Bone Marrow Derived Adult Stem Cells: Characterization and Application in Cell Therapy

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D7

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Tag der mündlichen Prüfung:

Dedicated to my beloved grandmother

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ABBREVIATIONS

ES cells, Embryonic Stem cells

MSC, Mesenchymal Stem Cell

LIF, Leukemia Inhibitory Factor

PDGF, Platelet Derived Growth Factor

EGF, Epidermal Growth Factor

FGF2, Fibroblast Growth Factor 2

BM, Bone Marrow

GFP, Green Fluorescent Protein

EAE, Experimental Autoimmune Encephalomyelitis

MS, Multiple Sclerosis

BBB, Blood Brain Barrier

SCF, Stem Cell Factor

CNS, Central Nervous System

ICM, Inner Cell Mass

MEF, Mouse Embryonic Fibroblast

BMP4, Bone Morphogenic Protein 4

STAT proteins, Signal Transducers and Activators of Transcription proteins

TGFα/β, Transforming Growth Factor α/β

IL-2/6, Interleukin-2/6

HSCs, Hematopoietic Stem Cells

HPCs, Hematopoietic Progenitor Cells

SDF-1, Stromal Derived Factor-1

MAPC, Multipotent Adult Progenitor Cells

DNA, Deoxyribonucleic Acid

RNA, Ribonucleic Acid

BDNF, Brain Derived Neurotrophic Factor

HIF-1, Hypoxia Induced Factor

RT, Reverse Transcriptase

IP-10, Interferon γ inducible Protein10

Mig, Monokine induced by interferon γ

IFN α/β/γ, Interferon α/β/γ

TNF-α, Tumor Necrosis Factor-α

MBP, Myelin Basic Protein

MOG, Myelin Oligodendrocyte Glycoprotein

SSEA-1, Stage Specific Embryonic Antigen

LTR region, Long Term Repeats region

PBS, Phosphate Buffered Saline

PCR, Polymerase Chain Reaction

FACS, Fluorescent Activated Cell Sorting

hCG, human Chorionic Gonadotropin

PMS, Pregnant Mare's Serum

IRES, Internal Ribosomal Entry Site

TAT, Trans-actin Activator of Transcription

NLS, Nuclear Localization Signal

pMSCV, Murine Stem Cell Vector

Ig, Immunoglobulin

BSA, Bovine Serum Albumin

CFA, Complete Freund's Adjuvant

PTX, Pertussis Toxin

FITC, Fluoro-isothiocyanate

FCS, Fetal Calf Serum

MHC, Major Histocompatibility Complex

PFA, Paraformaldehyde

VLA-4, Very Late Antigen-4

INTRODUCTION

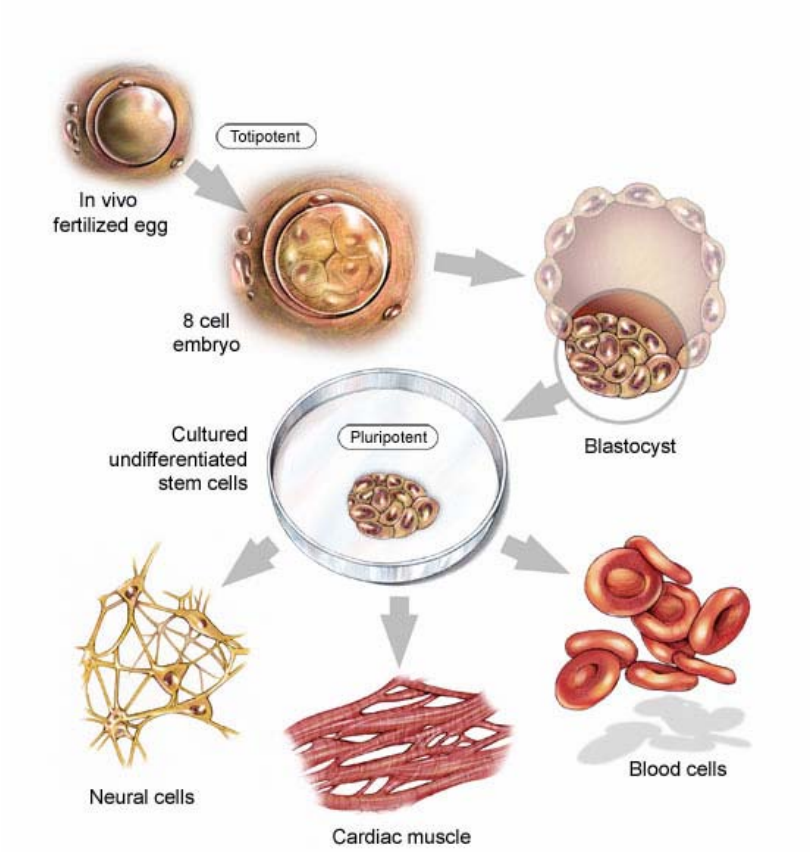
Stem Cells

Stem cells are unspecialized cells having a unique ability to self-renew, proliferate and the potential to give rise to other specialized cells. The potential of stem cells to differentiate into different type of cells is determined regarding their potency. Cells produced by the first divisions of a fertilized egg are totipotent stem cells and they can differentiate into all embryonic and extraembryonic lineages. Pluripotent stem cells are derived from further stages of embryo (ex. Blastocyst). They can give rise to all three embryonic germ layers excluding extraembryonic tissues. Multipotent stem cells have a more restricted differentiation profile. Many adult tissues have been discovered to have residing multipotent stem cells that can differentiate into several tissue types. A unipotent stem cell still has a property of self-renewal for which it can be considered a stem cell; however, unipotent stem cell only can differentiate to one type of cell. Regarding their source there are two types of stem cells: embryonic and adult stem cells.

Embryonic Stem (ES) Cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocyst (a four or five day old embryo) and can give rise to all three germ layers (ectoderm, mesoderm and endoderm) which are the embryonic source of all the tissues in the adult organism. Ectoderm is the upper external layer of the embryo which forms skin and nerve tissue. Mesoderm is the middle layer of cells of the embryonic disk which is known to be a precursor for bone, muscle and connective tissue. Endoderm is the lower layer of cells from the inner cell mass which gives rise to lungs and digestive organs at later stages.

Blastocyst includes three structures. Trophoblast, a layer of cells surrounding the blastocyst, forms the extraembryonic tissue responsible for implantation of the embryo and developing of placenta. Blastocoel is the fluid-filled hollow cavity inside the blastocyst. The inner cell mass is a cluster of approximately 30 cells located at one end of blastocoel and forms the embryo. The inner cell mass cells are the pluripotent stem cell population, from which ES cells can be generated (Figure 1).



Source: http://www.stemcellresearchfoundation.org/WhatsNew/Pluripotent.htm

Figure 1: *Derivation of ES cells from inner cell mass (ICM) of blastocyst. Once derived and cultured in vitro on MEF cells, ICM cells can grow extensively without losing their pluripotent properties. ES cells are pluripotent stem cells derived from ICM cells. They easily can be differentiated to any kind of tissue under proper differentiation signals.*

Mouse embryonic stem cells can be maintained on mouse embryonic fibroblast (MEF) cells for very long time (years) without differentiation. It has been previously described that MEFs prevent differentiation of mouse ES cells via producing various factors, like cytokines IL-6 and Leukemia Inhibitory Factor (LIF) (Williams et al., 1988;Smith et al., 1988). It is known that addition of recombinant LIF in culture medium can support the mouse ES cell self-renewal without MEF support.

Pluripotency of ES cells can be confirmed via transplantation of these cells into ICM of blastocyst and analysis of the integration capacity of these cells into various tissues. Pluripotent cells integrate completely into a developing embryo after intra-blastocyst injection and produce a high rate of chimerism in the tissues of the

developing fetus. Various transcription factors have been associated with pluripotency of the cell.

Octamer 4 (Oct4) is a homeodomain transcription factor of the POU family. This protein is critically involved with self-renewal of undifferentiated embryonic stem cells. In mice Oct4 is expressed in oocyte and preimplantation embryo; however, later it is restricted only to inner cell mass of blastocyst (Okamoto et al., 1990;Scholer et al., 1990) indicating that the expression of Oct4 is restricted to totipotent and pluripotent cells. Oct 4 deficient embryos lose the pluripotency of ICM, and differentiate towards a trophoblast lineage (Nichols et al., 1998). Downstream genes of Oct4 include the gene encoding the extracellular matrix (ECM) protein osteopontin (Spp1), which is expressed in the primitive endoderm; heart and neural crest derivatives expressed-1 (Hand1), which is expressed in early trophoectoderm; fibroblast-growth factor-4 (Fgf4) which is expressed in the ICM ; F-box protein-15 (Fbx15), which is expressed in embryonic stem cells and later in testis; and Rex1, which is also known as zinc-finger protein-42 (Zfp42).

Nanog is a transcription factor critically involved in self-renewal of pluripotent embryonic stem cells. It was first discovered by Wang in 2003 (Wang et al., 2003a) and, later, it was named Tir nan Og or Tir Na Nog, after the mythological Celtic land of the 'ever young' (Chambers et al., 2003). Nanog expression is restricted to the inner cell mass of blastocysts and primordial germ cells. Nanog deficient embryonic stem cells go towards extra-embryonic endoderm lineage differentiation. Little is known about the regulation of Nanog gene, except that the transcriptional activator and tumor suppressor p53 binds to the promoter of Nanog, thereby enabling p53-dependent suppression of Nanog expression (Lin et al., 2005).

Sox2 transcription factor is a member of the (high mobility group) HMG-domain Deoxiribonucleic acid (DNA)-binding-protein family that is implicated in the regulation of transcription and chromatin architecture. The Sox2 is expressed in ES cells as well as in neuronal stem cells. Oct4 binds to the octamer element in Sox2 forming a complex which have recently been shown to be required for the upregulation of mouse and human Nanog transcription (Kuroda et al., 2005).

Rex1 is a zinc finger protein (*Zfp42*). Zinc finger is a protein that can bind to DNA. The structure of each individual finger is highly conserved and consists of about 30 amino acid residues, constructed as two antiparallel β sheets and an α helix and held together by the zinc ion. Many transcription factors, regulatory proteins, and other

proteins that interact with DNA contain zinc fingers. Rex1 messenger Ribonucleic acid (mRNA) is detected in undifferentiated ES and embryonic carcinoma (EC) cells, blastocyst, trophoectoderm and meiotic germ cells of an adult mouse testis. Rex1 promoter contains an Octamer 4 binding region (ATTTGCAT) which requires Oct4 for its regulation. Upon differentiation the expression of Oct4 is downregulated which consequently interfere with Rex1 promoter regulation downregulating Rex1 expression.

Although proteins like Oct4, Nanog, Sox2 and Rex1 are necessary for pluripotency in embryonic stem cells, these proteins seem to be the downstream effectors of upstream signalling events (Figure 2).

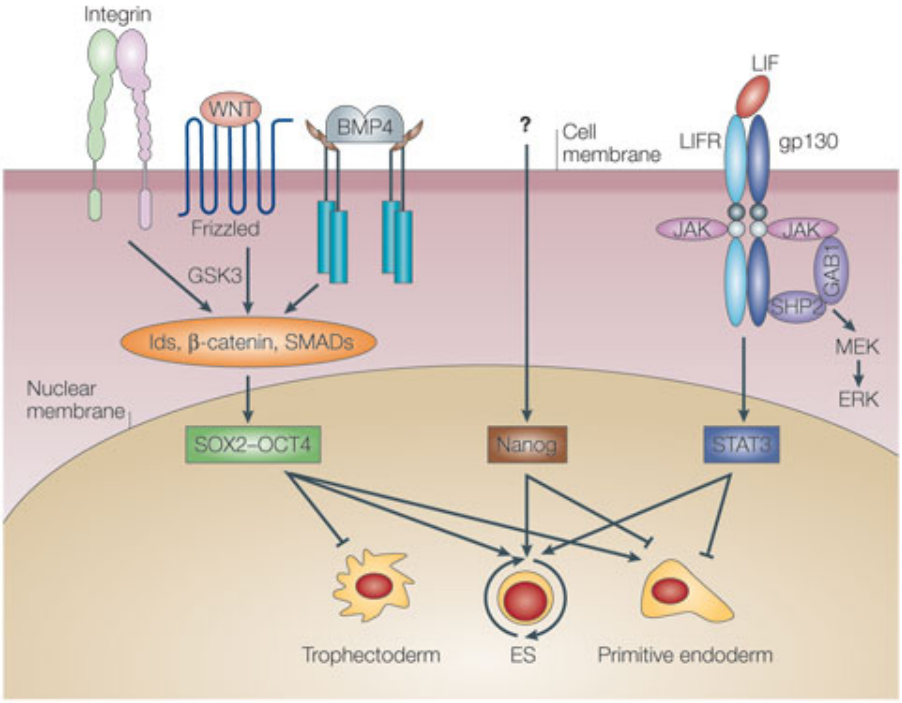


***Figure 2**: Signalling pathways potentially involved in maintaining mouse ES cell pluripotency. 1) LIF pathway is involved in activation of STAT3, 2) WNT and Bone Morphogenic Protein 4 (BMP4) signalling regulates Sox2 and Oct4 expression, and 3) Nanog although the molecular pathway is not defined are main pathways described to be important in maintaining the self-renewal and pluripotency of ES cells.*

Signalling Pathways Involved in Maintenance of Pluripotency in ES cells:

Leukemia Inhibitory Factor (LIF)

LIF, an interleukin (IL) 6 class cytokine, has a major role in self-renewal and proliferation. LIF derives its name from the ability to induce the terminal differentiation of myeloid leukemic cells. LIF is essential for maintaining the undifferentiated state of mouse ES cells. Interestingly, LIF is only able to maintain ES cells in the presence of serum, suggesting that additional factors are required. LIF signalling involves gp130 and LIF receptor complex. The tyrosine kinase Janus Kinase (JAK) is bound to the intracellular part of this receptor complex. Once the complex is activated via LIF binding, JAK phosphorylates the tyrosine residues of both receptors which activate the signal transducers and activators of transcription (STAT), STAT1 and STAT3. Activated STAT proteins form homo or heterodimers and translocate to the nucleus. Once the activated transcription factors reach the nucleus, they bind to a DNA-recognition motif called gamma activated sites (GAS) in the promoter region of cytokine-inducible genes and activate transcription of these genes. (Okita and Yamanaka, 2006). The treatment of ESCs with LIF also induces the phosphorylation of extracellular signal-regulated protein kinases, ERK1 and ERK2 (Ref. 23), and increases mitogen-activated protein kinase (MAPK) activity (Auernhammer et al., 2000) (Figure 2).

Bone morphogenic protein 4

BMP4 is a polypeptide belonging to the Transforming Growth Factor beta (TGF-β) protein superfamily. Like other bone morphogenetic proteins, it is involved in bone and cartilage development, specifically in tooth and limb development and fracture repair. BMP4 is another signalling molecule involved in self-renewal and proliferation during embryonic development. However, not much is known about its function. In the presence of LIF, BMP4 enhances self-renewal and proliferation of stem cells by activating the gene encoding for the transcription factor SMAD 4 (similar to mothers against decapentaplegic homologue 4) which, in turn, activates member of Id (inhibitor of differentiation) gene family. This interaction is facilitated in serum containing conditions. By contrast, in the absence of LIF, BMP4 counteracts the LIF cascade, interacting with different SMAD transcription factors (for example, SMAD1, 5 and 8) that have an inhibitory effect on the Id genes. Furthermore, BMP4

can regulate cell fate in relation to cell density (Rajan et al., 2003) by activating distinct cytoplasmic signals (Figure 2).

WNT

Wnt is another pathway known to be involved in the signalling cascade required for self-renewal and proliferation of mouse ES cells. Wnt proteins bind to cell-surface receptors of the Frizzled family, causing the receptors to activate Dishevelled family proteins and ultimately resulting in a change in the amount of β-catenin that reaches the nucleus. Dishevelled is a key component of a membrane-associated Wnt receptor complex which, when activated by Wnt binding, inhibits a second complex of proteins that includes axin, GSK-3, and the protein APC. The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signalling molecule. After this "β-catenin destruction complex" is inhibited, a pool of cytoplasmic β-catenin stabilizes, and some β-catenin is able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression (Figure 2).

Since ES cells have unrestricted differentiation capacity, they represent a very important tool for future cell therapy applications. Moreover, the discovery of ES cells enables the development of gene targeting technology via generation of knockout mice for studying the gene function. Despite the outstanding contributions to the science, there are some controversial points to be solved in ES cell research. The use of human ES cells in therapeutic treatments requires direct isolation of these cells from blastocyst embryo. Opponents of ES cell research hold that the human life begins as soon as an egg is fertilized, which brings the ES research to a serious ethical discussions. Besides, there are also critics on therapeutical use of ES cells. As a matter of fact, ES cells induce tumor formation in many therapeutic studies performed with mice.

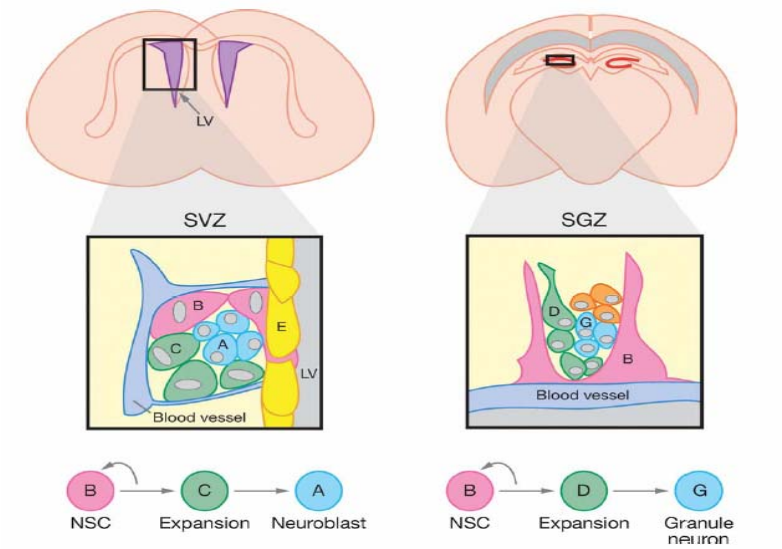
Adult Stem Cells

The abovementioned debates focused the search for derivation of multi- or pluripotent cell lines from adult tissues. Adult stem cells are active constructors during organogenesis, of tissue regeneration and homeostasis in an adult life. Adult stem cells have been identified in many tissues, where they continuously generate and regenerate mature tissues either as part of the normal physiology or in response to injury. Adult (or somatic) stem cells described so far include neural, epidermal, germ,

hematopoietic and mesenchymal stem cells. In postnatal life, adult stem cells reside in a special microenvironment called “niche”, which varies in nature and location depending on the tissue type. The “Niche” concept was first introduced by Schofield in 1978. It was proposed that Hematopoietic Stem Cells (HSCs) are in an intimate contact with the bone, and cell-to-cell contact is required to support the proliferation capacity of the HSCs (Schofield, 1978). However, the research work for the validation of the “niche” hypothesis is more recent. According to the most accepted definition, a stem cell “niche” contains microenvironmental niche cells that nurture the stem cells, provide a sheltering environment in order to protect the stem cells against differentiation, apoptotic signalling and many other stimuli that can change the “stemness” of the cells. The niche safeguards also against excessive cell proliferation that can potentially lead to cancer (Moore and Lemischka, 2006). Stem cells should periodically activate in order to maintain the tissue homeostasis and self-renew to keep the stem cell balance in the “niche”.

Neural Stem Cells (NSC)

Neural stem cells were first discovered in 1990 (varez-Buylla et al., 1990). Although they can be isolated from various regions from the adult brain, the subventricular zone (SVZ) and subgranular zone (SGZ) of hippocampus region are well-characterized germinal regions where NSCs reside and support neurogenesis (Lois and varez-Buylla, 1993;Kirschenbaum et al., 1999). There are four types of cells in SVZ (Figure 3). Immature astrocytes (B) in SVZ have stem cell features. They can self-renew and give rise to transient amplifying cells (C), which later generate neuroblasts (A). Neuroblasts differentiate into neurons and oligodendrocytes which later migrate to the olfactory bulb or other regions in the brain (Doetsch, 2003) . In SGZ region neurogenesis occurs locally in direct contact with blood vessels. Astrocytes (B) in this region show comparatively high self-renewal properties, proliferate and produce daughter cells (D) which further generates granule neurons (G).



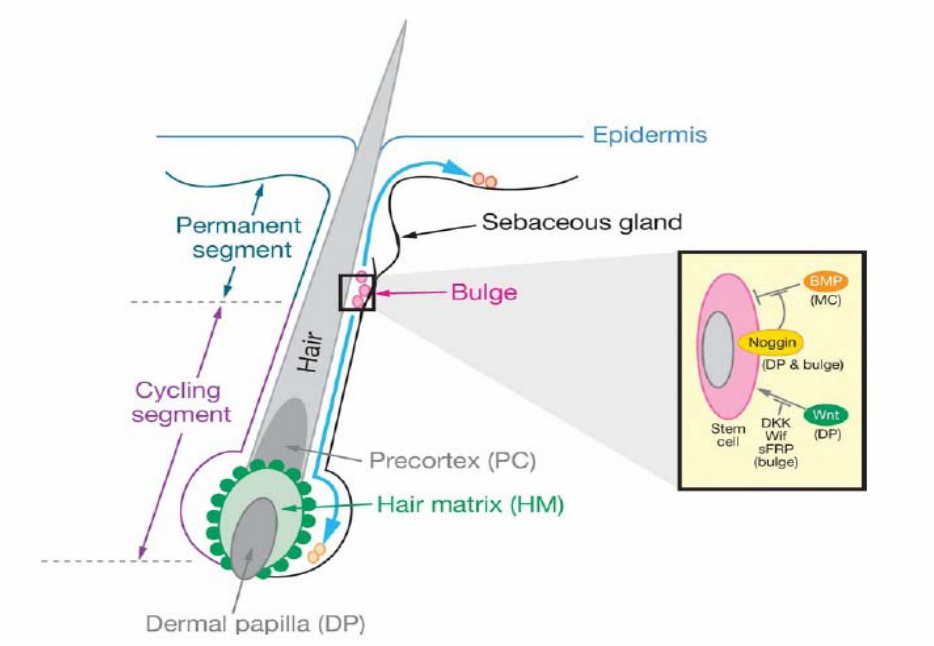
(Li and Xie, 2005)

***Figure 3**: Neural stem cells were discovered in SVZ and SGZ parts in the brain. Astrocytes (B cells) residing in that region proliferate and form daughter cells with high self-renewal properties (C and D cells) which further differentiate into neuronal lineages (A and G cells).*

Endothelial cells and specialized basal laminal cells in both SVZ and SGZ are known to be important components of the neural stem cell niche. Many signalling factors, generated from niche environment including BMPs, Noggin, fibroblast growth factors (FGFs), insulin like growth factor (IGF), vascular endothelial growth factor (VEGF), TGFα, brain-derived neurotrophic factor (BDNF), regulate neurogenesis (Shen et al., 2004) .

Epithelial Stem Cells (ESCs)

Epidermal stem cells are located in the bulge area in between the permanent and cycling segment of a hair follicle. The epidermal stem cells are self-renewing and highly proliferative cells that give rise to daughter cells that either can migrate upward generating epidermal progenitor cells and contribute to regeneration processes, or can migrate down forming hair-matrix progenitor cells (Niemann and Watt, 2002). The bulge area where the stem cells are located is an environment that restricts cell growth and differentiation by expressing Wnt inhibitors. Moreover, BMP, FGF, Notch, Noggin signalling pathways are important for epithelial stem cell maintenance in its niche (Li and Xie, 2005).

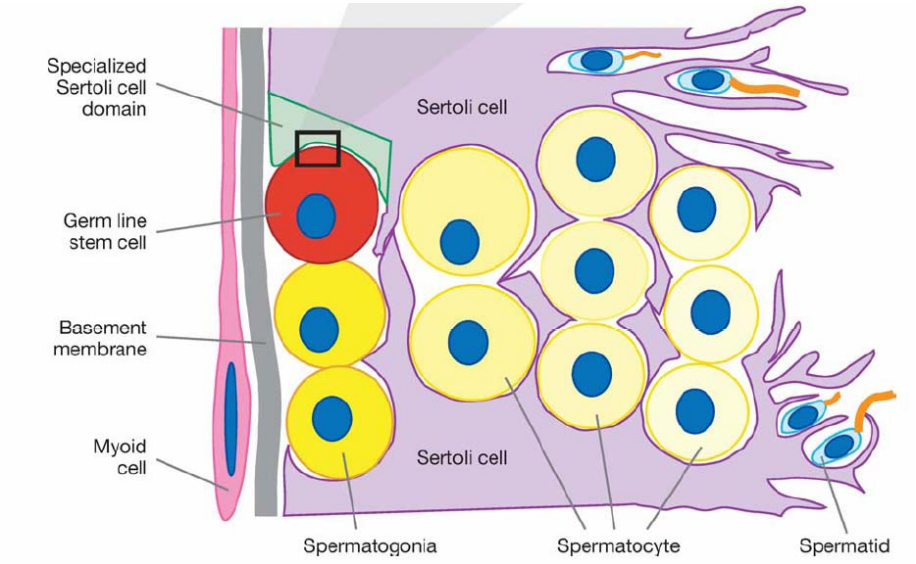


(Li and Xie, 2005)

***Figure 4**: Hair follicle. An epidermal stem cell resides in the Bulge region in the hair follicle. These stem cells with high self renewal properties can migrate either to upwards and takes place in regeneration of epidermis or migrates towards the dermal part and becomes a matrix cell.*

Germ Stem Cells

Germ stem cells are located in the periphery of seminiferous tubules. Germ stem cells (2n) are very rare; they divide and give rise to spermatogonial cells (2n). Spermatogonial cells divide and give rise to spermatocyte that later undergo meiosis and form immature sperm cells called spermatids (n) which finally become mature sperm cells. Germ stem cells are in close contact with the basement membrane. Steroli cells are the niche cells that maintain the germ stem cells and supports spermatogenesis (Shinohara et al., 2000).



