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When you have eliminated the impossible, whatever remains, however improable, must be the truth?

(Sherlock Holmes)

Content

Conclusions

Mesenchymal stem cells (MSCs) reside in various connective tissues and have multilineage potential, which makes them attractive candidates for regenerative medicine. MSCs are heterogeneous and many investigators are seeking for markers, which could predict and monitor multipotency. However, currently there are no markers, which can uniformly describe distinct progenitors of the mesenchymal lineage. Neither is there sufficient information for dissecting the mesenchymal hierarchy.

The present study compared the *in vitro* and *in vivo* potential of putative MSC from bone marrow (BM-MSC) and umbilical cord vein (UVSC). Although *in vitro* function and phenotype of these two cell sources was similar, only BM-MSC showed stem cell like behavior *in vivo*. These data highlight the importance of assessing multipotency of stromal progenitors *in vivo*, and shows that the analysis of phenotype and differentiation *in vitro* does not predict regenerative potential. Since many investigators found that MSC express transcripts for markers such as POU5F1 we investigated whether POU5F1 expression was associated with multipotency *in vivo*. In our efforts to find out whether monitoring expression of POU5F1 could have predicted the difference in *in vivo* activity of BM-MSC and UVSC, we determined that over 90% of all POU5F1 transcripts represented pseudogenes, so that “true” POU5F1 is expressed at a very low level which did not translate into detectable levels of POU5F1 protein. Thus, the study presented in this thesis challenges the detection of POU5F1 as regulator of multipotency in adult postnatal tissue and proposes the involvement of further processes or respectively molecules.

Since expression of POU5F1 could not be taken as a sign of multipotency, we started studies to define novel positive markers of multipotency using gene expression studies. In these studies, we compared BM-MSC to the cell populations without *in vivo* repopulating activity as well as hematopoietic cells. This comparison identified a number of surface antigens preferentially expressed on BM-MSC. Some of these, could be used to enrich for CFU-F. The novel antigen markers NOTCH3, FGFR2 and ITGA11 were used on single marker level as well as in combination of a marker previously described in the literature: CD146.

Our data highlight that NOTCH3 and FGFR2 positive BM-MSC show notable adipogenic and osteogenic potential while ITGA11 positive BM-MSC demonstrate strong osteogenic capacity. These results emphasize novel markers of mesenchymal stromal cells of the bone marrow and more important identify subpopulations with various differentiation potential that presents new insights in the understanding of the mesenchymal cells and their system. The results demonstrate moreover the identification of new molecules for characterization of multipotent MSC and give the first prospective view for defining multipotent and unipotent progenitors of the mesenchymal system. Taken together, the study stresses the need to establish new markers to predict MSC multipotency, which are studied in use of novel surface antigens.

Future studies (see above) should clear whether the new markers allow the definitive establishment of the existence of a mesenchymal hierarchy, which includes stem cell-like MSC as well as more mature progenitor populations (Figure 30).

The purpose to use MSC in clinical application is mainly interfered by a mixture heterogeneous MSC population with various differentiation potential where homogeneous population are needed. This study will present an approach for identification of progenitors with different lineage commitment. Based on the present method, the isolation of defined cell population are possible in exclusion of contaminating cells, which will have interfering effects in engraftment in vivo. The enumeration of populations with defined lineage-potential can be applied in targeted application like tissue repair or regeneration. Furthermore, it could possibly help to improve therapeutical approaches by reducing effects of chemo-, or radiotherapy or enable processes for better bone formations. The present study give first approaches, but further refinements may facilitate development of more homogeneous cell populations for mesenchymal cell therapy.

However, the classification of lineage-specific progenitors in the mesenchymal hierarchy, could not only help for better understanding of the MSC system but also form the basis for full clinical application and provide the possibility for novel therapeutical approaches.

Zusammenfassung

Mesenchymale Stammzellen (MSC) sind in unterschiedlichen Bindegeweben aufzufinden und besitzen multilineares Potential, die sie zu einem attraktiven Kandidaten für die regenerative Medizin machen. Da MSCs heterogen sind, suchen viele Wissenschaftler nach Markern, die Multipotenz vorhersagen und auch kontrollieren können. Jedoch gibt es zur Zeit weder Marker, die bestimmte Progenitoren der mesenchymalen Linie einheitlich beschreiben, noch gibt es hinreichend Informationen zur Unterteilung der mesenchymalen Hierarchie. In der vorliegenden Studie werden das *in vitro* und das *in vivo* Potential von putativen MSCs aus dem Knochenmark (BM-MSC) und der Nabelschnurvene (UVSC) verglichen. Obwohl die *in vitro* Funktion und der Phänotyp dieser zwei Zellquellen ähnlich sind, zeigen nur BM-MSC *in vivo* Stammzellverhalten. Diese Daten heben die Wichtigkeit hervor Multipotenz in Stromaprogenitoren in vivo zu beurteilen und zeigen, dass die Analyse des Phänotyps und der Differenzierung in vitro keine Aussage über das regenerative Potential ergeben.

Seitdem viele Wissenschaftler herausfanden, dass MSC Transkripte exprimieren wie von Marker wie POU5F1, untersuchten wir, ob die Expression von POU5F1 mit der Multipotenz in vivo verbunden werden kann.

In unseren Untersuchungen herauszufinden, ob eine messbare Expression von POU5F1 einen Unterschied in der in vivo Aktivität zwischen BMMSC und UVSC voraussagen kann, stellten wir fest, dass 90% aller POU5F1 Transkripte Pseudogenen entsprechen und dass das „wahre“ POU5F1 in einem so niedrigen Level exprimiert wird, dass es zu keinem detektierbaren POU5F1 Protein translatiert wird.
Daher stellt die vorliegende Studie in dieser Doktorarbeit die Detektion von POU5F1 als Regulator der Multipotenz in postnatalen Gewebe in Frage und empfiehlt die Einbeziehung von weiteren Prozessen bzw. Molekülen. Da die Expression von POU5F1 nicht als ein Indiz für Multipotenz aufgefasst werden kann, haben wir begonnen unter Verwendung von Genexpressions Studien neue positive Multipotenz-Marker zu definieren.

In dieser Studie wurden BM-MSC mit Zellpopulationen ohne *in vivo* Repopulations-Aktivität sowie hämatopoietischen Zellen verglichen. Anhand dieses Vergleichs wurden eine Reihe von Oberflächenantigenen identifiziert, die vorzugsweise in BM-MSC exprimiert werden. Einige davon können zur CFU-F Anreicherung verwendet werden. Die neuen Antigenmarker NOTCH3, FGFR2 und ITGA11 wurden sowohl als Einzelmarker als auch in Kombination mit dem aus der Literatur bekanntem Marker: CD146 verwendet.

Unsere Daten verdeutlichen, dass NOTCH3 und FGFR2 positive BM-MSCs ein auffallend adipogenes und osteogenes Differenzierungspotential zeigen, während ITGA11 positive BM-MSC eine starke osteogene Kapazität aufweisen. Diese Ergebnisse weisen auf neue Marker mesenchymaler Stromazellen aus dem Knochenmark hin und - viel wichtiger - sie identifizieren Subpopulationen mit unterschiedlichen Differenzierungspotential, welche neue Erkenntnisse im Verständnis mesenchymalen Zellen und ihres Systems darstellen.

Zudem zeigen unsere Ergebnisse die Identifizierung von neuen Molekülen zur Charakterisierung von multipotenten MSC. Sie geben einen ersten aussichtsreichen Einblick multipotente und unipotente Progenitoren des mesenchymalen Systems zu definieren.

Zusammenfassend soll diese Untersuchung die Notwendigkeit verdeutlichen, neue Marker zu etablieren um die Multipotenz von MSC vorrauszudeuten, welche unter Verwendung von neuen Oberflächenantigenen untersucht wurden.

Zukünftige Studien (siehe oben) sollen aufklären, ob die neuen Marker eine definitive Etablierung der mesenchymalen Hierarchie ermöglichen, die sowohl Stammzell ähnliche MSCs als auch eine reifere Progenitor-Population miteinbeziehen.

Das Ziel, MSC in der klinischen Anwendung einzusetzen, wird meistens durch ein Gemisch einer heterogenen MSC Population mit unterschiedlichen Differenzierungspotential gestört. Diese Untersuchung stellt einen Ansatz dar zur Identifizierung von Progenitoren mit unterschiedlicher Abstammungsrichtung.
Basierend auf der momentanen Methode, ist die Isolierung von bestimmten Zellpopulationen durch den Ausschluss von kontaminierten Zellen, die einen störenden Effekt auf das Anwachsen *in vivo* haben, möglich. Das Zusammenstellen von Populationen mit definierten Linienpotential kann in gezielten Anwendungen wie Gewebe Reparatur oder Regeneration eingesetzt werden.

Darüber hinaus könnten sie helfen therapeutische Ansätze durch die Minderung der Auswirkungen von Chemo- und Radiotherapie zu verbesseren oder den Prozess einer besseren Knochenbildung zu ermöglichen.

Die vorliegende Studie präsentiert die ersten Lösungsvorschläge, jedoch weitere Verfeinerungen würden eventuell die Entwicklung einer homogeneren Zellpopulation für die mesenchymale Zelltherapie erleichtern. Eine Klassifikation der abstammungs-spezifischer Progenitoren in der mesenchymalen Hierarchie könnte nicht nur zu einem besserem Verständnis des MSC-Systems verhelfen, sondern auch die Basis für eine vollständige klinische Applikation bilden und neue therapeutische Behandlungen liefern.

Chapter 1: Introduction

1.1 Definition of the Term „Mesenchymal Stem Cells“

The history of the “mesenchymal stem cells” (MSC) starts with the first suggestion of an undifferentiated non-hematopoietic cell type in the human body by Virchow et al. [Virchow et al, 1855]. In his studies and the studies of Conheim, a student of Virchow, they hypothesized similar histology of tumor and embryonic tissues concluded in “embryonic-rest” theory [Virchow, 1855; reviewed in Siggins et al., 2008]. However, Friedenstein and coworkers were the first to demonstrate that cells of the bone marrow are able of forming fibroblastic colonies [Friedenstein et al., 1970; Friedenstein et al., 1974]. When cells derived from single colonies were transplanted under the kidney capsule, many of these showed the capability of both ectopic bone formation and constitution of the hematopoietic microenvironment including adipocytes [Friedenstein et al., 1974]. These findings demonstrate the multipotentiality of the “mesenchymal stem cells”.

The concept of mesenchymal stem cells that is still used nowadays, originates from Arnold Caplan [Caplan et al., 1991]. Caplan and coworkers described culture conditions and differentiation experiments to analysis the development of muscle and cartilage of chick embryonic limb buds [Caplan et al., 1981; Caplan et al., 1984]. Moreover, they perform *in vivo* transplantation experiments of bone marrow and observed the formation of bone and ectopic cartilage. These experiments induced Caplan to call these adult cells “mesenchymal stem cells” (MSCs) and concluded fundamental aspects of multilineage pathways in the “mesengenic process” [Caplan et al., 1989] (Figure 1).

Using the term “mesenchymal stem cells” is confusing with regard to the stem cell characteristics originated from embryonic stem cells. The description of calling these cells “stem cells” based on the observation of their differentiation potential in mesenchymal lineage and therefore that these cells “stemmed” from a common precursor [reviewed in Caplan et al., 2004]. The term was adopted and used in multiple publications until today without considering that the understandings of stem cells have to be revised and adapted to the achievements in the recent years.

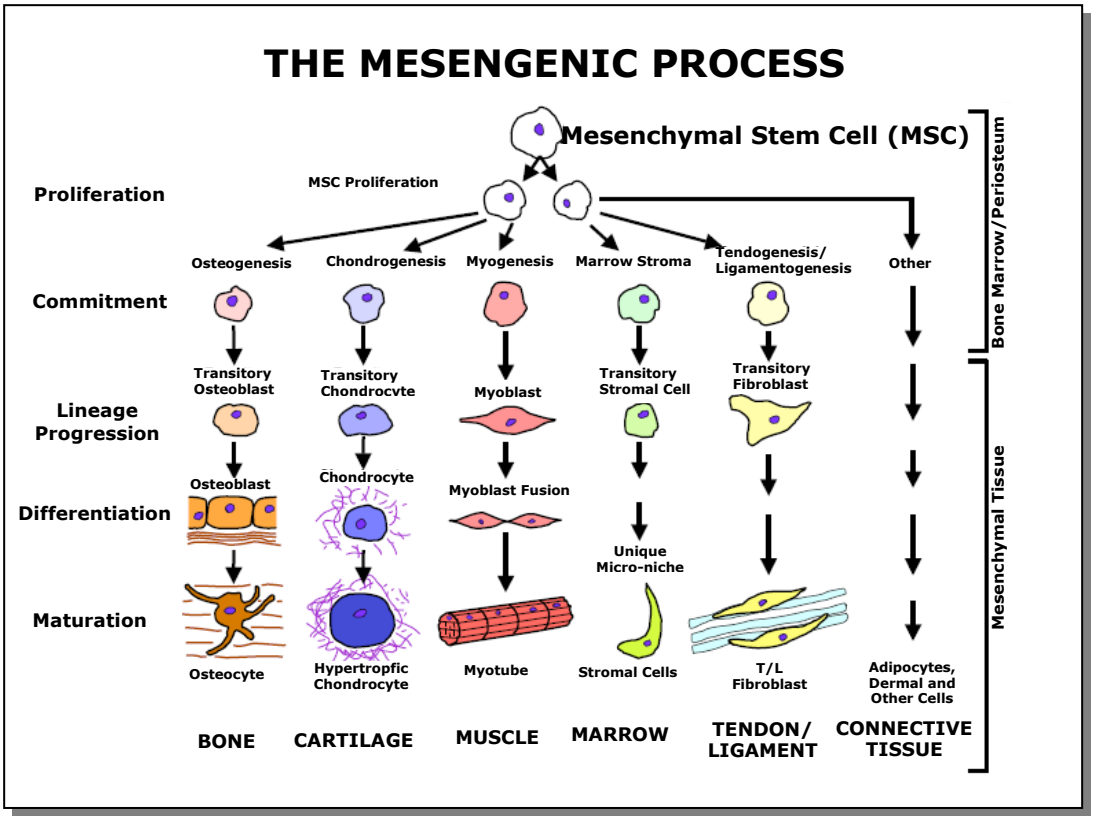


Figure 1*:* The "Mesengenic Process"

MSC have the potential to differentiate in various lineages like bone, cartilage, muscle, tendon, fat, marrow and dermis. The MSC commitment is passing different stages beginning with the proliferation and finally ended in fully determined, maturated, and lineage-specific cell type. The process is influenced by multiple growth factors and cytokines that causing the cell to a specific direction passing through various intermediate stages. During the different stages the synthesis of several proteins will be up-regulated while other protein will be suppressed for precise single directed cell fate. The terminally differentiated cell remains in the dedicated location until it will be replaced by a new one [image source Caplan et al., 1989].

Therefore, it is necessary to enlarge on stem cells characteristics and whether these attributes are transferable to mesenchymal stem cells. The definition of stem cells in certain tissues are summarized in Loeffler et al. and Zipori et al. [Loeffler et al., 2002; Zipori et al., 2005]. At this, stem cells are characterized as an undifferentiated population able to home in an designated microenvironment with explicit proliferation capacity and potential to differentiate in multiple lineages. Furthermore, stem cells possess regenerative potential and the ability to self-renew or self-maintenance.

Recent work of Sachetti et al. did not only show multipotentiality of single fibroblastoid colonies *in* vivo, but also demonstrated that single colonies isolated from osteogenic patches *in vivo*, could form secondary structures of osteogenesis and recruitment of the hematopoietic microenviroenment *in vivo* [Sacchetti et al., 2007]. This finding strongly suggests that primary CFU-F (fibroblasts colony forming units) could form secondary CFU-F capable of *in vivo* osteogenic activity. However elegant,

these experiments still do not provide definitive experimental evidence to prove the self-renewal capacity in the mesenchymal system. For this evidence, single CFU-F-derived cells, should be shown to form secondary CFU-F with *in vivo* multipotent activity. Thus, although very likely to happen, formal proof of self-renewal of multipotent MSC is still missing.

Taken together, the term of mesenchymal stem cells is misleading regarding to the stem cells characteristic self-renewal and self-maintenance.

However, to avoid the term of mesenchymal stem cells many investigators have used different names that has led to an inconsistent nomenclature of the MSCs [reviewed by Bianco et al., 2008]. Thus, several different designations like mesenchymal stem cells (Caplan, described above), as well as “stromal stem cells” which described cells derived from the cells between marrow and bone surface [Owen et al., 1988a; Owen et al., 1988b], or “Mesenchymal progenitor cells” [Dennis et al., 1999] meaning multipotential marrow derived cells but with restricted lineage properties and state these cells as progenitors instead of stem cells, which have been used side-by-side for a long period of time. In addition, Jiang et al. describe in his study “Multipotent Adult Progenitor Cells” (MAPC) as more primitive cell type with high plasticity which give rise to all mesenchymal lineages including visceral mesoderm, neuroectoderm and endoderm [Jiang et al., 2002]. The lineage relationships between MSC, “stromal stem cells”, “mesenchymal progenitor cells”, and MAPC remain unclear. Also, these descriptions represent only a few of the used terminologies for MSCs [Bianco et al., 2008].

Table 1: Definition of used Terms

Term	Definition
CFU-F	Fibroblast colony-forming cells single cells of the mesenchymal stromal lineage, capable of forming fibroblast-like colonies in culture
MSC	multipotent stromal cells (mesenchymal stem cells) (single) cells derived from CFU-F capable of differentiating in at least three mesenchymal lineage: osteogenic cells, chonrogenic cells and adipocytes in vitro

All these names have one commonality regarding to their tissue of origin - the bone marrow. In this thesis, we will only use the term CFU-F for single cells capable of forming fibroblastoid colonies with unknown differentiation potential, and MSCs for the collection of CFU-F-derived cells capable of multipotential differentiation both *in vivo* and *in vitro*.

