Investigation of the effect of freeze-dried human serum albumin on the biocompatibility of cancellous bone allograft

Ph.D. Thesis

Miklós Weszl

Semmelweis University
PhD School of Basic Medicine

Supervisor:	Zsombor Lacza, MD, DSc
Official Reviewers:	József Piffkó, DMD, MD, PhD Tibor Glasz, MD, PhD
Head of the Complex Examination Committee:	István Antal, D.Pharm, PhD
Members of the Complex Examination Committee:	Zoltán Benyó, MD, DSc Emília Madarász, Professor emerita Éva Szökő, D.Pharm, DSc

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Table of contents

1 LIST OF ABBREVIATIONS 5

2 INTRODUCTION 7

2.1 Overview of bone replacement 9

2.1.1 Aetiology of bone defects 10

2.1.2 Categorization of bone defects 10

2.1.2.1 Traumatic and long bone defects 11

2.1.2.2 Maxillary and mandibular alveolar bone defects 11

2.1.2.3 Vertebral compression fracture 12

2.1.2.4 Nonunions 13

2.1.3 Management strategy of bone defects 13

2.1.3.1 Replacement of traumatic bone losses and long bone defects 14

2.1.3.2 Fixation of orthopaedic implants 14

2.1.3.3 Replacement of maxillary and mandibular alveolar bone defects 15

2.1.3.4 Compression fracture repair 15

2.1.3.5 Clinical trial of bone grafts 17

2.2 Bone remodelling and fracture healing 17

2.3 Bone grafts 22

2.3.1 Bone graft incorporation 22

2.3.1.1 Osteoinduction 23

2.3.1.2 Osteoconduction 23

2.3.1.3 Osteogenesis 24

2.3.1.4 Mechanical environment 24

2.3.1.5 Complications associated with bone grafts 24

2.3.2 Bone graft types 27

2.3.2.1 Autogenic bone grafts 27

2.3.2.2 Allogeneic bone grafts 28

2.3.2.3 Xenogeneic bone grafts 29

2.3.2.4 Synthetic bone graft materials 29

2.3.2.5 Presentations of bone grafts 30

2.3.3 Growth factors 31

2.3.3.1 Bone morphogenetic proteins 31

2.3.3.2 Vascular endothelial growth factor (VEGF) 33

2.3.3.3 Platelet-derived growth factor (PDGF) 34

2.3.3.4 Complications associated with growth factors 35

2.3.4 Trends in bone graft development 35

2.3.4.1 Nanotechnology 35

2.3.4.2 Coatings 37

2.3.4.3 Tissue engineering 38

2.3.4.4 Bioreactors 39

2.4 Evaluation of the pre-clinical performance of bone grafts 39

2.4.1 Biomechanics of bone 39

2.4.2 Mechanotransduction 42

2.4.2.1 Chemical composition 45

2.4.2.2 Porosity 46

2.4.2.3 Hardness 47

2.4.2.4 Topography 48

2.4.3 Biological assessment of bone graft materials 50

2.4.3.1 *In vitro* biocompatibility studies 51

2.4.3.2 *In vitro* differentiation studies 51

2.4.3.3 *In vivo* osseointegration studies 52

2.4.4 Preliminary results of our research group 53

3 OBJECTIVES 58

3.1 Investigation of the *in vivo* biocompatibility of chemically sterilized, antigen-extracted freeze-dried human bone grafts 58
3.2 Identification of a coating substance to improve the biocompatibility of the chemically sterilized, antigen-extracted freeze-dried cancellous allogeneic bone grafts 58
3.3 Investigation of the *in vitro* and *in vivo* biocompatibility coated freeze-dried cancellous allogeneic bone grafts 59

4 MATERIALS AND METHODS 60
4.1 In vivo investigation of the biocompatibility of chemically sterilized, antigen extracted freeze-dried human bone graft 60
4.1.1 Surgical procedure 60
4.1.2 *In vivo* multimodal imaging 61
4.1.3 Ex-vivo µCT analysis 61
4.1.4 Histology 61
4.2 *In vitro* experimental design 62
4.2.1 Bone graft types 63
4.2.2 *In vitro* experimental groups 64
4.2.3 Coating substances 64
4.2.4 Coating methods 64
4.2.4.1 Aqueous coating 65
4.2.4.2 Freeze-dried coating 65
4.2.5 Physical characterization of the bone grafts 65
4.2.5.1 Micro-hardness measurement 65
4.2.5.2 Scanning Electron Microscopy 66
4.3 *In vitro* biocompatibility study of coated human bone grafts 66
4.3.1 Isolation and cultivation of bone marrow derived MSCs 66
4.3.2 Isolation and cultivation of dental pulp derived MSCs 67
4.3.3 Characterization of the stem cells 67
4.3.4 Labelling of MSCs 67
4.3.5 Seeding of MSCs under standard conditions 68
4.3.6 Seeding of cells under dynamic conditions 68
4.3.7 Assessment of the proliferation of BMSCs 68
4.3.8 Assessment of the proliferation of DPSCs 69
4.4 *In vivo* biocompatibility study of coated human bone grafts 69
4.4.1 Nonunion model and study design 70
4.4.2 Statistical analysis 71

5 RESULTS 73
5.1 *In vivo* biocompatibility of chemically sterilized, antigen extracted freeze-dried human bone allografts 73
5.1.1 NanoSPECT-CT analysis 73
5.1.2 Ex-vivo µCT analysis 74
5.1.3 Results of histology 76
5.2 Physical characteristics of bone grafts 76
5.2.1 Optical characteristics of bone grafts 76
5.2.2 Microhardness of bone grafts 79
5.3 *In vitro* biocompatibility of bone grafts 79
5.3.1 Characterization of stem cells 79
5.3.2 Adherence and survival of MSCs on bone grafts 80
5.3.2.1 Control group 80
5.3.2.2 Test group A 80
5.3.2.3 Test group B 81
5.3.2.4 Effect of dynamic seeding on MSC adherence 83
5.4 *In vivo* biocompatibility of freeze-dried albumin coated bone grafts 83

6 DISCUSSION 85

7 CONCLUSION 92

7.1 Investigation of the *in vivo* biocompatibility of chemically sterilized, antigen-extracted freeze-dried human bone grafts 92

7.2 Identification of a coating substance to improve the biocompatibility of the chemically sterilized, antigen-extracted freeze-dried cancellous allogeneic bone grafts 92

7.3 Investigation of the *in vitro* and *in vivo* biocompatibility coated freeze-dried cancellous allogeneic bone grafts 92

8 SUMMARY (English, Hungarian) 93

9 REFERENCES 95

10 PUBLICATION LIST 116

11 ACKNOWLEDGMENTS 118

1 LIST OF ABBREVIATIONS

α-MEM	alpha modification of Eagle's medium
β-cat	β-catenin
BA	Bone area
BMSC	Bone marrow derived mesenchymal stem cell
BMP	Bone morphogenetic protein
rhBMP	Recombinant human bone morphogenetic protein
BMU	Bone multicellular unit
BRC	Bone remodelling compartment
CD	Cluster of differentiation
CT	Computed tomography
CPC	Calcium phosphate cement
Dvl	Dishevelled
DPSC	Dental pulp derived stem cell
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
FA	Focal adhesion
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FYN	Src family tyrosine-protein kinase
Grb2	Growth factor receptor-bound protein 2
HAP	Hydroxyapatite
HV	Numeric value of Vickers hardness
LEF	Lymphoid enhancer factor
LTPB	Latent TGF-β-binding protein
MAKP	Mitogen-activated kinase
MDP	Methylene bisphosphonate
MEK	MAPK/Erk kinase.
MLC	Myosin light chain
MSC	Mesenchymal stem cell
NMMII	Non-muscle myosin II
OB	Osteoblast
OC	Osteoclast
Pax	Paxillin
pCPC	Premixed calcium phosphate cement
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PLA	Polylactide
PMMA	Poly(methyl methacrylate)
PPAR-γ	Peroxisome proliferator-activated receptor γ
PPF	Polypropylenefumarate
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rho GEFs	Rho guanine nucleotide exchange factors
ROCK	Rho-associated coiled-coil-containing protein kinase
ROI	Region of interest
Runx2	Runt-related transcription factor 2

RPM	Rotation per minute
SEM	Scanning electron microscopy
Shc	SH2-containing collagen-related proteins
Src	Rous sarcoma oncogene cellular homolog
SrCPS	Sr-doped calcium phosphate composite spheres
SUN	Sad1p and UNC-84 homology
VASP	Vasodilator-stimulated phosphoprotein
Vin	Vinculin
VOI	Volume of interest
VEGF	Vascular endothelial growth factor
TA	Tissue area
TAZ	Transcriptional co-activator with PDZ-binding motif
Tb.Th.	Trabecular thickness
Tb.Sp.	Trabecular separation
Tb.Pf.	Trabecular pattern factor
^{99m}Tc-MDP	Technetium 99m-methyl diphosphonate
YAP	Yes-associated protein

2 INTRODUCTION

A serious limitation of the clinical performance of bone grafts is their unreliable incorporation[1]. Bone grafts have the capability of turning into live bone, however sometimes the graft fails to coalesce with the host bone with no foreseeable reason. The pathophysiologic variability does not allow the simplification and regard bone defects as a uniform condition, but depending on their aetiology and anatomical location they must be evaluated and treated on a personalized manner. Age, sex, metabolism and physical activity are factors that may significantly influence the innate regenerative potential of the bone after fracture or bone replacement. Unfortunately, the biology of bone development and fracture healing is not fully understood yet, which is one of the major limitations of the development of bone replacement technologies that ensure reliable bone graft incorporation. Another limitation is the lack of potent biomaterials that may become alternatives of autogenic bone grafts. However, the biomimetic approach of tissue engineering (i.e. the development of artificial materials that mimic natural bone) and the discovery of the role of biophysical cues in bone remodelling may open a new chapter in the development of the next generation of bone graft materials. Pursuing biomimetic approach, the individual characteristics of the host bone and its environment should be taken into consideration, e.g. bone morphometric indices, elasticity, defect size and morphology, in order to develop personalized bone grafts with good clinical performance. However, the implementation of this approach presumes the existence of highly developed integrated technologies, such as diagnostic imaging and additive manufacturing. The development of diagnostic image processing that is able to provide data on the mechanical characteristics of host bone would be the prerequisite of personalized treatment. In the meantime, the development of additive manufacturing technologies would also be required because mass produced bone graft materials will always have biological weaknesses that limit their clinical performance[2]. However, the capability of producing artificial bone grafts that corresponds at least the physical environment (elasticity, mineral content, resorption rate, shape, etc.) of the host in order to activate biophysical cues may increase the reliability of graft incorporation.

At the current technology level the best biomimetic materials are of human origin, such as allogeneic bone graft. Allogeneic bone graft (allograft) is usually the

second choice for bone replacement after autogenic bone. Fresh, frozen, and freeze-dried allografts are most often used at load-bearing sites, however there are no exclusively applied protocols for their preparation. For patient safety the cadaveric and donor grafts are supposed to be subjected to disinfection to avoid the transfer of contagious agents from the donor to the recipient. Freeze-drying technique allows the disinfection of allogeneic bone grafts using volatile agents, such as ethylene-oxide and acids that form non-toxic residual salts[3]. As the disadvantageous effect of harsh disinfection the osteogenic cells are killed and most of the cell adhesive proteins become denatured, which impairs the biological value of the allograft. Thus, in such a way manufactured freeze-dried allograft can be characterized with good mechanical resistance and low biological value, which may be responsible for its unreliable incorporation. The re-establishment of the original or just quasi-original biological properties of the allografts would provide an unlimited source of potent bone grafts that could be an alternative to autogenic bone, which was the objective of the present doctoral work.

This thesis follows a bottom-up approach, meaning that the current achievements and challenges of bone replacement with bone grafts will be outlined first in order to put into context the objectives of the experimental work. The aim of the thematic introduction is to highlight the versatility of bone defects and clinical challenges that need to be solved by different management strategies. The different clinical challenges call for alternative approaches both at acute treatment and regenerative therapy levels. This is the underlying reason of the need for different bone grafts with various characteristics that could challenge the myth of the existence of an ultimate or 'ideal' bone graft. Each graft type has limitations – even the autogenic bone – in clinical applications, whereas there is poor extrapolation between the results of pre-clinical studies and clinical outcomes with bone grafts[4]. Therefore, the biological performance of a bone graft should be verified in each intended indication in controlled human studies, while the underlying biological mechanism should be evaluated in comprehensive pre-clinical studies. This thesis presents the first stage of the development of a 3-dimensional structural bone graft that has been designed for

orthopaedic use, which biological performance has been verified in subsequent animal and human studies by other members of our research group[5,6,7].

2.1 Overview of bone replacement

Bone possesses the intrinsic capability of regenerating itself after injuries through a well-orchestrated process involving multiple molecular signalling pathways that induce series of cellular and intercellular biological events. In the clinical setting, bone healing after fractures is the most common form of bone regeneration. In contrast to other tissues, most of the bone injuries heal without the formation of scar tissue, which is resulted in the total recovery of the pre-existing bone function, while the newly formed bone is undistinguishable from uninjured bone tissue. However, there are cases when the self-healing property of the bone is impaired or insufficient that results either in persistent bone defects or nonunion.

Bone defects may develop by various reasons at different anatomical locations in the human body that makes it difficult to conduct an exhaustive discussion that covers every aspect of the problem. The magnitude of the clinical problem of bone defects is highlighted by the fact that the human bone is the second most common transplant tissue after blood, which means ~2.2 million bone grafts used just in orthopaedic procedures annually worldwide[8,9]. The growing demand for a higher quality of life after bone and joint replacement has become an essential requirement from the patient side and the volume of research and development activities in the domain of bone tissue replacement has dramatically increased in the last decade. The driving force of the intense research of bone graft materials is to find alternative(s) of human bone tissue transplants (autografts), which has limited availability in general. In this endeavour several approaches have been published in the scientific literature concerning the construction and evaluation of artificial bone graft materials. However, there is consensus in the art concerning the desired biological properties of an ideal bone graft, it will be detailed later in paragraph 2.3, but there is controversy about how to achieve and measure those properties.

The underlying basis of the arguments may stem form that sometimes underemphasized fact that bone defects have different pathophysiology depending on

their aetiology. The diverse pathophysiology may require various approaches concerning the management of the bone defects in order to achieve the intended clinical outcome. There could be considerable differences in the clinical pictures when bone replacement is indicated, if we consider the different ends of the spectrum, for instance, critical size bone defects when the bone fails to bridge an oversized gap in the healing process, in contrast with the compression fracture of a vertebrae when a relatively small bone defect can result in serious complications. The different clinical need driven approaches are manifested in the development and evaluation strategies of the various bone graft materials.

2.1.1 Aetiology of bone defects

Bone defects may develop in several different ways: bone fracture can end up in the occurrence of a pseudoarthrosis in 5 – 10% of the cases, high energy trauma, prosthesis revision arthroplasty, treatment of musculoskeletal infections or excision of bone tumours can also result in large bone deficiencies. Depending on the aetiology, anatomical location and associated complications bone defects may be characterized with different pathophysiologic properties.

2.1.2 Categorization of bone defects

The importance of bone defect analysis and classification is to determine the best regenerative treatment for each specific defect. The categorization of bone defects can be performed according to various considerations, however the management-based approach may offer a clinically reasonable overview. Concerning clinical setting, perhaps the greatest need for enhanced bone repair is in the treatment of segmental bone defects and chronic nonunion of fractures. Even within these subcategories there are significant differences concerning the properties of bone defects depending on their aetiology and anatomical location of bone defects.

2.1.2.1 Traumatic and long bone defects

The overall incidence of long bone fractures in the Western world is estimated to be between 300 and 400 individuals per 100,000 per year[10]. The majority of trauma-induced fractures in adults will heal within nine months[11]. Apparently, 5–30% of the patients develop complications during the healing process, leading to delayed union or even nonunion of the fracture [12]. Traumatic bone loss implies a wide spectrum ranging from a small butterfly fragment through to complete loss of large sections of bone. The most commonly employed system for the classification of traumatic injuries was given by Gustilo and Anderson, which has been later modified by Gustilo, Mendoza and Williams; however, traumatic bone loss is not part of this classification[13,14]. Robinson and his colleagues proposed a classification of bone loss in tibial fractures that could be applied to all long bone diaphyseal fractures (Table 1). It must be noted that there is no widely recognized classification of traumatic bone loss.

Table 1. Classification of tibial bone loss according to Robinson and his colleagues[15].

Grade	Maximal bone loss (%)	Maximum length of bone loss (cm)
Trivial	Wedge < 25%	0
Minor	Wedge 25% to 50% Wedge > 50% to < 100%	< 2.5
Moderate	Wedge > 50% but < 100% Circumferential	2.5 to 10 < 2.5
Severe	Wedge > 50% but < 100% Circumferential	> 10 > 2.5

2.1.2.2 Maxillary and mandibular alveolar bone defects

Parameters that can describe alveolar bony defects are the anatomic position of defect in the jaws, dimensions and morphology of the defect (vertical, horizontal, ridge contour, etc.), defect base width and number of residual bony walls surrounding the defect[16]. Various classifications have been developed to describe alveolar ridge defects. Dimension based classification divided the alveolar ridge defects into 3 classes, such as horizontal defects (Class I), vertical defects (Class II) and a common variant of them, i.e. horizontal and vertical defects (Class 3)[17]. These classes can be subdivided based on

the amount of the deficiency[18]. The recently proposed more quantitative classification that takes into consideration the size and orientation is more descriptive and applicable in the diagnostic work-up of alveolar bone defects[19]. Perhaps, Khojasteh *et al*. proposed the most comprehensive classification that takes into consideration the recipient site characteristic, as well; (A: Two-wall defects, B: One-wall defects, C: A defect with no surrounding walls) and width of defect base (I: A bony defect with a base width of 5 mm or more, II: A bony defect with a base width of 3 mm or more, but less than 5 mm, III: A bony defect with a base width less than 3 mm)[16].

2.1.2.3 Vertebral compression fracture

Spine fractures are the most frequent fragility fractures and the second ones for morbidity and mortality in the elderly group after hip fractures[20]. The prevalence of vertebral fractures is increasing in the aging global population that is a consequence of the modern lifestyle that requires less movement, thus many elderly today have weak bone structure. Vertebral fractures are indicators for osteoporosis, which incidence will continue to rise and so will the incidence of osteoporotic vertebral fractures[21,22]. More than 25% of women 50 year of age and older will have one or more vertebral fractures by 2025[23]. The most frequent site is the lumbar spine, and the primary and major symptom is localized back pain that can be debilitating. Osteoporotic vertebral fractures happen for axial compression that is not always associated with trauma, but especially in old patients, a simple lateral bending or weight lifting can be the cause (Figure 1). Spinal deformity index has been introduced for the morphometric characterization of the fractures, which is important for fracture classification and treatment[24,25]. The risk of mortality is 2-fold higher in patients with osteoporotic vertebral fractures, while osteoporotic men are at higher risk for mortality than women. Compared to hip fractures, there is a 25% higher mortality risk after osteoporotic vertebral fractures[23].

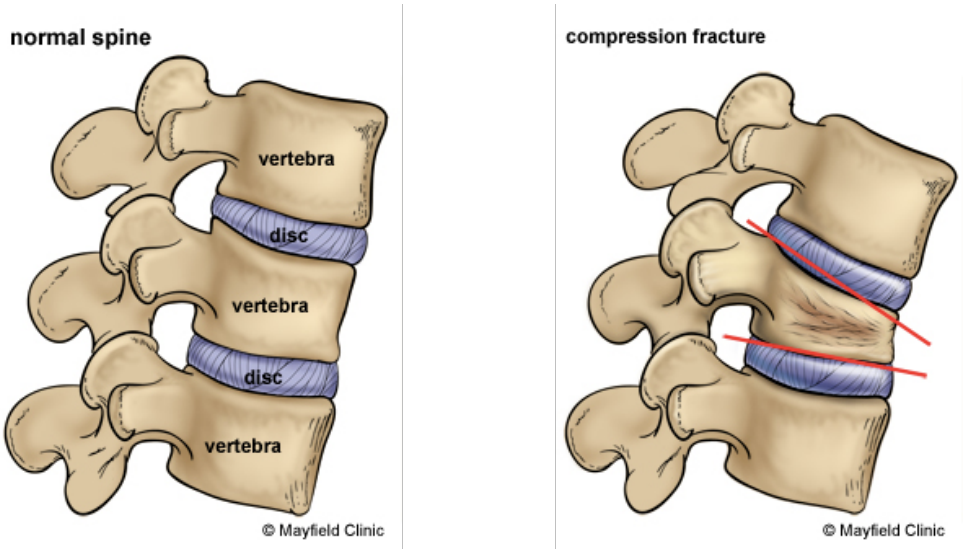

Figure 1. Side views of a normal spine and a spine with a compression fracture. An osteoporotic compression fracture causes the front of the vertebral body to collapse in a wedge-shape (red lines). The figure and legend were reprinted from the webpage of Mayfield Clinic[26].

2.1.2.4 Nonunions

The clinical symptoms and physical findings of nonunion include pain and motion at the fracture site as well as radiographic evidence of failure of union[27]. The incidence of nonunion can be as high as 5% to 20%, but varies by fracture site and is influenced by a number of factors[27]. Nonunions are classified as hypertrophic, and atrophic (oligotrophic). Hypertrophic nonunions have adequate vascularity and exuberant callus formation, and generally only require appropriate mechanical stabilization with fixation devices to support healing (this condition is also referred as pseudoarthrosis). In contrast, in oligotrophic or atrophic nonunions, there is minimal or no callus formation with diminished or absent vascularity. These types of nonunions may benefit most from bone grafting[28].

2.1.3 Management strategy of bone defects

The standard clinical approaches to stimulate or enhance bone regeneration include distraction osteogenesis, bone transport and various bone-grafting methods[29]. This thesis focuses on the discussion of bone-grafting methods from clinical point of

view and highlights the variety of the clinical approaches that need to comply with different clinical needs.

2.1.3.1 Replacement of traumatic bone losses and long bone defects

The goal in the management of any fracture with bone loss is to achieve solid bone union, adequate alignment, equal limb length and restoration of function. For fracture stabilization of traumatic bone losses *i*) plate fixation, *ii*) intramedullary fixation and *iii*) external fixation have been conventionally used[30,31]. Although, when severe traumatic bone loss is involved bone grafting can be applied as an alternative surgical technique. Vascularized free fibular graft may be used to bridge a bone defect > 12 cm in size; however, it is a technically demanding procedure that may cause undue burden to the patient[32,33]. Allografts can avoid the complication of donor-site morbidity but their use may be associated with lengthy recovery period, fracture and nonunion[34,35,36]. An alternative method of the reconstruction of long bone defects is a two-stage procedure (Masquelet technique) where the formation of a biological membrane is induced by the application of a cement spacer (first stage) that acts as a chamber for the insertion of a non-vascularized autogenic graft (second stage)[37,38,39].

2.1.3.2 Fixation of orthopaedic implants

Bone grafts may be used to fill bone voids in total joint (hip and knee) arthroplasty when the autologous bone tissue supply is limited, albeit there is still a debate on the cemented and cementless (biological) fixation[40]. There are pros and cons concerning each fixation techniques, however in some cases the biological fixation (using bone graft instead of cement) may provide more beneficial clinical outcome than cementation. For instance, in younger patients the cement-implant interface may alter in time due to the growth of the bone that may cause micro-fractures in the cement texture leading to osteolysis and implant loosening[41]. In contrast, biological fixation may allow tissue remodelling around the implant during the development and avoid the emergence of micro-fractures. In such indication the primary function of the bone graft, if applied, is to ensure the mechanical stability of the implant that is placed to a load-bearing site. The biological and mechanical features of the bone graft will influence the rate and

extent of bone remodelling and the quality of the biological fixation. Unfortunately, the scarcity of clinical data from controlled, randomized human studies makes it difficult to carry out the objective evaluation of the short-, and long-term clinical outcomes of cemented and cementless fixations techniques and even less the efficiency of (various) bone grafts in this application[41,42].

2.1.3.3 Replacement of maxillary and mandibular alveolar bone defects

The availability of adequate alveolar bone in terms of quality and quantity in all spatial dimensions is the prerequisite of successful dental implant placement. There are several surgical techniques for the vertical and horizontal augmentation of the alveolar bone, however guided bone regeneration and block graft techniques have become prevalent over distraction osteogenesis and osteoperiosteal flaps[45]. The block augmentation technique utilizes an autologous bone block that is fixed to the recipient ridge. The remodelling and histological performance of the bone block is affected primarily by its revascularization and the invasion rate by osteogenic cells. On the other hand, the principle of guided bone regeneration is to exclude encleftation (i.e. the proliferation of connective tissue into the sinus cavity) that would detrimentally affect the remodelling of particulate bone grafts, which occurs at a slower rate than soft tissue ingrowth[43]. In spite of the technical differences in the surgical techniques of block bone grafting and guided bone regeneration there are intrinsic similarities in their methodical principles, such as the exclusion of the epithelium and connective tissues, space maintenance, stability of fibrin clot and primary wound closure[44,45].

2.1.3.4 Compression fracture repair

The most frequent treatment of vertebral fractures is conservative because the majority of these fractures are stable and do not have radicular or medullar involvement. The indication of surgery depends on age, general conditions, fracture pattern and stability, involvement of medullary canal, bone quality, time elapsed from fracture[20]. The surgical options are vertebroplasty or kyphoplasty, vertebral stabilization and/or fusion with eventual decompression of the medullary canal. Vertebroplasty and kyphoplasty are minimal invasive interventions, which are performed through a hollow

needle that is driven directly into the fractured vertebra. In vertebroplasty, injectable bone filler is pressed through the hollow needle into the fractured bone. In kyphoplasty, a balloon is inserted first and inflated to expand the compressed vertebra to its normal height before filling the space with the bone filler[46]. The cemented bone filler in the vertebra allows to regain upright position, reduces pain, and prevents further fractures (Figure 2). Historically, poly(methyl methacrylate) (PMMA) cement has been used in these procedures first. However there have been complications associated with the use of PMMA, such as cement leakage and increased risk of adjacent vertebral fractures. Calcium phosphate, calcium sulphate and hydroxyapatite pastes have become the alternatives to PMMA because of their chemical resemblance to bone that renders good biocompatibility and biodegradability allowing both the biological and mechanical repair of the fracture. In clinical setting, there are two major obstacles in conjunction with calcium-based and other injectable bone fillers in spinal applications: handling and radiopacity[47]. Furthermore, the calcium-based injectable fillers are prone to syneresis and contraction as well as for brittleness after cementation[48]. However, most of these weaknesses of those injectable bone fillers can be eliminated by using additives, such as polymers that improve the mechanical properties and oxides that enhance the radiopacity[47,48,49].

