Plasticity of Murine Bone Marrow-Derived Adult Stem Cells: acquisition of specialized properties and contribution to embryonic development

Dissertation

zur Erlangung des akademischen Grades Dr. rer. nat.

vorgelegt der

Mathematisch-Naturwissenschaftlich-Technischen Fakultät

(mathematisch-naturwissenschaftlicher Bereich)

der Martin-Luther-Universität Halle-Wittenberg

von Fikru Belema Bedada

geb. am: 30.09.1971 in: Dillela west shoa, Ethiopia

Gutachter: Prof. Dr. Thomas Braun

Datum der Verteidigung: 01.11.2006, Halle (Saale)

urn:nbn:de:gbv:3-000011444
[http://nbn-resolving.de/urn/resolver.pl?urn=nbn%3Ade%3Agbv%3A3-000011444]

1 SUMMARY 5

2 INTRODUCTION 7

2.1 STEM CELLS 7
2.2 EMBRYONIC STEM (ES) CELLS 8
2.3 EMBRYONIC CARCINOMA (EC) CELLS 9
2.4 EMBRYONIC GERM (EG) CELLS 10
2.5 POSTNATAL OR SOMATIC ADULT STEM CELLS (ASCS) 11
2.6 LOCATION AND FUNCTION OF ADULT STEM CELLS 12
2.7 TRACKING ADULT STEM CELLS 13
2.8 DIFFERENTIATION OF ADULT STEM CELLS INTO SPECIFIC TISSUES 13
2.9 PLASTICITY OR TRANS-DIFFERENTIATION OF ADULT STEM CELLS 14
2.10 CRITICAL ISSUES TO BE ADDRESSED ABOUT ADULT STEM CELLS 16
2.11 SIMILARITIES AND DIFFERENCES BETWEEN EMBRYONIC AND ADULT STEM CELLS 17
2.12 HAEMATOPOIETIC STEM CELLS 17
2.13 MESENCHYMAL STEM CELLS 19
2.14 ANOTHER TYPE OF ADULT STEM CELLS: SP CELLS 20
2.15 HEART-DERIVED SCA-1^{+} ADULT STEM CELLS, H-ASCS 20
2.16 MUSCLE-DERIVED ADULT STEM CELLS, MS-ASCS 21
2.17 STEM CELLS MARKERS 21
2.17.1 Embryonic Stem (ES) Cells markers 22
2.17.2 Haematopoietic and other Stem Cells markers 23
2.17.3 Mesenchymal/Stromal Stem Cells Markers 25
2.17.4 Neural Stem Cells markers 25
2.18 INSTRUCTIVE SIGNALLING IN DEVELOPING VERTEBRATE EMBRYOS 26
2.18.1 Wnt signalling molecules 27
2.18.2 Vertebrate wnt signalling pathways 27
2.18.3 LEF: an intracellular mediator of Wnt-signalling 29
2.19 CDO 29
2.20 EPIGENETIC DNA MODIFICATION 30
2.21 INTERLEUKIN-3 (IL-3) 32
2.22 FGF-2 AND BMP-2 33
2.23 HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR (HGF/SF) 33

3 OBJECTIVES OF THE STUDY 34

3.1 GENERAL OBJECTIVE: 34
3.2 SPECIFIC OBJECTIVES: 34

4 RESULTS 36

4.1 ISOLATION, CULTIVATION AND CULTURE EXPANSION OF MBM-MASCS 36
4.2 CHARACTERIZATION OF MBM-MASCS 37
4.3 GENETICALLY LABELLED MBM-MASCS1 AND MBM-MASCS2 EXPRESS THE LENTIVIRAL EGFP TRANSGENE STABLY 38
4.4 DISTINCT WNT SIGNALLING MOLECULES AND CA-LEF ACTIVATE THE MYOGENIC PROGRAM IN MBM-MASCS 39
4.5 5-AZACYTIDINE AND/OR TRICHOSTATIN A ACTIVATE THE MYOGENIC PROGRAM IN MBM-MASCS 44
4.6 CDO POSITIVELY MEDIATES MYOGENESIS IN MBM-MASCS 45
4.7 ESTABLISHMENT OF MYOGENIC LINEAGES FROM UNCOMMITTED MBM-MASCS1 AND MBM-MASCS2 46
4.8 THE MBM-MASCS1, MBM-MASCS2, ML-MBM-MASCS1 AND ML-MBM-MASCS2 FUSE WITH C2C12 CELLS *IN VITRO* 48
4.9 INDUCTION OF CARDIOMYOCYTE DIFFERENTIATION IN MBM-MASCS BY WNT11 BUT NOT CDO, WNT7A, WNT7B, WNT4 AND CA-LEF 49
4.10 INHIBITION OF PKC ACTIVITY ATTENUATES THE EFFECT OF WNT-11 AND ABROGATES CARDIOMYOCYTE GENE EXPRESSION IN MBM-MASCS1 AND MBM-MASCS2 51
4.11 5-AZACYTIDINE AND/OR TSA AUGMENT THE ACQUISITION OF CARDIOMYOCYTE MARKERS IN MBM-MASCS 52
4.12 FGF-2 ALONE OR IN COMBINATION WITH BMP-2 INDUCES EXPRESSION OF CTNI AND CTNT PROTEINS IN MBM-MASCS1 AND 2 55

4.13 CARDIOGENIC POTENTIAL OF LONG TERM PASSAGED SCA-1 POSITIVE ADULT STEM CELLS ISOLATED FROM THE ADULT HEART, H-ASCS 57
4.14 EXPRESSION OF HAEMATOPOIETIC MARKERS BY MBM-MASCS1 AND MBM-MASCS2 57
4.15 BMP-2 OR 5-AZACYTIDINE EVOKES OSTEOGENESIS IN MBM-MASCS 58
4.16 FGF-2 CONFERS ECTODERMAL COMPETENCE TO MBM-MASCS 59
4.17 HGF/SF INDUCES THE INITIAL PHASE OF HEPATOGENESIS IN MBM-MASCS 61
4.18 GENETICALLY LABELLED MBM-MASCS1 (MBM-MASCS1-EGFP) CONTRIBUTED TO THE DEVELOPMENT OF SEVERAL TISSUES IN CHIMERIC EMBRYOS 62

5 DISCUSSION 64

5.1 ISOLATION, CULTIVATION, CULTURE EXPANSION AND CHARACTERIZATION OF MBM-MASCS 64
5.2 PROPERTIES OF MBM-MASCS 65
5.3 MBM-MASCS1 AND MBM-MASCS2 CAN BE LABELLED EFFICIENTLY AND MAY SERVE AS A VECTOR FOR DELIVERING GENES OF THERAPEUTIC VALUE 65
5.4 THE MBM-MASCS HAVE COMPETENCE TO DIFFERENTIATE INTO DIVERSE MESODERMAL LINEAGES 66
5.5 DISTINCT WNTS (WNT7A, 7B, 4 AND WNT11) AND CA-LEF ACTIVATE THE MYOGENIC PROGRAM IN MBM-MASCS 66
5.6 5-AZACYTIDINE (AZA) AND/OR TRICHOSTATIN A (TSA) ACTIVATE MYOGENIC PROGRAM IN MBM-MASCS 68
5.7 CDO POSITIVELY MEDIATES MYOGENESIS IN MBM-MASCS 69
5.8 MYOGENIC LINEAGES CAN BE DERIVED FROM MBM-MASCS1 AND MBM-MASCS2 *IN VITRO* 70
5.9 FUSION OF MBM-MASCS1, MBM-MASCS2, ML-MBM-MASCS1 AND ML-MBM-MASCS2 WITH C2C12 CELLS *IN VITRO* 70
5.10 ONLY WNT11 BUT NOT CDO OR THE CANONICAL-WNT PATHWAY MOLECULES LIKE WNT7A, WNT7B, WNT4 AND CA-LEF INITIATE THE CARDIOMYOCYTE PROGRAMME IN MBM-MASCS 71
5.11 ATTENUATION OF THE EFFECT OF WNT-11 BY PKC INHIBITORS IS FOLLOWED BY ABROGATION OF THE PROGRESSION OF CARDIOMYOCYTE DIFFERENTIATION PROGRAMME 72
5.12 FGF-2 INDUCES EXPRESSION OF CTNI AND CTNT PROTEINS IN MBM-MASCS1 AND 2 UPON PROLONGED TREATMENT 73
5.13 BMP-2 OR 5-AZACYTIDIN INDUCES OSTEOGENESIS IN MBM-MASCS 74
5.14 FGF-2 IMPARTS NEUROECTODERMAL COMPETENCE TO MBM-MASCS 75
5.15 HGF SPECIFIES THE INITIAL PHASE OF HEPATOGENESIS IN MBM-MASCS 75
5.16 CONTRIBUTION OF GENETICALLY LABELLED MBM-MASCS1-EGFP TO MYOGENIC, CARDIAC AND ENDOTHELIAL DEVELOPMENT IN CHIMERIC EMBRYO 76

6 CONCLUSIONS 77

7 MATERIALS AND METHODS 79

7.1 MATERIALS 79
7.1.1 Cell culture materials 80
7.1.2 Cytokines or growth factors 81
7.1.3 Epigenetic drugs 81
7.1.4 Protein kinase C inhibitors 81
7.1.5 Cell lines 81
7.1.6 Enzymes 83
7.1.7 Antibodies 84
7.1.8 Kits 85
7.1.9 PCR primers 85
7.1.10 Plasmids or vectors 87
7.1.11 Solutions 90
7.1.12 Microbiology materials 92
7.1.13 Animals 92
7.2 METHODS 93
7.2.1 Isolation, cultivation and culture expansion of mBM-MASCs 93
7.2.2 Characterization of mBM-MASCs 93
7.2.3 Lentiviral labelling of mBM-MASCs1 and mBM-MASCs2 94
7.2.4 Total RNA isolation and RT-PCR 95
7.2.5 Immunofluorescence staining 95
7.2.6 Detection of myogenin using diaminobenzidine (DAB) substrate 96
7.2.7 Detection of alkaline phosphatase (ALP) 97
7.2.8 DNA sequencing 97

7.2.9 Cloning, sequencing, tranfection and preparation of retroviral expression packaging lines 97
7.2.10 Preparation of feeder cells with distinct wnts, CDO, CA-LEF, co-culture and filter experiments 98
7.2.11 Myogenic differentiation with 5-aza cytidine and/or Trichostatin A (TSA).....99
7.2.12 Establishment of myogenic lineage in mBM-MASCs1 and mBM-MASCs2.....99
7.2.13 Co-culture of mBM-MASCs1, mBM-MASCs2, ML-mBM-MASCs1 or ML-mBM-MASCs2.....99
7.2.14 Inhibition of protein kinase C.....100
7.2.15 Cardiogenic differentiation with 5-aza cytidine and/or Trichostatin A (TSA).....100
7.2.16 Cardiogenic differentiation with FGF-2 and/or BMP-2.....100
7.2.17 Osteogenic differentiation with 5-azacytidine and BMP-2.....101
7.2.18 Haematopoietic differentiation with IL-3.....101
7.2.19 Neuroectodermal differentiation with FGF-2.....101
7.2.20 Nkx5-1-LacZ reporter construct transfection and LacZ staining.....101
7.2.21 Endodermal differentiation with hepatocyte growth factor/Scatter Factor (HGF/SF).....102
7.2.22 Injection of mBM-MASCs1 into blastocysts and analysis of chimeric mice.....102

8 ABBREVIATIONS.....103

9 CURRICULUM VITAE (CV).....107

10 PUBLICATIONS AND PRESENTATION.....109

11 ACKNOWLEDGEMENTS.....111

12 ERKLÄRUNG.....112

13 ZUSAMMENFASSUNG.....113

14 REFERENCE LIST.....115

1 Summary

Although we have seen much excitement in recent years about the possibility that adult mammalian stem cells may be able to differentiate across lineage boundaries, such stem cells have not been fully characterized. In particular, the potential for trans-differentiation has not been assessed comprehensively by subjecting characterized cells to various stimuli. In the course of this study, I have isolated two long term self-renewing murine adult stem cell populations termed mBM-MASCs1 and mBM-MASCs2 and characterized their multi-lineage differentiation responses to various bioactive molecules *in vitro* as well as their contribution to the development of chimeric embryos *in vivo*. FACS characterization revealed that both cell populations differ in their CD34 and Sca-1 expression levels but are virtually indistinguishable with respect to other surface markers. In addition, they express various pluripotency or stemness genes such as Oct3/4, Nanog, SSEA-1, Rex-1 and B-Myb typical for undifferentiated ES cells.

Treatment with various stimuli including wnt7a, wnt7b, wnt4, wnt11 CA-LEF and CDO or epigenetic reprogramming with 5-azacytidine or TSA or both induced expression of myogenic markers such as Myf5, MyoD, Pax7, Myogenin, and MRF4. Several structural proteins like sarcomeric MyHC and TnI were also detected by immunohistochemistry. Furthermore, distinct wnts and CA-LEF not only induced muscle programme but also localized β-catenin within the nucleus of mBM-MASCs, suggesting the requirement of β-catenin for the myogenic programme. Differentiation of myogenic lineages were also monitored by infection of mBM-MASCs with myogenin-eGFP containing lentivirus reporter construct, induction with Wnt 7A and expansion of FACS sorted GFP positive cells. The majority of these sorted cells became positive for sarcomeric MyHC (MF-20) and also fused readily with the *bona fide* muscle cell line C2C12 when kept in differentiation medium.

Interestingly, treatment with wnt7a, wnt7b, wnt4 and CA-LEF did not produce expression of cardiomyocyte markers such as α-MHC, myocardin A, β-MHC, ANP and BNP while, treatment with wnt11 led to the expression of Nkx2-5, myocardin A, GATA-4, β-MHC and BNP. Addition of PKC inhibitors attenuated the effect of wnt-11 as manifested by the abrogation of expression of the majority of cardiomyocyte specific genes involved in the initiation as well as progression of differentiation, suggesting the requirement of PKC dependent pathway in wnt11 signalling. Epigenetic reprogramming with 5-azacytidine, TSA or both additionally induced the expression of α-MHC as demonstrated by RT-PCR and α-MHC-eGFP reporter gene expression. Progressive erythroid and myeloid differentiation of mBM-MASCs was monitored by Ter119 and CD45 up-regulation after interleukine-3

treatment using FACS which showed a 2-fold increase compared to basal levels, arguing for a specific induction by IL-3. 5-azacytidine or BMP-2 treatments of mBM-MASCs also contributed to the expression of alkaline phosphatase (ALP), a characteristic marker of osteocytes.

Both adult murine stem cell populations were also shown to express the neuronal markers DBH and TH by RT-PCR and β_{III}-tubulin, neurofilament, GFAP by immunohistochemistry and Nkx5.1-LacZ reporter gene after FGF-2 treatment. In addition, hepatocyte growth factor treatment induced albumin and SEK-1 expression and an acquisition of epitheloid-like morphology. Finally, genetically labelled mBM-MASCs contributed to the development of somites, heart and endothelium in chimeric embryos. Taken together, these data demonstrate that the two isolated murine mesenchymal stem cell populations have the competence to recapitulate several aspects of the mesodermal, neuroectodermal and endodermal lineage *in vitro* and contribute to the development of chimeric embryos *in vivo.*

2 Introduction

2.1 Stem Cells

Stem cells are generally defined as primitive, unspecialised, clonogenic cells capable of both self-renewal and multi-lineage differentiation into specialised cells of various types of tissues (Till and McCULLOCH, 1961). The ability of self-renewal is characterized by a special type of cell division which will give rise to at least one daughter cell that maintains the multipotent character of its parent (the stem cell pool). To do this, stem cells undergo asymmetric cell divisions. Asymmetric cell division serves the purpose of maintaining the stem cell pool as well as generating a differentiated progeny. On the other hand, a symmetric cell division allows stem cell self-renewal and terminal differentiation of a progeny (Fig. 1). Stem cells can be divided into a long-term subset, capable of indefinite self-renewal, as well as short-term subset that self-renew for a defined period of time. The latter cell populations might give rise to non-self renewing oligolineage progenitors, which generate progeny that are increasingly restricted in their differentiation potential and finally to functionally mature cells. The earliest stem cells in ontogeny are totipotent, extending from the zygote to the inner cell mass (ICM) of blastocysts, which give rise to pluripotent ES cells *in vitro*. Soon after, totipotent stem cells differentiate into somatic stem cells/progenitor cells and primitive germ line stem cells. Today there are several stem cells lines of embryonic origin available in contrast to only very few cell lines of postnatal or adult tissue origin.

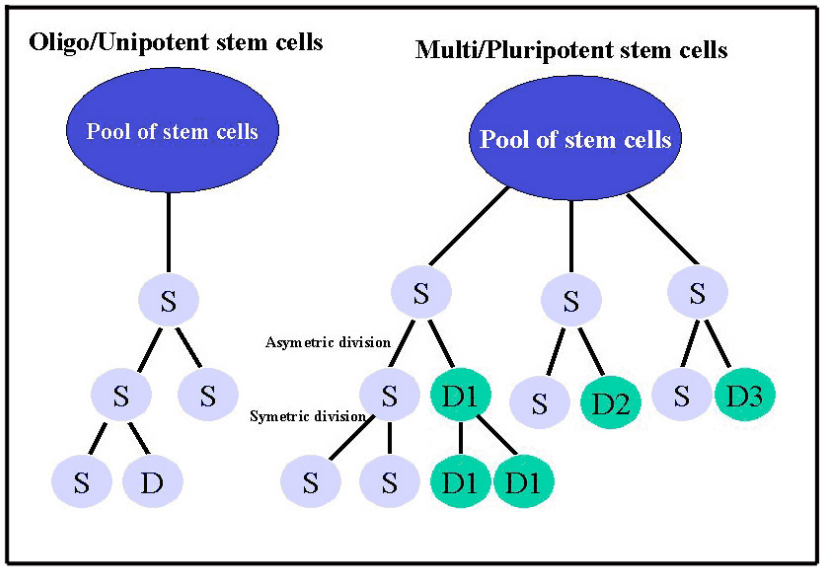

Fig.1 Schematic presentation of symmetric and asymmetric division of a putative oligo/unipotent and multi/pluripotent stem cell

2.2 Embryonic Stem (ES) Cells

In the early 1980s, several laboratories were able to cultivate cells from the 3.5 day mouse blastocysts inner cell mass (ICM) of approximately 50 cells by growing them on feeder cell layers of embryonic fibroblast or in conditioned medium that we now know contained an inhibitor of differentiation, leukaemia inhibitory factor (LIF). Since that time, ES cells have been derived from a number of other species (Prelle et al., 1999) including, the human blastocysts (Thomson et al., 1998a). Mouse ES cells have been studied most extensively. They were initially used primarily to create null mutations in mouse embryos via gene targeting and homologous recombination (Capecchi, 1989). In addition, they have also been employed as a model system to understand lineage differentiation and as source of cells for transplantation. ES cells exhibit all of the characteristics of a "stem cell," including long-term self-renewal. Unlike most tissue stem cells, when combined with normal blastocysts, they can differentiate into all cell types *in vivo* and into many derivatives *in vitro* (Bradley et al., 1984). ES cells have high levels of telomerase activity (Amit et al., 2000), have a very short G1 cell cycle, and initiate DNA replication without external stimulation (Savatier et al., 1996). Because they are widely available and technologies have been developed to delete, replace, or over-express genes of interest in a temporally and spatially controlled manner, ES cells provide a powerful means to study an otherwise largely inaccessible period of development. ES cells are also a good source of cells for gene and cell replacement therapies, particularly if differentiation of ES cell can be precisely controlled.

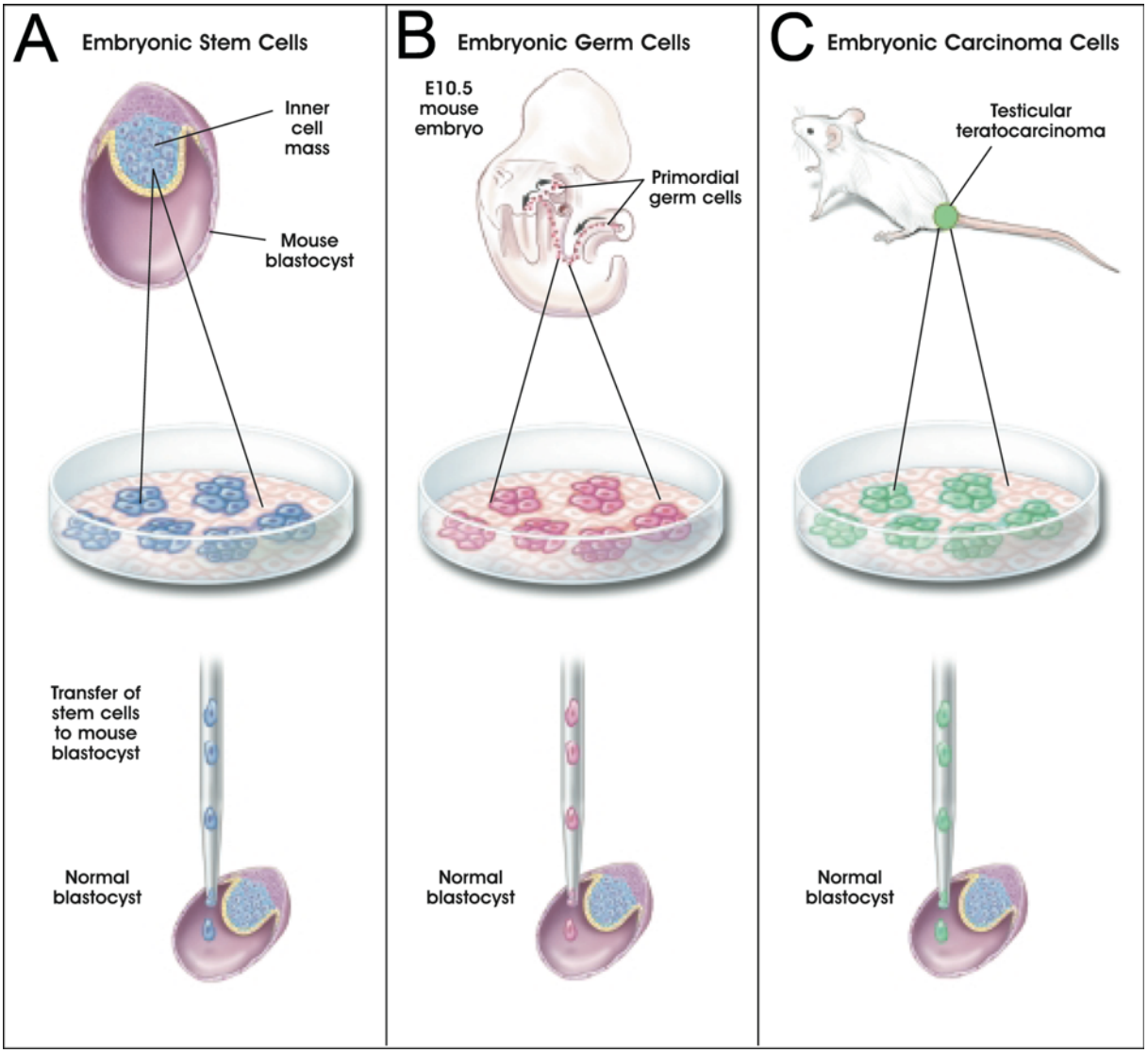

Fig. 2 Derivation of mouse pluripotent stems cells.

Derivation of embryonic stem cells (A), derivation of embryonic germ cells (B) and derivation of embryonic carcinoma cells (C). Source: stem cell information, NIH.

2.3 Embryonic Carcinoma (EC) Cells

Pluripotent cell lines have been described by their origin. For instance, embryonic carcinoma (EC) cells are derived from spontaneous teratocarcinoma of testicular and ovarian origin. Teratocarcinoma cell lines have also been produced by grafting normal blastocysts or fetal gonadal ridges into adult mice, dissociation of the malignant teratocarcinoma, and passage of resulting cell lines (Dewey et al., 1977). EC cell lines are pluripotent and have been shown to integrate into the mouse germ line, albeit with low frequency (Stewart and Mintz, 1981). Many of the lines have restricted developmental potential and can form only one or two differentiated derivatives (Martin and Evans, 1975) while others, interestingly, are nullipotent and form only teratomas (Martin, 1980). EC cells have been widely employed as important

models in the study of growth factor-mediated differentiation (Rudnicki et al., 1990). Unlike ES cells that must be constantly passaged to inhibit differentiation, EC cells typically must be stimulated to differentiate (Rudnicki et al., 1990).

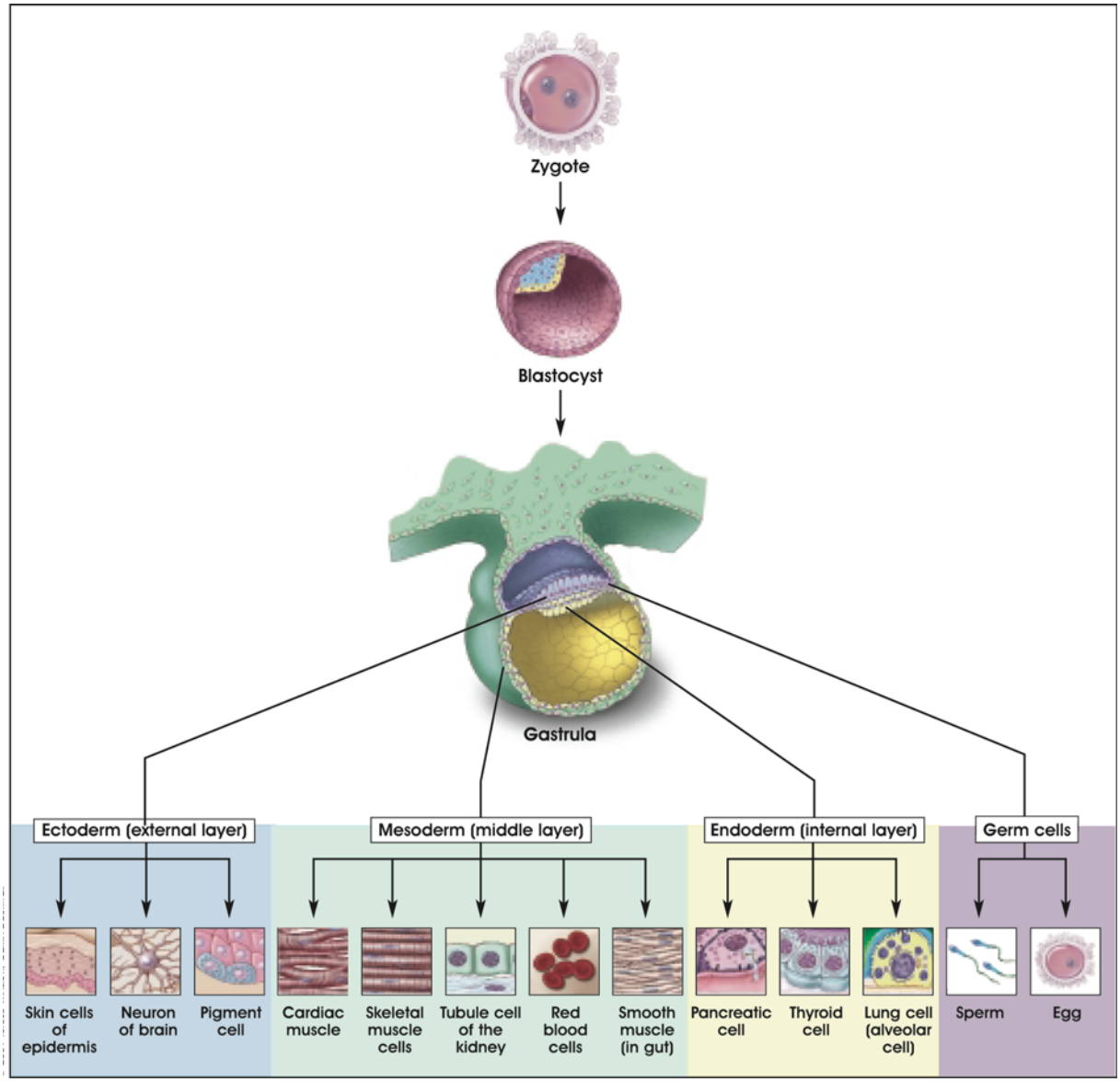

Fig. 3 Differentiation potential of ES cells into Ectoderm (external layer), mesoderm (middle layer), endoderm (internal layer) and germ cells. Source: stem cell information, NIH.

2.4 Embryonic Germ (EG) Cells

Embryonic germ cells have been developed by "reprogramming" primordial germ cells, which are destined to form egg and sperm, into pluripotent embryonic germ (EG) cells by culturing in medium containing leukaemia inhibitory factor (LIF), stem cell factor, and fibroblast growth factor-2 (FGF-2**)** (Resnick et al., 1992). These cells have been shown to contribute to the germ line in transgenic animals**,** (Stewart et al., 1994; Labosky et al., 1994a)

but appear to have limited growth and differentiation capabilities. Both mouse (Labosky et al., 1994b) and human (Shamblott et al., 2001) EG cell lines have been developed and have been differentiated as embryoid bodies (EBs) into many cell types (Rohwedel et al., 1996). Like tissue stem cells, they can also provide useful information in addressing specific questions regarding embryonic development.

2.5 Postnatal or Somatic Adult Stem Cells (ASCs)

Adult stem cells are undifferentiated cells found as a minor population among differentiated cells of a tissue or organ. They can self-renew and differentiate to yield the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are thought to be maintenance and repair of the tissue in which they are found. Some scientists now prefer to use the term somatic or postnatal stem cells instead of adult stem cells as they represent cells from postnatal life of the organism. Unlike ES, EC and EG cells, the origin of adult stem cells in mature tissues is largely unknown. Research on adult stem cells has recently generated a major focus of interest. Numerous studies found potential adult stem cells in many more tissues than once thought possible. This finding has led researchers to ask whether adult stem cells could be used for transplantation therapy. In fact, adult blood forming stem cells from bone marrow have been used in transplantations for 30 years (Dexter, 1990). Certain kinds of adult stem cells seem to have the ability to differentiate at least partially into a number of different cell types (Woodbury et al., 2002). If the differentiation of adult stem cells into various cell types and tissues could be controlled in the laboratory, they may become the basis of therapies for many serious common diseases.

The history of research on adult stem cells began about 40 years ago (Dexter, 1990). In the 1960s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoetic stem cells, forms all types of blood cells in the body (Dexter, 1990). A second population, called bone marrow stromal cells, was discovered a few years later. Stromal cells are a mixed cell population that generates bone, cartilage, fat, and fibrous connective tissue (Woodbury et al., 2002).

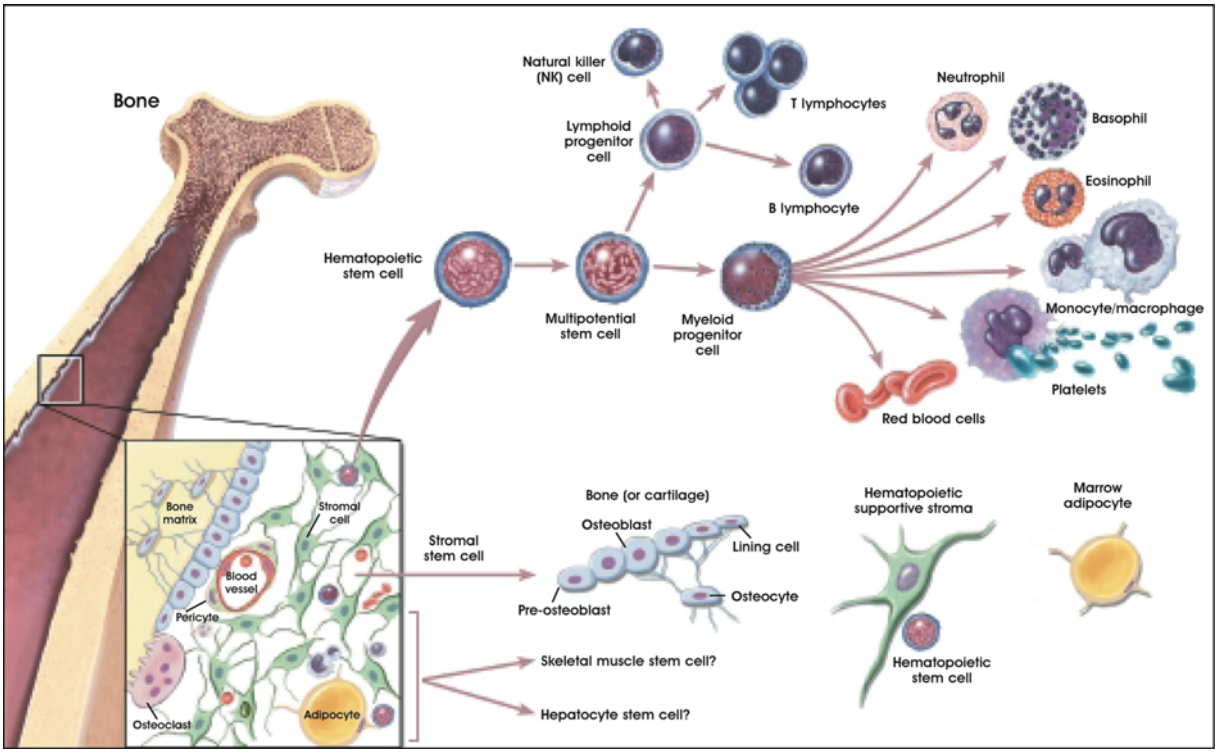

Fig. 4 Schematics of the differentiation pathway of hematopoietic and stromal stem cells. Source: stem cell information, NIH.

As indicated above, adult stem cells occur in many tissues and enter normal differentiation pathways to form specialized cell types of the tissue in which they reside. Adult stem cells may also exhibit the ability to form specialized cell types of other tissues, which is known as trans-differentiation or plasticity (Krause et al., 2001). Also in the 1960s, other investigators studying rats discovered two regions of the brain that contained dividing cells, which become nerve cells. Nevertheless, until recently it was believed that new nerve cells could not be generated in the adult brain. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types, astrocytes and, oligodendrocytes which are non-neuronal cells, and, neurons or nerve cells (Pagano et al., 2000).

2.6 Location and function of Adult Stem Cells

Adult stem cells have been identified in many organs and tissues. One confusing but important feature of adult stem cells is their presence in very small numbers in any tissue. Stem cells are thought to reside in a specific area (stem cell niche) of each tissue where they may remain quiescent (non-dividing) for many years until they are activated by disease or tissue injury. Adult tissues reported to contain stem cells include brain, bone marrow,

peripheral blood (Abuljadayel, 2003), blood vessels (Minasi et al., 2002), skeletal muscle, skin (Toma et al., 2001), liver (Yang et al., 2002) and recently heart (Beltrami et al., 2001).
Today many laboratories are trying to find ways to grow adult stem cells in cell culture and manipulate them to generate specific cell types so that they can be used for treatment of various injuries or diseases. Some examples of potential therapies include replenishing damaged muscle as in Duchene muscular dystrophy (DMD) (Torrente et al., 2001), repairing damaged heart muscle following a heart attack with cardiac muscle cells (Beltrami et al., 2001), replacing the dopamine-producing cells in the brains of Parkinson's patients (Kim et al., 2003), or developing insulin-producing cells for type I diabetes (Ramiya et al., 2000) .

2.7 Tracking Adult Stem Cells

There is no general agreement about the criteria that should be used to identify and test adult stem cells. Usually one or more of the following three strategies are applied: (1) labeling the cells in a living tissue with molecular markers and then determining specialized cell types they generate; (2) removing cells from a living animal, labeling them in cell culture, and transplanting them back into another animal to determine whether the cells repopulate their tissue of origin; and (3) isolating the cells, growing them in cell culture, and manipulating them, often by adding growth factors or introducing new genes, to determine what differentiated cells types they might form by checking the molecular marker they express. Furthermore, a single adult stem cell should be able to generate a line of genetically identical cells, known as a clone, which then gives rise to all the appropriate differentiated cell types of the tissue. Researchers tend to show either that a stem cell can give rise to a clone of cells in cell culture, or that a purified population of candidate stem cells can repopulate the tissue after transplantation into an animal. Recently, by infecting adult stem cells with a virus that gives a unique identifier to each individual cell, scientists have been able to demonstrate that individual adult stem cell clones have the ability to repopulate injured tissues in a living animal.

2.8 Differentiation of Adult Stem Cells into specific tissues

In a living animal, adult stem cells can divide for a long period and can give rise to mature cell types that have characteristic shapes, specialized structures and functions of a particular tissue in which they reside normally. They follow pathways of increasing lineage

commitments, with little or no trans-differentiation occurring naturally. These are a few examples of differentiation of adult stem cells into specific tissues.

Hematopoietic stem cells give rise to all types of blood cells including red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophiles, eosinophiles, monocytes, macrophages, and platelets (Phillips et al., 2000). Bone marrow stromal cells (mesenchymal stem cells) give rise to a variety of cell types: bone cells (osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and other kinds of connective tissue cells such as those in tendons (Pittenger et al., 1999b). Neural stem cells in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells, astrocytes and oligodendrocytes (Galli et al., 2000). Epithelial stem cells in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells (Owns and Watt, 2003). Skin stem cells occur in the basal layer of the epidermis and at the base of hair follicles. Epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer (Toma et al., 2001). Follicular stem cells can give rise to both the hair follicle and to the epidermis (Taylor et al., 2000).

2.9 Plasticity or trans-differentiation of Adult Stem Cells

Several recent findings have suggested that certain adult stem cell types are pluripotent. This ability to differentiate into multiple cell types by crossing lineage boundaries is called plasticity. The alteration in fate could involve either trans-differentiation or de-differentiation depending on the state of the stem cells. The following list offers examples of adult stem cell plasticity that have been reported during the past few years.

Haematopoietic stem cells may differentiate into: three major types of brain cells (neurons, oligodendrocytes, and astrocytes) (Brazilton TR et al) skeletal muscle cells; cardiac muscle cells; and liver cells (Zhao et al., 2003; Abuljadayel, 2003). Bone marrow stromal cells may differentiate into cardiac muscle cells and skeletal muscle cells (Pittenger et al., 1999a; Orlic et al., 2003). Brain stem cells may differentiate into blood cells and skeletal muscle cells (Bjornson et al., 1999; Galli et al., 2000).

Current research is aimed at determining the mechanisms that underline adult stem cell plasticity. If such mechanisms can be identified and controlled, existing stem cells from a healthy tissue might be induced to repopulate and repair a diseased tissue. Unfortunately, several of the parameters, which are of critical importance for the isolation and

characterization of distinct stromal cell populations, might vary from one lab to another. Since the stroma consists of various different mesenchymal cell types, it is usually necessary to separate distinct cell populations based on fluorescence-activated cell sorting (FACS) and/or the adherence properties of these cells to cell culture dishes, subsequent culture conditions, and other treatments (Jiang et al., 2002). Obviously, such procedures might lead to the isolation and growth of slightly different cell types with different properties in various assays. In addition, co-cultures of different cell types and transplantation of cells into host animals are prone to all types of labelling and detection artefacts. At present, it is not clear whether bone marrow-derived cells or other circulating cells play any significant role that can be attributed to the incorporation of these cells into diseased tissues. Alternatively, it seems possible that some of the beneficial effects observed after the infusion of stem cells rely on the induction of proliferation of resident cells by grafted cells.

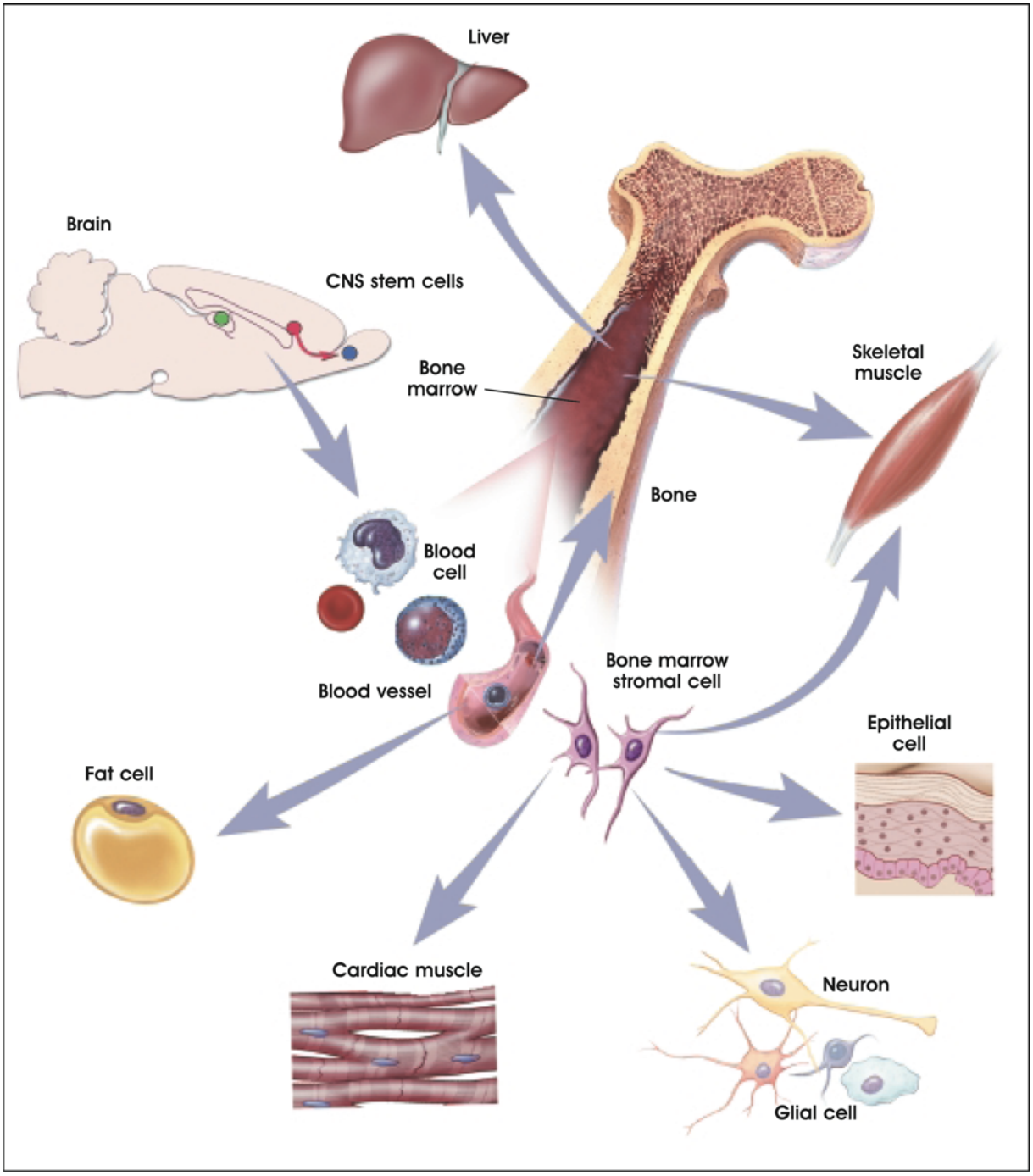

Fig. 5 Plasticity or trans-differentiation potential of adult stem cells in various tissues. Source: stem cell information, NIH.