In the present work CFU-F and MSC are defined as indicated in Table1.

1.2 Characterization of Multipotent Stroma Cells

1.2.1 Growth of Multipotent Stromal Cells

In the first transplantation experiments of marrow-derived stromal cells were performed by Friedenstein et al. [Friedenstein et al., 1970; Friedenstein et al., 1974]. These experiments showed important characteristics of the cells, which Caplan later defined as MSC. Friedenstein and coworkers seeded MSC in adherence to the plastic surface. Further along the culture, the stromal cells formed non-hematopoietic, non-phagocytotic, highly proliferative fibroblastoid colonies (CFU-F) [Friedenstein et al., 1970; Owen et al., 1988]. The description of CFU-F traces back to the spindle-shape morphology of the cells within the colonies. At the beginning of culture on plastic surfaces, the cells are small and round. But, after an initial lag phase, the fibroblastoid cells start to divide until the cytoplasmatic lumen increases [Castro-Malaspina et al., 1980].

The proportion of CFU-F in the postnatal tissue can vary depending on the source. The range of CFU-F derived from human bone marrow are reported to 1-10 MSC in 10^6 total bone marrow cells [Castro-Malaspina et al., 1980] while the CFU-F isolated from human umbilical cord blood range between one CFU-F per 3 in 10^6 to one in 10^8 mononuclear cells respectively [Goodwin et al., 2001, and Bieback et al., 2004]. This indicates that the CFU-F represent a rare population of cells in the stromal environment, which can vary in different MSC types and isolation methods.

1.2.2. Factors affecting Growth of CFU-F

The formation of CFU-F depends on various factors like culture conditions, seeding density as well as the age of the donor. Furthermore, the use of growth factors supplemented to media can affect the growth and the colony-forming efficiency of single CFU-F [Kuznetsov et al., 1997] or respectively show implications *in vivo*. However, the effect of culture supplements on CFU-F and their subsequent multipotent differentiation behaviour are still not completely understood. Basic fibroblast growth factor 2 (FGF-2), for example is a known mitogenic effector that can enhance the proliferative behaviour of CFU-F [Hanada K, 1997; Delorme et al., 2009]. At the same time, FGF2 also improves differentiation potential in the

chondrogenic and osteogenic lineage [Tsutsumi et al., 2001]. Additionally, Muraglia et al. demonstrated multilineage potential of MSC treated with and without FGF2 *in vitro* [Muraglia et al., 2000]. In constrast, *in vivo* studies demonstrated that FGF2 cultured BM-MSC have shown the formation of ossicles but no hematopoietic microenvironment [Sacchetti et al., 2007].

Another example is the single use Platelet-derived growth factor (PDGF mainly as homodimer of isoform B) and Epidermal growth factor (EGF) that are similarly mitogenic as FGF2. However, PDGF-BB and EGF can also inhibit colony formation by activation of Interleukin 4 (IL-4) in a dose-dependend manner [Gronthos et al., 1995; Owen et al., 1987]. Another important factor in the growth of CFU-F is the medium used to grow them in. The choice of medium has been shown to influence growth as well as differentiation potential of CFU-F [Dellavalle et al., 2007].

1.2.3 Surface Markers of the Multipotent Stromal Cells

The identity of CFU-F, their precursor or respectively their progenitors has not been defined. In other stem cell systems, like the hematopoietic system, different stages of differentiation have been precisely pinpointed using combinations of different antibodies [Uchida et al., 1992; Muller-Sieburg et al., 1986; Spangrude et al., 1988]. Despite a long record of research, a similar system of antigens, which define different stages is lacking in the mesenchymal stem cell system. The first antibody which specifically identified CFU-F, is STRO-1 [Simmons et al., 1991]. This antibody marks a relatively homogeneous subpopulation of CFU-F, which is capable of multilineage differentiation and, more importantly, of bone formation and establishment of the hematopoietic microenvironment in vivo [Gronthos et al., 1994]. Interestingly, the FACS-Analysis reveal that the best results for stem-cell like in vivo behaviour was presented in a STRO-1^{high} positive fraction that are not equally comparable to the other subpopulations and was also not expressed by other cell types [Jones et al., 2006]. This suggests once more that the bone marrow-derived stromal cells consist of a heterogeneous population, even within a more homogeneous population of STRO-1+ cells, whereas only a small percentage of cells are capable of formation of complete ossicles (bony construct including hematopoiec microenvironment and the differentiation of MSCs in the various mesenchymal lineages).

Due to the multilineage capacity and in vivo bone formation of CFU-F-derived progeny, the characterization and enrichment of CFU-F using antibody based isolation is a promising method. Unfortunately, in the case of STRO-1, the antigenic target molecule is not defined which means that further analysis on cellular and molecular level are restricted to protein applications and the use for clinical application is questionable.

Nevertheless, further analysis of STRO-1^+ cells allowed the analysis of CFU-F-derived progeny MSCs and demonstrated that these cells do not express the typical hematopoietic antigens CD34, CD45 and CD14, CD11b [Xu et al., 2004; Conget et al., 1999; Baddoo et al., 2003] or the endothelial marker CD31. This indicates that MSCs can be separated from the hematopoietic and endothelial cells.

Around the same time that STRO-1 was developed, the lab of Caplan developed SH-2, SH-3, and SH-4 which recognize antigens on the cell surface of marrow-derived mesenchymal cells, but fail to react with marrow-derived hemopoietic cells [Hayensworth et al., 1992]. Later studies showed that SH-2 recognizes the CD105 antigen [Barry et al., 1999], and that both SH-3 and SH-4 recognize CD73 [Barry et al., 2001]. These three SH markers subsequently developed to “classical” MSC markers. However, the isolation of SH-2 (CD105) positive subpopulation shows not only enrichment in the CFU-F which differentiate into bone through a cartilage intermediate known as endochondreal ossification. This developmental process is necessary for formation of the HSC niche [Chan et al., 2009]. SH-3 and SH-4 identifying the ecto-5-prime-nucleotidase CD73 which is not only expressed by CFU-F, but was also demonstrated to be involved in B-cell activation [Barry et al., 2001]. Besides STRO-1, SH-2, SH-3, and SH-4, a number of other possible markers have been described which could be useful for the isolation of CFU-F (Table2). One of those markers, Thy-1 (CD90), was originally identified as a hematopoietic marker. The glycoprotein CD90 or Thy-1 was first detected on T cells in mice [Raff et al., 1971] and is highly presented in MSCs. CD90 is highly expressed by fibroblasts and was recently shown to also present a good marker for CFU-F enrichment [Delorme et al., 2008].

Several investigators have studied surface antigen expression of MSC to define additional markers for enrichment of CFU-F. Deschaseaux et al. reported that adhesion molecules of the integrin alpha family (ITGA, also known as CD49) are

expressed on stroma cells of the bone marrow, and that CD49a represent a marker for mature and precursor in the stroma while CD49b and CD49d only expressed on immature cells [Deschaseaux et al., 2000]. In this regard, it is of interest to note that the CD49a subfraction co-expressed STRO-1 [Steward et al., 2003]. Adhesion molecules from other molecules were shown to be expressed by MSC and be involved in differentiation and self-maintenance processes. CD106, also known as VCAM-1(vascular cell adhesion molecule), and CD166 (SB-10 or activated leukocyte cell adhesion molecule; ALCAM) belong to the immunoglobulin-domain members of adhesion molecules (Table2). Both CD106 and CD166 are reported to be expressed on undifferentiated MSCs and to be downregulated upon induction of differentiation [Bruder et al., 1998; Liu et al., 2008]. While VCAM-1 can be used as a marker to study the development of the differentiation, ALCAM seem to be useful for identification of undifferentiated osteoblastic precursors [Bruder et al., 1997].

An additional marker useful for the isolation of CFU-F is the melanoma cell adhesion molecule MCAM, also known as Muc18 or CD146 (Table2). It has been clearly demonstrated that non-hematopoietic CD146+ stromal cells show bone formation with establishment of the hematopoietic microenvironment, so-called ossicles, *in vivo*. More impressively, the CD146+ fraction of these ossicles can form secondary ossicles *in vivo* [Sacchetti et al., 2007].

Recent studies suppose that alkaline phosphatase (ALPL, TNSALP), is also present on immature BM-MSC *in vivo* [Gronthos et al., 2007]. ALPL is a glycoprotein and mainly known to be associated to osteoblastic lineages and used for detection of osteogenic differentiation but also represented in liver, bone, as well as kidney [Goldstein et al, 1980] and skin fibroblasts [Fedde et al., 1990]. Previous *in vitro* studies of Gronthos et al. have confirm ALPL as an early osteogenic marker and suggest further on that STRO-1+/ALP+ cells represent an immature preosteoblastic stage in contrast to cells with ALP+ expression only [Gronthos et al., 1999; Asfandiiarov et al., 1985]. Additionally, in studies of Steward et al. a certain subset of BM-MSC are ALPL positive co-expressing further on a member of the tetraspanin family CD63 [Steward et al., 2003], which will be recognized by the monoclonal antibody HOP-26 [Zannettino et al., 2003].

Marker	Gene Symbol	CFU-F enrichment	Previous culture?	Trilineage differen-tiation?	*In vivo* ossicles?		Reference
					Bone	HME	
CD49a	*ITGA1*	*18*	*No*	*NI*	*NI*	*NI*	*Stewart et al., 2003*
CD49b	*ITGA2*	*23*	*Yes*	*NI*	*NI*	*NI*	*Delorme et al., 2008*
CD63	*CD63*	*6*	*No*	*NI*	*NI*	*NI*	*Stewart et al., 2003*
CD73	*NT5E*	*100*	*Yes*	*NI*	*NI*	*NI*	*Delorme et al., 2008*
CD90	*THY1*	*60*	*Yes*	*NI*	*NI*	*NI*	*Delorme et al., 2008*
CD105	*ENG*	*50*	*Yes*	*NI*	*NI*	*NI*	*Delorme et al., 2008*
CD130	*IL6ST*	*256*	*Yes*	*NI*	*NI*	*NI*	*Delorme et al., 2008*
CD146	*MCAM*	*830* *278*	*No* *Yes*	*Yes* *NI*	*Yes* *NI*	*Yes* *NI*	*Sachetti et al., 2007* *Delorme et al., 2008*
CD166	*ALCAM*	*4*	*No*	*NI*	*NI*	*NI*	*Stewart et al., 2003*
CD200	*CD200*	*333*	*Yes*	*Yes*	*Yes*	*No*	*Delorme et al., 2008*
CD271	*NGFR*	*45*	*No*	*Yes*	*NI*	*NI*	*Quirici et al., 2002*
CD349	*FZD9*	*30*	*No*	*Yes*	*NI*	*NI*	*Batula et al., 2007*
?	*ALPL*	*?*	*No*	*Yes*	*Yes*	*Yes*	*Gronthos et al., 2007*
ITGAV/B	*ITGAV/B*	*1750*	*Yes*	*NI*	*NI*	*NI*	*Delorme et al., 2008*
D7-Fib	*?*	*100*	*No*	*Yes*	*NI*	*NI*	*Jones et al., 2002*
STRO-1	*?*	*950* *9*	*No* *No*	*Yes* *Yes*	*Yes* *NI*	*Yes* *NI*	*Gronthos et al., 2003;* *Stewart et al., 2003*

Table 2: Molecules used to enrich CFU-F

Enrichment factor was calculated as the number of CFU-F per 10^5 enriched cells divided by the number of CFU-F per 10^5 mononuclear marrow cells. P0: cells not previously subjected to culture. P1: First passage cells from cultured marrow cells. HME: hematopoietic microenvironment consisting of bone marrow, blood vessels, and adipocytes. NI: not investigated.

Similar to ALCAM, also CD63 identify lineage specific cells from the bone marrow at early developmental stage.

Next to STRO-1 and CD146, also α-LNGFR (CD271) marks bone marrow stromal cells and isolates them in high purity [Quirici et al., 2002]. Studies with CD271 antibodies demonstrated that, similar to STRO-1, only the $CD271^{bright}$ population enriches for CFU-F and showed to be positive for other MSC markers, including CD73 and CD105 [Bühring et al., 2007]. However, CD271 is not detected on all MSC or populations, possibly depending on growth factor supplements which have been shown to downregulate the expression of the CD271 receptor [Quirici et al., 2002].

In Table 2 is a summary of several of the markers used for the enrichment of CFU-F described above as well as formation of ossicles.

Some researchers have recently started to combine new markers with already known ones. The idea of these experiments is to characterize MSC more precisely in comparison to the use of single markers only as well as to enable negative selection

by excluding hematopoietic (CD45, glycophorin A) and endothelial (CD31) markers. Such a strategy should result in a more homogenous and possibly uncommitted population of MSC [Battula et al., 2009], for instance, combines the myogenic marker CD56 with a recently identified MSC-staining antibody MSCA-1, to show that MSCA-$1^{+}CD56^{\pm}$ subpopulation represents strong chondrogenic differentiation while MSCA-$1^{+}CD56^{-}$ subfraction exclusively form adipogenic potential. This study is the first to isolate a homogenous population to either chondrogenic or adipogenic lineages.

The main problem of all of the markers studies presented above is that it has, so far, not been possible to produce a framework of expressed surface markers, which describes early uncommitted cells and separates them from later lineage-restricted mature progenitor subpopulations. Such a framework would be useful for quality-control in clinical production as well as define subpopulations useful for clinical applications. Thus, it is not surprising that the search for further markers is still continuing. Therefore, new markers and combination of markers should arise, which could help to characterize the stromal cells but are also helpful for the understanding of lineage relationships and plasticity within the mesenchymal system.

1.2.4 Various Multipotent Stromal Cells and their Differentiation Potential

The typical differentiation potential of MSCs is into the classical lineages adipogenic, osteogenic and chondrogenic (AOC). Investigators have taken either CFU-F-derived MSC for such studies, or defined other subpopulations of cells which may, or may not be similar to MSC. For instance, different research groups show populations of bone marrow derived-cells, which not only show AOC differentiation, but present the additional ability to differentiate into neuron and endothelium. This has, for instance been shown for the population of adherent bone marrow cells described as MAPCs [Jiang et al., 2002]. More interestingly, the culture-expanded cells were transplanted in blastocysts and show the commitment to different embryonic lineages which suggest that these cells may represent a more primitive cell type in comparison to MSC. Similarly, marrow isolated adult multilineage inducible cells (MIAMI) may also be slightly more primitive than CFU-F-derived MSC [D'Ippolito et al., 2004]. Both MAPC and MIAMI can be cultivated long term with over 50 population doublings with no morphological changes. Colter et al. and Smith et al. investigate in their experiments small plastic adherent cells, able of rapid self-renewal and high

clonogenity which were isolated using flow cytometry in a forward scatter (FS) low and side scatter low (FS^{low}/SS^{low}) fraction named them RS-cells (recycling stem cells, rapidly-self-renewing stem cells) [Colter et al., 2001; Smith et al., 2004]. In contrast to MAPCs and MIAMI the RS-cells were isolated primarily due their size and granularity in the FACS. The different testing of surface antigens and *in vivo* transplantation studies suggests that differences reported for these cell types cannot exclude that these cells may be linked with regard to their origin and development.

It is a continuing issue of debate whether CFU-F and their derived progeny, MSC, can be isolated from other tissues than bone marrow. MSC have been described to be present in different tissues like synovium [De Bari et al., 2001], periosteum, adipose tissue [Zuk et al., 2002], umbilical cord including Wharton's jelly and perivascular cells of the umbilical cord vein [Romanov et al., 2003] as well as cord blood [Rogers et al., 2004].

Experiments with cells from various tissues revealed that cells of synovium, periosteum and adipose-derived tissue contain a higher number of CFU-F than bone marrow [Sakaguchi et al., 2005]. The synovium is thin layer between the joint capsules of bones which can be fibrous or adipose tissue. Studies have shown that cells of the bone marrow, periosteum and synovium have a high osteogenic differentiation potential in common, whereas adipose-derived tissue cells and synovial cells represent a stronger adipogenic differentiation. Only the cells derived from the bone marrow and the synovium seem to show a strong ability of chondrogenic potential [Sakaguchi et al., 2005].

Due to the multilineage differentiation capacity with high chondrogenic potential the synovial MSCs are excellent source of osteoprogenitors for the purpose of regenerative medicine, especially in applications for osteochondreal defects. MSC derived from adipose tissue, on the other hand, are heterogeneous and may express CD34, in contrast to all other tissues tested. The adipose-derived MSC contain a $CD105^{+}$ subpopulation which is able to differentiate into hepatocytes including the expression of typical hepatogenic markers [Banas et al., 2007]. At this, these cells may represent a source for the need of cells in liver transplantations.

Finally, I like to introduce further important cell type- mural cells or also known under the more commonly used the pericytes.

Mural cells or pericytes resides abluminal, subendothelial, and closely associated

with endothelial cells in the microvascular wall surrounding the veins and capillaries. These MSCs represent putative progenitors of connective tissue and were often handled collectively under the term perivascular cells. The perivascular cells are synonymously used for mesenchymal cells of the peripherial vasculature including pericytes.

The pericytes represent the ALPL positive fraction in smooth muscle cells and have the potential to differentiate into chondrocytes, adipocytes [Farrington-Rock et al., 2004] as well as osteogenic lineage [Crisan et al., 2008a] and also a source of multinucleated myotubes [Crisan et al., 2008b]. Moreover, pericytes are able to maintain their differentiation in culturing of five month and show in *in vivo* mouse models of heart attacks reduced scar tissue, greater contractile volume, and more capillaries in the damaged region of the heart.

