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The influences of injury pattern, gender and age on the function and proliferation of marrow stromal cells

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Last but not least, I am grateful to all the patients who volunteered their support (and bone marrow!)

1 Introduction

Mesenchymal stem cells (MSC) are multipotent stem cells from human bone marrow. These MSC, also referred to as marrow stromal cells, maintain the capacity to differentiate into multiple mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes, myoblasts, stromal, neural and endothelial cells (Bianco et al. 2001a; Tremain et al. 2001; Roufosse et al. 2004). The process of differentiation is regulated by intrinsic growth factors and extrinsic signals (Long et al. 1995; Quesenberry et al. 2002; Moreau et al. 2006).

The use of autologous MSC has generated considerable interest on account of their developing use in regenerative medicine and tissue engineering in orthopedic surgery, which has been illustrated in numerous promising pre-clinical and clinical trials (Bruder et al. 1998a; Barry & Murphy 2004; DeRubeis & Cancedda 2004).

Cell transplantations date back to the sixteenth century when the Italian surgeon Gaspare Tagliacozzi, detailed in his work “De curatorum chirurgica per institionem”, attached a skin flap from a patient’s forearm to the nose and released the arm several weeks later, allowing the skin graft to heal on the nose (Romero-y-Huesca et al. 2005; Soto-Miranda et al. 2006). Currently such a procedure, considered a free flap transfer, is routinely performed successfully in reconstructive surgery (Bozikov & Arnez 2006). It is an example of autologous grafting, a method using a cell population to repair a defect at a different anatomical location of the same individual. Initiated by complications and limitations of autologous grafting, tissue engineering has emerged as a viable alternative. In short, functional tissue is generated *in vitro* using endogenous cells and a synthetic matrix for cellular delivery.

MSC are among the cells necessary for successful implementation of such applications. Little information is currently available concerning the donor characteristics for tissue engineering growth of skeletal tissue. As new methods are developed, there are several aspects regarding donor gender, age, injury pattern and regulatory factors which need to be explored.

1.1 Marrow stromal cells

Almost 140 years ago, the German pathologist Julius Cohnheim first suggested the existence of non-hematopoietic, undifferentiated cells in bone marrow (Cohnheim 1867). The development of such “stem” cells was later described in detail in the pioneering studies of Friedenstein, Piatetzky-Shapiro and Petrakova (1966). They reported the isolation of bone-forming progenitor cells from rat marrow. These cells, originally referred to as colony forming units-fibroblasts (CFU-F), retained the ability for bone and cartilage formation following successive passaging of single-colony and multicolonies. In addition, the content of osteogenic precursors in the cultured progeny significantly outnumbered the initiating CFU-F, establishing their highly proliferative and stem cell-like properties (Friedenstein et al. 1987). Finally named mesenchymal stem cells (Caplan 1991), proof of their actual stem cell nature was established in the diligent works of Pittenger et al. (1999) and later by Halleux et al. (2001). Direct experimental evidence was finally provided that cultures of bone marrow derived mesenchymal cells contain individual cells which possess extensive self-renewal capacity and multilineage potential.

In current literature, not only can a variety of nomenclature be found referring to marrow stromal cells (mesenchymal stem cells, mesenchymal precursor cells, autologous progenitor cells) but the methods of isolation, expansion and characterization differ as well. To address the resulting difficulty in comparing various research outcomes, the *Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy* proposed minimal criteria to define human MSC. First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD73, CD90, CD105 and lack expression of CD11b, CD14, CD19, CD34, CD45 or CD79-α and HLA-DR surface molecules. Third, MSC must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006). Although this definition certainly may be modified in the future, it does present guidelines by which the multiple parameters of MSC can be characterized and compared.

MSC are generally isolated from an aspirate of adult bone marrow harvested from the superior iliac crest of the pelvis, although various mesenchymal tissue sources have been identified. Such alternative sources include tibial and femoral marrow

compartments (Murphy et al. 2002; Ciapetti et al. 2006), thoracic and lumbar spine (D'Ippolito et al. 1999), adipose tissue (DeUgarte et al. 2003), synovium (DeBari et al. 2003) and skeletal muscle (Jankowski et al. 2002) as well as umbilical cord and embryonic tissue (Erices et al. 2000; Campagnoli et al. 2001). There is support for the existence of MSC circulating in blood, although only a small population has been identified (Huss et al. 2000; Zvaifler et al. 2000; Kuznetsov et al. 2001; He et al. 2006; Khosla et al. 2006). However, MSC can be routinely harvested easily, efficiently and in large numbers from adult marrow, also eliminating ethical issues inherent in using embryonic or fetal cells. The frequency of MSC residing within the stromal compartment of bone marrow is 0.001-0.01% of nucleated cells (Pittenger et al. 1999). These typically fusiform cells can be expanded in culture through many generations while retaining the capacity to differentiate (Pittenger et al. 1999; Majumdar 2000). Following isolation, they are initially fractioned by a density gradient and plated. During the period of primary culture, the nonadherent hematopoietic cell fraction is depleted while the remaining adherent MSC population forms colonies can be further expanded. Their multilineage differentiation potential has been described and characterized by multiple laboratories. Under defined culture conditions, they can differentiate into osteoblasts, chondrocytes, adipocytes, myoblasts and stromal cells as well as nonmesenchymal lineages unrelated to the cells in their tissue of origin such as neural cells and endothelial cells, a demonstration of the transgermal plasticity of MSC (Sanchez-Ramos et al. 2000; Bianco et al. 2001a; Tremain et al. 2001; Roufosse et al. 2004).

The manifold attributes of MSC have been extensively analyzed. They can for example be functionally characterized by their osteogenic differentiation. In the presence of β-glycerol-phosphate, ascorbic acid-2-phosphate and dexamethasone, cells acquire an osteoblastic morphology with upregulation of alkaline phosphate (ALP) activity and deposition of a calcium-rich mineralized extracellular matrix (Bruder et al. 1997a; Jaiswal et al. 1997). Using established techniques such as flow cytometry, MSC show heterogeneous surface markers and have variable potential for mesenchymal tissue development. Numerous investigations have been conducted not only to identify markers of mesenchymal and bone progenitors (Bruder et al. 1994; Barry et al. 1999), but also to address further issues regarding their heterogeneous properties. Castro-Malaspina et al. (1980) demonstrated in their

pioneering investigations the accurate determination of expansion limitations of MSC in culture for large numbers of cells, which has been confirmed in further studies (Bruder et al. 1997a; Wexler et al. 2003). However, the proliferative capacity declines with repetitive passaging (Caplan et al. 1997; Jaiswal et al. 1997).

The homing mechanism, a further attribute demonstrated by MSC, has also been investigated, whereby systemic or local delivery of MSC results in specific migration not only to bone marrow, but also to the site of injury (Pereira et al. 1995; Prockop 1997). In an animal model, marked MSC were shown to colonize rejected cardiac transplant tissue following intravenous injection (Wu et al. 2003). Murphy et al. (2003) demonstrated similar results in a human study where MSC delivered through an intraarticular injection to the knee joint after traumatic injury exhibited the capability of engraftment and repair of damaged meniscus and cartilage. Further compelling studies evaluated female allograft hearts transplanted into male patients, using the Y chromosome to detect migrated undifferentiated cells and differentiate between donor and recipient origin. Laflamme et al. (2002) and Quaini et al. (2002) demonstrated the translocation of undifferentiated cells and their repopulation of damaged myocardial tissue. In addition to cells originating from the donor, the presence of Y chromosome-positive cells confirms the capacity of such extracardiac progenitor cells to migrate to sites of tissue injury.

1.2 Factors influencing MSC

Numerous factors appear to influence the reservoir and proliferative capacity of MSC in bone marrow. Age is one factor which has generated considerable investigation. The concentration of MSC in bone marrow has been shown in many studies to be age-related. Many investigators suggest a decrease with age (D'Ippolito et al. 1999; Chen 2004; Abdallah et al. 2006; Mareschi et al. 2006) which would coincide with established knowledge of skeletal maturation and degeneration, while others found no change (Stenderup et al. 2001; Justesen et al. 2002). As with age, there is evidence that the MSC frequency in bone marrow is also gender-dependent. In women, CFU expressing ALP obtained from bone marrow aspirate was decreased (Muschler et al. 2001) as well as the cortical bone mass in postmenopausal women (Brockstedt et al. 1993).

Furthermore, investigators have identified interesting mediators influencing progenitor cell mobilization in trauma situations. Elevated concentrations of VEGF and TGF-β in serum derived from multiple trauma patients have been shown to promote differentiation of endothelial progenitor cells (Henrich et al. 2004). Hormones, growth factors and cytokines are likewise involved in MSC proliferation. Numerous influential factors have been discussed, including growth factors (Moreau et al. 2006), estrogen and testosterone (DiSilvio et al. 2006), prolactin (Ogueta et al. 2002) and interleukins (Strecker et al. 2003).

1.3 Therapeutic applications and relevance in orthopaedic surgery

The therapeutic value of stem cells is well documented in numerous medical fields and has been applied over 40 years. Following the identification of human leukocyte antigens (HLA) by the French medical researcher Jean Dausset, the first successful bone marrow transplantation was reported by Gatti et al. in 1968 in Minneapolis, MN (Gatti et al. 1968; Fischer 1999). Since then, stem cell therapy, in which autologous or allogenic undifferentiated stem cells are either locally or systemically delivered and ultimately differentiate to the appropriate phenotype under local signals, has provided a treatment alternative for many diseases and organ dysfunctions (Meyer et al. 2006). In this context, MSC can also be therapeutically applied systematically or with site-specific delivery vehicles to repair various tissues damaged by disease or trauma (Caplan 2005). An abundance of diseases have been targeted for potential MSC use, including diabetes mellitus (Lee RH et al. 2006), myocardial infarction (Orlic et al. 2003), stroke (Li et al. 2005), Parkinson’s disease (Ye et al. 2006) and muscular dystrophy (DeBari et al. 2003). Although much of this research is still focused on animal models, preliminary results are promising.

Orthopaedic surgery encompasses a wide spectrum of musculoskeletal conditions from acute injury to disease to congenital deformities. Many challenges are encountered in the treatment of multiple traumas, delayed wound healing, atrophic nonunions or increasingly common degenerative diseases such as osteoporosis. The Center for Disease Control in the U.S. reported an average of 1,028,600 hospitalizations due to fractures per year for 2002-2004 (National Center for Health Statistics 2006), 1.5 million of the fractures due to osteoporosis (Orsini et al. 2005) and 110,000 patients suffer nonhealing long bone defects (Ringe et al. 2002). Each

year in the U.S. 450,000 bone grafts are performed following various indications (Service 2000). Further demands result from the continuously growing elderly population. In a report focusing on adults older than 65 years, fractures were the most frequent and expensive type of injury in 2000, accounting for 35% of non-fatal injuries and 61% of direct medical costs which totaled $19 billion. Additionally, the incidence of fall injuries for women was 2.3 times higher and the medical cost 2.8 times higher than for men (Stevens & Sogolo 2005; Stevens et al. 2006). In Germany alone, 1.5 million people with degenerative joint diseases are under medical treatment (Ringe et al. 2002). Considering the demographics, incidence and costs of skeletal injury and disease, development of safe and more effective functional tissue replacement becomes increasingly necessary.

Osteogenic cells are a prerequisite for such methods of bone reconstruction. MSC have successfully been implemented for generation of replacement tissue for orthopedic procedures in numerous studies (Gao & Caplan 2003; Niemeyer et al. 2004b). The benefit of MSC use in many orthopedic surgery situations has been recently documented, such as segmental bone defects (Quarto et al. 2001), cartilage (Wakitani et al. 2002), tendon (Ouyang et al. 2004), wound repair (McFarlin et al. 2006), spinal fusion (Muschler et al. 2005), osteogenesis imperfecta (Horwitz et al. 2002) and in osteoarthritis (Murphy et al. 2003). Currently, autologous bone grafting is often achieved by transferring cancellous bone tissue from the iliac crest of the pelvis to the fracture site, providing mechanical stabilization as well as osteogenic progenitor cell enrichment. It can be used in fracture treatment, to induce joint fusion or fill segmental bone defects (Caterson et al. 2001). Such methods are still considered standard for osteogenic bone replacement in extended defects (Kneser et al. 2006). However, the clinical use of autologous osseous transplants is limited by donor site morbidity, increasing with the amount of harvested bone. Bone substitutes used in combination with autologous bone grafts can decrease the amount of bone tissue needed for reconstruction (Kneser et al. 2006). Recent progress in the isolation and characterization of MSC has led to extensive research in therapeutic strategies in a variety of orthopaedic surgery applications, including tissue engineering.

Tissue engineering is a viable alternative to allogen and autologous grafting in terms of reconstructive surgery, as it is associated with fewer limitations such as tissue availability, harvesting difficulties, donor site morbidity, immune reaction or transmission of disease (Schaefer et al. 2000). In tissue engineering procedures, a combination of extracellular scaffolds, bioactive factors and patient cells is necessary to generate functional replacement tissue *in vitro* for clinical use (Schaefer et al. 2000; Caplan & Bruder 2001). Pluripotent MSC in adult tissues have been shown to play a significant role in tissue regeneration and homeostasis (Minguell et al. 2001; Niemeyer et al. 2004a) and are therefore a promising cell population for such tissue engineering applications. Many new developments in the management of skeletal injury with osteogenic progenitor cells have been reported. Kuroda et al. (2006) recently presented a case in which successful full-thickness cartilage repair in a 31 year old male was achieved by collage-gel-assisted transplantation of *in vitro* expanded autologous MSC from the iliac crest. In this way, tissue engineering advances demonstrate how the body's own rejuvenative potential can be utilized by applying endogenous *in vitro* expanded reparative MSC (Caplan & Dennis 2006).

2 Aims of this thesis

To date there have been no studies published addressing the effect of age, gender, site of harvest and injury severity on the quantity, quality and osteogenic differentiation of mesenchymal stromal cells in injury situations. The goals of this thesis concentrate therefore on the influence of such donor characteristics on MSC from trauma patients. MSC concentration in bone marrow will be verified using the CFU-F assay. To determine the proliferative capacity of MSC, densitometric analysis will be applied. The results of this study can be used to determine the applicability of MSC from various patient groups for tissue engineering procedures.