2.10 Critical issues to be addressed about Adult Stem Cells

Albeit intensive research efforts in the field of adult stem cells were done, many important questions remain unanswered. Such questions include: how many kinds of adult stem cells exist, and in which tissues do they exist? What is the origin of these adult stem cells? Are they "leftovers" of embryonic stem cells, or do they arise in some other way? Why do they remain in an undifferentiated state when all the cells around them have differentiated? Do adult stem

cells normally exhibit plasticity, or do they only trans-differentiate when they are manipulated experimentally? What are the signals that regulate the proliferation and differentiation of ´´plastic´´ stem cells? Is it possible to manipulate adult stem cells to enhance their proliferation so that sufficient cells for transplants can be produced? Does a single type of stem cell exist, possibly in the bone marrow or circulating in the blood, which can generate cells of any organ or tissue? What are the factors that stimulate stem cells to relocate to sites of injury or damage?

2.11 Similarities and differences between Embryonic and Adult Stem Cells

ES cells and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. Of course, adult and ES cells differ in the number and type of differentiated cell types they can become. ES cells can become all cell types of the body because they are pluripotent. Adult stem cells are generally limited to differentiate into distinct cell types though some evidence suggests that adult stem cell plasticity may exist, even paralleling ES cells (Jiang et al., 2002b). Large numbers of embryonic stem cells can be relatively easily grown in culture, while adult stem cells are rare in mature tissues and methods for expanding their numbers in cell culture have not yet been worked out. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies. A potential advantage of using stem cells from an adult is that the patient's own cells could be expanded in culture and then reintroduced into the patient. The use of the patient's own adult stem cells would mean that the cells would not be rejected by the immune system. This represents a significant advantage as immune rejection is a difficult problem that so far can only be circumvented with immunosuppressive drugs. Embryonic stem cells from a donor introduced into a patient might cause transplant rejection. However, whether the recipient would reject donor embryonic stem cells has not been determined in human experimental therapies.

2.12 Haematopoietic Stem Cells

The molecular pathways of haematopoiesis are the most thoroughly explored of all stem cell systems. HSCs are pluripotent stem cells with the capacity for radioprotection, self-renewal and differentiation to generate a hierarchy of progenitors. Blood cell production is a dynamic process executed by immature precursors emanating from pluripotent haematopoietic stem cells (PHSCs), which through a series of developmental events culminate in the production of

mature cells of the erythroid, myeloid and lymphoid lineages (Phillips et al., 2000). In both human and mice, PHSCs lack cell surface determinants expressed by the committed myeloid and lymphoid lineages. PHSCs account for only 0.05-0.1% of total bone marrow nucleated cells and are characterized by the complete absence of any haematopoietic lineage markers (Lin^-). Advance in monoclonal antibody (mAb) production and flow cytometry has made it possible to isolate these lineage negative (Lin^-) rare cell populations from the bone marrow based on the expression of Thy-1^{low} and stem cell antigen-1 (Sca-1^+) in the mouse (Spangrude et al., 1988), and CD34 in human (Kim et al., 2002a). The progeny of PHSCs include common lymphoid progenitors (CLPs) from which B, T and NK cells descend, and common myeloid progenitors (CMPs), from which all myeloid and erythroid cells are derived (Kondo et al., 1997; Akashi et al., 2000). Reconstitution studies revealed that only PHSCs can mediate stable, long term haematopoietic reconstitution of the recipient. More differentiated (committed) progenitors provide only immediate and transient reconstitution. In addition to the bone marrow, HSCs can also be obtained from the peripheral blood, so called PBSCs (Abuljadayel, 2003), by mobilization with chemotherapy and growth factors or cytokines, fetal liver (Uchida et al., 2001) and umbilical cord blood (Broxmeyer et al., 2003).

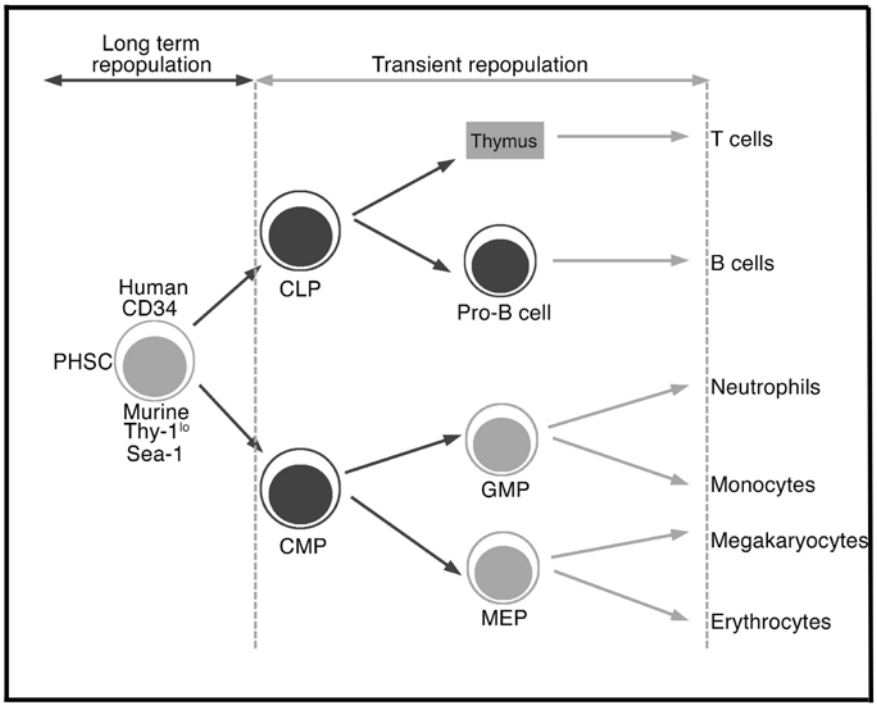

Fig. 6 Hierarchy of haematopoietic stem cells
The figure depicts selected cell surface determinants used to isolate PHSCs. Common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) can also be defined in the mouse. The figure shows that only PHSCs provide long-term, stable repopulation of recipients, while other cells mediate different degrees of transient reconstitution. The radioprotective megakaryocyte/erythroid progenitor in the mouse is indicated (Paquette and Dorshkind, 2002).

2.13 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) or marrow stromal cells are non-circulating multi-potent cells (Friedenstein et al., 1968) readily isolated from the cavity of adult bone marrow. They account for <0.05% of the bone marrow population. They generate single-cell-derived colonies and have the potential for multi-lineage differentiation along various mesenchymal tissues both *in vitro* (Pittenger et al., 1999b) and *in vivo* (Liechty et al., 2000). MSCs might also be engrafted into myocardium where they have been claimed to trans-differentiate into cardiac like cells (Wang et al., 2000). However, to differentiate into cardiac like and muscle cells they have to be treated with 5-azacytidine, a drug which causes demethylation of DNA. In culture, MSCs maintain an undifferentiated stable phenotype, which changes upon induction of differentiation into multiple mesenchymal lineages. There is still an ongoing discussion whether there is a common precursor cell of the marrow microenvironment and haematopoiesis (Simmons and Torok-Storb, 1991a). There is also more and more evidence that $CD34^-$ resting or quiescent HSC along with other fibroblast-like cells serve as supportive cells and provide necessary growth factors and even cell-cell contact to equilibrate the sensitive balance of differentiation and proliferation of haematopoietic progeny (Huss, 2000). In addition, there are indications that so-called "mesenchymal stem cells" reconstitute the marrow stroma and release committed progenitor cells into the circulation basically participating in the "stem cell cycle." Mesenchymal stem cells might be precursor cells of other mesenchymal organ systems, such as chondrocytes, osteoblasts, and myoblasts (Pittenger et al., 1999a). First clinical trials were already performed using bone marrow-derived mesenchymal cells to treat children with osteogenesis imperfecta (Horwitz et al., 1999). $CD34^-$ mesenchymal stem cells might also generate more specified cell types, such as endothelial cells (Makino et al., 1999b). It also seems to be possible that a common progenitor cell of $CD34^-$ haematopoietic and mesenchymal stem cells exists. This type of cell might give rise to various specified tissues, depending on growth factor-mediated signals and an internal signal control. By using novel vector systems for quiescent cells (Reiser et al., 1996), $CD34^-$ haematopoietic and mesenchymal stem cells might be used efficiently for cell and gene therapy with a wide spectrum of applications. Recent work showed that rare cells termed multi-potent adult progenitor cells (MPCs) can be selected from the whole bone marrow, muscle and brain with the capacity to differentiate not only into mesenchymal lineage but also endothelium, neuroectoderm and endoderm (Jiang et al., 2002c; Jiang et al., 2002a). These

MPCs require the presence of epidermal growth factor (EGF), platelet derived growth factors (PDGF) and Leukaemia inhibitory factor (LIF) to grow and expand.

2.14 Another type of Adult Stem Cells: SP Cells

Recent studies have identified novel populations of adult stem cells with a verapamil-sensitivity and Hoechst 33342 fluorescent dye-effluxing property known as side population (SP) cells. For instance in adult mouse, HSCs with long term multi-lineage reconstitution ability are contained in the dye-effluxing Hoechst SP of the bone marrow and umbilical cord (Goodell et al., 1997a). The dye extruding ability of this HSCs population is not confined to the mouse as pigs, rhesus monkey, and human bone marrow also contain SP cells with enriched haematopoietic ability (Goodell et al., 1997b). SP cells are also observed in other adult tissues, namely skeletal muscle (Majka et al., 2003), mammary gland (Welm et al., 2002) and liver (Uchida et al., 2001) where they also demonstrate stem cell activity.

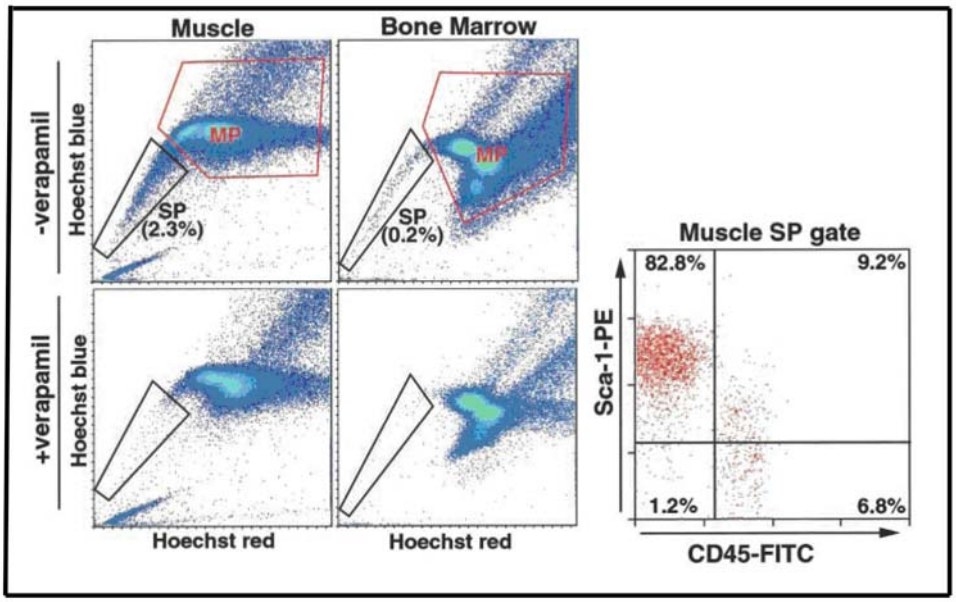

Fig. 7 Hoechst dye effluxing property of SP cells from bone marrow and muscle. FACS fractionation of SP cells from bone marrow and muscle was used for the isolation of adult stem cells by Hoechst dye exclusion. Both muscle and bone marrow SP cells stained with Hoechst dye were sensitive to verapamil. x- and y-axes indicate intensity of CD45-FITC and Sca-1-PE, respectively, taken from (Asakura et al., 2002).

2.15 Heart-derived Sca-1$^+$ Adult Stem Cells, H-ASCs

Although somatic stem cells have been documented to exist in various adult organs, the search for stem cells in the adult heart has been futile. This is because of the accepted paradigm in cardiac biology that considered the adult mammalian heart to be a post mitotic organ characterized by meagre capacity for proliferation and regeneration. Recently, however,

evidence that challenges the accepted view has been slowly emerging as stem cell like/progenitors have been identified in the adult heart (Oh et al., 2003). Such cells might be a reservoir to renew the myocardium other than the rare occurrence of cycling ventricular muscle cells. An important source of these stem/progenitor cells might be progenitors in the heart itself that were set-aside during development or recruited from extra cardiac niches.

2.16 Muscle-derived Adult Stem Cells, Ms-ASCs

Muscle contains satellite cells that normally participate in the replacement of myoblasts and myofibers. There are also indications that the muscle may harbour other stem cells, either haematopoietic migrants from bone marrow and peripheral blood or intrinsic stem cells of muscle tissue. Multi-potent adult stem cells have been reported to exist in the muscle (Jiang et al., 2002c). Based on similarities between skeletal and heart muscle cells, muscle-derived stem cells have also been proposed to be useful for the repair of cardiac damage, with evidence that mechanical beating is necessary for full differentiation of skeletal muscle stem cells into cardiomyocytes (Iijima et al., 2003). Qu-petersen et al. have shown that skeletal muscle-derived stem cells, which highly express Sca-1, contribute to the regeneration of the skeletal muscle in a mouse model of Duchene muscular dystrophy and differentiate into adipocytes, endothelial, and myogenic cells *in vitro* (Blau and Blakely, 1999). They also have demonstrated that skeletal muscle-derived stem cells were able to differentiate into neuronal and endothelial cells. Asakura et al have reported that approximately 90% of SP cell in skeletal muscle express Sca-1^+. It has been shown that Sca-1^+ and $CD34^+$ skeletal muscle-derived stem cells restore dystrophin in mdx mice (Orlic et al., 2001). These finding suggest that Sca-1 might be an important marker for somatic stem cells.

2.17 Stem Cells markers

It seems reasonable to assume that during differentiation of any cell type, stem cell markers that are expressed at high levels in the starting population are down regulated upon differentiation. It seems critical to determine if there is a residual stem cell population that could either be unintentionally implanted, or through differential cell survival could selectively skew the results of differentiation experiments. Good differentiation markers have to be employed to characterize differentiated cells. It is necessary to use a panel of genes typical of multiple differentiated cell types and not only genes expected to be expressed by a single lineage. Unfortunately, a unique marker of stem cells does not exist. While stem cells

are best defined functionally, a number of molecular markers have been used to characterize various stem cell populations. Although functions have yet to be ascertained for many of these early markers, their characteristic expression pattern and timing provide a useful tool to identify and isolate stem cells. For most of the molecules discussed, studies performed both *in vitro* and *in vivo* support their significant role in characterizing stem cells. However, the significance of these markers alone or in various combinations for the identification and isolation of stem cells needs to be proven. This is particularly important because they are not unique to the stem cells but also expressed by other cells from other tissues as well.

2.17.1 Embryonic Stem (ES) Cells markers

Embryonic stem cells express a number of genes, which although not restricted to ES cells, are expressed by undifferentiated versus differentiated ES cells and are useful in assessing the commitment for differentiation in a mixed population. Such markers include:

Oct-4: Oct-4 (also termed Oct-3 or Oct3/4), one of the POU transcription factors, was originally identified as a DNA-binding protein that activates gene transcription via a *cis*-element containing octamer motif. It is expressed in totipotent embryonic stem and germ cells (Scholer et al., 1990; Rosner et al., 1990). A critical level of Oct-4 expression is required to sustain stem cell self-renewal and pluripotency. Differentiation of embryonic stem (ES) cells results in down-regulation of Oct-4, an event essential for a proper and divergent developmental program. Oct-4 is not only a master regulator of pluripotency that controls lineage commitment, but is also the first and most recognised marker used for the identification of totipotent ES cells.

Rex-1: (zfp-42) gene encodes an acidic zinc finger protein and contains an octamer motif within its promoter. It is expressed highly in undifferentiated embryonic carcinoma (EC) cell and in the ICM of blastocysts and diminishes during ES, EC cells differentiation and normal embryonic development (Ben Shushan et al., 1998).

SSEA-1 (Stage Specific Embryonic Antigen-1): SSEA-1 is a cellular marker commonly expressed on the surface of pre-implantation-stage murine embryos (*i.e.* at the eight cell stage) and has been found on the surface of teratocarcinoma stem cells, but not on their differentiated derivatives (Solter and Knowles, 1978). The biological roles of these carbohydrate-associated molecules might be found in controlling cell surface interactions during development. Undifferentiated primate ES cells, human EC and ES cells express SSEA-3 and SSEA-4, but not SSEA-1. In contrast, undifferentiated mouse ES, EC and EG

cells express SSEA-1, but not SSEA-3 or SSEA-4 (Thomson et al., 1998b)**,** suggesting that SSEA-1 is a specific cell surface marker for undifferentiated cells of murine origin.

B-Myb: The Myb gene family has 3 members, A-Myb, B-Myb and C-Myb. Examination of its developmental role in B-Myb deficient mice has indicated that it is required for ICM formation at early stages of development. The expression of B-Myb correlates with cellular proliferation, and is not expressed in resting cells. The mRNA level decreases when cells are induced to differentiate. Inhibition of B-Myb expression by the introduction of a B-Myb antisenes construct diminished cell proliferation, but constitutive expression induced a transformed phenotype (Tanaka et al., 1999).

Nanog: The murine Nanog gene, a member of the homeobox family of DNA binding transcription factors, has been recently shown to maintain pluripotency of ES cells (Chambers et al 2003). Mouse Nanog expression is high in undifferentiated ES cells and down regulated during their differentiation, concomitant with the loss of pluripotency. Murine Nanog expression is detected in the ICM of the blastocysts (Mitsui et al., 2003)**.** Its expression also marks pluripotent germ cells and is highly expressed in germ cell and teratoma derived cell lines**.**

2.17.2 Haematopoietic and other Stem Cells markers

CD34: The cell surface sialomucin CD34 has been a focus of interest since it was found expressed on a small fraction of human bone marrow cells (Civin et al., 1984). The $CD34^+$-enriched cell population from bone marrow or mobilized peripheral blood appears to be responsible for most of the haematopoietic activity (Civin et al., 1984). CD34 has therefore been considered to be the most critical marker for haematopoietic stem cells (HSCs). CD34 expression on primitive cells is down regulated as they differentiate into mature cells. It is also found on clonogenic progenitors. Although its precise function is still unknown, the pattern of expression of CD34 suggests that it plays a significant role in early haematopoiesis (Sutherland and Keating, 1992). The theory of CD34 being the most primitive HSC marker, however, has recently been challenged. Osawa et al. first demonstrated that murine HSCs could be CD34 negative (Osawa et al., 1996). In addition, a low level of engraftment and haematopoietic capacity has been demonstrated in human $CD34^-$ cells. Additionally, studies have shown that both murine and human $CD34^+$ cells may be derived from $CD34^-$ cells. Collectively, these reports suggest the possibility that HSCs may be $CD34^+$ or $CD34^-$ and that selection of cells expressing CD34 might result in exclusion of more primitive stem cells.

Clinical and experimental protocols including *ex vivo* culture, gene therapy, and HSC transplantation are currently designed for cell populations enriched in $CD34^+$ cells.

Hoechst dye exclusion: CD34 class antigens have been useful to flow sort many types of haematopoietic stem cells. Alternatively, Hoechst dye exclusion is a very useful tool for the identification of SP cells. Many stem cells from various adult and embryonic tissues exclude Hoechst 33342 dye. For instance, FACS fractionation of SP cells from bone marrow, muscle and liver has been used for the isolation of adult stem cells by Hoechst dye exclusion. Given these findings, Hoechst dye efflux represents a general property of stem cells and progenitors in various tissues and various species.

CD133: CD133, a 120 kDa, glycosylated protein containing five transmembrane domains was identified initially by the AC133 monoclonal Ab, which recognizes a $CD34^+$ subset of human HSCs (Yin et al., 1997; Miraglia et al., 1998). CD133 may provide an alternative to CD34 for HSCs selection and *ex vivo* expansion. A $CD133^+$ enriched subset can be expanded in a similar manner as a $CD34^+$ enriched subset, retaining its multi-lineage capacity. Recent studies have offered evidence that CD133 expression is not limited to primitive blood cells, but defines unique cell populations in non-haematopoietic tissues as well. $CD133^+$ progenitor cells from peripheral blood can be induced to differentiate into endothelial cells *in vitro* (Gehling et al., 2000)**.** In addition, human neural stem cells can be directly isolated by using an anti-CD133 Ab (Uchida et al., 2000).

ABCG2: ABCG2 (ATP-binding cassette super-family G member 2) is a determinant of the Hoechst-negative phenotype of side population (SP) and found in a wide variety of stem cells, including HSCs (Zhou et al., 2001; Kim et al., 2002b). ABCG2 is a member of the family of ABC transporters and was first identified in a breast cancer cell line. It belongs to the half-transporter group and is unique as it is localised to the plasma membrane. The expression of ABCG2 appears highest in $CD34^-$ cells and is down-regulated with the acquisition of CD34 on the cell surface (Zhou et al., 2001). Down-regulation in ABCG2 expression is also observed in various committed haematopoietic progenitors. ABCG2 may therefore serve as a more promising marker than CD34 for primitive HSC isolation and characterisation. The expression pattern of ABCG2, however, is not limited to HSC. ABCG2 expression exclusively characterises the Hoechst SP phenotype in cells from diverse sources, including monkey bone marrow, mouse skeletal muscle and ES cells (Zhou et al., 2001). The potential plasticity of SP cells has been demonstrated by studies showing that cardiomyocytes and muscle can be regenerated from transplanted bone marrow-derived SP cells (Jackson et al., 2001; Gussoni et al., 1999). Exclusive expression of ABCG2 on SP cells suggests that

ABCG2 might be a potential marker for positive selection of pluripotent stem cells from various adult sources. ABCG2 has been implicated in playing a functional role in developmental stem cell biology (Bunting, 2002).

Sca-1: Sca-1 (stem cell antigen 1, Ly-6A/E), an 18 kDa phosphatidylinositol-anchored protein, is a member of the Ly-6 antigen family (van de et al., 1989). Sca-1 is the most recognized HSC marker in mice with both Ly-6 haplotypes as it is expressed on multi-potent HSCs. An anti-Sca-1 Ab is frequently used in combination with negative selection for expression of a number of cell surface markers characteristic of differentiated cells of hematolymphoid lineages (Lin-) to identify and isolate murine HSCs. Sca-1^+ HSCs can be found in the adult bone marrow, fetal liver and is mobilized from peripheral blood and spleen within the adult animal. Sca-1 has also been discovered in several non-haematopoietic tissues (van de et al., 1989), and can be used to enrich progenitor cell populations other than HSCs.

2.17.3 Mesenchymal/Stromal Stem Cells Markers

STRO-1: The murine IgM monoclonal Ab STRO-1, produced from an immunization with a population of human $CD34^+$ bone marrow cells, can identify a cell surface antigen expressed by Stromal elements in human bone marrow (Simmons and Torok-Storb, 1991b). From bone marrow cells, the frequency of fibroblast colony-forming cells (CFU-F) is enriched approximately 100-fold in the STRO-1^+/GlycophorinA^- population compared to the STRO-1^+/Glycophorin A^+ population (Simmons and Torok-Storb, 1991b). A STRO-1^+ enriched subset of marrow cells is capable of differentiating into multiple mesenchymal lineages including haematopoiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts and chondrocytes. STRO-1 is a valuable Ab for the identification, isolation and functional characterization of human bone marrow stromal cell precursors, which are quite distinct from those of primitive HSCs.

2.17.4 Neural Stem Cells markers

Nestin: It is a class VI intermediate filament protein. Although it is expressed predominantly in stem cells of the central nervous system (CNS) (Frederiksen and McKay, 1988), its expression is absent from nearly all mature CNS cells. Nestin is the most extensively used marker to identify CNS stem cells within various areas of the developing nervous system and in cultured cells *in vitro* (Uchida et al., 2000). Nestin expression has also been discovered in

non-neural stem cell populations, such as pancreatic islet progenitors as well as haematopoietic progenitors (Shih et al., 2001).

PSA-NCAM (Polysialic acid-neural cell adhesion molecule): The regulated expression of neural cell adhesion molecule (NCAM) isoforms in the brain is critical for many neural developmental processes. The embryonic form of NCAM, PSA-NCAM, is highly polysialylated and is mainly expressed in the developing nervous system (Kiss and Muller, 2001). PSA-NCAM expression may be related to synaptic rearrangement and plasticity (Muller et al., 1996). In the adult, PSA-NCAM expression is restricted to regions that retain plasticity. A neuronal-restricted precursor identified by its high expression of PSA-NCAM can undergo self-renewal and differentiate into multiple neuronal phenotypes. PSA-NCAM$^+$ neonatal brain precursors are restricted to a glial fate and thyroid hormone can derive them into an oligodendrocyte fate. Polysialic acid modification significantly decreases NCAM adhesiveness. Originally, it was suggested that PSA-NCAM works as a purely anti-adhesive factor that modulates cell-cell interactions in promoting brain plasticity. Increasing evidence indicates that PSA-NCAM may interact with secreted signalling molecules to perform an instructive role in development.

p75 Neurotrophin R (NTR): p75 NTR, also named low affinity nerve growth factor (NGF) receptor, is a type I transmembrane protein that belongs to the tumour necrosis factor receptor super family (Barker and Murphy, 1992)**.** It binds to NGF, BDNF, NT-3 and NT-4 equally well (with low affinity). p75NTR, when activated in the presence of Trk, enhances responses to neurotrophin. TrkC receptors which interact with p75 NTR have been suggested to serve critical functions during the development of the nervous system (Hapner et al., 1998).

Neural crest stem cells (NCSCs) have been isolated based on their surface expression of p75NTR (Stemple and Anderson, 1993). Freshly isolated p75NTR$^+$ NCSCs from peripheral nerve tissues can self-renew and generate neurons and glial both *in vitro* and *in vivo*. In addition, neuroepithelial-derived p75NTR$^+$ cells are also able to differentiate into neurons, smooth muscle and Schwann cells in culture (Mujtaba et al., 1998).

Recently, p75 NTR has been employed as a useful marker in the identification of mesenchymal precursors as well as hepatic stellate cells (Campagnolo et al., 2001; Cassiman etal.,2001).

2.18 Instructive signalling in developing vertebrate embryos

Cell differentiation is a consequence of changes in gene expression that are primarily controlled at the level of transcription. This implies that regulated expression of specific

transcription factors in response to extracellular signals is an important determinant of cell differentiation. In addition, organogenesis or morphogenesis requires cell-cell contact and a complex interplay of several temporally regulated signalling cascades and epigenetic reprogramming or modification steps. Exposure to a variety of such growth and differentiation signals or epigenetic reprogramming initiates expression and repression of cascades of genes that shape the developing embryo. While there has been considerable progress in understanding the molecular basis of morphogenesis, it is clear that many developmental control genes have not yet been identified. Basic questions regarding lineage specification are still unanswered. Because of the difficulty of manipulating the mammalian embryo, researchers have employed cell lines with multi-lineage potential as an alternative model to unravel this critical phase of development.

2.18.1 Wnt signalling molecules

The Wnt family of genes encodes for over twenty cysteine-rich secreted glycoproteins that act by binding to the frizzled (Fzd) receptors on target cells. They have been implicated in a variety of developmental processes such as cell differentiation, cell polarity, cell migration and cell proliferation. Wnt signalling directs cell fate determination in various tissues, including haematopoietic (Van Den Berg et al., 1998), central nervous system (Patapoutian and Reichardt, 2000) and kidney (Vainio and Uusitalo, 2000). The Wnt proteins initiate myogenesis in explants of mouse paraxial mesoderm by activating the expression of Myf5 and MyoD (Tajbakhsh et al., 1998) hence they may act by regulating both myogenic commitment and expansion of committed cells. As they are molecular cues implicated in embryonic myogenesis, it may be anticipated that they may have similar function in adult tissues.

2.18.2 Vertebrate wnt signalling pathways

Based on their biological activities, vertebrate wnts have been divided into several distinct signalling pathways, leading to distinct cellular and embryonic responses, namely the canonical Wnt/β-catenin (Wnt-1 class) and the non-canonical wnt/Ca^{2+} and Wnt/Jun N-terminal kinase, JNK (wnt-5A class) pathways (Tada and Smith, 2000; Miller et al., 1999; Kuhl et al., 2000b). The former occurs when Wnt binds to Frizzled (Fzd) receptors and activate Dishevelled (Dvl), leading to the inactivation of a cytoplasmic serine-threonine kinase, Glycogen synthase kinase-3β (GSK-3β). This stabilizes the cytoplasmic β-catenin protein which is a target of GSK-3β and ultimately translocates and accumulates to the

nucleus where it binds and forms a complex with family members of HMG transcription factors such as TCF/LEF. These complexes can for instance induce expression of Somite factors, including Pax3, Mox, Gli2 and Six1 during embryogenesis (Borycki et al., 2000; McDermott et al., 2005; Fan et al., 1997; Capdevila et al., 1998).

In contrast, wnt/Ca^{2+} stimulates intracellular Ca^{2+} release and activates two kinases, calcium calmodulin dependent kinase two (CamKII) and protein kinase C (PKC) in a G-protein dependent manner. A recent study showed that xwnt-11 dependent activation of a non-canonical Wnt signalling is required for heart formation in Xenopus embryo. It is also sufficient to induce a contractile phenotype in embryonic explants. In addition, treatment of mouse embryonic carcinoma stem cell line P19 with murine wnt-11 conditioned medium triggers carcinogenesis (Pandur et al., 2002; Kuhl et al., 2000a).

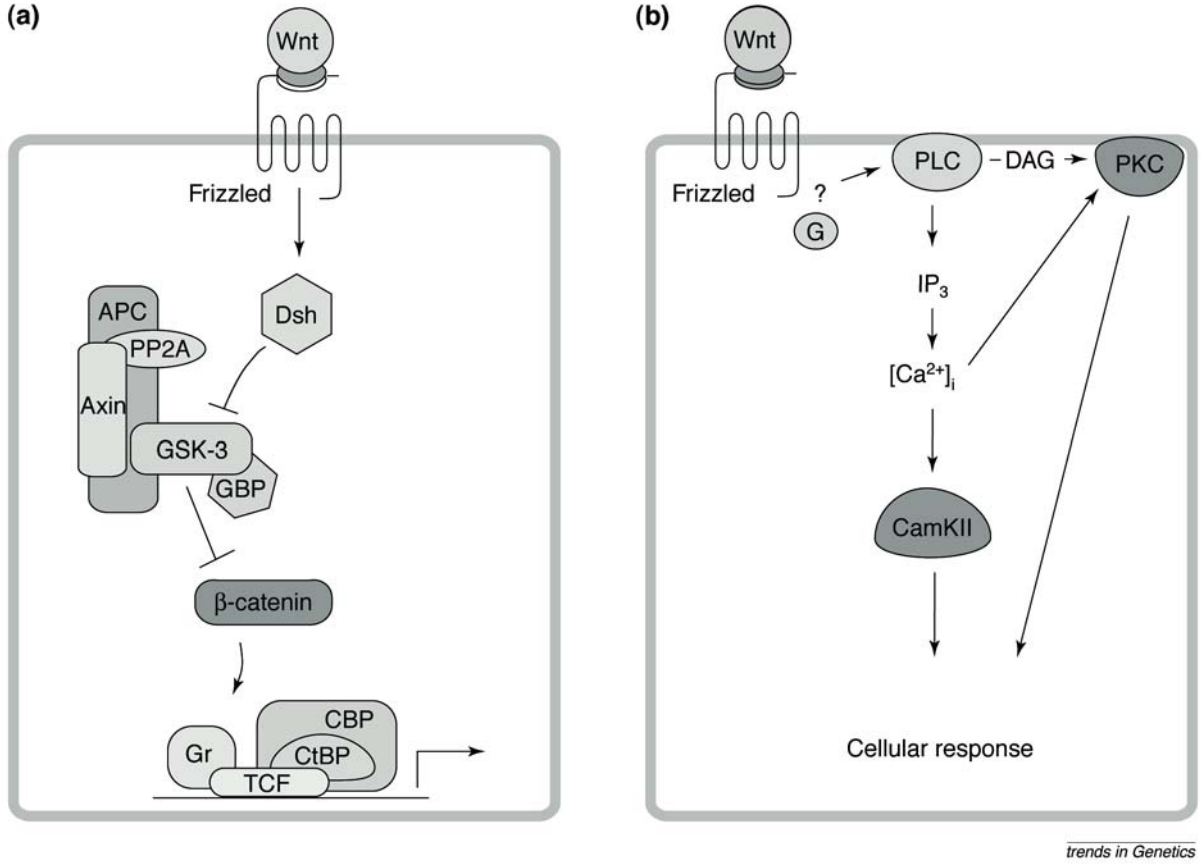

Fig. 8 Vertebrate Wnt signalling pathways
(a) The canonical Wnt/β-catenin signalling pathway. Interaction of different Wnts with an appropriate receptor of the Frizzled family leads to stabilization of cytoplasmic β-catenin and thus to activation of target genes. (b) The proposed vertebrate Wnt/Ca^{2+}pathway. Activation of the Wnt/Ca^{2+} pathway results in intracellular Ca^{2+} release and activation of the Ca^{2+}-sensitive enzymes Ca^{2+}-calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC) in a β-catenin-independent manner. Abbreviations: APC, adenomatous polyposis coli protein; CBP, CREB-binding proteins; CtBP, C-terminal binding protein; DAG, Diacylglycerol; Dsh, Dishevelled; G, heterotrimeric G proteins; Gr, Groucho; GBP, GSK-3 binding protein/Frat-1; GSK-3, Glycogen synthase kinase-3; IP3, inositol-1,4,5-trisphosphate; PP2A, Protein phosphatase 2A; PLC, Phospholipase C; TCF, T cell factor-1 (Kuhl et al., 2000c)

2.18.3 LEF: an intracellular mediator of Wnt-signalling

LEF1 is a high mobility group (HMG) protein and the nuclear effectors of the canonical Wnt signalling pathway (Hsu et al., 1998). LEF1 transcriptional activity is regulated by interactions with transcriptional co-activators and co-repressors. In the absence of Wnt signals, LEF1 binds to transcriptional co-repressors TLE, CtBP, and HDACs to inhibit gene expression (van Noort and Clevers, 2002). Wnt convert LEF1 into a transcriptional activator by stimulating the disassembly of GSK-3-Axin-APC multiprotein destruction complex to prevent ubiquitin-mediated degradation of β-catenin (van Noort and Clevers, 2002). Sequences encoding the LEF-DN lack certain amino acids of murine LEF-1, and are often described as the truncated form of LEF-1. However, sequences encoding the LEF-CA contain amino acids from β-catenin fused to the C-terminus of LEF-DN. The β-catenin is a multifunctional protein with an important role in cell adhesion and signal transduction. It is a member of the armadillo (arm) family of proteins and downstream effector of the Wnt signalling pathway (Dierick and Bejsovec, 1999; Miller et al., 1999). β-catenin binds to the transcription factors of the LEF-1/TCF family by displacing co-repressors from the LEF1, translocates to the nucleus, where it recruits co-activators and activates the expression of specific target genes (Brantjes et al., 2002; van Noort and Clevers, 2002). The DN-LEF contains the DNA binding domain of mouse LEF-1, but lacks the amino-terminal activation domain. This fusion protein is unable to bind β-catenin and functions as a feedback inhibitor of Wnt signalling *in vivo* (Kitagaki et al., 2003). The CA-LEF is a fusion protein consisting of the DNA binding domain of LEF-1 and a transactivation domain of β-catenin. LEF-DN and LEF-CA function as dominant negative and constitutive active molecules of β-catenin-LEF/TCF-dependent Wnt signalling respectively (Vleminckx et al., 1999).

2.19 CDO

CDO is an acronym for cell adhesion molecule (CAM) related, down regulated by oncogenes. It was identified on the basis of its down regulation by the Ras oncogenes. It has been described that oncogenes, serum growth factors, and cell substratum adhesions regulate the expression of CDO in fibroblasts (Kang et al., 1997). CDO is characterized by an extracellular region that contains five Ig-like repeats followed by three fibronectin type III (FNIII)-like repeats, a transmembrane segment and a long cytoplasmic tail. Oncogenic Ras inhibits myogenic differentiation in a manner dependent on the range of CDO expressions. During early embryogenesis, it is expressed in the CNS and early myogenic compartments,

including newly formed somite, dermomyotome and myotome (Kang et al., 1998). It is also expressed in myoblast lines derived by treating CH310T1/2 fibroblast cells with 5-azacytidine as well as proliferating and differentiating C2C12 myoblasts.

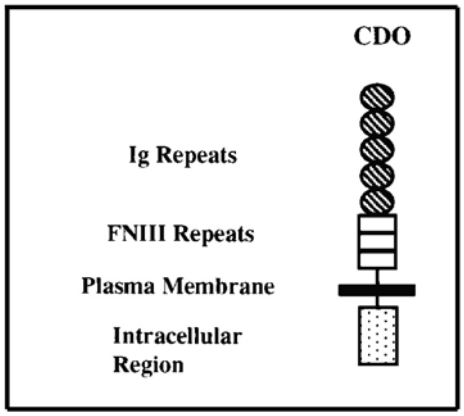

Fig. 9 Domains of the CDO molecule

CDO is characterized by an extra cellular region that contains five immunoglobulin (Ig)-like repeats followed by three fibronectin type III (FNIII)-like repeats, a transmembrane segment and a long cytoplasmic tail (Kang et al., 1998).

2.20 Epigenetic DNA modification

Epigenetics refers to alterations in gene expression that occur without a change in DNA sequence. It was more than 20 years ago that Shirley Taylor and Peter Jones from the University of Southern California first showed a connection between DNA methylation and gene expression. In their experiments, they treated undifferentiated cells from mouse embryos with 5-azacytidine, a potent inhibitor of DNA methylation. To their surprise, these developed into cells such as muscle and fat, and the changes were inherited by the next generation of cells. It became obvious that reducing DNA methylation reactivated certain genes, allowing the development of new cell types from the embryo. This opened the door to an array of experiments, which showed that a large number of genes could be reactivated by 5-azacytidine and its deoxy version, 5-aza-2-deoxycytidine. Both compounds are cytosine analogues that work by inhibiting methyl group transfer and trapping DNA methyltransferase.

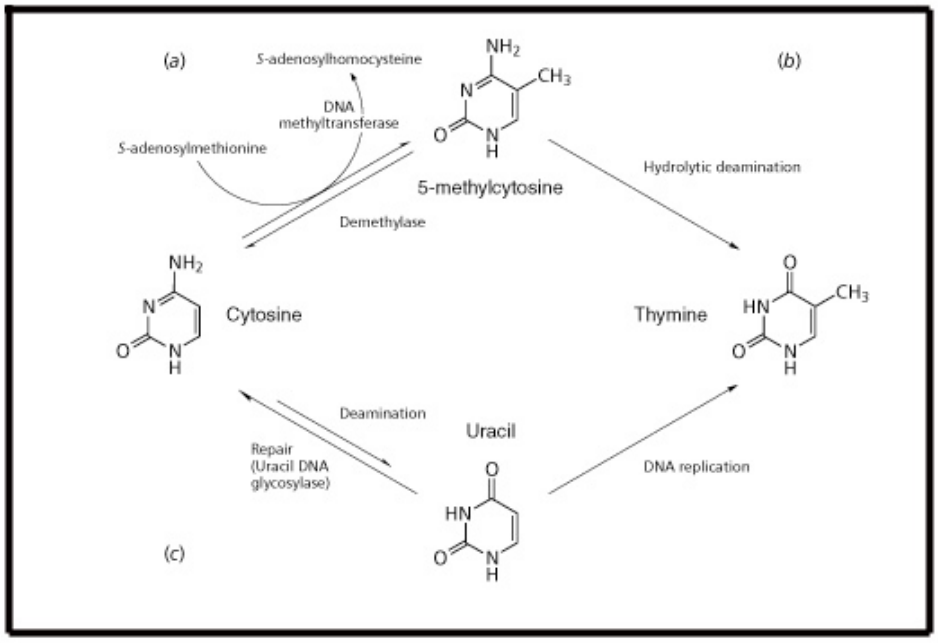

Fig. 10 Biochemical pathway for cytosine methylation and demethylation. The methylation reaction involves flipping the target cytosine out of the intact double helix, so that the transfer of the methyl group from the methyl donor (*S*-adenosylmethionine) can take place. An enzyme DNA methyltransferase catalyze this reaction *(*Maria Pufulete 2001)

There is evidence that DNA methylation plays a major role for carcinogenesis. It can activate proto-oncogenes and silence tumour suppresser genes. A growing body of evidence indicates that methylation is a major mechanism of silencing of SYK gene expression, a tumour suppressor whose absence is implicated in breast cancer. For instance, in an experiment conducted to determine whether methylation was responsible for loss of SYK expression, SYK-methylated cell lines were treated with methylation inhibitor, 5-aza-2-deoxycytdine (Jones, 1985)**,** consequently 5-aza-2-deoxycytdine reactivated the SYK expression in all of the methylation positive cell line as detected by RT-PCR. In contrast, treatment with 1µM TSA, a histone deacetylase inhibitor (Yoshida et al., 1990) was unable to reactivate SYK gene expression. Taken together, these results indicated that aberrant 5´ hypermethylation, not deacetylation plays a causal role in silencing the SYK gene. This establishes a strong correlation between SYK 5´ CpG hypermethylation and its loss of expression in cell lines (epigenetic alteration). Hypermethylated DNA is believed to interact with several methyl-CpG binding proteins. The interaction helps to assemble or recruit a repressive complex, including histone deacetylase, and forms an inactive chromatin context that leads to gene silencing (Nan et al., 1998; Wade et al., 1999)**.** Interestingly, the expression of some methylation-repressed genes can be reactivated by TSA treatment (Ferguson et al., 2000; Yang et al., 2000) in other words; demethylation-induced gene re-expression can be potentiated by TSA (Cameron et al., 1999b)**.** Together, these data demonstrate that both DNA

methylation and histone deacetylation are involved in the regulation of transcriptional inactivity, which occurs through recruitment of transcriptional silencing machinery to the promoter of target genes**.** The finding that methyl cytosine-binding protein (MeCP2) binds to histone deacetylases and repress transcription *in vivo*, supports a model in which MeCP2 recruits histone deacetylases to methylated DNA resulting in histone deacetylation, chromatin condensation and transcriptional silencing. Therefore, inhibition of histone deacetylases with TSA increases the acetylation level of histones and activation of gene transcription by restoring acetylated histones to the promoter region of a target gene. Dual treatment of cells with TSA and 5-azacytidine might lead to synergistic effects compared to the usage of individual drugs (Cameron et al., 1999a)**.**

2.21 Interleukin-3 (IL-3)

Recombinant human interleukin-3 (rhIL-3) is a haematopoietic growth factor with multilineage stimulatory activity *in vitro*. *In vivo*, a multilineage effect is observed showing an increase in leukocytes, neutrophils, eosinophiles, monocytes, reticulocytes and platelets. IL-3 is a glycoprotein that stimulates the proliferation and differentiation of multipotent as well as committed progenitors of various haematopoietic lineages (Saeland et al., 1988; Sonoda et al., 1988). In the body it is produced by T-lymphocytes, natural killer cells, mast cells and eosinophiles (Yang and Clark, 1990)**.** The human IL-3 has receptors (IL-3R) consisting of alpha and common beta (beta c) subunits (Kitamura and Miyajima, 1992). *In vitro* IL-3 promotes survival, proliferation and differentiation of multipotent haematopoietic stem cells and of the committed progenitors cells of the megakaryocytes, granulocyte/macrophage, erythroid, eosinophil, basophil and mast cell lineage (Leary et al., 1987). Furthermore, IL-3 has been shown to be a potent stimulator of the megakaryopoiesis *in vitro* (Bruno et al., 1988; Teramura et al., 1988). Studies in murine and primate models with recombinant human interleukine-3 (rhIL-3) showed an effect on myelopoiesis, megakaryopoiesis and erythtropoiesis (Wagemaker et al., 1990; Broxmeyer et al., 1987). In addition there was also a clear effect on basophiles and eosinophiles (Donahue et al., 1988b; Briddell et al., 1991). When IL-3 was administered for 7 days followed by GM-CSF for 4 days, a pronounced effect was observed on leukocytes with an increase in neutrophils, banded neutrophiles, eosinophiles, lymphocytes, monocytes, and platelets (Donahue et al., 1988a).