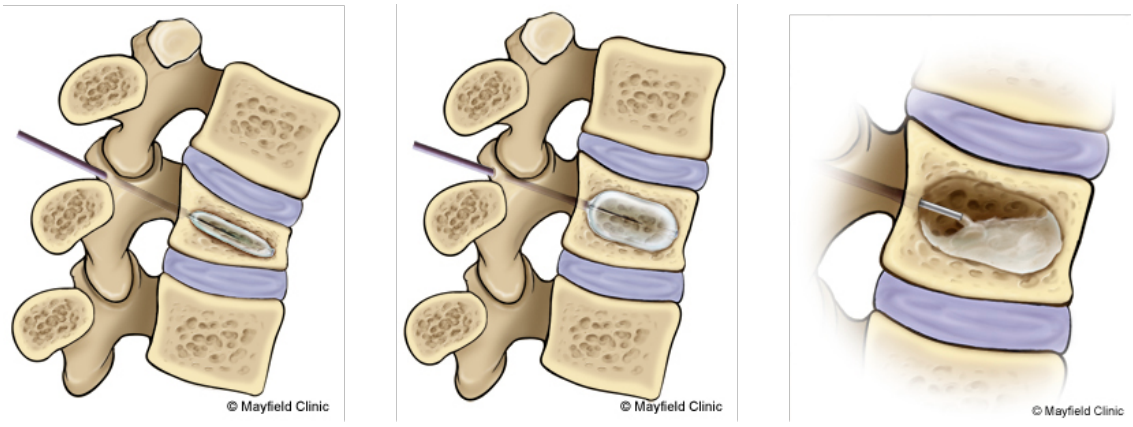

Figure 2. Illustration of compression fracture repair by kyphoplasty. The balloon is inserted into the working channel inside the vertebra, and then inflated to raise the vertebra to the appropriate height. The balloon is removed and bone cement is injected into the cavity. Fluoroscopic x-ray shows cement in upper vertebra (red arrow) and needle inserted in lower vertebra. Figure and legend were reprinted from reference 46.

2.1.3.5 Clinical trial of bone grafts

There are a large number of reported clinical trials concerning bone grafts but most of them have never been completed[50]. Furthermore, only a small fraction of the completed clinical studies have been published in peer-reviewed scientific journals. Hence, it may be concluded that the clinical safety and/or efficacy of the vast majority of bone grafts have not been established in controlled clinical studies. As a consequence, there are a lot of bone graft brands in clinical application, which have the risk of severe complications that may be associated with their use, e.g. rejection, early resorption or fracture. Concerning clinical study design, controlled, randomized, blinded, parallel assignment and multicentre arrangement would be appropriate to investigate the clinical performance of a bone substitute when the test graft is compared to a widely accepted comparator in selected patient groups. Interestingly, there are considerable amount of reliable and credible clinical data available for bone grafts that are intended for alveolar bone replacement, but concerning other indications and bone grafts the availability of such high quality clinical data is relatively low[50,51,52,53]. However, the extrapolation to the clinical performance of a bone graft based on pre-clinical experiments is questionable mainly due the lack of adequate animal models. Furthermore, even the results of a registered clinical study should be critically appraised and special emphasis should be taken to the evaluation of the correct study design and patient selection criteria[54,55]. It should also be kept in mind that the clinical performance of a bone graft in a selected indication may not be predictive for its performance in other indications (e.g. alveolar bone versus long bone replacement)[56].

2.2 Bone remodelling and fracture healing

Bone is constantly being remodelled throughout life in a sequence characterized by removal of old bone by osteoclasts and its replacement by osteoblasts. The main reason for this physiologic process is likely the removal of fatigue microcracks that occur in the skeleton as the result of daily physiological load. The remodelling process is driven in the basic multicellular unit (BMU) that comprises osteoclasts, osteoblasts, osteocytes, and lining cells (Figure 3). Under two-dimensional light microscopic

evaluation, bone remodelling compartments appear as narrow cleavages between trabecular bone and the bone marrow, which are linked by a layer of flattened lining cells on the marrow side and by the bone remodelling surface on the trabecular side. Given their tent-like appearance, the cells separating the bone remodelling compartment (BRC) from the bone marrow are thus termed the BRC canopies[57]. Osteoclasts are observed directly underneath canopies at the edges of the bone remodelling compartment (Figure 3)[58]. These canopy cells are connected to lining cells on the quiescent bone surface that are connected to osteocytes that reside in the bone matrix via gap junctions and cannaliculi[59]. The osteocytes have the capability of sensing biomechanical signals, such as mechanical strain and microcracks and initiate bone remodelling in respond to these signals presumably via its communication with lining cells[60]. In turn, the bone lining cells begins to form the BRC canopy and regulate osteoclast recruitment and differentiation by expressing receptor activator of nuclear factor κ B ligand (RANKL). Some recent studies show that activated osteoclasts may stimulate angiogenesis by secreting matrix metalloproteinase-9 that is able to release extracellular matrix (ECM)-bound vascular endothelial growth factor (VEGF)[61,62]. The ingrowth of a marrow capillary by penetrating the canopy of lining cells may serve as a conduit for the cells needed for the remodelling. At tissue level, mesenchymal stem cells (MSCs) reside in perivascular location close to sheets of osteoblast, as a cellular component of the hematopoietic niche or as an inactive marrow stromal cell[63]. When angiogenic stimuli occur the MSCs of bone marrow and fat has the capability of becoming pericytes on newly forming blood vessels (Figure 4). In the BRC, the pericytes are detached and act as MSCs that is driven by chemotactic factors released by inflammatory and other cells in the callus (Figure 5). There is emerging evidence that perivascular cells within the bone marrow exhibit mesenchymal lineage specific characteristics[64]. These mesenchymal cell-like perivascular cells form a unique niche, which possess self-renewing potential and the ability to commit to osteogenic, chondrogenic and adipogenic lineages (Figure 5)[65,66]. Thus, recent evidence indicates that the presence of blood vessels associated with the BRC may be a prerequisite for the appearance of osteoblasts within the BRC[67]. The same process is supposed to drive fracture healing that occurs after trauma. In the process of fracture healing the sequential stages of embryonic endochondral bone formation are reiterated, which is

called secondary bone healing. Secondary bone healing occurs in the vast majority of bony injuries, involving both intramembranous and endochondral ossification that lead to callus formation. Callus is a physiological reaction to inter-fragmentary movement and requires the existence of residual cell vitality and adequate blood flow (Figure 6)[68]. The fracture hematoma has been proven to be a source of signalling molecules, such as interleukins, tumour necrosis factor-α, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, vascular endothelial growth factor, and the transforming growth factor β superfamily members that are supposed to induce a cascade of cellular events that initiate healing[69,71]. Along with these biological cues the progressive union of a fracture requires the presence of four factors combined in the so-called diamond concept: an adequate cellular environment, sufficient growth factors, a bone matrix and mechanical stability[68]. Intriguingly, it appears that the initial cartilaginous callus forms even in the absence of a blood vessel, but the replacement of cartilage by bone only occurs following the penetration of blood vessels into the callus[70]. It is worth to note that oligotrophic and atrophic nonunions are characterized by the absence of blood vessels, which contain calcified cartilage that has not made the conversion step to bone, presumably due to the failure of the ingrowth of blood vessels and associated lack of appropriate osteoblast progenitor cells[71].

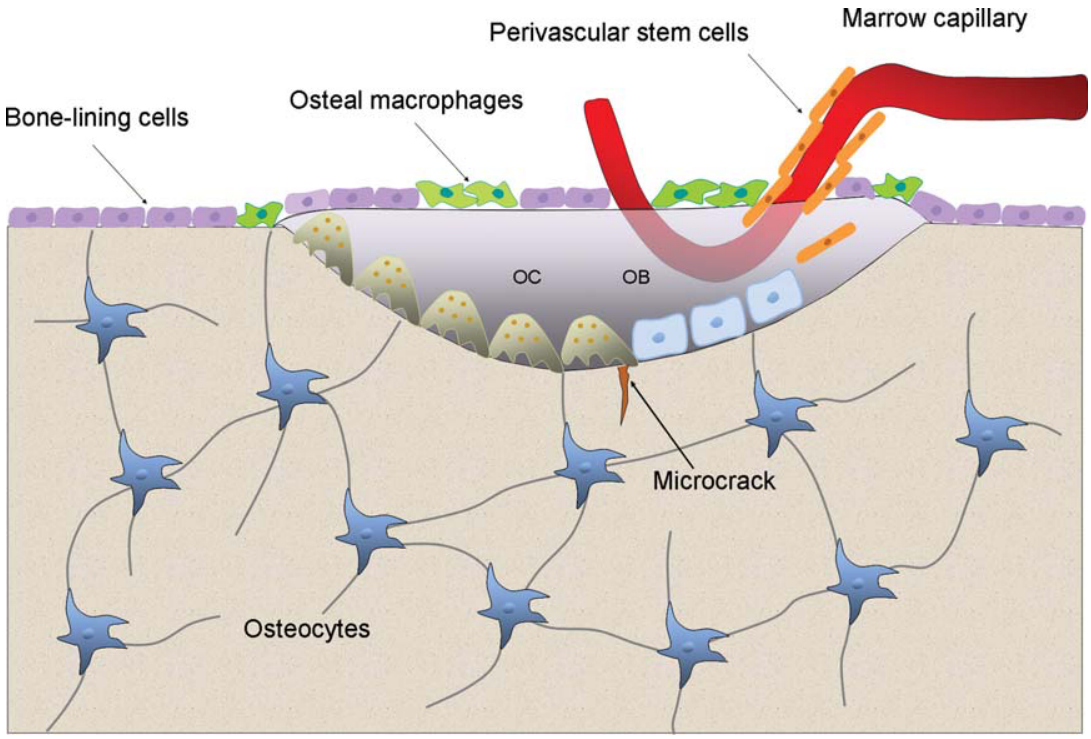

Figure 3. Schematic drawing of the basic multicellular unit within bone remodelling compartment (BRC). The figure shows the key cells involved in normal bone remodelling, including the osteocytes embedded within bone, osteoclasts (OC), osteoblasts (OB), bone lining cells, and, at least in mice, osteal macrophages. As depicted, normal bone remodelling may largely serve to repair fatigue microcracks in bone. Note also the close relationship between the BRC and the blood vessel, which likely carries the perivascular stem cells destined to become osteoblasts on the bone surface. Image and legend were reprinted from reference 28.

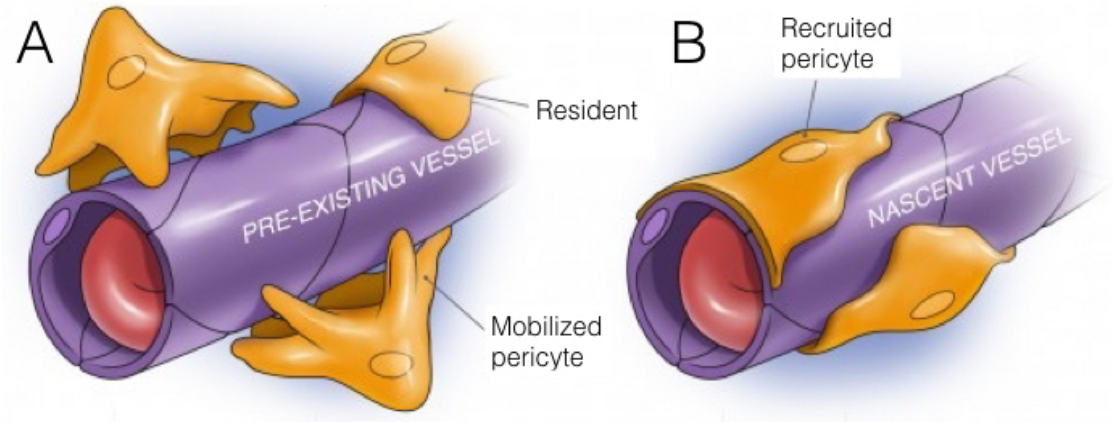

Figure 4. Mobilization and transfer of pericytes[72]. The residing MSCs have the capability of associating with bone pre-existing marrow vessels as pericytes for angiogenic stimuli (A). The pericytes are transferred to the BRC on the surface a newly forming marrow capillary (B).

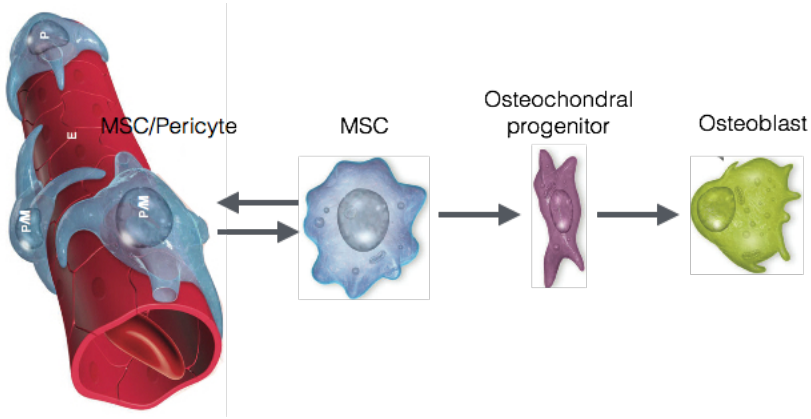

Figure 5. Pericyte - MSC transitions. MSCs reside in situ as perivascular cells, which can be released to enter an osteoblastic differentiation program and develop into secretory osteoblasts/embedded osteocytes. Alternatively, the released perivascular cells can become activated to exert trophic and immunomodulatory effects. Image is an adaptation from reference 63.

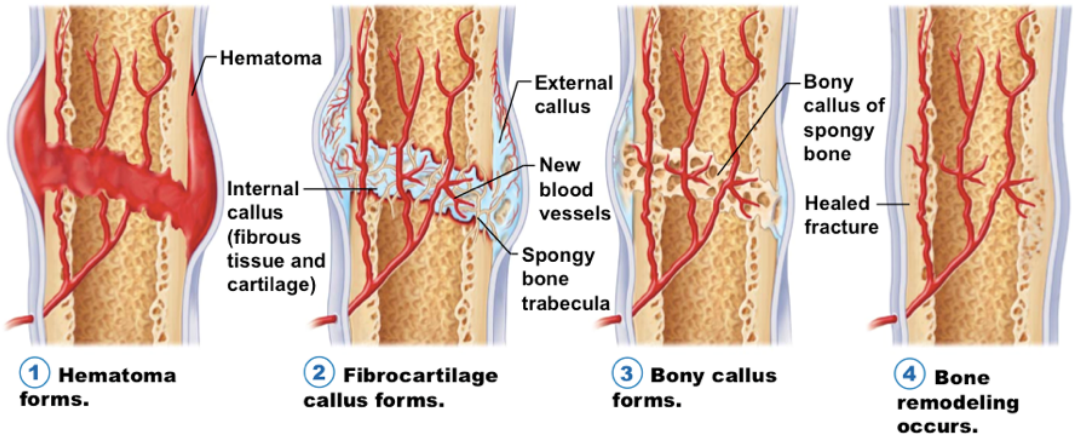

Figure 6. The stages of fracture repair. (1) Hematoma formation: following injury, fracture disrupts bony blood supply leading to hematoma formation in and around the bony defect; (2) Fibrocartilage (soft) callus formation: fracture hematoma is rich in VEGF, which promotes blood vessel ingrowth from surrounding vessels (angiogenesis) along with the formation of a cartilage intermediate by endochondral ossification (internal callus) and the external callus (intramembranous ossification); (3) Bony (hard) callus formation: the callus is mineralized as hypertrophic chondrocytes undergo apoptosis (partially regulated by VEGF) and woven bone is formed and eventually replaced by lamellar bone; (4) Bone remodelling: the fracture callus composed of primary lamellar bone is remodelled to secondary lamellar bone, and the vascular supply returns to normal. Image was reprinted from the internet[73] and legend is an adaptation from reference 62.

2.3 Bone grafts

A bone graft can be defined as either an inorganic or an organic 3-dimensional structure, or the combinations thereof that is intended for the replacement of a bone defect. Bone grafts can be used: i) to fill bone cavities and defects that emerged after cyst or tumour resection; ii) to bridge joints by creating arthrodesis; iii) to replace major defects and establish the continuity of a long bone; iv) to create bone block in order to limit joint motion; v) to establish union in pseudoarthrosis; vi) to promote union or fill defects in delayed union, malunion, fresh fractures and osteotomies; vii) to fix implants.

2.3.1 Bone graft incorporation

The successful incorporation of a bone graft depends on new bone formation that is driven by adaptive remodelling in response to mechanical stress. This process takes places in sequential phases that is supposed to be similar to those in fracture healing (Figure 6)[74,80]. The diagnostic follow-up of bone graft by computed tomography revealed that the discrete boundary between host and graft is initially identifiable; however, as union progresses, the graft-host junction is obliterated as a result of trabecular ingrowth, and the medullary canal is replaced by fibrous tissue, which may be attributed to fibrocartilage (soft) callus formation[80]. Morone and his co-workers gave a general description concerning the incorporation process of a bone graft into a host, e.g. the ‘‘process of envelopment and interdigitation of the donor bone tissue with new bone deposited by the recipient’’[75]. Campana and his colleagues gave an explanatory overview on the multiple stage process of bone graft incorporation[76]:

i) “Initially, the bone graft induces a response that leads to the accumulation of inflammatory cells that is followed by the chemotaxis of host mesenchymal cells to the graft site;

ii) Thereafter, the primitive host cells differentiate into chondroblasts and osteoblasts in a process that is directed by the cohort of osteoinductive factors;

iii) In the subsequent processes bone graft revascularization and necrotic graft resorption occur concurrently;

iv) Finally, bone remodelling carries out that directs the formation of new bone tissue in response to the mechanical stress.”

In order to stimulate this process a bone graft is supposed to possess particular biological and mechanical features, e.g. osteoinductive, osteoconductive and osteogenic properties similar to native bone, however this terms should be critically appraised or maybe overruled in this context.

2.3.1.1 Osteoinduction

Osteoinduction refers to the recruitment and stimulation of undifferentiated cell types to develop into osteogenic cell lineages[77]. This is a basic biological mechanism that occurs regularly during bone remodelling, fracture healing and bone graft incorporation. Even if pre-existing osteoblasts may help to form new bone, it is getting generally agreed that such pre-existing cells only contribute to a minor portion of the new bone formation after bone graft placement. The instant bony injury induces intense inflammatory response at the fracture site, where inflammatory and other cells release signalling molecules, such as growth factors that attract cell types needed for bone repair. The fate of the recruited stem and other cell types is modulated locally by both soluble biological and insoluble biophysical cues. In summary, the initial part of the healing response includes osteoinduction, a process that starts immediately after the injury and is very active during the first week thereafter, even though the action of the newly recruited pre-osteoblasts is not obvious until several weeks later, in the callus stage[77].

2.3.1.2 Osteoconduction

When bone graft placement is indicated either the size of the bone defect or the insufficient local supply of osteogenic cells compromise the physiologic fracture healing. In such cases, due to the lack of native bone structure the recruited bone forming need a matrix or scaffold to adhere, migrate, proliferate and differentiate. Hence, an osteoconductive bone graft may be defined as a scaffold that permits bone growth on its surface, including down into pores, channels or pipes[77].

2.3.1.3 Osteogenesis

It is often said that the osteogenic property of a bone graft derives from dwelling cells that synthetize bone at the recipient site. This interpretation assumes the pre-existence of dwelling stem cells or other osteogenic cells on the surface of a bone graft that are supposed to contribute to the new bone formation. In contrast, compelling studies support that pre-existing cells cannot survive on a bone graft *in vivo* because of the lack of proper blood supply[78]. There is growing evidence that the major source of bone forming cells in physiological bone remodelling and fracture healing process is delivered by blood vessels to the repair site days or weeks after the bone graft placement. Therefore, the osteogenic property of a bone graft may be interpreted as the consequence of its osteoconductive property and biophysical cues that are mediated by the mechanical features of the graft, such as hardness and topography that support the adherence, proliferation and differentiation of stem cells.

2.3.1.4 Mechanical environment

Mechanical stability is of primary importance to support the vascularisation and angiogenesis during bone regeneration[63]. In many cases, the bone defects are mechanically unstable that requires additional fixation by using metallic devices. The metallic fixation should cause minimal destruction in the local blood supply, supplement and protect the implanted bone graft from undue mechanical load. However, the optimal instrumentation allows small intramedullary movements that do not compromise the formation and integrity of marrow capillary and vessels within the callus. In such a mechanically stable environment fragile blood vessels are able to span distances and form anastomoses, while the MSCs both stabilize the blood vessels and form sheets of osteoblasts that generate osteoid, which becomes calcified into trabecular bone[68]. This newly formed bone is restructured as controlled by its loading dynamics.

2.3.1.5 Complications associated with bone grafts

In clinical settings, there are various situations that may compromise the success of a bone graft. In general, bone graft placement is absolutely contraindicated in bone

metabolic diseases (e.g. osteoporosis), under medication or treatment that suppresses bone metabolism (bisphosphonate, radiation treatment), and septic conditions, like osteomyelitis and other generic infections. Relative contraindications of bone graft placement may defer depending on the aetiology and the anatomical location of the bone defect that need to be taken into consideration on case-by-case basis by the medical team. Adverse events may emerge even if absolute and relative contraindications do not compromise the clinical outcome of the bone graft placement. The most common complications that may be associated with bone graft placement include early graft resorption, nonunion or delayed union of bone fragments, graft fracture, graft extrusion, and infection[79,80].

In surgical reconstruction, bone grafts are often placed along with implants and fixation devices at load-bearing sites. Early graft resorption and graft fracture allow excessive mechanical load on the hardware that may lead to consequential hardware failure (Figure 7). The extrusion of allografts or synthetic bone grafts may also be a major failure mode as it is shown on Figure 8. The early resorption of human and synthetic bone grafts are shown on Figure 9 and Figure 10, respectively.

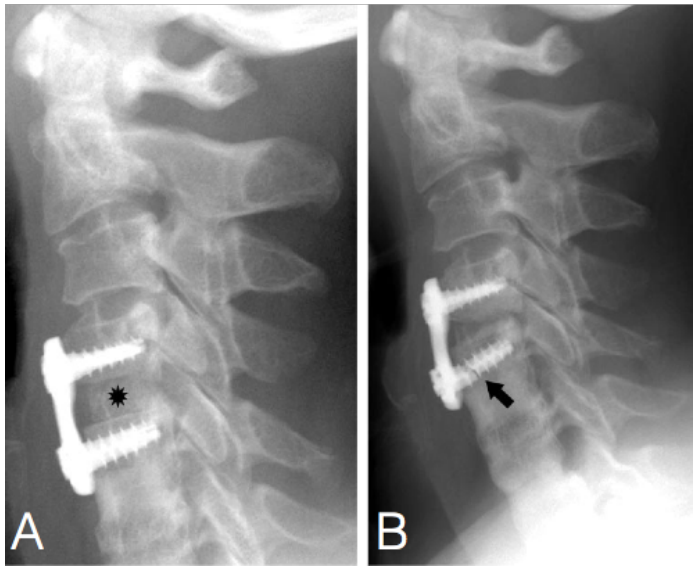

Figure 7. Cancellous bone allograft resorption with hardware loosening and failure in a 46-year old woman. Panel A: Lateral radiograph obtained on postoperative day 1 shows the allograft (∗) as an area of high opacity in the C4-C5 interspace and C4-C5 anterior cervical plate (Atlantis Vision; Medtronic Sofamor Danek, Memphis, Tenn). The graft was coated with injectable bone paste (Osteofil; Regeneration Technologies. Panel B: One-year follow-up radiograph shows focal allograft resorption, hardware loosening, and failure of the inferior screw (arrow). Figure and legend are adaptations from reference 80.

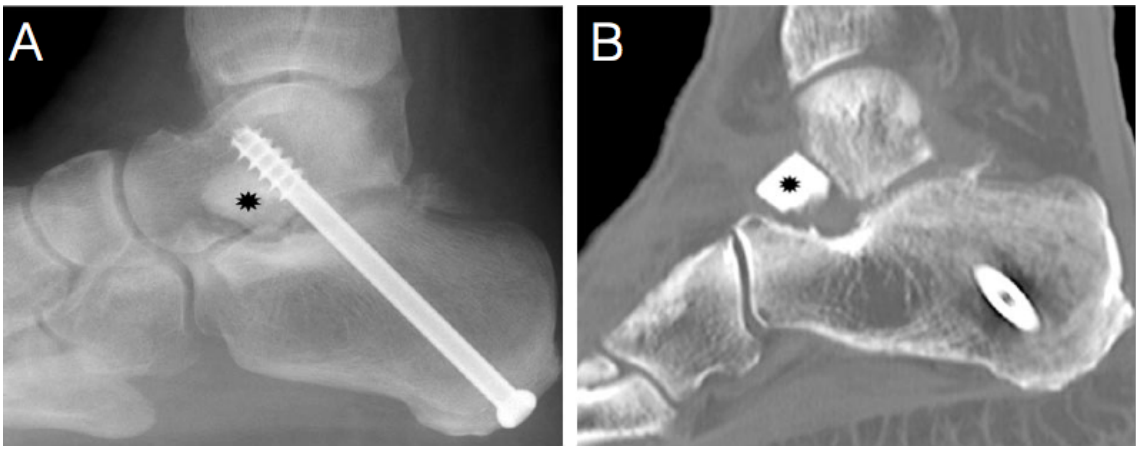

Figure 8. Extrusion of a cortical allograft and failure of fusion in a 61-year-old woman. Panel A: Lateral radiograph of the right foot, obtained 9 months after graft placement, shows the bone graft (∗) and a screw bridging the subtalar joint fragments. Panel B: Sagittal reconstruction CT image, obtained 4 days after 'A', shows extrusion of the bone graft (∗) into the sinus tarsi and persistence of the subtalar joint fracture, with no osseous union. Figure and legend are adaptation from reference 80.

