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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*.

3.1 *In vitro* 3D culture, choice of scaffold

Tissue engineering aims at regenerating or replacing tissues or even organs. Therefore a complex architecture is needed, which cannot be generated by simple two-dimensional (2D) cultures. Investigation on MSC concentrates on characterization *in vitro* in a 2D culture, as mentioned above, to assess both the differentiation potential and the influence of the biomaterial surface on growth and development. MSC can be driven towards osteogenic differentiation by use of dexamethasone, β-glycerophosphate and ascorbate in addition to osteogenic basal medium (Jaiswal, Haynesworth et al. 1997; Pittenger, Mackay et al. 1999; Augello and De Bari 2010). Cells can be used as undifferentiated, pre- or terminally differentiated cells in combinations with scaffolds to achieve tissue-like conditions. Compared to 2D, 3D cultures better mimic physiological conditions. Static 3D cultures are mainly used to investigate the suitability of a certain biomaterial (Bernhardt, Lode et al. 2009). Increasing attention is recently been paid to dynamic 3D culture, assuring a more homogenous cell distribution within a scaffold, a higher number of cells and all in all less manipulation (Diederichs, Roker et al. 2009; Stiehler, Bunger et al. 2009). Flow perfusion cultures itself, even in absence of dexamethasone, may lead to differentiation into bone tissue (Holtorf, Jansen et al. 2005). Nevertheless, cell expansion of MSC in order to achieve a high cell dose prior to use in animal or humans may not always be advantageous, since uncontrolled growth can also lead to benign or malign tumours.

There is a broad choice of biomaterials for scaffolds for clinical applications. However, only bone cement and bone of bone banks are regularly favoured for bone defect surgeries, when available. Bone itself has become the biomaterial per se as a natural scaffold supply. Bone cement on the other hand can be stored as powder, provides immediate stability and is easily prepared and applied during an operation. Within a few minutes, the cement becomes firm (Gruner and Heller 2009). Although acellular scaffolds prove stability immediately following implantation, a better option would be to seed them with cells. For MSC application a great variety of materials, ranging from sterilised original bone to nanostructures and bioglass-collagen composites are being utilised (Karageorgiou and Kaplan 2005; Tanner 2010). Eventually, in order to approach the therapeutic effect of scaffold-MSC composites, studies are currently being performed on several stages: cell culture either in a dish or in a bioreactor, animal models and individual attempts in human (Bernstein, Bornhauser et al. 2009; Diederichs, Roker et al. 2009; Diederichs, Bohm et al. 2010). Further key parameters for the choice of the suitable biomaterial is the ability to support cell growth, cellular ingrowth, osteogenic differentiation and antimicrobial functions (Costantino, Hiltzik et al. 2002; Bernstein, Bornhauser et al. 2009). For that reason, additional osteogenic cytokines such as bone morphogenetic proteins (BMP) or bioactive peptides that become integrated into scaffolds are of interest (Keibl, Fugl et al. 2011).

An optimum scaffold must allow bone cells to grow into it. Pores of 300 to 500µm are requested (De Long, Einhorn et al. 2007; Stiehler, Bunger et al. 2009). Apart from this an optimum scaffold has to be adapted to bone structures. Defects in facial areas, in the skull, femur or hip require different stabilities and shapes. Only hip re-implantation seems to provide some standardised features (Gruner and Heller 2009).

Building suitable biomaterials to be combined with MSC has led to very different approaches: Collagen as a basis of any bone tissue was modified and calcified at all pore

sizes. Integration of MSC is easily achieved but primary stability is comparably low (Bernhardt, Lode et al. 2009; Nienhuijs, Walboomers et al. 2011). Hydroxyapatite is a ubiquitous part of the vertebrate bone. Hydroxyapatite ceramics become easily integrated and also prove enough primary stability (John, Varma et al. 2009; Nair, Bernhardt et al. 2009; Nair, Varma et al. 2009). Beta-tricalciumposphate is a completely resorbable scaffold with high purity. It is available at all sizes, all porous degrees, it can be supplied as granules or as plates and therefore serves as comparison to newly developed biomaterials (Wiedmann-Al-Ahmad, Gutwald et al. 2007). Due to its low tissue reactivity and good stability titanium based structures not only serve well as implants but also as scaffolds. Titanium or TiO_2 does not become degraded or resorbed, instead as a whole it becomes very firmly integrated into any tissue (Gotman 1997; Olmedo, Tasat et al. 2009). Due to the fact that titanium is not resorbable it holds the risks of infection, be it acute or slowly increasing, so that an explantation must be performed. Since titanium becomes very well integrated into the host's body, an explantation is often associated with a great tissue loss. Application of titanium has to be carefully considered. In sum, since tissue reactions to titanium are quite well characterised as an implant it serves well as an example of future challenges and possibilities of other biomaterials. Silver nanoparticles are matter of current discussion due to their antimicrobial and toxic effects that can also be used within polymeric nanocomposites. Titanium nanostructures alone have been proven to act antimicrobially (Dallas, Sharma et al. 2011; Ercan, Taylor et al. 2011).