Aim #1: Evaluate the influence of injury pattern on the quantity and quality of MSC in trauma patients

Aim #2: Evaluate the influence of gender and age on the quantity and quality of MSC in trauma patients

Aim #3: Evaluate the influence of other mediatory factors on the quantity and quality of MSC in trauma patients

3 Materials and Methods

3.1 Materials

Table 1: *Materials*

Equipment:	
Centrifuge	Heraeus, Hanau, Germany
Incubator	Jouan, Winchester, VA, USA
Laminar airflow work bench	Antares, Hartmannsdorf, Germany
Microscope	Carl Zeiss, Göttingen, Germany
Water bath	GFL 1083, Anunak
Digital camera, Nikon DR Math. and Meth. 2 Coolpix 4500	Nikon, Düsseldorf, Germany
Refrigerator, +4 to -20℃	Bosch, Stuttgart, Germany
Freezer, -80℃	Heraeus, Hanau, Germany
Counting chamber, Neubauer	Optik Labor, Friedrichshofen, Germany
Vortex *MSI Minishaker*	Ika Inst., Wilmington, NC, USA
Transfer pipettes	Hirschmann Laborgeräte, Eberstadt, Germany
Eppendorf pipettes	Eppendorf, Wesseling-Berzdorf, Germany Star Lab, Ahrensburg, Germany
Magnetic heating / stirring plate	IKA Labortechnik, Staufen, Germany
FACScan (Fluorescent activated cell sorting)	Becton-Dickinson, Heidelberg, Germany
Multi Analyst software	Bio-Rad, Munich, Germany
Cell Explorer software	BioSciTek, Frankfurt, Germany
Disposable material:	
Tubes	Greiner Bio-One, Solingen, Germany
Culture flasks	Sarstedt, Nümbrecht, Germany
Transfer pipette tips	Costar, Corning, NY, USA
Eppendorf pipette tips	Star Lab, Ahrensburg, Germany

Syringes	Becton Dickinson, Fraga, Spain
Blood tubes	Sarstedt, Nümbrecht, Germany
Falcon tubes	Sarstedt, Nümbrecht, Germany
Insulin syringes	Becton Dickinson, Madrid, Spain
Cryovials	Nalge Nunc, Roskilde, Denmark
Pasteur pipettes	Sarstedt, Nümbrecht, Germany
Cell culture plates	Nalge Nunc, Roskilde, Denmark
Kendall monoject iliac aspiration needles	Tyco Healthcare, Neustadt, Germany
Buffers and solutions:	
Distilled water (Aqua dest.)	Braun, Melsungen, Germany
Accutase	PAA Laboratories, Pasching, Austria
Ficoll 1.077 g	Biochrom, Berlin, Germany
Erythrocyte lysis solution	self-preparation
Dulbeccos's Phosphate Buffered Saline without Mg^{2+} and Ca^{2+} (PBS w/o)	PAA Laboratories, Pasching, Austria
Fetal Bovine Serum (FBS)	Gibco, Paisley, Scotland
Culture mediums:	
MesenCult Basal Medium for Human Mesenchymal Stem Cells	StemCell Technologies, Vancouver, Canada
MesenCult Mesenchymal Stem Cell Stimulatory Supplements	StemCell Technologies, Vancouver, Canada
MesenCult Osteogenic Kit	StemCell Technologies, Vancouver, Canada
Chemicals and reagents:	
Diff-Quick	Baxter, Düdingen, Switzerland
Sigma Fast Alkaline Phosphatase Sustrate Tablets Set	Sigma, St. Louis MO, USA
Alizarin red dye	Chroma, Fürstenfeldbruck, Germany
Fast red dye	University Clinic pharmacy
Türks dye	Merck, Darmstadt, Germany
5% Silver nitrate solution	self-preparation, see appendix
5% Sodium thiosulfate solution	self-preparation, see appendix
β-Mercaptoethanol	Merk, Darmstadt, Germany

Dimethylsulfoxide (DMSO)	Sigma, Heidelberg, Germany
Ethanol	Merk, Darmstadt, Germany
Formaldehyde	Sigma-Aldrich, Seelze, Germany
Antibodies:	
Iso-PE	BD Biosciences, Heidelberg, Germany
Iso-FITC	BD Biosciences, Heidelberg, Germany
Iso-PE-Cy5	BD Biosciences, Heidelberg, Germany
CD 34-FITC	BD Biosciences, Heidelberg, Germany
CD 45-FITC	BD Biosciences, Heidelberg, Germany
CD 71-PE-Cy5	BD Biosciences, Heidelberg, Germany
CD 73-PE	BD Biosciences, Heidelberg, Germany
CD 90-PE	BD Biosciences, Heidelberg, Germany
CD 105-FITC	Serotec, Düsseldorf, Germany
Medications:	
Heparin 10,000 IU/ml	Ratiopharm, Ulm, Germany
NaCl 0.9% ampulles	Braun, Melsungen, Germany
Kits for the detection of hormones and cytokines:	
17β-estradiol, testosterone, prolactin	Bayer Healthcare, Leverkusen, Germany
Interleukin-6-ELISA	RayBiotech, Hoelzel Diagnostik GmbH, Köln, Germany

3.2 Experimental subjects

Trauma patients who were scheduled to undergo operative procedures on the pelvis, vertebrae, tibia or hip as well as cancellous bone autografts for reconstruction of various bone defects were included in the study. A total of 58 individuals (51 patients and 7 healthy volunteers) participated in the study, ranging from 18 to 64 years of age. Criteria for exclusion were pathological fractures due to carcinoma, bacterial or viral infections and infectious diseases including Hepatitis A, B, C, CMV (cytomegalovirus infection) and HIV (human immune deficiency virus). Patients were discharged from the study if the cell cultures showed signs of contamination. 5 samples obtained from patients were not included in statistical analysis due to insufficient growth (see Results 4.1.2 and Discussion 5.2). The remaining 53 participants were then divided into 8 groups according to injury pattern, gender and age ***(Table 2)***. 18 female and 35 male patients were included. The injury pattern was determined as follows: the first group (single fracture) included patients with 1 isolated, primarily treated fracture; the second group (multiple trauma) included polytraumatized patients with an injury severity score (ISS) of 16 or greater; the third group (atrophic nonunion) included patients with fractures over 6 months old with radiographic signs of insufficient healing. 7 healthy volunteers were included in the control group. All participants and the parents or guardians of minors, as well as all healthy volunteers were informed of all procedures before signing a consent form. The ethics committee of the University Hospital of Frankfurt approved all procedures.

***Table 2**: Participant groups (n=53)*

group	injury pattern	gender	age	number of patients (n)
1	single fracture	female	38±23	3
2	multiple trauma	female	55±18	4
3	atrophic nonunion	female	42±13	5
4	healthy volunteers	female	33±6	4
5	single fracture	male	46±17	11
6	multiple trauma	male	37±10	9
7	atrophic nonunion	male	41±15	14
8	healthy volunteers	male	32±9	3

3.3 Bone marrow extraction

For the extraction of human marrow stromal cells (MSC), a minimum of 6 ml bone marrow was aspirated intraoperatively under sterile conditions. Depending on the procedure, the cells were obtained from an aspirate of the iliac crest (n=46), proximal extremity of the tibia (n=3), greater trochanter (n=1), vertebral body (n=1) in trauma patients or pelvic bone puncture in health volunteers (n=7). 5,000 IU Heparin and 4.5 ml 0.9% saline solution were added, gently mixed and the sample was immediately processed.

3.4 Isolation of human marrow stromal cells

The following procedures for processing the MSC were conducted under sterile conditions. The original volume of bone marrow was noted before washing the suspension twice with an equal amount of Phosphate Buffered Saline, without Mg^{2+} and Ca^{2+} (PBS w/o). The sample was centrifuged between wash cycles at 850 g for 10 minutes at room temperature. The cells were then separated by density gradient centrifugation. Briefly, the cells were resuspended in a volume of PBS w/o equal to the original sample and overlaid onto a Ficoll density gradient. The mononuclear cell suspension was isolated by centrifugation at 1000 g for 30 min. Following careful removal of the cell layer, the cells were washed in PBS w/o containing 2% Fetal Bovine Serum (FBS), pelleted by centrifugation at 850 g for 10 minutes and resuspended in 3 ml MesentCult Basal Medium for Human Mesenchymal Stem Cells containing MesenCult Mesenchymal Stem Cell Stimulatory Supplements (450 ml:50 ml). The contaminating red blood cells were lysed in lysis solution (1:3 v/v) for 5 minutes before staining the remaining cells with Türks dye. The cells could then be accurately counted using a Neubauer counting chamber and seeded in culture flasks. See also **Protocol 1** and **Protocol 2** in the appendix.

3.5 Cultivation and expansion of human marrow stromal cells

The cells were cultivated in 25 cm^2 culture flasks with 10 ml of MesenCult medium plus supplements each at 37℃ and 5% CO_2. Adherent cells were allowed to reach approximately 30% confluence before the cultures were split and passaged. The medium was changed 3 times a week. 3 culture flasks were allocated for the colony forming unit-fibroblast (CFU-F) assay, with cells plated at densities of 5×10^5, 1×10^6 and 2×10^6. Any additional cells were seeded at a density of 2×10^6 for further analyses. The criterion of 30% confluence is based on a survey conducted parallel to standard cultivation. To observe at which confluence the cells could be optimally passaged, cultures from 5 patients were chosen randomly and 2 culture flasks from each patient were cultivated according to procedures described in **Protocol 1**. The cultures were divided into 2 groups: low and high density and passaged after 30% or 80% confluence respectively until proliferation stagnated. The maximum number of proliferative passages could then be observed.

3.6 Characterization of human marrow stromal cells

3.6.1 Colony forming unit-fibroblast (CFU-F) assay

For the CFU-F assay, the culture flask with cells plated at a density of 1 x 10^6 as mentioned above were used. The cells were allowed to adhere for 14 days during which the medium was changed 3 times a week. On day 14 the cells were stained using Diff-Quick. After medium removal, the cells were rinsed with distilled water and fixed and stained with the 3 formulas included in the Diff-Quick staining set (methanol, buffered eosin, buffered azure). They were rinsed again with distilled water and allowed to air-dry. Each culture flask was then photographed, digitized and converted to an 8 bit grey level TIF-format. See also **Protocol 3** in the appendix.

3.6.2 Mean colony number, mean colony area, and mean cell number per microscopic field of view

Using the digitized CFU-F assay, the mean colony number, mean colony area and mean cell number per microscopic field of view (FOV) could be determined. The software *Cell Explorer* was used to assess the colony number and mean colony area. To establish mean cell number per microscopic FOV the following procedures were applied: first, the mean cell number per colony was determined using 15 randomly chosen stained colonies from a total of 3 patients. Within each colony, the cells in 4 peripheral and 1 central randomly chosen area were counted microscopically (100 x magnification, FOV 0.1 x 0.1 mm) using an ocular grid and the mean cell density was calculated. In a second adjacent step, the colonies processed above were marked, photographed and digitally converted into an 8 bit gray level format. The mean gray level was then assessed (including background correction) using the software *Multi Analyst*. The mean cell number per FOV from the first step was then plotted against the corresponding gray level from the second step and a calibration curve was generated. Consequently, the mean cell number per colony could be calculated by applying linear regression.

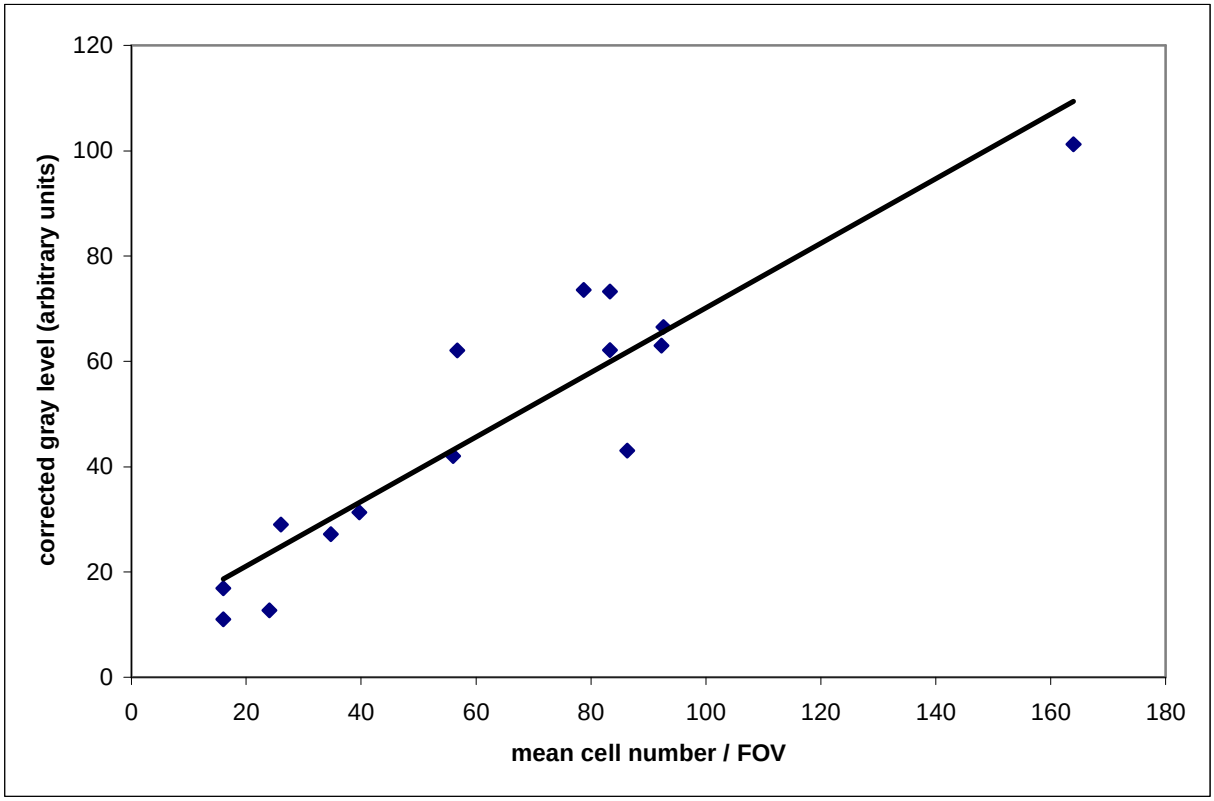


Figure 1*: Gray level and mean cell number per FOV (field of view)*
The mean cell density is plotted against the mean gray level. By applying linear regression, the mean cell number per colony can be calculated.

The mean cell number per FOV (cell density) correlated significantly with the corresponding gray level of the colony (***Fig. 1***, r=0.92, p<0.00001). Thus, the optical CFU-F density reflects the true cell number per colony and represents an appropriate surrogate of the proliferative capacity.

3.6.3 Fluorescent activated cell sorting (FACS)

Data was collected by analyzing 10,000-50,000 events and FACS analysis was performed to characterize the surface antigen expression of CD34, CD45, CD71, CD73, CD90 and CD105. Cells acquired under identical conditions as above were first expanded over 3 passages after a confluence of 30% was attained for each cell passage. The cells were detached through incubation with Accutase at 37℃ for 10 minutes, washed once in PBS w/o, centrifuged at 300 g for 1 minute and resuspended in PBS w/o containing 0.5% FBS at a concentration of 3 x 10^5 cell/ml. 100 µl aliquots of the cell suspension were incubated in the refrigerator at 4℃ for 20

minutes with 7.5 µl each of the following antibodies: anti-CD34, anti-CD45, anti-CD71, anti-CD73, anti-CD90, anti-CD105 and control isotypes. Cells were then washed in PBS w/o, suspended in 2 ml 0.5% formaldehyde and incubated for 10 minutes in the dark. Finally, the cells were centrifuged at 300 g for 10 minutes and resuspended in 250 µl PBS w/o for FACS analysis. A MSC-typical phenotype was required for inclusion of the CFU-F assay in the data.

