Bone and Cartilage Engineering

Ulrich Meyer · Hans Peter Wiesmann

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With 95 Figures and 55 Tables

With a Contribution by Thomas Meyer

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Preface

Bone and cartilage tissue engineering is a multidisciplinary, young and emerging field in biotechnology, which is expected to change medical practice profoundly, regenerating skeletal tissues and organs instead of just repairing them. This new medical discipline holds the promise of improved treatment possibilities, enhanced quality of the patient´s life and the ability to overcome, in the future, the need for major grafting procedures. It is expected that this biotechnology will also have a strong economical impact on clinical medicine. To fulfil these expectations several challenges concerning scientific, technological, clinical, ethical and also social issues need to be met. Basic research still requires the evaluation and elaboration of fundamental processes and procedures in multiple research fields. However, the first tissue engineered bone and cartilage products have already been introduced to the market, and many more are in the preclinical stage, and many companies are involved in this area.

Extracorporal bone and cartilage tissue engineering is a narrower definition of bone and cartilage reconstruction and regeneration by means of the implantation of a cell/scaffold complex. This kind of tissue engineering approach is a multidisciplinary subject bringing together various scientific fields, including material science, cell biology and clinical disciplines. The promising biotechnology, now introduced as a new clinical tool in the restoration of bone or joint defects, is expected to change treatment regimes and to contribute significantly to clinical medicine in future decades. Current limitations in tissue engineering seem most likely be overcome in the near future, suggesting that generating bone or cartilage tissue ex vivo will replace other therapies in routine clinical practice.

One of the reasons for the fast growth of tissue engineering is the large number of excellent research papers covering all aspects of bone and cartilage tissue engineering. Additionally, numerous high quality books are available describing in detail different aspects of bone and cartilage tissue engineering. Despite the fact that such literature is already available, we decided to write a book on bone and cartilage tissue engineering for two reasons. First, during the experimental and clinical work on tissue engineering carried out over more than 10 years in our clinic as well as in our biomineralisation and tissue engineering research group, we observed that many specialists from the different fields involved in tissue engineering had difficulties in overviewing the complexity of the field. We therefore intended to write a comprehensive book covering all aspects of bone and cartilage tissue engineering. Second, as tissue engineering brings together basic researchers, mainly having a biological, biophysical or material science oriented background, with clinically oriented physicians, we found that they differed in the language used. To overcome this problem, the demanding and complex aspects of tissue engineering were divided into the different aspects of tissue engineering on a level that provides extended information for specialist, but also gives usable access to non-specialists. The text of this book is therefore augmented by numerous tables, schematic illustrations and photographs in order to provide a better understanding of the information provided.

We hope this book will add further stimulus for all basic researchers and clinicians who are involved in investigating and applying tissue engineering and will contribute to making bone and cartilage tissue engineering an attractive and reliable alternative treatment option in reconstructive surgery.

Ulrich Meyer
Hans Peter Wiesman

Acknowledgements

This book would not have been possible without the help and support of a multitude of esteemed colleagues. First of all, we would like to thank Prof. Dr. Dr. Dr. hc U. Joos who created through his enormous clinical and scientific potential the logistic, scientific and personal environment as well as the enthusiasm to establish the Biomineralisation and Tissue Engineering Group in his clinic. The expertise and enthusiasm of the members of this group have set the basis for this book. In this respect, we are extremely grateful to Prof. Dr. H. J. Höhling. PD Dr. Dr. J. Kleinheinz, Dr. Dr. B. Kruse-Lösler, Dr. U. Plate, Dr. Th. Szuwart, Dr. R. Cankay, Prof. U. Stratmann, and PD Dr. A. Büchter, who shared our interst and enthusiasm in tissue engineering and inspired the research with their excellent studies. In this regard, we are also very grateful to the medical, scientific and laboratory staff of the Clinic for Cranio-, Maxillofacial Surgery in Münster. Additionally, the willingness and close cooperation with other departments has enabled us to perform complex and multidisciplinary research activities.

The outcome of this research was based on cooperation with a variety of commited colleagues (namely Prof. Dr. W. Schlegel, Department of Gynecology; Prof. Dr. K.-D. Richter, Department of Animal Studies; Prof. Dr. H. Arlinghaus, Prof. Dr. H. Kohl, Prof. Dr. L. Chi and Prof. Dr. H. Fuchs, Department of Physics; Prof. Dr. P. Bruckner, PD Dr. C. Runte and PD Dr. D. Dirksen, Institute of Biophysics, Prosthetic Department; PD Dr. T. Stamm, Orthodontic Department; Dr. E. Krefting and Prof. Dr. R. Reichelt, Department of Medical Physics and Biophysics; Dr. J. Libera, co.don AG, Teltow; Prof. Dr. Dr. D. Weingart, Katharienhospital, Stuttgart). We specially thank Dr. K. Reichenmiller of the Department of Conservative Dentistry, University Tübingen for many helpful discussions and for leaving the PCR images to us and we thank PD Dr. Meffert and Prof. Dr. Raschke of the Department of Reconstructive Surgery, who supported us with their scientific knowledge and who gave us the photographs on joint surgery. We thank Dr. Dr. J. Handschel and Prof. Dr. Dr. N.R. Kübler for their excellent scientific work in embryonic stem cell research at the University of Düsseldorf. Mister Sailer and Dipl.-Ing. N. Huda provided us with excellent schematic drawings. We are also very greatful to Dieter Sommer who supported us in the realisation of the book. Moreover we would like to express our gratitude to Springer, especially Ms. Irmela Bohn, for the professional accompaniment to this project.

We acknowledge with thanks the support of our work by a multitude of research grants over the last decade. Without the support of the German Research Foundation (DFG), the Federal Ministry of Education and Research (BMBF), grants from the University of Münster (IZKF, IMF) and grants given by biomedical companies it would not have been possible to extensively work on bone and cartilage enginnering.

Our most important advisor in the creation of this book was my brother Prof. Dr. Dr. Dr. T. Meyer. With his enormous scientific knowledge in clinical medicine, biochemistry and philosophy, his criticism and useful suggestions in the realisation of this book, as well as with his excellent chapter on ethical considerations in bone and cartilage tissue engineering, he markedly improved the quality of this book.

However, most of our gratitude goes to our families, our dear wives Janine and Andrea, as well as our children Johannes and Carla, who showed virtually endless patience and understanding for our research activities as well as for the writing of this book. Thus, they deserve the highest respect for the completion of this book.

Ulrich Meyer
Hans Peter Wiesmann

Contents

1 Introduction 1

2 Bone and Cartilage 7

2.1 Bone Structure and Function 7
2.2 Matrix Mineralisation 10
2.3 Osteoblast Structure and Function 17
2.4 Osteoclast Structure and Function 19
2.5 Bone Regeneration 20
2.6 Cartilage Structure and Function 25
2.7 Chondrocyte Structure and Function 30
2.8 Cartilage Regeneration 33
2.9 Skeletal Biomechanics 35
2.10 Bone Biomechanics 38
2.11 Cartilage Biomechanic 43

3 Principles of Bone and Cartilage Reconstruction 47

3.1 Bone Repair Strategies 48
3.2 Cartilage Repair Strategies 55
3.3 Extracorporal Strategies 62

4 Cell Development, Cell Sources 65

4.1 Cell Development 65
4.2 Cell Sources 66
4.3 Genetically Modified Cell Lines 70

5 Harvesting and Cultivation . . . 73

5.1 Marrow-derived Stem Cells . . . 73
5.2 Determined Bone Cells . . . 78
5.3 Determined Chondrogenic Cells . . . 83
5.4 Osteoclasts . . . 83
5.5 Endothelial Cells . . . 84
5.6 Co-cultures . . . 85

6 Materials . . . 121

6.1 Synthetic Organic Materials . . . 122
6.2 Synthetic Inorganic Materials . . . 124
6.3 Natural Organic Materials . . . 125
6.4 Natural Inorganic Materials . . . 128

7 Cells/Surface Interactions . . . 131

7.1 Protein Adsorption . . . 133
7.2 Cell Adhesion . . . 133
7.3 Surface Topography . . . 137
7.4 Surface Chemistry . . . 139
7.5 Induction of Mineralisation at Materials . . . 141
7.6 Functional Aspects of Biomaterial/Cell Interactions . . . 143
7.7 Surface Structuring . . . 143
7.8 Surface Patterning . . . 144
7.9 Peptide Adhesion . . . 145
7.10 Protein Coatings . . . 146

8 Bioreactors and Cell Stimulation . . . 149

8.1 Bioreactors . . . 149
8.2 Stimulation in Bioreactors . . . 156
8.3 Biomagnetic and Bioelectric Stimulation in Bioreactors . . . 159
8.4 Hormones and Cytokines . . . 161

9 Scaffold Structure and Fabrication 163

9.1 Scaffold Structure 163
9.2 Scaffold Fabrication 167
9.3 Scaffold-free Micromass Units 173

10 Tissue Reconstruction in vivo 179

10.1 Animal Models used for Bone Tissue Engineering 181
10.2 Animal Models used for Cartilage Tissue Engineering 186
10.3 Clinical Applications 187
10.4 Immunological Aspects 196

11 Clinical Handling and Control 199

11.1 Regulatory Issues 201

12 Ethical Considerations in Bone and Cartilage Tissue Engineering 203

13 Future Prospects 207

13.1 Customised Tissue Reconstruction 207
13.2 Vascularisation 208
13.3 Genetic Engineering 209

References 213

Subject Index 255

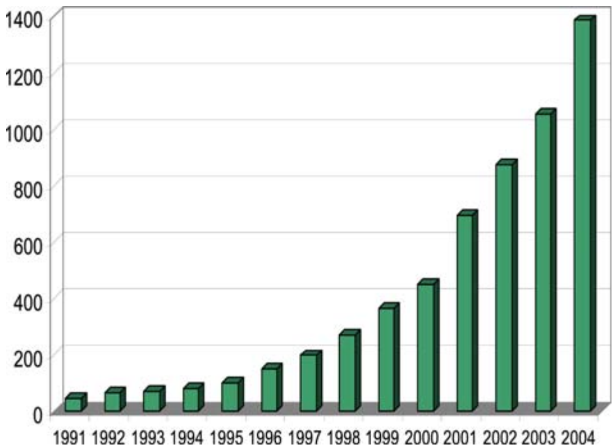
Introduction

1

With the advancement of the concept of regenerative medicine and in particular the field of tissue engineering, a completely new strategy of patient treatment can be expected, with the potential to change treatment regimes profoundly (Fodor, 2003). Tissue engineering aims at regenerating defective or lost tissues or organs, and signals a transition from the historically material-based approaches, in which mechanically stable, biocompatible materials were used, to focus on cell-based devices (Hunziker, 2002; Parikh, 2002). The new approach combines the biological properties of living cells and the physical properties of specially designed materials in order to create artificial organs (Orban et al., 2002). To rapidly restore full functionality in the case of lost organs, artificial tissues are created ex vivo and are implanted at the site of the lesion. Improved quality of life in the sense of accelerated healing and better treatment outcomes can be envisaged, ideally accompanied by overall lower treatment costs (Garfein et al., 2003). Tissue engineering is a rapidly growing discipline and research in this field is steadily increasing, indicated by the increasing number of publications in biological and material science journals as well as in medical journals (Fig. 1.1).

Whereas research effort in this field covers a broad area of biological and material science, clinical tissue engineering is still in its initial phase (Lavik and Langer, 2004). The first tissue-engineered product (cartilage) was approved for clinical use in 1996. Since then, developments in basic and clinical research have resulted in several commercialised products. Skeletal tissue engineering, especially cartilage tis-

Fig. 1.1. Year upon year increase in the number of relevant publications



sue engineering with some bone engineering products on the market, is the leading field in extracorporal creation of human substitute tissues, followed by replacement of skin by tissue-engineered skin products (Lysaght and Reyes, 2001). The reason for the early introduction of these artificial tissue substitutes is the relative simplicity of these tissues. While they are on the way to gain acceptance in routine clinical practice, more advanced products such as multicellular systems and scaffold-free matrix systems are currently under development and may soon be in the pipeline (Lysaght and Reyes, 2001; Stock and Vacanti, 2001).

The economically most important skeletal tissue substitute is cartilage. Cartilage is present in the human body in loaded (mainly joints and intervertebral discs) and unloaded locations (e.g. ear and nose). As cartilage has only a limited capacity to regenerate itself, regeneration-supporting therapies are necessary in most clinical cases (Kaufman and Tobias, 2003). Lesions in cartilage are a significant problem in medical practice, with approximately one million patients treated annually, and approximately 250,000 knee arthroplasty procedures per year (Hunziker, 2002). It is well known that lesions that are confined to the articular cartilage alone have little or no capacity to heal. In general, the patients become symptomatic and a significant progression to osteoarthritis is possible (Lohmander, 2003). However, those lesions that penetrate the subchondral bone have access to the bone marrow space and chondroprogenitor cells and therefore have a limited repair capacity.

Most therapies are aimed at healing hyaline cartilage defects in loaded locations, whereas only a few cases are intended to regenerate non-loaded cartilage defects. Over the last decade autologous chondrocyte transplantation (ACT) has attracted much scientific and commercial interest. ACT and its several modifications are the most widespread applications of skeletal tissue engineering. The use of autologous chondrocytes was primarily performed in traumatically damaged knee joints (Vangsness et al., 2004). Based on new findings in cartilage tissue engineering, preclinical studies suggest expansion to joints other than the knee, to treat also non-traumatic cartilage defects (arthrosis, arthritis defects), and to repair complex tissue defects (osteochondral defects) by a combination of bone and cartilage products. It is assumed that the use of tissue-engineered cartilage products will gain further importance in the near future, developing a significant market size (Table 1.1). The expectation of a steadily increasing tissue-engineered cartilage market is based on the assumption of some restrictions on the current state of ACT being overcome in future research:

- The restriction of ACT to traumatic knee injuries
- The restriction of the recently limited health insurance reimbursement
- The required cooperation of patients (preferred non-loading period after transplantation).

New developments in cartilage tissue engineering (self-adhesion scaffold-free micromass tissue) promise simplification of surgical procedures and therefore open up new application fields in the cartilage repair market (Fig. 1.2).

Tissue-engineered cartilage products have recently been extended to three-dimensional constructs with the aim of regenerating or replacing intervertebral discs (O'Driscoll, 2001). The combination of chondrocytes and biomaterials in the form of spheres is in the preclinical testing stage and may be a therapeutic option in arthritic joints. All the ongoing developments open up the possibility of larger, eco-

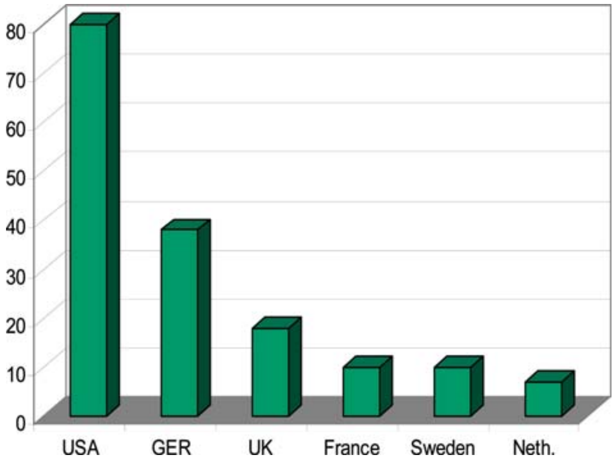


Fig. 1.2. Relevant applications by country

Table 1.1. Market for ACT procedures and companies involved

Country	ACT procedures/year	Calculated sales	Main companies
USA	2,000–3,000	€ 16–24 Mio	Genzyme Carticel
Germany	600	€ 3 Mio	Co.don Verigen ACI Chondrotransplant BioTissue
UK	300–850	€ 1.5–4.3 Mio	Verigen ACI
Italy	300–400	€ 1.5–2 Mio	Fidia Adv. Biomat.
Spain	40	€ 187,000	IsoTis
Total	3,240–4,850	€ 22.2–33.3 Mio	

nomically attractive market segments (Reddi, 1994). The promise of cartilage tissue engineering is mirrored by the number of core tissue engineering companies that are directly involved in cell-based tissue construction (Table 1.1).

In contrast to cartilage tissue engineering, commercial bone tissue engineering is at the beginning of clinical practice (Ripamonti and Tasker, 2000). Whereas many products claim to be tissue-engineered bone (biomaterial/growth factor products), most of them are not cell/biomaterial combinations and thus do not fully comply with the definition of tissue engineering used in this book (Calvert et al., 2003). Only a few companies are engaged in this area. The problems of commercial bone tissue engineering are based on:

- The established treatments in cases of small defects
- The need to reconstruct bone in acute cases (e.g. bone loss by accident)

- The required size and complexity of bone tissue substitutes (e.g. bone loss through ablative tumour surgery).

It is important to recognise that the worldwide market for bone replacement and repair strategies is estimated at about €300 million (Concord Corporate Finance Research). To date, synthetic bone materials, as well as xenogenic, allogenic or autologous materials are the preferred bone substitutes in clinical practice, but with improvements in tissue engineering technology the use of extracorporal grown bone tissue may cover a significant part of this market in the future (Hing, 2004; Salgado et al., 2004).

Tissue engineering is not a precisely defined term. Rather it is a multidisciplinary field of research, combining various aspects of medicine, biology, material science and engineering. Until recently, there were different definitions of tissue engineering, resulting in differences in what is meant by tissue engineering. Two main definitions of tissue engineering are in current use.

The US National Science Foundation defined tissue engineering as:

> The application of the principles and methods of engineering and the life sciences towards the fundamental understanding of structure/function relationship in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve function.

The European Commission (Scientific Committee on Medical Products and Medical Devices; SCMPMD) preferred a narrower definition:

> Tissue engineering is the regeneration of biological tissue through the use of cells, with the aid of supporting structures and/or biomolecules.

The main difference between the two definitions is that the US definition includes the various approaches of in vivo tissue engineering strategies (biological and biophysical stimulation with or without supporting materials) as well as the use of pure cell therapies (autogenic, allogenic, xenogenic), whereas the EU regulations relate to the idea of extracorporal tissue engineering.

This book covers bone and cartilage tissue engineering as it relates to the EU definition. Whereas the main focus is set on generating bone or cartilage tissue ex vivo, the present book considers also borderline approaches (cell therapies), and overviews alternative tissue engineering strategies in the broader meaning (in vivo strategies). As the EU definition covers products that combine cells with degradable materials and, if used, biomolecules such as differentiation or growth factors, the different chapters of the book follow, in a methodical approach, the various aspects of extracorporal bone and cartilage engineering. Basic information on skeletal science, cell biology, and material science (as the main areas for cell/scaffold development) is given in detail initially. In order to better understand the prerequisites of bone and cartilage tissue engineering, the functional features of both tissues are presented, and special focus is given to the histological and ultra-structural appearance of both tissues. Additionally, the principle mechanisms balancing skeletal formation and turnover are described in order to identify possible targets that can be exploited in tissue engineering strategies. It is important to note, that repair, as distinct from regeneration, involves replacement of the damaged, defective or lost tissue with functional tissue that is not necessarily identical to the normal original tissue. As tissue engineering is aimed at generating the original tissue, the terms regeneration and repair are sometimes used in a broader and more common sense. The description of tissue function is followed by biotechnological issues such as cell/material interactions, use of bioreactors, biophysical stimulation of cells and recent ex vivo tissue micromass developments. The in vivo evaluation of bone and cartilage engineering, mainly through animal models, as well as clinical use are highlighted in the final parts of this book. Special emphasis is also given to regulatory and ethical issues in this new and emerging medical field. In consideration of all aspects of bone and cartilage tissue engineering and the overwhelming body of literature covering experimental and clinical studies, it must be noted that no common or consensus approach has been advised. This book therefore considers the most important aspects of each partial issue.

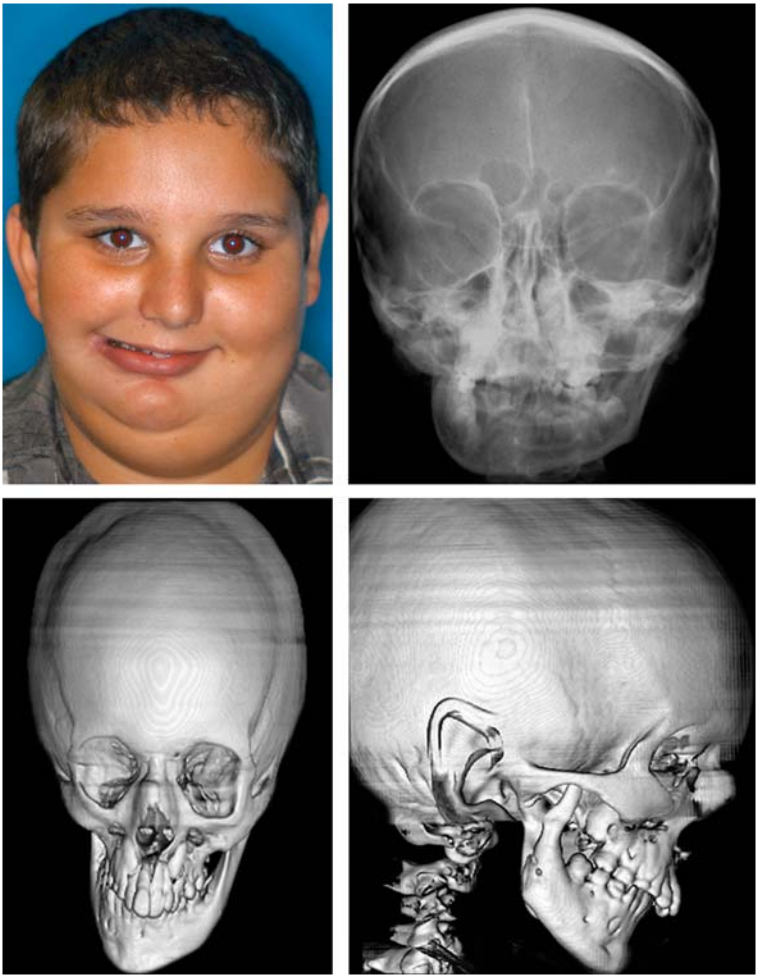
For the successful transfer of bone and cartilage tissue engineering to clinical practice it is important to have a clear and precise definition of clinical demands and of the problem being addressed (backward planning). Regardless of whether the application is focused on replacing or augmenting bone or cartilage tissue, the approach should start with identification of the individual clinical problem. The severity and location of the skeletal defect need to be precisely assessed before tissue engineering is planned. In particular, the complex anatomical nature of the skeletal defect to be substituted with extracorporally generated tissue constructs has to be taken into account (Fig. 1.3). This identification is the first step in defining the required properties of the extracorporally fabricated cellular hybrid material.

Fundamental aspects that should be considered in bone and cartilage tissue engineering include:

- The evaluation of the specific advantages of implanted cellular bone constructs over non-living materials
- Proven clinically applied models for skeletal tissue regeneration
- Mechanical properties of the implant materials
- Immunological reactions towards the cell/scaffold construct.

Whereas some of these aspects have been tested in preclinical and clinical studies, others have not or have only rarely been investigated in a standardised clinical setting. Therefore, when bone and cartilage tissue engineering is considered as an alternative surgical approach to the reconstruction of skeletal defects, experimental as well as clinical issues should be addressed in a comprehensive manner in order to improve the clinical outcome (for a review, see Rose and Oreffo, 2002).

Additionally, a functional definition of tissue may turn out to be more important than a biochemical or structural definition. Consequently, the focus has recently shifted towards functional tissue engineering to address issues that are relevant to the reconstruction of tissue that has a biomechanically relevant function (Reinholz et al., 2004).



Dge, /,1, The clinical problem, exemplified by a complex craniofacial malformation, encompassing a deficiency of mandibular bone and cartilage tissue

Bone and Cartilage

2

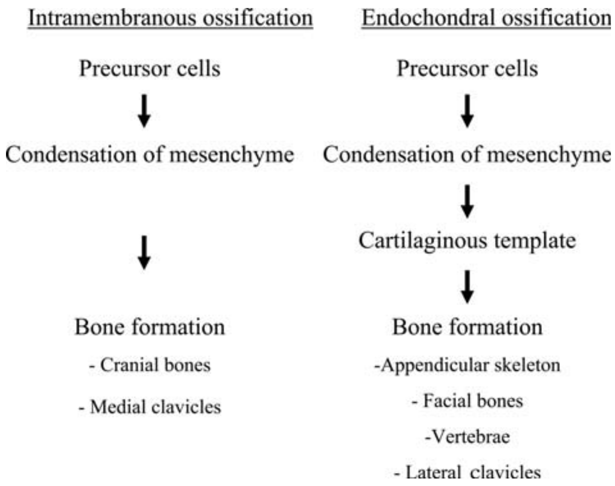
The first step in approaching bone and cartilage tissue engineering is the understanding of the structure and function of these tissues at the different hierarchial levels. The skeleton is a highly specialised form of connective tissue that is nature`s provision for an internal support system in all higher vertebrates. It is a complex living structure in which two main tissues are present: bone tissue and cartilage tissue. Cartilage and bone are specialised connective tissues that provide the body with mechanical support and protection. Both tissues have some common features in that they provide a unique combination of rigid, yet plastic, living tissue. Additionally, in endochondral ossification, one type of bone formation, cartilage models are converted to bone. In contrast to bone, where a variety of cell sources are responsible for the structural and functional integrity, cartilage tissue is a more uniform cellular tissue (Table 2.1). Human joints, as specialised parts of the skeleton, are composed of several different tissues (cartilage, calcified cartilage, bone) that interact in complex ways to allow joints to function. In addition to the structural properties, the skeleton actively participates in the body`s physiology (e.g. calcium homeostasis).

2.1 Bone Structure and Function

Bones are the principal support for the body. They provide a rigid structure for muscle attachment and constitute a system of levers that turn muscle contraction into purpouseful movements. Cartilage augments this function by providing flexible support. Bones are formed through two distinct developmental processes (Fig. 2.1). Endochondral ossification gives rise to long bones that comprise the appendicular skeleton, facial bones, vertebrae, and the lateral clavicles. Intramembranous ossification gives rise to the flat bones that comprise the cranium and medial clavicles.

R_`jc 0,/, Cells of bone and cartilage tissue

Amprga_j `mlc	A_lacjjmsq `mlc	A_prgj_ec
– Osteoblasts – Osteocytes – Lining cells – Osteoclasts – Endothelial cells – Fibroblasts	– Osteoblasts – Osteocytes – Lining cells – Osteoclasts – Endothelial cells – Fibroblasts – Marrow cells	– Chondrocytes

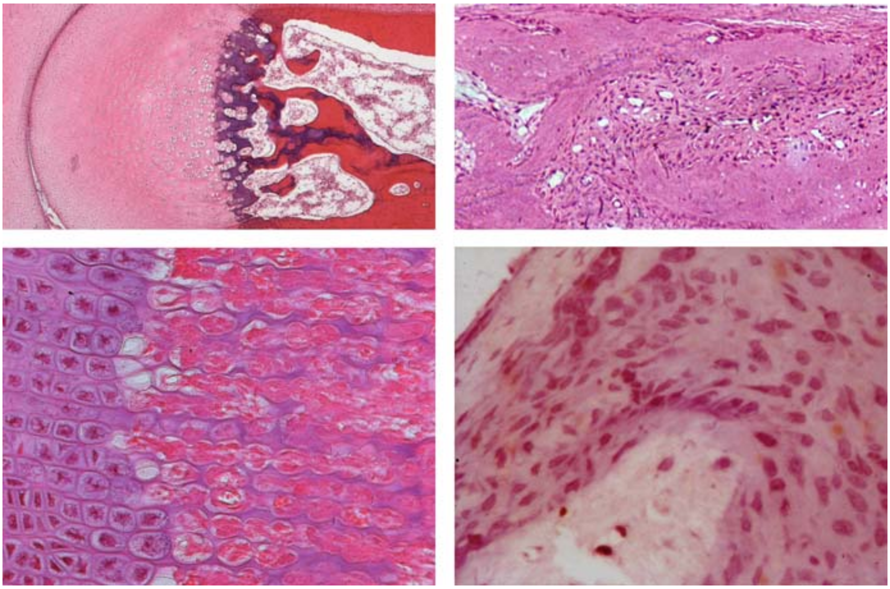


Dge, 0,/, Bone-forming pathways

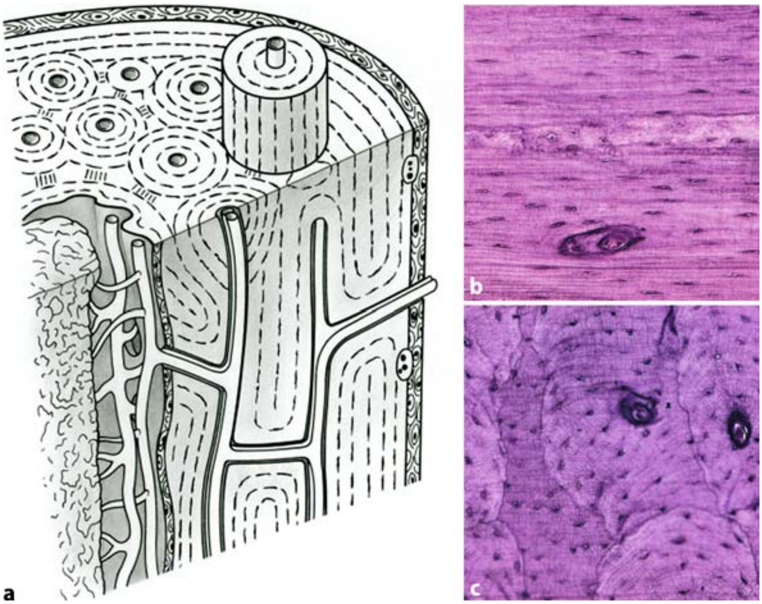
Both types of ossification involve an initial condensation of mesenchyme and the eventual formation of calcified bone. However, intramembranous bone formation accomplishes this directly, whereas endochondral ossification incorporates an intermediate step in which a cartilaginous template regulates the growth and patterning of the developing skeletal element (Fig. 2.2).

Bone is composed of a variety of cell types and an organic matrix that is strengthened by matrix-associated calcium minerals (primarily calcium and phosphate in the form of hydroxyapatite). The organic matrix consists mainly of collagen (approximately 95%), the remaining organic component of 5% is composed of proteoglycans and numerous non-collagenous proteins. Morphologically there are two forms of bone, which impose different structural and functional properties: cortical and cancellous bone. Fig. 2.3 visualises the structure of cortical bone: densely packed collagen fibrils form concentric lamellae, fibrils in adjacent lamellae run in perpendicular planes towards the concentric lamellae. Blood vessels form a three-dimensional network, located in the centre of osteons and penetrating the cortical bone layer perpendicular to the osteons. Blood vessels are also present in the outer layer of cortical bone. The main cellular component of cortical bone is the osteocyte, a mature osteoblast surrounded by mineralised matrix. The osteocytes have the capacity not only to synthesise, but also to resorb bone matrix to a limited extent. Osteocytes occupy a space (lacuna) within the matrix and extend filopodial processes through canaculi to other osteocytes. The cells are connected to each other by gap junctions. The network of osteocytes is assumed to play a fundamental role in cell communication, important in functional adaption of bone to external stimuli.

Cancellous bone has a loosely organised porous structure (Fig. 2.4). The structure of the trabecular system is optimised to transfer the loads through the bone by a dynamic feedback between load perception of cells and their subsequent cellular reaction. The trabeculae are covered by osteoblasts and bone-/lining cells. Whereas osteoblasts actively secrete the components of the extracellular matrix in order to



Dge, 0,0, Priciples of bone formation. *Top:* Overview. *eft:* Endochondral ossification of the epiphyseal growth plate. *Right:* Intermembraneous ossification of calvarian suture. *Bottom:* Detail aspect. *eft:* Endochondral ossification. *Right:* Intermembraneous ossification

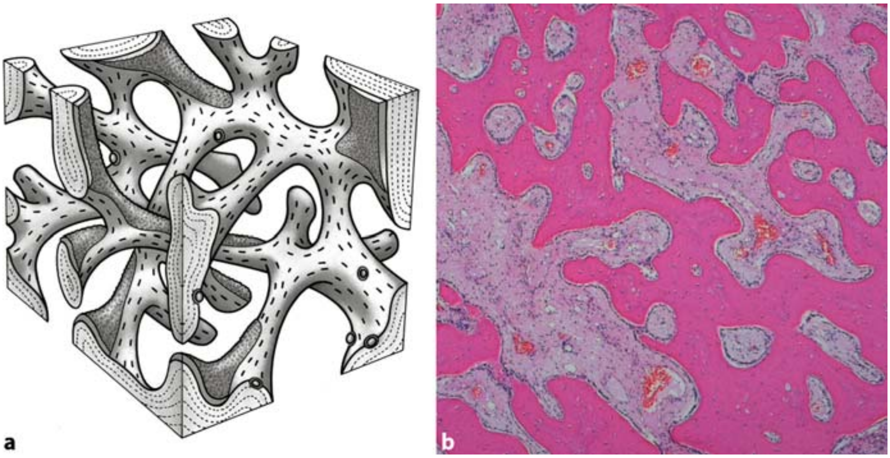


Dge, 0,1, Structure of cortical bone. _ Schematic. ` Longitudinal section. a Transversal section build up bone tissue, bone-lining cells are in an inactive state. They cover surfaces where neither bone formation nor resorption is present. In contrast to the former cells, which originate from local osteoprogenitor cells, osteoclasts arise from the fusion of mononuclear precursors. They can be located in cortical as well as in cancellous bone. Their presence is indicative of bone resorption activities.

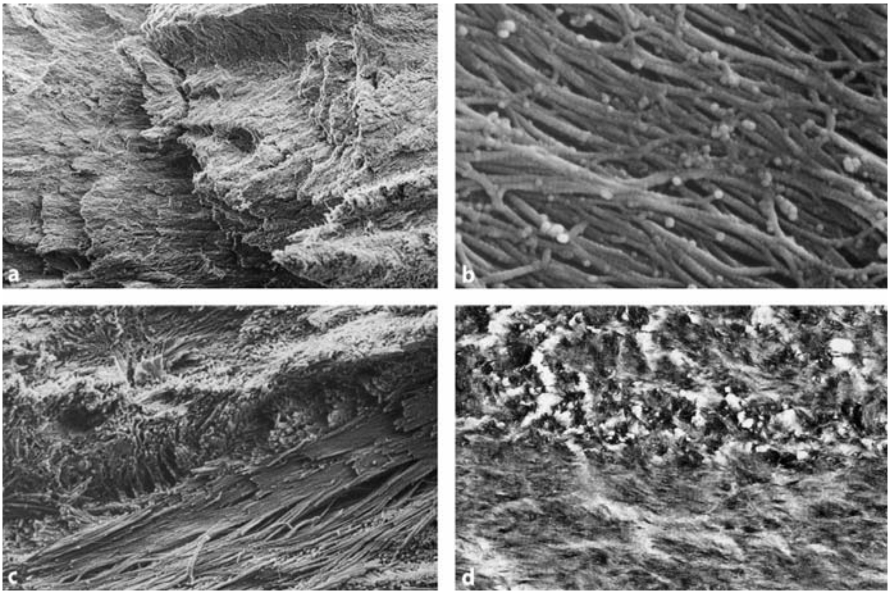
The difference between cortical and cancellous bone is not only structural but also functional. The differences in their histologic and ultrastructural appearance are related to their primary functions: the cortical part of bone provides mechanical and protective functions, whereas cancellous bone is more involved in metabolic functions (e.g. calcium homeostasis). Both aspects (structural and metabolic) are closely related to the features of the mineralised extracellular matrix. In order to engineer a bone-like tissue in vitro in an attempt to mimic the natural tissue structure (biomimetics), understanding of hard tissue formation and mineralisation processes in bone is fundamental. Additionally, as the ex vivo grown tissues interact with the host cells and the extracellular matrix at the implantation site, normal repair mechanisms have to be considered and even enhanced to accelerate the defect repair.

2.2 Matrix Mineralisation

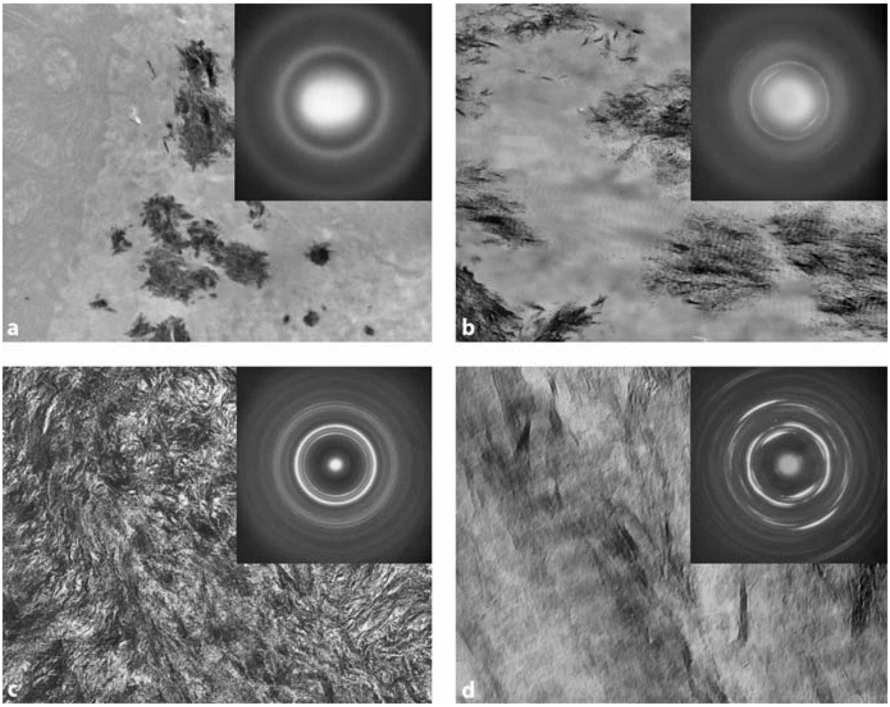
The final fate of bone repair and regeneration is the establishment of a mineralised, mechanically competent tissue. Skeletal mineralisation itself is closely related to the features of the extracellular matrix of bone and cartilage. Collagens, as the major constituent of the extracellular matrix network, are of major importance in the formation of a mineralised and therefore mechanically competent matrix (Fig. 2.5). Concerning the composition and structural organisation of these collagen-rich matrices of the human mineralising tissues, they form a microenvironment that favours apatite nucleation (Fig. 2.6). In the different types of calcified tissues, such as bone and calcified cartilage, structurally different types of collagen exist, with collagen type I being the dominant matrix protein in bone. Ultrastructural investigations of collagen mineralisation in these different calcifying tissues have revealed principal similarities in general but also some differences from the stage of crystal nucleation to tissue maturation (Höhling et al., 1990). It has been found that the first bone mineralisation is connected with matrix vesicles (Anderson, 1967; Bonucci, 1967), which spreads over the border of the vesicle membrane and includes extra- and intra-collagenous mineralisation. Extra- and intra-collagenous mineralisation are complex processes, connected with different characteristic non-collagenous proteins outside and on the collagen surface. This complex process of collagen mineralisation, not yet fully clarified in detail, is still under intensive research (Fig. 2.7). Insight into these fundamental processes may help in generating a mineralised cellular construct ex vivo and may therefore be valuable in generating a mechanical competent bone-like tissue in the laboratory. As various matrix proteins are used as a backbone in tissue engineering (in the sense of coating materials with these proteins or using them as scaffold materials), knowledge of their structure and function is of special relevance. Additionally, some of these proteins can be used as a marker for bone versus cartilage tissue, since some of these proteins are found, exceptionally, in bone or cartilage tissue. The most important structural matrix proteins of bone and cartilage are therefore described in more detail.



Dge, 0,2, Structure of cancellous bone. _ Schematic. ` Histological appearance



Dge, 0,3, _ Scanning electron microscopy (SEM) image of freeze fractured full mineralized bone tissue. ` SEM image of collagen fibres in bone. a*b Longitudinally and transversally oriented collagenous fibres in bone: c SEM of freeze-fractured specimen, d Transmission electron microscopy (TEM) image

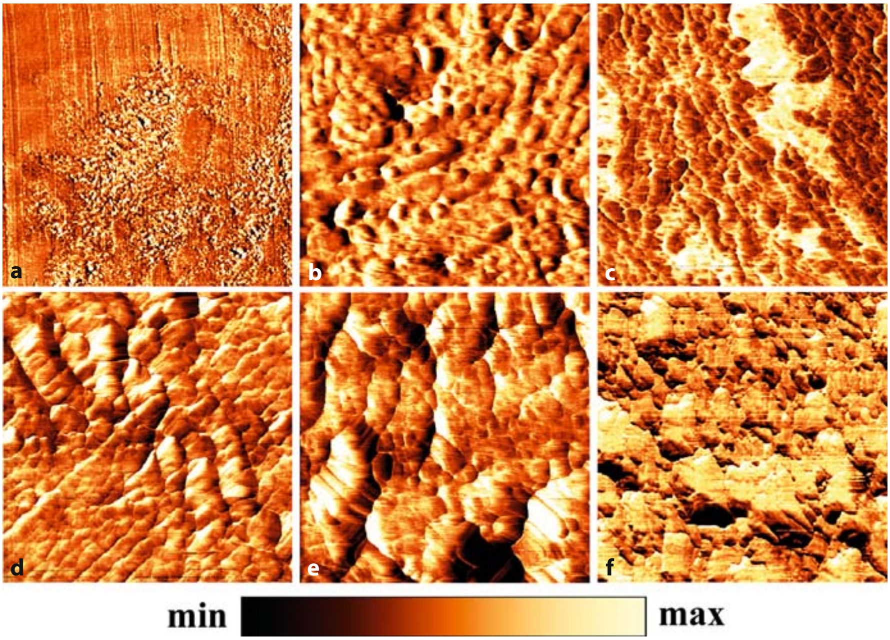


Dge, 0,4, Stages of mineral formation in bone as revealed by transmission electron microscopy (TEM) with electron diffraction analysis (EDA). _ Early mineral formation of matrix vesicles. ` early mineral formation at collagen fibres. a mature globular mineral formation. b mature collagen mineralization. The diffraction pattern of early mineral formation is diffuse; in contrast mature crystals represent a more clear-cut diffraction pattern. Further, in collagen mineralization the crystals are regular ordered and represent therefore a textured pattern

The collagens are the most abundant proteins in the human body, accounting for nearly 30% of all proteins. The different individual collagen types have been labelled with Roman numerals in the order of their discovery. These different collagen types provide the variety of structural units necessary to allow the connective tissue to adapt to the multitude of physiologic functions (Table 2.2).

The collagen types can be grouped according to variations in their structural features (Mayne and Brewton, 1993; Nielsen et al., 1994; Rehn and Pihlajaniemi, 1994; van der Rest and Garrone, 1991). The collagen types I to III, V and XI are banded, fibril-forming collagens, whereas the types IV, VI to X and XII to XIX are non-fibrillar. Additionally, the types IX, XII, XIV, XVI and XIX do not form supramolecular structures by their own, but are integrated in collagens of other types (Mayne and Brewton, 1993; Nielsen et al., 1994; Pan et al., 1992; Yamaguchi et al., 1992).

Collagen is the major constituent of the matrix of all mineralised tissues (except collagen-free enamel and otholites). The mineralising process of the different calcified tissues is characterised by a differentiated occurrence and distribution of the various types of collagen. Some proteins are present in bone and cartilage tissue,



Dge, 0,5, Scanning force microscopy of intramembraneous bone formation. _ Overview of mineralization front (image area 25×25 μm). ` Non-mineralised collagen fibres (image area 2×2 μm). a Pre-osteocyte surrounded by osteoid (image area 5×5 μm). b Early and c late stages of mineralized collagen fibres (image area 2×2 μm). d Fully mineralized bone (image area 5×5 μm)

whereas others are solely found in one of the two tissues. Lane et al. (1986) developed an expression for the sequence of the appearance of collagen types I, II and III in bone healing. Different post-translational modifications distinguish the hard tissue collagen from the type I collagen of soft tissues, including different glycosylation and cross-linking patterns, and possibly differences in phosphorylation (Robey et al., 1992; Robey and Boskey, 1996). Collagen type I is present throughout mature bone and collagen type II in the mineralising cartilage of the epiphysial growth plate. Collagen types II, III and V have specific locations within fracture callus development. Chondrocytes and osteoblasts produce type II and III collagen during the early stages of callus formation. Osteoblasts secrete a matrix of type III collagen fibres along the periosteal surface, which serves as a substrate for cell migration. Type I collagen is secreted in trabeculae of developing bone within the fibrous tissue, whereas type II is formed during endochondral ossification stages in the stage of cartilage mineralisation. Hiltunen et al. (1993) using Northern blotting to evaluate the regulation of the collagens during bone healing confirmed the coexpression of type IX collagen with type II collagen and the coexpression of type X collagen with type I during the stages of endochondral ossification. In particular, collagen type X,found in the hypertrophic cartilage zone of endochondral ossification (Ayad et al., 1987), is suggested to be connected with the mineralisation process.

R_`jc 0,0, Collagens of bone and cartilage tissue

@mlc	A_prgj_ec	Pcecl cp_rc rgqqsc
K_hmp am k nml c lrq « Amjj_ecl Rwnc G « Amjj_ecl Rwnc GGG « Amjj_ecl Rwnc V	K_hmp am k nml c lrq « Amjj_ecl Rwnc GG « Amjj_ecl Rwnc GV « Amjj_ecl Rwnc VG	K_hmp am k nml c lrq « Amjj_ecl Rwnc G « Amjj_ecl Rwnc GG « Amjj_ecl Rwnc GGG
Kglmp am k nml c lrq « Amjj_ecl Rwnc T « Amjj_ecl Rwnc TG « Amjj_ecl Rwnc VGGG « Amjj_ecl Rwnc VGT	Kglmp am k nml c lrq « Amjj_ecl Rwnc GGG « Amjj_ecl Rwnc TG « Amjj_ecl Rwnc VGG « Amjj_ecl Rwnc VGGG « Amjj_ecl Rwnc VGT	Kglmp am k nml c lrq « Amjj_ecl Rwnc T « Amjj_ecl Rwnc GV « Amjj_ecl Rwnc V

Collagen Type I

- Triple-helical glycoprotein
- Molecular weight: 406 kDa
- Fibrillar molecule with 67 nm periodicity
- Major collagen of bone
- Fibrils stabilised by cross-links
- Provides tensile strength to the tissue
- Interacts with decorin and fibromodulin.

Collagen Type VI

- Glycoprotein
- Short collagenous central domain
- 5 nm diameter supramolecular structure
- 100 nm periodicity
- Type VI tetramers are stabilised by disulphide bonds
- No cross-linking by lysine/hydroxylysine-derived bonds
- Present in nearly all connective tissues
- Interacts in vitro with various extracellular macromolecules (type II and XIV collagen, hyaluronan, decorin, biglycan).

Collagen Type XIII

- Short chain, non-fibrillar collagen
- Homotrimeric molecule
- Molecular weight: 180 kDa
- Present in a variety of tissues
- Expressed in bone and cartilage tissue
- May act as a transmembrane protein of focal adhesion sites.

Collagen Type XIV

- Member of a subfamily of collagens (FACIT)
- Disulphide-bonded homotrimeric molecule
- Molecular weight: 607 kDa
- Present mainly in collagen type I rich tissues
- Found also in collagen type II containing tissues
- Expressed in bone and cartilage tissue
- Present in locations of elevated mechanical stress

Concerning the mineralising process in bone and calcified cartilage, it is suggested that not only the type of collagen but the fibrillar network with non-collagenous proteins gives rise to a structure configuration that is necessary for the process of mineralisation. The non-collagenous proteins found in mineralised matrices or cartilage are members of diverse types of molecules (Tables 2.3 and 2.4). The main groups are: glycosaminoglycan-containing molecules so-called proteoglycans, glycoproteins and gamma-carboxy glutamic acid-containing molecules (Robey, 1996). The distribution of these proteins in the collagen- containing mineralised tissues such as bone and calcifying cartilage shows an overlap.

Concerning the role of these non-collagenous proteins in the mineral formation of collagen-containing hard tissues, several aspects have to be considered. Whereas numerous studies indicate that collagen alone is not a direct nucleator of hydroxyapatite mineralisation, rather providing a template for mineral accumulation, nevertheless it becomes mineralised in a characteristic way (Robey et al., 1992; Robey

R_`jc 0,1, Non-collagenous matrix proteins of bone

Ejwamnpmrcgl q	Ej_ npmrcgl q	Npmrcmejwa_l q
– Ostepontin – Osteonectin – Bone sialoprotein – Alkaline phosphatase – Fibronectin – Vitronectin – Thrombospondin	– Osteocalcin – Matrix Gla protein – Protein S	– Decorin – Biglycan – Fibromodulin

R_`jc 0,2, Non-collagenous matrix proteins of cartilage

Ejwamnpmrcgl q	Ej_ npmrcgl q	Npmrcmejwa_l q
– Fibronectin – Vitronectin – Link protein – Chondroadherin – Prolargin – Thrombospondin – Cartilage matrix protein (except articular cartilage, intervertebral disc)	– Matrix Gla protein	– Aggrecan – Biglycan – Decorin – Epiphycan – Fibromodulin – Lumican

and Boskey, 1996). So it is obvious that it is not possible to build up a tissue like bone without collagen. Investigations of the knock-out mouse model have revealed that no or low amounts of collagen type I result in defective bone development (Robey, 1996). These findings support the hypothesis that collagen plays the major role in orienting the mineralisation process, producing a tissue structure with increased biomechanical properties, important for the ex vivo fabrication of a mechanically competent bone-like tissue. A multitude of other matrix proteins are morphologically, structurally and functionally connected to the collagen fibres. Complex interactions between collagen and the non-collagenous proteins (Meyer et al., 1999b) seem to facilitate crystal formation and subsequent crystal growth. Three different non-collagenous proteins are of special relevance in bone formation: osteocalcin, osteonectin, and bone sialoprotein.

Osteocalcin

- Member of the Gla protein family
- Molecular weight: 11 kDa
- Most abundant non-collagenous protein in bone
- Binds tightly to hydroxyapatite
- Regulates apatite crystal growth
- Synthesised specifically by osteoblasts.

Osteonectin

- Single chain polypeptide
- Molecular weight: 32 kDa
- Present in a number of tissues
- Expressed early in bone development
- High concentrations in sites of bone remodelling
- Binds to hydroxyapatite
- Involved in osteoblast adhesion.

Bone Sialoprotein

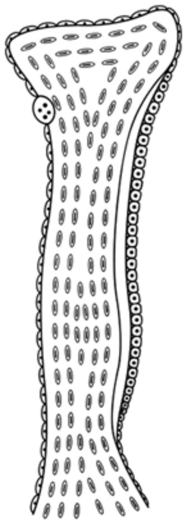
- Single polypeptide chain
- Molecular weight: 6×104 Da
- Comprises predominantly glutamic acid, aspartic acid and glycine
- Present in bone and mineralised connective tissue
- Deposited mainly at sites of de novo bone formation
- Promotes cell attachment via integrin binding
- Tightly connected to hydroxyapatite.

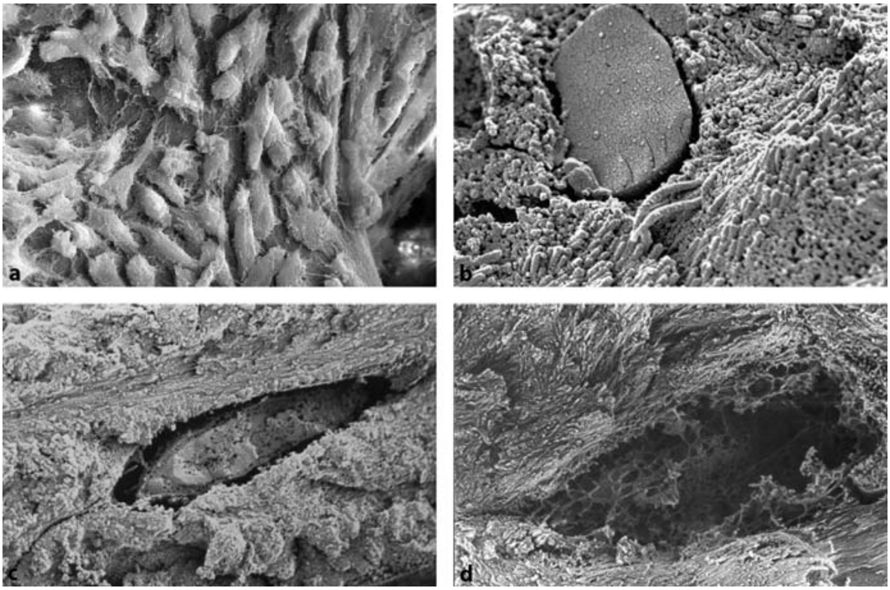
All three proteins are closely connected to hydroxyapatite. They do not only bind to hydroxyapatite but are also functionally involved in mineral formation. Osteocalcin is known to be involved in crystal growth, whereas the function of others (Osteonectin, Bone Sialoprotein) is not understood in detail. Despite their unknown role in the mineralising process, their localisation in the extracellular matrix of calcifying tissues is suggestive of important functional involvement in mineral formation (Butler and Ritchie, 1995).

2.3 Osteoblast Structure and Function

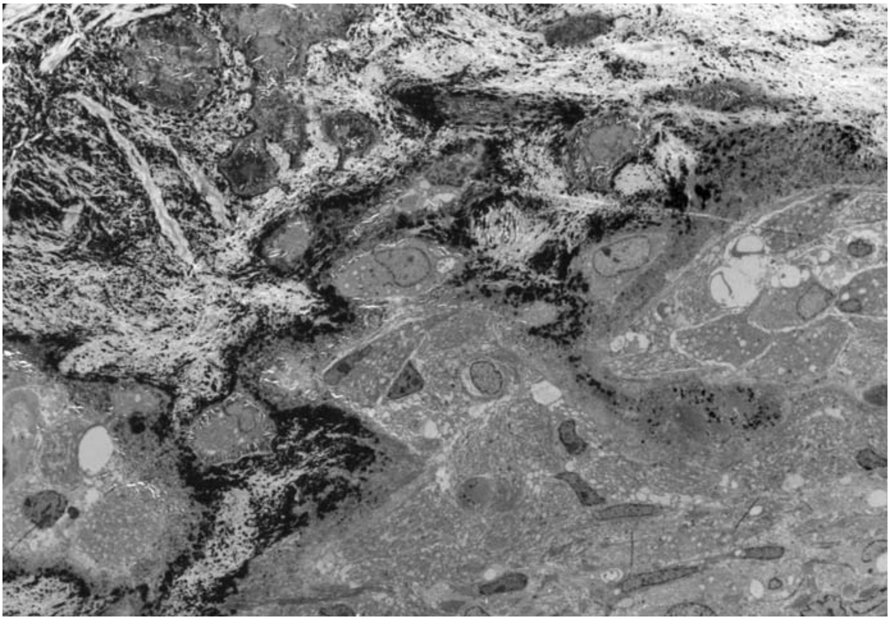
The osteoblast is defined as the critical cell type that forms bone tissue. Four maturation stages of osteoblast development can be distinguished: preosteoblasts, lining cells, osteoblasts and osteocytes (Fig. 2.8). The preosteoblast is considered as a precursor of osteoblasts and lining cells. Preosteoblasts share common phenotypical features of osteoblasts like alkaline-phosphatase activity, but they do not express all markers of mature osteoblasts (Aubin, 2001). Bone lining cells are, in contrast to osteoblasts, synthetically more inactive. The flat, thin and elongated cells cover bone surfaces where no significant remodelling takes place. A proportion of cells becomes embedded in bone; these cells, considered to represent the finally differentiated cell stage, are osteocytes (Figs 2.9 and 2.10). The mature osteoblast phenotype is characterised by the ability of the cells to sythesise a bone matrix that will finally mineralise (Fig. 2.11). Osteoblasts express various phenotypic markers such as high alkaline phosphatase (ALP) and synthesise collagenous and non-collagenous bone matrix proteins including osteocalcin (Aubin and Liu, 1996). Osteoblasts express receptors for various hormones including parathyroid hormonel (Dempster et al., 1993; 1995), 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (Lian et al., 1999), estrogen (Turner et al., 1994; Boyce et al., 1999), and glucocorticoids (Delany et al., 1994; Ishida and Heersche, 1998), which are involved in the regulation of osteoblast differentiation. Osteoblast differentiation is also regulated by various local factors in a paracrine and/or an autocrine fashion (Aubin and Liu, 1996) to enable the cell to perform its main function: the synthesis of a mechanically competent hard tissue. Osteocytes are the most abundant cell type of bone. It is assumed that there are approximately 10 times as many osteocytes as osteoblasts in adult human bone (Parfitt, 1977). During the transition from osteoblasts to mature osteocytes the cells lose a number of osteoblastic characteristics, but acquire other, osteocyte-specific ones, including their typical morphology (for a review see Liu et al., 1977). Mature osteocytes are stellate shaped or dendritic cells enclosed within the lacuno–canalicu-

Dge, 0,6, Scheme of bone trabecula containing different osteoblastic cells





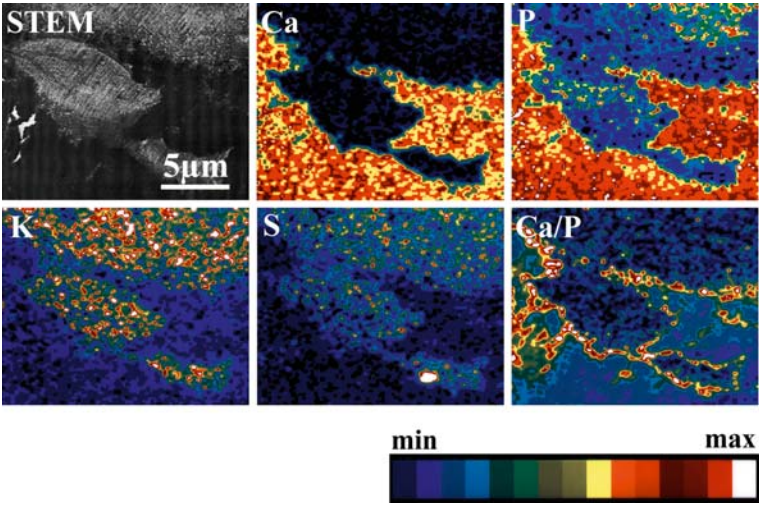
Dge, 0,7, Scanning electron microscopical (SEM) appearance of cancellous bone. _ Osteoblast layer at the surface of bone trabecula. ` Pre-osteocyte surrounded by extracellular matrix (freeze fractured specimen). a Mature osteocyte in mineralised bone matrix. d Pore containing osteocyte lacuna



Dge, 0,/ ., Transmission microscopical appearance of mineralising area demonstrating the transition of osteoblasts to osteocytes lar network of bone. Osteocytes are interconnected and connected with osteoblasts through cell contacts containing gap junctions (Aarden et al., 1994). Osteocytes have a main role in the blood-calcium homeostasis and in the functional adaptation of bones. Cells in all different stages of osteoblast development work together (and with other cells) in an interconnected fashion to dynamically regulate bone development, bone turnover and bone repair.

