

[a] *Third Department of Orthopaedic Surgery, Medical School, University of Athens, 'KAT' Accident's Hospital, Athens, Greece*
[b] *Department of Histology and Embryology, Cellular and Molecular Biomechanics Unit, Medical School, University of Athens, Athens, Greece*
[c] *Department of Biological Chemistry, Medical School, University of Athens, Athens, Greece*

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Abstract

Several conditions in clinical orthopaedic practice can lead to the development of a diaphyseal segmental bone defect, which cannot heal without intervention. Segmental bone defects have been traditionally treated with bone grafting and/or distraction osteogenesis, methods that have many advantages, but also major drawbacks, such as limited availability, risk of disease transmission and prolonged treatment. In order to overcome such limitations, biological treatments have been developed based on specific pathways of bone physiology and healing. Bone tissue engineering is a dynamic field of research, combining osteogenic cells, osteoinductive factors, such as bone morphogenetic proteins, and scaffolds with osteoconductive and osteoinductive attributes, to produce constructs that could be used as bone graft substitutes for the treatment of segmental bone defects. Scaffolds are usually made of ceramic or polymeric biomaterials, or combinations of both in composite materials. The purpose of the present review is to discuss in detail the molecular and cellular basis for the development of bone tissue engineering constructs.

Keywords: bone defects • tissue engineering • stem cells • scaffolds • growth factors

Clinical background

The bone has a unique healing potential after damage, resulting in tissue of ultimately the same quality, structure and architecture as before. High energy trauma, tumour resection, revision surgery, developmental deformities and infection can lead to significant bone loss and large defects with poor intrinsic healing potential. These large bone defects pose a major clinical and socioeconomic problem, as they have negative impact on patients' quality of life due to consecutive reoperations and prolonged hospitalizations. According to the American Academy of Orthopaedic Surgeons, there are approximately 6.3 million fractures each year in the United States, with more than 500,000 procedures of bone grafting, costing approximately $ 2.5 billion [1].

Critical size defects

A critical size defect is defined as the smallest size intra-osseous wound in a particular bone and species of animal that will not heal spontaneously [2], or as a defect that shows less than 10% bony regeneration during the lifetime of the animal [3]. Furthermore, a

*Correspondence to: Prof. Athanasios G. PAPAVASSILIOU, Department of Biological Chemistry, University of Athens Medical School, 75 Mikras Asias Str., 115 27 Athens, Greece.

Tel.: +30-210-7462508/9
Fax: +30-210-7791207
E-mail: papavas@med.uoa.gr

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defect can be characterized as 'critical size' when its length deficiency exceeds two to three times its diameter [4].

Bone physiology – molecular cues and cellular responses

All available treatments for segmental bone defects are based upon specific molecular and cellular mechanisms. Hence, their function is better understood with an insight of basic bone physiology.

The structural unit of bone is called bone multicellular unit and consists of bone-resorbing cells (osteoclasts), bone-forming cells (osteoblasts, osteocytes and bone-lining cells), their precursor cells and other associated cells, such as endothelial and nerve cells [5]. Osteoclasts form a bone-resorbing front and are followed by osteoblasts producing new bone. Those osteoblasts that are trapped within the newly formed bone finally differentiate into osteocytes, while those on the surface into lining cells.

Osteogenesis is the initial production of bone and includes intramembranous and endochondral ossification. Bone modelling involves the formation of bone's shape and structure by the independent actions of bone remodelling units. Furthermore, bone is being constantly renewed by bone remodelling *via* the coupled actions of osteoclasts and osteoblasts [6].

Osteoclasts are multinuclear cells and are formed by fusion of their progenitor cells, which circulate in the blood and, under the effect of specific signals, leave the circulation to the required site. It is known that these cells derive from the bone marrow haematopoietic lineage of cells, which also generate monocytes and granulocytes, with a central role in inflammatory processes. Colony stimulating factors (CSF), including granulocyte macrophage-CSF, granulocyte-CSF and macrophage-CSF and interleukin (IL)-3 regulate pre-osteoclast proliferation and differentiation. However, the most important role in bone resorption regulation is attributed to a cytokine of the tumour necrosis factor (TNF) group, the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and its two known receptors, RANK and osteoprotegerin (OPG) [7, 8]. RANKL stimulates pre-osteoclast differentiation, augments mature osteoclast activity and inhibits osteoclast apoptosis. It is expressed by osteogenic cells and its physiological role is induced by binding to RANK. On the other hand, OPG is a soluble receptor of RANKL, preventing RANKL–RANK binding. The control of RANK/OPG ratio by other cytokines appears to be the final stage of osteoclastogenesis regulation. These cytokines include IL-1, IL-3, IL-6, macrophage-CSF, granulocyte macrophage-CSF and TNF-α. Thus, inflammatory situations have a significant contribution to bone loss and bone defect formation, and can adversely affect therapeutic interventions [9, 10].

Osteoblasts are of mesenchymal origin, deriving from the non-haematopoietic part of bone marrow, which contains the mesenchymal stem cells (MSCs). These cells are capable of multilineage differentiation into osteoblasts, adipocytes and chondrocytes [11].

Osteoblast differentiation is under control by growth factors, hormones and transcription factors. Growth factors can be divided in the superfamily of the transforming growth factor β (TGF-β) and the group of the insulin-like growth factors (IGFs). The TGF-β superfamily includes the bone morphogenetic proteins (BMPs). All these factors have either a direct influence on osteoblast activity, or an effect to other bone growth regulators. BMPs' major effects are on osteoprogenitor cell proliferation and differentiation, while the rest of the TGF-βs seem to act predominantly on differentiated cells, promoting their proliferation and bone matrix synthesis [12]. The TGF-β family includes three isoforms: TGF-β_1, TGF-β_2 and TGF-β_3, with TGF-β_1 being the most abundant in bone, having a mean concentration of 200 μg/kg [13]. All isoforms show distinct roles during endochondral and intramembranous ossification. At sites of endochondral bone formation, TGF-β_2 is detected in all zones of the cartilage but mostly in the hypertrophic and mineralizing zone [14]. TGF-β_1 and TGF-β_3 are found in chondrocytes of the proliferative and hypertrophic zone [13]. On the other hand, during intramembranous ossification, TGF-β_1 and TGF-β_2 are detected in sites of mineralization, while TGF-β_3 shows a more diffuse distribution [14, 15]. The TGF-β isoforms *per se* are powerful inducers of endochondral bone formation in primates. This bone induction is site and tissue specific [16]. Furthermore, IGFs seem to enhance the effects of the previous factors [6, 17]. Finally, other growth factors include platelet-derived growth factor, fibroblast growth factor (FGF) and vascular endothelial growth factor [17]. Endocrine control of osteoblast differentiation includes glucocorticoids and sex steroids, PTH and PGE_2. All of them work in coordinance with the aforementioned growth factors.