3.6.4 Staining for osteogenic differentiation

To determine the potential of MSC for osteogenic differentiation, von Kossa, alkaline phosphatase (ALP) and alizarin staining was performed. Cells acquired under identical conditions as above were cultivated in 12-well plates at a density of 2 x 10^4 cells/well for 2 weeks. The osteogenic differentiation was induced using the MesenCult Osteogenic Kit by supplementation of 42.5 ml MesenCult medium with 7.5 ml Osteogenic supplements (final volume 15%), 5 µl dexamethasone 10^{-4} M (final concentration 10^{-8} M), 250 µl ascorbic acid 10 mg/ml (final concentration 50 µg/ml) and 175 µl β-glycerophosphate 1.0 M (final concentration 3.5 mM). The 1 ml medium/well was changed 3 times a week. On day 14 the calcium deposition was verified by the von Kossa staining procedure as follows: first the wells were rinsed 3 times with distilled water and incubated in 1 ml/well of ice cold ethanol for 20 minutes. After rinsing again 3 times with distilled water, the cell cultures were further incubated at room temperature in 1 ml/well 5% silver nitrate solution. Following one last rinse sequence, the un-reacted silver was removed by incubation with 1 ml/well 5% sodium thiosulfate and the cells were counterstained with 1 ml/well Fast red dye for 10 minutes. See also **Protocol 4** in the appendix. Alizarin staining for quantification of mineral deposition and ALP to verify osteoblastic activity were both performed using sets according to the instructions of the manufacturer. Subsequently the cells were photographed for assessment and documentation.

3.7 Patient serum analysis

Serum from a total of 39 randomly chosen participants from the 53 patients and 7 healthy individuals was collected on the day of the operative procedure and levels of the hormones 17β-estradiol, testosterone, prolactin and the cytokine interleukin-6 were tested as suggested by the manufacturers.

3.8 Statistical analysis

Values are expressed as a mean ± SD. Statistical analysis was performed using the non-parametric Kruskal Wallis test with Dunn-posthoc analysis with Holm's alpha correction for multiplicity. At most four groups were compared against each other, for example healthy male volunteers versus male patients with nonunions versus male patients with single fractures versus male patients with multiple traumas. A p value less than 0.05 was considered significant. Correction analysis was performed using the Spearman-rang test. SPSS V9.0 (SPSS, Chicago, IL, USA) was used for all statistical analysis.

4 Results

4.1 Characterization of human marrow stromal cells

4.1.1 Culture passaging at low and high density confluence

In order to obtain the highest possible number of MSC colonies and optimize the yield of cultivated MSC, the maximum number of proliferative passages was evaluated using 10 cultures from 5 different patients passaged after 30% and 80% confluence. MSC cultured at 30% confluence yielded a significantly higher number of maximum passages ($p<0.05$) than those cultured at high density **(*Fig. 2*)**. Average culture senescence began at passage 10±7. Additionally, the cells cultured at low density also exhibited a significantly higher rate of population doubling **(*Fig. 3*)**.

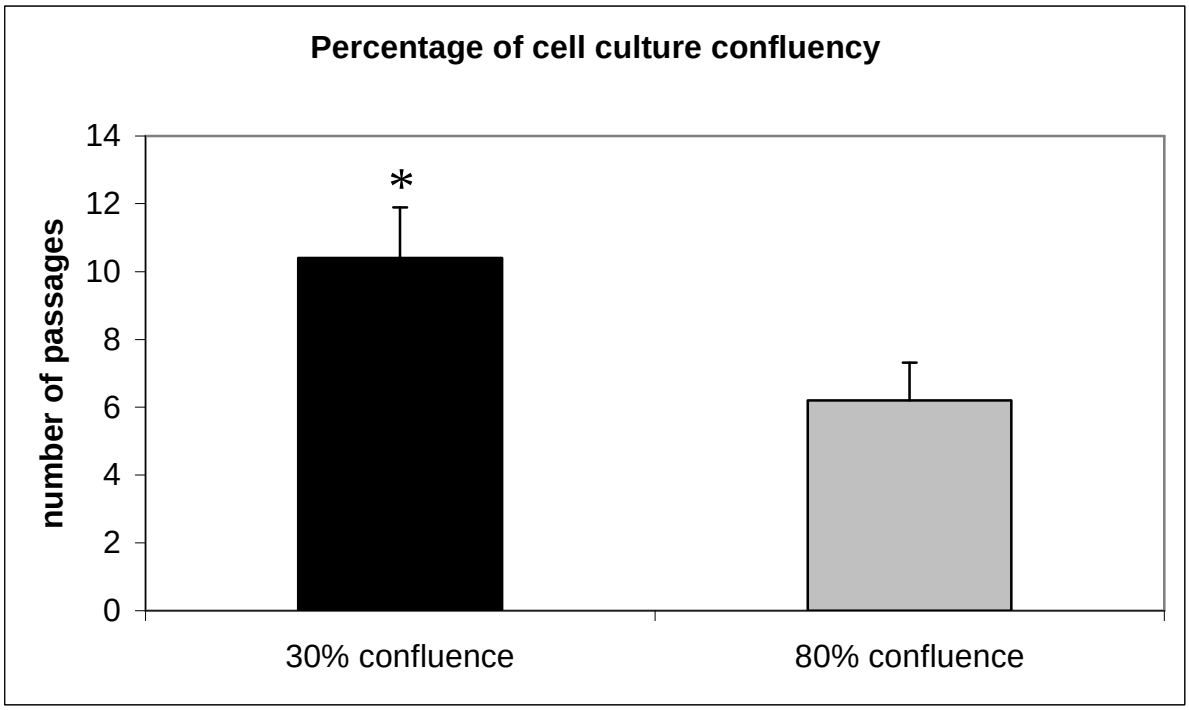


***Figure 2**: Mean maximum number of passages at 30% and 80% confluence*
(indicates significance)*
MSC cultured at 30% confluence yielded a significantly higher number of maximum passages than those cultured at high density.

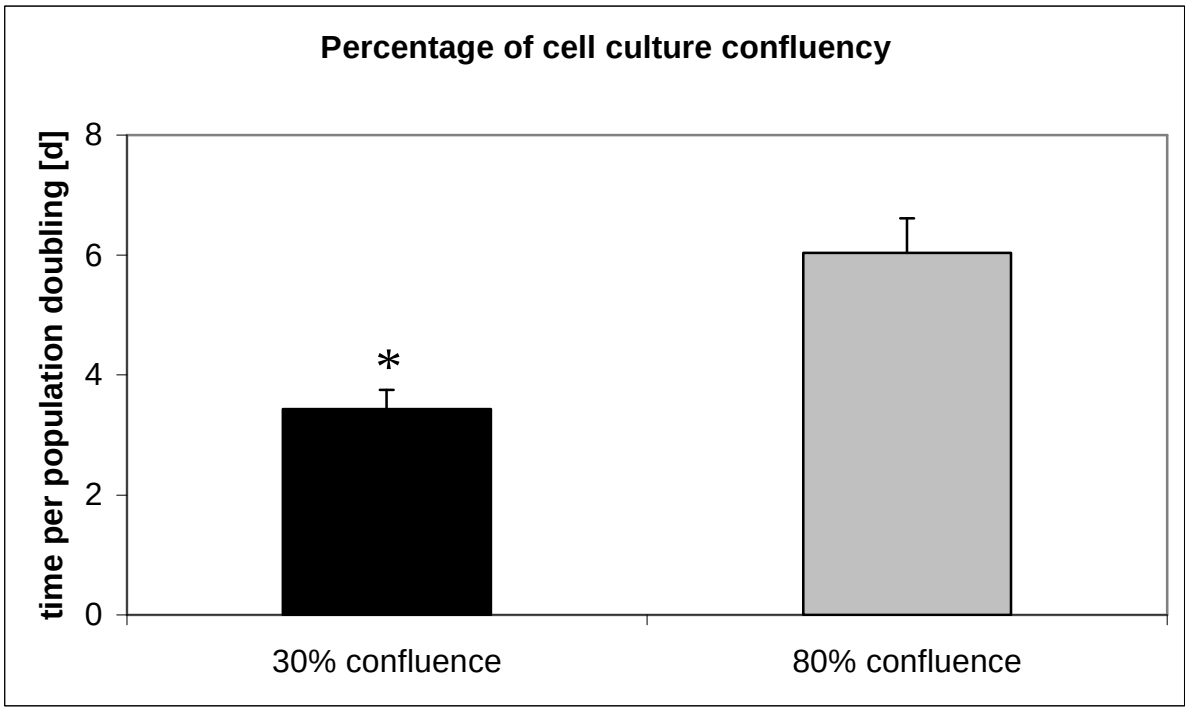


Figure 3*: Mean duration of proliferation at 30% and 80% confluence*
(indicates significance)*
Cells cultured at low density exhibited a significantly higher rate of population doubling.

4.1.2 Colony forming unit-fibroblast (CFU-F) assay

On day 14 following initial isolation and plating, the cells were stained using a Diff-Quick staining set. The resulting CFU-F exhibited morphology consistent with the expression of MSC, including typical homogeneous colony formation and individual fusiform cells ***(Fig. 4)***. The 5 cultures of cells obtained from the proximal extremity of the tibia, greater trochanter and vertebral body did not proliferate sufficiently enough to be included for further trials.

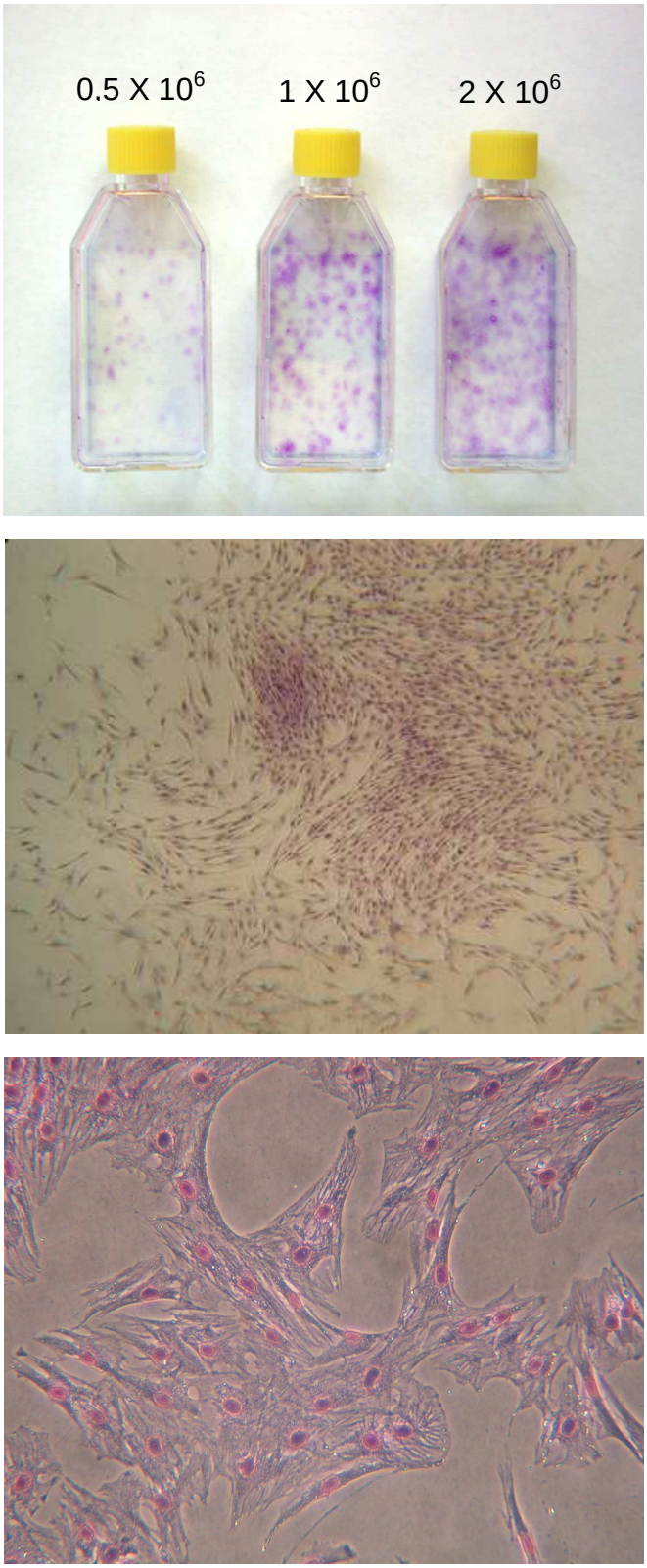


Figure 4: *CFU-F*

(a) culture flasks and initial cell densities (b) colony at x80 magnification (c) at x200 magnification

4.1.3 Confirmation of MSC phenotype

Phenotypical analysis of the cultures was performed using fluorescent activated cell sorting (FACS). All analyzed MSC samples expressed the surface antigens CD 71, CD73, CD90 and CD105 but did not express CD34 and CD45 ***(Fig. 5)***. This is consistent with the characterization of MSC. Control samples could be successfully differentiated to an osteogenic phenotype.