2.4 Osteoclast Structure and Function

In contrast to osteoblasts, osteoclasts are multinuclear cells derived from hematopoietic stem cells (Suda et al., 1992), thereby sharing common differentiation pathway of macrophages and dendritic cells (Fig. 2.12). The promyeloid precursor cell is able to differentiate into either an osteoclast, a macrophage or a dendritic cell. The differentiation pathway depends on whether the precursor cell is exposed to a receptor activator of various ligands (e.g. RANKL, OPGL, ODF) or colony-stimulating factors (M-CSF, GM-CSF) (Kong et al., 1999; Nutt et al., 1999; Rolink et al., 1999; Suda et al., 1999). The fact that bone marrow stromal cells and osteoblasts produce some of the above mentioned factors as membrane-bound and soluble substances is an indicater for the positive feedback mechanisms of osteoblasts on osteoclast formation (Lacey et al., 1998). Osteoprotegerin (OPG), as a counterpart of the above mentioned factors, strongly inhibits osteoclast formation in vitro and in vivo (Simonet et al., 1997). The inhibitory effect of OPG on osteoclast differentiation is due to the fact that it can prevent the binding of RANKL to its receptor, RANK (Hsu et al., 1999). Osteoclasts have a unique ability to dissolve mineral and degrade organic bone matrix (for a review see Väänänen et al., 2000). They are assumed to be also involved in the degradation process of foreign materials. After migration of the osteoclast to a resorption site, a specific membrane domain, the sealing zone, forms under the osteoclast. The plasma membrane attaches tightly to the bone matrix and seals the resorption site from its surroundings (Väänänen and Horton, 1995). Ultrastructural analysis indicates that osteoclasts contain in addition to the sealing zone, three other specialised membrane domains: a ruffled border, a functional secretory domain and a basolateral membrane. The ruffled border is a resorbing organelle. The fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone (Blair et al., 1989; Väänänen et al, 1990) and the subsequent outside transport of the vesicle content leads to a lowering of the pH below the osteoclast. The low pH in the resorption lacuna is achieved by the action of proton pumps at the ruffled border membrane. The main physiological function of osteoclasts is to degrade mineralised bone matrix. This involves dissolution of hydroxyapatite minerals and proteolytic cleavage of the organic matrix. After dissolution of the mineral phase, proteolytic enzymes degrade the extracellular matrix. The organic degradation products are removed from the resorption lacuna through a special transcytotic pathway in order to liberate them into the extracellular space (Nesbitt and Horton, 1997; Salo et al., 1997).



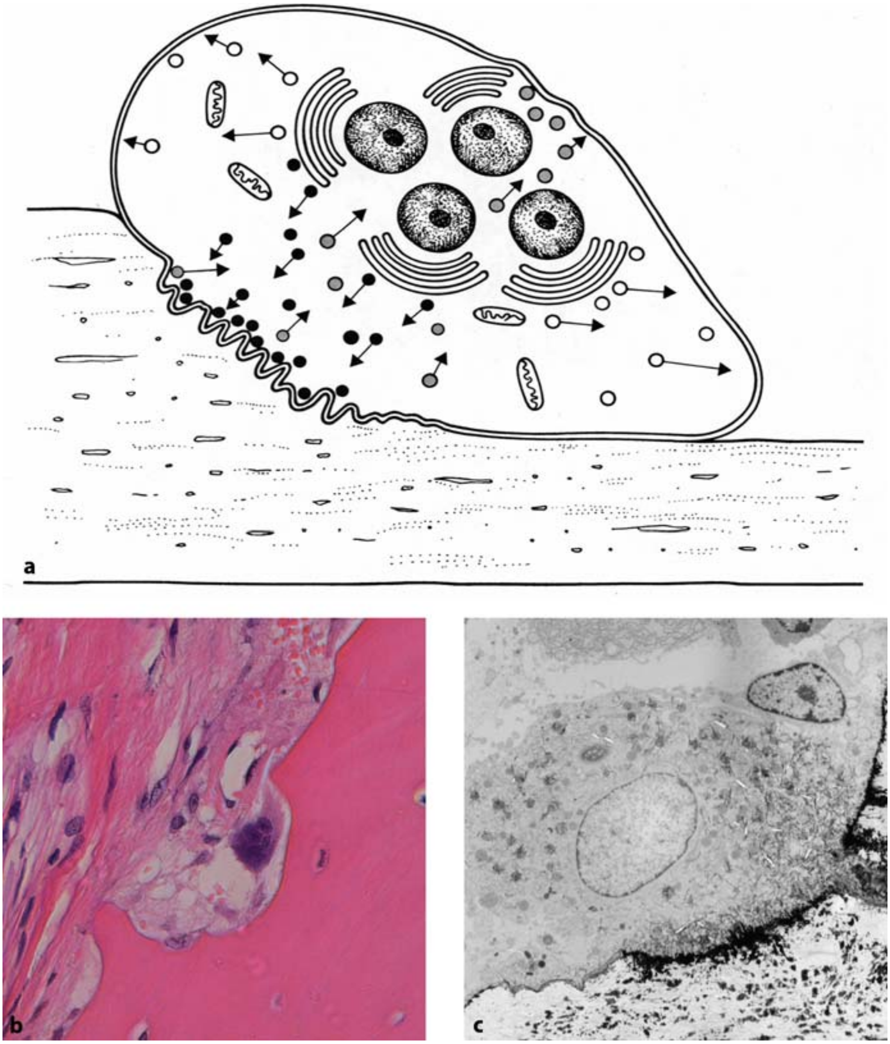
Dge, 0,//, Scanning transmission electron microscoy (STEM) image of bone mineral forming preosteocyte with energy dispersive X-ray analysis of element distribution for calcium (Ca), phosphorus (P), potassium (K), sulphorus (S) and the relation of calcium to phosphorus (Ca/P)

2.5 Bone Regeneration

Bone is one of the body´s tissues that have the ability to recapitulate specific aspects of their initial developmental processes and thereby undergo regeneration to a stage of repair, ad integrum under some circumstances. The repair of bone defects is one such regenerative process (Vortkamp et al., 1998; Ferguson et al., 1999). The post-natal tissue environment in which the regenerative processes of defect healing takes place is different in a number of specific respects from those present during embryological development, but the potential effects on the developmental process of defect healing are essential to our understanding of bone morphogenesis, and form the basis for bone tissue engineering approaches.

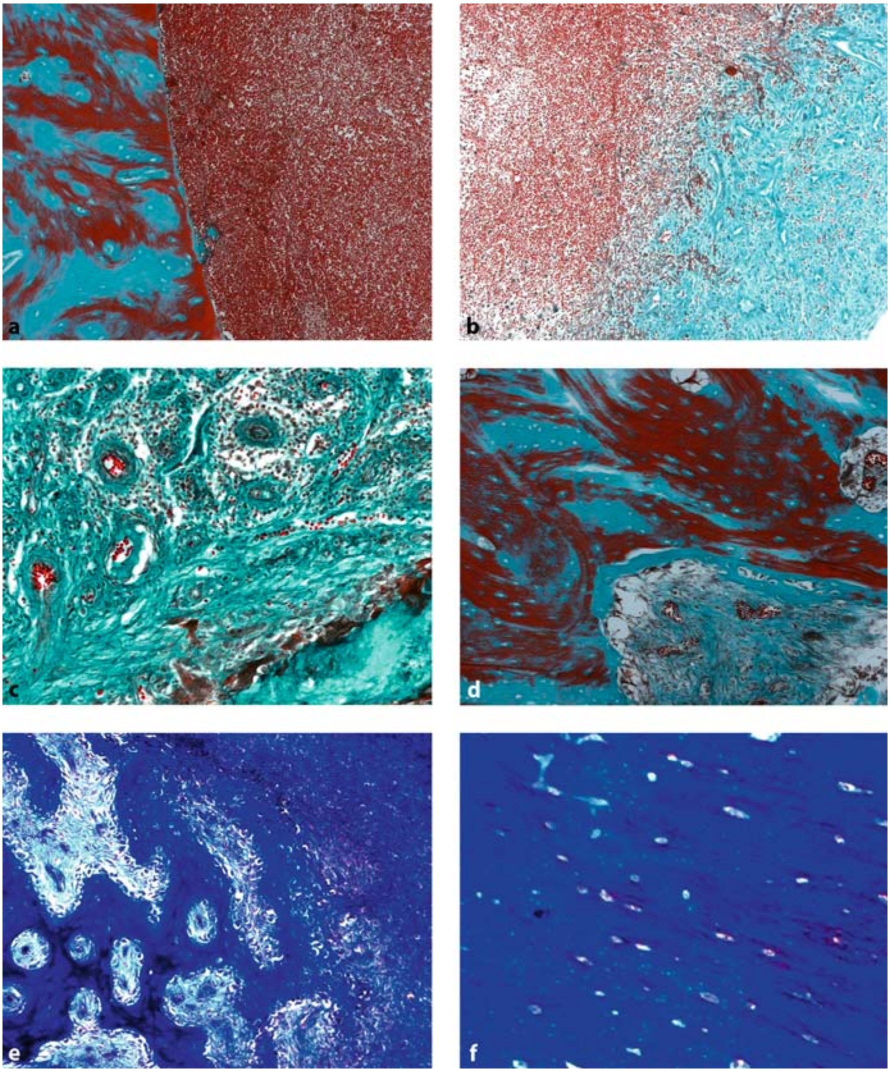
The instructive interactions between cells that are initiated within the bone repair and regeneration process must therefore occur between the newly formed tissue, the external soft tissues covering the defect site, and the adjacent cortical bone and bone marrow. Various sources of cells (periosteal cells, cortical cells, cells derived from the surrounding soft tissues, and marrow cells) and signals that set up these fields are responsible for the features of the repair tissue (Fig. 2.13). The primary tissue source of cells that form the repair tissue are believed to be from the periosteum (Nakahara et al., 1990a). Cells within the periosteum produce various morphogens starting from the onset of tissue disintegration (Bostrom et al., 1995), suggesting that these signal molecules act locally to recruit and induce skeletalgenic cells to proliferate and differentiate.

Other cells that contribute to repair tissue formation appear to be derived from the adjacent cortical and cancellous bone. It may be speculated that at least some of these cells are likely to have arisen from the proliferative expansion of committed



Dge, 0,/0, _ Schematic drawing of a resorbing osteclast, demonstrating the zonal structure at the resorption site. ` Osteoclast located in a resorption lacuna. a TEM appearance of active bone resorption at the ruffled border of an osteoclast

skeletal precursors. Mesenchymal stem cells, assumed to be derived from either the surrounding muscle tissue or the marrow space, are the third source of cells that participate in the formation of new bone. Cells synthesise a network of collagenous and non-collagenous proteins. The regenerate tissue contains, in addition to collagen type I and II, mainly collagen types III, V, IX and X.



Dge, 0,/1,Stages of bone repair. _ Blood clot formation. ` Substitution of blood cells by a matrix-rich repair tissue. a Invasion of blood vessels. b Organisation of predetermined osseous tissue. c Trabeculae formed by osteoblasts. d Final repair tissue

Collagen Type III

- Triple-helical glycoprotein
- Thin fibrillar molecule with 67-nm periodicity
- Fibrils stabilized by cross-links
- Molecular weight: 415 kDa
- Collagen present in small amounts in bone and cartilage

Collagen Type V

- Minor fibrillar collagen
- Thin fibrillar molecule with 67-nm periodicity
- Forms heterotypic fibrils with collagen type I or III
- Forms cross-type heterotrimers with type IX collagens
- Molecular weight: 421 kDa
- Interacts in vitro with various extracellular macromolecules (thrombospondin, decorin, biglycan)

Collagen Type X

- Short chain collagen
- Homodimer protein
- Molecular weight: 198 kDa
- Temporally and spatially regulated during fetal development
- Synthesised by hypertrophic chondrocytes
- Present in calcifying cartilage.

The mode of bone repair tissue critically depends on the defect size. Whereas non-critical size defects heal spontaneously, critical size defects will not completely fill with new bone tissue. As nutrition is another important parameter in determining the features of repair tissue formation, diffusion and vascularisation are two factors that influence to a great extent the outcome of histogenesis. Whereas the nature and origins of the initiating morphogenetic signals for the skeletogenic cells in the repair tissue are not completely determined, a multitude of signal factors have been identified (Table 2.5). Signals that initiate and maintain the bone-forming mechanisms are assumed to arise from the periosteum or bone marrow or are released from the

R_`jc 0,3, Signals involved in bone repair

Kmjcasjcq-Rgkc	Glgrg_j	C_pjw	J_rc	Pckmbcjjgle
Cytokines				
Interleukines	x			
Interferone-γ	x	x	x	
TNF-α	x			
TNF-β	x			
RANKL	x		x	
OPG	x	x		
MCSF	x	x		
Morphogens				
TGF-β (1–3)	x	x	x	
BMP (2–7, 8)	x	x	x	
ILGF (1,2)		x	x	
GDF (1,5,9,10)	x	x	x	
Various				
VEGF	x	x	x	x
Ang. (1,2)		x	x	x
MMP (2,8,9,13)		x	x	x

injured bone matrix, many of these signals are part of the inflammatory processes (Einhorn et al., 1995; Barnes et al., 1999). The presence and time of appearance of these signal molecules are of special relevance, since such molecules can be used in tissue engineering approaches to improve tissue formation in vitro and in vivo (for reviews, see Lieberman et al., 2002; Gerstenfeld et al., 2003a; 2003b).

The role of inflammatory cytokines, morphogens, proteases, and angiogenic factors in directing the repair response has recently given rise to increased scientific interest, yet the role these molecules play in the regulation of bone remodelling are not completely determined (Gowen et al., 1983; Kimble et al., 1994; 1997; Barnes et al., 1999). Beneath interleucines, interferones and tumornecrosis factor alpha, members of the TGF-β superfamilie are assumed to be of major relevance in skeletalgenesis and bone repair (Joyce et al., 1990; Rosier et al., 1998). There is recent evidence that specific members of the transforming growth factor-β superfamily, including multiple BMPs, GDFs, and TGF-β1-3, act in combinations with other factors (e.g. metalloproteases) to promote the various stages of repair tissue formation (Cho et al., 2002).

Bone healing creates a demand on the surrounding tissues to increase blood flow so that induction of bone regeneration can occur within the larger sized defects. Ingrowth of vascular tissues into the developing tissue proceeds with the development of the new periosteal bone and occurs mainly from where the periosteal response originates. Thus, there is evidence that the interaction of the vascular elements and the initiation and propagation of the periosteal response are the primary driving mechanisms that enhance the new bone formation, especially in larger defects. It has also been suggested that periovascular mesenchymal cells that exist in blood vessel walls contribute to the initiation and maintenance of defect healing (Bouletreau et al., 2002a; 2002b). The influence of vessel- formation-dependent adequate blood flow on optimal bone healing has been well established (Glowacki, 1998; Rowe et al., 1999; Gerber and Ferrara, 2000; Einhorn and Lee, 2001). Furthermore, cartilage tissue formation (as an intermediate or final state of repair tissue) is assumed to require the coordination of both the molecular mechanisms that regulate the bone`s extracellular matrix remodelling capacity and the influencial processes of vascular cells (Vu et al., 1998). Thus, matrix degradation and angiogenesis are either correlated or concurrent processes during tissue formation. The formation of new vessels is assumed to be regulated by two separate pathways: a vascular endothelial growth factor (VEGF)-dependent pathway and an angiopoietin-dependent pathway (Suri et al., 1996). It seems that both sets of regulatory pathways regulate and modulate defect repair. The VEGF related family of molecules are essential endothelial-cell-specific mediators of neo-angiogenesis (Ferrara and Davis-Smyth, 1997), whereas angiopoietin 1 and 2 are molecules that directly control vascular growth. The latter molecules are assumed to act during the whole period of new bone tissue formation. They seem to be related to the formation of larger vessel structures and the development of co-lateral branches from existent vessels. Evidence from a multitude of experimental studies indicates that a high number of signal molecules are essential for skeletal regeneration and suggest that there seems to be an intimate relationship between them. The orchestrated action of the various sources of cells and signal molecules allow for the coordinated regulation of events that lead to bone repair. The final outcome of defect repair is initiated and modulated by these factors, but whether such a repair process will lead to a complete bone repair is dependent on a number of boundary conditions (defect size, tissue biomechanics, host conditions).

2.6 Cartilage Structure and Function

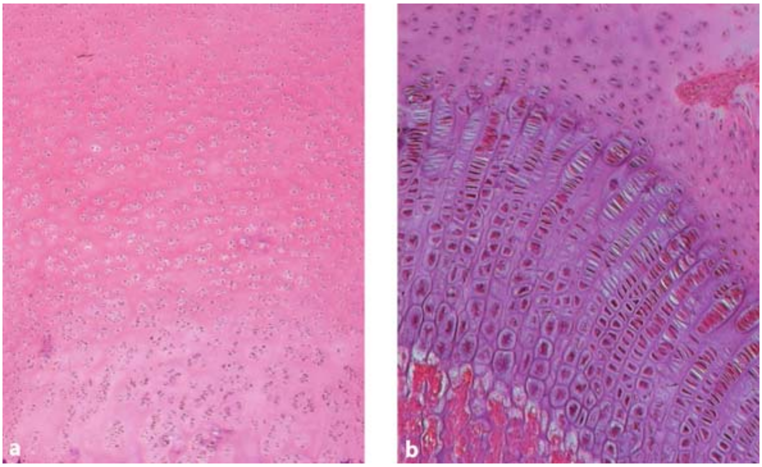
Adults have cartilage on their articular surface of long bones and in the trachea, bronchi, nose, ears, larynx, and intervertebral disks. Cartilage contains cells, fibres, and amorphous ground substance. It is dominated by the acellular elements and is devoid of blood vessels and nerves. Cartilage characteristics make it an excellent skeletal tissue for the fetus. Most adult bones existed as cartilaginous models during fetal life. Three types of cartilage can be destinguished: hyaline cartilage, elastic cartilage, and fibrocartilage. Hyaline cartilage is a bluish, opalescent tissue that is widely distributed and the most abundant type of cartilage present in the human body. Hyaline cartilage exists on the articular surface of bones, the ventral aspects of ribs, in the larynx, the trachea, and in the bronchial tree. Hyaline cartilage also exists at the epiphysal plates in the bones of fetuses and growing children. Elastic cartilage is present in the auricle of the external ear, the walls of the external auditory meatus, the eustachian tube, the epiglottis, and parts of the larynx. The main function of elastic cartilage is to ensure the patency of the lumina of tubes that are surrounded by this variety of cartilage. Fibrocartilage exists in the annulus fibrosus of intevertebral discs, the symphysis pubis, and the junctions between large tendons and articular cartilage in large joints. Intervertebral discs are specialised structures that consist of an outer layer of fibrocartilage (called annulus fibrosus) and an inner part of a special liquid connective tissue (nucleus pulposus). Despite the importance of all subsets of cartilage in maintaining the body`s function, articular cartilage has attracted the most scientific and economic interest in tissue engineering.

The primary bearing surface in a synovial joint is the articular cartilage (Fig. 2.14). Articular cartilage has a zonal assembly. In adults it is a comparatively acellular tissue, with cell volume averaging only approximately 2% of the total cartilage volume in human adults (Stockwell and Meachin, 1979). In contrast to the high cell volume during fetal life, the cartilage cell numbers decrease with age (Stockwell and Meachin, 1979), reaching its lowest point when the individual is 20 to 30 years. Chondrocytes are the most important cell source present in articular cartilage. All chondrocytes are surrounded by a narrow pericellular region. A territorial region surrounds this pericellular region which is present throughout the cartilage (Poole et al., 1982).

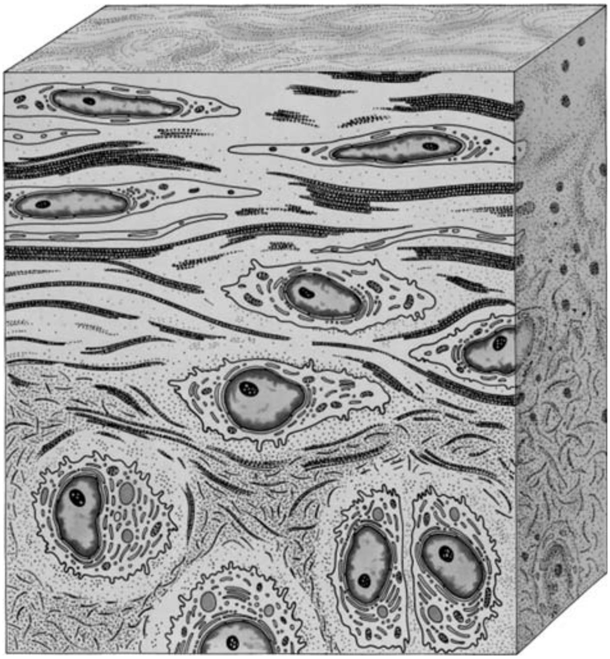
Cartilage has a zonal structure (Fig. 2.15).

Zonal Structure of Cartilage

- Superficial zone (SZ)
- Midzone (MZ)
- Deep zone (DZ)
- Calcified cartilage (CC)
- Subchondral bone plate (SBP)
- Subchondral trabecular bone (STB).



Dge, 0,/2, Different cartilage structures. _ Articular cartilage tissue. ` Hypertrophic cartilage zone



Dge, 0,/3, Scheme of zonal structure of articular cartilage, showing a detailed aspect of the tissue structure from the deep zone (*bottom*) to the surface (*top*)

Cartilage is occupied by an extensive extracellular matrix that is synthesised by these cells. At its free (superficial) surface, which is contacted by synovial fluid, the chondrocytes are flattened and aligned parallel to the surface (for a review see Poole et al., 2001). Below the superficial zone is the midzone where cell density is lower. The ultrastructure of the midzone reveals morphologic features more typical of a hyaline cartilage with more rounded cells and an extensive extracellular matrix. Between this midzone and the layer of calcified cartilage is the deep zone. Below the articular cartilage, and separated from it, is a layer of calcified cartilage. The calcified cartilage is not very vascular normally, and the remodelling process is therefore not as effective as in vascularised locations. Cell density is lowest in this zone. Aggrecan content and fibril diameter are maximal in the deep zone, whereas collagen content is minimal. The chondrocytes in this calcified zone usually express the hypertrophic phenotype, reaching a stage of differentiation that can also be found in fracture repair. A unique feature of hypertrophic cells is the ability to synthesise type X collagen and to calcify the extracellular matrix. Below the calcified cartilage is the subchondral bone plate. The calcified interface provides excellent structural integration with the subchondral bone. Subchondral trabecular bone underlies the subchondral plate. The structure and appearance of subchondral bone is critically dependent on the load situation of the joint. Subchondral bone may change its density by remodelling, but may also thicken through direct apposition of bone to its distal surface through bone modelling. Subchondral trabecular bone is not homogeneous and is not isotropic in nature. The trabeculae are oriented in different directions, and the mechanical properties of this tissue zone are therefore different in different planes. The subchondral plate and the trabecular bone are, despite their similarities, differently organised, adapt to mechanical loads in different ways, and have quite different mechanical properties (Hunziker, 2002).

The extracellular matrix of hyaline cartilages is composed of differentially distributed collagen fibrils and non-collagenous proteins that form an extensive network. Many of the molecules play a structural role, whereas others may be involved in regulating cell function. Different types of collagen are present in cartilage. Type II and type IX collagen form the main part of the fibrils; type XI collagen is present within and on the surface of the fibril (Mendler et al., 1989).

Collagen Type II

- Triple-helical glycoprotein
- Fibrillar molecule with 67-nm periodicity
- Molecular weight: 425 kDa
- Predominant collagen of cartilage and intervertebral disc
- Provides tensile strength
- Enables the tissue to resist shearing forces
- Supports chondrocyte adhesion
- Induces phenotypic differentiation of cells.

Collagen Type IX

- Member of subfamily of collagens termed FACIT (termed fibril associated collagens with interupted triplehelices)
- Disulphide-bonded heterodimer
- Molecular weight: 222 kDa
- Present in cartilage and intervertebral disc
- Bridges collagen fibrils with other macromolecules
- Associates with the surface of type II collagen
- Participates in the formation of type II collagen

Collagen Type XI

- Minor fibrillar collagen
- Forms heterotypic fibrils with type II and IX collagens
- Molecular weight: 545 kDa
- Predominantly present in cartilaginous tissue
- Also expressed by non-chondrogenic tissues

The ground substance of articular cartilage contains also a large variety of non-collagenous proteins and polysaccharides. The molecules vary in their abundance and structure with anatomical site or the person's age. There are no common features of non-collagenous proteins with respect to their distribution, structure and function. Many of the molecules are proteoglycans, bearing glycosaminoglycan chains, whereas others are glycoproteins or even non-glycosylated proteins. Among the non-collagenous proteins, aggrecan is of major importance.

Aggrecan

- Large chondroitin sulphate proteoglycan
- Molecular weight: 205 kDa
- Comprises 87% chondroitin sulphate, 6% keratan sulphate
- 10% of cartilage dry weight
- No presence in bone
- Interacts with hyaluronan
- Large number of fixed negatively charged side groups
- Responsible for high osmotic pressure
- Pimary role to swell and hydrate collagen framework.

The high aggrecan content in the extracellular matrix of cartilage is responsible for the unique ability of the tissue to resist compression in a specialised way. Aggrecan belongs to the family of aggregating proteoglycans that form large, multimolecular complexes with hyaluronan (Gomes et al., 2004). All members of the aggrecan family have an amino-terminal globular domain, which is responsible for interaction with hyaluronan. The molecules' ability to interact with hyaluronan is the basis for an undisturbed articular cartilage function, as they provide the rheological properties necessary for resisting compression. The interaction of aggrecan with hyaluronan is stabilised by the presence of link proteins. Link protein of aggrecan can be lost from the cartilage matrix during periods of tissue degeneration, but such loss is most likely due to depolymerisation of hyaluronan and involves concomitant loss of aggrecan. The structure and content of aggrecan is altered in many cartilage diseases. Aggrecan itself has, in addition to the functional relation with hyaluronan, a high chondroitinsulfate and keratansulfate content.

Link Protein

- Glycoprotein
- Molecular weight: 45 kDa
- Binds to aggrecan and hyaluronan.

Matrix Gla Protein

- Glycoprotein
- Molecular weight: 12 kDa
- Present in bone and cartilage
- Acts as an inhibitor of calcification.

Additionally, human cartilage contains three dermatan sulfate proteoglycans (biglycan, decorin, and epiphycan) and two potential keratan sulfate proteoglycans (fibromodulin and lumican). The presence of the above mentioned extracellular matrix proteins in articular cartilage is important for normal articular cartilage function. The extracellular matrix of other cartilage types contains numerous other proteins (e.g. cartilage matrix protein) that are neither collagens nor proteoglycans (Nomura et al., 1989), and several of these are thought to play a structural role in the matrix. Of special relevance for cartilage tissue is hyaluronan and S-100.

Biglycan

- Small proteoglycan
- Predominant small proteoglycan of cartilage
- Contains two extended glycosaminoglycans
- Leucine-rich proteoglycan
- Relatively abundant in the mineral compartment of bone.

Fibromodulin

- Small chondroitin sulphate/dermatan sulphate proteoglycan
- Molecular weight: 42 kDa
- Found in a variety of tissues
- Abundant in cartilage
- Compromises 0.1–0.3% of cartilage wet weight
- Binds to type I and type II collagen in vitro
- Inhibits collagen fibrillogenesis in vitro.

Cartilage Matrix Protein

- Disulphide-bonded multimer
- Molecular weight: 54 kDa
- Amino acid sequence closely related to von Willebrand factor
- Major component of non-articular cartilage
- Presence especially in tracheal, nasal, and epiphyseal cartilage
- 5% wet weight of tracheal cartilage.

Hyaluronan

- High molecular mass polysaccharide
- Copolymer of glucoronic acid and N-acetylglucosamine
- Present in a number of tissues
- Synthesised in the plasma membrane of cells
- Catabolised preferentially at local sites

- High overall turnover rate
- Important role during tissue development and differentiation
- Important for water and plasma protein homeostasis.

Hyaluronan is a high molecular-mass polysaccharide found in the extracellular matrix, especially of connective tissue. It is synthesised by cells and then excreted into the intercellular space. One special feature of this polysaccharide is its high turnover rate in the tissue. Hyaluronan has diverse functions. It acts as a mediator of tissue development and cell differentiation and possesses also important functions in water homeostasis. Through this action hyaluronan plays an important role for the functional and structural integrity of connective tissues, with special respect to cartilage tissue. S100 protein is an acidic calcium-binding protein (molecular weight 21 kDa) found in high concentrations in glial and Schwann cells.

S100 protein

- Acidic calcium-binding protein
- Molecular weight: 21 kDa
- Present in various conformations
- Unknown function
- Modulates a wide range of cellular processes
- Used as a chondrocyte marker protein
- Distinguishes cartilage from bone tissue.

It exists in various forms depending on alpha or beta unit configuration. The protein was found to be also present in human chondrocytes (Stefansson et al., 1982). The protein has been detected in chondrocytes of the skull, vertebrae, ribs, sternum, larynx and long bones of the human fetus, and in the larynx and xiphoid process of the human adult. The exact functions of S100 proteins are not well known. As S100 proteins are mostly present intracellularly, the majority of the molecules function as intracellular calcium receptor proteins. In addition, it has been suggested that S100 proteins modulate a wide range of intracellular processes including inter-cell communication, cell structure, growth, energy metabolism, contraction and intracellular signal transduction. S-100 is often used as a marker protein for cartilage tissue, in order to distinguish this tissue from bone.

Chondrocytes are the constituent cells of cartilage, and through their function in endochondral ossificatio

It is important to recognise that chondrocytes (like bone cells) are not compressible and they do not provide any special structural resistance to loading. Because of the structural and compositional characteristics of articular cartilage and the intrinsic coupling between the mechanical and physiochemical properties of the tissue (Lai et al., 1991), it has been, in contrast to bone tissue, more difficult to achieve a complete understanding of the mechanical signal transduction pathways used by chondrocytes. Fundamental to this issue is an understanding of the mechanical environment of the chondrocytes within the articular cartilage ECM. For example, compressive loading of the cartilage in the extracellular matrix exposes the chondrocytes to spatially- and time-varying stress, strain, fluid flow and pressure, osmotic pressure, and electric fields (Maroudas, 1979; Frank and Grodzinsky, 1987; Lai et al., 1991). The relative contribution of each of these factors to the regulation of chondrocyte activity is an important consideration which is being studied by a number of investigators (Guilak et al., 1997). It was found that under loading conditions shapes, pressures, and chemical environments of chondrocytes are altered by deformations created in the ECM as well as by local fluid pressure and fluid flow. Chondrocytes in articular cartilage undergo large changes in shape and intercellular spacing as the ECM is deformed. The cells were found to recover their morphology upon removal of compression and to have a stiffness that was less than or equal to that of the surrounding tissues. Furthermore, collagen fibre orientation was shown to change with compression and recover upon removal of compression. New microscope techniqes allowed three-dimensional imaging of fluorescently labelled chondrocytes (Guilak et al., 1995) or subcellular structures (Guilak, 1995) within a tissue explant. Volumetric images of chondrocytes recorded decreases in cell height of 26, 19, and 20% and decreases in cell volume of 22, 16, and 17% of control values in different zones of an osteochondral explant, thereby differing from mechanically induced deformations of bone cells. In responding to mechanical signals the chondrocyte responds to the several different types of information using various signal transduction mechanisms (for a review see Guilak et al., 2001). It was demonstrated by in vivo and in vitro studies that chondrocytes are able (in a similar manner to bone cells) to detect an array of physical parameters (tissue pressure, deformation, fluid flow). The sum of cellular reactions in response to loading is a gene activation state of cell that determines the histomorphology of articular cartilage remodelling and repair (Carter et al., 1987; Carter and Wong, 1990; Smith et al., 1992; Holmvall et al., 1995; Knudson et al., 1996; Buschmann et al., 1995; von der Mark and Mollenhauer, 1997; Beaupre et al., 2000; Lee et al., 2000; Loeser, 2000; Mauck et al., 2000; Ragan et al., 2000; Reid et al., 2000; Davisson et al., 2002; Giannoni et al., 2003). The factors, pathways, and receptors of cell sensing and the response of cells concerning the effect on its external environment have not yet been identified, but as indicated for bone cells, chondrocytes respond to a number of external signals. The chondrocyte perceives its mechanical environment through complex biological and biophysical interactions with the cartilage ECM. This matrix consists of several distinct regions, termed the pericellular, territorial, and interterritorial matrices (Hunziker, 1992). The bulk of the tissue is made up of the interterritorial matrix, which consists primarily of water dissolved with small electrolytes (Na^+, Cl^-, Ca^{2+}, etc.). The transfer of information proceeds to some extent in a similar manner as described for bone cells from the cell membrane to the cell nucleus, interacting also with other signalling pathways. Current experimental studies in tissue engineering try not only to evaluate the complex mechanobiology that underlies load-related cartilage histogenesis, but use biophysical stimuli to promote cartilage-like tissue growth in specially designed bioreactors (Meyer et al., 2005a).

Principles of Bone and Cartilage Reconstruction

3

Bone and cartilage repair is the subject of intensive investigation in reconstructive surgery (for a review, see Schultz et al., 2000). Current approaches to skeletal reconstructive surgery use biomaterials, autografts or allografts, although restrictions on all these techniques exist. These restrictions include donor site morbidity and donor shortages for autografts (Damien and Parsons, 1991), immunological barriers for allografts, and the risk of transmitting infectious diseases. Numerous artificial tissue substitutes containing metals, ceramics, and polymers have been introduced to maintain skeletal function (Binderman and Fin, 1990). Each material has specific disadvantages, and none of these can perfectly substitute autografts in current clinical practice. The use of biomaterials is a common treatment option in clinical practice. One important reason for the priority of tissue grafts over non-living biomaterials is that they contain living cells and tissue-inducing substances, thereby possessing biological plasticity. Research is currently in progress to develop cell-containing hybrid materials and to create replacement tissues that remain interactive after implantation, imparting physiological functions as well as structure to the tissue or organ damaged by disease or trauma (Alsberg et al., 2001).

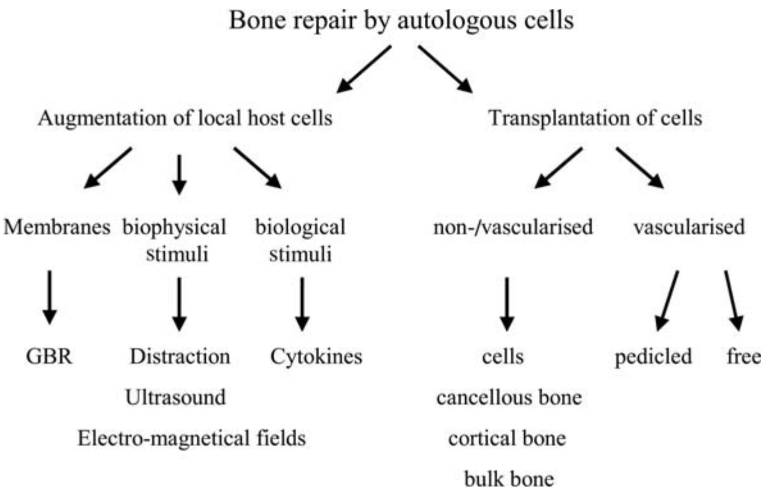
Bone and cartilage tissue engineering, as in most other tissue engineering areas, exploits living cells in a variety of ways to restore, maintain or enhance tissue functions (Langer and Vacanti, 1993; Lysaght and Reyes, 2001). There are three principal therapeutic strategies for treating diseased or lost tissue in patients: (1) in situ tissue regeneration; (2) implantation of freshly isolated or cultured cells; and (3) implantation of a bone-like or cartilage-like tissue assembled in vitro from cells and scaffolds. For in situ regeneration, new tissue formation is induced by specific scaffolds or external stimuli that are used to stimulate the body´s own cells and promote local tissue repair. Cellular implantation means that individual cells or small cellular aggregates from the patient or a donor are injected directly into the damaged or lost region with or without a degradable scaffold. For tissue implantation, a complete three-dimensional tissue is grown in vitro using autologous or donor cells within a scaffold* which has to be implanted once it has reached maturity (Loty et al., 2000; Schliephake et al., 2001; Meyer et al., 2004b). In this chapter we give some details on all of the above mentioned strategies and present alternatives to extracorporal approaches that are important in clinical decision making. Some of the techniques described here are clinically combined with extracorporal tissue engineering methods.

3.1 Bone Repair Strategies

The use of autologous bone in bone defect reconstruction can be considered to be the gold standard. There are two classical ways to repair bone defects by use of autologous cells: one utilises mechanisms of defect repair by enhancement of the local host cell population, while the other is based on transplantation of grafted bone (Fig. 3.1).

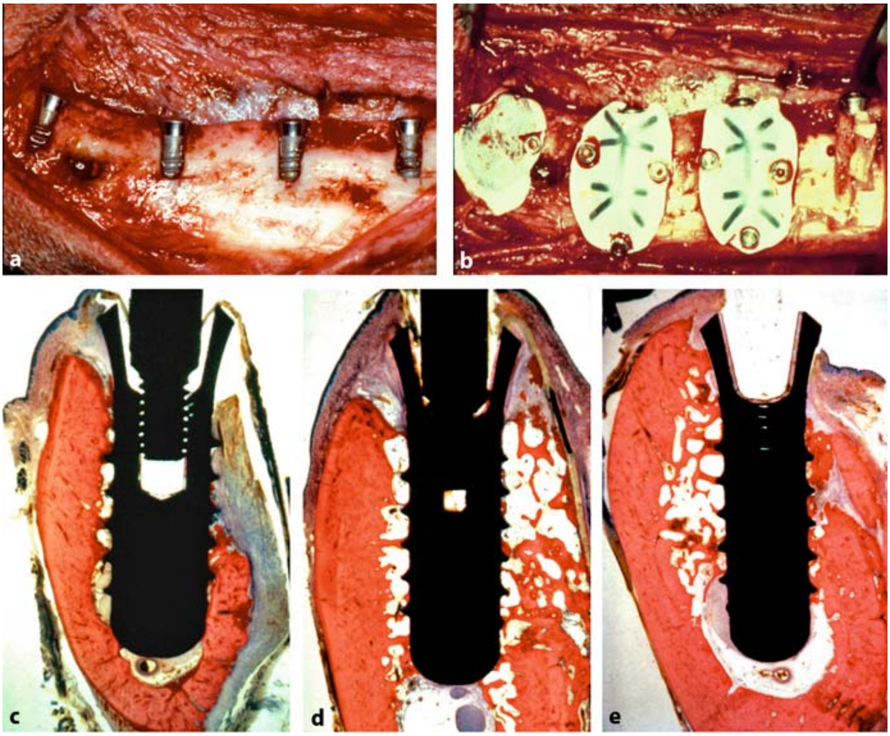
Augmentation of the host cell population can effectively be used to improve the healing of bone lesions. The ability to repair or reconstruct lost bone structure by this technique critically depends on the condition of the defect site. If the soft and hard tissues of the defect site are healthy, expansion of host cells is often successfully applied. In circumstances of impaired defect conditions such as wound infection and tissue necrosis or irritation, however, cellular augmentation of the local bone cell population will probably fail.

Membrane techniques can be used to improve defect healing by ingrowth of local host cells in the defect site (Fig. 3.2). This kind of defect repair procedure (guided bone regeneration, GBR) is mainly used in maxillofacial surgery to repair bony defects in the maxilla and mandible. The principle of GBR is to effectively separate bone tissue from the soft tissue ingrowth by a barrier (Lang et al., 1994). The application of the principle of guided bone regeneration (GBR) has proven to be successful in a number of controlled animal studies and clinical trials (Buser et al., 1996; Berglundh and Lindhe, 1997; Fiorellini et al., 1998). The healing pattern has been shown to involve all steps of de novo bone formation including blood clot formation, invasion by osteoprogenitor cells and their terminal differentiation into osteoblasts. Under these circumstances the extracellular matrix produced finally mineralises to form woven bone, and later is remodelled into lamellar bone (Hämmerle et al., 1998). The success of GBR depends critically on the defect size and geometry. Bone defects can be assumed to be repaired to a larger extent in cases where the defect is neighboured



Dge, 1,/, Bone repair by autologous cells by more than two bone walls. The main limitation of GBR is therefore the inability to reconstruct geometrically complex or large defect sites.

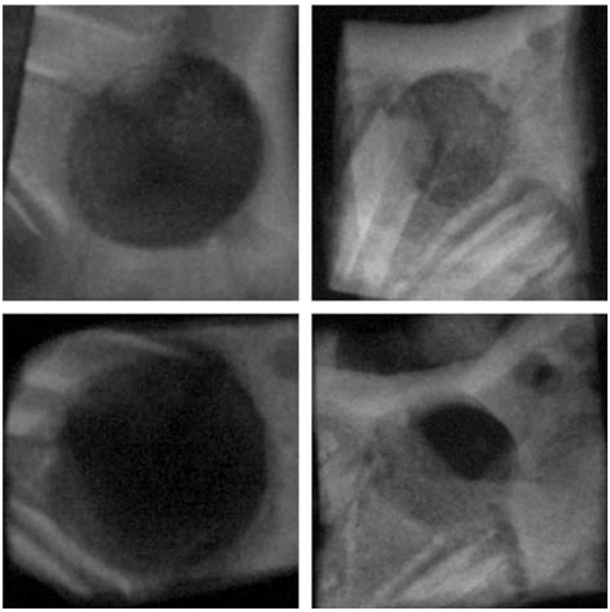
Biophysical effects, in particular mechanical loading and electromagnetic signals, are clearly important regulators of bone formation. Indeed, the regenerative capacity of bone tissue is largely due to the bone's capacity to recognise the functional environment required for the emergence and maintenance of a structurally intact bone tissue. Biophysical stimulation methods have therefore been introduced and have proved successful in clinical practice. Ultrasound application and electromagnetic field exposure are, besides distraction osteogenesis, considered to be a special form of mechanical stimulation, classical examples of biophysically driven approaches to influencing bone formation. Ample evidence from various prospective double-blinded, placebo-controlled clinical studies now documents the efficacy of mechanical and electrical stimulation (direct current, inductive coupling, capacitive coupling, and composite fields) and of mechanical stimulation (distraction osteogenesis, ultrasound stimulation, fracture activation) in enhancing bone repair



Dge, 1,0, Priciples of guided bone regeneration (GBR). _ Implants are inserted in bone having a defect at the vestibular aspect of the alveolar crest. ` Membranes are attached to the defect site, sealing the defect from tissue ingrowth of adjacent tissues. a«c Histology of bone regeneration. a Impaired bone regeneration without using a membrane. b New bone development by using the membrane technique. c Bone augmentation without the use of membranes led to an intermediate state of bone repair

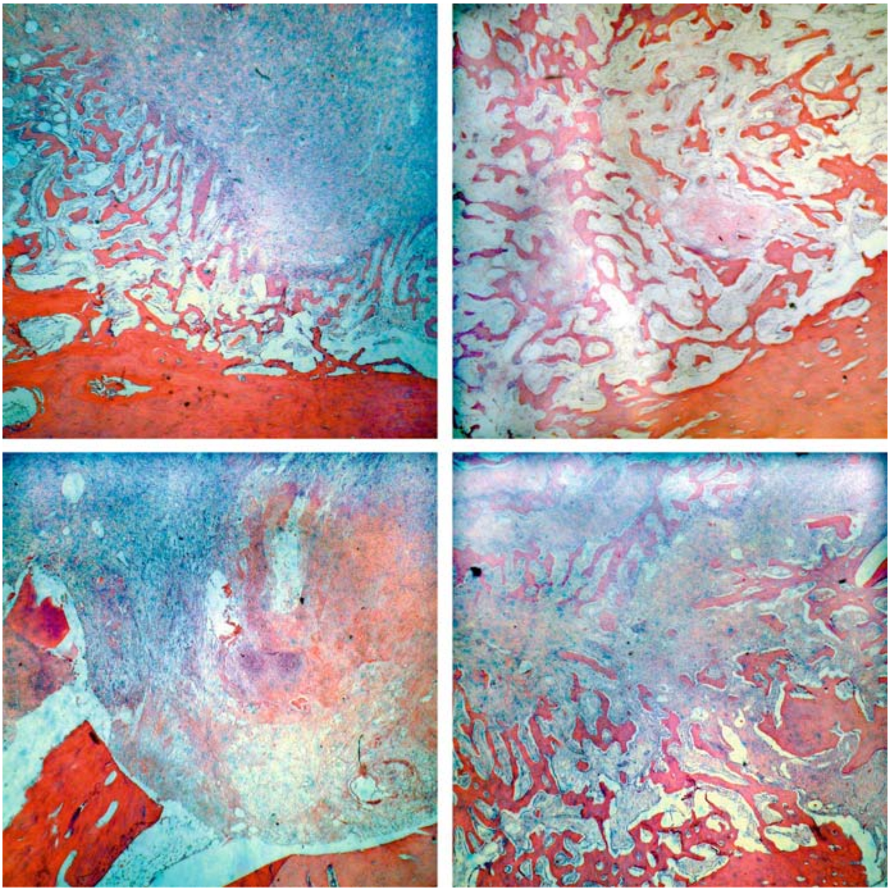
(Brighton, 1998). Because of its clinical efficacy, biophysical stimulation is now one of several methods used to enhance bone formation in patients.

When applied to a patient, biophysical stimuli exert their effects at distinct skeletal sites. The response to ultrasound stimuli is altered by the anatomy of the application site. Ultrasound-related deformations through adjacent structures (joints, ligaments, muscles) have a major impact on the resultant biophysical signal at the desired effector site. Both patient-specific and technique-specific factors play an important role. When biophysical treatment strategies are considered, the specific forms of biophysical stimulation and their related dose effect and application timing must be carefully determined and validated. Despite limited knowledge concerning the biological effects of biophysical stimulation, it has long been the goal of many scientists and clinicians to find applicable clinical tools for using biophysical stimuli in the context of bone healing. In recent decades, different treatment strategies have been reported to be successful in animal experiments as well as in clinical trials. This has led researchers and clinicians to conduct a multitude of defined experiments to assess the effects of different biophysical stimuli in facilitating new bone formation (for a review, see Chao and Inoue, 2003) (Figs 3.3 and 3.4). Numerous investigations have been performed on electrical field exposure. When electrical stimuli are applied iatrogenically to a patient in order to enhance bone formation, one has to be aware that electrical stimuli exert their effects within the various hierarchical structures of the skeleton. The influence of external stimuli in skeletal regeneration can generally be addressed at organ, tissue, cellular, and molecular levels. Understanding the overall process of biophysical signalling requires an appreciation of these various levels of study and a knowledge of how one level relates to and influences the next. Electrical stimuli can be analysed as they act on whole bones. Additionally, the contributions of electrical effects made on multicellular systems can be evalu-



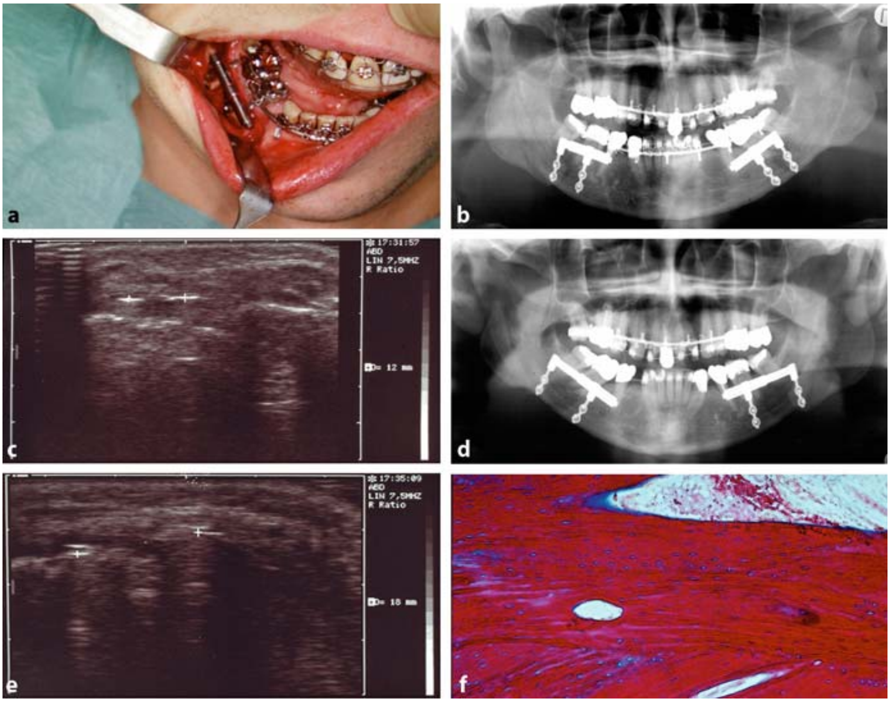
Dge, 1,1, X-ray images of bone defects with electrical stimulated bone regeneration *(top)* and without stimulation *(bottom)* after 1 *(left)* and 3 weeks *(right)*. Electrical stimulation improves biomineral formation in an in vivo setting of bone healing ated by examining the tissue reaction towards the biophysical microenvironment. Thirdly, cellular and molecular reactions critically shape the tissue response. The key requirement of using the effects of such biophysical forces is therefore to define the precise cellular response to the stimulation signal in an in vitro environment and to use well-established animal and clinical models to quantify and optimise the therapeutic stimulation approaches. Whereas this seems to be achievable through research collaboration among different disciplines using complementary approaches, electrical stimulation is not a standardised or common treatment option to regenerate skeletal defects.

Distraction osteogenesis is frequently used to augment local bone in clinical settings (Meyer et al., 2004d) and is now an established bone reconstructive treatment option (Fig. 3.5). Histologically, distraction osteogenesis shares many of the features



Dge, 1,2, Histology of bone defects with electrical stimulated bone regeneration (top) and without stimulation (*bottom*) after 1 (*left*) and 3 weeks (*right*). Critical size defects of mandibular bone were stimulated in rabbits by pulsed electric fields. Electric field application was able to enhance bone formation in critical size defects of fetal tissue growth and neonatal limb development, as well as normal fracture gap healing (Ilizarov, 1992; Sato et al., 1999). Although the biomechanical, histological, and ultrastructural changes associated with distraction osteogenesis have been widely described, the molecular mechanisms governing the formation of new bone in the interfragmental gap of gradually distracted bone segments remain largely unclear. A growing body of evidence has emphasised the contribution of both local bone or bone precursor cells and neovascularity to bone formation during distraction (Choi et al., 2000). Recent studies have implicated a growing number of cytokines that are intimately involved in the regulation of bone synthesis and turnover during distraction procedures (Meyer et al., 2001b). The gene regulation of numerous cytokines (transforming growth factor-beta1, -beta2, -beta3, bone morphogenetic proteins, insulin-like growth factor-1, and fibroblast growth factor-2) and extracellular matrix proteins (osteonectin, osteopontin) during distraction osteogenesis have been best characterised and reviewed by Bouletreau et al. (2002a; 2002b). Refinements in the surgical procedure of bone distraction have significantly improved clinical outcomes (Meyer et al., 2004d). Distraction osteogenesis has therefore become a mainstay in bone tissue engineering and has significantly improved bone reconstructive procedures.

Biomolecules applied with or without a carrier have been suggested to augment the local bone cell population (Schliephake, 2002).



Dge, 1,3, Distraction osteogenesis. _ Intraoperative view. ` Radiograph and a ultrasound at the beginning of distraction. b Radiograph and c ultrasound at the end of distraction. d Histological appearance of the regenerated tissue

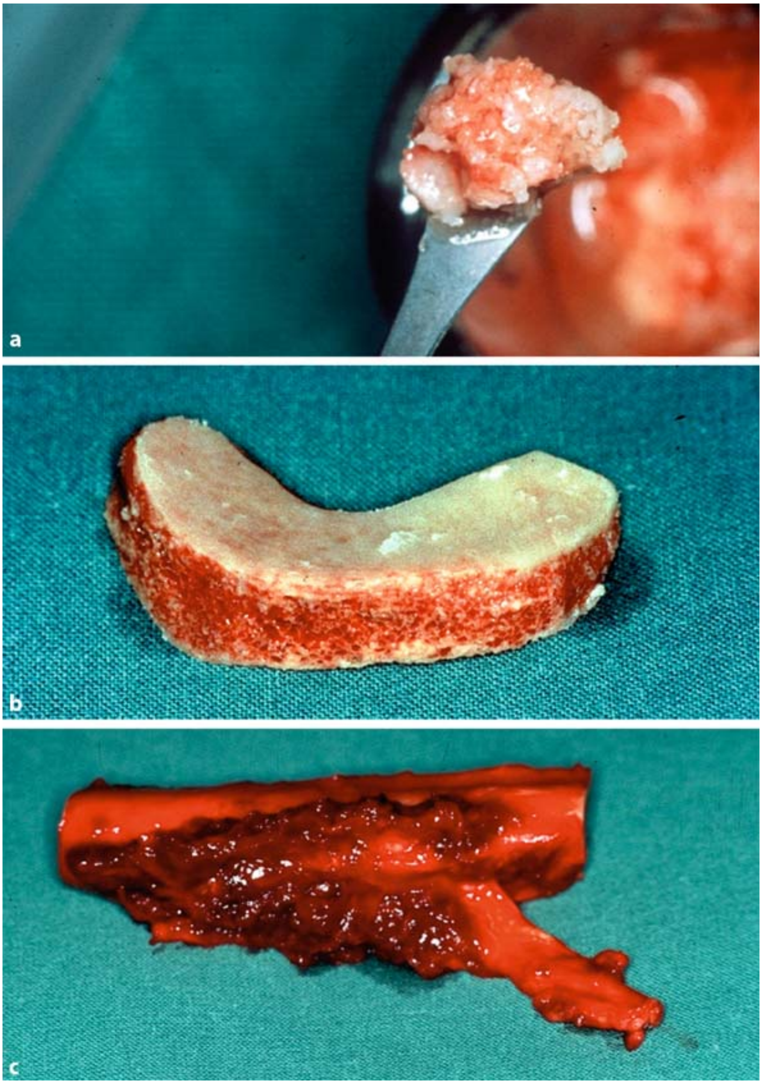
Biomolecules used to augment bone cell reaction

- Transforming growth factor beta (TGFß)
- Bone morphogenic proteins (BMPs)
- Insulin-like growth factors (IGFs)
- Platelet-derived growth factors (PDGFs)
- Fibroblast growth factors (FGFs).

It was suggested that bone reconstruction may be accomplished using various bioactive factors with varying potency and efficacy (Stevenson and Horowitz, 1992). Members of the TGF-ß superfamily and other growth factors, notably BMP, IGF, PDGF, and GDF are currently being tested for experimental use to engineer new bone. Evidence from a variety of studies indicates that exposure to osteoinductive growth factors can promote new bone formation (Hogan, 1996). Different healing environments require different types of bone tissue to be engineered. For example, a segmental long bone defect requires primarily cortical bone to be formed, whereas a maxillary alveolar crest augmentation requires primarily membranous (cancellous) bone. It is possible that different cascades of signalling molecules orchestrate these two healing processes. Alternatively, the same factors may be involved but have different outcomes because of the different mechanical environments or different host cell populations. It should be considered that different osteoinductive growth factors have a range of biological potencies and with increased efficacy comes increased cost and often increased risk. There are currently three strategies for delivery of osteoinductive growth factors, which are at various stages of development. The first strategy involves extracting and partially purifying a mixture of proteins from animal or human cortical bone, as described by Urist (1965) and Urist and Strates for BMPs (1971). The second strategy involves the use of recombinant proteins (e.g. rhBMPs), which was enabled by the cloning and sequencing of many of these genes (Wozney et al., 1988; Ozkaynak et al., 1990). The third and least developed strategy is that of gene therapy, which involves delivery of the DNA encoding a growth factor rather than delivery of the protein itself (Fang et al., 1996; Boden et al., 1998; Lieberman et al., 1998). It is also likely that there will not be one best strategy or one best growth factor and that success in the end may be determined by such factors as manufacturing cost and ease of use by the treating physicians or the flexibility to adapt to different bone tissue engineering environmental requirements. Regardless of the specific growth factor chosen, there are several common issues that, while unsolved, may place potential clinical limitations on all of the above strategies. These factors include dose, carrier, patient variability and confounding clinical factors.

Various donor sites allow the grafting of different types and amounts of bone. The use of autologous bone is accompanied by a grafting procedure and can be accompanied by donor site morbidity. Different surgical methods (Fig. 3.6) allow one to graft bone-forming tissue in the form of:

- Periosteal flaps
- Cancellous bone chips
- Cortical bone chips
- Bulk cortico-spongiosal grafts
- Vascularised bone.



Dge, 1,4, Different methods for bone reconstruction. _ Cancellous bone. ` Cortical bone. a Microvascular fibular flap

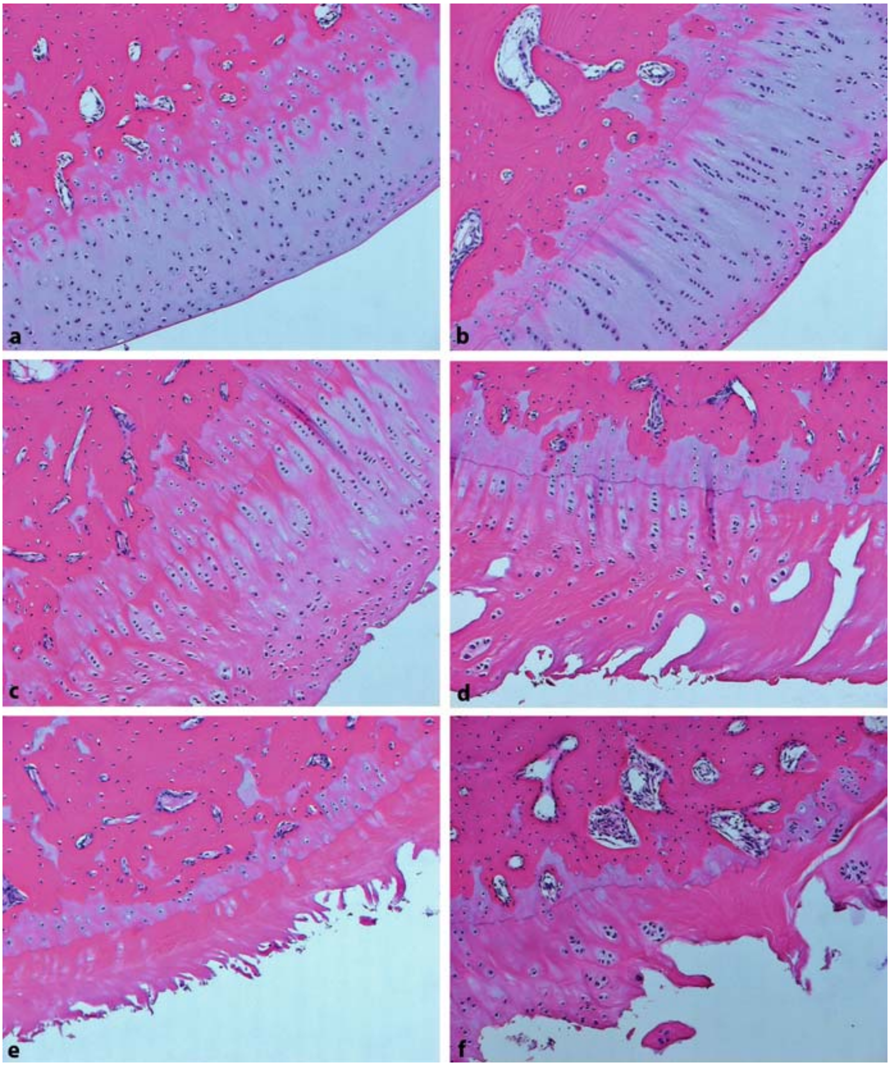
Small bone defects can be treated with small bone grafts (periosteal flaps, cancellous or cortical bone chips) provided local conditions are appropriate for bone healing. Endochondral bone from the ilium, tibia or rib can be harvested as well as membranous bone from the facial skeleton. Animal studies indicate that membranous bone is less prone to resorption than endochondral bone (Zins and Whitaker, 1983). However, when the bone defect is large, bulk cortico-spongiosal grafts are necessary. Effective rigid fixation of the graft facilitates graft survival and hematomas should be avoided. Problems with conventional grafts include their susceptibility to infection, the unknown survival of transplanted cells and the unpredictable degree of resorption. The extent of resorption is dependent on the graft volume, the delay time before loading and the condition of the transplant bed.