2.22 FGF-2 and BMP-2

Signals based on the release of Bone morphogenetic proteins (BMPs) and Fibroblast growth factors (FGFs) influence the genesis of many organs. Their ability to induce differentiation of adult stem/progenitor cells is of utmost importance. For instance, BMP-2 is implicated in heart induction because its inhibition blocks cardiogenesis. Mis-Expression of BMP-2 expands early cardiac gene expression into adjacent mesoderm. Both BMP-2 and FGF-2 synergistically augment heart induction and trigger proliferation of myocardial cells (Mima et al., 1995)**.** Investigations using early embryonic signals such as those indicated above might be very useful, as it might elicit a differentiation response in adult stem cells. For example, the cardiomyocyte potential of adult stem cells from neuronal, endothelial, liver and bone marrow haematopoietic or stromal cells can be investigated using these signals. The role of FGF-2 in inducing (Jiang et al., 2002c) and that of BMP-2 in inhibiting (Shou et al., 2000) neurogenesis has been documented. Moreover, BMP-2 initiates, promotes and regulates bone development, growth, remodelling and repair.

2.23 Hepatocyte Growth Factor/Scatter Factor (HGF/SF)

Developmental biologists have considered the liver to be a ´´mystery´´ organ because of the paucity of genetic data regarding its development. Such as the lungs, pancreas and intestine, the liver is also derived from gut premordia, making it difficult to discern patterning variants among natural animal populations. It has been reported that Sca-1 positive cells from the bone marrow differentiate into hepatocytes when treated with Hepatocyte growth factor (Okumoto et al., 2003). HGF/SF is a multifunctional polypeptide growth and motility factor whose receptor is a transmembrane tyrosine kinase, the c-met proto-oncogen product. Originally it was identified and characterized as two different factors, one with growth stimulatory activity (HGF) and the other with scatter factor activity (SF). These two activities were subsequently ascribed to the same factor (Clark, 1994)**.**

3 Objectives of the study

3.1 General objective:

The main objective of this study is to isolate, establish, culture, expand, characterise and follow the multilineage differentiation responses of mBM-MASCs1 and mBM-MASCs2 to various bioactive molecules *in vitro* as well as their contribution to the development of chimeric embryos *in vivo*.

3.2 Specific objectives:

The specific objectives of the present study are to:

- isolate and establish a permanent adult stem cell lines from mouse bone marrow
- characterize mBM-MASCs1 and mBM-MASCs2 with respect to surface molecules
- characterize mBM-MASCs1 and mBM-MASCs2 with respect to transcription factors that are expressed in adult pluripotent cells like ES cells
- assess the stable expression of the transgene in mBM-MASCs as a tool for genetic labelling as well as predicting their role as a cellular vehicle for administering genes of therapeutic value
- elucidate their differentiation potential into mesodermal lineages such as skeletal muscle using distinct Wnt signalling molecules and CA-LEF
- Analyse their differentiation potential into mesodermal lineages such as skeletal muscle using epigenetic drugs such as 5-azacytidine and Trichostatin A
- elucidate their differentiation potential into mesodermal lineages such as skeletal muscle after treatment with CDO
- Selection of myogenic lineages from mBM-MASCs1 and mBM-MASCs2 after infection with lenti-virus containing a skeletal muscle specific myogenin promoter that drives expression of nuclear eGFP and sorting eGFP positive cells after induction with wnt7A
- assess *in vitro* fusion potential of mBM-MASCs1 and mBM-MASCs2 after co-culture with the *bona fide* muscle cell line like C2C12
- elucidate their differentiation potential into mesodermal lineages such as cardiac muscle using wnt-11, other distinct wnts, CDO and CA-LEF signalling molecules.
- elucidate the dependence of cardiogenic wnt11 pathway on PKC activation

- elucidate their differentiation potential into mesodermal lineages such as cardiac muscle using epigenetic drugs such as 5-azacytidine and Trichostatin A
- elucidate their differentiation potential into mesodermal lineages such as cardiac muscle using recombinant FGF-2 and BMP-2
- elucidate their differentiation potential into mesodermal lineages such as osteocytes using recombinant BMP-2 and 5-azacytidine
- elucidate their differentiation potential into ectodermal lineages such as neuronal and non-neuronal cells like glial cells using recombinant FGF-2
- elucidate their differentiation potential into endodermal lineages such as hepatocytes using recombinant HGF/SF.
- assess the contribution of genetically labelled mBM-MASCs1 (mBM-MASCs1-eGFP) to development of chimeric embryos.

4 Results

4.1 Isolation, cultivation and culture expansion of mBM-MASCs

Two distinct permanent mouse bone marrow-derived multipotent adult stem cell populations (mBM-MASCs) have been isolated from two months old female ICR mice as described in (Prockop, 1997) by introducing some modifications.

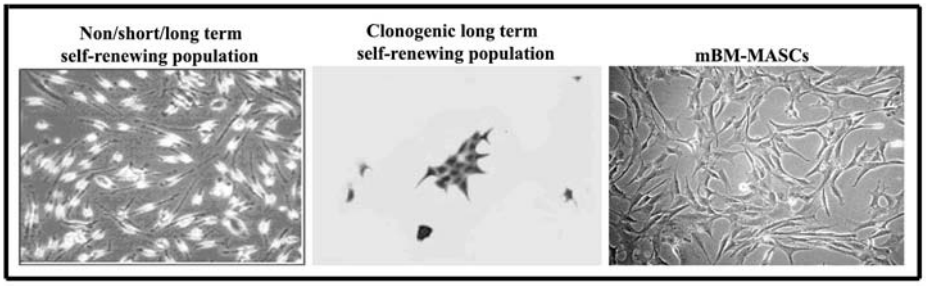

Fig. 1 Expansion of clonogenic long term self-renewing mBM-MASCs
Long term self-renewing mouse bone marrow multipotent adult stem cell (mBM-MASCs) could be enriched among none or short term self-renewing populations by differential adherence and survival, and the clonogenic long term self-renewing populations can then be expanded indefinitely using 10% FCS DMEM-LG.
Fig.1 Panel left to right, none or short or long term self-renewing population from primary preparation, clonally expanding long term self-renewing population after four weeks in culture and homogenous population of culture expanded mBM-MASCs.

The majority of non-adherent haematopoietic cells were eliminated during medium change (differential adherence). The majority of adherent cells containing none or short term self-renewing populations were eliminated after reaching confluency by keeping them without trypsinization for upto 4 weeks or until spot of newly expanding cells identified (differential survival). This modification resembles the concept of natural selection where dividing progenitor's cells can be eliminated by differentiation through cell cycle exit and exhaustion of nutrients. Therefore, only stem cell with long term self-renewing properties will eventually expand if present. At the beginning, the cells in the culture exhibited a phase of dormancy of nearly a month followed by exponential growth in one area of the plate and eventually expanded (Fig. 1). After a series of passages, mBM-MASCs became homogenous through a process of clonal expansion, and were devoid of non-adherent haematopoietic cells. These cells were expanded in 10% v/v FCS supplemented DMEM-LG without requiring additional growth promoting cytokines described in recent works (Jiang et al., 2002a; Jiang et al., 2002c). Microscopically, the two cell populations predominantly displayed large nuclei and scanty cytoplasm. To assess their expansion potential, equal numbers of starting populations were seeded where both populations reached confluence at the same time, suggesting that they have similar doubling time.

4.2 Characterization of mBM-MASCs

To define the phenotype of mBM-MASCs, both cell populations have been characterized with respect to surface molecule expression and pluripotency (stemness) markers.

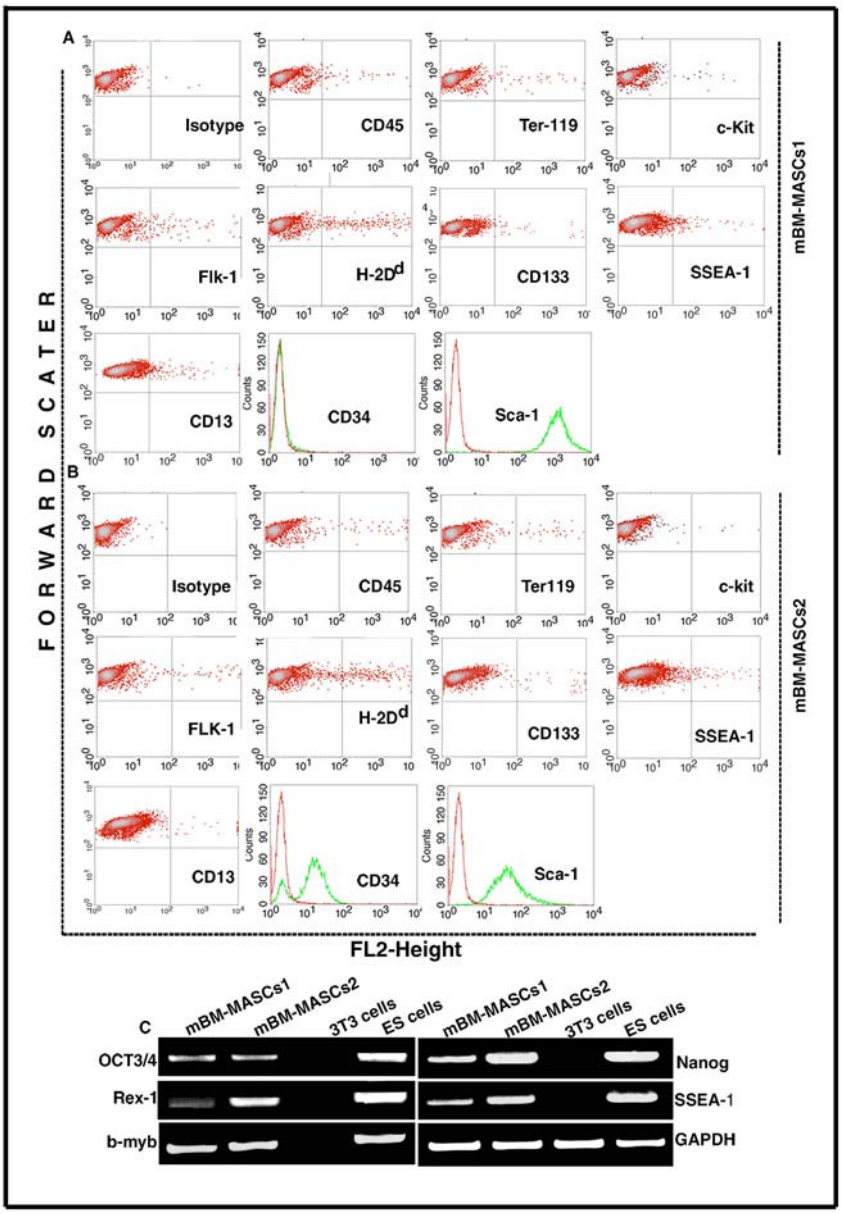

Fig. 2 Flowcytometric analysis of mBM-MASCs.
FACS analysis was performed on undifferentiated mBM-MASCs 1 and 2. The expression of CD45, Ter119/Glycophorin A, c-Kit, Flk-1, MHC1/H-2D^d), CD133/prominin, SSEA-1, CD13, CD34, and Sca-1 surface markers was analysed by direct staining with PE-conjugated monoclonal antibodies against these corresponding markers. The expression levels of all markers were virtually indistinguishable in both mBM-MASC 1 and 2, except for Sca-1 and CD34. The mBM-MASCs 1 expressed Sca-1 strongly but were negative for CD34, whereas mBM-MASCs 2 expressed Sca-1 moderately but were also positive for CD34.
Fig. 2A surface molecule expression of mBM-MASCs 1.
Top panel left to right, PE-Isotype-Control, PE-CD45, PE-Ter119/Glycophorin A, PE-c-Kit, **Middle panel left to right,** PE-Flk-1, PE-MHCI/H-2D^d, PE-CD133/prominin, PE-SSEA-1, **Bottom panel left to right,** PE-CD13, PE-CD34, and PE-Sca-1.
Fig. 2B Surface molecule expression of mBM-MASCs2 follows the same pattern as depicted in Fig. 1A.

Fig. 2C RT-PCR analysis of pluripotency markers, the expression levels of OCT3/4, Rex-1, B-Myb, Nanog, SSEA-1 and GAPDH (control) were analysed by standard PCR using cDNA synthesised from RNA isolated from 3T3 cells, ES cells and both stem cell populations.

According to the FACS analysis using the forward scatter (FSC) and sideward scatter (SSC), these cells contain a major population of large and moderately granular cells and a very minor population of small cells (not shown). Furthermore, immunocytometry for surface molecules as CD45, Ter119/Glycophorin A, c-Kit, Flk-1, MHC1/H-2D^{d}, CD133/prominin, SSEA-1, CD13, were virtually identical for both cell populations. However, mBM-MASCs1 did not express CD34 but expressed Sca-1 strongly. In contrast, mBM-MASCs2 expressed both CD34 and Sca-1 moderately. To demonstrate markers present at low concentration, dot blot graphics were used (Fig. 2A&B). Histogram graphics were used to show Sca-1 and CD34 expression in both cell populations (Fig. 2A&B). RT-PCR data showed detectable levels of transcripts of OCT3/4, Rex-1, B-Myb, Nanog, SSEA-1 in both cell populations and ES cells, but not in NIH3T3 fibroblast cells (Fig. 2C). However, when they differentiated, levels of these markers of stemness were virtually undetectable except for B-Myb which was still detectable albeit weakly (not shown).

4.3 Genetically labelled mBM-MASCs1 and mBM-MASCs2 express the lentiviral eGFP transgene stably

To assess stable expression of eGFP transgene in mBM-MASCs as a tool for genetic labelling as well as predicting their role as a cellular vehicle for administering genes of therapeutic value, a lentiviral expression packaging line was prepared following established protocols (Dull et al., 1998; Zufferey et al., 1998).

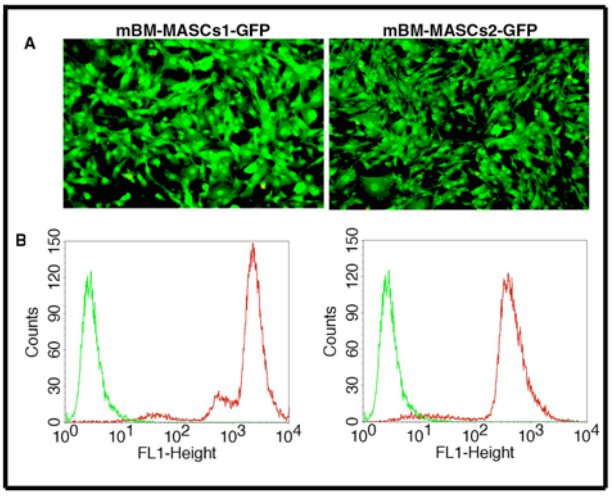

Fig. 3 Genetically labelled mBM-MASCs1 and mBM-MASCs2. Both cell populations were infected using lentiviral gene delivery system and the transduction efficiency determined by FACS analysis. The expression of

transgene was stronger in mBM-MASCs1-eGFP than mBM-MASCs2-eGFP. **Fig. 3A** Fluorescence microscopic analysis of eGFP expression. **Left panel,** mBM-MASCs1-eGFP **Right panel,** mBM-MASCs2-eGFP
Fig. 3B FACS analysis of eGFP expression, Histogram showing level of eGFP expression as measured in FL1-H Channel (x-axis). In all cases, green line shows eGFP negative control and red line shows eGFP positive cells. **Left panel,** mBM-MASCs1-eGFP **Right panel,** mBM-MASCs2-eGFP

Accordingly, after introduction into cultured mBM-MASCs1 and mBM-MASCs2 by lentiviral system, the expression of an eGFP transgene was found to be long term and stable after repeated cultivation and passaging. Initially, a transduction efficiency of 80-85% was achieved in these stem cells (not shown). The observed transduction efficiency was obtained by administering 1µl of viral suspension containing 10^8 infectious particles as determined by flowcytometry. To make the labelling more efficient, both lines were seeded at single cell concentrations using limited dilution and expanded. After FACS analysis of different sub-clones for highest expression of eGFP, those clone that completely expressed eGFP were termed mBM-MASCs1-eGFP and mBM-MASCs2-eGFP. The mBM-MASCs1-eGFP tended to show much higher eGFP fluorescence intensity than mBM-MASCs2-eGFP. This was evident both from fluorescence microscopy (Fig. 3A) and FACSs analysis (Fig. 3B) of the passaged cells.

4.4 Distinct Wnt signalling molecules and CA-LEF activate the myogenic program in mBM-MASCs

To investigate the capacity for commitment towards myogenic differentiation, a series of co-culture experiments of both mBM-MASCs and mitotically inactivated feeder cell lines producing wnt7a, wnt7b, wnt4 (Munsterberg et al., 1995), wnt11 signalling molecules and CA-LEF were initiated as described in the methods section. According to the microscopic observation, mBM-MASCs1 ($CD34^-/Sca\text{-}1^{high}$) changed their morphology from fibroblast-like to small, mono-nucleated cells after 7 days. The mBM-MASCs2 ($CD34^+/Sca\text{-}1^{moderate}$) needed 8 days for these differentiation-related morphologic changes. Neither giant, fused and multi-nucleated secondary myotubes nor contraction was observed under all experimental conditions (Fig. 4A & B).

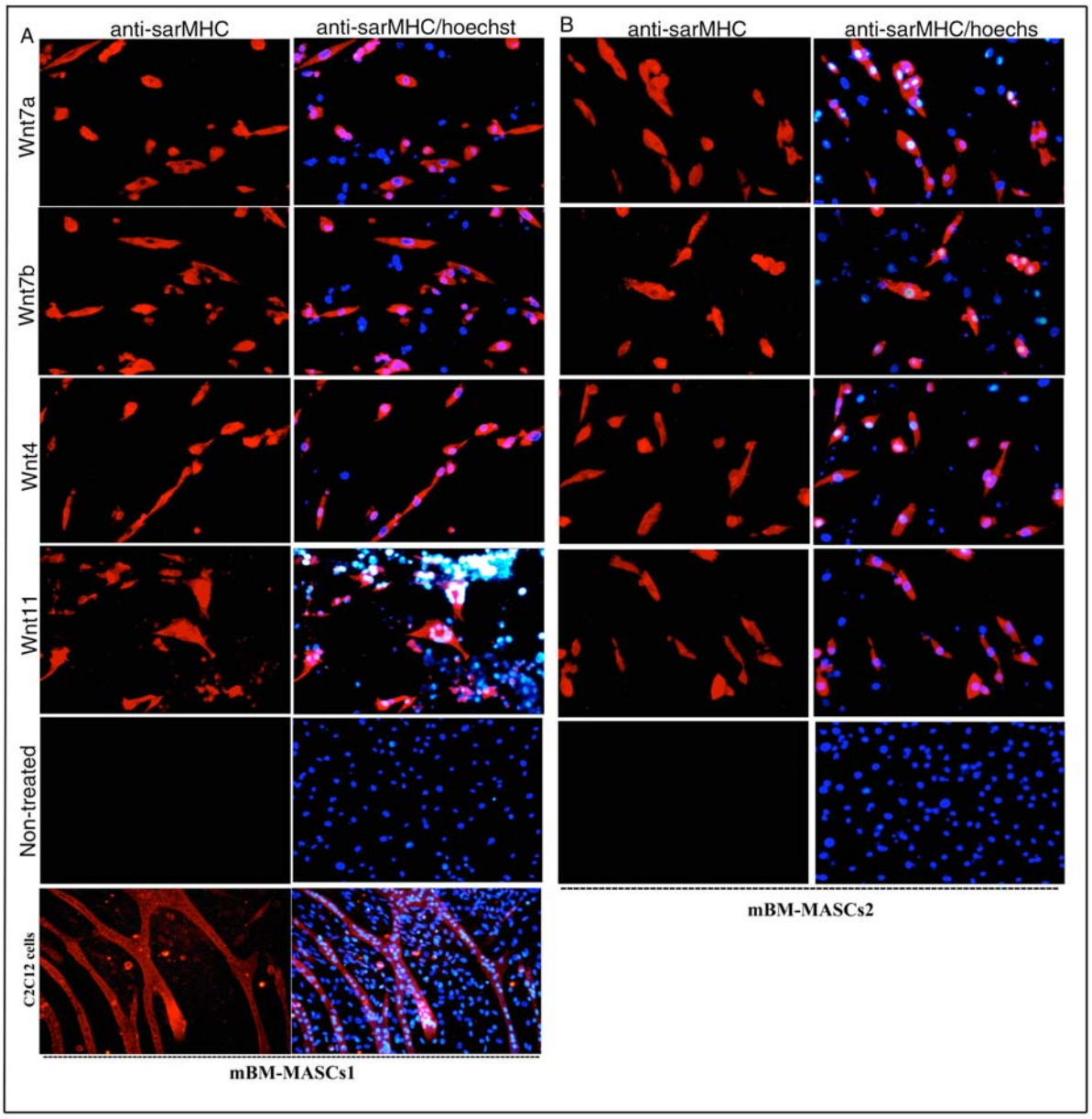

Fig. 4 Distinct vertebrate Wnt signalling molecules activate the myogenic program in mBM-MASCs
The mBM-MASCs 1 and 2 were co-cultured with amitotic retroviral packaging lines that produced wnt7a, wnt7b, wnt4 or wnt11 signalling molecules and CA-LEF. The cells were allowed to differentiate for 7 or 8 days. Myogenic cells were revealed by staining with an antibody against sarcomeric myosin heavy chain (MF20), followed by the secondary antibody Alexa594 anti-mouse and staining of the nuclei with Hoechst.
Fig. 4A Immunofluorescent staining for mBM-MASCs1
Panel Top to bottom represents wnt7a, wnt7b, wnt4, wnt11 and non-treated mBM-MASCs1 and C2C12 cells
Left panel, anti-sarcomeric MHC staining for mBM-MASCs 1, **right panel,** anti-sarcomeric MHC staining merged with Hoechst staining for mBM-MASCs1.
Fig. 4B Immunofluorescent staining for mBM-MASCs2
Panel Top to bottom represents wnt7a, wnt7b, wnt4, wnt11 and non-treated mBM-MASCs2.
Left panel, anti-sarcomeric MHC staining for mBM-MASCs2, **right panel,** anti-sarcomeric MHC staining merged with Hoechst staining for mBM-MASCs2

Immunofluorescent staining for sarcomeric myosin heavy chain protein using MF-20 antibody was positive for both lines exposed to each of the Wnt signalling molecules indicated above (Fig. 4A & B). The control (untreated mBM-MASCs) showed no MF-20

staining in both cell populations (Fig. 4A & B). Activation of the myogenic program was not observed in mBM-MASCs treated with conditioned medium containing Wnt molecules (not shown). However, a filter experiment which introduced a barrier with filters of different pore size (0.4, 3 and 8µm) where Wnt-secreting cells were placed at one side of the membranes and mBM-MASCs on the other also showed induction of the myogenic programme (Fig. 5A-L), suggesting that the induction required cell-cell contact but not cell fusion.

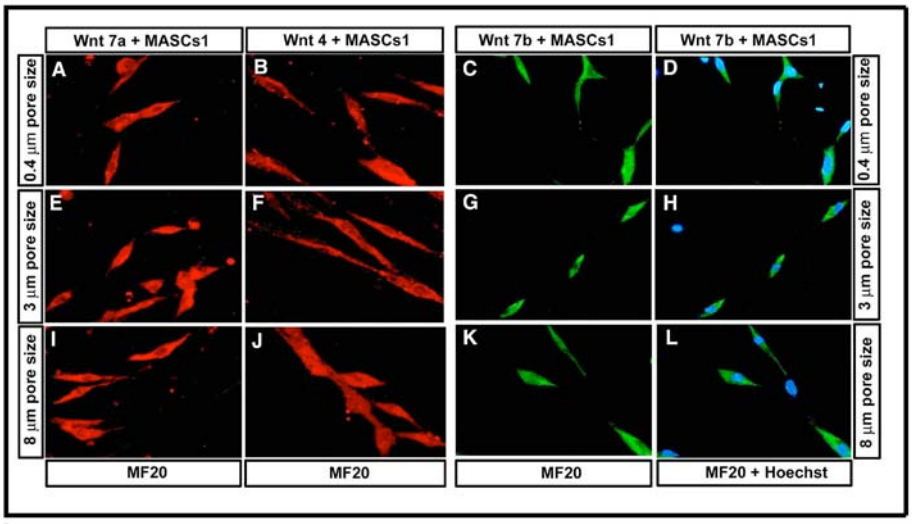

Fig. 5 Induction of sarcomeric MyHC expression in --- mBM-MASCs by Wnt-molecules does not require cell fusion. Wnt-secreting cells were placed at one side of membranes with 0.4 µm (A-D), 3µm (E-H) and 8 µm (I-L) pore sizes and the responding mBM-MASCs at the other side. After 7 to 8 days cultures were stained with the monoclonal MF20-antibody against sarcomeric myosin heavy chain and a secondary antibody coupled to Alexa594 (A, B, E, F, I, J) or Alexa488 (C, D, G, H, K, L). MyHC expression was indiscriminately activated by all Wnt molecules tested also when inducing cells did not pass the filter membrane (0.4 µm). In some experiments (D, H, L) nuclei were counter-stained with Hoechst33258 to locate all cells on the plate (D, H, and L).

RT-PCR analysis indicated mRNA expression of all four muscle specific transcription factors analyzed including Myf5 and MyoD, which are responsible for lineage determination or specification, Myogenin differentiation and lineage maintenance, and MRF4 (Myf6) terminal differentiation (Braun and Arnold, 1996).

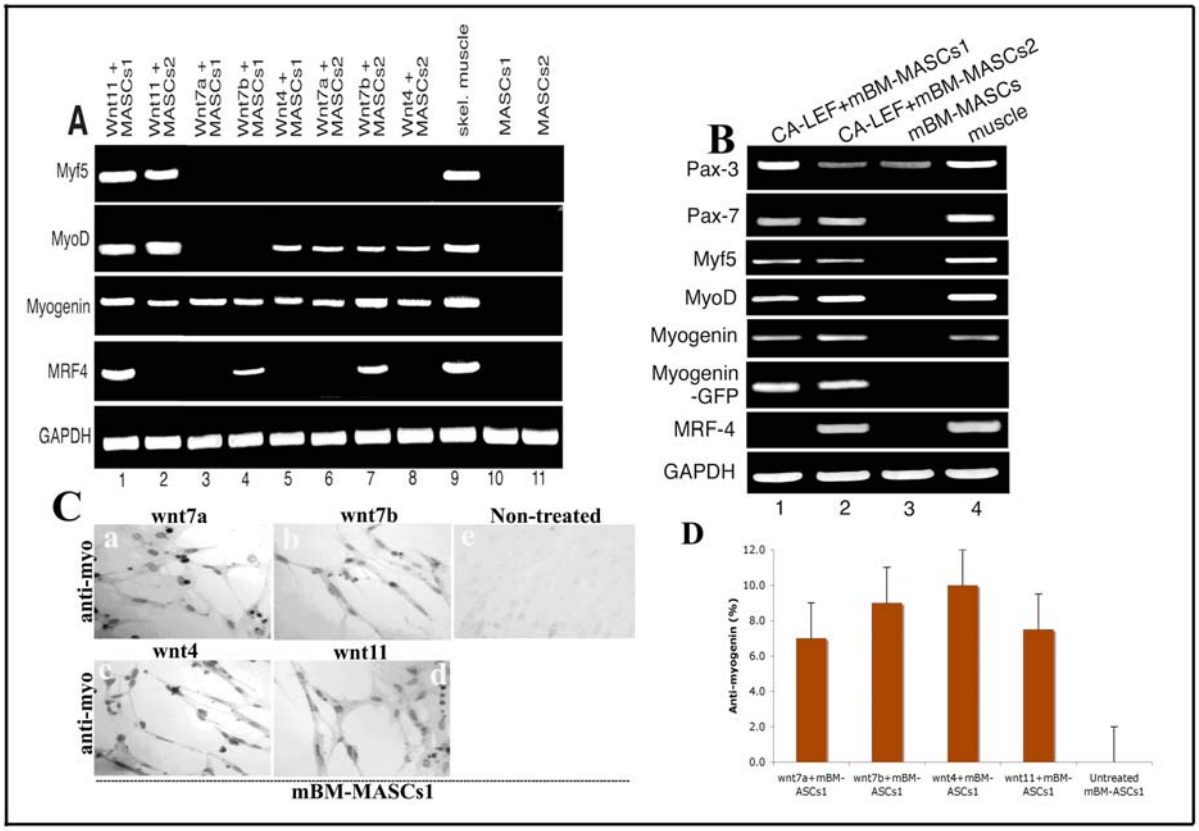

Fig. 6 Distinct Wnt molecules and CA-LEF induce the expression of muscle marker mRNAs differentially and myogenin can be detected by immuno staining 5-6 days after induction of mBM-MASCs.
RT-PCR analysis of muscle specific markers induced by wnt7a, wnt7b, wnt4 and wnt11 (A) and CA-LEF (B) in mBM-MASCs1 and mBM-MASCs2 The expression levels of transcripts of Pax-3, Myf5, MyoD, Pax-7, myogenin, myogenin-eGFP, MRF4, and GAPDH (control) were analyzed by standard PCR using cDNA synthesized from RNA isolated from muscle and both cell populations with and without induction.
Fig. 6C Immunofluorescent staining of myogenin for mBM-MASCs1
Panel Top left to right, represent wnt7a, wnt7b, and non-treated mBM-MASCs1.
Panel bottom left to right, represent wnt4 and wnt11. The staining of myogenin was done using anti-myogenin antibody that stains the nucleus and visualized by DAB plus 1% NiCl.

A closer look into the expression profiles of muscle specific genes revealed a varying expression of Myf5, MyoD and MRF4 (Myf6) and constitutive expression of myogenin (Fig. 6A&B). Myf5 and MyoD were detected in both cell populations after wnt11 and CA-LEF treatments. In general, mBM-MASCs2 expressed more myogenic factors after Wnt stimulation. In addition, MyoD expression was induced at higher levels by wnt7a, b, and 4 in mBM-MASCs2, but only with wnt4 in mBM-MASCs1 (Fig. 6A). The expressions of all four myogenic factors were observed in mBM-MASCs1 treated with wnt11 molecule as well as in mBM-MASCs2 treated with CA-LEF, thereby differing from other treatments (Fig. 6A&B). The expression of pax7 was also observed after treatment with CA-LEF. A weak expression of pax3 was found in untreated populations but markedly increased after CA-LEF treatment (Fig. 6B). Since the feeder cells secreting distinct wnts were mitotically inactive, they decreased significantly at the end of the incubation period (7

or 8 days). The contribution of RNA from the feeder cells that were co-cultured with both populations of cells was virtually negligible. None of the myogenic factors were detected in RNA isolated from feeder cells alone (not shown).

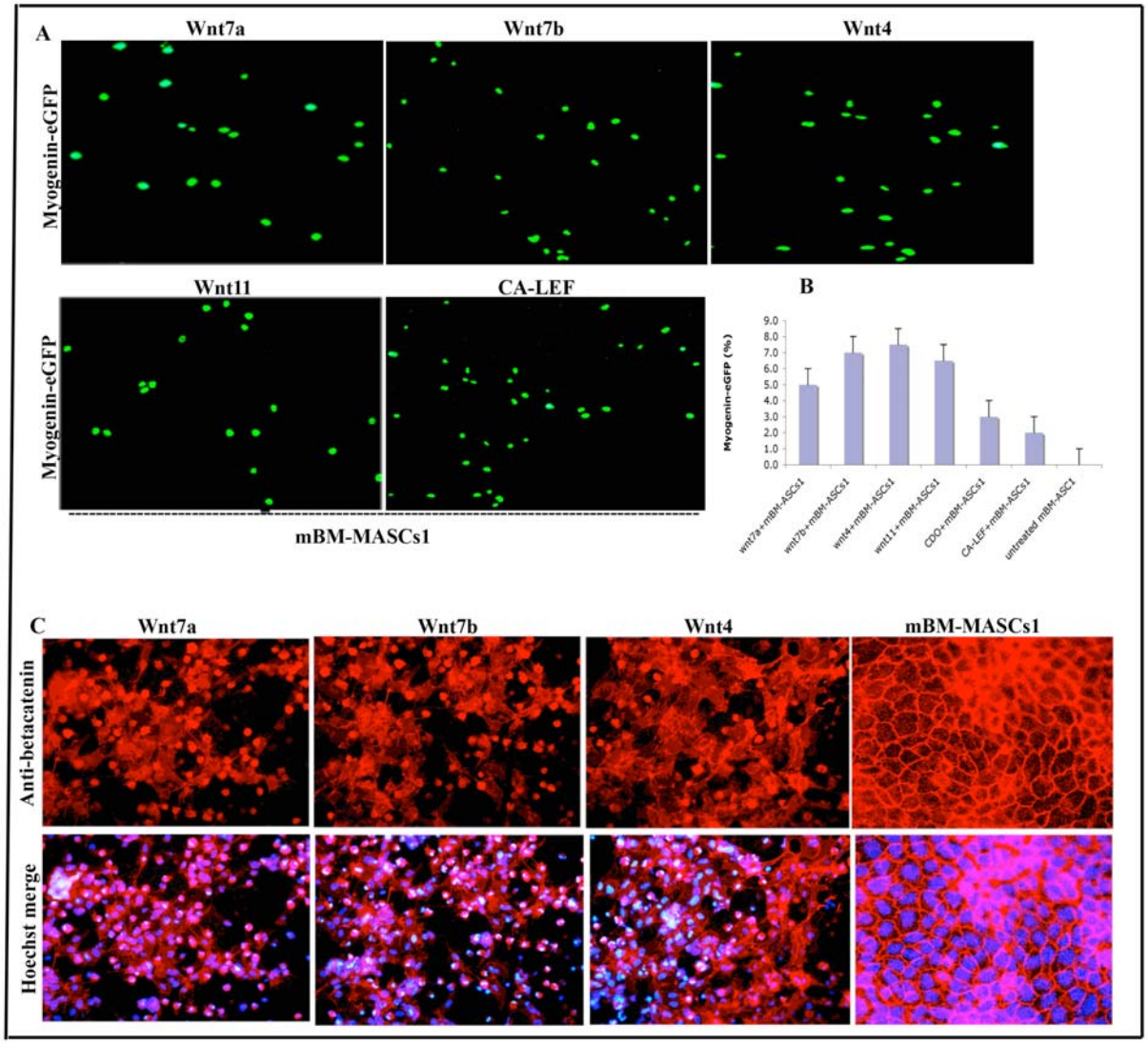

Fig. 7 Distinct Wnt molecules and CA-LEF induced the expression of nuclear eGFP driven by myogenin promoter and localized β-catenin into the nucleus of mBM-MASCs 4-6 days after induction.
Fig. 7A myogenin promoter driven nuclear eGFP expression of mBM-MASCs1
Top panel left to right, nuclear eGFP expression of mBM-MASCs1 after induction with wnt7a, 7b and wnt4.
Bottom panel left to right, nuclear eGFP expression of mBM-MASCs1 after induction with wnt11, and CA-LEF
Fig. 7B Comparison of the expression level of eGFP driven by myogenin promoter, mBM-MASCs1 and 2 were infected with lentiviral gene delivery system carrying the myogenin-eGFP construct and induced with different wnts, CDO and CA-LEF and FACS analysed four days after induction.
Fig. 7C Immunofluorescent staining of β-catenin for mBM-MASCs1
Top panel left to right, Staining of mBM-MASCs1 for β-catenin after induction with wnt7a, 7b and wnt4.
Bottom panel left to right, Staining of mBM-MASCs1 for β-catenin after induction with wnt7a, 7b and wnt4 and merged with Hoechst staining.

Although I have not looked into the expression pattern on a daily basis to identify which gene was expressed first and which followed, immuno staining for myogenin revealed an expression within 4-5 days (Fig. 6C and 7A). The percentage of cells that went into

myogenic programme varied between different inductive signals as assessed by a lentivirus containing the myogenin promoter that drives eGFP and induction with distinct wnts, CDO and CA-LEF (Fig. 7B). The level of inductions ranged from 1.5% for CA-LEF to nearly 8% for wnt4. A reporter construct containing myogenin promoter controlling the expression of nuclear eGFP delivered by lentiviral infection showed expression of eGFP in the nucleus of both cell populations induced by distinct wnts and CA-LEF (Fig. 7A). The subcellular localization of β-catenin was analyzed. As expected, I found nuclear localization of β-catenin in treated and membrane localization in untreated mBM-MASCs (Fig. 7C). The detection of myogenin by RT-PCR (Fig. 6A&B), immunostaining (Fig. 6C) and reporter gene expression (Fig. 7A) corroborated the activation of the myogenic programme. Together, my data suggest initiation of the skeletal muscle differentiation program in mBM-MASCs by inductive signals mediated by wnts and CA-LEF.

4.5 5-azacytidine and/or Trichostatin A activate the myogenic program in mBM-MASCs

To evaluate whether an inhibitor of DNA methylation, 5-azacytidine (AZA) or an inhibitor of histone deacetylase, Trichostatin A (TSA) or a combination of both drugs activate the myogenic programme, mBM-MASCs1 and mBM-MASCs2 were treated with different concentrations of AZA (5, 10 and 15 μM), TSA (0.1, 0.3 and 0.9 μM) and a combination of both drugs for 24 hrs as described in material and methods.

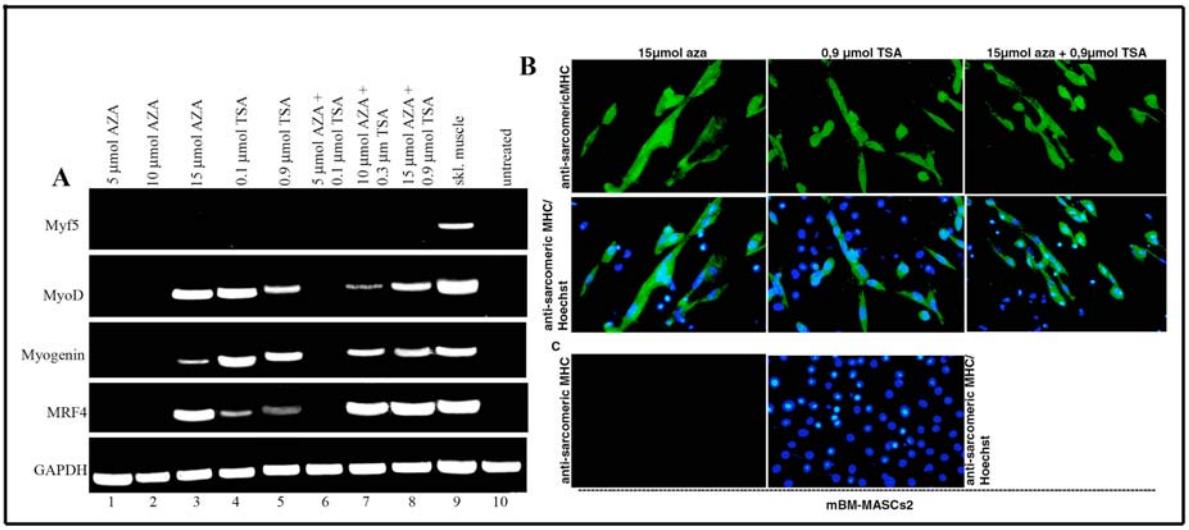

Fig. 8 Hypomethylating (5-azacytidine=AZA) or acetylating (trichostatin A=TSA) drugs activate the myogenic program in mBM-MASCs

Fig. 8A RT-PCR analysis of mBM-MASC2 treated with different concentrations of AZA or TSA or a combination of both for 24 hrs. The expression levels of transcripts of Myf5, MyoD, myogenin, MRF4, and GAPDH (control) were analysed by standard PCR using cDNA synthesized from RNA isolated from muscle and mBM-MASC2 with and without induction.

Fig. 8B MF-20 staining of mBM-MASCs2, followed by the secondary antibody Alexa488, after treatment with AZA, TSA or AZA+TSA
Top panel left to right, MF-20 staining for mBM-MASC2 treated with 15 µmol AZA, 0.9 µmol TSA and a combination of 15 µmol AZA and 0.9 µmol TSA
Bottom panel left to right, MF-20 staining for mBM-MASC2 treated with 15 µmol AZA, 0.9 µmol TSA and a combination of 15 µmol AZA and 0.9 µmol TSA merged with Hoechst nuclear staining.
Fig. 8C MF-20 staining of mBM-MASCs2 not treated with either of AZA, TSA or both.
Panel left to right, MF-20 staining and MF-20 staining merged with Hoechst nuclear staining.

Although the level of cell differentiation marker differed, all bHLH myogenic factors with the exception of Myf5 were detected by RT-PCR. The presence of AZA at 15 but not at 5 and 10 µmol favoured initiation of the myogenic program. TSA showed similar results at 0.1 and 0.9 µmol. Similarly, a combination of both drugs at 10+0.3 and 15+0.9 but not at 5+0.1 µmol (Fig. 8A) increased several myogenic marker molecules. In summary, myogenic factors were expressed at different concentrations in the presence of TSA or of TSA and AZA. In all cases, weaker expression of Myogenin was followed by strong expression of MRF-4 and vice versa. Furthermore, immunofluorescent staining for sarcomeric MHC (MF-20) showed a positive staining (Fig. 8B) suggesting that both drugs induced the myogenic conversion. Control cells (untreated mBM-MASCs) grew faster than those treated with AZA, TSA or both drugs and were negative for MF-20 staining (Fig. 8C). Cells treated with TSA or a combination of AZA and TSA grew slower than AZA treated cells. Similar results were obtained with mBM-MASCs1 (not shown).