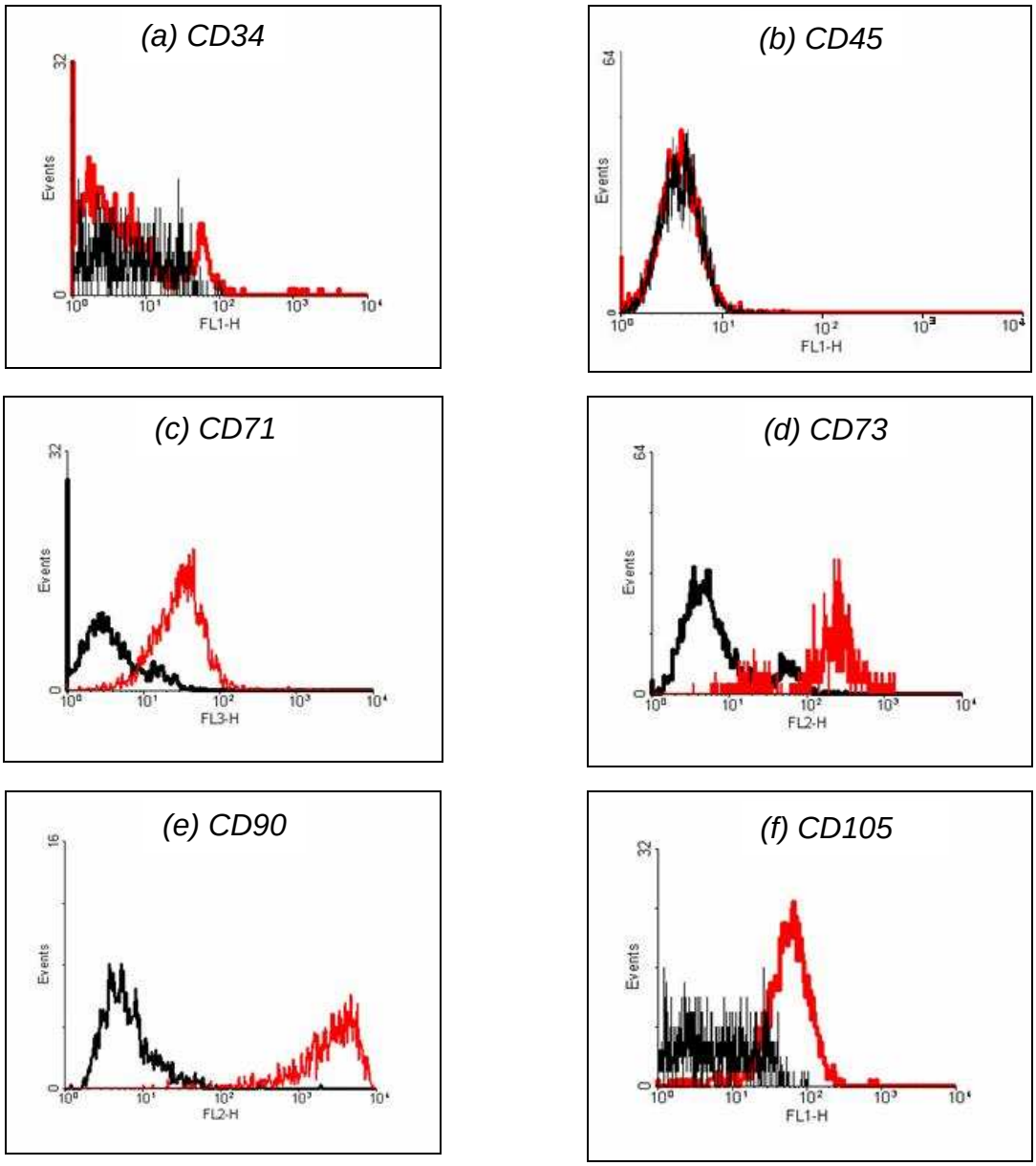
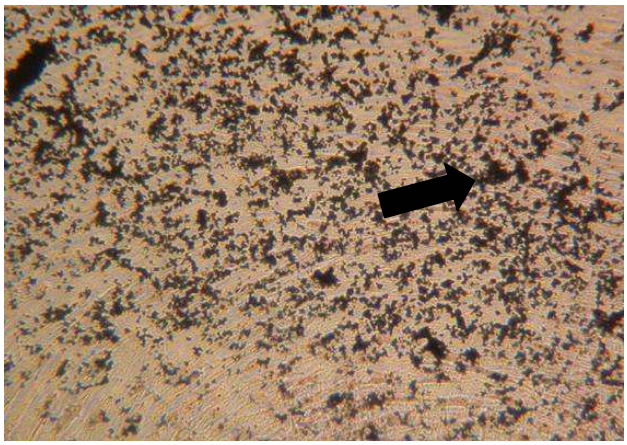


Figure 5: *Representative FACS analysis of MSC*
(a) CD34- (b) CD45- (c) CD71+ (d) CD73+ (e) CD90+ (f) CD105+. Black signifies control isotype, red signifies antibody

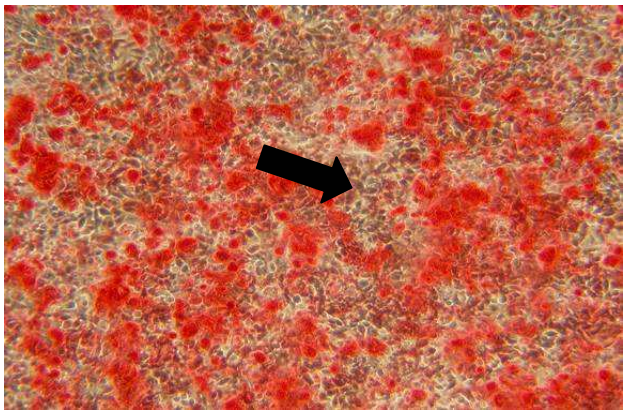
4.1.4 Staining for osteogenic differentiation

For verification of osteogenic differentiation, cells were stained following 14 days of supplemental incubation with dexamethasone, ascorbic acid, and β-glycerolphosphate. The von Kossa and alizarin stains quantified calcium deposition while alkaline phosphatase (ALP) expression confirmed osteoblastic differentiation ***(Fig. 6)***.

a



b



c

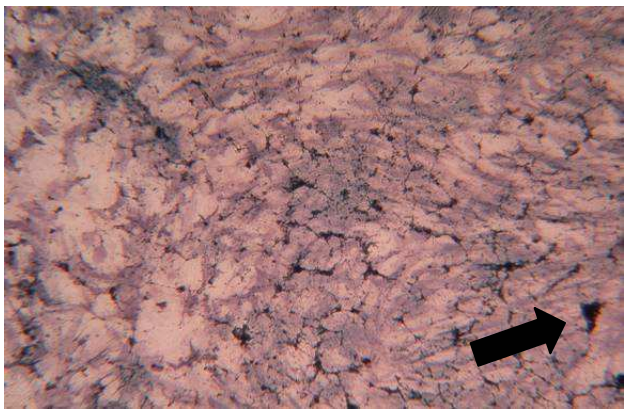


Figure 6: *MSC staining for osteogenic differentiation*
(a) Von Kossa (b) alizarin red (c) alkaline phosphatase (arrows indicate calcium deposition in a and b and alkaline phosphatase activity in c)

4.2 Evaluation of mean colony number

4.2.1 Mean colony number: females

The multiple trauma group demonstrated the highest mean colony number overall, with significant differences compared to the atrophic nonunion and control groups. Interestingly, the control group showed a significantly higher mean colony number than the atrophic nonunion group ($p<0.05$). The lowest mean colony number was found in the atrophic nonunion group ***(Fig. 7)***.

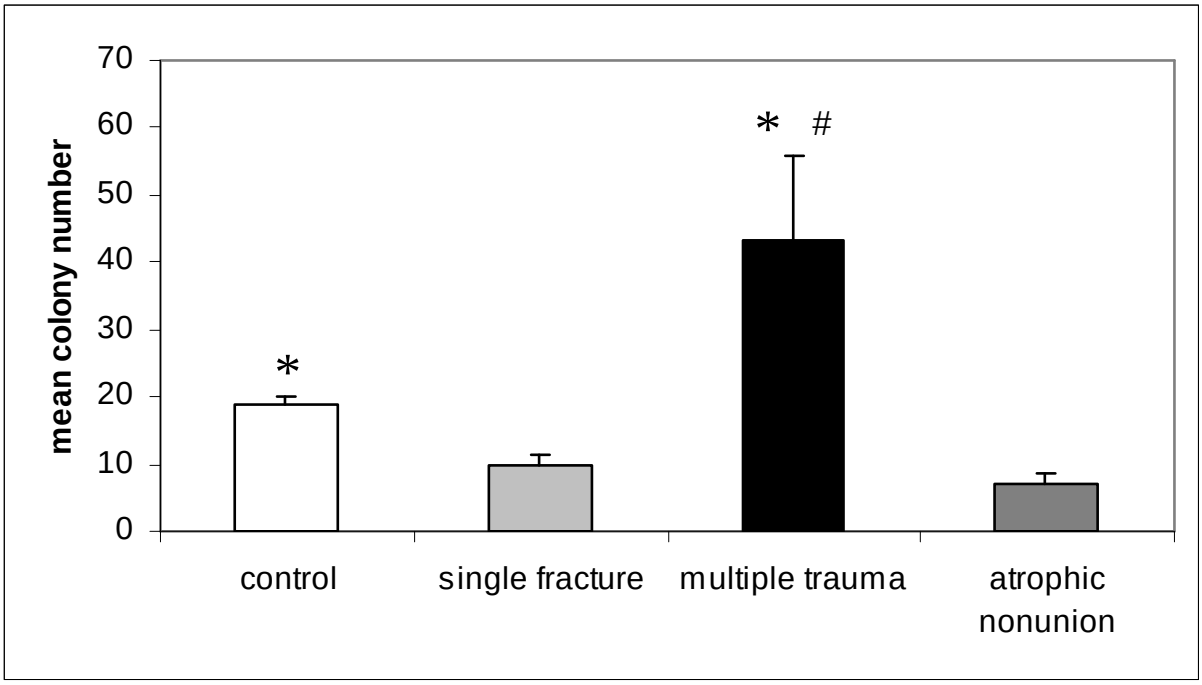


Figure 7: *Mean colony number: females*
(indicates significance vs. atrophic nonunion group; # indicates significance vs. control group)*
Total number of female patients n=18, control group n=4, single fracture n=3, multiple trauma n=4, atrophic nonunion n=5

4.2.2 Mean colony number: males

In the male groups, significant differences ($p<0.05$) were found in all subgroups. Again, the highest mean colony number overall was demonstrated in the multiple trauma group, with a significantly higher mean colony number compared to the single fracture and atrophic nonunion groups. As with females, a significantly higher mean colony number was found in the male control group compared to the atrophic nonunion as well as the single fracture groups. The lowest mean colony numbers were found in the atrophic nonunion and single fracture group ***(Fig. 8)***.

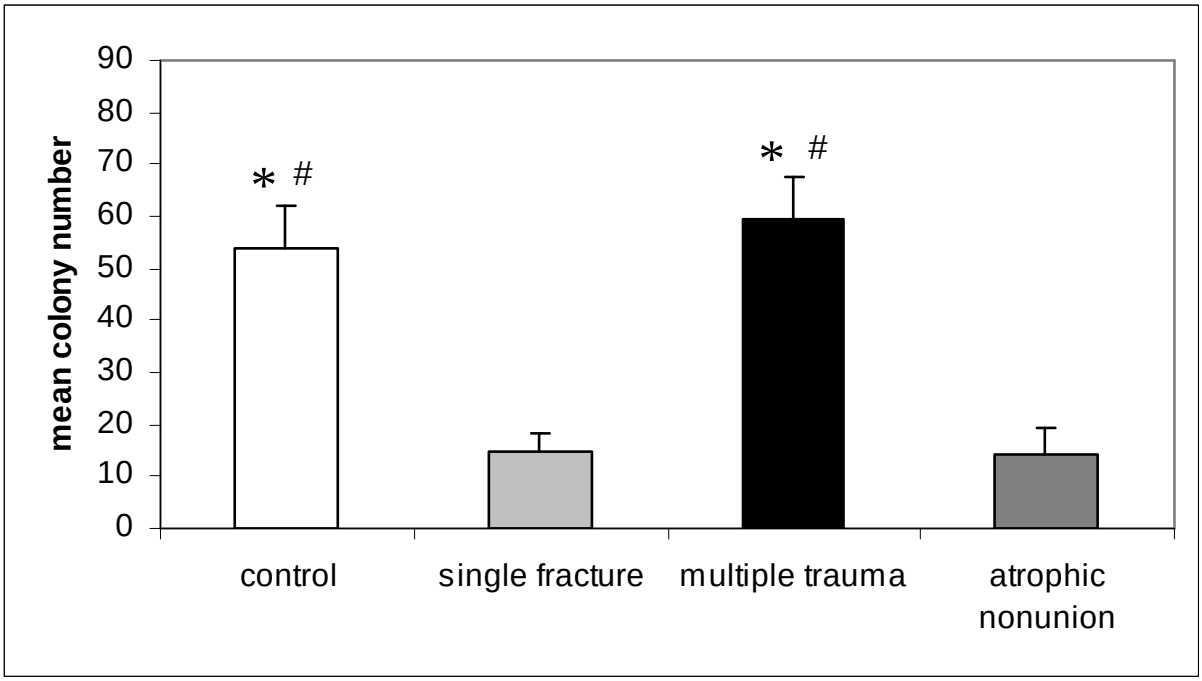


Figure 8: *Mean colony number: males*
(indicates significance vs. atrophic nonunion group; # indicates significance vs. single fracture group)*
Total number of male patients n=35, control group n=3, single fracture n=11, multiple trauma n=9, atrophic nonunion n=14

4.3 Evaluation of mean colony area

4.3.1 Mean colony area: females

Although the groups with the lowest mean colony area were found in the atrophic nonunion and single fracture groups, the differences were not significant ***(Fig. 9)***.

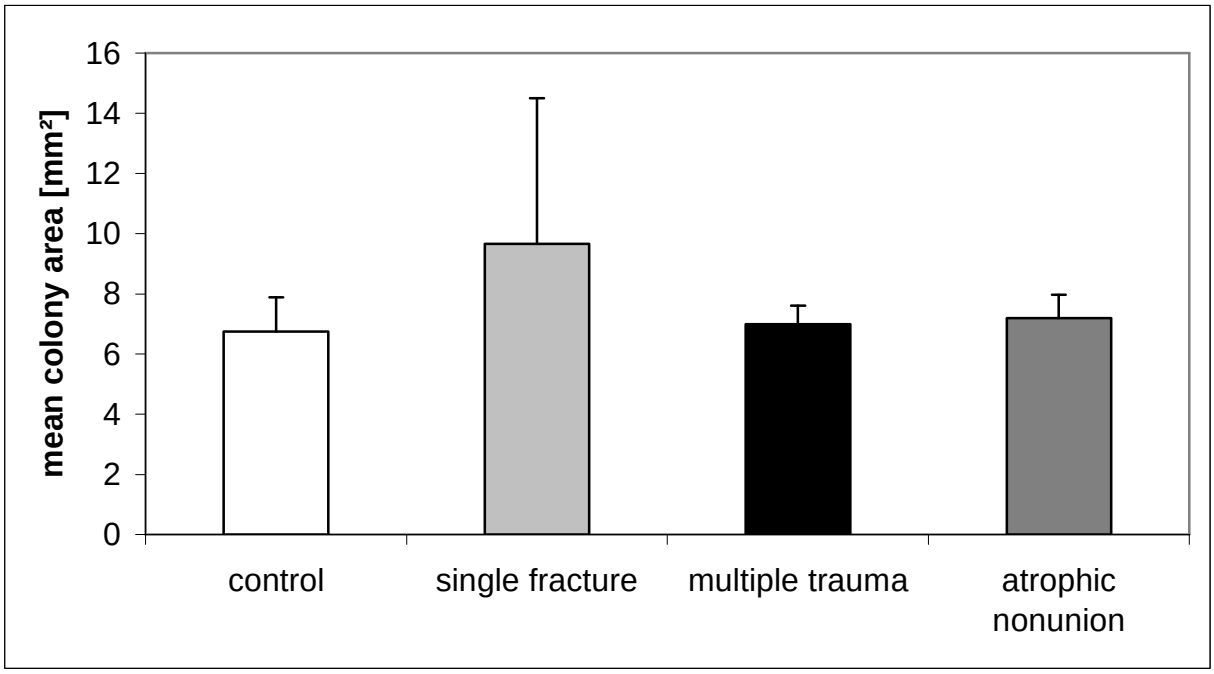


Figure 9: *Mean colony area: females*

Total number of female patients n=18, control group n=4, single fracture n=3, multiple trauma n=4, atrophic nonunion n=5

4.3.2 Mean colony area: males

Two groups demonstrated significantly higher mean colony areas ($p<0.05$): the multiple trauma compared to the single fracture group and the control compared to the single fracture group ***(Fig. 10)***.