A major improvement in bone reconstruction strategies was afforded by the use of vascularised bone tissue in combination with or without adjacent soft tissue (muscle, subdermal tissue, skin). The soft tissue of the flap can be used to obliterate the defect space to the desired extent, to prevent infection and to allow a better barrier against direct surface loads. Bone reconstruction by vascularised bone can be achieved through pedicled flaps or microvascular flaps. Several pedicled osteocutaneous or osteomuscular flaps have been established in clinical routine (Conley, 1972): sternocleidomastoid muscle combined with the clavicle (Siemssen et al., 1978), trapezius muscle with parts of the scapula (Demergasso and Piazza, 1979; Panje and Cutting, 1980; Guillamondegui and Larson, 1981), pectoralis muscle with part of a rib or sternum (Cuono and Ariyan, 1980; Green et al., 1981; Lam et al., 1984), latissimus dorsi osteomyocutaneous flap (Maruyama et al., 1985) and the temporalis muscle with calvarian bone (McCarthy and Zide, 1984; McCarthy et al., 1987; Rose and Norris, 1990). Free vascularised bone grafts provide the best possibility for reconstruction of large bone defects. Sufficient bone material with good quality cortico-cancellous bone can be harvested for use in reconstruction of large bone defects. A soft tissue paddle can be harvested together with the vascularised bone, as, for example, an osteocutaneous flap. The iliac crest (Taylor, 1982; Shenaq, 1988), the fibula (Taylor et al., 1975; Wei et al., 1986; 1994; Hidalgo, 1989) and the scapula (dos Santos, 1984; Swartz et al., 1986; Baker, 1989; Granick et al., 1990) are all well- /established donor sites from where a large amount of bone can be grafted (Frodel et al., 1993). This type of bone reconstruction combines the advantage of enlarging bone transplants while maintaining the nutritional needs of cells. In current clinical practice this technique overcomes the limitations of other approaches (free non-vascularised bone graft transfer, tissue engineered constructs) when large defects need to be reconstructed. The main disadvantage of free vascularised grafts is donor site morbidity, especially when large grafts are necessary.

3.2 Cartilage Repair Strategies

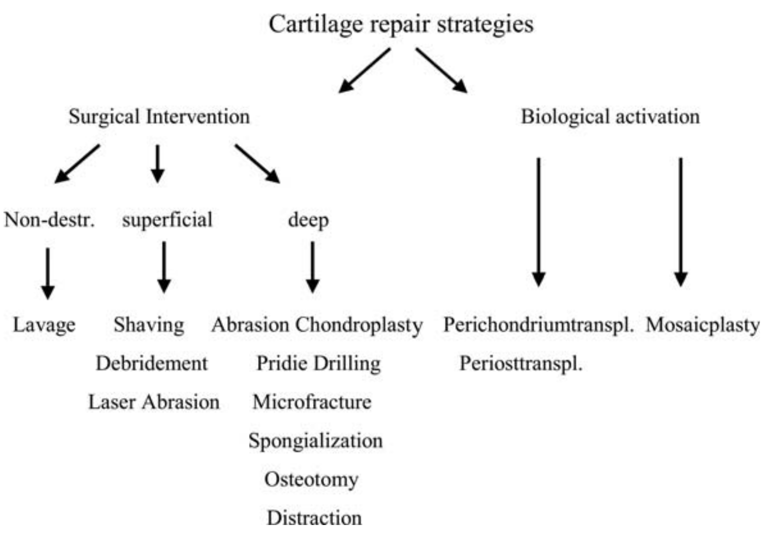
Most cartilage repair strategies have focused on healing articular cartilage, since this type of cartilage is the one most commonly diseased (Fig. 3.7), it is of special importance for an undisturbed joint function, and its disturbed function has a high socioeconomic impact. Various procedures have been introduced to induce the healing of histological and macroscopical lesions within mature articular cartilage in order to re-establish a structurally and functionally competent joint (Fig. 3.8).

Due to the multitude of pathogenical disturbances and based on the anatomy of cartilage joint, attempts to heal articular cartilage lesions span the whole range from symptomatic measures to extensive surgical interventions. There are two distinct goals for cartilage reconstruction. The first is the immediate need for clinical pain relief and restoration of joint function. The second goal is to prevent or at least delay the onset of arthritis. From a practical perspective, the current objective of articular cartilage repair is to delay joint replacement for a clinically significant



Dge, 1,5, Different states of chondroarthrosis. _ Healthy cartilage layer. ` Alteration of the superficial zone. a Destruction of the superficial zone accompanied by disturbance of deeper cartilage layers. b Destruction of cartilage down to the calcified cartilage layer. c Thinning of the cartilage layer. d Severe destruction of the basal cartilage zones

period (Reinholz et al., 2004). Besides non-surgical therapies that are based on the administration of drugs and biologicals, surgical options play a significant role, to provide pain relief, to restore joint functionality, and to prevent progression of joint destruction. In some instances drastic measures like total joint replacement by artificial materials are necessary to achieve clinical success. Most of the experimental and clinical attempts that have been made to restore articular cartilage structure aim at re-establishment of biomechanically competent tissue of an enduring nature. The



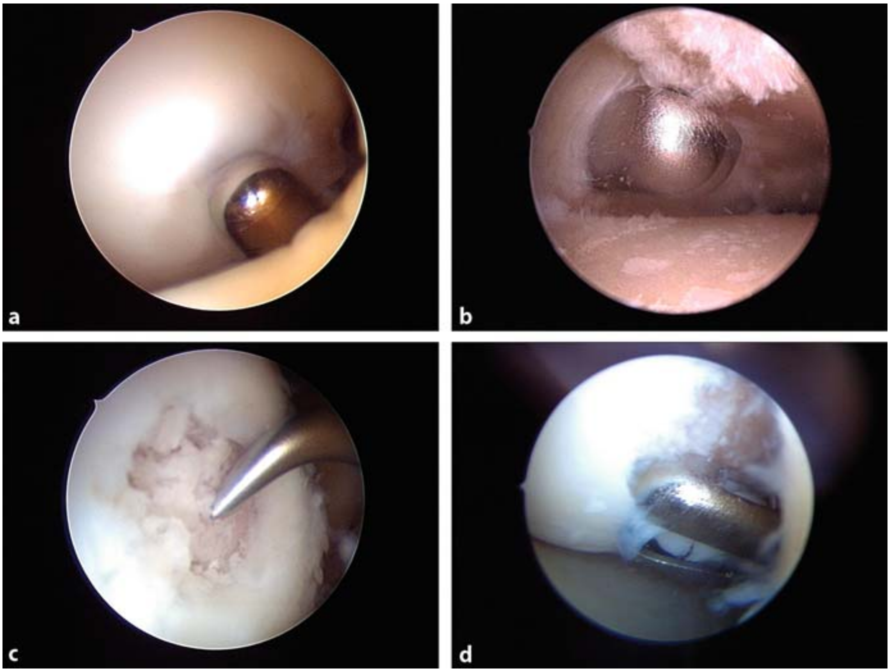
Dge, 1,6, Cartilage repair strategies

spontaneous repair response forms the basis and the rationale behind the surgical interventions. Unfortunately, when cartilage is damaged due to injury or disease, it has a limited capacity to heal (for a review, see Guilak et al., 2004). Articular defects larger than 2–4 mm in diameter rarely heal, even with continuous passive motion, due to the fact that adult articular cartilage contains no blood supply, neural network or lymphatic drainage. As a result, partial-thickness defects that do not reach the subchondral bone stimulate only a transient induction of cartilage repair (Reinholz et al., 2004). Since the physiological process of spontaneous repair is limited, new therapies are aimed at overcoming the biological restrictions on cartilage repair. As indicated in Chap. 2, different repair mechanisms are present in cartilage repair. It has been known for decades that large superficial lesions of cartilage tissue do not heal spontaneously. In contrast, once the bone and bone marrow spaces are involved, bleeding and hematoma formation start a repair cascade that leads to repair tissue formation. However, the resulting heterogenous repair tissue composition, which consists of a mixture of hyaline- and fibro-cartilage, contributes to an inferior biomechanical competence. Thus, histogenesis of the defect repair depends to a large extent on the defect size.

Therapeutical measures to improve joint structure and function without the use of biologically active substances can be conceptualised as methods to improve the condition of the joint fluids (lavage), to mechanically remove diseased or necrotic superficial chondral tissue (shaving, debridement, laser abrasion) and to gain access to the subchondral bone (abrasion chondroplasty, pridie drilling, microfracture techniques and spongialisation). The underlying reason for lavage or debridement is the removal of inflamed or diseased tissue, whereas gaining access to subchondral bone is aimed at initiating a spontaneous healing response. Arthroscopic lavage and debridement are often used to alleviate joint pain (Fig. 3.9). Lavage is mainly performed by arthroscopy. Through arthroscopy, debris may be removed from the joint space thereby alleviating pain. Both procedures routinely used to alleviate joint pain have been shown to be successful in treating early stages of osteoarthritis. It is important to consider, that both lavage and debridement do not induce repair of articular cartilage. A recent study has demonstrated that pain relief observed following debridement and lavage procedures may be no more than a placebo effect following surgery (Redman et al., 2005).

The provocation of bleeding from subchondral bone spaces and subsequent blood-clot formation has become a common therapy in cartilage surgery to induce spontaneous cartilage repair. Extensive surgical interventions like osteotomies or joint distractions are procedures for complex joint disturbances that exploit skeletal load transfers in cases of joint malposition. Despite the reported success rates, these invasive surgical options are reserved for severe joint diseases and are more beneficial if they are intended to correct malpositioning.

The use of cell-containing devices is performed by different measures. Transplants from either autologic or allogenic origin can be harvested in the form of perichondrial or periosteal tissue and as a bulk osteochondral part. Perichondrial or periosteal autotransplantation as a single procedure has been exploited in a variety of protocols elaborated for the treatment of articular cartilage defects. Other tissue engineering concepts such as autologous chondrocyte transplantation use a periosteal flap to cover the cell-filled defect in order to avoid the disappearance of injected cells. The basic biological principle behind the use of these transplants is the fact



Dge, 1,7, Endoscopical images of cartilage defect treatment in different situations in human knee. _ Lavage. `, b Debridement. a Microfracturing that perichondrial as well as periosteal tissue contains cells that possess a life-long chondrogenic or osteogenic activity. A pool of precursor or adult-type stem cells are assumed to be present in these tissues that render self-renewable capacity and are able to induce tissue healing. Whereas experimental in vitro and in vivo studies have demonstrated cartilage tissue formation, complete defect filling was only seldom reported. In most cases, however, the use of this treatment strategy did not lead to a complete restitution of hyaline cartilage layer, because it is commonly accompanied by a long-term mechanical instability of the newly formed tissue.

Implantation of chondral or osteochondral tissue (mosaicplasty) is routinely used in joint and bone surgery to repair chondral or osteochondral defects (Fig. 3.10). Experimental studies revealed that graft material persisted for a short time, however, long-term effects have not been extensively evaluated. It was demonstrated by ret-

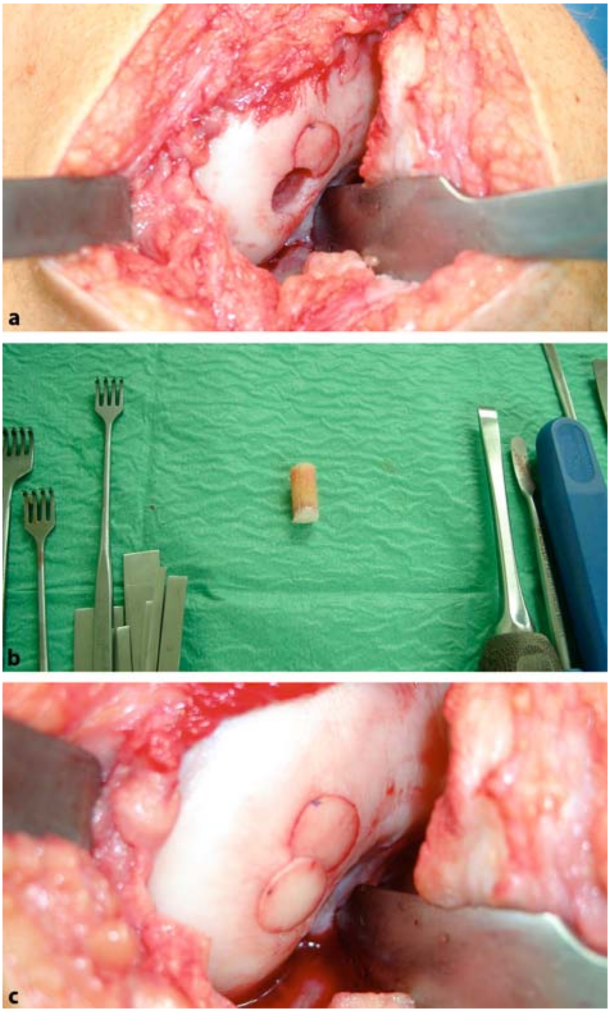


Fig. 3.10. Intraoperative views of a mosaic plasty procedure in the knee joint

rospective studies that clinical outcomes were acceptable in the sense of improved joint functionality and pain relief. Despite short-term clinical success, the use of autografts has a number of disadvantages. The donor site may experience severe morbidity since the explantation site will lose as much chondral or osteochondral tissue as the diseased implantation site will gain. Additionally, the surgical procedure to obtain the tissue is known to be accompanied by a loss of cells and matrix components at the transplant borders. Most important perhaps is the aspect that the translocation of tissue from a commonly used low-weight-bearing cartilage area to a weight-bearing-area (often accompanied by an incongruence between joint surfaces) may lead to overload- /induced degeneration of the transplant. The collateral damage to joint tissue and the problems inherent to this kind of tissue transfer are the main limitations on this autotransplantation technique.

The use of allogenic osteochondral grafts is another measure to substitute failed or lost cartilage tissue. The simplicity of filling cartilage defects with allogenic material retrieved from cadavers has made this therapy a common treatment option. In contrast to the relative ease of gaining grafts, immunologic barriers limit the use of allografts. Experimental investigations have revealed that allogenic tissue transplants survive longer under immunosuppression or when better histocompatibilty between the donor tissue and the host tissue is present. Immunologic reactions in humans have been reported not to be extensive, maybe based on the fact that cartilage is considered to occupy an immunologically privileged position within the joint. The kind of allogenic specimen preparation (fresh, cryopreserved, lyophilised) influences the clinical repair process. Fresh tissue possesses improved structural and mechanical competence as well as a longer life span compared to cryopreserved or lyophilised material, but transplantation of fresh tissue has an increased risk of transmitting diseases of the donor. Both, cryopreservation and lyophilisation techniques result in a limited biological activity and mechanical competence, since these techniques are prone to cause the death of chondrocytes. Despite these limitations, allogenic tissue transplantation is an established procedure, especially for patients with large osteochondral defects.

Autologous chondrocyte transplantation (ACT) is a recently established technique to treat cartilage defects. The use of single cells (chondrocytes multiplied in monolayers) for the restoration of tissue defects meets the US definition of tissue engineering but is not covered by the European definition, since this technique is devoid of a scaffold. Further developments of this technique (coating scaffolds with chondro-cytes, multilayer growth, scaffold-free chondrospheres) clearly fulfil the criteria of an ex vivo grown cartilage tissue. More details on this technique will be described in this and following chapters.

Articular chondrocytes are responsible for the unique features of articular cartilage; therefore, it seems rational to use committed chondrocytes to repair a cartilaginous defect. From arthroscopically harvested cartilage, chondrocytes can be isolated by enzymatic digestion and expanded in culture to 20–50 times the initial number of cells (Wei and Messner, 1999). Cells can be cultured in monolayers with serum supplementation in the culture media and during that time, the cells commence to dedifferentiate. The dedifferentiated chondrocytes share features of primitive mesenchymal cells and on implantation at high density the in vitro expanded primitive immature chondrocytes imitate prechondrogeneic cell condensation and cartilage formation (Brittberg, 1996; 1999). It was shown that in embryo-derived undifferentiated cells, a reduced rate of cell clustering and number of cell-to-cell contacts indicates a reduced number of cells that differentiate (Tacchetti et al., 1992). It was therefore suggested that there is a cascade of events, important in the early stages of chondrocyte differentiation, that consist of the acquisition of the ability to establish the cell to cell contacts, to form a permissive environment capable of starting the differentiation and to express differentiation markers. Furthermore, in an in vitro experiment with chondrocytes directly seeded to cartilage explants, the biosynthetic activity increased with the number of seeded chondrocytes, indicating how important the number of seeded cells is for the success of implantation (Chen et al., 1997). It was demonstrated that a high proportion of synthesised proteoglycan could be lost to the medium after cell seeding, which suggests that at least early in the culture an overlying membrane is important (Chen et al., 1997). In the clinical use of in vitro expanded autologous chondrocytes for cartilage repair the aim is to have an adequate number of expanded cells to implant and an overlying membrane to avoid cell and matrix loss. Brittberg et al. (1994) successfully reported autologous chondrocyte implantation using a monolayer culture system to treat cartilage defects. In this procedure, harvested autologous chondrocytes, expanded in a monolayer culture system, were transplanted to an osteochondral lesion, which was covered by a periosteal flap. This initial report had a high impact on cartilage surgery and was regarded as a breakthrough for cell-based cartilage repair strategies. The United States Food and Drug Administration approved in 1997 cell technology that uses the patient's own chondrocytes to repair cartilage injuries in the knee (Hauselmann et al., 1998). This was the first type of cell technology that was regulated by industry for use in expanding autologous cells for human transplantation. In the USA and Europe, cell processing in a monolayer culture is now being carried out on a commercial basis. An international autologous chondrocyte implantation study was initiated by Genzyme Tissue Repair to evaluate the clinical success of ACT. The study, monitored by a Registry Advisory Board and presenting 12 months of data on 249 patients and 24 months of data on 50 patients, revealed an overall significant improvement in patient and clinical scores from baseline scores through the ACT procedure (Mandelbaum et al., 1998).

In contrast to the clinical outcome rates, limited information is available on the histogenesis of cell-driven human repair tissue. Biopsy specimens from grafted areas in individuals obtained after autologous chondrocyte transplantation indicate that the ACT procedure helps to build up a tissue with hyaline and fibrocartilage-like features (Richardson et al., 1999; Peterson et al., 2000). Transarthroscopic biopsy specimens obtained from grafted areas demonstrated, in general, heterogeneity throughout the repair tissue (Richardson et al., 1999; Peterson et al., 2000). Although beneficial short- or middle-term clinical results were reported (Peterson et al., 2000; Minas, 2001), the ACT procedure has potential disadvantages, such as the risk of leakage of transplanted chondrocytes from the cartilage defects and an uneven distribution of chondrocytes in the transplanted site (Sohn et al., 2002). These limitations explain to some extent the finding of heterogenous tissue formation in the defect site. To overcome these limitations, further developments are focusing on the ex vivo growth of a three-dimensional cartilage-like tissue, which integrates intimately in the defect site after being implanted.

3.2.1 Flap Prefabrication

Flap prefabrication is one of the most exciting areas in reconstructive surgery because of its bridging role between conventional reconstructive surgery and tissue engineering (Tan et al., 2004). This new and exciting approach gained major public interest after Vacanti and colleagues fabricated a human ear on a mouse back (Cao et al., 1997). Using prefabrication technique, tissues such as bone or cartilage can be pre-assembled to form composite tissues that can fit even major defect sites. Prefabrication can expand the versatility of a graft. Existing organs can be transformed into a transplantable state, or completely new organs can be created. In its simple form, e.g. pre-lamination, a new tissue can be created by burying cartilage, bone, skin or mucosa underneath a fascia or in a muscle pouch, and later can be harvested as a composite tissue free flap to replace lost tissue (Alam et al., 2003; Jaquiery et al., 2004; Keser et al., 2004; Schultze-Mosgau et al., 2004; Staudenmaier et al., 2004; Tan et al., 2004; Terheyden et al., 2004; The Hoang et al., 2005; Top et al., 2005). Vascular induction is another approach in which new blood supply is generated in tissue to improve the survival of the transplanted construct. It was shown that bone chips wrapped in a vascular carrier such as muscular tissue became vascularised grafts (Fischer and Wood, 1987). The future of flap prefabrication can be envisioned to generate complex vascularised tissues in desired external shapes that mimic the defect to be replaced. The basis of such approaches may be the use of suitable vascular carriers, resorbable matrix components, attached cytokines, and incorporated cells. It was shown by various investigators that prefabrication can be started ex vivo and then transferred to the in vivo situation (Findlay et al., 2003), but can also be used solely in vivo. Warnke et al. (2004), for example, demonstrated the growth and transplantation of a custom vascularised bone graft in a man. The use of such complex tissue engineering strategies demonstrates the general feasibility of applying these therapies in humans, but problems with these therapy options have to be solved in detail before they can be used as routine treatment options.

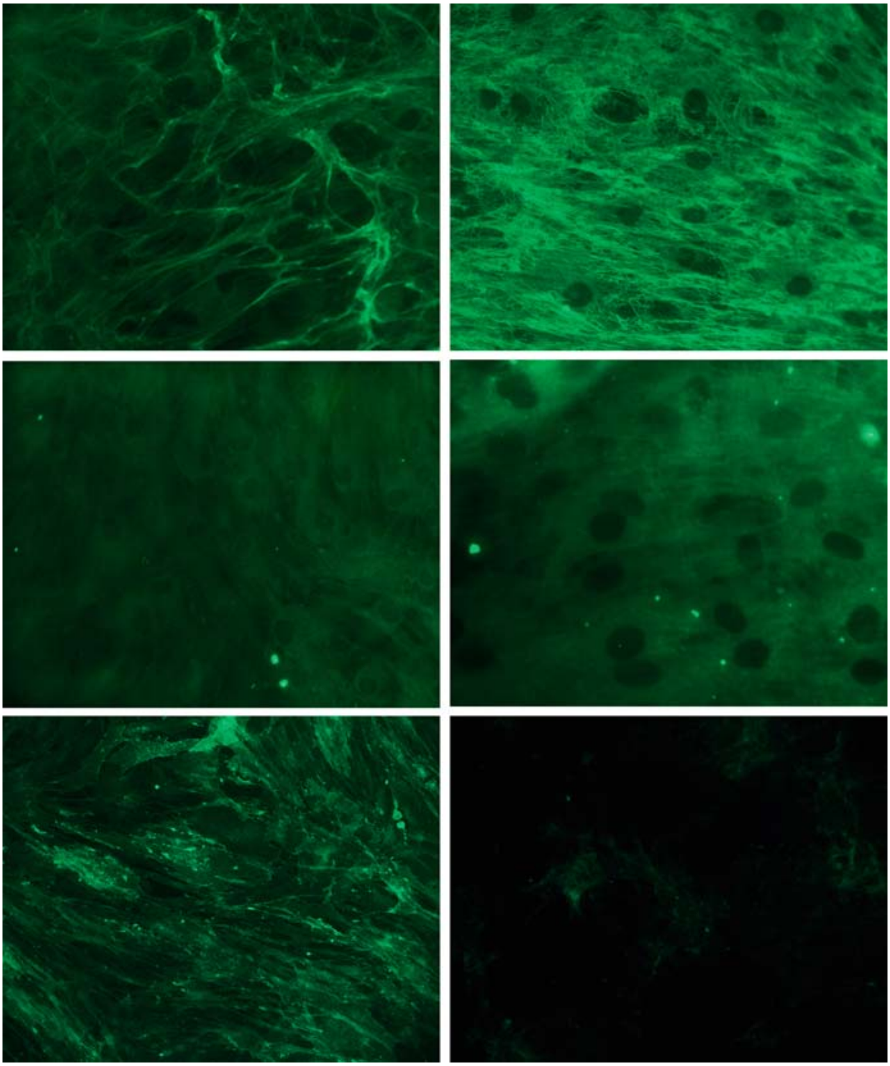
3.3 Extracorporal Strategies

Extracorporal tissue engineering in the narrower (EU) definition of bone and cartilage engineering requires not only living cells, but additionally the interaction of three biological components: bone cells, the extracellular scaffolds, and in some instances, growth factors. To engineer living tissues in vitro, cultured cells are commonly grown on two-dimensional or three-dimensional bioactive degradable biomaterials that provide the physical and chemical basis to guide their proliferation and differentiation. In bioreactors outside the body the biomaterial is assembled as a complex three-dimensional construct. New approaches in extracorporal tissue engineering strategies are aimed at fabricating scaffold-free three-dimensional micro-tissue constructs. The technique of micro-tissue formation is described in detail in Chap. 9. Additionally, the use of biomolecules seems not to be dispensable since the cells themselves may be stimulated to synthesise some of the needed biomolecules in an autocrine fashion. The assembly of cells into tissue substitutes is a highly orchestrated set of events that requires time scales ranging from seconds to weeks, and dimensions ranging from 0.0001 to 10 cm. At the moment the techniques are moving from an experimental stage to the level of clinical application. In the following chapters we will consider bone and cartilage tissue engineering as a new approach to generating extracorporally, cell-containing artificial materials designed as substitutes and implants for reconstructive surgery. Cell/scaffold-based approaches for tissue engineering of bone and cartilage offer perspectives on future treatment concepts. The skeletal system contains a variety of different cell types: vascular cells, bone marrow cells, pre-osteoblasts, osteocytes, chondroblasts, chondrocytes and osteoclasts, all executing distinct cellular functions to allow the skeleton to work as a highly dynamic, load bearing organ. Whereas all these cells are necessary to build up a real skeleton, limited cell sources are regarded to be sufficient for engineering a bone-like or cartilage-like construct in vitro.

Cell Development, Cell Sources 4

4.1 Cell Development

The skeletal tissue contains various types of mesenchymal cells, such as osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, adipocytes and bone marrow stromal cells. Every cell of the skeletal system, responsible for the regulated synthesis and deposition of extracellular matrix, is necessary to build up a real substitute tissue. The orchestrated work of the different cell types enables the skeleton to perform its unique function. To extracorporally fabricate a bone- or cartilage-like tissue, different cell sources and different cell types are principally suited to tissue-like substitution. The different cell lineages are believed to originate from common progenitors (Taylor and Jones, 1979; Grigoriadis et al., 1988; Owen, 1988; Caplan, 1991; Yamaguchi and Kahn, 1991; Aubin and Liu, 1996; Pittenger et al., 1999) called pluripotent stem cells (Owen, 1988; Caplan, 1991; Pittenger et al., 1999). These progenitor cells acquire specific phenotypes depending on their maturation during differentiation. Stem cells and mesenchymal precursors arise in the embryo and at least some of these appear to persist in the adult organism, where they contribute to the replacement of lost cells in skeletal tissue remodelling and repair. In addition to haematopoietic stem cells, the bone marrow comprises a population of marrow stromal cells or mesenchymal stem cells (MSCs). Mesenchymal stem cells serve as the major reservoir for different classes of stem cells. Stromal cells have multilineage differentiation capacity, thereby generating progenitors with restricted developmental potential, such as fibroblasts, osteoblasts, chondrocytes and adipocyte progenitors (Caplan, 1991; Pittenger et al., 1999) (Fig. 4.1). Maturation of cells happens through stages of proliferation, commitment, progression and differentiation in the organism in order to generate the various tissues (Fig. 4.2). As described in Chap. 2, various hormones and cytokines regulate the fate of the cell during the differentiation process from progenitor cells to mature cells. Among these, members of the transforming growth factor family are the most potent inducers and stimulators of osteogenic and chondrogenic differentiation. For bone development, BMPs as classical examples, not only stimulate osteoprogenitors to differentiate into mature osteoblasts but also induce non-osteogenic cells to differentiate into osteoblast lineage cells (for a review, see Kale and Long, 2000). Other factors (dexamethasone, prostaglandines) also play important roles in the regulation of osteoblast differentiation in an orchestrated way. In this context, it is important to note that molecules of the TGF-β and BMP superfamily are also main regulators of differentiation of stem and precursor cells towards chondrocytes. This is an indication of the complex situation of cell differentiation induction.



Dge, 2,/, Fluorescent staining of mesenchymal stem cells differentiated to osteoblasts, collagen type I (*top left*), fibronectin (*top right*), osteonectin (*midline left*), bone sialoprotein (*midline right*). For a significant characterisation of the osteoblast differentiation a combination of typical osteoblast and stem cell markers are necessary. For example, stem cells show a positive CD90 signal (*bottom left*) but differentiated osteoblast show no CD90 expression

4.2 Cell Sources

Principal sources of cells for tissue engineering include beneath xenogenic cells, autologous and allogeneic cells (Table 4.1). Each category can be subdivided according to whether the cells are stem cells (embryonic or adult) or whether the cells are in a more differentiated stage (Fig. 4.3). Various mature cell lines as well as multipo-

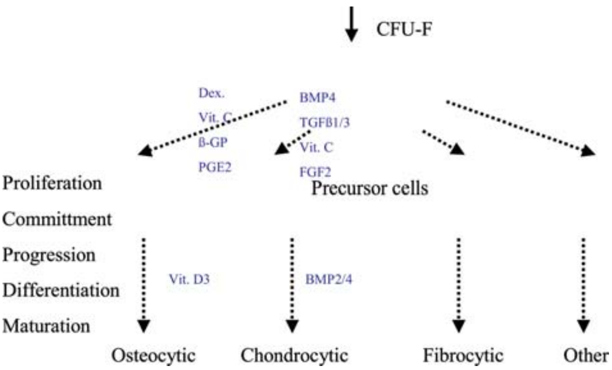


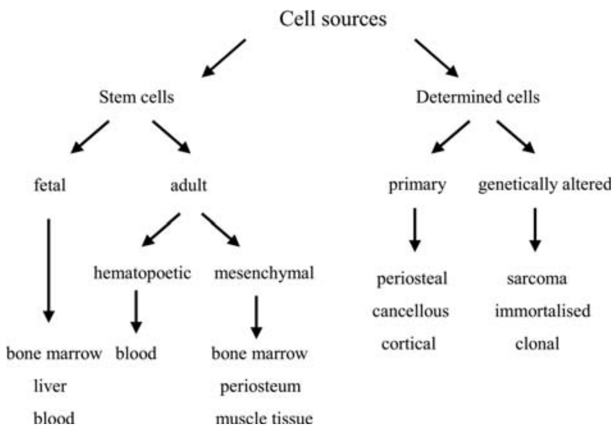
Fig. 4.2. Cell development pathways

Table 4.1. Principal cell sources used in tissue engineering studies

Experimental studies	Preclinical studies	Clinical studies
• Autologous • Allogeneic • Xenogeneic • Immortalised • Non-transformed clonal • Sarcoma	• Autologous • Allogeneic • Xenogeneic	• Autologous

tent mesenchymal progenitors have been successfully established (Yamaguchi et al., 2002) in bone and cartilage tissue engineering approaches. Additionally, other bone and cartilage cell lines like genetically altered cell lines (sarcoma cells, immortalised cells, non-transformed clonal cell lines) have been developed and used to evaluate basic aspects of in vitro cell behaviour in non-human settings.

The stem cell of skeletal tissue is a hypothetical concept with only circumstantial evidence for its existence, and indeed, there seems to be a hierarchy of stem cells each with variable self-renewal potentials (for a review, see Triffitt, 2002). Embryonic and adult stem cells can be distinguished. Embryonic stem cells reside in blastocysts. They were first isolated and grown in culture more than 20 years ago (Martin, 1981). The primitive stem cells renewing skeletal structures have been given a variety of names including connective tissue stem cells, osteogenic cells (Ham, 1969), stromal stem cells (Owen, 1988), stromal fibroblastic cells (Weinberg and Bell, 1986), and mesenchymal stem cells (Caplan, 1991). No nomenclature is entirely accurate based upon the developmental origins or differentiation capacities of these cells, but the latter term, although defective, appears to be in favour at the moment. Stem cells have the capacity for extensive replication without differentiation, and they possess, as mentioned, a multilineage developmental potential allowing them to give rise to not only bone and cartilage, but tendon, muscle, fat, and marrow stroma. This expansion of properties makes stem cells, whether derived from the hematopoetic system (HSCs) or marrow (MSCs), a very interesting source of cells for tissue engineering of bone and cartilage.



Dge, 2,1, Cell development pathways

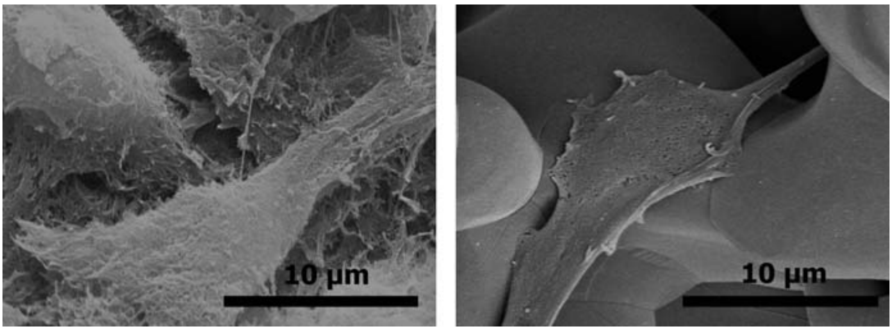
Differentiated Phenotypes Derived from MSCs

- Fibroblast
- Osteoblast
- Chondrocyte
- Adipocyte
- Skeletal myocyte
- Cardiac myocyte
- Myofibroblast
- Endothelial cell
- Pericyte
- Tenocyte
- Thymic stromacell

MSCs are present in fetal tissue, post-natal bone marrow and also in the bone marrow of adults (Caplan, 1991). A number of markers are expressed on MSCs. Some of these have been used not only to characterise these cells but also to enrich MSCs from populations of adherent bone marrow stromal cells. However, none of these markers seem to be specific for MSCs. Although the bone marrow serves as the primary reservoir for MSCs, their presence has also been reported in a variety of other tissues (periosteum, muscle connective tissue (Nathanson, 1985; Nakahara et al., 1991), fetal bone marrow, liver, and blood (Campagnoli et al., 2001)). Transfer of these tissues (e.g. coverage of cartilage defects by periosteum) may act also through stem cell-induced repair mechanisms. Whereas MSCs have been identified in fetal blood (Campagnoli et al., 2001) and infrequently observed in umbilical cord blood (Erices et al., 2000; Gutierrez-Rodriguez et al., 2000; Mareschi et al., 2001), it is not definitively solved whether MSCs are present in steady state peripheral blood of adults or not, but the number of stem cells in peripheral blood is probably extremely low (Zvaifler et al., 2000).

A main problem in using adult stem cells in a given clinical situation is the difficulty in obtaining a significant number of stem cells to generate cell constructs of large size. Limitations of adult stem cell harvesting have therefore given rise to the use of fetal stem cells, particularly on fetal bone marrow cells (FBM). This cell source seems to be a promising source for tissue engineering approaches, since they have a significantly reduced immunogenity, compared to adult stem cells. In contrast to adult tissues, fetal tissues produce more abundant trophic substances and growth factors, which promote to a greater extent cell growth and differentiation. Research on fetal tissue transfer in animal models and in human experimental treatments have confirmed distinct advantages over adult tissues, including lowered immunogenicity and a higher percentage of primitive cells. Due to the lowered immunogenity, fetus-derived stem cells appear to remove, to some extent, the problems of tissue typing. As such cells also have a high capacity to differentiate into the complete repertoire of mesenchymal cell lineages, combined with the ability for rapid cellular growth, differentiation and re-assembly, fetal cells may become a more important subject in tissue engineering strategies. (Fig 4.4) Fetal stem cells have, beneath their enormous potential for biomedical and tissue engineering applications, some serious limitations. There is a lack of methods that enable tissue engineers to direct the differentiation of embryonic stem cells and to induce specific functions of the embryonic cell-generated tissue after transplantation (Wobus, 2001). Two other issues are of main concern (Wobus, 2001). (1) It must be assured that the cultivation and transplantation of such stem cells are not accompanied by a tumorigenic differentiation. As it was shown that undifferentiated ES cells give rise to teratomas and teratocarcinomas after implantation in animals, this potential misdevelopment constitutes a major problem for the clinical use of such cells. (2) It must be assured that the in vitro generation of cells does not lead to an immunological incompatibility towards the host tissue. Immunological incompatibilities can be avoided by using the somatic cell nuclear transfer methodology (SCNT) (Alison et al., 2002). However, nuclear cloning methods are often criticised for this potential risk. The main problem in employing fetal cells for use in tissue engineering is not only obtaining such cells, but perhaps more important, the clinical, legal and ethical issues involved in such treatment strategies, which are probably the most difficult barrier to overcome (see Chap. 12).

Many attemps have been undertaken to refine procedures for the propagation and differentiation of stem cells. Despite the various advantages of using tissue-de-



Dge, 2,2, Fetal stem cell-based tissue engineering. Fetal stem cell cultured under defined conditions on an artificial material display the phenotypic appearance of mature bone cells. a Scanning electron microscopical picture of osteoblasts at the surface of a bone trabecula. b SEM view of a murine fetal stem cell cultured on a PLA/PGA scaffold rived adult stem cells over other sources of cells, there is some debate as to whether large enough populations of differentiated cells can be grown in vitro rapidly enough when needed clinically. At present, stem cells are, for example, not able to differentiate definitively into osteocyte-like cells, which are competent to mineralise the pericellular region in a bone-like manner under in vitro conditions (Jaiswal et al., 1997; Plate et al., 1998). This must be considered as one limitiation for the use of stem cells in extracorporal skeletal tissue engineering. Much more basic research is therefore necessary to assess the full potential of stem cell therapy to reconstitute skeletal mass. It is expected that many future studies will be directed towards the development of gene therapy protocols employing gene insertion strategies (Evans and Robbins, 1995). The concept that members of the bone morphogenetic proteins (BMP) and the transforming growth factor-beta (TGF-beta) superfamily will be particularly useful in this regard has already been tested by many investigators (Lieberman et al., 1998; Oakes and Lieberman, 2000). Genetic engineering to shape gene expression profiles may be, therefore, a future route for the use of stem cells in human tissue engineering, but this approach is at the moment a long way from clinical applications (see Chap. 12).

Differentiated osteoblast- or chondrocyte-like cells serve as common cell lines in evaluating preclinical and clinical aspects of bone and cartilage tissue engineering. Osteoblasts, derived from multiple sites of the skeleton, can be harvested in the form of precursor cells, lining cells, mature osteoblasts or osteocytes. Cell separation is then performed by various techniques in order to multiply distinct cell lines in culture (see Chap. 5). Chondrocytes can also be obtained from the various cartilage-containing tissue sites. They are easy to harvest and are, in most situations, in a mature differentiation stage.

A complementary approach in experimental settings can be distinguished from the use of primary cells: specifically modified cells. Whereas primary cells are commonly used in clinical cell-based engineering strategies, some of the genetic alterations of cells for use in bone or cartilage reconstruction have, up to now, been restricted to laboratory studies. Experiments using i*n vitro* assay systems have yielded not only much basic information concerning the cultivation of these cell lines, but can also be used for tissue engineering studies in basic and preclinical investigations. Each cell source has its inherent advantages and limitations.

4.3 Genetically Modified Cell Lines

Cell lines that are in use for evaluating aspects of extracorporal bone tissue engineering include, besides primary cells, the following cell types: (1) osteosarcoma cell lines; (2) intentionally immortalised cell lines; and (3) non-transformed clonal cell lines. (Jones et al., 1991). In the following we will describe these different cell types.

Osteosarcoma cell lines are known to display patterns of gene expression, modes of adhesion, and signal transduction pathways that in certain aspects resemble those of normal, non-transformed bone cells. Most of the osteosarcoma cell lines used, however, do not display a complete pattern of in vitro differentiation. The development of established clonal osteoblast-like cells from rat osteosarcomas (MG-63, UMR and ROS series) provided cell lines that were homogeneous, phenotypically stable, and easy to propagate and maintain in culture (Wada et al., 1998). They share many of the properties of non-transformed osteoblasts. But, as with cancer cells, these cells are transformed and display an aberrant genotype, have an uncoupled proliferation/differentiation relationship, and exhibit phenotypic instability in long-term culture. Therefore, these osteoblast-like cells do not reflect the normal phenotype of primary osteoblast-like cells. Therefore, as substrate-dependent cell reactions are difficult to assess, these cells seem not to be suitable to evaluate biomaterial-related aspects in tissue engineering and cannot be introduced into clinical engineering techniques.

Other approaches have been done by using clonally derived immortalised or spontaneously immortalised cell lines (neonatal mouse MC3T3E1 and fetal rat RCJ cell lines; Elgendy et al., 1993). Although none of these cell lines behave exactly alike and their behaviour in cell culture differs considerably (Aubin, 1998b), they do have some common features (alkaline phosphatase activity, collagen type I production, bone-like nodule formation). Despite these common features cells can be in different stages of growth and development under cell culture conditions. They have therefore various phenotypic features, depending on the cell culture situation. Conditionally transformed immortalised human osteoblast cell lines were developed by various researchers aimed to investigate the behaviour of osteoblasts towards external stimuli. Xiaoxue and co-workers (2004) for example assessed the generation of an immortilised human stromal cell line, which contains cells able to differentiate into the osteoblastic cells. Concerning the use of immortalise cells in ex vivo tissue engineering approaches, it is important to recognise that all cell lines impose the disadvantage of having unique phenotypes, so that the morphological sensitivity towards a changing environment (material surface, external stimuli) is impaired.

Considering the features of genetically altered cells, it becomes obvious that non-transformed and primary cultured osteoblasts are advantageous in extracorporal tissue engineering, since these cells display a well-defined inverse relationship of proliferation and differentiation (Owen et al., 1990). Measures of osteoblast-specific matrix protein expression define valuable reference points for the study of regulated osteoblast physiology, especially when a substratum-dependent reaction is under investigation. Oreffo and Triffitt (1999) demonstrated that primary cells are able to react sensitively to minor structural alterations in their surroundings, a key feature which is advantageous in tissue engineering concepts. To assess cellular reactions towards scaffolds the use of primary and non-transformed cells is advisable. It should be noticed that the reaction of cells towards the material is also dependent on the cellular maturation stage (Boyan et al., 1996). Additionally, it should be emphasised that the behaviour of osteoblasts on artificial surfaces is dependent on the experimental cell culture conditions (for a review, see Coehlo and Fernandes, 2000).

Clonal sarcoma cell lines with cartilage phenotypes were established from various sources (for a review, see Kudawara et al., 2004). The human chondrosarcoma cell line HCS-2/8 exhibits a polygonal to spherical morphology as the cells become confluent. After reaching confluence, the cells continue to proliferate slowly and form nodules, which show metachromatic features (for a review see Vautier et al., 2003). Electron microscopically, the cells resemble features of chondrocytes and produce an extracellular matrix consisting of thin collagen-like fibrils. Immortalised chondrocytes have been generated that serve as reproducible models for studying chondrocyte function. Immortalisation of chondrocytes increases the life span and proliferative capacity but does not necessarily stabilise the differentiated phenotype.

Immortalisation of primary human chondrocytes has been done with SV40-TAg, HPV-16 E6/E7, and telomerase by retrovirally mediated transduction, and selection for neomycin resistance are described. It was observed that stable integration of an immortalising gene stabilises proliferative capacity, but not the differentiated chondrocyte phenotype.

Harvesting and Cultivation

5

In order to obtain well-defined ex vivo multiplied cells, it is important to consider the various aspects of cell-based tissue engineering strategies. These aspects include the techniques used to harvest cells, the methods used to isolate and enrich cells, the standard conditions used for cell cultivation, and the methods used to screen cell culture outcomes. The parameters differ dependent on the cell source used for the tissue engineering approach.

5.1 Marrow-derived Stem Cells

Bone/marrow-derived stem cells are commonly obtained through a marrow aspiration procedure. Common locations to harvest bone marrow are the iliac crest and the sternum. (Bruder et al., 1997; Jaiswal et al., 1997; Joyner et al., 1997; Meyer et al., 1998; Bianco et al, 2001). In the first attempts to create tissue-engineered bone, unfractioned fresh autologous or syngeneic bone marrow was used (Ohgushi and Caplan, 1999; Grundel et al., 1991; Wolff et al., 1994). Because bone marrow is known to contain osteogenic and chondrogenic precursor cells, its use was perceived to potentially facilitate bone and cartilage regeneration. Principally, mesenchymal stem cells (MSCs) were harvested for these investigations by marrow aspiration, expanded in culture, and then re-implanted. Studies using this protocol have indicated that mouse marrow fibroblastic cells, obtained through the aspiration procedure, implanted locally or injected systemically, homed to bony sites and persisted there, participating in the regenerative processes (Perka et al., 2000; Wiesmann et al., 2003). Various investigations have used different methods to selectively isolate and enrich MSCs (Jackson et al., 1981; Ogushi et al., 1989a; 1989b). Isolation of MSCs is commonly based on density gradient centrifugation and cell culture techniques. Various cell culture techniques have been developed to allow MSCs to be cultured and expanded in number without undergoing differentiation (Nakahara et al., 1990a; 1990b; Bruder et al., 1997). The phenotype of the cells appeared to be stable throughout the culture period without loss in osteogenic, chondrogenic or adipogenic potential (Bruder et al., 1997; Pittenger et al., 1999). It is important to note that presently no unique phenotype has been identified that permits the reproducible isolation of MSC precursors with predictable developmental potential. The isolation of stromal cells is mainly dependent on their ability to adhere to plastic as well as their selective expansion potential. Human bone marrow progenitor cells were shown to be isolated and enriched by using selective markers (Simmons and Torok-Storb, 1991; Stewart et al., 1999). As a number of markers are expressed on MSCs some of these have been used to selectively obtain subpopulations of more determined cells. Phenotypic markers of MSC (as well as HSC) belong to different classes (according to Otto and Rao, 2004): surface antigens, secreted proteins, surface receptors, cytoskeletal proteins and ECM proteins. Phenotypic markers of osteogenic MSCs are listed below. Most of these are not specific or indicative for stem cells.

MSC

Qspd_ac _lrgeclq
CD13, CD29, CD44, CD49a, CD71, CD90, CD105, CD114,CD166, HLA-ABC, Glycophorin A, gp130, ICAM-1/2, Mab 1740,p75 NGF-R, SH3, SH4, Stro-1, HLA classII

Qcapcrcb npmrcglq
Interleukins IL1α, 6, 7, 8, 11, 12, 14, 15, LIF, SCF, FLT-3 ligand, GM-CSF, G-CSF, M-CSF

Qspd_ac pcacnrmpq
Il1-R, Il3-R, Il4-R, Il6-R, Il7-R, LIFR, SCFR, G-CSFR, VCAM-1, ALCAM1, LFA-3, IFNγR, TNF1R, TNF2R, TGFβ1R, TGFβ2R, bFGFR, PDGFR, EGFR

Awrmqicjcr_j npmrcglq
α-smooth muscle actin, GFAP

CAK amknmlclrq
Collagen type I, III, IV, V and VI, fibronectin, laminin, hyaluronan, proteoglycans

HSC express the following surface antigens:

Qspd_ac _lrgeclq
CD11a, CD11b, CD14, CD34, CD45, CD133, ABCG2, cKit, Sca-1

Human MSCs derived from bone marrow have not only been reported to maintain differentiation capacity into the osteogenic lineages for over 40 cell doublings (Reyes et al., 2001), but also when cultured in the presence of dexamethason and ascorbic acid, or selectively enriched through phenotypic markers, purified MSCs undergo a development characterised by the transient induction of alkaline phosphatase, expression of bone matrix protein mRNAs, and deposition of calcium (Pittenger et al., 1999). Cells were suggested to be able to form mineral-like foci indicative of an osteoprogenitor phenotype (Aubin, 1998a; 1998b), but it is not definitely solved whether this mineral formation resembles the mineral formation present in bone tissue. Phenotypic markers of chondrogenic MSC include also surface antigens, secreted proteins, surface receptors, cytoskeletal proteins, and ECM proteins.

Qspd_ac _lrgecl q
CD44, CD59, CDC42, HLA classIC

Qcapcrcb npmrcgl q
TGF β-3 IGF, BMP6

Qspd_ac pcacnrmpq
TGF β-3-R, BMP6-R

Awrmqi cjcr_j npmrcgl q
β-actin

CAK am k nml c l rq
Collagen type I, II, III, IX and XI, aggrecan, biglycan, chondromodulin, fibromodulin

As with osteogenic cells, many investigators have used enrichment techniques for MSCs for cartilage engineering because of their ability to differentiate into chondrocyte-like cells. Stem cells, which are the target cells for cartilage-inducing factors, retain thereby the ability to differentiate to more determined, functional chondrocytes. It was suggested that it is possible by various measures to induce MSCs to differentiate into chondrogenic lineage both in vivo and in culture (Johnstone et al., 1998; Caplan, 2000; Minguell et al., 2001). Various growth factors such as transforming growth factor-β3 (TGF-β3), bone morphogenic protein-6 (BMP-6), and insulin-like growth factor-1 (IGF-1) have been used to realise this goal (Frenz et al., 1994; Muraglia et al., 2000; Kucich et al., 2001; Martin et al., 2001; Sekiya et al., 2001; 2002; Worster et al., 2001; Rosado et al., 2002; Fukumoto et al., 2003; Schaefer et al., 2003). The in vitro chondrogenic differentiation of post-natal mammalian bone-marrow-derived progenitor cells has been described (Johnstone et al., 1998; Yoo et al., 1998). This in vitro system is applicable for evaluating the effects of particular factors on the chondrogenic process. The various growth factors can be used separately or together. Despite the finding of induced chondrogenic differentiation, the effects are currently only limited. It is assumed that the failure may be based on the fact that the in vivo environment contains a combination of various factors and matrices, with their content changing according to the cell differentiation process. The chondrogenic potential of these progenitor cells is therefore orchestrated by the combination of numerous factors. In vitro systems indicate that not only marrow-derived progenitor cells have the ability to differentiate into chondrocytes, but the cells differentiate into their terminal phenotype. The presence of hypertrophic chondrocytes, indicated by the presence of type X collagen mRNA and protein, is of special relevance (Johnstone et al., 1998; Yoo et al., 1998).

The presence of a metachromatic-staining matrix, the chondrocytic appearance of cells, and the immunochemical detection of type II collagen can be used in cell culture assays as indicators demonstrating that the tissue generated by marrow-derived cells is cartilage. Additionally, an immun-phenotypical characterisation is usually applied on culture-expanded cells and not on primary cells. However, none of the above mentioned markers are specific for MSCs, thus hampering the isolation of pure populations of MSCs. Commonly, after culture expansion, various stromal cell colonies are present. It seems that a proportion of the cultured cells remain multipotent and maintain multilineage potential into osteogenic, condrogenic, myogenic, endothelogenic, thymogenic and adipogenic lineages (Pittenger et al., 1999). These results indicate that even after isolation and enrichment strategies, some cells in culture retain multipotentiality.

As with differentiated cells, it is important to consider the basic cell culture conditions also for stem cell cultivation. In general, cell adhesion and cell differentiation is regulated by the conditions of the culture milieu. Cell adhesion is best, when cells are cultivated in a serum-containing media and when the surface of the culture dish is coated with proteins. In the absence of proteins, cells can hardly adhere to the surface as they have to synthesise the proteins to build up the proteinaceous interface (Fig. 5.1). Standard conditions for expansion of stem cells were established by a number of studies. These conditions include the cell density as well as the presence of serum (in most instances fetal bovine serum). As cell density is known to be a critical factor affecting the growth of cells, attempts to cultivate cells were done to enrich cells above a critical cell density. Additionally, cells can be grown directly – that is, unmanipulated after collection – or more often after density gradient separation. For clinical applications of stem cells used in extracorporal tissue engineering, it is imperative that in vitro culture protocols should be devoid of animal or human products, to avoid potential contamination with pathogens. By using defined standard conditions, it is possible to reduce the variability within the culture milieu, therefore providing also a more stringent level of quality control. A special problem of supplemented allogeneic animal or human proteins is the possibility that such proteins may enhance the antigenicity of cells upon transplantation. Ideally, the ideal culture milieu for stem cell differentiation in vitro should therefore be chemically defined, and either be serum-free, utilise synthetic serum replacements or use autologous serum (Wong and Tuan, 1993; Goldsborough et al., 1998). The possible supplementation of specific recom-

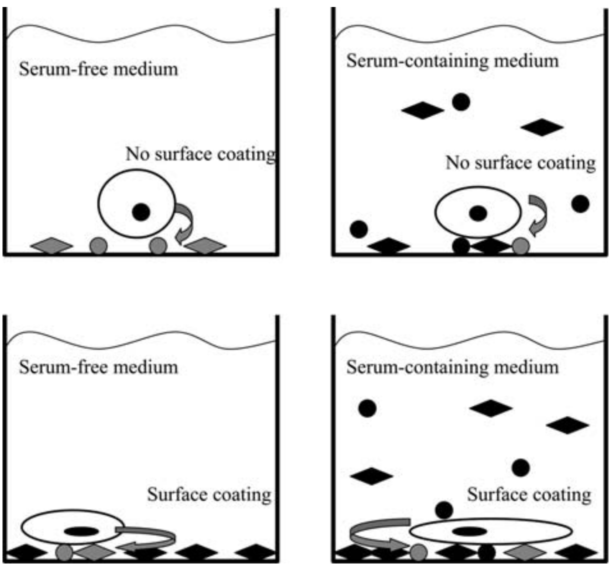


Fig. 5.1 Culture condition dependent cell attachment. *Top left*: Cell adhesion is impaired under serum-free culture conditions, especially when the substrate is not coated by adhesion proteins. Cell adhesion improves when a serum containing medium is in use (*top right*), or when the surface is coated prior sell seeding even in the absence of a serum containing medium (*bottom left*). Cell adhesion is best under conditions of surface coating in combination with the use of serum-containing medium (*bottom right*)

binant cytokines and growth factors may enhance the growth and differentiation of cells, especially when serum-free is in use. The major problems with culturing stem cells or more differentiated cells under serum-free conditions is that cells generally tend to have a lower mitotic index, become apoptotic, and display poor adhesion in the absence of serum compounds (Wong and Tuan, 1993). Serum supplementation is therefore usually required for in vitro culture of stem cells.

Autologous serum can be considered to be the ideal source of nutritional and stimulatory support of cells, when cells are used in immunocompetent animals or in clinical trials. For clinical applications, there are a number of reasons that serum should in future applications be completely eliminated from the in vitro culture milieu. The use of serum is accompanied by some disadvantages: the composition of serum is generally poorly defined. Serum batches possess a considerable degree of individual variation, even when obtained from the same patient. Additionally it is important to note that even serum is not completely physiological, since it is essentially a pathological fluid formed in response to blood clotting. Based on the individual patient situation, serum contains variable levels of growth and differentiation factors. The presence of these factors may therefore influence to an unknown extent the desired differentiation of stem cells into specific and well-defined lineages.

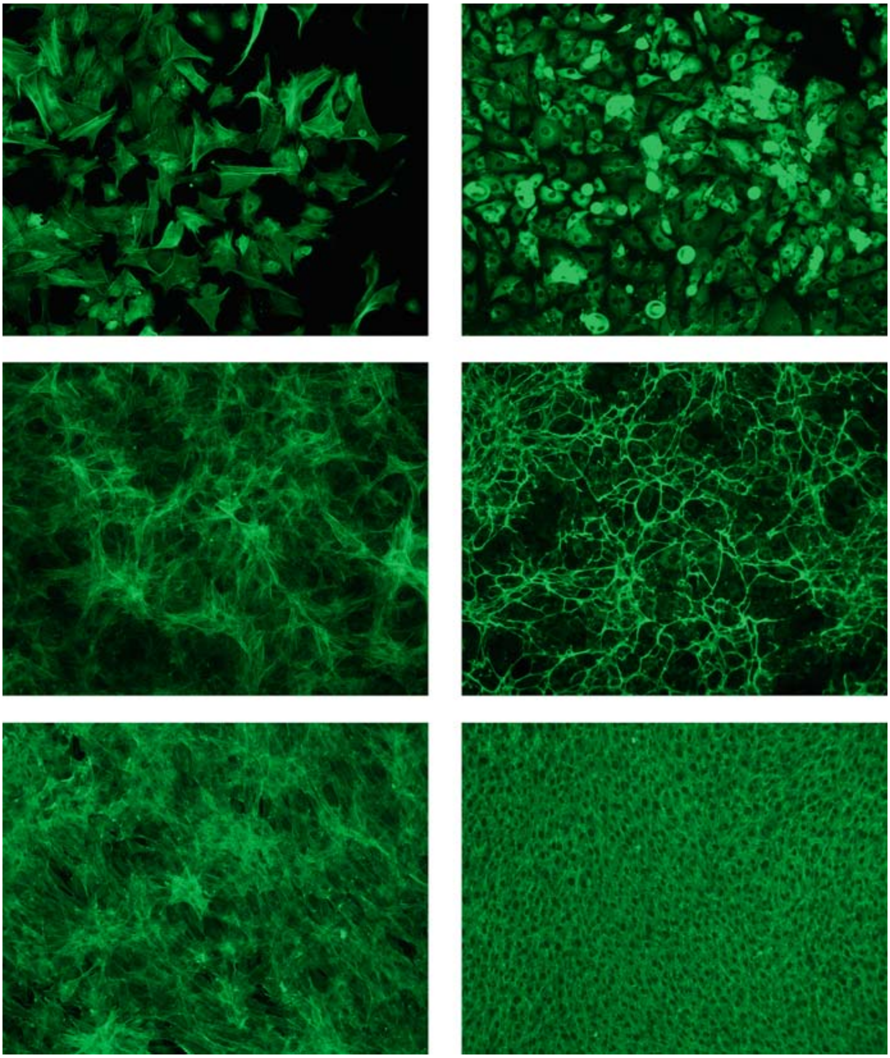
The development of chemically defined synthetic serum substitutes is therefore of special relevance. Commercially available synthetic serum substitutes (Wong and Tuan, 1993; Goldsborough et al., 1998) containing protein-based cytokines and growth factors or synthetic chemical compounds are currently under intensive investigation (Rogers et al., 1995; Schinstine and Iacovitti, 1997; Wobus et al., 1997; Liu et al., 2000; Buttery et al., 2001; Guan et al., 2001; Akita et al., 2002; Fukuda, 2002; Kim et al., 2002; Phillips et al., 2003; Rangappa et al., 2003). Such synthetic chemicals, possessing the advantage to be more stable compared to cell-based cytokines and growth factors, may improve not only the outcome of stem cell based tissue engineering approaches, but also strategies that use maturated cells.

Despite the success that has been obtained using bone-marrow-derived stem cells, one biological consideration limits its widespread application. Frequently, it is not feasible to obtain sufficient amounts of bone marrow with the requisite number of osteoprogenitor cells by marrow aspiration. In addition, the age-related decrease in bone marrow components, accompanied by a partial loss of precursor cells (Egrise et al., 1992; Quarto et al., 1995), is a frequent clinical limitation on obtaining sufficient numbers of stem cells. As mentioned before, the outcome of the in vitro use of bone marrow explants is critically dependent on the transfer of sufficient numbers of these progenitors. Therefore, the use of marrow-derived stem cells may be least applicable in those situations where it is most needed. It was in this respect shown that osteoprogenitors represent approximately 0.001% of the nucleated cells in healthy adult marrow (Haynesworth et al., 1992; Bruder et al., 1997), an indication of the practical problems obtaining pure stem cell sources by the cell culture strategies described. Therefore, improvements in all aspects of stem cell culture are necessary to further select, expand, and administer the progenitor marrow cell fraction in order to obtain clinically relevant numbers of osteogenic or chondrogenic stem cells.

5.2 Determined Bone Cells

It has long been known that the vast capacity for regeneration of bone is based on the presence of differentiated osteoblasts (Ashton et al., 1980). As the use of determined osteoblast-like cells does not raise (in contrast to the use of stem cells) legal issues and no problems of immune rejection, determined bone cells can be considered to be currently the most important cell source in bone tissue engineering. Therefore, in current clinical practice, differentiated autologous osteoblast-like cells are the most desirable cell source (Fig. 5.2). However, these cells may be insufficient to rebuild damaged bone tissue in a reasonable time. A considerable number of cell divisions is needed to bulk the tissue to its correct size. Former studies have regarded the propagation of adult mature cells in culture as a serious problem, because it was thought that most adult tissues contained only a minority of cells capable of effective expansion. However, in numerous recent investigations it has been shown that bone cells proliferate in culture without losing their viability. Various sources of determined bone cells can be used for cultivation. Committed osteoprogenitors, i.e. progenitor cells restricted to osteoblast development and bone formation, can be identified by functional assays of their differentiation capacity in vitro, such as the colony forming/units assay (CFU-O assay). A wide variety of systemic, local and positional factors regulate also proliferation and differentiation of determined cells, a sequence that is characterised by a series of cellular and molecular events distinguished by differential expression of osteoblast-associated genes, including those for specific transcription factors, cell cycle-related proteins, adhesion molecules and matrix proteins (for a review, see Yamaguchi et al., 2000). The mature osteoblast phenotype is in itself heterogeneous with subpopulations of osteoblasts expressing only subsets of the known osteoblast markers, including those for cytokine, hormones, and growth factor receptors, raising the intriguing possibility that only certain osteoblasts are competent to respond to regulatory agents at particular points in time.