(Li and Xie, 2005)

***Figure 5**: Germ stem cells are found in very close contact with the basement membrane. Steroli cells are the supportive niche cells for the germ stem cells. Germ stem cells (2n) give rise to Spermatogonial cells (2n) and further on to Spermatocyte (2n) which finally undergoes meiosis and forms gametes (n).*

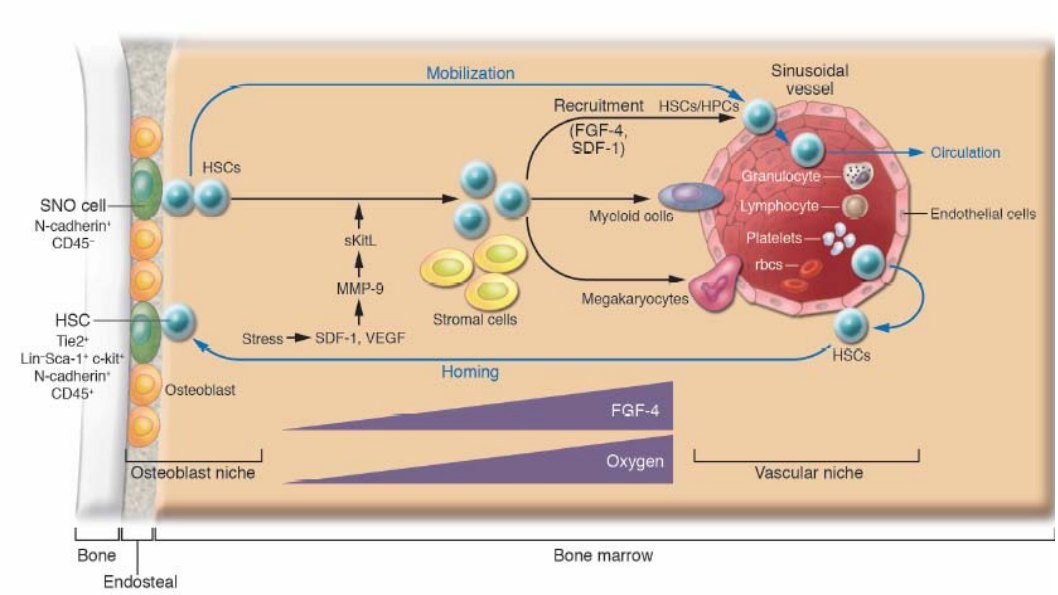
Bone Marrow Derived Stem cells

Among adult stem cells, bone marrow derived stem cells and their niches are the most widely studied. Bone has an important role in stability and movement of the body. The bone is composed of many bone cells (osteoblasts and osteoclasts), collagen fibrils, and mineral deposits like calcium and phosphate. Bone marrow, the largest organ in the body, is a very complex organ located inside of the bone cavity and contains blood vessels and many types of cells including variety of progenitor cells and stem cells. Two kind of stem cells have been identified in the bone marrow (BM): Hematopoietic Stem Cells (MCCULLOCH and TILL, 1960) and Mesenchymal Stem Cells (MSCs) (Friedenstein, 1976). Of these, HSCs are among the most studied stem cells.

Hematopoietic Stem Cells

HSC are the precursor cells which give rise to all the blood cell types of both the myeloid and lymphoid lineages. Murine HSCs are present at very low frequency (from 1/10 000 to 1/ 1 000 000) in the bone marrow depending on the age of the organism. There are 2 types of HSCs identified: short-term hematopoietic stem cells which have a short reconstitution ability limited to several weeks; and long-term hematopoietic stem cells which can contribute to hematopoiesis for a long time even for lifetime. Homing studies performed to trace the migration of the Green Fluorescent Protein (GFP)-labeled HSCs revealed that the endosteal surface of the trabecular bone might be the possible hematopoietic stem cell niche in the BM (Nilsson et al., 2001). Recent studies reported that an increase in the osteoblast number directly correlates with the number and activity of HSCs indicating that osteoblasts are an essential part of the niche (Zhang et al., 2003); (Calvi et al., 2003;Visnjic et al., 2004). Detection of HSCs adjacent to sinusoidal endothelial cells (Kiel et al., 2005), suggests the existence of a second HSC niche in the bone marrow. Studies supporting the presence of both stem cell niches in the bone marrow suggest a model in which osteoblastic niche in the endosteum provides a quiescent microenvironment, while sinusoidal endothelial niche promotes proliferation and differentiation of stem cells providing nutrient-rich microenvironment and higher oxygen concentration (Figure 6). Many adhesion molecules like N-cadherin/β-catenin, VCAM/integrin, and osteopontin/β1 integrin are important in the niche function. They serve for stem cell-niche cell adhesion and communication and stem cell migration. One of the known signalling pathways to promote self-renewal and proliferation of HSCs is Stem Cell Factor (SCF)/c-kit signalling. SCF is secreted by stromal cells of the niche which can bind to the c-kit receptor of HSCs and promote proliferation. In a similar way, the Notch receptor expressed by HSCs interacts with its ligand Jag 1, secreted by osteoblasts, and maintains undifferentiated state of stem cells in the niche. HSCs also express FGF receptor and FGF has been shown to stimulate HSC self-renewal and proliferation *in vitro* (de et al., 2003). Therefore, it is possible that the FGF signals coming from the vascular niche play a role in the recruitment of HSCs and Hematopoietic Progenitor Cells (HPCs) by a gradient between the osteoblastic niche (lower FGF expression) and the vascular niche (higher FGF expression) (Figure 6).

Endothelial cells, osteoblasts, and other stromal cells constitutively express stromal derived factor 1 (SDF-1), and HSCs express CXCR4, the only known receptor for this factor. Mobilization of HSCs from the osteogenic niche to the endothelial niche, to the blood stream and back again to the BM, depends strongly on SDF-1/CXCR4 interaction. The highest SDF-1 concentration is found on the surface of osteoblasts which is an important driving force for the HSC return to their osteoblastic niches. HSC mobilization will be described in detail further.



(Yin and Li, 2006)

Figure 6: *Endosteal and Vascular HSC niche in the bone marrow. Recruitment of HSCs from endosteum towards vascular niche occurs in response to different FGF-4 and SDF-1 concentration. High FGF4 and low SDF-1 direct the cells towards vascular niche and induce their proliferation. High SDF-1 secreted by niche stromal cells maintains the HSCs in the endosteum.*

Recent studies have revealed that most of the described adult stem cells divide infrequently and can be quiescent even for months (Fleming et al., 1993;Zhang et al., 2003;Tumbar et al., 2004). If so, these quiescent HSCs do not constitutively act in the hematopoiesis, instead they might be activated only during injury or stress. The proposed model for self-renewal in HSC niches reveals that there is an interface between the niche and non-niche environment where the dividing stem cells are affected already by the differentiation signals coming from adjacent non-niche

environment. The cells closer to that region become committed to differentiate while the daughter cell which remains in close proximity with niche cells retain its undifferentiated state (Wilson and Trumpp, 2006). It is not completely clear yet if the stem cells can be found only in a single cell niche or it is a network of interacting stem cell niches that maintain and store the quiescent stem cells.

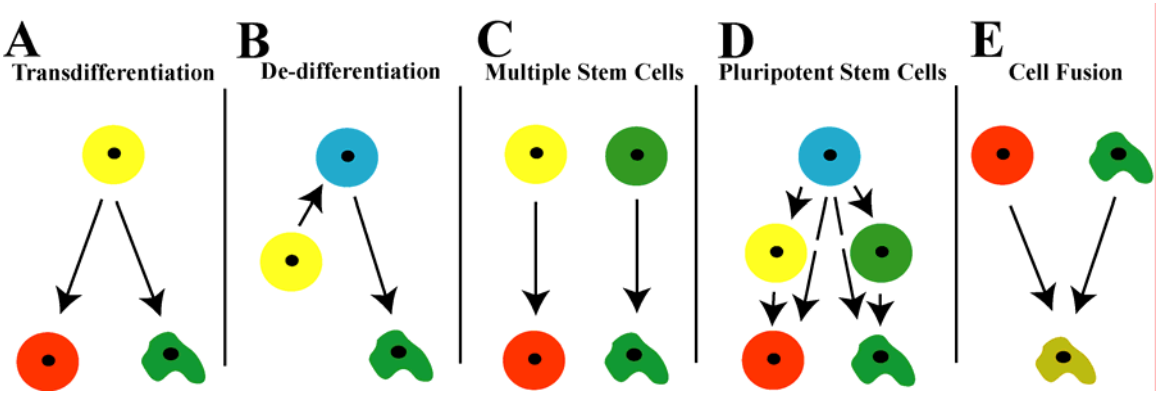
Mesenchymal Stem Cells

Another stem cell source derived from the bone marrow is the MSC source, first described by Friedenstein, AJ at 1976. Together with his co-workers Friedenstein demonstrated that a small fraction of the bone marrow derived cells has the capacity to adhere to the tissue culture dish and that these cells can be differentiated both *in vitro* and *in vivo* into osteoblasts, chondrocytes and adipocytes. Many investigators confirmed the observations of Friedenstein et al. and they further differentiated the bone marrow derived MSCs into cells from different lineages like muscle and early precursor of neural cells, liver and cardiomyocites. There are no well defined markers for isolation of MSCs. Therefore, the major problem in MSC isolation via adherence to the plastic dish is the high heterogeneity of the isolated culture, which contains osteoblasts, adipocytes, fibroblasts, macrophages, endothelial cells and, in early cultures, HSCs and HPCs. Long term culturing of MSCs can reduce the heterogeneity of the culture.

Recent works on the plasticity of adult stem cells, suggested that various adult stem cells, previously accepted as tissue committed stem cells, may cross the borders and differentiate to a much wider spectrum of cell types when cultured under defined conditions (Wagers and Weissman, 2004). Many proposed mechanisms were discussed in order to explain this phenomenon (Figure 7). “Transdifferentiation” could occur where lineage conversion occurs directly by the activation of differentiation programs leading to specific cell type commitment (Bjornson et al., 1999). Lineage conversion can be also explained via dedifferentiation of the adult stem cell to a more primitive state and according to the molecular signals redifferentiate to another cell lineage. Although there are no clear evidences for dedifferentiation in mammals, studies on amphibians (Brockes and Kumar, 2002) show that dedifferentiation of mature cells might be involved in the regeneration processes. Plasticity of adult stem cells can be explained also with the coexistence of several multiple, distinct stem cell types within the isolated stem cell population

which might explain the multipotentiality of the stem cell population. In order to exclude this possibility the methods for stem cell isolation should be improved to favor more homogeneous stem cell populations. Recently, a hypothesis of the existence of a very rare pluripotent stem cell population in the bone marrow was suggested by the group of Catherine Verfaillie from Minnesota (Jiang et al., 2002a). They isolated so called Multipotent Adult Progenitor Cells (MAPCs) from the bone marrow with high plasticity. It was reported about the possibility to isolate these MAPCs not only from the BM but as well from different organs like brain and muscle tissue (Jiang et al., 2002b). It is not known if these cells actually exist in adult organisms, or such properties were gain under long *in vitro* culture conditions. The last proposed mechanism explaining cell plasticity relies on cell-cell fusion event. Cell-cell fusion has been observed in several BM transplantation studies, where BM cells contribute to hepatocytes, cardiomyocites, Purkinje neurons (Wang et al., 2003b;Weimann et al., 2003;varez-Dolado et al., 2003). However, the observed frequency of this event was less than 1% which implies that this rare phenomenon makes it unlikely to be considered as a physiological repair mechanism.

There have been many controversial results so far, which is the main reason why the hypothesis above does not hold enough evidences.



(Wagers and Weissman, 2004)

Figure 7: *Schematic diagram representing different hypothesis used to explain adult stem cell plasticity.*

When compared to embryonic stem cells, adult stem cells show a clear difference in plasticity. Embryonic stem cells can be easily directed towards the lineage of interest, their epigenetic state is much more suitable for switching on and off the desired genes. Adult stem cells, on the other hand, are difficult to expand and to differentiate. The

epigenetic state of adult stem cells is less flexible. Recent studies developed new techniques to reprogram the adult nucleus into the embryonic state and to understand better the epigenetic changes that an embryonic stem cell undergoes during development and control better the adult stem cell differentiation.

Nuclear Reprogramming

The genetic information of an adult cell and an embryonic stem cell are the same; however, the differential gene expression is a result of reversible epigenetic changes that occur during development. The reversal of the differentiation state of an adult cell to one that is characteristic of the undifferentiated embryonic state is defined as nuclear 'reprogramming'. Different reprogramming approaches have been studied so far.

Reprogramming using Somatic Nuclear Transfer

This technique is based on the injection of a somatic nucleus into an enucleated oocyte which upon transfer into a pseudopregnant mother can give rise to a clone (reproductive cloning), or upon explanation in culture, can give rise to genetically matched ES cells. This method, however, do not solve the ethical problems which arise from ES cell isolation since it requires the usage of donor oocytes. Furthermore, the clones derived by somatic nuclear transfer die very soon after implantation and the ones who survived till birth often show severe abnormalities like obesity (Ogonuki et al., 2002). The reason lays in the inefficient reprogramming due to the differentiation state of the donor cell (donor nuclei). The persistence of donor-cell-specific gene expression indicates that the preserved "epigenetic memory" of the donor nucleus can lead to the observed abnormalities (Hochedlinger and Jaenisch, 2006).

Reprogramming via cell fusion

Cell fusion involves the fusion of differentiated cells with pluripotent ES cells which results in the generation of hybrids that show characteristics of pluripotent ES cells. Also in this case ethical problems are not circumvented because of the need for ES cells. Moreover, the generation of tetraploid cells limits the application of this method.

Reprogramming by cell extract

In this method somatic cells are exposed to cell extract derived from oocyte or ES cells inducing a transient modification of the gene expression profile in somatic cells. It is a potentially attractive approach, however, the data analysis can not exclude yet the possibilities that the detected gene products (Oct4, Nanog) following treatment might be from the ES cells used for extract preparation (Hakelien et al., 2002). So far no functional reprogramming was achieved using this technique.

Culture induced reprogramming

All the methods described so far, required extraction of oocyte or ES cells. In order to avoid the ethical complications, there have been many studies focused on adult stem cell reprogramming, using different growth factor combinations *in vitro.* Recently, neonatal (Kanatsu-Shinohara et al., 2004) and adult (Guan et al., 2006) testis cells were shown to gain characteristics similar to ES cells when exposed to specific combinations of growth factors. They express all the pluripotent markers of ES cells; they give rise to chimeras once injected into blastocyst, and formed teratomas after transplantation (like ES cells). However their potential therapeutic application is still debated due to unbalanced genomic imprinting. Lately discovered adult progenitor cells in the bone marrow (Jiang et al., 2002a) are found to express some important embryonic genes: Oct4, Nanog, and Rex1 when cultured with medium containing different growth factor combinations. Furthermore they were shown to have the ability to trans-differentiate to cells of all three germ layers. Having similar characteristics to embryonic stem cells, residing in the patients own bone marrow, and not leading to teratoma formation after injection in vivo, this so called multipotent adult progenitor cells may offer the possibility to overcome the ethical and clinical problems which restrict embryonic stem cell applications. Although these results are intriguing they await confirmation from independent laboratories.

Culture induced reprogramming was the main method on which our study was based on. Bone marrow cells cultured in a medium supplemented with various growth factors like LIF, platelet derived growth factor (PDGF) generated a stem cell culture with high plasticity. Plasticity of the stem cell population was confirmed using *in vitro* differentiation and *in vivo* aggregation with morulae. The studies went further with detection and analysis of the real source of the stem cell population in the

heterogeneous BM culture. Consequently, the multilineage potential of BM derived stem cells, their ability to elude detection by the host's immune system, and their relative ease of expansion in culture make these stem cells a very promising source of stem cells in cell therapy applications.

Bone marrow derived stem cells and their potential clinical application

After their discovery by Alexander Friedenstein in 1976, mesenchymal stem cells (MSCs) drew attention of many researchers. Many studies since then reported the potential of these bone marrow derived MSCs to differentiate into different tissues including bone, cartilage, lipid, epithelial, muscle and nerve tissues. High plasticity and self-renewal of these cells provides a great potential for the clinical tissue regeneration. Therapeutic application of MSCs initiated with bone and cartilage regeneration studies involving assistance of suitable supportive scaffolds (Goshima et al., 1991). The main limitation in this approach is the insufficient cell number. Several novel approaches like serum treatment (Shahdadfar et al., 2005;Bruinink et al., 2004), application of osteogenic growth factors like BMPs and FGFs (Noel et al., 2004;Reddi and Cunningham, 1990) have been used to improve the culture conditions in order to increase the proliferation of MSCs and prevent their loss of differentiation. Another interesting approach used was over-expression of telomerase, an enzyme that adds specific DNA sequences to the 3' ends of the DNA strands and prevents the DNA shortening due to cell division. This approach led to extensive proliferation of MSCs (Kratchmarova et al., 2005). In patients suffering from an inherited disease called osteogenesis imperfecta approaches like allogenic MSC transplantation, and gene therapy using transplantation of autologous MSCs transfected with the corrected form of the gene gave promising results (Le et al., 2005;Chamberlain et al., 2004). Other exciting potential clinical application of MSCs was reported for heart tissue regeneration (Amado et al., 2005). Studies performed with systemic or local delivery of MSCs to different animal disease with cardiomyopathy and ischemic injury have clearly showed the engraftment of the cells to the heart tissue leading to improved cardiac function. The discussion related to the mechanism of the healing process is still ongoing. It needs to be proved if the MSCs can autonomously differentiate into cardiomyocytes or the tissue regeneration is achieved due to cell fusion. Recent studies, however, propose that the MSCs have a protective effect in myocardial

infarction due to their paracrine activity rather than replacement of cardyomiocytes (Tang et al., 2005;Gnecchi et al., 2005).

In parallel to autologous cells source, MSCs can be used for their immunoregulatory role. Several studies demonstrated that MSCs avoid allogenic rejection in human and many animal models (Aggarwal and Pittenger, 2005;Le et al., 2004). Exact mechanism still being unknown, it was shown that MSCs lack major histocompatibility complex (MHC-II) which are antigen presenting proteins and initiators of the specific immunity in the body (Schoeberlein et al., 2005). Moreover, MSCs can show an immunosuppressive effect on T cells *in vitro*, also inducing apoptosis in activated T cells. Several studies suggest the treatment of graft-*versus*-host diseases with MSCs co-injection which was shown to clearly reduce the graft rejection complications (Bartholomew et al., 2002).

Animal studies illustrate the engraftment of MSCs to different tissues which can be enhanced via local tissue damage factors. This suggested that MSCs can be used as a targeting vehicle in gene therapy with various therapeutic molecules. Transplantation of MSCs genetically modified to express interleukin 2, responsible for T cell proliferation, into malignant gliomas inhibited the tumor growth and increased the survival of the rats with tumors (Nakamura et al., 2004). On the other hand MSCs over-expressing interferon (IFN) β were found to decrease the growth of pulmonary metastasis (Studeny et al., 2004). Treatment with human MSCs retrovirally transduced to express BDNF improved recovery in the rat transient middle cerebral occlusion model (Kurozumi et al., 2005). These studies demonstrate that gene therapy using MSCs as a targeting vehicle is a promising therapy approach in the treatment of many diseases.

Success of gene therapy strongly depends on organ or site specific production of therapeutical proteins. Bone marrow derived stem cells are an accessible source for a cellular vehicle for gene therapy. Identifying the molecular cues important for cell migration is necessary to understand the developmental mechanism and to develop new therapy approaches. In our study, we investigate the effect of chemokine trafficking in organ specific targeting of BM derived stem cells.

Chemokines and Chemokine Receptors

Chemokines are a family of structurally related glycoproteins that function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other

effector cells from the blood to sites of infection or damage. They can be released by many different cell types and serve to guide cells involved in innate immunity and also the lymphocytes in adaptive immunity. Some chemokines also have roles in the development of lymphocytes, migration and angiogenesis. Chemokines promote cell migration via inducing integrin activation and activating several intracellular pathways linked with cell proliferation and apoptosis (Ansel and Cyster, 2001). Chemokines are low molecular mass (8- 10 kDa) proteins, classified into four families: C, CC, CXC, and CX3C according to the number and spacing (X) of cysteine (C) residues in the amino- terminal part of each molecule. The α chemokines, also known as CXC chemokines, contain a single amino acid between the first and second cysteine residues; β, or CC, chemokines have adjacent cysteine residues. Most CXC chemokines are chemoattractants for neutrophils whereas CC chemokines generally attract monocytes, lymphocytes, basophils, and eosinophils. There are also 2 other small sub-groups. The C group has one member (lymphotactin). It lacks one of the cysteines in the four-cysteine motif, but shares homology at its carboxyl terminus with the C-C chemokines. The C chemokine seems to be lymphocyte specific. The fourth subgroup is the CX3C subgroup. The CX3C chemokine (fractalkine/neurotactin) has three amino acid residues between the first two cysteines. It is bound directly to the cell membrane via a long mucin stalk and induces both adhesion and migration of leukocytes.

More that 50 chemokines and more than 20 chemokine receptors were identified so far. Chemokines can bind to multiple chemokine receptors, as well as receptors can respond to more than one chemokine; however, the only exception of this rule is SDF-1, which binds only to the chemokine CXC receptor 4 (CXCR4) and is the unique ligand of this receptor. This unique bond suggests that SDF-1-CXCR4 pathway may play an important role during the developmental process.

Chemokine Receptor CXCR4

CXCR4 is a member of the seven- transmembrane domain G- protein- coupled receptors, and is mainly expressed by cells in the immune system and the central nervous system. This receptor is also used by T- tropic Human Immunodeficiency Virus (HIV) strains to infect CD4+ T-cells. The ligand for this receptor a CXC chemokine stromal derived factor 1, known also as CXCL12, is reported as a chemoattractant for lymphocytes, CD34+ progenitor cells, pre- and pro- B cells.

Binding of SDF-1 to its receptor activates G-protein mediated signalling, including downstream pathways such as Ras, and phosphatidylinositol3 (PI3) kinase. PI3 kinase activated by SDF-1 and CXCR4 plays a role in lymphocyte chemotaxis. CXCR4 signalling activates also extracellular-signal regulated kinases 1 and 2 (Erk1/Erk2) which are important for regulation of cell growth and proliferation. Janus Kinase and Signal Transducers and Activation of Transcription (JAK/STAT) signalling pathways also appear to play a role in SDF-1/CXCR4 signalling (Figure 8).

SDF-1-CXCR4 axis is the major regulator of trafficking and homing of CXCR4 expressing HSCs, progenitor cells, B and T lymphocytes. Beside HSCs, CXCR4 expression was as well detected in many tissue stem cells, primordial germ cells and even murine embryonic stem cells, which points out that CXCR4 can be a cell surface marker also of various stem cell populations.

There are 2 splice variant forms of SDF-1: α and β. SDF-1α is more abundant, and is expressed mainly by BM stromal cells, endothelial cells and fibroblasts. It reaches its highest concentration in the BM; however, it is widely secreted in other organs like heart, muscle, kidney and brain. SDF-1 secretion is upregulated in hypoxic conditions. Low oxygen levels triggers the expression of hypoxia induced factor 1 (HIF-1). Upregulation of HIF-1 increases directly the expression of SDF-1. Molecular analysis showed that SDF-1 promoter region contains 2 binding sites for HIF-1 (Ceradini et al., 2004). The shortage of oxygen in damaged tissues, like ischemia, triggers HIF-1 expression which consequently elevates SDF-1 expression. This leads to chemoattraction of CXCR4+ stem or progenitor cells to the site of injury where they participate to the regeneration process. HIF-1 upregulates also the expression of CXCR4 receptor (Helbig et al., 2003) which shows that HIF-1 regulates SDF-1-CXCR4 signalling from both ligand and receptor level.

