Tissue Regeneration

Biological Theory, Modeling and Applications

Shay Fisher

Volume II

Tissue Regeneration: Biological Theory, Modeling and Applications

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Edited by **Shay Fisher**

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Preface

In a majority of incidents involving damage or injuries, the healing process involves formation of a scar, which is like a patch, reinstating structural integrity of the damaged tissue without reviving physiological functions. A far better option for a patient affected by tissue damage would be to replace the damaged tissue with something functionally similar. Increasing amount of study and research is being undertaken across the world in search for such a technology. This book provides a timely overview on crucial topics in tissue regeneration, in a well-researched manner, written by experts from around the globe. It facilitates better understanding of the application of stem cells, uses of scaffolds and modelling and evaluation of regeneration. This book will be a great source of reference for study and work undertaken by students, experts and medical professionals.

Various studies have approached the subject by analyzing it with a single perspective, but the present book provides diverse methodologies and techniques to address this field. This book contains theories and applications needed for understanding the subject from different perspectives. The aim is to keep the readers informed about the progress in the field; therefore, the contributions were carefully examined to compile novel researches by specialists from across the globe.

Indeed, the job of the editor is the most crucial and challenging in compiling all chapters into a single book. In the end, I would extend my sincere thanks to the chapter authors for their profound work. I am also thankful for the support provided by my family and colleagues during the compilation of this book.

Editor

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Part 1

Application of Stem Cells

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Towards Clinical Application of Mesenchymal Stromal Cells: Perspectives and Requirements for Orthopaedic Applications

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1. Introduction

Mesenchymal stromal cells (MSC) possess a wide spectrum of interacting properties that contribute to their broad therapeutic potential: In pre- and clinical settings MSC have been demonstrated to reduce tissue damage, to activate the endogenous regenerative potential of tissues and to participate in tissue regeneration (Noort, Feye et al. 2010). Initially, MSC have been described to differentiate into derivates of the mesoderm: bone, adipose and cartilage tissue and were therefore applied to restore damaged tissue (Frohlich, Grayson et al. 2008). Subsequent analyses, however, indicated that the repair process does not only lay in the differentiation potential and plasticity of MSC. As demonstrated in later studies even if only few cells were detectable after MSC transplantation, the therapeutic effect was obvious (Fuchs, Baffour et al. 2001; Shake, Gruber et al. 2002). This could be attributed to paracrine properties with consecutive modification of the tissue microenvironment to decrease inflammatory and immune reactions. MSC are therefore beyond doubt promising candidates for cell therapy in various settings (Horwitz, Prockop et al. 2001; Le Blanc, Rasmusson et al. 2004; Prockop 2009; Pontikoglou, Deschaseaux et al. 2011).

The broad therapeutic efficacy of MSC renders them attractive candidates for cell therapy. However, translating basic research into clinical application is a complex multistep process (Bieback, Karagianni et al. 2011). It necessitates product regulation by the regulatory authorities and accurate management of the expected therapeutic benefits with the potential risks in order to balance the speed of clinical trials with a time-consuming, cautious risk assessment (Sensebe, Bourin et al. 2011). Despite their use in clinical studies, some questions remain open: What are the deviations among the MSC from different tissue sources? How shall MSC be adequately procured, isolated and cultivated? How should their therapeutic propensity, e.g. their homing properties, the secretion of bioactive factors, the differentiation pattern *in vivo* and their plasticity, be defined?

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It is obvious that MSC need to be further characterised in clinical studies with standardized protocols (Bieback, Karagianni et al. 2011; Sensebe, Bourin et al. 2011). Furthermore, despite immense work, still MSC cannot be identified as a distinct cell population by a set of marker proteins as CD34 defines hematopoietic stem cells. The field currently uses "minimal criteria" for MSC to describe them according to their *in vitro* behaviour (osteo-, adipo- and chondrogenic differentiation) and morphology (fibroblastoid, expressing a set of markers) (Dominici, Le Blanc et al. 2006). Nevertheless it has to be taken into account that *in vitro* data do not necessarily predict *in vivo* behaviour: MSC seem to alter their *in vitro* traits after *in vivo* transplantation and this might affect a future therapeutic outcome severely. For example MSC can express HLA-class II antigens and can therefore possibly trigger an immunreaction in the host after transplantation (Vassalli and Moccetti 2011) or may calcify spontaneously in uremic conditions and cause vessel occlusion in case of intravenous application (Kramann, Couson et al. 2011).