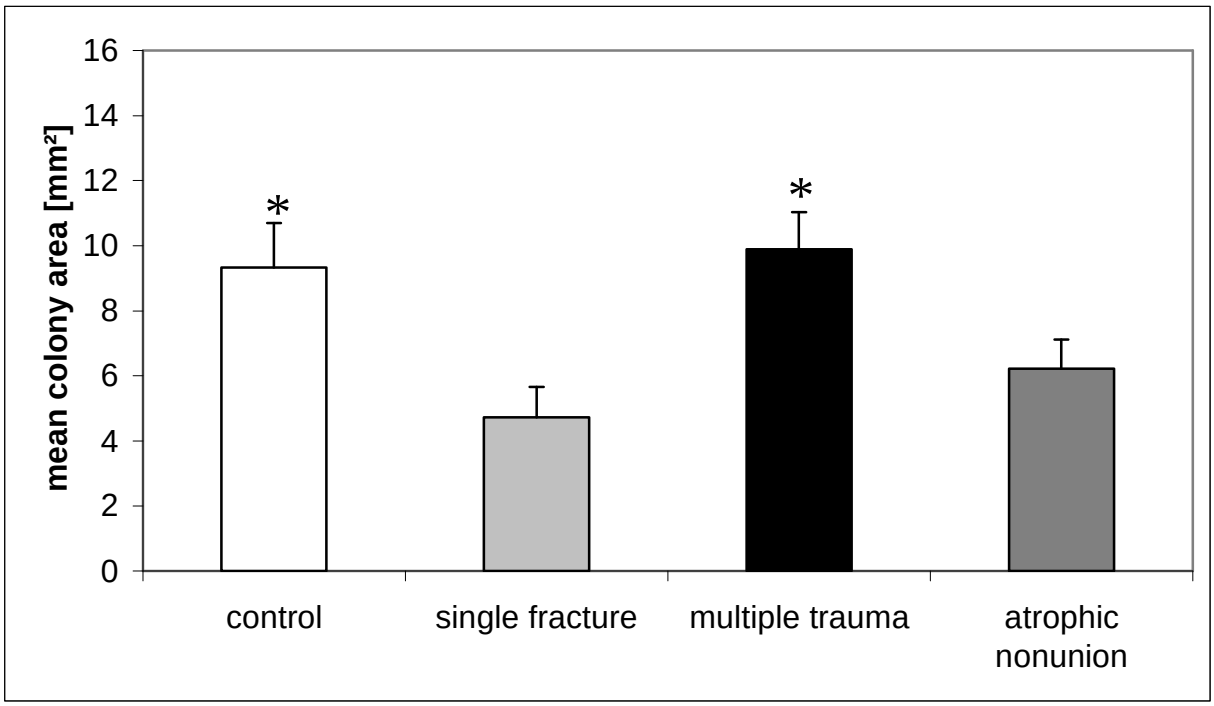


Figure 10: *Mean colony area: males*
(indicates significance vs. single fracture group)*
Total number of male patients n=35, control group n=3, single fracture n=11, multiple trauma n=9, atrophic nonunion n=14

4.4 Evaluation of proliferative capacity

4.4.1 Proliferative capacity: females

Although the multiple trauma group demonstrated the highest, and the atrophic nonunion and control groups the lowest mean cell number / FOV, no significant differences were found regarding the female proliferative capacity groups ***(Fig. 11)***.

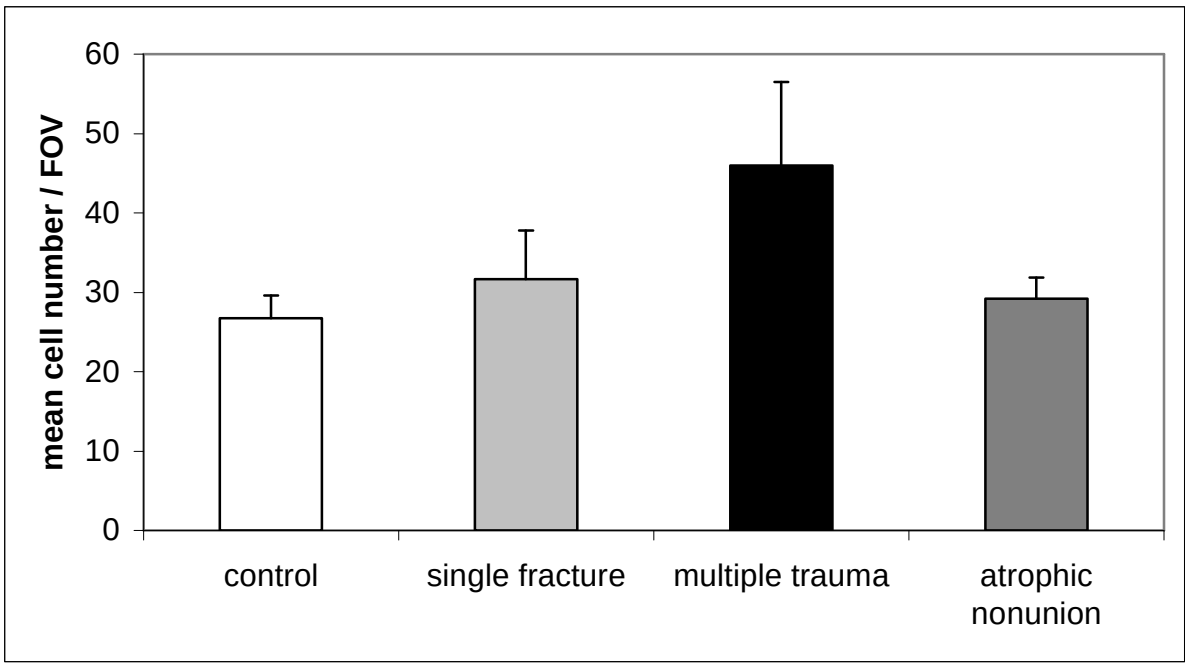


Figure 11: *Proliferative capacity: females*
Values are based on the established significant correlation between cell density and corresponding gray level, determined by using the optical CFU-F density values.
total number of female patients n=18, control group n=4, single fracture n=3, multiple trauma n=4, atrophic nonunion n=5

4.4.2 Proliferative capacity: males

The highest proliferative capacity was demonstrated in the multiple trauma group, with significance ($p<0.05$) compared to the single fracture group ***(Fig. 12)***.

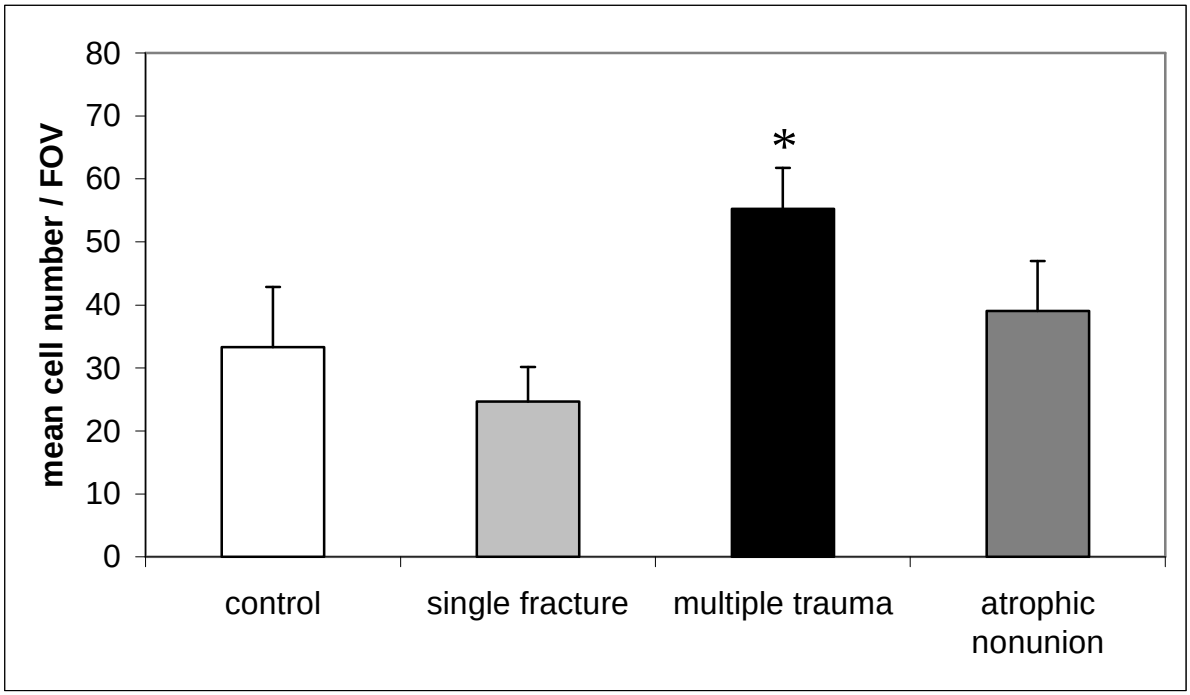


Figure 12: *Proliferative capacity: males*
(indicates significance vs. single fracture)*
Values are based on the established significant correlation between cell density and corresponding gray level, determined by using the optical CFU-F density values.
total number of male patients n=35, control group n=3, single fracture n=11, multiple trauma n=9, atrophic nonunion n=14

4.5 Evaluation of gender

4.5.1 Evaluation of gender: mean colony number

The male group demonstrated a significantly higher mean colony number ($p<0.05$) compared to the female group in healthy volunteers ***(Fig. 13)***.

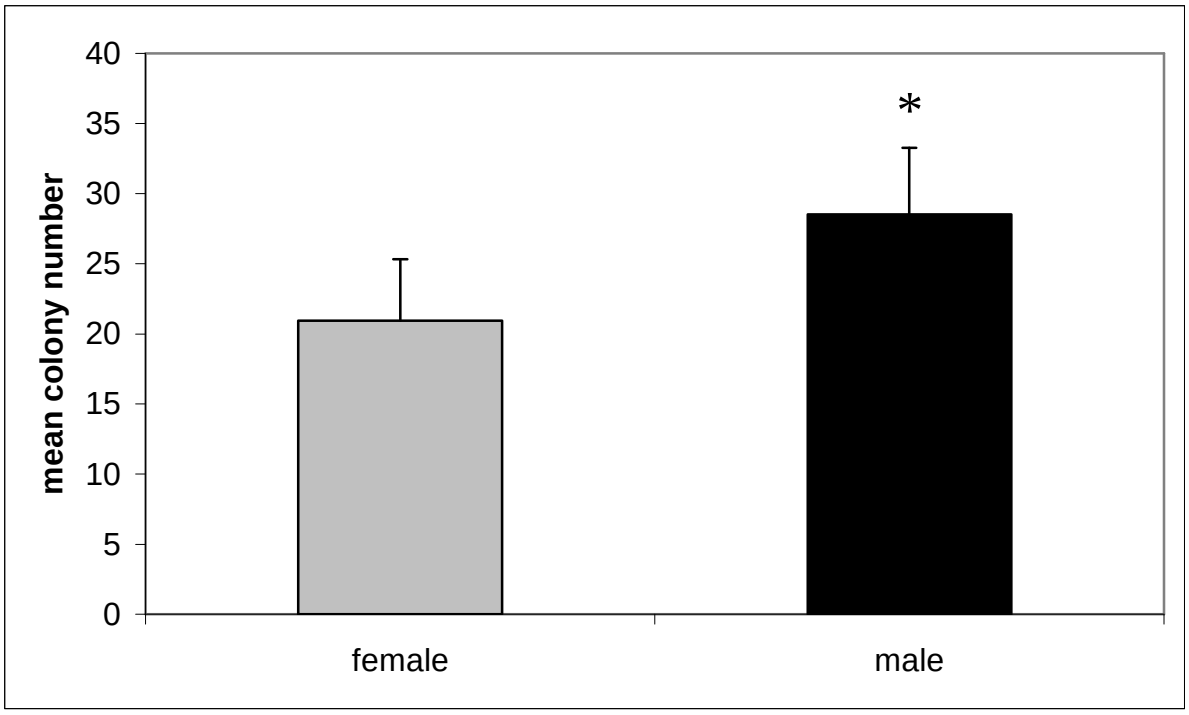


Figure 13: *Influence of gender: mean colony number*

(indicates significance)*

Total number of female patients n=18, total number of male patients n=35

4.5.2 Evaluation of gender: mean colony area

Although the mean colony area was higher in the male group, the difference was not significant ***(Fig. 14)***.