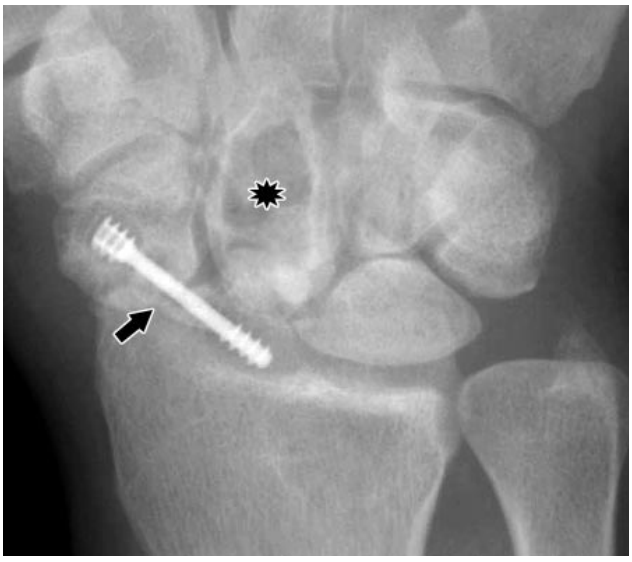

Figure 9. Failure of an autograft in the wrist of a 24 year old man. Anteroposterior radiograph shows a Herbert screw that bridges an old nonunited scaphoid fracture deformity (arrow), accompanied by evidence of scapholunate advanced collapse. The autograft that was initially placed to aid in fracture union has failed and cannot be seen. The proximal pole of the scaphoid is diminutive and not well defined, and there is marked cystic change of the capitate (∗) and distal radius, with ulnar positive variance. Figure and legend are adaptations from reference 80.

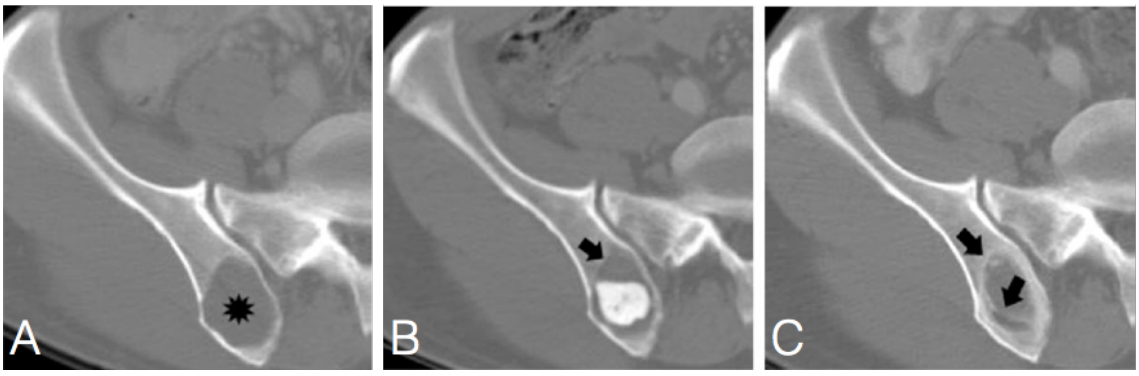

Figure 10. Calcium sulphate ceramic bone graft substitute used for joint repair in a 42-year-old man. Panel A: Preprocedural axial CT image shows a unicameral bone cyst (∗) in the right posterior ilium at the level of the superior sacroiliac joint. Panel B: Axial CT image, obtained 1 month after graft placement, shows partial resorption of the graft material (arrow). Panel C: Axial CT image, obtained 2 years after graft placement, shows complete resorption of the graft material and minimal ingrowth of bone (arrows). Figure and legend are adaptations from reference 80.

2.3.2 Bone graft types

Various classifications of bone grafts exist but none of them is comprehensive enough to outline the whole spectrum of the available products in detail. Perhaps the most plausible categorization is based on the origin of the bone grafts because this approach provides an illustrative description of their clinical performance and limitations.

2.3.2.1 Autogenic bone grafts

The first use of autogenic bone grafts in large volume is dated back to World War II when massive cancellous bone grafts constituted the mainstay of treatment of bone loss in war injuries[81]. Based on the experience of these early procedures the development of grafting techniques has been commenced and volume of treatment methods in the surgeon's armamentarium is still growing[82]. Autogenic bone grafts (autografts) are harvested from a patient who is in need of bone replacement, thus it is more likely to be incorporated than a foreign graft material. Autograft can be harvested from non-essential bones, like iliac crest, fibula, chin, rib, mandible or the skull depending on the type of the surgery and the size of the bone defect. The unique bone healing potential of the autologous bone graft derives from its good osteoconductive,

osteoinductive and osteogenic potential, while the risk of immune reaction or the transmission of diseases is not implicated. The osteogenic property of an autograft is provided by the osteoblasts, osteoclasts, and stem cells residing between the lamellae and trabeculae of the freshly harvested autologous bone grafts. Albeit, the complications associated with the harvest of autologous bone, like chronic pain at the donor site and its limited availability, especially in paediatric and elder patients often impede their use[83]. The incidence of autograft donor site morbidity and associated complications can be as high as 25.3% even exceeding the complication rate of the grafting procedure in the second surgery[84].

2.3.2.2 Allogeneic bone grafts

Allogeneic bone graft is supposed to be a good alternative of autografts because of its human origin[85]. The off-the-shelf availability and the lack of donor site morbidity due to the elimination of need for a second surgery (bone harvesting) are the undisputed advantages of the allograft compared to autograft[86,87]. Allografts[88] may be collected either from patients who are subjected to total joint replacement surgery or from cadavers. The allografts must be processed in a certified bone tissue bank before distribution. Fresh, frozen and freeze-dried allografts are seemed to be the most popular, however there are not exclusively applied protocols for their preparation[87,89]. For the patient safety the allografts should be subjected to disinfection so as to avoid the transfer of contagious agents from the donor to the host[90,91]. Freeze-drying technique allows the preliminary disinfection of allogeneic bone grafts with chemicals, like acids and ethylene-oxide because these agents eliminate from the allograft in the rinsing and freeze-drying process. An unwanted effect of such disinfection is that, the osteogenic cells are killed on the allograft and most of the osteoinductive proteins become denatured, which impairs the biological value of the allograft[91,92]. On the other hand, allografts are often subjected to gamma-sterilization that may detrimentally affect the mechanical property of the bone, while deactivates proteins that are normally found in the bone tissue. Thus, the 3-dimensional (structural) freeze-dried and irradiated allografts can be characterized, in general, by good osteoconductivity but low osteoinductive and osteogenic properties that ultimately result in unreliable

incorporation, which is the greatest disadvantage of bone allografts compared to autografts.

2.3.2.3 Xenogeneic bone grafts

Xenogeneic bone grafts (xenografts) are collected from non-human species, such as bovine bone, porcine bone or coralline grafts[93]. The advantage of xenografts is their easy availability, good osteoconductivity, mechanical property and low cost. In spite of these advantages xenografts are rarely used in clinical practice. The reason of the neglect may be the contradictory results, however favourable data have also been published with these kinds of grafts[94,95]. Perhaps bovine bone has achieved the highest penetration in oral surgery and has become an alternative of autogenic block bone grafts , but scarce validation in orthopaedics[93]. There are continuous arguments over the safety of xenogeneic grafts because the transmission of diseases ("zoonosis") may not be excluded for sure[96].

2.3.2.4 Synthetic bone graft materials

Synthetic bone grafts are supposed to be the alternatives of the human and xenogeneic bone grafts by taking the advantage of their unlimited availability and the complete elimination of the risk associated with the transfer of diseases. Such bone grafts are synthetized in chemical reactions with the goal to create artificial materials that support the replacement of specific types of bone defects. Although, the ultimate goal is to create an artificial bone graft material that possesses autograft-like properties, but in reality the engineering projects are driven by a specific clinical problem, which solution relies on only a few specific features of a synthetic bone graft. From practical point of view, either the mechanical or the biological features of a synthetic bone graft material can be enhanced depending on the type of the bone defect. For instance, at load-bearing sites the mechanical strength is the most important feature of a bone graft, while its biodegradability is a secondary attribute. In contrast, at non-load-bearing sites the biodegradability is the paramount feature of a bone graft, while its poor mechanical resistance, such as brittleness has lower clinical relevance. This kind of appraisal concerning the biological and mechanical properties of a synthetic bone graft is

unavoidable because the currently known bone graft materials have their own specific strengths and weaknesses compared to an autograft, which is still the gold standard to replace a bone defect. Based on their chemical compositions the synthetic bone graft materials can be categorized into three main groups, e.g. ceramics, polymers and composites. Many other subgroups are possible within the main categories of synthetic bone graft materials and further characterized according to their biological, chemical and mechanical properties, however its relevance is questionable. The mechanical and biological properties of a synthetic bone graft strongly depends on multiple factors, such as the raw material, the method and process parameters of manufacturing, the presence of additives and so on and forth. This means that depending on the manufacturing parameters, for instance, a ceramic bone graft material may show remarkably different mechanical properties that will influence its biological behaviour. Therefore, it cannot be concluded that there are well-defined sets of physical properties that are distinctive concerning the biological behaviour of the ceramic, polymer and composite bone graft materials. On the other hand, the composition-based categorization of the synthetic bone graft materials is still predominant in the scientific publications. However, an indication-based categorization system of the synthetic bone graft materials may also be useful in order to support the decision making of the surgeons.

2.3.2.5 Presentations of bone grafts

The availability of bone grafts in various forms may be necessary depending on the characteristic of the bone defect to be replaced. Bohner gave a practical overview about the specific features of the four most common forms of bone grafts, such as granules, blocks, cements and putties (Table 2)[174]. The forms of bone defects are categorized based on their dimensions in Table 2. 'Open' refers to open cancellous bone defects; 'defined shape' refers to osteotomy site; 'closed' refers for cavital defect that is surrounded by bone substance.

Table 2. Specific features of the four most common forms of bone grafts[174]

Presentation of bone graft	Form of bone defect	Mechanical stability	Handling
Granules (0.1 – 5mm in diameter)	Open	Poor	Fair (granule migration during and after surgery)
(Macroporous) block	Open and defined shape	Fair, if there is press-fitting into the defect	Very good (problems might arise to fit the block within the defect)
Injectable paste	Closed	Fair	Fair to good (the paste might set too fast or might be poorly injectable)
Putty	Open or closed	Poor	Very good for pastes that have to be mixed in the operating room to excellent for ready-mixed pastes (the paste might be poorly-injectable)

2.3.3 Growth factors

There are various strategies to enhance the osseointegration of bone grafts. To reach this goal, one possibility is to improve the osteoinductive property of bone grafts by loading them with biologically active molecules. Growth factors are often used for this purpose because they have the capability of directing the fate and action of various cells via cell-surface receptor binding and activation. Growth factors naturally occur within the bone matrix or are expressed during fracture healing to direct the development of structures, vascularization and differentiation of bone cells[97]. There are well-known growth factors that influence bone remodelling, such as transforming growth factor-βs, bone morphogenetic proteins, insulin-like growth factor, platelet-derived growth factor and vascular endothelial growth factor.

2.3.3.1 Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are a subfamily of the transforming growth factors-β superfamily. BMPs are expressed by osteoprogenitor cells, osteoblasts, chondrocytes and platelets in the native bone tissue[98]. BMPs induce a sequential cascade of events that guides to chondrogenesis, osteogenesis, angiogenesis and

controlled synthesis of extracellular matrix (Figure 11)[99]. BMPs exert their effects through binding as dimers to type I and type II serine/threonine kinase receptors[12]. The activity of BMPs is locally regulated by a number of extracellular (noggin, chordin, twisted gastrulation, gremlin, follistatin, etc.) and intracellular (Smad6, Smad7, Smad8b, Smurf1, Smurf2, etc.) antagonists[100,101]. Therefore, the mere presence of BMPs is not guarantee of the efficient bone healing but it is strongly affected by the local presence of various activity regulating inhibitors and stimulators[102,103,104]. Up to now recombinant human BMP-2 has received FDA approval for clinical application under different brand names (InductOs®, InFUSE), whereas BMP-7 only received Humanitarian Device Exemption approval from the FDA[105]. In spite of the potency as treatment option several side effects may be associated with the use of recombinant human bone morphogenetic proteins (rhBMPs) in fracture healing[106]. Ectopic bone formation in fracture treatment and critical soft tissue swelling for cervical spine fusions have been observed in association with the use of BMPs[12,107]. The administration of larger amounts of BMPs in an effort to enhance the efficacy might result in the opposite effect. In contrast, the controlled release of BMP from novel carriers shows better results with lower doses that corresponds more with physiological concentrations[108]. In summary, the dosage and side effect profile of BMPs is little known, which limits their clinical application.

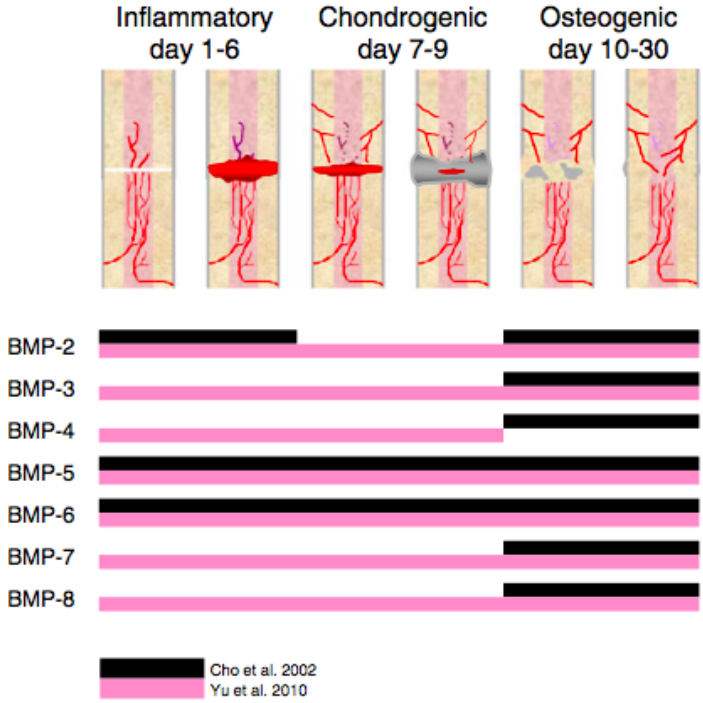

Figure 11. Schematic overview of BMP expression during different stages of fracture healing. The indicated days are dependent on the bone and fracture type. The black lanes indicate the sequence of the expression of various BMPs in the different stages of fracture healing according to Cho *et al*, while the pink lanes indicates the expression sequence according to Yu *et al*. Image and legend were reprinted from reference 12.

2.3.3.2 Vascular endothelial growth factor (VEGF)

VEGF is key regulator in re-establishing blood supply to a site of fracture. In general, VEGF is released in response to hypoxia or tissue damage from both extracellular (ECM-bound VEGF) and intracellular sources, such as endothelial cells, macrophages, fibroblasts, smooth muscle cells, osteoblasts and hypertrophic chondrocytes and many other cell types[62]. The locally and systemically elevated VEGF drives the vasculogenesis and angiogenesis that allow the delivery of pericyte and mesenchymal stem cells to the BRC that are capable of differentiating into additional osteoblasts. Similar to most peptide growth factors, VEGF binds to receptors (VEGFR-1 and 2) on the surface of its target cells[109]. Through VEGFR-2, VEGF induces activation, migration, proliferation, and differentiation of endotheliocytes and their progenitor cells, increasing cell survival, are essential for the formation of capillary-like structures and subsequent remodelling into mature vessels[110]. Primary human osteoblasts also express high levels of VEGFR-1 and signalling through VEGFR-1 on

osteoblasts induces a strong chemotactic response, while indirectly induces proliferation and differentiation of osteoblast precursor cells[111]. This induction is achieved by the secretion of osteoanabolic factors, such as endothelin-I and insulin-like growth factor -I by VEGF stimulated endothelial cells[62].

2.3.3.3 Platelet-derived growth factor (PDGF)

Angiogenesis, osteogenesis and mesengenesis are often studied as separate processes; however, recent findings suggest that PDGF-BB may act as a central connector via interacting signalling pathways[63]. Concerning fracture healing process, when bony injury occurs rapid and active inflammatory response floods the injury zone with blood cells, platelets, monocytes, macrophages and other cells of the inflammatory cascade[112]. The result of this process is that the injury site gets isolated from the rest of the body, becoming avascular to insure that the local injury environment does not propagate to the rest of the body. These segregation processes that occur at the bone break or injury sites eventually result in the repair blastema and outer surrounding reparative callus[63]. Within this isolated environment platelets and macrophages release a lot of regulating molecules, including PDGF that stimulates the secretion of VEGF by pericytes bringing new endothelial cells into the angiogenic injury site[112]. In turn, in mechanically stabile environment, newly formed blood vessels invade the repair tissue and vessel-associated MSCs form sheets of osteoblasts that fabricate bone as oriented by the invading blood vessels (Figure 6)[112]. PDGF has various isoforms (AA, AB, BB, CC, and DD) that bind to two distinct dimerized receptors (PDGFR-α/β) with different affinities. Despite these various isoforms, PDGF-BB is recognized as the 'universal' PDGF because of its ability to bind to all known receptor isotypes and due to its physiological functions[63]. PDGF-BB/PDGFR-β signalling constitutes the principal pathway responsible for pericyte recruitment and attachment to vasculature, their subsequent maturation and detachment, while it also regulates the sequence of osteogenic stimuli[63]. The detached pericytes act as 'free' MSCs at the injury site, however PDGF modulate their responsiveness to BMPs. In a later phase, MSCs can become pericytes again in the site of injury to stabilize newly formed capillaries, which

highlights their multiple role and highly flexible plasticity concerning bone remodelling and fracture healing (Figure 5)[113].

2.3.3.4 Complications associated with growth factors

Myriad of canonical and cross-talking signalling pathways are mediated by growth factors that orchestrate the sequence of cellular events in the repair tissue. These signalling pathways constitute a fine-balanced system where secretion of growth factors might be separated temporally or even spatially in compartments, such as BRC. The local delivery and uncontrolled release of exogenic growth factors might perturb this balanced system of soluble cues. Such an external intervention may lead to fatal consequences, like the development of malignancies, if the growth factor is used in concentrations that exceeds the physiological level[114,115,116]. Therefore, the clinical application of exogenic growth factor to enhance bone regeneration should be performed with extreme caution and long-term follow-ups of those patients should be established who are exposed to such treatments.

2.3.4 Trends in bone graft development

There is increasing need for bone grafts that has inherent capability of supporting new bone formation and the complete remodelling of the grafts. The biological fixation of an implant by virtue of native bone is supposed to ensure the longer survival and prolong the need for the revision of orthopaedic and maxillofacial implants compared to inanimate bone grafts. In order to reach this goal various strategies have been developed to enhance the osteoinductive and osteogenic potential of bone grafts by taking the advantage of the recent achievements of nanotechnology and coating techniques that are exploited by tissue engineers.

2.3.4.1 Nanotechnology

When bone substitutes are placed into the human body, interactions between the surface of the bone substitute and the surrounding bone and soft tissues are critical to MSC differentiation and osseointegration. MSCs appear to be one of the first cell types

involved when a nanophase biomaterial is introduced into the host[117]. Thus, mimicking the nanoscale, three-dimensional extracellular matrix and cell topography may improve the osseointegration by promoting the adhesion, proliferation and differentiation of MSCs[118]. Supplied with state-of-the-art nanofabrication techniques (nanolithography), scientists have become able to produce well-controlled nano-scale topography on 2-dimensional planar substrates to investigate their effect on stem cell fate under standardized experimental conditions. Nanolithography is a branch of methods that are suitable to control the size, shape, spacing and organizational symmetry of nano-scale features on the surface of various planar materials at least in one lateral dimension between 1-100 nm, like nanopits, nanopillars and nanochannels (Figure 12)[119,120,121,122]. For instance, the diameter and spacing of nano-fibres or nano-channels produced by electrospinning can be varied in ranges that approach the dimensions of natural basement-membrane fibre sizes of 5–200 nm and pore sizes of 3–80 nm[123,124,125]. Channels and pillars formed by lithographic techniques can also be varied in ranges that mimic the porosity of natural ECM[126]. Various nano-patterned materials have been shown to enhance osteogenic differentiation of MSCs compared to micro-rough surfaces, including nanophase ceramics, aluminium-oxide, titanium-oxide and titanium alloy, carbon nanotubes and cobalt–chrome alloys[127]. Nanocrystalline hydroxyapatite paste has been used as a filler of bone defects with good clinical outcome[128]. Nano-composite scaffolds that consists of type I collagen and nano-crystalline hydroxyapatite are currently being used in the treatment of osteochondral defects of the knee with encouraging short-term clinical and radiological results[129]. Nanophase delivery systems have recently been studied for the local and precision delivery of drugs and growth factors. For instance, type-2 bone morphogenetic protein loaded nanofibre poly-L-lactic acid enhanced the closure of large calvarial bony defects[130].

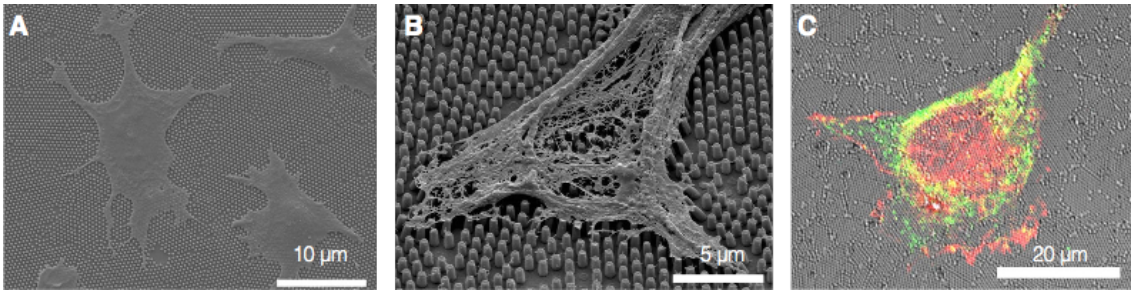

Figure 12. Cultured cells on the 400 nm polymeric nanopillar arrays. Panel A-B show the SEM image of an adhered cell on the array. Panel C shows the combined confocal image of a cell cultured on a 200-nm nanopillar array immunofluorescently stained with tensin (green) and FAK (red) after 24 hours of incubation. Images are adaptations from reference 125.

2.3.4.2 Coatings

The coating of bone grafts with peptides, proteins and other molecules in order to enhance their ability to incorporate into the host tissue is one of the first biomimetic approaches[131,132]. More recently novel coatings address the local and controlled delivery of therapeutic molecules at the fracture site. The advance of polymer and composite techniques may enable bone grafts to carry and sustain the release of molecules in appropriate dosage for days or weeks[133]. For the purpose of delivery of therapeutic molecules synthetic (poly-lactic-acid, poly lactic-*co*-glycolic acid, polycapronolactone, etc.) and natural (chitosan, silk, alginate, etc.) polymers or their composite with inorganic substances can be used either for the fabrication of scaffolds or for the coating of bone grafts[134,135,136]. In general, such composite scaffolds or coatings are utilized to carry either antimicrobial agents to prevent local infections, or growth factors and adjuvants that enhance bone regeneration[137,138]. The latter approach is also known as *in situ* tissue engineering, which utilizes the own regenerating capacity of the body by mobilizing host endogenous stem cells or tissue-specific progenitor cells to the site of injury. This approach relies on the development of target-specific biomaterial scaffolding and coating systems that have the capability of controlling the host microenvironment by mechanical and soluble cues that directs the fate of recruited host cells[139]. The release of the therapeutic molecules can be optimized by various methods, including their encapsulation into nano-, or micro-carriers and by the degradation rate of the polymer bed through its chemical composition[140,141,142,143]. From regulatory point of view, coated bone grafts that are loaded with therapeutic molecules are regarded as

combination products that fall under the scope both of the directives of medical devices and the medicinal products. Growth factors are relatively new as therapeutic molecules, while most of the drugs, such as antimicrobial agents are administered through different routes (oral or parenteral); therefore, the clinical safety and efficacy of such novel combination products are supposed to be proven in controlled, randomized human studies before clinical use.

2.3.4.3 Tissue engineering

The approach of tissue engineering is to generate new, functional tissues with living cells instead of placing non-living scaffolds into bone defects in order to enhance bone regeneration, especially in the case of large bone defects where bone grafting is essential[144]. The objective of bone tissue engineering is to populate three-dimensional scaffolds with progenitor and/or mature cells, such as mesenchymal stem cells and epithelial cells so that enhance bone remodelling[145]. The recently constructed engineered bone tissues are the artworks of multidisciplinary science that utilize the achievements of biology, material sciences, physics and engineering[194]. By the development of nanotechnology and the discovery of biophysical cues the biomimetic approach has spread in the field of tissue engineering[153]. These achievements allowed the developed three-dimensional porous scaffolds to have specific surface topography ranging from the micro-, to nano-size with optimized surface chemistry to improve cell adherence, proliferation and differentiation. The development of additive manufacturing, e.g. 3D printing, stereolithography, fused deposition modelling, and selective laser sintering may also have a significant impact on the engineering of scaffolds that mimic more the mechanical properties of the host bone[146]. Dynamic cell culture instruments have been designed to support the continuous oxygen and nutrient supply of the cells in the 3-dimensional scaffolds. Albeit, in clinical setting, the large artificial bone grafts still have a significant weakness because their blood supply is insufficient due to the lack of indwelling blood vessels. The adequate blood supply is essential to ensure the physiologic metabolism of the dwelling cells otherwise they are not able to contribute to the incorporation of the artificial bone graft. This give rise to

the question of the clinical relevance of bone tissue engineering; or it should be used as an *in vitro* testing method for the evaluation of the novel biomimetic scaffolds.