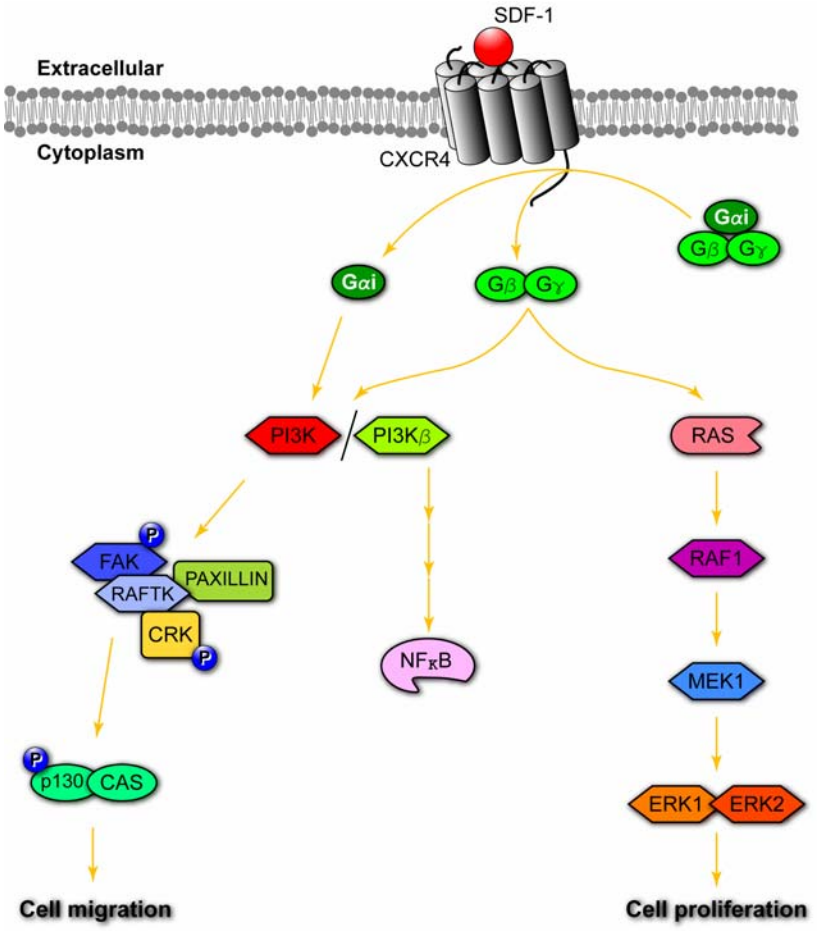
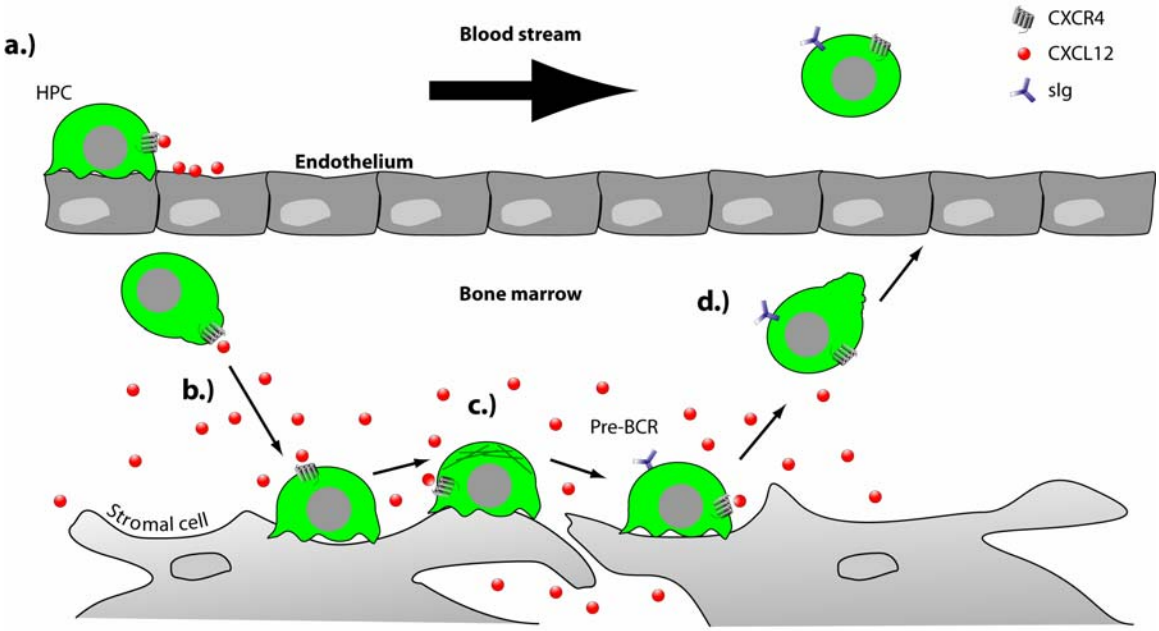


***Figure 8**: SDF-1-CXCR4 signalling pathway. Activation of CXCR4 receptor via SDF-1 leads to activation of PI3 pathway regulating the cell migration. Cell growth and proliferation is maintained via Erk1/Erk2 pathway activated through CXCR4 signalling.*

CXCR4-SDF-1 axis and Hematopoiesis

The CXCR4-SDF-1 axis plays an important role during embryonic stages and adulthood. It is known that CXCR4-SDF-1 pathway is important in the development of cerebellum, hippocampus and neocortex (Jazin et al., 1997;Ma et al., 1998;Zou et al., 1998). Furthermore, this signalling pathway is necessary for the growth and survival of germ cells and it is suggested to play role in guiding the primordial germ cells (PGCs) into their gonadal niches during early mouse development (Molyneaux et al., 2003). Gene silencing experiments shows that SDF-1/CXCR4 signalling is crucial for colonization and formation of the bone marrow starting from E15 in early organogenesis. Fetal liver is colonized by HSCs at mouse embryonic day E9.5. Between E10.5-E14.5 colonization of HSCs from fetal liver towards the bone marrow initiates (McGrath et al., 1999). HSCs expressing CXCR4 are attracted towards the

BM due to high SDF-1 levels (Figure 9). Migrated HSCs reside within the bone marrow niches (endosteum). During differentiation process HSCs move out of endosteum region towards the inner parts of the bone attaching to the stromal cells via their $\alpha_4\beta_1$ integrins (also known as Very Late Antigen (VLA-4) receptor). In B-cell maturation, surface-immunoglobulin -expressing premature B cells become desensitized towards the SDF-1; they either internalize their CXCR4 receptors and downregulate the expression due to high SDF-1 or the receptor becomes insensitive to its ligand during the differentiation process. Either way leads to the release of the pro-B cells to the bloodstream. During the adult life SDF-1-CXCR4 axis plays a crucial role in the retention/homing of HSCs in the BM microenvironment.



Modified from Ansel et al., 2001

***Figure 9**: Hematopoiesis. During early organogenesis, hematopoietic stem cells expressing CXCR4 receptor migrate from fetal liver to the BM due to high SDF-1 concentration in the BM. During maturation the cells lose the sensitivity to the SDF-1. They may internalize their CXCR4 receptor or downregulate its expression regulated by high SDF-1 levels, or due to differentiation process they might have desensitized the receptor towards its ligand. As a result the committed stem cell overtakes the CXCR4-SDF-1 force and escapes to the bloodstream.*

High hypoxic conditions are known to enhance SDF-1 expression. During injury, organs like heart, liver and brain secrete high levels of SDF-1 due to depletion of

oxygen. This becomes the driving force for CXCR4 expressing stem and endothelial cells to migrate from the circulation to the lesion sites and contribute to the regeneration process.

In addition to the defects in homing of HSCs in BM, the SDF-1 and CXCR4 knockout studies revealed also an impaired development of heart, brain and vessels which points that this signalling pathway may have a more general role during organogenesis.

In addition to HSCs, CXCR4 expression has been detected in some non-hematopoietic stem cells in the bone marrow (Kucia et al., 2005a) and other tissues (brain-neural stem cells, (Bagri et al., 2002)), murine ES cells (Kucia et al., 2005b) as well as cancer cells. Tumor stem cells expressing CXCR4 are mainly involved in metastasis. They migrate to the bloodstream due to the SDF-1 gradient, adhere to the endothelium, invade the tissues, proliferate and grow new tumors in suitable environment for them. Several therapeutic approaches targeting the SDF-1-CXCR4 axis have been experimentally applied in order to prevent the metastasis in cancer: like using CXCR4 inhibitor AMD3100; strategies based on RNA interference in order to downregulate expression of CXCR4 in metastatic tumor cells (Chen et al., 2003), or similarly downregulation of HIF-1 (Mazure et al., 2004) in order to reduce SDF-1 expression in certain tissues and prevent CXCR4 expressing tumor cell invasion.

Chemokine Receptor CXCR3

Chemokine CXC receptor 3 (CXCR3) is expressed on the surface of many cell types including activated T cells, NK cells, dendritic cells, macrophages and B cells. It is a 7 transmembrane G-protein linked receptor activation of which is inducing a number of signalling pathways including leukocyte trafficking, integrin activation, cytoskeletal changes and chemotactic migration. Three ligands, Mig (monokine induced by interferon γ) (CXCL9), IP-10 (interferon γ inducible protein10) (CXCL10), and I-TAC (interferon inducible T-cell α-chemoattractant) (CXCL11) are known to activate the CXCR3 receptor. IP-10, Mig and I-TAC share similar characteristics. They are produced by macrophages and other cell types like fibroblasts and endothelial cells and are stimulated by interferon-γ. In addition, IP10, Mig and I-TAC are produced by the local cells in inflamed tissues suggesting that CXCR3 is involved in recruitment of inflammatory cells (Piali et al., 1998;Cole et al., 1998).

CXCR3 has been indicated as a polarization marker of the T helper cells towards Th1 phenotype (Bonecchi et al., 1998). Resting CD8+ cytotoxic T cells express CXCR3 receptor and IFN α/β in small amounts. With stimulation of CD8+ T cells by antigen presenting cells, the IFN expression increases and subsequently leads to the induction of CXCR3 receptor and its ligands (Cole et al., 1998). The ligands bound to CXCR3 activate a protein tyrosine kinase Src, which leads to recruitment of Ras and activation of Erk pathway. It is reported that, in parallel to Erk pathway, PI3K/Akt pathway is also activated. Whether activation of Src is somehow related with the regulation of PI3K pathway is not defined yet (Bonacchi et al., 2001). On the other hand the CXCR3 signal contributes to the expression of activation markers CD25, CD69 enabling an active proliferation of reactive CD8+ T cells.

In contrary to CXCR4 signalling, the signalling pathway for CXCR3 is not described in detail. It is well known, however, that chemokines like IP-10 and Mig together with their receptor CXCR3, are primarily involved in autoimmune diseases like multiple sclerosis (MS).

Multiple Sclerosis and its murine model Experimental Autoimmune Encephalomyelitis (EAE)

There is no exact defined pathogen as a cause for MS. However, it is known for certain that it is an autoimmune inflammatory disorder affecting the central nervous system (CNS). More precisely the immune attack is raised against the myelin sheath, the lipoprotein produced by oligodendrocytes, covering the neuronal axons. Myelin sheaths, wrapping the neuronal axons, are separated by gaps called nodes of Ranvier. This structure of the axon facilitates the signal transmission along the axons. With the damage to the myelin, the conduction is impaired and transmission of impulses along the axons is critically delayed.

MS affects 0.05% to 0.15% of the white population, leading over time to severe disability in half of the affected people. MS occurs twice as often in women as men, and typically starts between the ages of 20 and 40 years. In the majority of patients, the disease course is characterized by onset and remission of neurological symptoms originating from different areas in the CNS (relapsing remitting [RR-MS]). In acute MS lesions, demyelination of axons, activation of microglia, and infiltration of immune cells are key features. The infiltrates mostly consist of T cells and macrophages. B cells and plasma cells are also found, but at lower numbers. Two

kind of molecules direct leukocytes to the inflammatory sites, the adhesion molecules and chemoattractants. Among chemoattractants, chemokines received more interest since they have been known for their role in inflammation. During MS attacks, levels of three chemokines that act toward T cells and mononuclear phagocytes: IP-10; Mig; and regulated on activation, normal T-cell expressed and secreted (RANTES) have been elevated (Sorensen et al., 1999). They investigated whether specific chemokine receptors were expressed by infiltrating cells in demyelinating MS brain lesions and in cerebrospinal fluid. CXCR3, an IP-10/Mig receptor, was expressed on lymphocytes in virtually every perivascular inflammatory infiltrate in active MS lesions. Once T cells recognize the myelin antigen as non-self, they remain in perivascular space and produce proinflamatory cytokines like tumor necrosis factor (TNF-α) and IFN-γ, which will upregulate the chemokine expression in leukocytes and resident glial cells in CNS, microglia and astrocytes (Oh et al., 1999;Oppenheim et al., 1991). The blood brain barrier (BBB) loses its integrity and a large scale of leukocyte (expressing chemokine receptors) trafficking occurs towards the chemokine gradient within the CNS (Cuzner et al., 1996). Resident cells, activated T cells and infiltrating leukocytes can further promote the inflammatory response. The chemokines mostly involved in MS are CCL2, CCL3, CCL4, CCL5 and CXCL10. Within the cerebrospinal fluid the high levels of T cell chemoattractants CCL2, CCL5 and CXCL10 and high expression of CCR5 and CXCR3 indicated that these chemokines are involved in T cell infiltration within the CNS (Muller et al., 2004). The pathogenic role of chemokine-chemokine receptor pathway in MS suggests new therapeutical strategies.

Most of the studies related to MS have been done on the murine model for MS known as experimental autoimmune encephalomyelitis. EAE can be induced in mice by immunization with immunodominant peptides from myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocytes glycoprotein (MOG) emulsified in complete Freund's adjuvant followed by injection of pertussis toxin as an additional adjuvant for certain mouse strains (Whitham et al., 1991;Amor et al., 1994). Disease score progression is variable from strain to strain. In SJL/J mice, PLP or MBP induce a relapsing-remitting progression, C57BL/6 mice on the other hand are resistant to MBP disease induction but develop a chronic EAE induced by MOG. EAE starts with the activation and differentiation of Th1 cells (van, V and Stohlman, 1993;Segal et al., 1998) that leave the lymph nodes and enter the CNS via crossing BBB (Brennan et al., 1999). Whether chemokines are involved in

this initial entry of the reactive T cells is not clear. EAE also can be induced by injection of antigen specific CD4+ T cells into recipient mice (Mokhtarian et al., 1984). Adoptively-transferred, activated T cells migrate to the CNS within 24 hours (Hickey, 1991) and initiates the CNS inflammation.

Chemokines and chemokine receptors are the main regulators in MS disease. Recent studies have focused on therapeutical approaches targeting this specific pathway. IFNβ administration for example, which already is approved for clinical uses, leads to a downregulation of CCR5 and CXCR3 receptor in CD4+ and CD8+ T cells in MS patients (Teleshova et al., 2002;Sorensen et al., 1999). Many studies report the amelioration of symptoms in experimental models (*i.e.* EAE) is observed when treated with neutralizing antibodies against chemokines CCL2 (Kennedy et al., 1998), CCL3 and CXCL10 (Karpus et al., 1995). Modified chemokines were reported to be used successfully in EAE in order to interfere with the signalling pathway of these chemokines (Gong et al., 1996).

In our study, we investigated the therapeutic potential of bone marrow derived stem cells targeted to the CNS using retroviral expression of the chemokine receptor CXCR3.

Retroviral Systems

Retroviral vectors are widely used due to their safe and highly effective gene delivery. In order to create safe and effective gene expression systems, wild-type murine retroviruses have been modified to ensure that a replication-incompetent viral particle is generated. A basic retroviral vector contains elements required for replication as a virus (*cis* element); however, it lacks all the viral genes (Mann et al., 1983). RNA from retroviruses consists of three major coding domains; *gag* which includes the proteins making up the matrix, the capsid and the nucleoproteins; *pol*, which encodes the reverse transcriptase and integrase and *env*, which codes for the proteins making up the viral envelope. An additional smaller coding domain, *pro* encodes the viral protease. Retroviruses include most oncogenic viruses, for example Moloney Murine Leukemia Virus (MMLV) (MOLONEY, 1960) and Mouse Mammary Tumor Virus (MMTV) (Bittner, 1936). For an efficient packaging of the retroviral vector special packaging cell lines are generated that supply all the packaging genes of the virus (*gag, pol, env*).

The retrovirus first binds to the surface of an uninfected cell by recognizing a cell receptor (Figure 10). After binding, the virus particle is brought into the cytoplasm of the cell. During this process, the viral envelope is removed, leaving the core particle. Once this happens, a unique virus-specified enzyme called reverse transcriptase is activated. This enzyme reads the viral RNA and makes viral DNA. The host cell lacks such an enzyme. The viral DNA then moves to the nucleus of the cell, where it is incorporated into the chromosomal DNA of the host cell using the long term repeats regions (LTRs). Once this viral DNA is integrated into the chromosome, it mimics the host cell gene. As a result, the normal cell machinery reads the integrated viral DNA to make more copies of viral RNA. This viral RNA is then used for two purposes: 1) some of the viral RNA moves to the cytoplasm and functions as viral messenger RNA to program the formation of viral proteins; 2) the rest of the viral RNA becomes genetic material for new virus particles by moving to the cytoplasm and combining with viral proteins. These virus particles are formed at the cell surface and leave the cell by a process called budding.

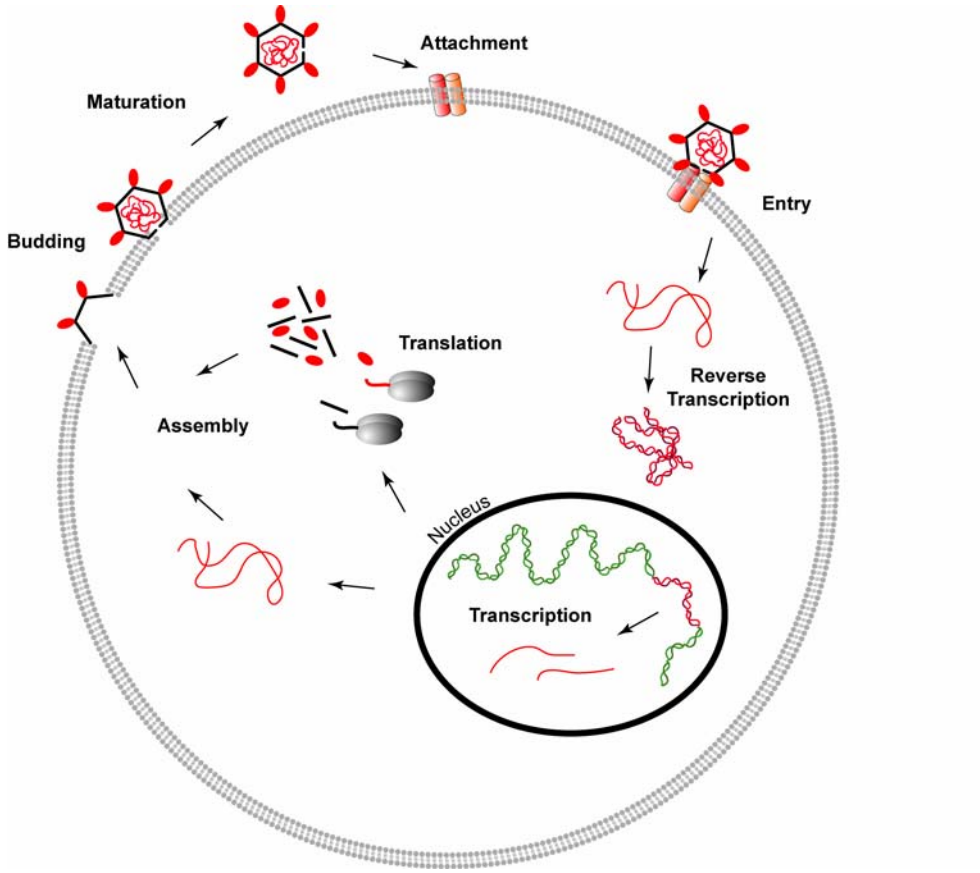


Figure 10*: Life cycle of a retrovirus. Once the viral RNA enters the cells, the viral reverse transcriptase enzyme converts the viral RNA into a DNA. The DNA fragment enters the nucleus and integrates into the host genomic DNA.*

This viral DNA is transcribed through the cell transcription machinery into the viral RNA, which forms the necessary viral coat proteins and a part of this RNA serves as a genetic material to the new viruses. The new viral particles are packed and leave the host cell surface via budding.

Retroviral systems can efficiently deliver a gene to any actively dividing cell. The envelop proteins (*env*) produced by the packaging cell lines, targeting specific cell surface receptors) determines the infectivity (tropism) of the virus.

EcoPack 293 cell lines (Clontech) are Ecotropic and can produce viral particles that can infect both mouse and rat cells. This cell line is derived from human embryonic kidney (HEK) 293 cell line and can produce high viral titer 10^6 virus/ml/48 h.

Viruses produced by RetroPack PT 67 cell line has dualtropic envelops, which has a broader range of infection (mouse, rat, hamster, rabbit, cat, dog, monkey, human).

Pantropic expression systems produce an envelop glycoprotein, inducing the virus entry via plasma membrane fusion (Burns et al., 1993). These viruses have the broadest range of infection including mammalian and non-mammalian hosts.

In our study we used retroviral delivery tool to express chemokine receptors CXCR4 and CXCR3 in BM derived stem cells. A commercially available Murine Stem Cell Virus vector (pMSCV) was used as a backbone vector for insertion of CXCR3 (or CXCR4) -Internal Ribosomal Entry Site (IRES)-GFP sequence in construction of the retroviral vectors. Genetically modified BM cells were used for *in vivo* targeting in response to their chemokine gradients. CXCR4 transduced BM cells were targeted *in vivo* into BM of lethally irradiated mice following high SDF-1 concentrations, while CXCR3 transduced BM cells were used for targeting the lesion sites in EAE animal models following elevated IP10 expression in CNS tissues.

AIMS OF THE STUDY

Lately there have been many reports regarding the plasticity of bone marrow derived stem cells; however, the multi- or pluripotency of these cells is still debated. Hereby, we characterized in detail BM derived stem cells cultured with various growth factors like LIF, PDGF and EGF. Furthermore, we investigated their capacity to differentiate to neurogenic, adipogenic and osteogenic lineages *in vitro* and to generate chimeric mice. Since the BM is a very heterogeneous population, we used Oct4GiP transgenic mice, where the GFP is expressed under Oct4 promoter, in order to isolate only the multipotent stem cell population from the bone marrow. This allowed us to enrich the multipotent stem cells expressing the Oct4 pluripotent gene *in vitro* cultures and to expand them maintaining their plasticity. Protein transduction with recombinant Nanog protein, culturing with Fibroblast Growth Factor 2 (FGF2), and other methods were used in order to expand the Oct4 expressing multipotent population maintaining their plasticity.

A successful gene therapy relies on organ or site specific production of therapeutical proteins. We used the motility control properties of chemokines to achieve an organ specific migration and integration of BM derived stem cells via gene transfer of chemokine receptors. Retroviral vector system was used to transduce BM derived stem cells with chemokine receptors CXCR4 and CXCR3. CXCR4 transduced BM stem cells have been administered to lethally irradiated and various organs of the injected mice were analyzed 3 to 5 weeks post injection. Mice injected with only GFP transduced BM stem cells was used as a control.

In addition, we used CXCR3 transduced cells in EAE animal models for a therapy of multiple sclerosis, since it has been shown that the chemokines IP10 and Mig (the ligands for the CXC3 receptor) are among the main regulators during the development of MS disease. During the onset of the disease (10 days post immunization) EAE induced mice were injected with CXCR3 transduced BM derived stem cells and the score was followed up to 15 days post injection. Scores were compared with mice injected with Phosphate Buffered Saline and BM stem cells transduced only with GFP. Using fluorescence microscopy, brain and spinal cord were analyzed to detect the cell migration to the lesion sites in EAE models.

MATERIALS AND METHODS

Isolation and expansion of bone marrow derived stem cells

Bone marrow was collected from 6-8 week- old C57BL6 mice (Charles River). The hind limbs were removed and, using a syringe, the bone marrow was flushed out with PBS from the medullary cavities of the tibia and femur bones. Removal of erythrocytes was performed by treatment with lysis buffer (See Appendix to Materials and Methods); cells were suspended in 5ml lysis buffer for 30sec followed by the addition of 5ml PBS to prevent further lysis of white blood cells. Isolated cells were cultured in stem cell expansion medium consisting of 54% Dulbecco's Modified Eagle Medium- Low Glucose (DMEM- LG) (Gibco), 40% MCDB-201 (Sigma), supplemented with Insulin-Transferrin-Selenium (ITS, Gibco), 1X Linoleic- Acid-Bovine- Serum- Albumin (LA-BSA, Sigma), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2- phosphate (Sigma), 100U penicillin, 1000U streptomycin (Gibco), 2% Fetal Calf Serum (FCS) (Gibco), 10 ng/ml Epidermal Growth Factor (EGF, Sigma), 10 ng/ml Platelet Derived Growth Factor (PDGF, R&D) and 10 ng/ml Leukemia Inhibitory Factor (LIF) (Chemicon) on Fibronectin (Sigma) (5ng/ml) coated flasks (Appendix).

Adherent cells were cultured up to two 20 passages under above described conditions, splitted once or twice a week at 70% confluency. Passaged cells were seeded at a density of 4000 cells/cm^2. More than 80% of presumed hematopoietic cells died during the first week of culture and only the cells which are resistant to long cell culture conditions and have a high proliferative capacity survived.

Characterization of BM derived stem cells by Flow Cytometry, immunohistochemistry and RT-PCR

The multipotency of generated BM derived stem cells was analyzed by immunological and molecular detection of various cell surface and intracellular markers specific for ES Cells. Flow cytometry analysis was performed for detection of various cell surface markers (See Appendix to Materials and Methods).