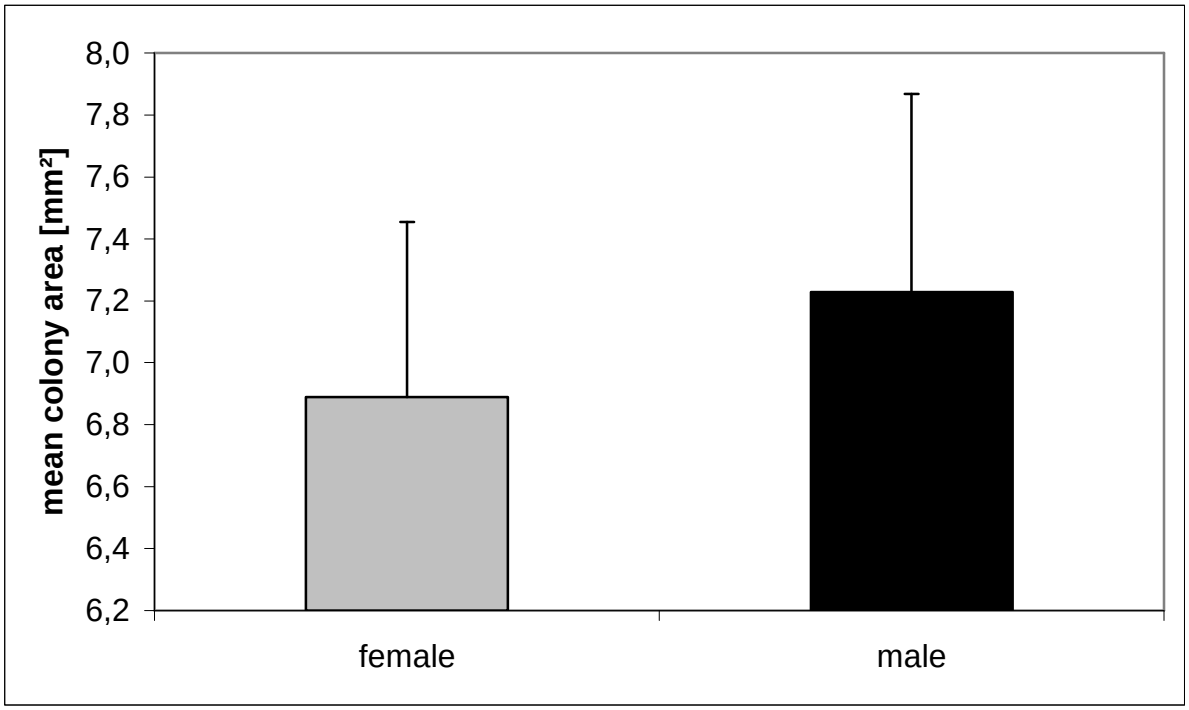


Figure 14: *Influence of gender: mean colony area*

Total number of female patients n=18, total number of male patients n=35

4.5.3 Evaluation of gender: proliferative capacity

Although the mean cell number / FOV was higher in the male group, the difference was not significant ***(Fig. 15)***.

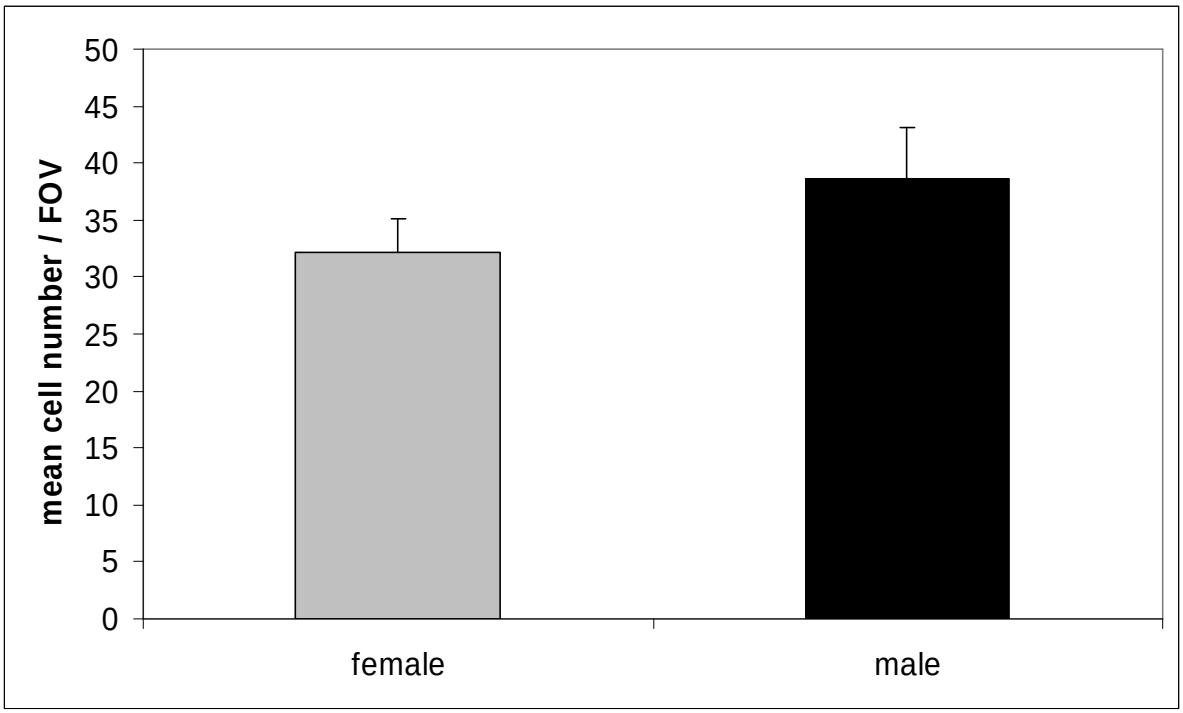


Figure 15: *Influence of gender: proliferative capacity*

Total number of female patients n=18, total number of male patients n=35

4.6 Evaluation of age

Single fracture and control groups were chosen to assess the influence of age on MSC reservoir and proliferative capacity to eliminate unrelated factors such as dysfunctions in wound healing or excessive injury found in atrophic nonunion and multiple trauma situations. Colony number ***(Fig. 16)*** decreased significantly ($p<0.02$) in patients over 40 years of age, whereas the proliferative capacity demonstrated only a tendency ($p<0.09$) towards decreasing values in older patients ***(Fig. 17)***.

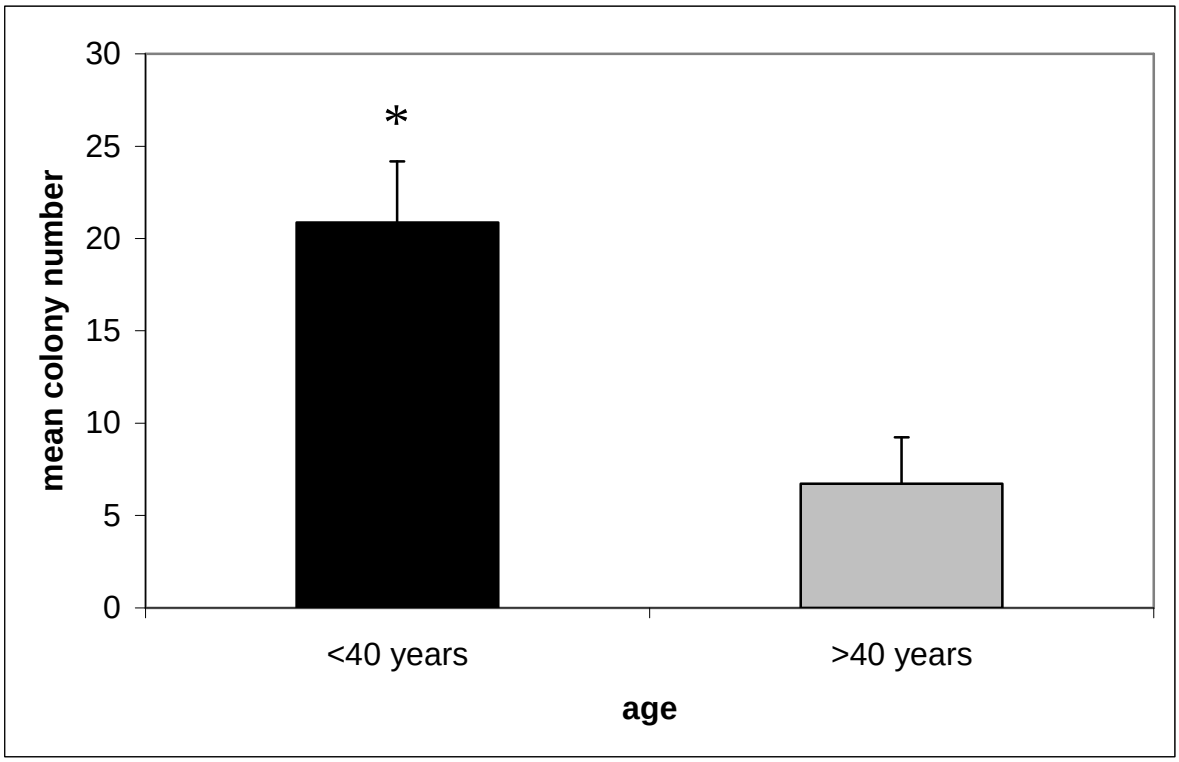


Figure 16: *Influence of age in single fracture and control groups: colony number*
Total number of patients <40 years of age n=7; total number of patients >40 years of age n=7
(indicates significance)*

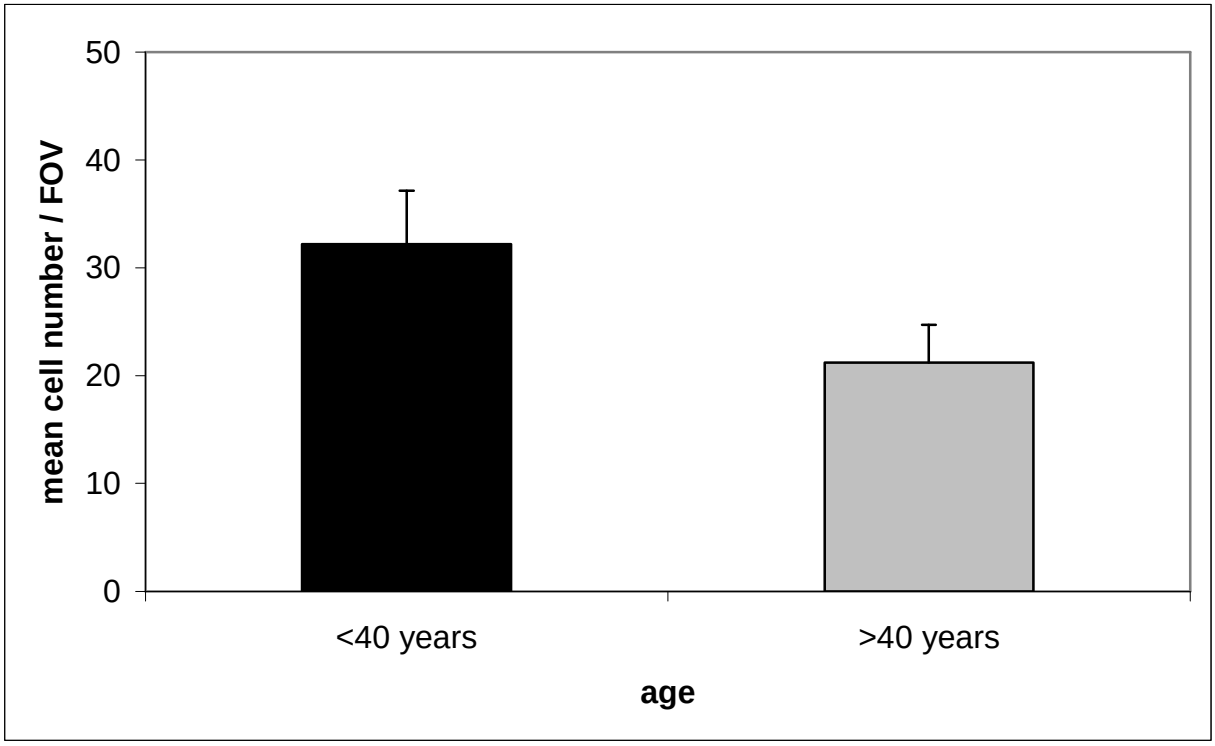


Figure 16: *Influence of age in single fracture and control groups: proliferative capacity*

Total number of patients <40 years of age n=7; total number of patients >40 years of age n=7

4.7 Evaluation of patient serum

No significant correlations were observed between the number of CFU-F and the serum levels of estradiol, prolactin or IL-6. The serum concentration of testosterone was significantly elevated in male participants compared to female participants (440±250 versus 48±41, $p<0.05$).

5 Discussion

This study clearly demonstrates the effect of injury pattern (single fracture, multiple trauma, atrophic nonunion) on the reservoir and proliferative capacity of MSC in human bone marrow. To date, the influence of injury pattern and severity on MSC frequency has not been described. Further donor characteristics such as age and gender were also shown to influence MSC number and proliferation, as in previous studies (Muschler et al. 2001; Phinney et al. 1999). While current literature supports the general influence of physiological status, age and gender, a diversity of donor characteristic-related outcomes can be found.

5.1 Characterization of MSC

An important attribute of MSC is their rapid *in vitro* expansion. Detailed culture conditions which promote the most efficient expansion however, have yet to be established. In the additional, parallel trial to optimize the MSC yield, cultures plated at low density generated more proliferative passages. A higher CFU-F frequency was observed at 30% than at 80% confluence. Additionally, the cells cultured at low density exhibited a higher rate of population doubling. Sotiropoulou et al. (2006) evaluated multiple culture conditions for characterization of optimal parameters for large clinical scale MSC production and confirmed that the MSC plating and passaging density are sensitive to the community effect in their microenvironment. In this regard, various components of the cultivation process can be examined. Similar investigations, although measuring only initial seeding density, report diverse findings. Colter et al. (2000) documented accelerated cell growth when plated at low density and suggested that this behavior could be explained by either cell-to-cell contact or factors that cells secrete into the medium. Conversely, other authors have found no significant effect on MSC proliferation but greater mineral deposit at higher densities (Jaiswal et al. 1997) or an increased osteoprogenitor (murine) cell proliferation at high initial cell densities (Purpura et al. 2004).

Despite the variant outcomes, fundamental advances have provided useful information for effective MSC expansion. Bruder et al. (1997a), while passaging cells at 80-90% confluence, established the decline in cell proliferation as a function of increasing passage number. Culture senescence occurred between passages 10-15. As in other investigations (Banfi et al. 2000; Mendes et al. 2004), this observation was also supported in our study in which the average culture senescence began at

passage 10±7, depending on the donor. Moreover, passage at 30% confluence is expectedly more effective for maximal cell expansion by limiting differentiation, as increasing MSC density is known to trigger differentiation (Caplan et al. 1983). Furthermore, cell proximity is not only important between individual MSC. Cell-to-cell contact between MSC and other cell types (cardiomyocytes, smooth muscle cells, endothelial cells) has been shown to induce MSC differentiation into those phenotypes (Rangappa et al. 2003; Ball et al. 2004; Wang et al. 2006). It is evident however, that further observations are necessary in order to maximize the efficiency of MSC expansion in large numbers.

The CFU-F assay has become a standard *in vitro* assay for MSC concentration in bone marrow, providing a reliable quantitative measure for the occurrence of cells (Castro-Malaspina et al. 1980; Perkins & Fleischman 1990). The frequency of CFU-F can be used as a surrogate marker for MSC frequency (Wexler et al. 2003). Proliferative potential of the CFU-F can be further quantified by the mean cell density within a cell colony. Following assessment of the cell number per FOV (cell density) and corresponding gray level, we demonstrated that the optical CFU-F density correlates with the number of cells within a colony. The value for cell density in turn most likely reflects the true proliferative capacity, thus facilitating the evaluation and comparison of donor colonies.