Bone formation is regulated through a hierarchical expression of transcription factors. Runx2 is essential for endochondral bone formation. It is a member of the *runx* family of genes, which includes *runx1*, *runx2* and *runx3*. Runx2 expression in MSCs results in up-regulation of osteoblast-specific genes, including *osteocalcin*, *alkaline phosphatase*, *bone sialoprotein* and *collagen type I α1* (*collα1*). Intramembranous and endochondral ossification are lost in Runx2 knockout mice. On the other hand, overexpression of Runx2 leads to osteopenia. Hence, its activity is under tight control by several other transcription factors [6].

Osterix (Osx) is specifically expressed by osteoblasts and functions downstream of Runx2. It forms a complex with the nuclear factor of activated T cells, thus potentiating the *collα1* promoter, or, according to other studies, the Wnt signalling pathway [18, 19].

The Wnt–β-catenin signalling pathway results in translocation of β-catenin into the nucleus and activation of genes, including *runx2*, which lead to osteoblast differentiation. Activating transcription factor 4 and distal-less homeobox 5 also play important roles in osteoblastogenesis. Finally, NF-κB probably activates the transcription of osteoblast-specific genes and through RANKL promotes osteoclast differentiation, coupling the actions of bone-producing and bone-resorbing cells [20].

Osteoblast differentiation can be triggered by mechanical stimuli, which are transduced by osteocytes. Osteocytes are stimulated through streaming potentials by fluid flow strain in the lacuno-canalicular system. Mechanical stimulation ultimately

leads to up-regulation of IGF-I, growth-related genes *c-fos*, *erg-1* and basic FGF [21].

Standard treatment of segmental bone defects

Vascularized bone graft

Vascularized bone grafts are the first choice for surgical reconstruction of critical sized bone defects, as they contain an internal vascular network supplied by a vascular pedicle. Unlike non-vascularized bone grafts which are discussed further on (subsections 'Autologous bone graft' and 'Allograft'), integration with the host bone resembles the process of fracture healing, resulting in short union times (3–5 months) [22].

In clinical practice, however, integration of the transferred to native bone is not reliable and frequently the procedure is supplemented with autologous grafting. The technique's disadvantages are donor site morbidity, the need for prolonged non-weight-bearing period and the fact that it is technically challenging [23]. Furthermore, vascularized bone grafts often do not meet the mechanical demands of the site in which they are transferred, leading to stress fractures in up to 25% of reconstructions [22].

A recent technique that combines a free fibular flap with a large structural allograft, for surgical reconstruction of large bone defects, provides the mechanical strength of cortical allografts, along with the potential for remodelling through the vascularized fibular graft [24]. In paediatric or adolescent patients with a greater potential of bone remodelling, the application of the technique is very promising [25].

Distraction osteogenesis

Distraction osteogenesis in its modern form was developed by Ilizarov, who, in contrast to previous proposals, used an external fixator, allowing the patient to be ambulatory, and extended its use from limb lengthening to various conditions including segmental defects [26]. The technique's basic concept is osteogenesis during distraction through a corticotomy, *i.e.* cutting the cortex while leaving the medullary vessels and the periosteum intact to form bone *de novo*. The forming callus can be distracted, by longitudinally translating the bone segment included between the corticotomy site (usually in the proximal or distal metaphysis) and the bone defect, with the use of an external fixator and a rate of 1 mm/day [27, 28]. With distraction osteogenesis, massive grafting is not necessary, early weight bearing is possible and local blood flow is increased through stimulation of angiogenesis [29]. On the other hand, patient's compliance is an important issue. Moreover, the application of the external fixator is technically demanding and the whole treatment requires a prolonged follow-up period for the frame adjustments to be done.

Autologous bone graft

Autologous bone graft is considered as the 'gold standard' material for grafting, because it consists of all three elements needed for bone formation, which include the structural lattice, growth factors and, to some extent, osteoprogenitor cells. Its scaffold-like structure allows for cell migration and proliferation. Its disadvantages include donor site morbidity, extended operating time, the risks of infection and injury of vessels and nerves and autologous graft's limited availability, especially in elderly patients [30]. The lack of vasculature is a major drawback, as the process of osseointegration proceeds by creeping substitution and the diffusion of oxygen and nutrients is reversely proportional to the distance from the healthy bone tissue [22].

Allograft

Allografts have been introduced in order to overcome the donor morbidity of autologous bone grafts, can be available in the desired quantities and their application does not require sophisticated surgical techniques. The lack of osteogenicity and the risks of disease transmission and immunogenic response are drawbacks of this technique. Furthermore, the maintenance of allograft banks is rather expensive. Finally, as with autologous bone grafts, allografts are non-vascularized and have similar limitations concerning integration with host bone, while infection, non-union and fracture are complications related to their avascular nature [23].

Demineralized bone matrix

Demineralized bone matrix (DBM) is human cortical and cancellous allograft subjected to decalcification with the use of numerous weak and strong acids, such as hydrochloric acid, as well as acidic buffers. When hydrochloric acid is used, the main components of bone's inorganic phase, *i.e.* hydroxyapatite, calcium phosphate (CaP) and calcium carbonate react to form calcium chloride [31]. This procedure preserves collagen and non-collagenous proteins including growth factors. As with allografts, disease transmission and immunogenic reaction remain as disadvantages. DBM lacks structural strength hence its mechanical properties and handling characteristics can be enhanced by addition of hydroxyapatite, autograft, allograft or bone marrow aspirate [30].