2.3.4.4 Bioreactors

In vitro bioreactors have been designed to ensure the homogeneous cell distribution on the surface of 3-dimensional biomaterials and their appropriate oxygen and nutrient supply. Under static cell culture condition the insufficient nutrient and oxygen transport, and waste removal may cause decreased proliferation and differentiation and non-uniform cell distribution [147]. Therefore, more complex instruments needed to improve culture media circulation and convective transport of nutrients to the dwelling cells allowing the development of a more uniform tissue. Bioreactors have been designed to: a) allow controlled and fast cell expansion, b) support the efficient exchange of nutrients, oxygen and metabolites in all parts of the scaffold, and c) enhanced cell seeding and provision of physical or biochemical stimuli. The most common bioreactor types are spinner flasks, rotating wall vessels and perfusion systems. Chronologically, spinner flasks and rotating-wall vessels have been the first alternatives to static culture that tried to minimize gradients in nutrient and metabolite concentrations. These types of bioreactors utilize convection to improve the nutrient transport into the porous structure of the scaffold. Perfusion systems constitute a newer generation of bioreactors that are more complex, they can perfuse fluid directly through the pores and interconnecting channels ensuring good mass transport inside the scaffold[147].

2.4 Evaluation of the pre-clinical performance of bone grafts

2.4.1 Biomechanics of bone

Bone is a load-bearing tissue and mechanical forces play key roles in the development and maintenance of its structure. Mechanical forces are converted into mechanical cues that stimulate the expression of osteogenic phenotype by enhancing matrix and mineral deposition, and influence the physiologic organization of the bone structure. Osteocytes are the cells primarily responsible for the transduction of physical

signals into specific biological responses in bone. Concerning anatomical location, osteocytes are encased within fluid filled voids or "lacunae" in the bone matrix and they are interconnected with each other and with osteoblasts at the bone surface via their cell processes that extend through cannaliculi (Figure 13). In this interconnected canalicular network interstitial fluid flows that is driven by pressure difference, which emerges when compressive loading of bone occurs in the course of daily physical activity, like walking or running. The interstitial fluid flow imparts sheer stress on the osteocytes in the range of 1 to 3 Pa and the osteocytes transduce this mechanical force into biological signals via the primary cilium (this process also called mechanotransduction)[148]. The mechanical cues are relayed to effector cells, such as osteoblasts and osteoclasts by different molecular mechanisms. Osteocytes are connected to osteoblasts through gap junctions, while the osteoclast differentiation and activation is regulated through OPG/RANKL ratio. RANKL stimulates osteoclast formation by binding to its receptor on the surface of osteoclast precursors, while OPG, as a decoy receptor for RANKL, inhibits osteoclastogenesis by competitively binding RANKL[148,149].

The compressive load induced interstitial fluid flow in the interconnected canalicular network may originate from the elastic property of the bone tissue. Elasticity is the property of solid materials to return to their original shape and size after external deforming force has been discontinued. For the better understanding, the mechanical property of bone can be characterised by the diagram of compressive test that provides illustrative explanation of the elasticity and strength (Figure 14).

During daily activity the bone is subjected to series of compressive and tensile forces (tensegrity) yielding the periodic elastic strain of the bone. This periodic reversible deformation of bone may be the driving force of the interstitial fluid flow in the canalicular network – as a 'pumping mechanism'. This peculiar biomechanical behaviour of bone may be explained by its composite nature. If we treat bone tissue as a nanometer-scale composite the brittle hydroxyapatite acts as a stiffening phase, while the ductile collagen provides a strong matrix. It should be kept in mind that unlike any engineered composite material bone tissue is not a constant substance but alters its structure in response to physiological loading. Consequently, cortical and cancellous bones have ranges of associated mechanical properties rather than sets of unique values depending on their location, orientation and age[150].

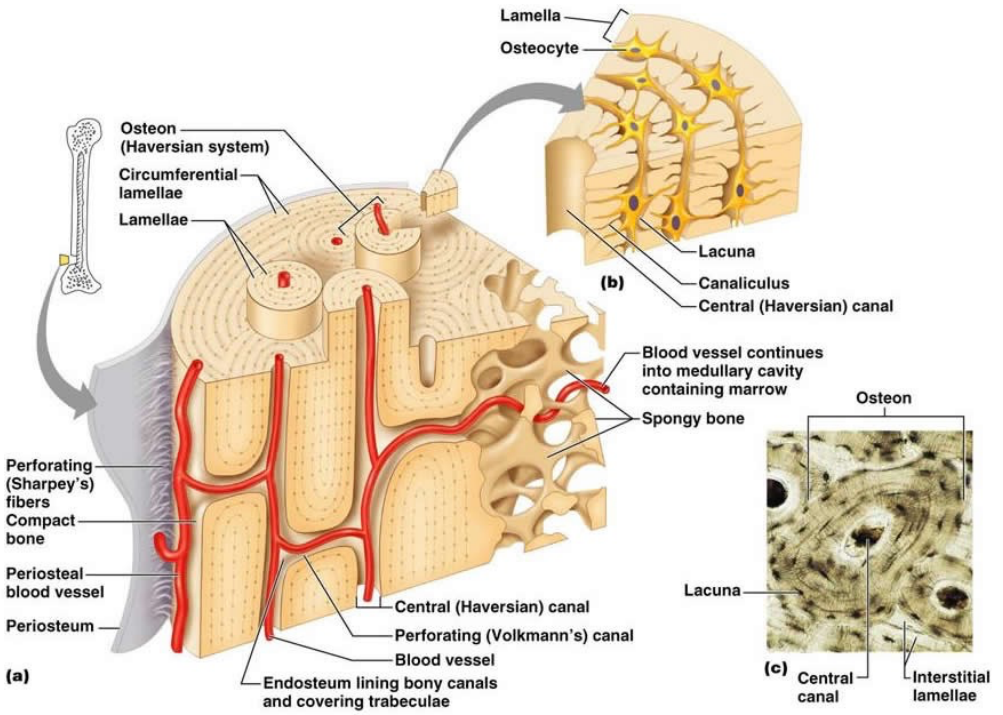

Figure 13. Schematic representation of bone, depicting gross overview, and cellular distribution [151].

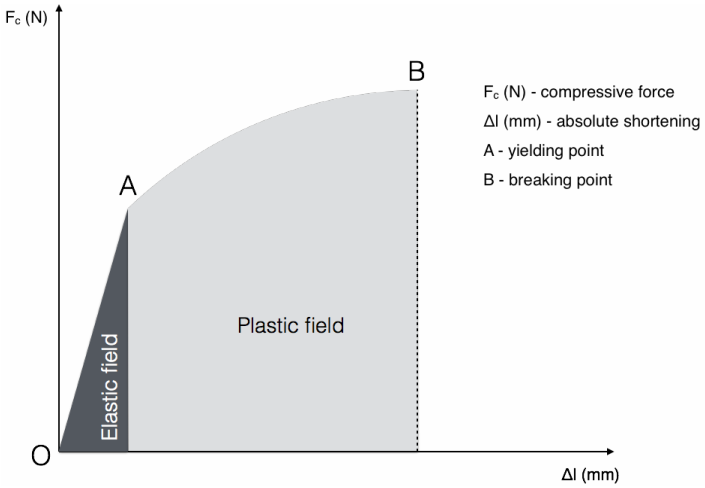

Figure 14. Diagram of compression test for bone. The diagram shows that the increasing compression force shortens the bone up to the point (A), which is called yielding point[152]. Between point (O) and (A) the shortening of the bone is directly proportional to the magnitude of the applied compressive force. Whenever the application of the compressive force stops the bone regains its original size and shape, i.e. the deformation is temporary (reversible). This part of the diagram called bone elasticity. Increasing the compressive force beyond point (A) permanent deformation occurs, which is called plastic deformation of bone. If the compressive force is further increased the bone begins to crack at point (B), which is called breaking point. If the compressive force exceeds the breaking point the bone fractures. The figure is an adaptation from reference 152.

2.4.2 Mechanotransduction

Stem cell niches create specialized *in vivo* microenvironments consisting of soluble and surface-bound signalling factors, cell-cell contacts, stem cell niche support cells, extracellular matrix and local mechanical microenvironment[126]. Recent studies show that beside stem cell niche signals, such as growth factors and cytokines there are coexisting and inherent mechanical and topological cues in the environment of the cells[153]. These insoluble cues that may originate from the fabric of a synthetic bone graft material can influence or even induce, lineage-specific stem cell differentiation by virtue of its stiffness, nanotopography (refers to surfaces and structures with nanoscale topological features), cell adhesiveness, binding affinity, chemical functionality, degradability and/or degradation by-products, in a process known as mechanotransduction (Figure 15)[154,155,156,157]. The transduction of the mechanical cues is performed via canonical and cross-talking signalling pathways that directly or indirectly regulate proliferation and differentiation of cells. So far, the role of Ras/MAPK, Rhoa/ROCK, Wnt/β-catenin, TGF-β signalling pathways and mechanosensitive ion channels have been revealed that influence stem cells fate (Figure 16)[153]. The focal adhesions (FA) mediated activation of Ras/MAPK and PI3K (phosphatidylinositol 3-kinase)/Act stimulate downstream signalling pathways that are important to the self-renewing potential and lineage specification of stem cells[155,158,159]. The RhoA/ROCK pathway is a key molecular regulator of actin cytoskeleton tension and the osteogenic linage commitment of MSCs by up-regulating Runx2 expression[160]. The Wnt/β-catenin signalling pathway have important role in the regulation of stem cell fate[161]. The TGF-β is linked to ECM, its release is activated by mechanical forces; its most remarkable role is to inhibit cell proliferation[162]. Mechanosensitive ion channels can be linked to ECM and/or cytoskeleton, the relative displacement of the channels to ECM or cytoskeleton is responsible for the gating of channels that modulates cytoplasmic calcium concentration and oscillation, which has important role in the differentiation of MSCs[163,153]. Even though there was no cause and effect feedback loop revealed concerning the individual mechanical and chemical cues; however, combining the results of the recent studies, it may be reasonable to speculate that stem cell fate is mediated by a combination of soluble factors and insoluble biophysical signals in the local stem cell microenvironment[153].

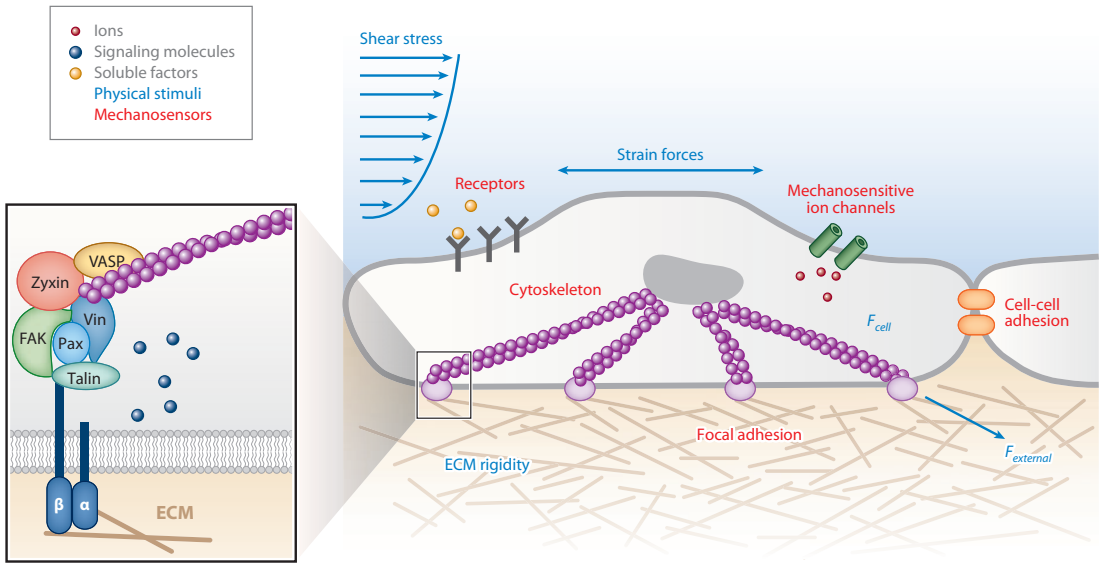

Figure 15. Schematic drawing of insoluble cues in the stem cell niche and the intricate reciprocal molecular interactions between stem cells and their microenvironment to regulate stem cell fate. The extracellular microenvironment of stem cells is a hydrated protein- and proteoglycan-based gel network comprising soluble and physically bound signals as well as signals arising from cell-cell interactions. Biophysical signals in the stem cell niche include matrix rigidity and topography, flow shear stress, strain forces, and other mechanical forces exerted by adjacent support cells (blue text). Stem cells can sense these biophysical stimuli through mechanosensors such as ion channels, focal adhesions, cell surface receptors, actin cytoskeleton, and cell-cell adhesions (red text). A magnified view of the focal adhesion structure is also shown, which includes transmembrane heterodimeric integrin, paxillin (Pax), talin, focal adhesion kinase (FAK), vinculin (Vin), zyxin, and vasodilator-stimulated phosphoprotein (VASP). Figure and legend were reprinted from reference 153.

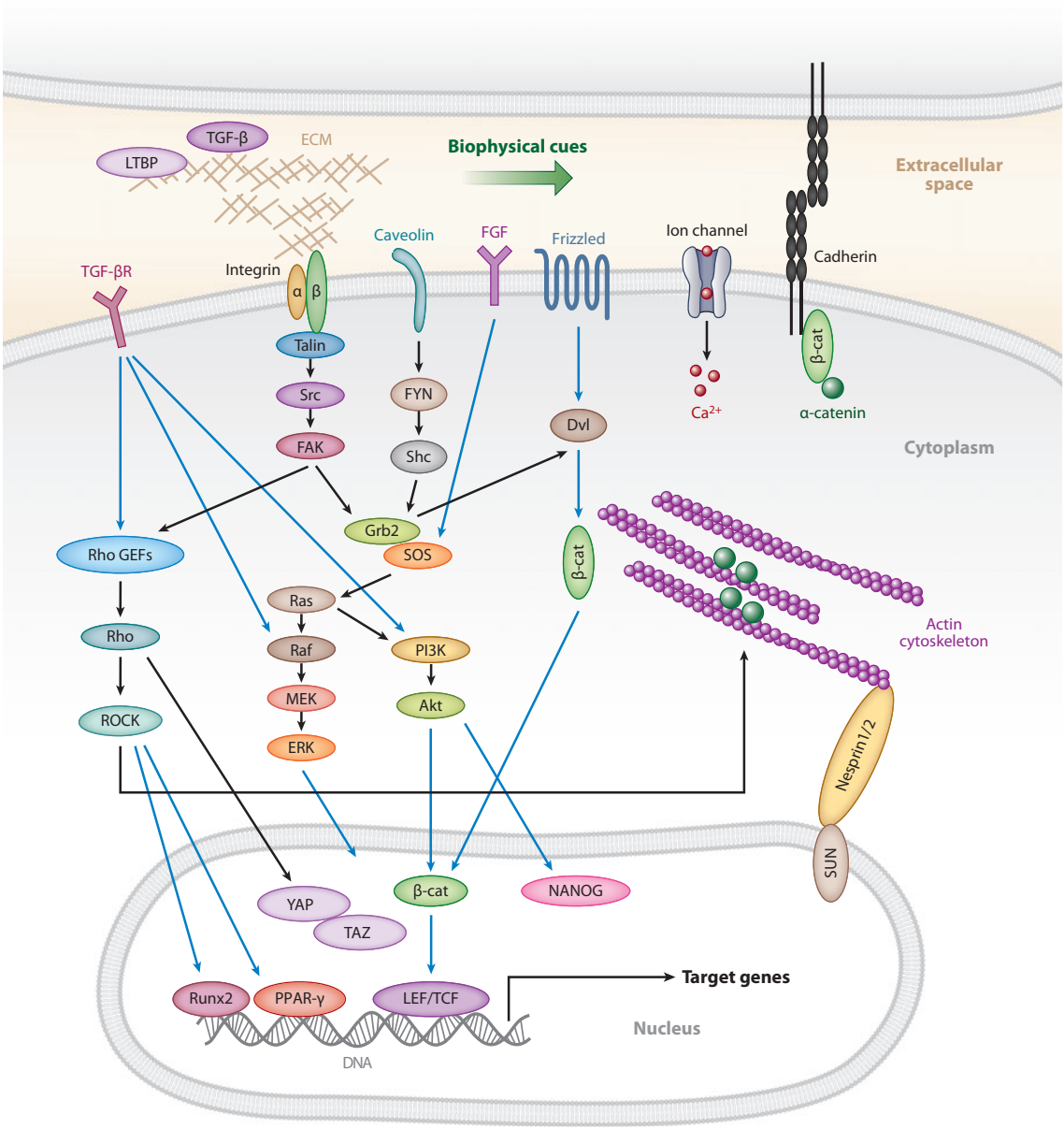

Figure 16. Schematic of signalling cross-talk between the mechanotransductive processes (*black arrows*) and other known soluble factor-mediated signalling pathways regulating the fate decisions of stem cells (*blue arrows*). Abbreviations: TGF-β, transforming growth factor β; LTPB, latent TGF-β-binding protein; TGF-βR, transforming growth factor β receptor; Rho GEFs, Rho guanine nucleotide exchange factors; ROCK, Rho-associated kinase; FAK, focal adhesion kinase; Grb2, growth factor receptor-bound protein 2; SOS, Son of sevenless; PI3K, phosphoinositide 3-kinase; FGF, fibroblast growth factor; Dvl, Dishevelled; β-cat, β-catenin; YAP, Yes-associated protein; TAZ, transcriptional co-activator with PDZ-binding motif; Runx2, Runt-related transcription factor 2; PPAR-γ, peroxisome proliferator-activated receptor γ; LEF, lymphoid enhancer factor; Ca^{2+}, calcium ion; ECM, extracellular matrix; Src, Rous sarcoma oncogene cellular homolog; Shc, SH2-containing collagen-related proteins; FYN, a Src family tyrosine-protein kinase; SUN, Sad1p and UNC-84 homology; MEK, MAPK/Erk kinase. Figure and legend were reprinted from reference 153.

2.4.2.1 Chemical composition

The chemical composition of a synthetic bone graft material determines its mechanical properties that influence the biological behaviour and the clinical applicability eventually. Historically, non-degradable poly(methyl methacrylate)-based (PMMA) cements have been used for bone replacement procedures first[47]. However, there have been complications associated with the use of PMMA-based cements, such as cement leakage and increased risk of adjacent fractures[164,165], so there was increased interest in finding alternative materials. Acrylic (non-degradable) resins have been developed to reduce problems encountered with PMMA-based cements; however, the lack of porosity, stress shielding associated with strong mechanics and the unpredictable biocompatibility limits the widespread clinical application of acrylic polymers[166]. Adjustable porosity, biological properties and biomechanics have become essential requirements so that achieve appropriate mechanical stability and interface between a bone graft and the bone tissue. Calcium phosphate cements (CPCs), which were first presented in the beginning of the 80s[167], have been the real alternatives to PMMA that have had the capability of fulfilling the abovementioned requirements[168,169]. Since the mid of '90s biodegradable polymer composites have been studied for the application of tissue engineering[166]. Typically, a polymer composite consists of a matrix polymer, like polylactide (PLA), polycaprolactone (PCL), polypropylenefumarate (PPF) that is mixed with bioactive compounds and delivered as viscous liquid or paste[170,171,172]. Injectable bone grafts constitute a new generation of polymer composites that have aroused high interest because of their *in situ* curing potential, especially in vertebroplasty[47,173]. The chemical composition primarily affects the rate of (bio)degradability of a synthetic bone graft (it must be noted here that the degradability of a bone graft is also affected by the dimension and interconnectivity of pores[174]. The mechanism of degradation is different depending on the actual chemical composition. For instance, polylactides and polyglycolides are hydrolysed in aqueous media; collagen, fibrin glue and hyaluronan are decomposed enzymatically; beta-tricalcium-phosphate is resorbed by the action of osteoclasts[166,175,176]. The various degradation mechanisms can be exploited to control the rate of remodelling and to utilize bone grafts as delivery systems[177]. Concerning

bone grafts of natural origin, they are composite materials, in which elastic protein filament cages hold inorganic calcium and magnesium during bone formation. Although such bone grafts mimic the host bone best but even their osseointegration and resorption rate is unpredictable.

2.4.2.2 Porosity

The size, shape, interconnectivity and distribution of pores are characteristics that directly affect the biological properties of a three-dimensional bone graft (or scaffold for tissue engineering)[178]. It must be noted that our understanding is still low concerning causality between the pore structure and the biological performance of bone grafts, however the recent findings suggest the inherent significance of this mechanical feature. It has been demonstrated that the pore structure of a three-dimensional structural bone graft influences *i*) the diffusion of oxygen and nutrients; *ii*) cell attachment and migration; and *iii*) mechanical stability[179,180,181]. Currently, the biggest challenge is the induction of angiogenesis in the deeper layers of a three-dimensional structural bone graft that is still an unmet need and the ultimate barrier for the widespread clinical use of artificial bone tissues[182]. The underlying problem is the difficulty to maintain sufficient nutrient and oxygen supply in the newly formed tissue. Recently, it has been realized that interconnected pores enhance the overall flow permeability of a structural bone graft that may allow sufficient nutrient and gas exchange. However, the definition of the optimal pore size and interconnectivity for a bone graft is still a missing chapter in the art. If the pore size is too small it may inhibit the inward migration of cells, while the too large pore size reduce the surface are for cell attachment[183,184]. Interestingly, the shape of the pores may be utilized to regulate the oxygen supply. Ahn *et al*. found that significantly higher oxygen concentration and cell proliferation were associated with diameter gradient (cone-shape) pores than identical diameter pores[185]. On the other hand, when oxygen supply is reduced to a degree the angiogenesis is facilitated via the hypoxia-induced factor-1 pathway[186]. Speculatively, the pore dimensions - as a mechanical parameter - may be utilized to control oxygen supply throughout the pores. Concerning mechanical integrity, the increase in porosity may be associated with increased absorption rate and weaker mechanical resistance[187].

In spite of the advancements in scaffold fabrication technologies it is almost impossible to take into consideration all these factors currently; therefore, an engineered bone graft should resemble natural cancellous bone as much as possible (in the lack of a more appropriate approach).

2.4.2.3 Hardness

Bone grafts that are placed into load-bearing sites are subjected to physiological loading; therefore, it is important to know what the response to dynamic, non-heterogeneous load of a bone graft is before its clinical use[188,]. From practical point of view, a bone graft – either granular or block – *i*) should not crunch under impaction or under dynamic load; and *ii*) should present elastic deformation similar to that of native bone in order to allow the flow of interstitial fluid and blood around and in the graft[189]. In a clinical setting, the low fracture resistance or the proneness for plastic deformation of a bone graft may result in the migration of the prosthesis after operation[190]. Concerning biological aspects, following implantation the bone graft is invaded by MSCs along with many other cell types. The osteogenic lineage specific differentiation of MSCs is supposed to be influenced by two biomechanical cues in conjunction with the elasticity of the bone graft, such as *a*) the hardness of the bone graft, and *b*) fluid-flow induced shear stress. It has been demonstrated that when MSCs are cultured on the surface a substrate with elastic moduli mimicking soft tissues, such as brain and muscle the MSCs showed neuronal and myogenic phenotypes; whereas, MSCs cultured on substrate with elastic modulus mimicking bone tissue responded by adopting phenotypic characteristics of osteogenic lineage (Figure 17) [191,192]. However, the molecular pathway and the nature of mechanical signals that initiate the differentiation of MSCs are still remained undiscovered[193,194]. It has also been demonstrated that MSCs respond to strain-induced fluid shear stress by increasing the expression of the bone markers BMP-2, bone sialoprotein, alkaline phosphatase, calcium deposition and osteopontin[195]. The sensitivity of MSCs to shear stress appeared to be greater after they have a longer attachment time prior to shear stress exposure[196]. This *in vitro* result may support the finding that the temporal regulation of the loading applied to a bone graft may also affect the graft-host integration and impact the remodelling process. Animal

studies show that shielding the bone grafts from loading by internal plate fixation for the first 4 weeks post-implantation (load was transferred to the graft afterward) enhanced the infiltration of neo-vasculature and improved bone volume and integration[197,198]. The plate fixation of the bone graft may allow the inward migration, attachment and proliferation of MSCs that will respond to the mechanical stress leading to enhanced ossification[199].

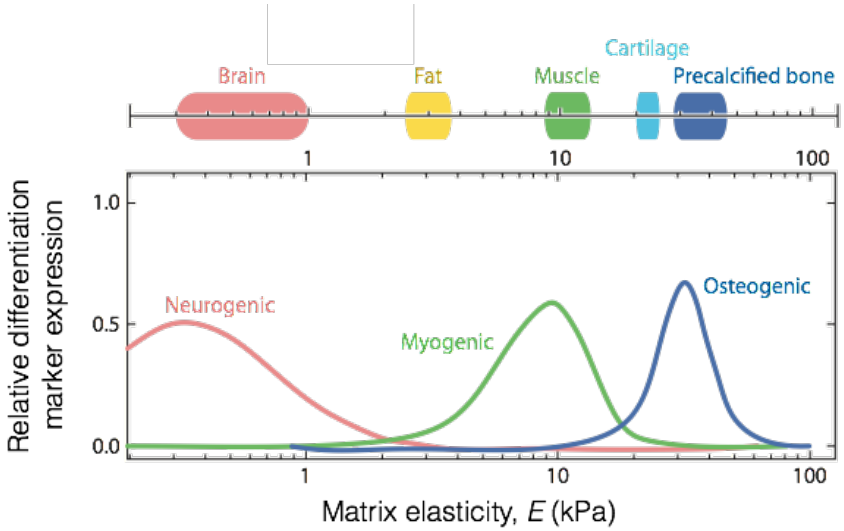

Figure 17. Matrix mechanics directs stem cell fate. Varying matrix elasticity or rigidity can induce multipotent MSCs to differentiate into different tissue cell types corresponding to the tissues' relative mechanical elasticity *in vivo*. Figure and legend were reprinted from reference 153.