4.6 CDO positively mediates myogenesis in mBM-MASCs

To investigate the role of CDO during stem cell differentiation, mBM-MASCs1 and mBM-MASCs2 were co-cultured with mitotically inactive phoenix feeder cells that produced amphotrophic retroviruses containing CDO for 7 or 8 days, and were analysed as described in the methods.

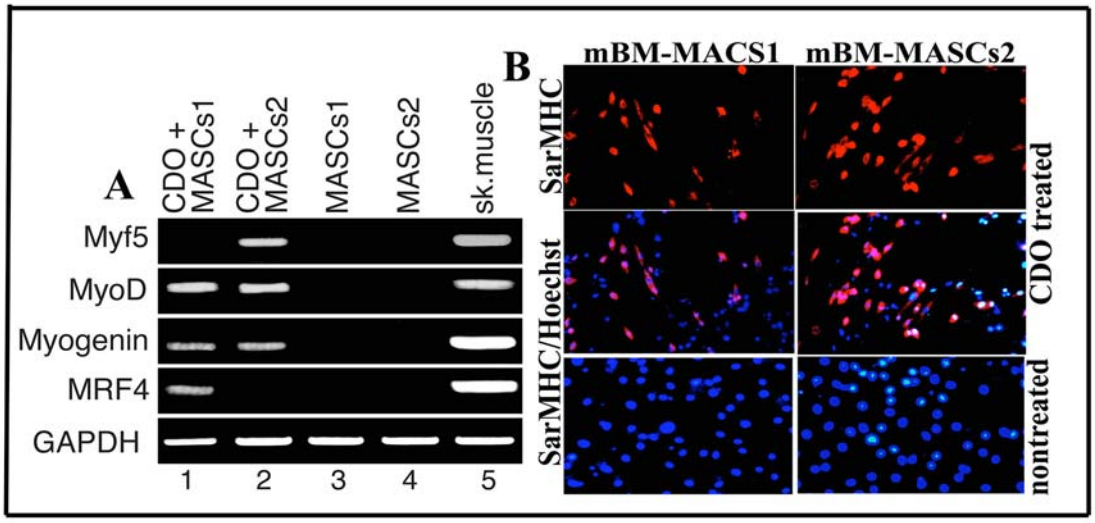

Fig. 9 CDO mediated activation of the myogenic program in mBM-MASCs 1 or 2
The mBM-MASCs were co-cultured with CDO producing cells for 7 or 8 days.
Fig. 9A RT-PCR analysis, the expression levels of transcripts of Myf5, MyoD, myogenin, MRF4 and GAPDH (control) were analyzed by standard PCR using cDNA synthesized from RNA isolated from muscle and both cell populations with or without treatment.
Fig. 9B Immunofluorescent staining of sarcomeric MHC for mBM-MASCs1 and 2 treated with or without CDO. **Top panel,** sarcomeric MHC staining of mBM-MASCs1&2 treated with CDO. **Middle panel,** sarcomeric MHC staining of mBM-MASCs1&2 treated with CDO merged with Hoechst staining. **Bottom panel,** sarcomeric MHC staining of mBM-MASCs1&2 without CDO treatment merged with Hoechst staining.

Under the conditions used in this study, mBM-MASC1 and mBM-MASCs2 cultures expressed MyoD and Myogenin after co-culture with cells producing CDO (Fig. 9A). Moreover, immunofluorescent staining for sarcomeric MyHC (MF-20) revealed positive staining for both cell lines suggesting an induction of myogenesis by CDO in mBM-MASCs (Fig. 9B). Control cells (mBM-MASCs without CDO) did not show any sign of myogenesis. CDO treated cells displayed a morphology different from the undifferentiated parental cells. Taken together, these data show that CDO can trigger expression of muscle specific genes.

4.7 Establishment of myogenic lineages from uncommitted mBM-MASCs1 and mBM-MASCs2

To isolate the responding cells, mBM-MASCs1 and mBM-MASCs2 were infected with reporter construct expressing eGFP under the transcriptional control of a skeletal muscle specific myogenin promoter, which allows isolation of responding cells by FACS.

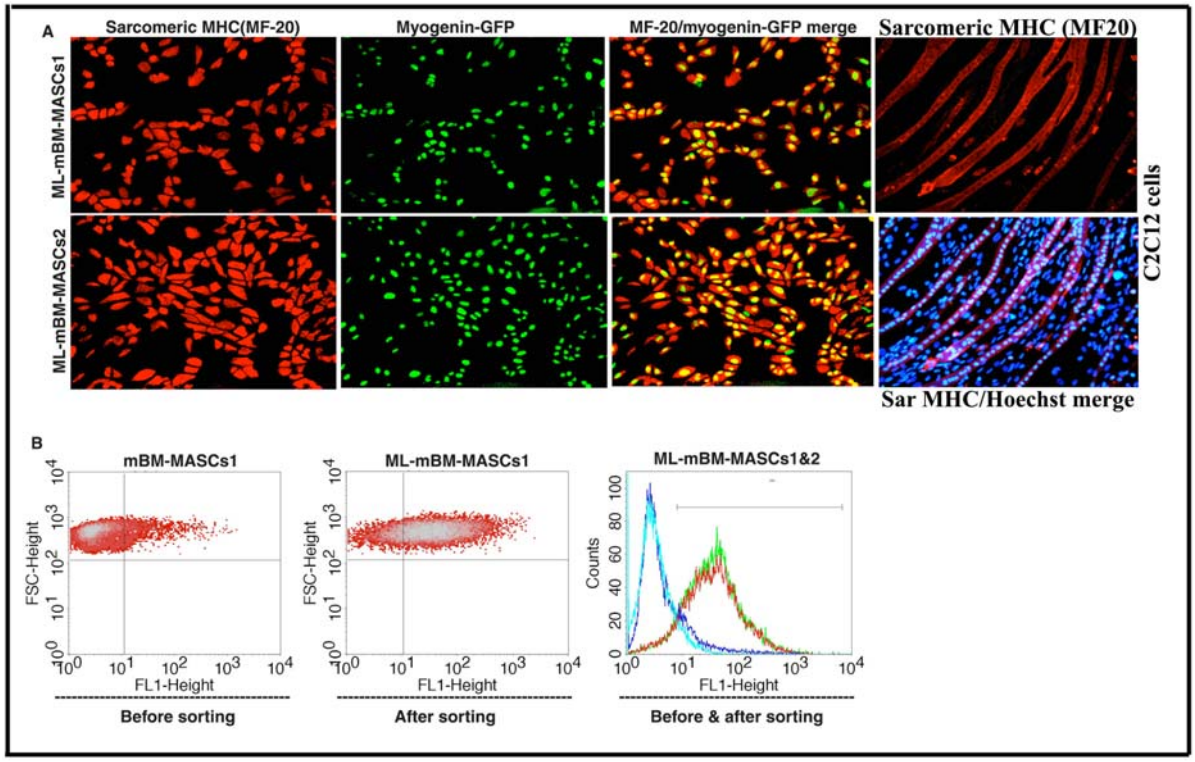

Fig. 10 Myogenic lineages can be established from mBM-MASCs1 and mBM-MASCs2. Induction with the wnt-7a signalling molecule resulted in the expression of nuclear eGFP in both mBM-MASCs1 and mBM-MASCs2 within 4 days after induction and these eGFP positive cells were sorted by FACS.
Fig, 10A Immuno fluorescent staining for MF-20 in ML-mBM-MASCs1, ML-mBM-MASCs2 and C2C12 cells **Left,** MF-20 staining for ML-mBM-MASCs1&2 after being cultivated in 2% horse serum for 7 or 8 days **Middle,** ML-mBM-MASCs1&2 expressing nuclear eGFP. **Right,** MF-20 staining for ML-mBM-MASCs1&2 merged with ML-mBM-MASCs1&2 expressing nuclear eGFP. **Far right top,** MF-20 staining for C2C12 cells and **far right bottom,** MF-20 staining for C2C12 cells merged with Hoechst staining
Fig. 10B FACS analysis of ML-mBM-MASCs1 and ML-mBM-MASCs2 before and after sorting, the histogram shows difference between sorted, unsorted and negative control cells.
Left panel, wnt7a induced mBM-MASCs1 before sorting. **Middle panel,** wnt7a induced mBM-MASCs1 after sorting (ML-mBM-MASCs1). **Right panel,** Histogram showing wnt7a induced mBM-MASCs1 and mBM-MASCs2 before sorting (strong blue mBM-MASCs1and light blue, negative control) and after sorting (yellow, ML-mBM-MASCs1 and red, ML-mBM-MASCs2).

Although both mBM-MASCs1 and 2 respond to inductive stimuli by expression of myogenic marker molecules, the response seemed to be limited to a subset of cells (~ 8% at best). The lentivirus carrying this tissue specific reporter construct (see materials for detail) was designed to express eGFP in the nucleus. Induction with the wnt-7a signalling molecule delivered through co-culture with mitotically inactive feeder cells resulted in the expression of nuclear eGFP in both mBM-MASCs1 and mBM-MASCs2 within 4 days after induction (Fig. 10A). Before sorting, the fractions of positive cells were 20% and 15% for mBM-MASCs1 (Fig. 10B) and mBM-MASCs2 (not shown) respectively. After sorting most cells were still viable and could be expanded easily. The eGFP expression was stably maintained at the initial level of 85% even after repeated expansion and passaging (Fig. 10B). When kept in a differentiation medium (DMEM-LG supplemented with 2% horse serum) for 7 days, the

majority of sorted cells became positive for sarcomeric MyHC (MF-20) (Fig. 10A). They also fused more efficiently than the parental cells with the *bona fide* muscle line C2C12 (Fig. 11B, C & D).

4.8 The mBM-MASCs1, mBM-MASCs2, ML-mBM-MASCs1 and ML-mBM-MASCs2 fuse with C2C12 cells *in vitro*

To investigate fusion potential of mBM-MASCs *in vitro*, mBM-MASCs1-eGFP, mBM-MASCs2-eGFP, ML-mBM-MASCs1 and ML-mBM-MASCs2 were co-cultured with C2C12 cells. Each cell line was seeded at the same time at an equal cell density together with C2C12 cells and analysed for cell fusion.

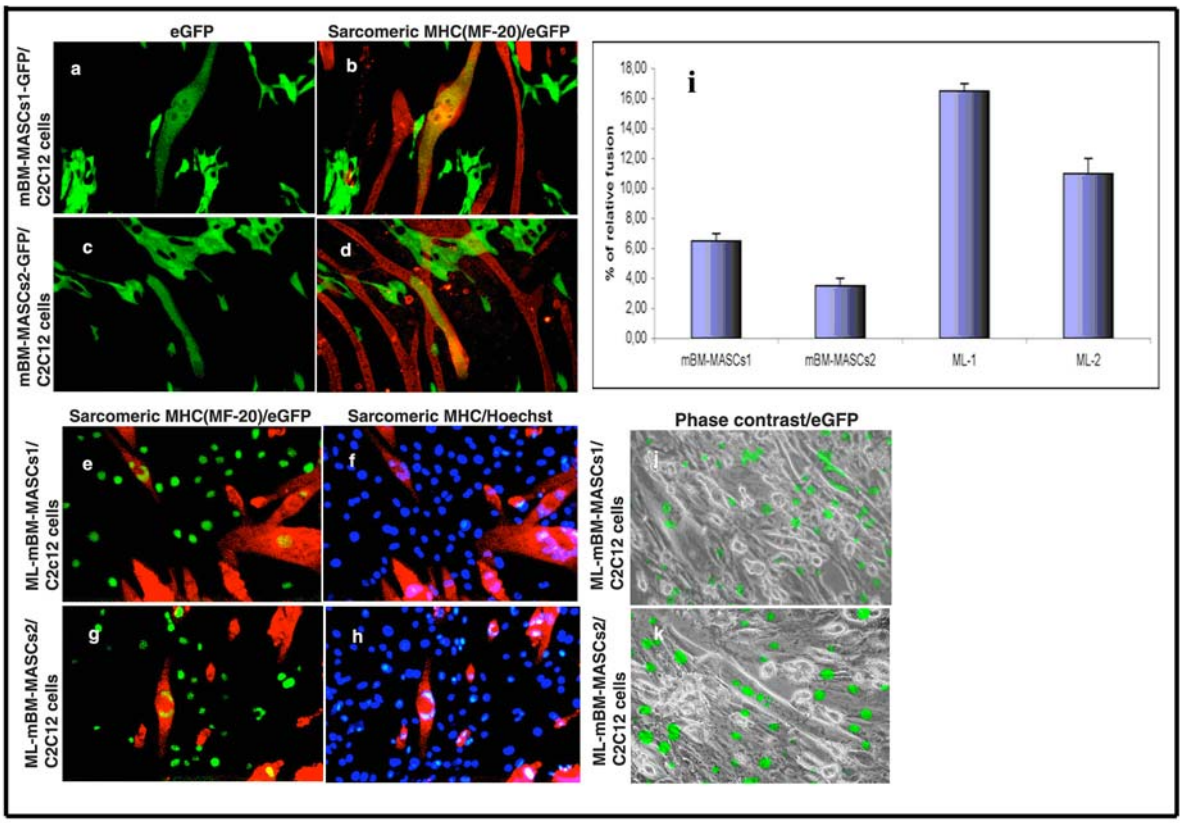

Fig. 11 Assessment of the fusion potential of mBM-MASCs1-eGFP, mBM-MASCs2-eGFP, ML-mBM-MASCs1 and ML-mBM-MASCs2 cells by co-culture with C2C12 cells. Fusion of uncommitted mBM-MASCs1-eGFP and mBM-MASCs2-eGFP cells co-cultured with C2C12 cells **(a-d)**. The eGFP expression in hybrid myotube and mBM-MASCs1-eGFP and mBM-MASCs2-eGFP cells **(a, c)** eGFP expression in hybrid myotube and mBM-MASCs1-eGFP cells and mBM-MASCs2-eGFP cells merged with MF-20 staining **(b, d).**
Fusion of ML-mBM-MASCs1 and ML-mBM-MASCs2 co-cultured with C2C12 cells **(e-h)**. MF-20 staining of hybrid, non-hybrid myotubes and ML-mBM-MASCs1 **(e)** ML-mBM-MASCs2 **(g),** Hoechst staining merged with MF-20 staining of hybrid, non-hybrid myotubes and ML-mBM-MASCs1 **(f)** ML-mBM-MASCs2 **(h).**
Comparison of fusion efficiency between mBM-MASCs1-eGFP, mBM-MASCs2-eGFP, ML-mBM-MASCs1 and ML-mBM-MASCs2 cells after MF-20 staining **(i),** for ML-mBM-MASCs1 and ML-mBM-MASCs2 number of nuclear eGFP in MF-20 positive hybrid myotubes was counted in 6 well plates. For mBM- MASCs1-eGFP and mBM-MASCs2-eGFP, number of MF-20 positive hybrid myotubes was counted in 6 well plates.
Phase contrast microscopy of hybrid and non-hybrid myotubes merged with ML-mBM-MASCs1 **(j)** and ML-mBM-MASCs2 **(k).**

Uncommitted mBM-MASCs1-eGFP and mBM-MASCs2-eGFP were marked with eGFP expressed under the transcriptional control of the human ubiquitin C promoter, whereas the myogenic lineages, ML-mBM-MASCs1 and ML-mBM-MASCs2 were marked with eGFP that is expressed under the transcriptional control of the myogenin promoter. Fusion of mBM-MASCs derived cells with C2C12 cells was identified by the presence of nuclear eGFP in the fused hybrid myotube. The number of nuclear eGFP ranged from one to 6 in a single fused hybrid myotube. The extent of fusion was determined by staining for sarcomeric MyHC and counting the number of double positives (nuclear eGFP in MF-20 positive hybrid myotubes) in case of fusion of myogenic lineages with the C2C12 or (cytoplasmic eGFP in MF-20 positive hybrid myotubes) in case of uncommitted mBM-MASCs1 and 2-eGFP (Fig. 11a-d and e-h). Interestingly, ML-mBM-MASCs1 and ML-mBM-MASCs2 tended to fuse more readily than uncommitted parental lines mBM-MASCs1-eGFP and mBM-MASCs2-eGFP (Fig. 11i). The highest fusion rate was achieved with ML-mBM-MASCs1. Both uncommitted parental lines also displayed some fusion potential with the C2C12 cells (Fig 11a-d). The eGFP expression was displayed throughout the hybrid myotube because of the cytoplasmic expression of eGFP in parental lines as opposed to fusion with the myogenic lineages, which was nuclear (Fig 11a-d, e-h & j-k). In summary, the data from the *in vitro* differentiation and fusion experiments indicate that mBM-MASCs1-eGFP, mBM-MASCs2-eGFP; ML-mBM-MASCs1 and ML-mBM-MASCs2 possess a certain myogenic differentiation as well as fusion capacity *in vitro*.

4.9 Induction of cardiomyocyte differentiation in mBM-MASCs by Wnt11 but not CDO, wnt7a, wnt7b, wnt4 and CA-LEF

To investigate the activation of the cardiomyogenic program in mBM-MASCs, co-culture experiments of mBM-MASCs and mitotically in active feeder cell lines producing wnt7a, wnt7b, wnt4 (Munsterberg et al., 1995) CA-LEF, wnt11, CDO or a combination of both wnt11 and CDO were initiated.

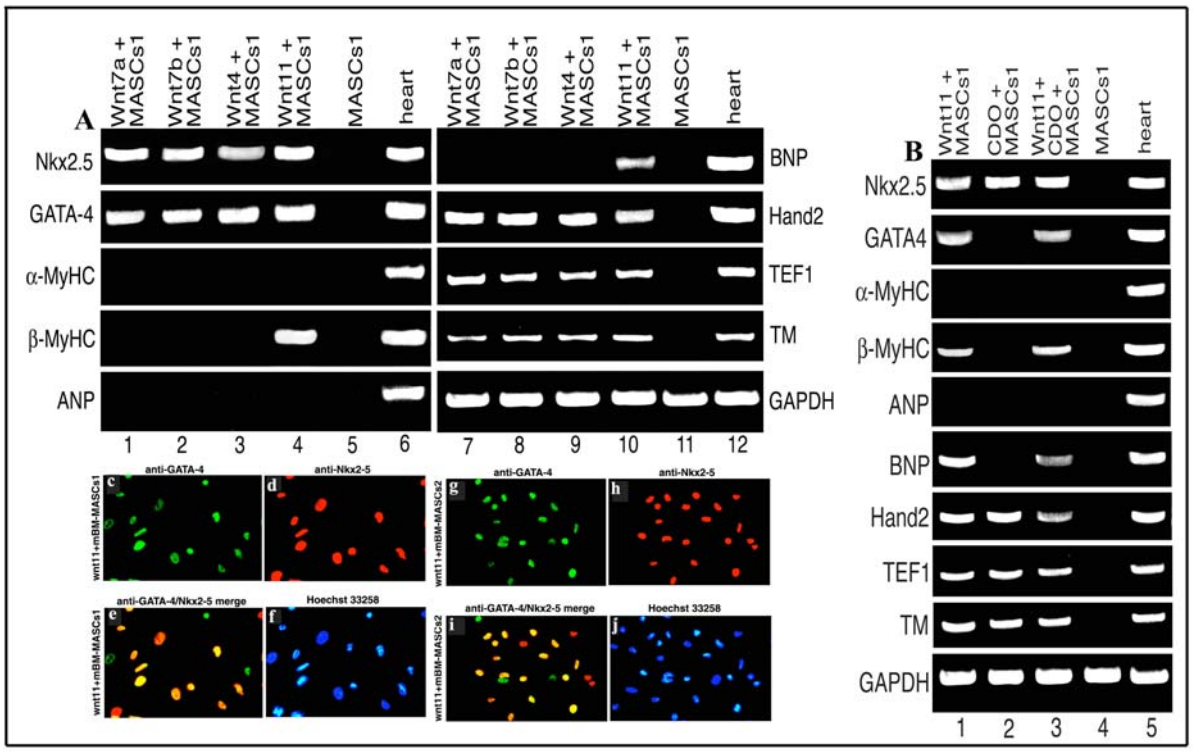

Fig. 12 Only wnt11 but not wnt7a, wnt7b, wnt4, CA-LEF and CDO induced cardiac markers in mBM-MASCs 1 and 2.
mBM-MASCs were co-cultured with wnt7a, wnt7b, wnt4, CA-LEF, wnt11, CDO or a combination of wnt11 and CDO producing cells for 7-8 days.
The expression of Nkx2-5, GATA-4, α-MHC, β-MHC, ANP, BNP, Hand2, Tef-1, Tropomyosin and GAPDH (control) were analysed by standard PCR using cDNA synthesised from RNA isolated from heart and both cell populations with or without treatment but shown for mBM-MASCs1 **(A&B).**
(c-j) double immunofluorescent staining of GATA-4 and Nkx2-5 for mBM-MASCs1 and 2 treated with wnt11. GATA-4 **(c)** and Nkx2-5 **(d)** staining, GATA-4/Nkx2-5 staining merged **(e)** and Hoechst 33258 staining **(f)** for mBM-MASCs1. GATA-4 **(g)** and Nkx2-5 **(h)** staining, GATA-4/Nkx2-5 staining merged **(i)** and Hoechst 33258 staining **(j)** for mBM-MASCs2.

The treatment with wnt7a, wnt7b, wnt4 (Fig. 12A) and CA-LEF (not shown) did not induce expression of cardiomyocyte markers such as α-MHC, β-MHC, ANP and BNP. However, RT-PCR data showed transcripts for Nkx2-5, GATA-4, β-MHC, BNP and Hand2 in mBM-MASCs1, which received wnt11 signals either alone, or in combination with CDO, but not in cells which received CDO alone or the untreated mBM-MASCs1 (Fig. 12A&B). Surprisingly, however, the expression of Nkx2-5, GATA-4, Hand2, Tef-1 and tropomyosin did not vary significantly between distinct wnts (Fig. 12A&B).

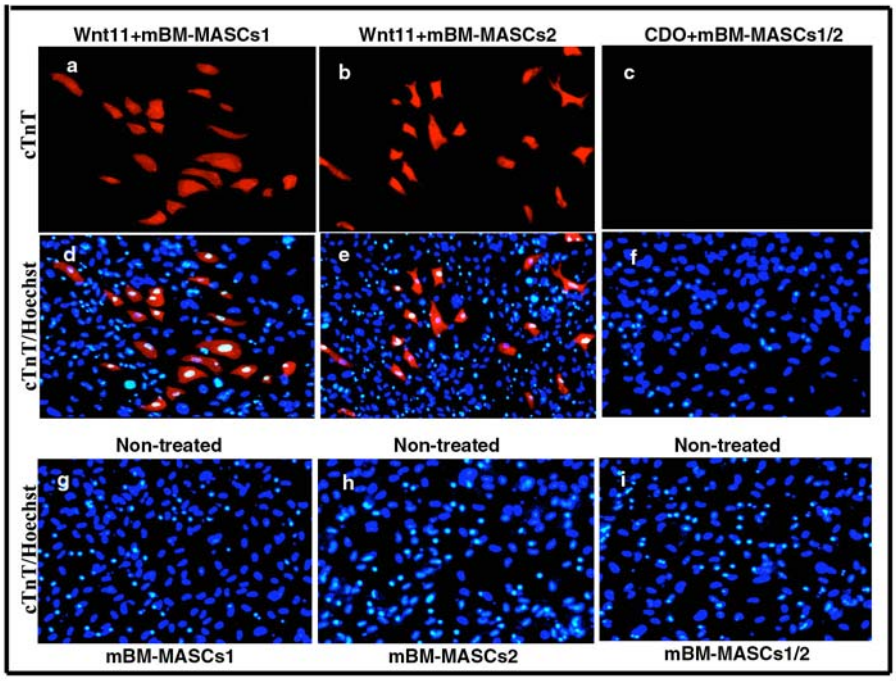

Fig. 13 Immunofluorescent staining of induced mBM-MASCs for cTnT.
Immunofluorescent staining for cTnT with or without treatment of mBM-MASCs1&2 with either wnt11 or CDO molecule **(a-i)**. Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 **(a),** mBM-MASCs2 **(b)** with wnt11 and mBM-MASCs1/2 with CDO **(c)** Hoechst staining merged with immuno fluorescent staining for cTnT after treatment of mBM-MASCs1 **(d),** mBM-MASCs2 **(e)** with wnt11 and mBM-MASCs1/2 with CDO **(f).** Hoechst staining merged with immuno fluorescent staining for cTnT without treatment of mBM-MASCs1 **(g),** mBM-MASCs2 **(h)** with wnt11 and mBM-MASCs1/2 with CDO **(i).**

Neither contraction nor expression of transcripts of ANP and α-MHC was observed in any of the treated lines. Nkx2-5 and GATA-4 antibodies stained both cell populations after treatment with wnt11. The majority of cells co-expressed both transcription factors as revealed by double staining using these antibodies. However, there were individual cells expressing each transcription factor alone (Fig. 12c-i). The expression of cTnT was observed using immunofluorescent staining after wnt11 but not CDO treatment (Fig. 13a-i). Taken together these data suggest that only wnt11 but not CDO, wnt7a, wnt7b, wnt4 and CA-LEF induce differentiation towards cardiomyocyte-like cells. It should be pointed out that the induction of differentiation was incomplete, probably due to the absence of crucial regulatory factors.

4.10 Inhibition of PKC activity attenuates the effect of wnt-11 and abrogates cardiomyocyte gene expression in mBM-MASCs1 and mBM-MASCs2

The induction of the cardiomyocyte programme by wnt-11, a supposedly non-canonical Wnt signalling molecule had been suggested to depend on the activity of PKC (Pandur et al., 2002). To reveal the role of PKC for the induction of the cardiomyogenic program, both

mBM-MASCs1 and mBM-MASCs2 were treated with different concentrations of two PKC inhibitor compounds, Staurosporine (8 nM) and Bisindolylmalemeimide I (0.1, 1 and 2μM).

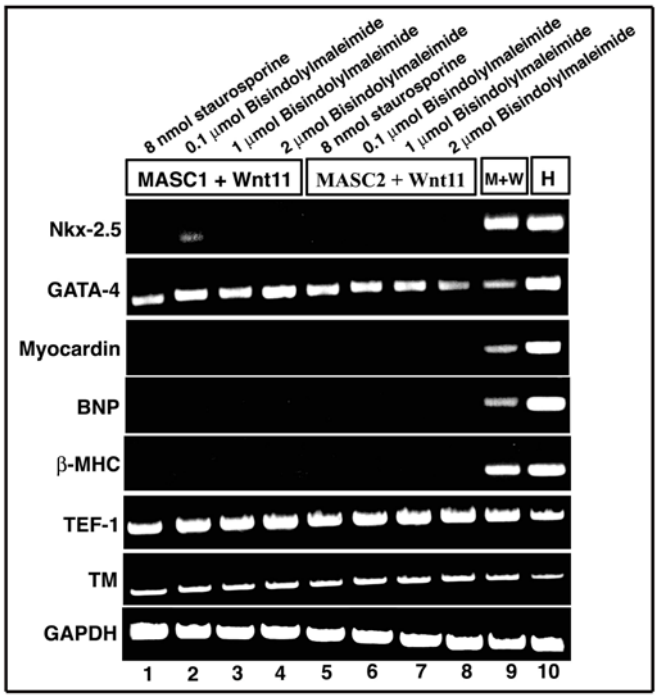

Fig. 14 RT-PCR analysis of effects of inhibition of PKC activity with staurosporine and bisindolylmalemeimide I. mBM-MASCs1 and mBM-MASCs2 were treated with different concentrations of two PKC inhibitors, Staurosporine (8 nM) and Bisindolylmalemeide I (0.1, 1 and 2 μM). PCR for cardiomyocyte specific genes was performed under standard conditions using cDNA synthesized from RNA isolated from heart, mBM-MASCs1 and mBM-MASCs2 treated with PKC inhibitors and wnt-11, and cells treated with wnt-11 only. Inhibition of PKC activity attenuated the cardiogenic effect of wnt-11 and abrogated cardiomyocyte gene expression in both stem cell populations.

By adding PKC inhibitors to the cell culture, the effect of wnt-11 was attenuated or completely antagonized as indicated by the abrogation of expression of the majority of cardiomyocyte specific genes (Fig. 14). mBM-MASCs1 and mBM-MASCs2, which were treated with wnt-11 without PKC inhibitors, expressed several cardiomycyte specific genes. The only cardiomyocyte genes which were not affected by the treatment with PKC inhibitors were GATA-4, Tef-1 and tropomyosin (Fig. 14). Taken together, my results clearly demonstrate a role of PKC in the non-canonical wnt-11 signalling pathway.

4.11 5-azacytidine and/or TSA augment the acquisition of cardiomyocyte markers in mBM-MASCs

The role of an inhibitor of DNA methyl transferase, 5-azacytidine (AZA) to induce cardiomyocyte programme has been reported (Makino et al., 1999). To substantiate whether an inhibitor of histone deacetylase, Trichostatin A (TSA) or a combination of both drugs activate the cardiogenic program, mBM-MASCs1 and mBM-MASCs2 were treated with

different concentrations of AZA or TSA or a combination of both drugs for 24 hrs. After testing different concentrations of these drugs, the concentration where most of the cardiomyocyte genes were expressed was considered optimum and indicated. Microscopically, mBM-MASCs displayed progressive morphological changes after treatment with these drugs. The process of differentiation was accompanied by death of some cells possibly due to cell cycle exit and terminal differentiation. None of the treated cells did show any beating phenotype even after extended cultivation times of up to 21 days.

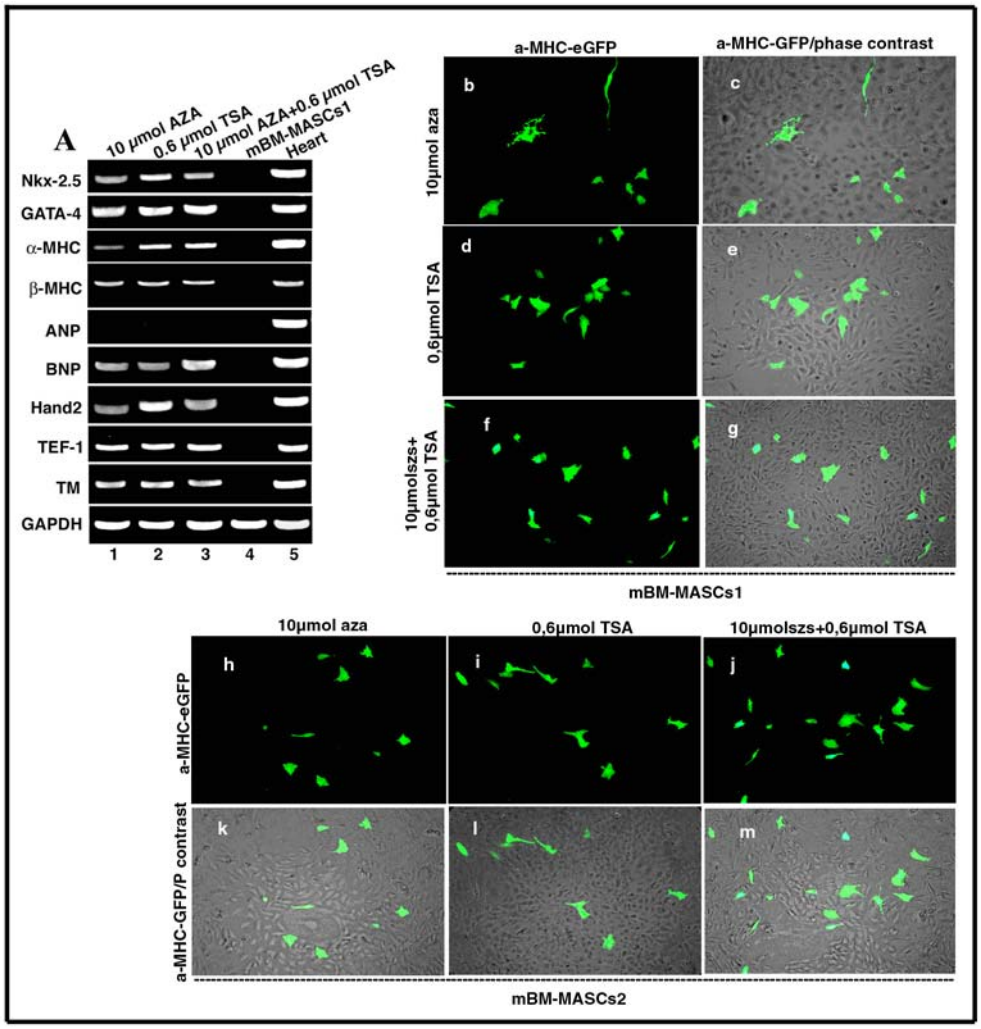

Fig. 15 Epigenetic reprogramming leads to the progression towards a cardiomyocyte phenotype in mBM-MASCs. The mBM-MASCs1&2 were treated with optimum concentrations of AZA or TSA or both in DMEM-LG/3% FCS for 24 hrs and allowed to differentiate for 8- 15 days with medium change every 3 days.
The expression levels of Nkx2-5, GATA-4, α-MHC, β-MHC, ANP, BNP, Hand2, Tef-1, Tropomyosin and GAPDH (control) were analysed by standard PCR using cDNA synthesised from RNA isolated from heart and both cell populations with or without treatment of AZA, TSA or both but shown for mBM-MASCs1 **(A).**
The α-MHC promoter controlled expression of eGFP in mBM-MASCs1and 2 after treatment with epigenetic drugs **(b-m)**. The α-MHC promoter controlled expression of eGFP in mBM-MASCs1 after treatment with AZA **(b),** TSA **(d)** or both **(f)** for 8 days. Phase contrast microscopy merged with α-MHC promoter controlled expression of eGFP in mBM-MASCs1 after treatment with AZA **(c),** TSA **(e)** or both **(g).**
The α-MHC promoter controlled expression of eGFP in mBM-MASCs2 after treatment with AZA **(h),** TSA **(i)** or both **(j)** for 8 days. Phase contrast microscopy merged with α-MHC promoter controlled expression of eGFP in mBM-MASCs2 after treatment with AZA **(k),** TSA **(l)** or both **(m).**

RT-PCR data revealed expression of Nkx2-5, GATA-4, α-MHC, β-MHC, BNP, Hand2, Tef-1 and tropomyosin in mBM-MASCs1 (Fig. 15A) and mBM-MASCs2 (not shown) which were treated with 10 µM of AZA, 0.6 µM of TSA and a combination of both drugs. The untreated mBM-MASCs did not show expression of cardiomyocyte markers with the exception of Tef-1 and tropomyosin.

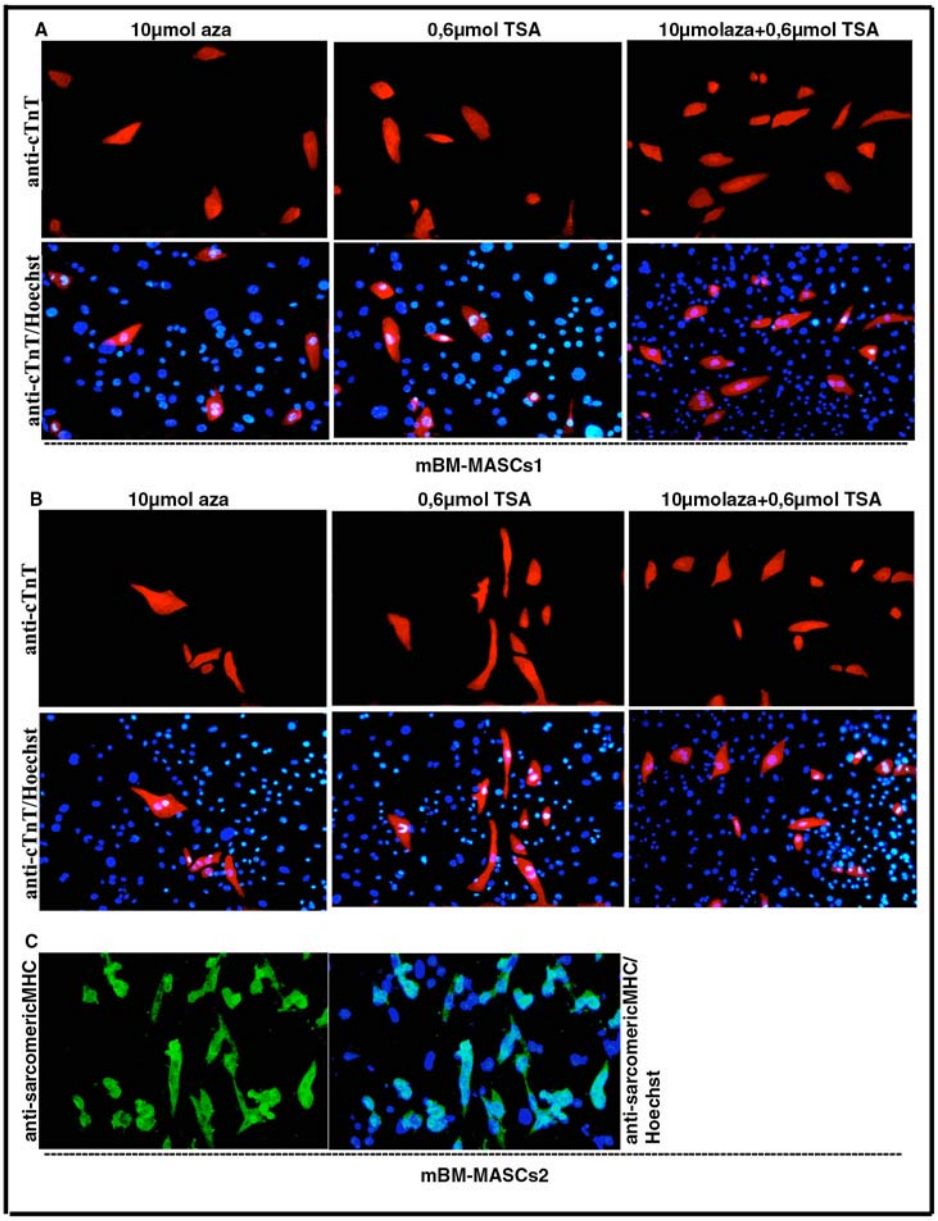

Fig. 16 Immunofluorescent staining of mBM-MASCs1 and mBM-MASCs2
Fig. 16A Immunofluorescent staining of mBM-MASCs1 for cTnT after treatment with optimum concentrations of AZA, TSA or both for 10 days
Top panel left, Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 with 10 µM of AZA. **Bottom panel left,** Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 with 10 µM of AZA merged with Hoechst staining. **Top panel middle,** Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 with 0.6 µM of TSA **Bottom panel middle,** Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 with 0.6 µM of TSA merged with Hoechst staining. **Top panel right** Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 with 0.6 µM of TSA and 10 µM of AZA. **Bottom panel right,** Immunofluorescent staining for cTnT after treatment of mBM-MASCs1 with 0.6 µM of TSA and 10 µM of AZA merged with Hoechst staining.

Fig. 16B Immunofluorescent staining of mBM-MASCs2 for cTnT after treatment with optimum concentrations of AZA, TSA or both for 10 days, all panels are similar to panels in Fig. 12A but for mBM-MASCs2.
Fig. 16C Immunofluorescent staining of mBM-MASCs2 for MF-20 after treatment with 10 μM of AZA for 10 days, **Left panel,** MF-20 staining and **right panel** MF-20 staining merged with Hoechst staining for mBM-MASCs2

Interestingly, I never found expression of α-MHC after wnt11 treatment alone (Fig. 12A). However, this situation changed when either mBM-MASCs1 or 2 were treated with AZA, TSA or both drugs (Fig.15A). Transfection experiments with reporter constructs containing the αMHC promoter driving expression of eGFP resulted in the presence of more eGFP positive cells after combination of AZA and TSA treatment although GFP was also expressed in the presence of individual drugs (Fig. 15b-m). RT-PCR data were consistent with the robust expression of eGFP driven by α-MHC promoter when both epigenetic drugs were used (Fig. 15A&b-m). The data from the expression of eGFP driven by α-MHC promoter and RT-PCR corroborate the expression of α-MHC in both cell populations. Furthermore, an expression of cTnT was observed after treatment with both epigenetic drugs (Fig. 16A&B), confirming the progression of differentiation towards cardiomyocyte like cells. Neither RT-PCR nor immunostaining for cardiac actin showed expression of this cardiomyocyte marker in both cell populations (not shown). Additionally, neither contraction nor expression of transcripts of ANP was observed in both lines. Taken together my data suggest that AZA and TSA promote the differentiation of mBM-MASCs1 and mBM-MASCs2 towards cardiomyocyte-like cells.

4.12 FGF-2 alone or in combination with BMP-2 induces expression of cTnI and cTnT proteins in mBM-MASCs1 and 2

FGF-2 induced some neuronal markers in mBM-MASCs after treatment for 10 days. Since FGFs and BMPs have also been shown to initiate cardiomyogenesis during embryogenesis, mBM-MASCs1 and 2 were treated continuously with different concentrations of FGF-2 (1, 3, and 5 ng/ml) or combination of both FGF-2 and BMP-2 (1+0.2), (3+0.6) and (5+1 ng/ml) for 15- 21 days.