Tissue engineering

According to Langer and Vacanti, tissue engineering is 'an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function' [32]. Its main purpose is to produce fully functional tissues that could replace damaged ones. In order for that to be accomplished, cells,

extracellular matrix, blood vessels, nerves, intercellular communication and cell–matrix interaction need to be combined in an appropriate spatial- and time-dependent fashion [33].

An ideally engineered bone tissue should exhibit three main properties: osteogenicity, osteoinduction and osteoconduction. Osteogenicity is defined as an artificial tissue's capability of forming bone tissue *de novo*. Osteoinduction is the ability to induce bone formation by stimulating the proliferation and differentiation of host's osteogenic cells. Finally, osteoconductivity is the capacity to guide bone tissue formation. Consequently, engineered bone tissue is composed of osteogenic cells, osteoinductive growth factors and osteoconductive scaffolds. Ease of use, mechanical stability and vascularization are other properties of an ideal bioartificial bone tissue [33].

Osteogenic cells

The main source of MSCs is bone marrow. A bone marrow aspirate is easy to obtain and the contained stromal cells can be used in bone tissue engineering to create autologous bone tissue, without the ethical issues raised by the use of cells from different sources [34].

The concentration of stem cells in the bone marrow is approximately 1:100,000 nucleated cells. Centrifugation of aspirated bone marrow separates the marrow cells from plasma and preserves the osteogenic potential of the cells, decreasing the volume of the material injected [23]. Human bone marrow-derived MSCs can be subcultured for as many as 15 passages without losing differentiation potential [35].

The skeletal stem cells, originally termed colony forming units-fibroblasts are of stromal nature, meaning that they do not bear any haematopoietic or endothelial characteristics and are thus found to the non-haematopoietic tissue which is included in the intact bone marrow [36, 37].

Furthermore, bone marrow stromal cells form clonal colonies under certain conditions [38]. Multipotency is another characteristic of skeletal stem cells. They have the potential to differentiate into bone, cartilage, tendon, muscle and fat both *in vitro* and *in vivo* [36, 39, 40].

In vivo, stem cells undergo asymmetric division, leading to two daughter cells, one that clonally expands and differentiates and another one that remains a stem cell. *In vitro*, all non-transformed postnatal cells are subject to asymmetric kinetics and ultimately show replicative senescence. Thus, a culture initiated by a single stromal colony forming units-fibroblasts, which undergoes asymmetric division, includes the expanding and differentiating progeny of the stem cell, and the original stem cell, without expansion. As a result, any bone marrow stromal cell strain is actually never a pure culture of MSCs, as they cannot be culture expanded [41]. During long-term *in vitro* culture of human MSCs, the cells ultimately enter a state of growth arrest, which is called replicative senescence and is caused by several factors, such as accumulation of DNA damage or mitochondrial alterations [42]. Progressive telomere shortening due to absence of telomerase activity is considered as the most important factor [43]. In order to overcome this limitation, telomerization can be used and a large number of cultured cells can be obtained. Nevertheless, this procedure can lead to genomic instability and after approximately 250 population doublings to cell transformation [44]. Transient induction of human telomerase reverse transcriptase can be used as an alternative [11]. Besides telomerization, growth factors, such as FGF-2, or the culture of cells under low oxygen tension have been employed to extend the lifespan of the MSCs *in vitro* [45].

Bone marrow stromal cell strains exhibit a characteristic profile of surface markers [41]. These markers show high sensitivity but lack specificity, as they cannot distinguish between the multipotent MSCs and their differentiated progeny or other 'mesenchymal' cells. Typical markers of MSCs include CD71, CD105, CD166, CD44, Thy1, CD29 and CD63, whereas a total of 29 integrins and cell adhesion molecules, 20 receptors and 18 Ras-related small GTPases were also identified [11].

In addition to surface markers, the master gene of osteogenic commitment, *runx2* [46], is constitutively expressed in stromal cell cultures. Furthermore, bone marrow stromal strains express Osx and the chondrogenesis-controlling transcription factor Sox9, transcription factors regulating adipogenesis, such as peroxisome proliferator-activated receptor γ, and other proteins, which characterize the osteoblastic lineage, such as types I and III collagens [41].

It is necessary to limit the differentiation potential of MSCs into a particular lineage before their use in therapy, because of the theoretical danger of serious complications (*e.g.* tumour formation) by the use of undifferentiated stem cells. This limitation can be achieved by the use of lineage-specific transcription factors or growth factors, hormones and extracellular matrix proteins [11, 47].

Skeletal stem cells can be transduced using oncoretrovectors and lentivectors, which have been proved very efficient and neutral in respect to the cells' growth and differentiation properties [48]. The use of molecular engineering allows for the production of stromal cell strains that overexpress specific genes [36].

After *in vivo* transplantation of skeletal stem cells, formation of bone and bone marrow occurs. MSCs are able to regenerate segmental bone defects by direct orthotopic placement in conjunction with appropriate scaffolds [41]. They have been used successfully in the treatment of segmental bone defects in various animal models [49, 50]. Additionally, there have been reported cases of patients with large bone defects who were treated with locally injected autologous MSCs [11].

However, it should be mentioned that clinically, the main drawback of MSCs use is the low number of cells obtained upon harvest. This has led research to alternative sources of MSCs. Adipose tissue is also of mesenchymal origin and is characterized by a supportive stroma that can be processed with homogenization, enzymatic digestion, differential centrifugation, red blood cells lysis and washing, to isolate the stromal vascular fraction (SVF). The latter represents a heterogeneous cell population that includes multipotent adipose tissue MSCs. Adipose tissue MSCs can be cultured and expanded, preserving multipotency and making them an attractive solution for regenerative medicine. Interestingly, it has been proven that the frequency of mesenchymal progenitors

in the stromal vascular fraction is approximately 1/4880, much greater than the respective frequency in the bone marrow and comparable to that in the umbilical cord blood. Consequently, this leads to a higher yield of harvested cells and much less time required for cell expansion, with potentially beneficial clinical implications [51–54].