2.4.2.4 Topography

Compelling studies support that cell shape is a key regulator of stem cell fate through RhoA-dependent actomyosin contractibility[200]. RhoA is a member of Rho family small GTPases involved in cellular signalling and cytoskeletal organization, and it stimulates cytoskeleton tension through its effector, ROCK, which directly phosphorylates both non-muscle myosin II (NMMII) regulatory myosin light chain (MLC) and MLC phosphatase to synergistically increase MLC phosphorylation and thus myosin II contractility (Figure 16)[201]. Recent studies on nanotopography[202] have suggested that instead of directly affecting cytoskeleton tension, nanotopographical cues appear to elicit their effect on stem cells by directly modulating the molecular arrangement, dynamic organization, and signalling of the cellular adhesion

machinery[153]. *In vivo*, stem cells adhere to nanotopographical ECM is mediated via heterodimeric transmembrane receptors, i.e. α- and β-integrins. Upon binding ECM, integrins can cluster to form dynamic adhesion structures called focal adhesions (FAs) [153]. On the cytoplasmic side of FA the integrins are linked to adaptor proteins, such as talin, vinculin, paxillin, and α-actinin that establish direct linkage to the actin cytoskeleton (Figure 18) [203]. Furthermore, binding to FAs tyrosine kinase and phosphatase signalling pathways may be activated that elicit downstream biochemical signals important for gene expression and stem cell fate. However, FA signalling is essential for many cellular functions but it also autoregulates its turnover and cytoskeletal organization, thereby controlling stem cell responsiveness to nanotopograpy. The responsiveness of various cells was observed for nanotopography, including fibroblasts, osteoblasts, osteoclasts and endotehlial cells and the findings support that nanotopography provides a useful tool to guide the osteogenic differentiation of MSCs [204,205,206,207]. Therefore, it seems reasonable to speculate that MSCs and other cells adhere to the nanoscale topological features of a biomaterial via FAs. In conclusion, it appears that (stem) cells are sensitive and responsive to biophysical cues through a modulated delicate force balance between endogenous cytoskeleton and external mechanical strain transmitted across cell-ECM and/or cell-nanotopography adhesions. The biophysical cues are sensed at the FA sites where integrins provide the mechanical linkage between the ECM and the actin cytoskeleton[153].

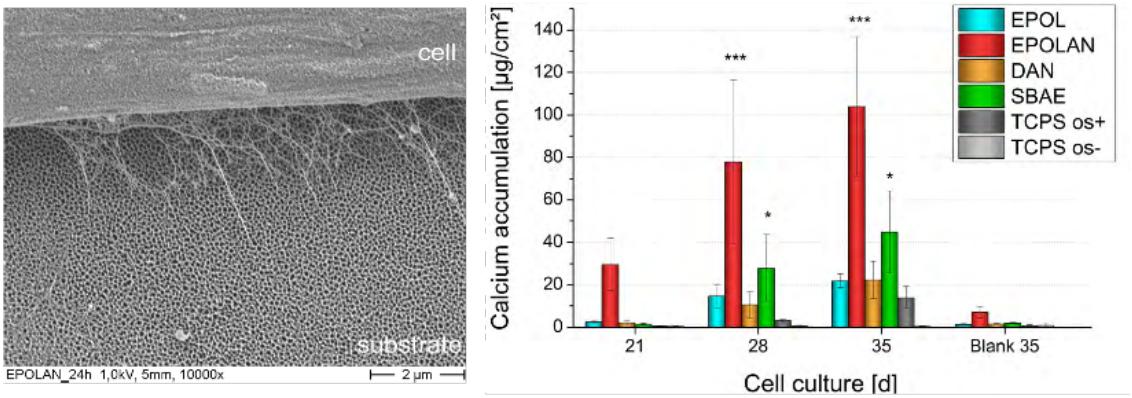

Figure 18. Adherence and osteogenic differentiation of MSC on titanium-oxide nanotubular array. The SEM image shows that MSCs adhere to the nanosurface through nanotubular projections of the ECM. The bar diagram shows singificantly higher mineralization on nanotubular array (induced by soluble cues) than on other nano-, and micro-rough surfaces. EPOL: electromechically polished (featurless) surface; EPOLAN: nanotubular array; DAN: nanopitted surface; SBAE: sand-blasted/acid etched microrough surface; TCPS: tissue culture polystyrene (internal reference)[208].

2.4.3 Biological assessment of bone graft materials

The demonstrated biocompatibility of a bone graft material is an essential requirement before clinical use. A wide-range of *in vitro* and *in vivo* techniques have been developed for the testing of biomaterials, including *in vitro* cell culture and microbiology studies and, if applicable, *in vivo* animal and human clinical studies. These studies are aligned into a hierarchical order starting from *in vitro* cell culture tests to human clinical studies in order to investigate the efficacy and safety of a prospective bone graft material. It must be noted that generic bone graft materials (materials that have already been used in clinical application) may not need such extensive studies before human use, but the demonstration of the pre-clinical biocompatibility according to ISO 10993 or other relevant standards is required by the Competent Authorities. However, the methodology of the biological examinations of original and generic materials is based on the same scientific principles, the applied test methods may vary on a wide-spectrum. In this chapter these scientific principles are discussed through the introduction of some well-established recent methodological approaches.

2.4.3.1 *In vitro* biocompatibility studies

The biocompatibility of a material that is intended for medical use can be investigated comprehensively in *in vitro* experimental settings. In the lack of a widely accepted definition, biocompatibility might be described by the ability of a medical device or a component material that has the capability of integrating into the human body without evoking inflammation or cytotoxic reaction. Cell culture is regarded as a fine-tuned biological 'measuring tool' because in the lack of immune system the cells are very sensitive even to the trace of soluble and insoluble cytotoxic cues that disturb their normal function (shape, proliferation, viability), which is easily detectable.

The survival of osteogenic cells on the surface of a bone graft material is essential to establish evidence base for clinical safety. In contrast to the human body, the isolated cells do not have an immune system, which makes them a sensitive tool to detect toxic cues in *in vitro* experimental settings. The cell shape, adherence and proliferation rate are important diagnostic signs in the judgment of the biocompatibility or the toxicity of a bone graft material. The labelling of the cells with fluorescent dyes allows the quantitative and qualitative analysis of cells by using imaging techniques, like confocal microscopy, fluorescent microscopy and so on and forth. The *in vitro* biocompatibility studies give the most cost-effective way of the quality check or the optimization of a bone graft in the research and development phase. Although, *in vitro* biocompatibility studies may be suitable for the comprehensive characterisation of the cell-biomaterial interaction, however they do not provide enough information to extrapolate the clinical performance of a bone graft.

2.4.3.2 *In vitro* differentiation studies

MSCs are the most often used cells both for the *in vitro* biocompatibility and differentiation studies. The currently applied protocols mostly rely only on soluble factors that initiate the differentiation of MSCs, such as dexamethasone, beta-glycerophosphate and L-ascorbic acid-2-phosphate; however, other factors may also be used, for instance, bone morphogenetic proteins. The expression of various markers may be analysed to follow-up the osteogenic differentiation of MSCs, like alkaline

phosphatase, osteopontin, osteocalcin, collagen type I, bone sialoprotein and bone morphogenetic proteins[209]. According to the current approach the expression of such markers are used as qualitative and quantitative diagnostic signs to evaluate and predict the biological performance of bone graft materials in clinical applications. However, these studies do not take into consideration the effect of insoluble cues that coexists with soluble factors *in vivo*, neither the fact that these cues co-regulate the fate of stem cells. Therefore, those *in vitro* differentiation studies where only soluble differentiation factors are used have serious limitations and they may be insufficient for the evaluation of the biological properties of a bone graft material.

2.4.3.3 *In vivo* osseointegration studies

There is an increasing need for animal models that are suitable for the evaluation of bone grafts in forms that they are used in human clinical applications. This gives rise to questions, including the type and breed of the animal, the shape and size of the bone defect and the fixation technique of the bone graft. Animal studies are supposed to be performed in sequence starting from small animals proceeding towards larger animals. The advantage of small animals is their fast recovery rate and adaptability to various *in vivo* and *ex vivo* diagnostic tools, like micro-CT, nanoSPECT-CT and PET-MRI. Thus, rat and rabbit are often used for the *in vivo* test of bone grafts. In contrast, the advantage of large animals is that they are suitable to test the bone grafts under conditions that are closer to the human clinical setting than small animal models. The location, shape and size of the bone defect profoundly influence the healing and may lead to artefacts. Critical size bone defect (the smallest bone defect that cannot heal spontaneously) models have spread in the last few years the most, however there are arguments over the extensive clinical applicability of these models. These arguments are based upon a clinical situation when the size of the bone defect is not critical but the innate healing potential of the host bone is compromised. The lack of reliable and widely accepted animal models for the *in vivo* evaluation of bone grafts generates a serious gap between the experimental and clinical settings concerning bone replacement. This may be a possible reason of the poor extrapolation between the results of animal studies and clinical outcomes concerning bone grafts.

2.4.4 Preliminary results of our research group

We have developed and standardized a bone defect model, where bone healing was compromised without a critical size gap, and allow the testing of bone graft materials[210]. Critical size model has been extensively used to investigate bone defects where the regenerative process fails to bridge an oversized gap. However, the orthopaedic surgeon commonly faces smaller defects with compromised healing capacity, where the size of the deficiency is usually not challenging, but the time necessary to bony consolidation and to full weight-bearing is of paramount importance. In these situations the critical size model has limited applicability that necessitated the development of a more appropriate model that reflects the relevant clinical needs. In this chapter only the theoretical basis of the experimental model is described in order to support that the applied experimental design and μCT analysis (4.4) are suitable for the intended purpose, that is, the evaluation of the *in vivo* performance of bone grafts. The detailed experimental protocol, including the surgical procedure is detailed later in paragraph 4.4. The experimental model is described below in brief.

Adult male Wistar rats (n=26) of 459 to 692 grams were housed and maintained at 12/12 day/night cycles and were provided with water and lab chow ad libitum. The animals were separated into four experimental groups as it is shown in Table 3, while Figure 19 shows the experimental design.

Table 3. The experimental groups.

Group 1	A 6 mm thick osteoperiosteal defect was created in the femur of 8 rats (classical critical size defect). After metallic plate and screw fixation the osteotomy gap was left empty. The animals were sacrificed after 4 weeks.
Group 2	A 2 mm mid-diaphyseal osteoperiosteal defect was created in the femur of 6 rats in order to follow-up the normal regenerative capacity of the bone. Therefore, after metallic plate and screw fixation the osteotomy gap was left empty. The animals were euthanized after four weeks.
Group 3	A 2 mm mid-diaphyseal osteoperiosteal defect was created in the femur of 6 animals. After plate and screw fixation a 2 mm thick bone cement (PMMA) spacer was interposed into the osteotomy gap in order to block normal bone healing. The animals were sacrificed after four weeks.
Group 4	Six (6) animals were operated as in Group 3, however the interposed cement bone spacer was taken out after 4 weeks and the defect was left empty for additional 4 weeks, when the animals sacrificed after 8 weeks. This experimental group was meant to investigate the self-healing ability of the bone defect after the removal of the spacer.

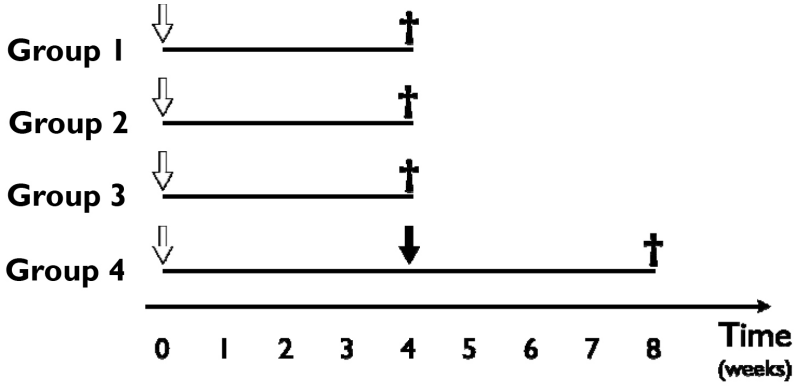

Figure 19. Experimental design. White arrow (⇩) indicates the time of the first procedure in each group. Black cross (†) marks the time when the animals were sacrificed. Black arrow (🡻) shows the time of the removal of the spacer in the Group 4.

For μCT analysis, a cylindrical region of interest (ROI) was placed in the mid-diaphyseal region of the femur as it is shown on Figure 20. Before μCT image acquisition, metallic plates and screws were removed leaving screw holes in the femur. The screw holes were used as representative landmarks allowing the reproducible set of ROI between the screw holes neighbouring the osteotomy site. The ROI was positioned in such a way that the base of the 'cylinder' started at the distal end of the proximal screw hole and ended at the proximal end of the distal screw hole on the other side of the osteotomy gap. Within the ROI three sub-regions (VOI) were determined in order to be able to investigate new bone formation in the osteotomy gap. After visualizing the margins of the bone defect, the VOI 1 was set from the proximal screw hole to the osteotomy site, the VOI 2 covered the osteotomy gap, while VOI 3 was set from the osteotomy site to the distal screw hole. Thus, VOI 1 and VOI 3 represented the original bone substance of the femur that was used as internal references. New bone formation and union/nonunion was assessed using 3D reconstruction according to Schmidhammer[211]. It has been proved by Schmidhammer and his colleagues that there is 100% correlation between μCT measurement and biomechanical testing, – which is the most accurate reference method – concerning bone healing assessment, if the abovementioned approach of μCT analysis is applied.

Multimodal imaging was used to confirm the results of μCT analysis, which is briefly described here on Figure 21. Radiophosphonate imaging using ^{99m}Tc-methylydene diphosphonate (^{99m}Tc-MDP, Skeleton®, Medi-Radiopharma Ltd, Hungary)

and SPECT/CT imaging with a NanoSPECT/CT imaging system (NanoSPECT/CT®, Mediso Ltd-Bioscan Inc, Hungary-US) was performed weekly until the 4th week. One of the most widely used radiopharmaceuticals, ^{99m}Tc-MDP is considered to accumulate in sites of elevated osteoblast activity, and thus can be used both in clinical settings and in experimental animals to evaluate and quantitatively characterize osseous regenerative processes[212].

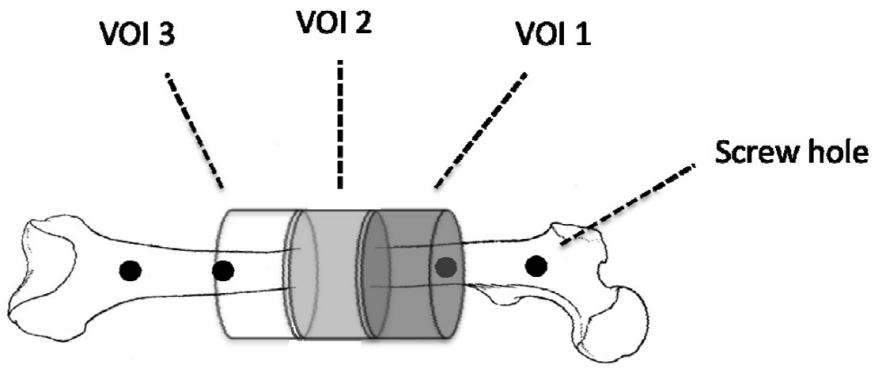

Figure 20. **Micro-CT analysis of rat femur**. The black circles represent screw holes in the bone after the removal of the plate and screws. A cylindrical volume of interest (VOI) is placed between the screw holes neighbouring the osteotomy site. The VOI starts at the distal end of the proximal screw hole and ends at the proximal end of the distal screw hole on the other side of the defect. Within this total VOI region three sub-VOI regions are determined: VOI 1 is set form the proximal screw hole to the osteotomy site; VOI 2 covers osteotomy gap; and VOI 3 is set from the osteotomy site to the distal screw hole.

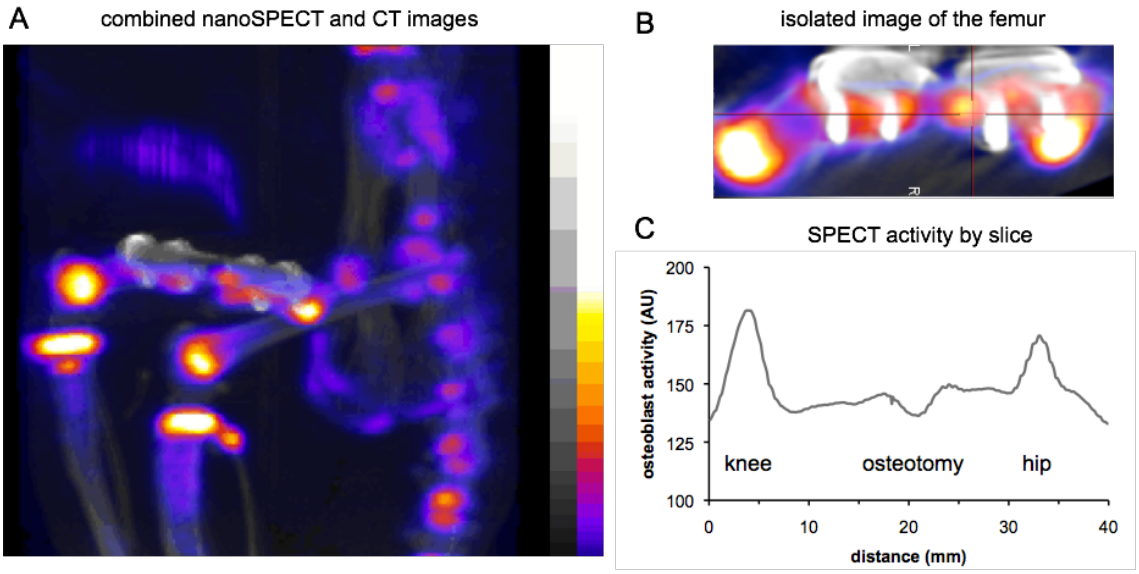

Figure 21. Osteoblast activity of an osteotomized femur. The ^{99m}Tc-MDP isotope specifically labels active osteoblasts. In Panel A the combined μCT and nanoSPECT images are seen of a rat 1 week after osteotomy. The epiphyseal parts of the knee joints at both the operated and intact sides have similar osteoblast activity. Panel B shows the isolated image of the plated femur, while Panel C represents the calculated osteoblast activity at consecutive slices of the image. The knee and hip joints are easily distinguishable as well as the osteotomy in the mid-diaphysis. The bone ends at the osteotomy site show a slightly increased osteoblast activity compared to the intact parts of the diaphysis, which suggests that new bone formation is depressed in the osteotomy gap.

Our results showed that the classical critical size model (group I) had a relative bone volume of -7.85±1.47 % with a 12.5 % union rate (1 out of 8). Group II, which served as control osteotomy group, we measured -2.47±0.88 % relative bone volume in the defect site with 83.33 % union rate (5 out of 6). Group III, where a spacer was interposed into the defect non-union developed in all cases, and callous formation did not stabilize the spacer in place. The relative bone volume was -7.9±1.06 % in the defect site. However, Group IV, where the spacer was removed and the bone was left to heal for additional 4 weeks a bone defect occurred in 5 out of 6 cases (83.33 %) and the relative bone volume remained at a low level (-4.73±1.36 %. Histological analysis confirmed bony consolidation of the defect in cases of a union, while an essentially bone-free zone was microscopically seen at the defect site in cases of non-union determined by μCT (Figure 22; Figure 23). The relative bone volume was significantly ($p<0.05$) lower in Groups I and III compared to that of group II. Based on the results it has been concluded that the compromised bone defect model was suitable for the testing

of the *in vivo* performance of bone graft materials and it provided a clinically relevant experimental setting.

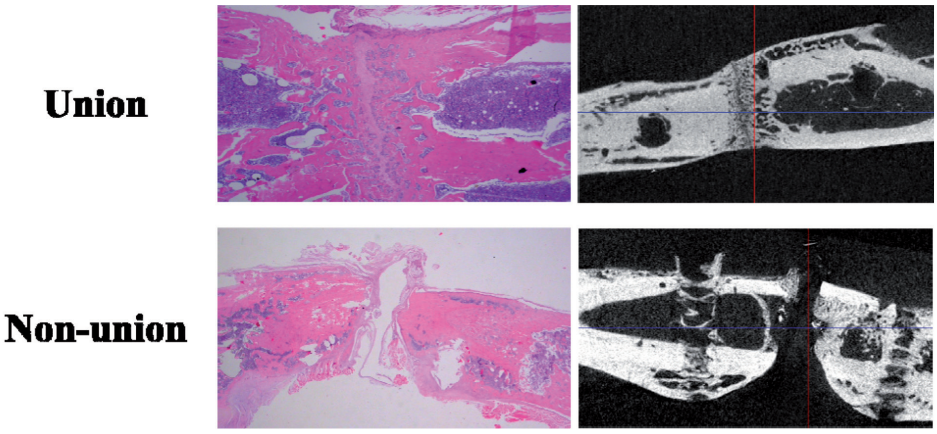

Figure 22. Histology and µCT image of bony consolidation. In Group 2 bony healing occurred in the 83.33 % of the cases. Complete bone regeneration is demonstrated by 2D µCT and by histology on the upper row (union). On the other hand, PMMA spacer interposition resulted in non-union in all of the cases concerning experimental Group 3 and 4, as it is presented in the lower row (non-union).

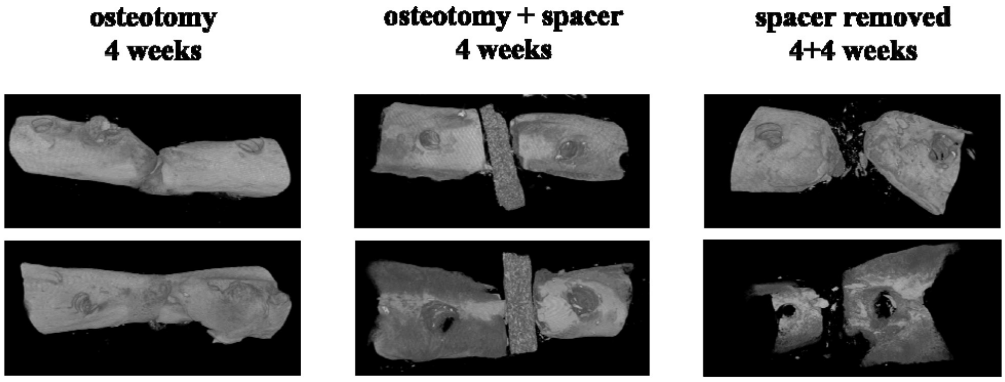

Figure 23. 3D reconstruction of the excited femur and the spacer that is inserted into the osteotomy gap. It is shown that the spacer impeded bony consolidation even 4 weeks after its removal. Representative images show that a 2 mm osteotomy is completely healed after 4 weeks in Group 2, however healing is compromised when there is a PMMA spacer interposition in Group 3 and 4. After the removal of the spacer the osteotomy is not consolidated yet another 4 weeks later, resulting in a permanent bone defect.

3 OBJECTIVES

The main objective of this thesis was to develop a coating technology for the enhancement of allogeneic bone grafts and investigate their *in vitro* and *in vivo* biocompatibility.

3.1 Investigation of the *in vivo* biocompatibility of chemically sterilized, antigen-extracted freeze-dried human bone grafts

The vigorous chemical treatment of allografts may eliminate not only the pathogenic microorganisms and reduce the quantity of antigens but it also could destroy the osteoinductive and osteogenic molecules turning bone grafts into mineralized scaffolds with reduced biological value.

The first objective of the present doctoral work was to investigate the *in vivo* biocompatibility of chemically sterilized, antigen-extracted freeze-dried human cancellous bone allografts in a compromised bone healing model in comparison to novel injectable synthetic bone fillers.

3.2 Identification of a coating substance to improve the biocompatibility of the chemically sterilized, antigen-extracted freeze-dried cancellous allogeneic bone grafts

Various strategies and technological approaches have been applied in order to improve the adherence and viability of osteogenic cells on the surface of 3-dimensional structural bone grafts. Polymers and ceramics of natural and synthetic origin have been used to engineer or coat scaffolds to make them potent alternatives of autogenic bone grafts. According to our current understanding, synthetic or xenogeneic materials are not comparable to materials of human origin as bone substitutes or coating materials in clinical applications. Thus, allogeneic bone may provide an unlimited source of grafts with good mechanical properties. A wide range of coating proteins have been proposed to improve the biocompatibility of 3-dimensional structural bone grafts, such as cancellous human allografts, including bone structure proteins, like fibronectin, collagen, and serum derived proteins, like platelet-rich plasma.

The second objective of the present doctoral work was to investigate the homogeneity, durability and reproducibility of aqueous and freeze-dried fibronectin, collagen and human serum albumin coatings on freeze-dried cancellous bone allografts.

3.3 Investigation of the *in vitro* and *in vivo* biocompatibility coated freeze-dried cancellous allogeneic bone grafts

In order to improve the biocompatibility of allogeneic cancellous bone grafts the coating should allow the fast adherence and proliferation of osteogenic cells. The cell adherence strongly depends on the temporal and spatial cell supply, while the proliferation of cells is a consequence of the good biocompatibility of the surface of the bone graft. Culture conditions also may influence the survival of cells that are dwelling the deeper layers of the bone graft where the oxygen and nutrient supply is limited.

More credible data can be obtained on the biological performance of a bone graft an in *in vivo* experimental setting that mimic real clinical conditions than *in vitro* studies. In clinical setting, the replacement of 3-dimensional segmental bone defects is a challenge, especially when the time necessary for bony consolidation and to full load bearing is delayed or compromised.

The third objective of the present doctoral work was to investigate the *in vitro* and *in vivo* biocompatibility coated freeze-dried cancellous allogeneic bone grafts.