To eliminate complications due to heterogeneous cultures, FACS analysis was performed and confirmed mesenchymal cell phenotype. Although no monospecific and unique marker to unequivocally identify MSC has been ascertained, various markers have been identified which facilitate isolation. Early surface markers were identified including STRO-1 (Simmons & Torok-Storb 1991) and endoglin, or CD105 (Barry et al. 1999). After prolonged investigation, numerous markers have been identified to verify or negate mesenchymal origin: MSC express CD29 (β1-integrin), CD73 (endonucleotidase), CD117, which is the receptor for the stem cell factor ckit (Haynesworth et al. 1992; Pittenger et al. 1999), CD 70, CD90 and HLA class I antigens but do not express typical hematopoietic markers such as CD34 and CD45 or HLA class II antigens (Bruder et al. 1997b; Campagnoli et al. 2001; DeUgarte et al. 2003; Fibbe & Noort 2003; Risbud et al. 2006). CD271, also known as low-affinity nerve growth factor receptor (LNGFR), has been shown to be an efficient marker to

isolate MSC from bone marrow (Quirici et al. 2002; Jones et al. 2006). To facilitate the analysis and comparison of MSC research, the *Mesenchymal and Tissue Stem Cell Committee* proposed the following surface markers for their definition of MSC: cells must express CD73, CD90 and CD105 lack expression of CD11b, CD14, CD 19, CD34, CD79α and HLA-DR. As research in this area progresses quickly, there may be subsequent changes in definition. Our analyses however, confirmed the expression of a large selection of the above suggested markers.

Plasticity, a further criterion of the *Mesenchymal and Tissue Stem Cell Committee*, describes the multipotent capacity of MSC to differentiate into other cell types. This was assessed through induction of osteogenic differentiation by supplementing the culture medium with dexamethasone, ascorbic acid and β-glycerophosphate. These supplements have been extensively reported to induce and enhance osteogenic lineage differentiation from precursor cells (Jaiswal et al. 1997; Bruder et al. 1998b; Mendes et al. 2004). Alkaline phosphatase (ALP) expression verified osteoblastic differentiation. The significant use of ALP in MSC characterization was described almost 10 years ago (Jaiswal et al. 1997; D'Ippolito et al. 1999) and is now the most widely recognized marker for osteoblastic activity (Majumdar et al. 2000; Mendes et al. 2004). In addition, calcium deposition was confirmed and quantified by von Kossa and alizarin red staining, as in many previous investigations (Bianco et al. 2001a; Ciapetti et al. 2006).

5.2 The influence of injury pattern and donor characteristics on MSC

These analyses demonstrated several significant trauma-related modifications in MSC reservoir and proliferation, in both male and female patients. Injury severity has a substantial effect on MSC colony number (reservoir). In multiple trauma patients, the highest CFU-F frequency of MSC was found, independent of gender and age. Proliferative capacity was also highest in multiple trauma patients, although data for female patients provided insufficient significance. Furthermore, in the case of atrophic nonunion, the lowest CFU-frequency was detected, independent of gender.

The stimulated reservoir and proliferative capacity in patients with multiple trauma can be explained by a higher skeletal cell turnover necessary for bone remodeling and suggests a systemically-increased osteogenic potential through enhanced

differentiation, recruitment and migration of MSC. Numerous studies support the influence of injury on such osteogenic processes. In patients with both head injury and limb fracture there is evidence of an increased concentration of osteoblastic cells in serum (Bidner et al. 1990) as well as more rapid bone union and enhanced fracture healing (Spencer 1987; Giannoudis et al. 2006), compared to limb fracture only. In the later 2 studies heterotopic bone formation was detected in those patients with twofold injuries as well, giving rise to questions concerning the mechanism involved in the accelerated healing: injury severity or heterotopic bone formation. It is evident that central nervous system injury is frequently associated with heterotopic ossification (HO) and enhanced fracture healing (Andermahr et al. 2006; da Paz et al. 2007). Although it remains incompletely understood if it is truly enhanced healing or a form of heterotopic ossification, an increased osteoprogenitor activity is apparent (Balboni et al. 2006).

In severe trauma other types of progenitor cells are also activated, such as hematopoietic bone marrow cells in spinal trauma (Chernykh et al. 2006) and endothelial progenitor cells in acute lung injury (Burnham & Moss 2006). Additionally, enhanced concentrations of the mediators VEGF and TGF-β in serum derived from multiple trauma patients promote the differentiation of endothelial progenitor cells (Henrich et al. 2004). Serum-mediated changes in osteogenic processes can also be observed in severe trauma. Klein et al. (1999) detected an increased ALP activity in murine MSC following head injury. In a recent study of serum from polytraumatized patients, MSC stimulation and inhibition of osteoprogenitor apoptosis provided evidence of enhanced osteogenesis (Eid et al. 2006). Not surprisingly, enhancement of MSC is not limited to trauma patients, but is evident in complex regenerative processes as well. Mansilla et al. (2006) documented significantly higher quantities of circulating MSC in patients during acute burn situations, and the percentage of MSC correlated with the size and severity of the burn. The summation of these results present a model in which, in response to severe injury, increased differentiation and recruitment of MSC and other progenitor cells are induced, supplemented by elevated concentrations of various mediators which further promote osteogenic processes and ultimately osseous tissue healing.

While the extensive influence of injury severity on MSC is apparent, one of the many biochemical mediators involved, cytokines, must be considered concurrently.

Cytokines are a group of endogenous, pleiotropic proteins which have an essential function in immune response and are released by many different cells relatively rapidly after injury (Lin et al. 2000; Keel & Trentz 2005). There is evidence that excessive amounts of proinflammatory mediators have a detrimental effect on progenitor cells (Henrich et al. 2006). To evaluate the systemic inflammation, serum concentrations of the cytokine IL-6 were assessed. Contrary to expected results, no correlation with the CFU-F frequency or cell density was found. The increase in IL-6 following severe trauma has been reported by many investigators (Klein et al. 1999; Fonseca et al. 2005; Keel & Trentz 2005; Lin et al. 2000) and a further correlation between the concentration of IL-6 and injury severity has also been established (Gebhard et al. 2000; Strecker et al. 2003). Fonseca et al. (2005) documented the dose- and time-dependent stimulating effect of epinephrine and norepinephrine on bone marrow cell release of IL-6 and suggested a correlation with the persistent catecholamine elevation seen following severe injury. This trauma-induced hypercatecholamine effect on MSC was not analyzed in this study but an interesting premise for further studies. These diverse findings confirm the extended involvement of IL-6 in the trauma situation but its effect on MSC necessitates future investigations.

Nonunions present an unfortunate outcome of trauma, where a fracture fails to unite within 6 months (depending on the definition). The etiology is multifactorial, the incidence varies by fracture site and they are often treated by autologous bone marrow grafting (Babhulkar et al. 2005; Hernigou et al. 2006). In this regard, the processes in bone marrow involved in bone healing and bone union are therefore of great interest in orthopedic surgery. In patients with atrophic nonunions, the MSC reservoir was significantly decreased, independent of gender. Additionally, there was no compensatory increase detected in the proliferative capacity of those patients. Similar results were found in patients with single fractures; MSC reservoir was decreased with no compensatory increase in proliferation. It is interesting to note that in patients with multiple trauma, both reservoir and proliferative capacity were elevated. It appears that in the case of local bone injury, the absence of systemic stimulation present in multiple trauma results in reduced activation of proliferative capacity. Due to the paucity of studies on MSC and atrophic nonunions, it is difficult to compare the results of this investigation with others. In an interesting recent

examination of percutaneous autologous grafting for tibial atrophic nonunions, the concentration and number of CFU-F from the bone marrow-derived cells were evaluated (Hernigou et al. 2005). CFU-F was decreased in unsuccessful bone unions and negatively associated with longer time necessary for bone union. Moreover, CFU-F was increased in transplants in which the volume of mineralized callus was also increased. They concluded that the addition of bone marrow-derived cells enhances bone healing. The negative outcome observed in unsuccessful bone union confirms our findings. It appears that the reservoir of progenitor cells in bone marrow exert a stimulating effect on bone union processes.

It is well known that age and gender effect bone growth, development and regeneration. A remodeling balance between bone formation through osteoblasts and resorption through osteoclasts is necessary to maintain bone mass. As skeletal maturation is reached around the age of 30, the dynamics shift toward resorption and bone mass gradually declines (Parfitt et al. 2000; Adachi & Takayanagi 2006), which can result in age-related fractures, osteopenia or osteoporosis (Raisz 2005; Russell et al. 2006). However, the exact function of MSC in these processes as well as in the trauma setting has yet to be elucidated. In this study, CFU-F occurrence in trauma patients is clearly both age- and gender-dependent. Colony number and proliferative capacity declined significantly with increasing patient age. In a review of the literature, there are vast discrepancies. Some authors describe a decline in CFU-F occurrence (D'Ippolito et al. 1999; Nishida et al. 1999; Muschler et al. 2001) while others have found additional decreasing ALP expression (Quarto et al. 1995; Baxter et al. 2004) or osteoblastic differentiation (Abdallah et al. 2006) with increasing age. Oreffo et al. (1998) evaluated MSC changes in patients with osteoarthritis and reported no decrease in colony frequency but a decrease in colony size in older patients, which implies a loss of proliferation. Alternatively, other evaluations provided no correlation between MSC growth (Phinney et al. 1999) and age. In further studies including patients with osteoporosis (Stenderup et al. 2001; Justensen et al. 2002), no significant differences were detected in MSC colony number, cell density and mineralized matrix formation in young, old or patients with osteoporosis.
An elegant method of evaluating the aging process in MSC is determining the telomere length. Telomeres are specialized regions of highly repetitive DNA at the end of eukaryotic chromosomes which participate in cell replication. Their shortening

is involved in the cellular aging process (Blackburn 2005) and their fragment length is used to analyze, in this case, MSC replicative potential and lifespan. For instance, Parsch et al. (2004) found in their study comparing MSC with chondrocytes that the telomere length of MSC was not related to the age of the donor. But MSC appear to age themselves as soon as they begin expanding (Bonab et al. 2006). Another recent publication described not only a loss of proliferative and differentiation capacity, a slower growth rate and a lower number of total population doublings (PD) with increasing donor age, but demonstrated that even minimal *in vitro* expansion (10 PD) induces rapid losses of MSC replicative lifespan (Baxter et al. 2004). It is possible that the MSC frequency in bone marrow is maintained with age, but the cells lack the capabilities to form sufficient colonies.

In consideration of these diverse findings, it still remains to be determined if age has an effect on MSC concentration in bone marrow. Many findings, including ours, are consistent with an age-dependent depletion in osteoprogenitor cells in bone marrow and their decreased proliferation potential, which possibly contribute to reduced bone mass and repair. It is feasible to assert that the age-dependent changes discussed above are a result of gender difference, as estrogen is known to have a critical role in bone loss (Raisz 2005). In a comparison of MSC donor properties, Phinney et al. (1999) presented no correlation between MSC growth rate and gender, in contrast to results confirming a decrease in CFU-F in women (Muschler et al. 2001) and postmenopausal loss of bone mass (Brockstedt et al. 1993). We found that the MSC reservoir in male patients was indeed significantly higher than in female, but analyses of estradiol levels, the primary estrogen in men and women, resulted in no significant differences. Moreover, the MSC frequency increased in female patients with multiple trauma in comparison to that of males, which can be explained by the effects of injury severity discussed above.

Nevertheless, age- and gender-associated aspects of the results suggest a dependence on sex hormones or other biochemical mediators. Receptors for sex hormones such as testosterone and estrogen mediate their interactions with osteoblast and osteoclast precursors in bone formation and resorption processes (Michael et al. 2005). Changes in the levels of such sex hormones can in turn result in pathological changes in bone remodeling (Lee K et al. 2003; Raisz 2005). The regulatory actions of estrogen on osteoprogenitor cells have been reported to

promote their phenotypic expression in the form of increased ALP expression (Holzer et al. 2002), improved calcium deposition (Hong et al. 2006) and increased proliferation (DiSilvio et al. 2006). Prolactin, a hormone synthesized in the pituitary gland and ectopically, regulates milk production in the mammary gland, lipid metabolism, insulin secretion stimulation and citrate production in the prostate (Ben-Jonathan et al. 2006). It has been shown to influence bone mass formation and maintenance (Freemark et al. 1997; Clement-Lacroix et al. 1999), MSC differentiation (Ogueta et al. 2002) and improve osteogenesis with heterotopic ossification in severe head injury (Wildburger et al. 1998). Testosterone, the primary androgen in men and women inhibits osteoclasts activity and bone resorption (Hofbauer et al. 2002; Michael et al. 2005). Therefore, in addition to the estradiol and IL-6 analyses described above, prolactin and testosterone values were evaluated as well but yielded no correlations with MSC occurrence. The testosterone levels were, not surprisingly, significantly elevated in male patients. It is important to note however, that estrogen, more than testosterone, regulates bone resorption in both men and women (Hofbauer et al. 2002; Van Pottelbergh et al. 2004). In summation, age and gender account for only a fraction of variability; Thus, MSC can be obtained from patients of both genders and all ages, provided methods for stimulating proliferation are further identified and implemented.

In addition to injury severity, age, gender and hormones, various methods of isolation and cultivation have been found to influence MSC. The source of MSC for instance has been the subject of considerable examination. The use of cells of different origin such as cancellous bone fragments (Noth et al. 2002; Bertram et al. 2005), femoral bone marrow (Lee HS et al. 2003), lumbar vertebral body (Risbud et al. 2006) and umbilical cord and peripheral blood (Wexler et al. 2003) pose difficulties in comparing outcomes. Bone marrow aspirates from the iliac crest are currently the standard site of isolation. In our study, MSC isolated from the proximal extremity of the tibia, greater trochanter and vertebral body did not proliferate sufficiently, supporting the use of the iliac crest for efficient expansion of MSC. Other investigators have examined novel methods of optimizing expansion. Since medium containing fetal bovine serum (FBS) can cause immune reactions or transmit bovine diseases, autologous serum is a viable, less expensive alternative. It was shown to be as effective in stimulating MSC growth as FBS, more effective in promoting cell motility

(Kobayashi et al. 2005) and maintain differentiation potential (Yamamoto et al. 2003; Stute et al. 2004).

These diverse methods of isolation and cultivation contribute to the discrepancies which arise in the abundant publications available. Harvest site, donor variability, nonstandardized assessment of various parameters and general dissimilar experimental conditions are potential sources for limitations in analyses. Establishment of uniform approaches for MSC cultivation is therefore essential for future research.

5.3 Conclusion

The frequency and proliferative capacity of MSC in trauma patients are regulated by the interactions of an extensive spectrum of various factors. In multiple trauma, yet unidentified processes in bone marrow and serum induce the release of factors which stimulate the reservoir and proliferation of MSC. Furthermore, in the case of atrophic nonunion, the MSC concentration in bone marrow is depleted and the absence of systemic stimulation present in multiple trauma results in reduced activation of proliferative capacity. Such patients, with severe injury or atrophic nonunion, present clinically higher necessities for a cell therapy - multiple trauma patients due to their extensive injuries, encompassing often multiple and medically challenging fractures, and atrophic nonunion patients due to their insufficient MSC resources. Future research is therefore essential to identify further factors which regulate MSC activity in trauma and nonunion situations. Moreover, in order to enhance MSC number and proliferative capacity for therapeutic applications in these cases, development of methods to stimulate such factors is necessary. Overall, MSC are a viable cellular resource for a multitude of therapies including degenerative diseases, congenital deformities, trauma-related wound management and acute injury. They present an exciting source of cells for future regeneration and restoration of musculoskeletal tissues damaged by disease or trauma.