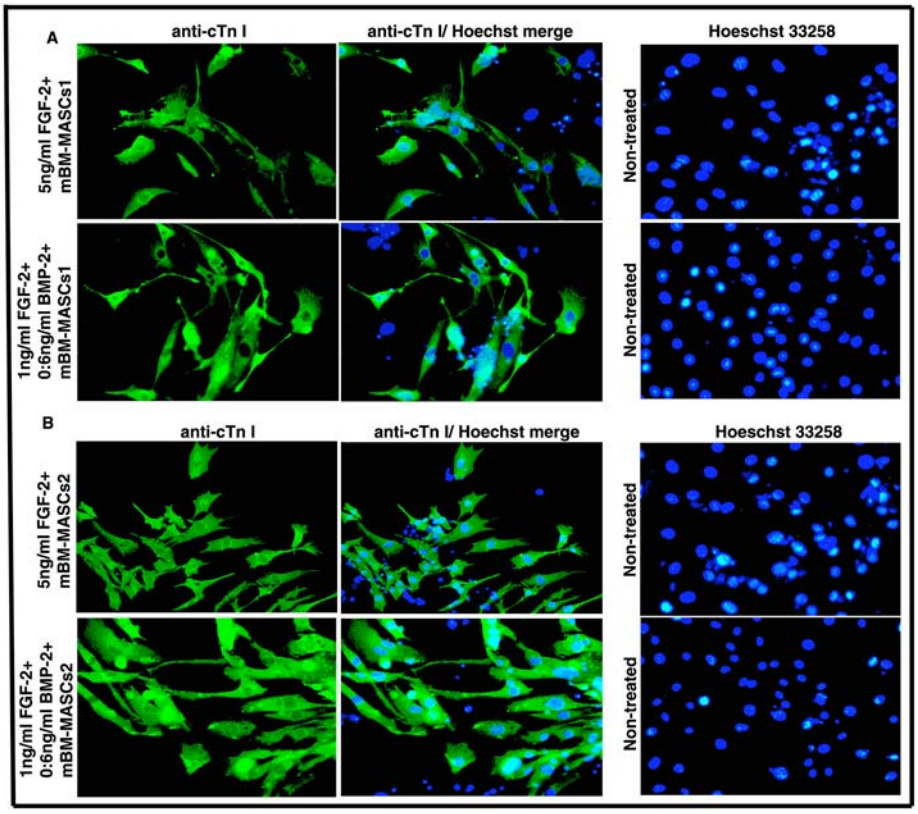

Fig. 17 FGF-2 alone or in combination with BMP-2 triggers cardiac trooping I (cTnI) expression, mBM-MASCs 1 or 2 were treated with either 5 ng/ml FGF-2 or combination of 1 ng/ml FGF-2 + 0,6 ng/ml BMP-2 and allowed to differentiate along with the untreated control cells for 15-21 days with medium change every 3 days.
Fig. 17A Immunofluorescent staining of mBM-MASCs1 for cTnI
Top panel left, Immunofluorescent staining of mBM-MASCs1 for cTnI after treated with 5 ng/ml FGF-2. **Top panel middle,** merge with Hoechst nuclear staining. **Top panel right,** Immunofluorescent staining of untreated mBM-MASCs1 for cTnI merged with Hoechst nuclear staining.
Bottom panel left, Immunofluorescent staining of mBM-MASCs1 for cTnI after treated with 3 ng/ml FGF-2 and 0,6 ng/ml BMP-2. **Bottom panel middle,** merge with Hoechst nuclear staining. **Bottom panel right,** Immunofluorescent staining of untreated mBM-MASCs1 for cTnI merged with Hoechst nuclear staining.
Fig. 17B Immunofluorescent staining of mBM-MASCs2 for cTnI

Continuous treatment for additional 5-11 days with FGF-2 or a combination of both FGF-2 and BMP-2 resulted in dramatic shift in the morphology of both populations of cells. The cells started to divide and expand clonally. This led to the loss of cells with neuronal like morphology which was evident after FGF-2 treatment (Fig. 19A, B&D). RT-PCR revealed up-regulation of transcripts of Nkx2.5 (not shown). Moreover, expression of cTnT (not shown) and cTnI (Fig. 17A & B) were observed using immunofluorescent staining. Importantly, the combined treatment of FGF-2 and BMP-2 resulted in robust induction of cTnI (Fig. 17A & B). Taken together, these observations suggest that mBM-MASCs can be reprogrammed to follow different developmental pathways using the same inductive signals but different differentiation conditions.

4.13 Cardiogenic potential of long term passaged Sca-1 positive adult stem cells isolated from the adult heart, H-ASCs

Although somatic stem cells have been documented to exist in various adult organs, the search for stem cells in the adult heart has been futile until recently (Oh et al., 2003). In the past the adult mammalian heart was viewed as an exclusive postmitotic organ which has lost its capacity for proliferation and regeneration. Recently, however, the accepted view was challenged by the description of stem cell like/progenitors in the adult heart (Oh et al., 2003). This may provide an explanation distinct from cell cycle re-entry for the reported rare occurrence of cycling ventricular muscle cells. An important source of these stem/progenitor cells might be progenitors in the heart itself that were set-aside during development or recruited from extra cardiac niches. In the course of my work I isolated and characterized an adult stem cell line from the heart which was designated H-ASCs. This cell line expressed Sca-1 strongly and several pluripotency markers such as OCT3/4, Nanog, SSEA-1 and B-Myb but not Rex-1. According to FACS analysis, H-ASCs have similar surface molecule expression profile with mBM-MASCs2 except that they express Sca-1 at higher levels. They are also similar to mBM-MASCs1 except that they express CD34. However, it remains to be assessed whether these cells have the same multilineage differentiation characteristics as mBM-MASCs1 and mBM-MASCs2.

4.14 Expression of haematopoietic markers by mBM-MASCs1 and mBM-MASCs2

A potential haematopoietic potential of BM derived mesenchymal stem cells has been a subject of heated debate due to possible contaminations from marrow. To exclude contribution of haematopoietic stem cells to the derivation of mBM-MASCs, undifferentiated mBM-MASCs were analysed by immunocytometry for expression of haematopoietic and other stem cell markers. It should also be noted that culture conditions in which mBM-MASCs were expanded did not favour expansion of haematopoietic cells. FACS analysis revealed the presence of a small population of cells (< 0.5%) which expressed surface markers such as CD45, a specific marker of all myeloid lineages and Glycophorin A or Ter119, a specific marker for the erythroid lineage, c-Kit, a haematopoietic stem cell marker and Flk-1, an endothelial marker. To investigate whether CD45 and Ter119 markers were up-regulated during induction of mBM-MASCs1 and mBM-MASCs2 with IL-3, both cell lines were grown in the presence of different concentrations of IL-3. Progressive erythroid and myeloid differentiation was monitored by Ter119 and CD45 up-regulation using FACS. A 2-fold

increase of these haematopoietic markers compared to basal levels were found in both cell populations, arguing for a specific induction by IL-3. Furthermore, spectrophotometric monitoring of haemoglobin expression using the Benzidine test showed an up to 6-fold increase for mBM-MASCs1 and upto 3-fold for mBM-MASCs2 compared to untreated controls. The difference in the expression of haemoglobin between IL-3 treated and untreated mBM-MASCs1 and mBM-MASCs2 was statistically significant. Immunostainings using antibodies against the haematopoietic factors LMO-2 and LMO-4 revealed a nuclear localisation of these transcriptional co-factors corroborating an induction of haematopoietic markers by IL-3 in mBM-MASCs1 and mBM-MASCs2. In addition, RT-PCR analysis showed an up-regulation of haematopoietic transcription factors such as GATA-1, AML-1, PU.1 and EKLF, which are instrumental for proper haematopoiesis. Transcription factors involved in primitive as well as definitive haematopoiesis such as SCL (tal-1), LMO-2 and GATA-2 were also detected by RT-PCR after IL-3 treatment. Furthermore, cells with morphological characteristics of monocytes, lymphocytes, granulocytes and mast cells were found after May-Grünwald staining, a haematological dye widely used for differential staining of blood cells. Induction of haematopoietic differentiation might also be stimulated by using a combination of other lineage specific cytokines such as IL-6, erythropoietin, stem cell factor and others. Given the fact that haematopoietic stem cells or progenitors do not grow under normal culture conditions and since committed haematopoietic cells have a short life span, it seems likely that cells with haematopoietic characteristics were not derived from contaminating haematopoietic stem cells but originated from undifferentiated mBM-MASCs. The minor fraction of cells which express CD45 and Ter119 in the absence of exogenously supplied IL-3 might have been induced by bioactive molecules present in the fetal calf serum used to grow mBM-MASCs. Taken together, it seems unlikely that contaminating haematopoietic cells remain viable after extensive passage of mBM-MASCs1 and mBM-MASCs2. Together, these observations make mBM-MASCs versatile in their properties and an excellent model allowing further characterization of haematopoietic and other lineage commitment potential.

4.15 BMP-2 or 5-azacytidine evokes osteogenesis in mBM-MASCs

The differentiation potential of mBM-MASCs into different mesodermal cell types has been demonstrated by expression of markers for myocytes, cardiomyocytes and blood cells. To further assess the mesodermal differentiation potential, both lines were treated with 5-azacytidine and BMP-2.

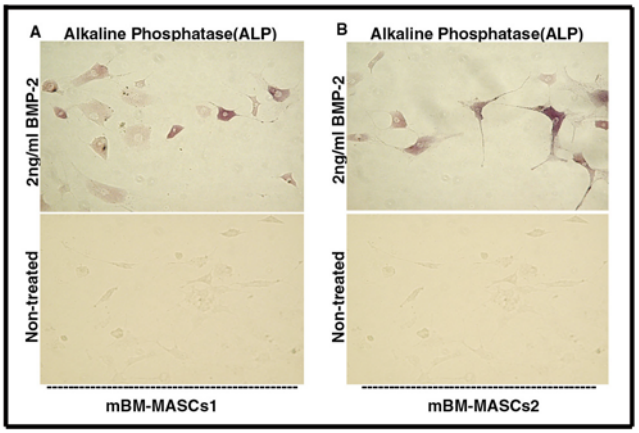

Fig. 18 Epigenetic reprogramming and embryonic signalling molecules like BMP-2 commit mBM-MASCs towards the bone phenotype. The mBM-MASCs were treated with 10 µM AZA and 2 ng/ml BMP-2 in DMEM-LG/3% FCS, with medium change every 3 days.
Fig. 18A Alkaline phosphatase (ALP) staining of mBM-MASCs1
Top panel left, Alkaline phosphatase (ALP) staining of mBM-MASCs1 treated with 2ng/ml BMP-2.
Bottom panel left, Alkaline phosphatase (ALP) staining of untreated mBM-MASCs1
Fig. 18B Alkaline phosphatase (ALP) staining of mBM-MASCs2
Top panel right, same as top panel left in Fig. 16A but for mBM-MASCs2.
Bottom panel right, same as bottom panel left in Fig. 16A but for mBM-MASCs2.

5-AZA treatments of mBM-MASCs led to the expression of alkaline phosphatase (ALP), a characteristic marker of osteocytes at 10 µmol (not shown). Similar results were obtained with 2 ng/ml of BMP-2 (Fig. 18A & B). Interestingly, the dosage of BMP-2 required to induce a bone phenotype was 10 times higher than the concentration needed to induce cardiogenesis in ES cells (not shown) and 3 times higher than the concentration needed to induce cardiogenesis in mBM-MASCs (not shown). This implies that the same cells react differently depending on the concentration of inductive signals provided to them. There was also a marked morphologic change observed between treated cells and non-treated controls (Fig. 18A & B). The treated mBM-MASCs tended to show an extended cell soma and a round morphology. Some of those cells had cytoplasmic processes. Taken together these experiments demonstrate that mBM-MASCs are able to acquire characteristics of diverse mesenchymal tissue as long as the right signal is presented to them.

4.16 FGF-2 confers ectodermal competence to mBM-MASCs

The *in vitro* differentiation ability of mBM-MASCs into neuroectoderm was investigated using different concentrations of FGF-2 for 10 days as described in the methods.

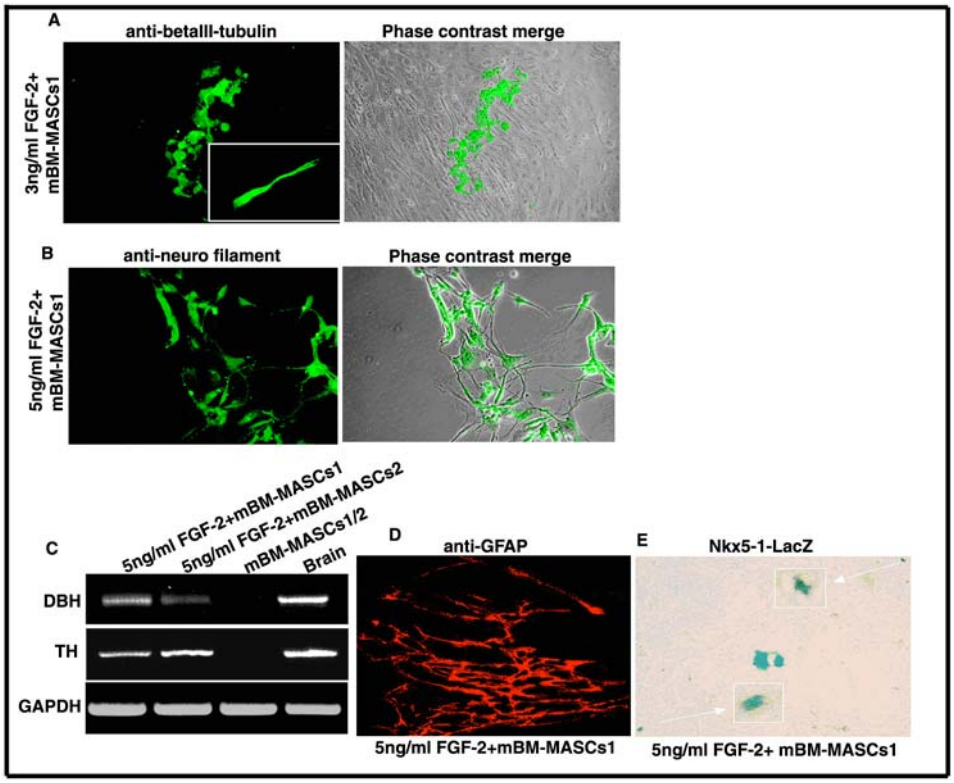

Fig. 19 FGF-2 induces the neuroectodermal phenotype in mBM-MASCs
The mBM-MASCs were treated with 3 ng/ml or 5 ng/ml of FGF-2 for 10 days with medium change every 3 days, as indicated in the method section.
Fig. 19A β_{III}-tubulin staining of mBM-MASCs1 treated with 3 ng/ml of FGF-2.
Top panel left, β_{III}-tubulin staining with unique positive cell boxed. **Top panel right,** β_{III}-tubulin staining merged with phase-contrast microscopy.
Fig. 19B neurofilament staining of mBM-MASCs1 treated with 5 ng/ml of FGF-2.
Top panel left, neurofilament (NF-200) staining. **Top panel right,** neuro filament (NF-200) staining merged with phase contrast microscopy.
Fig. 19C RT-PCR analysis of neuronal markers in mBM-MASCs1 and 2 The expression levels of DBH, selective marker of adrenergic and noradrenergic neurons, TH and GAPDH (control) were analysed by standard PCR using cDNA synthesised from RNA isolated from brain and both cell populations with or without treatment of FGF-2.
Fig. 19D GFAP staining of mBM-MASCs1 treated with 5 ng/ml of FGF-2.
Fig. 19E LacZ staining of mBM-MASCs1 treated with 5 ng/ml of FGF-2. The lacZ was expressed in the nucleus after activation of the tissue specific promoter, Nkx5-1. Nuclei of small cells were enlarged and boxed

Microscopically, changes in cell morphology were observed following the addition of FGF-2. After extended culture, these changes became more pronounced and resulted in the appearance of cells with cytoplasmic processes. After 10 days, cells were stained for the expression of neuronal markers that are typical for immature neurones (β_{III}-tubulin) (Fig. 19A) and a marker of mature neurones (200 kDa neurofilament, NF-200) (Fig. 19B). Furthermore, the expression of transcripts of neuroectodermal markers such as dopamine beta-hydroxylase (DBH) selective marker of adrenergic and noradrenergic neurons and thyroxin hydroxylase (TH) were demonstrated by RT-PCR (Fig. 19C) in both cell populations. In addition, immunofluorescent staining showed the presence of glial fibrially acidic protein (GFAP), indicating the induction of non-neuronal or glial cells in mBM-MASCs1 with 5 ng/ml of

FGF-2 (Fig. 19D). This observation was also confirmed by transfection of Nkx5-1-LacZ reporter construct into mBM-MASCs1 and 2. The expression of LacZ indicated the activation of this neuroectodermal promoter and hence the induction of neurogenic programs in these cells. Only FGF-2 treated but not untreated mBM-MASCs1 (Fig. 19E) expressed the LacZ reporter gene. Similar results were obtained with mBM-MACSs2 (not shown).

4.17 HGF/SF induces the initial phase of hepatogenesis in mBM-MASCs

Previous studies had suggested an ability of HGF/SF to induce hepatogenesis. To investigate the differentiation potential of mBM-MASCs towards the endodermal lineage, cells were grown in the presence of different concentrations of HGF (also called scatter factor, SF) for 15 days.

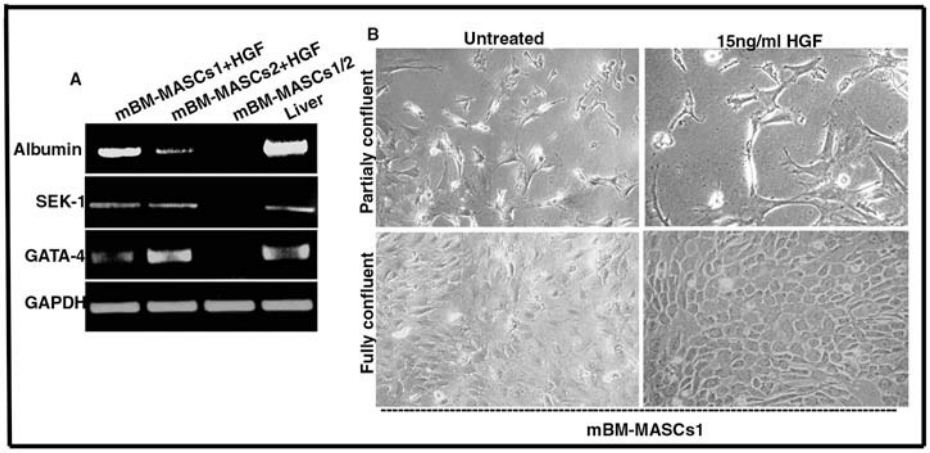

Fig. 20 Hepatocyte growth factor confers hepatocyte phenotype on mBM-MASCs. mBM-MASCs were treated with 15 ng/ml HGF/SF for 15 days and the progression of differentiation was followed over the course of days.
Fig. 20A RT-PCR analysis of hepatoblast markers in mBM-MASCs1 and 2, the expression levels of transcripts of albumin, SEK-1, GATA-4 and GAPDH (control) were analysed by standard PCR using cDNA synthesized from RNA isolated from liver and both cell populations with or without treatment of HGF/SF.
Fig. 20B Phase contrast microscopy of mBM-MASCs1
Top panel left, partially confluent untreated mBM-MASCs1. **Bottom panel left,** fully confluent untreated mBM-MASCs1.
Top panel right, partially confluent mBM-MASCs1 treated with 15 ng/ml of HGF. **Bottom panel right,** fully confluent mBM-MASCs1 treated with 15 ng/ml of HGF.

HGF caused the expression of transcripts of liver specific genes such as albumin-1, a characteristic marker of hepatoblasts, stress signalling kinase-1, SEK-1 and GATA-4 (Fig. 20A). Microscopically, HGF treated mBM-MASCs formed colonies with a marked difference in morphology, giving rise to polygonal or epitheloid-like cells (Fig. 20B). In contrast, the untreated control did not show such appearance throughout the observation period (Fig. 20B). Taken together, these data indicate that treatments of mBM-MASCs with HGF induces specification of the initial phase of hepatocyte phenotype by up-regulating the expression of early liver markers (Bedada et al., 2006).

4.18 Genetically labelled mBM-MASCs1 (mBM-MASCs1-eGFP) contributed to the development of several tissues in chimeric embryos

To assess if the *in vitro* observation of broad developmental potential of mBM-MASCs can be recapitulated *in vivo*, 10-20 mBM-MASCs1-eGFP cells were administered into 3.5 days old C57/BL6 mouse blastocysts.

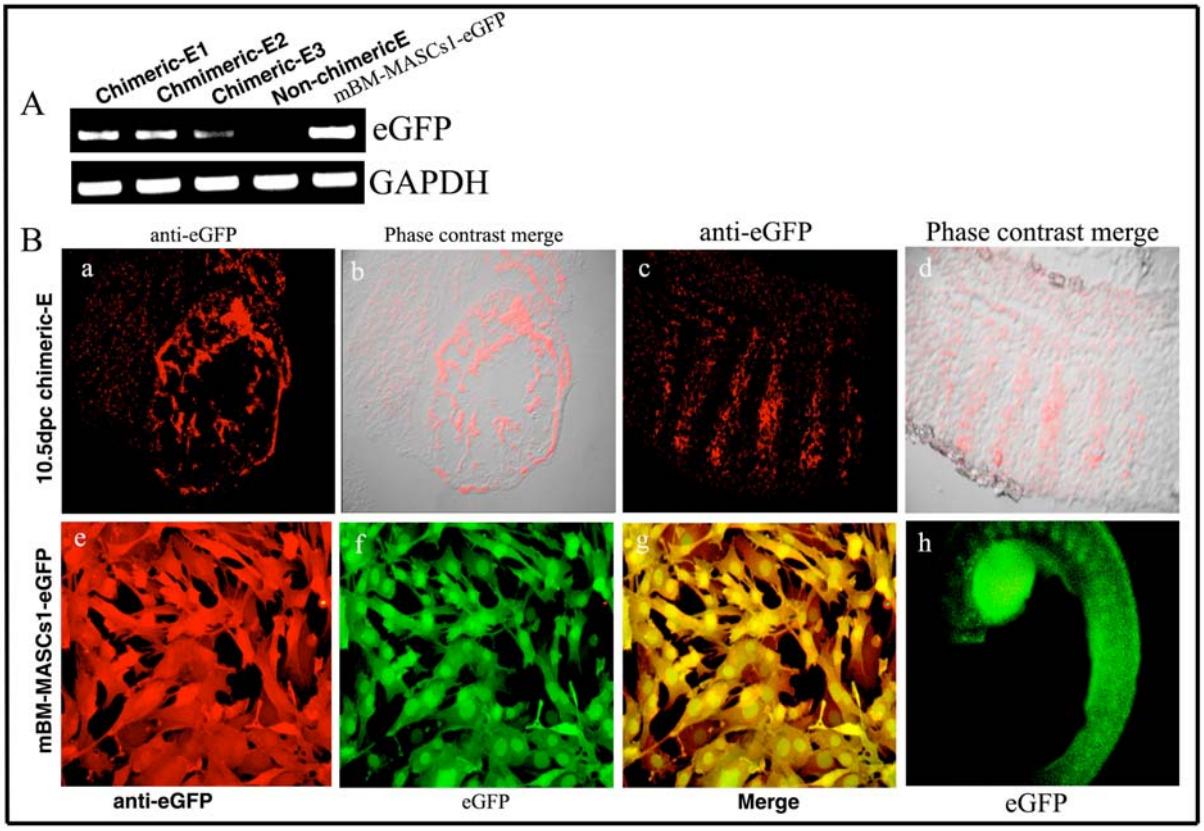

Fig. 21 MBM-MASCs contribute to the development of E10.5 mouse embryos after injection into C57BL/6 blastocysts. (A) RT-PCR analysis of eGFP expression in chimeric embryos and in mBM-MASCs1-eGFP cells. (B) Immunofluorescence staining and fluorescence microscopy. To identify the contribution of mBM-MASCs1-eGFP cells to mouse embryonic development chimeric embryos were illuminated with fluorescence light and viewed with an eGFP-filter (f and h). In addition, 10 µm thick sections were prepared from chimeric embryos and stained for eGFP protein using a polyclonal rabbit anti-eGFP primary antibody and Alexa594 anti-rabbit secondary antibody (a–e). Merged images of eGFP antibody staining and eGFP fluorescence microscopy (g). Note the presence of eGFP-positive cells in various tissues.

pFUGW, a lentiviral reporter construct was used to express eGFP in mBM-MASCs (see material plasmid section). Fluoresence microscopy of whole chimeric embryos that had been injected with eGFP labelled mBM-MASCs (Fig. 21B) revealed the presence of eGFP positive cells in somites and the heart (Fig. 21h). To additionally confirm the presence of mBM-MASCs in injected embryos, antibody staining and PCR based detection of the transgene was accomplished. As expected, the eGFP protein was detected using cDNA synthesized from RNA isolated from the whole embryos as well as in mBM-MASCs1-eGFP cells, which

served as a positive control. Apparently, the level of expression of the transgene varied between the different chimeric embryos (Fig 21A). All injected embryos which were considered to be chimeric were positive for the transgene. Non-chimeric embryos did not express the transgene (Fig 21A). To identify the specific part of the embryos to which mBM-MASCs1-eGFP have contributed, 10µm thick sections were stained for the presence of eGFP protein using polyclonal rabbit anti-eGFP primary antibody and Alexa 594 anti-rabbit secondary antibody. The contribution of mBM-MASCs1-eGFP to the somites, heart and endothelium can be seen clearly from the immunofluorescence staining (Fig 21B). With regard to contribution to endothelium, a close look at the pattern of staining indicated that the signals were collected in the part of the heart where there are many large blood vessels following the direction of bifurcation. Similarly, as a positive control, the injected cells (mBM-MASCs1-eGFP) were stained with the anti-eGFP antibody where the antibody detected all eGFP positive cells (Fig. 21B). The contribution of mMB-MASCs1-eGFP to somite, heart and endothelium development of chimeric embryos confirmed the observation made *in vitro* and indicate a broad developmental potential of mBM-MASCs.

5 Discussion

5.1 Isolation, cultivation, culture expansion and characterization of mBM-MASCs

Many laboratories are working on the isolation of mesenchymal stem cells from the mouse bone marrow. To date, very few mesenchymal stem cells lines have been established. In addition, several studies suffered from a superficial analysis of the respective differentiation programme. In this work, two populations of long term self-renewing mesenchymal stem cells termed mBM-MASCs1 and mBM-MASCs2 have been established from mouse bone marrow and were characterized thoroughly (Belema Bedada et al., 2005). Some modifications were introduced into the method to establish these lines. The initial phase of cultivation of bone marrow derived stem cells, where the adherent cells are allowed to reach confluency and then trypsinized was omitted. The necessity for this modification emanated from the fact that stem cells are very rare (1:15,000-100,000) compared with the predominant non-stem cell populations. During trypsinization, the rare slowly dividing stem cells can be lost easily. Therefore, heterogeneous adherent cells from the primary preparation were kept for upto four weeks without trypsinization until clonally expanding cells emerged (Fig. 1). Since the likelihood that several stem cells are located together at same spot in the culture plate is very low although not impossible, one might expect that expanding populations originated from a single clone of cells (Fig. 1). This assumption was further supported by the finding that there was no difference between the parent cells and subclones. The identified clonally expanding cells at one spot in the tissue culture plate (Fig. 1) were then expanded in 10% v/v FCS supplemented DMEM-LG without addition of additional growth promoting cytokines as suggested in recent works (Jiang et al., 2002a; Jiang et al., 2002c). Isolated cells were characterized extensively by FACS for the presence of surface molecules including CD45, Ter119/Glycophorin A, c-Kit, Flk-1, MHC1/H-2D^{d}, CD133/prominin, SSEA-1, CD13, CD34, and Sca-1. With respect to expression of such surface molecules, the different clones were virtually indistinguishable from one another with the notable exception of CD34 expression and differences in the level of Sca-1 expression (Fig. 2A&B). In addition, the expression of several pluripotency (stemness) marker genes such as OCT3/4, Rex-1, SSEA-1, Nanog, and B-Myb were assessed by RT-PCR. The expression of pluripotency marker genes in the mBM-MASCs1, mBM-MASCs2 and ES cells but not in NIH3T3 cells suggests that these cells have features similar to ES cells but distinct from NIH3T3 cells, a fibroblast cell line (Fig 2C). Similar mouse adult stem cells have been established from other tissues such as heart, termed

mH-ASCs and muscle, mMs-ASCs and also based on microbeads cell sorting for Sca-1 antigen from mouse bone marrow, mBM-ASCs-Sca-1 using a similar approach.

5.2 Properties of mBM-MASCs

When mBM-MASCs were induced to differentiate, dividing cells were still present among differentiated cells that expanded with morphologic and phenotypic characteristics similar to the original parent cells. This implies that some stem cells retained their ability to self renew and maintain the stem cell pool. Subsequent clonal analysis showed that both parent cells and sub-clones did not show differences in the expression of surface marker molecules and pluripotency (stemness) markers. Interestingly, my attempts to isolate mBM-MASCs by a different approach, which was based on the initial cloning of cells failed. Since the frequency of stem cells is very low (probably 1:15,000-1:100,000) such an experiment might only work if a very large number of (>200,000-300,000) cells is cloned, which is technically difficult to achieve. However, if one identifies stem cells using the concept of natural selection or differential survival out of large pool of heterogeneous cells as used in this work, stem cells can easily be expanded and clonal analysis can be done. This way of generating permanently dividing lines is not so frequent but one can always get these stem cells from numerous cell preparations.

Since stem cells have the potential to self renew, they are not lost by cell cycle exit as most progenitors. They have the potential to survive harsh conditions as compared to the highly proliferative cell populations. In our hand, bone marrow derived MSCs did not divide after the 3rd passage, after which they changed morphology and failed to expand continuously. MSCs which were derived by traditional means usually failed to acquire different developmental fates after induction (Belema Bedada et al., 2005). mBM-MASCs have been injected into SCID mice and also used to generate chimera after blastocysts injection. Since they did not form tumours *in vivo*, it is unlikely that they have been transformed. My different mBM-MASCs cell lines have been in culture for more than 2 years and have not shown signs of senescence or a failure to maintain their multi-differentiation potential.

5.3 mBM-MASCs1 and mBM-MASCs2 can be labelled efficiently and may serve as a vector for delivering genes of therapeutic value

The concept of gene therapy involves the introduction of a functional gene into cells within the body to correct a genetic defect. Retroviral gene transfer is presently one of the most

powerful techniques to introduce genetic material into mammalian cells. The observations that lentivirally transduced mBM-MASCs1 and mBM-MASCs2 maintained the transgene expression after multiple passages suggest stability of expression. Besides genetic labelling, this has important implications specially when using these adult stem cells as a cellular vehicle for delivering or administering genes of clinical and therapeutic potential. Although stable eGFP expression in these cells has no therapeutic potential, it serves as a model for the delivery of substances that are needed for an extended period of time.

5.4 The mBM-MASCs have competence to differentiate into diverse mesodermal lineages

Several previous reports (Pereira et al., 1995; Pereira et al., 1998; Reyes et al., 2001) showed the ability of mesenchymal stem cells isolated from the bone marrow to differentiate into mesodermal tissues such as bone, tendon, cartilage, muscle and fat cells. However, these cells were poorly characterized in several respects. A more recent study (Jiang et al., 2002c; Jiang et al., 2002a) has characterized bone marrow, muscle and brain tissue isolated MAPCs and reported their multi-potentiality. To assess the differentiation potential of mBM-MASCs into diverse mesodermal lineages, several effector molecules such as vertebrate Wnt signalling molecules (wnt7a, wnt7b, wnt4 and wnt11), CA-LEF, CDO molecule, hypomethylating drug like 5-azacytidine, acetylating drug like TSA and cytokines such as FGF-2, BMP-2, and IL-3 have been employed in this study. Several of these effector molecules can stimulate at least a partial differentiation of naïve mBM-MASCs towards different mesodermal lineages (Belema Bedada et al., 2005).

5.5 Distinct wnts (wnt7a, 7b, 4 and wnt11) and CA-LEF activate the myogenic program in mBM-MASCs

Wnts are secreted signalling ligands that control diverse biological processes including cell proliferation, migration, differentiation and polarity. The observation that wnts induce myogenic differentiation in mBM-MASCs supports their role in cell specification. The only myogenic bHLH factor consistently induced by all wnts used in this study was myogenin (Fig. 6A, B&C). The expression levels of other myogenic factors varied between individual experiments. Whether or not the expression of myogenin is a direct consequence of wnts, without activating other myogenic factors like Myf5, Pax3, Pax7 and MyoD upstream of myogenin remains to be established. However, the possibility that myogenin might be directly

activated by wnts in the absence of other myogenic factors cannot be ruled out. Although Pax3 was already present in un-induced mBM-MASCs, Pax7 was detected only after induction with constitutive active LEF, a molecule that mimics the effects of wnts (Fig. 6B). In un-segmented paraxial mesoderm of the early mouse embryo, wnt7a was reported (Tajbakhsh et al., 1998) to preferentially activate MyoD. In this study, MyoD was activated by all wnts and CA-LEF in mBM-MASC2 (Fig. 6A&B), but also by wnt4, wnt11 and CA-LEF in mBM-MASC1 (Fig. 6A&B) suggesting differential responses. Studies so far have not directly implicated wnt11 to the activation of the myogenic program as it is a molecule involved in non-classical Wnt signal transduction. The expression of Myf5, MyoD and myogenin was detected after induction with wnt11 and CA-LEF in both lines but MRF4 was found only in mBM-MASCs1 after Wnt 11 inductions and in mBM-MASCs2 after CA-LEF treatment (Fig. 6A&B). The distinct expression profile of myogenic factors observed after induction with wnt11 might be due to the activation of alternate intracellular pathways by wnt11. Although CD34 expression is a sign of activated stem cells, my results showed a delay of at least one day in the differentiation time of mBM-MASCs2 expressing CD34 compared to mBM-MASCs1. However, since both lines entered the myogenic pathway after induction by wnts, the role of CD34 remains elusive at this point. Whether or not CD34 is potentially involved in the regulation of stem cell function remains to be established. Although we have not looked into the expression pattern of myogenic factors at a daily basis to identify which gene was expressed first and which followed, it seems likely that expression followed a developmental continuum reminiscent of the situation at embryogenesis as revealed from the pattern of myogenic factor expression (Tajbakhsh et al., 1998). The higher expression levels of myogenic factors in mBM-MASCs2 may be related to its CD34 content in contrast to mBM-MASCs1, which lacks it. A reporter construct containing the myogenin promoter controlling the expression of nuclear eGFP delivered by lentiviral infection showed expression of eGFP in the nucleus of both cell populations induced by all distinct wnts and CA-LEF (Fig.7A). The detection of myogenin by RT-PCR (Fig. 6A&B), immunostaining (Fig. 6C) and reporter gene expression (Fig.7A) corroborate the activation of the myogenic programme in these cell populations. It has been established that cell proliferation and differentiation are mutually exclusive, and myogenic bHLH factors activate muscle specific genes by co-ordinating withdrawal from the cell cycle during differentiation (Kang et al., 1998). As these muscle specific genes are also targets of growth factors, which regulate myogenesis negatively, the experiment were conducted in 3% FCS so that growth did not negatively affect differentiation of these cells. One of the features of stem cells is their ability

to self-renew which allows them to maintain the stem cell pool. This is a continuous process which occurs through asymmetric or symmetric cell divisions (Anderson, 2001). In resting uninduced mBM-MASCs, in the absence of Wnt activation, β-catenin is localized at adherent junctions of the cell membrane. Free cytoplasmic β-catenin levels are very low because it is rapidly destroyed by ubiqutin-proteasome degradation (Dierick and Bejsovec, 1999; Miller et al., 1999). Wnt binding to the cell surface receptors of the Frizzled protein family antagonizes degradation of the β-catenin that enters the nucleus where it stimulates expression of specific target genes (Dierick and Bejsovec, 1999; Miller et al., 1999). Consistent with this model, nuclear localization of β-catenin was observed in target cells only after Wnt treatment, suggesting the activation of the Wnt pathway in a β-catenin dependent manner (Fig 7C). This finding confirms the role of the Wnt-signalling molecule to the activation of myogenesis. Induction of the myogenic program was not observed in the Wnt releasing feeder cells containing distinct Wnt molecules (not shown), suggesting that the cells are not responsive to Wnt signals. Furthermore, an activation of myogenic program was not observed in mBM-MASCs treated with conditioned medium containing Wnt molecules (not shown). This might be due to a rapid degradation of secreted Wnt molecules in culture or an insufficient concentration to elicit biological effects (Yamamoto et al., 1999). The small amount of biologically active Wnt protein that had seeped into the medium may not have been sufficient to trigger the myogenic program, which may be dependent on a higher dose of the biologically active Wnt proteins to initiate the program. Although sarcomeric MHC is detected clearly in both cell populations in a similar manner as in C2C12 cells, a *bona fide* muscle lineage, no contraction or fusion of induced mBM-MASCs was observed. On the other hand, a filter experiment in which barriers with filters of different pore sizes were used to separate inducing from responding cells demonstrated that close contact of inducing and responding cells is required for the induction of the myogenic program (Fig. 5) and also that induction of this myogenic programme does not depend on cell fusion.

5.6 5-azacytidine (AZA) and/or Trichostatin A (TSA) activate myogenic program in mBM-MASCs

It has been shown that promoter/gene hypermethylation by DNA methyltransferase and chromatin remodelling of histone by histone deacetylase account for the silencing of certain genes (Bharadwaj and Prasad, 2002). The use of hypomethylating agent or inhibitors of DNA methyltransferase (5-aza-cytidine) and acetylating agent or inhibitor of histone deacetylase

(TSA) can reactivate certain genes. In general, myogenic factors were induced in mBM-MASCs upon treatment with different concentrations of TSA or a combination of TSA and AZA (Fig. 8A**)**. This indicates that deacetylation as well as hypermethylation might be involved in the silencing of these genes. 5-azacytidine was required at a higher concentration as compared to TSA to activate myogenic induction. However, a lower concentration of 5-azacytidine was required to effect differentiation into cardiomyocyte programme (Fig. 15A) (Belema Bedada et al., 2005). This suggests that the same molecule affects different differentiation programs at different concentration within the same cells. It has been postulated (Bharadwaj and Prasad, 2002) that gene methylation and histone deacetylation act as two layers in ensuring silencing of certain important genes. Consistent with this notion, combined treatment of AZA and TSA resulted in robust reactivation of MRF4 gene indicating cellular differentiation (Fig. 8A). Both drugs enhanced the expression of myogenic genes synergistically. Contrary to the observation with wnts and CA-LEF, no induction of Myf5 was found in mBM-MASCs after treatment with these drugs. In all cases weaker expression of myogenin was followed by strong expression of MRF-4 and vice versa (Fig. 8A). This might be related to a hierarchical activation of myogenic factors. Furthermore, these finding might indicate that hypermethylation and chromatin compaction by histone deacetylation are the major mechanism by which myogenic gene expression is kept silent in stem cells.

5.7 CDO positively mediates myogenesis in mBM-MASCs

CDO is a novel member of the Immunoglobulin (Ig) super-family characterized by an extracellular region that contains 5 Ig like repeats followed by three fibronectin type III (FN III)-like repeats, a transmembrane segment and a long cytoplasmic tail (Kang et al., 1998). Treatment of mBM-MASCs2 with CDO resulted in a weak expression of myogenin followed by the activation of MRF4 (Belema Bedada et al., 2005). The observation that CDO is robustly expressed in the early myogenic compartment suggests its pivotal role during determination or differentiation of cells in the myogenic lineage (Kang et al., 1998). Moreover, CDO was reported (Kang et al., 1998) in a study with C2C12, a *bona fide* myogenic line to act upstream of MyoD and myogenin. The expression of MyoD and myogenin in both lines is consistent with these observations. The presence of all the myogenic factors with the exception of Myf5 for mBM-MASCs1 and of MRF4 for mBM-MASCs2 might indicate that differentiation has progressed terminally in the former rather than in the latter line (Fig. 9A). Previous studies indicated that C2C12/Ras cells retain undetectable levels of MyoD and Myf5 which might have been kept inactive by some poorly

understood post-translational mechanism (Kong et al., 1995). The variability in the expression of Myf5 observed between both stem cell lines used might be related to this fact. In somites, the temporal expression pattern of CDO closely resembles Myf5 expression (Ott et al., 1991). The strong expression of Myf5, the earliest recognizable bHLH myogenic factor during embryogenesis (Megeney and Rudnicki, 1995) in mBM-MASCs2 is consistent with this observation.

5.8 Myogenic lineages can be derived from mBM-MASCs1 and mBM-MASCs2 *in vitro*

As mentioned above, not all mBM-MASCs responded to myogenic signals. To separate responding from non-responding cells, I established a protocol to enrich for those mBM-MASCs which are able to activate the myogenic programme (Fig.10A, 11a-d, e-h and j-k). In mouse ES cell system, strategies for the generation of enriched preparations of proliferating progenitors have been developed (Okabe et al., 1996; Li et al., 1998). In the current study, I have employed the myogenin promoter which drives the expression of eGFP to select myogenic mBM-MASCs1 and mBM-MASCs2 after induction with wnt7a. The eGFP expression was exploited to enrich myogenic cells through FACS cell sorting. Since their establishment, these cells have been passaged 10 times and the expression of eGFP driven by muscle specific promoter was maintained, corroborating the stability of the muscle phenotype. These progenitor cells did not express sarcomeric MHC unless they are pushed to differentiate in the presence of differentiation medium for 7 days indicating that they respond to differentiating signals. Although these cells seem to recapitulate some characteristics of muscle line, they do not fuse with each other albeit they fuse with the *bona fide* muscle line like C2C12 cells. Whether such progenitor cells respond appropriately to normal developmental cues *in vivo* is currently unknown but might help to discern their developmental competence. Selected myogenic lines may serve as *in vitro* model for the study of myogenesis and identification of novel genes, growth and differentiation factors that have a role in muscle development.

5.9 Fusion of mBM-MASCs1, mBM-MASCs2, ML-mBM-MASCs1 and ML-mBM-MASCs2 with C2C12 cells *in vitro*

Although bone marrow derived adult stem cells such as mBM-MASCs can activate numerous muscle markers *in vitro* some of the effects of mBM-MASCs *in vivo* might be mediated by

cell fusion (Schulze et al., 2005). In the course of this study, it could be shown that both committed, ML-mBM-MASCs1 and ML-mBM-MASCs2, and uncommitted mBM-MASCs1 and mBM-MASCs2 cell lines can fuse with myofibers *in vitro* albeit with variable efficiency (Fig. 11a-d, e-h and j-k). The highest fusion rate was associated with ML-mBM-MASCs1. This might be explained by the ability of C2C12 to fuse more readily with the muscle like ML-mBM-MASCs1 than with other non-myogenic lines. Indeed more hybrid myotubes were observed when C2C12 cells were co-cultured with ML-mBM-MASCs1 (Bedada et al., 2006). Recent work has shown that secretion of Interleukin-4 (IL-4) by newly formed myotubes stimulates the IL-4 signalling pathway in surrounding myoblast and promotes their fusion with existing myotubes (Horsley et al., 2003). This *in vitro* interaction (fusion) might not be a culture artefact but an interesting indication of the potential of these cell lines to replenish injured or damaged muscle. Furthermore, our *in vitro* model might reflect the *in vivo* contribution to tissue reconstitution of these cell lines either through differentiation by becoming *bona fide* mononuclear muscle progenitor cells or fusion to newly forming myofibers (Schulze et al., 2005). Whether mBM-MASCs, which have been implanted into regenerating muscles, differentiate independently of fusion or fuse autonomously or both remain to be investigated.