Cells were trypsinized, washed with PBS and, in order to prevent unspecific binding, incubated with CD16/32 F_c blocking antibody for 5 min at 4˚C. First antibody was applied afterwards (at v/v ratio 1:100) for 30 min at 4˚C. Following washing, the cells were incubated with the selected secondary antibody (Appendix)

for 30 min at 4˚C. After washing, the samples were used immediately for flow cytometry analysis. Immunostaining for Oct- 4 transcription factor was performed using mouse anti-mouse Oct- 4 monoclonal antibody (Santa Cruz) (at a 1:100 dilution) followed by Fluoro-sothiocyanate (FITC) conjugated goat anti- mouse IgG/IgM (Dianova) incubation (overnight at a 1:200 dilution) as a secondary antibody. Reverse Transcriptase- Polymerase Chain Reaction (RT- PCR) was used for detection of pluripotency transcription factors Oct- 4, Nanog, Rex- 1. RNA was extracted from cells by using the RNeasy kit (Qiagen); mRNA was reverse-transcribed and cDNA underwent 40 rounds of amplification with the following conditions: 94°C for 1`, 60°C for 40``, and 72°C for 50``. Amplifications without reverse transcriptase and reactions without addition of cDNA were used as a control. The housekeeping gene GAPDH was amplified using the PCR conditions above.

In vitro differentiation of bone marrow derived stem cells into neuronal, adipogenic and osteogenic lineages

BM cells were cultured in stem cell expansion medium for 2 weeks. Neuronal differentiation was induced via addition of FGF2 (100 ng/ml) to the stem cell expansion medium for 7 days followed by 10ng/ml FGF and 100 ng/ml Sonig Hedgehog (SHH) for 7 more days in the medium (Jiang et al., 2003). After 2 weeks in culture with differentiation medium the cells were stained with anti β- tubulin isotype III (Sigma) (dilution of 1:100) followed by incubation with the secondary antibody Cy3 conjugated goat anti mouse IgG (Dianova) (dilution of 1:200) (Table2).

Adipogenic differentiation of bone marrow derived cells was induced via removal of the growth factors (PDGF, EGF, LIF) from the stem cells expansion medium and addition of 10% horse serum and 10^{-6}M dexamethasone (Laharrague et al., 1998). 14 days after differentiation the cells were stained with Oil Red O (Sigma) (0.3%) in 60% isopropanol for 1 h at room temperature after fixation with 4% paraformaldehyde for 30 min at 4˚C.

Osteogenic differentiation was performed using DMEM medium containing 10% FCS (Gibco), 10 mM β- glycerophosphate (Sigma), 10^{-7} M dexamethasone (Sigma), and 0.2 mM ascorbic acid (Sigma). 14 days after differentiation calcium deposits were detected using Alizarin Red S (Sigma) staining. The cells were washed and fixed in 4% paraformaldehyde and stained for 5 min with 1% alizarin red in 2% ethanol solution to reveal mineralization.

Aggregation with 8-cell stage morulae and chimera generation

Aggregation of BM derived stem cells with 8-cell stage morulae was performed in order to confirm their *in vivo* differentiation potential. 8-cell stage morulae embryos were obtained from 6 weeks old CD1 female mice (Charles River). Pregnant mare's serum (PMS), which is used to mimic follicle-stimulating hormone (FSH), and 46-48 hours later human chorionic gonadotropin (hCG), which is used to mimic luteinizing hormone (LH), were administered intraperitoneally each at a dose of 5 IU/mice in order to increase the number of the ovulating eggs. After the administration of hCG, one female was placed in a cage with one stud male, and the next morning the female was checked for a copulation plug. Two days after, oviducts from sacrificed females were collected. The 8-cell embryos were flushed out and collected from the oviduct. The zona pellucida of the embryos was removed using an acidic tyrode solution. Small drops (50 ul) of M16 medium (Sigma) were placed on the bottom of a 35-mm sterile plastic culture dish. 3-4 holes were punched with special aggregation needles in each microdrop. The dish was flooded with light paraffin oil. Zona pellucida free embryos were transferred into these microdrops, each embryo in one well. Nearly 10 BM derived stem cells expressing GFP were subsequently transferred on top of each embryo. The next day, blastocyts were transferred to the uterus of a pseudopregnant recipient female mouse. Embryos were analyzed at day 12.5.

Enrichment of Oct4 expressing BM stem cells

Mechanical isolation: In order to establish a more homogenous multipotent BM stem cell population, a transgenic mouse (Oct4GiP), in which GFP is expressed under the Oct4 promoter was used (Figure 11). The transgenic mice were generated via microinjection of the transgene into the pronuclei of a fertilized oocyte (Ying et al., 2002). The transgenic line was kindly provided by laboratory of Prof. Austin Smith, ISCR, Edinburgh, UK.

***Figure 11**: Construct used for transgenic generation. GFP is expressed under Oct4 promoter, followed by IRES and puromycine.*

Bone marrow derived from these transgenic mice was cultured with stem cell expansion medium. After 2 weeks, GFP fluorescence was detected in some colonies in the cultures. The colonies were manually picked using a Pasteur pipette; and real time PCR was performed to quantify the relative concentration of pluripotency gene transcripts Oct4 and Nanog.

Nanog protein transduction of BM derived stem cells: Recombinant Nanog protein transduction was used to enhance the proliferation of the stem cell population derived from the bone marrow. The TAT-Nanog fusion protein contains a Nuclear Localization Signal (NLS) (important for the translocation of the protein into the nucleus), the recombinant Nanog protein, and the positively charged Trans-actin Activator of Transcription (TAT) peptide which mediates the entry of the protein to the cell due to positive charge of TAT and negative charge of the cell membrane (Figure 12).

NLS: Nuclear Localisation Signal

TAT: Trans-actin Activator of Transcription Peptide (+ve charge)

***Figure 12**: TAT-Nanog construct. TAT (positively charged) promotes the entrance of the protein through the negatively charged plasma membrane via macropinocytosis. NLS region promotes the protein diffused from macropinocytic vehicle to the cytoplasm to enter the nucleus through the nuclear pores.*

The purified TAT- Nanog fusion protein was kindly provided by Frank Edenhover (RNB, Life&Brain Center, University of Bonn). The protein was added to the stem cell expansion medium at a concentration of 100nM. Cells were cultured for 3 weeks and analyzed for pluripotency markers.

Growth Factor Application: 2-3 weeks after isolation of BM cells, the effect of the addition of FGF2 (25 ng/ml) and SCF (10 ng/ml) to the stem cell expansion medium was analyzed after 24h, 72h and 1 week post-administration. GFP fluorescence was detected in the cultured colonies and RT-PCR of treated (FGF2 and SCF added) and non-treated (stem cell expansion medium only) cultures for Oct4 expression was compared.

Retroviral expression of CXCR3 and CXCR4 in BM derived stem cells

Construction of the retroviral vectors

The retroviral vectors were constructed using a commercial pMSCVneo (Clontech) (Figure 13). The CXCR3 and CXCR4 genes were inserted using the XhoI and MluI restriction sites. IRES and GFP coding sequences were inserted in the multicloning site, between the MluI/XbaI and XbaI/BlgII restriction sites, respectively.

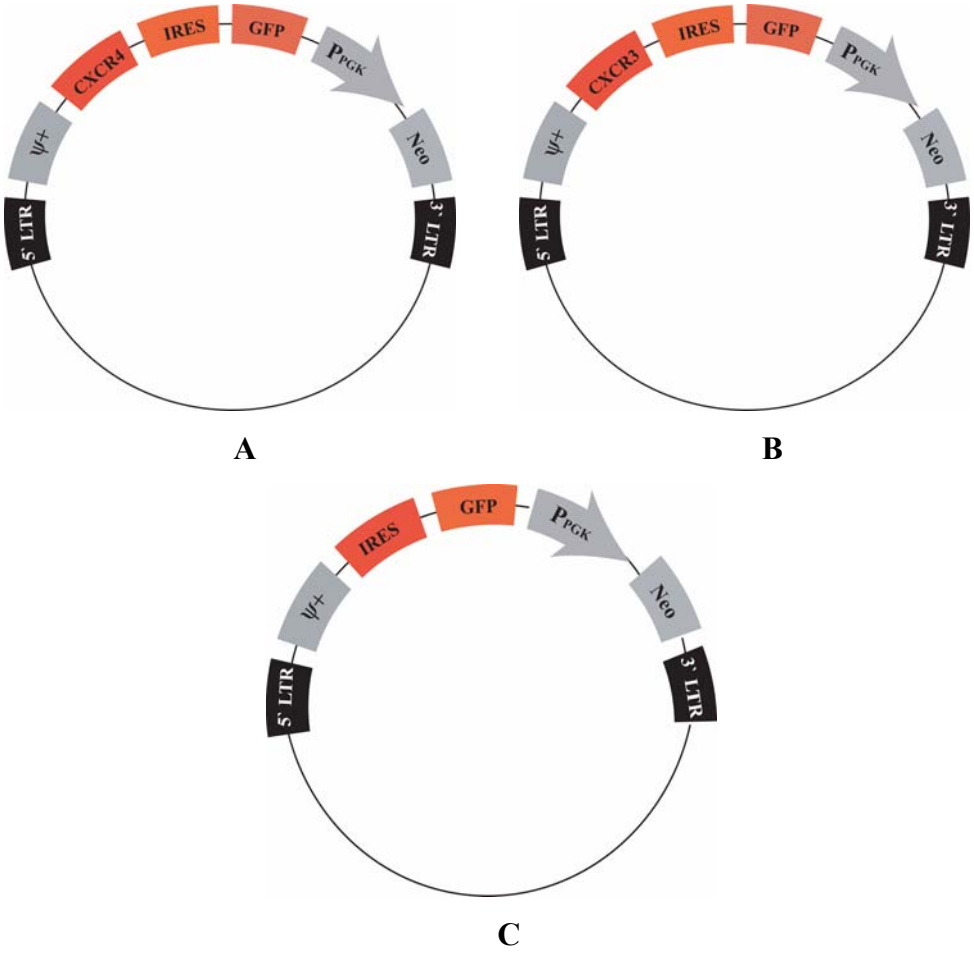


Figure 13: *Retroviral plasmids constructed via insertion of CXCR4 (or CXCR3), IRES and GFP into the MCS of the original construct pMSCVneo. pMSCV-CXCR4-IRES-GFP (A), pMSCV-CXCR3-IRES-GFP (B) and control pMSCV-IRES-GFP (C) used for transduction of BM cells.*

Effectene transfection of EcoPack 293 cells and generation of a stable cell line (PT67) for a viral production

The DNA constructs used during the experiments were pMSCV-CXCR4-IRES-GFP, pMSCV-CXCR3-IRES-GFP and pMSCV-IRES-GFP retroviral expression vectors.

EcoPack 293 (Clontech) cells, plated on 10 cm tissue culture dish, were transfected using Effectene Transfection Reagent (Qiagen). The transfection was performed according to the Qiagen transfection protocol. Fresh medium was added 7 hours after transfection. 48 hours later supernatant was collected, filtered using 0.45 um filters to avoid the cell debris and used for transduction of RetroPack PT 67 cells plated on 6 well plates. PT 67 cells were treated with 1 ml of fresh viral supernatant/well using spin infection method, centrifuged at 2500 rpm, 37˚C for 45 min. The transduction was repeated a second time with a fresh viral batch and the cells were incubated overnight at 37˚C and 5% CO_2. Fresh medium was added the next day. CXCR4 or CXCR3 transduced PT 67 cells were subjected several times to magnetic bead selection. Cells were trypsinized, washed with PBS, incubated with rat anti-mouse CXCR4 or CXCR3 monoclonal antibody (Pharmingen) for 1 h at 4˚C, washed with PBS and followed by incubation with secondary antibody Dynabeads- conjugated sheep anti- rat IgG (Dynal) for 30min at 4˚C. Following collection and washing of the magnetic fraction the cells were cultured till at least 2 passages in order to get a magnetic bead free culture. The PT 67 stable cell line was consequently producing retroviruses.

Retroviral transduction of BM derived stem cells

48 hours after confluency of the PT 67 cell line, the collected viral supernatant was filtered through 0.45 um filter and directly used for transduction of BM derived stem cells. Bone marrow stem cells, at a confluency of 40-50% (70 000 cells/well of a 6 well plate) were treated twice with 1 ml fresh viral supernatant by spin infection method. Expression of GFP reached its peak, around 48 hours after transduction.

Analysis of CXCR4 transduced BM derived stem cells

Immunolabelling and flow cytometry analysis of CXCR4 transduced BM derived cells

Transduced BM stem cells were fixed with 4% paraformaldehyde for 30 minutes, washed twice in PBS and incubated with 1% BSA for 1 hour at 4˚C to reduce the nonspecific binding of the antibody. The samples were then incubated overnight with rat anti-mouse CXCR4 monoclonal antibody (Pharmingen) at 4˚C. Cells were washed two times in PBS and incubated with goat anti-rat Cy3 secondary antibody (1:200, diluted with 1% BSA). For flow cytometry analysis the cells were trypsinized, washed

with PBS, incubated with primary antibody as mentioned above for 30 min at 4˚C, after rinsing with PBS incubated with secondary antibody for 30 min at 4˚C.

In vitro migration assay

The function of BM derived stem cells transduced with CXCR4 receptor was analyzed using the migratory property of CXCR4 transduced cells towards SDF- 1. 100 000 BM derived stem cells transduced with CXCR4-GFP retroviral vector were seeded on top of each Millicell filter (Millipore, USA) immersed into a 24- well plate containing 600 ul medium with varying concentrations of SDF- 1(0 to 60 ng/ml) (Figure 14). The same amount of BM derived stem cells transduced with only GFP retroviral vector was used as a control. The migration of BM stem cells from the membrane towards the medium was allowed to proceed for 4 hours at 37˚C and 5% CO_2. Migrated cells were estimated by counting the cells in the well and on the opposite (downward) side of the membrane.

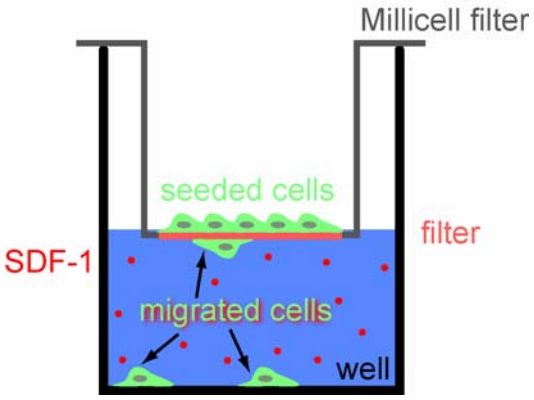


***Figure 14**: Representative scheme for migration assay. The cells were seeded on top of a Millicell filter immersed into a well containing medium supplemented with SDF-1 at different concentrations. Cells were incubated for 4h at 37˚C allowed to migrate towards SDF-1 containing medium.*

Tracking CXCR4 transduced BM derived stem cells after injection to irradiated mice

6 to 8 week- old C57BL6 mice were subjected to irradiation at 8 Gray. 1 million CXCR4 transduced BM stem cells together with 1.5 million freshly isolated hematopoietic stem cells (lin- c-Kit+ Sca1+ selected) from BM were injected intravenously to the mice the same day after irradiation. Control was injected with 1 million IRES-GFP transduced cells together with 1.5 HSCs selected for lin-/c-Kit+/Sca1+ markers. Freshly isolated HSCs are necessary for the recovery of the mice

from irradiation; they serve as an immediate source for HSC replacement. BM was flushed and erythrocytes were removed as described above. Enrichment of c-Kit+Sca-1+ HSCs was performed by negative selection with the use of a mixture of purified rat anti-mouse monoclonal antibodies specific for the mature cell lineage antigens CDB220 (B lymphocytes), Gr-1 (granulocytes), CD4 (T lymphocytes), CD8 (T lymphocytes), TER119 (erythrocytes) and Mac-1 (macrophages) (all from Pharmingen, at a concentration of 1:100 for each antibody). After incubating for 1 hour at 4˚C with this primary antibody mixture, cells were washed with PBS and incubated with rat anti-mouse immuno-magnetic beads ($1x10^7$/ml) (Dynal) for 30min at 4˚C. After selection, the non-magnetic fraction was washed and used together with transduced BM stem cells for intravenous injection. As a control injection, GFP transduced BM stem cells were used in combination with HSCs (lin- c-Kit+ Sca1 +). 3 to 5 weeks postinjection, the animals were anesthetized and transcardially perfused with 0.125M PBS followed by 4% paraformaldehyde. Spinal cord, brain, leg, heart, spleen, liver, lung, thymus were removed and kept overnight in 4% paraformaldehyde followed by overnight incubation in glycerol solution (10% Glycerol and 1% DMSO) for dehydration. The organs were embedded in Tissue- Tek (SAKURA, NL), frozen at -80 ˚C for 2-3 hours and cryosectioned to slices of 20 um thickness. Tissue slices were fixed on microscope slides with 4% paraformaldehyde and migration was detected using fluorescence microscopy.

Analysis of CXCR3 transduced BM derived stem cells

BM derived stem cells were transduced with fresh PT67 supernatant from PT67 cell line expressing viral particles containing the CXCR3-IRES-GFP sequence. For immunostaining, transduced cells were washed and fixed with 4% Paraformaldehyde (PFA) for 30 min at 4˚C, and blocked with 1% BSA for 30 min at 4˚C to prevent unspecific binding. Primary antibody (rat ant-mouse CXCR3) was incubated overnight followed by 30 min incubation with Cy3 anti-rat secondary antibody for 30 min at room temperature. The percentage of transduction efficiency was estimated by flow cytometry analysis. The cells were trypsinized, washed with PBS, incubated with rat ant-mouse CXCR3 for 30 min at 4˚C, washed with PBS and followed by incubation with Cy3 anti-rat secondary antibody for additional 30 min at 4˚C.

The CXCR3 transduced BM derived stem cells were used for injection into EAE induced mice.

EAE Induction

6 weeks old C57/BL6 mice (Charles River) were immunized with the myelin oligodendrocyte glycoprotein peptide (MOG35-55, SeqLab). Complete Freund's adjuvant (CFA) was prepared by suspending 1 ampoule (100mg) of desiccated M.Tuberculosis (Difco Laboratories) in 10ml incomplete Freund's adjuvant (Difco Laboratories). Using CFA, MOG35-55 (2mg/ml) was diluted 1:1 and emulsified. 100ul of the emulsion was injected subcutaneously into two sites at the base of the tail. 100ul of lyophilized pertussis toxin (PTX, 1ng/ul PBS; List Biological Laboratories Inc.) was injected intravenously to accelerate progression of the disease. 48 hours later, a second injection of PTX was given intraperitoneally (100 ul). The weight and the clinical score (Figure 15) was followed up every day. Chronic, nonremitting EAE was induced normally within 10-12 days.

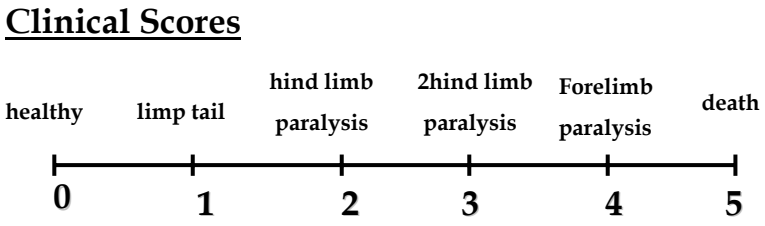


Figure 15*: Clinical Scores for EAE. 10-12 days post immunization mice show the first symptoms of the disease, weak tail, score 1. In 1-2 days the mice reach score 2 where they further show one hind limb paralysis while at score 3 both hind limbs are paralyzed. Score 4 involves further forelimb paralysis which further leads to death (score 5).*

Injection of CXCR3 transduced BM derived stem cells into EAE mice

0.5 Million CXCR3-IRES-GFP transduced BM cells were injected intravenously at 2 different time points, at the onset of the disease (10 days postimmunization) and at the peak of the disease (15-20 days post immunization). Clinical score was followed up immediately after immunization till 10 days post-injection of the cells. 10 days post-injection animals were anesthetized and transcardially perfused with 0.125M PBS followed by 4% paraformaldehyde. Organs were removed and kept overnight in 4% paraformaldehyde followed by overnight incubation in glycerol solution (10% Glycerol and 1% DMSO) for dehydration. The organs were embedded in Tissue- Tek (SAKURA, NL), frozen at -80 ˚C for 2-3 hours and cryosectioned to slices of 20 um

thickness. Tissue slices were fixed on microscope slides with 4% paraformaldehyde and migration was detected using fluorescence microscopy.

APPENDIX TO MATERIALS AND METHODS

Materials

Antibodies

Primary Antibodies

Antigen	Specificity	Source	Class	Company
SSEA-1	Multipotent Cells	Mouse	Monoclonal	Chemicon,USA
Oct4	ES cells	Mouse	Monoclonal	Santa Cruz, DE
β- III tubulin	Neurons	Mouse	Monoclonal	Sigma, DE
CD11b	Macrophages and Microglia	Rat	Monoclonal	Pharmingen, DE
CD11c	Macrophages	Rat	Monoclonal	Pharmingen, DE
Sca1	HSCs	Rat	Monoclonal	Pharmingen, DE
CD49d	BM cells (α4 integrin)	Rat	Monoclonal	Pharmingen, DE
CXCR4	HSCs, B cells	Rat	Monoclonal	Pharmingen, DE
CD29	BM cells (β1integrin)	Rat	Monoclonal	Pharmingen, DE
c-Kit	HSCs	Rat	Monoclonal	Pharmingen, DE
CD133	Somatic Stem Cells	Rat	Monoclonal	Pharmingen, DE

Secondary Antibodies

Fluorophore	Specificity	Source	Company
FITC	Mouse	Goat	Dianova, DE
FITC	Rat	Goat	Dianova, DE
Cy3	Rat	Goat	Sigma, DE
Dynabeads M-450	Rat	Goat	Dynal, USA

Primers

Primers Used for RT-PCR

Gene	Forward Primer	Reverse Primer
Oct4	5'-AGTGGAAAGCAACTCAGAGGG- 3'	5' -GGTTCTCATTGTTGTCGGCTT- 3'
Nanog	5' -GGAGAACAAGGTCCTTGCCAG- 3'	5' -AGTTGGGTTGGTCCAAGTCTG- 3'
Rex-1	5' -GGAAGATGGCTTCCCTGACG- 3'	5' -TGAGGACACTCCAGCATCGAT- 3'
β-3 tub	5'-CAGCGTATACTACAATGAGG-3'	5' -ATCGACAATGAAGCCCTCTACG- 3'
Sox2	5' –AACCAGAAGAACAGCCCG- 3'	5' –GCATCGGTTGCATCTGTG-3'
Gapdh	5'-ACGACCCCTTCATTGACCTCAACT-3'	5'-ATATTTCTCGTGGTTCACACCCAT-3'

Primers Used for Cloning:

CXCR4^{XhoI}: 5'-CCGCTCGAGCGGATGGAACCGATCAGTGTGAGTATA-3'

CXCR4^{MluI}: 5'-CGACGCGTCGTTAGCTGGAGTGAAAACTGGAGG-3'

CXCR3^{XhoI}: 5'-CCGCTCGAGCGGATGTACCTTGAGGTTAGTGAACGTC-3'

CXCR3^{MluI}: 5'-CGACGCGTCGTTACAAGCCCAGGTAGGAGGCCACA-3'

Growth Factors (GF), Cytokines and Chemokines

GF, Cytokines and Chemokines	Source	Affected Receptor	Primary Activity
LIF (Chemicon,USA)	Wide range of cells (ex. stromal cells, astrocytes)	LIFR	Maintain self-renewal in ES and other stem cell populations, while it can also promotes differentiation in different cell types.
PDGF (R&D Systems, DE)	Platelets, Endothelial Cells, Placenta	PDGF receptor	Promotes proliferation, induces expression of some nuclear localized proto-oncogenes (Fos, Myc , Jun).
EGF (Sigma, DE)	Submaxillary gland, Brunners Gland	EGF receptor	Proliferative effect on cells from mesodermal and ectodermal origin, Induction of proto-oncogenes (Fos, Myc and Jun).
FGF (Chemicon,USA)	Wide range of Cells	4 distinct receptors	Promotes proliferation of many cells, Inhibition of some stem cells, Induction of mesoderm to form in early embryos.
SCF (R&D Syst,DE)	Bone marrow stromal cells	c-kit	Support cell growth and proliferation of HSCs in the BM.
SDF-1α (R&D Syst, DE)	HSCs,astrocytes, Endothelial&ES cells	CXCR4	Cell migration, Homing.

Buffers, Solutions and Medium

0.125M Phosphate Buffered Saline (10XPBS)- pH 7.3

Component	Concentration	Company
$NaH_2PO_4.H_2O$	0.007M	Roth, DE
Na_2HPO_4	0.034M	Roth, DE
NaCl	0.6M	Roth, DE

Lysis Buffer

Component	Concentration	Company
NH_4Cl	0.156M	Roth, DE
$KHCO_3$	0.01M	Roth, DE
EDTA	$5x10^{-6}$M	Roth, DE

TBE Buffer (10X)

Component	Concentration	Company
Tris-Base	1.78M	Roth, DE
Boric Acid	1.78M	Sigma, DE
EDTA	0.04M	Roth, DE

4% Paraformaldehyde pH 7.3

Component	Amount	Company
PFA	20g	Sigma, DE
PBS (0.125M)	50 ml	See PBS preparation
ddH_2O	450 ml	Roth, DE

Agarose Gel (1%)

Component	Amount	Company
Agarose	0.5 g	SeaKem, Cambrex, USA
EtBr	1.25 ul	Roth, DE
Gel Star	4 ul	BioWhittaker Mol Appl, USA
TBE(1X)	50 ml	See TBE preparation

Fibronectin (Fn) Stock Solution

Component	Amount	Company
Fibronectin	1 mg	Sigma, DE
ddH_2O	1 ml	Roth, DE

Working solution is 5ng/ml prepared with 1XPBS.