Osteoinductive factors

The most extensively studied and most widely used osteoinductive factors for the treatment of bone defects are BMPs. Based on the observation of previous researchers that intramuscular injection of bone extracts can induce ectopic bone formation, Urist discovered that a mix of proteins was responsible for bone regeneration, which he named BMPs [55–57]. Later, Sampath and Reddi developed a BMP bioassay for ectopic bone formation, based on the activity of alkaline phosphatase and calcium content in the newly formed bone [58]. Reddi and Huggins proposed that BMPs induce bone marrow progenitor cells to produce bone cells, leading to bone regeneration [59, 60]. In the late 1980s, BMPs were produced and isolated using recombinant DNA methodologies [61].

BMPs are members of the TGF-β superfamily and include 18 known proteins, among which BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9 have full osteoinductive potential. They are active as homodimers or heterodimers. Heterodimers have been found to be more effective in inducing bone formation, as shown by the higher yields of alkaline phosphatase in cell cultures [62].

BMPs bind to two types of cell surface receptors, BMPRI and BMPRII [63]. These receptors are serine-threonine kinases and trigger specific intracellular pathways. Different combinations of type I and type II receptors provide different and specific signals which lead to distinct cell effects [64].

BMP activity is subjected to regulation by specific proteins that act as antagonists and include noggin, chordin, follistatin, gremlin, betaglycan and crypto [12]. These proteins are extracellular and inhibit BMP binding to cell receptors.

Upon BMP binding, BMP receptors phosphorylate certain intracellular signal transducing proteins, called receptor-regulated Smads (R-Smads), which include Smad1, Smad5, Smad8 and Smad9. R-Smads form a complex with a common-partner Smad (Co-Smad). Smad4 is so far the only described Co-Smad. Two R-Smads and one Co-Smad form a heterotrimeric complex, which is translocated into the nucleus and modulates the function of transcription factors, among which Runx2 is the most important one [65], and ultimately gene expression. Inhibitory Smads (I-Smads) negatively regulate Smad signal transduction. Additionally, Smad ubiquitin regulatory factors (Smurfs) induce the degradation of Smads and control BMP signal transduction [12]. BMP signalling also involves MAPK pathways, including the ERK, the c-Jun N-terminal kinase and the p38 MAPK cascades [66].

Osx is another transcription factor induced by BMP signalling and probably other pathways including MAPK pathway [67]. Osx and Runx2 are the most thoroughly studied transcription factors activated by BMP signalling. Finally, menin also regulates Runx2-induced gene expression during the commitment of MSCs into osteoblast differentiation [68].

The TGF-β–activin and BMP pathways share similar signalling molecules and thus compete with each other. Expression of TGF-β induces I-Smads, which regulate the BMP signalling effects [69]. The Notch, EGF, Wnt, IGF and FGF pathways also interact with the BMP–Smad pathway [12].

Several preclinical studies in animal models have evaluated the effectiveness of BMPs in the treatment of segmental bone defects [70–72]. These studies encouraged researchers to evaluate the usefulness of BMPs in treating bone defects in human beings [73–78].

Recombinant BMPs are currently derived from mammalian cell cultures, elevating the costs of the products containing them. In the future, the use of bacterial cells is expected to lower the production costs. Recombinant technology allows molecular modifications in order to improve BMPs' effectiveness. For instance, by adding heparin-binding domains to the rhBMP, one can decrease its specific activity *in vitro*, but also increase bone formation *in vivo* [12]. The production of recombinant proteins containing only the binding sites to cell receptors is another approach [79]. Another way of improving BMP effectiveness is chemical modification [12]. Finally, BMPs' bioactivity could be enhanced by the production of rhBMP heterodimers.

IGFs have also been studied for their effectiveness in the treatment of segmental bone defects. Their anabolic role in bone formation has been outlined by many researchers [80, 81]. According to other studies, IGF-1 induces both bone resorption and formation [82].

Prostaglandins (PGs) are another category of substances found in large quantities in bone tissue. Among them, PGE_2 is the most potent one [83]. There are four prostaglandin receptor types, EP1, EP2, EP3 and EP4 [84]. Depending on their type and the receptor type they activate, PGs show both stimulatory and inhibitory effects. Application of PGE_2 enhances bone healing. The presence of PGE_2 in a culture medium during the first 21 days increases its osteoinductive effect. It has been suggested that this effect reaches its peak mainly during the first 24 hrs [85]. The application of PGE_2 to a fracture site has been found to significantly stimulate osteoblastic activity [86].

A novel approach for the promotion of osteoinductive growth factors effectiveness and the enhancement of bioartificial bone tissues is gene therapy [87]. Cell transfection has been achieved with viral vectors, which provide efficient and stable transfection [88]. Non-viral vectors, such as transfection reagents, and matrix-mediated gene transfer are approaches that avoid the use of potentially harmful viral vectors [89]. For the time being, issues like phenotypical stability, application techniques and long-term effects hamper extensive application of gene therapy [90].

Biomaterials and scaffolds

The use of synthetic materials that possess bioactivity has been applied in clinical practice since the mid-1980s. These bioactive

materials can promote certain cellular responses and include ceramics and polymers.

Ceramic materials have been extensively used as bone defect fillers. Bioglasses, glass ceramics and CaPs belong to this category. Unlike metallic implants, ceramics can integrate with bone without the formation of fibrous connective tissue. This is due to their unique structure and surface features, which resemble the bone mineral phase [91]. These materials provide an environment where bone matrix proteins are absorbed, resulting in osteoblast adhesion and proliferation [92]. The most commonly used ceramics are hydroxyapatite, b-tricalcium phosphate, their derivatives and their combinations.

Polymers are resorbable biomaterials that are characterized by a controlled degradation. They have been successfully employed for the treatment of bone defects, for healing of bone fractures, as well as for the production of biodegradable sutures, screws, plates, rods and pins [93]. Polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymer PLGA are the most widely used polymers for the treatment of segmental bone defects. Degradation is mainly caused by hydrolysis and enzymatic disintegration. As the proliferation of cells proceeds into the scaffold, the latter is gradually degraded and the mechanical stress and strain is distributed to the newly formed tissue. Lactic and glycolic acid are the final products of PLA and PGA degradation and they are used in the metabolic pathway of the tricarboxylic acid cycle in the mitochondria. Ultimately, ATP, H_2O and CO_2 are produced and excreted by the lungs and kidneys [94].