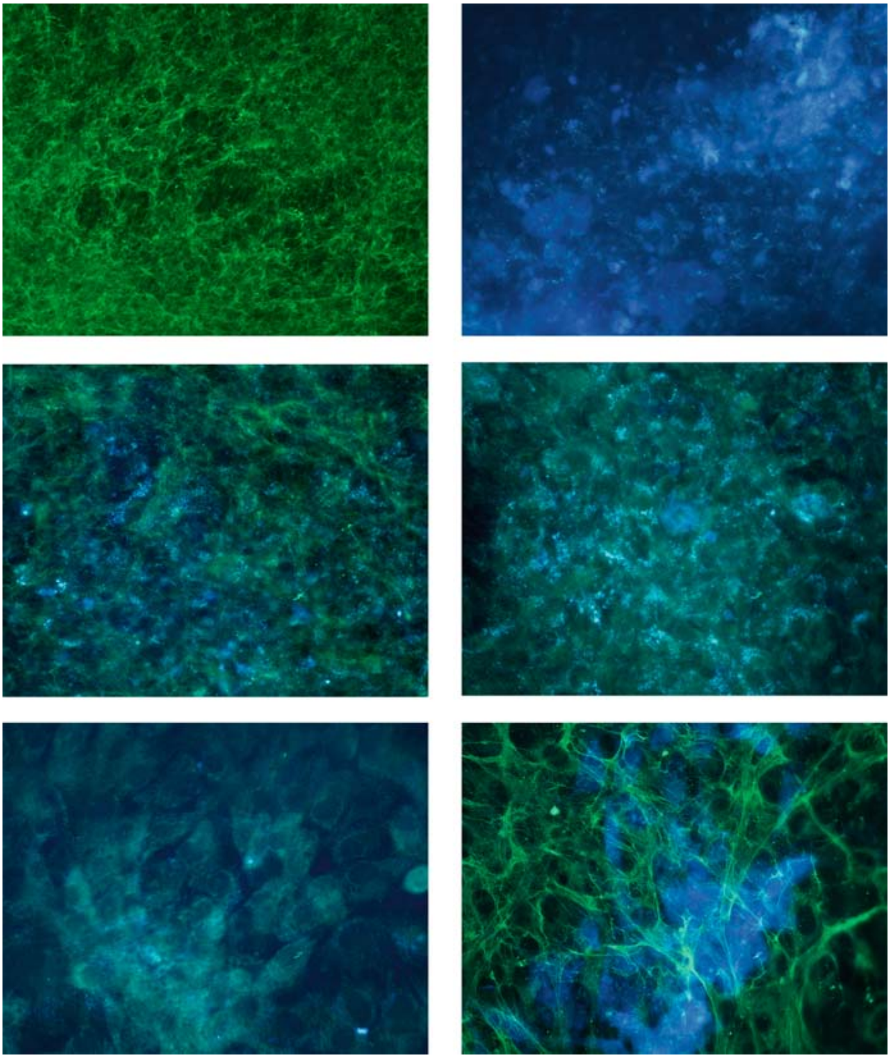
Cultures containing determined osteoblastic or osteoblast-like cells have been established from different cell populations in the lineage of osteogenic cells (osteoprogenitor cells, lining cells, osteoblasts, and osteocytes) (for a review, see Hutmacher and Sittinger, 2003). They can be derived from several anatomical sites, using different explant procedures. Bone cell populations may be derived from the cortical or cancellous bone, bone marrow, periosteum, and in some instances from other tissues. Isolation of cells can be performed by a variety of techniques, including mechanical disruption, explant outgrowth, and enzyme digestion (Vacanti et al., 1995). Commonly used procedures to gain cells are digestive or outgrowth measures. Outgrowth of bone-like cells can be achieved through culturing of periosteum pieces or bone explants. Cells located within the periosteum and bone can differentiate into fibroblastic, osteogenic or reticular cells (Friedenstein, 1976; Nuttall et al., 1998; Triffitt and Oreffo, 1998; Dahir et al., 2000; Bianco et al., 2001) (Fig. 5.3). Periosteal-derived mesenchymal precursor cells generate progenitor cells committed to one or more cell lines with an apparent degree of plasticity and interconversion (Nakahara et al., 1992; Park et al., 1999; Bahrami et al., 2000; Schantz et al., 2002a; 2002b). In culture expanded periosteum cells were shown to retain the ability to heal a segmental bone defect after being reimplanted and induce osteogenic tissue when seeded into diffusion chambers (Ashton et al., 1980; Ohgushi et al., 1989a; 1989b; Nakahara



Dge, 3,0, Fluorescence microscopy of cell behaviour and extracellular matrix formation in osteoblast culture. During culture, subconfluent located cells (top left) proliferate over time (top right) in an attempt to form a confluent cell layer (bottom right). During the same time, a dense extracellular matrix formation can be observed between cells (midline, bottom left)

et al., 1990a). Outgrowth cultures of periosteum pieces favour the co-culture of different cell types (Osteoplasts and Osteolasts) /(Meyer et al., 1993b).

It remains controversial whether cortical or spongy bone, gained by different post-surgical processing techniques, is a better material of choice (Girdler and Hosseini, 1992; Chen et al. 1994; Schwipper et al. 1997) to outgrow cells in culture. It has been suggested that particulate culturing is superior to bone chip culturing (Marx et al. 1984; Shirota et al. 1996), based on the assumption that when particle

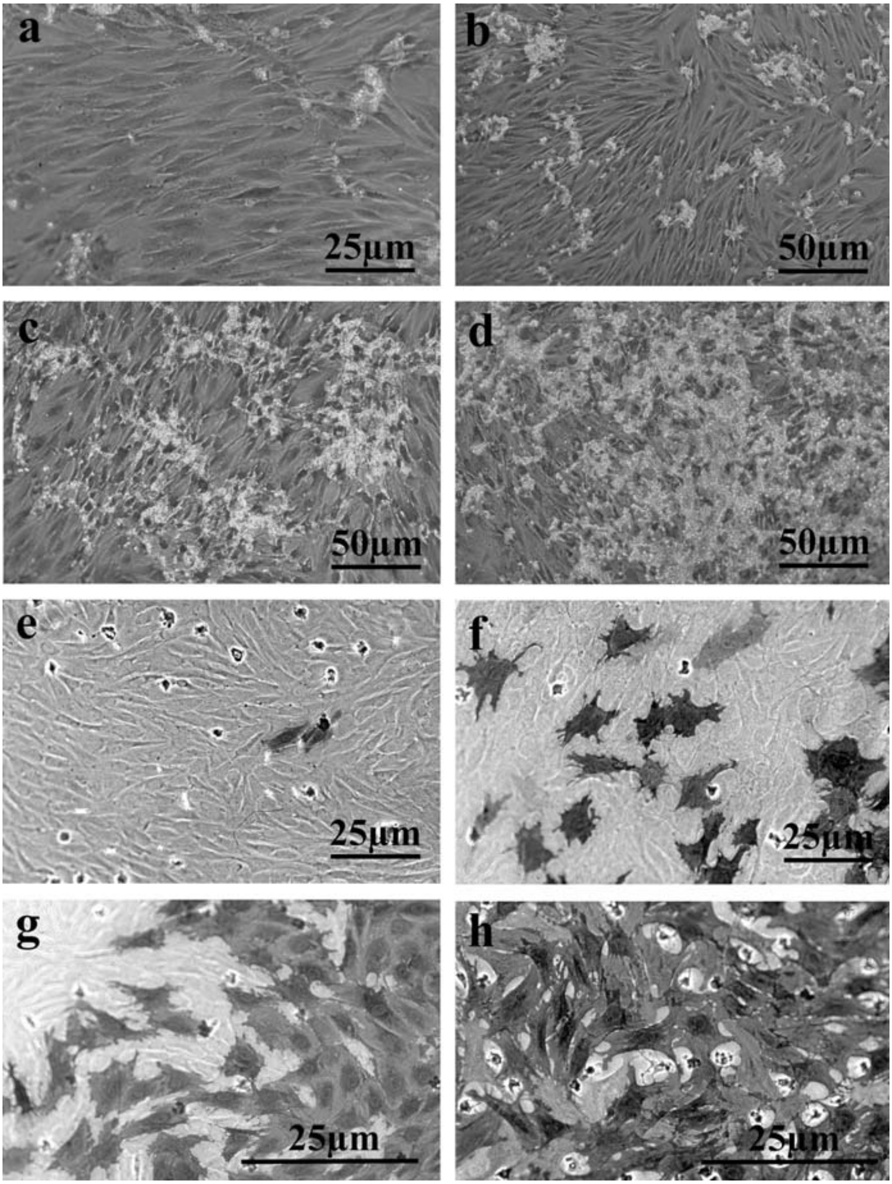


Dge, 3,1, Fluorescence microscopy of the mineral-matrix relationship in osteoblast culture. Mineral is deposited in direct contact to the extracellular matrix network. The green dye visualises the presence of immunostained proteins, blue colouring is indicative for the formation of hydroxyapatite. The colocalization approach gives insight into aspects of biomineral formation in osteoblast culture

size decreases, the absolute square measure of the surface of the tissue specimens in the culture dish increases. An increased absolute square measure of the transplant surface would increase the number of living cells released. Springer et al. (2004) demonstrated in an experimental study, that bone chips obtained from trabecular bone provided a higher cell number than those raised from cortical bone. Surprisingly, they found that processing of spongy bone graft in the bone mill (leading to bone particulates) results in lower absolute amounts of osteoblast-like cells, whereas use of the bone mill on cortical bone has much less impact on the number of cells counted. The authors speculated that the treatment of transplants with the bone mill or raising of transplants by rotating instruments should reduce the number of bone cells supplied, suggesting that a decreased particle size is disadvanageous when cell outgrowth methods are in use. Ecarot-Charrier and colleagues (1983) were the first to present a method for isolating osteoblasts from newborn mouse calvaria using digestive enzymes in solution. As it was demonstrated that isolated osteoblasts gained through this method retained their unique properties in culture, tissue digestion became a common method of cell harvesting for in vitro purposes.

Once such matured cells have been isolated from the tissue, there are also in such culture strategies a number of parameters that influence the expression of the osteoblastic phenotype in cell culture, most important the culture medium, culture time, number of passages and the presence of compounds. The presence of ascorbic acid, ß-glycerophosphate and dexamethasone influence the expression of the osteoblastic phenotype in a differentiated manner. β-Glycerophosphate for example induces phenotypic matrix maturation by enabling mineral formation in osteoblast-like cell cultures (Fig. 5.4). Dexamethasone, as an additional factor, is described as inducing cell differentiation but imposing a negative effect on cell proliferation, indicative of a reciprocal and functionally coupled relationship between proliferation and differentiation. Therefore, it is convenient to select suitable experimental conditions for cultivation. As with stem cell cultivation, the culture conditions should be well defined in order to standardise the ex vivo grown product.

Because endochondral bone formation and, frequently, fracture repair proceed through a cartilaginous intermediate, some investigators have suggested that the transplantation of committed chondrocytes would also ameliorate bone regeneration (Bahrami et al., 2000). Vacanti and colleagues (1995) compared the ability of periosteal progenitors and articular chondrocytes to effect bone repair. They showed that periosteal cells from newborn calves seeded on a scaffold and implanted in critical-sized calvarial defects generated new bone. Specimens examined at early times contained material that grossly and histologically appeared to be cartilage. The scaffold seeded with chondrocytes also formed cartilage. However, no endochondral ossification was observed, since the transplanted specimens remained in a cartilaginous state. Therefore, chondrocytes proved ineffective as a cell-based therapy for tissue engineering of bone. Because mature cartilage is thought to produce factors that inhibit angiogenesis, implants seeded with committed chondrocytes may prevent the endochondral cascade by preventing vascular invasion. Cells derived from cartilage seem to be committed to retaining their phenotype and, therefore, are unable to differentiate towards hypertrophic chondrocytes under the experimental conditions tested so far. In contrast, when precursor cells from the periosteum are provided, their primitive developmental state allows them to proceed through the entire chondrogenic lineage, ultimately becoming hypertrophic chondrocytes. The molecular basis for the difference in the phenotypic potential of these different cell types remains mysterious and is an area of intensive investigation.



Dge, 3,2, Mineral formation and alkaline phosphatase (ALP) expression in osteoblast culture. _«b The amount of biomineral formation can be observed through the deposition of calcium phosphate-containing minerals *(bright re s)* after 1, 2, 3, and 4 weeks, respectively. c«f The extent of ALP expression *(d rk re s)* correlates with the biomineral deposition after 1, 2, 3, and 4 weeks of culture

5.3 Determined Chondrogenic cells

Clinical cartilage engineering strategies have, to date, predominantly focused on the use of an unselected source of chondrocytes (Brittberg et al., 1994). In the ongoing search to improve cohondrocyte cell lines, the use of specific chondrocyte populations are now being considered to investigate whether an improved cartilaginous structure would be generated in vivo and in vitro by these specifically selected populations of determined chondrocytes (Redman et al., 2005). As distinct phenotypic and functional properties of chondrocytes across the zones of articular cartilage are present, it seems reasonable to search for the best source of chondrocyte subpopulations (Waldman et al., 2003). It was reported in this respect that a combination of mid- and deep-zone chondrocytes seems to be more suitable for the ex vivo generation of a hyaline-like cartilage tissue. Dowthwaite et al. (2004) recently reported on an isolation technique for chondrocytes that reside in the superficial zone of immature bovine articular cartilage. These cells, characterised as determined chondrogenic cells, were shown to allow appositional growth of the articular cartilage from the articular surface (Hayes et al., 2001). Therefore, when chondrocytes are aimed to generate a cartilage-like structure ex vivo, it seems to be reasonable not to gain full thickness cartilage implants but to use subpopulations of chondrocytes. Separation of cartilage zones after explantation and before cultivation with a selective subpopulation may therefore provide a tool to improve tissue engineering strategies using determined cells. Phenotypic plasticity was tested by a series of in ovo injections where colony-derived populations of these chondroprogenitors were engrafted into a variety of connective tissue lineages, thus confirming that this population of cells have properties akin to those of a progenitor cell. The high colony-forming ability and the capacity to successfully expand these progenitor populations in vitro (Dowthwaite et al., 2004) may further aid our knowledge of cartilage development and growth and may provide novel solutions in ex vivo cartilage tissue engineering strategies.

5.4 Osteoclasts

Osteoclasts may be used in bone tissue engineering strategies, since these cells play an important role in bone physiology and since a culture containing both cell lines would more closely mimic the in vivo bone situation. Osteoclasts are derived from mononuclear cells of hemopoietic bone marrow and peripheral blood. The potential to differentiate into mature osteoclasts in culture is maintained by a variety of cells (Hayashi et al., 1998; Chambers, 2000), including hematopoietic stem cells and colony-forming precursors cells, elicited by hematopoietic colony-stimulating factors, and their progeny in the colonies (Yamazaki et al., 2001). Moreover, well-differentiated cells, such as monocytes and even mature macrophages, are capable of differentiating into osteoclasts (Akagawa et al., 1996). As the differentiation pathway is common to that of macrophages and dendritic cells, factors can be used to selectively induce differentiation. A promyeloid precursor can differentiate into an osteoclast, a macrophage or a dendritic cell, depending on whether it is exposed to some osteoclast-inducing factors (RANKL, OPGL, ODF, M-CSF, GM-CSF). As some groups demonstrated that bone marrow stromal cells and osteoblasts produce membrane-bound and soluble RANKL/TRANCE/OPGL/ODF factors, an important positive regulator of osteoclast formation (Lacey et al., 1998) is a co-culture of osteoclasts with osteoblasts. Alternatively, culturing of osteoclasts can be performed by enrichment of osteoclast precursor cells from the peripheral blood or from bone marrow. Periosteum outgrowth techniques allow also the propagation of osteoclastic cells from monocytes located in the periosteum (Szulczewski et al., 1993). Recent research suggests that multinucleated osteoclasts can be cultivated by adding alveolar mononuclear cells to new-born rat calvaria osteoblasts in vitro.

5.5 Endothelial Cells

The additional use of vascular cells offers several theoretical advantages over approaches of extracorporal bone tissue engineering exploiting only bone cells as a single cell type. As a cell-based strategy, endothelial cell therapy promises to deliver at the same time both substrate (endothelial cells) and cytokines and growth factors. Endothelial progenitor cells as well as mature endothelial cells are capable of settling in areas of bone neovascularisation, thus exerting their effects in sites in need of new blood vessel growth (Asahara et al., 1999). As these cells are present in nearly all sites of the body, they exhibit no unfavourable side effects when transplanted autologously. The induction of angiogenesis as well as their participiation in new vessel formation makes them appealing components for bone tissue engineering. Endothelial cells promote synergistic vasculogenesis and bone formation. The action of endothelial cells on osteoblast function was demonstrated recently through various cell culture studies.

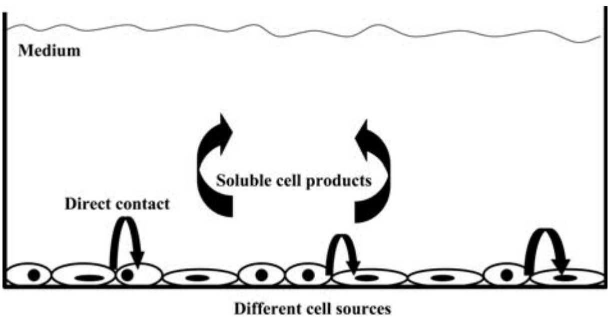
Endothelial progenitor cells were first described by Asahara et al. in 1997. Since then, significant progress has been made in defining the origin and lineage of these cells. A number of studies gave evidence that endothelial progenitors originate in the bone marrow and are then selectively recruited to sites of neovascularisation. Therefore, bone marrow cells as well as cells obtained from peripheral vessels are in use for tissue engineering approaches.

The relative ease of isolating and expanding mature endothelial cells (from explanted blood vessels) or endothelial progenitor cells (from bone marrow) makes them an attractive source of autologous vascular cells for the generation of a vascularised scaffold complex in vitro. Studies have demonstrated that endothelial cells form ring-like structures in two-dimensional cultures and tubules in three-dimensional extracellular matrices in vitro. It is assured that they are able to induce vascular invasion in the host tissue if implanted (Al-Khadi et al., 2001; Murayama et al., 2002). Studies with various cell lines indicated that patency rates were strongly correlated with the amount of host cells (smooth muscle cells and endothelial cells) incorporated into the graft (Jarrell et al., 1986; Weinberg and Bell, 1986; L´Heureux et al., 1998; Deutsch et al., 1999). Whereas most of these investigations were not intended to induce the formation of vascular matrices for tissue engineering, the findings are encouraging in light of recent work showing the potential of axial vessels to vascularise cellular scaffolds in vitro and in vivo (Chung et al., 2003).

By using osteoblast-like cells and endothelial cells it seems to be possible to create complex tissue-engineered vascularised bone constructs. In recent studies tissue-engineered bone constructs have been fabricated by combining autologous vascular cells and bone cells in a porous scaffold structure. Endothelial cells may accelerate the defect-healing process by improving cell nutrition in the scaffolds as well as at the defect site. It was shown that the success of grafts seeded with endothelial cells was significantly greater than that of non-seeded grafts (Kaushal et al., 2001). Vacanti and co-authors (1995) demonstrated early on in vivo studies that bone replacement materials could be effectively combined with autologous endothelial cells. An additional advantage of using endothelial cells is their potential for thrombus regression. As thrombosis is a serious problem in tissue engineering, prevention of microvascular failures is of major importance after a tissue-engineered bone construct is implanted in vivo. In the future, endothelial cells offer new perspectives to improve the ex vivo formation of a more mature bone construct, thereby accelerating the process of new blood vessel formation in vivo. With our recent understanding of the physiological roles of endothelial cells, the importance of vasculogenesis in extracorporal bone tissue engineering has come into focus, especially in light of the fact that cell survival balanced by nutrition is one of the main limiting steps in scaling up bone constructs for clinical use.

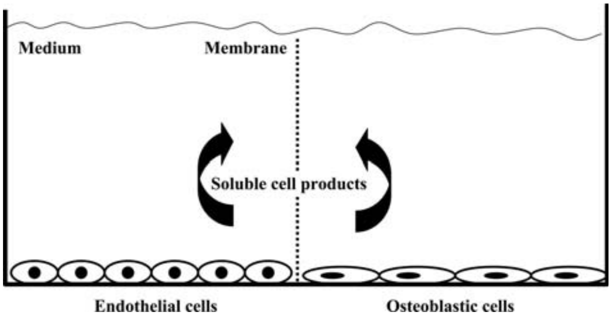
5.6 Co-cultures

Co-cultures of various cell lines (osteoblasts and osteoclasts, osteoblasts and endothelial cells as well as the co-culture of differentiated and stem cells) can be used to improve extracorporeal tissue engineering strategies (Richards et al., 2002; Yamaguchi et al., 2002). The major advantage of co-cultures of differentiated cells and stem cells for use in extracorporal tissue engineering strategies is that the pool of stem cells allows the renewing of cells and, via efficient signal transduction pathways, the induction of a differentiated cell population. As with pure differentiated cell co-culture systems, autocrine and paracrine factors secreted by one cell type readily interact with other cell types when different cell lines are in use. Hence, co-cultures provide a more physiological environment for cell regeneration in vitro that more closely resembles the in vivo conditions, especially in bone tissue engineering. However, recently it has been reported that under certain circumstances intimate physical contact between different cells during co-culture may lead to the fusion of different cell types, resulting in the formation of heterokaryons (Terada et al., 2002; Ying et al. 2002). Three different approaches for a co-culture of cells are possible:

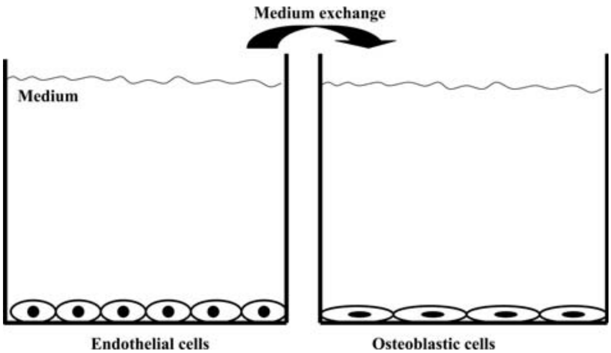


Dge, 3,3, Co-culture: two cell types in one chamber

Dge, 3,4,Co-culture: two cell types in one chamber, separated by a permeable membrane



Dge, 3,5, Co-culture: different cell types are cultivated in different chapters followed by a medium exchange



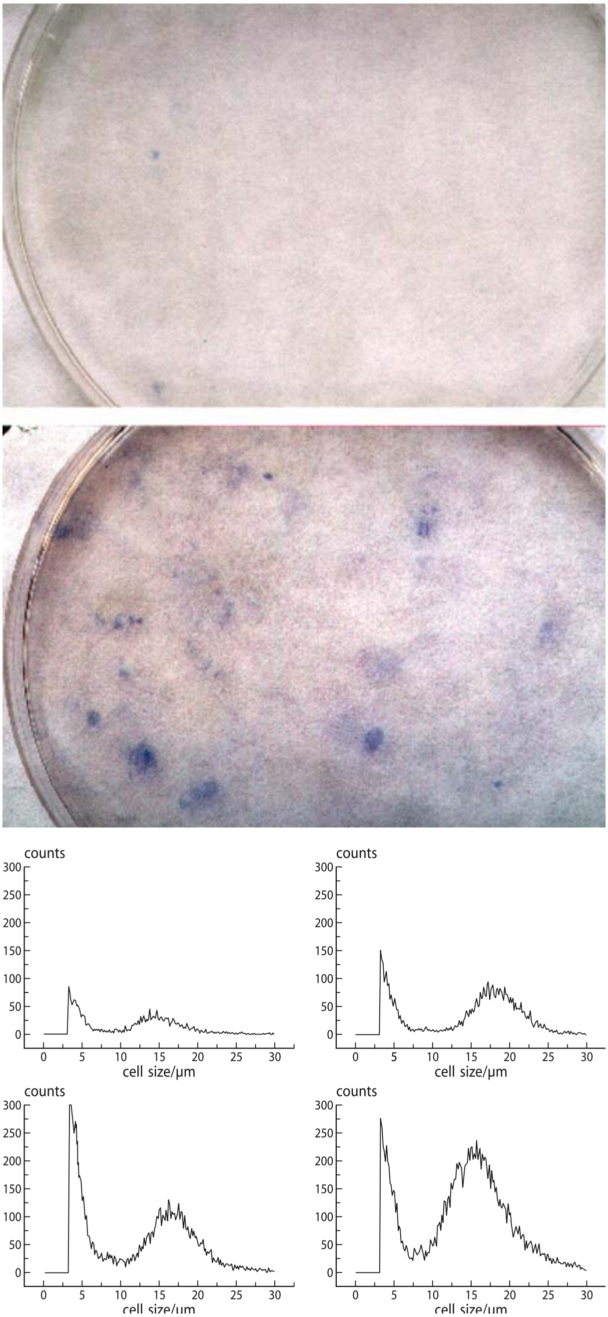
first, the culture of two or more cell lines in one chamber (Fig. 5.5); second, the culture of two cells in one chamber, separated by a selectively permeable membrane (Fig. 5.6); and third, the exchange of medium secreted by cells (when cells are cultivated in different chambers) (Fig. 5.7). It is known that the last two approaches work through a culture media conditioned by one type of cells that contain various soluble factors, capable of inducing cell differentiation. As such factors are easy to transfer, a conditioned media seems to be desirable for cell scaffold maturation in vitro. The advantage of using cell-conditioned media or the separation of two different cell lines by a permeable membrane over pure co-culture techniques is that there is no risk of heterokaryon cell function. Additionally, filtering the conditioned media can significantly reduce the risk of contamination with a different cell type, and decreases the risk of disease transmission. The major disadvantage of using membranes or exchange medium, as opposed to conventional co-culture, is that there is no intimate physical contact and regulatory cross-talk between different cell types. The subsequent limitation of differentiating signal transduction between the various cells is also not close to the in vivo situation. Indeed, a number of studies have shown that, in some cases, conditioned media failed to elicit cell proliferation and differentiation, when compared to a pure co-culture (Rangappa et al., 2003).

5.6.1 Techniques of Cell Culture Evaluation

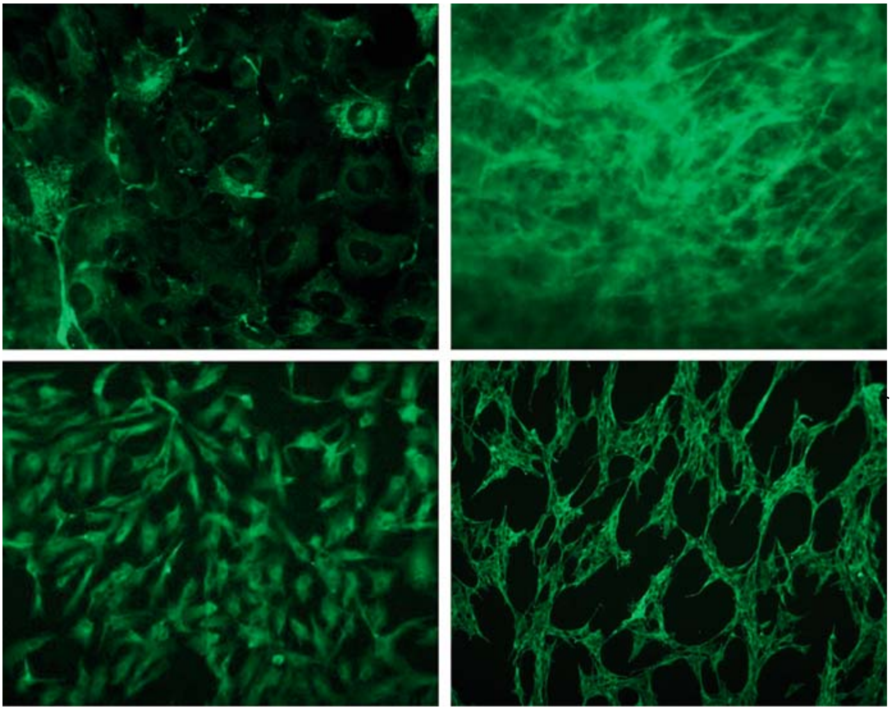
The success of cell culturing is monitored by both biochemical and morphological investigations (Figs 5.8–5.15). Biochemical criteria include all aspects from molecular features of the cell to the composition of the extracellular matrix (Table 5.1). Morphological aspects range from ultrastructural analysis of cells and matrix components to the spatial relationships of cells in a three-dimensional tissue construct (Table 5.2). Initially, biochemical assays used traditional methods of protein chemistry (e.g. immunostaining, electrophoresis and blot techniques) (Fig 5.8; Fig 5.9). More recently, the availability of genetic probes for many of the bones and cartilages proteins has made it easy to assess gene expression of multiple phenotypic markers (Fig 5.10). Advances in imaging and analytical techniques allow morphological insight into the creation of engineered tissue by using high resolution pictures (transmission electron microscopy, scanning electron microscopy, X-ray diffraction, atomic force microscopy) (for a review, see Manso et al., 2002). In addition, some of the new techniques enable combined biochemical and morphological analysis of probes (TOF-SIMS).

In addition, some of the new techniques enable combined biochemical and morphological analysis of probes (TOF-SIMS). Biomechanical features of cells in culture can be determined by special investigations. Cell adhesion strength, for example, can indirectly be assessed by cell detachment evaluations. The relative strength of cell adhesion can be determined by trypsination experiments (Fig. 5.11). The amount of adhered cells is an indicator for the number and strength of focal adhesions and can provide information concering the biology of cell–material interaction.

Generally, it is important to consider that the outcome of individual cell culture determinations is dependant on the chosen cell culture parameters. Characterisation of cells can be well performed by determination of the scope of synthesised extracellular matrix proteins (Fig. 5.12). The results of such immunostaining determinations are dependant not only on the preferred cell source but also on the chemicals used in the cell culture. The expression and the visualisation of synthesised matrix proteins, for example, differ in dependence of the preferred blocking system (Fig. 5.13) and serum batches (Fig. 5.14). One of the most influencing factors in cell differentiation is, as mentioned before, the spacial placement of cells. The phenotypical appearance as well as the differentiation capacity of chondrocytes differs significantly between culture of cells in a two- or three-dimensional cell culture system (Fig. 5.15), indicative for the complex reactivity of cells towards their cell culture microenvironment. Advancements in genetic analysis techniques allow the determination of cell differentiation not only at the protein level, but also at the level of gene expression (Fig. 5.11). Nowadays, the use of gene chips enables a comprehensive insight in gene activity of cells (Fig. 5.16, Table 5.3). The application of gene chip technology can therefore be expected to significantly improve the possibility to get a more detailed insight in cell behaviour, especially when the screening results of gene chip technology are confirmed by the PCR technique. Evaluation of cell-based tissue engineering strategies in vitro and in vivo can now be determined more precisely by gene analysis technology (Table 5.4, Appendix). In general it is mandatory, in order to compare results of different in vitro experiments, to consider the cell culture outcomes in light of the individual experimental setting and the chosen analytical techniques.



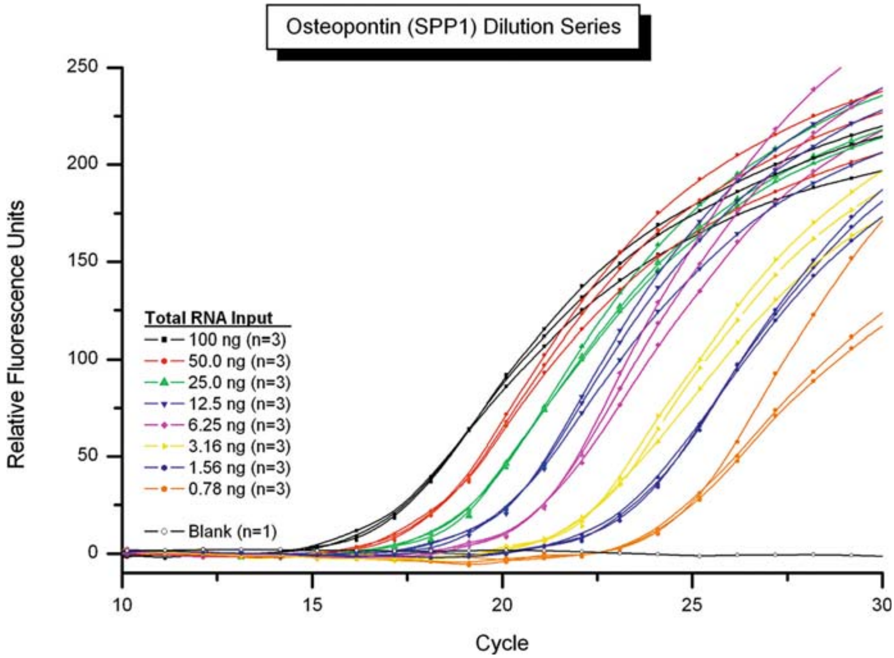
Dge, 3,6, Alkaline Phosphotase expression in petri dish and size distribution of osteoblasts in vitro. Alkaline phosphatase activity increases over time in culture. Additionally, the cell size increases during growth of cells in the culture dish (Coulter Counter measurements, graphics on right side). Both parameters are indicative of viable osteoblast-like cells



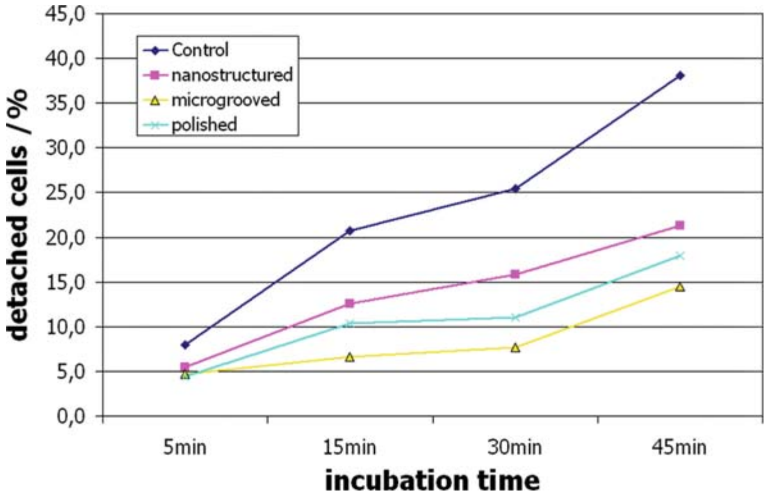
Dge, 3,7, Co-culture of osteoblasts and endothelial cells. Cells have no direct contact but can interact over the media. Top: collagen type I expression of osteoblasts in single (*left*) and in co-culture (*right*) after one week; bottom: actin expression of endothelial cells in single (*left*) and in co-culture (*right*) demonstrates ring format only in co-culture

R_`jc 3,/, Biochemical evaluation methods

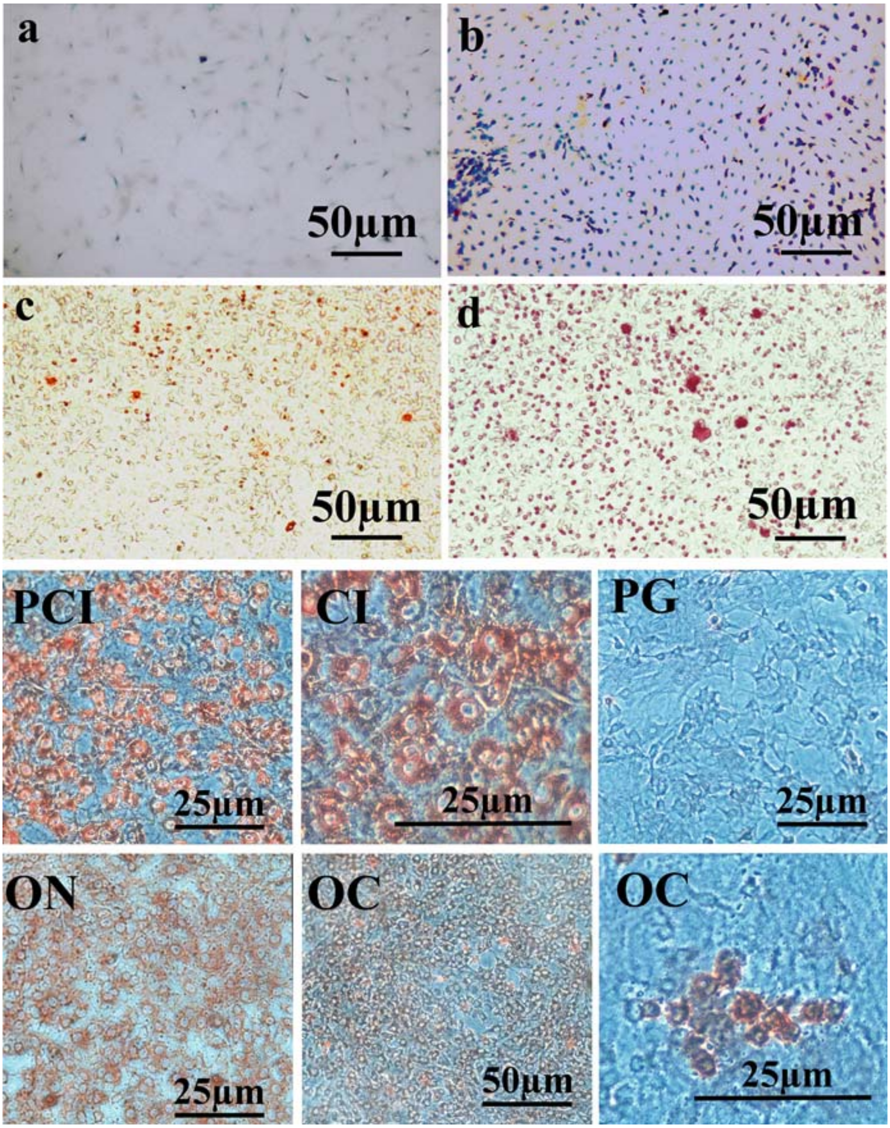
Kcrfmb	Bcrcpkgl_rgml
Cell counter	Cell number, cell volume
Colorimetric assays	Cell viability, calcium-phosphate content
Fluorescence staining	Cytoskeletal proteins, Minerals
Immunostaining	Cell and matrix components
Gelectrophoresis	Proteins, non-proteinaceous components
HPLC	Proteins, non-proteinaceous components
Capillary electrophoresis	Proteins, non-proteinaceous components
Western-blot	Proteins
ELISA	Proteins, non-proteinaceous components
IRMA	Proteins, non-proteinaceous components
Radioimmunoassay	Proteins, non-proteinaceous components
DNA analysis (PCR)	DNA
Gene chips	Gene composition



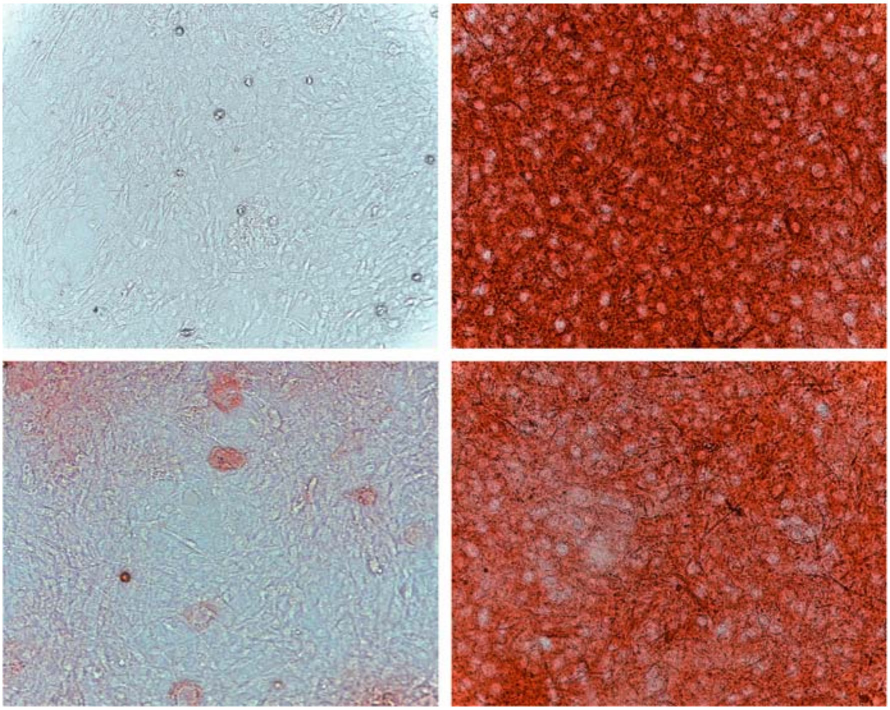
Dge, 3,/ ., Quantitative osteopontin detection by polymerase chain reaction (PCR). The quantitative analysis of gene expression by the PCR technology is a valuable tool in the determination of cell activity states in culture



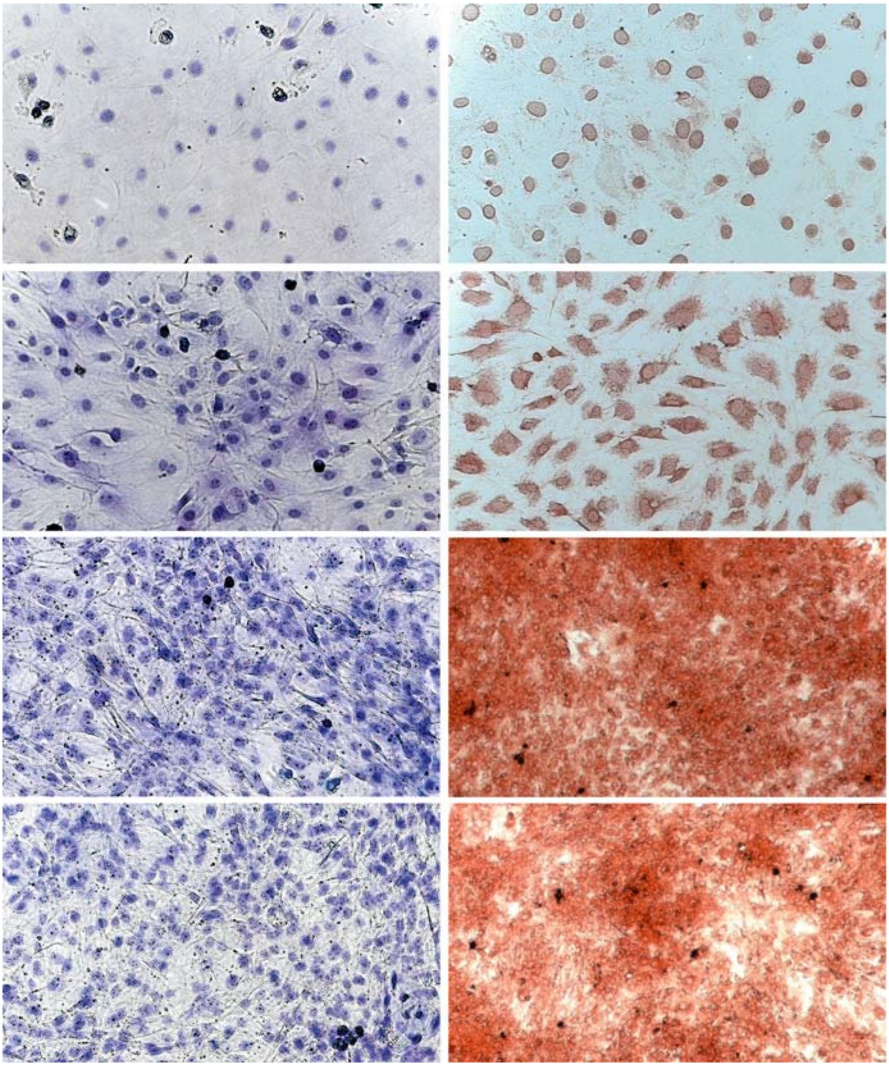
Dge, 3,//, Relative strength of cell adhesion at different surfaces can be determined by trypsinisation with diluted trypsin at different time points, plotting the percentage of detached cells versus trypsinisation time. Example shows cell attachment at a control compared with attachment at different surface nanostructures of titanium



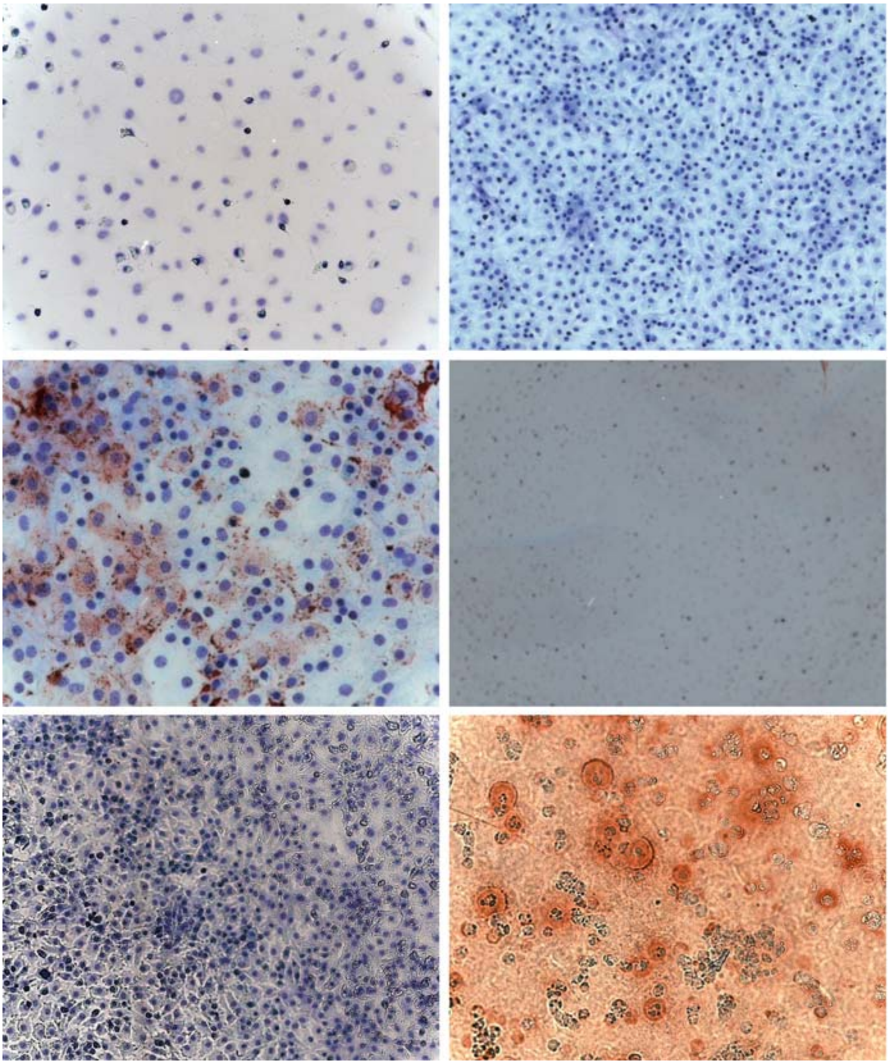
Dge, 3,/0, Cell cultures can be well characterised by various staining procedures (*top,* _«b) and a comprehensive analysis of the array of proteins synthesised by cells (*bottom:* immunostaining). The presence of procollagen type I (PCI), collagen type I (CI), proteoglycans (PG), osteonectin (ON), and osteocalcin (OC) is indicative for a mature osteoblast cell culture



Dge, 3,/1, Osteocalcin expression of osteoblasts. The extent of staining intensity depends on the used blocking systems, indicative for the difficulties to quantitatively compare results of different cell culture investigations. Defining the culture condition is a main requirement in cell biology research. *eft:* Candor blocking solution provides the correct osteocalcin expression in 1 day (*top, left*) and 7 days (*bottom, left*) and 14 days (*bottom, right*) in comparison to the overstaining by using a BSY blocking solution



Dge, 3,/2, Osteoblast monolayer culture. The outcome of cell cultures is dependent on different serum concentrations. Different serum concentrations lead to a significant difference in the growth and differentiation characteristics of cells during cell culture. 1. row: Richardson`s stain (*left*) and osteonectin expression (*right*) in serum-free culture; 2. row: Richardson`s stain (*left*) and osteonectin expression (*right*) in 2% serum culture; 3. row: Richardson`s stain (*left*) and osteonectin expression (*right*) in 10% serum culture; 4. row: Richardson`s stain (*left*) and osteonectin expression (*right*) in 15% serum culture show no prominent difference to 10% serum culture



Dge, 3,/3, Staining of chondrocytes in culture. The appearance of chondrocytes differs significant between cells cultured in a monolayer and in a three-dimensional gel system. *Top*: Monolayer culture with low (*left*) and high cell density (*right*); midline: collagen type I (*left*) and collagen type II (*right*) expression in monolayer culture; *bottom*: collagen type I (*left*) and collagen type II (*right*) expression in a three-dimensional gel culture. Chondrocyte differentiation is sensitive to the spacial environment. The different collagen expression of monolayer and gel culture represents the unequal cell differentiation

Table 5.2. Microscopical methods used in tissue engineering

Method	Resolution (nm)	Immuno-staining	Element analysis	Molecule analysis
Phase contrast microscopy	100	–	–	–
Conventional microscopy	100	+	–	–
Fluorescence microscopy	100	+	–	–
Scanning electron microscopy	10	+	+ (EDX)	–
Transmission electron microscopy	0.1	+	+ (EDX)	–
Atomic force microscopy	0.01	–	–	–
Laser SMS	10	–	+	–
ToF SIMS	10	–	+	–

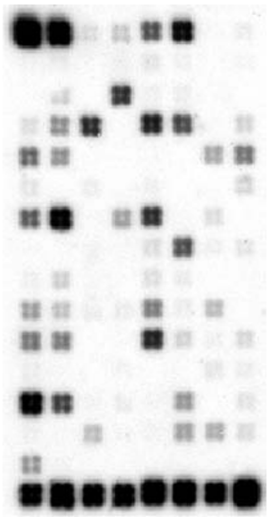


Fig. 5.16. View of staining pattern on an osteogenesis array gene chip (Super Array Bioscience Corporation, USA). Expression is measured on this array as intensity of the spots. Genes with higher expression are dark on the image

R_`jc 3,1, Location of the different genes on the osteogenesis array gene chip (Fig. 5.16., Super Array Bioscience Corporation, USA)

?pp_w J_wmsr							
Gapdh	Ahsg	Akp2	Ambn	Anxa5	Bgn	Bmp1	Bmp2
1	2	3	4	5	6	7	8
Bmp3	Bmp4	Bmp5	Bmp6	Bmp7	Bmp8a	Bmp8b	Bmpr1a
9	10	11	12	13	14	15	16
Bmpr1b	Bmpr2	Calcr	Calcr	Cdh11	Col10a1	Col11a1	Col11a1
17	18	19	20	21	22	23	24
Col14a1	Col15a1	Col18a1	Col19a1	Col1a1	Col1a2	Col2a1	Col3a1
25	26	27	28	29	30	31	32
Col4a1	Col4a2	Col4a3	Col4a4	Col4a5	Col4a6	Col5a1	Col6a1
33	34	35	36	37	38	39	40
Col6a2	Col7a1	Col8a1	Col9a1	Col9a3	Comp	Csf2	Csf3
41	42	43	44	45	46	47	48
Ctsk	Dcn	Dmp1	Dspp	Egf	Enam	Fgf1	Fgf2
49	50	51	52	53	54	55	56
Fgf3	Fgfr1	Fgfr2	Fgfr3	Flt1	Fn1	Gdf10	Ibsp
57	58	59	60	61	62	63	64
Icam1	Igf1	Igf1r	Itga2	Itga2b	Itga3	Itgam	Itgav
65	66	67	68	69	70	71	72
Itgb1	Mglap	Mmp10	Mmp13	Mmp2	Mmp8	Mmp9	Msx1
73	74	75	76	77	78	79	80
Nfkb1	Pdgfa	Phex	Runx2	Scarb1	Ser-pinh1	Smad1	Smad2
81	82	83	84	85	86	87	88
Smad3	Smad4	Smad5	Smad6	Smad7	Smad9	Sost	Sox9
89	90	91	92	93	94	95	96
Sparc	Spp1	Tfip11	Tgfb1	Tgfb2	Tgfb3	Tgfbr1	Tgfbr2
97	98	99	100	101	102	103	104
Tgfbr3	Tnf	Tuft1	Twist1	Twist2	Vcam1	Vdr	Vegfa
105	106	107	108	109	110	111	112
Vegfb	Vegfc	PUC18	Blank	Blank	AS1R2	AS1R1	AS1
113	114	115	116	117	118	119	120
Rps27a	B2m	Hspcb	Hspcb	Ppia	Ppia	BAS2C	BAS2C
121	122	123	124	125	126	127	128

R_`jc 3,2, Details on the proteins of the osteogenesis array gene chip (Fig. 5.16. and Table 5.3., Super Array Bioscience Corporation, USA).