Crisan et al. demonstrated in his studies that perivascular cells are highly similar to MSC and propose a common origin of both cell types [Crisan et al., 2008a]. The location of perivascular cells at blood vessel in the bone marrow connected to endothelial cells and closely in linked to the hematopoietic microenvironment, support this hypotheses.

1.2.5 Multilineage Differentiation Potential of Multipotent Stromal Cells

In late embryonic developmental stages the epiblast will form the three embryonic layers (endoderm, mesoderm, and ectoderm). These germ layers then form the origin of the various organs. During the developmental program from an uncommitted to committed cell type, the MSC will become more restricted in their capacity. *In vivo* the MSC become determined in their function regarding to the tissue they reside. However, some investigators proposed that a rare population of “embryonic-rest cells” also exists in different tissues that may represent a more undifferentiated, uncommitted cell type with similar attributes as stem cells.

The properties of stem cells include their pluripotent differentiation capacity to all tissue-specific cell types. The mesenchymal cells derived classically from the bone marrow show multipotential activity and form all mesenchymal cells find in the skeletal system: bone cells, chondrogenic cells and adipose cells (Figure 2). These three different mesenchymal lineages have been demonstrated from MSC in both *in*

vitro and in *in vivo* transplantation experiments.

While *in vivo*, the uncommitted quiescent MSC will divide into multipotent daughter cells which will differentiate by responding to environmental cues, *in vitro*, the stimulus is mostly induced by supplementation of distinct differentiation reagents. Several studies show that MSC may also differentiate into further lineages which can be achieved by placing the cells in distinct locations or treating the cells with other stimuli like neuronal or endothelial direction. However, before I start with other differentiation ways of the MSC I will first describe the “classical” potential.

As we described, single cells forming fibroblast-like colonies CFU-F give rise to MSC, which possess multipotent differentiation potential. In studies of Friedenstein et al. and Owen et al. the CFU-F-derived cells demonstrably show the expression of alkaline phosphatase and undergo morphological changes during culture [Friedenstein et al., 1987; Owen et al., 1988]. These cells represent a dominant osteogenic differentiated cell type. However, transplantation studies reveal not only the osteogenic lineage but also adipogenic cells and hematopoietic supporting stroma [Friedenstein et al., 1970; Friedenstein et al., 1974].

To facilitate the studies of developmental steps until terminally differentiation on the cellular and molecular level, detailed analyses of the differentiation events was performed *in vitro*.

1.2.5.1 Osteogenic Differentiation

In vitro differentiation of osteogenic lineage (Figure 2) was shown to require dexamethasone, ascorbic-acid and β-glycerolphosphate [Jaiswal et al., 1997]. The several reagents act in concert at different time points of osteogenic induction and function as inducer or for maintenance of the differentiation [Jaiswal et al., 1997]. Dexamethasone induces both osteogenic and adipogenic differentiation. Beresford et al. demonstrate that the combined use of dexamethasone and 1,25-dihydroxyvitamin D3 enhances osteogenic and inhibits adipogenic differentiation and mRNAs involved in this process like alkaline phosphatase (ALP), osteopontin and osteocalcin [Beresford et al., 1994].

During the osteogenic differentiation, the spindle-shaped morphology of the MSCs changes into cuboidal, and mineralization can be noted at the cell membrane as well as more general deposition of calcium. The calcified cells can be detected in use of histochemical methods like von Kossa. Studies of the signalling processes involved show that the protein kinases ERK and JNK play important role at different time points in the osteogenic differentiation. Jaiswal et al. demonstrate that ERK2 is activated in early stages and inducing mineralization as well as up-regulation of osteopontin [Jaiswal et al., 1999]. JNK activation causes synthesis of the extracellular membrane and increased calcium deposition [Jaiswal et al., 1999]. RUNX2, CBFA-1 and AML3. The linkage of WNT with the canonical mediators β-catenin/TCF1 acts regulatory of the bone formation mediated by the activation of RUNX2 [Gaur et al., 2005]. In addition, the studies of Boland et al. suggest linkage between the canonical and non-canonical WNT pathway by differentially regulating the osteogenesis [Boland et al., 2004]. At the one hand the canonical WNT stimulator WNT3a inhibits osteogenic processes by reducing the mineralization and activity of ALP. On the other hand, activators of the non-canonical WNT pathway like WNT5a promote the osteogenesis and suppress the expression of PPAR-γ (Peroxisome proliferators-activated receptor gamma) [Takada et al., 2007].
These contrary effects of canonical and non-canonical WNT signalling suggest an important role of WNT in the control of differentiation events in MSC and demonstrate that the both WNT signalling pathways are important for cell fate decision in

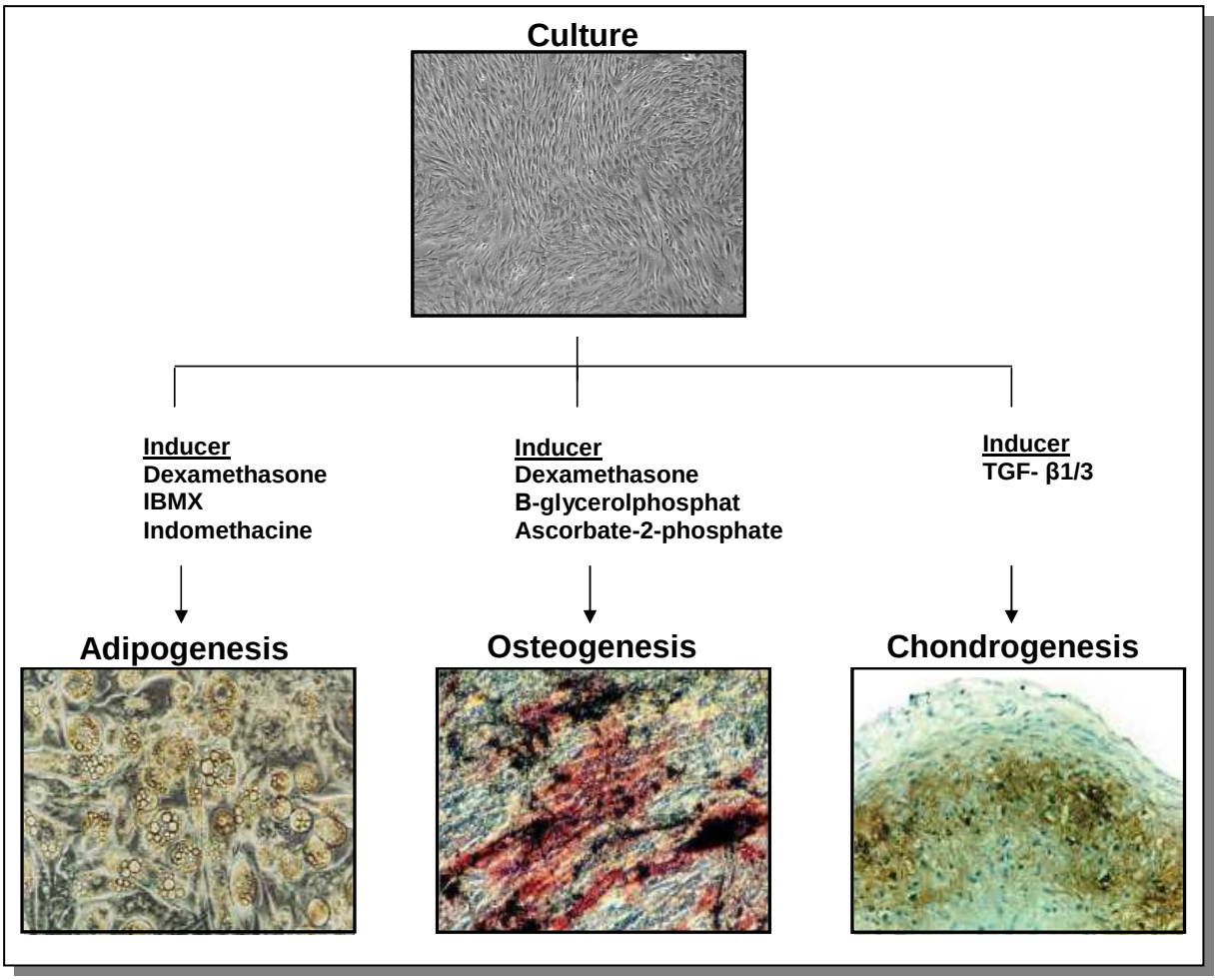


Figure 2: Classical mesenchymal Differentiation in vitro

The typical mesenchymal differentiation is represented in adipogenic, osteogenic and chondrogenic lineages. Clonal expanded MSC grown to monolayer culture and will be induced by lineage-specific differentiation reagents. In each differentiation process morphological alterations are observed. Adipogenic induced cells (dexamethasone and IBMX (3-Isobutyl-1-methylxanthine) or indomethacine) show enlarged lumen and the formation of lipid droplets. The osteogenic differentiated cells (induced by dexamethasone, β-glycerolphosphat, and ascorbate-2-phosphate) show mineralized matrix which can be detected by von Kossa (black) and alkaline phosphatase staining (red). Chondrogenic differentiation is performed in densed micropellet cultures and detected with collagen labelling (toluidine blue).
Pictures of adipogenic differentiation from [Yeh et al., 1995], osteogenic and chondrogenic differentiation from [Pittenger et al., 1999].

adipogenic or osteogenic differentiation. JNK is part of the so-called non-canonical Wingless signalling (WNT) pathway. The WNT pathway is involved in the osteogenesis by activation of the transcription factors. In view to cellular signaling, these studies also propose a common precursor for osteogenic and adipogenic cells, which may be different from chondrogenic precursor cells [DiGirolamo et al., 1999].

1.2.5.2 Adipogenic Differentiation

The different stages of adipogenic lineage start with growth to monolayer cultures. Due to the contact inhibition, the cells will remain in growth arrest and respond to exogenous stimuli with introducing the adipogenic determination process. This first steps will results in a pre-adipogenic cell type, which is not distinguishable to further forms until terminally differentiation. Finally, the cells will mature to the fully differentiated cell type with expanded lumen by accumulation of fat droplets in the cytoplasmatic space (Figure 2).

The activation of the ERK (also used together with MEK) pathway is not only presented in early osteogenic differentiation phase but further on important for activation of the key molecule in adipogenesis *in vitro* and *in vivo*, PPAR-γ [Adams et al., 1997]. In early events of the adipogenic differentiation (1-2h) the activation of ERK1 and ERK2 influenced by adipogenic stimuli is necessary for the expression of PPAR-γ and another transcription factor C/EBPα. Both molecules are important for terminally formation of adipocytes [Prusty et al., 2002]. The key role of PPAR-γ was demonstrated in overexpression experiments of double negative mutants which results in loss of sustaining the differentiation in molecular and morphological view [Tamori et al., 2002]. This means that the adipogenic differentiation process cannot fully terminated and maintain without the support of PPAR-γ. C/EBPs (CCAAT-enhancer binding protein) are a family of transcription factors which interact together with PPAR-γ. Both molecules are needed for the different processes in the developing adipogenesis and are closely linked. Studies have demonstrated that C/EBP α ($^{-}/^{-}$) mice show several defects in the adipogenic differentiation including lipid accumulation and fail to interact with PPAR-γ [Wu et al., 1999].

Similar to the *in vitro* stimulation of the osteogenic differentiation also the adipogenic differentiation reacting to the molecules dexamethasone, ascorbic acid and β-glycerolphosphat. However, several studies have shown that the enhancement of the adipogenesis can be achieved with the use of 1-methyl-3-isobutylxanthine (MIX) or respectively the more common form 3-isobutyl-1-methylxanthine (IBMX). Finally, MIX together with dexamethasone was shown as a poptent inducer for adipogenesis and C/EBP [Yeh et al., 1995].

1.2.5.3 Chondrogenic Differentiation

In contrast to the osteogenic and adipogenic differentiation, the chondrogenic differentiation *in vitro* is performed mainly in use of high-density micromass (pellets). The differences here consist of non-adherent cells, accumulated to a dense spherule. The use of these highly accumulated cells origin in studies and observations of chondrogenesis in embryonic limb development demonstrating that pre-chondrogenic processes are performed by accumulation of mesenchymal cells [Thorogood et al., 1975]. *In vivo* studies presented that TGF-βs are formed by embryonic cartilage and involved in the cell-condensation [Hall et al., 1995]. Finally, Johnstone et al. have presented a three-dimensional constructs of condensed MSCs, which effectively underwent chondrogenic differentiation in appropriate medium supplemented with TGF-β1 and additionally demonstrated a useful system for studying chondrogenesis [Johnstone et al., 1998].

In most studies today the micropellet model is used for chondrogenic differentiation. The development of cartilage is part of an intermediated step to bone formation. In the growing and the persisting bone, several locations remain chondrogenic like synovium, periosteum, and high specialised connective tissue like tendon and ligaments. These regions are rich of different types of collagen and aggrecan. The pleiotrophic effects of the WNT-signalling as already mentioned before is a main actor in this mesenchymal differentiation orchestra. At this, it is not very surprising that besides Sonig hedgehog and FGF as well as homeobox (HOX) transcription factors and BMPs influencing differentiation also influencing WNT signalling. The cooperation of WNT is inevitable if not mandatory for differentiation decision. In the (Figure 2) the key molecules of the mesenchymal differentiation ways are delineated. Here it can be noted that the osteochondro-progenitor and not the adipogenic differentiation way are induced by WNT10b [Ross et al., 2000]. Moreover, WNT10b inhibit the expression of PPAR-γ and C/EBP but promotes vice versa the induction of RUNX2 [Bennett et al., 2005] a key transcription factor in the osteogenic differentiation (Figure 3). Equally as already demonstrated members of the non-canonical pathway as there are WNT5a and WNT5b induce osteogenic differentiation but also involved in chondrogenic lineage [Church et al., 2002]. These relationships are clearly indicates that early differentiation processes of both osteogenic and chondrogenic cells origin from a common precursor (Figure 3).

However, the markers which dominate the chondrogenic differentiation from early time points are from the Sox family. The transcription factor belongs to the HMG (high mobility group) box family. The expression of Sox9 in the early chondrogenic differentiation (Figure 3) causing upregulation of collagen type II gene (Col2a1) and for its deposition in the matrix [Bell et al., 1997; Ng et al., 1997]. Sox9 was also shown to be involved in promotion of aggrecan acitivity in chondrogenic cell lines [Sekiya et al., 2000]. Furthermore, studies show that also Sox5 and Sox6 are able to induce the expression of Col2a1 and it will be suggest that all three are involved in the control of the collagen expression [Lefebvre et al., 1998].

The *in vitro* stimulation of the chondrogenic differentiation can be induced by transforming growth factor TGF-β (TGF-β1 and TGF-β3) [Sekiya et al., 2002; Worster et al., 2001]. By this stimulation also another signalling pathway to WNT is activated which belongs to the SMAD family. Furumatsu et al.demonstrate that the SMAD2/3 pathway controls early event of the chondrogenic differentiation by promoting the expression of Sox9 and Col2a1 mediated by TGF-β stimulation [Furumatsu et al., 2005].

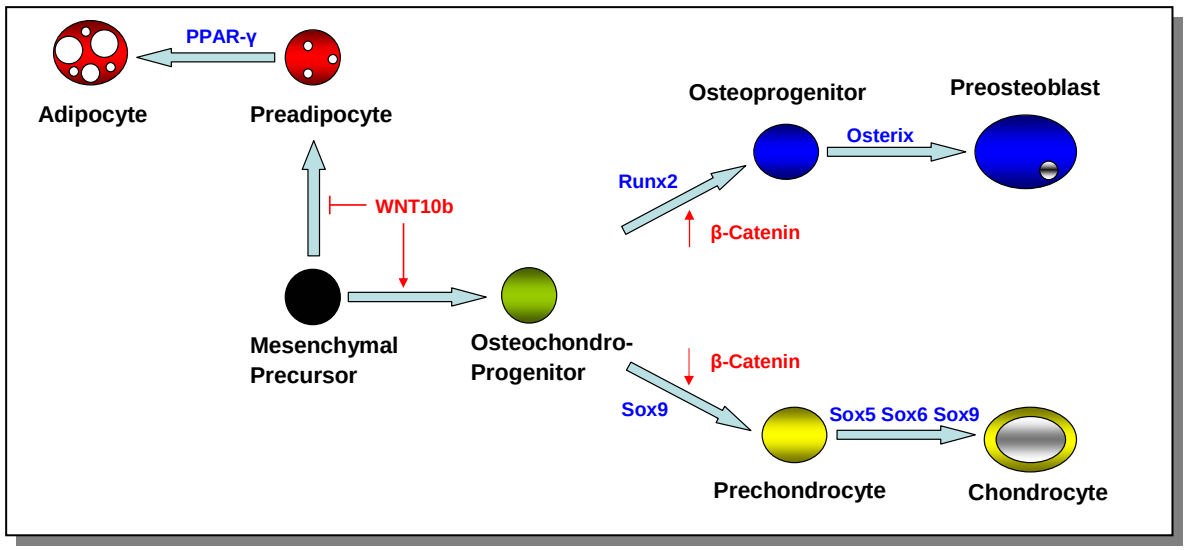


Figure 3: The role of canonical WNT-signalling in the differentiation process

Mesenchymal stem cells are able to differentiate in various lineages. The differentiation process will be influenced by extrinsic and intrinsic factors. The presented WNT signalling pathway constitutes a major regulating process in adipogenic and osteogenic-chondrogenic decision. Expression of WNT10b influence both differentiation ways by inhibiting PPAR-γ expression for adipogenic differentiation and in contrast to promote osteochondro-progenitor formation. The osteogenic and chondrogenic lineage will be regulated by differently expression of β-catenin to the respective progenitors. Finally, the chondrogenic precursor will be differentiated into chondrocytes with influence of SOX9 while Osterix will lead to the formation of prosteoblasts. [picture changed after Glass et al., 2007]

Finally, at the end of the chondrogenic differentiation process the three-dimensional aggregate and will immunhistochemical stained in use of toluidine blue for collagen detection [Johnstone et al., 1998] (Figure 2).The detection of these differentiation lineages is necessary to conclude the present mesenchymal cell type in multi-, bi-, or unipotent.