6 Summary

Mesenchymal stem cells (MSC), also referred to as marrow stromal cells, maintain the capacity to differentiate into multiple mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes, myoblasts, stromal, neural and endothelial cells. The use of autologous MSC has generated widespread interest due to their developing application in regenerative medicine and tissue engineering in orthopedic surgery. They have become an indispensable cell source for successful implementation in many bone reconstruction procedures. In addition to their multipotency and self-renewal capacity, they are easily harvested, have demonstrated a homing mechanism and can be efficiently expanded *in vitro*, thus providing a safe and cost-efficient tissue replacement for patients with skeletal injury or disease.

Little information is currently available concerning donor characteristics for tissue engineering growth of osseous tissue. This study examines the influences of such donor characteristics, including injury pattern, gender, age, and site of harvest on the quantity, quality and osteogenic differentiation of MSC. The goal is to evaluate whether certain patient groups are practically suitable for an *ex vivo* expansion and therapeutic reimplantation of MSC.

The effect of injury pattern on the reservoir and proliferative capacity of MSC in human bone marrow is clearly demonstrated in this analysis. Age and gender were also shown to influence MSC number and proliferation, as in previous studies.

A total of 53 participants (46 patients and 7 healthy volunteers ranging from 18 to 64 years of age), who were scheduled to undergo operative procedures on the pelvis, vertebrae, tibia or hip as well as cancellous bone autografts for reconstruction of various bone defects, were included in the study. Participants were divided into 4 groups for each gender: single fracture, multiple trauma, atrophic nonunion and healthy volunteers. A minimum of 6 ml bone marrow samples were aspirated intraoperatively and processed immediately according to protocol. Following cultivation and expansion for 14 days, the cells were then stained for the colony forming unit-fibroblast (CFU-F) assay and each culture flask was photographed, digitized and converted to an 8 bit grey level TIF-format. Using the digitized CFU-F assay, the mean colony number, mean colony area and mean cell number per microscopic field of view (cell density) could be determined. In addition, confirmation of MSC phenotype was established using fluorescent activated cell sorting (FACS).

MSC potential for osteogenic differentiation was quantified by von Kossa, alkaline phosphatase and alizarin staining. Furthermore, serum from a total of 39 randomly chosen participants was collected and tested for hormone levels of 17β-estradiol, testosterone and prolactin as well as the cytokine interleukin-6.

These analyses demonstrate several significant trauma-related modifications in MSC reservoir and proliferation, in both male and female patients. In multiple trauma patients, the highest MSC frequency was found, independent of gender and age. Proliferative capacity was also highest in male multiple trauma patients. In the case of atrophic nonunion, the lowest MSC reservoir was detected, independent of gender. Furthermore, MSC frequency in male patients was significantly higher than in female, although analyses of hormone and interleukin-6 levels provided no correlation. Age-related changes in MSC reservoir could also be observed, whereas the proliferative capacity produced only a tendency toward decreasing values with increasing age. Concerning the site of cell harvest, MSC isolated from the proximal extremity of the tibia, greater trochanter and vertebral body did not proliferate sufficiently enough to be included in statistical analysis, supporting the use of the iliac crest for efficient expansion of MSC.

This data suggests the interaction of yet to be identified processes in bone marrow in multiple trauma situations which stimulate the activation and mobilization of MSC. Moreover, in the case of atrophic nonunion, the concentration in bone marrow is depleted and the absence of systemic stimulation present in multiple trauma results in reduced activation of proliferative capacity. Such patients, with severe injury or atrophic nonunion, represent a group of patients with an especially acute necessity for effective and successful bone reconstruction. This data can be used to determine the applicability of MSC from various patient groups for osseous tissue replacement procedures. Especially in such medically challenging situations, further research is essential not only to delineate the factors involved in MSC regulation but also to develop methods to stimulate MSC expansion and proliferation.

7 Zusammenfassung

Mesenchymale Stammzellen (MSC), auch als „marrow stromal cells“ bezeichnet, besitzen die Kapazität zur Differenzierung in unterschiedliche Zellreihen wie Osteoblasten, Chondrozyten, Adipozyten, Myoblasten, Stromazellen, neurale Zellen und Endothellzellen. Der mögliche klinische Einsatz autologer MSC für die Gewebetechnik (tissue engineering) im Bereich der Wiederherstellungschirurgie, Traumatologie und Orthopädie ist mittlerweile das Thema von zahlreichen wissenschaftlichen und medizinischen Studien. Charakteristisch für MSC sind nicht nur Multipotenz und die Kapazität zur Selbsterneuerung, sie sind auch leicht isolierbar, besitzen einen Homing-Mechanismus und können effektiv *in vitro* expandiert werden. Dadurch stellen sie eine sichere und kosteneffiziente Option für Gewebsersatz für Patienten mit ossären Verletzungen oder Erkrankungen dar.

Derzeit gibt es nur unzureichende Informationen bezüglich der Eigenschaften von MSC in Abhängigkeit des Spenders. Diese Arbeit untersucht den Einfluss von Spendereigenschaften wie Verletzungsmuster, Geschlecht, Alter und Entnahmestelle auf die Quantität, Qualität und osteogene Differenzierung mesenchymaler Stammzellen. Ziel der Evaluation ist Aufschluss zu geben, ob MSC bestimmter Patientengruppen überhaupt für eine *ex vivo* Expansion und anschließende therapeutische Reimplantation geeignet sind.

Der signifikante Einfluss des Verletzungsmusters auf das Reservoir und die proliferative Kapazität von MSC im menschlichen Knochenmark wird in dieser Analyse eindeutig nachgewiesen. Analog zu früheren Studien korrelierten Alter und Geschlecht mit der MSC-Konzentration und –Proliferation.

Insgesamt 53 Personen (46 Patienten und 7 gesunde Freiwilligen im Alter von 18 bis 64 Jahren) wurden in die Studie eingeschlossen. Die Knochenmarkpunktion erfolgte während einer ohnehin geplanten Operation an Beckenkamm, Wirbelsäule oder Tibia. Die Teilnehmer wurden gemäß ihres Verletzungsmusters in 4 Gruppen eingeteilt: Monofraktur, Polytrauma, Pseudarthrose und Kontrolle. Mindestens 6 ml Knochenmark wurden intraoperativ aspiriert und sofort nach einem Standardprotokoll verarbeitet. Nach 14 Tage Kultivation und Expansion wurden Zellen für den *Colony Forming Unit-Fibroblast (CFU-F)*-Test gefärbt und anschließend fotografiert, digitalisiert und in 8-Bit-Graustufen-TIF formatiert. Anhand der digitalisierten CFU-Assays konnten die mittlere Koloniezahl, Koloniegröße und Zellzahl pro Kolonie

bestimmt werden. Der Phänotyp der kultivierten MSC wurde mittels Durchflusszytometrie bestätigt und das Potential zur osteogenen Differenzierung durch von Kossa-, Alkalische Phosphatase- und Alizarin-Färbung überprüft. Weiterhin wurde in Serumproben von insgesamt 39 zufällig ausgewählte Patienten 17β-Estradiol-, Testosteron-, Prolaktin und Interleukin-6 Spiegel bestimmt.

Diese Arbeit zeigt bei Männer und Frauen multiple, signifikante, verletzungsbezogene Modifizierungen im MSC Reservoir und in der MSC-Proliferation. Polytraumen zeigten die höchste MSC-Konzentration im Knochenmark, unabhängig vom Geschlecht oder Alter. Die proliferative Kapazität war am höchsten bei männlichen Patienten mit Polytrauma. Bei Pseudarthrose konnte unabhängig vom Geschlecht die niedrigste MSC-Konzentration im Knochenmark festgestellt werden. Des Weiteren war die MSC Frequenz bei männlichen Patienten signifikant höher als bei weiblichen Patienten. Die Hormon- bzw der Interlekin-6-Spiegel korrelierten jedoch nicht mit den Stammzellparametern. Altersabhängige Veränderungen im MSC Reservoir wurden beobachtet, wobei die proliferative Kapazität nur eine Tendenz zu verminderten Werten mit zunehmendem Alter aufwies. Untersuchungen zum Einfluss der Entnahmestelle ergaben, dass MSC aus dem Tibiakopf oder Wirbelkörper nur ungenügend wachsen. Dieses Ergebnis unterstützt die Hypothese, dass hauptsächlich MSC aus dem Knochenmark des Beckenkamms zur effizienten MSC Expansion geeignet sind.

Die Ergebnisse weisen auf noch unklare Prozesse im Knochenmark hin, welche bei Polytrauma die Aktivierung und Mobilisierung von MSC stimulieren. Weiterhin ist bei Pseudarthrose die MSC-Konzentration im Knochenmark reduziert. In diesem Fall bewirkt wahrscheinlich die fehlende systemische Stimulation eine verminderte Aktivität. Besonders Patienten mit schweren Verletzungen oder Pseudarthrose haben höhere Risiken für Knochenheilungsstörungen und benötigen deshalb effektive und sichere Methoden zur Knochenrekonstruktion und gegebenenfalls für Knochenersatz. Für die zukünftige Verwendung von autologen *ex vivo* expandierten MSC geben diese Erkenntnisse Aufschluss, bei welchen Patientengruppen MSC eine ausreichende Qualität für eine therapeutische Anwendung aufweisen. Besonders für problematische Fälle sind weitere Studien notwendig, um die detaillierte Regulation von MSC zu untersuchen und Kultivierungs- und Expandierungsbedingungen zu verbessern.

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9 List of abbreviations

ALP: alkaline phosphatase
CD: cluster of differentiation
CFU-F: colony forming unit-fibroblast
CMV: cytomegalovirus
FACS: fluorescent activated cell sorting
FBS: fetal bovine serum
FOV: field of view
HIV: human immune deficiency virus
HLA: human leukocyte antigen
HO: heterotopic ossification
IL: interleukin
ISS: injury severity score
LNGFR: low-affinity nerve growth factor receptor
MSC: marrow stromal cell, mesenchymal stem cell
PBS: phosphate buffered saline
PD: population doubling
TGF-β: transforming growth factor beta
TIFF: tagged image file format
VEGF: vascular endothelial growth factor

10 Appendix

Self preparation: 5% silver nitrate solution

5 gram silver nitrate dissolved in 100 ml distilled water

Self-preparation: 5% sodium thiosulfate solution

5 gram thiosulfate dissolved in 100 ml distilled water

Protocol 1: Isolation of MSC from bone marrow aspirate

a) wash cells (1:1 v/v) with PBS w/o Mg^{2+} and Ca^{2+}
b) centrifuge at 850 g for 10 minutes
c) aspirate the supernatant
d) repeat steps a) to c)
e) fill with PBS w/o to original volume from a)
f) fill a Falcon tube of Ficoll 1.073 g to original volume from a)
g) pipet the cells carefully onto the Ficoll
h) centrifuge at 1000 g for 30 minutes
i) transfer the opaque interface into a clean Falcon tube
j) add 30 ml PBS w/o containing FBS 2% to the cells
k) pellet cells by centrifugation at 850 g for 10 minutes
l) aspirate the supernatant
m) resuspend the cells in 3 ml MesenCult Basal Medium for counting

Protocol 2: Cell count

a) combine 150 µl erythrocyte lysis solution with 50 µl of the cell suspension, vortex
b) place in water bath at 37℃ for 5 minutes
c) add 50 µl Türks dye, vortex
d) count cells using Neubauer counting chamber and adjust density for plating

Protocol 3: CFU-Assay

a) using cells from Protocol 1, plate cells in 3 culture flasks, at a density of 5 x 10^5, 1 x 10^6 and 2 x 10^6 in 10 ml each of MesenCult Basal Medium
b) incubate cells for 14 days in humidified incubator at 37°C, 5% CO_2, changing medium 3 times per week
c) on day 14 remove medium and rinse cells with distilled water
d) fix cells with 1 ml/culture flask Diff-Quick formula 1 (methanol) for 5 seconds
e) stain cells with 1 ml/culture flask Diff-Quick formula 2 (buffered eosin) for 5 seconds
f) stain cells with 1 ml/culture flask Diff-Quick formula 3 (buffered azure) for 5 seconds
g) rinse cells thoroughly but gently with distilled water and allow to air-dry

Protocol 4: Von Kossa staining

a) wash cells 3 times with distilled water
b) set the cells with 1 ml ice cold ethanol for 20 minutes (in freezer)
c) wash again 3 times with distilled water
d) incubate cells with 1 ml 5% silver nitrate solution for 1 hour (room temperature)
e) wash again 3 times with distilled water
f) remove un-reacted silver with 1 ml 5% sodium thiosulfate for 5 minutes
g) counterstain with 1 ml nuclear fast red for 10 min

11 Curriculum vitae

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Publikationen / Poster

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R. Tewksbury, C. Seebach, D. Henrich, K. Wilhelm, I. Marzi. Influence of age, gender and extraction site on the colony number of mesenchymal stem cells. *Langenbeck's Archives of Surgery, Vol. 390, Nr. 5, Sept 2005, Abstract 104, 9. Chirurgische Forschungstage*, Johann Wolfgang Goethe-Universität, Frankfurt	09/2005
A. Scherzed, R. Tewksbury, C. Seebach, D. Henrich, K. Wilhelm, I. Marzi. Comparison of the osteogenic capacity of osteogenic differentiating media. *Langenbeck's Archives of Surgery, Vol. 390, Nr. 5, Sept 2005 Abstract 89, 9. Chirurgische Forschungstage*, Johann Wolfgang Goethe-Universität, Frankfurt	09/2005

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12 Schriftliche Erklärung

Ich erkläre, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

„The influences of injury pattern, gender and age on the function and proliferation of marrow stromal cells”

in der
Klinik für Unfall-, Hand- und Wiederherstellungschirurgie

unter Betreuung und Anleitung von Herrn Professor Dr. med. I. Marzi mit Unterstützung von Herrn Dr. phil. nat. D. Henrich

ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.
Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht.
Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.
Ausschnitte aus der vorliegenden Arbeit wurde in folgendem Publikationsorgan veröffentlicht:
C. Seebach, D. Henrich, R. Tewksbury, K. Wilhelm, I. Marzi. Number and proliferative capacity of human mesenchymal stem cells (MSC) are positively modulated in multiple trauma patients and negatively in atrophic nonunions. *Calcified Tissue International, 2007 April;80(4):294-300*

Frankfurt am Main, 20. September 2007 Robyn Tewksbury