Nmqg+rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
1	NM_008084	Gapdh	Glyceral-dehyde-3-phosphate de-hydrogenase	Oxidoreductase activity; Mito-chondrion; Glyceraldehyde-3-phosphate dehydrogenase (phos-phorylating) activity; Glucose metabolism; Glycolysis; Glycer-aldehyde-3-phosphate dehydroge-nase activity
2	NM_013465	Ahsg	Alpha-2-HS-glycoprotein	Extracellular space; Ossifica-tion; Cysteine protease inhibitor activity
3	NM_007431	Akp	Alkaline phosphatase 2, liver	Hydrolase activity; Membrane; Extracellular space; Metabolism; Magnesium ion binding; Alkaline phosphatase activity
4	NM_009664	Ambn	Ameloblastin	Extracellular space; Odontogene-sis (sensu Vertebrata); Ossification; Extracellular matrix organiza-tion and biogenesis; Extracellular matrix (sensu Metazoa); Struc-tural constituent of tooth enamel; Extracellular matrix structural constituent conferring compres-sion resistance
5	NM_009673	Anxa5	Annexin A5	Calcium ion binding; Blood coagulation; Calcium-dependent phospholipid binding
6	NM_007542	Bgn	Biglycan	Extracellular space; Extracellular matrix (sensu Metazoa)
7	NM_009755	Bmp1	Bone mor-phogenetic protein 1	Hydrolase activity; Calcium ion binding; Proteolysis and peptidol-ysis; Zinc ion binding; Organogen-esis; Growth factor activity; Metal-loendopeptidase activity; Cytokine activity; Metallopeptidase activity; Astacin activity
8	NM_007553	Bmp2	Bone mor-phogenetic protein 2	Protein binding; Extracellular space; Organogenesis; Growth factor activity; Cytokine activity; Transforming growth factor beta receptor signalling pathway; Em-bryonic development; BMP signal-ling pathway; Growth; Cardiac cell differentiation; Cell fate commit-ment; Epithelial to mesenchymal transition

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
9	NM_173404	Bmp3	Bone morphogenetic protein 3	Growth factor activity; Cytokine activity; Transforming growth factor beta receptor signalling pathway
10	NM_007554	Bmp4	Bone morphogenetic protein 4	Protein binding; Extracellular space; Growth factor activity; Skeletal development; Angiogenesis; Cytokine activity; Heart development; Mesoderm cell fate determination; BMP signalling pathway; Growth; Eye morphogenesis (sensu Mammalia); Cell fate commitment; Metanephros development; Ureteric bud development; Forebrain development; Histogenesis
11	NM_007555	Bmp5	Bone morphogenetic protein 5	Protein binding; Extracellular space; Growth factor activity; Skeletal development; Cytokine activity; Transforming growth factor beta receptor signalling pathway; Growth; Pattern specification
12	NM_007556	Bmp6	Bone morphogenetic protein 6	Extracellular; Protein binding; Cell growth and/or maintenance; Extracellular space; Growth factor activity; Cell differentiation; Cytokine activity; BMP signalling pathway; Growth
13	NM_007557	Bmp7	Bone morphogenetic protein 7	Protein binding; Extracellular space; Organogenesis; Growth factor activity; Cytokine activity; BMP signalling pathway; Axon guidance; Growth; Pattern specification; Salivary gland morphogenesis
14	NM_007558	Bmp8a	Bone morphogenetic protein 8a	Development; Extracellular space; Growth factor activity; Cell differentiation; Spermatogenesis; Cytokine activity; Transforming growth factor beta receptor signalling pathway; Growth

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
15	NM_007559	Bmp8b	Bone morphogenetic protein 8b	Extracellular space; Growth factor activity; Spermatogenesis; Cytokine activity; Transforming growth factor beta receptor signalling pathway; Growth
16	NM_009758	Bmpr1a	Bone morphogenetic protein receptor, type 1A	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Membrane; Protein kinase activity; Extracellular space; Kinase activity; Cell differentiation; Transforming growth factor beta receptor signalling pathway; Transforming growth factor beta receptor activity; Transmembrane receptor protein serine/threonine kinase activity; Anterior/posterior pattern formation; Endoderm development; Ectoderm development; Mullerian duct regression; Dorsal/ventral axis specification; Mesendoderm development
17	NM_007560	Bmpr1b	Bone morphogenetic protein receptor, type 1B	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor signalling protein serine/threonine kinase activity; Receptor activity; Membrane; Protein kinase activity; Kinase activity; Cell differentiation; Type I transforming growth factor beta receptor activity; Transmembrane receptor protein serine/threonine kinase signalling pathway; Transforming growth factor beta receptor signalling pathway; Transforming growth factor beta receptor activity; BMP signalling pathway; Transmembrane receptor protein serine/threonine kinase activity; Cartilage condensation; Eye morphogenesis

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
18	NM_007561	Bmpr2	Bone morphogenic protein receptor, type II (serine/threonine kinase)	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Membrane; Protein kinase activity; Extracellular space; Transforming growth factor beta receptor signalling pathway; Cell surface; Transforming growth factor beta receptor activity; Transmembrane receptor protein serine/threonine kinase activity
19	NM_007588	Calcr	Calcitonin receptor	Integral to membrane; G-protein coupled receptor protein signalling pathway; Receptor activity; Membrane; Extracellular space; G-protein coupled receptor activity; Calcitonin receptor activity
20	NM_007643	Cd36	CD36 antigen	Plasma membrane; Integral to membrane; Cell adhesion; Protein binding; Receptor activity; Membrane; Transport;
21	NM_009866	Cdh11	Cadherin 11	Plasma membrane; Integral to membrane; Calcium ion binding; Cell adhesion; Protein binding; Cytoplasm; Homophilic cell adhesion; Membrane
22	NM_009925	Col10a1	Procollagen, type X, alpha 1	Structural molecule activity; Cell adhesion; Cytoplasm; Extracellular space; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
23	NM_007729	Col11a1	Procollagen, type XI, alpha 1	Structural molecule activity; Cell adhesion; Cytoplasm; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Cartilage condensation; Proteoglycan metabolism; Extracellular matrix (sensu Metazoa); Collagen fibril organization;

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
24	NM_007730	Col12a1	Procollagen, type XII, alpha 1	Structural molecule activity; Cell adhesion; Protein binding; Cytoplasm; Extracellular space; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
25	NM_181277	Col14a1	Procollagen, type XIV, alpha 1	Structural molecule activity; Cell adhesion; Protein binding; Cytoplasm; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
26	NM_009928	Col15a1	Procollagen, type XV	Structural molecule activity; Cell adhesion; Extracellular space; Collagen; Extracellular matrix structural constituent conferring tensile strength
27	NM_009929	Col18a1	Procollagen, type XVIII, alpha 1	Structural molecule activity; Cell adhesion; Protein binding; Cytoplasm; Positive regulation of cell proliferation; Development; Membrane; Extracellular space; Transmembrane receptor activity; Extracellular matrix structural constituent; Angiogenesis; Positive regulation of apoptosis; Phosphate transport; Collagen; Positive regulation of cell migration; Basement membrane; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa); Endothelial cell morphogenesis
28	NM_007733	Col19a1	Procollagen, type XIX, alpha 1	Structural molecule activity; Extracellular space; Collagen; Myogenesis; Extracellular matrix organization and biogenesis

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
29	NM_007742	Col1a1	Procollagen, type I, alpha 1	Cell adhesion; Cytoplasm; Extracellular matrix; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
30	NM_007743	Col1a2	Procollagen, type I, alpha 2	Structural molecule activity; Cell adhesion; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
31	NM_031163	Col2a1	Procollagen, type II, alpha 1	Cell adhesion; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Cartilage condensation; Proteoglycan metabolism; Extracellular matrix (sensu Metazoa); Collagen fibril organization
32	NM_009930	Col3a1	Procollagen, type III, alpha 1	Cell adhesion; Cytoplasm; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
33	NM_009931	Col4a1	Procollagen, type IV, alpha 1	Cell adhesion; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Basement membrane; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
34	NM_009932	Col4a2	Procollagen, type IV, alpha 2	Cell adhesion; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Basement membrane; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
35	NM_007734	Col4a3	Procollagen, type IV, alpha 3	Nucleic acid binding; Cell adhesion; Extracellular space; Extracellular matrix structural constituent; Collagen; Basement membrane; Extracellular matrix structural constituent conferring tensile strength
36	NM_007735	Col4a4	Procollagen, type IV, alpha 4	Structural molecule activity; Cell adhesion; Protein binding; Collagen; Basement membrane; Extracellular matrix structural constituent conferring tensile strength;
37	NM_007736	Col4a5	Procollagen, type IV, alpha 5	Structural molecule activity; Cell adhesion; Protein binding; Membrane; Extracellular space; Extracellular matrix structural constituent; Collagen; Basement membrane; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa); Collagen type IV
38	NM_053185	Col4a6	Procollagen, type IV, alpha 6	Structural molecule activity; Extracellular space; Extracellular matrix structural constituent; Collagen; Basement membrane; Extracellular matrix organization and biogenesis; Collagen type IV
39	NM_015734	Col5a1	Procollagen, type V, alpha 1	Structural molecule activity; Cell adhesion; Extracellular space; Extracellular matrix structural constituent; Heparin binding; Collagen; Collagen type V; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
40	NM_009933	Col6a1	Procollagen, type VI, alpha 1	Cell adhesion; Proteolysis and peptidolysis; Protein binding; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Subtilase activity; Extracellular matrix (sensu Metazoa)
41	NM_146007	Col6a2	Procollagen, type VI, alpha 2	Cell adhesion; Protein binding; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
42	NM_007738	Col7a1	Procollagen, type VII, alpha 1	Cell adhesion; Extracellular space; Collagen; Extracellular matrix structural constituent conferring tensile strength
43	NM_007739	Col8a1	Procollagen, type VIII, alpha 1	Cell adhesion; Protein binding; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)
44	NM_007740	Col9a1	Procollagen, type IX, alpha 1	Cell adhesion; Cytoplasm; Extracellular space; Extracellular matrix structural constituent; Phosphate transport; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa)

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
45	NM_009936	Col9a3	Procollagen, type IX, alpha 3	Structural molecule activity; Cell adhesion; Protein binding; Extracellular space; Extracellular matrix structural constituent; Collagen; Extracellular matrix structural constituent conferring tensile strength; Extracellular matrix (sensu Metazoa); Collagen type IX
46	NM_016685	Comp	Cartilage oligomeric matrix protein	Calcium ion binding; Cell adhesion; Protein binding; Extracellular space; Extracellular region
47	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	Extracellular; Immune response; Extracellular space; Growth factor activity; Cytokine and chemokine mediated signalling pathway; Cytokine activity; Granulocyte macrophage colony-stimulating factor receptor binding; Dendritic cell differentiation
48	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	Extracellular; Immune response; Extracellular space; Growth factor activity; Cytokine activity
49	NM_007802	Ctsk	Cathepsin K	Hydrolase activity; Proteolysis and peptidolysis; Cysteine-type peptidase activity; Extracellular space; Cysteine-type endopeptidase activity; Cathepsin K activity
50	NM_007833	Dcn	Decorin	Extracellular space; Extracellular matrix (sensu Metazoa)
51	NM_016779	Dmp1	Dentin matrix protein 1	Extracellular space; Ossification; Extracellular matrix organization and biogenesis; Extracellular matrix (sensu Metazoa)
52	NM_010080	Dspp	Dentin sialophosphoprotein	Extracellular space; Ossification; Extracellular matrix (sensu Metazoa)

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
53	NM_010113	Egf	Epidermal growth factor	Integral to membrane; Calcium ion binding; Membrane; Extracellular space; Growth factor activity; Regulation of peptidyl-tyrosine phosphorylation;
54	NM_017468	Enam	Enamelin	Extracellular space; Ossification; Extracellular matrix (sensu Metazoa)
55	NM_010197	Fgf1	Fibroblast growth factor 1	Cell proliferation; Signal transduction; Regulation of cell cycle; Heparin binding; Growth factor activity; Angiogenesis; Fibroblast growth factor receptor bindin
56	NM_008006	Fgf2	Fibroblast growth factor 2	Cell proliferation; Signal transduction; Protein binding; Regulation of cell cycle; Extracellular space; Heparin binding; Growth factor activity; Angiogenesis; Positive regulation of cell differentiation; Regulation of retinal programmed cell death; Glial cell differentiation; Fibroblast growth factor receptor binding
57	NM_008007	Fgf3	Fibroblast growth factor 3	Cell proliferation; Signal transduction; Nucleus; Regulation of cell cycle; Extracellular space; Growth factor activity; Fibroblast growth factor receptor binding; Induction of an organ
58	NM_010206	Fgfr1	Fibroblast growth factor receptor 1	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Signal transduction; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Protein kinase activity; Extracellular space; Heparin binding; Kinase activity; Regulation of cell proliferation; Fibroblast growth factor receptor activity; Salivary gland morphogenesis; Brain development; Branching morphogenesis; Lung development; Induction of an organ; Inner ear morphogenesis

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
59	NM_010207	Fgfr2	Fibroblast growth factor receptor 2	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Signal transduction; Nucleus; Protein binding; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Positive regulation of cell proliferation; Receptor activity; Protein kinase activity; Extracellular space; Cell-cell signalling; Regulation of cell proliferation; Fibroblast growth factor receptor activity; Salivary gland morphogenesis; Bone mineralization
60	NM_008010	Fgfr3	Fibroblast growth factor receptor 3	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Signal transduction; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Protein kinase activity; Extracellular space; Kinase activity; Positive regulation of cell differentiation; Fibroblast growth factor receptor activity
61	NM_010228	Flt1	FMS-like tyrosine kinase 1	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Transmembrane receptor protein tyrosine kinase activity; Membrane; Protein kinase activity; Extracellular space; Transmembrane receptor protein tyrosine kinase signalling pathway; Kinase activity; Vascular endothelial growth factor receptor activity; Angiogenesis; Patterning of blood vessels

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
62	NM_010233	Fn1	Fibronectin 1	Cell adhesion; Extracellular; Protein binding; Oxidoreductase activity; Extracellular space; Heparin binding; Acute-phase response; Metabolism; Cell-substrate junction assembly; Wound healing; Extracellular matrix (sensu Metazoa)
63	NM_145741	Gdf10	Growth differentiation factor 10	Extracellular space; Growth factor activity; Cytokine activity
64	NM_008318	Ibsp	Integrin binding sialoprotein	Cell adhesion; Protein binding; Extracellular space; Integrin-mediated signalling pathway; Ossification; Extracellular region
65	NM_010493	Icam1	H1 histone family, member 0	Plasma membrane; Integral to membrane; Cell adhesion; Protein binding; Membrane; Cell-cell adhesion; Defence response
66	NM_010512	Igf1	Insulin-like growth factor 1	Extracellular; Anti-apoptosis; Organogenesis; Growth factor activity; Hormone activity; Physiological process; Neurogenesis; Glial cell differentiation; Insulin-like growth factor receptor signalling pathway
67	NM_010513	Igf1r	Insulin-like growth factor I receptor	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein binding; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Transmembrane receptor protein tyrosine kinase activity; Membrane; Protein kinase activity; Extracellular space; Epidermal growth factor receptor activity; Organogenesis; Transmembrane receptor protein tyrosine kinase signalling pathway; Insulin-like growth factor receptor signalling pathway; Brain development; Male sex determination

R_`jc 3,2, *Continued*

Nmqg+rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
68	NM_008396	Itga2	Integrin alpha 2	Integral to membrane; Cell adhesion; Protein binding; Receptor activity; Cell-matrix adhesion; Extracellular space; Integrin-mediated signalling pathway; Integrin complex; Magnesium ion binding
69	NM_010575	Itga2b	Integrin alpha 2b	Integral to membrane; Cell adhesion; Proteolysis and peptidolysis; Protein binding; Receptor activity; Cell-matrix adhesion; Extracellular space; Integrin-mediated signalling pathway; Integrin complex; External side of plasma membrane; Carboxypeptidase A activity
70	NM_013565	Itga3	Integrin alpha 3	Integral to membrane; Cell adhesion; Protein binding; Cell-matrix adhesion; Integrin-mediated signalling pathway; Integrin complex; Synaptosome; Basolateral plasma membrane; Memory; NOT fusion of sperm to egg plasma membrane; Neuronal migration
71	NM_008401	Itgam	Integrin alpha M	Integral to membrane; Cell adhesion; Protein binding; Receptor activity; Cell-matrix adhesion; Integrin-mediated signalling pathway; Integrin complex; Neutrophil chemotaxis; Cellular extravasation; External side of plasma membrane; Opsonin binding
72	NM_008402	Itgav	Integrin alpha V	Integral to membrane; Cell adhesion; Protein binding; Receptor activity; Cell-matrix adhesion; Integrin-mediated signalling pathway; Integrin complex; Angiogenesis; Blood vessel development
73	NM_010578	Itgb1	Integrin beta 1 (fibronectin receptor beta)	Plasma membrane; Integral to membrane; Cell adhesion; Protein binding; Oxidoreductase activity; Regulation of cell cycle; Receptor activity; Cell-matrix adhesion; G1/S transition of mitotic cell cycle; Extracellular space; Metabolism; Integrin-mediated signalling pathway; Integrin complex; Synaptosome

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EMRcpk
74	NM_008597	Mglap	Matrix gamma-carboxyglutamate (gla) protein	Calcium ion binding; Development; Extracellular space; Cell differentiation; Extracellular region; Regulation of bone mineralization
75	NM_019471	Mmp10	Matrix metalloproteinase 10	Hydrolase activity; Extracellular; Proteolysis and peptidolysis; Zinc ion binding; Collagen catabolism; Extracellular space; Metalloendopeptidase activity; Metallopeptidase activity; Extracellular matrix (sensu Metazoa)
76	NM_008607	Mmp13	Matrix metalloproteinase 13	Hydrolase activity; Calcium ion binding; Proteolysis and peptidolysis; Zinc ion binding; Interstitial collagenase activity; Collagen catabolism; Extracellular space; Metalloendopeptidase activity; Metallopeptidase activity; Extracellular matrix (sensu Metazoa)
77	NM_008610	Mmp2	Matrix metalloproteinase 2	Hydrolase activity; Calcium ion binding; Proteolysis and peptidolysis; Zinc ion binding; Collagen catabolism; Extracellular matrix; Extracellular space; Gelatinase A activity; Metalloendopeptidase activity; Metallopeptidase activity; Extracellular matrix (sensu Metazoa)
78	NM_008611	Mmp8	Matrix metalloproteinase 8	Hydrolase activity; Calcium ion binding; Proteolysis and peptidolysis; Zinc ion binding; Collagen catabolism; Extracellular space; Metalloendopeptidase activity; Metallopeptidase activity; Extracellular matrix (sensu Metazoa)
79	NM_013599	Mmp9	Matrix metalloproteinase 9	Hydrolase activity; Proteolysis and peptidolysis; Zinc ion binding; Collagen catabolism; Extracellular space; Metalloendopeptidase activity; Metallopeptidase activity; Gelatinase B activity; Extracellular matrix (sensu Metazoa)

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EMRcpk
80	NM_010835	Msx1	Homeo box, msh-like 1	DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Transcription factor activity; Development; Transcription factor complex; Transcription; Forebrain development; Midbrain development
81	NM_008689	Nfkb1	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Signal transduction; DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Apoptosis; Transcription factor activity; Lymph gland development; Specific transcriptional repressor activity; Negative regulation of interleukin-12 biosynthesis; Negative regulation of transcription, DNA-dependent
82	NM_008808	Pdgfa	Platelet derived growth factor, alpha	Cell proliferation; Cell growth and/or maintenance; Regulation of cell cycle; Membrane; Extracellular space; Organogenesis; Growth factor activity; Regulation of peptidyl-tyrosine phosphorylation; Actin cytoskeleton organization and biogenesis; Cell projection biogenesis;
83	NM_011077	Phex	Phosphate regulating gene with homologies to endopeptidases on the X chromosome	Hydrolase activity; Integral to membrane; Proteolysis and peptidolysis; Zinc ion binding; Membrane; Metallopeptidase activity; Neprilysin activity; Peptidase activity; Ossification

R_`jc 3,2, *Continued*

Nmqg+ rgml	Ec1c@_1i	Qwk`mj	Bcqapgnrgml	EM Rcpk
84	NM_009820	Runx2	Runt related transcription factor 2	ATP binding; DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Cytoplasm; Transcription factor activity; Skeletal development; Transcription; Odontogenesis (sensu Vertebrata); Chromatin binding; Regulation of fibroblast growth factor receptor signalling pathway; Positive regulation of transcription from RNA polymerase II promoter; Cell maturation; Osteoblast differentiation; Regulation of odontogenesis (sensu Vertebrata)
85	NM_016741	Scarb1	Scavenger receptor class B, member 1	Integral to membrane; Cell adhesion; Receptor activity; Membrane
86	NM_009825	Serpinh1	Serine (or cysteine) proteinase inhibitor, clade H, member 1	Response to heat; Extracellular space; Heat shock protein activity; Endoplasmic reticulum; Serine-type endopeptidase inhibitor activity
87	NM_008539	Smad1	MAD homolog 1 (Drosophila)	Regulation of transcription, DNA-dependent; Protein binding; Transcription factor activity; Regulation of transcription from Pol II promoter; RNA polymerase II transcription factor activity; Intracellular; Transcription factor complex; Organogenesis; MAPKKK cascade; BMP signalling pathway; Gametogenesis; Common-partner SMAD protein phosphorylation; SMAD protein heteromerization; Homeostasis

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
88	NM_010754	Smad2	MAD homolog 2 (Drosophila)	Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Intracellular; Transcription factor complex; Organogenesis; Transcription factor binding; Transforming growth factor beta receptor signalling pathway; Positive regulation of transcription from Pol II promoter; Cell fate commitment; Mesoderm formation; Anterior/posterior pattern formation; Common-partner SMAD protein phosphorylation; SMAD protein heteromerization; Paraxial mesoderm morphogenesis
89	NM_016769	Smad3	MAD homolog 3 (Drosophila)	Plasma membrane; Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Transcription factor activity; Negative regulation of transcription from Pol II promoter; Transcription from Pol II promoter; Intracellular; Organogenesis; Transcription factor binding; Transforming growth factor beta receptor signalling pathway; Positive regulation of transcription from Pol II promoter; Mesoderm formation; Regulation of myogenesis; Common-partner SMAD protein phosphorylation; SMAD protein heteromerization; Paraxial mesoderm morphogenesis; Regulation of epithelial cell proliferation; Regulation of transforming growth factor beta receptor signalling pathway

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
90	NM_008540	Smad4	MAD homolog 4 (Drosophila)	DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Transcription factor activity; Regulation of transcription from Pol II promoter; Intracellular; Transcription factor complex; Organogenesis; Positive regulation of transcription from Pol II promoter; SMAD protein nuclear translocation; Common-partner SMAD protein phosphorylation;
91	NM_008541	Smad5	MAD homolog 5 (Drosophila)	Regulation of transcription, DNA-dependent; Protein binding; Intracellular; Organogenesis; Angiogenesis; Transforming growth factor beta receptor signalling pathway; BMP signalling pathway; Common-partner SMAD protein phosphorylation; SMAD protein heteromerization
92	NM_008542	Smad6	MAD homolog 6 (Drosophila)	Regulation of transcription, DNA-dependent; Intracellular; Common-partner SMAD protein phosphorylation
93	NM_008543	Smad7	MAD homolog 7 (Drosophila)	Regulation of transcription, DNA-dependent; Intracellular; Transforming growth factor beta receptor signalling pathway; Common-partner SMAD protein phosphorylation
94	NM_019483	Smad9	MAD homolog 9 (Drosophila)	Regulation of transcription, DNA-dependent; Protein binding; Intracellular
95	NM_024449	Sost	Sclerostin	Extracellular space; Ossification; Negative regulation of BMP signalling pathway

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EMRcpk
96	NM_011448	Sox9	SRY-box containing gene 9	DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Transcription factor activity; Transcription factor complex; Cell differentiation; Transcription; Heart development; Epithelial to mesenchymal transition; Cartilage condensation; Cell fate specification; Male gonad development; Positive regulation of transcription from RNA polymerase II promoter; Male germ-line sex determination
97	NM_009242	Sparc	Secreted acidic cysteine rich glycoprotein	Calcium ion binding; Extracellular space; Basement membrane; Extracellular matrix (sensu Metazoa)
98	NM_009263	Spp1	Secreted phosphoprotein 1	Cell adhesion; Protein binding; Extracellular space; Cytokine activity; Ossification
99	NM_018783	Tfip11	Tuftelin interacting protein 11	Nucleic acid binding; Nucleus; Cytoplasm; Intracellular; Ossification; Extracellular matrix (sensu Metazoa); Rhythmic process
100	NM_011577	Tgfb1	Transforming growth factor, beta 1	Protein amino acid phosphorylation; Cell proliferation; Protein binding; Negative regulation of cell proliferation; Regulation of cell cycle; Inflammatory response; Extracellular matrix; Extracellular space; Organogenesis; Growth factor activity; Skeletal development; Defence response; Regulation of cell proliferation; Necrosis; Transforming growth factor beta receptor signalling pathway; Transforming growth factor beta receptor binding; Cell growth; Lymph gland development; Growth; Myogenesis; Regulation of myogenesis; Regulation of protein-nucleus import

R_`jc 3,2, *Continued*

Nmqg+rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
101	NM_009367	Tgfb2	Transforming growth factor, beta 2	Cell proliferation; Regulation of cell cycle; Extracellular space; Growth factor activity; Transforming growth factor beta receptor binding; Cell growth; Growth; Extracellular matrix organization and biogenesis
102	NM_009368	Tgfb3	Transforming growth factor, beta 3	Cell proliferation; Negative regulation of cell proliferation; Regulation of cell cycle; Extracellular space; Organogenesis; Growth factor activity; Transforming growth factor beta receptor binding; Cell growth; Growth; Embryonic development (sensu Mammalia); Embryonic morphogenesis
103	NM_009370	Tgfbr1	Transforming growth factor, beta receptor I	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein binding; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor activity; Membrane; Protein kinase activity; Extracellular space; Kinase activity; Transforming growth factor beta receptor signalling pathway; Transforming growth factor beta receptor activity; Transmembrane receptor protein serine/threonine kinase activity

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
104	NM_009371	Tgfbr2	Transforming growth factor, beta receptor II	ATP binding; Transferase activity; Protein amino acid phosphorylation; Integral to membrane; Protein serine/threonine kinase activity; Protein-tyrosine kinase activity; Receptor signalling protein serine/threonine kinase activity; Receptor activity; Membrane; Protein kinase activity; Kinase activity; Regulation of cell proliferation; Transmembrane receptor protein serine/threonine kinase signalling pathway; Transforming growth factor beta receptor activity; Cell fate commitment; Transmembrane receptor protein serine/threonine kinase activity
105	NM_011578	Tgfbr3	Similar to Ornithine decarboxylase (ODC)	Integral to membrane; Receptor activity; Extracellular space
106	NM_013693	Tnf	Tumour necrosis factor	Plasma membrane; Cell proliferation; Immune response; Cell growth and/or maintenance; Humoral immune response; Inflammatory response; Positive regulation of cell proliferation; Induction of apoptosis via death domain receptors; Signal transducer activity; Development; Positive regulation of I-kappaB kinase/NF-kappaB cascade; Integral to plasma membrane; Organogenesis; Tumour necrosis factor receptor binding; Defence response; Secretory granule; Regulation of cell proliferation; Cytokine and chemokine mediated signalling pathway; Cellular defence response; Lymph gland development; Cellular extravasation; Positive regulation of osteoclast differentiation; Programmed cell death, transformed cells; Regulation of osteoclast differentiation
107	NM_011656	Tuft1	Tuftelin 1	Ossification

R_`jc 3,2, *Continued*

Nmqg+rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
108	NM_011658	Twist1	Twist gene homolog 1 (Drosophila)	DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Development; Cell differentiation; Embryonic limb morphogenesis; Neuronal migration; Hindlimb morphogenesis; Neural tube closure
109	NM_007855	Twist2	Twist homolog 2 (Drosophila)	DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Transcription factor activity; Development; Transcription factor complex; Cell differentiation; Transcription; Transcriptional repressor activity; Regulation of transcription from RNA polymerase II promoter
110	NM_011693	Vcam1	Vascular cell adhesion molecule 1	Plasma membrane; Integral to membrane; Cell adhesion; Protein binding; Membrane; Cell-cell adhesion; Extracellular space
111	NM_009504	Vdr	Vitamin D receptor	DNA binding; Regulation of transcription, DNA-dependent; Nucleus; Protein binding; Transcription factor activity; Development; Receptor activity; Organogenesis; Skeletal development; Calcium ion homeostasis; Steroid hormone receptor activity; Transcription; Calcium ion transport; Ligand-dependent nuclear receptor activity; Transcription regulator activity; Negative regulation of transcription; Intestinal absorption
112	NM_009505	Vegfa	Vascular endothelial growth factor A	Cell proliferation; Cell growth and/or maintenance; Anti-apoptosis; Regulation of cell cycle; Mesoderm development; Membrane; Extracellular space; Heparin binding; Growth factor activity; Angiogenesis

R_`jc 3,2, *Continued*

Nmqg+ rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EM Rcpk
113	NM_011697	Vegfb	Vascular endothelial growth factor B	Cell proliferation; Cell growth and/or maintenance; Regulation of cell cycle; Membrane; Extracellular space; Heparin binding; Growth factor activity; Angiogenesis
114	NM_009506	Vegfc	Vascular endothelial growth factor C	Cell proliferation; Cell growth and/or maintenance; Regulation of cell cycle; Membrane; Extracellular space; Organogenesis; Growth factor activity; Angiogenesis; Morphogenesis of embryonic epithelium
115	L08752	PUC18	PUC18 Plasmid DNA	
116	SA_00005	AS1R2	Artificial Sequence 1 Related 2 (80% identity)(48/60)	
117	SA_00004	AS1R1	Artificial Sequence 1 Related 1 (90% identity)(54/60)	
118	SA_00003	AS1	Artificial Sequence 1	
119	NM_024277	Rps27a	Ribosomal protein S27a	Intracellular; Protein biosynthesis; Ribosome
120	NM_009735	B2m	Beta-2 microglobulin	Plasma membrane; Integral to plasma membrane; Extracellular space; Defence response; Cellular defence response; Antigen processing, endogenous antigen via MHC class I; MHC class I receptor activity; Antigen presentation, endogenous antigen

R_`jc 3,2, *Continued*

Nmqg+rgml	Eclc@_li	Qwk`mj	Bcqapgnrgml	EMRcpk
121	NM_008302	Hspcb	Heat shock protein 1, beta	ATP binding; Protein binding; Mitochondrion; Response to heat; Protein folding; Unfolded protein binding; Response to unfolded protein; ATP binding; Protein binding; Mitochondrion; Response to heat; Protein folding; Unfolded protein binding; Response to unfolded protein
122	NM_008302	Hspcb	Heat shock protein 1, beta	ATP binding; Protein binding; Mitochondrion; Response to heat; Protein folding; Unfolded protein binding; Response to unfolded protein; ATP binding; Protein binding; Mitochondrion; Response to heat; Protein folding; Unfolded protein binding; Response to unfolded protein
123	NM_008907	Ppia	Peptidylprolyl isomerase A	Cytosol; Protein folding; Isomerase activity; Peptidyl-prolyl cis-trans isomerase activity; Cytosol; Protein folding; Isomerase activity; Peptidyl-prolyl cis-trans isomerase activity
124	NM_008907	Ppia	Peptidylprolyl isomerase A	Cytosol; Protein folding; Isomerase activity; Peptidyl-prolyl cis-trans isomerase activity; Cytosol; Protein folding; Isomerase activity; Peptidyl-prolyl cis-trans isomerase activity
125	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	
126	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	

Materials

6

Application of non-living biomaterials can be conceptualised as the use of materials to replace lost structures, augment existing structures or promote new tissue formation (Burg et al., 2000a; 2000b). Common degradable and non-degradable implant materials can be divided into synthetically produced metals and metallic alloys, ceramics, polymers, and composites or modified natural materials (Meyer et al., 2004a). Whereas non-resorbable materials like steel or titanium alloys are commonly used for prosthetic devices, resorbable substitute materials are currently being investigated for their utility in bone and cartilage replacement therapies. Whether or not a material is biodegradable, its surface properties will influence the initial cellular events at the cell–material interface. A major difference between degradable and non-degradable implants is that the surface adhesion towards osteoblasts or chondrocytes changes in degradable materials, while it remains constant in non-degradable implants. The clinical fate of implants, substitute materials and scaffolds used in tissue engineering strategies critically depends on the underlying material (Meyer et al., 2004b) and the mechanical properties of the material-based scaffold (Table 6.1). In the design process of cell-based implants and engineered bone and cartilage substitutes it is important to consider the cellular behaviour of the desired cell source towards the material (for a review, see Shin et al, 2003).

Four types of materials have been experimentally and/or clinically studied as bone and cartilage substitute materials or scaffold materials for applications in tissue engineering:

- Various groups of synthetic organic materials, including (1) biodegradable and bioresorbable polymers, which have been used for clinically established products, such as polyglycolide, optically active and racemic polylactides, polydioxanone,

Table 6.1. Mechanical properties of materials

	Young's modulus (GPa)	Strength (MPa) Compressive	Bending
Cortical bone	7–30	100–230	50–150
Cancellous bone	0.05–0.5	2–12	–
Cartilage	0.2–0.3	0.01–3	–
Synthetic HA	80–110	500–1000	115–200
PLGA 85/15	2	0.34	–
PLGA 75/25	2	0.34	–
A-W glass-ceramic	118	1080	220
Collagen tendon	1,5	0.14	–

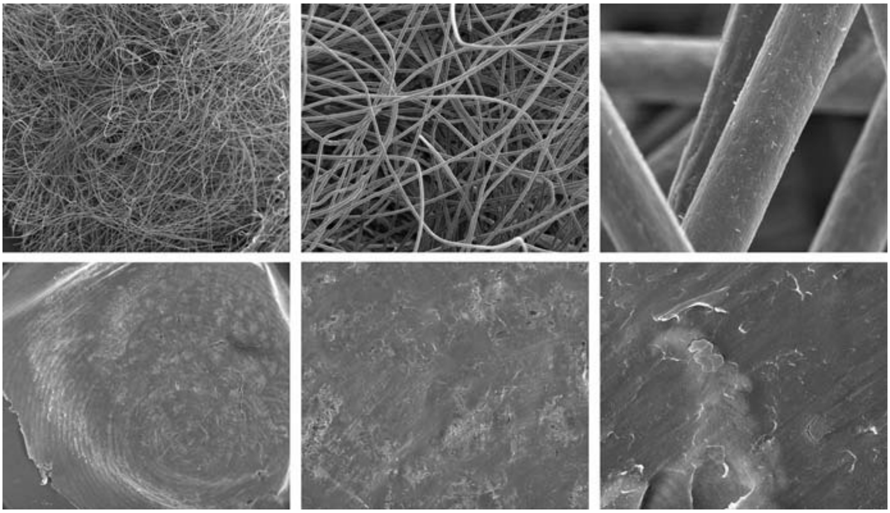
and polycaprolactone; (2) polymers currently under clinical investigation, such as polyorthoester, polyanhydrides, and polyhydroxyalkanoate; and (3) entrepreneurial polymeric biomaterials, such as poly (lactic acid-co-lysine)
- Synthetic inorganic materials (e.g. hydroxyapatite, calcium/phosphate composites, glass ceramics)
- Organic materials of natural origin (e.g. collagen, fibrin, hyaluronic acid)
- Inorganic material of natural origin (e.g. coralline hydroxyapatite).

Several reports have been published on the physico-chemical properties and surface characteristics of these biodegradable and bioresorbable polymers (Hutmacher, 2000). It was found that features of the material surface are important for correct implantation and coverage by autochtonic cells.

A comprehensive overview of the materials used in bone and cartilage tissue engineering is given in this chapter. Additionally, surface properties of materials will be discussed, since material characteristics and surface properties of materials are closely related. The list of materials used for bone tissue engineering differs from that of cartilage engineering, in that organic materials are preferentially used for bone tissue engineering.

6.1 Synthetic Organic Materials

Various polymers are used extensively for the preparation of bone and cartilage scaffolds (Fig. 6.1). Most of them are fabricated on the basis of polyhydroxyacids, such as polylactides, polyglycolides, and their copolymers. Other synthetic materials include polyethylene oxide, polyvinyl alcohol, polyacrylic acid, and polypropylene



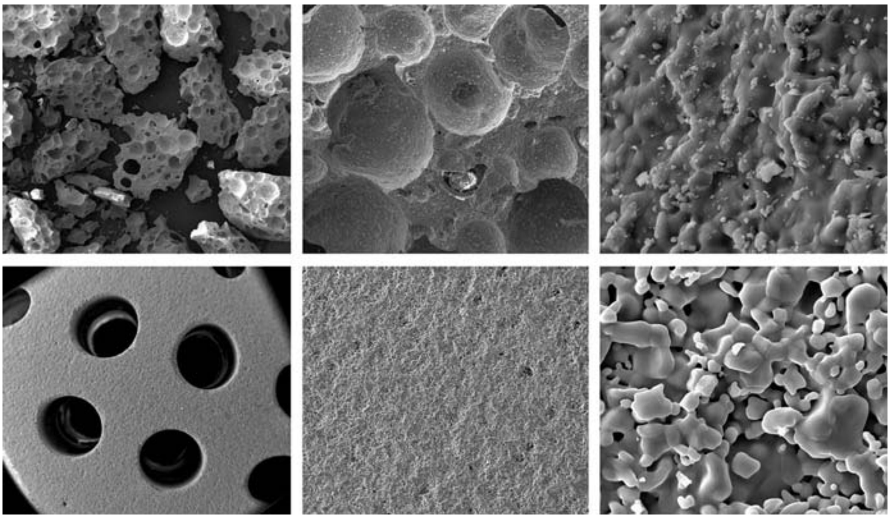
Dge, 4,/, Scanning electron microscopical view of hyaluronic acid-based biomaterials. The biomaterial can be processed as a fibre-based scaffold *(top row)* or as a bulk material. *From left to right:* detailed aspects of material structure and surface furmarate-co-ethylene glycol. Scaffolds based on biodegradable polyhydroxyacids, such as poly-lactide-glycolide copolymers, have been widely used as materials for bone and cartilage tissue engineering, since these polymers support the attachment and proliferation of both cell types (Vacanti et al., 1991; Freed et al., 1993; 1994; Kim et al., 1994; Chu et al., 1995; Sittinger et al., 1996; Grande et al., 1997; Gugala and Gogolewski., 2000). Polylactic acid and polyglycolic acid (PLA/PGA) copolymers degrade primarily via chemical hydrolysis of the hydrolytically unstable ester bonds into lactic acid and glycolic acid, which are non-toxic and can be removed from the body by normal metabolic pathways (Athanasiou et al., 2001). Additionally, enzymatic degradation has been reported for those copolymers (Freed et al., 1993). It was found that the degradation behaviour of polyhydroxyacids depends on the polymer structure and surface properties (molecular weight, copolymer composition, crystallinity, overall material surface, etc.) and environmental conditions (medium exchange, temperature, polymer/host interaction, and pH) (Table 6.2). The degradation rate of polyhydroxyacids can be adjusted by changes of the PLA/PGA ratio, the molecular weight of each component, the crystallinity, and other factors in order to support the slow degradation rate. This degradation rate may be similar or slightly lower than the rate of tissue formation in the defect site (Burkart et al., 2001). It is important to note in this respect that the repair or remodelling turnover time of cartilage and bone differs significantly. Limited resistance of polyhydroxyacid materials to loading, as well as their limited deformability and elasticity are disadvantageous in their use for bone and cartilage tissue engineering strategies, especially if mechanical competence is required (Grad et al., 2003). Elastomeric polyurethanes are materials with enhanced molecular stability in vivo. Newly designed biodegradable polyurethanes were shown to degrade in vivo to non-toxic by-products (Gogolewski and Pennings, 1982; 1983; Gogolewski et al., 1983; 1987a; 1987b; Gogolewski and Galetti, 1986; Galetti et al., 1989; Elema et al., 1990; Klompmaker et al., 1991). Based on these findings, biodegradable polyurethanes have been used successfully as cancellous bone graft substitutes in animals (Gogolewski et al., 2001; 2002). However, very little attention has been paid to biodegradable polyurethanes as scaffolding materials for chondrocytes. To date, only hydroxybutyrate-co-valerate polyol-based polyurethane was used to culture rat chondrocytes (Saad et al., 1999).

R_`jc 4,0, Properties of synthetic polymers

Nmjwkcp	Qrpclerf &EN_'	Bcep_b_rgmlrgkc rgkc&kmlrf'	Bcep_b_rgml npmbsarq
Polyester (polyglycolic acid)	7.0	6–12	Glycolic acid
Polyester (poly(l)-lactic acid)	2.7	>>24	l-Lactic acid
Polyester (poly(d,l-lactic-co-glycolic) acid (85/15))	2.0	1–6	d,l-Lactic acid, glycolic acid
Polyester (polycaprolactone)	0.4	>>24	Caproic acid
Polyester (polydioxanone)		–	
Polyester (polypropylenefumarat)	2–30	–	Fumaric acid, propylene glycol

6.2 Synthetic Inorganic Materials

Various types of synthetic inorganic materials have been developed in skeletal replacement therapies (Table 6.4). These synthetic substrates, capable of supporting the natural process of bone remodelling, were mainly used in bone tissue engineering applications, which include the ex vivo generation of cell–scaffold complexes, in vivo resorbable bone cements, implantable coatings to enhance the bonding of natural bone to the implant, various forms of implantable prostheses and bone repair agents (Friedman et al., 1998; LeGeros, 2002; Ohgushi et al., 2003; Uemura et al., 2003). Synthetic inorganic materials are seldom used in cartilage tissue engineering strategies, since the matrix of cartilage does not contain crystalline inorganic materials (except for the calcified cartilage layer). Among the materials used as scaffolds for bone tissue engineering, calcium phosphate containing ceramics are most frequently exploited in bone replacement strategies (Fig. 6.2). These are: hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$), β-tricalcium phosphate (TCP) ($Ca_3(PO_4)_2$) and hydroxyapatite/β-tricalcium phosphate bi-phase ceramics. Calcium phosphate materials have differing degrees of stoichiometry. Hydroxyapatite is the most widely used of a number of calcium–phosphorous (Ca–P) compounds, which are near the primary ionic component of natural bone. Pure hydroxyapatite has the stoichiometric Ca to P ratio of 1.67, lattice parameters: a axis = 0.94 nm and c axis = 0.69 nm, and the presence of only the OH and PO4 absorption bands in their infrared spectra. Other CaP materials also recommended for scaffolds in bone tissue engineering include octacalcium phosphate (Kamakura et al., 1999), whitlockite, or Mg-substituted tricalcium phosphates (Gatti et al.,1998), Zn-substituted tricalcium phosphate (Kawamura et al., 2000), carbonate-substituted apatites (Okazaki et al., 1998), and fluoride-substituted apatites (Monroe et al., 1971; Kazimiroff et al., 1996; Frondoza



Dge, 4,0, Scanning electron microscopical view of two different tricalciumphosphate-based biomaterials *(top vs. bottom)*, showing different gross morphologies. *From left to right:* detailed aspects of material structure and surface et al., 1998; Gatti et al.,1998). Substitution of single elements in the calcium phosphate or apatite structure affects the crystal and dissociation properties of CaP. Carbonate substitution for example causes not only the formation of smaller and more soluble apatite particles, but also better pH stability, whereas fluoride incorporation has the opposite effects upon material degradation while having no effect on the pH stability (Zapanta-LeGeros, 1965; Moreno et al., 1977; LeGeros, 1981; 1991; LeGeros et al., 1967; 1971; 1996). Magnesium incorporation in apatite is limited but causes reduction in crystallinity (smaller crystal size) and increases the extent of dissolution (LeGeros, 1981; 1991; LeGeros et al., 1989; 1995a; 1996). Magnesium or zinc substitution in different calcium materials also affects the properties (LeGeros, 1991; Okazaki and LeGeros, 1992; LeGeros et al., 1999).

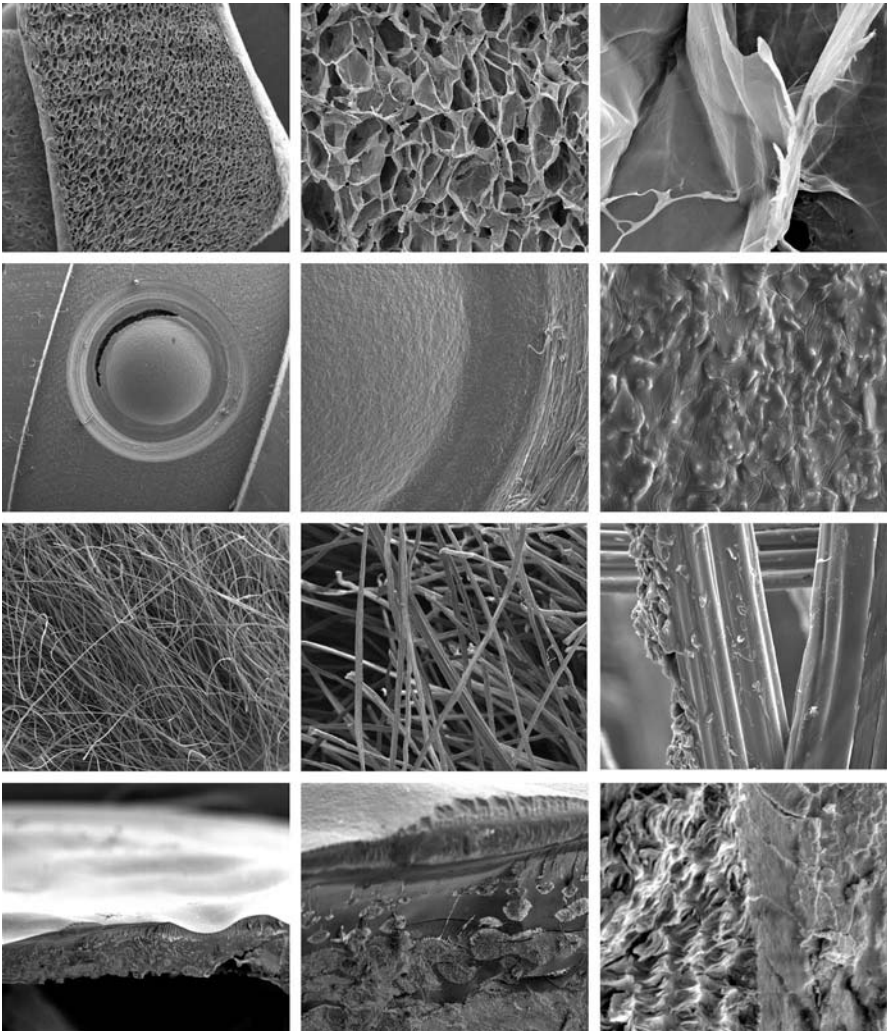
Properties of ceramic materials can be manipulated by the fabrication process. Parameters like porosity, crystal size, composition, and dissolution properties determine to a great extent the fate of the material in the in vivo and in vitro situation (LeGeros, 1988; 1991; LeGeros et al., 1991). Due to their ionic, hydrophilic composition, ceramic materials have a particular affinity to bind proteins. They may therefore be suitable carriers for bioactive peptides or bone growth factors (Ohgushi and Caplan, 1999; Toquet et al., 1999; Reddi, 2000). However, it is important to note that although CaP biomaterials are osteoconductive they do not have osteoinductive properties meaning that they are unable to support de novo bone tissue generation at non-bony sites.

6.3 Natural Organic Materials

Naturally derived materials have frequently been exploited in tissue engineering applications because they are either components of or have macromolecular properties similar to the natural ECM (Table 6.3). For example, collagens, fibrin, hyaluronan or some proteoglycans are main proteins of hard tissue tissue ECM of vertebrates (Fig. 6.3). Additionally, non-mammalian molecules like alginate and chitosan are candidates for scaffold materials. Despite the fact that the latter materials are not present

R_`jc 4,1, Naturally occurring and artificial polymeric scaffold materials

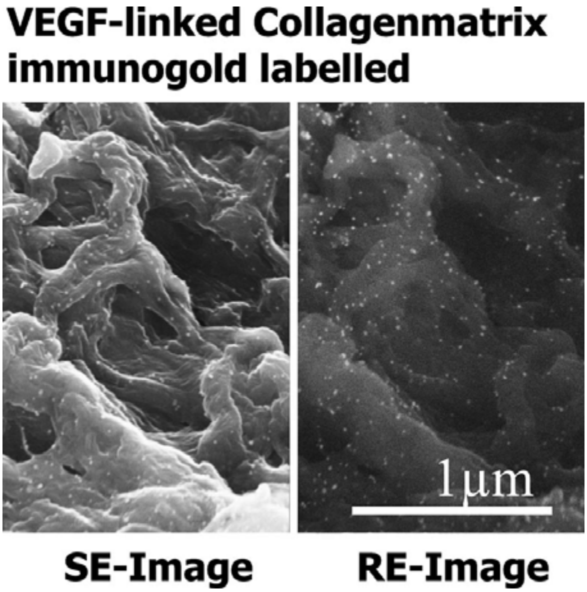
L_rsp_j		
– Collagen – Fibrin	– Hyaluronan	
?prĨag_j		
– Gelatine	– Polylactic acid – Polyglycolic acid – Chitosan – Agarose – Alginate	– Polymethlymethacrylate – Polyethylene – Polytetrafluoroethylene – Polybutyric acid – Polycarbonate – Polyesterurethane
Npmrcgl+`_qcb	A_p`mfwbp_rc+`_qcb	Qwlrfcrga



Dge, 4,1, Scanning electron microscopical view of differently structured biodegradable materials for bone and cartilage reconstruction. Materials can have a spongiosa-like *(top row)*, a bulk *(second row)*, a fibre-like *(third row)*, or a compound *(bottom row)* structure. *From left to right:* detailed aspects of material structure and surface

in human tissues, they have also been shown to interact in a favourable manner with the surface of implant devices and have been utilised as scaffold materials for bone and cartilage tissue engineering.

Collagens are attractive materials for bone and cartilage tissue engineering as this group of secreted proteins is present in skeletal tissues, where they constitute a main substrate of the ECM (Höhling et al., 1995; Sanchez et al., 2005; Wiesmann et al., 2005). Details of the ECM structure of bone and cartilage are described in detail



Dge, 5,6, Immunogold-labelled detection of VEGF attached to a collagen matrix by scanning electron microscopy (*left:* scanning mode, *right:* backscattered mode)

faces with plasmids encoding human genes is a future perspective (Thorwarth et al., 2004) in an effort to improve biomaterial surfaces, but biological, legal and ethical aspects have to be solved before this approach can be used in clinical trials.

Bioreactors and Cell Stimulation 8

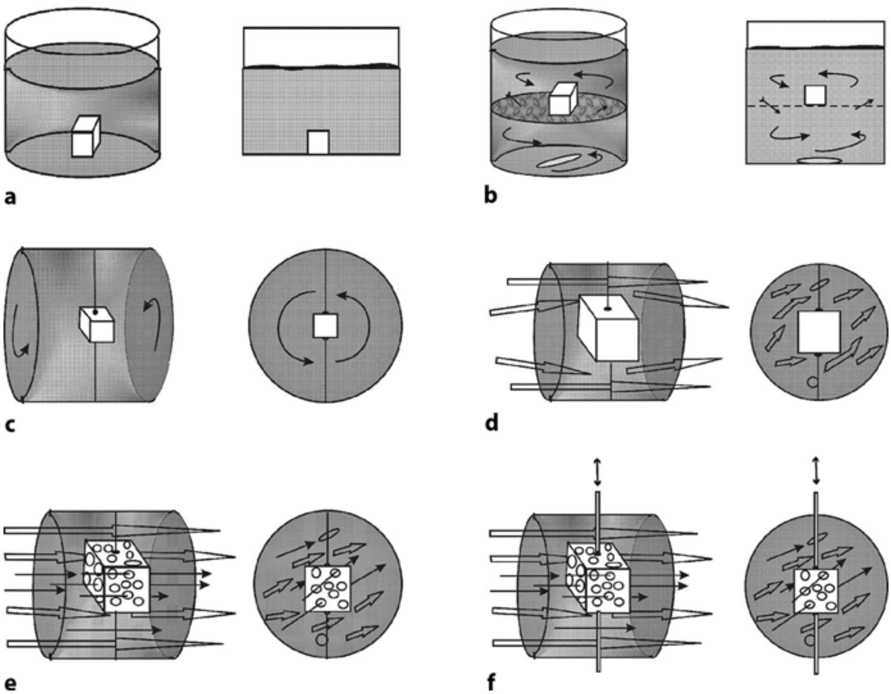
8.1 Bioreactors

The current challenge for bone and cartilage tissue engineering is to raise research-scale products to a level of reproducible tissue substitute fabrication that is clinically effective by the use of bioreactors (Salgado et al., 2004). Various types of bioreactors have been tested for their utility in skeletal tissue engineering (Martin et al., 2004). Bioreactors can be considered as devices in which biological and/or biochemical processes are performed under controlled conditions (e.g. pH, temperature, pressure, oxygen supply, nutrient supply, and waste removal). Most bioreactors were initially developed to test biomaterials (Meyer et al., 1993b), but some were also constructed to allow extracorporal bone and cartilage tissue fabrication. The ex vivo generation of large three-dimensional tissues requires the development of new devices which can meet the physico-chemical requirements of large cell masses commonly coaxed with a scaffold (Griffith and Naughton, 2002). The main aspects of bioreactor development and usage are the following:

- Nutrition of cells in the scaffold
- Control over environmental conditions
- Long-term culture
- Biophysical and biological stimulation of cell/scaffold constructs
- On-line evaluation of biological parameters
- Automation of bioprocesses.

The control of these various aspects of bioreactor technology is necessary to improve the ex vivo development of tissues and to reduce manufacturing costs in order to facilitate clinical use. Cell seeding on scaffolds – that is, short-term coaxing of cells with a scaffold – and long-term cell ingrowth of cells in scaffolds are the two principle ways to generate a three-dimensional cell/scaffold complex. An alternative and recently established method of generating three-dimensional tissues is the use of scaffold-free micromass technology in bioreactors, described in more detail in Chap. 9. The design and performance of bioreactors is of special relevance to bone and cartilage tissue engineering. As both tissues are stimulated by mechanical forces, but react to the microenvironment very differently (e.g. O_2 tension), bioreactors with defined properties are desirable to optimise the ex vivo growth of the desired cell/scaffold constructs (Malda et al., 2003). It is therefore important to consider the in vivo situation of the different cell sources in skeletal tissues. The survival of cells in vivo as well as in bioreactors depends on the response of the paticular cells to the environment. A higher oxygen tension, for example, is needed for osteoblastic differentiation, whereas prolonged hypoxia favours formation of cartilage or fibrous tissue (Malda et al., 2003). Experimental findings have revealed that at least some connective tissue progenitors in bone and bone marrow are prone to survive in hypoxic conditions. Experimental data have shown that many stem and progenitor cells, including connective tissue progenitors in bone, exhibit a remarkable tolerance to, and are even stimulated by, hypoxia, not unlike endothelial cells. In metabolically active tissues such as trabecular bone and bone marrow, the distance that, for example, oxygen must diffuse between a capillary lumen and a cell membrane is almost never more than 40 to 200 μm. This diffusion distance is critical in maintaining the balance between oxygen delivery to a bone cell and consumption of oxygen by cells, both in native tissues and in extracorporal engineered tissue. The adjustment of oxygen tension in bioreactors is therefore a critical aspect of bioreactor design.

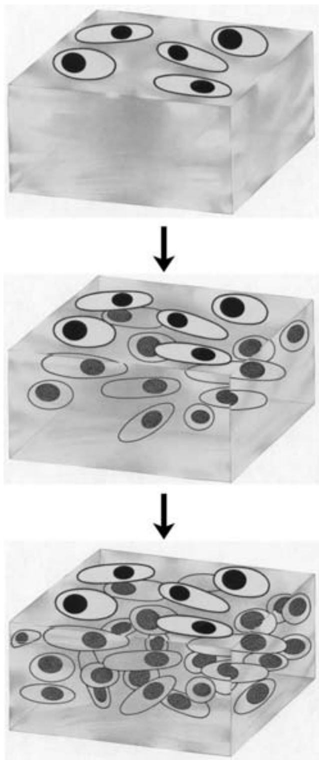
The simplest and most widely used bioreactor for bone and cartilage tissue engineering today is the culture dish (Fig. 8.1). It provides an environment that is easy



Dge, 6,/, Different principles of bioreactor systems. _ Petri dish as a simple biorector system. ` Stirring the medium improves cell nutrition. a Rotating bioreactor. b Rotating bioreactor with laminar flow as a more complex biophysical bioreactor system. c A porous scaffold improves the nutrition of cells located within that scaffold. d Mechanical loading of the bioreactor system described in (c) leads to the most complex bioreactor to handle and economical to manufacture. Additionally, flasks are also commonly used bioreactors today (Langer and Vacanti, 1993), but both types of bioreactor are of limited value, especially when a three-dimensional bone construct has to be fabricated. The cultivation of cell monolayers to multiply the initial cell numbers has various advantages. Cells in monolayer culture are not generally nutrient-limited. Passive diffusion is more than adequate to supply the cell layers but the supply of oxygen and soluble nutrients becomes critical when the diffusion distance becomes wider than 100–200 μm. Cell cultivation in monolayers allows rapid multiplication of cells. This major advantage is accompanied by limitations on creating a three-dimensional construct.

In all settings in which cells are desired to grow in multiple layers or in which cells are located in scaffolds (Fig. 8.2), access to substrate as well as signalling molecules, growth factors, and nutrients (oxygen, glucose, amino acids and proteins) and clearance from metabolic products of metabolism (CO_2, lactate, and urea) are critical for cell survival (Vunjak-Novakovic, 2003). The diffusion of these substrates in and out of the cell-containing microenvironment is a prerequisite to scale up cell/material devices to tissue-like products. The transport of the various substrates can be mediated by inducing fluid flow in the cell-containing culture dishes or flasks, thereby creating more complex (e.g. multilayer) cell–scaffold constructs (Fig. 8.1).

Dge, 6,0, Cell invasion in hydrogel



The pressure gradients driving this fluid flow can be induced by various techniques. The nutrient supply of cells can be, in part, improved by stirring the culture medium. As one of the most basic bioreactors, the stirred flask induces mixing of oxygen and nutrients throughout the medium and reduces the concentration boundary layer at the construct surface. Cell growth in a spinner flask therefore provides continuous exposure of the cells to various nutrients by convection. Convection is of special relevance in skeletal tissue engineering since cells are embedded in a dense extracellular matrix, which plays a regulatory role in enhancing the transport of large molecules (e.g. proteins and growth factors). However, in tissues like cartilage, passive diffusion along concentration gradients is another mechanism for mass transport, particularly for small molecules. Importantly, the turbulent flow generated within stirred-flask bioreactors leads to unpredictable flow effects in different regions of the multilayered cell/scaffold complex, which complicates the standardised evaluation of nutrient-dependent cell effects.

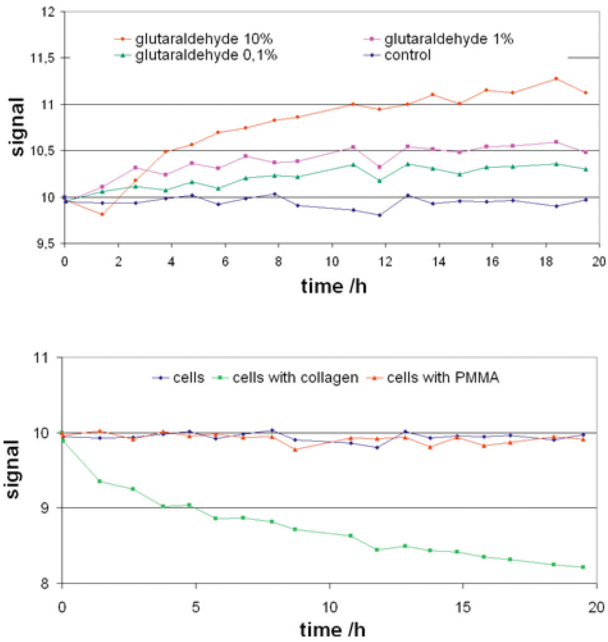
When cells are located in a more complex, scaled-up scaffold, the medium that delivers oxygen and other nutrients is commonly confined to the outer surfaces or the pores of the scaffold, even when the medium is stirred. As one moves deeper into the bulk scaffold material, each cell competes for oxygen, nutrients and signal molecules with other cells located in the same microenvironment. When complex cell/scaffold constructs (e.g. by co-culturing osteoblasts and endothelial cells) are fabricated, cells compete with the various cell sources that are located within a scaffold. More complex bioreactor systems thus need to be developed to improve cell survival in scaled-up scaffolds. Factors influencing and regulating cell viability in elaborated bioreactors include, besides the structure of the scaffold, the following:

- Oxygen concentration
- Oxygen need and consumption of cells
- Nutrient concentration
- Metabolite removal
- Medium/cell volume ratio
- Overall cell/scaffold construct size
- Material porosity
- Diffusion through the biomaterial
- Fluid flow within the site.

Each of these factors has a dramatic impact on the growth and survival of the tissue substitute, and when controlled can be used as major positive modulators. Providing such scaled-up three-dimensional tissues with nutrients is of major importance, since cells are aimed not to colonise the surface of scaffolds but should also be located within the scaffold in a viable state. In complex systems nutrients may therefore often be actively delivered by direct perfusion (Curtis and Riehle, 2001). A dynamic laminar flow environment is a desirable and efficient way to reduce diffusional limitations of nutrients and metabolites. If laminar flow can be adjusted in an ideal fashion, cells may, in addition to gaining sufficient nutrition, be stimulated through optimised levels of shear. The efficacy of laminar flow bioreactors for the generation of tissue equivalents has been demonstrated using chondrocytes and osteoblasts (Risbud and Sittinger, 2002). After a long period of cultivation under laminar flow, cartilaginous constructs can adopt biochemical and biomechanical properties superior to those of static or stirred-flask cultures, and approaching those of native cartilage (Darling and Athanasiou, 2003). Advances in bioreactor design are therefore closely related to improvements of scaffold design that are aimed to increase the nutrition of cells in complex constructs by combining laminar flow with tubular structures located in the scaffold. Bioreactors that perfuse medium either through or around semi-permeable tubes have been used successfully to enhance the metabolic function of cells by increasing the mass transport of nutrients and oxygen in central parts of cell/scaffold complexes. This concept has been extended to engineered tissues by perfusing culture medium not only at the surface but also directly through prefabricated interconnecting tubes and pores of scaffolds, thereby reducing mass transfer limitations, especially in the central parts of scaffolds. Direct perfusion bioreactors have been shown to enhance growth, differentiation and deposition of mineralised matrix by bone cells, and cartilage-like matrix synthesis by chondrocytes. It is obvious that the effects of medium perfusion depend to a great extent on the flow rate in the microenvironment of the cells. Therefore, in optimising a perfusion bioreactor for skeletal tissue engineering, one must carefully address the balance between the extent of nutrient supply, the transport of metabolites to and away from cells, and the fluid-induced shear stress effects on cells located at the surface and in the porous structures of the scaffold (Ratcliffe and Niklason, 2002).

Based on experimental data, oxygen is demonstrated to be a main limiting factor in cell survival in bioreactors containing scaled-up artificial tissues. As few cells tolerate diffusion distances greater than 200 μm, materials should be limited in their bulk size (especially in the case of bone tissue engineering) (Muschler et al., 2004). It was shown that osteoblasts seeded on porous scaffolds in vitro form a viable tissue that is no thicker than 0.2 mm. Similar observations were reported for different cell types cultured in three-dimensional scaffolds under diffusion conditions. Deposition of mineralised matrix by stromal osteoblasts cultured into poly(DL-lactic-co-glycolic acid) foams reached a maximum penetration depth of 240 μm from the top surface. Cartilage is, in contrast to bone and most other tissues, exceptional, since chondrocytes retain viability or may even be stimulated by low oxygen concentration. It must be stressed that, as with osteoblasts, chondrocyte function is not only dependent on the oxygen concentration present, but also on various other nutrients. It was shown that below a distinct level of nutritional supply chondrocyte function is disturbed. For example, glycosaminoglycan deposition by chondrocytes cultured on poly(glycolic acid) meshes was found to be poor in the central parts of three-dimensional constructs. Stirring-induced fluid flow in the flask was accompanied by an increase in the synthesis of glucosaminoglycans and fragments thereof, indicative of an improved chondrocyte function through enhanced delivery of metabolites.

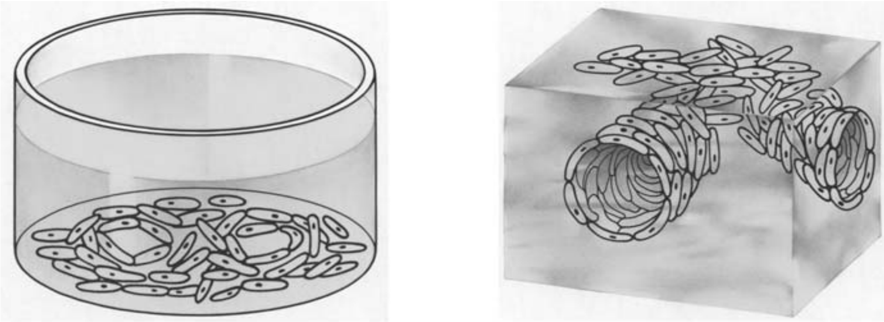
In light of the importance of optimised cell nutrition, it is important to have some means of measuring/assessing levels of oxygen and other nutritive factors of cells located in the scaffold structure (Fig. 8.3). One way to optimise the nutrient supply in cell/scaffold constructs and to determine the diffusion-related flow conditions in deep structures of bulk materials is by mathematical modelling. Computational tools have recently become available to calculate parameters such as flow fields, shear stresses and mass transport in scaffold-



Dge, 6,1. Measurement of cell oxygen consumption is a sufficient tool for characterisation of cell promotion or impaired cell activity by biomaterials in vitro

containing bioreactors. These models have also been used to determine the relationship between mass transport and cell viability. Mathematical modelling enables researchers, for example, to calculate the momentum and oxygen transport within concentric cylinder bioreactors for cartilage tissue engineering and to design and characterise a scaled-up version. Theoretical modelling was also used to explore the relationships between cell density, diffusion distance, and cell viability within a cell/scaffold construct under a given nutrient supply. Muschler et al. (2004) presented a system of differential equations that can be used to calculate the relationship between cell density, diffusion distance, and cell viability in materials. Modelling of these parameters was performed to estimate when conditions of hypoxia will appear. It was calculated that increasing the scaffold dimension by a factor of 5 will decrease the maximum concentration of cells that can be delivered by oxygen by a factor of 25. This theoretical assumption confirms the finding that flat or porous scaffold structures work better than bulk materials. A combined approach of using both mathematical models (calculating flow and diffusion conditions) seems to be promising in order to most closely reflect the real in vivo situation for oxygen supply and consumption in complex scaffold systems.

The complexity of cell nutrition modelling is even greater when considering that not only oxygen tension but also the delivery, consumption and removal of nutrients and metabolic products are critical for cell survival. Since diffusion of oxygen is relatively slow in some of the biomaterials, while oxygen consumption is high, and the transport of other nutrients (e.g. glucose and amino acids) to and away from cells is more favourable in some biomaterials than in others, modelling of complex systems is difficult. Little, therefore, is known about the balance between diffusion, consumption and removal of nutrients and waste products on the one hand, and cell



Dge, 6,2 . Schematic drawing of endothelial cell behaviour in cell monolayer and 3D culture

proliferation on the other. As this complexity is difficult to overcome by mathematical modelling, experimental data are necessary to gain a more detailed insight into nutrition-related cell reactions.