In the clinical application cartilage, tendon, and ligament will be used previously at patients with injuries from sportive activities, physically stress and age-related weakening of bones and muscles. At this point the tissue engeneering hold various techniques whereas MSCs were used together with biological or artificial matrices or scaffolds. Here, chondrocyte implantation can be introduced for repair of articular cartilage or for meniscus repair [Oakes et al., 2004]. Similar to cartilage repair also tendon repair is used in combination of matrices which origin from differentiation studies of tendogenesis.

1.2.5.4 Tendogenic Differentiation

The differentiation process of tendon is performed in a three-dimensional collagen construct under contractionary movement [Archambault et al., 2000]. Awad et al. demonstrated that the seeding density of the cultured MSCs lead to different morphological and kinetically effects [Awad et al., 2000]. The cells at higher density were elongated and aligned to one direction. The MSCs grown under these conditions and loaded together with collagen gel show *in vivo* increased structural and mechanical adaptation as well regeneration effects on tendon as compared to cell without collagen load [Wakitani et al., 1994].

Further MSC differentiation ways

However, several studies show that MSC are also able to differentiate further non-classical lineages. The plasticity of the MSC was shown for example for, myocytes, and nerves (glia cells). Some of these differentiation ways are familiar with concept of the “mesengenic process” from Caplan and are supposed to be expected.

1.2.5.5 Myogenesis

The myogenic differentiation process is separated into smooth muscle, skeletal muscle and cardiomyocytes. Muscle stem cells in adult organism are known as satellite cells. The properties of these adult muscle stem cells are the reparation processes of muscle injuries [Bryson-Richardson et al., 2008]. Moreover Ferrari et al. reported that cells able to form muscle also reside in the bone marrow [Ferrari et al., 1998].

Transplantation of bone marrow cells in Mdx mice which represent a model for defective muscle development have shown that the bone marrow cell harbour population with expression of typical muscle markers like myogenin and Myf5 and also dystrophin [Bittner et al., 1999]. Mdx stands for spontaneous X- chromosome linked mutation in the dystrophin gene and used for studying human muscle dystrophy (Duchenne muscular dystrophy).

Recent studies show that pericytes derived from the skeletal muscle show only expression of myogenic markers after terminal differentiation and differ from satellite cells but show similar *in vivo* behaviour as demonstrated in experiments by [Bittner et al., 1999; Dellavalle et al., 2007]. Due to the engraftment experiments and the detection of myogenic markers of the transplanted MSC the myogenic potential is no mistaking. Nevertheless, the suggestion that muscle cells derived from satellite cells only is disproved by [Dellavalle et al., 2007]. The background of their studies arise from the experiments for identification of a vessel derived stem cell and the hypothesis of the origin of MSC from vascular development [Minasi et al., 2002; Cossu et al., 2003].

1.2.5.6 Neurogenesis

A further differentiation potential of bone marrow derived cells is the ability to generate neural cells [Kopen et al., 1999]. Gage et al. propose that bone marrow-derived cells can differentiate into various neural cell types like neurons, oligodendendrocytes, and astrocytes [Gage et al., 2000]. Induced will be the neurogenic differentiation potential in human MSC by IBMX, which the putative inducer for adipogenic differentiation mentioned above, and cAMP [Deng et al., 2001]. Interestingly, transplantation experiments of bone marrow cells have shown

that donor derived neuronal cells were detected after one year in the recipient [Mezey et al., 2003]. Whether the MSC-derived neural cells are functionally active within the neural system is an area of intense research. The use of MSC in neuronal differentiation has so far, only been proven *in vitro* and the cellular and molecular events in MSC neurogenesis still need to be elucidated.

The untypical plasticity of MSCs shows the possibilities of a wide differentiation potential and suggests these cells as highly potent cell type for the regenerative medicine in several areas. However, there is still great demand in understanding the cellular and molecular mechanisms of MSC differentiation as well as in the understanding of the lineage relationship in the concept of mesenchymal hierarchy.

1.2.6 Is the Umbilical Cord an alternative Source of MSC?

The morphological profile of a typical umbilical cord consists of two arteries and one vein both embedded by stroma called Wharton's jelly consisting of proteoglycan and mucopolysaccharide [Sobolewski et al., 1997]. All of it is enclosed by the subamionepithel and the amnion outer layer. The umbilical cord is the source of two different types of cells: the cord (or placental) blood, and the cord itself, which contain the stromal Wharton's jelly, as well as the blood vessels.

The umbilical cord does, in contrast to the other tissues described above, not require invasive surgical procedures for its isolation. Also, as a by-product of natural child-birth, the umbilical cord will normally be disposed. The MSCs can be isolated from different compartments of the umbilical cord like the arteries, the vein, umbilical blood, and Wharton's jelly. Furthermore, analysis have shown that MSCs derived from umbilical cord compared with BM-MSC reveal high similarity in morphology, phenotypical appearance and differentiation potential in MSC lineages. Based on the simple collection of MSCs without impairing mother or newborne, as well as the detection of typical mesenchymal attributes, MSCs derived from umbilical cord represent an alternative autologous cell source and possible replacement for bone marrow derived MSCs in regenerative medicine and therapeutical applications. Studies have shown the role for umbilical stromal cells and cord-derived endothelial precursors in cardiovascular tissue engeneering [Schmidt et al., 2005; Kadner et al., 2002]. Due to the differentiation potential in chondrogenic lineage, a possible field of application for umbilical stromal cells can be suggested in cartilage defects. Here,

already MSCs from bone marrow showed to be useful for cartilage defects in knee joints after stimulation with chondrogenic inducing cytokins [Gelse et al., 2003].

Furthermore, MSCs derived from umbilical cord blood are used for allogeneic transplantation and low risk of graft versus host disease (GvHD). Additionally, hematopoietic stem and progenitor cells can be isolated from primary umbilical cord [Rubinstein et al., 1995; Wyrsch et al., 1999]. In differentiation experiments the cord blood-derived MSCs show similar multilineage potential to BM-MSC. Gang et al. demonstrate skeletal myogenic differentiation potential of the cord blood cells [Gang et al., 2004]. The cord blood-derived MSC demonstrate the expression of classical MSC markers like CD90, CD105 and no detection of the hematopoietic or endothelilal markers CD34, CD45 and CD31 [Bieback et al., 2004; Erices et al., 2000]. Further phenotypic studies show also the detection of CD146 and STRO-1, common markers of MSC.

It is also supposed that the MSCs derived from the cord blood and Wharton's jelly presents a more primitive population in comparison to that of bone marrow with regard to their rapid dividing behaviour [Troyer et al., 2008]. In consideration of the studies with Wharton's jelly compared to vein-derived stromal cells (UVSC) presents that the UVSC have a less differentiation potential and also lower population doubling [Karahuseyinoglu et al., 2007]. Based on these facts the stromal cells of the Wharton's jelly are proposed to be more uncommitted than cells derived from the bone marrow. CFU-F-derived stromal cells from Wharton's jelly or the umbilical vein also have been shown to express MSC markers like CD146 and STRO-1 [Sarugaser et al., 2005; Baksh et al., 2007].

In several publications the stromal cells derived from Wharton's jelly, umbilical arteries and vein are proclaimed to contain more CFU-F which more efficiently produce MSC in comparison to bone marrow cells. In comparative proteomics analysis of MSC from different sources, UVSC showed a closely related proteome to BM-derived MSC and embryonic stem cells [Roche et al., 2009]. However, analysis have shown that adipogenic potential of both cord-blood and umbilical tissue-derived MSC may be impaired [Karahuseyinoglu et al., 2007; Lu et al., 2006; Bieback et al., 2004]. Thus, true multipotentiality of umbilical cord-derived MSC may not be the same as those derived from bone marrow.

In particularly, stromal cells derived from the umbilical vein- the UVSC- represent in comparison to MSCs derived from umbilical artery or Wharton's jelly, strong differentiation potential to the mesenchymal lineages [Ishige et al., 2009].

As described above, there are no clearly defined surface markers which can distinguish primitive uncommitted MSC from their more differentiated progeny. To prove the primitive nature of MSC, many investigators used surrogate markers for primitiveness and tested MSC for the expression of embryonic stem cell markers. These so called "stemness" markers include transcription factors and other molecules known to be involved in self-renewal of embryonic stem cells. One of such factors, like the POU-domain transcription factor OCT4 (POU5F1) has been detected in umbilical blood [Tondreau et al., 2005], but also in MSCs of the umbilical vein and Wharton's jelly [Kermani et al., 2008; Carlin et al., 2006]. Due to the expression of embryonic stem cell markers like POU5F1, the MSCs of the umbilical cord have been proposed to be more undifferentiated, and possibly stem-cell like than those derived from bone marrow.

Taken together, the stromal cells of cord blood, Wharton's jelly and the umbilical vein may represent promising alternative source of MSC [Bieback et al., 2004].

Due to the high similarities to BM-MSC, the umbilical cord derived MSCs are not sufficient tested which limited the umbilical cord stromal cells in their use in clinical or therapeutical application. The different isolation methods and culture conditions influence the output and interpretation of the results. Here, Bieback et al. also demonstrate that the efficiency of isolated UCB derived MSCs depends on the time of collection and isolation as well as the age of the donor, which suggest to reduce the differentiation potential [Bieback et al., 2004]. In gene expression analysis of UVSC compared to BM-MSC [Panepucci et al., 2004] suppose that expression UVSC or BM-MSC related genes will explained by the tissue of origin. The fact that MSCs are detected in several tissues may also be reasoned by their different characteristic and functions.

However, whether the umbilical-cord-derived cells represent the same MSC as those derived from bone marrow remains to be established.

1.3 Molecular Signature in embryonic and adult Stem Cells

The regulation and the maintenance of the self-renewing stem cell state is managed by key molecules. The best investifgated type of stem cell in this respect is the embryonic stem cell (ESC). Few of the molecules required for ESC self-renewal were demonstrated to be also expressed in adult postnatal tissues. The molecular mechanisms involved in MSC self-renewal are not known. In fact, formal proof of MSC self-renewal is still lacking [Sacchetti et al., 2007]. Yet, it has been clearly demonstrated that MSC express transcripts for several different molecules known to be involved in ESC self-renewal. In search of the molecular mechanism of self-renewal processes the expression of these transcripts has been taken as proof of the stem cell activity of MSC.

The understanding of self-renewal and stem cell properties in general would facilitate studies on various aspects of MSC biology required for its efficient use in regenerative medicine. Also, knowledge about self-renewal and differentiation mechanisms would enable study of phenomena noted in stem cell biology. First, the self-renewal capacity of stem cells is decreasing with age, which means that the regenerative potential may be restricted to young donors [Rando et al., 2006]. Second, the regulation of the self-renewal process itself needs to be tightly controlled because of the strong potential of malignant transformation [Reya et al., 2001]. Thus, the knowledge of genes involved in the maintenance, self-renewal, and differentiation as well as other functional characteristics of stem cells is important to promote understanding in this field.

The use of “stemness” Markers to predict Stem Cell Activity

Several factors have been shown to be required for ESC self-renewal. Some of these factors like REX-1 and NANOG are known to be expressed in ESC and for maintenance of the stem cell state (Figure 4). REX-1 or ZFP42 (zinc finger protein 42) act as transcription factor and is expressed only in ESC, embryonic carcinoma cells (ECC), mouse embryonic cells at blastocyste stage, trophoectoderm, and meiotic germ cells of the adult mouse testis [Rogers et al., 1991]. Further studies reveal that REX-1 is also present in MAPCs [Moriscot et al., 2005] and MSCs [Roche et al., 2007]. NANOG, however, is more intensively studied and was shown as to be

reduced during differentiation whereas deletion of NANOG benefits the differentiation in ESC. These results conclude [Chambers et al., 2003] as possible factors sustaining self-renewal in ESC.

Both genes are regulated by POU5F1 a well studied marker in the field of ESC genes which is also known as OCT3, OCT4, or OTF3. POU5F1 is a key regulator of the stem cell state involving the differentiation to all germ layers and the maintenance of self-renewal in ESC [Niwa et al., 2000]. Several studies were performed to investigate the self-renewal and differentiation processes with POU5F1 as model marker (Figure 4).

POU5F1 is part of the core self-renewal circuit which also contains SOX2 and NANOG [Niwa et al.; 2000 Ivanova et al, 2006; Chavez et al., 2009]. Together, these three transcription factors regulate a network of target genes which not only self-renew and demonstrate maintenance of the pluripotent state, but also inhibit the formation of the germ layers. In this thesis, we will describe POU5F1 in more detail as a model “stemness” marker. Also, in accordance to this function and influence in ESC on other markers, POU5F1 is thought to be a key regulator of stem cell characteristics in all types of cells where POU5F1 can be detected.

The POU5F1 is a transcription factor located on human chromosome 6 (6p21.31). The POU5F1 gene consists of 5 Exons with a TATA-lacking promoter region only containing proximal and distal regulatory elements. The POU-binding domain is subdivided in POU_S and the POU_{HD} and allows by configuration changes the interaction with different target molecules [reviewed in Pesce et al., 2001].

Due to an alternative start codon POU5F1 gene can give rise to two main isoforms POU5F1A and POU5F1B (OTF3A and OTF3B in [Takeda et al., 1992]). Both isoforms can be distinguished by their amino acid length (OCT3A 360AA, OCT3B 265AA). Also, both isoforms differ in their location: while POU5F1A is detected mainly in the nucleus, POU5F1B appears only in the cytoplasmatic region [Cauffman et al., 2006]. Functional studies on the two isoforms suggest that POU5F1B is unable to sustain ESC self-renewal [Lee et al., 2006]. This finding points to an important functional role of the POU5F1 N-terminal domain which is only expressed by POU5F1A.

In studies POU5F1 mutations in early developmental stages, it was found that ESC with a Null mutation for POU5F1 cannot form ICM and results in pre-implant lethality. *In vitro* studies demonstrate that POU5F1 deficient ESC show increased trophoectoderm differentiation [Nichols et al., 1998] and upregulation of GATA4 and GATA6 [Hay et al., 2004]. In contrast, induced POU5F1 overexpression causes inhibition of differentiation but also results in dysplastic growth in epithelial tissues, which show high similarities to cancer formation by defective WNT/β-catenin signalling [Hochedlinger et al., 2005].

Sato et al. previously reported the importance of the canonical WNT signalling pathway in the maintenance of the embryonic stem cell state by blocking GSK-3 (glyocogen synthase kinase-3) and therefore supporting the expression of POU5F1, REX-1 and NANOG [Sato et al., 2004]. This study suggests that POU5F1 expression may be regulated by canonical WNT signals. The activity of POU5F1 is shown to depend on its dose [Niwa et al., 2000]; the undifferentiated state linked with POU5F1 is dependent on an optimal dose window above as well as below which ESC differentiate and maintenance of pluripotency is impaired. Thus, an increase over 50% induces differentiation while repression causes trophoectodermal cell fate.
The POU5F1 promotor in ESC is regulated through an enhancer region including an octamer motif for feedback regulation [Okumura-Nakanishi et al., 2005; Chew et al., 2005]. In addition, it is probably the distal enhancer of POU5F1, which consist of four highly conserved regions (CR1-CR4) (Figure 4), which are responsible for the expression of POU5F1 in the inner cell mass of blastocysts [Yeom et al., 1996]. Additionally, the POU5F1 promoter also contains elements for repression of POU5F1. These motifs are in contrast to the positive regulatory elements not part of the distal enhancer region but part of the proximal promoter region. At this proximal region, the germ cell nuclear factor (GCNF) binds and negatively regulates POU5F1 expression by facilitating methylation of CpG islands of the distal promotor region, thereby blocking POU5F1 expression [Fuhrmann et al., 2001] (Figure 4).

Other factors of the key regulators of the ESC self-renewal circuitry are NANOG and SOX2. Deficiency or overexpression of these factors has the same effects as similar manipulations of POU5F1, suggesting that all three factors are required for the self-renewal circuit to function properly. Besides POU5F1, NANOG, and SOX2 in human and in mouse embryonic stem cells, further molecules were found to be required for

ESC self-renewal, probably as down-stream factors of the key circuit. One of those putative down-stream factors is DDPA3 also known as STELLA [Bortvin et al., 2004; Bowles et al., 2003]. The ESC factors sustain methylation events in epigenetic reprogramming in early embryogenesis [Nakamura et al., 2007]. In general, the DNA methylation is necessary for mammalian development for activation and inhibition of gene expression.

The characterization of ESC with regard to defined markers and their function is still ongoing. One of the main efforts in this endeavor is the identification of surface markers for isolation of stem cells from various tissues. In studies of undifferentiated human embryonic stem cells [Draper et al., 2002] found surface antigens, which were equally expressed in embryonic carcinoma cells like Stage Specific Embryonic Antigen-3 (SSEA-3), SSEA-4 as well as TRA-1-81 and TRA-1-60. In comparative analysis between human and mouse ESC it was shown that SSEA-1 is expressed in mouse ESC whereas ESC markers SSEA-4, TRA-1-60, TRA-1-81 primarily detected in human [Ginis et al., 2004; Henderson et al., 2002].