Using the example of bone defect regeneration, we will emphasize key parameters relevant for the translation of experimental data to clinical application. The focus on bone defect regeneration exemplifies the possibilities and challenges for MSC in combination with biomaterials in the light of regulatory frameworks in Europe, where MSC may be classified as "Advanced Therapy Medicinal Product - ATMP", or the US, where MSC fall under the term "Human Cells, Tissues, and Cellular and Tissue-Based Products -HCT/Ps". In this context, questions that need to be answered concern an adequate MSC tissue source with superior osteogenic potential compared to other tissues, the degree of cell differentiation prior to implantation and the adequate scaffold for tissue engineering (Seong, Kim et al. 2010).

1.1 MSC definition

Mesenchymal stromal cells (MSC) were initially isolated from bone marrow (BM) as described by Friedenstein and co-workers in 1968 (Friedenstein, Petrakova et al. 1968). They were identified as non hematopoietic, fibroblast-like cells adherent to plastic, with a colony-forming capacity (Friedenstein, Deriglasova et al. 1974), also as feeder cells for hematopoietic precursors (Eaves, Cashman et al. 1991; Wagner, Saffrich et al. 2008). Subsequent characterisation revealed their mesodermal differentiation and immune modulatory capacity, raising the interest in these cells (Le Blanc, Rasmusson et al. 2004; Bieback, Hecker et al. 2009; Mosna, Sensebe et al. 2010). Consequently, numerous terms for these cells were established: mesenchymal stem cells, mesenchymal stromal cells, adult stromal cells, multipotent and non hematopoietic adult precursor cells (Horwitz, Le Blanc et al. 2005; Dominici, Le Blanc et al. 2006). These conflicting nomenclature suggestions in the literature lead to a complex information exchange upon MSC (Prockop 2009). In an attempt to clarify and define the nomenclature, the ISCT (International Society for Cell Therapy) set "minimal criteria" for MSC, such as:

- adherence to plastic when maintained in standard culture conditions,
- expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules,
- as well as differentiation ability into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici, Le Blanc et al. 2006).

In the last decade there has been rapid movement from bench to bedside. Based on their stromal origin, MSC were initially applied in co-transplantation studies with hematopoietic

precursor cells (Koc, Day et al. 2002). Later, due to their mesodermal differentiation potential, Horwitz et al. were able to perform seminal studies applying MSC to children with osteogenesis imperfecta (Horwitz, Prockop et al. 2001). MSC were then applied as immunosuppressants in patients with graft versus host disease (Le Blanc, Rasmusson et al. 2004). Further studies introduced them as promising candidates for tissue regeneration in bone and cartilage repair (Frohlich, Grayson et al. 2008), epithelial regeneration (Long, Zuk et al. 2010), cardiovascular regeneration (Noort, Feye et al. 2010; Rangappa, Makkar et al. 2010), immunomodulation in graft versus host disease (GvHD) (Ringden, Uzunel et al. 2006), and inflammatory neurological diseases (Momin, Mohyeldin et al. 2010). MSC are expected to reduce tissue damage, to activate the endogenous regenerative potential of tissues and to participate in the regeneration (Noort, Feye et al. 2010). However, in all these studies it became apparent that MSC function mainly through paracrine effects rather than differentiating into cells or tissues (Caplan and Correa 2011).