A totally different approach to improve the nutrition of cells is to enforce the formation of a vascular-like tissue in bioreactors (Fig. 8.4). Neovascularisation may be reached by the use of a co-culture system of bone cells and endothelial cells. However, since this approach introduces a new level of complexity, the technical challenges are significant. Aspects of ex vivo vascular formation are described in more detail in Chap. 13.

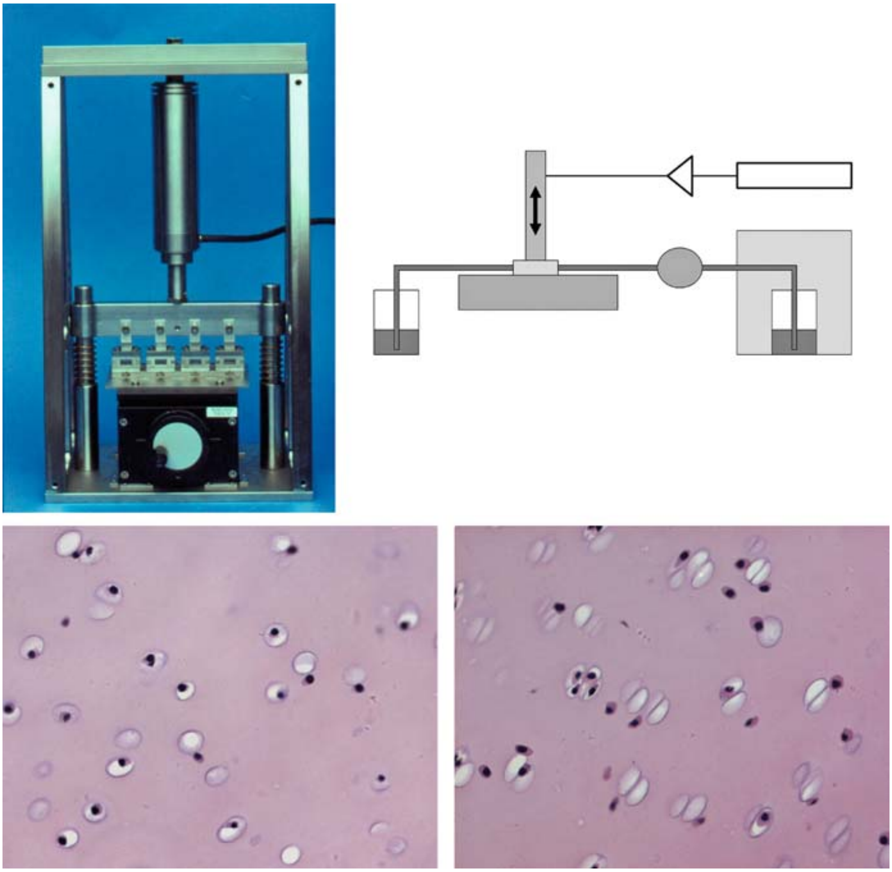
From a technical point of view, bioreactors can be classified according to their design properties. Bioreactors, connected to ports and filters for gas exchange, can be regarded as more open systems compared to conventional dishes and flasks. However, the fact that the culture dish requires individual manual handling for medium exchange, cell seeding, etc., ultimately limits its usefulness when large cell numbers are required. Closed bioreactor systems offer major advantages for manufacturing, since sterility can be assured and viability of the tissue product maintained. Closed and automated bioreactors should contain state-of-the-art systems for monitoring and adjusting physico-chemical as well as mechanical parameters. For reproducibility and standardisation, the culture conditions (e.g. temperature, pH, nutrient and oxygen supply) should be adjustable. An improvement in bioreactor design would be the addition of devices which allow online evaluation of the cell/scaffold maturation. The introduction of non-destructive analysis tools to monitor material parameters (e-modulus, degradation), biological parameters (cell number, cell differentiation) and metabolic parameters (pH, oxygen concentration, nutrient concentration) is helpful in adjusting culture conditions towards tissue needs. Advanced techniques (oxygen measurements, substrate monitoring, flow determination, fluorescence microscopy, micro-computerised tomography) aimed to predict the development of the tissue construct over time, have now been introduced in the manufacture of skeletal tissue-engineered products.

8.2 Mechanical Stimulation in Bioreactors

The skeletal system is continuously subjected to mechanical forces imposed by muscular contractions, body movement and various other external loadings. Similarly, externally applied mechanical forces in ex vivo generated bone tissue may elicit effects on osteoblast and chondrocyte proliferation, cell orientation, gene activity and other features of cellular activity (Cheng et al., 1996). It has been shown that mechanical forces applied from outside or organised from within a scaffold are beneficial (Schmelzeisen et al., 2003). Engineering bone and cartilage substitutes in vitro has been developed towards advanced bioreactor systems that mimic not only the three-dimensional morphology (Lee et al., 1996) but also the mechanical situation of bones (Cheng et al., 1997) or joints. Various studies have demonstrated the validity of this principle, particularly in the context of musculoskeletal tissue engineering (Hung et al., 2004). Despite numerous proof-of-principle studies showing that mechanical conditioning can improve the structural and functional properties of engineered bone and cartilage tissue, little is known about the specific mechanical parameters or regimes of application (i.e. magnitude, frequency, duration and mode of load application) that provide an optimal stimulation for bone or cartilage tissue (Butler et al., 2000). Most studies on the effects of load on osteoblasts were performed in monolayer cultures. Investigations of load-related chondrocyte reactions were done preferentially in explant cultures. In the context of bioreactor design for mechanically supported bone and cartilage tissues some aspects have to be considered here:

- Tissues at different stages of development or skeletal sites might require different regimes of mechanical conditioning.
- Cell deformations resulting from mechanical loading inside a bulk material may differ from deformations of surface-bound cells (Casser-Bette et al., 1990).
- The physiological strain environment of bone cells is much lower than that of other types of cells and they are correspondingly far more sensitive to mechanical deformations than most other cell types (e.g. chondrocytes).

In this highly complex field, a comprehensive understanding can only be achieved through experimental and theoretical investigations at different structural levels (monolayers, multiple layers, three-dimensional constructs). As load transfer through the scaffold and the substrate surface to cells (Rubin and Lanyon, 1987) exhibiting small deformations will have profound effects on cell behaviour, creating load devices in bioreactors has to take this aspect into account. Precise control over deformation at the various levels of hierarchy is most important. Bioreactors for mechanically supporting skeletal tissues have been custom-designed to provide defined deformations. Measuring strain and stresses in culture dishes and bioreactors can be done experimentally as well as theoretically (Fig. 8.5), however limitations on both approaches exist. Different authors have developed experimental systems aimed at applying defined mechanical loads on cells in culture (Granet et al., 1998; Hung et al., 2004). Some techniques used biaxial strain devices in an effort to apply homogeneous, isotropic strain to cells cultured in a monolayer, and used finite element analysis to predict the appropriate geometry for a uniform strain field. The accuracy of the strain field calculations was experimentally confirmed (Bottlang



Dge, 6,3, Bioreactor for mechanical stimulation of cartilage or bone. Four tissue chambers can be loaded by a piezo-electric driven lever arm. Homogenuous strain fields can be applied. Use of the bioractor to stimulate explanted cartilage tissue (*bottom*). Histology of cartilage after mechanical loading: non-stimulated (*left*) and stimulated (*right*) cartilage

et al., 1997). However, it is not possible to measure the loading of cells in a three-dimensional space with the cell culture system used. Pressure application through lever-arm connected stamps is a common method to apply loads to three-dimensional tissue specimens. Although the stamp movement with subsequent deformation of the scaffold may be applied with high precision, determination of strain and stress fields within a specimen is much more complex than with biaxial systems. Assessment of the stress and strain fields by finite element analysis (FEA) is a commonly used approach, since other experimental measures are not able to determine load-related deformations in the microenvironment of a cell-containing scaffold, but the accuracy of FEA is limited by some simplifications. The matrix structure is, in most approaches, considered to be a homogenous incompressible structure, whereas in reality the cell-containing scaffold is generally more complex. Another limitation is that bioreactor and scaffold parameters are implemented on a simplified database.

Despite these simplifications, calculated strain fields seem to reflect real deformations in the tissue chamber. At least, the calculated magnitude that is biologically relevant allows one to discriminate between no, physiological and hyperphysiological load (Winston et al., 1989). Jones et al. (2003) developed an advanced loading device for application of specific compressive strains to tissue cylinders, which can be used to measure the resulting deformations. The device allows one to load the specimen at frequencies between 0.1 Hz and 50 Hz and amplitudes over 7,000 μstrain. This system combines accurate measurements of mechanical loading in real time with exact assessment of visco-elastic properties and may be useful in both, bone and cartilage tissue engineering applications.

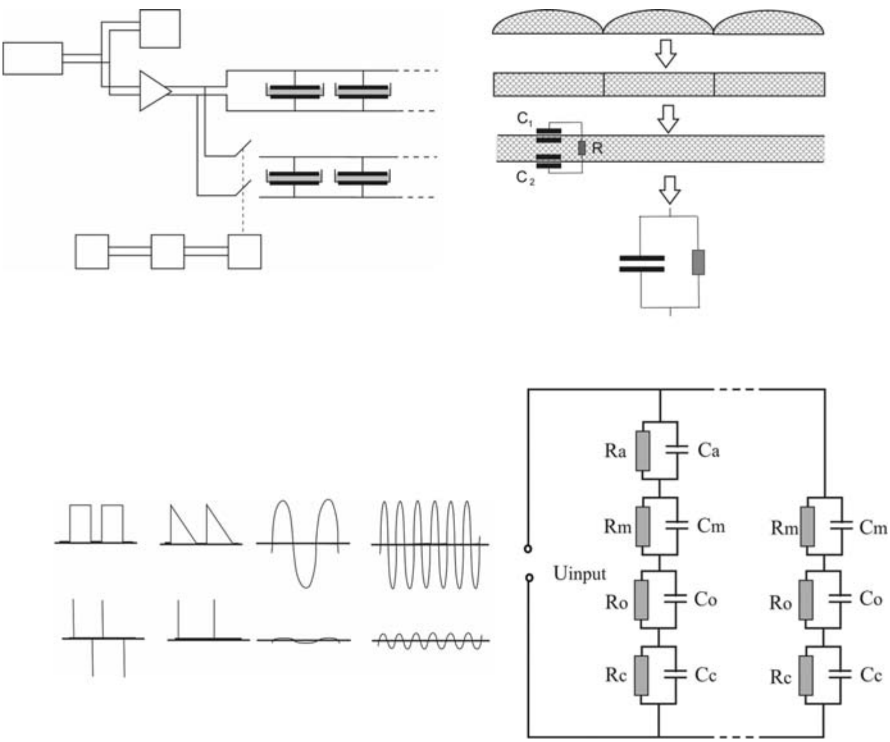
Other investigators embedded osteoblast-like cells in gels that were subjected to cyclical tension forces and found that cell orientation and gene activity were altered as a result of cyclical mechanical loading. The most effective frequency of this loading lay around 1 Hz (Buschmann et al., 1995; Jones et al., 1995; Meyer et al., 2004c). Most in vitro studies indicate that mechanical stress is able to stimulate the proliferation of osteoblasts (Stein and Lian, 1993; Buschmann et al., 1995; Brown, 2000). The optimal strain magnitude in vivo and in vitro was found to be in the range from 1000 to 3000 μstrain (Meyer et al., 2004c). Mechanical stimulation was shown to result in an altered expression of bone-specific proteins, such as alkaline phosphatase, osteopontin, and osteocalcin. Concerning load-related cell effects in bioreactors, it is important to note, that the effects on protein synthesis (e.g. alkaline phosphatase, osteocalcin, or collagen expression) vary depending on the techniques used for loading (Masi et al., 1992; Krishnan et al., 2004). Some authors reported improved bone cell behaviour when cells were cultured under fluid flow-induced mechanical loads in spinner flasks. The underlying reason is assumed to be the existence of forces in the spinner flask exposing cells to physiological strains (Toma et al., 1997; Pavalko et al., 1998; Ogata, 2000). The sensitivity of osteoblasts to fluid shear stress is well established for cell cultures within flow chambers (Hillsley and Frangos, 1994). Rotating wall vessel reactors, originally designed to simulate a microgravity environment, were introduced and assessed for their relevance to skeletal tissue engineering. Rotating wall vessels have been tested mainly in cartilage and bone tissue engineering (Botchwey et al., 2001). This type of bioreactor rotates at a definable and controlled rate (Schwarz et al., 1992). By defining the centrifugal forces in these bioreactors, cells can be mechanically stimulated. Recent investigations showed that bone cells seeded on polymer constructs and grown in rotating bioreactors displayed minimal differentiation towards the osteoblastic phenotype. A low alkaline phosphatase activity compared to the static controls, a decreased extracellular matrix protein synthesis in the medium during long-term cultivation, and a reduced calcium deposition were found. These findings contradict earlier reports on the beneficial effects on osteoblastic cells cultured in rotating wall vessels (Botchwey et al., 2001). The underlying causes of the low inductive properties of some of the rotating wall vessel bioreactors in skeletal tissue formation are not understood, but non-physiological forces may be responsible for the experimental findings. The mechanisms whereby mechanical stimulation leads to cell proliferation and expression of tissue-specific genes reflect the in vivo situation of load-induced osteoblast behaviour (Duncan and Turner, 1995). In general, all bioreactor systems that expose cell/polymer constructs to biophysical stimuli not exceeding a physiologically tolerable range may support the differentiation of precursor cells towards the mature phenotype and enhance the function of mature cell types (Fig. 8.5). The sum of experimental data confirm that engineered skeletal tissue provides a good example that mimicking the native mechanical environment of cells can be beneficial for the ex vivo fabrication of bone or cartilage tissues (Meyer et al., 2004c). Under conditions of optimised periodic strains, the mechanical properties of engineered tissues appeared to improve significantly. In addition, the synthesis of matrix proteins and collagen, both being components of the secreted cell environment, can be enhanced by dynamic loading. Scaffold deformation on a physiological scale appears therefore to improve extracorporeal cell/scaffold maturation in bioreactors.

It is important to consider that, whereas the physiological microstrain environment for bone cells is well defined (Frost, 2000b), less is known about the physiological load environment of cartilage (Freed and Vunjak-Novakovic, 1997). What is generally accepted is that an important issue for cartilage bioprocessing relates to the development of bioreactors that produce a mechanically favourable load environment. Many bioreactors were introduced to load cartilage explants by external forcers, but only a few were developed to provide a uniform and quantifiable mechanical environment to promote chondrocyte proliferation and matrix growth under well-defined and controllable conditions. In contrast to bone tissue engineering, where porous scaffold structures are frequently in use, cartilage tissue engineering is commonly performed in bulk scaffold materials. Some of the scaffold matrices are in a gel-like condition. It was shown by some authors that more uniform cartilage tissue production is obtained in mechanically stimulated tissue cylinders, an effect attributed to the homogeneous hydrodynamic and favourable mass transport kinetics in the bioreactor (Martin et al., 2000). Load-related cartilage tissue maturation was reached by cartilage culturing in various bioreactor types, including spinner flasks, rotating wall vessel bioreactors and perfusion flow bioreactors. The observations and experience from various laboratories suggest that cartilage tissue should be dynamically loaded in order to support chondrocyte proliferation and differentiation (Buschmann et al., 1995; Freed and Vunjak-Novakovic, 1997; Martin et al., 2000). The application of micro-movements in extracorporal tissue chambers is therefore commonly considered as a promising approach in facilitating skeletal tissue engineering.

In addition to external mechanical stimulation as one strategy to promote tissue formation, another approach is the development of mechano-active scaffolds with optimised inherent physical properties (Yang et al., 2002b).

8.3 Biomagnetic and Bioelectric Stimulation in Bioreactors

Based on the discovery of piezoelectric potentials in bone tissue in the late 1960s, it was assumed that osteoblast physiology is influenced by electric fields (Domen et al., 2000). Many experiments have suggested that indeed electric fields modify the behaviour of bone cells. The effects of electromagnetic fields on bone cell behaviour have been the focus of many studies, however, cellular mechanisms of the action are not understood. Electromagnetic effects at biomaterial surfaces were considered to be an influencing factor in cell behaviour, since material-specific effects work through electrical changes at the surface. Within the scope of their in vitro studies, many investigators reported on osteoblast-like primary cultures, giving detailed description of cell proliferation and differentiation and of initialisation of mineral formation, with some researches focusing on these effects at material interfaces. Recent investigations demonstrated that the presence of electric fields in bioreactors has distinct effects on osteoblasts in vitro (Hartig et al., 2000). Furthermore, electrical effects on osteoblast cell cultures in long-term experiments are well suited to altering biochemical processes at artificial surfaces resulting in a newly formed extracellular matrix leading to improved biomineral formation (Wiesmann et al., 2001) (Fig. 8.6). Recently, it has been demonstrated that mineral formation in cultured osteoblasts is enhanced when cells are exposed to an electric field. Long-term electrical stimulation of osteoblasts in bioreactors appears to alter the pattern of gene expression resulting in enhanced extracellular matrix synthesis. In this respect the application of electric fields in bioreactors seems to be a promising approach to extracorporal bone tissue engineering. Piezoelectric potentials are, of course, present in loaded cartilage, but the knowledge of these effects on cartilage tissue is very limited. Approaches to stimulate chondrocyte-like cells by electric fields in bioreactors are therefore seldom performed.



Dge, 6,4, Electrical stimulation of cell culture in vitro. Schematic drawing of the generation of pulsed electromagnetic fields. Cells can be exposed to differently pulsed electromagnetic fields in specially designed bioreactors

8.4 Hormones and Cytokines

Extracorporal bone and cartilage tissue formation can be enhanced through the action of several cytokines and bioactive proteins supplemented into the medium or released as scaffold-bound proteins (Schliephake, 2002) in bioreactors. Therefore, the addition of extracellular signalling molecules in ex vivo bone and cartilage generation may be beneficial (Salgado et al., 2004; Tuan, 2004). The effect of cytokines on cells in bioreactors depends on various factors (dose, release kinetics, activity, cell differentiation and maturity state). The release kinetics of different growth factors varies depending on their chemistry and the delivery system used (Leach and Mooney, 2004). Selecting an appropriate carrier or delivery system has to take the following issues into account: (1) the release kinetics, which means the release of the growth factor at an appropriate time, rate, and in the proper dose; (2) the ability of the system to deliver the protein in the microenvironment of cells; and (3) the properties of the substratum that will influence cell recruitment, attachment, and potentiate chemotaxis. Different carrier and delivery systems have been used to deliver proteins in experimental and clinical models.(Fig. 7.7.) The delivery of these proteins is aimed at improving tissue function by enhancing the cell differentiation process.

As described in Chap. 2, numerous active molecules with different biological functions are expressed during bone and cartilage formation (Rose et al., 2004). Some of these factors have been used as biological agents to promote tissue formation in ex vivo strategies. Among these growth factors are transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMP), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF), to mention the most popular ones. Besides the above mentioned factors, a multitude of other cytokines or growth factors have been used as single factors or in combination with other factors to enhance and accelerate ex vivo bone or cartilage formation. The administration of the various molecules is a matter of bioreactor design and factor pharmacology. The complexity of factor supplementation, biochemical kinetics, the rate of degradation and the dose–effect relationship, makes the proper release technique difficult. This area of research is under intensive investigation. An overview of this complex field is given in some detailed reviews (Bouletrau et al., 2002; Schliephake, 2002; Leach and Mooney, 2004). One aspect of protein delivery studies is of special pharmacological concern. Although several molecules may soon be available as recombinant or non-recombinant growth factors for therapeutical purposes, there is concern that a single dose of exogenous protein added to the medium will induce a sustained biologic response, particularly in situations in which the protein will be lost in the bioreactor system. To overcome this problem a new approach to protein delivery may be gene therapy. Gene therapy involves the transfer and expression of genetic information to target cells. With gene therapy the genetic message is delivered to a particular cell, which then expresses the transfected gene product. In general, the duration of protein synthesis after gene therapy depends on the techniques used to deliver the gene to the target cell and the promoter. Both short-term and long-term expressions are possible. Alongside the potential use of gene therapy in tissue engineering, gene therapy also involves technical and legal problems that have not yet been fully solved.

Scaffold Structure and Fabrication 9

9.1 Scaffold Structure

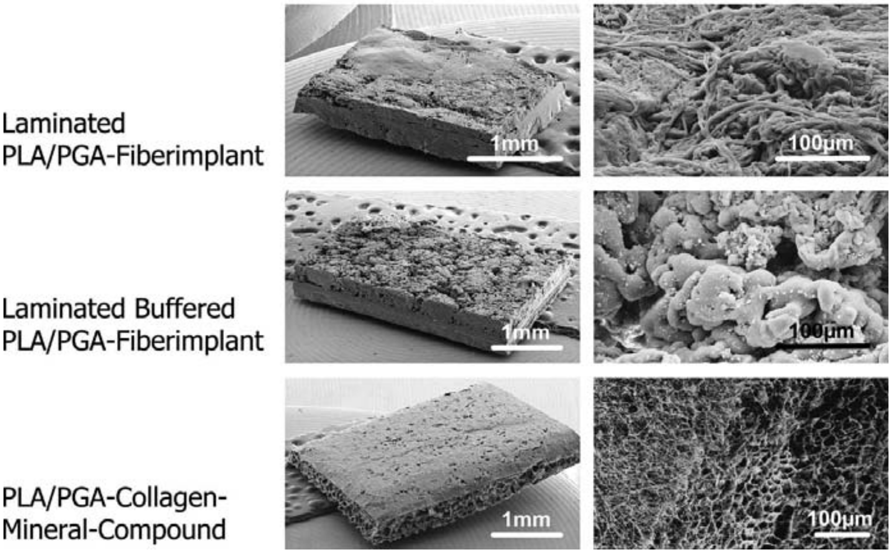
Whereas the properties of the material surface directly influence the behaviour at the single cell level, the three-dimensional scaffold structure plays a critical role in orchestration of tissue formation both in vitro and in vivo. Whereas the microstructure of the material refers to characteristics of a material at the nanoscale or microscale level (mainly used to characterise material surfaces, see Chap. 7), scaffold architecture defines the structure of the material in space at the tissue-length scale. Scaffolds do not only provide the structural basis that is available for cells to form a three-dimensional tissue-like construct in vitro, but also determine the features of mass transport (diffusion and convection). The scaffold architecture affects both single cell parameters (e.g. cell viability, cell migration, cell differentiation), and the composition of the generated tissue substitute.

Scaffolds can be grouped into different categories: underlying material, macrostructure (spacial geometry), micro-structure (bulk versus porous), mechanical properties, and degradation characteristics (Hutmacher, 2001).

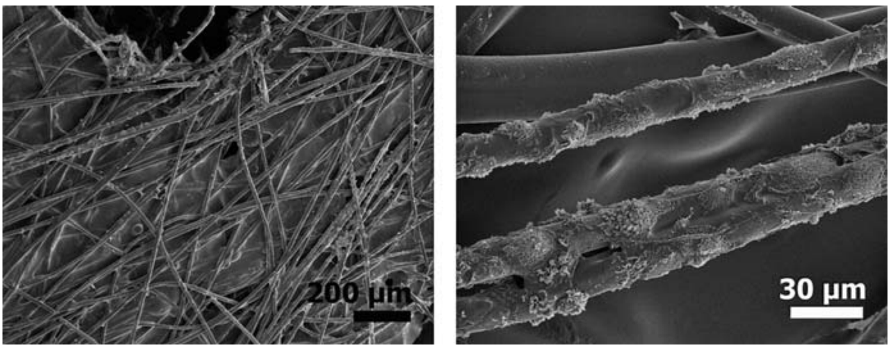
Scaffold Characteristics

- Scaffold composition
- External geometry
- Macrostructure
- Microstructure
- Interconnectivity
- Surface/volume ratio
- Mechanical competence
- Degradation characteristics
- Chemical properties.

Whereas micro- and nano-properties are, to a great extent, related to the material surface and are described in detail in Chap. 7, micro- and macro-properties are also critical for tissue development. Scaffolds serve as space-holders for cells and allow ingrowth of surrounding tissues into the reconstruction site after transplantation. Thus, they provide structures that facilitate the three-dimensional proliferation, differentiation, and orientation of cells in order to enable a tissue-like construct growth ex vivo. Scaffolds facilitate the transfer of loads to surrounding tissues and ideally allow the reconstruction site to be mechanically competent directly after insertion. Scaffolds also provide a space in which tissue development and maturation towards complex multicellular systems can occur (Tsang and Bhatia, 2004). (Fig. 9.1) Two classical ways are in use to fabricate cell-containing scaffolds. One commonly used method is to coat cells with the scaffold by seeding cells on the scaffold. This approach is simple and fast, since cells attach to the scaffold within the first 24 hours. In contrast to the second method (to cultivate and maturate cells within a scaffold in a bioreactor), this approach is limited by the inhomogeneous distribution of cells. Various techniques to coat cells with scaffolds are in use. Static cell seeding techniques are the most commonly used methods, and involve placement of the scaffold in a bioreactor followed by adding a cell suspension to allow the adhesion of cells. (Fig. 9.2) However, the resulting cell distribution in the scaffold is random, and de-



Dge, 7,/, Scanning electron microscopical view of different scaffolds (*left*: overview, *right*: detail), fabricated by conventional polymer fabrication technique. Polymer scaffolds can be altered by inclusion of buffer systems or mineral compounds in order to optimise their properties.

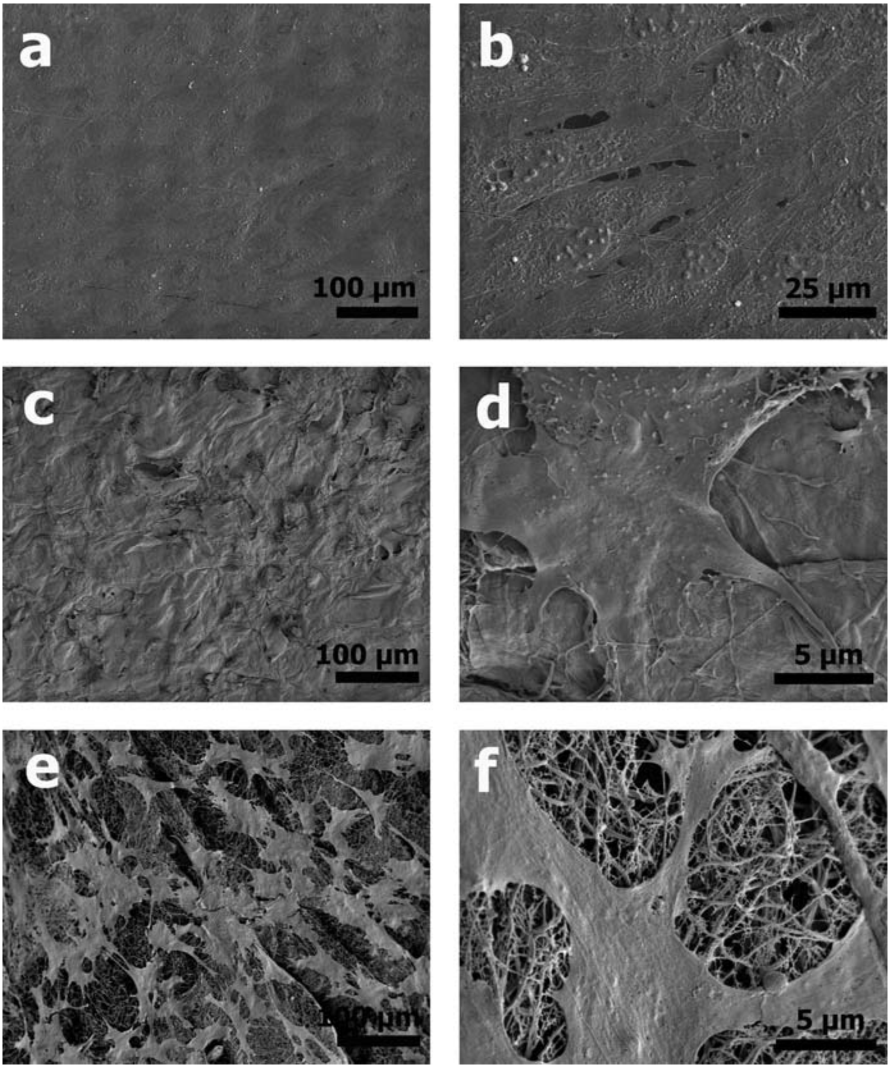


Dge, 7,0, Scanning electron microscopical view of cell/scaffold construct. Seeding of osteoblasts to a fiber scaffold lead to an adhesion of cells at the fiber surface without filling the space between fibers.

pendent on the gross scaffold structure, but in general with the majority of cells attached only to the outer surfaces. Wetting techniques applied to scaffolds prior to cell seeding allow for displacement of air-filled pores with water and facilitate penetration by the cell suspension into these pores. In contrast to simple seeding techniques, cells can also be coated with scaffolds by injection or by applying a vacuum to ensure penetration of the cell suspension. Using these techniques cells can be placed more homogenously throughout the three-dimensional space. However, uniformity is lost under static culture conditions because of nutrient and oxygen diffusion limitations within the scaffold. When cells are seeded on scaffolds, high cell densities are necessary to achieve uniform tissue regeneration. The effects of cell seeding density on cartilage formation indicates that high numbers of cells are required to obtain mature tissue formation (Vunjak-Novakovic et al., 1998; 1999; Burg et al., 2000a; Holy et al., 2000).

The second approach to coating cells with scaffolds is long-term culture of cells with scaffolds. (Fig. 9.3) Using this technique, cells have to proliferate, migrate and differentiate during culture with the scaffold. This longer-lasting method has the main advantage that cells are not restricted to the outer surface of the scaffold but may grow deep into scaffold structures. As bone and cartilage differs significantly in tissue composition at the different levels (cellular level, supracellular level, tissue level), optimal scaffold properties can be assumed to be different for scaffolds used for bone and scaffold used for cartilage tissue engineering (Wiesmann et al., 2004). Reviewing the literature reveals that bio-organic scaffold materials (e.g. collagen, fibrin, hyaluronan, alginate, chitosan) and polymer materials (e.g. polyhydroxy acids) are commonly used for both (cartilage and bone) tissue engineering strategies (Agrawal and Ray, 2001), whereas organic materials (hydroxyapatite, tricalcium phosphates) are preferably used in bone tissue engineering. Independent of the underlying material, various basic scaffold characteristics can be distinguished:

- *Sc ffold composition*: Scaffold materials can be distinguished by the chemical composition. Pure non-organic materials can de distinguished from composite materials (containing also organic materials) and solely organic materials. Materials can also be grouped into solid or gel-like conditions (hydrogels, for example, contain a water content greater than 30% by weight).
- *crostructure*: The macrostructure reflects the external geometry and gross internal structure of the scaffold. A three-dimensional scaffold that is congruent to the external geometry of the tissue to be replaced is required for scaffold placement and fixation in the clinical situation. Direct contact between the defect borders and the scaffold will enhance the interaction between the ex vivo generated construct and the host site. An optimal internal scaffold structure will promote tissue formation by mimicking the native environment.
- *Porosity nd pore interconnectivity*: Scaffolds are constituted of either bulk materials or they have a pore or tube geometry. Pores or tubes can be introduced in scaffold in an isolated fashion or they can be interconnected. An advantage of an interconnected porous or tubular system is the improved nutritional supply (by diffusion, convection or directed fluid flow) in deeper scaffold areas, thereby enabling cells to survive in these regions. A high interconnectivity of pores or tubes facilitates a hydrodynamic microenvironment with minimal diffusion constraints, but at the same time will reduce the mechanical strength. As spongiosal



Dge, 7,1, Scanning electron microscopical view of osteoblasts cultured on a petri dish (top row), a collagen matrix (middle row), and a fibrin matrix (bottom row). Cells with an osteoblastic appearance cover the surface of scaffolds.

bone tissue has a porous structure these scaffold features seem to be advantageous, especially in the context of ex vivo bone tissue engineering.

- *Pore size/tube di meter*: Tissue generation ex vivo and regeneration in vivo may be achieved using scaffolds with optimal hollow structures (tube/pore combinations). The size of these hollow structures does not only improve the fluid flow through the scaffold but may also allow vascular ingrowth (in vitro and after transplantation in vivo) and therefore improve the outcome of tissue engineering

R_`jc 7,/, Parameters affecting scaffold degradation

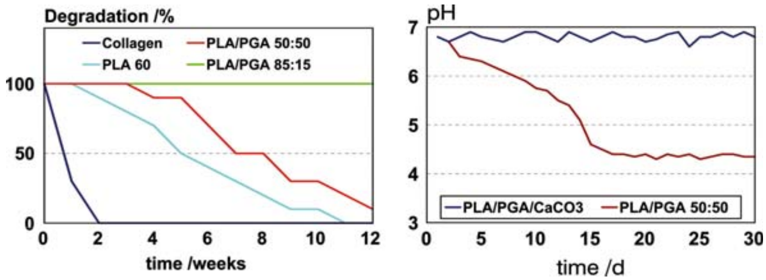
K_rcpg_j	Qa_Őmjb	Gl tgrpm	Gl tgtm
– Composition – Structure – Configuration – Molecular weight – Molecular orientation – Cross-links – Chain motility	– Size – Mass – Porosity – Surface/volume ratio – Shape – Surface topography – Surface chemistry	– Medium – pH – Fluid flow – Biomechanics – Temperature – Cell sources – Cell number – Cell activity – Ionic strength	– Defect site – Species – Inflammation – Immunology – Biomechanics – Tissue turnover – Enzymes – Vascularisation

treatments. From a conceptual point of view, cartilage tissue as a non-vascular tissue, does not benefit from porous scaffolds, whereas bone formation critically depends on vascularisation. As researchers have suggested the need for pore sizes ranging from 200 to 500 μm for vascular ingrowth, scaffolds containing tubular structures of such diameters seem to be beneficial in bone tissue engineering applications.

- *Surf ce/volume r tio*: A high overall material surface area to volume ratio is beneficial, allowing large numbers of cells to attach and migrate into porous scaffolds. Since pore diameter and internal surface area are related linearly, a compromise between surface gain and mechanical stability has to be found depending on the application of the scaffold.
- *ech nic l properties*: Scaffolds should ideally have sufficient mechanical strength during in vitro culturing to resist the physiological mechanical environment in the regenerating load-bearing tissues (cartilage, bone) at the desired implantation site.
- *Degr d tion ch r cteristics*: The ideal scaffold degradation must be adjusted appropriately such that it parallels the rate of new tissue formation and at the same time retains sufficient structural integrity until the newly grown tissue has replaced the scaffold supporting function (for a review see Lu et al., 2001). Additionally, degradation of the scaffold should not be accompanied by a lowering of the physiological pH in the in vitro environment or at the defect site. Moreover, during the degradation process there should be no release of toxic degradation products. Multiple parameters determine the degradationrate of a scaffold in the in vitro and in vivo situation (Table 9.1), (Fig. 9.4)
- *Chemic l properties*: A scaffold should ideally be used as a carrier for proteins. The proteins, bound to the scaffold material, should be biologically active and they should be released in a predetermined fashion. (Fig. 7.7)

9.2 Scaffold Fabrication

A major technical challenge in scaffold production is to maintain accurate control over the desired macrostructural (e.g. external geometry, spatial composition, mechanical strength, density, porosity), microstructural (e.g. pore size, pore interconnectivity, degradation characteristics), and nanostructural (e.g. surface topography



Dge, 7,2, Scaffold characteristics can be adjusted by chemical modification techniques. The degradation kinetics of polyesters can be adjusted by a defined co-polymerisation of different polymers (e.g. PLA and PGA). Additionally, a pH stability during scaffold degradation can be reached through an inclusion of a carbonate buffer system in the scaffold.

or physico-chemical surface characteristics) properties at the same time. Ideally, the processing technique used to fabricate the scaffolds should also allow use of various underlying materials. Besides the fabrication techniques that largely define the scaffold macro- and microstructure, there is a variety of natural or synthetic scaffolding materials to consider. It is important to note, that each underlying material possesses different processing requirements and varying degrees of processability to form scaffolds. The design of complex scaffolds composed of different materials adds a further step in complexity in scaffold fabrication. Various engineering parameters (technical procedure, fabrication accuracy, automation) determine the result of scaffold production.

- *Technic l procedure*: The processing technique should not change the chemical and biophysical properties of the scaffold nor cause any damage to the material structure.
- *F bric tion ccur cy*: The fabrication process should create spatially accurate three-dimensional scaffolds at the macro- and microscale level.
- *Autom tion*: The applied technique should be automated in order to ease the production process, reduce interbatch inconsistency, ideally reduce fabrication time, and at the same time be cost effective.

As mentioned earlier, the scaffold fabrication technique applied is primarily determined by the underlying material. It is obvious that naturally occurring non-organic materials are commonly processed by different methods to, for example, synthetic organic materials. Additionally, naturally occurring non-organic materials are generated by arrays of techniques different from those for synthetic organic materials. Fabrication of non-organic materials is performed by techniques commonly used in ceramic technology, whereas a wide range of techniques have been developed to process synthetic and natural (organic) polymer materials into scaffold structures (Peter et al., 1998a; 1998b). Conventional polymer fabrication techniques can be distinguished from the newly introduced solid-free form fabrication techniques (SFF) (Sachlos and Czernuszka, 2003) (Table 9.2). Conventional fabrication techniques are defined as processes that create scaffolds having a bulk or porous (interconnected or non-interconnected) structure that lacks any long-range channelling microstructure (Yang et al., 2002a; 2002b). (Fig. 9.1) Solid-free form fabrication, in contrast, uses

R_`jc 7,0, Scaffold fabrication techniques

Aml tclrgml_j	P_ngb npmrmrwngl e
– Solvent casting/particulate leaching – Phase inversion/particulate leaching – Fibre meshing/bonding – Melt moulding – Gas foaming – Membrane lamination – Hydrocarbon templating – Freeze drying – Emulsion freeze drying – Solution casting	– Fused deposition modelling – Three-dimensional printing – Three-dimensional plotting – Selective laser sintering – Laminated object manufacture – Stereolithography – Multiphase jet solidification

layer manufacturing processes to form scaffolds directly from computer-generated models, thereby enabling the introduction of hollow or tubular structures in scaffolds. Additionally, SFF enables one to create the external geometry of the scaffold with high precision (Yang et al., 2001). Conventional scaffold fabrication is often used in scaffold fabrication for bone and cartilage tissue engineering. Commonly used techniques are: solvent casting/particulate leaching, phase inversion/particulate leaching, fibre meshing/bonding, melt moulding, gas foaming/high pressure processing, membrane lamination, hydrocarbon templating, freeze drying, emulsion freeze drying, solution casting, and combinations of these techniques. The principles and technical procedures of these techniques can be found in various reviews (Hutmacher, 2001; Nishimura et al., 2003; Liu and Ma, 2004). A wide variety of scaffolds has been successfully applied to engineer bone and cartilage tissue ex vivo and investigated for their success in animal experimental and clinical situations. In order to understand the advantages and limitations of each processing method, a brief description is given of the technical aspects involved.

Solvent casting/particulate leaching is a commonly used and widely investigated method for the preparation of tissue engineered scaffolds. Scaffolds produced by this technique have been used in a multitude of studies for bone and cartilage tissue engineering, with favourable results (Ochi et al., 2003). This method, first described by Mikos et al. in 1994, is based on dispersing mineral (e.g. sodium chloride, sodium tartrate and sodium citrate) or organic (e.g. saccharose) particles in a polymer solution. The dispersion process is then performed by casting or by freeze-drying in order to produce porous scaffolds. By modulation of the basic process, different researchers (Mikos et al,. 1994; Agraval et al., 2000; Yoon and Park, 2001; Murphy et al., 2002) aimed to improve the scaffold structure. They introduced variations of the basic processing principle to alter the polymer structure to that desired: i.e. to increase the porogen/polymer ratio, avoid crystal deposition, increase the pore interconnectivity, and obtain fully interconnected scaffolds with a high degree of porosity (Agraval et al., 2000; Murphy et al., 2002). Solvent casting/particulate leaching has been used, in particular, to fabricate poly(L-lactic acid) (PLLA) or poly(lactide-*co*-glycolide) (PLGA) scaffolds, but the method has also been applied to process other polymers (Gomes et al., 2002b). Despite the relative ease of fabricating scaffolds using this approach, the method has some inherent disadvantages such as the possible release of highly toxic solvents (Hutmacher, 2000; Yang et al., 2001; Gomes et al., 2002a), the limitation of producing only thin scaffolds (Yang et al., 2001; Gomes et al., 2002b), and impaired mechanical properties (Gomes et al., 2002a).

Phase inversion/particulate leaching is a closely related technique to solvent casting. The difference from the previously described method is that instead of allowing the solvent to evaporate, the solution film is placed in water. The subsequent phase inversion causes the polymer to precipitate (Holy et al., 2000). The main advantage for tissue engineering purposes when compared to the basic solvent casting method is that crystal deposition is avoided, and this enables the production of thicker scaffolds. It was shown technically that using phase inversion, scaffolds with improved interconnectivity and morphologies mimicking trabecular bone could be fabricated (Holy et al., 2000). Based on experimental biological studies, these scaffolds have, on a number of occasions, been shown to support the growth of osteoblast-like cells and facilitate consequent bone matrix formation (Holy et al., 2000; Karp et al., 2003).

Fibre bonding is based on the presence of fibre-like polymer structures. In fibre bonding, individual fibres are woven or knitted into three-dimensional scaffolds having variable pore size. By using fibres with different diameters and by connecting the fibres in different special configurations, a high degree of variability in the scaffold structure can be achieved. Although this technique enables one to create large surface areas thereby improving nutrient diffusion (Mikos et al., 1993; Kim and Mooney, 1998), it is technically difficult to obtain the desired porosity and pore size. Additionally, there is a possibility of solvent residues in the scaffold that may be harmful to cells or cells at the host site (Lu and Mikos, 1996; Hutmacher, 2000; Yang et al., 2001; Leong et al., 2003).

Melt-based technologies are often used when it is desired to fabricate porous scaffolds. Melt moulding/particulate leaching is, of all the melt-based techniques, the most commonly used. Using this technique a polymer is mixed with a porogen prior loading into a mould (Thomson et al., 1995). The mould, shaped in the form of the desired defect geometry, is then heated above the glass transition temperature of the polymer used. After reaching the glass transition temperature, the composite material is immersed in a solvent for selective dissolution of the porogen (Thomson et al., 1995). As this technique enables one to fabricate porous scaffolds of defined shapes by creating a defined (external) mould geometry (Thomson et al., 1995; Lu and Mikos, 1996; Leong et al., 2003), this method has become popular in clinical tissue engineering strategies (Schiller et al., 2004; Meyer et al., 2005b). Furthermore, this method also offers independent control of the pore size and porosity, by varying the amount and size of the porogen crystals (Thomson et al., 1995; Lu and Mikos, 1996; Leong et al., 2003). Extrusion and injection moulding are other melt-based techniques used for the processing of scaffolds (Gomes et al., 2001; 2002a; 2002b). As with particulate leaching, generation of a scaffold (Gomes et al., 2002a) with a high degree of porosity and interconnectivity is possible. By resembling the porous structure of trabecular bone, the mechanical properties of such scaffolds can be adjusted in order to mimic the compressive modulus and compressive strength of trabecular bone. As the control of pore distribution is impaired using this method, nutritional demands of cells are difficult to meet.

Gas foaming under high pressure as another processing technique is based on the CO_2 saturation of polymer discs, through their exposure to high-pressure CO_2 (Mooney et al., 1996). An advantage of this method is the possibility of obtaining scaffolds with a high degree of porosity and at the same time, producing pores with a pore size in the range of 100 μm (Mooney et al., 1996). Scaffolds produced by this technique have the disadvantages of low mechanical strength and poorly defined pore structure, which limits widespread use of this technique (Shea et al., 2000).

A further method is based on thermally induced phase separation (freeze-drying), which occurs when the temperature of a homogeneous polymer solution, previously poured into a mould, is decreased. After the phase-separated system is stabilised, the solvent-rich phase is removed by vacuum sublimation leaving behind the polymeric foam. This technique allows the production of scaffolds consisting of natural and synthetic polymers (Malafaya and Reis, 2003; Mao et al., 2003). As the processing conditions are technically challenging and the scaffolds obtained have low mechanical competence accompanied by reduced pore size, application of this technique is rare (Hutmacher, 2000; Yang et al., 2001).

The varying success rates of scaffold fabrication by the above mentioned methods is, to some extent, technique-inherent. Additional limitations of conventional techniques are the need for manual intervention and the inconsistent and inflexible processing procedures. As an ideal scaffold material for bone and cartilage tissue engineering is unknown, it is speculative to comment on the feasibility of each processing technique. Much experimental work has to be performed to gain a more detailed insight into the success of the various conventionally fabricated scaffolds in terms of the biological and clinical outcome in bone and certilage tissue engineering strategies.

New processing technologies have been developed in recent decades, and have been applied in tissue engineering for scaffold fabrication to overcome the limitations of conventional processing techniques. Rapid prototyping (RP) (Hutmacher, 2000), a synonym for solid free-form fabrication (SFF) (Leong et al., 2003), is the most popular technique. Rapid prototyping is a computer-based design and fabrication techniques that enables one to produce ordered external and internal scaffold structures. The main advantages of the SFF techniques over conventional techniques are: the possibility of defined external and internal scaffold structure fabrication, computer-controlled fabrication processes, improved processing accuracy, and, eventually, the coating of scaffolds with cells. As with other computer-based techniques, automation and standardisation of processing protocols can be more easily achieved. The data sets used to design and fabricate scaffolds can be based on virtual data sets or computer-based medical imaging technologies (e.g. CT scans), and other technologies (Hutmacher, 2000; Leong et al., 2003). Rapid prototyping is a method that can not only individualise the external and internal defect geometry but can be envisioned in the future to produce controlled anisotrophic scaffold nanostructures. The solid free-form technique can be considered as a method of layered manufacturing. In the case of scaffold fabrication, the three-dimensional geometry is created layer-by-layer via defined processing techniques. Through the possibility of implementing macroscopic, microscopic and likely in the future, nanoscopic structural features in different areas of the scaffold, tissue-like structures may be obtained. A complex scaffold micro- and nanostructure, closely related to the desired biological defeet tissue, can be assumed to be advantageous when cell/scaffold constructs with multiple cell types (e.g. bone and endothelial cells), arranged in hierarchical orders, are desirable (Quarto et al., 2001; Vacanti et al., 2001). Solid free-form fabrication techniques currently used for tissue engineering purposes include: fused deposition modelling (FDM) (Hutmacher et al., 2001a; 2001b; Zein et al., 2002); three-dimensional printing (Cima et al., 1991; Park et al., 1998; Shen et al., 2000; Lam et al., 2002; Sherwood et al., 2002); three-dimensional plotting (Landers and Mühlhaupt, 2000; Ang et al., 2002; Landers et al., 2002) and a variety of other approaches (Fedchenko, 1996; Chu et al., 2001; Taboas et al., 2003). Whereas extensive research has been done at a material-based or cell biologically based level, only a small number of SFF techniques have been exploited for the clinical use of extracorporally grown tissues. As the advantages of these techniques are impressive and the logistic and technical problems seem capable of solution in the near future, it can be assumed that these techniques will become standard measures to create scaffolds.

Using fused deposition modelling, scaffolds with a highly ordered microstructure can be fabricated. The avoidance of organic solvents is the main advantage of this technique from a technical point of view. In contrast, incorporation of growth factors is difficult, because elevated temperatures during processing destroy the structure and activity of such growth factors (Leong et al., 2003). Fused deposition modelling was shown to generate scaffolds with adequate porosity supporting the ingrowth and survival of cells and tissues (Hutmacher et al., 2001a; Zein et al., 2002). It was demonstrated by Schantz et al. (2002) that osteoid formation could be obtained when these scaffolds, coated with periostal cells under osteogenic conditions in vitro, were implanted in vivo in a subcutaneous animal model. As the processing techniques leads to elevated material temperature, cells can be coated with the material only after the scaffold is produced.

From the viewpoint of tissue engineering, three-dimensional printing is a promising prototyping technique (Cima et al., 1991; Shen et al., 2000). The main advantage of three-dimensional printing is the fact that scaffolds can be created at room temperature. This method thus allows the seeding of cells or the incorporation of growth factors during the fabrication process (Hutmacher, 2000). The technique, based on the printing of a liquid binder onto thin layers of powder, enables one to coat cells with the polymer material during layered manufacturing and therefore locate cells in deeper structures of the scaffold (Park et al., 1998; Hutmacher, 2000; Leong et al., 2003). By stacking multiple cells containing material layers in a scaffold with a defined tunnel structure, the viability of cells in central parts of the scaffold can be envisioned. This promising technique has been successfully exploited in bone tissue engineering (Park et al., 1998; Hutmacher, 2000; Leong et al., 2003). In addition to the cell biological advantages of processing at room temperature, the deposition of cells in central areas of the scaffold and improved tube and pore geometry provide the further advantage of the possibility of fabricating complex scaffolds containing different materials with different substructures (Cima et al., 1991; Park et al., 1998; Lam et al., 2002; Sherwood et al., 2002). A main disadvantage of the three-dimensional printing technique is the fact that the microstructure of the scaffolds is dependent on the powder size of the stock material. An additional technical problem is the technique-related closure of the pores by the underlying material.

Three-dimensional plotting is a commonly used method of SFF fabrication for processing hydrogels (Landers and Mülhaupt, 2000; Ang et al., 2002; Landers et al., 2002). A hydrogel-based scaffold formation of special relevance in cartilage tissue engineering is achieved by chemical reactions of co-reactive components that are placed in two component dispensers, or one component can be plotted in a liquid medium containing a coreactive component (Landers et al., 2002). This technique has, like three-dimensional printing, the advantage of operating under physiological conditions (e.g. physiological temperature). Scaffolds can also be enriched homogenously throughout the scaffold volume with cells or growth factors during the fabrication process.

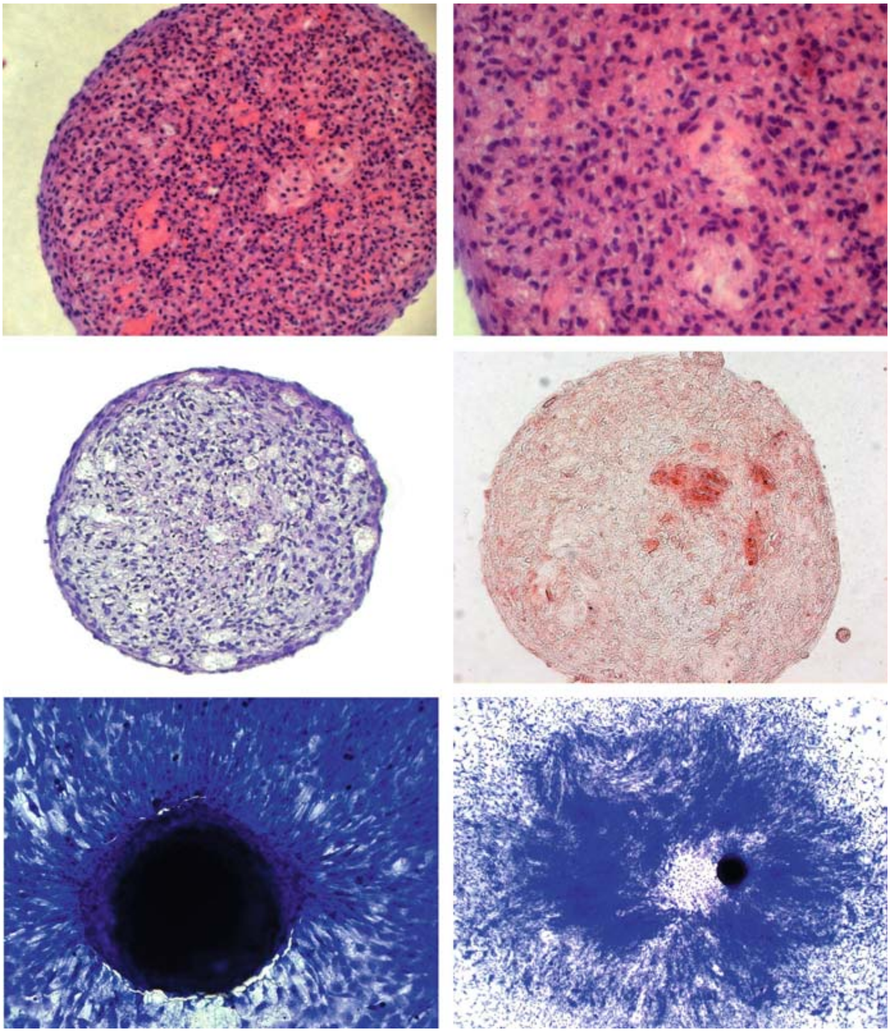
9.3 Scaffold-free Micromass Units

The in vitro formation of bone- or cartilage-like tissue for subsequent implantation (Lindenhayn et al., 1999; Hutmacher, 2000; Anderer and Libera, 2002) is, as described, commonly performed by using scaffolds. Recently, there has been controversy concerning the use of artificial scaffolds compared to the use of a natural matrix (van der Kraan et al., 2002). Skeletal defect regeneration by extracorporally created tissues commonly exploits a three-dimensional cell-containing artificial scaffold. As indicated earlier, a number of in vitro studies have been performed to evaluate the cell behaviour in various three-dimensional artificial scaffold materials. Whereas most of these materials were generally shown to allow spacing of skeletal cells in a three-dimensional space, not all materials promote the ingrowth of cells within the scaffolds (Freed et al., 1998). Rather, supporting cellular function depends, as described, on multiple parameters such as the chosen cell line, the underlying material, the surface properties and the scaffold structure. Some in vitro studies indicate that a material itself may impair the outcome of ex vivo tissue formation, when compared to a natural tissue-containing matrix. Additionally, in the in vivo situation defect regeneration can be critically impaired by the immunogenity of the material, the unpredictable degradation time and by side effects caused by degradation products (van der Kraan et al., 2002) (see Chap. 10). Based on these considerations, some researchers regard matrices close to the natural extracellular matrix as most promising for skeletal tissue engineering. A recently elaborated approach in extracorporal tissue engineering is therefore the avoidance of non-degradable scaffolds. These are resorbed at a different rate to skeletal tissue regeneration, which proceeds by itself. Therefore, new approaches have been invented to overcome these problems by renouncing scaffolds.

It is well known that tissue explants can be dissociated into dispersed cells and then reaggregated in vitro to regenerate complete organisms (Kelm and Fussenegger, 2004). Basic research has indicated that regeneration of simple animals, and of whole vertebrate tissues, can be achieved by reaggregation approaches using micromass technique. Investigations on skeletal development gave first insight into this micromass biology. The micromass technology relies to a great extent on the presence of the proteinacious extracellular matrix. As described earlier, the ECM may exert both direct and indirect influences on cells and consequently modulate their behaviour. At the same time, these cells alter the composition of the ECM. This may be accomplished in a variety of fashions, including differential expression of particular ECM components and/or proteases such as metalloproteinases by cells in the local microenvironment. A large body of evidence has confirmed that a minimal cell number is required in three-dimensional tissue-like constructs to induce the differentiation of mesenchymal precursors along the chondrogenic and osteogenic pathways (reviewed in Hall and Miyake, 1992). In contrast, mesenchymal precursors seeded in low-density micromasses adopt features of a fibroblastic phenotype and abolish cell differentiation, when mimicking a low-density condensation (Hattori and Ide, 1984; Cottrill et al., 1987; Hurle et al., 1989). These findings indicate that a critical cell mass is necessary to proceed with a specific ECM formation. A threshold amount of precursor cells is necessary to form a three-dimensional ECM structure around these cell masses promoting their differentiation. The ECM in the microenvironment then interacts with cells to further develop towards a specific tissue. The absence of the requisite ECM components would lead to decreased recruitment of precursors to the condensations, causing a subsequent deficiency in chondrocyte or osteoblast differentiation. In vitro studies with chondrocytes confirmed these findings, showing that the ability of mesenchymal precursors to initiate chondrogenic differentiation is dependent upon cell configuration within a condensation process, which varies with the density of the condensation (Archer et al., 1985).

In the context of tissue engineering, ex vivo tissue generation may be optimised by the use of cell reaggregation technology. The reaggregate approach is a method to generate, in an attempt to mimic the in vivo situation, a tissue-like construct from dispersed cells, under special culture conditions (Fig. 9.5). Therefore, the self-renewal (cell amplification), spatial sorting and self-organisation of multipotent stem cells in combination with the self-assembly of determined cells are the basis for such an engineering design option. Technically, cells are dissociated as indicated in the cell cultivation section, and the dispersed cells are then reaggregated into cellular spheres. In order to technically refine scaffold-free spheres, cells are kept either in regular culture dishes (as gravitory cultures), in spinner flasks, or in more sophisticated bioreactors. In contrast to conventional monolayer cell cultures, in which cells grow in only two dimensions on the flat surface of a plastic dish, suspension cultures allow tissue growth in all three dimensions. It has been observed that cells in spheres have higher proliferation rates than cells in monolayer cultures, and their differentiation more closely resembles that seen in situ. These findings may be based on the spatial configuration in a three-dimensional matrix network.

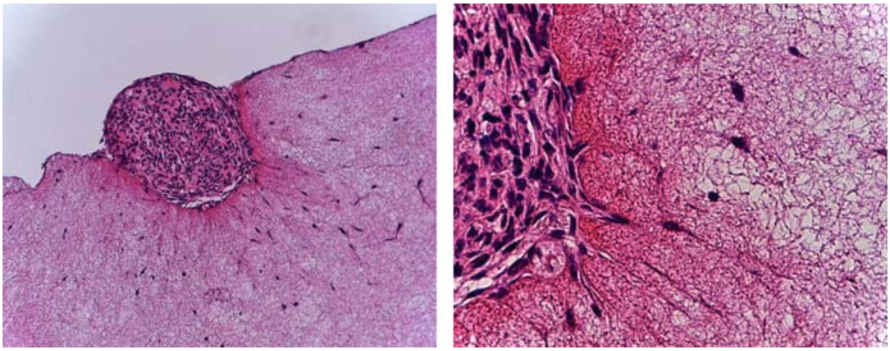
Different culture parameters (sizes of the culture plate, movement in a bioreactor, coating of culture walls) are all crucial to the process. Roller tube culture systems have been shown to be suitable for cultivation of tissue explants in suspension. The cultivated and fabricated tissues may be used to study the primary mixing of cells and the patterns of cell differentiation and growth within growing spheres in order to improve the outcome of microsphere cultivation. In addition, some culture conditions could aid the development of high-throughput systems and allow manipulation of individual spheres. It seems worthwhile elaborating new bioreactor technologies and culture techniques to improve the ex vivo growth of scaffold-free tissues. Technically, short-term reaggregation experiments, which last from minutes to a few hours, can be distinguished from long-term studies. Short-term reaggregation has been used widely to evaluate basic principles of cell–cell interactions and cell–matrix interactions, whereas long-term cultivation (days to several weeks) is suitable in ex vivo tissue engineering strategies. Recent studies of the reaggregation approach aim to solve two aspects: to fabricate scaffold-free, three-dimensional tissue formation and at the same time to investigate basic principles of cellular self-assembly. As in monolayer cultures, which facilitate the study of cell–material interactions, suspension cultures allow the evaluation of cell reactions towards a three-dimensional space. The reaggregate approach enables one to follow tissue formation from single cell sources to organised spheres in a controlled environment (Fig. 9.6). Thus, the inherent fundamentals of tissue engineering are better revealed. Additionally, as the



Dge, 7,3, Micromass culture of chondrocytes (*top*) (toluidine blue O staining) and osteoblasts (*middle*) (left: Richardson staining, right: Ostecalein expression). Cell outgrowth of osteoblasts from spheres to Petri dish (bottom) (Richardson son staining). Cultures are 100–300 μm in diameter

newly formed tissue is devoid of any artificial material, it more closely resembles the in vivo situation.