Pregnant Mare Serum Gonadotropin

Component	Amount	Company
PMS	1000 IU	Intervet, DE
NaCl (0.9%)	20 ml	Roth, DE

100 ul/mice used for injection.

Human Choriongonadotropin

Component	Amount	Company
hCG	5000 IU	Intervet, DE
Sterile ddH_2O	5 ml	Intervet, DE
NaCl (0.9%)	45 ml	Roth, DE

100 ul/mice used for injection.

Avertin

Component	Amount	Company
Tribromethanol	2g	Intervet, DE
Tert-Amylalcohol	1g	Intervet, DE
ddH_2O	158g	Roth, DE

Injected according to the mouse body weight (50 ul/g).

Tyrod's Solution (g/100ml) pH 2.5

Component	Amount	Company
NaCl	0.800g	Intervet, DE
KCl	0.020g	Intervet, DE
$CaCl_2 2H_2O$	0.024g	Roth, DE
$MgCl_2 6H_2O$	0.010g	Roth, DE
Glucose	0.100g	Sigma, DE
Polyvinylpyrrolidone (PVP)	0.400g	Sigma, DE

Plasmid Restriction-Digestion (Total volume 20 ul)

Component	Amount	Company
Enzyme 1	0.5ul	Roche, DE
Enzyme 2	0.5ul	Roche, DE
Buffer	2ul	Roche, DE
Plasmid	17ul plasmid in ddH_2O (up to 1 ug)	

Plasmid Ligation (Total volume 10 ul)

Component	Amount	Company
T4 Ligase	0.5 g	Roche, DE
Ligation Buffer	1.25 ul	Roche, DE
Total DNA	8 ul (1:3 or 1:15 ratio of insert:plasmid)	

PCR Mix(1 sample tube-50 ul)

Component	Amount	Company
ddH_2O	41 ul	Roth, DE
dNTP (10mM)	1 ul	Amersham Biosciences, USA
Buffer	5 ul	Roche, DE
Forward Primer (100pmol)	0.5 ul	MWG, DE
Reverse Primer (100pmol)	0.5 u	MWG, DE
DNA (<500 ng)	1 ul	
DNA Polymerase	1 ul	Roche, DE

Reverse Transcription Mix(1 sample tube- 20 ul)

Component	Amount	Company
RNA (<400 ng)	11 ul	
dNTP (10mM)	1 ul	Amersham Biosciences, USA
Hexa-nucleotide Solution	1 ul	Roche, DE
5XRT Buffer	4 ul	Invitrogen, DE
DTT Buffer	2 ul	Invitrogen, DE
Reverse Transcriptase	1 ul	Invitrogen, DE

Real time PCR Mix (1 sample tube- 50 ul)

Component	Amount	Company
SYBR Green	25 ul	Applied Biosystems, UK
Forward Primer(10pmol)	3 ul	MWG, DE
Reverse Primer(10pmol)	3 ul	MWG, DE
cDNA	1 ul	
dNTP (0.4mM)	1 ul	Amersham Biosciences, USA
ddH_2O	17 ul	Roth, DE

Medium

Stem Cell Expansion Medium

Component	Amount	Company
Dexamethasone	50 nM	Sigma, DE
L-Ascorbic acid	100 uM	Sigma, DE
LA-BSA	0.5 mg/ml	Sigma, DE
BSA	0.5 mg/ml	Sigma, DE
ITS	1%	Gibco, DE
MCDB 201	40%	Sigma, DE
Penicillin/Streptomycine	1%	Gibco, DE
FCS	2%	Gibco, DE
DMEM/Low Glucose	54%	Gibco, DE
EGF	10 ng/ml	Sigma, DE
rhPDGF-BB	10 ng/ml	R&D Systems, DE
mLIF	1000 U/ml	Chemicon, USA

PT67 and EcoPack 293 Medium

Component	Amount	Company
DMEM/Low Glucose	87%	Gibco, DE
FCS	10%	Gibco, DE
L-Glutamine	1%	Sigma, DE
Penicillin/Streptomycine	1%	Gibco, DE
Glucose	1%	Sigma, DE

Medium Used During Aggregation

M2 –Sigma, DE

M16-Sigma, DE

Equipments

Fluorescence Microscope- Axiovert 40 CFL, Zeiss, DE

Confocal Microscope- TSC-SP2 AOBS, Leica Microsystems GmbH, DE

Stereomicroscope- SZ51, Olympus GmbH, DE

BD FACS Calibur, BD Biosciences, DE

Bio-Rad i-cycler, Bio-Rad Laboratories, DE

Cryostat- Microm HM 560, Microm International GmbH, DE

Filters used

FITC- Excitation 450-490; Emission 515-565

Cy3- Excitation 546/12; Emission 575-640

DAPI- Excitation 365; Emission 420

Methods

RNA Isolation

Isolation of RNA was performed using the RNeasy Mini Kit (Qiagen). The protocol used is summarized as follows:

i. Take eppendorf with tissue (e.g. 20-30mg in PBS).
ii. Add 600ul lysis buffer (inc. 1:100 B-mercaptoethanol), use 300ul if less than 20mg of tissue (lysis buffer lasts approx. 1 month).
iii. Homogenise with small syringe (use large first if a lot of tissue). Centrifuge (3min, max).
iv. Mix 600ul of supernatant with 600ul EtOH+DEPC H2O.
v. Add 700ul into column and centrifuge (15s, 10000rpm), throw away waste, add rest of mixture and repeat.
vi. Add 350ul wash buffer RW1 and centrifuge (15s, 1000rpm), throw away waste.
vii. Mix 10ul DNase1 with 70ul RDD buffer, add to filter and leave for 15min.
viii. Add 350ul wash buffer RW1 and centrifuge (15s, 10000rpm), throw away waste.
ix. Add 500ul wash buffer RPE (+EtOH) and centrifuge (15s, 10000rpm), remove waste, repeat and centrifuge (2min, max). Remove waste and centrifuge again to dry (1min, max).
x. Place column in eppendorf, add 35ul RNase free H2O (onto filter), leave for 3min, centrifuge (1min, 10000), throw away column. RNA is collected in eppendorf.
xi. Measure concentration (ug/ml) and RNA/protein absorption (260/280) using photometer (Eppendorf), and dilute with DEPC H2O accordingly:

RNA conc. (ug/ml)	**DEPC H2O**
<300	30
300-400	40
400-500	50
>500	60

Transfection

Transfection of cell lines was performed to produce retroviral particles as well as to confirm the expression of genes cloned into plasmids. Retroviral particles were produced using Effectene transfection protocol (Qiagen):

i. Plate $5x10^6$ EcoPack293 cells in a poly-L-lysine coated 10cm dish one day before transfection to obtain a culture of 80-90% confluency.
ii. On the day of transfection add antibiotic-free medium to the cells (10ml).
iii. Prepare 425 ul EC-Buffer, 2.5 ul of plasmid, 20 ul of Enhancer (ratio Enhancer:DNA 8:1) in an eppendorf.
iv. Vortex and leave for 2-5 min at RT.
v. Add 50 ul Effectene to the mixture, vortex and leave for 5-10 min at RT.
vi. Add 500 ul medium to the mix.
vii. Add transfection mix to cells dropwise, mix gently and leave for 6 hours.
viii. Add medium containing antibiotics and 2% serum to the transfected cells
ix. Remove supernatant 36-72hrs post-transfection and pellet the debris.

The supernatant may be stored overnight at 4ºC or frozen at -80ºC.

Transduction

Supernatant Spin Infection

Cells at a confluency of 30-50% in 6-well dishes were treated with retroviral supernatant, centrifuged twice (2500rpm, 37˚C, 45 min) and kept at 37ºC overnight. Fresh medium was added to the cells on the

following day. Expression normally reached its peak 48hr post-transduction and was generally higher than that observed following regular supernatant treatment.

Chimera Generation

i. 6 weeks CD1 female mice were intraperitoneally injected with PMS.
ii. 48 h later hCG hormone was administered intraperitoneally.
iii. Female mice were placed in a cage with one stud male, and the next morning the female was checked for a copulation plug.
iv. 2 days later the female mice were sacrificed and 8-cell morula stage embryos are collected by flushing the oviduct with M2 medium.
v. Zona pellucida is removed from the collected embryos using acidic Tyrod's solution.
vi. The zona pellucida free 8-cell stage embryos are placed on previously prepared separate wells containing M16 medium.
vii. 8-10 BM derived stem cells are transferred to each well containing a single 8-cell stage embryo and the cells were incubated overnight till blastocyst stage.
viii. The next day the aggregated blastocysts were transferred to foster mice (max. 40 blastocysts per mice).

RESULTS

Characterization of BM derived cells

Total bone marrow was isolated from femur and tibia bones of C57Bl6 mice and cultured on fibronectin coated dishes in stem cell expansion medium containing LIF. Erythrocytes were removed with a hypotonic lysis buffer treatment for a very short time (30 seconds). Medium was changed every two days. The cells start to adhere to the culture dish after one week. The first passage is done after 7 days. Once adherent, the cells show high proliferation rate, 36 hours of doubling time. Cells were passaged twice a week at 60-70% confluency and plated at a density of 4000 cells/cm^2. Adherent BM derived cells were cultured for up to 20 passages. More than 80% of presumed hematopoietic cells died during the first week of culture and only the cells which are resistant to long cell culture conditions and have a high proliferative capacity survived. Cultured with LIF, BM derived cell cultures form a heterogeneous population containing cells with various size and morphologies (Figure16).

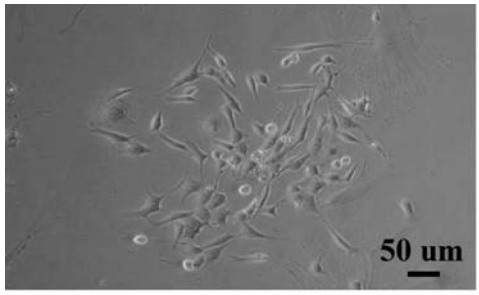


Figure 16: *Heterogeneous Bone Marrow population cultured in LIF containing medium conditions.*

Detailed characterization using flow cytometry analysis (Figure 17) confirmed the high heterogeneity of the BM culture after 7 passages. Cells were collected, and immunostained against surface markers: CXCR4, Sca1, cKit, CD11b, CD11c, CD29, CD49d, SSEA-1 and CD133. Analysis via flow cytometry revealed cell population expressing 31±2% Sca-1, 13±1% c-kit and 15±2% CXCR4 surface receptors which are markers for hematopoietic stem cells. Some cells (13±0.5%) were expressing Alpha 4 integrin-CD49d and β1 integrin-CD29 (20±2%) which are important components of VLA4 integrins involved in stem cell mobilization. Nearly 45±3.5% of the cells were expressing CD 11b, a cell surface integrin of macrophages,

granulocytes and natural killer cells, demonstrate the presence of immune cells in the culture.

Around 44±3% of cells in this heterogeneous population were expressing surface marker for murine ES cells, stage specific embryonic antigen 1 (SSEA-1) (Figure 17). In order to obtain a more homogenous stem cell population, SSEA-1 expressing cells were sorted and cultured on fibronectin coated dishes in stem cell expansion medium. However, the cells did not attach to the fibronectin coated dish and did not expand; they showed almost no proliferation and they died within one week after selection.

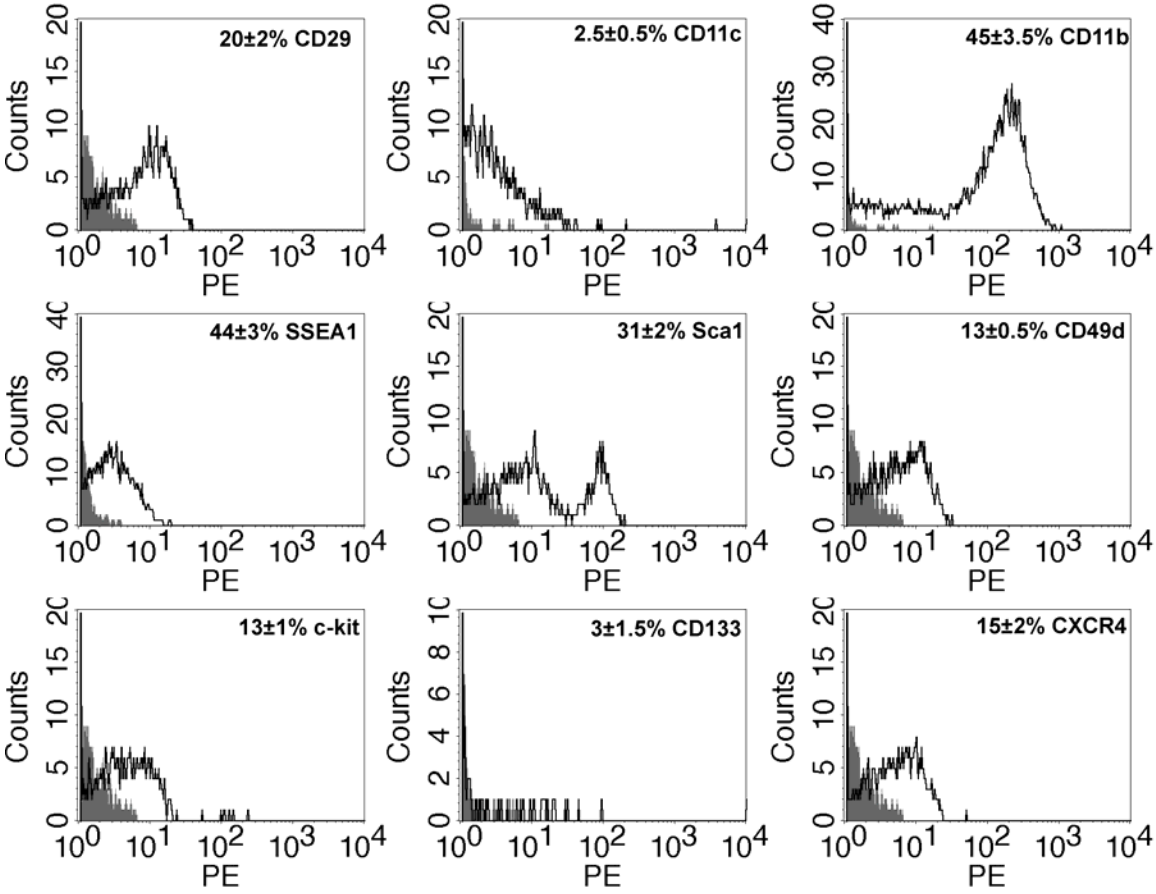


Figure 17: *Flow cytometry analysis of Bone Marrow derived stem cells after 7 passages. Results reveal a high heterogeneity in the population. Many cells express hematopoietic stem cell markers (Sca1, c-Kit, CXCR4), some express early macrophage markers (CD11b), expression of stem cell adhesion markers (CD29, CD49d) confirmed the presence of stromal cells. Population was also enriched (44±3%) for SSEA-1, an early Embryonic Stem cell marker, expressing stem cells in BM cultures.*

The SSEA-1 marker is a marker specific for ES cells. Its detection suggested the presence of a non-hematopoietic stem cell population in the BM culture. Analysis of other ES cell markers (Oct4, Nanog, and Rex1) was performed via RT-PCR and immunocytochemistry

Reverse transcriptase data after 7 passages showed a significant expression of pluripotency genes like Oct4, and Rex1 followed by weak Nanog expression (Figure 18). The expression was followed up to 20 passages after which the amount of these transcription factors was undetectable.

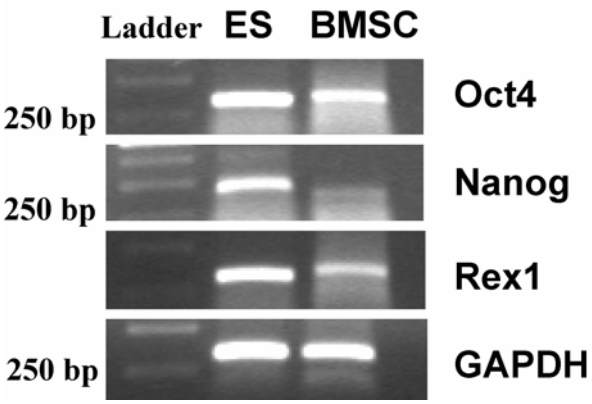
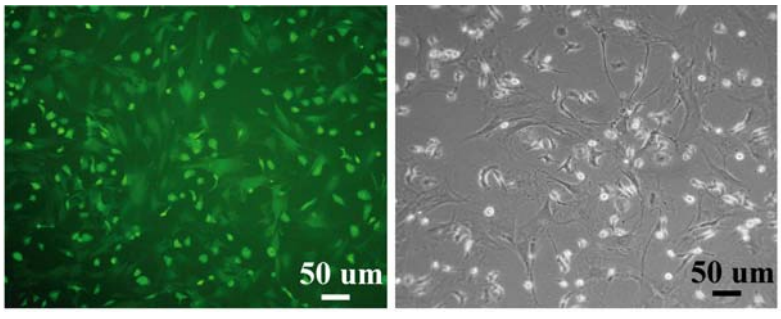


Figure 18: *Gene transcripts for pluripotency genes Oct4, Nanog and Rex1 in BM derived cell cultures (BMSC) and ES cells (ES) after 7 passages detected by RT-PCR (40 cycles). Oct4 and Rex1 are detected at higher levels, while Nanog shows weak expression.*

Immunolabelling for Oct4 of BM derived stem cells after 7 passages showed that cells with round shaped morphology and smaller in size (10-15 um) are the source for the Oct4 gene transcript (Figure 19).



A **B**

Figure 19*: Transmission (A) and fluorescence (B) images of BM derived stem cells after 7 passages stained for Oct4 transcription factor (green*

fluorescence). Cells stained for Oct4 have high nuclei to cytoplasm ratio revealing stem cell-typical morphology.

In vitro differentiation of BM derived stem cells

Neuronal differentiation of the BM derived stem cells was induced by FGF2 and Shh supplemented to the culture medium for 2 weeks. Initially, Nestin negative BM cells initiated Nestin expression after 1-2 weeks in stem cell expansion medium. Together with Nestin, β-III tubulin expression was also detected after 1 week in differentiation medium via RT-PCR (Figure 20B). Immunostaining experiments revealed that after 2 weeks in neuronal differentiation medium most of the cells in culture expressed the early neuronal marker β-III tubulin (Figure 20A) initiating some cytoplasmic projections.

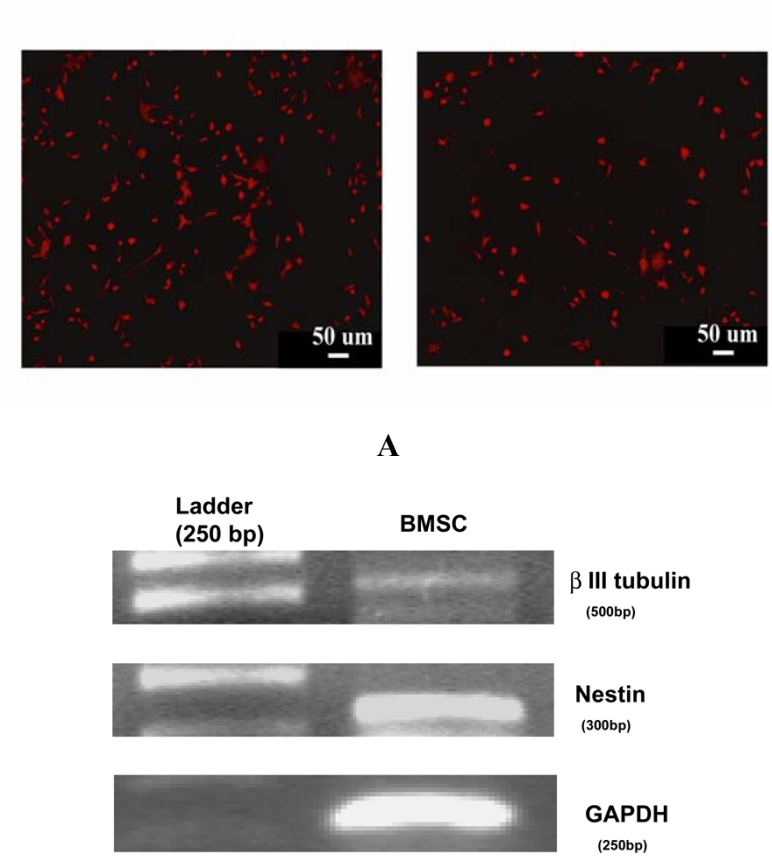
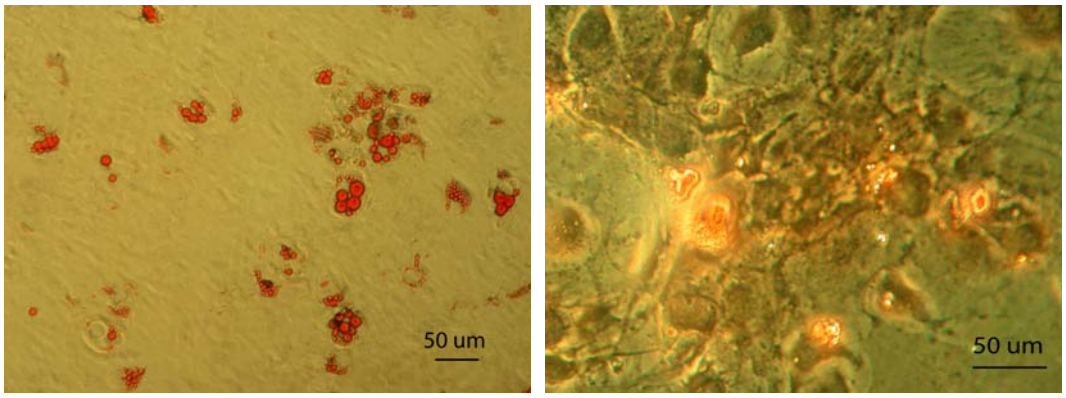


Figure 20: *Cells stained for β -III tubulin after 2 weeks in neuronal differentiation medium (A). RT-PCR for β -III tubulin, Nestin and GAPDH housekeeping gene expression was detected in BM derived stem cells (BMSC) following differentiation (B).*

Adipogenic differentiation was performed for 2 weeks under adipogenic differentiation conditions. Oil red, a lipid soluble dye, was used for staining the fat droplets formed in the differentiated adipocytes (Figure 21 A). Osteogenic induction for 2 weeks allowed a detection of calcium deposits in differentiated osteocyte cultures. Alizarin Red, forming a complex with Calcium ions, was used to detect the mineralization in the cultures (Figure 21 B).

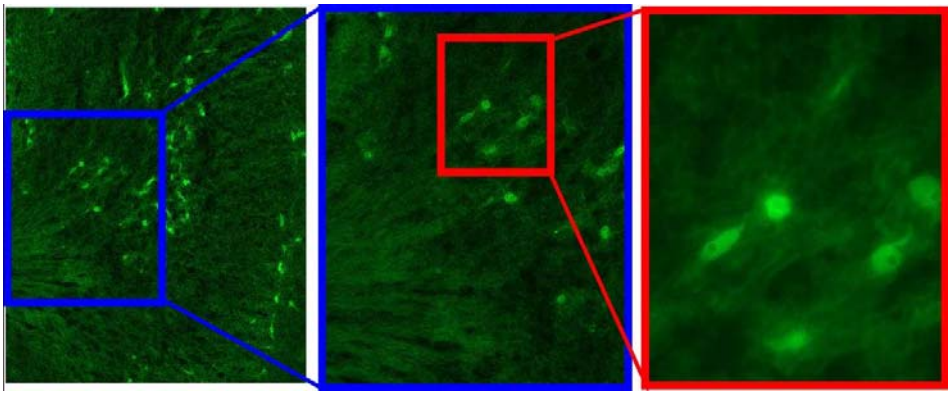


A **B**

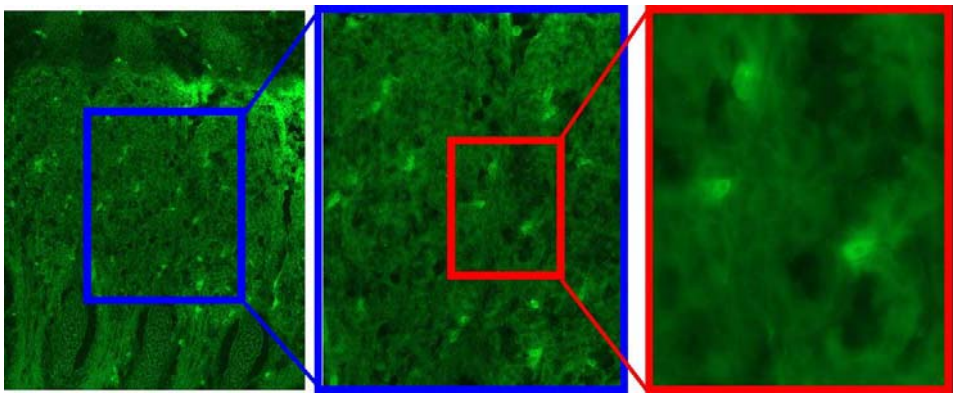
Figure 21: *Oil red staining for lipid droplets indicating adipocyte differentiation (A); Mineralization detected via Alizarin Red staining in osteoblast differentiation cultures (B).*

In vivo plasticity of BM derived stem cells

8-cell stage morulae derived from CD1 mouse were used to aggregate with GFP labeled BM derived cells. Embryos were analyzed at embryonic day 12.5 (E12.5).GFP positive cells were detected in various parts of the embryos like brain, dorsal root and muscle tissues (Figure 22). Aggregation experiments revealed up to 5 % contribution of BM derived stem cells in tissues of different embryonic lineages which suggest the presence of ES cell like population in the BM having capacity to contribute to different embryonic tissues.