1.2 MSC from different tissue sources

Bone marrow (BM) was the first source of MSC identified by Friedenstein and co-workers (Friedenstein, Gorskaja et al. 1976). BM-MSC are already being tested worldwide in clinical studies with currently over 1500 found in the Clinical Trials registry of the NIH (www.clinicaltrials.gov). Due to the long lasting research on BM-MSC they became the gold standard for any MSC research and therapeutic application. Nevertheless, a limitation for BM MSC clinical application is the low cell frequency in source tissue. Thus large volume bone marrow aspiration is necessary even in autologous settings, feasible only in general anaesthesia which is associated with an additional patient morbidity. In consequence, investigators have developed protocols for isolating MSC from a variety of different tissues and sources other than bone marrow. Latest studies led to the conclusion that MSC are not limited to a certain tissue source: the MSC niche is rather localized in the perivascular area of virtually all tissues (Crisan, Yap et al. 2008; da Silva Meirelles, Caplan et al. 2008). Thus numerous tissues containing MSC have been identified, for example adipose tissue (AT), cord blood (CB), fetal membranes and amniotic fluid, pancreatic islet, lung parenchyma, intestinal lamina propria, oral and nasal mucosa, eye limbus, dental tissues and synovial fluid (Jakob, Hemeda et al. ; Karaoz, Ayhan et al. ; Marynka-Kalmani, Treves et al. ; Pinchuk, Mifflin et al. ; Powell, Pinchuk et al. ; Zuk, Zhu et al. 2002; Kern, Eichler et al. 2006; Phinney and Prockop 2007; Jones, Crawford et al. 2008; Polisetty, Fatima et al. 2008; Huang, Gronthos et al. 2009; Ilancheran, Moodley et al. 2009; Karoubi, Cortes-Dericks et al. 2009).

Among all tissue sources, AT shows several important clinical advantages compared to BM: AT procurement can be achieved via tumescent-lipoaspiration in local anaesthesia, a lower risk operating procedure. Adipose tissue is abundant even in older individuals. AT-MSC are shown to have similar functional properties to BM-MSC while their frequency is definitely higher than in BM (Zuk, Zhu et al. 2002; Kern, Eichler et al. 2006). AT-MSC are currently being applied in clinical trials, at least 33 trials can be found in the NIH registry. The high frequency of MSC in AT renders it possible to isolate the mononuclear cell fraction directly at the patients bedside without the need for expansion in a GMP facility (Duckers, Pinkernell et al. 2006). There are divergent outcomes in those studies directly comparing freshly isolated with expanded cells (Garcia-Olmo, Herreros et al. 2009). Despite the advantages of processing at the patient's bedside, direct application of the freshly isolated

mononuclear cells in one session procedure gives no opportunity to control the clinical outcome, for an amount of diverse undefined cell populations are effective in these settings. However, this is still being exercised as autologous treatment.

Studies are being performed in order to compare BM-MSC, AT-MSC and MSC of other tissue sources. They show that MSC are not one distinct cell population. Among their tissue sources MSC differ concerning their isolating rate, their expansion potential, their differentiating capacities (Kern, Eichler et al. 2006), their immunosuppressive and migratory properties (Najar, Raicevic et al. ; Constantin, Marconi et al. 2009). These differences have probably an impact on their quality and therapeutic ability, which only can be definitely clarified in "*in vivo*" studies. Summarizing, there is a complex algorithm, which should be followed in order to find the adequate tissue source for MSC cell therapy. Very important are:

- the patient's risk associated with the tissue procurement,
- the MSC frequency in the origin tissue stroma,
- the potential of MSC to be enrolled in its therapeutic function *in vivo*.

All this can rather be answered gradually applying standardized protocols. After procurement and expansion MSC have to be analysed regarding their functional properties through well defined *in vitro* potency assays. Finally functional properties have to be compared *in vivo* through animal studies and phase I clinical trials.

2. MSC protocols for clinical applications

Translating MSC into cell therapy settings requires a manufacturing process and manufacturing authorisation congruent to the local regulatory framework. Regulatory standards in the EU and USA comply with the good manufacturing practice (GMP) regulations and are set in order to control the therapeutics' safety process, e.g. tissue procurement, cell isolation, selection and expansion and have to be validated according to the quality criteria as defined by the manufacturer. Furthermore it is essential to control the quality, purity and potency of the cell product prior to their administration by well defined and validated quality control and potency assays to ensure safety.

2.1 Isolation and expansion of MSC for clinical applications

For clinical applications, MSC shall be isolated under aseptic conditions in GMP facilities. MSC are a subpopulation among the mononuclear cell fraction. They can be isolated after density gradient centrifugation or if MSC are embedded in extracellular matrix after enzymatic digestion. In general, the low frequency of human MSC within their origin tissues necessitates their expansion prior to clinical use. This raises the risk for contaminations (Bieback, Karagianni et al. 2011; Sensebe, Bourin et al. 2011). Furthermore, in long term cell culture the proliferation rate decays, the cell size increases, differentiation potential becomes affected and chromosomal instabilities and neoplastic transformation may arise (Prockop, Brenner et al.; Lepperdinger, Brunauer et al. 2008; Wagner, Horn et al. 2008) raising the risk for adverse reactions.