Moreover, the expression of POU5F1, SSEA-4 and REX-1 was found in MIAMI of the bone marrow. The authors took this as proof that MIAMI cells present a more undifferentiated state than MSC [D'Ippolito et al., 2006]. Also, the detection of embryonic characteristics stem cell markers among others like SSEA-4, SSEA-3, and POU5F1 were demonstrated in umbilical cord blood cells, which additionally show no expression in SSEA-1 as previously presented in ESC [McGuckin et al., 2005].

The isolated VSEL-cells (very small embryonic-like) may represent a subpopulation of stromal cells in bone marrow and in cord blood which expresses “stemness” factors like POU5F1, NANOG and SSEA4 and were proposed to be a source of embryonic stem cells [Kucia et al., 2006; Kucia et al., 2007]. However, the experiments are shown only in mouse and there are no clonal studies or more importantly detection of self-renewal capacity. Furthermore, the possible pluripotency of these cells wasn’t proved which means there is no differentiation shown into all three germ layer (endoderm, ectoderm, and mesoderm) as shown in comparison to the study of Jiang et al. [Jiang et al., 2002].

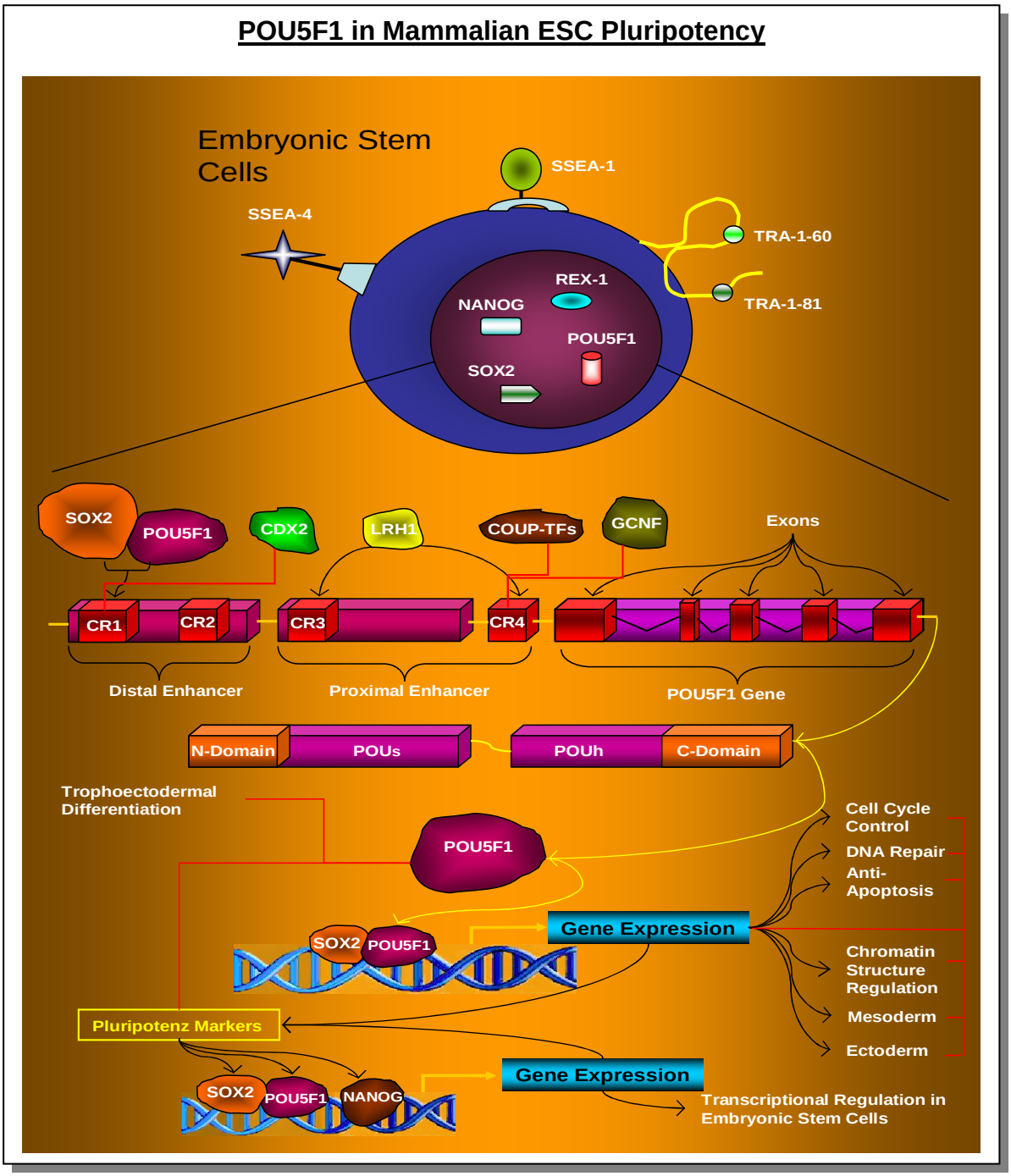


Figure 4: POU5F1 Properties in ESC Pluripotency

Shown are a choice of embryonic markers and the various influence areas of POU5F1 in the mammalian pluripotency. POU5F1 is subdivided in the different binding sites which interact with different co-activators like SOX2, LRH1 and POU5F1 itself or repressors like CDX2, COUP-TFs, GCNF at conserved regions in the distal or proximal enhancer region. The POU5F1 protein can influence different gene expression events by regulation of cell cycle, apotosis, DNA repair and chromatin structure. Cell fate determination processes will be also effected by differential expression of POU5F1 which cause promotion of the ectodermal and mesodermal progenitors. Moreover, POU5F1 repress lineage-specific differention like trophoectodermal. The most common interaction partners of POU5F1 are SOX2 and NANOG, which form a regulatory circuit for maintaining pluripotency and directly stimulate the expression of further pluripotency factors.
(Repression — Regulation, Activation ⟶)

The “stemness” factor SSEA-4 was detected on the surface of MSC and it was shown that the SSEA-4^+ subfraction was multipotent [Gang et al., 2007]. These studies suggest that expression of “stemness” factors may predict stem cell properties or characterize a very primitive population of MSC. However, protein data for POU5F1 and NANOG is conflicting, and the function of these “stemness” proteins in MSC biology has not been elucidated.

With regard to this issue Greco et al. demonstrated that undifferentiated human MSC express transcripts for all three ESC self-renewal circuitry genes POU5F1, NANOG, and SOX2 and that POU5F1 expression may promote the expression of mesodermal genes [Greco et al., 2007]. Thus, it is possible that POU5F1 expression is, in fact a mesodermal lineage-promoting factor.

1.4 Hierachical Model of Multipotent Stromal Cells

An alternative approach to distinguish early from late mature cells is to find out the lineage relationships between the different stages of mesenchymal differentiation. Since the mesenchymal concept induced by Caplan et al. the characterization of MSC changed with ongoing development of new protocols and optimization methods [Caplan et al., 1991]. Different subpopulations of MSC were described with more undifferentiated stem cell-like, or rather lineage-restricted progenitor behaviour. All these studies suggest that there may be a hierarchy of cells within the mesenchymal system. In comparison to the hematopoietic system the mesenchymal system is not dissected in as much detail.

Whether the comparison of the hematopoietic and the mesenchymal systems is, if at all, appropriate a matter of debate. There are obvious differences between the cells involved in the two interacting systems.

If we regard the lifespan of HSC in comparison to MSC, a high turnover of short-lived cells can be noted in the hematopoietic system while the mesenchymal system appears to consist of long-lived cells.

While HSC develop in the bone marrow in close proximity with the supporting stromal cell system, which is required for stem cell maintenance a similar dependency of CFU-F maintenance on microenvironmental cells is unclear [Bianco et al., 2000].

In studies of the mesenchymal lineage Muraglia et al. suggest a hierarchical subdivision demonstrating that not all CFU-F are endowed with an equal differentiation potential and therefore the CFU-Fs can be distinguished with tri-, bi-, or unipotent differentiation capacity [Muraglia et al., 2000].

Hence, Minguell et al. [Minguell et al., 2001] and other MSC hierarchical models too, have categorized CFU-F on the basis of various differentiation potentials, as well as the expression of, for instance, STRO-1 [Gronthos et al., 1994] or expression of several antigens in different developmental stages of osteoprogenitors [Bruder et al., 1997] (Figure 7). These authors, classify MSC based on their differentiation potential and proposed a hierarchical model between uncommitted and committed mesenchymal cell types (Figure 5 and 6).

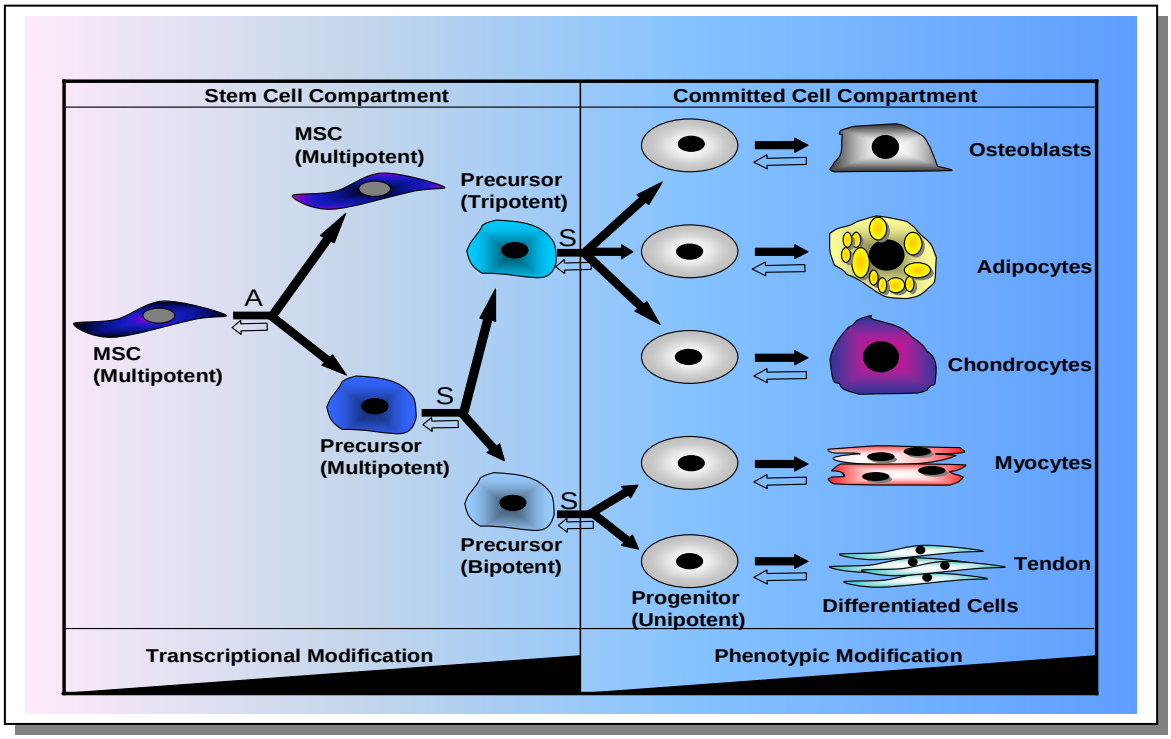


Figure 5: Hierarchical Model of Mesenchymal Stem Cells I

Hierarchical concept of the MSC system modified from [Baksh et al., 2004]. The concepts is subdivided in stem cell compartment and committed cell compartment. In the stem cell compartment, multipotent MSC give rise to less potent progenitors via asymmetric (A) and symmetric (S) division. The bi-and tripotent precursor develop to unipotent progenitors with determined differentiation potential to distinct differentiated cell type. During the different processes the MSC undergo various molecular and phenotypical modifications. Several studies suggest that under appropriate conditions committed cells are able to dedifferentiate in a more potent precursor (open arrow).

However, due to the classification based on the differentiation potential, the studies above do not include a clear identification or definition of a certain lineage/stage-specific MSC typus like it is known from the hematopoietic system. The approaches here define a heterogeneous population among them uncommitted and more committed MSC consist. This uncommitted MSC type in most hierarchical models, is described as precursor to the multipotent MSC. Where in such a scheme, even more undifferentiated cells like RS cells [Colter et al., 2001], MAPC [Jiang et al., 2002] and MIAMI cells [D'Ippolito et al., 2004] fit, remains to be determined. As the hematopoietic system has shown, detailed knowledge of lineage relationships between precursor and progenitor population allows detailed studies of cell fate decisions. The effective manipulation of cell fate of MSC is required for a proper application of these cells for regenerative medicine purposes. It is clear that differentiated progeny can be induced from MSC. However, MSC are very heterogeneous, and it is unclear whether within the MSC population MSC will be present with a differentially active or repressed molecular profile as compared to a system.

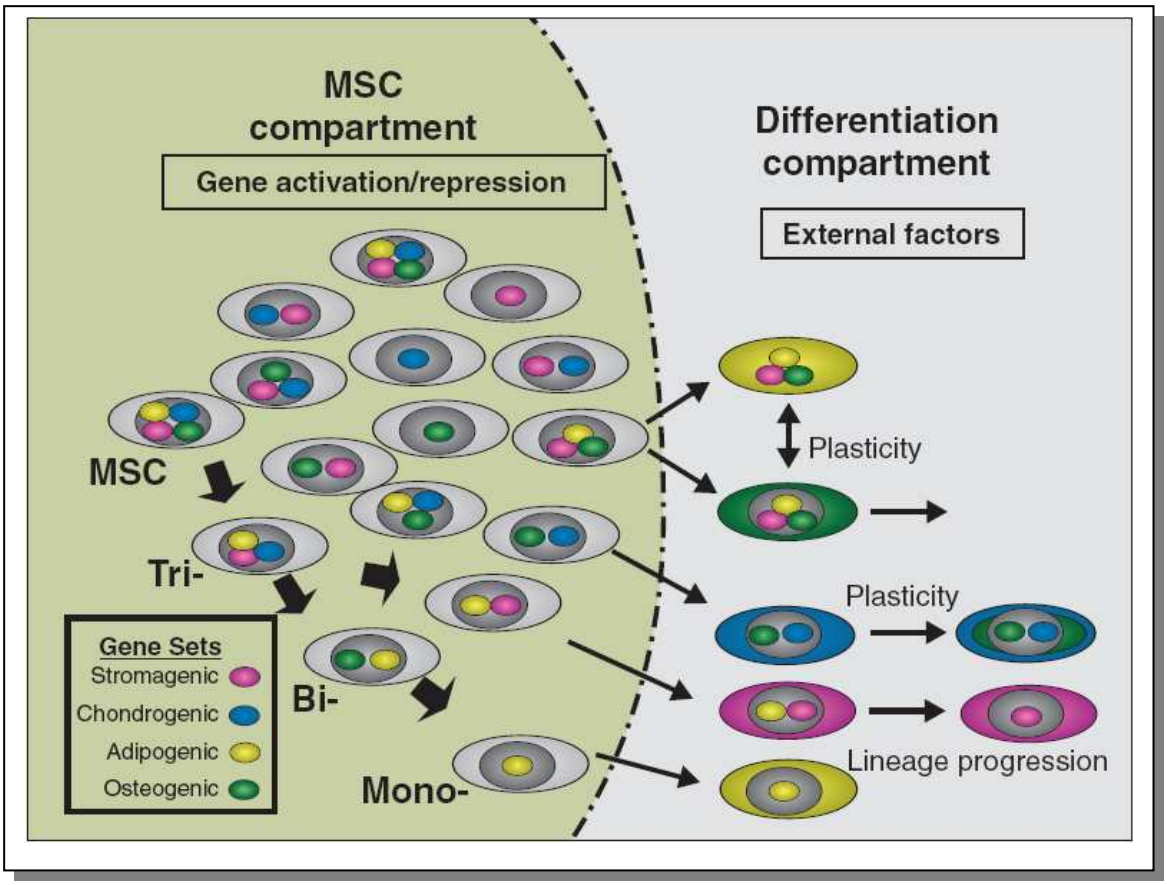


Figure 6: Hierachical Model of Mesenchymal Stem Cells II

Mesenchymal concept by [Dennis et al., 2002] distinguish between two compartments whereas one part include the uncommitted cell (MSC compartment) with different lineage potential and a further part the differentiation compartment comprising more committed cells, which differentiate with regard to their external factors and plasticity to terminally determined cell types. The different commitment processes undergo different modifications following stochastic activation and/or repression events as described in [Dennis et al., 2002].

Based on the active or repressed gene expression profiles, the MSC can response differently to various lineage-inducing extrinsic factors. Like in the hematopoietic system, what cues are dominant in cell fate decisions: extrinsic or intrinsic stochastic factors is unclear. Dennis et al. prefers a model in which activation and repression processes are determined in a stochastic manner in which cells in the MSC compartment are capable of differentiating into several different lineages, whereas cells induced to differentiate slowly gain the transcriptional profile of committed lineages within the differentiation compartment (Figure 6) [Dennis et al., 2002]. A similar subdivision of uncommitted and committed compartments has been proposed by Baksh et al. [Baksh et al., 2004] (Figure 5).