A



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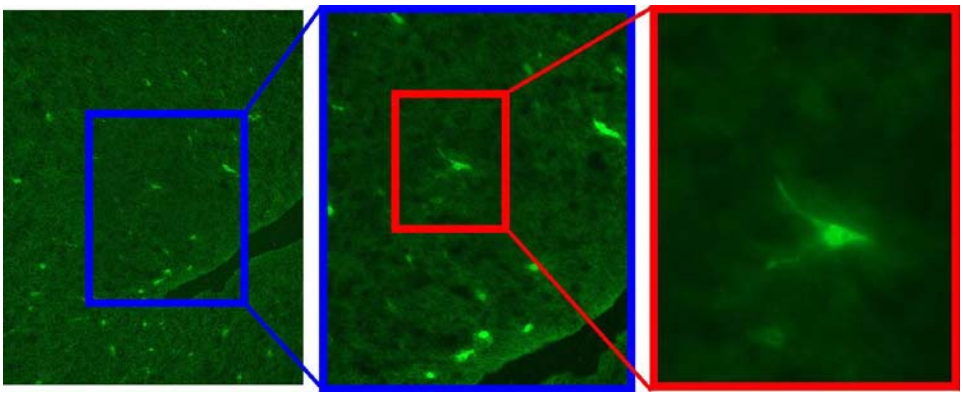
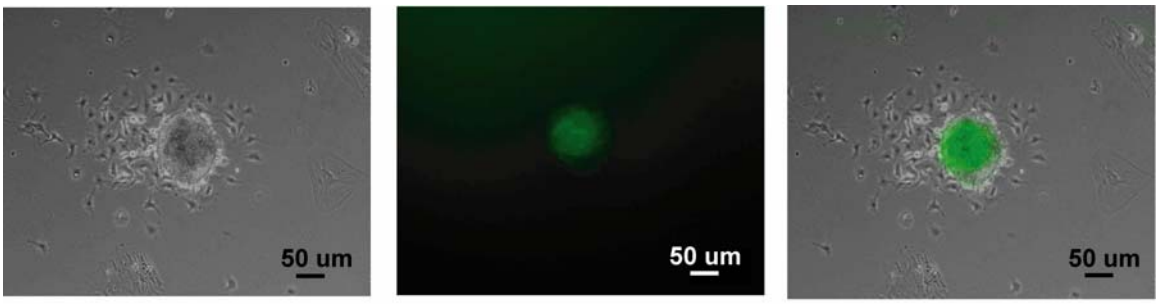


C

Figure 22: *Aggregation of 8-cell CD1 morulae with GFP labeled BM derived cells from C57BL6 mice. GFP cells were detected in muscle (from tongue) (A), dorsal root (B), and brain (C) of embryos at 12.5 day. Overall contribution of GFP positive BM cells to the total embryo was in the range of (1-5%).*

Selection of Oct4 expressing BM derived stem cells

In order to enrich multipotent stem cells derived from the BM, we used transgenic mice expressing GFP under Oct4 promoter. GFP positive clusters have been detected within early cultures of BM derived cells cultured with stem cell expansion medium (Figure 23 A). GFP cluster formation was rare, 0.5% of the BM cells isolated from a mouse can form GFP clusters. GFP expression was mostly localized in the core of the cluster where cells were having a round shaped morphology, while there was no GFP signal in the periphery of the clusters where the differentiation had already started. Selected clusters were analyzed for Oct4 and Nanog expression levels via Real time PCR and compared with ES cells. Relative quantification by real time PCR revealed nearly 30 fold lower Oct 4 and 10 fold lower Nanog expression in BM derived GFP clusters in comparison to ES cells expression values (Figure 23 B).



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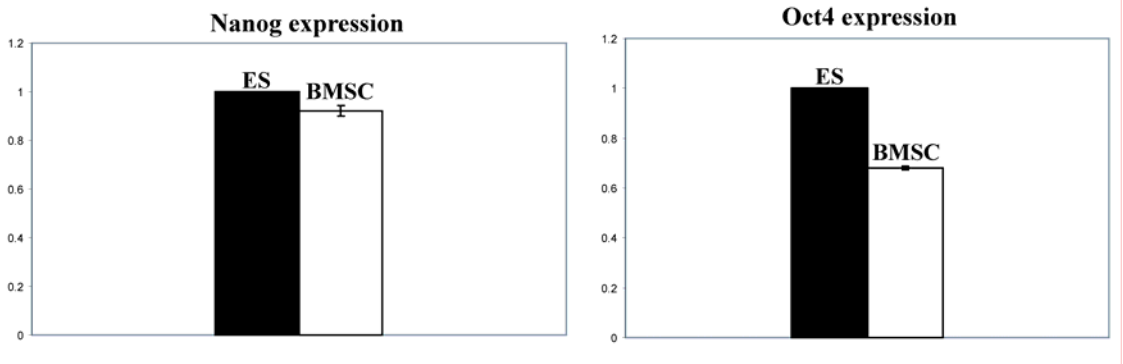
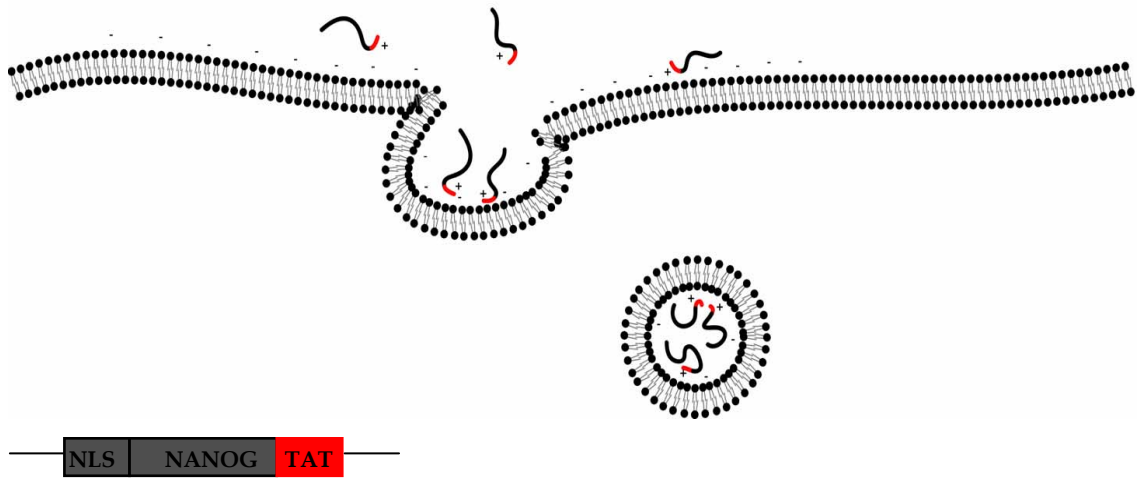


B

Figure 23: *GFP positive clusters derived from BM of Oct4 promoter GFP transgenic mice (Oct4GiP) (A). GFP positive clusters have a low frequency, less than 0.5% /mouse. Real time PCR data represents Oct4 and Nanog gene transcript levels in BM derived GFP clusters (BMSC) in comparison with ES cell expression levels (ES) (B). Nanog shows 10 fold and Oct4 30 fold lower expression in comparison to the ES expression levels. Y-axis shows relative values of real time RT PCR normalized by GAPDH gene transcripts.*

TAT-Nanog protein transduction

Expansion of mechanically isolated GFP positive clusters in stem cell expansion medium without inducing differentiation was not possible. In order to rescue the cells from their quiescent state and improve their proliferation efficiency we used several approaches. Nanog, a pluripotency gene transcript, maintains self renewal in ES cells. In order to induce the further proliferation of the GFP clusters we introduced TAT-Nanog fusion protein (Figure 24) to the stem cell expansion medium.



NLS: Nuclear Localisation Signal

TAT: Trans-actin Activator of Transcription Peptide (+ve charge)

Figure 24: *NLS-Nanog-TAT protein construct. TAT (positively charged) is involved in the endocytosis (macropinocytosis). NLS is involved in nuclear transportation of the protein. The scheme is a representation of TAT-Nanog protein entering the cell via macropinocytosis. Once taken to the cell, very low amount of protein diffuses to the cytoplasm.*

The recombinant Nanog protein contains positively charged TAT on C terminus which allowed the attraction between the protein and the plasma membrane and enhanced the protein uptake via macropinocytosis. The nuclear localization signal was added to the N terminus which allowed the entrance of the protein to the nucleus. Most of the protein taken to the cell does not diffuse through the vesicle, only a very low amount is released to the cytoplasm.

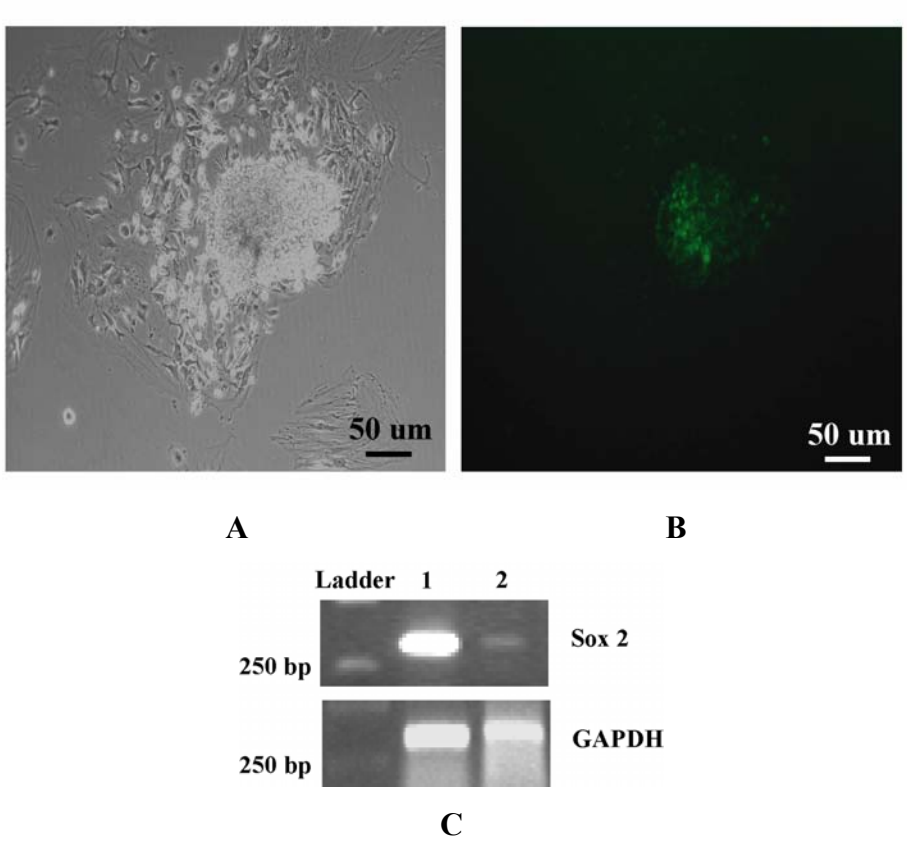


Figure 25*: Transmission (A) and Fluorescence (B) images of clusters formed in medium containing Nanog protein. Total cell number and the rate of cluster formation were increased, however the GFP signal indicating Oct4 expression decreased. Higher Sox 2 expression was detected in cells cultured with Nanog (1) in comparison to cells cultured in stem cell expansion medium (2) (C).*

Introduction of the TAT-Nanog protein to the medium significantly increased total cell proliferation in freshly isolated cultures. The frequency of cluster formation was higher (10%) in comparison to the cell cultured in only stem cell expansion medium, however the early analysis (4^{th} passage) showed very low Oct4 (and GFP) expression (Figure 25B) and strong Sox2 expression (Figure 25C) which is another early transcription marker in ES cells known to make complexes with Oct4 and activate other pluripotent genes like Rex1 and Nanog. Expansion of cells for 2 weeks (4 passages) under Nanog containing conditions did not prolong the pluripotency gene expression.

FGF2 and SCF application

Since FGF2 is an important regulator for maintenance in pluripotency in human ES and Primordial Germ Cell (PGC) cultures, we supplemented the stem cell expansion medium with FGF2 in order to expand the GFP positive multipotent stem cells and maintain their plasticity. The addition of the growth factors SCF (10 ng/ml) and FGF2 (25 ng/ml) 2 weeks after the initiation of the BM cell culture induced an earlier expression of Oct 4 at 2nd passage (Figure 26). The effect was observed at 72 h after addition of the growth factors. The culture expressing Oct 4, was maintained up to 8 passages.

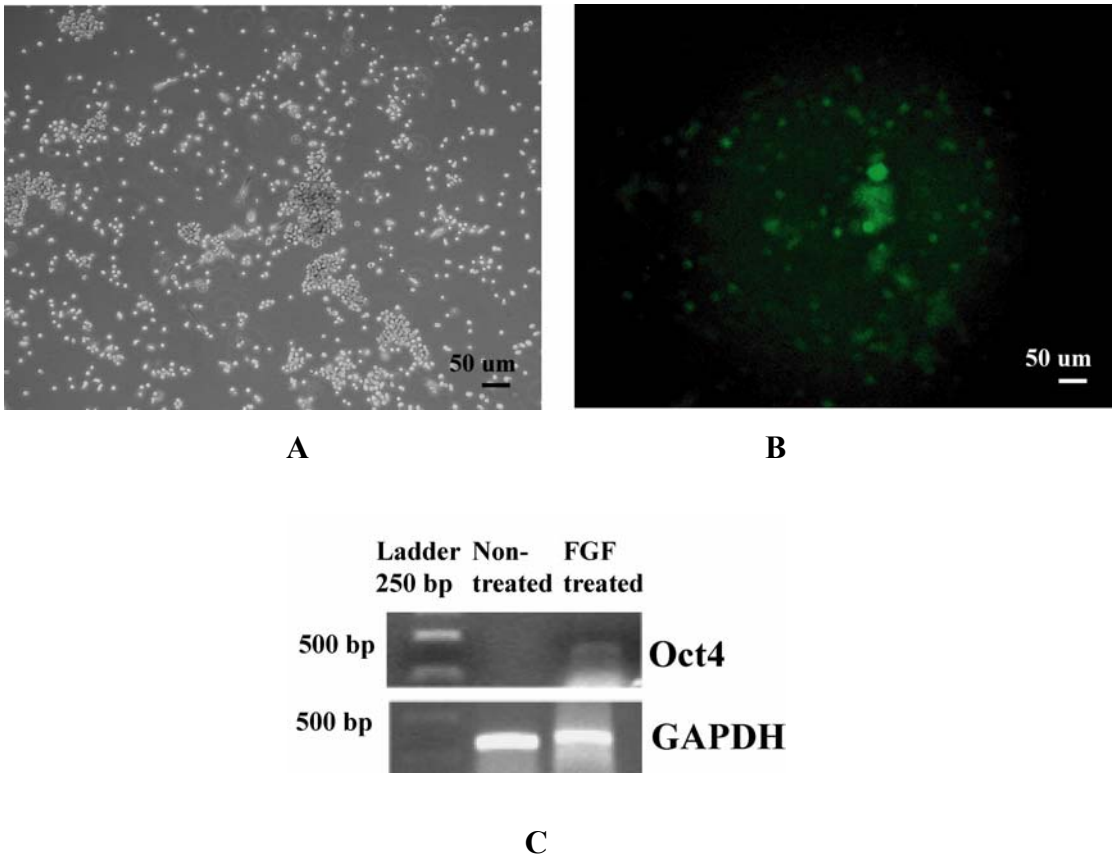
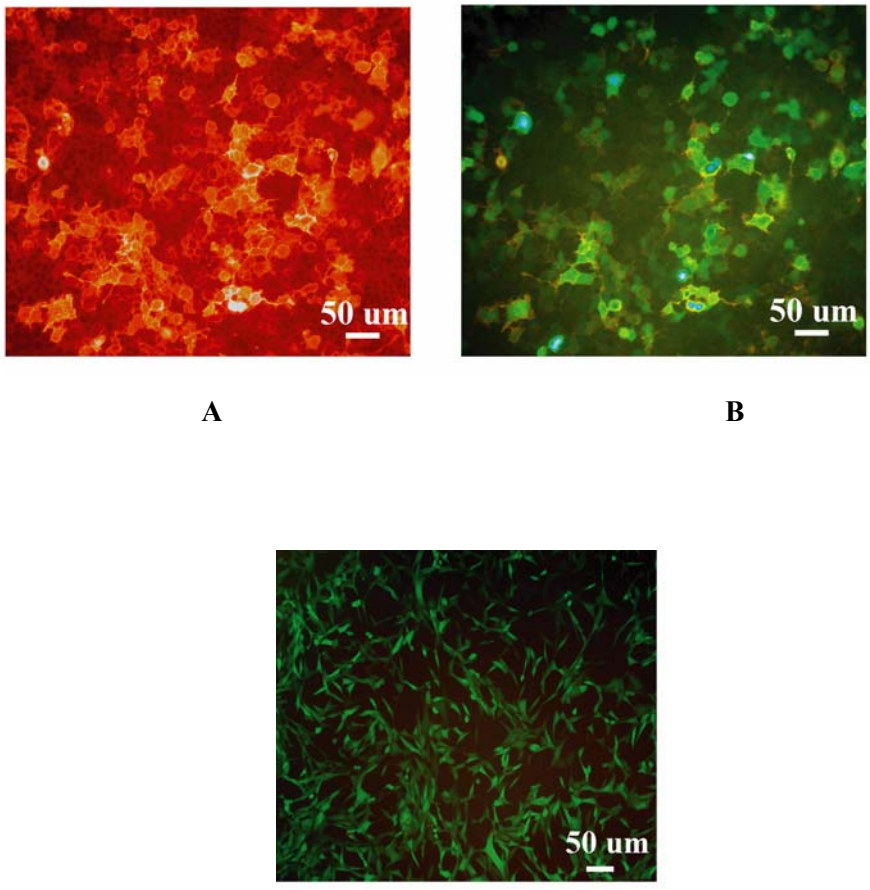


***Figure 26**: Transmission (A) and Fluorescence (B) images of BM derived cells at 2nd passage cultured with SCF and FGF2 growth factors for 72 hours. Many small-sized GFP clusters were detected. The GFP signal was maintained up to 8 passages. Application of FGF and SCF induced early expression of Oct4 transcription factor, while no Oct4 was detected in cells cultured in stem cell expansion medium after 3 passage (C).*

Bone Marrow Derived Stem Cells in Cell Therapy Application

Generation of a stable cell line for constitutive retroviral expression

EcoPack 293 cell line was transfected with the pMSCV-CXCR4-IRES-GFP vector using Effectene transfection kit (Qiagen). Transfection efficiency was >90% (Figure 27 B). Immunostaining for CXCR4 marked the cell surface indicating the expression of the receptor on the membrane, while GFP was localized within the cell body (Figure 27 A and B). The retroviral supernatant collected from EcoPack cells was used for transduction of RetroPack PT 67 cells. After positive magnetic bead selection of PT 67 cells using monoclonal rat anti- mouse CXCR4 antibody, a highly enriched stably viral particle producing PT 67 cell line was generated (Figure 27 C). Similar results were obtained with EcoPack transfection using CXCR3-IRES-GFP, and IRES-GFP vectors. PT67 cell lines expressing CXCR3-GFP and PT67 expressing only GFP were generated with same efficiency.



A **B**

C

Figure 27: *A- Immunostaining for CXCR4 receptor in EcoPack 293 cells transfected with pMSCV-CXCR4-IRES-GFP; B- Overlay for CXCR4 immunostaining and GFP expression of transfected EcoPack 293 cells; C-*

Stably transduced PT67 cell line transduced with CXCR4-IRES-GFP transfected EcoPack 293 viral supernatant.

Retroviral CXCR4 Transduction of BM derived stem cells

BM cells isolated from C57B6 mice were cultured for 7-8 passages in stem cell expansion medium prior to transduction. Transduction of BM derived stem cells was performed with fresh supernatant provided from PT 67 cell line producing retroviruses carrying the CXCR4-IRES-GFP sequence, 48h after confluency. The transduction process was repeated twice. Confocal images of CXCR4 transduced BM stem cells showed the labeling of the CXCR4 receptor (red labeling) on the surface of the cell membrane while GFP is mostly localized in a perinuclear region in the cytoplasm (Figure 28). Retroviral system using IRES gives the advantage of expressing both the gene of interest and the reporter gene simultaneously, avoiding interference with the function of the protein of interest, as might occur with fusion constructs.

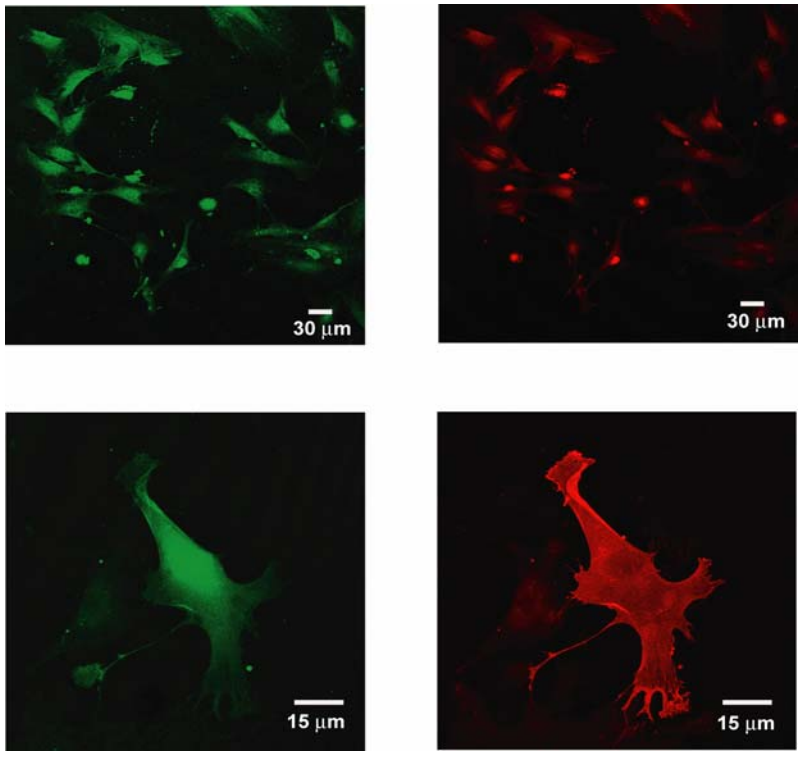


Figure 28: *BM derived stem cells transduced with pMSCV-CXCR4-IRES-GFP vector. GFP expression (A) and immunostaining for CXCR4 receptor (B) in BM derived stem cells.*

Flow cytometry analysis was performed in order to detect the transduction efficiency of our expression system. BM stem cell population is very heterogeneous. In fact, there is a cell population around 15% that already express the CXCR4 receptor. Upon transduction, we were able to increase this cellular fraction up to 48±3% (Figure 29). Cells used for experiments were enriched by sorting the GFP positive cells up to 90%.

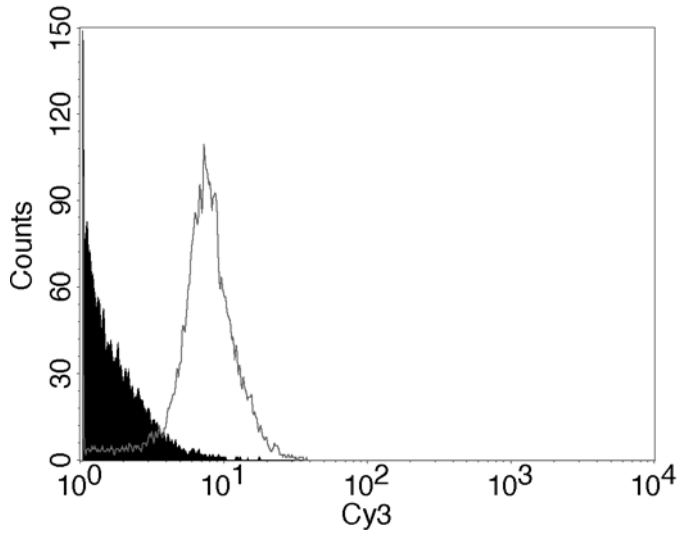


Figure 29: *Flow cytometry results of CXCR4-IRES-GFP transduced BM stem cells. 48±3% transduction efficiency was achieved. Isotype control is shown as filled black histogram.*

In vitro functional analysis for CXCR4 transduced BM derived stem cells

In vitro functional analysis for the CXCR4 expressed in BM stem cells was performed using a MilliCell chamber filter set up, where cells were seeded over the 0.45 um filters embedded into a medium containing varying concentrations of the receptor ligand SDF-1. Migration was allowed to proceed for up to 4 hours. The results from three independent experiments revealed an increased migration potential of CXCR4 transduced cells in comparison to only GFP transduced cells (Figure 30). As a matter of fact, the number of GFP transduced cells was quite constant (around 100 cells) at every SDF-1 concentration. Migrated CXCR4 transduced BM stem cells showed an increase between 120 up to 800 for SDF-1 concentrations between 0 and 30 ng/ml. The dose dependent increase is almost linear, inducing an increase up to 8 fold at a concentration of 30 ng/ml. Higher concentrations of SDF-1 (50 and 60

ng/ml) exhibit a limiting effect, reducing the migration levels similar to the control experiments (~100 cells).