Similarly, the cultivation media potentially affect MSC, exposing them to pathogens and immunogens (Heiskanen, Satomaa et al. 2007; Sundin, Ringden et al. 2007; Bieback, Hecker et al. 2009). In order to achieve controlled conditions and a safe cell product for clinical

use it is necessary to define quality criteria to monitor the cell product (Bieback, Schallmoser et al. 2008; Bieback, Karagianni et al. 2011; Sensebe, Bourin et al. 2011). For expansion aiming at clinical application it is obligatory to use GMP-grade supplements and sera if available. However, these reagents are just under development. Accordingly we, amongst others, tested human blood-derived components, like human serum or platelet derivatives to replace fetal bovine serum commonly used to expand MSC (Kocaoemer, Kern et al. 2007; Mannello and Tonti 2007; Bieback, Schallmoser et al. 2008; Bieback, Hecker et al. 2009). Human blood components offer the advantage that they are both well controlled and already in clinical use for decades. Still, human serum as well as platelet lysate is a very crude protein cocktail. Essential growth factors for optimal MSC culture have not yet been defined. Platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF-ß), and insulin growth factor (IGF) have been subjected to investigation. Basic fibroblast growth factor (bFGF) has demonstrated most promising effects in expanding MSC whilst maintaining stem cell properties and reducing replicative senescence (Tsutsumi, Shimazu et al. 2001). Recently, Pytlik et al described a human serum and growth factor supplemented clinical-grade medium, which allowed high cell expansion mediated by loss of contact inhibition (Pytlik, Stehlik et al. 2009). Anyhow, the ideal solution is a chemically defined clinical-grade medium permitting both adhesion and expansion of MSC and numerous attempts are ongoing to develop this (Mannello and Tonti 2007).

2.2 Quality control

In order to obtain a manufacturing authorization for cell therapeutics the quality criteria ought to meet the regulatory standards. Quality controls are instrumented within the manufacturing process to prove according to the set quality criteria. Essential quality criteria are the traceability of the cell product through donor identification and product labelling, the prevention of introduction and spreading of infection and communicable diseases through donor screening and aseptic cell processing and proof of the therapeutic safety, lot consistency, potency and purity of the cell product (European Parliament 2007; FDA 2010).

2.2.1 Therapeutic safety, purity and potency

Safety is a key issue in cell therapy. In addition to the above mentioned aspects regarding reagents (fetal bovine serum has been elaborated on) and sterility testing (bacterial, fungal, viral, mycoplasma), cellular aspects have to be considered as well. In long term cell culture current testing methods of chromosomal aberrations and neoplastic transformation are fluoerescence in situ hybridization (FISH), karyotype analysis or detection of proto-oncogenes or activators of tumorigenesis like myc-assosiated proteins (Agrawal, Yu et al. 2010). Further lately developed testing methods are BAC-based (Bacterial Artificial Chromosome) Array to detect DNA copy number or oligonucleotide-based Array CGH (Chromosomal Comparative Genomic Hybridization) to detect small genomic regions with amplification or deletion (Wicker, Carles et al. 2007). Additionally, detection of telomerase activation is often performed, as telomerase plays a role in malignant transformation *in vitro* (Yamaoka, Hiyama et al. 2011). All these assays indicate that there is a low risk of transformation of MSC in *in vitro* expansion. However, more safety studies - especially long term follow up *in vivo* - are required to exclude risks and to enable to value risks against therapeutic value.

Further aspects that are critical for the therapeutic safety and need to be analysed are the spontaneous or the induced *in vivo* differentiation potential of MSC. It has to be proven that MSC after *in vivo* application serve their therapeutic function and do not develop into unwanted cell types for example BM-MSC into adipocytes or osteocytes when intended for epithelial or myogenic regeneration. The latter could possibly lead to threatening thrombembolic incidents after intravascular application. In general, intravascular injection is associated with a higher risk than direct application into the site of injury or into the neighbouring parenchyma (Furlani, Ugurlucan et al. 2009).