5.10 Only Wnt11 but not CDO or the canonical-Wnt pathway molecules like wnt7a, wnt7b, wnt4 and CA-LEF initiate the cardiomyocyte programme in mBM-MASCs

Wnt11 is involved in the non-classical (wnt/Ca^{2+}) Wnt signal transduction pathway which is dependent on the release of intracellular Ca^{2+} ions (Pandur et al., 2002). The observation that only wnt11 but not CDO or the canonical-Wnt pathway molecules like wnt7a, wnt7b, wnt4 and CA-LEF initiated the cardiomyocyte programme in mBM-MASCs implicates the non-classical pathway in the cardiogenic differentiation program. A cardiogenic role of CDO has not been reported so far although it is implicated in the myogenic program. Cardiac transcription factors expressed early in cells fated to become cardiomyocytes as well as late cardiac transcription factors were detected after wnt11 induction in mBM-MASCs (Fig. 12A). It has been discussed that mWnt-11 is not inhibited by DKK-1 or Crescent and act positively in cardiac induction (Pandur et al., 2002). In addition, a recently published study has demonstrated that wnt-11 is positively required for heart induction through a non-canonical Wnt signalling pathway (Pandur et al., 2002). In my hands, treatment of mBM-MASCs with wnt7a, wnt7b, wnt4 (Fig. 12A) and CA-LEF (not shown) did not produce expression of such cardiomyocyte markers as α-MHC, β-MHC, myocardin A, ANP and BNP which is in

agreement with studies in vertebrate embryos which have proposed a role of Wnt signalling in blocking cardiogenesis (Marvin et al., 2001). However, this is contrary to the finding in Drosophila, where wingless is essential for cardiac induction (Schneider and Mercola, 2001). On the other hand, Nkx2-5, GATA-4, Hand2, Tef-1 and tropomyosin were expressed in the presence of distinct wnts or CDO (Fig. 12A&B). It may be that their expression was positively regulated by the presence of growth factors in the medium. Furthermore, the expression of such early cardiac gene seems to be less demanding than the late genes which demand the presence of potent inducers. In addition, that there was no variation in the expression of transcripts of Tef-1 and Tropomyosin in both mBM-MASCs, which received signals from distinct wnts or CDO (Fig. 12A&B) correlated with the activation of skeletal muscle differentiation programme. It is worth noting that their expression is not restricted to cardiomyocytes as they are also detected in skeletal muscle. In the present work they have also been detected in mBM-MASCs induced to express skeletal muscle specific myogenic factors (not shown). Although efforts to similarly induce progression of cardiogenic differentiation programme using wnt7a, wnt7b, wnt4, CA-LEF and CDO were not successful, these molecules including wnt-11 can induce skeletal muscle programme in these two stem cell populations. This suggests the dual role of wnt-11 as cardiogenic as well as myogenic agents in mBM-MASCs. α-MHC was only expressed in mBM-MASCs after additional treatment with (Fig. 15A) 5-azacytidine, TSA or a combination of both (Fig. 15A). Obviously, the presence of wnt-11 alone was not sufficient to allow mBM-MASCs to acquire features of more mature cardiomyocyte-like cells.

5.11 Attenuation of the effect of wnt-11 by PKC inhibitors is followed by abrogation of the progression of cardiomyocyte differentiation programme

Wnt-11 has been implicated to act through the non-canonical Wnt signal transduction pathway which is dependent on PKC activity to initiate the cardiomyocyte programme (Pandur et al., 2002). In the present work, I interfered with one of the components of this pathway, the protein kinase C (PKC) with two inhibitor compounds. Upon treatments of mBM-MASCs with compounds that inhibit PKC activity (staurosporine (general inhibitor) and bisindolylmalemeimide I (specific inhibitor)), the effect of wnt-11 was neutralized (Fig. 14). This was proven by the loss of expression of several cardiomyocyte specific genes. This finding was in agreement with the involvement of PKC in the non-canonical wnt-11 signalling pathway (Pandur et al., 2002; Kuhl et al., 2000a). Interestingly, some cardiomyocyte genes including GATA-4, Tef-1 and tropomyosin (Fig. 14) were not

influenced by the treatment of the inhibitors. These genes might be targets of growth factors in the culture medium, which regulate their expression positively or activated by the canonical Wnt signalling pathway. Based on the profile of cardiomyocyte genes expression, three categories of genes seem to exist: first those genes which are induced easily by growth factor related signals in the culture medium (GATA-4, Tef-1 and tropomyosin), second those genes which are induced only in the presence of signals like wnt-11 (Nkx2-5, myocardin A, BNP, β-MHC) and third, those genes which are not induced by growth factor signalling (ANP, α-MHC) (Fig. 14). Interestingly, a gene from the last group such as α-MHC which was not induced by wnt-11 (Fig. 12A), was induced by drugs such as 5-azacytidine or trichostatin A (TSA) that evoke epigenetic remodelling (Fig. 15A&b-m). It seems clear that both mBM-MASCs1 and mBM-MASCs2 respond to a diverse array of effector molecules or compounds and react differently according to the efficiency of the effector molecules or compounds. Despite the importance of GATA-4 in early cardiogenesis, its presence in the absence of PKC seemed to have no effect on the progression of the cardiomyocyte differentiation programme. Nkx2-5 in contrast seems to be a major player in the initiation of cardiomyocyte programme as its absence was always associated by the absence of other cardiomyocyte genes (myocardin A, BNP, β-MHC, HAND2). Together, wnt-11 induces expression of both early cardiac genes as well as myocardial structural proteins, which indicate that wnt11 is sufficient to activate parts of the cardiomyocyte programme in mBM-MASCs

5.12 FGF-2 induces expression of cTnI and cTnT proteins in mBM-MASCs1 and 2 upon prolonged treatment

Cell commitment or specification is a process by which an undifferentiated cell enters a developmental pathway. Induction of uncommitted cells with members of growth factors of FGFs, BMPs family (Wolpert, 1989) might induce differentiation. My finding revealed that cells change their fate upon continuous treatment with FGFs. Initially, cells showed a neural morphology upon treatment with FGF-2 but not BMP-2 for 10 days. An extended treatment for 5-11 more days with FGF-2 or both FGF-2 and BMP-2 resulted in dramatic shift in the morphology, probably by allowing cells to divide and expand clonally. Continuous treatment with FGFs resulted not only in the loss of the original neuronal cell morphology but also in an up-regulation of Nkx2.5 (not shown), expression of cTnT (not shown) and cTnI (Fig. 17A&B) (Schulze et al., 2005). The detection of transcripts of Nkx2.5 is consistent with the observation that FGF-2/FGF-4 and BMP-2/BMP-4 induced cardiac differentiation via regulation of the transcription factors cNkx2.5 and SRF in non-precardiac mesoderm (Barron

et al., 2000). The expression of mesodermal markers by cells which initially showed a neuronal morphology might imply plasticity of primitive neuronal progenitors that gave rise to cells of other lineages (Tropepe et al., 2001; Clarke et al., 2000). It might be possible however, also that non-neuronal cells that have persisted within the cultures were fated to cardiomyocytes, as FGF-2 or both FGF-2 and BMP-2 treatment continued and neuronal cells were lost. Yet, reprogramming or de-differentiation followed by re-differentiation might contribute to a developmental potential under certain culture conditions *in vitro* (Palmer et al., 1999; Dupin et al., 2000). The synergistic effect of a combination of BMP and FGF signalling has been reported in many developmental processes and both operate co-ordinately during cardiac development (Barron et al., 2000). Moreover, the finding that more cTnI and cTnT positive cells were present when both growth factors were applied might indicate a strong anti-neurogenic effect of BMP-2 which allowed more cells to express cardiomyocyte markers (Shou et al., 2000).

5.13 BMP-2 or 5-azacytidin induces osteogenesis in mBM-MASCs

Inductive signals or agents are known to direct MSCs along several mesodermal lineage pathways including bone cells or osteocytes, cartilage and heart. The differentiation of mBM-MASCs into osteocytes or bone cells using BMP-2 is in line with the known potential of BMP-2 and other BMP proteins to induce and accelerate the ossification of bone lesions. The fact that BMP-2 induced both cardiogenic and osteogenic markers in mBM-MASCs correlates with its *in vivo* osteogenic and cardiogenic activity, suggesting its involvement in a variety of developmental processes. The prevalence of osteogenesis in mBM-MASCs was demonstrated by detection of ALP, which is typical for osteoblasts (Rodan and Noda, 1991). In other systems such as fetal rat calvarial cells and mouse marrow stromal cell lines, BMP-2 greatly stimulated ALP expression, osteocalcin production and bone nodule formation at concentration of 20-100 ng/ml (Thies et al., 1992). Contrary to this finding, a very low concentration of BMP-2 (2 ng/ml) was sufficient to induce the expression of ALP in mBM-MASCs, suggesting a high responsiveness of mBM-MASCs1 and mBM-MASCs2 to BMP-2 (Fig.18 A&B). The induction of osteogenic markers by the epigenetic drug 5-azacytidine has also been reported (Bruder et al., 1994) as was corroborated in the present work (not shown).

5.14 FGF-2 imparts neuroectodermal competence to mBM-MASCs

The derivation of cells with morphologic and phenotypic features of neurons from mBM-ASCs might help study neurogenesis and might eventually result in the creation of donor cells for a neural transplantation therapy. The detection of cells expressing neuronal markers such as β_{III}-tubulin (Fig. 19A), the 200 kDa neurofilament, NF-200 (Fig. 19B), dopamine beta-hydroxylase (DBH) and thyroxin hydroxylase (TH) by RT-PCR (Fig. 19C) confirms the differentiation of mBM-MASCs into immature and mature stages of neurons. It has been reported that the 200 kDa neurofilament is present in mature neurons. The detection of 200 kDa NF at the relatively high dose of FGF-2 (5 ng/ml) might reflect the requirement of higher doses of FGF-2 for the acquisition of mature neuronal phenotypes. This correlates with detection of β_{III}-tubulin, a marker for immature neurons at low dose of FGF-2 (3 ng/ml). Importantly, the detection of GFAP by immuno staining (Fig. 19D) indicates that mBM-MASCs might also be induced to form non-neuronal or glial cells. Similar to the 200 kDa neurofilament, higher doses of FGF-2 (5 ng/ml) were necessary to induce GFAP expression. The detection of neuronal and non-neuronal markers is consistent with observation raised by others (Jiang et al., 2002a; Jiang et al., 2002c) who reported that MAPCs contributed to the development of neuroectodermal cells. As indicated above, FGF-2 activated neurogenic programme in both mBM-MASCs1 and mBM-MASCs2 (Bedada et al., 2006). To provide additional evidence for the neurogenic potential of mBM-MASCs, a neuron specific Nkx5-1-LacZ reporter construct was employed. In this system, the expression of LacZ indicates activation of the promoter, concomitant with the induction of the neurogenic program. Only FGF-2 treated but not non-treated mBM-MASCs expressed the reporter gene, indicating the specificity of the expression (Fig. 19E). However, the efficiency of expression was rather low which might in part also be explained by the difficulty with which adult stem cells can be transfected by the conventional $CaPO_4$ method. The use of a lentiviral system, which can transduce both dividing and non-dividing cells with high efficiency, might bypass such technical problems. The expression of Nkx5-1-LacZ reporter construct is clearly an additional line of evidence that indicates the activation of the neuroectoderm programme in mBM-MASCs.

5.15 HGF specifies the initial phase of hepatogenesis in mBM-MASCs

The liver, similar to the lungs, pancreas and intestine is derived from the endoderm. Its location within the body and its vital function for survival makes it difficult to identify

mutations or naturally occurring variants (Zaret, 2002). mBM-MASCs serve therefore as an *in vitro* model system to study developmental decisions of primordial liver cells. The detection of transcripts for albumin-1 (Fig. 20A) is in good agreement with the observation that albumin is among the first and liver specific markers induced during mouse liver development (Gualdi et al., 1996; Jung et al., 1999). It was reported that the albumin enhancer has essential binding sites for the transcription factors GATA-4, HNF3, NF-1 and others (Bossard and Zaret, 1998). The detection of GATA-4 after HGF treatment suggested that HGF might control liver gene expression via stimulation of GATA-4 expression. The detection of transcripts of the stress signalling kinase-1, SEK-1 is another line of evidence that indicates activation of the hepatogenic program in mBM-MASCs (Fig. 20A). SEK-1 regulates liver formation and its absence results in defective liver formation and death of the embryo (Bossard and Zaret, 1998). Moreover, various growth factors such as SF/HGF, EGF, IL-1 and TNFα have been shown to play a role in hepatogenesis and liver regeneration following hepatoectomy (Diehl and Rai, 1996; Naldini et al., 1991) possibly through the activation of SEK-1 and SAPK pathways (Bossard and Zaret, 1998).

5.16 Contribution of genetically labelled mBM-MASCs1-eGFP to myogenic, cardiac and endothelial development in chimeric embryo

After the analysis of the differentiation potential of mBM-MASCs lines into cells of the mesoderm, endoderm and neuroectoderm, the developmental potentials of these cells *in vivo* were examined. Genetically labelled mBM-MASCs1 (mBM-MASCs1-eGFP) contributed to somites, heart and blood vessels during development of chimeric embryos (Fig. 21B) (Bedada et al., 2006). The apparent contribution of mMB-MASCs1-eGFP to somites, heart and endothelial development of the chimeric embryo is in line with the *in vitro* observation of the current study (Belema Bedada et al., 2005). Other groups have also reported the contribution of MAPCs to most somatic tissues when injected into an early blastocysts (Jiang et al., 2002a), where they differentiated into tissue specific cell types in response to signals provided by different organs. Other studies proposed that neuronal stem cells when injected into blastocysts contribute to a number of tissues of the chimeric mouse embryo (Clarke et al., 2000).

6 Conclusions

The competence of mBM-MASCs to differentiate at least partially into a variety of cell types representative of all three germ layers (mesodermal, neuroectodermal and endodermal) is the key finding of the present work. I conclude that mBM-MASCs might serve as valuable *in vitro* models for the study of developmental programs and the creation of donor cells for the correction of different genetic and acquired disorders through gene and cell therapy.

mBM-MASCs might serve as a renewable source of cells for the cure of neurodegenerative, muscular, cardiovascular and hepatocyte disorders. Differentiated populations of mBM-MASCs might be purified specifically or enriched for single progenitor cells on the basis of different surface marker molecules. Further technical improvements might give rise to homogenous populations of differentiated progeny in response to lineage specific instructive signals that might be used for clinical studies. Interestingly, it was always possible to isolate dividing undifferentiated cells that maintained the properties of parental cells in cultures that were induced to differentiate. Apparently, mBM-MASCs possess a strong bias to keep their original stem cell programme and to self renew. Subsequent clonal analysis showed that both parental lines and sub-clones share expression of surface marker molecules and of several pluripotency (stemness) markers.

The derivation of muscle progenitor cells from mBM-MASCs1 and mBM-MASCs2 will facilitate the study of early myogenesis and will help create an unlimited source of donor cells for muscle transplantation therapy. Enriched and expandable populations of proliferating muscle progenitors can be generated from mBM-MASCs and might set the stage for future developments that will allow the use of ML-mBM-MASCs1 and ML-mBM-MASCs2 not only for the treatment of muscular disorders but also as an *in vitro* model for the study of myogenesis and identification of novel genes, growth and differentiation factors that have a role in muscle development.

Given the fact that haematopoietic stem cells or progenitors do not grow under normal culture conditions and since committed haematopoietic cells have a short life span, it seems likely that cells with haematopoietic characteristics were not derived from contaminating haematopoietic stem cells but originated from undifferentiated mBM-MASCs. The minor fraction of cells which express CD45 and Ter119 in the absence of exogenously supplied IL-3 might have been induced by bioactive molecules present in the fetal calf serum used to grow mBM-MASCs. It seems unlikely that contaminating haematopoietic cells remain viable after extensive passage of mBM-MASCs1 and mBM-MASCs2. Taken together, these observations

make mBM-MASCs versatile in their properties and excellent model allowing further characterisation of haematopoietic and other lineage commitment potential.

Despite their remarkable plasticity, mBM-MASCs were not able to recapitulate all aspects of cardiomyocytes without modifications of the epigenetic programme. Only the addition of AZA or TSA, which eradicate the ´´histone code´´ initiated, epigenetically allowed the expression of mature cardiomyocyte markers.

Although the differentiation of genetically labelled mBM-MASCs into different cell types *in vivo* in chimeric embryos was not analysed thoroughly in this study, the presence of mBM-MASCs derivatives in various organs of chimeric embryos suggests a remarkable plasticity of mBM-MASCs *in vivo*. The apparent contribution of genetically labelled mBM-MASCs or mBM-MASCs1-eGFP to somite, heart and endothelium development of the chimeric embryos support the *in vitro* observations and serve as an additional line of evidence for a multi-lineage differentiation potential of mBM-MASCs.

7 Materials and methods

7.1 Materials

The majority of chemicals, reagents, cell culture materials, cytokines, growth factors, antibodies, enzymes, oligonucleotides and etc used in the current work were procured from one of the following companies: Sigma (Deisenhofen, Germany), Roth (Hamburg/Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), cell system biotechnology, (Germany), Research Diagnostic, Inc. (NJ, USA), Eppendorf (Wessening-Berzdorf, Germany), New England Biolabs (Frankfurt/Main, Germany), Jena Biosciences, (Germany), Promega (Mannheim, Germany), Stratagene (Heidelberg, Germany), Boehringer (Mannheim, Germany) Quiagen (Hilden, Germany), Molecular Probes-Invitrogen (Karlsruhe, Germany), Pharmacia (Freiburg, Germany), Roche Diagnostics (Mannheim, Germany), Miltenyi Biotec (Bergisch Gladbach, Germany), Life Science (Köln, Germany), BD Biosciences (Heidelberg, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany).

100x BCIP	Roth (Hamburg/Karlsruhe, Germany)
2, 2, 2-Tribromoethanol	Sigma (Deisenhofen, Germany)
30% H_2O_2	Roth (Hamburg/Karlsruhe, Germany)
50x NBT	Roth (Hamburg/Karlsruhe, Germany)
Agarose	Roth (Hamburg/Karlsruhe, Germany)
Bovin serum albumin	Roth (Hamburg/Karlsruhe, Germany)
Chloroform	Roth (Hamburg/Karlsruhe, Germany)
Diaminobenzidine (DAB)	Sigma (Deisenhofen, Germany)
Dithyotreithol (DTT)	Promega (Mannheim, Germany)
Ethidium bromide	Applichen, Germany
Fluorescein	Bio-Rad (Munich, Germany)
Glass slides and cover slides	Plano (Wetzlar, Germany)
Glassware	Schütt (Göttingen, Germany)
Hoechst 33258 dye	Molecular Probes (Karlsruhe, Germany)
Ion exchange resin	Bio-Rad (Munich, Germany)
Isopropanol	Roth (Hamburg/Karlsruhe, Germany)
K_3 [Fe (CN) $_6$], $K_4[Fe(CN)_6]$	Sigma (Deisenhofen, Germany)
Kodak D-19 developer	Kodak (Frankfurt/Main, Germany)
Mowiol	Merck (Darmstadt, Germany)

OligoDT primers	Promega (Mannheim, Germany)
Paraformaldehyde (PFA)	Roth (Hamburg/Karlsruhe, Germany)
Phenol, pH 4-4.5	Roth (Karlsruhe, Germany)
Plastic ware	Nunc (Wiesbaden, Germany)
	Polysciences (Warrington PA, USA)
Random primers	Promega (Mannheim, Germany)
SDS	Bio-Rad (Munich, Germany)
SYBR green	Sigma (Deisenhofen, Germany)
Triton-x	Roth (Hamburg /Karlsruhe, Germany)
Trizol reagent	Invitrogen (Karlsruhe, Germany)
Tween-20	USB (Bad Homburg, Germany)
Vectabond reagent	Camon (Wiesbaden, Germany)
X-gal	Sigma (Deisenhofen, Germany)

7.1.1 Cell culture materials

DMEM-LG	Invitrogen (Karlsruhe, Germany)
DMEM-HG	Invitrogen (Karlsruhe, Germany)
M2 and M16 Media	Sigma (Deisenhofen, Germany)
Fetal calf serum	PAA Laboratories (Haidmannweg)
Horse serum	Invitrogen (Karlsruhe, Germany)
Penicillin G sodium, Streptomycin sulfate and L-Glutamine	Invitrogen (Karlsruhe, Germany)
Penicillin G sodium and Streptomycin sulfate	Invitrogen (Karlsruhe, Germany)
Trypsin-EDTA (TE) mix	Invitrogen (Karlsruhe, Germany)
2.5% Trypsin	Invitrogen (Karlsruhe, Germany)
Non- essential amino acids	Invitrogen (Karlsruhe, Germany)
2-mercaptoethanol	Sigma (Deisenhofen, Germany)
G418 (Geneticin)	Invitrogen (Karlsruhe, Germany)
Dimethyl sulfoxide (DMSO)	Sigma (Deisenhofen, Germany)
Gelatine	Sigma (Deisenhofen, Germany
Filters Minisart NML (0.2 und 0.45 μm)	Sartorius (Göttingen, Germany)
Filters	Schleicher & Schüll (Hannover)
Neubauer counting chamber and cover slips	Optics
Cell scraper	Nunc

DiI	Molecular Probes (Karlsruhe, Germany)

7.1.2 Cytokines or growth factors

RhFGF-2	Cell system biotechnology
RhBMP-2	Research Diagnostic, INC. NJ, USA
RhIL-3	cell system biotechnology
RhHGF/SF	Invitrogen (Karlsruhe, Germany)
Leukaemia inhibitory factor	Laboratory preparation

7.1.3 Epigenetic drugs

5-azacytidine	Sigma-Aldrich (Schnelldorf, Germany)
5-aza-2-deoxycytidine	Sigma-Aldrich (Schnelldorf, Germany)
Trichostatin A	Alexis Biochemicals (Humberg, Germany)

7.1.4 Protein kinase C inhibitors

Staurosporine	Santa Cruz Biotech. Inc.,
Bisindolylmalemeide I	Santa Cruz Biotech. Inc.,

7.1.5 Cell lines

Cell lines established in this work includes

mBM-MASCs1, a mouse bone marrow derived multipotent adult stem cell line. The phenotype of this cell line is $CD34^{-}/Sca\text{-}1^{high}$

mBM-MASCs2 a mouse bone marrow derived multipotent adult stem cell line. The phenotype of this cell line is $CD34^{+}/Sca\text{-}1^{moderate}$

mBM-MASCs1-GFP, A ubiqutin promoter controlled eGFP expressing clonal line derived from mBM-MASCs1 after introducing stably the reporter construct using lenti-viral gene delivery system.

mBM-MASCs2-GFP, A ubiqutin promoter controlled eGFP expressing clonal line derived from mBM-MASCs2 after introducing stably the reporter construct using lenti-viral gene delivery system

ML- mBM-MASCs1, myogenic cell line derived from mBM-MASCs1 after infection with lenti-virus containing a skeletal muscle specific myogenin promoter that drives expression of nuclear eGFP and allows sorting of eGFP positive cells after induction with wnt7A

ML- mBM-MASCs2, myogenic cell line derived from mBM-MASCs2 after infection with lenti-virus containing a skeletal muscle specific myogenin promoter that drives expression of nuclear eGFP and allows sorting of eGFP positive cells after induction with wnt7A.

mBM-ASCs-Sca-1, mouse bone marrow derived adult stem cells enriched for Sca-1 using magnetic cell sorting containing microbeads conjugated to monoclonal rat anti-mouse Sca-1 antibody. The phenotype of this cell line is $CD34^{-}/Sca\text{-}1^{high}$

mH-ASCs, mouse heart-derived adult stem cells. The phenotype of this cell line is $CD34^{+}/Sca\text{-}1^{high}$

mMs-ASCs, mouse muscle-derived adult stem cells.

C2BAC-Wnt-11 cell line, which was established by introducing a gene encoding Wnt-11 using an amphotrophic retroviral gene delivery system into C2BAC packaging cell line.

Phoenix-CDO cell line was established by introducing a gene encoding CDO using a retroviral gene delivery system into a HEK293T based amphotrophic host range packaging cell line.

Phoenix-CA-LEF cell line was established by introducing a gene encoding CA-LEF using a retroviral gene delivery system into a HEK293T based amphotrophic host range packaging cell line.

Phoenix-DN-LEF cell line was established by introducing a gene encoding DN-LEF using a retroviral gene delivery system into a HEK293T based amphotrophic host range packaging cell line.

Cell lines obtained from other sources

Phoenix-Ecotropic cell line is a HEK293 based packaging cell line into which ecotrophic host range retrovirus is packaged. It is designed to infect a narrow host range, including mice and rat strains (American type culture collection, ATCC)

Phoenix-amphotrophic cell line is a HEK293 based packaging cell line into which amphotrophic host range retrovirus is packaged. It is designed to infect a wide variety of mammalian cell types including human and mice (American type culture collection, ATCC)

Phoenix-gp (gag pol) cell line is a HEK293T based packaging cell line into which pantropic or polytropic host range retrovirus could be packaged. It is designed to infect virtually all cell types by pseudotyping with other envelope proteins such as gibbon ape leukaemia virus envelope or Vesicular Stomatitus VSV-G protein (American type culture collection, ATCC)

NIH3T3-Wnt1 cell line was established by introducing a gene encoding Wnt-1 using retroviral gene delivery system into NIH-3T3 cells (Munsterberg et al., 1995).

NIH3T3-Wnt3 cell line was established by introducing a gene encoding Wnt-3 using retroviral gene delivery system into NIH-3T3 cells (Munsterberg et al., 1995).

NIH3T3-Wnt4 cell line was established by introducing a gene encoding Wnt-4 using retroviral gene delivery system into NIH-3T3 cells (Munsterberg et al., 1995).

NIH3T3-Wnt7a cell line was established by introducing a gene encoding Wnt-7a using retroviral gene delivery system into NIH-3T3 cells (Munsterberg et al., 1995).

NIH3T3-Wnt7b cell line was established by introducing a gene encoding Wnt-7b using retroviral gene delivery system into NIH-3T3 cells (Munsterberg et al., 1995).

C2C12, laboratory collection

CH310T1/2 cells, laboratory collection

NIH 3T3 cells (American type culture collection, ATCC), laboratory collection

C2BAC (American type culture collection, ATCC)

Embryonic stem (ES) cells, laboratory collection

Embryonic mouse fibroblast, Emfi (Laboratory collection)

SNHL a cell line containing neomycin and hygromycin resistance genes and LIF gene used as a feeder cell during neomycin and hygromycin selection of ES cells

7.1.6 Enzymes

Collagenase P	Roche (Mannheim, Germany)
DNA polymerase	Promega (Mannheim, Germany)
DNA polymerase I large (Klenow enzyme)	Promega (Mannheim, Germany)
Expand Reverse Transcriptase	Boehringer (Mannheim, Germany)
Protease from Streptomyces griseus	Sigma (Deisenhofen, Germany)
Proteinase K	Boehringer (Mannheim, Germany)
Restriction endonucleases	Boehringer (Mannheim, Germany), Jena Biosciences, Germany New England Biolabs (Schwalbach, Germany)
RNase A	Boehringer (Mannheim, Germany)
RNase H	Promega (Heidelberg, Germany)
RNasin Plus (RNase Inhibitor)	Promega (Heidelberg, Germany)
RQ1 Rnase-free DNase	Promega (Heidelberg, Germany)
Shrimp alkaline phosphatase	Roche (Mannheim, Germany)
Superscript II	Invitrogen (Karlsruhe, Germany)
Taq-DNA-Polymerase	Eppendorf (Wesseling-Berzdorf, Germany)

Trypsin Sigma (Deisenhofen, Germany)

7.1.7 Antibodies

Antibodies for FACS analysis

PE-anti Sca-1 PharMingen, Becton-Dickinson Biosciences

PE-anti c-kit PharMingen, Becton-Dickinson Biosciences

PE- anti CD34 PharMingen, Becton-Dickinson Biosciences

PE- anti CD45 PharMingen, Becton-Dickinson Biosciences

PE- anti CD13 PharMingen, Becton-Dickinson Biosciences

PE- anti Flk-1 PharMingen, Becton-Dickinson Biosciences

PE- anti SSEA-1 PharMingen, Becton-Dickinson Biosciences

PE- anti MHCI (H-2D^{d}) PharMingen, Becton-Dickinson Biosciences

PE- anti CD133 (Prominin) PharMingen, Becton-Dickinson Biosciences

PE- anti Ter119 (glychophorinA) PharMingen, Becton-Dickinson Biosciences

Primary antibodies for immunohistochemsistry

Monoclonal rat anti-mouse Sca-1 antibody, Miltenyi Biotec

Goat ploYclonal anti-Nkx2-5, Santa Cruz Biotech. Inc. 1:300

Mouse monoclonal anti-GATA-4, Santa Cruz Biotech. Inc. 1:300

Mouse monoclonal anti-connexin 43, Zymed 1:1000

Mouse monoclonal anti-cTnI (DPC Biermann) 1:500

Mouse monoclonal anti-cTnT (DPC Biermann) 1:500

Mouse anti-cardiac actin (DPC Biermann) 1:500

Mouse monoclonal anti-sarcomeric myosin heavy chain (MF-20), Developmental studies hybridoma bank, 1:20

Mouse monoclonal anti-myogenin, Developmental studies hybridoma bank. 1:15

Mouse monoclonal anti-β-catenin, Molecular probes 1:500

Mouse monoclonal anti-βIII-tubulin (TuJ1 Ab), BABCO, 1:400

Mouse monoclonal ant-neurofilament 200 clone N52, Sigma, 1:400

Rabbit monoclonal anti-GFAP, DAKO, 1:500

Mouse monoclonal anti-LMO-2, Santa Cruz Biotech. Inc. 1:200

Mouse monoclonal anti-LMO-4, Santa Cruz Biotech. Inc.1:200

Rabbit polyclonal anti-eGFP, Molecular probes 1:1000

Secondary antibodies

Alexa Fluor 488 labelled chicken anti-rabbit IgG, Molecular Probes, 1:1000

Alexa Fluor 594 labelled chicken anti-rabbit IgG, Molecular Probes, 1:1000

Alexa Fluor 488 labelled chicken anti-mouse IgG, Molecular Probes, 1:1000

Alexa Fluor 594 labelled chicken anti-mouse IgG, Molecular Probes, 1:1000

Alexa Fluor 594 labelled chicken anti-goat IgG, Molecular Probes, 1:1000

Biotinylated horse anti-mouse, anti-rabbit IgG, Vector Laboratories, 1:300

7.1.8 Kits

MACS Sca-1 Multisort Kit (Miltenyi Biotec, Bergisch Gladbach)

DNA Cycle Sequencing Kit (Abi, Weiterstadt)

Qiagen® Plasmid Mini- und Maxi-Kit (Qiagen, Hilden)

Vectastain Elite ABC Kit (Vector Laboratories, CA, USA)

Quiagex II® Agarose Gel Extraction Kit (Quiagen Köln)

7.1.9 PCR primers

Primers for amplification of pluripotency marker genes		Anealing temps	cycles
Rex-1	Forward: 5´-GATTTCCACTGTGGCTCTG	56°C	30-35
	Reverse: 5´-CCTTTCTGGCCACTTGTC		
OCT3/4	Forward: 5´-ATACGAGTTCTGCGGAGGGATG	60°C	30-35
	Reverse: 5´-CGGGCACTTCAGAAACATGG		
SSEA-1	Forward: 5´-GCGTTGACCACCTTCATCTG	62°C	30-35
	Reverse: 5´-TCATCCACACCCACCTCTGC		
Nanog	Forward: 5´-TTTCAGAAATCCCTTCCCTC	58°C	30-35
	Reverse: 5´-GCTGAGGTACTTCTGCTTCTG		
B-Myb	Forward: 5´-TGCGAGGATCTGGATGAGTTAC	62°C	30-35
	Reverse: 5´-CGGTCCAGCATGACTTCTTC		

Primers for amplification of muscle specific genes

CW-MyoD	IR: 5´-GGT CTG GGT TCC CTG TTC TGT GT	67°C	30-35
	IF: 5´-CCC CGG CGG CAG AAT GGC TAC G		
CW-Myf5	IR: 5´-CGC TGG TCG CTG GAG AG	58°C	30-35
	IF: 5´-GAG GGA ACA GGT GGA GAA CTA TTA		
CW-MRF4	OR: 5´-ATG GAA GAA AGG CGC TGA AGA CTG	67°C	30-35

	OF: 5´-CTG CGC GAA AGG AGG AGA CTA AAG		
CW-myogenin	OR: 5´-AGG AGG CGC TGT GGG AGT T	58°C	30-35
	OF: 5´-GGG CCC CTG GAA GAA AAG		
Pax3	OR: 5´-GAT CCG CCT CCT CCT CTT CTC CTT	60°C	30-35
	OF: 5´-GCC AGG GCC GAG TCA ACC AG		
Pax-7	Forward: 5´-CCAGCCCCTTCCGCCATCAAC	63°C	30-35
	Reverse: 5´-GTA GCC AGCCACAGGGTCCACACT		
GAPDH	Forward: 5´-GTG GCA AAG TGG AGA TTG TTG CC	61°C	30-35
	Reverse: 5´-GAT GAT GAC CCG TTT GGC TCC		

Primers for amplification of cardiac muscle specific genes

Nkx2-5	Forward: 5´- TTCAAGCCCGAGGCCTACTCTG	64°C	30-35
	Reverse: 5´-TCTTGACCTGCGTGGACGTG		
GATA-4	Forward: 5´-TCAATTGTGGGGCCATGTCCA	62°C	30-35
	Reverse: 5´-TGAATCCCCTCCTTCCGCATT		
Myocardin A	Forward: 5´-ACTGAGGTGAGCCTCTCCAAG	60°C	30-35
	Reverse: 5´-TCTCTGCCTTCTGGTCTGG		
ANP	Forward: 5´-ACCTGCTAGACCACCTGGAGGAG	67°C	30-35
	Reverse: 5´-CCTTGGCTGTTATCTTCGGTACCGG		
BNP	Forward: 5´-ATCTCCTGCAGGTGCTGTCCCAG	67°C	30-35
	Reverse: 5´-GGTCTTCCTACAACAACTTCAGTGCGTTAC		
α-MHC	Forward: 5´-GAATGACGGACGCCCAGATG	64°C	30-35
	Reverse: 5´-ACTGGCAGCCACTTGTAGGG		
β-MHC	Forward: 5´-CTTCAACCACCACATGTTCG	61°C	30-35
	Reverse: 5´-CTTTCTTTGCCTTGCCTTTG		
Hand-2	Forward: 5´-TCCACGGCTGGCTTATTG	67°C	30-35
	Reverse: 5´-ACGTCGGTCTTCTTGATCTC		
TEF-1	Forward: 5´-AAGACGTCAAGCCCTTTGTG	60°C	30-35
	Reverse: 5´-AAAGGAGCACACTTTGGTGG		
Tropomyosin	Forward: 5´-AAGATGCAGATGCTGAAGCTCGAC	67°C	30-35
	Reverse: 5´-CTCCAGCTTCTGCAGAGCTGTG		

Primers for amplification of neuron specific genes

Dopamine beta hydroxylase Forward: 5´-CTTTGCGGATGCCTGGAGTGACC 60°C 30-35

Reverse: 5´-CCGGAAGTGGGGGCTGTAGTGG

Thyroxin hydroxylase Forward: 5´-TACTGTCTGCCCGTGATTTTCTGG 58°C 30-35

Reverse: 5´-AGCGCCGGATGGTGTGAGGACT

Primers for amplification of liver specific genes

Albumin Forward: 5´-CAAGTTCCGCCCTGTCATCTG 64°C 30-35

Reverse: 5´-AACCCCAGCCTGCCACCATT

SEK-1 Forward: 5´-TGTATGGAGCTCATGTCTACC 58°C 30-35

Reverse: 5´-GTCTATTCTTTCAGGTGCCA

Primers for amplification of eGFP gene

Forward: 5´-AAGTTCATCTGCACCACCG 60°C 30-35

Reverse: 5´-TGCTCAGGTAGTGGTTGTCG

Primers for sequencing

T3 5´-ATTAACCCTCACTAAAG 46°C 25

T7 5´- -CGCGCGTAATACGACTCACTTATAG 52°C 25

SP6 5´-CATACGATTTAGGTGACACTATAG 46°C 25

MSCV-neo Forward: 5´-CCCTTGAACCTCCTCGTTCGACC 67°C 25

Reverse: 5´-GAGACGTGCTACTTCCATTTGTC

Used for sequencing Wnt-11, CDO, CA-LEF, DN-LEF cDNAs cloned into MSCV-neo vector

7.1.10 Plasmids or vectors

pGEM-T vector (Promega, Heidelberg)

pBluescript KSII+ (pKSII+) (GenBank-Nr.:X52327 [KS(+)]), Stratagene, Heidelberg)

pBSKSII-α-MHC-c26, a pBSKSII based plasmid containing a 5,5kb α-MHC promoter, which is released by BamHI enzyme (Provided by Jim Gulick)

pBSKSII-α-MHC-c26-eGFP, a pBSKSII based plasmid containing a 5,5kb α-MHC promoter into which eGFP cDNA has been cloned (constructed by the author of this thesis)

pT7T3D-PacI-Wnt-11, A pT7T3D-PacI vector based plasmid containing cDNA of wnt-11 that can be released by NotI and EcoRI (procured from RZPD, clone ID IMAGp 998G23800)

pCMV-SPORT6-CDO, A pCMV-SPORT6 vector based plasmid containing cDNA of CDO that can be released by Sal I and Not I enzymes (procured from RZPD, clone ID IMAGp 998C168558Q3)

pRCAS (A) L14-LEF-CA, an RCAS-L14 based plasmid containing cDNA of CA-LEF that can be released by Nsi and Not I (Laboratory collection)

pRCAS (A) L14-LEF-DN, an RCAS-L14 based plasmid containing cDNA of DN-LEF that can be released by Nsi and Not I (Laboratory collection)

pBM20-α-MHC-neo-pGK-hygro, a pBM20 based vector carrying both an α-cardiac myosin heavy chain-aminoglycoside phosphotransferase (MHC-neo^r) and phosphoglycerate kinase (pGK)-hygromycin resistant genes in a common pBM20 vector backbone (Klug et al., 1996)

pBM20-Nkx2-5-neo-pGK-hygro, a pBM20 based vector carrying both an Nkx2-5-aminoglycoside phosphotransferase (Nkx2-5-neo^r) and phophoglycerate kinase (pGK)-hygromycin resistant genes in a common pBM20 vector backbone (constructed in the laboratory by Sonja Krueger).

pNkx5-1-lac-Z a vector carrying Nkx5-1 promoter driving expression of Lac-Z gene (laboratory collection)

Retroviral vectors

pMSCVneo, is a murine stem cell virus cloning vector containing a neomycin resistant gene (CLONTECH Laboratories, Inc.)

pM57 (gag pol), this retroviral vector expresses the gag pol part of the virus **(**laboratory collection**)**

pM108 (amphotropic), this retroviral vector expresses an amphotropic host range envelope protein **(**laboratory collection**)**

pMSCVneo-eGFP this retroviral transfer vector expresses a reporter gene (eGFP) to be delivered by the virus for marking a putative cell after packaging and infection **(**Laboratory collection**)**

pMSCVneo-wnt11 this retroviral transfer vector is based on a murine stem cell virus cloning vector and expresses the wnt11 to be delivered by the virus during infection after packaging (constructed in this work).

pMSCVneo-CDO this retroviral transfer vector is based on a murine stem cell virus cloning vector and expresses the CDO to be delivered by the virus during infection after packaging (constructed in this work).

pMSCVneo-CA-LEF this retroviral transfer vector is based on a murine stem cell virus cloning vector and expresses the CA-LEF to be delivered by the virus during infection after packaging (constructed in this work).

pMSCVneo-DN-LEF this retroviral transfer vector is based on a murine stem cell virus cloning vector and expresses the DN-LEF to be delivered by the virus during infection after packaging (constructed in this work).

Lentiviral vectors

pHCMV-VSV-G, this lentiviral vector expresses the Vesicular Stomatitus Virus glycoprotein (VSV-G) which has been used to pseudotype a virus. It contains HCMV, the immediate early promoter of human cytomegalovirus. VSV-G, the cDNA encoding VSV-G protein. PA, the polyadenylation signal of the rabbit β-globin gene. Am^r, the ampicilin resistant gene of pBR323 (Dull et al., 1998; Zufferey et al., 1998).

pMDLg/pRRE, this lenti viral vector expresses the gag pol part of the virus (Dull et al., 1998; Zufferey et al., 1998).

pRSV-Rev, this retroviral vector expresses the reverse transcriptase part of the virus (Dull et al., 1998; Zufferey et al., 1998).

pFUGW, this lentiviral backbone is based on the self inactivating vector described in (Miyoshi et al., 1998; Lois et al., 2002). An internal human ubiquitin C promoter drives the expression of the GFP reporter gene. The wood-chuck hepatitis virus post-transcriptional regulatory element (WRE) was inserted downstream of GFP gene to increase the level of transcription (Zufferey et al., 1999). To increase the titter of the virus, the human immunodeficiency virus-1 (HIV-1) flap element (Zennou et al., 2000) was inserted between the 5´ long terminal repeat (LTR) and the human ubiquitin C internal promoter, hence the name FUGW.

pFMHGW, this lentiviral vector was used to express gene of interest in a tissue specific manner. The pFMHGW vector is essentially based on the pFUGW system but engineered in such a way that myogenin promoter drives the expression of the histone 2B-GFP (H2B-GFP) fusion gene, the activity of which is specific to skeletal muscle (Yee and Rigby, 1993). The H2B-GFP reporter gene concentrates the GFP fluorescence in the nucleus.