Skeletal cells were found to be a suitable cell source for such ex vivo reaggregate approaches. Anderer and Libera (2002) developed an autologous spheroid system to culture chondrocytes and osteoblasts without adding xenogenous serum, growth factors, or scaffolds, in consideration of the fact that several growth factors and scaffolds are not permitted for use in clinical applications. It was demonstrated that by such an approach autologous chondrocytes and osteoblasts cultered in the presence of autologous serum form a three-dimensional micro-tissue that had generated its



Dge, 7,4, Cell migration of micromass cultures of osteoblasts in a fibrin membrane

own extracellular matrix. Chondrocyte-based micro-tissues have a characteristic extracellular space that was similar to the natural matrix of hyaline cartilage. Osteoblasts were also able to build up a micro-tissue similar to that of bone repair tissue without collagen-associated mineral formation. The fabrication of a self-assembled skeletal tissue seems not to be limited to certain species, as results from bovine and porcine chondrocyte and osteoblast cultivation led to the formation of species-related cartilage-like or bone-like tissue.

It was recently observed that even complex cellular systems can be generated ex vivo without the use of scaffolds. Co-cultures of osteoblasts and endothelial cells, for example, resulted in the formation of a bi-cellular micromass tissue renouncing any other materials. Other organotypic cultures, used to develop engineered tissues other than of skeletal origin, confirm that it is feasible to create tissue substitutes based on reaggregated spheres technology. Examples of these strategies include liver reconstruction, synthesis of an artificial pancreas, restoration of heart valve tissue and cardiac organogenesis in vitro.

Several investigations have suggested that after in vivo transfer of such reaggregates, tissue healing is improved in the sense that the repair tissue mimics the features of the original skeletal tissue (Anderer and Libera, 2002; Meyer et al., 2005c). Preclinical and clinical cartilage repair studies, in particular, have demonstrated that tissue formation resembled more closely the natural situation. The transplantation of reassembled chondrogenic micro-tissues is able to impair the formation of fibrocartilage by suppression of type I collagen expression, while promoting the formation of proteoglycan accompanied by a distinct expression of type II collagen. It can be assumed that the volume of the observed repair tissue was formed by the implanted chondrospheres itself as well as by host cells located in the superficial cartilage defect. The mechanisms by which chondrospheres promote defect healing are complex and not completely understood. Van der Kraan et al. (2002) reviewed the role of the extracellular matrix in the regulation of chondrocyte function in the defect site and the relevance for cartilage tissue engineering. Numerous other studies have confirmed that extracellular matrix of articular cartilage can be maintained by a distinct number of chondrocytes and that the extracellular matrix plays an important role in the regulation of chondrocyte function. In in vitro generated cartilage-like tissue a time-dependent increase in the expression of collagen type II, S-100, and cartilage-specific proteoglycans paralleled by a reduced cell–matrix ratio was observed in the microspheres (Meyer et al., 2005c). The transplanted cell/matrix complex was attributed to be responsible for the observed chondrocyte proliferation, differentiation and hyaline cartilage-like matrix maturation in vivo.

The inductive properties of the implantation site may also be beneficial when a stem cell-based micro-tissue strategy is chosen. Stem cell tissue engineering using fetal or adult stem cells in combination with sphere technologies leads to implantable stem cell driven tissues. Typically, stem cells must be amplified to large quantities in suspension cultures and have access to appropriate growth factors to establish specially organised histotypical spheres. These spheres can then be implanted into the lesioned skeletal site. Although adult stem cells of various origins can transdifferentiate into distinct cell types, the transformation of these cell types into functioning tissues and their successful implantation by reaggregation technology needs further elaboration.

Tissue Reconstruction in vivo

10

Despite the advances in extracorporal tissue engineering that have been achieved over the last few years, there are several clinical situations in which the implantation of a non-living biomaterial or a protein-containing matrix without exogenous cells will result in an acceptable clinical outcome (Einhorn, 1999). Supplementing an implantable matrix with cells is not required in all clinical settings for the success of the surgical treatment. However, when a reduced number, viability, and distribution of cell progenitors at the defect site impairs the outcome of tissue healing, it is necessary to augment the local population of cells. The addition of bone marrow-derived cells injected alone or seeded onto materials has been shown to enhance the performance of a wide variety of implanted graft materials. Most studies have used young animals and graft sites that are surrounded by normal healthy tissues or wound sites that almost surely would heal without intervention (Perka et al., 2000; Schantz et al., 2002a). Generally, bone or cartilage healing seems to be more rapid, complete and reliable, when a cell-based therapy is employed (Kon et al., 2000; Schliephake et al., 2001). However, experimental studies have also demonstrated that bone healing is accelerated by using extracts from cultured human osteosarcoma cells (Hunt et al., 1996), indicating that the addition of components of non-viable cells improves the healing of matrix-filled bone defects.

For extracorporal tissue engineering, one has to take into account that the implant tissue requires a critical balance between the local metabolic demand of the transplanted bone cells and the capacity for oxygen and nutrients to diffuse into and out of the implant not only during ex vivo growth but also directly after implantation. In most clinical grafts, the diffusion distance for oxygen and other metabolites from the edge of the graft to the centre of the graft is a minimum of 5 mm, which is approximately fifty times the normal diffusion distance. In this setting, diffusion is able to support only a limited number of transplanted cells before the balance between metabolic demand and diffusion creates a zone in the centre of the graft where oxygen tension is too low to support viable cells, resulting in central necrosis. The size of the necrotic region and the number, distribution, and type of cells that do survive in the deeper regions of the graft site are a function of many variables. As the in vivo situation is more complex than the controllable bioreactor situation, assessment of the nutritional situation is difficult. The principal variables are listed as below:

Principal Parameters of Nutritional Supply in vivo

- Vascularisation
- Oxygen tension/consumption
- Nutrient concentration/consumption

- Number of cells
- Inflammatory state
- Fluid flow situation
- Diffusion constants
- Biological state of cells (resting, proliferative, synthetic).

If a cell-containing material is used, it is important to ensure that after implantation the local environment supports the survival of the grafted cells. The survival of transplanted cells also depends on the response of these cells to the transplantation environment after surgery. Many observations have long supported the concept that at least some connective tissue progenitors are located in tissues surrounding the defect site. Also, such cells may have a high capacity to survive in hypoxic conditions (Burwell, 1964; 1966; 1985). Experimental data have shown that many connective tissue stem and progenitor cells exhibit a remarkable tolerance to, and are even stimulated by hypoxia (Ivanovic et al., 2000; Morrison et al., 2000; Studer et al., 2000; Lennon et al., 2001; Ramirez-Bergeron and Simon, 2001), hypoxia, not unlike endothelial cells (Shweiki et al., 1992; Mogi et al., 2001). It is assumed that the capacity to convert the biochemical state of cells to glycolysis transiently in a hypoxic situation is a possible adaptive mechanism (Abdel-Aleem et al., 1999). The vascularisation of the defect site as well as the rate and extent of revascularisation are of major importance. A good vascularisation and fast formation of new vessels favours osteoblastic differentiation, whereas the absence of vessels and a prolonged hypoxia favours the formation of cartilage or fibrous tissue (Connolly, 1981; Jargiello and Caplan, 1983; Caplan, 1984; 1991). In the context of bone and cartilage tissue engineering, these findings can be converted into several in vitro or in vivo strategies to optimise tissue function in clinical grafts. One in vitro method is to adjust the concentration of transplanted cells. Another is to limit the transplanted cells to only superficially located scaffold areas. Both concentration (Connolly et al., 1989) and selection strategies (Muschler et al., 2003) have been shown to alter graft performance. Finally, in the future culture-expanded cells might be pre-adapted to hypoxic conditions prior to transplantation and/or selected to enrich for those most likely to survive. Unfortunately, to date there are limited data on the survival and fate of transplanted cells at the graft site during bone or cartilage healing.

Appropriate model systems are required to assess the clinical outcome of extracorporal bone and cartilage tissue engineering strategies. In general, an ideal model system is one which closely mimics the clinical situation in the patient. Animal models are therefore widely used to develop and evaluate tissue-engineering techniques for the reconstruction of damaged human bone or cartilage sites (Table 10.1). With in vitro culture of cells and cell scaffold complexes, heterotopic models of histogenesis and animal defect models are widely used. While no animal model permits direct application to humans, each is capable of yielding principles on which decisions can be made that might eventually translate into a human application. Therefore, the use of animal models has and will continue to play a significant role in the advancement of tissue engineering. Each animal model has specific advantages and disadvantages. The key issue in the selection and evaluation of an appropriate animal model is to match the model to the question being investigated and the hypothesis to be tested (Reinholz et al., 2004) (Table 10.2). Basic research can be performed on small animal species, whereas a clinical approach requires larger animals of a higher phylogenetic scale.

R_`jc /.,/, In vivo model systems

? lgk_j kmbcjq
– Heterotopic immunocompetent animals – Heterotopic immunodeficient animals
Qk_jj _lgk_j qncagcq
– Mouse – Rat – Rabbit – Cat
J_pec _lgk_j qncagcq
– Dog – Swine – Goat – Horse – Ape

R_`jc /.,0, Experimental animal and clinical evaluation methods

Kcrfmb &gl tgtm'	Bcrcpkgl_rgml
– Clinical	– Form, function
– X-ray	– Hard tissue structure/overview
– Microradiography	– Hard tissue structure/details
– CT	– Hard and soft tissue structure/overview
– µCT	– Hard and soft tissue structure/details
– NMR	– Hard and soft tissue structure
– Densiometry	– Mineral content
– Serum analysis	– Bone formation and resorption marker, hormones, cytokines
– Urine analysis	
Kcrfmb &gl tgrpm'	
– Histology	– Tissue structure

10.1 Animal Models used for Bone Tissue Engineering

For the purpose of testing ex vivo generated bone tissue substitutes, the experimental model should allow manipulation of the mode of defect healing, the size and location of bone defects, and the various forms of regeneration disturbances. A careful experimental design and an extended evaluation including the biomechanical characteristics of the newly formed tissue will improve our understanding of the biological basis and the clinical implications of extracorporal bone tissue engineering. A test of the conditions under which bone tissue engineering is advantageous over other techniques relies greatly on the experimental model selected (Einhorn, 1999). Extracorporal bone tissue engineering is aimed at restoring large segments of the skeletal bone lost for various reasons, improving the healing of complicated fractures, and hopefully in the near future, fully regenerating complex skeletal defects (Puelacher et al., 1996; Bruder et al., 1998a; 1998b). Animal models should effectively mimic the clinical situation in human bone healing. However, it is often desired that bone defects in animal models should fail to heal unless they are treated with a cell-based tissue engineering strategy. Clinicians should be aware that bone tissue engineering approaches have unique features, as they differ in different fields of surgery. For example, bony regeneration is often observed after a sinus lift procedure in the maxillary sinus region, even in the absence of implanted biomaterial. In this case, elevation of the sinus mucosa seems to be the crucial procedure that induces bone growth. Bone regeneration in the maxillofacial area appears to take place in part by mechanisms distinct from those in the orthopaedic field. In particular, the response to scaffold materials may be different depending on the nature of the surrounding intact bone and soft tissue. As an initial approach to determine the osteogenic potential of tissue-engineered bone constructs in vivo, heterotopic animal models are of special interest. They allow the evaluation of the biological performance of the hybrid material commonly implanted in ectopic sites. Implantation is performed for example in muscle pouches, fascial pouches or subcutaneously. The investigation can be performed in immunocompetent as well as in immunocompromised animals. The next step of preclinical evaluation is the in vivo defect model. Schmitz and Hollinger (1986) were the first to postulate a rationale for testing bone tissue engineering by using critical size defects in a hierarchy of animal models. It is known that the rate of bone repair varies inversely with order along the phylogenetic scale (Enneking and Morris, 1972; Prolo et al., 1982). Moreover, bone regenerative capacities differ significantly among animals of the same species (Harris et al., 1968; Enneking et al., 1975) and among loaded and non-loaded skeletal sites (Figs 10.1 and 10.2). Bone defects regenerate more actively in immature animals of a given species than in older ones. Therefore, from a clinical point of view it is advisable to test cellular hybrid materials for their ability to repair an osseous defect in a mature, adult animal (Harris et al., 1968) (Figs 10.3–10.5). Mature dogs, goats, pigs and

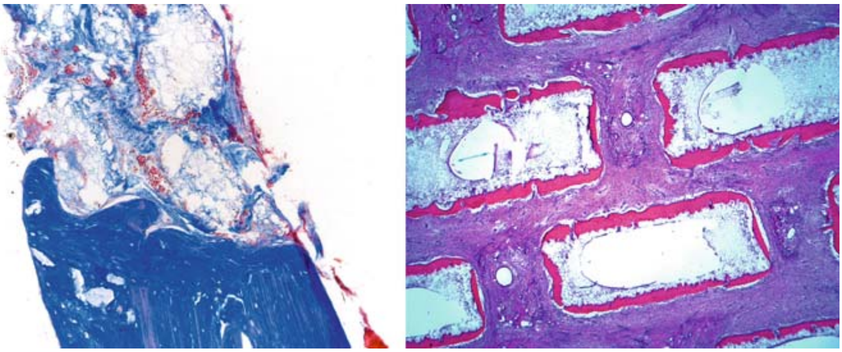
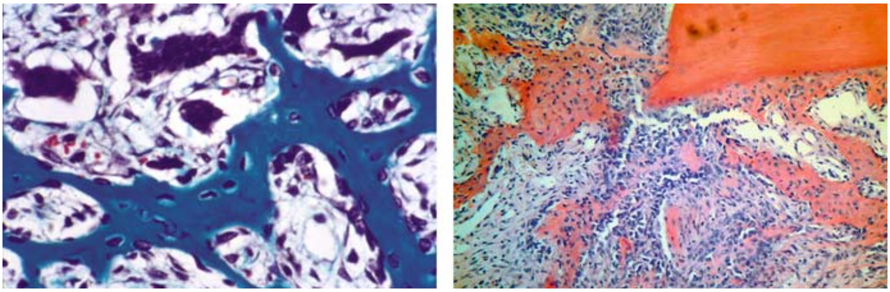
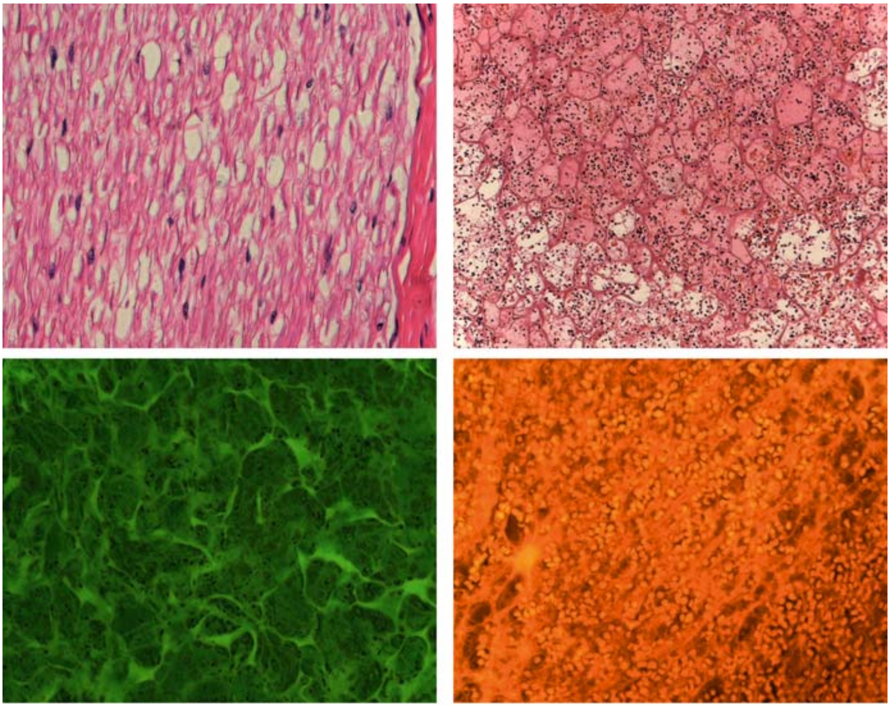


Fig. 10.1 Bone containing tricalcium phosphate-based bone substitutes after 4–6 months in rat calvaria (*left*) and sheep long bone (*right*)

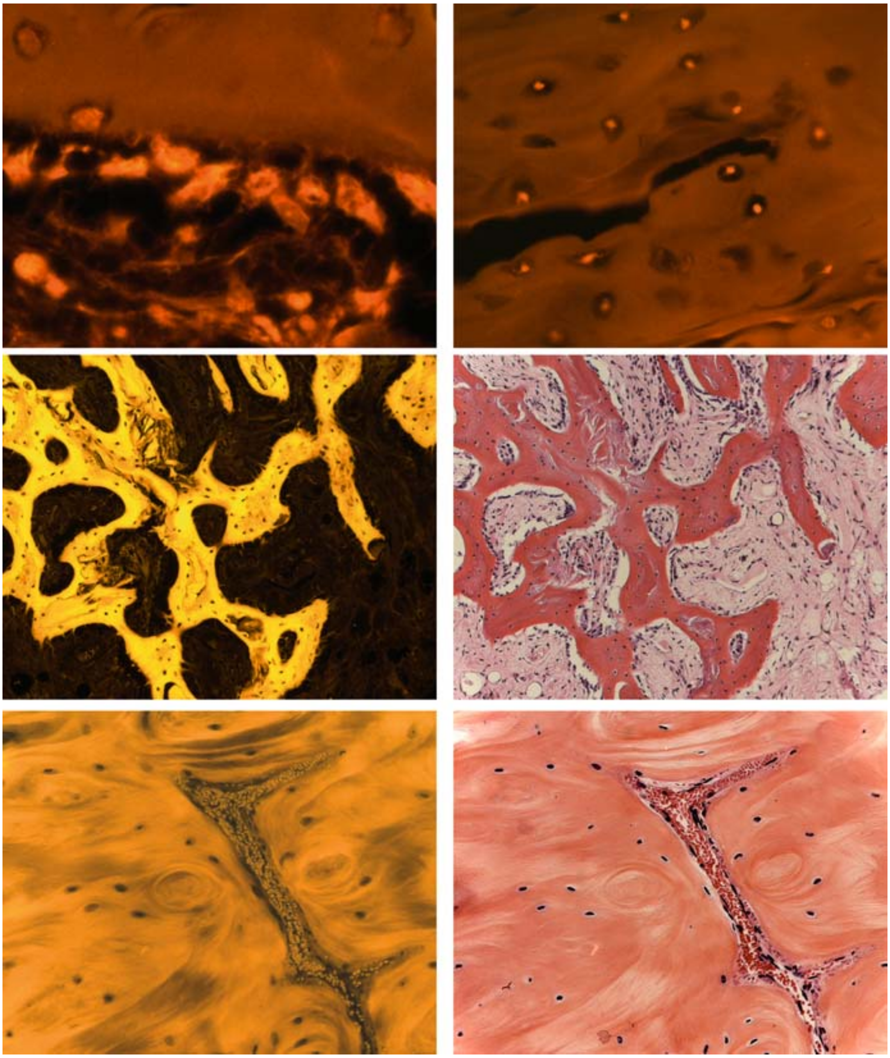
non-human primates have been successfully used to examine the regeneration of mandibular discontinuity defects (Fennis et al., 2002). These animal models allow us to follow the fate of the implanted material in a functional environment which is comparable to the human situation. This is particularly important in preparation for future Food and Drug Administration trials (Slavkin, 2000). Furthermore, transgenic knock-in or knock-out animals may be attractive candidates for studying the functional role of a protein of interest in bone (or certilage) healing.



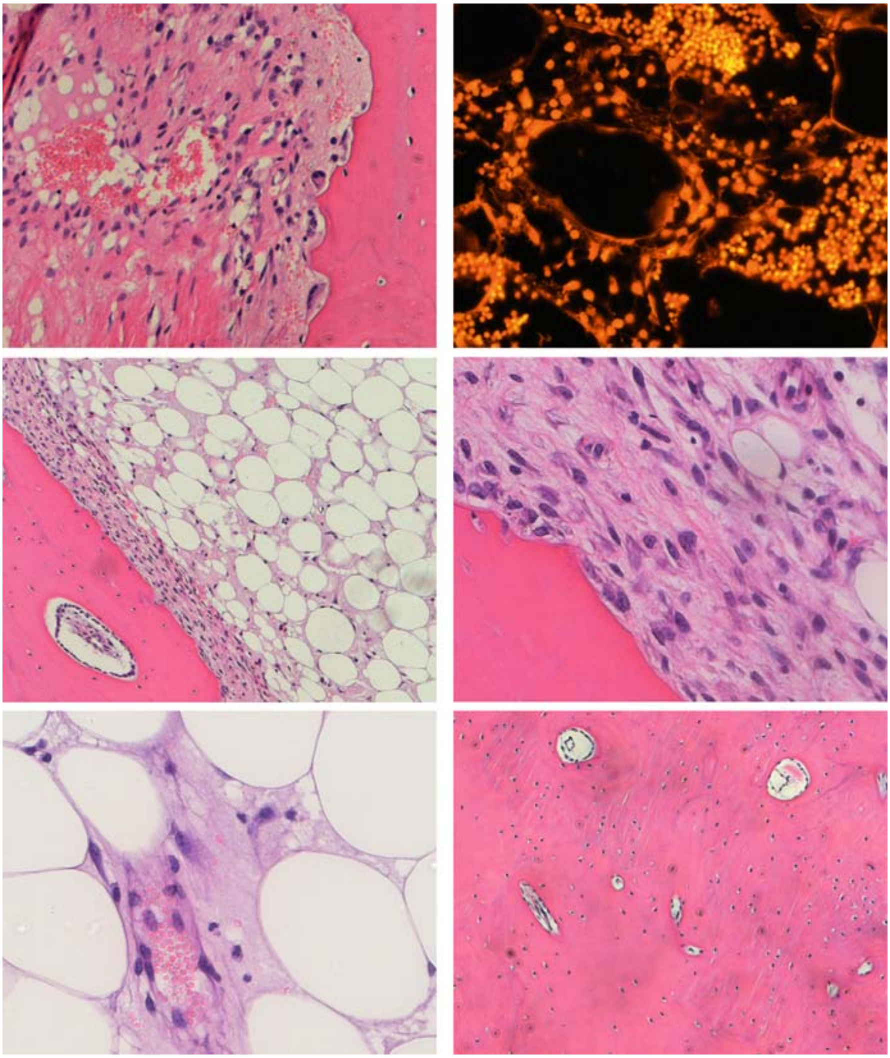
Dge, / .,0, New bone formation after extracorporal shock wave therapy



Dge, / .,1, Different matrices of biodegradable implants



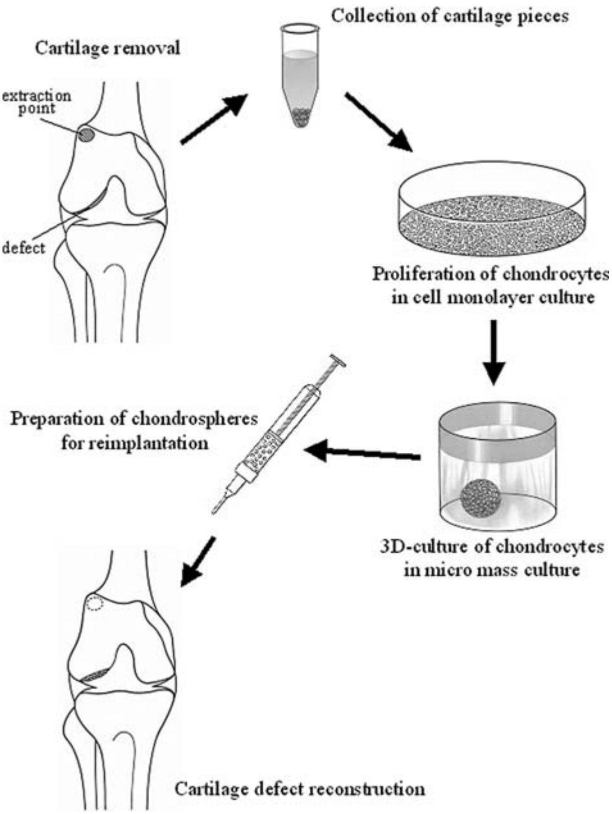
Dge, / .,2, Fluorescence and standard histology of bone healing at the scaffold/bone interface. *Top*: Autologous osteoblasts were fluorescence marked by a fluorescence dye in vitro and settled down on scaffolds. After cell/scaffold transplantation in minipigs, fluorescent cells indicate their ancestry from the ex vivo generated tissue-like construct. The technique enables not only to distinguish between resident and transplanted cells, but also to investigate the survival of cells after transplantation. In vivo, the marked cells are obvious during early bone formation (after 2 weeks) at the mineralization front (*left*) and in the newly formed bone (*right*). Midline: Auto fluorescence of hydroxyapatite (*left*) and alizarin red S staining in standard histology (*right*) can indicate the newly formed bone. Bottom: Newly formed blood vesicles can make out by auto fluorescence of erythrocytes (*left*) and in standard histology (*right*)



Dge, / .,3, Histological images of bone regeneration after cell/scaffold implantation. The bone repair tissue at early stages of bone regeneration (one week after transplantation) differs between various areas of the defect site (central scaffold area, superficial scaffold area). After 30 days bone regeneration is partly finished (*bottom, right*)

10.2 Animal Models used for Cartilage Tissue Engineering

As for bone tissue engineering, as a first step in determining the chondrogenic potential of tissue-engineered constructs in vivo, investigators have used small animal species in orthotopic sites or heterotopic animal models (Lipman et al., 1983; Nakahara et al., 1990b; Vacanti et al., 1991; Freed et al., 1993; Puelacher et al., 1994; Paige et al., 1996; Madry et al., 2002). Tissue-engineered cartilage constructs have, for example, been implanted in dorsal subcutaneous pouches of immunocompromised nude mice, since Vacanti and colleages were able to grow an ear on the back of a mouse. While heterotopic models may provide the first evidence of the nature of the tissue-engineered cartilage under in vivo conditions, different results may be observed when the construct is implanted into a cartilage defect or tested in immunocompetent animals. Therefore, further in vivo studies using articular cartilage defect models in a hierarchy of animal species should be conducted. However, heterotopic chondrogenesis models may provide some useful information concerning the basic biological phenomena of histogenesis. Due to the physiological and anatomical differences between the clinical situation of patients and those of experimental animals, there is no animal defect model that directly mimics the human situation. Additionally, many other variables are relevant when considering these experiments, such as age, gender, size and depth of the defect, age of the defect (i.e. intra-articular environment), post-operative treatment, etc. (An and Friedman, 1999; Hunziker, 1999; 2002). Whereas animals of a high phylogenetical scale like dogs, goats, pigs and horses more closely resemble the human situation compared to smaller animal models, it is usually not possible to conduct extended experimental series in larger animals. Therefore, the choice of a small-animal model for fundamental investigations (O´Driscoll, 2001) is well accepted. However, the preclinical assessment of an articular cartilage tissue engineering technique requires confirmation in a large animal model (Figs 10.6 and 10.7). The potential for cartilage repair depends to a great extent on the age of the animal. As is true for bone regeneration, cartilage defects can be repaired more successfully in immature animals than in skeletally mature animals. The capacity of articular cartilage repair potential is dependent on the state of the tissue (cartilage extracellular matrix, cell content), the inflammation at the defect site, and cartilage biomechanics (Stockwell, 1965; Stockwell and Scott, 1965; Meachim, 1969; Bonner et al., 1975; Roughley, 2001a; 2001b). In this respect it is of special concern in cartilage tissue engineering that most basic and preclinical results published originate from an optimally controlled laboratory environment or animal experimental work with healthy joint tissue where a surgically induced cartilage defect was treated using tissue engineering (Figs 10.7 and 10.8). In the clinical situation, the majority of patients have a chronic defect situation with a diseased peri-defect tissue before they are treated for their joint damage. This altered tissue environment is concomitant with an altered joint physiology. The impaired regenerative capacity of inflamed joint tissue, especially in cases of osteoarthritis, often associated with a disturbed biomechanical situation at the defect site, could provide an explanation for the confounding data concerning basic scientific results and the variable clinical results reported in the current literature (for a review see Salter, 1994). As mechanical factors influence chondrogenesis and articular cartilage healing in the in vivo situation, it is important to consider the post-operative treatment used after defect repair in designing experiments using animal defect models. Since

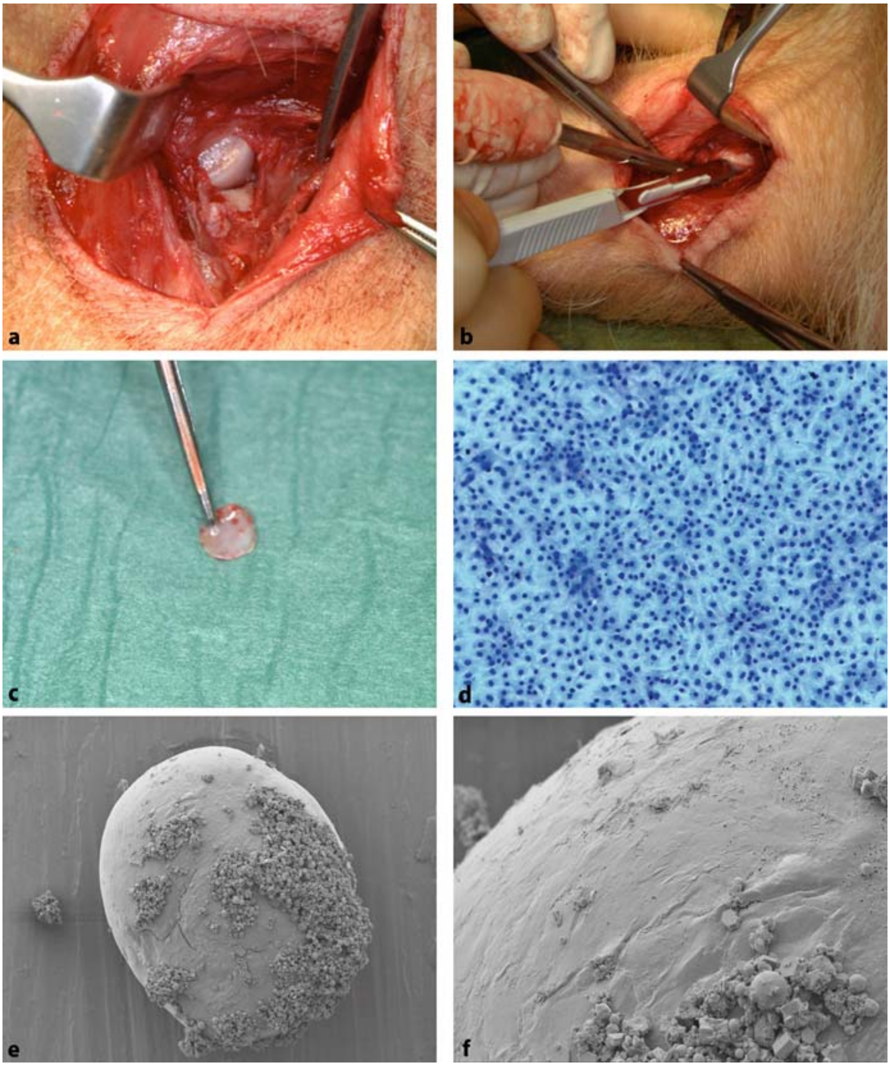


Dge, / .,4, Principle of cartilage tissue engineering

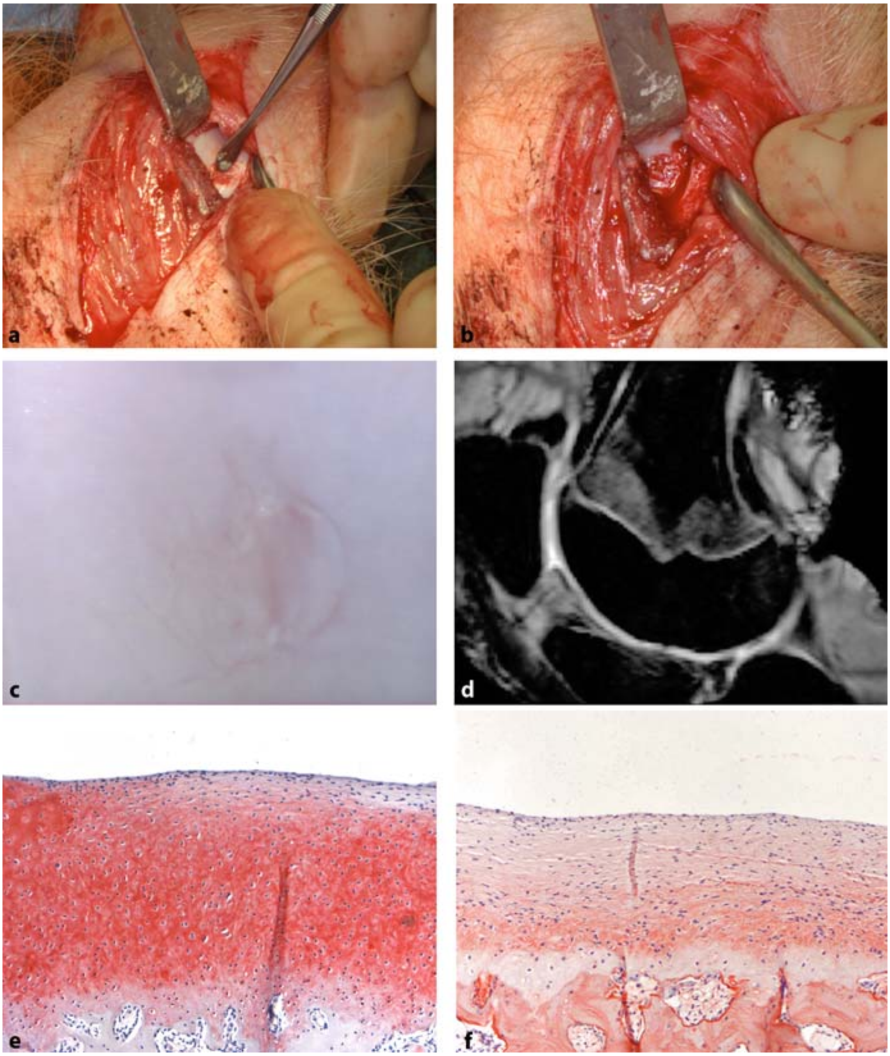
the use of continuous passive motion has been experimentally demonstrated to enhance the healing of articular cartilage in rabbits (Salter et al., 1980; O´Driscoll et al., 1983a; 1983b; O´Driscoll, 1984; O´Driscoll and Salter, 1984; 1986; Salter et al., 1984; Dhert et al., 1988; O´Driscoll et al., 1988; Salter, 1989; Kim et al., 1991; Moran et al., 1992) and in contrast, joint immobilisation has shown to decrease articular cartilage thickness, adjustment of the mechanical loading during the post-operation phase is a matter of relevance.

10.3 Clinical Applications

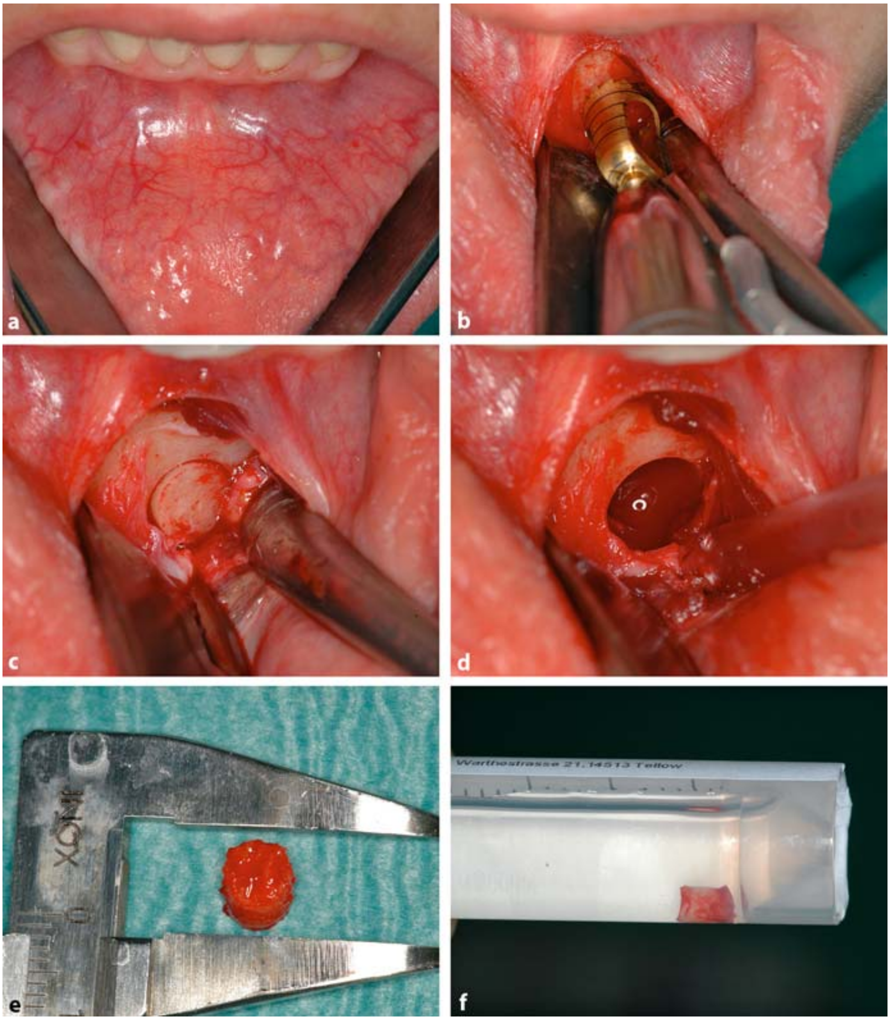
Tissue engineering demands that clinicians participate actively in the design, development, clinical introduction and informed use of this treatment option. The active participation requires that clinicians have a solid understanding of the biological and material-based principles of ex vivo tissue generation. To assess the efficacy and safety of tissue engineering, clinical studies must include a number of defined outcome measures. In contrast to animal studies in which the sacrificed animals are subjected to a variety of different investigations ranging from macroscopic to molecular level, clinical studies in humans use restricted outcome parameters (Figs 10.9 to 10.13).



Dge, / .,5, Animal experiment using micromass culture for cartilage reconstruction in the knee; part I. _ Exposure of joint prior to tissue harvesting. ` Surgical removal of cartilage. a Explanted cartilage piece. b Cell growth in monolayer. c Gross and d detailed appearance of chondrospeheres, generated by micromass culture (c, d SEM micrographs)

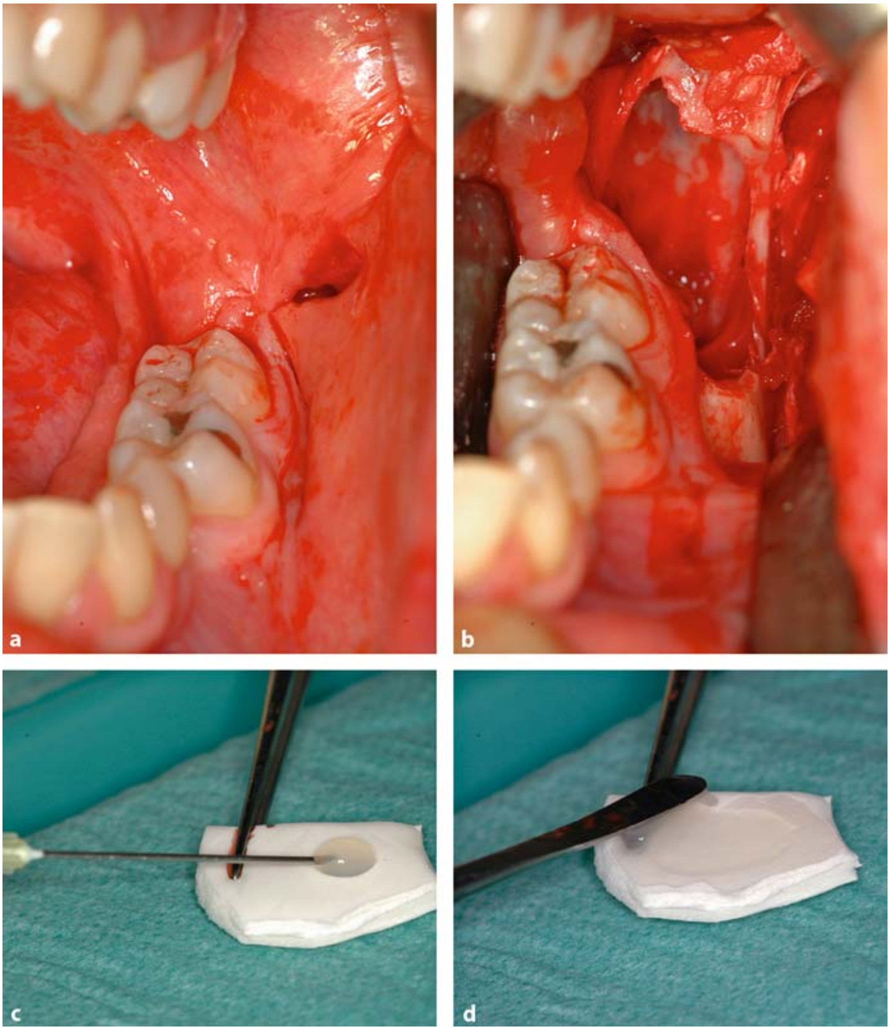


Dge, / .,6. Animal experiment using micromass culture for cartilage reconstruction in the knee; part II. _ Overview. ` Detail of the clinical replantation procedure. 20–25 chondrospheres are attached to the defect site. a Clinical and b NMR picture of the regenerate site. c Immunhistology revealed an enhanced expression of type II collagen, with d minor amounts of type I collagen, indicative of hyaline cartilage regeneration

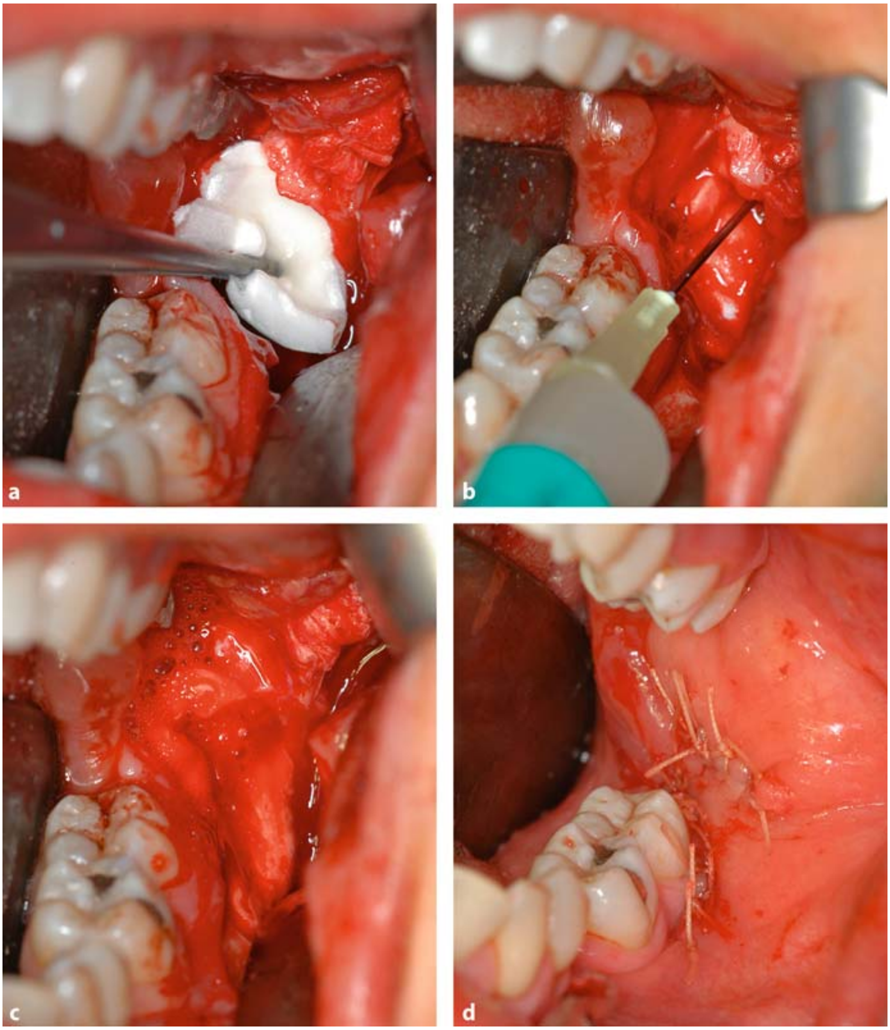


Dge, / .,7, Clinical bone tissue engineering procedure for bone reconstruction; part I. _ View of the mucosa. `, a, b Different steps of bone harvesting by a trephane burr. c Size of the explanted bone piece. d Bone piece located in transport medium

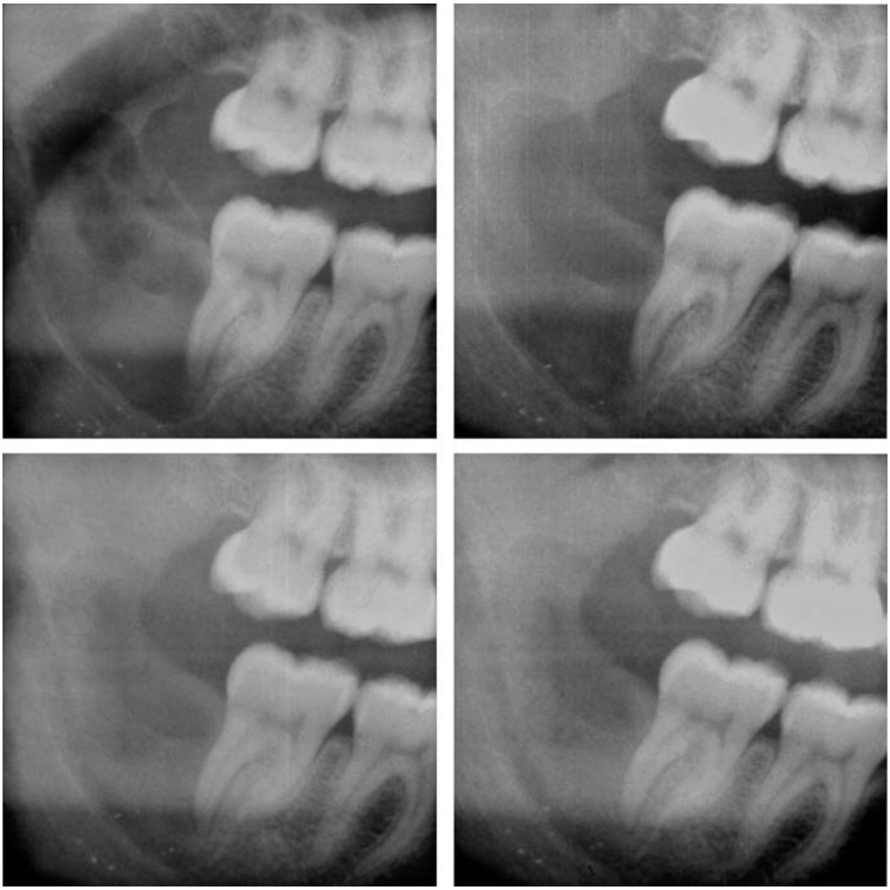
Dge, / .,/ ., Clinical bone tissue engineering procedure for bone reconstruction; part II. Different steps of autologous serum sample preparation



Dge, / . ,/ / , Clinical bone tissue engineering procedure for bone reconstruction; part III. _ Intraoral incision of the vestibular mucosa. ` Critical size defect at the mandibular angle. a Attachment of the bone cell suspension to a collagen scaffold. b Coating the complete scaffold surface with cells



Dge, / .,/0, Clinical bone tissue engineering procedure for bone reconstruction; part IV. _ Defect filling by the cell/scaffold complex. ` Injection of cells between defect border and scaffold. Clinical view after (a) defect filling and (b) wound closure



Dge, /.,/1, Radiological control of bone regeneration. Time-dependent regeneration of bone. Pictures demonstrate successive mineralisation of a critical size defect from day of implatation to 3 months after

Animal studies should therefore include in addition to outcome measures similar to those that are anticipated in clinical studies also histological and biomechanical evaluations (Tables 10.3 and 10.4). When incorporating a cellular bone substitute in reconstructive surgery, radiographs and computed tomography should be routinely performed in order to link high resolution data with clinical findings. The repair response to a cellular bone construct varies substantially according to the mechanical stability of the defect site. In vivo remodelling either reinforces, maintains, or degrades the tissue formed during implant healing. Both the initial and long-term biological responses to tissue-engineered constructs are strongly influenced by mechanical interactions at the implant interface. The load transduction at the interface critically determines the fate of the implanted tissue substitute (Meyer et al., 1999b). In a changing strain environment these mechanical factors represent a key challenge in the surgical treatment of bone and cartilage defects.

R_`jc / .,1, Biomechanical evaluation methods

Kcrfmb
– Young`s modulus
– Compressive strength
– Bending strength
– Shear stress
– Hardness
– Deformation
– Dislocation

R_`jc / .,2, Evaluation of in vivo models

Ajglga_jjw
– Form
– Function
P_bgmep_nfga_jjw
– X-rays
– Ultrasound
– Computed tomography
– Magnetic resonance tomography
– Positron emission tomography
– Szintigraphy
Qrpsarsp_jjw
– Histology
– Immunohistochemistry
– Ultrastructure
Kcaf_lga_jjw
– E-modulus
– Compression resistance

The integration of the extracorporally generated hybrid material into the surrounding tissue depends clinically, mainly on sufficient fixation of the transplanted construct, and biologically, mainly on the subsequent remodelling phase. Rigid fixation of bone constructs can be performed by plating, suturing or by a fit-in-fit system, covering the cell-containing hybrid material with membranes or soft tissues (e.g. periosteal flaps). In contrast, extracorporally grown cartilage tissue is mainly fixed by soft tissue flaps or artificial membranes. Loosening or loss of the transplanted construct should be considered as a currently unsolved aspect of tissue engineering. Additionally, whereas bone has a higher capacity for regeneration than cartilage tissue, both tissues retain their inherent biochemical property of forming new tissue in the presence of externally applied forces and strains. Implant integration into the host site depends on the strain and stress environment acting on the scaffold construct as well as on the surrounding implantation bed. Hyperphysiological loading may result in the destruction of scaffold integrity and also may induce cellular dedifferentiation, ultimately leading to fibrous tissue formation (Meyer et al., 2004b). Repeated disruption of vascular invasion at the construct interface resulting in impaired tissue formation is a clinically relevant problem mainly observed in bone tissue engineering. In the complete absence of adequate mechanical stimuli, however, implant integration may fail due to impaired bone or cartilage stimulation. Thus, mechanical factors can elicit both positive and negative effects on the biological response to implanted cell/scaffold constructs.

Guldberg (2002) was the first to study in vivo the influence of the mechanical environment on the formation and retention of bone within tissue-engineered constructs implanted into hydraulic bone chambers. He tested the hypothesis that tissue-engineered constructs under dynamic loading would promote bone repair in vivo. And indeed, it was found that, relative to the baseline bone formation, mechanical loading significantly increased the amount of mineralised matrix components. In contrast, the amount of mineralised matrix synthesised in the non-load control chambers was decreased. For load-bearing maxillofacial or orthopaedic defects, the implanted construct must provide adequate mechanical stability at the defect site, even under normal load when compressive strains have to be transferred to the newly formed tissue (Davy, 1999; Kon et al., 2000). Constructs possessing insufficient mechanical stability and fatigue resistance are at risk of plastic deformation or brittle fracture under functional loads, ultimately resulting in the collapse of the scaffold template. Only a very few in vivo applications in skeletal tissue engineering are non-load bearing. Calvarial critical sized defects for bone repair and non-load bearing sinus lift procedures for bone augmentation are two examples (Shang et al., 2001; Bidic et al., 2003). Although the impact of the local mechanical environment on bone remodelling has been appreciated (Meyer et al., 1999a), in the context of tissue-engineered constructs mechanical factors have not been systematically studied (Perka et al., 2000). Based on various clinical and experimental investigations it has been postulated that mechanical effects may overrule material-specific effects when biomaterials are tested in vivo (Handschel et al., 2002). Thus, mechanical effects should be anticipated when substitution with extracorporal bone or cartilage tissue is planned (Guldberg, 2002). An understanding of how mechanical signals affect the integration of the tissue construct may provide clinically relevant microstructural information for improved three-dimensional scaffold architecture. Models that take these three-dimensional, load-bearing functional requirements of skeletal tissues into account may be more predictive for human clinical trails.

10.4 Immunological Aspects

Despite the remarkable progress towards the generation of tissue-engineered bone or cartilage substitutes for therapeutical use, immunological problems associated with the recipient´s response to the bioengineered tissue will have to be overcome. Although little has been published on the immune reaction toward bioengineered bone or cartilage tissue, it is known that the cell/scaffold complex will most likely induce a host reaction (Harlan et al., 2002). Both inflammatory and immune reactions can be anticipated, even with autologous tissue (Table 10.5). Clinical trials with allografts and xenografts have demonstrated that innate and adaptive immu-

R_`jc / .,3, Inflammatory/immune responses

?qncar	Amknmlclrq
– Non-specific (innate)	– Cellular (granulocytes, macrophages, lymphocytes)
	– Non-cellular (complement system, lysozyme)
– Specific (acquired)	– Humoral (B-cells, immunoglobulins)
– Naturally acquired	– Cell-mediated (T-cells)
– Artificially acquired	

nity are serious barriers. Inflammation likewise influences cell/scaffold engraftment, and may also be pivotal for proper tissue remodelling. Therefore, it should not be assumed that an extracorporally bioengineered tissue will be inert. Rather, the host immune system will have a profound impact on the implanted bioengineered construct, and vice versa.

The two main components of an extracorporal tissue-engineered hybrid construct are the transplanted cells and the scaffold material. The implantation of a bone-like or cartilage-like polymer/cell construct combines concepts of both biomaterials and cell transplantation. Immune rejection is a common host response towards the cellular component of tissue-engineered devices containing allogeneic, xenogeneic or ex vivo manipulated immunogenic cells (Mikos et al., 1998).

The implantation of preformed scaffold materials without cells can initiate a sequence of events related to a foreign body reaction. Clinically, it may present as an acute inflammatory response and in other situations as a chronic inflammatory rejection (Anderson, 1993). The duration and intensity of the clinical response is dependent on patient-associated parameters (extent of tissue injury, age, immunological status) and scaffold parameters (chemical composition, porosity, roughness, implant size and shape) (Anderson, 1988). It has been shown that the intensity of the response is modulated by the biodegradation process itself (Lam et al., 1993; Nakaoka et al., 1996), determining to a great extent the clinical fate of the hybrid material (den Dunnen et al., 1997; Winet and Bao, 1997; Peter et al., 1998b). The immune response to common scaffold materials implanted into a defect region may result in a fibrous capsule containing macrophages and foreign body giant cells with needle-like, crystalline particles (Rozema et al., 1994). Some in vivo studies have suggested that particles released from a degrading implant may affect tissue regeneration or repair by direct interaction with cells (Lam et al., 1995; Winet and Bao, 1997). Indirect interactions through inflammatory cytokines released by macrophages upon particle phagocytosis will have to be examined in more detail.

Immunologic reactions against the transplanted cells and the host´s response towards the hybrid material are critical after transplantation of the extracorporally fabricated construct (Mikos et al., 1998). Implant rejection can be avoided or at least diminished in clinical practice by using autologous or isogeneic cells as the principal cell source. Obviously, cells derived from an autologous source can be rendered immunogenic if they are genetically modified (Verna and Somia, 1997) or extensively cultured in vitro (Sato, 1997) prior to their transplantation. If allogeneic or xenogeneic cells are incorporated into the device, the prevention of immune rejection by immune suppressive treatment, the induction of tolerance in the host

R_`jc / .,4, Factors contributing to immunity

Lml+qncagÏa	QncagÏa
– Mechanical barriers (ext. surface) – Chemical barriers (pH) – Physiological factors (temperature) – Phagocytosis – Complement – Beta lysin	– T-cells – B-cells – Immunoglobulins – Interferons – Cytoriner – Chemotactic factors

or immuno-modulation of the graft become important clinical issues (Meiser and Reichart, 1994).

Immune rejection is the most important host response to the cellular component of tissue-engineered constructs containing non-autologous cells (Table 10.6). Xenografts were rejected hyperacutely by preformed natural antibodies and complement activation (Platt, 1992) or more slowly by cellular immunity (Dorling et al., 1996), when preventive measures were not taken. It should be emphasised that the immune response to a cellular bone or cartilage construct is critical for clinical success. Thus, the immunogenic nature of the biomaterial and the cellular components must be considered in the selection of both the scaffold material and the cell source. Future animal studies or clinical trials should evaluate thoroughly the immunological host response in order to improve clinical outcomes.

The use of allogeneic or xenogeneic cells has the clinical advantage of avoiding cell harvesting from the patient. Additionally, a time delay between the initial cell explantation and the final implantation does not occur when allogeneic or xenogeneic cells are employed. Methods for the use of these cells in extracorporal tissue engineering have been developed. Microencapsulation is a clinically applied method to prevent immune reaction against allogeneic or xenogeneic cells (Sefton et al., 2000). It places cells within a core such that the cells are sequestered by a material from direct contact with host cells and huge macromolecules. The wall thus formed, typically consisting of a polymer, is thought to provide immunoisolation by preventing contact between the encapsulated cells and the host immune system to allow for the transplantation of allogeneic or xenogeneic cells without extensive immunosuppressive therapy. Cartilage repair by xenogeneic chondrocytes placed in a polymer core is a recently established method in joint reconstruction. Similar approaches may also be successful in cellular bone tissue engineering despite the different cellular requirements between osteoblasts and chondrocytes.

Clinical Handling and Control 11

In order to provide safe cell/scaffold products of reliable quality, the principles of good manufacturing practice (GMP) must be followed in the selection of donors, retrieval of cells, testing of cells (e.g. after subculture), processing of cell/scaffold constructs, storage and delivery of finished tissues (for a review, see Hellman, 1997). To date, regulations governing these matters have been set up by individual countries. In Germany, for example, manufacturing is regulated by the German drug law and by the German operation ordinance for pharmaceutical entrepreneurs, if autologous cells are used.

Additionally, some institutions have set regulatory frameworks to guide the use of cell-containing products, initially implemented for tissue bank products. Codes of Practice (COP) for tissue banks specify the requirements for the activities of tissue banks that store and/or process human tissues for therapeutic use. The scope of these codes includes, in most instances, all human tissues used for therapeutic purposes including those tested in clinical trials. Musculoskeletal tissues (e.g. bone, cartilage, osteochondral tissues) or haemopoietic progenitor cells (e.g. bone marrow, peripheral blood, cord/placental blood) are typical examples of related items. Professional institutions and specialist societies have, in many instances, prepared standards and guidelines for specific procedures in order to introduce a framework for developing and supporting pre-existing standards and guidelines. An area where there is currently no specific guidance is that of tissue engineering, but where therapeutic products (including cells of human origin as is the case for tissue engineering applications) are processed by commercial or non-commercial organisations, this code applies. In addition the following principles apply.

- If autologous cells are cultured in vitro prior to implantation, the procedures must be validated or monitored, e.g. to ensure vitality of cells and sterility as well as identification of samples. In addition, lack of malignant transformation, and maintenance of relevant biological properties are desired properties of autologous cells that should ideally be documented by proper test systems.
- If allogeneic cells are used in tissue engineering applications (a strategy that has not yet been introduced into clinical bone or cartilage procedures), donors should be thoroughly evaluated to reduce the risk of transmitting diseases and immune rejections. Allogenic stem cells are commonly regulated by laws and regulations set up for tissue banks.

The intention to establish a code is based on Quality Management Systems (in Germany for example ISO 9000 series: ISO 9001–2000) This system refers to cell-based therapeuticals. The principles of good manufacturing practice concerning tissue banks differ in detail between countries (for example in Rules and Guidance for

Pharmaceutical Manufacturers and Distributors, Department of Health, 1997). The complexity of the ex vivo generation of artificial tissues and organs emphasises the need for compliance with an integrated quality assurance programme. As is the case for tissue banks, commercial or non-commercial organisations working in the cell-based tissue engineering field must adhere to the principles set out in other documents (e.g. Guidance on the Microbiological Safety of Human Organs, Tissues and Cells used in Transplantation; Department of Health, 2000). All stages of the selection and testing of donors, retrieval (harvesting) of cells or tissues, culturing, coating with materials, processing, storage and delivery of cell/scaffold constructs should meet the specified criteria. It is important to note that all institutions working in this field have a general duty and responsibility to comply with other statutory legislation of the countries that are involved in the performance and processing of tissue engineered products. Physicians and involved personnel must ensure that proper consent has been obtained for the retrieval of cells or tissue, as addressed by the UK Human Tissue Act 1961, the Human Fertilisation and Embryology Act 1990, the Human Fertilisation and Embryology (disclosure of information) Act 1992 and other professional guidelines.