MSC are not a distinct cell fraction in fresh tissue isolates. Accordingly purity is a key issue to be taken into account. To isolate MSC, mononuclear cells of fresh tissue isolates are seeded on plastic culture dishes, MSC adhere, proliferate and form colonies. Those expanded MSC should have a distinct immune phenotype, defined by the ISCT, they do not express haematopoietic markers and have a characteristic fibroblastoid morphology (Dominici, Le Blanc et al. 2006). Based on these criteria, contaminations of MSC with hematopoietic or endothelial cells can be assessed and consequently purity of the MSC cell product can be proven via flow cytometry. This is further amended by description of expanded MSC morphology and colony assays (CFU-F-assay) to quantify the precursor frequency. Quality controls of MSC expanded in scaffolds or in bioreactors vs. 2D cell culture regarding population purity is probably more complex.

MSC are applied in various clinical settings, as they possess a variety of functional properties. MSC can work as progenitor cells in tissue modelling, due to their adipo-, osteo-, chondrogenic potential, or as immunomodulatory agents in GvHD, autoimmune disease or as anti-inflammatory agents through their paracrine abilities. Due to this extremely broad range it is difficult to establish potency assays. These standardized *in vitro* functional assays have to be performed to predict the consistency of the manufacturing process and the functionality of the cell product. Quality control assays, including potency assays, have to be well established and validated to be capable of addressing the consistent quality of the cellular product. It is certainly difficult to reproduce the *in vivo* setting within *in vitro* conditions,. This is probably why *in vitro* potency assays often fail to predict the *in vivo* outcome (Sensebe, Bourin et al. 2011). Anyhow, it is a demand for the manufacturing facility to implement potency assays capable of predicting therapeutic capacity. These assays have to be quantitative and directly related to the mechanism of action. Where possible surrogate assays can replace time-consuming functional assays (e.g. cell surface marker expression, growth factor release, gene or protein expression analysis). Finally, the manufacturing process in order to conduct clinical trials in Europe and the US has to be validated and approved by the authorities in accordance to the pharmaceutical regulations.

2.3 Pharmaceutical guidelines

2.3.1 Advanced therapy medicinal products as described in the Regulation (EC) No 1394/2007 of the European Parliament

In cases where MSC are to be used in a medicinal product the donation, procurement and testing of the cells are covered in Europe by the Tissues and Cells Directive (2004/23/EC). To make innovative treatments available to patients, and to ensure that these novel treatments are safe, the EU institutions agreed on a "regulation on advanced therapies"

(EC1394/2007). Furthermore, a number of products also combine biological materials, cells and tissues with scaffolds. This regulation defines those products as "advanced therapy medicinal products (ATMP)" that are:

- "a gene therapy medicinal product" (Part IV of Annex I to Directive 2001/83/EC),
- "a somatic cell therapy medicinal product" (Part IV of Annex I to Directive 2001/83/EC) and
- "a tissue engineered product".

Cells or tissues shall be considered 'engineered' if they fulfil at least one of the following conditions:

- "the cells or tissues have been subject to substantial manipulation, in order to unfold their biological characteristics, physiological functions or structural properties" or
- "the cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor" (Official Journal of the European Union 10.12.2007).

The scope of this regulation is to set standards for advanced therapy medicinal products which are intended to be placed on the market in European member states. It indicates the setting of manufacturing guidelines specific for ATMP as to properly reflect the particular nature of their manufacturing process. The directive 2004/23/EC amends to this regulation setting standards of quality and safety in tissue procurement and donor testing. Regarding clinical trials on ATMP, they should be conducted in accordance with the Directive 2001/20/EC. Additionally Directive 2005/28/EC laid down principles and detailed guidelines for good clinical practice as well as the requirements for authorisation of the manufacturing and importation of ATMP. Considering tissue engineered cell products, medicinal devices incorporated in the ATMP (combined medicinal products) are regulated by the directive 93/42/and the directive 90/385/ EEC.