The bioabsorbable polymer can be reinforced with a bioactive ceramic already described [95, 96]. The aim is to obtain a material that displays similar mechanical properties to bone, can integrate effectively with bone tissue and shows a degradation rate that matches the lesion's healing period. Finally, the coupling of polymers and proteins on the surface of the material can improve its bioactivity [91].

Scaffolds are three-dimensional porous structures that need to fulfil certain criteria in order to be used in tissue engineering. These include biocompatibility, biodegradability, porosity and mechanical analogy to the load-bearing bone [97].

A wide spectrum of both natural and synthetic materials is being investigated for the construction of scaffolds for bone tissue engineering, including the aforementioned ceramics and polymers. PLA and PGA are the most widely used polymers for bone tissue engineering, as they provide better control of their physicochemical properties and have been successfully employed in clinical applications [98]. Interestingly, it has been shown that certain CaP scaffolds exhibit intrinsic potential to induce bone formation, even without the presence of exogenous osteogenic growth factors. This ability depends on the biomaterial's macro- and microstructure, as well as chemical composition (*i.e.* presence of CaP) [99, 100].

An ideal scaffold must combine bioactivity and biodegradability. In order to simulate the extracellular matrix environment and function, the scaffold's surface can be coupled with biomolecules that influence cell adhesion, migration, proliferation and differentiation. The incorporation of an inorganic phase into the polymer modifies both the mechanical properties and degradation pattern of the material, but also improves its bioactivity [91].

Pore distribution, interconnectivity and size are crucial for the proper function of the material as a scaffold for bone tissue engineering. The optimal pore size has been determined between 100 and 350 μm [101]. Evaluating the scaffold's permeability gives an assessment of its ability to maintain cell viability and extracellular matrix production. Furthermore, oxygen and nutrient transport inside the scaffolds must be also assessed. Porosity is also of critical importance for the transmission of changes in hydrodynamic pressure or streaming potentials throughout the bulk of the material [102].

Composite materials have been developed in order to combine the properties of each individual material. Their further combination with MSCs led to the production of injectable cements with osteogenic potential [103]. The results were encouraging, as the material exhibited good osseointegration and vascularization *in vivo*. When combined with growth factors, scaffolds are effective in maintaining a controlled release of these factors. For this approach various factors have been used, including FGF, BMPs, platelet-derived growth factor and IGF [91].

The challenge of angiogenesis

Synthetic bone constructs lack vasculature. Blood perfusion and interstitial fluid diffusion are the ways oxygen and nutrients are delivered to the implant. Diffusion can support cell survival within a maximum range of 200 μm into the matrix [104]. Prolonged hypoxia and lack of nutrients ultimately result in cell death. Local environmental factors, such as trauma and infection further impair implant's integration with bone [90].

Adding a vascularized periosteal flap to the scaffold led to a significant increase in bone formation. Co-implantation of perivascular cell precursors and endothelial cells in engineered constructs leads to long-lasting, stable microvessels *in vivo*, which are fully functional for more than 1 year [105]. Recent studies have shown that the combination of angiogenic and osteogenic factors can stimulate bone healing and regeneration [106, 107]. In a rat animal model, the application of BMP-3 on a muscle flap, covered with DBM and enclosed in pre-shaped moulds, led to the transformation of the muscle tissue into vascularized bone grafts, in the shape of femoral heads and mandibles [108]. A pedicled bone graft can be generated with BMPs embedded on appropriate carriers around a vascular bed. This graft could be grown in one part of the body and then transplanted to the site of the defect, in case an orthotopic procedure is contra-indicated because of extensive trauma, loss of vascularity or infection [109, 110]. Based on this concept of flap pre-fabrication, a group of researchers developed a technique, in which a porous ceramic scaffold seeded with BMSCs was wrapped by a panniculus carnosus flap that provided vascular supply [22]. Another developed technique involves the placement of a functioning arteriovenous loop inside a semi-sealed polycarbonate chamber, thus

generating a functional vascular network, along with a supportive extracellular matrix [111].

Conclusions

Bone tissue engineering is a continuously evolving field. The use of tissue engineering constructs for the treatment of segmental bone defects has been promising. The combination of MSCs and growth factors along with appropriate scaffolds is based on specific physiological mechanisms and aims at mimicking the behaviour of bone tissue during repair. On the other hand, issues like safety, cost and heterogeneity in construct preparation between different laboratories need to be further evaluated. Importantly, a deeper understanding of the cellular and molecular pathways implicated in bone–construct integration is necessary for the development of more efficient products.