7.1.11 Solutions

Growth medium	DMEM-LG supplemented with 10% FCS and 1X PSG mix
ES-Growth medium	DMEM-HG supplemented with 15% FCS, 1x PS mix, 1x non-essential amino acids, 0,1 mM 2-mercaptoethanol and 100 u/ml LIF.
Pre-implantation stage	
Embryo medium	M16 medium supplemented with 15% FCS.
Blastocysts transfer buffer	M2 medium supplemented with 15% FCS
Differentiation medium	DMEM-LG supplemented with 3% FCS, 1X PSG mix and differentiation inducing agents (cytokines, growth factors, epigenetic drugs or Wnt molecules)
Differentiation medium	DMEM-LG supplemented with 2% horse serum and 1X PSG mix
0.25%Trypsin-EDTA	contains 0.25 % Trypsin from a 2.5% stock, mycoplasma screened and 0.1 mM EDTA mix in Hepes buffer, for ES cells
rhFGF-2	contains stock concentration of 0.1 mg/mL in 0.1%BSA, aliquoted and kept at –20^{o}C
rhBMP-2	contains a stock concentration of 0.1 mg/mL in 0.1%BSA, aliquoted and kept at –20^{o}C
rhIL-3	contains a stock concentration of 0.1 mg/mL in 0.1%BSA, aliquoted and kept at –20^{o}C
rhHGF/SF)	contains a stock concentration of 0.1 mg/mL in 0.1%BSA, aliquoted and kept at –20^{o}C
Leukaemia inhibitory factor	contains a working concentration of 100 U/ml, aliquoted and kept at –20^{o}C.
5-azacytidine	contains a stock concentration of 0.5 mM in DMSO, aliquoted and kept at –20^{o}C
5-aza-2-deoxycytidine	contains a stock concentration of 10 mM in DMSO, aliquoted and kept at –20^{o}C
Trichostatin A	contains a stock concentration of 0.5 mM in DMSO, aliquoted and kept at –20^{o}C
Staurosporine	contains a stock concentration of 0.2 mM in DMSO, aliquoted and kept at –20^{o}C
Bisindolylmalemeimide I	contain a stock concentration of 1.2 mM in DMSO, aliquoted

	and kept at –20°C
2x HBS	contains 8.0 g NaCl, 6.5 g HEPES (sodium salt), 10 ml of Na2HPO4 stock solution (5.25 g in 500 ml of water). pH adjusted to 7 using NaOH or HCl and volume to 500 ml
Chloroquine	stock (50 mM) and final concentration is 25 µM, aliquoted and kept at –20°C
HEPES buffer	contains 42.42 g NaCl, 2.4 g KCl, 0.36 g KH_2PO_4, 0.48 g $Na_2HPO_4.7H_2O$, 6.9 g glucose, 0.06 g phenol red and 28.6 g Hepes. pH adjusted to 7.3 using NaOH or HCl and volume to 6 litre and autoclaved and kept at –20°C.
Hepes-EDTA	contains 0.073 g of EDTA and 2 L of Hepes buffer and kept at –20°C.
Protamin sulphate	8 mg/ml stock and 8 µg/ml final concentration, aliquoted and kept at –20°C.
Mitomycin C	2mg mitomycin C in 200ml DMEM-LG supplemented with 5%FCS
Mowiol	6 g of glycerol, 2.4 g of Mowiol and 6 ml of H2O mixed and incubated for 4 hours at room temperature, 12 ml of 0.2 M Tris, pH 8,5 added, heated 10 minutes at 50°C, aliquoted and stored at –20°C.
10 x PBS buffer	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4
TE buffer	10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0
50x TAE buffer	contains 2M Tris-base, 1M CH3COOH and 0.1M EDTA, pH adjusted to 8.3 with acetic acid
4% paraformaldehyde (PFA)	
	20 g of PFA was dissolved in 400 ml of water, heated at 60°C with stirring, a few drops of 10 M NaOH were added to clear the solution. After adding 50 ml of 10 x PBS buffer, the volume adjusted to 500 ml and pH to 7.4, filtered and aliquots kept at –20°C.
100x BCIP	5-bromo-4-chloro-3-indolyl phosphate (10 mg/ml in H_2O) and kept at -20°C.
50x NBT	nitro blue tetrazolium chloride (50 mg/ml in 70% dimethyl

	formamide/30% H_2O and kept at -20^0C.
ALP detection buffer 3	contains 100 mM Tris pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$
ALP detection solution	contains 100 µL of 50x NBT, 50 µL of 100x BCIP-x Phos and 5 mL of detection buffer 3.
DAB staining solution	dissolve 1 tablet (1 mg/ml) in 10 ml 0.1 M Tris-HCL pH 7.2 and 5 µl of 30% H_2O_2.
10x Loading buffer	1ml Bromophenol blue, 2.5%ig in H2O; 1ml xylencyanol, 2.5%ig in H2O; 2.5g Ficoll Type 4000 in total volume of 10ml

7.1.12 Microbiology materials

Competent bacteria	E. coli XL1-blue, Stratagene, Heidelberg
LB broth	5 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract/L of distilled water, autoclaved and kept at–20^oC.
LB agar	contains LB broth and 15 g Agar/L
Antibiotics	Ampicilin, stock of 50 mg/ml and working concentration of 100 µg/ml Kanamycin, stock of 10 mg/ml and working concentration of 30 µg/ml Tetracycline, stock of 12.5 mg/ml and working concentration of 12.5 µg/ml
Selective agars	LB-amp-agar, contains ampiciline with a working concentration of 100 µg/ml
LB-kan-agar	contains kanamycin with a working concentration of 30 µg/ml LB-tet-agar, contains tetracycline with a working concentration of 12.5 µg/ml.

7.1.13 Animals

ICR mice: used for the preparation of bone marrow derived adult stem cells, mBM-MASCs1, mBM-MASCs2 and mBM-ASCs-Sca-1 (enriched for Sca-1 expression)

SCID mice: severely compromised immune deficient (SCID) mice were used for transplantation experiments with mBM-MASCs1 and mBM-MASCs2

TgN (beta-act-EGFP): transgenic mouse line expressing eGFP driven by beta-actin promoter and cytomegalovirus enhancer, beta-actin intron and bovine globin poly-adenylation signal

(Okabe et al., 1997) used for the isolation of heart-derived adult stem cells, H-ASCs and muscle-derived adult stem cells, Ms-ASCs.

C57BL/6: has been used for blastocysts injection of mBM-MASCs1-GFP.

B6D2F1: mice have been used as a foster mother to transfer the mBM-MASCs1-eGFP injected blastocysts.

7.2 Methods

7.2.1 Isolation, cultivation and culture expansion of mBM-MASCs

The procedures were performed in accordance with the guidelines for animal experimentation of the Martin Luther University, Halle, Germany. The mBM-MASCs were isolated from the bone marrow of 2 months old female ICR mice as described (Prockop, 1997). Briefly, femur bones were collected and dissected meticulously to ensure that myogenic precursors were not included in the preparation. Both ends of the bones were cut from the diaphysis using bone scissors. Marrow plugs were expelled by inserting a 22-gauge needle fitted to 10 ml syringe containing complete medium (Dulbecco´s modified Eagle medium-low glucose (DMEM-LG) supplemented with 10% (v/v) filtered FCS and 1% (v/v) Penicillin/Streptomycin/ Glutamine (PSG), all from GIBCO) into the proximal end of the tibia or distal end of the femur. The cells were centrifuged at 1000 RPM for 5 min after thorough disaggregation of the marrow plugs, and seeded into tissue culture plates at $6x10^6$ cells per 10 cm plate and allowed to grow at 37 ^{0}C in a humid air with 10% CO_2. The mBM-MASCs were isolated and cultures were expanded by exploiting their adherence to tissue culture plates. Haematopoietic cells were removed through subsequent washing steps and medium changes every 3 days. In contrast to the routine method of cultivation of bone marrow derived stem cells, where the adherent cells were trypsinized after reaching confluency, the culture was continued without subcultivation. Keeping confluent cells for many days without trypsinization allowed some cells to expand clonally (differential survival). Four permanent stem cell populations from different mouse whole bone marrow preparations and other tissues like heart and muscle were established using an identical approach. Two of the permanent cell populations, designated mBM-MASCs1 and mBM-MASCs2 were further characterized and investigated.

7.2.2 Characterization of mBM-MASCs

The cultured mBM-MASCs were observed using a binocular phase contrast microscope (Zeiss) equipped with a digital camera system (Nikon, Japan). A fluorescent activated cell

sorter (FACS)-BD (Becton Dickinson) was used for the phenotypic characterization of these cells. Flow cytometry was performed as described in the manufacturer's manual supplied with the monoclonal antibodies. The expression of CD45, Ter119/Glycophorin A, c-Kit, Flk-1, MHC1/H-2D^{d}), CD133/prominin, SSEA-1, CD13, CD34, and Sca-1 surface markers were assessed at various time points by direct staining with PE-conjugated monoclonal antibodies (mAbs) against CD45, Ter119/GlycophorinA, c-Kit, Flk-1, MHC1/H-2D^{d}), CD133/prominin, SSEA-1, CD13, CD34, and Sca-1, all purchased from BD-Biosciences/Pharmingen. Briefly, undifferentiated cells were trypsinised at approximately 90% confluence and washed twice in Phosphate-Buffered-Saline (PBS) to completely remove the medium, and blocked for 20 min in 5% horse serum plus PBS. The cells were incubated with 1 µg phycoerythrin (PE)-conjugated mAb /10^{6}cells, and incubated for 15 min at 4^{0}C in the dark. The cells were fixed with 2% PFA (Sigma) for 5 min after washing with PBS, and resuspended in 500 µl 5% horse serum in PBS, following a final washing step. Isotype-matched controls were used to assess non-specific fluorescence. The data collected from 10, 000 cells were expressed as percentage of positive cells per total gated cells. The data analysis was made using cell Quest pro software.

7.2.3 Lentiviral labelling of mBM-MASCs1 and mBM-MASCs2

To mark mBM-MASCs1 and mBM-MASCs2 genetically, a lentiviral expression packaging line was prepared following established protocols (Dull et al., 1998; Zufferey et al., 1998). Briefly, 20 µg of pFUGW (see material for detail) containing reporter gene, eGFP were introduced into HEK-293T packaging cells (ATCC) through $CaPO_4$ transfection. Since the pFUGW transfer vector carrying the gene of interest does not contain the gag, pol and env structural genes necessary for particle formation, and eventual infection, 10 µg of pMDLg/pRRE and pRSV-REV (structural vectors) containing gag pol and the enzyme reverse transcriptase and the same amount of pHCMV-VSV-G (envelope vector) carrying envelope protein (polytrophic) for pseudotyping were provided through co-transfection into the same packaging line. 48 hrs post co-transfection, the viral suspension was collected and concentrated using ultra-centrifugation and the titter was determined by FACS analysis using a gradient dilutions. This system yielded a viral titter of 10^{8} infectious viral particles per ml. In practical terms, 1 µl of this viral concentrate was able to infect 75-80% of both mBM-MASCs1 and mBM-MASCs2 in the presence of 8 µg/ml of protamin sulphate in a 6 cm dish. To extend the labelling to 100%, both lines were seeded at single cell level using a limited dilution into 96 wells, expanded, and checked for 100% labelling using FACSs analysis.

7.2.4 Total RNA isolation and RT-PCR

Total RNA was isolated from adult mouse skeletal muscle, heart, liver, brain tissues, packaging lines, differentiated and undifferentiated mBM-MASCs, chimeric and non-chimeric whole embryos using Trizol (Invitrogen) following the manufacturers instruction. RT-PCR for skeletal muscle specific genes, such as Myf-5, MyoD, myogenin and MRF4 (Myf-6 or herculin), pax-3 and pax-7 was performed using 1 μg of DNAase treated RNA isolated from skeletal muscle tissue, differentiated and undifferentiated mBM-MASCs. RT-PCR for cardiomyocyte specific genes, such as Nkx2.5, GATA-4, myocardin A, α-MHC, β-MHC, ANP, BNP, Hand2, Tef-1, and Tropomyosin, for brain tissue specific genes, such as dopamine beta hydroxylase (DBH) and thyroxin hydroxylase (TH), for liver specific genes, such as albumin-1 and stress signalling kinase-1, SEK-1, for pluripotency (stemness) marking transcription factors such as OCT3/4, Rex-1, B-Myb, Nanog and SSEA-1, and for eGFP was performed using 1 μg of DNAase treated RNA isolated from heart, brain, liver tissues, differentiated and undifferentiated mBM-MASCs, chimeric and non-chimeric whole embryos. In all cases, a house keeping gene, GAPDH was used as an internal control for the integrity of RNA. The amplified PCR products were size fractionated on 2% agarose gel electrophoresis stained with ethidium bromide. All primers used for amplification of all genes were purchased from Roth. PCR was performed at standard PCR conditions. The annealing temperatures, number of cycles and specific primers are indicated in the material section.

7.2.5 Immunofluorescence staining

Monoclonal antibodies against sarcomeric myosin heavy chain (MF-20, 1:20) and myogenin (1:15) were prepared from hybridoma cells (Developmental studies hybridoma bank). Monoclonal antibody against β-catenin was a kind gift from Prof. Hartzfeld (MLU, Halle). Mouse monoclonal antibodies against GATA-4 (anti-GATA-4, 1:300) and goat polyclonal against Nkx2.5 (anti-Nkx2.5, 1:300) were purchased from Santa Cruz Biotech Inc. Monoclonal antibodies against cardiac troponin T (anti-cTnT 1:500) Troponin I (anti-cTnI, 1:500) and cardiac actin (1:500) were obtained from DPC Biermann. Monoclonal antibodies against β_{III}-tubulin were from BABCO, 1:400; neurofilament (NF-200 clone N52, 1:400) was purchased from Sigma and anti-GFAP (1:500) was from DAKO. Monoclonal antibodies against LMO-2 and LMO-4 (1:200) were obtained from Santa Cruz Biotech Inc. A rabbit polyclonal anti-eGFP (1:1000) was from Molecular probes. Differentiated cultured cells from

6-well plates were washed three times with Phosphate-Buffered-Saline (PBS), and fixed in 4% PFA (Sigma) for 15-20 min at room temperature. The fixed cells were permeablized with 0.1% Triton X-100 (Sigma) in PBS, and non-specific binding sites were blocked for 45 min in 1% BSA (Sigma) + 5% of a serum from an animal in which the secondary antibody was raised + 0.1% Triton X-100 in PBS. The cells were incubated with appropriate dilutions of respective primary antibodies in blocking solution overnight at 4^0C. After 3 times washing with 1% BSA + 0.1% Triton X-100 in PBS, the secondary antibodies Alexa flour 488 (anti-mouse IgG), 594 (anti-mouse IgG), 488 (anti-rabbit IgG), 594 (anti-rabbit IgG), 594 (anti-goat IgG) all from Molecular probes were added at 1:1000 dilution and incubated for 1 hr at room temperature in the dark. The nuclear staining was performed using Hoechst33258 dye (Dako) at 5 μg/ml for 10 min. The resulting fluorescence was examined with a fluorescence microscope (Zeiss) and photographs were taken with a Nikon digital Camera (Nikon, Japan) at 100 or 200 fold magnification.

7.2.6 Detection of myogenin using diaminobenzidine (DAB) substrate

A monoclonal antibody against myogenin was prepared from hybridoma cells (Developmental studies hybridoma bank). Differentiated cultured cells were washed 3x with Phosphate-Buffered-Saline (PBS), and fixed in 4% PFA (Merck) for 20 min at room temperature. Fixed cells were permeablized in 0.1% Triton X-100 (Roth) /PBS. Endogenous peroxidase activity was reduced by incubation with 3% H_2O_2 (Roth) in methanol (Roth) for 10 minutes. Non-specific binding sites were blocked for 45-60 min in 1% BSA (Roth) + 5% horse serum (Vector Laboratories INC.) + 0.1% Triton X-100 /PBS. The cells were incubated with the 1:15 diluted anti-myogenin primary antibody overnight at 4^0C. After 3x washing in 1% BSA + 0.1% Triton X-100/PBS, the secondary antibody, biotinylated anti-rabbit and anti-mouse IgG (ABC-kit, Vector Laboratories, CA, USA) were added at 1:200 dilution in blocking solution and incubated for 1hr at room temperature in the dark. Washing was done serially with 1% BSA + 0.1% Triton X-100/PBS for 5min, 0.1% Triton X-100/PBS for 5min, 2x, and in PBS for 5 min, 2x. Incubation in ABC reagent containing avidine-coupled horseradish peroxidase (Vector Laboratories INC.) was done for 30 min, while the biotin-coupled antibody complexed with avidine-biotin-horseradish peroxidase. After a final 4 times washing in PBS for 5 min, cells were stained with 1% NiCl (8% stock) plus Diaminobenzidine (DAB) (Sigma) for 2-5 min. After removal of the staining solution, cells were washed in PBS and water, and kept in PBS for microscopic observation. Images were captured with the Nikon digital Camera (Nikon, Japan) at 100-fold magnification.

7.2.7 Detection of alkaline phosphatase (ALP)

BMP-2 treated mBM-MASCs1 and mBM-MASCs2 were washed in PBS 3 times and fixed with 4% PFA for 10 min. After 4 times washing in PBS, they were treated with detection buffer 3 (100 mM Tris, 100 mM NaCl and 50 mM $MgCl_2$) for 15 min. After treatment with detection solution (100 µL of 50x NBT, 50 µL of 100x BCIP-x Phos and 5 mL of detection buffer 3) for 15-20 min, cells were placed in 10 mM TE for 5 min. Finally, specimens were washed in PBS for 5 min, 3x and post fixed in 4% PFA for 5 min. Images were captured with the Nikon digital Camera (Nikon, Japan) at 100 fold magnification.

7.2.8 DNA sequencing

PCR amplified cDNA products and cloned DNA fragments were sequenced using the polymerase chain reaction and subjected to Abi Prism TM 310 Genetic Analyser (Perkin Elmer). A sequencing reaction typically contained 300 ng of plasmid DNA; the respective primers designed to recognize sequences from the vector backbone and cloned gene of interest and components of the DNA sequencing Kit (Abi, Weiterstadt). The reaction was performed following the manufacturers instruction. Perkin Elmer Cetus Cycler "Gene Amp PCR-System 9600" was used for amplification of DNA.

7.2.9 Cloning, sequencing, tranfection and preparation of retroviral expression packaging lines

A mouse EST clone containing a cDNA of cell adhesion molecule CDO (Down regulated by Oncogen) IMAGp 998C168558Q3, and another mouse EST clone containing a cDNA of wingless related MMTV integration site II (wnt11) IMAGp998G23800 were purchased from Deutsches Ressourcenzentrum für Genomforschung, GmBH. The sequence of wnt-11 from pT7T3D pacI vector was verified with T3 and T7 sequencing primers. A 1.8 kb wnt11 cDNA fragment was released from pT7T3D pacI vector using Not I and EcoR I restriction endonucleases, and cloned into a 6.5 kb murine stem cell virus carrying the neomycin resistance gene (pMSCVneo) vector (Clontech). The sequence of CDO from pCMV-SPORT 6 vector was analysed using SP6 sequencing primers. A 3.9 kb cDNA fragment of CDO gene was released from pCMV-SPORT 6 vector using Sal I and Not I restriction endonucleases and cloned into pMSCVneo vector (Clontech). To clone CA-LEF, a 0.8 kb CA-LEF cDNA fragment was released from pRCAS (A) L14 vector using Nsi and Not I restriction endonucleases modified with Bgl II linkers and cloned into a Bgl II cloning site of the 6.5 kb

murine stem cell virus vector carrying the neomycin resistance gene (pMSCVneo) (Clontech). To clone DN-LEF, a 0.6 kb DN-LEF cDNA fragment was released from the pRCAS (A) L14 vector using Nsi and Not I restriction endonucleases modified with Bgl II linkers to create a Bgl II site and cloned into a Bgl II cloning site of 6.5 kb murine stem cell virus vector carrying the neomycin resistance gene (pMSCVneo) (Clontech). In all cases, the success of the cloning procedure was controlled by DNA sequencing. To clone eGFP under the transcriptional control of αMHC promoter, a 0.8 kb eGFP fragment cloned into Sal I site of pBSKSII plasmid vector containing αMHC promoter. The correct orientation was checked with Hind III and sequence analysis. Wnt-11, CDO, CA-LEF and DN-LEF retroviral expression-packaging lines were prepared following the protocol developed from the Nolan laboratory, http://www.stanford.edu/group/nolan/protocols/pro_helper_free.html. Briefly, 15 μg of pMSCVneo containing wnt11, CDO, CA-LEF or DN-LEF coding region were delivered into C2BAC for wnt-11, and for CDO, CA-LEF or DN-LEF phoenix packaging cell line (ATCC) through transfection. Since the pMSCVneo transfer vector carrying gene of interest does not contain the gag, pol and env structural genes necessary for particle formation, and replication, 15 μg of pM57 vector (structural vector) containing gag pol and the same amount of pM108 vector (envelope vector) carrying env (amphotrophic) were provided through co-transfection into the same packaging line. The two packaging cell lines, C2BAC and phoenix were placed under G418 selection (1-1.5 mg/ml) 48 hrs after transfections for 1 week. The success of packaging and protein expression was evaluated by isolating total RNA from the respective packaging lines and RT-PCR analysis using appropriate primers.

7.2.10 Preparation of feeder cells with distinct wnts, CDO, CA-LEF, co-culture and filter experiments

Retroviral packaging cell lines producing wnt7A, wnt7B, wnt4 (Munsterberg et al., 1995), wnt11, CDO and CA-LEF were expanded in DMEM-LG supplemented with 10% FCS v/v and treated with mitomycin C (GIBCO BRL). For co-culture experiments, mBM-MASCs1 and 2 were seeded in 6-well tissue culture plates (Nunc) at a density of $1x10^5$ cells/well. After 24 hrs, equal amounts of amitotic feeder cells producing either the Wnt signalling molecules or CDO or CA-LEF were added, and maintained in DMEM-LG supplemented with 3% FCS v/v and allowed to differentiate for 7 or 8 days with a medium change every three days. The cells were observed under a microscope each day for signs of morphologic changes. For filter-experiments polycarbonate-filter (Nunc) with different pore-sizes were used (0.4 μm,

3.0 μm and 8.0 μm). Feeder cells containing distinct wnts and mBM-MASCs were plated on opposite sites of the filters and allowed to attach before differentiation medium was added. After 7-8 days cells on the filters were stained with the MyHC-specific, MF20 antibody. In all cases the experiments were done in duplicate, one well was used for immunostaining and total RNA was isolated from the other well.

7.2.11 Myogenic differentiation with 5-aza cytidine and/or Trichostatin A (TSA)

To initiate the skeletal muscle differentiation program by epigenetic reprogramming, mBM-MASCs1 and 2 were seeded in 6-well tissue culture plates (Nunc) at a density of $1x10^5$ cells/well. The next day, they were treated with different concentrations of 5-azacytidine (5, 10, and15 μM) or Trichostatin A (0.1, 0.3, and 0.9 μM or combination of both 5-aza and TSA (5+0.1), (10+0.3), (15+0.9 μM), obtained from Sigma and Alexis. After 24 hrs of treatment, medium was changed and the cells were maintained in DMEM-LG supplemented with 3% FCS v/v and allowed to differentiate for 7-10 days with medium change every three days.

7.2.12 Establishment of myogenic lineage in mBM-MASCs1 and mBM-MASCs2

To establish myogenic lineages from mesenchymal stem cells, both mBM-MASCs1 and mBM-MASCs2 were infected with lentiviral gene delivery system carrying a skeletal muscle specific myogenin promoter that controls the expression of eGFP. The virus carrying this tissue specific reporter construct pFMHGW (see materials for detail) is produced essentially in the same manner as pFUGW, a tissue non-specific reporter construct used for cell tracking. After infection with a tissue specific reporter construct, mBM-MASCs1 and mBM-MASCs2 were co-cultured with mitotically inactivated feeder cell expressing wnt-7a. The expression of nuclear eGFP was monitored daily. After analysing the number of eGFP positive cells using FACS analysis, mBM-MASCs1 and mBM-MASCs2 expressing eGFP were sorted using a FACS. Sorted cells were expanded and termed ML- mBM-MASCs1 and ML-mBM-MASCs2 in accordance with their parent cell line from which they were derived.

7.2.13 Co-culture of mBM-MASCs1, mBM-MASCs2, ML-mBM-MASCs1 or ML-mBM-MASCs2

To address the question if these cell lines show fusion capabilities *in vitro*, each of the committed myogenic lineages (ML-mBM-MASCs1 or ML-mBM-MASCs2) and uncommitted parent lines (mBM-MASCs1 or mBM-MASCs2) were co-cultured with C2C12 cells, a *bona fide* muscle cell line. The co-culture was initiated by seeding equal cell density

($7.5x10^4$) of both the inducer and responding cell lines, and followed for evidence of fusion on the daily basis.

7.2.14 Inhibition of protein kinase C

To analyse the effects of an inhibition of PKC activity, both mBM-MASCs1 and mBM-MASCs2 were treated with different concentrations of two PKC inhibitors, Staurosporine (8 nM) and Bisindolylmalemeimide I (0.1, 1 and 2 μM). After 12 hours of inhibition, mBM-MASCs1 and mBM-MASCs2 were co-cultured with mitotically inactive C2BAC feeder cells carrying wnt-11 and allowed to differentiate for 7-8 days with medium and inhibitors change every 3 days**.** To monitor the effect of PKC inhibition on the expression of cardiomyocyte genes, PCR was performed for cardiomyocyte specific genes under standard conditions. The cDNA was synthesised from RNA isolated from heart, mBM-MASCs1 and mBM-MASCs2 treated with inhibitors and wnt-11 as well as those treated with wnt-11 only. The primers that specifically amplify cardiomyocyte genes and the corresponding annealing temperatures are indicated in the material section.

7.2.15 Cardiogenic differentiation with 5-aza cytidine and/or Trichostatin A (TSA)

To initiate the cardiogenic differentiation program, mBM-MASCs1 and 2 were seeded in 6-well tissue culture plates (Nunc) at a density of $1x10^5$ cells/well. The next day, they were treated with 10 μM of 5-azacytidine, 0.6 μM of Trichostatin A or a combination of 10 μM of 5-azacytidine and 0.6 μM of Trichostatin A obtained from Sigma and Alexis respectively. After 24 hrs of treatment, medium was changed and cells were maintained in DMEM-LG supplemented with 3% FCS v/v and allowed to differentiate for 8-15 days with medium change every three days.

7.2.16 Cardiogenic differentiation with FGF-2 and/or BMP-2

To initiate the cardiomyogenic differentiation program, mBM-MASCs1 and 2 were seeded in 6-well tissue culture plate (Nunc) at a density of $1x10^5$ cells/well. The next day, they were treated with different concentrations of FGF-2 (1,3, and 5 ng/ml) or a combination of both FGF-2 and BMP-2 (1+0,2), (3+0,6), (5+1 ng/ml) in DMEM-LG supplemented with 3% FCS v/v (differentiation medium, DM) and allowed to differentiate for 15- 21 days with DM change every 3 days.

7.2.17 Osteogenic differentiation with 5-azacytidine and BMP-2

To induce the osteogenic differentiation program, mBM-MASCs1 and 2 were seeded in 6 well tissue culture plate (Nunc) at a density of $1x10^5$ cells/well. The next day, they were treated with 2 ng/ml BMP-2 (RDI) and 10 μM 5-azacytidine in DMEM-LG supplemented with 3% FCS v/v (differentiation medium, DM) and allowed to differentiate for 15 days with DM change every 3 days.

7.2.18 Haematopoietic differentiation with IL-3

To induce expression of haematopoietic markers, mBM-MASCs1 and 2 were seeded in 6-well tissue culture plate (Nunc) at a density of $1x10^5$ cells/well. The next day, they were treated with different concentration of IL-3 (Cell System) at 1, 3, and 5 ng/ml in DMEM-LG supplemented with 3% FCS v/v (differentiation medium, DM) and allowed to differentiate for 21 days with DM change every 3 days. Haematopoietic like cells were identified by staining with May-Grunwald Giemsa (Boehringer Mannheim). Expression of haemoglobin was measured spectrophotometrically using the benzidine test. Cells were further characterized by immunofluoresence using antibodies against various cell surface molecules and by RT-PCR using primers for GATA-1, AML-1, PU.1, EKLF, SCL (tal-1), LMO2 and GATA-2.

7.2.19 Neuroectodermal differentiation with FGF-2

To induce neuronal and glial cells markers, mBM-MASCs1 and 2 were seeded in 6 well tissue culture plate (Nunc) at a density of $1x10^5$ cells/well. The next day, they were treated with different concentrations of FGF-2 (Cell Systems) 1, 3, 5 ng/ml in DMEM-LG supplemented with 3% FCS v/v (differentiation medium, DM) and allowed to differentiate for 10 days with DM change every 3 days.

7.2.20 Nkx5-1-LacZ reporter construct transfection and LacZ staining

To analyze the neurogenic potential of mBM-MASCs, 8 μg of Nkx5-1-LacZ reporter construct was transfected into mBM-MASCs1&2 via $CaPO_4$ transfection method. 24 hrs post-transfection, both cell lines were treated with 5 ng/ml of FGF-2 and allowed to differentiate for 10-14 days with differentiation medium (DMEM-LG supplemented with 3% FCS and 5 ng/ml FGF-2) change every three days. To monitor the expression of LacZ and hence the activity of the Nkx-5-1 promoter, mBM-MASCs1&2 were stained with X-GAL. Cells were washed 3x in PBS, fixed for 5min at room temperature in solution B (0.2% glutaraldehyde +

solution A (5 mM EGTA and 2 mM $MgCl_2$ in PBS) and washed 3x in solution C (0.01% Na desoxycholate and 0.02% Nonidet P40 in solution A). After washing, cells were incubated overnight at 37^0C in solution D (0.01% Na desoxycholate, 0.02% Nonidet P40, 10 mM K_3 [Fe (CN) $_6$], 10 mM K_4 [Fe (CN) $_6$] and 1.5-2 mg/ml X-gal in solution A). After final wash in solution C, the cells were observed under microscope for evidence of LacZ staining.

7.2.21 Endodermal differentiation with hepatocyte growth factor/Scatter Factor (HGF/SF)

To monitor the induction of hepatogenic markers, mBM-MASCs1 and 2 were seeded in 6-well tissue culture plates (Nunc) at a density of $1x10^5$cells/well. The next day, they were treated with different concentration of HGF/SF (GIBCO BRL) such as, 10, 15, and 20 ng/ml in DMEM-LG supplemented with 3% FCS v/v (differentiation medium, DM) and allowed to differentiate for 15 days with DM change every 3 days.

7.2.22 Injection of mBM-MASCs1 into blastocysts and analysis of chimeric mice

To asses the contribution of mBM-MASCs to chimeric embryos, 10-20 mBM-MACs1-eGFP were delivered into 3,5 days old blastocysts isolated from C57/BL6 mice as described previously (Braun et al., 1992). The injected blastocysts were transplanted into foster mothers. At E10.5 dpc, the embryos were isolated and checked for eGFP expression using fluorescence microscope. After fixing the embryos for 1hr in 4% PFA and cryoprotection in 30% sucrose, the embryos were embedded in embedding boxes containing polyfreeze tissue freezing medium and kept in dry ice until freezing medium solidified. The blocks were kept at -20^0C and sectioned at 10µm using a cryotome (Leica, Germany). Cryosections were collected on Vectabond-coated glass slides, dried at room temperature and stored at -20^0C. To identify the part of embryos where mBM-MASCs1-eGFP were present, sections were stained for eGFP protein using polyclonal rabbit anti-eGFP primary antibody and Alexa 594 anti-rabbit secondary antibody (both from Molecular probes INC) using a dilution of 1:1000 for both antibodies. In addition, a PCR was performed to detect expression of the eGFP marker gene using cDNA synthesized from RNA isolated from the chimeric and non-chimeric whole embryos and mBM-MASCs1-GFP as a positive control. The PCR was run for 30-35 cycles at 60^oC annealing temperature using primers that specifically amplify the eGFP gene.

8 Abbreviations

ABCG2	ATP-binding cassette super family G member 2
ANP	Atrial nitriuretic peptide
APC	Adenomatous polyposis coli protein
ATCC	American type culture collection
BDNF	Brain derived neurotrophic factor
BNP	Brain nitriuretic peptide
BSA	Bovine serum albumin
CA-LEF	Constitutive active- Lymphocyte enhancer factor
CAM	Cell adhesion molecule
CamKII	Ca^{2+}-calmodulin-dependent protein kinase II
CBP	CREB-binding proteins
CD	Clusters of differentiation
cDNA	Complimentary DNA
CDO	Cell adhesion related down regulated by oncogen
CFU-F	Fibroblast colony forming unit
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CNS	Central nervous system
CtBP	C-terminal binding protein
DAB	Diaminobenzidine
DAG	Diacylglycerol
DBH	Dopamine beta hydroxylase
DMD	Duchenne muscular dystrophy
DMEM-HG	Dulbecco´s modified Eagle medium-high glucose
DMEM-LG	Dulbecco´s modified Eagle medium-low glucose
DNA	Deoxyribonucleic acid
DN-LEF	Dominant negative- Lymphocyte enhancer factor
Dpc	Days post coitum
Dvl	Deshiveled
EB	Embryonic bodies
EC cells	Embryonic carcinoma cells
EG cells	Embryonic germ cells
EGF	Epidermal growth factor

eGFP	Enhanced green fluorescence protein
ES cells	Embryonic stem cells
FACS	Fluorescent activated cell sorter
FITC	Fluorescein isothiocyanate
FLK-1	Fetal liver kinase-1
FNIII	Fibronectin III
G	Heterotrimeric G proteins
GBP	GSK-3 binding protein/Frat-1
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony stimulating factor
Gr	Groucho
GSK-3β	Glycogen synthase kinase-3beta
HAND2	Heart- and neural crest derivatives-expressed protein 2
HMG	High mobility group
HNF3	Hepatocyte nuclear factor 3
HSCs	Haematopoietic stem cells
ICM	Inner cell mass
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-3R	Interleukin-3 receptor
IL-4	Interleukin-4
IP3	Inositol-1, 4, 5-trisphosphate
JNK	Jun N-terminal kinase
KDa	Killodalton
LIF	Leukemia inhibitory factor
Lin^-	Lineage negative
LMO-1	Lim only-1
LMO-2	Lim only-2
LMO-4	Lim only-4
mAbs	Monoclonal antibodies
MAPKs	Mitogen activated protein kinase
mBM-ASCs-Sca-1	Mouse bone marrow derived adult stem cells enriched for Sca-1
mBM-MASCs1	Mouse bone marrow derived multipotent adult stem cells 1
mBM-MASCs1-eGFP	eGFP marked mBM-MASCs1

mBM-MASCs2	Mouse bone marrow derived multipotent adult stem cells 2
mBM-MASCs2-eGFP	eGFP marked mBM-MASCs2
MeCP2	Methyl CpG binding proteins
mH-ASCs	Mouse heart-derived adult stem cells
MHC	Myosin heavy chain
MHCI	Major Histocompatibility complex I
ML- mBM-MASCs1	Myogenic lineage derived from mBM-MASCs1
ML- mBM-MASCs2	Myogenic lineage derived from mBM-MASCs2
mMs-ASCs	Mouse muscle-derived adult stem cells
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MSCV	Murine stem cell virus
mWnt-11	Mouse wnt-11
Myb	Myeloblastoma
NCSCs	Neural crest stem cells
NF-1	Neurofilament-1
NGF	Nerve growth factor
NIH	National institute of health
NK cells	Natural killer cells
NTR	Neurotrophin R
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
Pax-3	Paired box-3
Pax-7	Paired box-7
PBS	Phosphate buffer saline
PBSCs	Peripheral blood stem cells
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PHSCs	Pluripotent haematopoietic stem cells
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PSA-NCAM	Polysialic acid-neural cell adhesion molecule

rhBMP-2	Recombinant human bone morphogenetic protein-2
rhFGF-2	Recombinant human fibroblast growth factor-2
rhHGF/SF	Recombinant human hepatocyte growth factor/scatter factor
rhIL-3	Recombinant human interleukin-3
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAPK	Stress activated protein kinase
Sca-1	Stem cell antigen-1
SEK-1	Stress signalling kinase-1
SP cells	Side population cells
SSEA-1	Stage specific embryonic antigen-1
TCF/LEF	T-cell factor/Lymphocyte enhancer factor
TEF-1	Transcription enhancer factor-1
TH	Thyroxin hydroxylase
TNF2	Tumer neucrosis factor 2
TrkC	Tyrosine kinase C
TSA	Trichostatin A
xWnt-11	Xenopus wnt-11
α-MHC	Alpha myosin heavy chain
β-MHC	Beta myosin heavy chain

9 Curriculum Vitae (CV)

I personal information

Name: Fikru Belema Bedada

Date of birth: Sep 30, 1971

Country of birth: Ethiopia

Nationality: Ethiopian

Ethnicity: Oromo

Native language: Oromic and Amharic

Contact address: Fikru Belema Bedada

Max-Planck-Institute for Heart and Lung Research

(W.G. Kerckhoff- Institute)

Department of Cardiac Development and Remodelling

Parkstrasse 1, D-61231, Bad Nauheim, Germany

Email fikru.belema@kerckhoff.mpg.de

Phone +49/6032/ 705-267

Fax +49/6032/ 705-419

II Education

Diploma in Medical Laboratory Technology (MeLT), awarded by Jimma Institute of Health Sciences (JIHS) in 1990, Ethiopia

BSc in MeLT, awarded in 1998 by JIHS, now Jimma University (JU), Ethiopia

MSc in Molecular Biology, Human health, awarded on sep 21, 2001 by free University of Brussels (VUB) with grade of great distinction.

PhD work: Plasticity of murine Bone Marrow-Derived Adult Stem Cells: acquisition of specialized properties and contribution to embryonic development from 2002-2006.

Academic award: student of the year 1996/1997

Msc dissertation: *In vitro* differentiation and maturation of human myeloid dendritic cells from CD14+ precursors cells in peripheral and cord blood cells.

PhD dissertation: Plasticity of murine Bone Marrow-Derived Adult Stem Cells: acquisition of specialized properties and contribution to embryonic development.

III work experience

Technical assistance and instructor (1990-1995) at JIHS, Ethiopia

-Conducted practical laboratory work

-Instructed courses such as Immunology, Immuno-haematology, Clinical Chemistry and Microbiology
-Assistant lecturer (1998-1999) at JIHS, Ethiopia
-Advised students in their research project
-Assisted as senior supervisor in Team Training Program (TTP) and Community Based Training Program (CBTP)
-Taught different laboratory courses
-Involved in research

IV. Techniques

Molecular Biology Techniques

DNA isolation and manipulation, DNA cloning and sub-cloning, RT-PCR, Colony-PCR, SDS-PAGE, Transfection, Infection, Electrophoresis, Protein Purification, Affinity chromatography, Western Blotting.

Cellular Biology Techniques

Culture and maintenances of embryonic stem cells (ES cells) and adult stem cells (ASCs) and their differentiation. Tail vein injection of stem cells into mice, isolation of cells from different tissues and their characterization, Flowcytometry (FACS), Immunohistochemistry, genetic labelling of cells using retroviruses and lentiviruses.

Knowledge of English language

Sufficient knowledge to understand, read, write and speak. It is the working and Teaching language in the higher Education system of my country.

10 Publications and presentation

Conference participation:

BioMEMS and BIOMEDICAL NANOTECHNOLOGY WORLD 2001 organized by Cambridge Healthtech Institute and the Ohio state University from Sept 22-25, 2001.

Poster Präsentation

Belema Bedada, F., Technau, A., Ebelt, H., Schulze, M., and Braun, T. (2005). Two populations of murine Bone Marrow-Derived Multi-potent Adult Stem Cells (mBM-MASCs) have the competence to produce cells representative of the three germ layers in vitro and contribute to development of chimeric embryos in vivo: Evidence for their plasticity. At Free University of Brussels, Follow-up meeting for the Inter-university programme molecular biology (IPMB) Brussels, August 22-27, 2005

Belema Bedada, F., Braun, T. (2006). Activation of myogenic differentiation in adult stem cells (mBM-MASCs) by different signalling molecules and epigenetic reporgraming. At 5th International Ascona Workshop on cardiomyocyte cell biology, Differentiation, stability of cytoarchitecture and therapeutic potential of heart muscle cells, Monte Verta, Ascona, Switzerland, April 2-6, 2006

Martire, A., Belema Bedada, F., Wietelmann, A., Braun, T. (2006). Peripheral injection of mesenchymal adult stem cells prevents left atrium dilatation and improves cardiac function in MCP-1 induced cardiomyopathy. At 5th International Ascona Workshop on cardiomyocyte cell biology, Differentiation, stability of cytoarchitecture and therapeutic potential of heart muscle cells, Monte Verta, Ascona, Switzerland, April 2-6, 2006

Martire, A., Belema Bedada, F., Wietelmann, A., Braun, T. (2006). Peripheral injection of mesenchymal adult stem cells prevents left atrium dilatation and improves cardiac function in MCP-1 induced cardiomyopathy. At International Society for Heart Research 26th European Section Meeting, Manchester, UK, Jun 14-17 2006.

Oral Präsentation

Belema Bedada, F., Technau, A., Ebelt, H., Schulze, M., and Braun, T. (2005). Molecular cues guided specification and maintenance of myogenic program in non-committed adult

stem cells (mBM-MASCs). At 6th annual meeting of the DFG priority program 1109 Embryonic and somatic stem cells together with the Priority program 1129 Epigenetics in Dresden, October 27-29/30, 2005. Max-Planck-Institute of Molecular Cell Biology and genetics

Belema Bedada, F., Braun, T. (2006). Activation of myogenic differentiation in adult stem cells (mBM-MASCs) by different signalling molecules and epigenetic reporgraming. At 5th International Ascona Workshop on cardiomyocyte cell biology, Differentiation, stability of cytoarchitecture and therapeutic potential of heart muscle cells, Monte Verta, Ascona, Switzerland, April 2-6, 2006

Publications

Schulze, M., Belema-Bedada, F., Technau, A., and Braun, T. (2005). Mesenchymal stem cells are recruited to striated muscle by NFAT/IL-4-mediated cell fusion. Genes Dev *19*, 1787-1798.

Belema Bedada, F., Technau, A., Ebelt, H., Schulze, M., and Braun, T. (2005). Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. Mol Cell Biol *25*, 9509-9519.

Bedada, F. B., Gunther, S., Kubin, T., and Braun, T. (2006). Differentiation versus Plasticity: Fixing the Fate of Undetermined Adult Stem Cells. Cell Cycle *5*.

I certify that all the information indicated above are true and corret to the best of my Knowledge and belief.

Signature Date

11 Acknowledgements

First of all, praise shall be to the almighty GOD for giving me the strength and hope which has brought my PhD work to a success. I owe special gratitude to my promoter Prof. Dr. Thomas Braun for sharing his accumulated experience, unwavering full support and help to the last minute of my work. To my families who owed me their affection and followed my progress enthusiastically. To my wife Mihret Nigusse whose presence by my side all the time contiues to inspire me.

I would like to thank Dr. Thilo Borchardt, Dr. Thomas Kubin and Dr. Matthias Heil for reading my thesis and constructive suggestions. I would like to acknowledge Dr. Henning Ebelt for his invaluable help in introducing me to important laboratory techniques. To Dr. Marcus and Sonja Krüger for their assistance in the lab and acquainting me to the new social environment during the first year of my PhD life. A very special thanks goes to Gabriele Liebert-Hoang for solving all administrative works with amazing patience. To Stefan Gunther for his invaluable help in time of need. A big thanks goes to Undine Ziese for her excellent lab support.

I have been benefited enormously from the following people who are my colleagues and work mates, whose enormous contribution I can not acknowledge in person due to space limitation, Manja Schulze, Dr Antje Technau, Katja Zabel, Svetlana Ustanina, Olesya Vakhrusheva, Katja Kolditz, Dr. Eva Bober, Michal Mielcarek, Izabela Piotrowska, Tomek Loch, Robert Kramek,Karen Ruschke, Thomas Schmidt, Cristian Smolka, Jens-Uwe Hartmann, Susan Weinlich, Daniela Bräuer, Dr. Petra Neuhaus, Dr. Herbert Neuhaus, Julia Kruse, Annelies Wolter, Anett Thate, Dr. Dagobert Glanz, Dr. Friedeman Laube, Dr. Beate Fricke, Dr. Axel Kaul, Dawaasuren Agambai, Dr Alessandra Martire, Shizuka Uchida, Monika Euler, Kerstin Richter, Jutta Wetzel and others.