4 MATERIALS AND METHODS

4.1 In vivo investigation of the biocompatibility of chemically sterilized, antigen extracted freeze-dried human bone graft

4.1.1 Surgical procedure

Male Wistar rats (Toxi-Coop, Hungary) weighing 500–600 g were anesthetized with 1.5 L/min oxygen, 200 cm^3/min halothane (Sigma Aldrich, St Louis, MO). The tail was washed three times with braunol (Braun Medical, Bethlehem PA) and ligatured at the tail root for the prevention of bleeding. The tip of the tail was surgically removed after which a standardized defect was created by drilling through the distal side of the caudal vertebrae (C4-C5) by using a custom made drill with 2 mm diameter, and with a shoulder at 3.5 mm to ensure a standardized depth. To prevent the self-regeneration of the vertebra a stainless steel spacer was implanted into the drill hole. The wound was sutured and the animals were returned to their cages. After 12 weeks, the animals were anesthetized and the spacer was replaced. The hole was filled either with PMMA (Heraeus Palacos R) (n=5), premixed calcium phosphate cement (pCPC; n=5), Sr-doped calcium phosphate composite spheres (SrCPS; n=5), with impacted chemically sterilized, antigen extracted human lyophilized bone chips (n=5) or left empty (n=7), and the wound was closed. At this time point a third group (n=5) was added to serve as a positive control. Within this group, a defect was created as previously described, but the defect was left to heal normally without any spacer (Figure 24). The wound was closed by the same procedure as mentioned earlier. Twelve weeks later, all animals were over-anesthetized and euthanized by exsanguination. The last two vertebrae (last operated vertebra plus one healthy vertebra) were fixed in 4% formaldehyde and analyzed with micro-CT and histology. One rat from the group with no spacer in the defect, that is a ‘‘positive control,’’ died before the endpoint and was excluded from the study. The animal experiment had been approved by the Local Committee of Animal Research Ethics according to the Federation of European Laboratory Animal Science Associations guidelines[47,173].

4.1.2 *In vivo* multimodal imaging

Single isotope nano- SPECT/CT (Bioscan) imaging acquisitions were performed on 2-3 rats from each group in order to follow the integration of the bone substitutes and bone regeneration. Nano-SPECT/CT was carried out weekly for 6 weeks and once again on the 12th week. The rats were inoculated with 0.5 mL, 150 MBq of 99m Tc-methyl diphosphonate (99mTc-MDP) through the tail vein under halothane anesthesia. Metastable technetium (99mTc) is tagged onto a phosphonate compound such as MDP to generate 99mTc- MDP, which selectively concentrates in osteoblasts. The selective accumulation of 99mTc-MDP is ensured by both chemical adsorption onto the surface of the hydroxyapatite in bone and incorporation into the crystalline structure of hydroxyapatite[213]. Two hours later the animals were anesthetized with euthasol intraperitoneally, and 30-minute image acquisitions were performed. Regeneration activity was expressed as a percentage compared to the proximal healthy vertebra depending on isotope density[47,173].

4.1.3 Ex-vivo μCT analysis

The operated vertebrae were examined with microtomography (Skyscan 1172 X-ray micro- tomography Skyscan, Kontich, Belgium). Scans were carried out applying a 60 kV voltage and an Al-filter. Reconstruction was done with a modified Feldkamp algorithm using Skyscan Nrecon software. Microtomographical reconstruction was obtained by rotating the view through 180 degrees (rotation step 0.5 degrees). SkyScan CTvox (Kontich, Belgium) was used for the 3D visualization[47,173].

4.1.4 Histology

The formaldehyde-fixed 4th and 5th vertebrae were decalcified by immersing the samples in Biodec-R solution for 1 week. Five micron longitudinal sections were cut from the paraffin blocks and mounted on glass slides. Conventional hematoxylin-eosin (Merck & Co) staining was used to confirm the results of the micro-CT measurements[47,173].

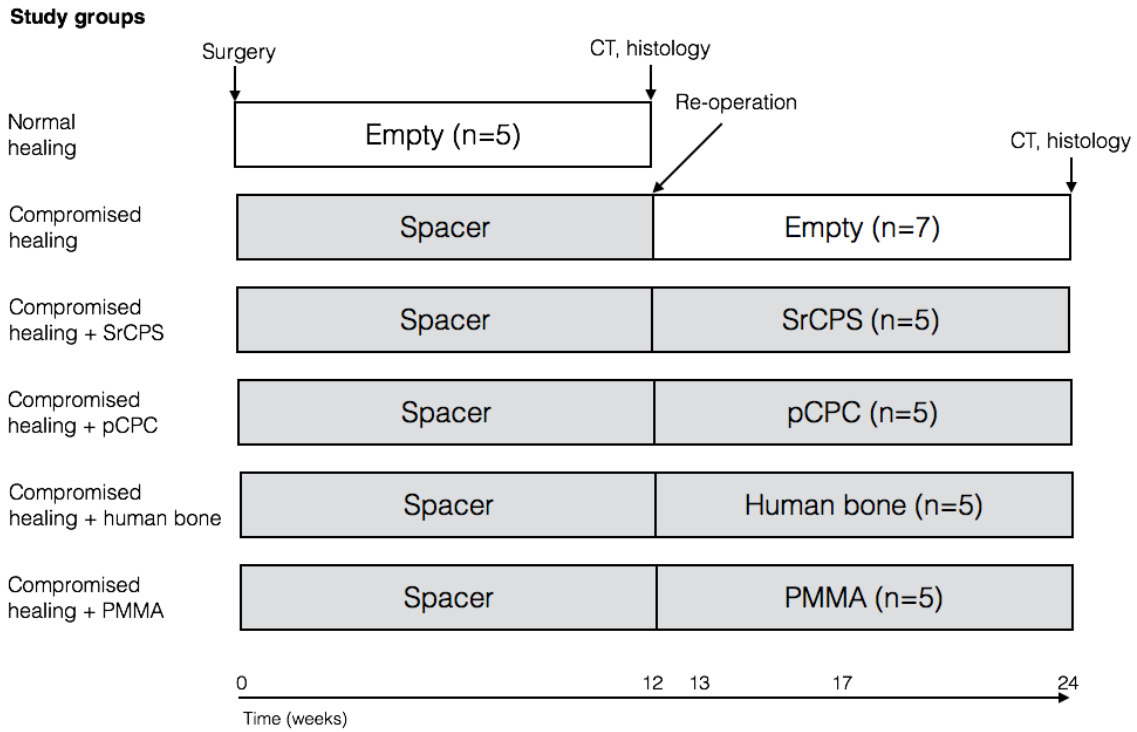

Figure 24. Schematic representation of the *in vivo* study design and protocol. Cavital bone defects were created in the vertebrae of the animals and a stainless steel spacer was implanted into the drill hole in order to reduce the self-healing potential of the bone even after the removal of the spacer (compromised healing). After 12 weeks the animals were re-operated and the spacers were either removed, or replaced with SrCPS, pCPC, human bone chips or PMMA and the they were followed-up by nanoSPECT-CT for additional 12 weeks. After 12+12 weeks the animals were sacrificed, the operated and the adjacent vertebrae were removed and subjected to *ex-vivo* μCT analysis and histology. In a group of animals empty cavital bone defects were created in order to follow-up normal bone healing and use this group as positive control.

4.2 *In vitro* experimental design

Test A and Test B subgroups (Table 4) were investigated in an observational study with longitudinal design where the mutual exposure was cell seeding onto the surface of bone grafts and the survival (effect of the exposure) of the seeded cells was measured at two time points during the experiments. The possible outcomes of the exposure were classified according to pre-set verification criteria that also constituted the basis of the evaluation of the performance of the coated bone grafts in the course of the *in vitro* study (Table 4). Those bone grafts were progressively excluded from further investigations that did not facilitate the adherence or proliferation of seeded cells under any culture conditions (Figure 35).

Table 4. Verification criteria to evaluate the *in vitro* performance of coated bone grafts. Those bone grafts were progressively dropped out from further investigations that did not facilitate the adherence or proliferation compared to their uncoated counterparts. Those bone grafts were excluded from further experiments that felt into at least one exclusion category.

	Baseline	**Decrease**	**Stagnate**	**Increase**
Adherence	Quantity of attached cells on uncoated bone grafts	Decrease in the quantity of adhered cells compared to baseline **Exclude**	Does not affect the quantity of adhered cells compared to baseline **Exclude**	Increase in the quantity of adhered cells compared to baseline **Include**
Proliferation	Proliferation rate of cells on uncoated bone grafts	Decrease in the proliferation rate compared to baseline **Exclude**	Does not affect the proliferation rate compared to baseline **Exclude**	Increase in the proliferation rate compared to baseline **Include**

4.2.1 Bone graft types

The following bone graft types were used as scaffolds in the experiments:

- Chemically sterilized, antigen-extracted freeze-dried human bone graft (West Hungarian Regional Tissue Bank),
- Freeze-dried bovine cancellous bone graft (Bio-Oss, Geistlich Pharma AG),
- Porous hydroxyapatite (META BIOMED).

Before further processing, the bone grafts were cut into 0.3-0.5 cm^3 cubes with an orthopaedic saw under aseptic conditions.

Production of chemically sterilized, antigen-extracted freeze-dried human bone graft: cadaveric bones were washed in methanol for 4 hours, then they were digested in a solution of 0.1 M phosphate buffer saline, 10mM sodium-azide and 10mM monoiodineacetic acid for 24 hours. Next, the bones were subjected to partial decalcification using 0.6 M HCl at room temperature for 4 to 6 hours. The as-produced mineralized bone grafts were sterilized in ethylene-dioxide at 27°C, then they were freeze-dried aseptically (primer drying: 32°C, 2Pa, 12h; second drying: 32°C, 0Pa, 12h).

4.2.2 *In vitro* experimental groups

The bone grafts were divided into three main experimental groups, i.e. Control, Test A and Test B groups as it is shown in the Table 5 below. Test A and Test B groups were further divided into subgroups according to the method of coating, e.g. aqueous coating and freeze-dried coating. As control, uncoated bone grafts were used in the experiments. In each experimental subgroup 12 or more samples were investigated (N≥12).

Table 5. *In vitro* experimental groups. In Test group A, the bone grafts were soaked into the aqueous solution of either fibronectin or human serum derived albumin or collagen. In Test group B, the bone grafts were incubated in the aqueous protein solution overnight and then freeze-dried onto the surface of the grafts.

	Allograft	**BioOss**	**Hydroxyapatite**
Control	Uncoated	Uncoated	Uncoated
Test A	Aqueous albumin	Aqueous albumin	Aqueous albumin
	Aqueous fibronectin	Aqueous fibronectin	Aqueous fibronectin
	Aqueous collagen	Aqueous collagen	Aqueous collagen
Test B	Freeze-dried albumin	Freeze-dried albumin	Freeze-dried albumin
	Freeze-dried fibronectin	Freeze-dried fibronectin	Freeze-dried fibronectin
	Freeze-dried collagen	Freeze-dried collagen	Freeze-dried collagen

4.2.3 Coating substances

As coating substances the following proteins were used in the experiments:

i) albumin of human serum origin (200g/1000ml, BIOTEST), and

ii) fibronectin of human serum origin (20µg/ml, Sigma Aldrich),

iii) 1,5% porcine type I collagen (Biom' up).

The albumin was used in 50% dilution for the coating of bone grafts. The dilution was made in our laboratory using phosphate buffered saline.

4.2.4 Coating methods

In the following the coating methods will be detailed that constitute the basis of the categorization of the samples into Test group A and Test group B.

4.2.4.1 Aqueous coating

In Test group A, the bone grafts were soaked into the aqueous solution of either fibronectin or human serum derived albumin or collagen and incubated at + 4°C overnight. Upon the elapse of the incubation period the bone grafts were removed from the protein solutions and placed into cell culture dishes where MSCs were seeded onto their surfaces instantly[214].

4.2.4.2 Freeze-dried coating

In Test group B, bone grafts were incubated overnight in aqueous protein solutions, as it was detailed in the case of Test group A. However, after overnight incubation the bone grafts were removed from the aqueous protein solution and they were freeze-dried at 32 °C, at 1 Pa for 24 hours. After freeze-drying the bone grafts were placed into cell culture dishes where cells were seeded onto their surfaces immediately[214].

4.2.5 Physical characterization of the bone grafts

Samples before cell seeding were taken from each batch of Test A and Test B groups for mechanical and optical characterization. For mechanical characterization the Vickers hardness test method was applied, while scanning electron microscopic images were acquired for structural characterization.

4.2.5.1 Micro-hardness measurement

The micro-hardness of synthetic hydroxyapatite (HAP), lyophilized bovine bone (Bio-Oss), and the cortical phase of freeze-dried albumin coated and uncoated mineralized allografts was measured[215]. The HV Vickers – hardness measurement is performed with a 136^{o} angle of the vertex and square based diamond-pyramid. Flat surface areas were selected for micro-hardness measurement where the diamond-pyramid was pressed into with 50 g load weight for a period of 5 seconds. On each sample at least five measurements were carried out, while the two diagonals of the impression and the micro-hardness values were measured and averaged. The numeric

value (HV) of the Vickers – hardness was determined based on the following formula: the load force (F) explicit in Newton (N) was divided by the surface area (A) of the impression in mm^2, and then the result was multiplied with a constant (C = 0,102) [214].

4.2.5.2 Scanning Electron Microscopy

The surface characteristic of the bone grafts was investigated by scanning electron microscope (SEM) (Philips XL 30). An argentiferous adhesive was applied on the bottom of the samples that were coated with an electrically conductive gold layer using a vacuum-pulverisation method. The procedure was then performed in vacuum. The photographs were taken in the secunder electron (SE) mode with 15kV accelerating voltage. The secunder electrons are able to emerge from the uppermost layers, having a thickness of 5-50 nm, meaning that they are extremely sensitive to the disproportionate surface. The full surface area of the samples was investigated and representative microscopic images were acquired at 50x, 200x and 1000x magnifications[214].

4.3 *In vitro* biocompatibility study of coated human bone grafts

In the following the *in vitro* experimental methods are detailed.

4.3.1 Isolation and cultivation of bone marrow derived MSCs

Human bone marrow samples were obtained from young patients (aged 2-20) during standard orthopaedic surgical procedures, with the informed consent of the patients or their parents under approved ethical guidelines set by the Ethical Committee of the Hungarian Medical Research Council. Only such tissues were used that otherwise would have been discarded. The bone marrow was taken into T75 flasks and diluted with Dulbecco's Modified Eagle's Medium (DMEM) culture medium containing 10% foetal calf serum (FCS), 100 U/ml penicillin and 10 µg/ml streptomycin, 2mM L-glutamine and 1g/l glucose. The flasks were incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 for 3 days. After the incubation period the BMSCs adhered to the surface of the flasks and the remaining components of bone marrow were eliminated

by washing with PBS. The used BMSCs were between passages 1 and 5 in the experiments[214].

4.3.2 Isolation and cultivation of dental pulp derived MSCs

The protocol that we followed for the isolation and culturing of dental pulp derived mesenchymal stem cells (DPSCs) was based on the procedure of Gronthos *et al*, however it was implemented with minor modifications in our experiment[66]. Human impacted third molars were collected from adults (18–26 years of age). The tooth was cut around the cemento-enamel junction by sterile dental fissure burs to expose the pulp chamber. The pulp tissue had been removed from the crown and the roots then digested in a solution of collagenase type I (3 mg/ml) and dispase (4 mg/ml) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer and were seeded into 6-well plates with alpha modification of Eagle's medium (α-MEM) supplemented with 20% FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, then grown under standard cell culture conditions[214].

4.3.3 Characterization of the stem cells

The linage specificity of cells was confirmed by the presence of lineage-specific cell surface markers with flow cytometry (BD® FacsCalibur, Becton Dickinson, NJ, USA). Haematopoietic linage-specific surface markers (CD34, CD45) and mesenchymal surface markers (CD73, CD90, CD105 and CD166) were investigated[214].

4.3.4 Labelling of MSCs

The BMSCs were labelled with the fluorescent membrane dye Vybrant DiD (excitation/emission: 644/665 nm, Molecular Probes, Invitrogen, USA) for 30 minutes at 37°C in monolayer. As opposed to BMSCs, the DPSCs did not take up the Vybrant DiD dye to allow uniform staining, thus their proliferation was followed up with UV-VIS spectrophotometer (BIOTEK Powerwave XS) using Alamar Blue assay (Biosource, Invitrogen, USA) [214].

4.3.5 Seeding of MSCs under standard conditions

The MSCs had been trypsinized and suspended in culture medium then applied with pipette to the surface of the test and control scaffolds (100.000 cells per scaffold). After seeding, the cells were expanded on the scaffolds under standard cell culture conditions for 18 days and their proliferation was investigated at the 3rd and 18th days[214].

4.3.6 Seeding of cells under dynamic conditions

First, 100.000 cells per scaffold were seeded on the surface of freeze-dried albumin coated bone allografts and stored under standard culture conditions for 24 hours. Following the incubation period, the bone grafts were placed into a bioreactor tube, which had been filled with 25 ml cell culture medium comprising 1,5 million MSCs in suspension. The bone grafts had been incubated in bioreactor under dynamic cell culture conditions for 24 hours then the cells were further expanded on the surface of bone grafts under standard culture conditions for 18 days. The viability and quantity of attached MSCs on the surface was investigated after 3 and 18 days of incubation[214].

A custom-made rotating bioreactor was built based on Terai's and Hannouche's Rational Oxygen-Permeable Bioreactor System[216]. The bioreactor was designed to provide sufficient oxygen tension to cell culture media under dynamic cell culture conditions. A thin coating of silicone polymer was applied inside a 50-ml polypropylene centrifuge tube, which is permeable for gas through a window and does not support cell attachment. The filled tubes with the stem cell suspension and scaffolds were placed into a rotator apparatus. The apparatus was placed into an incubator and maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air. The rotating speed was set to 8 RPM to allow the mild rolling of the scaffolds on the wall of the tube without the occurrence of turbulence and swirl.

4.3.7 Assessment of the proliferation of BMSCs

The survival of the fluorescent dye labelled BMSCs was observed with confocal microscopy (LSM 510 META, Zeiss) on the surface of control and test groups. Three

individual view fields were randomly selected on the surface of the grafts where the quantity of pixels belonging to the fluorescent BMSCs was measured. The quantity of the cells was assessed on the 3rd and 18th days[214].

4.3.8 Assessment of the proliferation of DPSCs

The survival of the DPSCs was measured by spectrophotometry at the 3rd and 18th days on the surface of bone grafts. Alamar Blue was added in 10 w/w% to α-MEM cell culture medium supplemented with 10 w/w% FCS, 2 mM L-glutamine, ascorbic-2-phosphate acid 100 μmol/l, 100 U/ml penicillin and 100 μg/ml streptomycin and 1g/l glucose. The bone grafts were incubated in this medium for 4 hours in cell culture incubator under standard cell culture condition. After incubation 200 μl of supernatant was pipetted into 96-well plate and the absorbance was measured at 570 nm and 600 nm wavelengths[214].

4.4 *In vivo* biocompatibility study of coated human bone grafts

The objective evaluation of the *in vivo* performance of bone grafts required the development of a standardized segmental bone defect, where bone healing is compromised without a critical size osteotomy gap (hereinafter referred as nonunion model)[210]. Critical size models have been extensively used to investigate bone defects where the regenerative process fails to bridge an oversized gap. However, the orthopaedic surgeon commonly faces smaller defects with compromised healing capacity, where the size of the deficiency is usually not challenging, but the time necessary to bony consolidation and to full weight-bearing is of paramount importance. In these situations, the critical size model has limited applicability that necessitated the development of a more appropriate experimental animal model that reflects the relevant clinical needs.

Only those grafts were further investigated in the nonunion model that facilitated the long-term survival and proliferation of the MSCs in the *in vitro* experiments. In the following the *in vivo* experimental methods will be detailed.

4.4.1 Nonunion model and study design

The animals were anaesthetized with halothane in a 1:1 mixture of N_2O and O_2. The surgical site on the thigh around the femur was shaved and disinfected. The skin, the subcutaneous layer and the fascia were incised, the tensor fascia latae, and the vastus lateralis muscles were separated from the biceps femoris muscle. The femur was exposed from the hip joint to the knee, with special care to preserve the periosteum. A 5 hole steel plate (Mini plate; Sanatmetal, Eger, Hungary) was fixed to the diaphysis of the femur by four 1.5 mm wide and 8 mm long cortical screws (Sanatmetal, Eger, Hungary) using two proximal and two distal holes, while leaving the middle hole empty. After fixing the plate by the screws, an osteoperiosteal segment was removed at the level of the middle hole using a reciprocating saw (Electric Pen Drive, Synthes GmbH, Oberdorf, Switzerland). The bone was cut precisely through both cortical layers together with the periosteum. The size of the defect was 6 mm in the critical size group and 2 mm in the nonunion group[217].

Adult male Wistar rats (n=39) weighing 496–692 g were housed and maintained at 12/12 day/night cycles and were provided with water and lab chow ad libitum. The animals were separated into six groups as it is summarized on Figure 25. In the first moiety of the animals the classical critical size model was established by creating a six millimetres wide mid-shaft defect, which was either left empty or filled with an uncoated or freeze-dried albumin coated cancellous bone allograft block[218]. In the second moiety of the animals a nonunion bone defect was created by blocking bone healing for 4 weeks by the interposition of a spacer into the osteotomy gap (for details see 4.4.1)[219]. The interposed spacer was removed after 4 weeks and the osteotomy gap was either left empty or filled with uncoated or freeze-dried albumin coated cancellous bone allograft block. Each animal was sacrificed 4 weeks after the grafting procedure. The surgical protocol of the animal study was approved by the Institutional Review Board of Semmelweis University. In the critical size groups the defect was either left empty, or filled with an uncoated or a freeze-dried albumin coated graft. In the nonunion group 2 mm preformed thick surgical-grade sterile PMMA (Heraeus Medical, Wehrheim, Germany) bone cement spacer was interposed into the gap for four weeks.

The PMMA spacer was secured to the plate by 3-0 non-absorbable sutures to avoid displacement. Then, the muscle lobes were stitched by 3-0 interrupted absorbable sutures over the femur, and the skin was closed by 3-0 interrupted nylon sutures. The animals were placed back to their cages and observed daily. The weight of the animals was measured and recorded weekly. After 4 weeks a second procedure was performed to allow bone grafting. In the first step, the sutures were removed and the femur was exposed as previously described. Then the PMMA spacer was removed and the defect was either left empty, or filled with an uncoated or a freeze-dried albumin coated bone allograft block. After graft insertion the wound was closed in the same manner as detailed earlier. Four weeks after completion of the protocol the animals were sacrificed by exsanguination under anaesthesia and the femora were harvested; the plate and screws were removed to allow radiographic analysis (see the method in details in section 4.4.3).

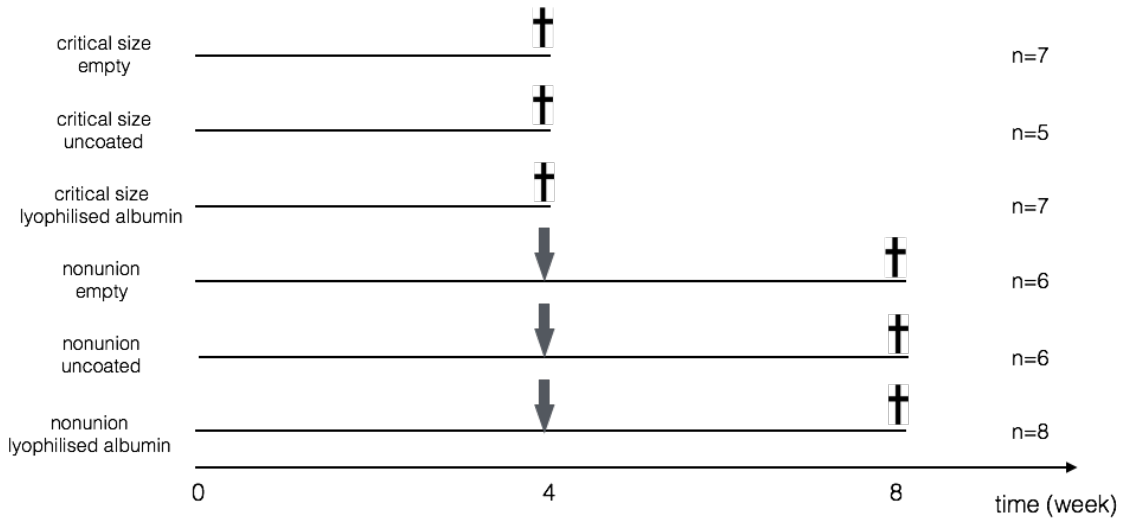

Figure 25. Experimental design of the animal study. White arrow indicates the time of the first procedure in each group. Black arrow shows the time of the second procedure, i.e. removal of the spacer in the control nonunion group, or removal of the spacer and implantation of the uncoated graft or coated graft. The black cross represents the time when the animals were sacrificed and the bone harvested for CT morphometric analysis.

4.4.2 Statistical analysis

Repeated measures one-way ANOVA analysis was performed (Tukey's post hoc test) to compare the quantity of MSCs on the scaffolds. One-way ANOVA analysis was

used (Tukey's post hoc test) to compare the effect of dynamic and standard conditions on the seeding efficiency of MSCs. A p value < 0.05 was considered significant[214].

5 RESULTS

5.1 *In vivo* biocompatibility of chemically sterilized, antigen extracted freeze-dried human bone allografts

5.1.1 NanoSPECT-CT analysis

The osteoblast activity was monitored during 12 weeks after implantation in case of pCPC, PMMA and human bone chip experimental groups (Figure 26). The activity was calculated as the percentage of the next healthy vertebra. The CT graph (blue line on Panel A) follows the structure of the two vertebrae, while the pixel intensity is much higher in the operated vertebra compared to the healthy control vertebra when looking at the red line in Figure 26. The same trend was observed in all animals. In all three groups the values are higher than those of the normal bone (adjacent vertebra on Panel B). The osteoblast activity in this cavital defect is higher than the normal bone and remains high during the first 5 weeks after implantation. The overall osteoblast activity is lower in the human bone chip group than the pCPC group, while a slight decrease can be observed both in the bone chips and the PMMA groups. The osteoblast activity did not return to normal levels (unit 100 in the graph) in any animals in any group during the study[47].