2.3.2 Human cells, tissues, and cellular and tissue-based products (HCT/P's) as described by the US Food and Drug Administration (FDA)

The quality system for Food and Drug Administration (FDA) regulated products is known as current good manufacturing practices (cGMP). For globally operating pharmaceutical facilities it is mandatory to fulfil the requirements of both FDA and EU. The Code of Federal Regulation (CFR) Title 21, part 1271 has the purpose to create a unified registration and listing system for human cells, tissues, and cellular and tissue-based products (HCT/P's) and to establish donor-eligibility, current good tissue practice, and other procedures to "prevent the introduction, transmission, and spread of communicable diseases by HCT/P's" (www.FDA.gov).

Whereas cell products, only minimally manipulated or subjected to homologous use without systemic effect, are regulated solely by the Public Health Service (PHS) Act Section 361 and do not require to undergo premarket review (GEN Mar. 15, vol 25, no 6), they still must comply with Good Tissue practice (GTP) (Burger 2003). Clinical trials of higher-risk involving "more-than-minimally manipulated" HCT/P's require the Investigational New Drug (IND) mechanism.

3. Example for MSC in regenerative medicine: Attempts for orthopaedic applications in bone defect healing

Orthopaedic surgery provides a fascinating field for the application of MSC (Horwitz, Prockop et al. 2001; Le Blanc, Gotherstrom et al. 2005; Bernhardt, Lode et al. 2009; Chanda, Kumar et al. 2010; Diederichs, Bohm et al. 2010; Mosna, Sensebe et al. 2010; Parekkadan and Milwid 2010; Levi and Longaker 2011). Bone defects appear in increasing numbers in orthopaedic clinics due to aseptic loosening of hip endoprosthesis after 10 to 20 years. These defects are then covered primarily with either bone cement or acellular bone from a bone bank prior to insertion of a new endoprosthesis in order to provide primary stability - that is immediate mechanical support of a new implant (Gruner and Heller 2009).

An ideal scaffold must offer osteoinduction - induction of bone growth - and osteoconduction - providing the guiding structure that paves the way for future bone growth - and eventually osteointegration, becoming part of the bone architecture of a body (Frohlich, Grayson et al. 2008; Ferretti, Ripamonti et al. 2010). The advantages and disadvantages of bone cement have been controversially discussed regarding different rates of implant failure in follow up examinations (Kavanagh, Ilstrup et al. 1985; Izquierdo and Northmore-Ball 1994; Stromberg and Herberts 1996). Recent works suggest to proceed without use of bone cement if possible, and recommend other surgical techniques to implant a total hip endoprosthesis. Bone cement is stiff and strong with a gradual increasing resorption area at its limits. Where bone cement is placed, immediate primary stability is provided, however, at the expense of bone regeneration that does not take place anymore (Izquierdo and Northmore-Ball 1994; Gruner and Heller 2009). Depending on the localization of the bone cement and the mechanical stress, this can gradually lead to a decreased stability. In case another revision operation is needed but great bone defects and osteolysis can impede or even inhibit surgical possibilities (Kavanagh, Ilstrup et al. 1985; Izquierdo and Northmore-Ball 1994; Stromberg and Herberts 1996; Gruner and Heller 2009). Fresh autologous bone or allogenous acellular bone from a bone bank can support bone growth. These preparations are osteoconductive and are, if preserved as a cancellous bone even osteoinductive but fail to provide immediate stability alone. These scaffolds have osteoconductive potential, however regular radiological controls often demonstrate gradually increasing resorption at sites of the implanted acellular bone. In the consequence, stability may be compromised (Gruner and Heller 2009).

Given the potential of MSC to differentiate into bone, MSC became attractive candidates. For hard tissue replacement, cells alone are not adequate. Thus surgical procedures treating bone defects in which a combination of MSC and scaffolds are applied, may provide both immediate stability and permanent integration into the recipient's bone. Different techniques are described for the implantation of MSC. Still it remains unclear if implants shall carry completely osteogenically differentiated MSC, or more likely optimize adaptive possibilities within the host organism. The more differentiated the MSC the more initial stability they provide for implants in areas with high mechanical force exposure (Bernhardt, Lode et al. 2008). Less differentiated MSC on the other prove more plasticity (Niemeyer, Krause et al. 2004; Bieback, Kern et al. 2008). In the worst case, undesired differentiation or even dedifferentiation might occur. Medication, integrated drugs or even genetically engineered cells may prove a possible control *in vivo*.