A special gratitude to all my instructors in Jimma Institute of Health Sciences (JIHS) now Jimma University (JU) who provided me with good academic background. To my friends specially Zeleke Mekonnen and those in Europe, USA and Ethiopia for their encouragement. I would like to thank everybody in the interuniversity programme molecular biology (IPMB), especially Prof. E. Van Driessche, Prof. Dr. W. Stevens and Dr.J.Pinxteren and those who have taught me molecular biology courses during MSc study which served as a spring board for my PhD study. Special thanks go to Mr. Rudi Willems for his consistent encouragement. Last but, not list, I would like to thank the Max-Planck-Society, the DFG (priority program "stem cells"), the BMBF, and the Wilhelm-Roux-Program for Research of the Martin-Luther-University for funding this work.

12 Erklärung

Hiermit erkläre ich, daß ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt und die den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle/Saale, 2006 Fikru Belema Bedada

13 Zusammenfassung

Obwohl es in den letzten Jahren Enthusiasmus über die Möglichkeiten gab, daß adulte Stammzellen aus Säugetieren fähig sein können, über ihre Abstammungsgrenzen hinaus zu differenzieren, so sind solche Stammzellen noch nicht vollständig charakterisiert worden. Im Besonderen ist das Potential zur Transdifferenzierung durch Behandeln definierter Zellen mit verschiedenen Stimuli nicht vollständig erforscht worden. Im Rahmen dieser Studie isolierte ich zwei sich selbst langzeitig erneuernde Populationen von Mäusestammzellen und nannte sie BM-MASCS1 und mBM-MASCs2. Ich charakterisierte ihre multipotenten Differenzierungsreaktionen auf verschiedene bioaktive Moleküle *in vitro* und ihren Beitrag zur Entwicklung von schimären Embryonen *in vivo*. Die durchflußzytometrische Charakterisierung zeigte, daß beide Zellpopulationen sich in ihrer CD34 und Sca-1 Expressionsstärke unterscheiden, aber in Bezug auf andere Oberflächenmarker tatsächlich nicht unterscheidbar sind. Zusätzlich exprimieren sie verschiedene pluripotente oder für Stammzellen charakteristische Gene wie Oct3/4, nanog, SSEA-1 Rex-1 und B-myb, alle typisch für undifferenzierte ES Zellen.

Die Inkubation mit verschiedenen Stimuli wie die wnt7a, wnt7b, wnt4, wnt11, CA-LEF und CDO oder epigenetisches Reprogrammieren mit 5-Azazytidine oder TSA (einzeln und in Kombination) induzierten die Expression von myogenen Markern wie Myf5, MyoD, Pax7, Myogenin und MRF4. Mehrere Strukturproteine wie sarkomerisches MyHC und TnI wurden auch immunhistochemisch nachgewiesen. Weiterhin induzierten unterschiedliche wnts und CA-LEF nicht nur das Muskelprogramm sondern translozierten ß-Catenin in den Nukleus von mBM-MASCS, auf die Notwendigkeit von ß-Catenin für das myogene Programm hindeutend. Die Differenzierung der myogenen Linien wurde auch durch die Infektion von mBM-MASCs mit einem Myogenin-eGFP enthaltendem lentiviralen Reporterkonstrukt untersucht, durch Induktion mit wnt7A und durch die Expansion über FACS sortierte positive Zellen. Die Mehrheit dieser sortierten Zellen war für das sarkomerische MyHC (MF-20) positiv und fusionierte prompt mit der *bona fide* Muskelzelllinie C2C12, wenn sie in Differenzierungsmedium gehalten wurden.

Interessanterweise führte die Behandlung mit wnt7a, wnt7b, wnt4 und CA-LEF nicht zur Expression von Kardiomyozytenmarkern wie α-MHC, myocardin A, ß-MHC, ANP und BNP, während die Behandlung mit wnt11 zur Expression von Nkx-2.5, Myocardin A, GATA-4, ß-MHC und BNP führte. Die Zugabe von PKC Inhibitoren verzögerte die Wirkung von wnt-11, was sich durch den Verlust der Expression der Mehrheit der kardiomyozytenspezifischen Gene manifestierte, die in der Initiation genauso wie in der Progression der Differenzierung

involviert sind und die Notwendigkeit eines vom PKC-Pathway abhängigen wnt11 „Signalling“ wahrscheinlich machen. Epigenetisches Reprogrammieren mit 5-Azazytidine, TSA oder beidem, induzierte zusätzlich die Expression von α-MHC, was durch RT-PCR und α-MHC-eGFP Reportergenexpression demonstriert wurde. Die Behandlung von mBM-MASCs mit 5-Azacytidine oder BMP-2 trug auch zur Expression der basischen Phosphatase (ALP) bei, einem charakteristischen Marker der Osteozyten.

Bei beiden adulten Stamzellpopulationen wurde auch immunhistochemisch nach FGF-2 Behandlung die Expression der neuronalen Marker DBH und TH durch RT-PCR und ßIII-tubulin, neurofilament NF-200, GFAP und Nkx5.1-LacZ reporter gene gezeigt. Zusätzlich induzierte „Hepatocyte Growth Factor“ die Expression von Albumin und SEK-1 und eine epitheloidähnliche Morphologie. Letztendlich trugen genetisch markierte mBM-MASCs zur Entwicklung der Somiten, des Herzens und des Endotheliums in schimären Embryonen bei.

Zusammengefaßt zeigen diese Daten, daß die zwei isolierten mesenchymalen Stammzellpopulationen die Fähigkeit besitzen, mehrere Aspekte von mesodermalen, neuroektodermalen und endodermalen Linien *in vitro* zu erlangen und zur Entwicklung von schimären Embryonen *in vivo* beitragen.

14 Reference List

Abuljadayel,I.S. (2003). Induction of stem cell-like plasticity in mononuclear cells derived from unmobilised adult human peripheral blood. Curr. Med. Res. Opin. *19*, 355-375.

Akashi,K., Traver,D., Miyamoto,T., and Weissman,I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Amit,M., Carpenter,M.K., Inokuma,M.S., Chiu,C.P., Harris,C.P., Waknitz,M.A., Itskovitz-Eldor,J., and Thomson,J.A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev. Biol. *227*, 271-278.

Anderson,D.J. (2001). Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron *30*, 19-35.

Asakura,A., Seale,P., Girgis-Gabardo,A., and Rudnicki,M.A. (2002). Myogenic specification of side population cells in skeletal muscle. J. Cell Biol. *159*, 123-134.

Barker,P.A. and Murphy,R.A. (1992). The nerve growth factor receptor: a multicomponent system that mediates the actions of the neurotrophin family of proteins. Mol. Cell Biochem. *110*, 1-15.

Barron,M., Gao,M., and Lough,J. (2000). Requirement for BMP and FGF signalling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. Dev. Dyn. *218*, 383-393.

Beltrami,A.P., Urbanek,K., Kajstura,J., Yan,S.M., Finato,N., Bussani,R., Nadal-Ginard,B., Silvestri,F., Leri,A., Beltrami,C.A., and Anversa,P. (2001). Evidence that human cardiac myocytes divide after myocardial infarction. N. Engl. J. Med. *344*, 1750-1757.

Ben Shushan,E., Thompson,J.R., Gudas,L.J., and Bergman,Y. (1998). Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. Mol. Cell Biol. *18*, 1866-1878.

Bharadwaj,S. and Prasad,G.L. (2002). Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells. Cancer Lett. *183*, 205-213.

Bjornson,C.R., Rietze,R.L., Reynolds,B.A., Magli,M.C., and Vescovi,A.L. (1999). Turning brain into blood: a haematopoietic fate adopted by adult neural stem cells in vivo. Science *283*, 534-537.

Blau,H.M. and Blakely,B.T. (1999). Plasticity of cell fate: insights from heterokaryons. Semin. Cell Dev. Biol. *10*, 267-272.

Borycki,A., Brown,A.M., and Emerson,C.P., Jr. (2000). Shh and Wnt signalling pathways converge to control Gli gene activation in avian somites. Development *127*, 2075-2087.

Bossard,P. and Zaret,K.S. (1998). GATA transcription factors as potentiators of gut endoderm differentiation. Development *125*, 4909-4917.

Bradley,A., Evans,M., Kaufman,M.H., and Robertson,E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature *309*, 255-256.

Braun,T., Rudnicki,M.A., Arnold,H.H., and Jaenisch,R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell *71*, 369-382.

Briddell,R.A., Bruno,E., Cooper,R.J., Brandt,J.E., and Hoffman,R. (1991). Effect of c-kit ligand on in vitro human megakaryocytopoiesis. Blood *78*, 2854-2859.

Broxmeyer,H.E., Srour,E.F., Hangoc,G., Cooper,S., Anderson,S.A., and Bodine,D.M. (2003). High-efficiency recovery of functional hematopoietic progenitor and stem cells from human cord blood cryopreserved for 15 years. Proc. Natl. Acad. Sci. U. S. A *100*, 645-650.

Broxmeyer,H.E., Williams,D.E., Hangoc,G., Cooper,S., Gillis,S., Shadduck,R.K., and Bicknell,D.C. (1987). Synergistic myelopoietic actions in vivo after administration to mice of combinations of purified natural murine colony-stimulating factor 1, recombinant murine interleukin 3, and recombinant murine granulocyte/macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. U. S. A *84*, 3871-3875.

Bruno,E., Briddell,R., and Hoffman,R. (1988). Effect of recombinant and purified hematopoietic growth factors on human megakaryocyte colony formation. Exp. Hematol. *16*, 371-377.

Bunting,K.D. (2002). ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells *20*, 11-20.

Cameron,E.E., Bachman,K.E., Myohanen,S., Herman,J.G., and Baylin,S.B. (1999b). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat. Genet. *21*, 103-107.

Cameron,E.E., Bachman,K.E., Myohanen,S., Herman,J.G., and Baylin,S.B. (1999a). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat. Genet. *21*, 103-107.

Campagnolo,L., Russo,M.A., Puglianiello,A., Favale,A., and Siracusa,G. (2001). Mesenchymal cell precursors of peritubular smooth muscle cells of the mouse testis can be identified by the presence of the p75 neurotrophin receptor. Biol. Reprod. *64*, 464-472.

Capdevila,J., Tabin,C., and Johnson,R.L. (1998). Control of dorsoventral somite patterning by Wnt-1 and beta-catenin. Dev. Biol. *193*, 182-194.

Cassiman,D., Denef,C., Desmet,V.J., and Roskams,T. (2001). Human and rat hepatic stellate cells express neurotrophins and neurotrophin receptors. Hepatology *33*, 148-158.

Chen,C.Y. and Schwartz,R.J. (1995). Identification of novel DNA binding targets and regulatory domains of a murine tinman homeodomain factor, nkx-2.5
9. J. Biol. Chem. *270*, 15628-15633.

Civin,C.I., Strauss,L.C., Brovall,C., Fackler,M.J., Schwartz,J.F., and Shaper,J.H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J. Immunol. *133*, 157-165.

Clarke,D.L., Johansson,C.B., Wilbertz,J., Veress,B., Nilsson,E., Karlstrom,H., Lendahl,U., and Frisen,J. (2000). Generalized potential of adult neural stem cells. Science *288*, 1660-1663.

Cleaver,O.B., Patterson,K.D., and Krieg,P.A. (1996). Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in Xenopus embryos results in myocardial hyperplasia
1. Development *122*, 3549-3556.

Dewey,M.J., Martin,D.W., Jr., Martin,G.R., and Mintz,B. (1977). Mosaic mice with teratocarcinoma-derived mutant cells deficient in hypoxanthine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U. S. A *74*, 5564-5568.

Dexter,T.M. (1990). Introduction to the haemopoietic system. Cancer Surv. *9*, 1-5.

Diehl,A.M. and Rai,R.M. (1996). Liver regeneration 3: Regulation of signal transduction during liver regeneration. FASEB J. *10*, 215-227.

Donahue,R.E., Seehra,J., Metzger,M., Lefebvre,D., Rock,B., Carbone,S., Nathan,D.G., Garnick,M., Sehgal,P.K., Laston,D., and . (1988a). Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science *241*, 1820-1823.

Donahue,R.E., Seehra,J., Metzger,M., Lefebvre,D., Rock,B., Carbone,S., Nathan,D.G., Garnick,M., Sehgal,P.K., Laston,D., and . (1988b). Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science *241*, 1820-1823.

Dupin,E., Glavieux,C., Vaigot,P., and Le Douarin,N.M. (2000). Endothelin 3 induces the reversion of melanocytes to glia through a neural crest-derived glial-melanocytic progenitor. Proc. Natl. Acad. Sci. U. S. A *97*, 7882-7887.

Durocher,D., Charron,F., Warren,R., Schwartz,R.J., and Nemer,M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. EMBO J. *16*, 5687-5696.

Fan,C.M., Lee,C.S., and Tessier-Lavigne,M. (1997). A role for WNT proteins in induction of dermomyotome. Dev. Biol. *191*, 160-165.

Ferguson,A.T., Evron,E., Umbricht,C.B., Pandita,T.K., Chan,T.A., Hermeking,H., Marks,J.R., Lambers,A.R., Futreal,P.A., Stampfer,M.R., and Sukumar,S. (2000). High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc. Natl. Acad. Sci. U. S. A *97*, 6049-6054.

Frederiksen,K. and McKay,R.D. (1988). Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. J. Neurosci. *8*, 1144-1151.

Friedenstein,A.J., Petrakova,K.V., Kurolesova,A.I., and Frolova,G.P. (1968). Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation *6*, 230-247.

Galli,R., Borello,U., Gritti,A., Minasi,M.G., Bjornson,C., Coletta,M., Mora,M., De Angelis,M.G., Fiocco,R., Cossu,G., and Vescovi,A.L. (2000). Skeletal myogenic potential of human and mouse neural stem cells. Nat. Neurosci. *3*, 986-991.

Gehling,U.M., Ergun,S., Schumacher,U., Wagener,C., Pantel,K., Otte,M., Schuch,G., Schafhausen,P., Mende,T., Kilic,N., Kluge,K., Schafer,B., Hossfeld,D.K., and Fiedler,W. (2000). In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood *95*, 3106-3112.

Goodell,M.A., Rosenzweig,M., Kim,H., Marks,D.F., DeMaria,M., Paradis,G., Grupp,S.A., Sieff,C.A., Mulligan,R.C., and Johnson,R.P. (1997a). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med. *3*, 1337-1345.

Goodell,M.A., Rosenzweig,M., Kim,H., Marks,D.F., DeMaria,M., Paradis,G., Grupp,S.A., Sieff,C.A., Mulligan,R.C., and Johnson,R.P. (1997b). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med. *3*, 1337-1345.

Grepin,C., Nemer,G., and Nemer,M. (1997). Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor

1. Development *124*, 2387-2395.

Gualdi,R., Bossard,P., Zheng,M., Hamada,Y., Coleman,J.R., and Zaret,K.S. (1996). Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev. *10*, 1670-1682.

Gussoni,E., Soneoka,Y., Strickland,C.D., Buzney,E.A., Khan,M.K., Flint,A.F., Kunkel,L.M., and Mulligan,R.C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature *401*, 390-394.

Hapner,S.J., Boeshore,K.L., Large,T.H., and Lefcort,F. (1998). Neural differentiation promoted by truncated trkC receptors in collaboration with p75(NTR). Dev. Biol. *201*, 90-100.

Horsley,V., Jansen,K.M., Mills,S.T., and Pavlath,G.K. (2003). IL-4 acts as a myoblast recruitment factor during mammalian muscle growth
1. Cell *113*, 483-494.

Horwitz,E.M., Prockop,D.J., Fitzpatrick,L.A., Koo,W.W., Gordon,P.L., Neel,M., Sussman,M., Orchard,P., Marx,J.C., Pyeritz,R.E., and Brenner,M.K. (1999). Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat. Med. *5*, 309-313.

Huss,R. (2000). Isolation of primary and immortalized CD34-hematopoietic and mesenchymal stem cells from various sources. Stem Cells *18*, 1-9.

Iijima,Y., Nagai,T., Mizukami,M., Matsuura,K., Ogura,T., Wada,H., Toko,H., Akazawa,H., Takano,H., Nakaya,H., and Komuro,I. (2003). Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. FASEB J. *17*, 1361-1363.

Jackson,K.A., Majka,S.M., Wang,H., Pocius,J., Hartley,C.J., Majesky,M.W., Entman,M.L., Michael,L.H., Hirschi,K.K., and Goodell,M.A. (2001). Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J. Clin. Invest *107*, 1395-1402.

Jiang,Y. and Evans,T. (1996). The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis
3. Dev. Biol. *174*, 258-270.

Jiang,Y., Jahagirdar,B.N., Reinhardt,R.L., Schwartz,R.E., Keene,C.D., Ortiz-Gonzalez,X.R., Reyes,M., Lenvik,T., Lund,T., Blackstad,M., Du,J., Aldrich,S., Lisberg,A., Low,W.C., Largaespada,D.A., and Verfaillie,C.M. (2002a). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature *418*, 41-49.

Jiang,Y., Jahagirdar,B.N., Reinhardt,R.L., Schwartz,R.E., Keene,C.D., Ortiz-Gonzalez,X.R., Reyes,M., Lenvik,T., Lund,T., Blackstad,M., Du,J., Aldrich,S., Lisberg,A., Low,W.C., Largaespada,D.A., and Verfaillie,C.M. (2002b). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature *418*, 41-49.

Jiang,Y., Vaessen,B., Lenvik,T., Blackstad,M., Reyes,M., and Verfaillie,C.M. (2002c). Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp. Hematol. *30*, 896-904.

Jones,P.A. (1985). Altering gene expression with 5-azacytidine. Cell *40*, 485-486.

Jung,J., Zheng,M., Goldfarb,M., and Zaret,K.S. (1999). Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science *284*, 1998-2003.

Kang,J.S., Gao,M., Feinleib,J.L., Cotter,P.D., Guadagno,S.N., and Krauss,R.S. (1997). CDO: an oncogene-, serum-, and anchorage-regulated member of the Ig/fibronectin type III repeat family. J. Cell Biol. *138*, 203-213.

Kang,J.S., Mulieri,P.J., Miller,C., Sassoon,D.A., and Krauss,R.S. (1998). CDO, a robo-related cell surface protein that mediates myogenic differentiation. J. Cell Biol. *143*, 403-413.

Kim,H., Whartenby,K.A., Georgantas,R.W., III, Wingard,J., and Civin,C.I. (2002a). Human CD34+ hematopoietic stem/progenitor cells express high levels of FLIP and are resistant to Fas-mediated apoptosis. Stem Cells *20*, 174-182.

Kim,M., Turnquist,H., Jackson,J., Sgagias,M., Yan,Y., Gong,M., Dean,M., Sharp,J.G., and Cowan,K. (2002b). The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin. Cancer Res. *8*, 22-28.

Kim,T.E., Lee,H.S., Lee,Y.B., Hong,S.H., Lee,Y.S., Ichinose,H., Kim,S.U., and Lee,M.A. (2003). Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell. Biochem. Biophys. Res. Commun. *305*, 1040-1048.

Kiss,J.Z. and Muller,D. (2001). Contribution of the neural cell adhesion molecule to neuronal and synaptic plasticity. Rev. Neurosci. *12*, 297-310.

Kitamura,T. and Miyajima,A. (1992). Functional reconstitution of the human interleukin-3 receptor. Blood *80*, 84-90.

Kondo,M., Weissman,I.L., and Akashi,K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Kong,Y., Johnson,S.E., Taparowsky,E.J., and Konieczny,S.F. (1995). Ras p21Val inhibits myogenesis without altering the DNA binding or transcriptional activities of the myogenic basic helix-loop-helix factors. Mol. Cell Biol. *15*, 5205-5213.

Krause,D.S., Theise,N.D., Collector,M.I., Henegariu,O., Hwang,S., Gardner,R., Neutzel,S., and Sharkis,S.J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell *105*, 369-377.

Kuhl,M., Sheldahl,L.C., Park,M., Miller,J.R., and Moon,R.T. (2000c). The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet. *16*, 279-283.

Kuhl,M., Sheldahl,L.C., Park,M., Miller,J.R., and Moon,R.T. (2000a). The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet. *16*, 279-283.

Kuhl,M., Sheldahl,L.C., Park,M., Miller,J.R., and Moon,R.T. (2000b). The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet. *16*, 279-283.

Labosky,P.A., Barlow,D.P., and Hogan,B.L. (1994b). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development *120*, 3197-3204.

Labosky,P.A., Barlow,D.P., and Hogan,B.L. (1994a). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development *120*, 3197-3204.

Leary,A.G., Yang,Y.C., Clark,S.C., Gasson,J.C., Golde,D.W., and Ogawa,M. (1987). Recombinant gibbon interleukin 3 supports formation of human multilineage colonies and blast cell colonies in culture: comparison with recombinant human granulocyte-macrophage colony-stimulating factor

2. Blood *70*, 1343-1348.

Li,M., Pevny,L., Lovell-Badge,R., and Smith,A. (1998). Generation of purified neural precursors from embryonic stem cells by lineage selection

6. Curr. Biol. *8*, 971-974.

Liechty,K.W., MacKenzie,T.C., Shaaban,A.F., Radu,A., Moseley,A.M., Deans,R., Marshak,D.R., and Flake,A.W. (2000). Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat. Med. *6*, 1282-1286.

Lois,C., Hong,E.J., Pease,S., Brown,E.J., and Baltimore,D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Lyons,I., Parsons,L.M., Hartley,L., Li,R., Andrews,J.E., Robb,L., and Harvey,R.P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev. *9*, 1654-1666.

Majka,S.M., Jackson,K.A., Kienstra,K.A., Majesky,M.W., Goodell,M.A., and Hirschi,K.K. (2003). Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. J. Clin. Invest *111*, 71-79.

Makino,S., Fukuda,K., Miyoshi,S., Konishi,F., Kodama,H., Pan,J., Sano,M., Takahashi,T., Hori,S., Abe,H., Hata,J., Umezawa,A., and Ogawa,S. (1999a). Cardiomyocytes can be generated from marrow stromal cells in vitro. J. Clin. Invest *103*, 697-705.

Makino,S., Fukuda,K., Miyoshi,S., Konishi,F., Kodama,H., Pan,J., Sano,M., Takahashi,T., Hori,S., Abe,H., Hata,J., Umezawa,A., and Ogawa,S. (1999b). Cardiomyocytes can be generated from marrow stromal cells in vitro. J. Clin. Invest *103*, 697-705.

Martin,G.R. (1980). Teratocarcinomas and mammalian embryogenesis. Science *209*, 768-776.

Martin,G.R. and Evans,M.J. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc. Natl. Acad. Sci. U. S. A *72*, 1441-1445.

McDermott,A., Gustafsson,M., Elsam,T., Hui,C.C., Emerson,C.P., Jr., and Borycki,A.G. (2005). Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. Development *132*, 345-357.

Megeney,L.A. and Rudnicki,M.A. (1995). Determination versus differentiation and the MyoD family of transcription factors. Biochem. Cell Biol. *73*, 723-732.

Miller,J.R., Hocking,A.M., Brown,J.D., and Moon,R.T. (1999). Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. Oncogene *18*, 7860-7872.

Mima,T., Ueno,H., Fischman,D.A., Williams,L.T., and Mikawa,T. (1995). Fibroblast growth factor receptor is required for in vivo cardiac myocyte proliferation at early embryonic stages of heart development. Proc. Natl. Acad. Sci. U. S. A *92*, 467-471.

Minasi,M.G., Riminucci,M., De Angelis,L., Borello,U., Berarducci,B., Innocenzi,A., Caprioli,A., Sirabella,D., Baiocchi,M., De Maria,R., Boratto,R., Jaffredo,T., Broccoli,V., Bianco,P., and Cossu,G. (2002). The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development *129*, 2773-2783.

Miraglia,S., Godfrey,W., and Buck,D. (1998). A response to AC133 hematopoietic stem cell antigen: human homologue of mouse kidney prominin or distinct member of a novel protein family? Blood *91*, 4390-4391.

Mitsui,K., Tokuzawa,Y., Itoh,H., Segawa,K., Murakami,M., Takahashi,K., Maruyama,M., Maeda,M., and Yamanaka,S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.

Miyoshi,H., Blomer,U., Takahashi,M., Gage,F.H., and Verma,I.M. (1998). Development of a self-inactivating lentivirus vector. J. Virol. *72*, 8150-8157.

Mujtaba,T., Mayer-Proschel,M., and Rao,M.S. (1998). A common neural progenitor for the CNS and PNS. Dev. Biol. *200*, 1-15.

Muller,D., Wang,C., Skibo,G., Toni,N., Cremer,H., Calaora,V., Rougon,G., and Kiss,J.Z. (1996). PSA-NCAM is required for activity-induced synaptic plasticity. Neuron *17*, 413-422.

Naldini,L., Weidner,K.M., Vigna,E., Gaudino,G., Bardelli,A., Ponzetto,C., Narsimhan,R.P., Hartmann,G., Zarnegar,R., Michalopoulos,G.K., and . (1991). Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO J. *10*, 2867-2878.

Nan,X., Ng,H.H., Johnson,C.A., Laherty,C.D., Turner,B.M., Eisenman,R.N., and Bird,A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386-389.

Oh,H., Bradfute,S.B., Gallardo,T.D., Nakamura,T., Gaussin,V., Mishina,Y., Pocius,J., Michael,L.H., Behringer,R.R., Garry,D.J., Entman,M.L., and Schneider,M.D. (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc. Natl. Acad. Sci. U. S. A *100*, 12313-12318.

Okabe,S., Forsberg-Nilsson,K., Spiro,A.C., Segal,M., and McKay,R.D. (1996). Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro

1. Mech. Dev. *59*, 89-102.

Okumoto,K., Saito,T., Hattori,E., Ito,J.I., Adachi,T., Takeda,T., Sugahara,K., Watanabe,H., Saito,K., Togashi,H., and Kawata,S. (2003). Differentiation of bone marrow cells into cells that express liver-specific genes in vitro: implication of the Notch signals in differentiation. Biochem. Biophys. Res. Commun. *304*, 691-695.

Orlic,D., Kajstura,J., Chimenti,S., Bodine,D.M., Leri,A., and Anversa,P. (2003). Bone marrow stem cells regenerate infarcted myocardium. Pediatr. Transplant. *7 Suppl 3*, 86-88.

Orlic,D., Kajstura,J., Chimenti,S., Limana,F., Jakoniuk,I., Quaini,F., Nadal-Ginard,B., Bodine,D.M., Leri,A., and Anversa,P. (2001). Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc. Natl. Acad. Sci. U. S. A *98*, 10344-10349.

Osawa,M., Hanada,K., Hamada,H., and Nakauchi,H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Ott,M.O., Bober,E., Lyons,G., Arnold,H., and Buckingham,M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development *111*, 1097-1107.

Pagano,S.F., Impagnatiello,F., Girelli,M., Cova,L., Grioni,E., Onofri,M., Cavallaro,M., Etteri,S., Vitello,F., Giombini,S., Solero,C.L., and Parati,E.A. (2000). Isolation and characterization of neural stem cells from the adult human olfactory bulb. Stem Cells *18*, 295-300.

Palmer,T.D., Markakis,E.A., Willhoite,A.R., Safar,F., and Gage,F.H. (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J. Neurosci. *19*, 8487-8497.

Pandur,P., Lasche,M., Eisenberg,L.M., and Kuhl,M. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature *418*, 636-641.

Paquette,R. and Dorshkind,K. (2002). Optimizing hematopoietic recovery following bone marrow transplantation. J. Clin. Invest *109*, 1527-1528.

Patapoutian,A. and Reichardt,L.F. (2000). Roles of Wnt proteins in neural development and maintenance. Curr. Opin. Neurobiol. *10*, 392-399.

Pereira,R.F., Halford,K.W., O'Hara,M.D., Leeper,D.B., Sokolov,B.P., Pollard,M.D., Bagasra,O., and Prockop,D.J. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Natl. Acad. Sci. U. S. A *92*, 4857-4861.

Pereira,R.F., O'Hara,M.D., Laptev,A.V., Halford,K.W., Pollard,M.D., Class,R., Simon,D., Livezey,K., and Prockop,D.J. (1998). Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proc. Natl. Acad. Sci. U. S. A *95*, 1142-1147.

Phillips,R.L., Ernst,R.E., Brunk,B., Ivanova,N., Mahan,M.A., Deanehan,J.K., Moore,K.A., Overton,G.C., and Lemischka,I.R. (2000). The genetic program of hematopoietic stem cells. Science *288*, 1635-1640.

Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., and Marshak,D.R. (1999a). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., and Marshak,D.R. (1999b). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Prelle,K., Vassiliev,I.M., Vassilieva,S.G., Wolf,E., and Wobus,A.M. (1999). Establishment of pluripotent cell lines from vertebrate species--present status and future prospects. Cells Tissues. Organs *165*, 220-236.

Ramiya,V.K., Maraist,M., Arfors,K.E., Schatz,D.A., Peck,A.B., and Cornelius,J.G. (2000). Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat. Med. *6*, 278-282.

Reiser,J., Harmison,G., Kluepfel-Stahl,S., Brady,R.O., Karlsson,S., and Schubert,M. (1996). Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc. Natl. Acad. Sci. U. S. A *93*, 15266-15271.

Resnick,J.L., Bixler,L.S., Cheng,L., and Donovan,P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature *359*, 550-551.

Reyes,M., Lund,T., Lenvik,T., Aguiar,D., Koodie,L., and Verfaillie,C.M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood *98*, 2615-2625.

Rohwedel,J., Sehlmeyer,U., Shan,J., Meister,A., and Wobus,A.M. (1996). Primordial germ cell-derived mouse embryonic germ (EG) cells in vitro resemble undifferentiated stem cells with respect to differentiation capacity and cell cycle distribution. Cell Biol. Int. *20*, 579-587.

Rosner,M.H., Vigano,M.A., Ozato,K., Timmons,P.M., Poirier,F., Rigby,P.W., and Staudt,L.M. (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature *345*, 686-692.

Rudnicki,M.A., Sawtell,N.M., Reuhl,K.R., Berg,R., Craig,J.C., Jardine,K., Lessard,J.L., and McBurney,M.W. (1990). Smooth muscle actin expression during P19 embryonal carcinoma differentiation in cell culture. J. Cell Physiol *142*, 89-98.

Saeland,S., Caux,C., Favre,C., Aubry,J.P., Mannoni,P., Pebusque,M.J., Gentilhomme,O., Otsuka,T., Yokota,T., Arai,N., and . (1988). Effects of recombinant human interleukin-3 on CD34-enriched normal hematopoietic progenitors and on myeloblastic leukemia cells
3. Blood *72*, 1580-1588.

Savatier,P., Lapillonne,H., van Grunsven,L.A., Rudkin,B.B., and Samarut,J. (1996). Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. Oncogene *12*, 309-322.

Scholer,H.R., Dressler,G.R., Balling,R., Rohdewohld,H., and Gruss,P. (1990). Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J. *9*, 2185-2195.

Shamblott,M.J., Axelman,J., Littlefield,J.W., Blumenthal,P.D., Huggins,G.R., Cui,Y., Cheng,L., and Gearhart,J.D. (2001). Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. Proc. Natl. Acad. Sci. U. S. A *98*, 113-118.

Shih,C.C., Weng,Y., Mamelak,A., LeBon,T., Hu,M.C., and Forman,S.J. (2001). Identification of a candidate human neurohematopoietic stem-cell population. Blood *98*, 2412-2422.

Simmons,P.J. and Torok-Storb,B. (1991a). CD34 expression by stromal precursors in normal human adult bone marrow. Blood *78*, 2848-2853.

Simmons,P.J. and Torok-Storb,B. (1991b). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood *78*, 55-62.

Solter,D. and Knowles,B.B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc. Natl. Acad. Sci. U. S. A *75*, 5565-5569.

Sonoda,Y., Yang,Y.C., Wong,G.G., Clark,S.C., and Ogawa,M. (1988). Analysis in serum-free culture of the targets of recombinant human hemopoietic growth factors: interleukin 3 and granulocyte/macrophage-colony-stimulating factor are specific for early developmental stages. Proc. Natl. Acad. Sci. U. S. A *85*, 4360-4364.

Spangrude,G.J., Heimfeld,S., and Weissman,I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58-62.

Stemple,D.L. and Anderson,D.J. (1993). Lineage diversification of the neural crest: in vitro investigations. Dev. Biol. *159*, 12-23.

Stewart,C.L., Gadi,I., and Bhatt,H. (1994). Stem cells from primordial germ cells can reenter the germ line. Dev. Biol. *161*, 626-628.

Stewart,T.A. and Mintz,B. (1981). Successive generations of mice produced from an established culture line of euploid teratocarcinoma cells. Proc. Natl. Acad. Sci. U. S. A *78*, 6314-6318.

Sutherland,D.R. and Keating,A. (1992). The CD34 antigen: structure, biology, and potential clinical applications. J. Hematother. *1*, 115-129.

Tada,M. and Smith,J.C. (2000). Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. Development *127*, 2227-2238.

Tajbakhsh,S., Borello,U., Vivarelli,E., Kelly,R., Papkoff,J., Duprez,D., Buckingham,M., and Cossu,G. (1998). Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development *125*, 4155-4162.

Tanaka,Y., Patestos,N.P., Maekawa,T., and Ishii,S. (1999). B-myb is required for inner cell mass formation at an early stage of development. J. Biol. Chem. *274*, 28067-28070.

Taylor,G., Lehrer,M.S., Jensen,P.J., Sun,T.T., and Lavker,R.M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell *102*, 451-461.

Teramura,M., Katahira,J., Hoshino,S., Motoji,T., Oshimi,K., and Mizoguchi,H. (1988). Clonal growth of human megakaryocyte progenitors in serum-free cultures: effect of recombinant human interleukin 3. Exp. Hematol. *16*, 843-848.

Thomson,J.A., Itskovitz-Eldor,J., Shapiro,S.S., Waknitz,M.A., Swiergiel,J.J., Marshall,V.S., and Jones,J.M. (1998a). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Thomson,J.A., Itskovitz-Eldor,J., Shapiro,S.S., Waknitz,M.A., Swiergiel,J.J., Marshall,V.S., and Jones,J.M. (1998b). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Till,J.E. and McCULLOCH,E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. *14*, 213-222.

Toma,J.G., Akhavan,M., Fernandes,K.J., Barnabe-Heider,F., Sadikot,A., Kaplan,D.R., and Miller,F.D. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat. Cell Biol. *3*, 778-784.

Torrente,Y., Tremblay,J.P., Pisati,F., Belicchi,M., Rossi,B., Sironi,M., Fortunato,F., El Fahime,M., D'Angelo,M.G., Caron,N.J., Constantin,G., Paulin,D., Scarlato,G., and Bresolin,N. (2001). Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. J. Cell Biol. *152*, 335-348.

Tropepe,V., Hitoshi,S., Sirard,C., Mak,T.W., Rossant,J., and van der,K.D. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism

1. Neuron *30*, 65-78.

Uchida,N., Buck,D.W., He,D., Reitsma,M.J., Masek,M., Phan,T.V., Tsukamoto,A.S., Gage,F.H., and Weissman,I.L. (2000). Direct isolation of human central nervous system stem cells. Proc. Natl. Acad. Sci. U. S. A *97*, 14720-14725.

Uchida,N., Fujisaki,T., Eaves,A.C., and Eaves,C.J. (2001). Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP)phenotype. J. Clin. Invest *108*, 1071-1077.

Vainio,S.J. and Uusitalo,M.S. (2000). A road to kidney tubules via the Wnt pathway. Pediatr. Nephrol. *15*, 151-156.

van de,R.M., Heimfeld,S., Spangrude,G.J., and Weissman,I.L. (1989). Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci. U. S. A *86*, 4634-4638.

Van Den Berg,D.J., Sharma,A.K., Bruno,E., and Hoffman,R. (1998). Role of members of the Wnt gene family in human hematopoiesis. Blood *92*, 3189-3202.

Wade,P.A., Gegonne,A., Jones,P.L., Ballestar,E., Aubry,F., and Wolffe,A.P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat. Genet. *23*, 62-66.

Wagemaker,G., van Gils,F.C., Burger,H., Dorssers,L.C., van Leen,R.W., Persoon,N.L., Wielenga,J.J., Heeney,J.L., and Knol,E. (1990). Highly increased production of bone marrow-derived blood cells by administration of homologous interleukin-3 to rhesus monkeys 2. Blood *76*, 2235-2241.

Wang,J.S., Shum-Tim,D., Galipeau,J., Chedrawy,E., Eliopoulos,N., and Chiu,R.C. (2000). Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. J. Thorac. Cardiovasc. Surg. *120*, 999-1005.

Welm,B.E., Tepera,S.B., Venezia,T., Graubert,T.A., Rosen,J.M., and Goodell,M.A. (2002). Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev. Biol. *245*, 42-56.

Woodbury,D., Reynolds,K., and Black,I.B. (2002). Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res. *69*, 908-917.

Yamamoto,H., Kishida,S., Kishida,M., Ikeda,S., Takada,S., and Kikuchi,A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. J. Biol. Chem. *274*, 10681-10684.

Yang,L., Li,S., Hatch,H., Ahrens,K., Cornelius,J.G., Petersen,B.E., and Peck,A.B. (2002). In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc. Natl. Acad. Sci. U. S. A *99*, 8078-8083.

Yang,X., Ferguson,A.T., Nass,S.J., Phillips,D.L., Butash,K.A., Wang,S.M., Herman,J.G., and Davidson,N.E. (2000). Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. Cancer Res. *60*, 6890-6894.

Yang,Y.C. and Clark,S.C. (1990). Human interleukin 3: analysis of the gene and its role in the regulation of hematopoiesis. Int. J. Cell Cloning *8 Suppl 1*, 121-128.

Yee,S.P. and Rigby,P.W. (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev. *7*, 1277-1289.

Yin,A.H., Miraglia,S., Zanjani,E.D., Almeida-Porada,G., Ogawa,M., Leary,A.G., Olweus,J., Kearney,J., and Buck,D.W. (1997). AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood *90*, 5002-5012.

Yoshida,M., Kijima,M., Akita,M., and Beppu,T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. *265*, 17174-17179.

Zaret,K.S. (2002). Regulatory phases of early liver development: paradigms of organogenesis. Nat. Rev. Genet. *3*, 499-512.

Zennou,V., Petit,C., Guetard,D., Nerhbass,U., Montagnier,L., and Charneau,P. (2000). HIV-1 genome nuclear import is mediated by a central DNA flap. Cell *101*, 173-185.

Zhao,Y., Glesne,D., and Huberman,E. (2003). A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A *100*, 2426-2431.

Zhou,S., Schuetz,J.D., Bunting,K.D., Colapietro,A.M., Sampath,J., Morris,J.J., Lagutina,I., Grosveld,G.C., Osawa,M., Nakauchi,H., and Sorrentino,B.P. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat. Med. *7*, 1028-1034.

Zufferey,R., Donello,J.E., Trono,D., and Hope,T.J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. *73*, 2886-2892.

Bedada, F. B., Gunther, S., Kubin, T., and Braun, T. (2006). Differentiation Versus Plasticity: Fixing the Fate of Undetermined Adult Stem Cells. Cell Cycle *5*.

Belema Bedada, F., Technau, A., Ebelt, H., Schulze, M., and Braun, T. (2005). Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. Mol Cell Biol *25*, 9509-9519.

Brantjes, H., Barker, N., van Es, J., and Clevers, H. (2002). TCF: Lady Justice casting the final verdict on the outcome of Wnt signalling. Biol Chem *383*, 255-261.

Braun, T., and Arnold, H. H. (1996). Myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. EMBO J *15*, 310.

Bruder, S. P., Fink, D. J., and Caplan, A. I. (1994). Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem *56*, 283-294.

Capecchi, M. R. (1989). The new mouse genetics: altering the genome by gene targeting. Trends Genet *5*, 70-76.

Clark, P. (1994). Modulation of scatter factor/hepatocyte growth factor activity by cell-substratum adhesion. J Cell Sci *107 (Pt 5)*, 1265-1275.

Dierick, H., and Bejsovec, A. (1999). Cellular mechanisms of wingless/Wnt signal transduction. Curr Top Dev Biol *43*, 153-190.

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. J Virol *72*, 8463-8471.

Galli, R., Borello, U., Gritti, A., Minasi, M. G., Bjornson, C., Coletta, M., Mora, M., De Angelis, M. G., Fiocco, R., Cossu, G., and Vescovi, A. L. (2000). Skeletal myogenic potential of human and mouse neural stem cells. NatNeurosci *3*, 986.

Hsu, S. C., Galceran, J., and Grosschedl, R. (1998). Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol *18*, 4807-4818.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., *et al.* (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature *418*, 41.

Kitagaki, J., Iwamoto, M., Liu, J. G., Tamamura, Y., Pacifci, M., and Enomoto-Iwamoto, M. (2003). Activation of beta-catenin-LEF/TCF signal pathway in chondrocytes stimulates ectopic endochondral ossification. Osteoarthritis Cartilage *11*, 36-43.

Klug, M. G., Soonpaa, M. H., Koh, G. Y., and Field, L. J. (1996). Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. J Clin Invest *98*, 216-224.

Makino, S., Fukuda, K., Miyoshi, S., Konishi, F., Kodama, H., Pan, J., Sano, M., Takahashi, T., Hori, S., Abe, H., *et al.* (1999). Cardiomyocytes can be generated from marrow stromal cells in vitro. JClinInvest *103*, 697.

Miller, J. R., Hocking, A. M., Brown, J. D., and Moon, R. T. (1999). Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. Oncogene *18*, 7860.

Munsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P., and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. Genes Dev *9*, 2911-2922.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. FEBS Lett *407*, 313-319.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science *276*, 71.

Rodan, G. A., and Noda, M. (1991). Gene expression in osteoblastic cells. Crit Rev Eukaryot Gene Expr *1*, 85-98.

Schulze, M., Belema-Bedada, F., Technau, A., and Braun, T. (2005). Mesenchymal stem cells are recruited to striated muscle by NFAT/IL-4-mediated cell fusion. Genes Dev *19*, 1787-1798.

Shou, J., Murray, R. C., Rim, P. C., and Calof, A. L. (2000). Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. Development *127*, 5403-5413.

Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M., and Cossu, G. (1998). Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development *125*, 4155.

Thies, R. S., Bauduy, M., Ashton, B. A., Kurtzberg, L., Wozney, J. M., and Rosen, V. (1992). Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology *130*, 1318-1324.

Toma, J. G., Akhavan, M., Fernandes, K. J., Barnabe-Heider, F., Sadikot, A., Kaplan, D. R., and Miller, F. D. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. NatCell Biol *3*, 778.

van Noort, M., and Clevers, H. (2002). TCF transcription factors, mediators of Wnt-signaling in development and cancer. Dev Biol *244*, 1-8.

Vleminckx, K., Kemler, R., and Hecht, A. (1999). The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in Xenopus laevis. Mech Dev *81*, 65-74.

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol *72*, 9873-9880.