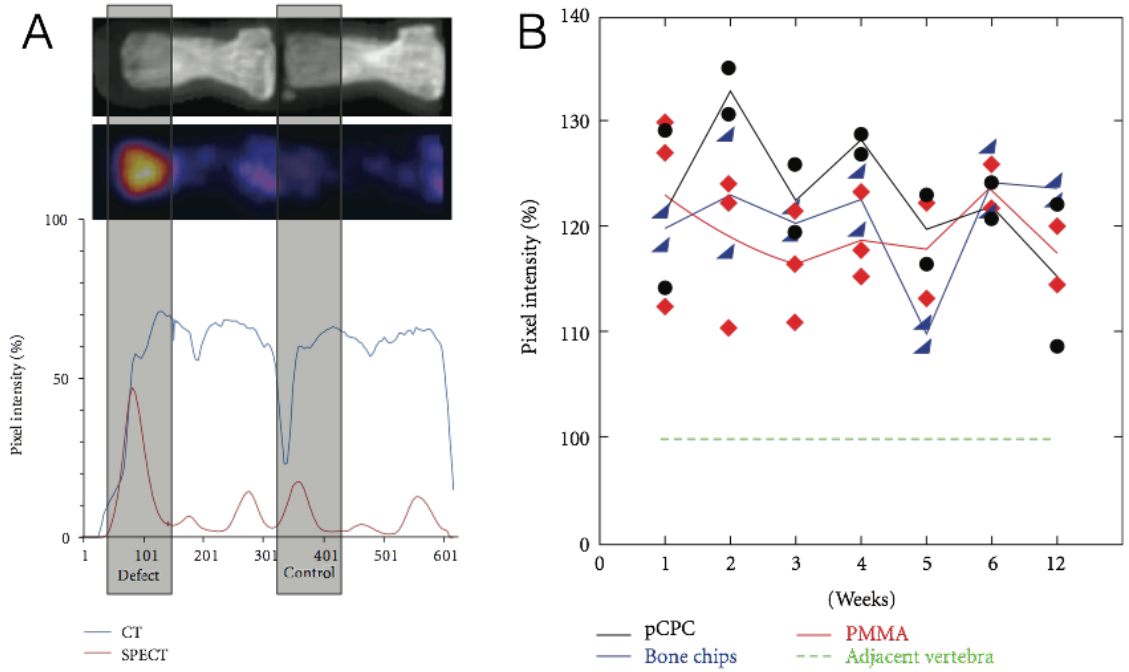

Figure 26. Quantitative measurement of the osteoblast activities by multimodal imaging. Panel A shows the comparison of intensities from CT (blue line) and nano-SPECT (red line) images. A representative image shows that the peak intensity of a vertebral epiphysis can be localized on the basis of the CT image and compared to the defect site. SPECT intensities reflect osteoblast activity. The defect site has a significantly higher osteoblast activity than the normal bone. Panel B shows the diagram of osteoblast activity in the operated vertebra compared to the adjacent vertebra. The lines represent the mean value and the dots the individual animals value. The 12-week nano-SPECT/CT follow-up showed considerably lower osteoblast activity in the bone chips group during the first 5 weeks compared to the pCPC group. Although the differences between the three groups level off after the 5th week, pixel intensity stays higher in all three groups compared to the neighboring healthy vertebra.

5.1.2 Ex-vivo µCT analysis

In Figure 27 representative 3D renderings of the filled defect along with cross-sections are presented. As seen in the figure, in the vertebrae filled with PMMA (upper image on Panel a), the defect was completely filled with PMMA and no bone formation was observed. The PMMA was demarcated from the bone. This result was consistent in all animals in the PMMA group. In the vertebrae filled with bone chips no or slow resorption could be observed, and limited bone healing. There was no bony connection between the bone chips and the new bone as seen in the cross-section CT image (bottom image on Panel a). In the case of pCPC in the vertebrae (middle image on Panel a) new bone formation can be observed (white arrow). The pCPC has been partially resorbed and replaced with bone. However the ZrO_2 present in the pCPC for radiopacity is not

resorbed, and thus residues of the cement can be observed in the newly formed bone. Bone defects without any spacer or filling showed good bone regeneration with new trabecular bone being formed in each sample providing a defect union rate of 100% (Figure 27, Panel b). In the delayed healing group, where the spacers were removed at 12 weeks and the defect was left empty for an additional 12 weeks, bone formation was observed in three defects while no or very limited bone formation was observed in the remaining four defects, where the union rate was 43% (Figure 27, Panel c). The μCT images demonstrated that trabecular bone was formed in the defects filled with SrCPS with a union rate of 80% with four being completely filled with new bone while in one specimen bone was formed but it did not fill the entire defect (Figure 27, Panel d) [47,173].

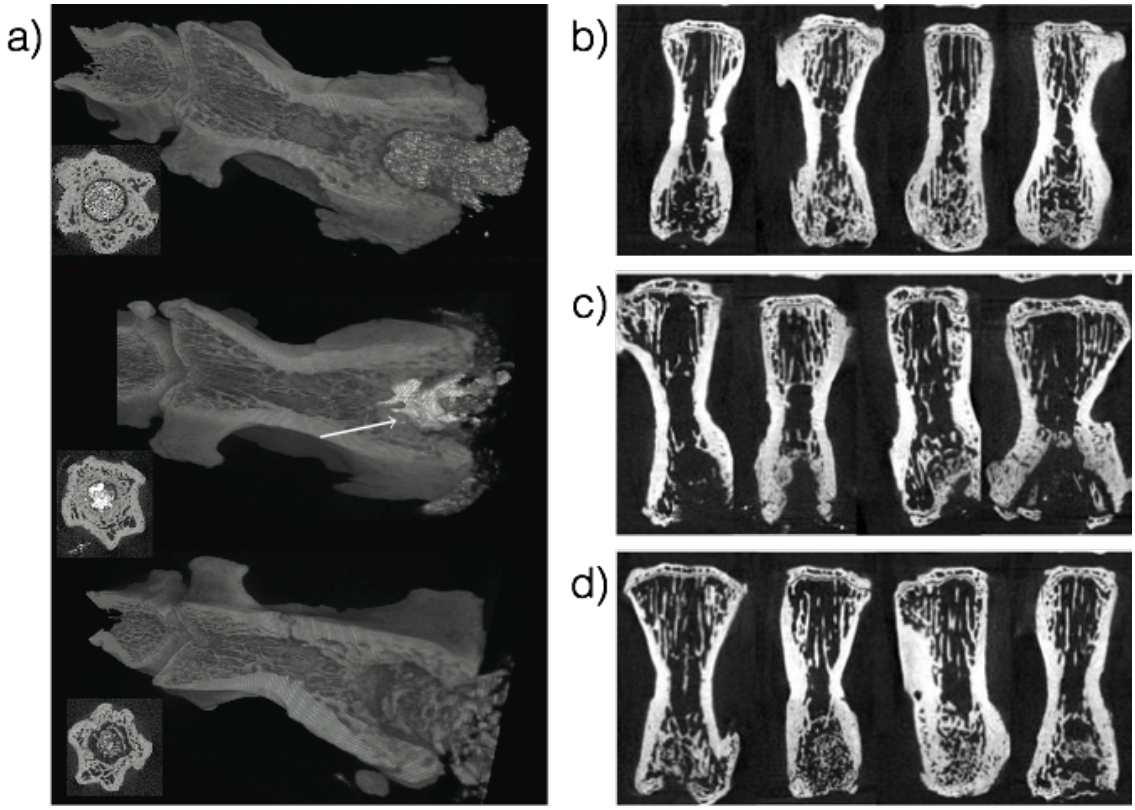

Figure 27. Representative micro-CT images of operated vertebrae after 12 weeks. Panel a) PMMA (upper image) is compacted in the hole, but no bone formation was observed; a radiolucent area is seen between the PMMA and the bone. The pCPC (middle image) is slowly resorbed, and new bone is formed in connection with the pCPC (arrow). There was some bone formation around the bone chips, however considerable radiolucent areas can also be observed. Panel b) In the normal healing group, when no spacer was used the drilled defect self-regenerated in all four cases (the fifth animal died during the study period and could not be evaluated). Panel c) In the empty (spacer) group three out of seven were healed, and showed similar bone structure in the previous defect as in the SrCPS group. Panel d) In the SrCPS group, four out of five were healed and trabecular bone formation was obvious in all cases.

5.1.3 Results of histology

Histological assessment showed that there was some bone formation in the bone chip group; however the bone chips are still demarcated from the bone. In the histology image bone chips are seen to have direct contact with newly formed bone (Figure 28). In contrast, the histological assessment confirmed that the SrCPS and pCPC had good biocompatibility and at the time of observation there were no visible signs of remaining SrCPS particles at light microscopy level indicating that the material was resorbed and built into the newly formed bone stock (Figure 28) [47,173].

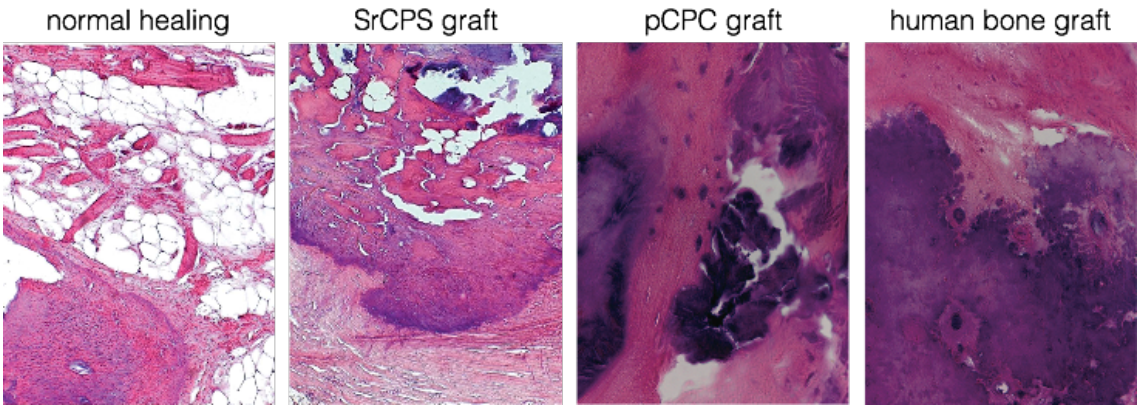

Figure 28. Representative hematoxylin and eosin stained histological section of the distal end of rat-tail vertebrae after 12 weeks of normal healing and SrCPS, pCPC and human bone graft implantation. Morphology of the bone was similar to healed trabecular bone, with lacunas and ossification centers. There was no sign of the nanosized SrCPS with light microscopy resolution. Fat tissue in the bone marrow and dense trabecular bone was present in all cases, implying bone formation properties and biocompatibility of the specimens with SrCPS. Enchondral bone formation was still present at this timepoint, indicating that remodeling was still underway. Concerning pCPC graft, newly formed bone is seen in contact with the slowly remodelling calcified bone fragment. In case of human bone graft there was some bone formation, too.

5.2 Physical characteristics of bone grafts

5.2.1 Optical characteristics of bone grafts

The SEM analysis revealed significant differences in the macro-, and microstructure of freeze-dried human bone allograft and hydroxyapatite or lyophilized bovine bone (BioOss) (Figure 29). Hydroxyapatite exhibited the most compact structure

with low number of micro-pores. In contrast, the texture of lyophilized bovine bone was rich in large diameter channels. The freeze-dried cancellous allogeneic bone graft exhibited segmented surface with pores of various sizes. The coating of the surface of bone grafts with freeze-dried albumin amorphous protein flakes masked the original structural differences. The albumin flakes showed the most homogeneous distribution on the surface of Bio-Oss, whereas the continuity of the albumin flakes was disrupted with random uncoated areas on allografts (Figure 29)[214].

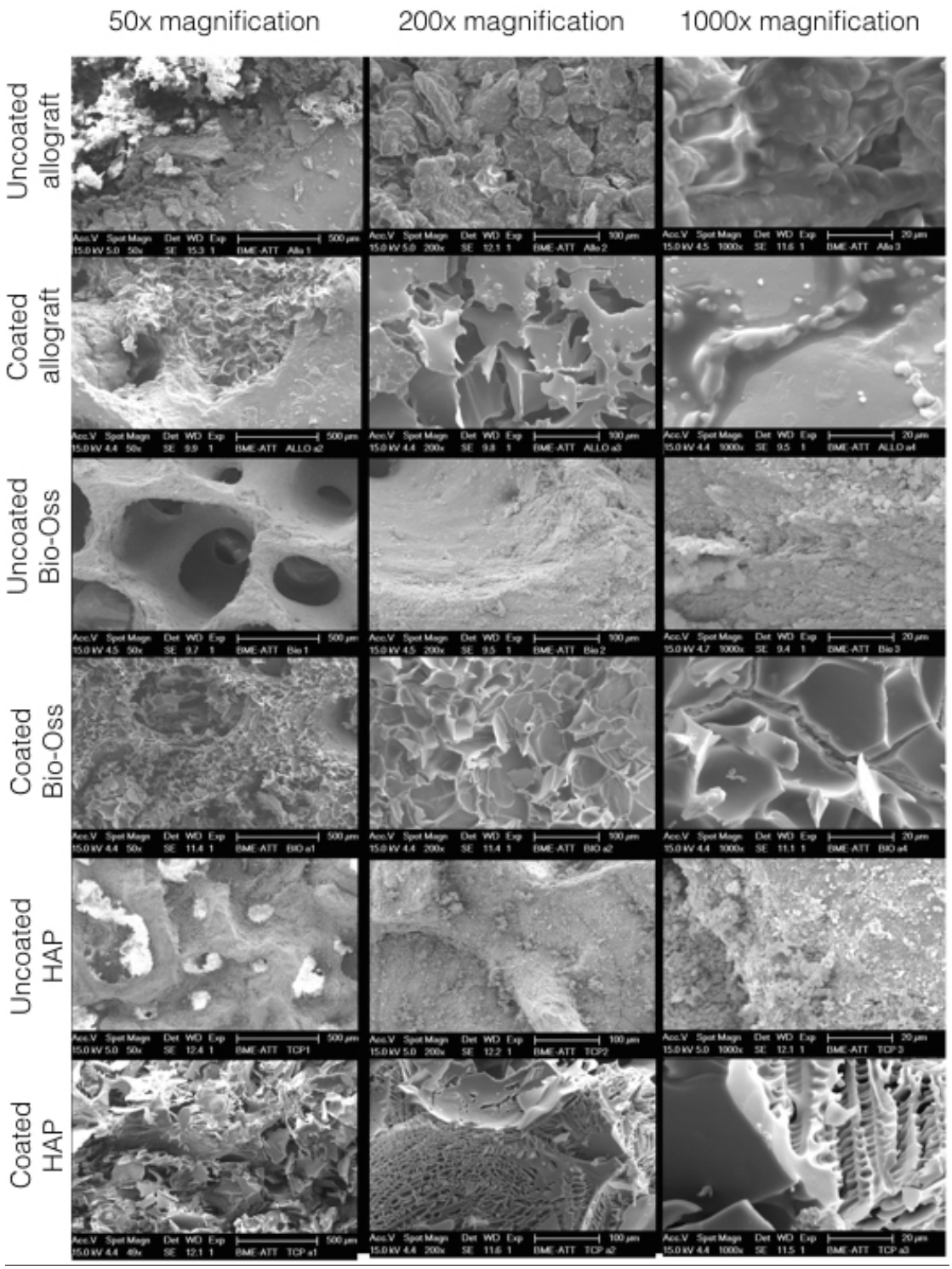

Figure 29. Macro- and microstructure of freeze-dried albumin coated and uncoated bone grafts. Scanning electron microscopy shows significant differences in the texture of allograft, hydroxyapatite (HAP), and lyophilized bovine bone graft (BioOss). Hydroxyapatite has the most compact structure with low porosity. On the other hand, high connectivity and thin wall-thickness typifies BioOss. The structure of human mineralized bone allograft is different from the other two. It is more compact than BioOss and its surface contains multiple micro-pores. By coating the surface of bone grafts with albumin amorphous protein chips mask the apparent differences in the micro- and nanostructure.

5.2.2 Microhardness of bone grafts

The xenogeneic BioOss showed the lowest Vickers-hardness (14,9 HV ± 4,1). The allogeneic bone graft showed higher values (55,1 HV ± 7,7) than BioOss, whereas the synthetic β-TCP was a magnitude harder than the other two grafts (320,4 HV ± 44,6). The micro-hardness values of freeze-dried albumin coated and uncoated allogeneic bone grafts were very similar (55.1N ± 7.7 vs. 53.9N ± 7.9, respectively)[214].

5.3 *In vitro* biocompatibility of bone grafts

5.3.1 Characterization of stem cells

The majority of the isolated cells exhibited mesenchymal lineage specific cell surface markers (CD73, CD90, CD105, CD166) and lacked haematopoietic markers (CD34, CD45) (Figure 30) [214].

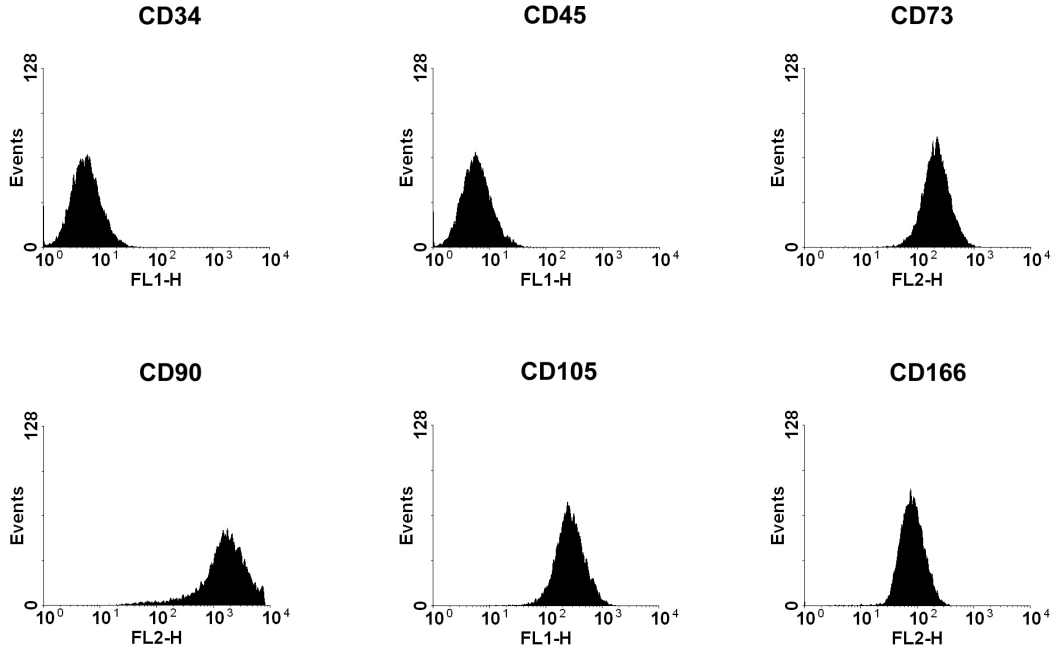

Figure 30. Result of flow cytometry analysis. The cultured cells exhibited MSC lineage specific cell surface markers ($CD73^+$, $CD90^+$, $CD105^+$, $CD\ 166^+$) but not haematopoietic markers ($CD34^-$, $CD45^-$).

5.3.2 Adherence and survival of MSCs on bone grafts

5.3.2.1 Control group

The uncoated mineralized allogeneic bone graft did not support the adherence and survival of BMSCs on the surface. Only a few cells adhered temporarily on the uncoated allografts but their quantity was decreasing and they completely diminished by the 18th day of the experiment (mean of pixels at the 3rd day: 183 ± 34; at the 18th day: 0) (Figure 31). The initial adherence of BMSCs on the surface of uncoated BioOss and HAP bone grafts did not show statistical significance compared to the allograft (Figure 33) [214].

5.3.2.2 Test group A

The aqueous collagen and fibronectin coating of the surface of mineralized allografts slightly increased the initial adherence of BMSCs compared to the uncoated allograft, but the quantity of the cells was decreasing between the 3rd and 18th days of the experiments (Figure 31). In contrast, the albumin coating of the surface of mineralized allografts markedly improved the initial adherence of BMSCs, however the cells disappeared from the surface by the 18th day (mean of pixels at the 3rd day: 2373 ± 142; at the 18th day: 0) [214].

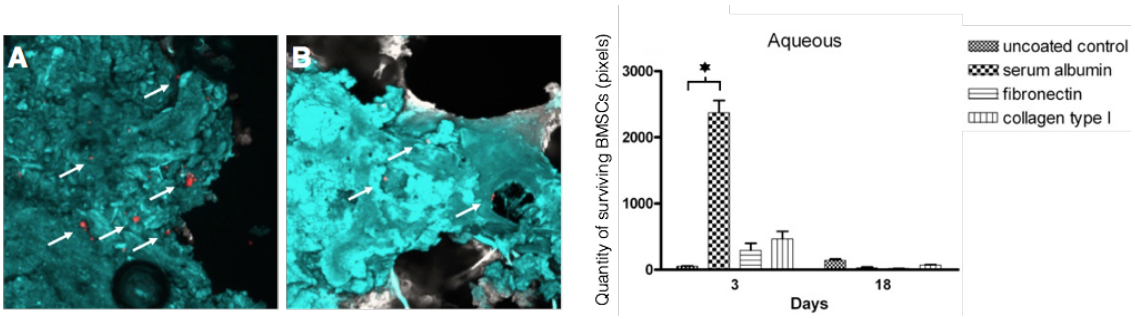

Figure 31. Adherence and proliferation of BMSCs on the surface of mineralized allogeneic bone grafts coated with aqueous proteins. The bar diagram shows that coating of mineralized allografts with aqueous albumin resulted in high cell density at day 3 ($p^* < 0.05$), whereas fibronectin and collagen slightly increased the initial cell adherence. Irrespective to the composition of aqueous coating proteins only a few cells were detectable on the surface of allografts at day 18. Panels A-B show representative confocal microscopic images of uncoated allografts (blue) with Vybrant-DiD labelled BMSCs (red). The few observed cells on the surface at day 3 diminished even more by the 18th day.

5.3.2.3 Test group B

Freeze-drying of serum albumin onto the surface of mineralized allografts reversed the tendency, thus the adhered BMSCs remained on the surface and showed moderate proliferation during the 18 day long experimental period (mean of pixels at the 3rd day: 1658 ± 278; mean of pixels at the 18th day: 2082, ± 110; $p < 0.05$) (Figure 32). Interestingly, the freeze-drying of fibronectin and collagen onto the surface of allografts did not affect positively either the initial adherence or the proliferation BMSCs compared to allografts coated with the aqueous form that of proteins. As opposed to allografts, freeze-dried albumin failed to improve the initial adherence and proliferation of BMSCs on the surface of BioOss and HAP grafts (Figure 33, Figure 34). In contrast to uncoated mineralized allograft, the initially adhered BMSCs did not lose their viability on the surface of uncoated BioOss and hydroxyapatite, albeit they did not show proliferation either (Figure 33, Figure 35) [214].

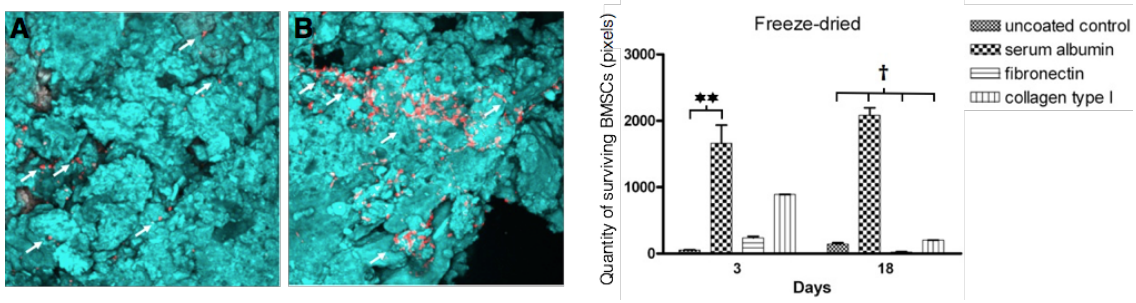

Figure 32. Adherence and proliferation of BMSCs on the surface of mineralized bone allografts coated with freeze-dried proteins. The bar diagram shows that freeze-drying of albumin onto the surface of bone allografts significantly increased the quantity of initially adhered cells at day 3 compared to freeze-dried collagen or fibronectin coating. However, the multiplication of the adhered cells was moderate on the surface of freeze-dried albumin coated allografts during the experimental period. Panel A-B show representative images of freeze-dried albumin coated bone. The proliferation of the attached cells was observed on the day 18.

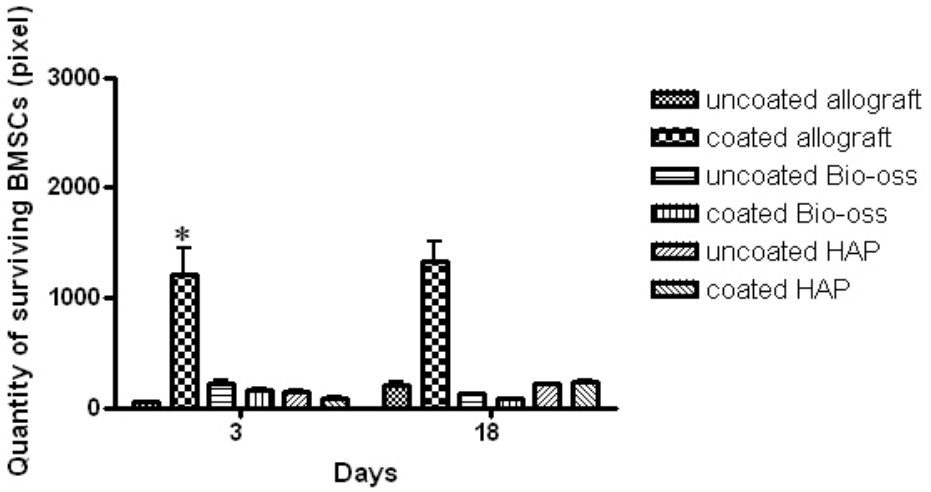

Figure 33. Adherence and proliferation of BMSCs on the surface of freeze-dried albumin coated and uncoated bone grafts. Freeze-dried albumin coating significantly improved the adherence of BMSCs on the surface of mineralized bone allografts compared to HAP and BioOss bone grafts ($p^* < 0.05$).