3.2 Analysis of 3D cultures and biomaterials

Once a 3D scaffold has been seeded, the efficiency of the seeding procedure, cell growth and differentiation must be determined, e.g. by quantifying the DNA content and mineralisation by histochemical stains or RT-PCR (Stiehler, Bunger et al. 2009; Peister, Woodruff et al. 2011). Homogeneic seeding and / or cell growth can be determined by fluorescence microscopy or µCT (Zou, Hunter et al. 2011). Mechanical tests are not standardized. For *in vitro* generated bone tissue from MSC crush tests, i.e. the use of a defined force until a scaffold breaks, are the most simple. For *in vivo* generated bone tissue shear and bending tests give additional data concerning the stability of the MSC composite within the animal's original bone. However, *in vitro* and *in vivo* experiments are only conclusive when scaffolds used are comparable in size and porosity (De Long, Einhorn et al. 2007; Stiehler, Bunger et al. 2009). The same applies to standardisation of surgical procedures and animal models used (Reichert, Saifzadeh et al. 2009).

3.3 Tissue source

As already mentioned, tissue engineering requires a scaffold next to the cells to seed it. Since MSC can be isolated from different tissue sources, the question remains: which cells are best suited? MSC derived from different tissues show different osteogenic differentiation properties: human embryonic stem cells (hESC), CB-MSC, AT-MSC, BM-MSC and even amniotic membrane-derived MSC can undergo osteogenic differentiation. Historically, most work had been performed on BM-MSC, so at least BM-MSC are the source to compare with, when MSC behaviour in a scaffold is analysed (Lindenmair, Wolbank et al. 2010; Guven, Mehrkens et al. 2011; Stockmann, Park et al. 2011; Weinand, Nabili et al. 2011). In recent studies, aspects of differentiation in 2D tissue culture and in 3D tissue culture have been

examined. Comparisons between BM-MSC and amniotic fluid derived stem cells (AFS) showed different properties in differentiation in 2D and 3D. In 2D tissue culture, AFS produce more mineralized matrix but delayed peaks in osteogenic markers. Differentiation towards bone tissue occurred faster in BM-MSC, however, after weeks mineralization slowed down. AFS differentiated more slowly but mineralized until the end of the observation period 15 weeks, producing 5 fold higher amounts of mineral matrix. Human term placenta derived MSC seem to be less prone to osteogenic differentiation than BM-MSC (Pilz, Ulrich et al. 2011). These characteristics might be of interest, when fast ingrowth is needed (Peister, Woodruff et al. 2011). As initially mentioned, for some groups AT-MSC are the most promising candidates in bone tissue engineering (Levi and Longaker 2011). Osteogenic capacity does not decrease with age in contrast to BM-MSC (Khan, Adesida et al. 2009). Also due to a relatively high and still increasing rate of obesity in the western hemisphere it can be considered that adipose tissue has a great potential as main source for MSC. So, metabolic disease can be of benefit when it comes to autologous MSC implantation (Diederichs, Bohm et al. 2010). All in all an ideal cell source has yet not been identified. Further research is important to compare the advantages of all tissue sources. Moreover, for each biomaterial the MSC differentiation properties have to be determined. The adequate MSC will depend both on availability and differentiating / functional properties.