References

1. **Laurencin C, Khan Y, El-Amin SF.** Bone graft substitutes. *Expert Rev Med Devices.* 2006; 3: 49–57.
2. **Schmitz JP, Hollinger JO.** The critical size defect as an experimental model for cranio-mandibular non unions. *Clin Orthop.* 1986; 205: 299–308.
3. **Gugala Z, Gogolewski S.** Regeneration of segmental diaphyseal defects in sheep tibiae using resorbable polymeric membranes: a preliminary study. *J Orthop Trauma.* 1999; 13: 187–95.
4. **Gugala Z, Lindsey RW, Gogolewski S.** New approaches in the treatment of critical-size segmental defects in long bones. *Macromol Symp.* 2007; 253: 147–61.
5. **Frost HM.** Bone's mechanostat: a 2003 update. *Anat Rec A Discov Mol Cell Evol Biol.* 2003; 275: 1081–101.
6. **Papachroni KK, Karatzas D, Papavassiliou KA, *et al.*** Mechanotransduction in osteoblast regulation and bone disease. *Trends Mol Med.* 2009; 15: 208–16.
7. **Anderson DM, Maraskovsky E, Billingsley WL, *et al.*** A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature.* 1997; 390: 175–9.
8. **Lacey DL, Timms E, Tan HL, *et al.*** Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 1998; 93: 165–76.
9. **Boyle WJ, Simonet WS, Lacey DL.** Osteoclast differentiation and activation. *Nature.* 2003; 423: 337–42.
10. **Blair HC, Athanasou NA.** Recent advances in osteoclast biology and pathological bone resorption. *Histol Histopathol.* 2004; 14: 255–70.
11. **Abdallah BM, Kassem M.** Human mesenchymal stem cells: from basic biology to clinical applications. *Gene Therapy.* 2008; 15: 109–16.
12. **Bessa PC, Casal M, Reis RL.** Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts). *J Tissue Eng Regen Med.* 2008; 2: 1–13.
13. **Janssens K, ten Dijke P, Janssens S, *et al.*** Transforming growth factor-β1 to the bone. *Endocr Rev.* 2005; 26: 743–74.
14. **Horner A, Kemp P, Summers C, *et al.*** Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. *Bone.* 1998; 23: 95–102.
15. **Opperman LA, Nolen AA, Ogle RC.** TGF-beta 1, TGF-beta 2, and TGF-beta 3 exhibit distinct patterns of expression during cranial suture formation and obliteration in vivo and in vitro. *J Bone Miner Res.* 1997; 12: 301–10.
16. **Ripamonti U.** Soluble osteogenic molecular signals and the induction of bone formation. *Biomaterials.* 2006; 27: 807–22.
17. **McCarthy TL, Ji C, Centrella M.** Links among growth factors, hormones, and nuclear factors with essential roles in bone formation. *Crit Rev Oral Biol Med.* 2000; 11: 409–22.
18. **Koga T, Matsui Y, Asagiri M, *et al.*** NFAT and Osterix cooperatively regulate bone formation. *Nat Med.* 2005; 11: 880–5.
19. **Winslow MM, Pan M, Starbuck M, *et al.*** Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev Cell.* 2006; 10: 771–82.
20. **Wada T, Nakashima T, Hiroshi N, *et al.*** RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med.* 2006; 12: 17–25.
21. **Papachristou DJ, Papachroni KK, Basdra EK, *et al.*** Signaling networks and transcription factors regulating mechanotransduction in bone. *BioEssays.* 2009; 31: 794–804.
22. **Scheufler O, Schaefer DJ, Jaquiery C, *et al.*** Spatial and temporal patterns of bone formation in ectopically pre-fabricated, autologous cell-based engineered bone flaps in rabbits. *J Cell Mol Med.* 2008; 12: 1238–49.
23. **Perry CR.** Bone repair techniques, bone graft, and bone graft substitutes. *Clin Orthop.* 1999; 360: 71–86.
24. **Ceruso M, Falcone C, Innocenti M, *et al.*** Skeletal reconstruction with a free vascularized fibula graft associated to bone allograft after resection of malignant bone tumor of limbs. *Handchir Mikrochir Plast Chir.* 2001; 33: 277–82.
25. **Moran SL, Shin AY, Bishop AT.** The use of massive bone allograft with intramedullary free fibular flap for limb salvage in a pediatric and adolescent population. *Plast Reconstr Surg.* 2006; 118: 413–9.
26. **Ilizarov GA.** Clinical application of the tension-stress effect for limb lengthening. *Clin Orthop Relat Res.* 1990; 250: 8–26.
27. **Ilizarov GA.** The tension-stress effect on the genesis and growth of tissues: part I. The influence of stability of fixation and soft-tissue preservation. *Clin Orthop Relat Res.*1989; 238: 249–81.
28. **Ilizarov GA.** The tension-stress effect on the genesis and growth of tissues: part II. The influence of the rate and frequency of distraction. *Clin Orthop Relat Res.*1989; 239: 263–85.
29. **Aronson J.** Temporal and spatial increases in blood flow during distraction osteogenesis. *Clin Orthop.* 1994; 301: 124–31.
30. **Mahendra A, Maclean AD.** Available biological treatments for complex nonunions. *Injury.* 2007; 38: S7–12.
31. **Lewandrowski KU, Venugopalan V, Tomford WW, *et al.*** Kinetics of cortical bone demineralization: controlled demineralization–a new method for modifying cortical bone allografts. *J Biomed Mater Res.* 1996; 31: 365–72.
32. **Langer R, Vacanti JP.** Tissue engineering. *Science.* 1993; 260: 920–6.
33. **Stock UA, Vacanti JP.** Tissue engineering: current state and prospects. *Annu Rev Med.* 2001; 52: 443–51.