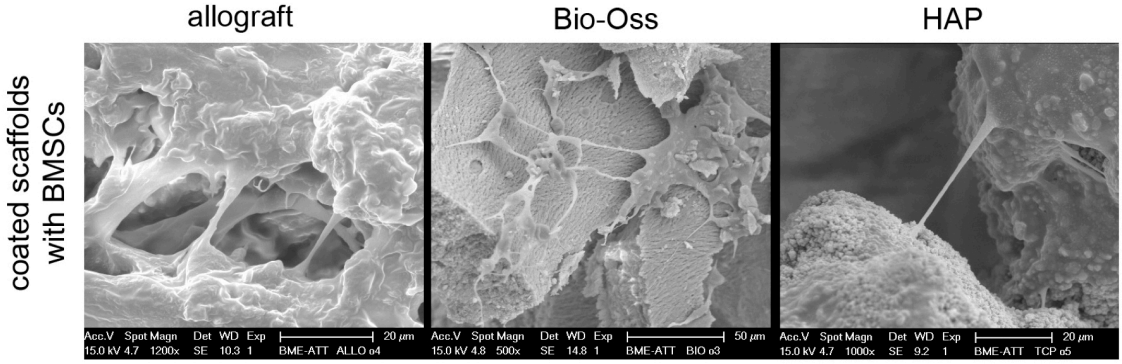

Figure 34. The attachment of bone marrow derived MSCs on the surface of freeze-dried albumin coated bone grafts. The MSCs do not exhibit flat cover on the surface in a monolayer but rather span the pores from side to side.

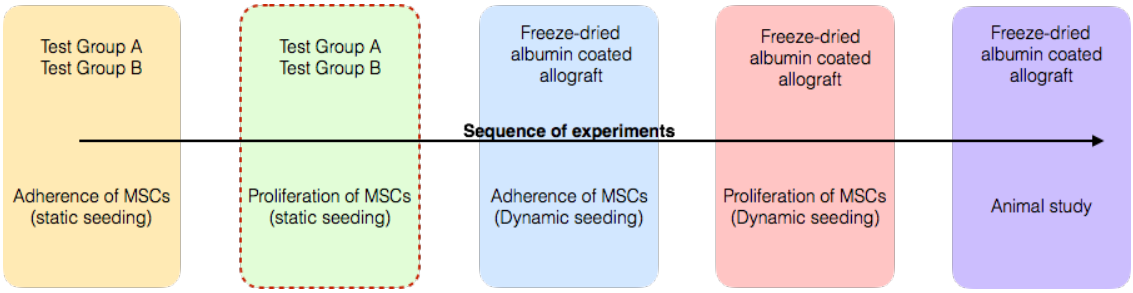

Figure 35. The progressive dropout of underperforming bone grafts. The figure shows the sequence of the experiments. According to the dropout criteria only freeze-dried albumin coated allografts were suitable improve the biocompatibility (adherence and/or proliferation) of MSCs compared to its uncoated counterpart (**Table**).

5.3.2.4 Effect of dynamic seeding on MSC adherence

Dynamic seeding significantly increased the quantity of initially adhered BMSCs on the surface of the freeze-dried albumin coated allografts compared to the standard culture conditions and the adhered cells showed intense proliferation (mean of pixels under standard condition at day 1: 197 ± 23, under dynamic conditions at day 1: 9825 ± 1208; at the 7th day: 15025 ± 1704) (Figure 36A). The same tendency was observed when DPSCs were seeded on the surface of freeze-dried albumin coated allografts under dynamic and standard culture conditions (standard condition: mean of reduced Alamar Blue (%) at 3rd day: 14.5 ± 2.23; mean at 7th day: 33.7 ± 0,06; dynamic condition: mean of reduced Alamar Blue (%) at 1st day: 33.5 ± 2.23; mean at 7th day: 60.9 ± 1,09) (Figure 36B) [214].

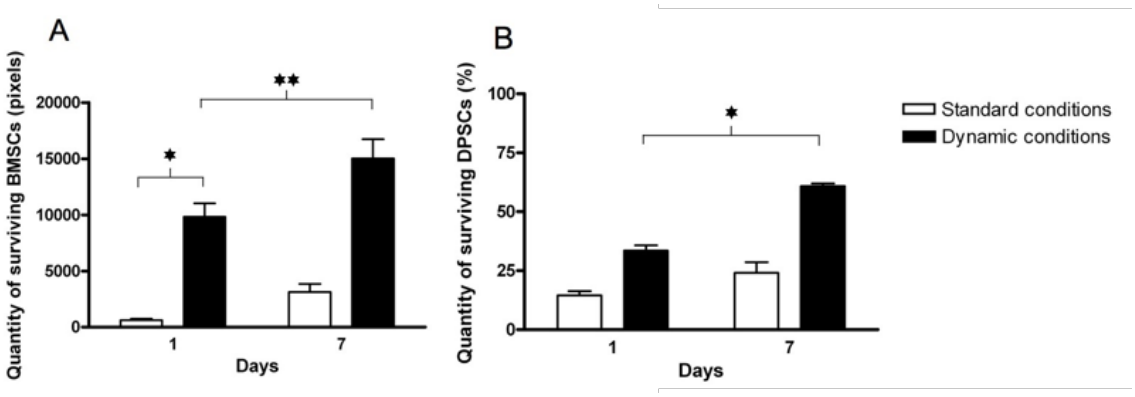

Figure 36AB. Adherence and proliferation of BMSCs and DPSCs onto the surface of freeze-dried albumin coated allografts under dynamic conditions in a rotating bioreactor. The dynamic cell culture conditions markedly improved the initial attachment of both BMSCs (panel A) and DPSCs (panel B) which showed intense proliferation.

5.4 *In vivo* biocompatibility of freeze-dried albumin coated bone grafts

Implantation of albumin coated bone grafts into a rat model of delayed bone healing resulted in better integration of the albumin-coated grafts than the native ones (Figure 37). The implanted graft was located in the defect in each case, but only the albumin-coated grafts had significant ingrowth of new bone from the host resulting in union of the defect. Other members of our researcher groups provided quantitative demonstration

on that the bone ends coalesce and bone remodelling occurs when freeze-dried albumin coated human bone graft is implanted into the nonunion based on micromorphometric analysis (i.e. bone volume, trabecular thickness, trabecular separation and trabecular pattern factor) [214].

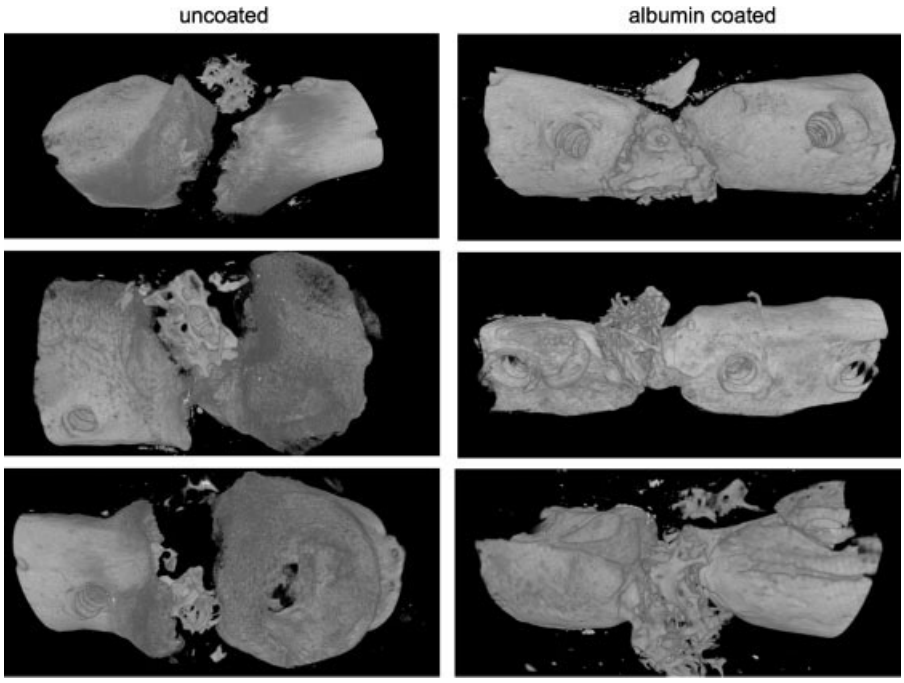

Figure 37. Reconstructed 3-dimensional µCT images of osteotomized rat femurs after 4 weeks of the grafting procedure. The left column shows that without freeze-dried albumin coating the allogeneic human bone graft does not coalesce with the bone ends and there is no bony consolidation. In contrast, when the grafts were coated with albumin there was apparent bone coalescence and a bony callus was formed.

6 DISCUSSION

The results show that the *in vivo* biocompatibility of the chemically sterilized, antigen extracted freeze-dried human bone grafts is inferior to the comparator synthetic bone substitutes. The results supported that human albumin is a suitable coating substance to enhance the biological performance that of human bone grafts in *in vitro* and *in vivo* experimental settings. The freeze-drying procedure supports the reproducible performance of albumin coating, however, the homogeneity of albumin flakes is poor between the trabeculae of the human bone graft. The albumin coating does not influence the microhardness of the freeze-dried allografts; the agitation under dynamic culture condition does not reduce its biological value. Intriguingly, the albumin coating increases mainly the initial adherence of MSCs on the surface of allografts, whereas significant proliferation is only seen after seeding under dynamic culture conditions. After implantation into a nonunion site, albumin coat improved the ingrowth of new bone from the host and resulted in the union of the bone ends. Interestingly, the albumin coat does not improve the *in vitro* biocompatibility of either the lyophilized bovine bone or the synthetic hydroxyapatite bone scaffolds.

It is often said that an ideal bone graft should have good osteoconductive, osteoinductive and osteogenic property, which statement have determined the orientation of bone replacement related researches after all. Historically, these terms came to birth in the second part of ’90s in order to explain better the process of osseointegration, which was described by Brånemark first in 1977[220,77]. It should be noted that these terms were applied first to define the incorporation of titanium dental implants into the jawbone. Later, they spread to other fields, such as orthopaedics, biomaterial sciences, and bone tissue engineering, where they have built into the jargon of these professions. However, the applicability of these terms to 3-dimensional porous biomaterials, like structural bone grafts so that characterize their biological properties might be challenged. It is easy to agree with the fact that a solid, cylindrical dental implant has 2-dimensional superficies to contact with host tissues, whereas a porous bone block has a 3-dimensional interface. It is more straightforward to support the adherence, migration and proliferation of host cells on a 2-dimensional surface, of which each point is in direct contact with host tissue providing unrestricted supply of

oxygen and nutrient. In contrast, a bone block with 3-dimensional interconnecting channels may have inner surfaces that are demarcated from the host tissues by the walls of the block, which creates barrier to oxygen and nutrient supply. The same principle holds true *in vitro*, when cells are cultured on 3-dimensional scaffolds and cells that are dwelling at deeper levels are subjected to constant oxygen and glucose deprivation compared to monolayers. It has been demonstrated that ischemic microenvironment along with serum and glucose deficiency may lead to the apoptosis of MSCs; albeit reduced hypoxic and serum levels may enhance their osteogenic potential and contribute to the induction of angiogenesis[221,222,223]. Nevertheless, the understanding of the delicate balance of such soluble cues that direct cell fate is beyond our reach at the time being. *In vivo*, soluble cues that are mediated by a wide range of molecules, like growth factors are responsible for the recruitment of cells that perform the fracture repair (the same cells are supposed to take part in graft incorporation). Beside MSCs, many other cell types are present in the repair tissue, i.e. osteoclasts, endothelial cells and macrophages that interact and modulate the activity of each other. However, in *in vitro* experimental settings only one or a few interactions can be modelled simultaneously, which is far behind the *in vivo* complexity of soluble cues that influence the viability of bone forming cells in the repair tissue. Therefore, at the current level of technology, concerning experimental tools, explanations of physiological and pathological processes relying only on *in vitro* experimental results should be critically appraised. On the other hand, perhaps *in vitro* settings are the most appropriate to investigate cell response on insoluble (biophysical) cues in isolated system. Furthermore, *in vitro* tests may be extremely useful to investigate the interrelation between the mechanical/physical properties and the biological performance of the bone grafts.

The 3-dimensional structure of a bone graft, especially the pore size and interconnectivity influences the coating efficiency and quality. The distribution of the coating agent, i.e. albumin depends on the surface characteristics of the bone graft and the physicochemical properties of the solution that is used as the vehicle of the coating agent. The transport of aqueous solutions into the pores and channels of a cancellous bone allograft should be supported by the wettability of the surface, which is

responsible for the related effect, i.e. capillarity action of a liquid. The albumin solution is a colloid that is characterised with relatively high viscosity in the concentration it was applied in our experiment[224]. The high viscosity reduces the dynamic wetting ability of the solution, which limits the accessible area by the albumin solution and so its capillary action[225]. Concerning surface characteristics, the SEM images show that pores and channels of cancellous allografts are extremely rough compared to Bio-Oss and hydroxyapatite. The asperities within the pores and channels of allograft might further reduce the motion of the albumin solution, especially towards inner layers, which lead to the inhomogeneous distribution of albumin flakes after freeze-drying (Figure 29). This inhomogeneity of the coating maybe another component of the low proliferation activity of MSCs. Speculatively, the migration and proliferation of cells might have been restricted towards uncoated sites creating limited area for cell survival. By the improvement of the manufacturing technique this problem may be eliminated, for example, using centrifuge before freeze-drying to ensure the complete penetration of the albumin solution through all pores and channels of the allograft. It must be noted here that the causality between wettability and viscosity is not straightforward. Surface tension is an important factor that affects the wettability, which is supposed to be low (i.e. should favour the wettability) in case of the albumin solution. Hence, the wettability should be a cumulative result of the surface roughness, viscosity and surface tension in our experimental setting[226].

The coating procedure was carried out under mild conditions that may be the reason for that the microhardness of allografts did not change after freeze-drying. The hardness gives the ability of a solid material to resist plastic deformation when a compressive force is applied. Vickers hardness test is one of the most commonly used indentation method to measure the hardness of solids. The hardness is a function of the force and size of the impression, thus the pressure (stress) used to create the impression can be related to both the yield and ultimate strengths of materials. The Vickers Hardness (VH) of a bone graft reflexes its tendency to crunch under impaction and dynamic load. The Vickers microhardness measurements ranked the synthetic ceramic bone graft an order of magnitude harder than the natural-source bone substitutes, which may be explicable by material-structure differences[188]. The natural-origin bone

substitutes are composite materials, in which elastic protein filament cages hold inorganic calcium and magnesium during bone formation. In contrast, synthetic ceramic is a single-phase compact material and lacks protein filaments that may explain the higher microhardness. From this perspective, the synthetic ceramic bone graft may be more suitable to apply at load-bearing sites than the allograft and Bio-Oss because it is associated with lower risk of compression and implant migration; whereas, human allograft and Bio-Oss seem more applicable at non load-bearing sites or they should be shielded by fixation devices at load-bearing sites. It should be noted here, that direct relation can not be established between the hardness, strength and toughness of the bone grafts based on a single measurement, thus the applicability of those bone grafts at load-bearing sites should be evaluated critically from various point-of-views before making such conclusion. In order to highlight the complexity of this issue it is an interesting property of the chemically sterilized, antigen-extracted human bone graft is that after *in vitro* incubation in aqueous media its fingering turns extremely elastic, like a rubber. This empirical finding suggests that the rehydration, which will occur after implantation in the body, may significantly alter the mechanical properties, especially the elastic modulus of allogeneic bone grafts. The versatility of the mechanical properties depending on the microenvironment should be either an innate property of the allogeneic bone or the result of its preparation method because such obvious change does not happen to Bio-Oss after *in vitro* rehydration.

The partial decalcification of the human bone grafts in the course of their preparation results in the dissolution of hydroxyapatite component that covers collagen filaments of the bone, which increases the exposure of collagen structure to environmental changes, i.e. hydration and dehydration. Therefore, it seems reasonable to assume that the deficiency of the hydroxyapatite content of the human bone grafts may increase the efficiency of freeze-drying allowing the complete removal of aqueous vapour from the deepest layers, which is essential to preserve the biological value of the allografts. In return, the rehydration of the collagen filaments also can be complete and fast, while these filaments regain their elasticity because they are easily accessible to water. This physical characteristic may explain the experienced change in the elasticity of allografts. Another possible consequence of the chemical treatment is that the

osteogenic cells are killed on the allograft and most of the osteoinductive proteins become denatured, which impairs the biological value of the allograft, eventually. The relatively low quantity of adhered cells and their poor proliferation of the three types of bone grafts suggest that inert scaffold may not have innate biophysical cues to stimulate the viability of MSCs. Presumably, the lack of cell-adhesive proteins, such as fibronectin and vitronectin on the surface of grafts might be a possible explanation, however the coating of freeze-dried human bone allografts with collagen and fibronectin is not appropriate to stimulate either the adherence or the proliferation of MSCs. Perhaps the surface of the chemically sterilized, antigen-extracted freeze-dried human bone grafts do not contain binding sites to anchor collagen and fibronectin to facilitate the adherence of MSCs. This theory may be supported, if physiologic endochondral ossification is taken into consideration, when collagen, and other structure proteins first build up the texture of the bone tissue followed by mineral deposition. Therefore, from retrospective view, it may not be surprising that working in the opposite direction, that is, putting structure proteins on top of an inorganic scaffold does not yield optimal results. This may be applicable to Bio-Oss and synthetic hydroxyapatite that may explain their low *in vitro* biocompatibility (Figure 33). It must be emphasized again that the *in vivo* environment is supposed to be more complex than *in vitro* experimental settings, thus direct *in vitro in vivo* extrapolation is not applicable, particularly because Bio-Oss is known as one of the best graft for alveolar bone replacement[227].

The cell capturing effect of albumin coating is not fully understood yet. It seems to be a possible explanation that freeze-dried albumin adsorbs water when the cells are seeded onto the surface of allografts in aqueous media. The water adsorption may be associated with the volume expansion of albumin yielding colloidal suspension that is temporally trapped in the inter-trabecular channels of the allograft due to its high viscosity. This colloidal suspension of albumin may embed MSCs and keep them in the pores and channels of the allograft providing enough time to them to establish focal adhesions with the surface. Presumably, cell morphology and biophysical cues may also influence cell proliferation, albeit the mechanistic description of MSC biology needs further refinement for the better understanding of this phenomenon. Unfortunately, the

effect of pore-size (trabecular separation) and surface chemistry on the establishment of focal adhesions is not well documented in the literature. Nevertheless, it is worth to note that MSCs do not have flat shape in the inter-trabecular channels of the 3-dimensional grafts as they do in monolayer, but they show spindle-like morphology with long projections that establish connections with the inner surface of channels (Figure 34). There is growing evidence that cytoplasmic actin filaments are essential factors in the modulation of nuclear shape and function. Recent findings indicate that large-scale cell shape changes may induce a drastic condensation of chromatin and dramatically affect cell proliferation[228]. Therefore, it seems plausible to assume that cell shape might influence the proliferation of the MSCs on the grafts by the tension in central actomyosin fibres.

The volume expansion (colloidal suspension) theory may also be applicable to explain the improved osseointegration of freeze-dried albumin coated allografts in animal study. After surgery, alike after injury, the cells of inflammatory cascade flood the surgery site where they release a number of signalling molecules that induce a cascade of cellular events that initiate healing. These signal molecules might be adsorbed by the colloidal suspension of albumin that increases the local concentration of such biological cues, while it provides a natural delivery system that allows the prolonged release of those signals. This assumption is based upon the high affinity of serum albumin to a wide range of biomolecules creating a high capacity natural buffer (reservoir) for them in the blood. The increased local concentration and prolonged availability of those soluble cues may enhance cellular events in the repair tissue allowing the union of the bone ends in a delayed union animal model. Later, other members of our research group have confirmed the *in vivo* performance of freeze-dried albumin coated bone allograft in a 12-month length observational case-control study in hip and knee revision surgery[7].

The preliminary *in vitro* colonization of freeze-dried albumin coated allografts with MSCs before implantation was not necessary in order to achieve good clinical outcome in terms of osseointegration when bone healing was compromised in the *in vivo* animal study. This supports the hypothesis that the contribution of pre-existing

cells to the osseointegration of a bone graft is negligible but the invading host cells orchestrate the healing process. On the other hand, our results suggest that *in situ* bone tissue engineering may be more feasible in clinical settings than the classic approach (i.e. *in vitro* grown tissue) that poses formidable technical and regulatory barriers limiting the smooth transition of the vitalized graft between *in vitro* and *in vivo* sites. Our results also support the viability of biomimetic approach in tissue engineering, given that human serum albumin coated allogeneic bone graft is a bone substitute of human origin, because it mimics human bone better than the other bone grafts. In the current stage of development freeze-dried albumin coated allograft may not be suitable to stimulate all biophysical cues, especially hardness and topography mediated ones. However, the interconnectivity of internal pores may support sheer-stress induced cues, while its chemical composition might allow an optimal resorption rate. These assumptions give rise to a series of questions, including the implication of biophysical cues and the verification of the local accumulation of soluble cues in the albumin colloid that need to be confirmed in future experiments.

7 CONCLUSION

7.1 Investigation of the *in vivo* biocompatibility of chemically sterilized, antigen-extracted freeze-dried human bone grafts

Chemically sterilized, antigen-extracted freeze-dried human bone grafts showed reduced *in vivo* osseointegration in compromised bone defect model compared to synthetic bone fillers.

7.2 Identification of a coating substance to improve the biocompatibility of the chemically sterilized, antigen-extracted freeze-dried cancellous allogeneic bone grafts

Freeze-dried human serum albumin is a suitable substance for the reproducible coating of cancellous human bone grafts.

7.3 Investigation of the *in vitro* and *in vivo* biocompatibility coated freeze-dried cancellous allogeneic bone grafts

The freeze-dried human serum albumin coating improved the *in vitro* and *in vivo* biocompatibility of the chemically sterilized, antigen-extracted human bone grafts.

8 SUMMARY (English, Hungarian)

The replacement of segmental bone defects is still a challenge for orthopaedic surgeons, especially when the self-healing ability of the bone is compromised. Under such circumstances bone grafts often fail to incorporate into the host tissue leading to the development of nonunion, which explains their unreliable incorporation. Currently, autogenic bone grafts are known as the best for the replacement of bony defects because they are immunologically identical to the host, thus their incorporation is more secure than materials of foreign origin. In spite of the beneficial properties the extensive clinical use of autografts is not possible owing to their limited availability, therefore there is an increasing need for donor bones (allografts) as a potent alternative of autografts. Freeze-drying of allografts is a common preparation method following chemical treatment in order to ensure their long-term shelf life. However, chemical treatment deteriorates the biological performance of the allografts that reduces their biocompatibility with osteogenic cells, such as mesenchymal stem cells that compromises the incorporation of allografts. Hence, the objective of my studies was to develop a coating method that significantly improves the biological performance of bone allografts and justify this enhancement through *in vitro* and *in vivo* experiments. We found that human serum albumin was the most suitable substance for coating that was delivered by freeze-drying technique, identical to the preparation method of the allografts. Our results showed that the coating did not affect the micro-hardness of the allografts, whereas the distribution of the albumin was not homogenous in the pores. Nonetheless, the albumin coating significantly increased the *in vitro* adherence of stem cells on the allografts compared to the uncoated counterparts. We also developed a clinically relevant compromised bone-healing (nonunion) model in order to investigate the incorporation of freeze-dried albumin coated structural bone grafts in rat femur. Our results showed that albumin coating significantly enhanced the incorporation of bone grafts in nonunion that was later confirmed in human studies by other members of our research group.

ÖSSZEFOGLALÁS

Az ortopédiai szegmentális csonthiányok pótlása nagy kihívást jelent olyan esetekben, amikor a csont saját regenerációs képessége csökkent. Ilyenkor gyakran tapasztalható, hogy a csontgraftok nem, vagy csak részben épülnek be és ún. *nonunion* alakul ki, ami miatt sokszor bizonytalan a rekonstrukciós bevatkozások kimenetele. A jelenleg legjobbnak tartott csontgraft a saját csont transzplant, ami immunológiailag azonos a befogadó szövettel, így beépülése valószínűbb az idegen eredetű csontpótlókhoz képest. Az előnyös tuljdonságok ellenére a saját csont széleskörű klinikai alkalmazásának gátat szab annak korlátozott elérhetősége, ami miatt gyakran donor csont (allograft) alkalmazása válik indokoltá. Az allograftok előkészítésének egyik módja a kémiai előkezelést követő fagyasztva szárítás (liofilizálás), ami biztosítja azok hosszútávú eltarthatóságát. Azonban az előkezelés hatására a liofilizált allograftok veszítenek biokompatibilitásukból, vagyis kevésbé képesek megtapadni a felületükön a csontképződésben fontos szerepet játszó sejttípusok (mesenchímális őssejtek), ami csökkenti a beépülésük esélyét. Ezért munkacsoportunk azt a célt tűzte ki, hogy kidolgozzon egy bevonatoló módszert, amely jelentősen képes javítani az allograftok biokompatibilitását, illetve igazolja azt *in vitro* és *in vivo* vizsgálatokkal. Erre a célra a humán szérum eredetű albumint találtunk a legalkalmasabbnak, amit integrálni tudtunk a csontgraftok rendszeresített előállításába, vagyis fagyasztva szárításos módszerrel sikerült reprodukálhatóan felvinnünk a felületre. Az eredményeink azt mutatták, hogy az eljárás nem befolyásolta az allograftok mikrokeménységét, viszont a bevonat eloszlása nem volt homogén az allograftok pórusaiban. Ennek ellenére az albumin bevonat szignifikánsan megnövelte az *in vitro* kitapadó őssejtek mennyiségét a bevonatot nem tartalmazó kontrollhoz képest. Kidolgoztunk egy klinikailag releváns állatmodellt, amelyben a saját csont gyógyulás jelentősen csökkent, és így lehetővé teszi a csontpótló anyagok beépülésének előzetes *in vivo* vizsgálatát patkány combcsontban. Ebben a modellben végzett vizsgálatok az mutatták, hogy a liofilizált albuminbevonat jelentősen javította a humán eredetű donor csongraftok beépülését, amit munkacsoportunk később humán vizsgálatokban is igazolt.

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10 PUBLICATION LIST

Publications related to the present thesis

1. **Weszl M**, Skaliczki G, Cselenyák A, Kiss L, Major T, Schandl K, Bognár E, Stadler G, Peterbauer A, Csönge L, Lacza Z. Freeze-dried human serum albumin improves the adherence and proliferation of mesenchymal stem cells on mineralized human bone allografts. J Orthop Res. 2012 Mar;30(3):489-96.

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