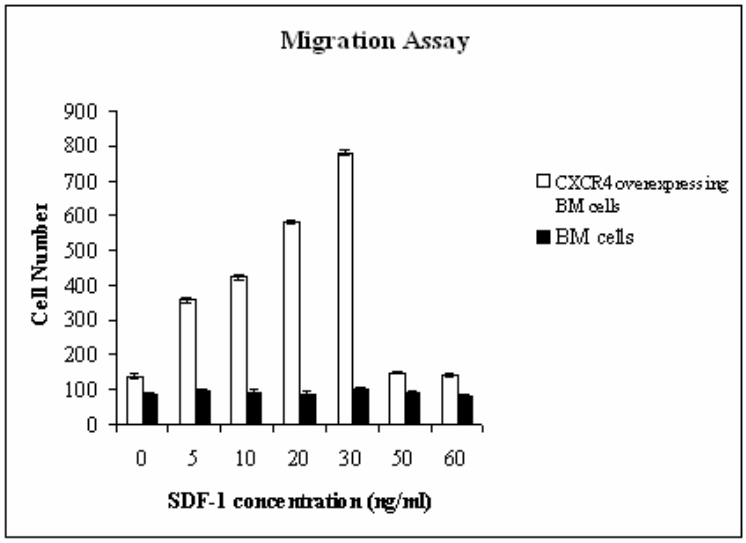
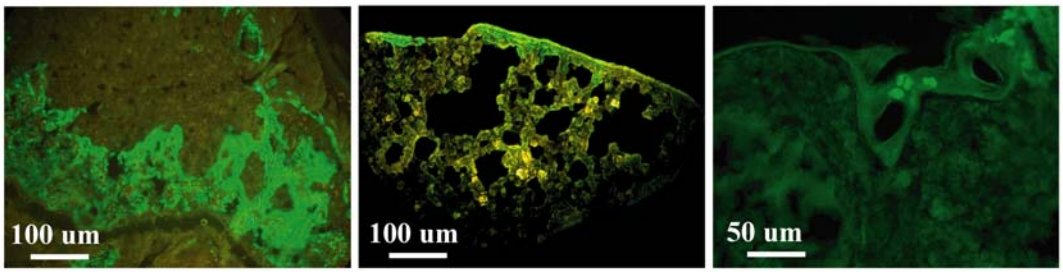


Figure 30: *Migration Assay of CXCR4 and only GFP transduced BM stem cells towards various SDF-1 concentrations (0-60 ng/ml). CXCR4 transduced cells show increased migration in the range 0-30 ng/ml SDF-1, while follows a decreased migration in higher concentrations. GFP transduced cells (BM cells) on the other hand do not show much deviation in migration towards different SDF-1 levels.*

In vivo targeted migration of CXCR4 transduced BM stem cells in irradiated mice

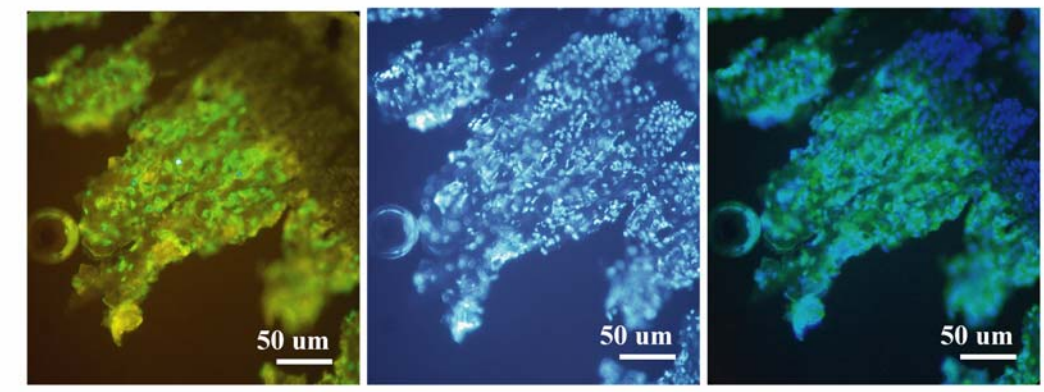
In vivo effect of CXCR4 expression was evaluated via injection of 1 million CXCR4-IRES-GFP transduced BM derived stem cells to lethally irradiated mice causing the depletion of their hematopoietic stem cells together with 1.5 million HSCs selected for lin-cKit+Sca1+ from freshly isolated BM of C57Bl6 mice. 1 million only IRES-GFP transduced BM cells together with 1.5 million HSCs were injected to a control irradiated mice. Majority of the engrafted CXCR4-IRES-GFP transduced BM cells were detected 3 weeks post injection within the endosteum (stem cell niche) of the bone marrow, and some within lung and spleen tissues (Figure 31). No cells were detected in organs from control mice using fluorescence imaging.



A **B** **C**

***Figure 31**: Fluorescence images of CXCR4-IRES-GFP transduced BM derved stem cells detected in the endosteum region of the Bone Marrow (A), Lung (B), and Spleen (C), 3 weeks post-injection*

Observations revealed the domination of the endosteal regions by CXCR4 transduced stem cells (Figure 32). More than 50% of the cells through the entire endosteum region were GFP positive cells.



A **B** **C**

***Figure 32:** Part of endosteum (stem cell niche of the bone marrow), migration of CXCR4-IRES-GFP transduced BM derived stem cells (A), DAPI staining (B), and overlay (C).*

In order to confirm migration to the spleen we performed flow cytometry analyses where results (Figure 33) show a low migration of engrafted CXCR4 transduced cells to the spleen (3%). On the other hand via flow cytometry we detected 2% GFP positive cells in the spleen of the control mice (Figure 33) which can be associated with the low number of CXCR4 expressing cells in the initial heterogeneous BM culture.

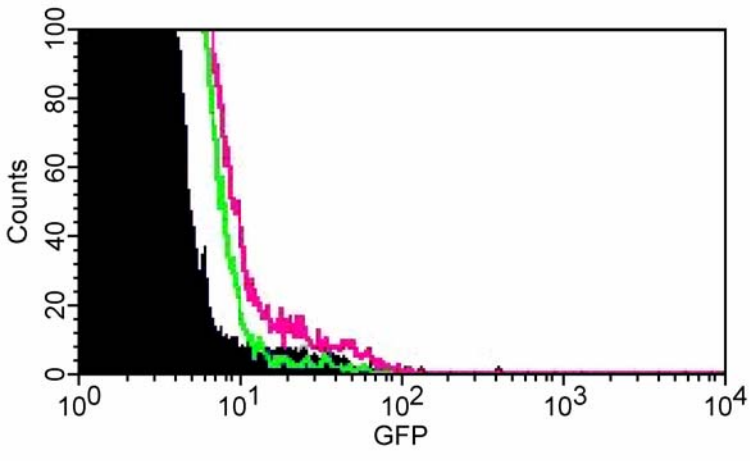


Figure 33: *Flow cytometry analysis of spleen taken from irradiated mouse (black), irradiated mouse 3 weeks after injection with GFP transduced (green) (2%) and with CXCR4 transduced BM stem cells (pink) (3%). Isotype control is shown as filled black histogram.*

Analysis 5 weeks post-injection revealed a higher reconstitution of the bone marrow via CXCR4 transduced BM stem cells (Figure 34), while there was no detected migration of injected IRES-GFP transduced BM cells except few cells detected in lung tissue.

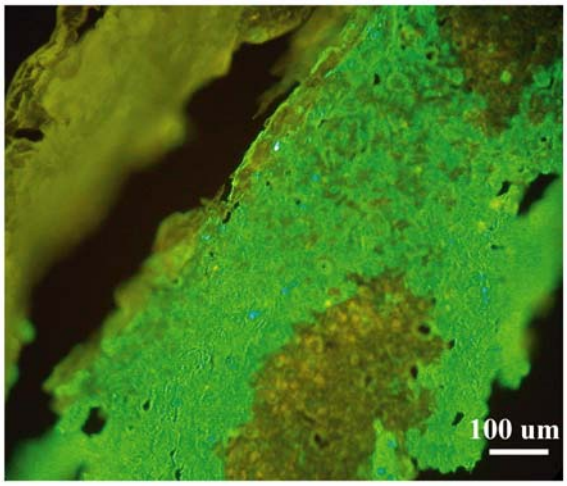
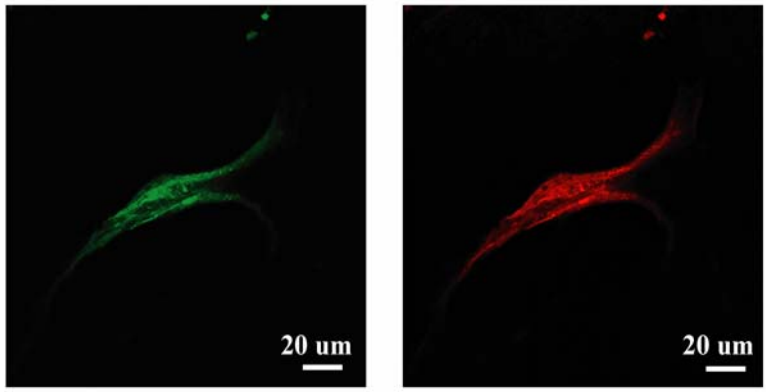


Figure 34*: Fluorescence image of CXCR4-GFP-IRES transduced BM derived stem cells detected in the bone marrow of lethally irradiated mice 5 weeks post-injection. High reconstitution of the bone marrow by CXCR4-IRES-GFP transduced BM derived stem cells was achieved after 5 weeks.*

Retroviral CXCR3 Transduction of BM derived stem cells

Similarly, the EcoPack 293 and RetroPack PT 67 cell lines were generated using CXCR3-IRES-GFP vector. The supernatant derived from stably transduced PT 67 cell line was used for transduction of BM derived stem cells (Figure 35).



A **B**

Figure 35: *Fluorescence images of CXCR3 transduced cells. GFP fluorescence of tranduced cells (A) and Cy3 immunostaining for CXCR3 receptor (B) in transduced BM stem cells.*

BM stem cells were cultured under stem cell expansion conditions 3 weeks prior transduction. The transduction efficiency of BM cells was 40±5%; non-transduced BM stem cells do not show CXCR3 receptor expression (Figure 36).

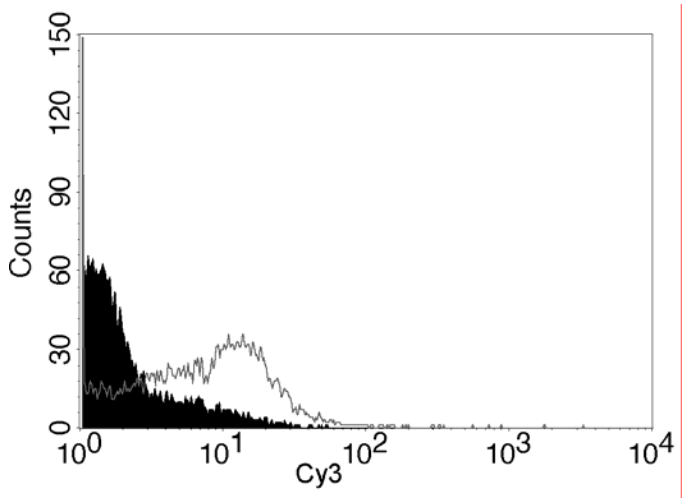


Figure 36*: Flow cytometry results of CXCR3-IRES-GFP transduced BM stem cells. Transduction efficiency was detected to be 40±5%. Isotype control is shown as filled black histogram.*

Injection of CXCR3 expressing BM stem cells into EAE mice

CXCR3 transduced BM derived cells were used in EAE animal models for multiple sclerosis to investigate its possible therapeutic effect. Cell injection was performed at the early onset of the disease, 10 days post-immunization of the mice using MOG emulsified in CFA and Pertussis Toxin. Immunized mice were injected with 0.5 million CXCR3-IRES-GFP transduced or IRES-GFP transduced BM stem cells and controls were injected with PBS. Clinical score of the mice were followed for 10-13 days post injection. Figure 37 (n=3) shows that in PBS injected controls, the disease showed a severe progression reaching up to score 4. Injection of GFP transduced BM cells did not prevent the progression of the disease for the first 7 days post-injection, however, it showed a subsequent amelioration of the symptoms. On the other hand, injection of CXCR3 expressing cells prevent the progression of the disease above score 2.

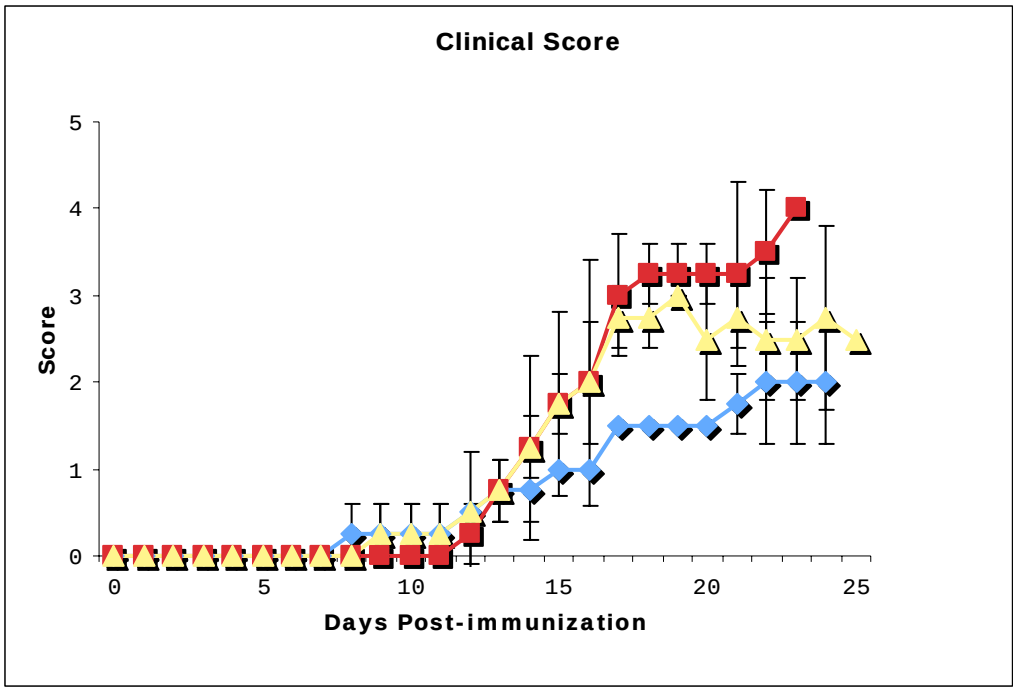


***Figure 37**: Clinical scores of EAE immunized mice showing progression of the disease after injection with PBS as a control (red), BM stem cells transduced with IRES-GFP (yellow) and CXCR3-IRES-GFP transduced BM stem cells (blue) 10 days post-immunization (n=3).*

13 days post-injection, the EAE mice were sacrificed and analyzed for migration of the GFP cells in CNS tissues. A low number of cells were detected in the brain and

the spinal cord of EAE mice injected with CXCR3 expressing BM stem cells (Figure 38).

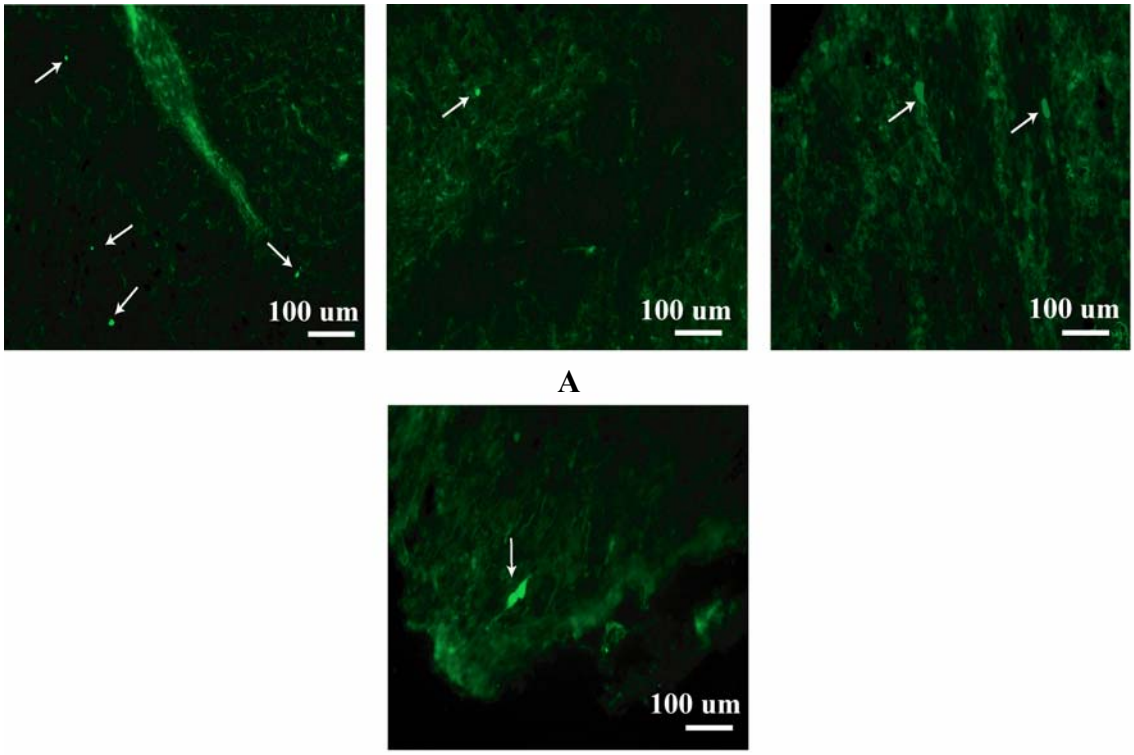


Figure 38: *Fluorescence images from brain (A) and spinal cord (B) of EAE mice injected with CXCR3-IRES-GFP transduced BM derived stem cells 10 days post-immunization. Low number of cells was detected in CNS tissues.*

DISCUSSION

Characterization of Bone Marrow Derived Stem Cells

Bone Marrow- A Home for Non-Hematopoietic Stem Cells

Bone marrow is the largest organ in the body. It is known as an enriched resource for adult stem cells. Various stem cells, progenitor cells, stromal and blood-forming cells reside within the BM. HSCs are the most studied population of adult stem cells so far. Therefore many markers have been identified, which makes these cells easy to isolate from the bone marrow. MSCs, the non-hematopoietic stem cells within the bone marrow, do not have characteristic surface markers. This makes them difficult to isolate and to obtain homogenous cultures. Many culture conditions for the enrichment of non-hematopoietic stem cells were described. In our study, the medium conditions have been modified from Jiang et al, 2002. Removal of many erythrocytes was achieved shortly after extraction of BM via standard hypotonic lysis buffer. The remaining cells were seeded on fibronectin coated dishes. BM contains HSCs that give rise to all lineages of hematopoietic system and MSCs that constitutes the adhesive part of the BM derived cells. Major part of HSCs was removed during subsequent medium changes since they grow in suspension. The adherent cells, however, still contain many different cell types (Figure 16). Flow cytometry analysis revealed cell population expressing markers like c-Kit (13±1%), Sca1 (31±2%), CXCR4 (15±2%) indicating the presence of some HSCs after 7-8 passages in cultures. The expression of CD11b marker by a large fraction of the cells (45±3.5%) indicates a large macrophage population (Figure 17). Stem cell adhesion molecules CD29 (20±2%) and CD49d (13±0.5%), which are important for the communication between stromal and stem cells, confirms also the presence of stromal cells within the cultures. On the other hand, 44±3% of the population was expressing stage specific embryonic antigen 1. SSEA-1 antigen is associated with embryonic stem cells. The expression is restricted to undifferentiated embryonic stem cells and primordial germ cells. The detection of this surface marker suggests the presence of another stem cell population beside HSCs within the BM culture. Sorted SSEA-1 positive population, however, did not proliferate when seeded on fibronectin coated dishes, which shows that they do not grow in the absence of supportive environment provided by the heterogeneous culture. Stem cells are “hidden” in special stem cell niches in the bone marrow. There

is a continuous interaction between the specialized niche cells and the stem cells. Molecular signalling, although still unknown, provides necessary conditions for maintenance of the stemness of these cells and their proliferation in case of injury. The amount of stem cells within the niche is kept constant. Their proliferation is only restricted to the need, allowing the protection against a continuous proliferation that might lead to cancer. The cells closer to the niche borders differentiate in response to the differentiation stimuli in the periphery. The difficulty of expanding SSEA-1 positive cells out of their "niches" is possibly related to this mechanism. This can show that stem cells need the interaction between different progenitor and stromal cells for their survival and proliferation. Understanding the molecular signalling between the niche cells and the resident stem cells will provide a big advantage for the expansion of BM derived stem cells *in vitro*.

Beside SSEA-1, we analyzed the expression of other pluripotency markers (Oct4, Nanog and Rex1) proteins exclusively expressed by embryonic stem cells. RT-PCR revealed a significant expression of Oct4 and Rex1 transcription factors and a weak Nanog expression in BM derived population after 7 passages in culture (Figure 18). Immunostaining for Oct4 transcription factor showed that small cells (diameter of 10-15 um) with a high nuclear to cytoplasm ratio, which is a common morphology of stem cells, express Oct4 (Figure 19). In various adult stem cells which have been studied so far, no detection of pluripotency markers like Oct4, Nanog, Rex1, and SSEA-1 was reported. In freshly isolated bone marrow we could not detect any of these markers. This might suggest either that the cells gain this phenotype as a result of *in vitro* culture conditions, or this culture conditions enrich the low number of multipotent stem cells which initially is undetectable. Recently, however, similar findings were reported regarding BM population. Jiang and his coworkers (Jiang et al., 2002a) were able to obtain a homogeneous BM stem cell population which they named MAPCs. MAPCs were expressing the above mentioned pluripotency markers at a very low relative concentration (1000 fold less compared with ES cell expression levels).

BM derived stem cells can show differentiation towards neurogenic, adipogenic and osteogenic lineages *in vitro*

Differentiation of bone marrow derived non-hematopoietic stem cells to multiple lineages have been studied for a long time. It has been widely accepted that bone marrow cells can differentiate to adipogenic, osteogenic and chondrogenic lineages. However, in the last 6 years, there has been accumulating evidence demonstrating the high plasticity of the BM from hepatocytes to neurons. Freshly isolated BM cells have been found to migrate to several brain regions and to adopt a neuronal phenotype after transplantation to irradiated mice (Mezey et al., 2000).

To investigate the plasticity of BM derived stem cell population, *in vitro* differentiation studies were performed. BM cells in stem cell expansion medium initiate a Nestin expression, which is an early neural marker. It is known that low serum conditions can initiate Nestin expression in BM cells (Wislet-Gendebien et al., 2003). Neuronal differentiation medium, containing FGF2 and Shh promoted neural differentiation and process outgrowth of BM stem cells, enhanced further the expression of Nestin and β-III tubulin as detected by RT-PCR (Figure 20B). Cells labeled for β-III tubulin show elongated processes resembling neural outgrowth (Figure 20A).

Differentiation of BM stem cells to adipocytes was performed using horse serum and dexamethasone. High cortisol content in horse serum has a positive effect on adipocyte differentiation. Dexamethasone in the absence of horse serum leads to differentiation to the bone lineages; however, in the presence of horse serum, it enhanced the differentiation and proliferation of adipocytes. Oil red O staining shows the oil droplets formed within adipocytes 2 weeks after differentiation (Figure 21A).

Osteogenic differentiation of BM stem cells was achieved using ascorbic acid and dexamethasone supplements. 14 days of differentiation induced calcium phosphate deposits formation, which was detected with Alizarin staining (Figure 21B). *In vitro* differentiation of BM derived stem cells to neurogenic, adipogenic and osteogenic lineages confirms an existence of multipotent stem cell population within BM derived cells which have a plasticity allowing them to respond to the external stimuli and program their differentiation.

Further studies involving appropriate *in vivo* experiments are in progress in order to explore the functional properties of these cells. Another point that needs exploration is whether multipotent stem cells(the most primitive source in adult organism) are a

very small fraction in the BM and have high differentiation capacity, or BM is a pool for all kind of progenitor cells that circulate and in case of injury they migrate to the damaged tissue and they differentiate. Since we are still dealing with heterogeneous BM cultures this question can have an answer once the different populations residing in the BM are investigated more in detail.

BM derived stem cells contribute in vivo to multiple embryonic tissues

Aggregation of ES cells with morulae (8-cell stage embryo) and chimera generation experiments are widely used for testing the pluripotent properties of ES cells. The higher the plasticity of the cells aggregated with morulae, the higher is the rate of contribution of these cells to different embryonic tissues. A similar experiment was performed to test the plasticity of BM derived stem cells *in vivo*. The contribution of BM derived stem cells into different embryonic tissues confirmed the multipotency of the stem cells residing in the bone marrow (Figure 22). GFP labeled BM stem cells contributed to tissues from mesodermal (Figure 22A) and ectodermal (Figure 22B, C) origin. The overall contribution to the whole embryo was about 5%. The low contribution of these cells can be associated with the heterogeneous population obtained under *in vitro* conditions. BM cultures cultured in defined medium conditions still contain many stromal and progenitor cells together with a very low amount of multipotent stem cells. Under these conditions, the aggregation experiments which require 10-12 BM cells, per embryo exhibited a low percentage of chimera generation.

In vitro differentiation experiments and chimera experiments confirmed the presence of a non-hematopoietic stem cell population derived from the BM that exhibit high plasticity.