Similar to the model from Dennis et al., commitment is mainly based on stochastic processes which lead up to asymmetric and symmetric divisions of an uncommitted (stem) cell, resulting in multipotent MSC which progressively looses lineage potential

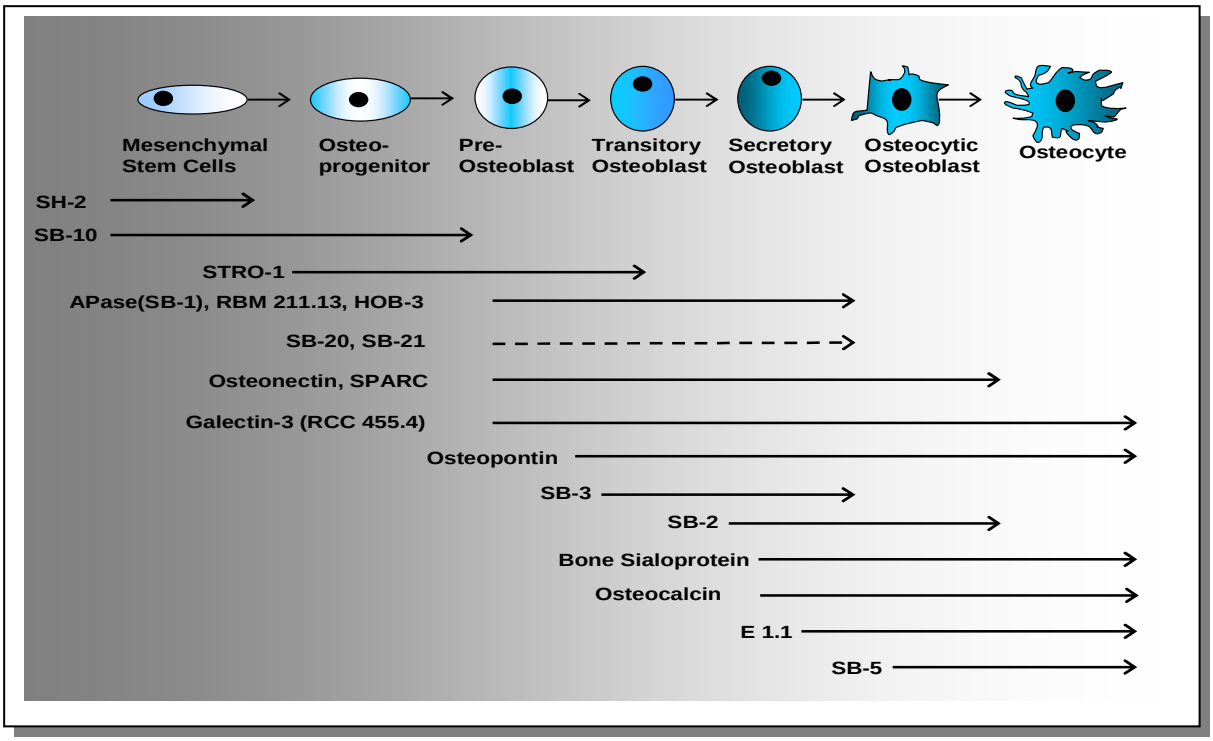


Figure 7: Known Markers of Osteogenic Differentiation process

Shown are known antibodies of the osteogenic lineage. This modified concept was established by [Bruder et al., 1997] and represents the recognition of several markers in course of the osteogenic differentiation. The scheme shows state specific expression of markers. While SH2 and SB10 as well as STRO-1 demonstrate to be expressed by undifferentiated MSC but cannot be detected in osteocytes, Galectin3, Osteopontin, Bone Sialoprotein, Osteocalcin, E11, and SB5 are later expressed in the differentiation but detecable until osteocytes. This concept represent first concluded antibody-based hierarchy of the various differentiation stages in osteogenic lineage.

with continuous divisions. Although the presented hierarchical mesenchymal systems will give an overview to the mesenchymal precursor and progenitor system of uncommitted and committed MSCs, a clear delineation of the different cell types in the system has, so far, not been achieved.

Bruder et al. has constructed initial attempts to define different stages within osteogenic lineage (Figure 7) [Bruder et al., 1997]. Such studies could be combined with recent analyses of novel antibodies and markers [Battula et al., 2009] or known markers [Steward et al., 2003]. To define different stages within the mesenchymal hierarchy based on surface markers. The problem underlying the definition of a mesenchymal system remains its high degree of heterogeneity. To date, there is no available marker, or system of markers, which can definitely distinguish between uncommitted and committed CFU-F *in vivo* or exclusively identified tri-lineage (AOC) potential MSC. In this thesis, I will try to contribute to this definition of uncommitted MSC.

1.5 MSC in clinical Application

The ability of the MSC to differentiate in fat, bone, and cartilage the simply isolation and cultivation method as well as good expansion capacity, make these cells to an interesting tool for regenerative medicine in various clinical and therapeutical applications. Additionally, the immunsuppressive and immunmodulatory function of MSC reduce the graft-versus host disease (GvHD) and make MSC useful in allogeneic and autologous cell therapy.

MSC avoid a T-lymphocyte immune response due to lack of expression of co-stimulatory molecules, like CD80 and CD86 [Di Nicola et al., 2002] which are necessary for introduce T-cell response. But also the interaction with CD8+ regulatory T cells, which inhibit the allogeneic lymphocyte proliferation [Djouad et al., 2003] and the secretion of factors like TGF-β, hepatocyte growth factor (HGF), Prostaglandin E2 (PGE2), Interferon-γ, Interleukin-6, and M-CSF have suppressive and inhibitory effects on T- and B-cell proliferation [Di Nicola et al., 2002; Tse et al., 2003; Krampera et al., 2006] and dendtritic cell maturation [Zhang et al., 2004]. Due to the immunsuppressive properties, the use in clinical application is not sufficiently tested regarding the regeneration of tissue for example. The bivalent clones prevail in the MSC population and influence the quality [Petite et al., 2000]

1.6 Aim of the phd thesis

MSCs has development to useful clinical tools in therapeutical and regenerative application. The interest of these cells is still limited due to the lack of informations concerning the definition of MSC and his progeny.

Already, the inconsistent nomenclature of MSCs is confusing. The use of the term “mesenchymal stem cell” does not agree with the actual potential. At this, the self-renewal potential of MSC is insufficiently proven and experimentally just emerge to perform on single cell level. Additionally, many studies claim the cells as multipotent (Multipotent stroma cells) without demonstrating *in vivo* transplantations. Only a few publications fulfill the nomenclature of MSC in regard of their location and potential.

However, due to lacking knowledge of commitment and senescence events the identification of homogeneous multipotent progenitors is still problematic. Therefore, the strong heterogen populations of cells including MSC of different developmental stage, complicating the isolation and engraftment of cells in *in vivo* experiments.

Multiple studies claim that multipotent progenitors, similar to those found in the bone marrow, can also be isolated from a variety of other sources, including the synovial membrane, periosteum, and umbilical cord. While the multipotential nature of these cells is undisputed, their "stemness" is controversial. Therefore, the expression of stemness markers like POU5F1, NANOG, REX-1 etc are often used to define the undifferentiated state of MSCs. However, whether these markers are also markers for undifferentiation in adult postnatal tissue and possibly connected to multipotent differentiation capacity is not shown.

There are currently no markers, which can be used to definitively detect multipotent MSC. In comparison to the well-defined hematopoietic cell system, no culture conditions have been described, which can maintain multipotency over time. Therefore, because of the lack of knowledge about the relation between multi-, bi- and unipotent cells, directed differentiation of MSC is currently unlikely to give homogeneous cell populations useful for regenerative purposes.

However, a number of studies show multilineage differentiation *in vitro*, but only a few can really show *in vivo* functionality of MSC subpopulations. The differentiation behaviour of MSC *in vivo* has no relation to the chemically enhanced differentiation effects *in vitro*. Therefore, also the self-renewal or self-maintenance of MSC can only

be tested *in vivo*. Recent studies show first approached, but do not supply definitive evidence [Sacchetti et al., 2007].

Moreover, the MSC concept originated from studies conducted on bone marrow-derived stromal cells [Friedenstein et al., 1974; Owen et al., 1988; Pittenger et al., 1999; Caplan et al., 1991], suggested that committed and less-committed MSC may exist in a mesenchymal hierarchy [Minguell et al., 2001]. Therefore, the dissection of the mesenchymal system is an important component for understanding of the MSC biology, for identification of distinct subpopulations and for special application in tissue engineering.

However, independent of the problems described above also the homing of MSC to an appropriate growth environment is not sufficiently solved. It was not show until yet that cultured MSC do home in contrast to primary cells. Also, the clinical potential of MSC, as described in the chapter before, is limited due to the existence of different clones with different potential, which reduce engraftment capacity or multipotent *in vivo* differentiation.

Based on the multiple problems in the understanding of the MSC biology, in the present thesis only a few of the problems can be attended. Therefore, the main aim of this thesis is to define markers to identify uncommitted MSC. For this purpose, I first set out to validate “stemness” markers as possible markers of uncommitted MSC. In a second series of experiments, I have performed gene expression studies to define new surface markers which could be used to distinguish uncommitted from committed MSC.

1.6.1 Validation of “stemness” Markers to define uncommitted MSC

Due to the role of stem cell markers in ESC, their properties were often used for defined cells positive for these markers like POU5F1 as more undifferentiated and suggested a greater potential of these populations. However, the expression of POU5F1 in different postnatal tissues like bone marrow, which means his role outside of the development state and pluripotency propose to reconsider his function in the adult stem cell system. Zangrossi et al. and Lengner et al. demonstrated in their studies that POU5F1 does not sustain ESC state and is not necessary for self-renewal and maintenance in adult cells [Zangrossi et al., 2007; Lengner et al., 2007].

Due to the controversial studies of the expression of “stemness” markers in adult postnatal cells as well as the lack of marker for identification and isolation of multipotent MSCs, one aim of the present phd thesis is the study of similar populations of multipotent mesenchymal progenitors that are found across different tissues, and whether transcriptional regulators of the pluripotent state, expressed in pluripotent ESCs, are in fact expressed in postnatal mesenchymal progenitors, as claimed in several recent studies.

1.6.2 Identification of novel surface Markers to isolate uncommitted MSC

The distinction of undifferentiated markers and more differentiated progeny on an antibody-based identification is already shown in the hematopoietic system. The precise definition of cells allows dissecting different stages of the hematopoietic system [Shizuru et al., 2005]. Due to the lack of clear markers distinguish different stages in the MSC commitment, the study of differentiation of the mesenchymal mechanism has not given unequivocal answers.

An important criterion of uncommitted precursor in MSC is the detection of multipotency based on *in vivo* transplantation including the formation of “ossicles”, differentiation to multiple mesenchymal lineages, and establishment of the hematopoietic niche. These attributes were demonstrated only for STRO-1 [Gronthos et al., 2003], CD146 [Sacchetti et al., 2007] and CD200 [Delorme et al., 2008].

However, several studies have shown promising candidates for further isolation of distinct MSC population of different commitment stages or even show approaches to undifferentiated subsets using marker combinations [Battula et al., 2009; Battula et al., 2007].

Here, podocalyxin-like protein (PODXL) could represent a prospective marker for early MSC progenitors. PODXL is normally expressed in early hematopoietical development stages [Doyonnas et al., 2005; Furness et al., 2006] and vascular endothelium [Li et al., 2001; Hara et al., 1999], but was also detected in MSC of early passaged stage [Larson et al., 2008; Lee et al., 2008]. In contrast, Battula et al represented a marker for isolation of unipotent MSC like MSCA-1 [Battula et al., 2009] or CD63 (HOP-26) [Steward et al., 2003]. These markers can be used for identification and isolation of more committed MSC and further on used in combination to exclude more differentiated progenitors. A common strategy to obtain

MSC populations is achieved by removing other lineages from MSC preparation like hematopoietic cells (CD45 and GYPA cells) and endothelial cells (CD31).

As it is known that MSC comprise a mixture of different cell types of which only a minority is therapeutically relevant, the need for protocols for specific enrichment of therapeutic cells becomes obvious. However, clinically useful markers specifically identifying non-differentiated MSC from their differentiated progeny have not been described in sufficient detail. Additionally, the lack of knowledge about the relationship between multi-, bi- and unipotent cells, directed differentiation of MSC is currently unlikely to give homogeneous cell populations useful for regenerative purposes.

In this second part of the phd thesis, I want to distinguish between multipotent MSC from their unipotent osteogenic progenitors with an antibody-based system. Here, I compare the gene expression patterns of BM-MSC with UVSC. This refined analysis of membrane associated markers identified new candidates of potentially novel markers of multipotent MSC. Finally, in functional experiments to prospectively isolate these cells, the novel markers shall be defined as candidates for multipotent cells. As such, these observations may facilitate the study of cell fate determination in MSC as well as the development of more homogeneous cell populations for mesenchymal cell therapy.

Chapter 2: Results

2.1 Isolation and Characterisation of BM-MSC and UVSC

In this study, different types of putative mesenchymal progenitor cells were used: BM-MSC (bone marrow-derived mesenchymal stromal cells), and UVSC (umbilical vein-derived stromal cells). BM-MSCs represent the original prototype of MSC and were isolated and cultured as described in the literature [Sacchetti et al., 2007; Delorme et al., 2008]. UVSC, reported as putatively equivalent to BM-derived MSC, were taken as an example of non-BM-derived MSC and isolated and cultured as described by others [Romanov et al., 2003; Panepucci et al., 2004]. The stromal cells of umbilical vein were isolated based on modified protocol of Marin et al. [Marin et al, 2001]. The isolation of BM-MSC was performed following the method of Hayensworth et al. [Hayensworth et al., 1992]. After plastic adherence of the stromal cells, the MSC will show clonal growth within two weeks.

The isolation and cultivation procedure simultaneously is important for selection of the correct cells. Here the typical characteristics of MSCs like plastic adherence and their fibroblastic, spindle-shape appearance are essential for later study of the MSCs. These features together with clonal expansion will reveal a more homogeneous and proliferative population.

2.1.1 Isolation of BM-MSC and UVSC

The UVSC were isolated from umbilical vein in a two-step procedure. Thereby the vein was digested two times with 0.5 mg/ml collagenase. In the second flush, contain the subendothelial UVSC. The first medium change was performed after 2-3 days to allow plastic adherence and to remove interfering cells. At these blood cells were removed by washing, while endothelial cells will disappear after first splitting. In accordance of further cultivation period and for enrichment of the cell number the medium was changed every third day. At this, only half of existing medium was replaced with new medium.

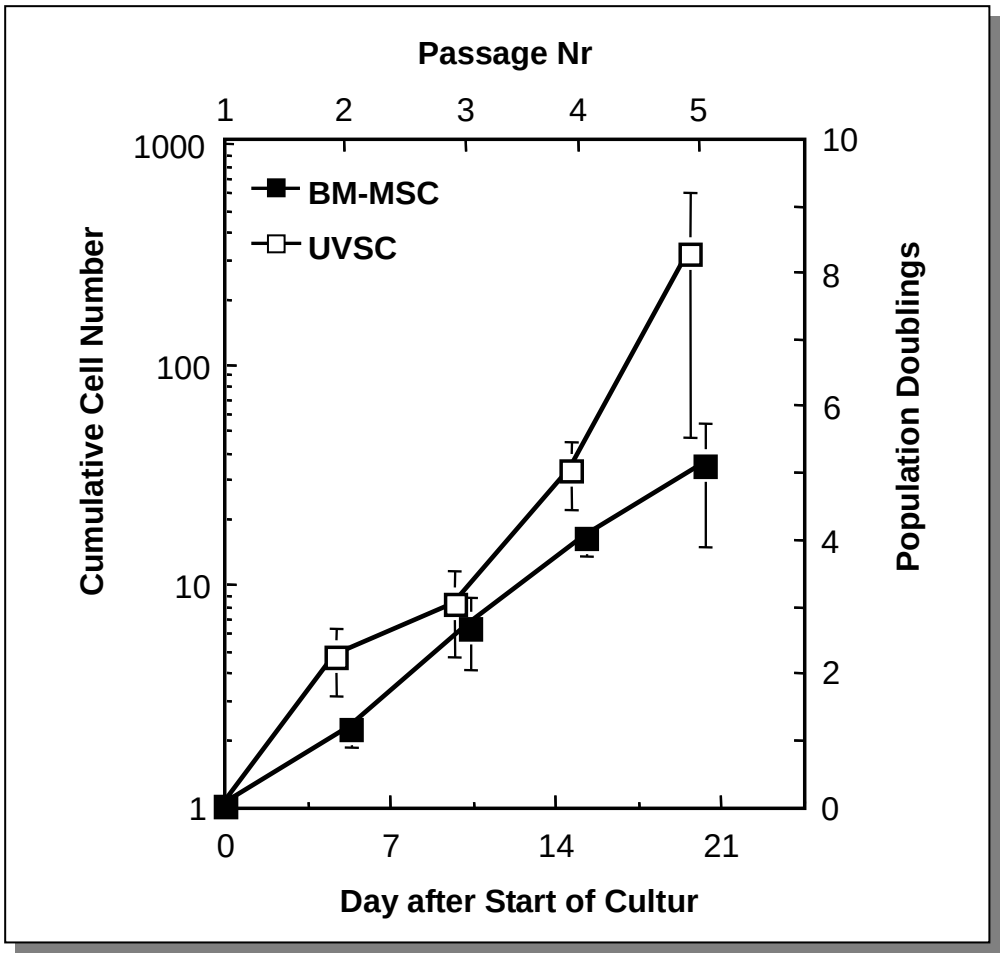


Figure 8: Growth kinetics of BM-MSC and UVSC

BM-MSC and UVSC were cultured as described in the Materials and Methods Section. The cumulative cell number in the first three weeks after subculture of CFU-F-derived cells is shown. Both BM-MSC and UVSC show similar growth kinetics.

Hence, the secreting factors of the MSCs themselves remain partly in culture and ensure better proliferation capacity and less mortality of the MSCs.

Primary cultures of fibroblastoid clones (CFU-F) were established at 0.1–10 x 10^3 cells per cm^2 (BM-MSC) or 0.5–20 x10^3 (UVSC). CFU-F-derived colonies were harvested using trypsin/EDTA, pooled to generate multiclonal strains, and replated for additional studies. In subcultures, UVSC were passaged at 50%–70% confluence. After 10 to 14 days colonies were formed. In further culture process, the cells were seeded at 5x10^3cell/cm^2.