3.4 Clinical trials

There is no on-going clinical trial that deals with the use of MSC and a suitable biomaterial in healing of bone defects in humans. Osteogenesis imperfecta has been successfully treated with MSC alone, even with allogenic MSC (Horwitz, Prockop et al. 2001; Le Blanc, Gotherstrom et al. 2005). The Iranian Royan Institute, Teheran, announced a clinical trial in 2008 (http://www.clinicaltrials.gov). The study aimed to establish the influence of MSC in non-union fracture healing. However, in 2011 the state of the study is still unknown and cannot be verified. One case report from 2009 refers to a clinical trial in preparation. The benefit of the use of decellularized bone and MSC was demonstrated in a case of large hip transplant loosening. Follow-up radiological exams could confirm the stable position of a new hip implant (Bernstein, Bornhauser et al. 2009). So far, no clinical trial on the use of MSC for bone fracture healing has been published. Various preclinical studies predict benefits in bone tissue healing and stability by use of MSC (Bernhardt, Lode et al. 2008; Bernhardt, Lode et al. 2009; John, Varma et al. 2009; Nair, Bernhardt et al. 2009; Nienhuijs, Walboomers et al. 2011). However the methods and more importantly the animal models to prove beneficial effects of MSC are not yet standardized. This is of great importance since the forces exerted on a fracture cannot be compared between animal species, nor can it be to humans. Comparisons between different procedures, cells and scaffolds are thus not reliable. A recent article proposes rules for comparable preclinical bone defects model that amongst others affect standardized surgical procedures and measurements. In this work tibia fracture and segmental defect models are preferred (Reichert, Saifzadeh et al. 2009).

3.5 Animal model and interpretation

Unfortunately, the criteria to evaluate the outcome of studies - be it *in vitro* or *in vivo* - differ considerably. Regarding the major requirement of mechanical stability, a variety of mechanical tests exist that determine stability. However, till date none of them has been defined as

standard (Hak, Makino et al. 2006; Jones, Atwood et al. 2009; Reichert, Saifzadeh et al. 2009). In animal models success criteria of implanted MSC and scaffold are restricted mainly to analysis of regenerated bone e.g. by histologiacal findings, CT-scan technology, x-ray or simply by measuring the weight of the created bone as well as by mechanical torsion tests (Zou, Hunter et al. 2011). The fate of implanted scaffold and MSC, in terms of material resorption and MSC engraftment into the host body, is rarely studied (Bernstein, Bornhauser et al. 2009). Since there is no standard in animal models, experiments are being carried out on various models. The rat model is broadly used because of availability. Bio-mechanical properties similar to humans are found in sheep, especially in hip arthroplasty (Korda, Blunn et al. 2008). Usually a fracture is induced as described by Matsumoto et al or Mifune et al (Matsumoto, Kawamoto et al. 2006; Mifune, Matsumoto et al. 2008). In a first step a tibia is fractured. Then a collagen scaffold is inserted containing saline and either BM-MSC or hESC. Then Undale et al compared the bone tissue healing properties of BM-MSC and hESC in rats after an induced fracture. BM-MSC resulted to be more efficient than hESC to bridge and heal a critical bone fracture. Moreover, in this setting hESC tended to produce benign bony tumours compromising the use of these cells in clinical settings (Undale, Fraser et al. 2011).

Bone fracture healing or integration into the animal's bone tissue can be demonstrated by follow-up conventional radiology in two weeks intervals. The limbs are both fully extended so that the broken and fractured limb can be compared. In recent studies μCT, a specialized CT for small animal structure, is used. Precise 3D models can be built from the data, allowing a comparison between the original and the newly built bone. Eight weeks after fracturing the animals can be euthanized and the limbs can be analysed histologically or biomechanically. Biomechanical stability of the fracture healing can be assessed by torsional load to evaluate normal and abnormal fracture healing (Undale, Fraser et al. 2011).

In summary, MSC from different sources appear as complementation to biomaterial implants. Depending on the tissue source and culture, different patterns of differentiation into bone, cartilage or fibre can be obtained. Depending on the precise situation different sorts of MSC-biocomposites may facilitate wound healing and functional regeneration of bone defects with high long term stability. However, the handling of biomaterial MSC composites is far more complex than conventional methods and oblige to adhere to regulatory standards: Since living cells are worked with, purity, a lack of bacterial contamination and absence of cell transformation has to be proven before clinical application. Conventional methods, that are acellular implants, may be limited because of rigidity and even lack of stability on the long run, but actually, in contrast to MSC biocomposites, they can be well compared regarding their advantages and disadvantages. MSC may differ much more as a matter of treatment, culture conditions and the cells itself need further investigation, experimental and clinical studies to evaluate their true potential at best in comparative studies. But the prospect of individual medicine with the patients' easily extractable and expandable own cells may support future research and applications in regenerative medicine.