Bone marrow harbor a very low number of "real" non-hematopoietic stem cell population

In order to select the stem cell population that express Oct4 transcription factor, we used a transgenic mouse model generated via microinjection of a transgene, which has GFP driven under Oct4 promoter, to the pronuclei of a fertilized oocyte. Extensive studies on the transgenic mouse confirmed that the GFP expression is only detected in ES cells derived from blastocyst embryos of the Oct4 transgenic mice (Ying et al., 2002).

In early cultures (4th passage) in stem cell expansion medium, we observed very low number (<0.5% /mouse BM) of GFP positive clusters (Figure 23A). We did not detect any GFP signal from freshly isolated BM by flow cytometry analysis. We note that fresh BM exhibits a high autofluorescence background; dealing with low cell number, the GFP fluorescence of these cells may be undetectable. However, *in vitro* culture conditions were clearly selective for these GFP positive cells. Mechanical isolation and real time PCR analysis for Oct4 and Nanog gene expression revealed very high levels of expression. In the clusters Oct 4 expression was nearly 30 fold less, and Nanog expression nearly 10 fold less than ES cell expression levels (Figure 23B). This finding confirms our previous findings that BM may harbor non-hematopoietic stem cells with high plasticity. The only report regarding Oct4 and Nanog expression profile of BM cells (Jiang et al., 2002a) shows a 1000 fold lower expression of Oct4 and Nanog in MAPCs in comparison to ES cell expression levels. Detected very high Oct4 and Nanog expression levels in our study suggests that our culturing conditions supported with the Oct4GiP reporter system supports the enrichment of more homogeneous population from the BM. We went further with expansion of these clusters; however, their low proliferation brought a major limitation for our further analysis. It is known that the quiescence of stem cells in the bone marrow is of critical importance in order to keep a constant stem cell number and to avoid their extensive proliferation that could lead to cancer. Their proliferation *in vivo* is stimulated according to the demand in the injured area. We used several approaches in order to expand the multipotent stem cells *in vitro*.

TAT-Nanog protein transduction induced cell proliferation in expense of Oct4 expression

Recombinant Nanog protein transduction was one of the methods which we used in order to expand GFP positive BM clusters (Figure 24). It has been reported that elevated Nanog expression is sufficient for clonal expansion of ES cells maintaining their Oct4 expression (Chambers et al., 2003). Application of Nanog protein for 2 weeks induced cell proliferation. Cluster formation rate was higher than the ones in stem cell medium; however, the GFP signal within the clusters was relatively low (Figure 25) in comparison to clusters formed in expansion medium (Figure 23A). Although there was induction in Sox2, a pluripotency marker, the GFP was not detectable after 2 weeks (4 passages) of Nanog application. This results shows that

Nanog transduction may induce cell proliferation, however at the detriment of Oct4 expression. This might indicate that Nanog *per se* is not enough for the maintenance and proliferation of BM multipotent stem cells. Adult stem cells differ from ES cells in differential gene expression. The chromatin structure of ES cells is much loose. The gene loci are easily accessible for the regulatory molecules. However, in various adult stem cells many gene loci have been described to be highly methylated and inaccessible to the transcription factors. In our particular example, Nanog transcription factor applied to the bone marrow derived stem cells certainly activates the cell proliferation cascade; however, some other unidentified downstream targets of Nanog closely related with the maintenance of "stemness" in the cell (like Oct4 expression) might be inaccessible due to excessive methylation. In a recent study it was reported about the induction of pluripotency in embryonic and adult mouse fibroblasts (Takahashi and Yamanaka, 2006). Combinatorial overexpression experiments were performed either alone or in combination of different pluripotency genes and oncogenes. The interesting finding was that from 24 different genes only 4 of them (Oct4, Sox-2 transcription factors, c-Myc (oncogene), and Klf4 (tumor suppressor) genes) have been described as being the genes sufficient for induction of pluripotency in adult cells. Nanog, on the other hand, was not sufficient to induce the pluripotency of adult cells.

Since the cells used in our experiment are neither fibroblasts nor ES cells, we do not know whether or not Nanog has an effect in pluripotency induction. However, it is also clear that Nanog alone is not enough for maintenance of pluripotency gene expression in our culture system. For this reason, we suggest that other signalling pathways in combination with Nanog may help for maintenance and proliferation of multi- or pluripotent stem cell population derived from the bone marrow. Oct4 recombinant protein might be another factor that can be used in combination with Nanog protein in BM cultures for the enrichment of multipotent stem cell colonies.

FGF2 and SCF induce early Oct4 and Nanog expression in BM derived stem cells

Primordial germ cells (PGCs) are detectable in embryos at E7. Once isolated this cells proliferate for up to 7 days *in vitro*, but their number eventually declines and their proliferative capacity is only a fraction of that seen *in vivo*. It has been reported previously (Resnick et al., 1992) that in order to enrich the pluripotent PCG

population, SCF (also known as steel factor), FGF2 and LIF were supplemented to their culture medium. This composition further enhanced their proliferation and stimulated the derivation of embryonic germ (EG) cells which has similar characteristics to ES cells. In parallel to this study, Durcova-Hills and her coworkers (Durcova-Hills et al., 2006) reported that PGC reprogramming towards EG cells can be induced via exogenous FGF2 addition for not more than 24 hours in the presence of LIF. In the light of these studies we used exogenous FGF2 and SCF supplements in our stem cell expansion medium containing LIF. The application for 24 hours did not show any effect, however 72 hours after application the cells showed an expression of Oct4 (Figure 26). FGF2 application induced an early expression of pluripotency factors (Figure 26C). The clusters formed were smaller in size however with a high GFP signal (Figure 26B). The cultures were maintained up to 8 passages.

It has been known that FGF2 is important for maintenance of pluripotency in human ES cells (Dvorak et al., 2005). A recent report brings a closer look to the FGF2 signalling (Greber et al., 2006). They suggest a regulatory circuit for the maintenance of pluripotency in hES cells where FGF2 is the most upstream self-renewal factor. Exogenous signalling of FGF2 sustains Oct4, Nanog and Sox2 expression via upregulating TGFβ1, Inhba (Activin A), Grem1, down regulates BMP4 genes and consequently activates the endogenous expression in human ES cells. TGFβ1 and Activin A prevent differentiation via activation of SMAD 2/3 (James et al., 2005;Vallier et al., 2005), while BMP4 initiates trophoblast and primitive endoderm differentiation via SMAD 1/5/8 signalling (Xu et al., 2002;Pera et al., 2004). On the other hand Gremlin1 is a BMP4 antagonist which is upregulated at high FGF2 levels.

FGF2 studies conclude that at defined concentrations it has a crucial role in maintenance of pluripotency in human ES cells and initiation of pluripotency in PGCs. This may clearly explain the early initiation of Oct4 expression in BM derived stem cell culture after 72 hours of FGF2 application. Short term maintenance of Oct4 expression, on the other hand, suggests the involvement of other signalling pathways necessary for the maintenance of adult stem cell plasticity.

Due to their high plasticity, ES cells offer a great promise in regenerative medicine; however, the ethical and serious clinical problems limit their application. On the other hand, there is an accumulating evidence regarding the multilineage potential of adult stem cells. Moreover, adult stem cells can be reprogrammed to embryonic-like cells via nuclear transfer to oocyte or fusion with ES cells. These methods, however, have

major disadvantages. Our findings suggest existence of a BM stem cell population with very similar characteristics to ES cells. Discovering the critical factors involved in epigenetic reprogramming may allow the derivation of pluripotent adult stem cells. Providing the advantages and lacking the limiting factors of ES cell properties, adult stem cells might open a new road in stem cell therapies.

Bone Marrow Derived Stem Cells in Cell Therapy Application

Specific targeting of therapeutical proteins is a crucial for a successful gene therapy. Since chemokines are the key regulators in cell migration, we have focused on organ specific targeting of BM derived stem cells by gene transfer of chemokine receptors.

Transfection and Transduction using Retroviral System

pMSCV, a retroviral plasmid having murine stem cell vector background was used for construction of the vectors. pMSCV-CXCR3(or CXCR4)-IRES-GFP vector encodes murine CXCR3 or CXCR4 receptor- IRES-GFP (Figure 13). This is a bicistronic vector in which both CXCR3(or CXCR4) and GFP are constitutively expressed through a specifically designed 5' LTR from murine stem cell virus. IRES of the encephalomyocarditis virus (ECMV) permits the translation of two open reading frames from one messenger RNA. Ribosomes can enter the bicistronic mRNA either at the 5' end to translate the gene of interest (CXCR3 or CXCR4) or at the ECMV IRES to translate the GFP.

The envelope protein made by packaging cell line determines the infectivity (tropism) of the virus. Ecotropic viruses, produced by EcoPack 293 cell lines, can recognize EcoR receptor on mouse and rat cells. PT 67 cells on the other hand, produce high titer dualtropic viruses which recognize both Ram-1 and EcoR receptors and can infect broad range of mammalian cells.

Cells successfully transduced with CXCR3 (or CXCR4)-IRES-GFP, shows clearly a CXCR4 membrane staining while GFP is localized in the cytoplasm (Figure 28 and 35). This expression system allows an expression of the desired gene and the reporter gene (i.e. GFP) without any alteration in the function of the gene of interest which is highly advantageous to fusion protein expression systems. In fusion protein approach two genes, the gene of interest and the reporter gene are linked together. Transcription of the fusion gene occurs under the control of definable upstream promoter/enhancer elements and the fusion protein is a single hybrid of two individual proteins: the protein of interest and the reporter protein. Thus, information obtained by imaging the

reporter component will provide information about the gene of interest (Hackman et al., 2002). However, fusion construct may not produce a functional product, the fused protein may interfere with conformational structure of the protein of interest resulting in alteration of the function or localization of the protein (Kien et al., 2003;Blasberg, 2003;Hart and Tarendeau, 2006)

CXCR4-IRES-GFP transduced cells express functional CXCR4 receptor

Functional analysis was performed in order to verify the CXCR4 activity. According to Figure 30, GFP transduced cells, showed a consistent migration pattern at different SDF-1 concentrations. On the other hand, the CXCR4 expressing BM cells showed a linear increase in migration towards elevating SDF-1 concentrations. 8 fold increase was observed in migration of the CXCR4 transduced cells at SDF-1 concentrations between 0-30 ng/ml, however there was a sharp decline in migration of these cells in concentrations ≥50 ng/ml. Decreased migration can be associated with high SDF-1 levels. In mice bone marrow 50 ng/ml fells in a range of non physiological concentration (Petit et al., 2002). It is known that high levels of SDF-1 can negatively regulate the production and expression of the receptor in the cell membrane. In high SDF-1 conditions cells expressing CXCR4 are known to either internalize their receptors or desensitize them towards their ligand (McGrath et al., 1999;Ansel and Cyster, 2001), which results to a reduced migration of BM stem cells towards SDF-1.

CXCR4 transduced BM derived stem cells can be successfully targeted in vivo towards high SDF-1 expressing organs

Targeting potential of receptor expressing BM cells was investigated via injection to lethally irradiated mice.

Irradiation damages stem cells and their microenvironment (Di et al., 1994) causes damage to the cellular plasma membrane, cytoplasmic organelles, and DNA synthesis and repair, partially due to free-radical generation (Harper et al., 1997;Nikkels et al., 1987). Primitive hematopoietic stem cells, responsible for life-long marrow stem cell reconstitution, are most significantly impacted by irradiation (Down et al., 1995). Jacobsen et al (JACOBSON et al., 1951) and Lorenz et al (LORENZ et al., 1951) have shown that transplantation of hematopoietic tissues is protective against lethal doses of radiation.

Nowadays, irradiation is a common method used in cancer therapy. The primary reason for that is to eliminate malignant cells since actively dividing cells are the most sensitive to the DNA-damaging agents which also leads to the elimination of host hematopoietic cells. On the other hand, in bone marrow transplantation studies the replacement of the stem cells is more effective via irradiation, since elimination of host HSCs creates a space in the marrow niches which improves the engraftment of healthy donor marrow cells (Ponomaryov et al., 2000). Irradiation also prevents the host immune response toward the graft (Weissman, 2000;Moore, 1999), and significantly increases IL-1a, IL-6, and TNF-α mRNA levels in mouse BM and spleen (Chang et al., 1997). It has been shown by Ponomaryov et al (Ponomaryov et al., 2000) that expression levels of SDF-1 increases in the bone marrow following irradiation, which unable the stem cell homing after transplantation.

A similar method could be used to target a renewable reservoir of genetically modified cells for gene therapy. BM derived stem cell reported in this study are an interesting population, since they contain multipotent stem cell that can be maintained in culture for up to 20 passages sustaining their multipotency and allowing adequate gene transfers.

Following irradiation, within 24h we injected mice with CXCR4-IRES-GFP transduced BM stem cells in assistance with freshly isolated HSCs selected for lin-cKit+Sca1+. Since injected BM stem cells do not have a hematopoietic origin, the mice need an immediate supply for HSCs due to the depletion of HSCs in the BM upon irradiation. Control mice were injected with only GFP transduced stem cells together with freshly isolated HSCs. Coinjection of transduced BM derived stem cells and HSCs might induce a competition in homing between these two types of stem cells. Cells with increased CXCR4 expression will dominantly populate the regions with high SDF-1 expression (Kahn et al., 2004;Brenner et al., 2004).

Migration to the lung, spleen and the BM was observed in CXCR4 transduced stem cell injected mice 3 weeks post-injection (Figure 31) CXCR4 expressing cells mainly migrated towards the BM due to high SDF-1 levels, however, low number of cells were detected in lung and spleen. It is not clear whether the cells detected in the spleen migrated directly after injection (since the cells can be attracted from the mixed chemokine pool of the spleen during circulation) or they were directed later from the bone marrow. Figure 33 shows around 3% of contribution of CXCR4-IRES-GFP transduced cells to the whole spleen. Very low migration to the lung reflects mainly a

generalized migration, due to the long circulation of the stem cells in the body, rather that a specific targeted migration. Interestingly the cells which migrated to the bone marrow localized in the endosteum, known as the stem cell niche where previously have been shown that the most primitive HSCs are localized (Wilson and Trumpp, 2006). Higher expression of SDF-1 is detected in these areas by immature osteoblasts (Ponomaryov et al., 2000). It is therefore possible that these stem cells expressing high levels of CXCR4 show high migration potential towards SDF-1 and home in the endosteum region (Figure 31A). More than 50% of the cells within the entire endosteum were GFP positive which indicates the preferential engraftment of CXCR4 transduced BM stem cells to the coinjected HSCs (Figure 32). We can see a striking difference between the bone marrow analyzed 3 weeks after and 5 weeks after injection with CXCR4-IRES-GFP transduced stem cells. 5 weeks post-injection the total BM is reconstituted via GFP positive CXCR4 expressing stem cells, which indicates a persistent engraftment of the cells (Figure 34).

Control mice injected with only GFP transduced stem cells together with HSCs did not show any migration towards the bone marrow. This is not surprising since lacking the CXCR4 expression they do not respond to the SDF-1 gradient. Low migration, however, was detected in the spleen (Figure 33) which suggests the presence of low number of CXCR4 expressing cells remaining in the BM cultures (Figure 17). The CXCR4 expression is mainly associated with HSCs/HPCs in the bone marrow. It is not easy however the maintenance of this population ex vivo. Prior to transduction, BM cultures have been expanded for up to 7-8 passages in vitro, which may suggest that culturing under LIF conditions expanded the ex vivo lifetime of HSCs/HPCs matching the findings of Shih et al (Shih et al., 2000). These cells however did not migrate to the bone marrow. The level of CXCR4 expression in those cells differs very much from those transduced with CXCR4. There is an upregulated CXCR4 expression in transduced cells while the other cells produce native levels of CXCR4, which can explain their lower migratory potential to the bone marrow. Furthermore, being low in number they hardly can compete with coinjected freshly isolated HSCs for BM homing.

Findings from CXCR4 experiments have shown that we can use genetically engineered BM derived stem cells and express CXCR4 receptor to successfully target tissues where its ligand SDF-1 is highly expressed. One possible application is ischemic model. Ischemia induces mobilization of bone marrow progenitors both in

animal models and in humans (Takahashi et al., 1999;Gill et al., 2001;Murayama et al., 2002). Some of the molecules involved in mobilization of bone marrow progenitors to the circulation are VEGF, PDGF, SCF, G-CSF, eNOS (De et al., 2004). SDF-1 is involved in mobilization of stem cells from the bone marrow. G-CSF-induced mobilization of hematopoietic progenitor cells requires a reduction of SDF-1 expression in the BM and elevated SDF-1 levels in the plasma and enhancement of CXCR4 expression on the HPCs. Recently, a possible role for SDF-1 in homing of stem cells to damaged sites has been reported by studies in animal models of liver, limb and heart damage (Yamaguchi et al., 2003;Ceradini et al., 2004;Kollet et al., 2003). Ceradini and coworkers showed that SDF-1 gene expression is regulated by the transcription factor HIF-1 in endothelial cells, resulting in selective in vivo expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tension. HIF-1-induced SDF-1 expression increases the adhesion, migration and homing of circulating CXCR4-positive progenitor cells to ischemic tissue.

In parallel to these studies, our findings suggest that CXCR4 transduced BM derived stem cells are good candidates for cell therapy in ischemic models, being able to target them specifically to the ischemic tissues. Moreover, they can be further engineered in order to deliver genes the production of which can enhance the regeneration process in damaged tissues.

CXCR3 transduced BM derived stem cells injected to EAE mice cause amelioration of the disease symptoms

EAE is the murine model for multiple sclerosis. It is an autoimmune disease where the target antigen for T cells is the myelin peptide found in the myelin sheath wrapping the neuronal axons. The disease begins with infiltration of cytotoxic T cells into the CNS. They recognize the myelin protein as “non-self” and induce an immune reaction which leads to more T cell infiltration and severe immune reaction. It is not known whether chemokines are involved during the first cytotoxic T cell infiltration; however, it is well known that chemokine signalling is one of the key regulatory elements involved in the progression of the disease. Once the reactive T cells recognize the myelin protein, they reside in the CNS and initiate a secretion of pro-inflammatory cytokines like TNF-α and IFN-γ. These cytokines upregulate the production of chemokines IP-10, Mig and Rantes. They are involved in infiltration of many other reactive lymphocytes through the already disrupted blood brain barrier.

Many studies have focused on therapies via treatment with neutralizing antibodies against chemokines CCL2 (Kennedy et al., 1998), CCL3 and CXCL10 (Karpus et al., 1995). Modified chemokines were reported to be used successfully in EAE in order to interfere with the signalling pathway of these chemokines (Gong et al., 1996).

We investigated the therapeutic potential of bone marrow derived stem cells targeted to the CNS using retroviral expression of the chemokine receptor CXCR3.

CXCR3 transduced BM derived cells were used for injection into EAE mice. BM stem cells transduced with IRES-GFP, the same vector used in CXCR4 experiments, were used as a control to test CXCR3 migratory effect. PBS was used as a general control. The cells were injected 10 days post induction in the early onset of the disease and the clinical score was followed up to 15 days post-injection. As followed in Figure 37, PBS injected control mice had a severe progress in the disease score reaching score 4, while mice injected with only GFP expressing BM stem cells, showed a score progression up to 3 until the day 17 post-immunization, however there was a clear decline in the score afterwards. On the other hand, injection of CXCR3 expressing BM stem cells evaded the progression of the disease further than score 2. Analysis of CNS tissues revealed a low migration of BM derived stem cells transduced with CXCR3 to the brain and spinal cord (Figure 38). This can be due to low cell number injection or the inability of the cells (due to their size or lack of necessary adhesion molecules) to cross the blood brain barrier. The low migration efficiency suggests that CXCR3 targeting to the CNS tissues maybe not the optimal strategy for a gene delivery. Nevertheless, this finding indicates that BM derived stem cells are involved in the amelioration of EAE symptoms not via direct migration to the CNS tissues, but they might rather show a paracrine effect. They may secrete certain cytokines that reduce the further progress of the disease. On the other hand, the results obtained by injection of CXCR3 transduced BM cells show that they may lead to better engraftment and targeting to the closer regions in CNS tissues, which could enhance the paracrine effect of BM derived stem cells. The findings are in line with the extensive work of Zappia et al (Zappia et al., 2005), where they inject MSCs into EAE mice and they observe the reduced clinical scores. They observed migration of the cells mainly within the lymphoid organs, concluding that MSCs might have a paracrine effect which causes the amelioration of disease symptoms. Our data shows consistence as well with the work of Zhang et al (Zhang et al., 2005), where they injected human bone marrow stromal cells into EAE mice and detected a significant

decrease in the maximal clinical score of cell transplanted mice in comparison to the PBS injected ones. They observed significant reduction in demyelination and reduction of inflammatory infiltrates.

Not many studies have been reported about BM stem cell based therapies in EAE. However, BM derived cells have an ascending potential in cell therapies. According to our preliminary findings we suggest that BM derived stem cells have a potential to be used in EAE therapies. Furthermore, CXCR3 expression seems to enhance the positive effect of BM stem cells in moderating the EAE symptoms, possibly due to better engraftment and targeting to the closer regions to CNS tissue which still needs to be confirmed. Accordingly we suggest that the therapeutical effect of BM derived stem cells can be further improved via genetically engineering them to express anti-inflammatory cytokines and be used as a gene delivery tool in EAE disease therapies.

Genetic manipulation of BM stem cells using chemokine receptors or similar surface markers can improve gene delivery efficiency via successful targeting of stem cells to the damaged area. In the light of this study, we suggest that BM derived stem cells can be isolated and expanded easily in culture allowing their genetic manipulation which makes them a powerful tool in gene therapy applications.

SUMMARY

Despite of having a short history, stem cell research had made a remarkable progress. ES cells are pluripotent stem cells which have unrestricted capacity of differentiation into all cell lineages. This makes them a particularly attractive choice for cell therapy applications. However, therapies involving ES cell application are facing certain ethical (destroying embryos) and clinical (tumor formation) difficulties. Therefore, scientists have focused on the search for possible "pluri- or multipotent" cells in various adult tissues. MSCs derived from the bone marrow were first described by Friedenstein and co-workers (Friedenstein, 1976). They demonstrated that a small fraction of the bone marrow derived cells has the capacity to adhere to the tissue culture dish and that these cells can be differentiated both *in vitro* and *in vivo* into osteoblasts, chondrocytes and adipocytes. Recent works on the "plasticity" of adult stem cells, suggested that various adult stem cells, previously accepted as tissue committed stem cells, may cross the borders and differentiate to a much wider spectrum of cell types when cultured under defined conditions (Wagers and Weissman, 2004). Lately discovered adult progenitor cells in the bone marrow (Jiang et al., 2002a) were found to express some important embryonic genes: Oct4 and Nanog. Furthermore, they were shown to have the ability to contribute to tissues of all three germ layers in chimera generation experiments.

Our study was mainly based on culture induced reprogramming. Bone marrow cells cultured in a medium supplemented with various growth factors like LIF and PDGF generated a stem cell culture with high plasticity. Plasticity of the stem cell population was confirmed using *in vitro* differentiation and *in vivo* aggregation with morulae. Furthermore, we detected and analyzed the high Oct4 and Nanog expressing stem cell population in the heterogeneous BM culture. Real-time PCR data revealed 30 fold less Oct4 and 10 fold less Nanog expression in comparison to ES cell expression levels. These high Oct4, Nanog expressing stem cell clusters were mechanically isolated and cultured with Nanog or FGF2 supplemented medium in order to expand them *in vitro*.

Success of gene therapy strongly depends on organ or site specific production of therapeutical proteins. The identification of the molecular cues important for cell migration is necessary to understand the developmental mechanism and to develop

new therapy approaches. Bone marrow derived mesenchymal stem cells are an accessible source for a cellular vehicle in targeted gene therapy.

In our study we asked whether it is possible to use trafficking properties of chemokine receptors in order to achieve a targeted migration using BM stem cells *in vivo* for cell therapy approach. We investigated the migratory effect of CXCR4 transduced BM derived stem cell in different tissues after injection in irradiated mice, to study their contribution in reconstitution of hematopoietic system. CXCR4 receptor was expressed in murine bone marrow derived mesenchymal cells by gene transfer with retroviral vector. Cells transduced with CXCR4 or with a GFP control retroviral vector were injected intravenously into lethally irradiated mice. At 3-5 weeks after injection CXCR4 transduced GFP+ cells were detected mostly in the bone marrow, some were detected in lung and spleen, and after 5 weeks a high reconstitution of the BM by CXCR4 transduced GFP+ cells was detect. The results demonstrate that gene transfer of chemokine receptors can target BM derived stem cells into the bone marrow opening new avenues for tissue specific gene delivery.

In parallel, we used the chemokine-chemokine receptor driving force as an alternative approach in therapy for EAE, a murine disease model for multiple sclerosis (MS). CXCR3 receptor, whose ligands were known to be upregulated during the onset of the disease, was expressed in BM derived stem cells via retroviral transduction and BM cells were targeted to the EAE lesion sites. Low cell migration was detected in the CNS lesion sites; more striking was the effect in amelioration of the symptoms during the disease score. This result suggests that BM stem cell delivery may improve the disease symptoms in EAE via a paracrine effect, such that they might secrete factors having an immunosuppressive effect on T-cell induced inflammation in EAE.

The multilineage potential of BM derived stem cells, their ability to elude detection by the host's immune system, and their relative ease of expansion in culture, make these stem cells a very promising source for cell therapy applications.

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This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

Winston Churchill (1874-1965)

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