Both BM-MSC and UVSC show a proportional growth curve whereas UVSC present a faster doubling time and more than 8 PD (population doublings) in 21 days of cultivation (Figure 8).

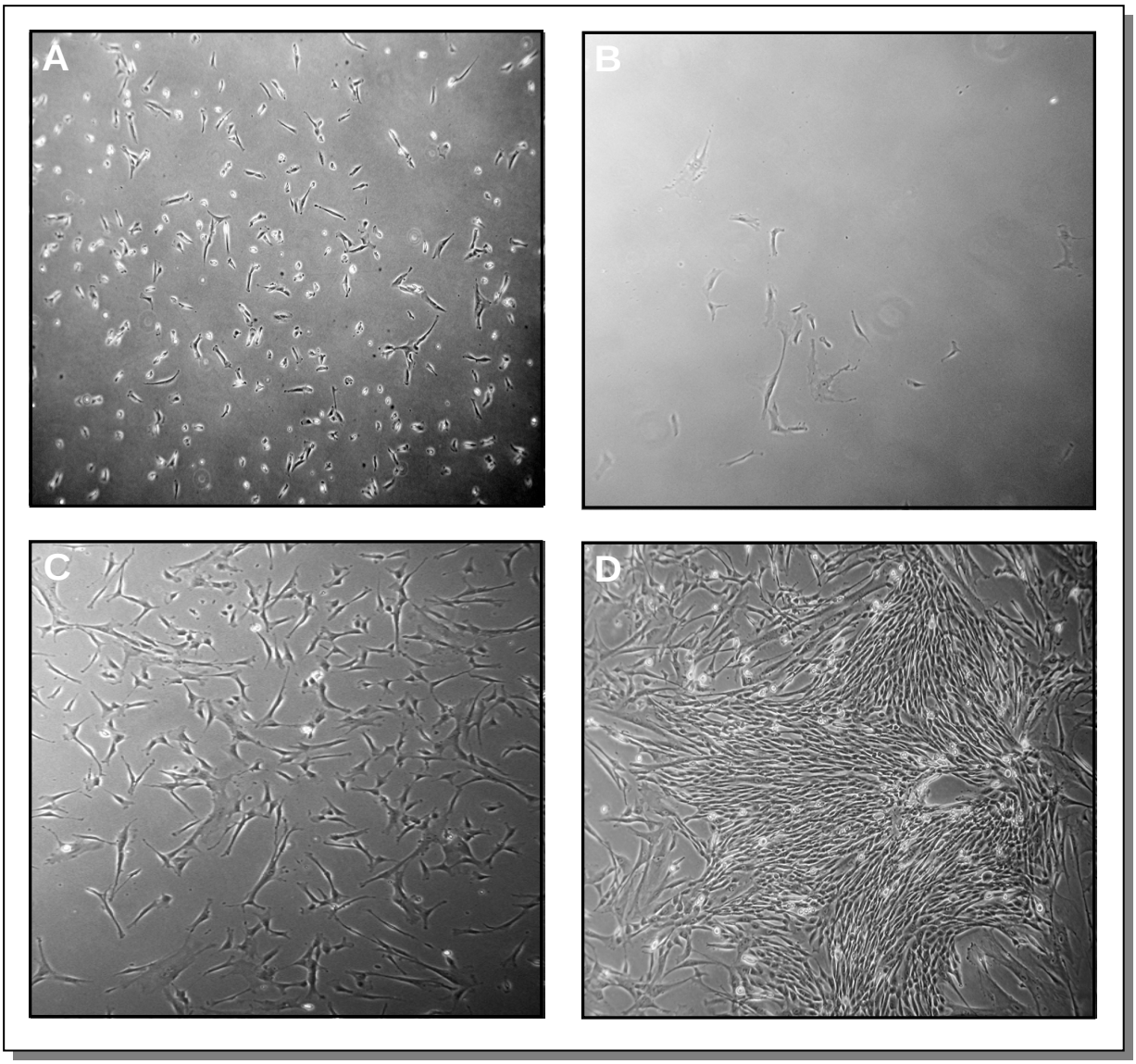


Figure 9: Morphology of UVSC

Primary isolated UVSC were culture at 0.5–20 x10^3 cells/cm^2. (A) Show small adherent UVSC P0 to P1 together with round, bright blood cells at day 2. (B) Day 4 interfering blood cells and endothelial cells are removed off culture and only the growing UVSC remain. (C) Day 8 expansions of the UVSC and alteration in cell morphology are observed in all cells. (D) Day 12 forming of distinct CFU-F of UVSC. Magnification: Picture A, B and C 10X and D 5X.

After passage, there is an initial lag phase in which the cells first get adherent to the plastic surface, which is followed by spreading into spindle-shaped fibroblastic cells (Figure 9 and 10). When the cells start to divide ,smaller round cells appear, which can be observed in both UVSC and BM-MSC (Figure 9 and 10).

Prior to passage, primary isolated BM-MSC and UVSC show also in the first days of adherence (1-3 day) floating residuals from the isolation involved hematopoietic cells and other tissue particle. These residuals will wash away after several media changes.

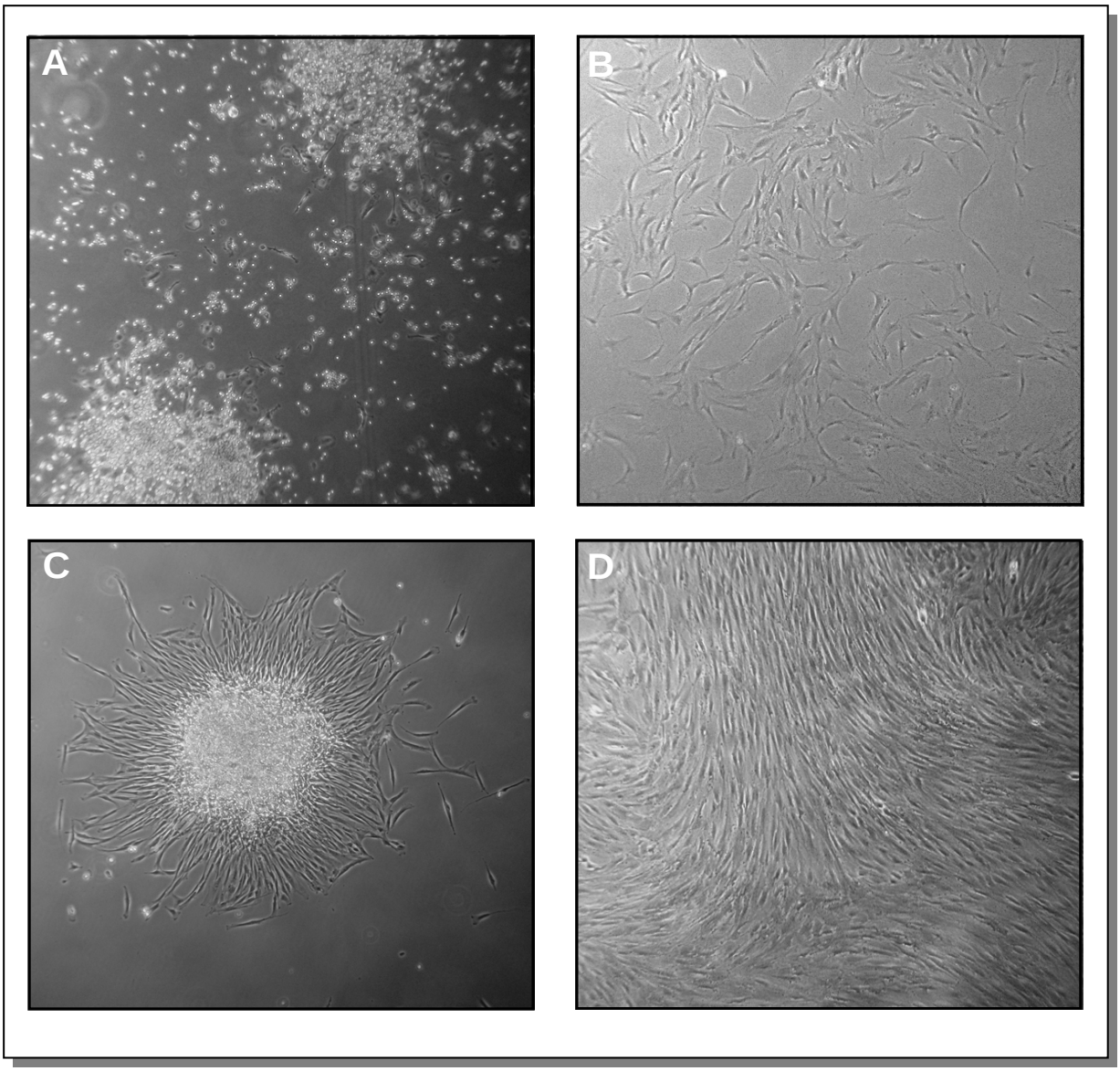


Figure 10: Morphology of BM-MSC

Isolation of BM-MSC via Ficoll gradient and direct cultivation show high percentage of remaining blood cells (A) day 4 shows distribution and high proliferation capacity of the BM-MSC (B) Day 12 CFU-F with proliferation centre are spreading over culture plate (C). Cultivation of the BM-MSC over 15-21 days results in huge CFU-F that growing together and forming monolayer (D). Magnification: Picture A,B and D in 10X B and C 5X.

At days 4 to 15 (Figure 9 and 10 picture B, C and D) CFU-F are slowly becoming discernible in BM-MSC and UVSC. The clonal expansion of the single CFU-F continues growing to monolayers (day 15-18). The average number of CFU-F counted 14 days after passaging range between 3 CFU-F per 100 cells in UVSC and 1-2 CFU-Fs per 100 cells in BM-MSC. The number of CFU-F can vary because of culture media and supplements (like growth factors, FCS-content) and donor age.

The cultivation of UVSC and BM-MSC shows phenotypical heterogeneity within the same culture. At the beginning of the culture period small round cells and the typical trapezoid cell shape are found (Figure 9 and 10A). Within the culture process appear

a more flattened, broader cell types with increased cytoplasmic volumina (Figure 9 and 10 B/C). The CFU-F between BM-MSC and UVSC are also different. Stromal cells of the umbilical vein show CFU-F with hundreds of cells and, in contrast, also colonies with only few cells of cuboidal shape (Figure 9 C/D and Figure 11). Taken together, my results demonstrate that the formation of colonies is not a uniform process and that several different cell types form colonies. Although growth kinetics of BM-MSC and UVSC are similar, the colonies from the bone marrow show a more homogeneous cell type. The phenotypical alterations in UVSC represent a heterogeneous population with different cells in the same culture. These observations are similar to Mets et al. for BM-MSC [Mets et al., 1981].

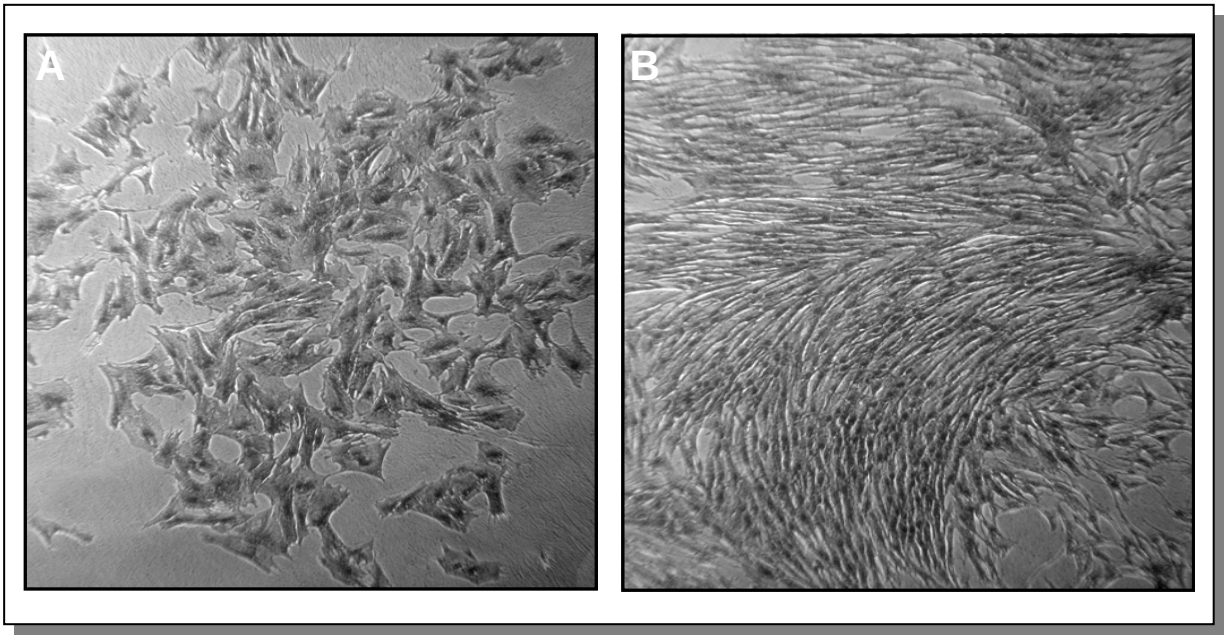


Figure 11: Heterogeneity in UVSC

Several cell types are found in UVSC cell culture. Typical spindle–shape fibroblastic cells are found in CFU-F (B). In same culture also are flattened,as well as cuboidal cells with large nuclei (A) demonstrated. Magnification picture A in 20X and B in 10X.

2.1.2 FACS-Analysis of BM-MSC and UVSC

The flow cytometric analysis is used to get information about the quality of the mesenchymal culture in respect of homogeneity and expression of characteristic surface molecules. The different culture conditions in various laboratories make it difficult to directly compare results between laboratories. Since culture conditions determine regulation of genes, the differences in culture conditions can lead to alterations in the surface expression of some of these surface molecules on stromal cells. Thus, the BM-MSC and UVSC used in this study were studied for expression of phenotypic markers in culture.

Both stromal cell types were cultured until 60 - 70 % confluence and harvested using trypsin digestion as described in chapter 5.1.4 and 5.1.7 in “Material and Methods”. The stromal cells were single stained with various antibodies (Figure 12) and supplemented with Propidiumiodid for excluding the apototic cells and dead cell debris from the viable cells, as described in “Materials and Methods”.

The results of the FACS-Analysis are represented in histogram plots (Figure 12). Each plot shows the antigen expression of a certain antibody with the corresponding isotype control in BM-MSC and UVSC.

Cells were stained with antibodies against the antigens indicated (see chapter 5.1.7). The antigens were divided into different categories: a. “classical” MSC antigens, hematopoietic and endothelial antigens, b. integrins and their ligands, c. pericyte markers miscellaneous molecules d. growth factor receptors.

Human MSCs are characterized as non-hematopoietic (CD34-, CD45-) and nonendothelial (CD31-) and molecules used by many investigators as “classical” MSC markers: SH2/CD105+, SH3/CD73+, ALCAM/CD166+ and CD90+ [Barry et al 2004; Pittenger et al, 1999].

All markers above were expressed as expected in both BM-MSC and UVSC like CD105 (also known as endoglin). This surface molecule is a component of the TGF-beta 3 complex. Furthermore, the expression of CD105 as well as CD90, a member of the glycol family, and CD73, an ecto-5-prime nucleotidase also known as NT5E, are described as good MSC markers.

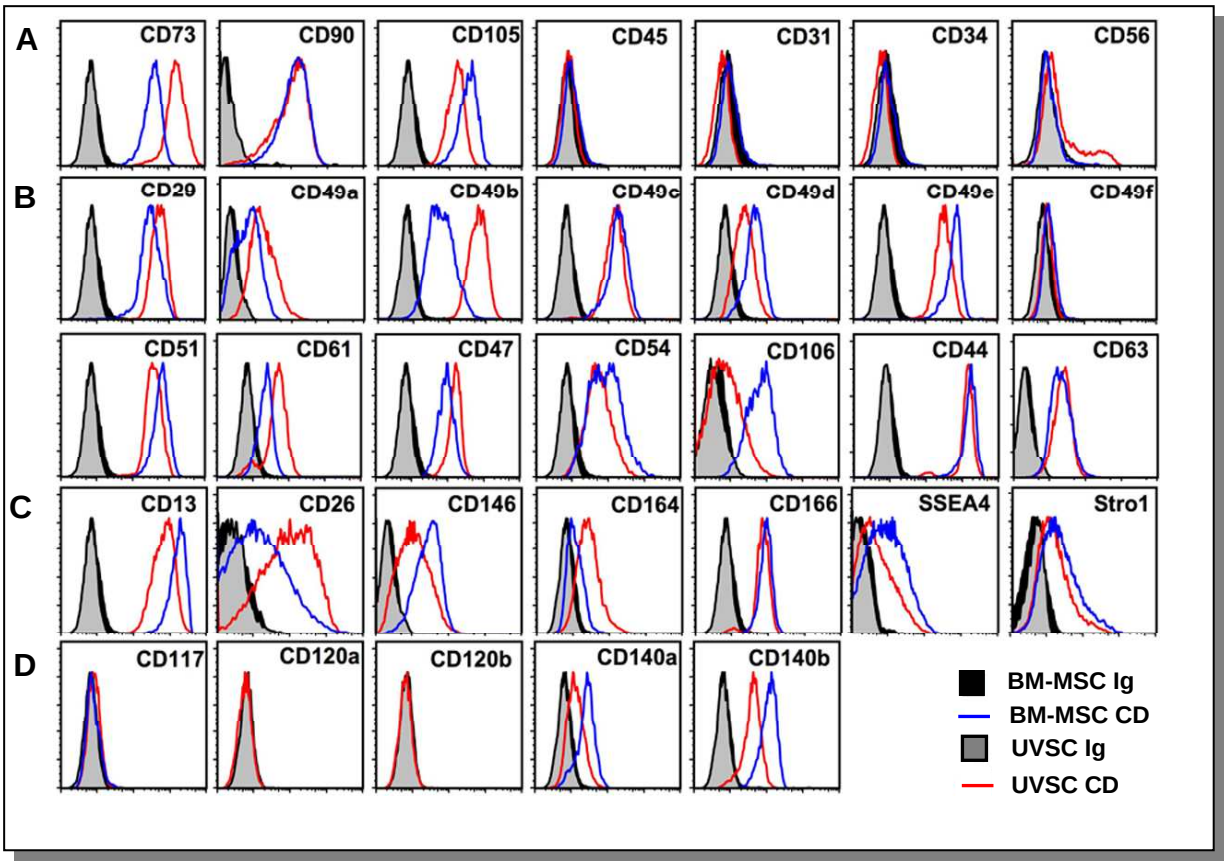


Figure 12: Flow Cytometric analysis of BM-MSC and UVSC

Cells were cultured until 70% confluency and harvested using trypsin digestion. BM-MSC and UVSC were stained with antibodies against the isotype controls and antigens indicated (Ig and CD see Material and Methods). The antigens were divided into different categories: (A) "classical" MSC antigens, hematopoietic and endothelial antigens, (B) integrins and their ligands, (C) pericyte markers, and (D) growth factor receptors.