3.6 Future prospects

Future orthopaedic research that may one day provide suitable personalized scaffolds to cover bone defects must integrate vascularisation as well. A balanced attempt to support both bone growth and blood supply must be established to create a stable long lasting graft

that becomes completely integrated into bone. Osteoinduction is difficult to obtain. Local application of osteoinductive factors such as FGF, the bone morphogenic proteins BMP-2, BMP-4, BMP-7 and vascular endothelial growth factor VEGF does either not lead to results due to degradation or does lead to too strong responses since it cannot be well regulated. Recent work shows promising results in this regard. However no standard can be proposed in terms of choice of growth factor, dose and modification (Keibl, Fugl et al. 2011). Recent work demonstrated the feasibility of plasmid DNA-integration into a scaffold that lead to a higher bone differentiation ratio (Hosseinkhani, Hosseinkhani et al. 2008). Future research must also deal with possibly breaking the border between autologous and allogenic MSC in treatment, in case patients cannot donate autologous MSC of any source. Allogeneic MSC in treatment of patients with osteogenesis imperfecta defects could be recently demonstrated (Le Blanc, Gotherstrom et al. 2005).

The optimal degree of differentiation in culture prior to implantation in an animal model or a human remains unclear: Should implants carry completely osteogenically differentiated MSC, or more likely quite the opposite to provide an optimum of adaptive possibilities within the host organism? The more differentiated the MSC the more initial stability they provide for implants in areas in which great forces act. Less differentiated MSC on the other hand prove more plasticity. In the worst case undesired differentiation or even dedifferentiation might occur. Medication, integrated drugs or even genetically engineered cells may provide a possible control *in vivo*.

The specifications defined by the regulatory framework focussing on the clinical use of MSC are becoming increasingly detailed (Burger 2003). These are more complex when it comes to MSC and biomaterial composites as there are no standards for quality controls. *In vitro* and *in vivo* interactions between scaffolds and in-growing cells, as well as between scaffolds and host tissues, need to be investigated further.

4. Conclusion

In vitro studies indicate that MSC possess a wide spectrum of properties in tissue regeneration as adult progenitor cells or by secreting immunomodulatory and antiinflammatory factors. Still various manufacturing protocols, cultivating media and methods hinder to correlate and interpret scientific findings. Nevertheless MSC are very promising candidates for cell therapy and have moved extremely quickly in the last ten years from the bench to the bedside. For controlled clinical trials there are several obstacles to overcome in order to define a safe and efficacious therapeutic. There is a need to determine factors that may influence the cell quality and consequently the clinical outcome in terms of the tissue source, the isolating, expansion and cultivating conditions. Above that, protocols and *in vitro* and safety animal studies need to be performed in compliance with GMP requirements. To be able to conduct clinical trials on MSC, the manufacturing process has to fulfil several regulatory standards. Advances in clinical application of MSC can be exemplified in the field of orthopaedic bone regeneration. The osteogenic potential of MSC is seen to be of great benefit in bone defect healing. However, only in rare conditions are MSC alone beneficial. The choice of a suitable biomaterial to both carry MSC and provide good primary stability is crucial for clinical applications in hard tissue regeneration. Different sources of MSC that have different differentiation properties can be used. To

assess compatibility of both MSC and biomaterial *in vitro*, MSC can be cultured on 2D or in 3D structures. Stability testing of seeded scaffolds helps determine the biomechanical properties of the biocomposite. Different animal models are being used, but no standard has yet been proposed that allows comparison of biomaterials and biomaterial/MSC. No biomaterial/MSC composite is in regular use in human for bone regeneration at present. Future efforts to establish treatments with these biocomposites must therefore concentrate on standardised procedures both in evaluation of tissue culture experiments and, more importantly, in animal models. The choice of the animal and the precise comparable procedures need to be defined. The prospect is individual autologous healing.

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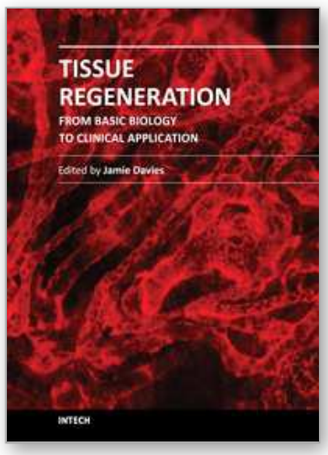

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When most types of human tissue are damaged, they repair themselves by forming a scar - a mechanically strong 'patch' that restores structural integrity to the tissue without restoring physiological function. Much better, for a patient, would be like-for-like replacement of damaged tissue with something functionally equivalent: there is currently an intense international research effort focused on this goal. This timely book addresses key topics in tissue regeneration in a sequence of linked chapters, each written by world experts; understanding normal healing; sources of, and methods of using, stem cells; construction and use of scaffolds; and modelling and assessment of regeneration. The book is intended for an audience consisting of advanced students, and research and medical professionals.

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