34. **Stevens B, Yang Y, Mohandas A, *et al.*** A review of materials, fabrication methods, and strategies used to enhance bone regeneration in engineered bone tissues. *J Biomed Mater Res.* 2008; 85B: 573–82.
35. **Bruder SP, Neelam J, Haynesworth SE.** Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biol.* l997; 64: 278–94.
36. **Tsuchida H, Hashimoto J, Crawford E, *et al.*** Engineered allogenic mesenchymal stem cells repair femoral segmental defect in rats. *J Orthop Research.* 2003; 21: 44–53.
37. **Bianco P, Robey PG.** Stem cells in tissue engineering. *Nature.* 2001; 414: 118–21.
38. **Friedenstein AJ, Deriglasova UF, Kulagina, *et al.*** Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp Hematol.* 1974; 2: 83–92.
39. **Caplan AI.** Mesenchymal stem cells. *J Orthop Res.* 1991; 9: 641–50.
40. **Pittenger MF, Mackey AM, Beck SC, *et al.*** Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999; 284: 143–7.
41. **Bianco P, Kuznetsov SA, Riminucci M, *et al.*** Postnatal skeletal stem cells. In: Klimanskaya I, Lanza R, editors. Methods in enzymology, Volume 419. New York: Academic Press; 2006. pp. 117–48.
42. **Stenderup K, Justesen J, Clausen C, *et al.*** Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone.* 2003; 33: 919–26.
43. **Harley CB.** Telomere loss: mitotic clock or genetic time bomb? *Mutat Res.* 1991; 256: 271–82.
44. **Burns JS, Abdallah BM, Guldberg P, *et al.*** Tumorigenic heterogeneity in cancer stem cells evolved from long-term cultures of telomerase-immortalized human mesenchymal stem cells. *Cancer Res.* 2005; 65: 3126–35.
45. **Bianchi G, Banfi A, Mastrogiacomo M, *et al.*** Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor. *Exp Cell Res.* 2003; 287: 98–105.
46. **Ducy P, Zhang R, Geoffroy V, *et al.*** Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell.* 1997; 89: 747–54.
47. **Rifas L.** The role of noggin in human mesenchymal stem cell differentiation. *J Cell Biochem.* 2007; 100: 824–34.
48. **Naldini L, Blomer U, Gallay P, *et al.*** *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science.* 1996; 272: 263–7.
49. **Chao W, Qiling H, Gang L.** Allogenic peripheral blood derived mesenchymal stem cells (MSCs) enhance bone regeneration in rabbit ulna critical-sized bone defect model. *J Orthop Res.* 2006; 24: 610–8.
50. **Ohgushi H, Goldberg VM, Caplan AI.** Repair of bone defects with marrow cells and porous ceramic. Experiments in rats. *Acta Orthop Scand.* 1989; 60: 334–9.
51. **Oedayrajsingh-Varma MJ, van Ham SM, Knippenberg M, *et al.*** Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. *Cytotherapy.* 2006; 8: 166–77.
52. **Astori G, Vignati F, Bardelli S, *et al.*** "In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. *J Transl Med.* 2007; 5: 55.
53. **Zuk PA, Zhu M, Ashjian P, *et al.*** Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002; 13: 4279–95.
54. **Aust L, Devlin B, Foster SJ, *et al.*** Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy.* 2004; 6: 7–14.
55. **Urist MR.** Bone: formation by autoinduction. *Science.* 1965; 150: 893–9.
56. **Urist MR, Mikulski A, Lietze A.** Solubilized and insolubilized bone morphogenetic protein. *Proc Natl Acad Sci USA.* 1979; 76: 1828–32.
57. **Urist MR, Sato K, Brownell AG, *et al.*** Human bone morphogenetic protein (hBMP). *Proc Soc Exp Biol Med.* 1983; 173: 194–9.
58. **Sampath TK, Reddi AH.** Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. *Proc Natl Acad Sci USA.* 1981; 78: 7599–603.
59. **Reddi AH, Huggins C.** Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. *Proc Natl Acad Sci USA.* 1972; 69: 1601–5.
60. **Reddi AH.** Morphogenesis and tissue engineering of bone and cartilage: inductive signals, stem cells, and biomimetic biomaterials. *Tissue Eng.* 2000; 6: 351–9.
61. **Wozney JM, Rosen V, Celeste AJ, *et al.*** Novel regulators of bone formation: molecular clones and activities. *Science.* 1988; 242: 1528–34.
62. **Aono A, Hazama M, Notoya K, *et al.*** Potent ectopic bone-inducing activity of bone morphogenetic protein–4/7 heterodimer. *Biochem Biophys Res Commun.*1995; 210: 670–7.
63. **Heldin CH, Miyazono K, ten Dijke P.** TGFβ signalling from cell membrane to nucleus through SMAD proteins. *Nature.* 1997; 390: 465–71.
64. **Sebald W, Nickel J, Zhang JL, *et al.*** Molecular recognition in bone morphogenetic protein (BMP)/receptor interaction. *Biol Chem.* 2004; 385: 697–710.
65. **Miyazono K, Maeda S, Imamura T.** Coordinate regulation of cell growth and differentiation by TGFβ superfamily and Runx proteins. *Oncogene.* 2004; 23: 4232–7.
66. **Derynck R, Zhang YE.** Smad-dependent and Smad independent pathways in TGFβ family signalling. *Nature.* 2003; 425: 577–84.
67. **Celil AB, Hollinger JO, Campbell PG.** Osx transcriptional regulation is mediated by additional pathways to BMP2/Smad signaling. *J Cell Biochem.* 2005; 95: 518–28.
68. **Sowa H, Kaji H, Hendy GN, *et al.*** Menin is required for bone morphogenetic protein 2- and transforming growth factor beta-regulated osteoblastic differentiation through interaction with Smads and Runx2. *J Biol Chem.* 2004; 279: 40267–75.
69. **Maeda S, Hayashi M, Komiya S, *et al.*** Endogenous TGFβ signaling suppresses maturation of osteoblastic mesenchymal cells. *EMBO J.* 2004; 23: 552–63.
70. **Cook SD, Baffes GC, Wolfe MW, *et al.*** Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. *Clin Orthop.* 1994; 301: 302–12.
71. **Cook SD, Baffes GC, Wolfe MW, *et al.*** The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. *J Bone Joint Surg Am.* 1994; 76: 827–8.
72. **Chen X, Kidder LS, Lew WD.** Osteogenic protein-1 induced bone formation in an infected segmental defect in the rat femur. *J Orthop Res.* 2002; 20: 142–50.
73. **Geesink RG, Hoefnagels NH, Bulstra SK.** Osteogenic activity of OP-1 bone morphogenetic protein (BMP-7) in a human fibular defect. *J Bone Joint Surg Br.* 1999; 81: 710–8.
74. **Jones AL, Bucholz RW, Bosse MJ, *et al.*** Recombinant human BMP-2 and allograft

compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. *J Bone Joint Surg Am.* 2006; 88: 1431–41.