The processes that are relevant for the clinical use of extracorporal fabricated bone and cartilage tissue constructs should be monitored by a quality system. Today, autologous cells are the sole cell source used in clinical bone and cartilage tissue engineering strategies. Quality management should include all aspects of tissue engineering: formal issues of tissue banks, managerial responsibilities, donor selection and testing, written agreements or contracts with third parties, specified testing of tissues, standards for the processing and storage of tissue, documentation and record keeping and transport of tissues. Formal issues of cell-based tissue engineering facilities include: buildings and premises, environmental controls, processing areas, personnel, ancillary materials, contamination from tissues of donors and equipment. A variety of donor criteria should be documented if allogenic or autologous cells are used, for example medical and behavioural history, pathology, microbiological testing, exclusion factors and consent. The above mentioned parameters are also relevant for donors donating tissue for autologous and/or directed allogeneic use. Ideally, the following parameters should be included in the whole process of clinical bone and cartilage tissue engineering, with respect to transplantation of an ex vivo grown cell/scaffold complex:

Manufacture

- Product consistency
- Product stability (functional, genetic)

Preclinical Safety

- Material sourcing
- Adventitious agents (testing process validation)
- Toxicity testing
- Sterility
- Sterilisation byproducts

Preclinical Activity

- Material characterisation (structural/functional)
- Biomaterial compatibility testing
- In vitro animal models (efficacy measures)

Clinical

- Indications
- Efficacy endpoints
- Safety monitoring
- Population exposure
- Post-market reporting

It is important to note that it is difficult to meet all of these criteria, since many of them are either ill or not defined in sufficient detail. Future developments will stress the need to monitor and eventually to refine such quality systems.

11.1 Regulatory Issues

Bone and cartilage tissue-engineered products act primarily in a functional way, thus resembling medical devices. As they act as pharmaceuticals (e.g. bone with its metabolic functions) they are regulated in some countries by drug-related laws (e.g. Germany: German Drug Law). As tissue-engineered products are a relatively new entity, existing regulatory frameworks are being reviewed and modified to ensure that these new procedures are covered.

Knowledge of the legal situation is important for all scientists and physicians working in all aspects of this new life science discipline. Currently, there is no common or specific regulation for human tissue-engineered products. The responsibilities as well as the regulations differ between countries. The regulatory framework in the USA and the European Community differs in various aspects, and this framework differs also between the various EU members. The Scientific Committee on Medicinal Products and Medical Devices (SCMPMD) of the European Commission stated that none of the existing European regulatory frameworks in their current form encompass tissue-engineered products appropriately (European Commission, 2001, http://europa.eu.int/). The special characteristics and the inherent risks concerning the cellular hybrid materials involved, the new kind of production process and the mode of action are only partially covered by existing regulations. Therefore, initiatives for a complementary regulatory framework have been commenced in the EU. The European Commission released in June 2002 (European Commission, 2002) a proposal for a directive on tissue banking, aimed at assuring quality and safety of human tissues and cells for medical purposes. The Directive is currently being discussed by the responsible institutions in order to implement common standards concerning the marketing of human tissue-engineered products, to safeguard consumers and to protect users (http://lifesciences.jrc.es). The Directive itself is supposed to cover only the donation, procurement, and testing of tissue in the context of tissue engineering, whereas processing, preservation, storage, and distribution are covered by other Community legislation. The current regulatory frameworks in some countries and the imposed problems of legal assessment of this new biotechnology can be gleaned from relevant society recommendations.

Ethical Considerations in Bone and Cartilage Tissue Engineering

12

T. Meyer

The rapid development of tissue engineering and in particular the introduction of gene therapy have elicited much interest among scientists, physicians, and the public in general. The implantation of artificially constructed bone-like tissue for research and therapeutical purposes raises major ethical and legal concerns, which must be considered by the medical profession as well as society. The recent acceleration of knowledge about tissue engineering, which stands currently at the threshold for routine clinical application, assures that the ethical problems presented will be significant and important (Longley and Lowford, 2001; Jenkins et al., 2003). However, it should be noted that from the standpoint of bioethics, scientific research on tissue engineering raises no completely novel ethical questions that have not been debated previously in the context of related advancements. This chapter deals with the stratification of risks and benefits in the development of artificial bone and cartilage tissue and focuses on appropriate and ethically acceptable ways of utilising the recent advances of stem cell derived therapies. Arguments are provided in support of the concept that bone and cartilage tissue engineering can be beneficial only when these ethical and moral issues are adequately addressed prior to its future clinical application. It is argued here that all research projects aimed at improving the outcome of bone and cartilage tissue engineering should be guided by ethical principles that respect and consider the patient´s interest and honour the moral, cultural, and religious values of society.

At the centre of all medical treatments, not limited to new or experimental therapies, stands the doctrine of informed consent, which is defined as the fundamental process of communication between a patient and physician that results in the patient´s authorisation or agreement to undergo a specific medical intervention (Churchill et al., 1998). Like all surgical procedures, this process begins long before the patient undergoes a surgical operation such as, for example, the implantation of extracorporally engineered bone and cartilage tissue. Moreover, the responsible physician has to assure the patient´s informed consent in all stages of the therapy starting from surgical planning to post-operative treatment. Informed consent in this respect means the interactive process by which a fully informed patient has the choice between newly developed techniques of tissue engineering and conventional methods of reconstructive bone surgery such as, for example, osteosynthesis of fractures. The treating physician has to comment on all relevant risks, benefits,

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and uncertainties related to each treatment strategy and must ensure that the patient is thereafter competent to decide which individual therapy should be favoured. To become a fully and emancipated participant, the patient must know about reasonable alternatives to the proposed treatment, since he holds the last decision on the nature of the intervention. Given that all possible and practicable alternatives are extensively discussed, the physician is not generally prohibited from providing a recommendation, but should share his/her reasoning process with the patient. Informed consent in this respect is regarded as an invitation to the patient to participate in his/her health care decisions. Provided that all relevant information are given and that comprehension is achieved, the patient has been offered the opportunity to either accept, refuse or modify the proposed intervention according to his/her own planning. This implies that the patient makes all therapeutical decisions on a rational and voluntary basis with the aid of professional medical advice. Given that the intervention is properly executed without negligence according to the prevailing standard of surgical care, the procedure of informed consent guarantees that the doctor is protected from financial liability.

Bone and cartilage tissue engineering holds great promise to ameliorate the regeneration of skeletal defects and has the potential to compensate for the loss of bone structures in cases when bone reconstruction is required to prevent a non-union defect. Currently, several groups worldwide are searching for the appropriate carriers and the right set of cells that, once retransplanted into the site of bone lesions, will ensure bony reunion (Lucarelli et al., 2004). Although a huge amount of knowledge has been gained so far, we are only at the beginning of our understanding, and the direction the field will take in the near future is not fully known. Nevertheless, it is inevitable that numerous ethical problems will have to be solved on the way. Generally, these ethical issues fall into three different categories: (1) conflicts arising from the allocation of donor cells and the general access to this surgical treatment; (2) problems concerning the use of stem cell technology, including foetal stem cells for ex vivo engineered bone and cartilage tissue; and (3) boundaries set by the genetic manipulation of transplanted cells.

In the first set of ethical issues we concentrate on bone cell allocation for use in reconstructive medicine and on general obligations associated with bone surgery. Two distinct treatment strategies are possible when attempting to engineer bone tissue substitutes for use in surgery. One approach involves the removal of osteoblasts from unaffected bones and their reimplantation in the patient´s body after they had been successfully seeded on bone-mimicking scaffolds (Lucarelli et al., 2004). The other approach is based on the allocation of bone-derived cells explanted from a donor and the reimplantation into a recipient after three-dimensional cultivation on devices (Strom et al., 2002). While the use of autologous cells is facilitated by less stringent ethical concerns, the transplantation of bone cells from a donor to a genetically different recipient gives rise to more serious ethical considerations (Lucarelli et al., 2004). In the former case there is no need for an immunosuppressive treatment, but the use of heterologous somatic or stem cells implies the long-term maintenance of an immunosuppressive state. The latter approach with heterologous cells carries the risk of graft rejection and graft failure depending on the histocompatibility match between the graft and the recipient. Grafting of engineered tissues derived from a genetically different donor requires the daily application of immunosuppressive drugs, which may compromise the patient´s resistance to infectious agents or result in otherwise serious side effects (Strom et al., 2002). These adverse reactions must be taken into account when deliberating on the use of grafting heterologous cells or tissues. Since reliance upon daily immunosuppressive medication is unacceptable for many clinical applications, it is important to develop new methods for the induction of immune tolerance. Moreover, the surgical explantation of bone cells from a living donor may cause harm by itself and is therefore only justified in severe cases where the surgical risk for the donor is acceptably low and when all other opportunities for bone reconstruction are either impracticable or have been exhausted. In addition to the usual iatrogenic risks attached to surgical procedures in general, such as local or systemic infections and damage to healthy tissues, certain potential health risks are specifically associated with the new approaches in regenerative medicine. Immune reactions against components of the device or against surface structures of the implanted allogeneic cell layer may cause graft rejection and finally result in an adverse outcome even under immunosuppressive treatment. Toxic effects or induction of auto-immune diseases may be other potentially harmful risks, which should be considered when cellular therapies using allogeneic cells are taken into account. Animal experimentation as an integrated part of tissue engineering helps to assess these risks (Nordgren, 2004).

The paucity of donor cells is another serious problem limiting the widespread application of such cell-based replacement therapies. This sourcing problem demands fair and transparent criteria for cell distribution and standardised procedures that assure equitable access to transplantation resources (Carter, 1999). It is advisable to fully exclude economic aspects of donor cell acquisition in order to prevent commercialism in handling and distribution of donor cells. However, there is some hope that the ability to establish stem cell lines in vitro will allow the production of large batches of terminally differentiated allogenic cells, which can be administered to patients for therapeutic grafting. When there is no need for a direct transplantation of freshly isolated cell preparations, the clinical utility of such cell lines is believed to ameliorate allocation problems and facilitate quality controls for minimalising the risk of disease transmission. Nevertheless, extensive controls of the cell-seeded devices are absolutely necessary to check that the applied donor cells are pathogen-free and unable to induce malignancies due to genetic transformation.

In this respect elaborated protocols for the handling, procurement, and delivery of cell-reconstituted devices would be helpful in order to avoid adverse side effects (Longley and Lawford, 2001). These protocols should follow strictly the guidelines for good manufacturing practice. It is important that for all stages of cellular replacement therapy, detailed protocols are established. Implementing guidelines for cell-derived replacement therapy will allow clinicians to pursue therapeutical advances in tissue engineering while maintaining the highest ethical and medical standards (Winter and Roger, 1995; Longley and Lawford, 2001). Provided all requirements for these new therapies fulfil the principles of good clinical practice and standardised quality control programmes exist, cell-based therapies may become acceptable and potentially important applications that in certain cases may be beneficial over conventional surgery. Furthermore, intervention-associated morbidity and mortality should be carefully documented, preferentially in central registries. Multicentre studies that are accompanied by a long-term scientific evaluation allow one to access the benefit of new therapies in a more reasonable timescale and are thus preferred over studies carried out at only one centre.

It is generally believed that human primary fetal bone cells could be of great interest for bone research, due to their fast growth rate and their ability to differentiate into mature osteoblasts (Cogle et al., 2003; Gilbert, 2004). A barrier, which hampers the progress in cell-derived replacement treatment, lies in the opposition to the use of embryonic stem cells because of moral and religious concerns over osteoblasts (Cogle et al., 2003; Gilbert, 2004). Objections are mainly raised because experimentation with embryonic stem cells derived from human blastocysts requires the destruction of human embryos. The current debate on the ethical status of the human embryo is focused on the fragility and defenceless of the human embryo, and the question as to what respect a human embryo should deserve in light of recent medical progress. It is generally accepted that the blastocyst contains a complete set of genetic instructions and the capacity for the epigenetic determinations needed to develop into a human being. However, there is a dispute on the ethical and legal implications of using human primordial stem cells for tissue engineering, which includes controversy over whether the absolute respect for individual human life begins already at conception osteoblasts (Cogle et al., 2003; Gilbert, 2004).

As it is evident that adult stem cells have the unexpected ability to differentiate into many specific cell types including those of skeletal origin, research with multipotent adult stem cells has become a promising alternative to embryonic stem cells for use in vivo (Colman and Burley, 2001; Gao et al, 2001; Im et al., 2001; Cogle et al., 2003; Tsuchida et al., 2003). Not all limitations on the use of adult stem cells have been solved so far, such as, for example, a comparably lower capacity to expand indefinitely without losing their differentiation potential. Furthermore, the initial number of adult stem cells obtained is lower than with stem cells of embryonic origin and it is apparently more difficult to genetically manipulate adult stem cells. Clearly, the ethical consequences of adult stem cell research are not so serious, making the use of stem cells obtained from adult tissue much less controversial.

The recent progress made in establishing stem cell lines in vitro implies that standardised therapeutical grafting of ex vivo generated bone substitutes will be applicable in the near future. In the context of the new cell-based therapies, genetic modification of stem cells used to cover implant carriers is a promising approach, which is expected to fulfil the requirements for better biocompatibility and immune tolerance (Jenkins et al., 2003). Up to now, experimental studies utilising somatic gene therapy have been restricted to treating serious, life-threatening or debilitating disorders and it is indeed questionable if clinical situations for which tissue-engineered implants are desired fall into this category. Nevertheless, despite the recent setbacks in the administration of gene constructs, there is some hope that future improvements in the efficacy and safety of somatic gene therapy will ultimately be integrated into the fast moving field of tissue engineering. Provided the problems with the design of the appropriate gene vectors are satisfactorily solved, it is assumed that future advances in somatic gene therapy will continue to shape the ethical debate and may influence the patient´s attitudes towards this therapeutical approach (Motulsky, 1983; Walters, 1991; Winter and Roger, 1995; Spink and Geddes, 2004). Genetic manipulation of cultured cells on carriers generated to be implanted into the body may be less harmful, compared to standard protocols for somatic gene therapy (Smith, 2003). This appears to be particularly true for the risk that the desired gene is unintentionally integrated into the germ line, however, this risk is commonly expected to be negligibly low.

Future Prospects 13

The most promising future areas in tissue engineering are in the fields of stem cell research, genetic engineering, customised tissue reconstructions and improvement of the nutritional features in the transplant.

13.1 Customised tissue reconstruction

A defined three-dimensional scaffold assembly of complex structure is needed for the development of unique bone or cartilage tissue substitutes. Present day computer-aided technologies, medical imaging, and modern techniques offer elegant solutions and create new possibilities in the design and application of bone tissue substitutes. Non-invasive computed tomography (CT) and magnetic resonance imaging (MRI) generate accurate anatomical information for scaffold design, and computer-aided design/computer-aided manufacturing (CAD/CAM) and rapid prototyping (RP) technology allow the production and insertion of individualised scaffolds (Coward et al., 1999; Zonneveld, 2000; Runte et al., 2002) (Table 13.1). Implant prostheses, custom-made according to the size and shape of an individual defect site, can thus be produced (Fig. 13.1). The utilisation of these technologies has evolved into a new emerging field of computer-aided tissue engineering (CATE) (Sun and Lal, 2002). Driven by computer imaging and modern manufacturing technology, regenerative surgery may in future be directed towards individualised fabrication of bone and cartilage substitutes (Meyer et al., 2003). The principle steps of computer aided tissue engineering are given in Fig. 13.2.

R_`jc /1,/, Aspects of computer aided tissue engineering

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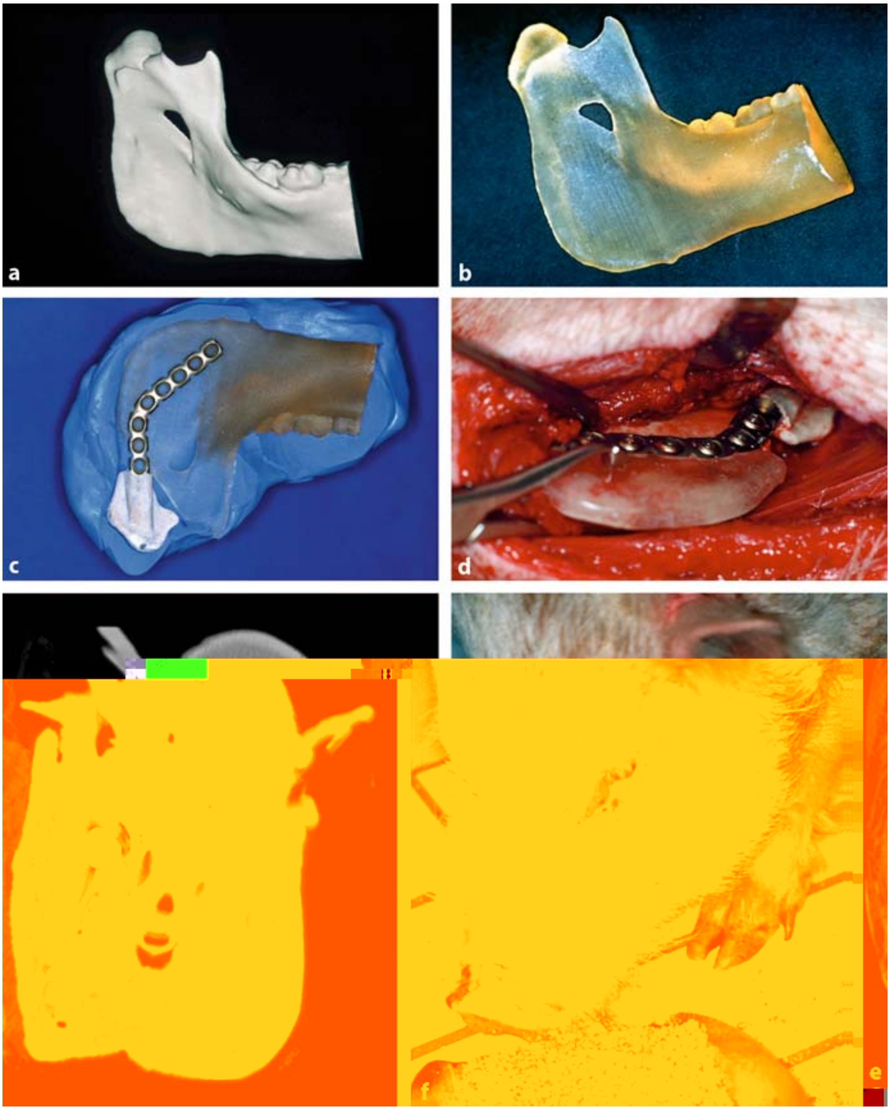
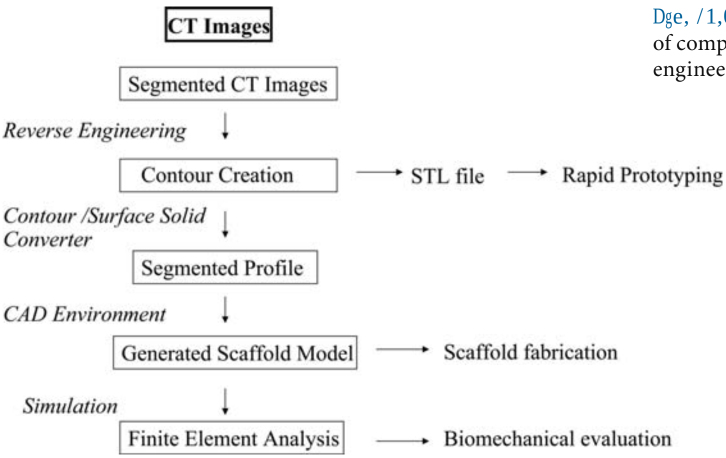
– Anatomical modelling – Physical modelling – Physiological modelling – CAD-based modelling – Prototype modelling	– Defect identification – 3D quantification – Tissue measurement – Tissue structure analysis	– Surgical planning – Surgical guidance – Robotics



Dge, /1,/, Individual computer-aided reconstruction of temporomandibular joint. _ STL model of the mandible. ` stereolithography model. a Fabrication of a PLA/PGA condyle attached to a reconstruction plate. b Template-driven insertion of the artificial TMJ. c CT scan one month after insertion. d Clinical view of masticating animal

13.2 Vascularisation

One future development in clinically applied bone tissue engineering will have to focus on the vascularisation of the regenerate tissue. Angiogenesis is crucial for the survival of tissue-engineered constructs. The need for a prompt restart of blood sup-



Dge, /1,0, Principal steps of computer-aided tissue engineering

ply is underlined by the observation that a delayed and inadequate neovascularisation of the graft implant is likely to account for implant failure. However, it appears unlikely that the final vascular structure can be predetermined in the micro-fabrication of the tissue construct in the near future, despite the improvements in vascular-like structure function ex vivo. Rather, the natural adaptive power of resident cells will be needed to achieve a vasculature that is sufficient for large, complex tissues. Recent advances indicate that the goal of providing a tissue-engineered vascular graft that will remain patent in vivo for substantial periods of time is achievable. It was shown by Ratcliffe that a non-degradable polyurethane scaffold cultured with smooth muscle cells generated a construct with mechanical properties similar to native vessels (Ratcliffe, 2000). These results show that tissue engineering of vascular grafts has true potential for application in the clinical situation. Adding growth factors such as vascular endothelial growth factor (VEGF) to the scaffold material is another promising approach to support vascular assembly (Nomi et al., 2002).

13.3 Genetic Engineering

The prospect of gene therapy in the context of tissue engineering is offered by recent advances in gene delivery techniques. Gene therapy itself can be defined as the introduction of nucleic acid into cells in order to alter their genetic constitution. Application of gene therapy to control cellular behaviour has a number of advantages above conventional, protein-based therapies (for a review, see Klamut et al., 2004). Genetic modification of cells has some advantages over protein delivery approaches to the ex vivo generation of a bone- or cartilage-like tissue. The advantages associated with genetic modification of cells are:

- Expression of the desired transgene is prolonged compared to the effects of added proteins
- Proteins often require hyperphysiological doses to execute specific effects
- Expression of protein restricted to forget cell

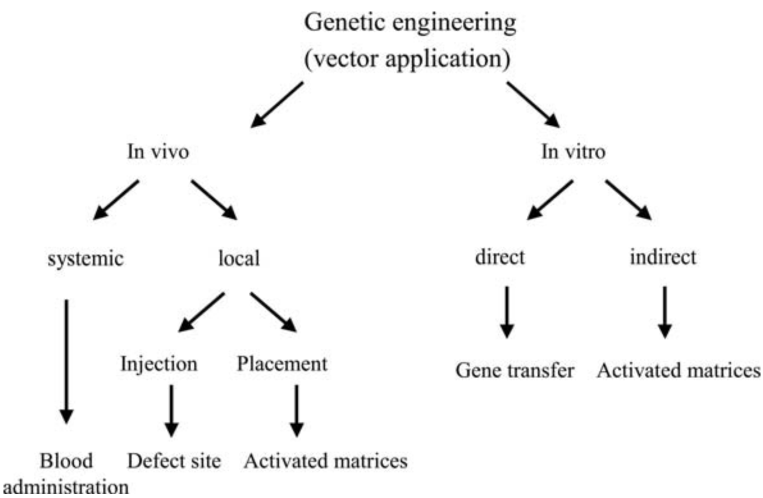
- Transgenes do not have to be produced in large amounts with recombinant expression technology
- Transgene production can be regulated by the use of controllable or tissue-specific promoters.

Various genetic engineering strategies can be used for gene transfer to tissues of bone and cartilage (Evans and Robbins, 1995; Robbins and Ghivizzani, 1998; Hannallah et al., 2002) (Fig. 13.3) The strategies include, beneath local or systemic delivery approaches in vivo, the transfection of cells ex vivo (for a review, see Baltzer and Lieberman, 2004; Partridge and Oreffo, 2004). Systemic delivery, performed by administration of the vector into the bloodstream, allows gene application and dissemination into all organs of the body. This method has advantages in bone or cartilage tissue engineering (as referred to in the extended definition of tissue engineering) only under some circumstances. It is a favourable approach when the target tissue is located in different regions of the body or when it cannot be reached directly.

Major limitations of systemic delivery include:

- The low specificity of gene expression
- The high vector concentration required to attain therapeutic effects
- The lack of blood supply in cartilage tissue
- The non-specific dissemination of the vectors to all areas of the body
- Immuno-reactions against components of the delivery systems (vectors).

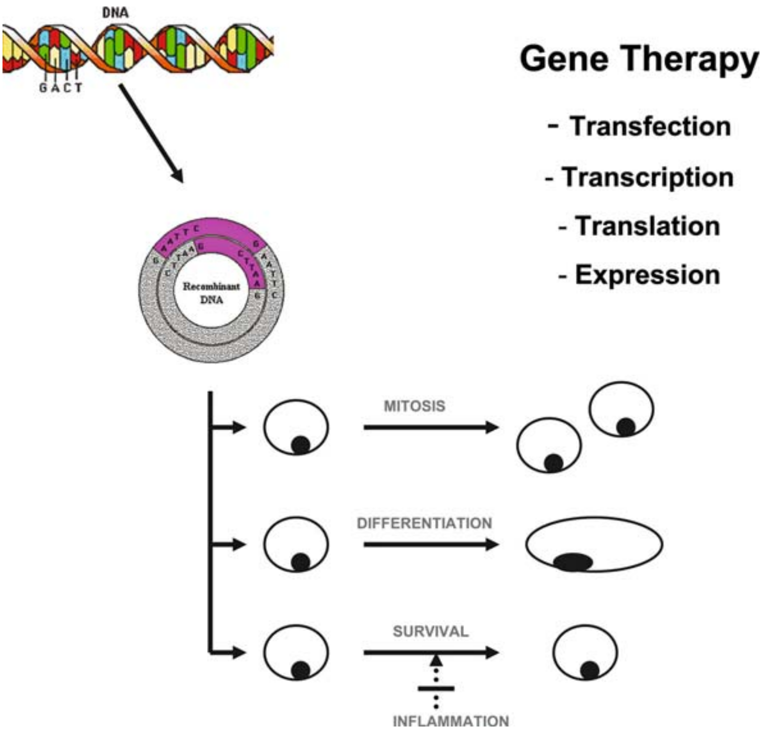
In contrast to systemic delivery, local gene therapy in bone and cartilage healing has been investigated in a number of studies (Evans and Robbins, 1995; Robbins and Ghivizzani, 1998; Hannallah et al., 2002). Two approaches can be distinguished: first, vectors can be applied directly into the host tissue (in vivo) (Alden et al., 1999; Musgrave et al., 1999; Baltzer et al., 2000; Okubo et al., 2000), and secondly, cells harvested from the patient can be genetically modified by DNA transfection in vitro.



Dge, /1,1, Scheme of genetic engineering approaches.

For the ex vivo tissue engineering approach, genetically altered cells are then coated with a scaffold in order to extracorporally fabricate bone or cartilage tissue. Ex vivo gene delivery can be considered as a more defined method of genetic engineering. In addition to the gene transfer under controlled in vitro conditions, this technique is safer since the gene manipulation takes place under controlled conditions outside the body. An additional advantage of this method is that nearly all cells can be transfected which is very hard to achieve in vivo. Successful gene therapy involves four key steps: effective transfection followed by transcription, translation, and sustained expression of the reccombinant target gene. The uptake of new genetic material and the expression of the introduced foreign gene have to be facilitated by advanced transfection protocols (Fig. 13.4.). Generally, virus-based vectors (adenovirus, adeno-associated virus, lentivirus and Herpes simplex virus) are more efficient than non-viral vector systems (e.g. liposomes). A variety of cDNAs have been cloned aimed to improve tissue healing by (1) inducing mitosis or the synthesis and deposition of tissue-specific extracellular matrix components by autochthonous cells, (2) induction of cell differentiation by mesenchymal progenitor cells, or (3) inhibiting cellular responses to inflammatory stimuli. (Fig. 13.4)

Experimental studies have shown that gene therapy is effective for both bone and cartilage tissue engineering. The effectiveness of gene therapy has been demonstrated by in vitro and in vivo studies. Several investigators have, for example,



Dge, /1,2, Scheme of gene transfer and cellular actions.

demonstrated that viral vectors can be used to deliver genes to chondrocytes in vitro (Arai et al., 1997; Wehling et al., 1997; Doherty et al., 1998; Smith et al., 2000). The genetically altered chondrocytes have been cultured ex vivo in a three-dimensional space without loss of cell viability or differentiation characteristics (Doherty et al., 1998; Ikeda et al., 2000). Cells were commonly transduced with genes that are involved in chondrocyte development. It was demonstrated that the in vitro delivery of these genes (encoding for BMP-2, IGF-1, and TGF-β) to articular chondrocytes leads to an increase in proteoglycan synthesis by the cells in culture (Arai et al., 1997; Smith et al., 2000). In addition to chondrocyte-based studies, Lieberman and collegues. (1999) were able to successfully present an ex vivo gene therapy approach to enhance the osteogenic potential of bone marrow cells. In general, cells from various mesenchymal cell lines were able to induce bone- or cartilage-like cell differentiation by incorporating cell differentiation genes.

The local gene therapy approach was also applied to experimental animal studies as revealed by investigations on in vitro cartilage or bone healing (Baragi et al., 1997; Kang et al., 1997; Mason et al., 1998; Goomer et al., 2000; 2001). Mason et al., for example, demonstrated improved healing of articular defects by using a retroviral vector to transduce mesenchymal stem cells with a gene for BMP-7 in a rabbit model (Mason et al., 1998). Gene delivery methods have also been successful in the promotion of bone healing in various animal experimental settings (Beresford, 1989; Fang et al., 1996; Bruder et al., 1998a; b; Bonadio et al., 1999). In this respect, gene therapy and the use of gene activated matrices offers great promise in the field of tissue engineering.

Importantly, gene therapy has not only advantages but also risks. The integration of viral vectors into the genome of cells bears the risk of mutagenesis and oncogenesis (Crystal, 1995). Abnormal regulation of cell growth, toxicity due to chronic overexpression of the gene product, dysregulated development and induction of malignant tumours are currently unsolved problems associated with gene therapy. Despite their promising expectations all gene therapy protocols should be regarded with extreme caution and should be conducted only in specialised centres with long-standing expertise in recombinant DNA technologies. The effect of genetic tissue engineering has therefore to be tested at different experimental levels. Basic evaluation can be performed in in vitro systems and in small animals in order to prove basic concepts of genetic engineering (such as the regulation of gene expression). Large animal models are the next step to study the effectiveness as well as to evaluate possible adverse effects of gene therapy at an in vivo level, before clinical trials can be started. A special model system that can be used as a proof of the system concept system is the immunodeficient mouse model, since this model allows one to study the effect of transgene expression on tissue formation without having additional interactions by immune mechanisms.

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Subject Index

(e.g. RANKL, OPGL, ODF) 19
1α,25-dihydroxyvitamin D3 17
ß-glycerophosphate 81
β-Glycerophosphate 81
β-tricalcium phosphate 124

A

aberrant genotype 71
abrasion chondroplasty 57
acidic vesicles 19
actin 32
activity 161
adaptive behaviour 38
adaptive immunity 196
adhesion forces 140
adhesion molecules 78, 134
adhesion phase 132
adhesive surfaces 146
adipocytes 65
adipogenic lineages 76
adult stem cells 67, 206
Aggrecan 27, 28
alginate 125, 127, 128, 165
alkaline phosphatase 17, 74, 158
allogeneic cells 66
allogenic osteochondral grafts 60
allogenic specimen 60
allografts 47, 196
altered cell lines 67
amino acids 151
analytical techniques 87, 89
anchorage structures 134
angiogenesis 24, 81, 84, 208
angiogenic factors 24
angiopoietin-dependent pathway 24
animal models 5, 180, 201
anisotrophic scaffold nanostructures 171
anisotropic texture 145
Anisotropic topographies 139
annulus fibrosus 25
antibodies 198
apatite 125
apoptosis 31
aprotinin 127
Arg–Gly–Asp (RGD) sequence, 133
arthritis 55
arthroscopy 57
articular cartilage 25, 186
articular surface defect 33
artificial organs 1
artificial pancreas 176
artilage Regeneration 33
ascorbic acid 74, 81
attachment phase 132
Augmentation 48
auto-immune diseases 205
autocrine 17, 85
autografts 47
autologous 66
autologous bone 48
autologous chondrocyte transplantation 2, 58, 60
autologous serum 76, 77
autologous tissue 196
autologous vascular cells 84
automated bioreactors 155
automation 168, 171

B

barrier 48
basement membranes 138
basic multicellular units 38, 39, 41
basolateral membrane 19
bi-cellular micromass 176
biaxial strain devices 156
Biglycan 29
bioactive peptides 125
biochemical 87, 89
biochemical coupling 40
biochemical signal 40
biocompatibility 206
biodegradation process 197
bioethics 203
biofunctional surfaces 146
biologicals 56
biological plasticity 47
biomaterials 47, 121
biomechanical competence 34
Biomechanics 35
biomimetics 10
biomineral 160
biomolecular engineering 146
biomolecules 4, 52
biophysical constraints 131, 143
Biophysical constraints under function. 131
Biophysical effects 49
biophysical microenvironment 41
biophysical signals 42
biophysical stimulation 5, 49, 50
Biophysical stimuli of cells 40
bioreactor 150, 164
bioreactors 5, 46, 149, 153, 156, 160, 174
bioreactor design 161
bioreactor technology 149
biotechnological issues 5
blastic phenotype 81
blood-calcium 19
blood flow 24
blood vesse 84
BMP-2 212
BMPs 24
bonding 169
bone-like mineral 141

bone-lining cells 10
bone augmentation 196
bone cements 124
bone chips 80
bone marrow 73
bone marrow cells 84
bone marrow stromal cells. 65
bone mill 80
bone mineral 128
bone morphogenesis 20
bone morphogenetic proteins 31, 52, 161
bone morphogenic protein-6 75
bone morphogenic proteins 34, 53
bone particulates 81
Bone Regeneration 20
bone resorption 10
Bone Sialoprotein 16
bone sialoprotein 133, 146
Bone Tissue Engineering 181
bone turnover 41
brittle fracture 196
bronchial tree 25
Bulk cortico-spongiosal grafts 53

C

calcified bone 8
calcified cartilage 10, 25, 27, 32
calcifying tissues 10
calcium homeostasis 10
calcium phosphate 141
Calvarial critical sized defects 196
canaculi 8
Cancellous bone chips 53
carbodiimide 127
carbonate-substituted apatites 124
carbonate hydroxyapatite 129
Cardiac myocyte 68
cardiac organogenesis 176
carrier 53, 125, 161
cartilage 34
cartilage biomechanics 43, 186
cartilage bioprocessing 159
Cartilage Matrix Protein 29
cartilage phenotypes 71
cartilage repair 47
cartilage repair strategies 55
cartilage tissue engineering 186
cartilaginous models 25
cartilaginous template 8
cell-based therapies 205, 206
cell-based therapy 179
cell-based tissue engineering 182
cell-flattening 136
cellular deformation 39
Cellular implantation 47
cellular spheres 174
cell adherence 132
cell adhesion 76, 133, 134, 136
Cell alignment 138
cell amplification 174
cell condensations 136
cell culture assays 75
cell culture conditions 76
cell culture techniques 73
cell cycle-related proteins 78
cell density 76, 154
cell differentiation 76, 161
cell harvesting 81
cell lines 138
cell membranes 138
cell migration 132, 163
cell movement 136
cell nuclear transfer methodology 69
cell seeding density 165
cell survival 153
cell therapies 4
cell viability 154, 163
cement lines 142
ceramics 47, 121
ceramic technology 168
charge density 140
chemically patterned stripes 139
chemical properties 163, 167
chemistry 143
chitosan 125, 128, 165
chondral defects 33
chondroblasts 65
chondrocyte precursor cell 30
chondrogenesis 186
chondrogenic differentiation 75
chondrogenic lineage 75, 81
chondrogenic pathway 31
chondroprogenitors 83
chondrospheres 176
cleavage 19
clinical application 203
clinical approach 180
clinical demands 5
clinical grafts 179
Clinical Handling 199
clinical outcome 5
clinical setting 5
clinical settings 179
clinical studies 187
clinical trials 77, 196
clinical use 200
Closed bioreactor systems 155
close contacts 134
co-culture 79, 84
Co-cultures 85
CO2 151
coatings 124
Codes of Practice 199
cohesion forces 140
collagen 8, 12, 127, 146, 165
collagenases 127
collagens 10, 12, 125, 126
collagen mineralisation 10
Collagen types II 13
Collagen types II, III and V 13
Collagen Type I 15
Collagen type I 13
Collagen Type II 13, 27, 176
Collagen Type III 22
Collagen Type IX 27
Collagen Type V 23
Collagen Type VI 12
Collagen Type X 23
Collagen Type XI 28
Collagen Type XIII 14
Collagen Type XIV 15
colony-forming ability 83
colony-forming precursors cells 83
colony-stimulating 19
colony-stimulating factors 83
colony forming/units assay 78
commercial bone tissue engineering 3
committed chondrocytes 81
Committed osteoprogenitors 78

compatibility testing 201
complement activation 198
composites 121
composite materials 165
composite tissues 62
composition 125
compression 28, 35
compressive modulus 170
compressive strains 158, 196
compressive strength 170
Computational tools 153
computed tomography 194, 207
computer-aided design/computer-aided manufacturing 207
Computer-aided simulation 37
computer-aided technologies 207
computer-aided tissue engineering 207
computer-based design 171
concentration gradients 152
condensation process 174
conditioned media 86
condrogenic 76
connective tissue 7
connective tissue stem cells 67
conservation mode 39
consumption 152, 179
contact guidance 138
Control 199
convection 152, 163, 165
copolymerisation 127
corals 128
coral mineral 128
Cortical bone chips 53
cortical cells 20
critical cell mass 174
critical size defects 182
cross-linkers 127
cross-linking 13
cryopreserved 60
crystal 125
crystallinity 123, 125
crystal nucleation 10
crystal size 125
crystal structure 141
culture artefacts 142
culture conditions 81
culture dish 150
culture dishes 156
culture medium 81
culture strategies 77
culture time 81
cytokines 24, 52, 65, 84, 161
cytoplasm 136
cytoskeletal proteins 74
cytoskeleton 32, 41, 134, 136

D

debridement 57
deep zone 25, 27
defect 25
deformability 123
deformation 37, 45
degradable materials 121
degradation 123, 161, 167
degradation characteristics 163, 167
degradation products 167, 173
delivery system 161
dendritic cells 19, 83
density 167
density gradient centrifugation 73
density gradient separation 76
Determined Bone Cells 78
determined cells 74
developmental processes 7
dexamethason 74
dexamethasone 65, 81
diaphysis 31
differentiation capacity 65
diffraction analysis 141
diffusion 151, 152 ,163, 165
diffusion chambers 78
Diffusion constants 180
diffusion constraints 165
diffusion distance 150, 153, 154
digestive enzymes 81
directed fluid flow 165
disease transmission 86, 205
disordered nanotopography 138
dispersion process 169
displacement 36
dissociation 125
dissolution 19, 125
dissolve mineral 19
Distortion 41
distraction osteogenesis 51, 49
disuse mode 39
documentation 200
dogs 182, 186
domain 19
donor selection 200
donor shortages 47
donor site 60
donor site morbidity 47, 55
donor tissue 60
dose 53, 161
dose–effect relationship 161
drugs 56

E

ECM proteins 74
ectopic sites 182
effector cell 40
efficacy 187
elasticity 123
elastic cartilage 25
elastic material 37
electrical field exposure 50
electrical interactions 42
electrical potentials 42
electrical stimuli 50
electric fields 42, 45, 159
Electro-mechanical effects 40
electrokinetic effects 42
electromagnetic field exposure 42, 49, 159
electromagnetic signals 49
electromechanical effects 143
element composition 131, 140
Element release 131
ell attachment 133
ell Development 65
ell differentiation 163
ell maturation state 139
ell Sources 66
embryogenesis 31
embryological development 20
embryonic stem cells 67, 206
emulsion freeze drying 169
enamel 12
endochondral ossification 7, 8, 13
endothelial cell 68, 84, 85, 150, 155
Endothelial progenitor cells 84
endothelogenic 76

Energy 37
Energy dissipation 41
engineering 211
Enhancement of molecular transport 41
enrichment 76
enzyme digestion 78
epiglottis 25
epiphysal plates 25
epiphysis 31
eptide Adhesion 145
estrogen 17
ethical issues 204
ethical principles 203
ethical status 206
European Commission 4, 201
eustachian tube 25
experimental studies 179
explant outgrowth 78
explant procedures 78
expression 211
external ear 25
external geometry 165, 167
extracellular matrix contacts 134
extracorporal tissue engineering 62, 179
Extrusion 170

F

fabrication accuracy 168
factors 19
factor pharmacology 161
factor supplementation 161
factor XIII 127
fashion 17
fatigue resistance 196
FEA methods 38
fetal bone cells 206
fetal bone marrow cells 68
fetal bovine serum 76
fetal stem cells 68, 69
fetal tissue growth 52
fetal tissue transfer 69
fibre 139
Fibre bonding 170
fibre meshing 169
fibrin 125, 127, 165
fibrinogen 127
Fibrin glue 127
fibro-cartilage 57
fibroblast growth factor 53, 161
fibroblast growth factor-2) 52
fibrocartilage 25, 34
Fibromodulin 29
Fibronectin 136
fibronectin 32, 133
fibronexus 32
filopodial processes 8
financial liability 204
finite element analysis 37, 156, 157
fixation 195
Flap prefabrication 62
flasks 151
flow chambers 158
flow determination 155
flow fields 153
fluid 43
fluid continuum 43
fluid flow 41, 42, 43, 45, 151, 152, 180
fluorescence microscopy 155
fluoride 125
fluoride-substituted apatites 124
focal adhesions 139, 146
focal adhesion kinase 136
focal contacts 134, 136
foetal stem cells 204
foreign body giant cells 197
foreign body reaction 197
formaldehyde 127
fracture gap healing 52
freeze drying 127, 169, 171
frequency 156
Fresh tissue 60
functional adaption 8
functional assays 78
functional secretory 19
functions 10
fused deposition modelling 171
Future Prospects 207

G

gap 19
gap junctions 8, 42
Gas foaming 170
gas foaming 169
GDFs 24
gel 127
genetically altered cells 71
Genetic engineering 70, 209
genetic engineering 207
genetic manipulation 204
genetic material 211
genetic modification 206
genetic tissue engineering 212
genetic transformation 205
Gene delivery 212
gene insertion 70
gene therapy 53, 70, 161, 203, 209
gene transfer 211
gene vectors 206
glass transition temperature 170
globular structure 133
glucocorticoids 17
glucose 151
glutaraldehyde 127
glycolysis 180
glycoproteins 15
glycosylation 13
GM-CSF 83
goats 182, 186
good manufacturing practice 199, 205
graft failure 204
graft performance 180
graft rejection 204, 205
graft volume 55
gravitory cultures 174
groove structures 138
gross morphology 137
growth factors 4, 77, 84, 125, 151, 175
growth plate 31
guided bone regeneration 48
guidelines 205

H

haematopoietic stem cells 65
hard tissue 13
HCS-2/8 71
heart valve 176
heating 127
hematopoietic stem cells 19, 83
heterokaryons 85
heterotopic animal models 182, 186
heterotopic chondrogenesis models 186
heterotopic models 180
high pressure 170
high pressure processing 169

histocompatibility match 204
histocompatibilty 60
histogenesis 180
hollow structures 166
holographic interferometry 37
homeostasis 19, 30
hormonal regulation 41
hormones 38, 65, 161
horses 186
host conditions 25
host reaction 196
host response 197, 198
host site 195
host tissue 60
HPV-16 E6/E7 72
human application 180
human embryos 206
hyaline 57
hyaline cartilage 25, 176
hyaluronan 28, 29, 30, 125, 127, 165
hyaluronidases 127
hybrid materials 47, 195
hydraulic bone chambers 196
hydrazide 128
hydrocarbon templating 169
hydrogels 127, 172
hydrolysis 123
hydrophilic 140
hydrophilic channels 145
hydrophobic 140
hydrophobic stripes 145
hydrostatic compression 41
hydrostatic pressure 41
Hydroxyapatite 128
hydroxyapatite 8, 15, 16, 124, 165
Hydroxyapatite coatings 132
hypergravity 41
hypertrophic chondrocytes 31, 81
hypertrophic phenotype 27
hypoxia 154, 180

I

IGF-1 212
iliac crest 73
imaging 87, 89
immature animals 182, 186
immortalised cells 67
Immortalised chondrocytes 71
immortalised human osteoblast cell lines 71
immune rejection 78, 197
immune suppressive treatment 197
immune system 197
immune toleranc 206
immune tolerance 205
immuno-modulation 198
immunocompetent animals 77, 186
immunogenity 69, 173
immunoisolation 198
immunological barriers 47
immunologic barriers 60
immunosuppression 60
immunosuppressive therapy 198
immunosuppressive treatment 204
implantation bed 195
implant failure 209
Implant integration 195
implant materials 121
Indian hedgehog 31
infectious diseases 47
inflammation 186, 197
inflammatory cytokines 197
inflammatory processes 24
Inflammatory state 180
Informed consent 204
informed consent 203
injection moulding 170
innate 196
Inorganic material 122
inorganic materials 124
insulin-like growth factor 161
insulin-like growth factor-1 52, 75
Insulin-like growth factors 53
insulin-like growth factor I and II 34
integrins 133, 134, 136, 146
intentionally immortalised cell lines 70
interconnecting tubes 153
interconnectivity 163, 170
interconversion 78
interfacial tension 139, 140
interferones 24
interleucines 24
internal resistance 35
internal structure 165
interstitial fluid 44
interterritorial matrices 45
intevertebral discs 25
intramembranous bone formation 8
in situ regeneration 47
ionic composition 133
ionic forces 133
ion exchange 140
ion release 131, 141
irritation 48
isolation 76
ixed charges 139

J

joints 35
joint degeneration 33
joint distractions 58
joint forces 43
joint function 55
joint loading 43
joint malposition 58
joint physiology 186
joint replacement 55
junctions 19

L

lactate 151
lamellar bone 48
lamellipodium 136
laminar flow 152
laminin 133
Langmuir Blodgett transfer 145
large animal model 186
larynx 25
laser abrasion 57
lavage 57
layered manufacturing 171
layer manufacturing 169
legal assessment 202
legal concerns 203
legislation 200
ligaments 35
ligand densities 146
limb bud mesenchyme 31
lining cells 17, 78
Link Protein 28
liquid-guiding channels 145
liver reconstruction 176
Load 35

loading 196
loading device 158
load application 37, 156
load perception 8
load transduction 194
low-density micromasses 173
lyophilised materia 60
lysozym 128

M

M-CSF 83
macromolecules 198
macrophage 83
macrophages 19, 83, 197
Macrostructure 163, 165
Magnesium 125
magnetic resonance imaging 207
magnetron sputtering 132
magnitude 156
malignant transformation 199
Manufacture 200
marker protein 30
market 2
marrow aspiration 73
marrow cells 20
mass transfer limitations 153
mass transport 152, 153, 163
material processing techniques 144
Material sourcing 200
material surface 131, 136
Mathematical modelling 154
matrix-vesicle 142
Matrix Gla Protein 29
matrix maturation 133
matrix proteins 78, 134
matrix vesicles 10, 141
mature animals 186
maturity state 161
MC3T3E1 71
Mechanical 42
mechanical disruption 78
Mechanical effects 40
mechanical effects 143
mechanical environment 38
mechanical factors 194
mechanical loading 49
Mechanical properties 167
mechanical stability 196
mechanical strain 42
mechanical strength 165, 167
mechanical support 7
mechanical tests 35
mechano-active scaffolds 159
mechanocoupling 39
mechanotransduction 39
medical imaging 207
medical imaging technologies 171
medical intervention 203
Melt-based technologies 170
melt moulding 169, 170
membrane lamination 169
membrane potential 140
mesenchymal cells 65
mesenchymal precursors 65
mesenchymal progenitors 67
mesenchymal stem cells 21, 65, 67, 73
metabolic 10
Metabolite 152
metachromatic-staining matrix 75
metallic alloys 121
metalloproteases 24
metalloproteinases 173
metals 47, 121
MG-63 70
Mg-substituted tricalcium phosphates 124
micro-computerised tomography 155
micro-tissues 176
microcontact printing 145
Microencapsulation 198
microfracture techniques and spongialisation 57
microgravity 158
microgrooves 137, 145
micromachining 145
micromass technology 149, 173
microsphere 174, 177
Microstructure 163
microtopographies 144
Microtopography 131
microtopography 138
microvascular failures is 85
microvascular flaps 55
midzone 25, 27
migration 134
mineral-like foci 74
mineralisation 15, 133, 141
mineralisation processes 142
mineralised matrix 8, 137
mineral formation 15, 74, 160
mineral spherites 141
mitotic index 77
mmunological Aspects 196
modelling 39, 42
modified cells 70
modulus of elasticity 36
molecular features 87, 89
molecular imprinting 146
molecular recognition 145
molecular transport mechanisms 42, 143
monocytes 83
mononuclear cells 83
mononuclear precursors 10
moral 206
morphogens 20, 24
Morphological aspects 87, 89
morphological investigations 87, 89
mosaicplasty 59
multicellular systems 2, 163
Multicentre studies 205
multilineage developmental potentia 67
multilineage potential 76
multinuclear 19
multinuclear cells 19
muscles 35
mutagenesis 212
myoblasts 65
Myofibroblast 68
myogenic 76

N

Nanotechniques 146
Nanotopography 131
nanotopography 138, 144
natural materials 121
necrosis 179
neo-angiogenesis 24
neonatal limb development 52
Neovascularisation 155
neovascularisation 84, 209
non-collagenous 15
non-collagenous proteins 8, 10
non-recombinant growth factors 161

non-transformed clonal cell lines 67, 70
nuclear cloning 69
nucleation core complexes 141
nucleator 15
nucleus pulposus 25
number of passages 81
nutrient 165
nutrient/waste exchange 32
Nutrient concentration 152, 179
nutrient supply 149
Nutrition 149
Nutritional Supply 179
nutritional supply 165

O

octacalcium phosphate 124
ODF 83
oestrogen receptor 41
oncogenesis 212
OPGL 83
ordered nanostructures 145
ordered topographical microstructures 138
Organic materials 122
organic matrix 129
organic solvents 172
organotypic cultures 176
orthotopic 186
osmotic pressure 45
osteoarthritis 186
osteoblast 8, 17
osteoblastic phenotype 81
osteoblasts 17, 19, 78, 85
osteoblast markers 78
osteocalcin 16, 17, 158
osteochondral defects 59
osteoclasts 10, 19, 83, 85
osteocyte 8, 17, 78
osteogenic 76
osteogenic cells 67
osteogenic pathway 31
osteoid-like matrix 141
osteoinductive growth factors 53
osteonectin 16, 52
osteons 8
osteopontin 52, 133, 158
osteoprogenitor cells 78
osteoprogenitor phenotype 74
Osteoprotegerin 19
osteosarcoma 138
osteosarcomas 70
osteosarcoma cell lines 70
outcome measures 187
Outgrowth cultures 79
overlying membrane 61
oxygen 151, 165
Oxygen concentration 152
oxygen consumption 154
oxygen delivery 150
oxygen measurements 155
Oxygen need 152
oxygen supply 149
oxygen tension 32, 150, 179
oxygen transport 154

P

paracrine 17
paracrine factors 85
parathyroid hormone (PTH)-related peptide (PTHrP) 31
partial-thickness defects 57
particle shape 137
particulate leaching 169, 170
passive diffusion 152
patency 84
Patterned material surfaces 144
patterned surfaces 133
patterned surface topographies 144
paxillin 134
pedicled flaps 55
perfusion flow bioreactors 159
pericellular 45
Pericyte 68
periosteal autotransplantation 58
periosteal cells 20
Periosteal flaps 53
periosteum 20
pH 133
phagocytosis 197
Phase inversion 170
phase inversion 169
phase separation 171
phenotype plasticity 38
phenotypic differentiation 134
phenotypic instability 71
Phenotypic markers 74
Phenotypic plasticity 83
phospholipid strips 145
phosphorylation 13
photolithography 145
phylogenetic scale 180, 182
physical signals 35
piezoelectric effects 42
piezoelectric potentials 159, 160
pigs 182, 186
planar surfaces 138
plasma spraying 132
plasmids 148
plasticity 78
plastic deformation 196
platelet-derived growth 34
platelet-derived growth factor 34, 53, 161
pluripotent stem cells 65
polyhydroxy acids 165
polymeric foam 171
polymers 47, 121
polymer fabrication techniques 168
polymer structure 123
polymethylmetacrylate 132
Pores 165
pore distribution 170
pore geometry 172
pore interconnectivity 165, 167
pore size 166, 167, 170
porogen 170
porosity 125, 152, 165, 167, 169, 170
pre-lamination 62
Prechondrogenic cells 31
precipitation 141
Preclinical Activity 201
Preclinical Safety 200
preosteoblasts 17
pressure 41, 45, 149, 157
pressure gradients 41, 152
Pressure magnitudes 43
pridie drilling 57
primary cells 70, 71
primates 183
processability 168
processing accuracy 171
processing technique 168
Product consistency 200
Product stability 200
progenitor cells 65, 150
proliferation 134
promyeloid precursor 83
promyeloid precursor cell 19

prostaglandines 65
proteases 24
protection 7
protective functions 10
proteinaceous interface 76
proteins 15
protein adsorption 131, 132
protein coatings 136, 146
protein kinase 136
protein molecules 133
protein recognition sites 146
proteoglycans 8, 15, 125, 177
proteolytic 19
proton pumps 19

Q

quality assurance programme 200
quality control 205
quality controls 205
Quality management 200
Quality Management Systems 199
quality system 200
quantitative element determinations 141

R

radial zone 44
Radiation effects 40
radiographs 194
RANK 19
RANKL 19, 83
Rapid prototyping 171
rapid prototyping 207
RCJ cell lines 71
rdered nanotopography 138
reaggregate approaches 111, 175
reaggregation technology 177
recombinant 161
recombinant cytokines 76
recombinant proteins 53
reconstructive bone surgery 203
reconstructive surgery 47, 194
regenerative medicine 1
regulations 199
regulatory frameworks 199, 201
Regulatory Issues 201
release kinetics 161
release technique 161
remodelling 41, 42
resilience 37
resorbable substitute materials 121
resorption lacuna 19
resorption site 19
revascularisation 180
RGD peptides 146
rheological properties 28
ribs 25
rigidity 37
rigid structure 7
Roller tube culture systems 174
ROS 70
rotating wall vessel bioreactors 158, 159
roughness 132
roughnesses 137
ruffled border 19

S

S-100 176
S/100 protein 30
safety 187
sarcoma cells 67
sarcoma cell lines 71
scaffold 153
scaffold-free spheres 174
scaffolds 121, 152
scaffold architecture 163
Scaffold composition 163, 165
Scaffold deformation 159
scaffold design 207
Scaffold Fabrication 167
scaffold integrity 196
scaffold structure 163
sealing zone 19
secreted proteins 74
seeding techniques 165
selection strategies 180
selective adsorption 145
selective markers 73
selective surface coating 145
self-renewal 174
series 70
serum 175
serum replacements 76
serum supplementation 77
shaving 57
shear 35, 152
shear stress 153, 158
side effects 205
signalling molecules 53, 151
signalling process 136
signal factors 23
signal molecules 24
signal transduction 45, 86, 134
sintered 129
sinus lift 182, 196
size 137
skeletal defects 182
skeletal formation 4
Skeletal mineralisation 10
soft tissue 35
soft tissues 13
soft tissue envelope 35
solid-free form fabrication techniques 168
solid constituents 43
solid free-form fabrication 171
soluble factors 86
soluble nutrients 151
solution casting 169
solvent casting 169
solvent residues 170
somatic gene therapy 206
spacial geometry 163
spatial composition 167
spatial sorting 174
spinner flask 152
spinner flasks 158, 159, 174
spreading 136
Static cell seeding 164
static compression 41
steel 121
stem 150
stem cell 65, 203
stem cell research 207
stem cell technology 204
steoblastic phenotype 138
sterility 199, 200
sternum 73
stiffness 37
stirred flask 152
stoichiometry 124
strain 43, 45, 156, 157, 194, 195
strains 40
strain environment 156
strain fields 158
strain gauges 37
strain magnitude 158
strain sensor 41

strain threshold 39
streaming potentials 42
stress 35, 43, 45, 156, 157
stress environment 195
stromal fibroblastic cells 67
stromal stem cells 67
structured surfaces 132
subchondral bone layer 44
subchondral bone plate 25, 27
Subchondral trabecular bone 25, 27
substrate deformation 41
substrate monitoring 155
superficial 44
superficial cartilage layers 43
Superficial zone 25
Surface/volume ratio 163, 167
surface antigens 74
surface characteristics 122
surface chemistry 131, 139, 140
surface coatings 146
surface energy 132
surface properties 121, 123, 131
surface receptors 74
surface roughness 139
surface structures 143
surface texture 137
Surface Topography 137
surface topography 131, 138, 143, 167
suspension cultures 174
SV40-TAg 72
symphysis pubis 25
Synthetic inorganic materials 122
synthetic organic materials 121
synthetic serum substitutes 77

T

talin 134, 146
technical procedure 168
temperature 133, 149
template molecules 146
Tenocyte 68
tensile strains 39
tensin 134
tension 35
tension forces 158
teratocarcinomas 69
teratomas 69
territorial 45
TGF-β 212
TGF-β1-3 24
TGF-β superfamilie 24
theoretical models 38
therapeutic use 199
thermic effects 40
three-dimensional morphology 131
three-dimensional plotting 172
three-dimensional plotting 172
three-dimensional printing 172
thrombin 127
thrombosis 85
thrombospondin 133
Thymic stromacell 68
thymogenic 76
tissue bank 199
tissue banks 200
tissue biomechanics 25
tissue engineering 187
tissue grafts 47
tissue implantation 47
tissue micromass 5
tissue necrosis 48
tissue pressure 45
tissue substitutes 2
tissue typing 69
titanium 121, 136
titanium implants 143
tolerance 197
topographical grooves 139
topographical sensors 37
topographies 138
total joint replacement 56
Toxicity testing 200
Toxic effects 205
toxic solvents 169
trabecular bone 27
trabecular system 8
trachea 25
traction 136
transcription 211
transcription factors, 78
transforming growth 161
transforming growth factor-beta 34
transforming growth factor-beta1, -beta2, -beta3 52
transforming growth factor-β3 75
Transforming growth factor beta 53
transforming growth factor family 146
Transgenes 210
translation 211
transmission of signals 40
transplantation resources 205
traumatic knee injuries 2
tricalcium phosphates 165
tubes 165
tube diameter 166
tube geometry 165
tubular structures 153
tumorigenic differentiation 69
tumornecrosis factor alpha 24
type II collagen 75
type IX collagen 13
type I collagen 133
type X collagen 13, 27

U

Ultrasound application 49
ultrastructural analysis 87, 89
UMR 70
Underlying material 131
unsintered 129
urea 151
urface Chemistry 139
US National Science Foundation 4
UV irradiation 127

V

vacuum sublimation 171
van der Waals forces 133
vapour deposition 145
vascular-like tissue 155
Vascularisation 179, 208
vascularisation 180
Vascularised bone 53
vascularised bone tissue 55
vascular assembly 209
vascular cells 84
vascular endothelial growth factor 24, 209
vascular growth 24

Vascular induction 62
vascular ingrowth 166
vascular invasion 84, 196
vascular tissues 24
vector concentration 210
vessel- formation 24
vinculin 32, 134, 136, 146
visco-elastic properties 158
vitronectin 133

W

waste products 154
waste removal 149
wettability 131, 140
wetting techniques 165
whitlockite 124
wound infection 48
woven bone 48

X

xenogenic cells 66
Xenografts 198
xenografts 196
xperimental model 182

Y

Young´s modulus 36

Z

zeta potential 131, 139, 140
zinc 125
Zn-substituted tricalcium
phosphate 124
zonal assembly 25
zonal structure 25, 43