75. **Riedel GE, Valentin-Opran A.** Clinical evaluation of rh-BMP-2/ ACS in orthopedic trauma: a progress report. *Orthopedics.* 1999; 22: 663–5.
76. **BESTT Study Group.** Recombinant human bone morphogenic protein-2 for the treatment of open tibial fractures: a prospective, controlled randomised study of four hundred and fifty patients. *J Bone Joint Surg Am.* 2002; 84: 2123–34.
77. **Swiontkowski MF, Aro HT, Donell S, *et al.*** Recombinant human bone morphogenetic protein-2 in open tibial fractures. A subgroup analysis of data combined from two prospective randomized studies. *J Bone Joint Surg Am.* 2006; 88: 1258–65.
78. **McKee MD, Schemitsch EH, Waddell JP, *et al.*** The effect of human recombinant bone morphogenic protein (rh-BMP-7) on the healing of open tibial shaft fractures: results of a multi-center, prospective, randomized clinical trial. In: Proceedings of the 18th Annual Meeting of the Orthopaedic Trauma Association. Toronto, Ontario, Canada; October 11–13; 2002. pp. 157–58.
79. **Saito A, Suzuki Y, Ogata S, *et al.*** Prolonged ectopic calcification induced by BMP-2-derived synthetic peptide. *J Biomed Mater Res.* 2004; 70: 115–21.
80. **Shinar DM, Endo N, Halperin D, *et al.*** Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology.* 1993; 132: 1158–67.
81. **Canalis E.** Insulin-like growth factors and the local regulation of bone formation. *Bone.* 1993; 14: 273–6.
82. **Mochizuki H, Hakeda Y, Wakatsuki N, *et al.*** Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology.* 1992; 131: 1075–80.
83. **Flanagan AM, Chambers TJ.** Stimulation of bone nodule formation in vitro by prostaglandins E1 and E2. *Endocrinology.* 1992; 130: 443–8.
84. **Narumiya S, Sugimoto Y, Ushikubi F.** Prostanoid receptors: structures, properties, and functions. *Physiol Rev.* 1999; 79: 1193–226.
85. **Weinreb M, Grosskopf A, Shir N.** The anabolic effect of PGE2 in rat bone marrow cultures is mediated via the EP4 receptor subtype. *Am J Physiol.* 1999; 276: E376–83.
86. **Senkoylu A, Uzunok D, Piskin E.** Treatment of segmental bone defects in rats by the stimulation of bone marrow osteo-progenitor cells with prostaglandin E2. *Int Orthop.* 2005; 29: 73–7.
87. **Wu D, Razzano P, Grande DA.** Gene therapy and tissue engineering in repair of the musculoskeletal system. *J Cell Biochem.* 2003; 88: 467–81.
88. **Chang SC, Chuang H, Chen YR, *et al.*** Cranial repair using BMP-2 gene engineered bone marrow stromal cells. *J Surg Res.* 2004; 119: 85–91.
89. **Bonadio J, Smiley E, Patil P, *et al.*** Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. *Nat Med.* 1999; 5: 753–9.
90. **Kneser U, Schaefer DJ, Polykandriotis E, *et al.*** Tissue engineering of bone: the reconstructive surgeon's point of view. *J Cell Mol Med.* 2006; 10: 7–19.
91. **Navarro M, Michiardi A, Castaño O, *et al.*** Biomaterials in orthopaedics. *J R Soc Interface.* 2008; 5: 1137–58.
92. **Ducheyne P, Qiu Q.** Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. *Biomaterials.* 1999; 20: 2287–303.
93. **Ciccone W, Motz C, Bentley C, *et al.*** Bioabsorbable implants in orthopaedics: new developments and clinical applications. *J Am Acad Orthop Surg.* 2001; 9: 280–8.
94. **Brady JM, Cutright DE, Miller RA, *et al.*** Resorption rate, route of elimination, and ultrastructure of implant site of polylactic acid in abdominal-wall of rat. *J Biomed Mater Res.* 1973; 7: 155–66.
95. **Kasuga T, Maeda H, Kato K, *et al.*** Preparation of poly(lactic acid) composites containing calcium carbonate (vaterite). *Biomaterials.* 2003; 24: 3247–53.
96. **Kunze C, Freier T, Heldwig E, *et al.*** Surface modification of tricalcium phosphate for improvement of the interfacial compatibility with biodegradable polymers. *Biomaterials.* 2003; 24: 967–74.
97. **Boccaccini AR, Roether JA, Hench LL, *et al.*** A composites approach to tissue engineering. *Ceram Eng Sci Proc.* 2002; 23: 805–16.
98. **Hutmacher DW, Schantz JT, Lam CXF, *et al.*** State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. *J Tissue Eng Regen Med.* 2007; 1: 245–60.
99. **Habibovic P, de Groot K.** Osteoinductive biomaterials – properties and relevance in bone repair. *J Tissue Eng Regen Med.* 2007; 1: 25–32.
100. **Ripamonti U.** Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. *Biomaterials.* 1996; 17: 31–5.
101. **Klawitter JJ, Hulbert SF.** Application of porous ceramics for the attachment of load-bearing internal orthopedic applications. *Cell Transplant.* 1971; 3: 339.
102. **Babis GC, Soucacos PN.** Bone scaffolds: the role of mechanical stability and instrumentation. *Injury.* 2005; 36S: S38–44.
103. **Trojani C, Boukhechba F, Scimeca JC, *et al.*** Ectopic bone formation using an injectable biphasic calcium phosphate/Si-HPMC hydrogel composite loaded with undifferentiated bone marrow stromal cells. *Biomaterials.* 2006; 27: 3256–64.
104. **Folkman J, Hochberg M.** Self-regulation of growth in three dimensions. *J Exp Med.* 1973; 138: 745–53.
105. **Koike N, Fukumura D, Gralla O, *et al.*** Tissue engineering: creation of long-lasting blood vessels. *Nature.* 2004; 428: 138–9.
106. **Geiger F, Bertram H, Berger I, *et al.*** Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. *J Bone Miner Res.* 2005; 20: 2028–35.
107. **Peng H, Usas A, Olshanski A, *et al.*** VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. *J Bone Miner Res.* 2005; 20: 2017–27.
108. **Khouri RK, Koudsi B, Reddi H.** Tissue transformation into bone in vivo. A potential practical application. *JAMA.* 1991; 266: 1953–5.
109. **Warnke PH, Springer ING, Wiltfang J, *et al.*** Growth transplantation of a custom vascularized bone graft in a man. *Lancet.* 2004; 364: 766–70.
110. **Heliotis M, Lavery KM, Ripamonti U, *et al.*** Transformation of a prefabricated hydroxyapatite/osteogenic protein-1 implant into a vascularized pedicled bone flap in the human chest. *Int J Oral Macillofac Surg.* 2006; 35: 265–9.
111. **Jiang F, Zhang G, Hashimoto I, *et al.*** Neovascularization in an arterio-venous loop-containing tissue engineering chamber: role of NADPH oxidase. *J Cell Mol Med.* 2008; 12: 2062–72.