Molecules of the integrin family are known to be associated with MSC expression and were already studied in freshly unexpanded BM-MSC like CD49f (ITGA6) and CD49a (ITGA1) in Boiret et al. [Boiret, et al., 2005]. Also in this study these markers represent a strong expression in both BM-MSC and UVSC.

The expression in STRO-1 and CD146 (or MCAM) can also be demonstrated here. Thus, STRO-1 and CD146 are markers for bona-fide bone-forming cells *in vivo* [Gronthos, et al., 2003; Sacchetti, et al., 2007].

This analysis confirms the expression of CD166 or ALCAM. The marker is in association with STRO-1 and CD49a described as potent candidates for isolation of primitive human bone marrow stromal cells and their more differentiated progenitors [Steward et al., 2003]. The FACS analysis shows that BM-MSC and UVSC express next to CD146 an further pericyte marker, endosialin (TEM-1 or CD248). The latter marker is detected on stromal fibroblasts during angiogenesis during embryonic and tumor development [Bagley, et al., 2008; MacFadyen, et al., 2005].

CD140a and CD140b are antigens of growth factors expressed in MSCs of both presented cell types here. These two molecules are known as PDGFR A/B and belong to the tyrosin kinase receptors of the platelet-derived growth factor family.

Schwab et al. documented the co-expression of the perivascular markers CD146 and PDGF-RB in a small population of endometrial stromal cells [Schwab et al., 2007]. CD140a is often observed in association with tumor malignance and described as an defined phenotyp of MSC with the ability to differentiate into cardiomyocytes, endothelial cells, pericytes and smooth muscle cells [Gojo et al., 2003].

The BM-MSC and UVSC used in this study, show the expression of multiple phenotypical MSC markers. The flowcytometric profiles did not reveal great differences in surface marker expression between UVSC and BM-MSC.

2.1.3 Multipotent Differentiation Capacity of Multipotent Stromal Cells

An essential characteristic of mesenchymal stromal cells is their ability to differentiate into adipogenic, osteogenic and chondrogenic (AOC) mesenchymal lineages [Friedenstein et al., 1982; Pittenger et al., 1999]. MSC have therefore the ability to differentiate in at least in three lineages. In addition, differentiation studies of MSCs showed further capacities under appropriate conditions like the differentiation into tenocytes, smooth and skeletal myocytes, neurons and visceral mesoderm [Pittenger et al., 1999; Woodbury et al., 2000; Reyes et al, 2001].

In this study standard protocols were performed for adipogenic [Pittenger et al., 1999], osteogenic [Jaiswal et al., 1997] and chondrogenic differentiation [Johnstone et al.,1998].

The adipogenic induction was performed in use of dexamethasone and 3-isobutyl-1-methylxanthine (IBMX). After 3 weeks, the cells form fatty vesicles of different size. These vesicles are distributed over the cell as perinuclear accumulations of fat vacuoles with different size (Figure 13C) . The staining with fat-soluble lysochrome diazo dye Oil Red O marks these cells as adipogenic by colouring the charactersistic adipogenic lipid vesicles in red (Figure 13C).

The induction of osteogenic differentiation was performed with Ascorbinacid-2-phosphat, β-Glycerolphsphat and dexamethasone. The composition is used as standart for osteogenic differentiation of monolayer cultures. After three weeks brownish, densed areas appeared indicating calcium accumulation (Figure 13B). For detection of calcium deposits, the von Kossa staining was used. At this, the calcium deposits were replaced by reduction of silver nitrate. Based on this process the mineralized cells are visualized in black. An alternative method is the detection of alkaline phosphatase using specific antibodies and reagents for visualization (see 5.2.4). Here the osteogenic differentiated cells appeared in red colouration. Adipogenic as well osteogenic differentiation was represented in BM-MSC and UVSC (Figure 14 B/C).

Chondrogenic differentiation was performed in high-density micropellet culture, as described in the Materials and Methods. The differentiation was induced using either TGF-β3 or BMP-2. In our experience, TGF- β3 gave the most complete chondrogenic differentiation, as was determined by, histochemically staining of micropellet tissue sections with Safrain O (Figure 13D). Safranin O is a cationic dye which is binding on the anionic groups of proteoglycans and glycosaminoglycans and not to collagen [Rosenberg et al., 1971]. The staining appear in a yellow red colour. Both BM-MSC and UVSC formed micropellets. But, chondrogenic differentiation was detected only in BM-MSC in this study. In addition PCR analysis was performed for detection of

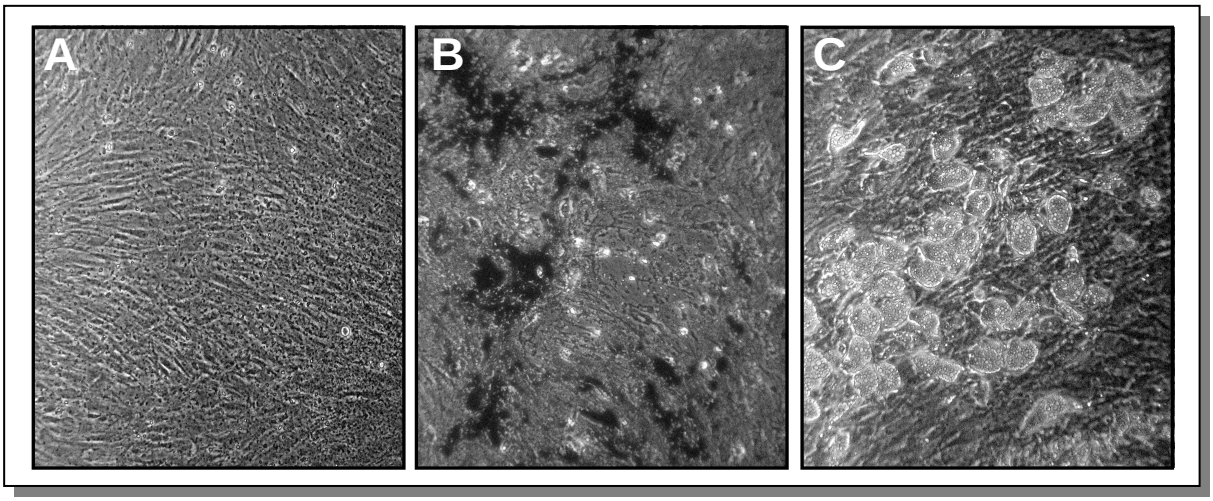


Figure 13: Morphological study of differentiated BM-MSC:

Osteogenic (B) and adipogenic (C) differentiated BM-MSC are compared to undifferentiated (A). Cells were grown to monolayer culture and induced with differentiation media described in Material and methods. The osteogenic differentiated MSCs appear cubiodial and show brownish (black) surface indicating mineralization. Adipogenic differentiated cells (C) form intracellular fatty vesicles. Magnification of picture (A), (B), and (C) 20x.

chondrogenic markers Aggrecan (ACAN), Collagen 2A1 (Col2A1) in differentiated BM-MSC and UVSC. However, the expression analysis showed, consistent with the histochemical evaluation, a detection of the typical chondrogenic markers only in BM-MSC but not in UVSC (Figure 14C).

The results demonstrate that AOC differentiation is more pronounced in BM-MSC in comparison to UVSC when exposed to conventional media. The detection of the three typical mesenchymal lineages marks the BM-MSC in vitro as multipotent.

The in vitro experiments above suggested that, although the two populations are very similar with respect to surface marker expression, UVSC may not display the same AOC differentiation ability as BM-MSC.

For evaluation of the stem cell behavior, multipotent MSCs as an in vivo model was used which allows multipotent osteoprogenitor cells to form adipocytes, osteoblasts, and osteocytes. In addition, in this model, murine hematopoietic cells and endothelial cells are recruited to form bone marrow [Sacchetti et al., 2007; Mankani et al., 2007].

Transplantation studies were performed in cooperation with GENOSTEM partners. Prior to transplantation, BM-MSC and UVSC were expanded as described in Bianco et al., Delorme et al. and Sacchetti et al. [Bianco et al., 2006; Delorme et al., 2008; Sacchetti et al., 2007]. Cultured BM-MSC and UVSC were attached to hydroxyapatite/tricalcium phosphate particles (see chapter 5.5 in Material and Methods) and transplanted subcutanously in BNX mice. For BM-MSC, cells from three different donors (ages 35–50 years) were used to generate 12 transplants in six mice. For UVSC, cells from three donors were used to generate 14 transplants in seven mice. Eight weeks after transplantation heterotopic ossicles were isolated and immunohistologically analyzed. The structures formed from the transplant, called ossicles which were removed and the presence of mesenchymal tissue assessed. The experiments showed that BM-MSC form all mesenchymal lineages expected (Figure 15). In addition, as previously reported in Sacchetti et al. [Sacchetti et al., 2007], BM-MSC-assisted ossicles recruit hematopoietic cells to form bona fide bone marrow.

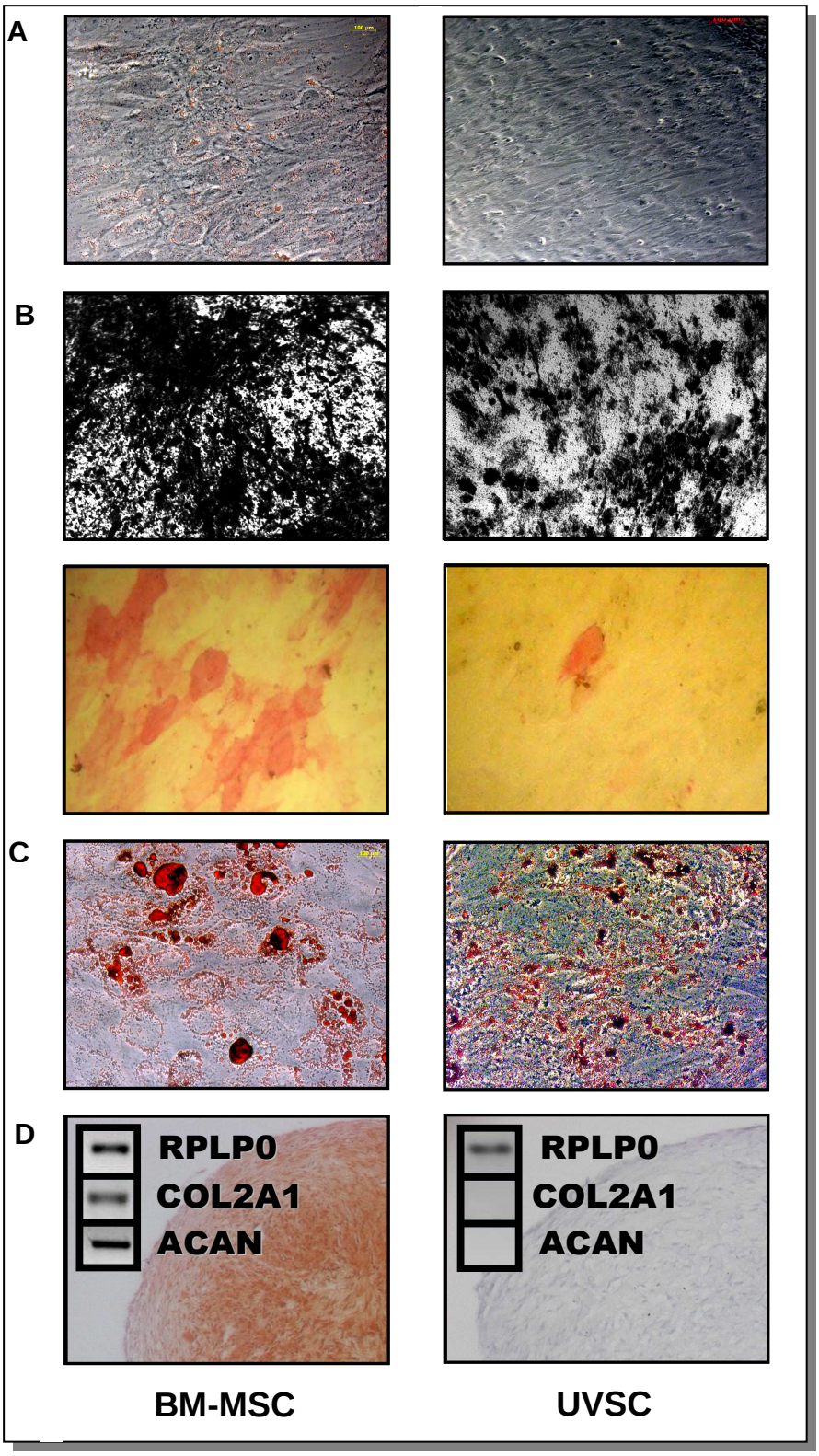


Figure 14: Differentiation of unfractionated BM-MSC and UVSC

Shown are undifferentiated (A) and differentiated BM-MSC and UVSC in osteogenic (B), adipogenic (C) and chondrogenic (D) lineage. (B) Osteogenic differentiation is detected using von Kossa staining (black staining) and Alkaline Phosphatase (red stained cells). (C) Adipogenic induced cells show fatty vesicles that were stained with Oil red O. (D) Chondrogenic differentiation was detected by Safarin O staining. Additionally PCR analysis with known chondrogenic markers were performed (D). Magnification 20x

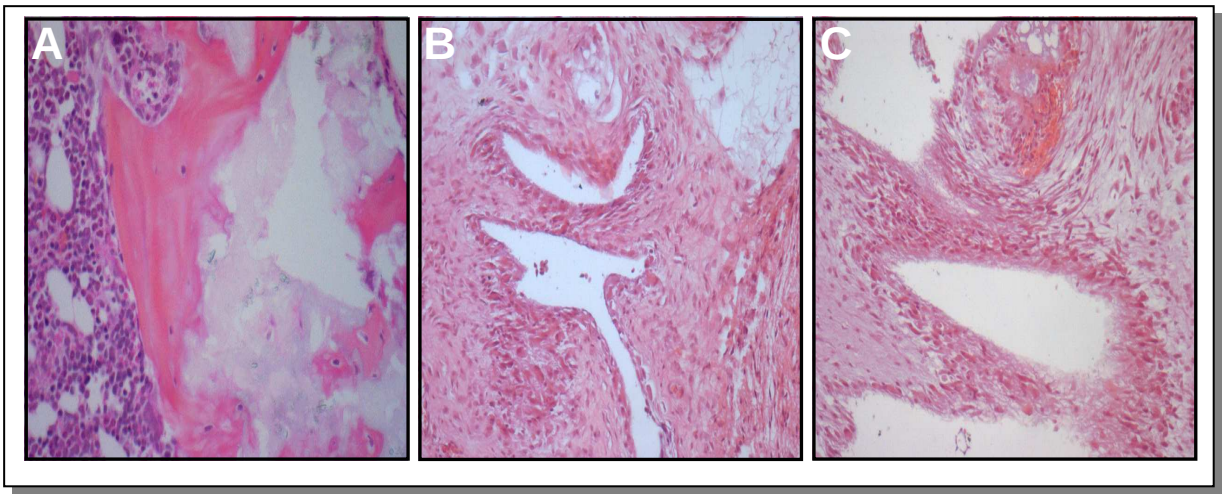


Figure 15: In vivo transplantation of BM-MSC and UVSC.

Cultured BM-MSC and UVSC were attached to HA/TCP particles (see 5.5) and transplanted subcutanously in BNX mice. 8 weeks after transplantation “ossicles” were isolated and immunohistologically analysed. Differentiation into mesenchymal lineages is only shown in BM-MSC (A). To see are marrow (purple) with fatty vacuoles (white spots) and adjected bone (reddish) (A). UVSC form mainly vascular-like structures (B, C).

In contrast, and in line with the above in vitro findings, none of the UVSC preparations showed osteogenic engraftment *in vivo* (Figure 15). Thus, despite similar or sometimes even higher levels of “stemness” transcripts in UVSC, only BM-MSC contains multipotent cells with stem cell-like repopulating properties *in vivo*.

2.2 “Stemness” Genes as Markers for the undifferentiated Cell State

The expression “stemness” markers like POU5F1, NANOG, STELLA-related etc. is often used for description of the undifferentiated cell state in the literature. However, studies have shown that these genes are also expressed in adult cells and therefore outside of the developmental process and pluripotency as demonstrated for ESC [Zangrossi et al., 2007]. Furthermore, several pseudogenes were identified in these markers like six for POU5F1, 10 for NANOG, and 16 for STELLA [Pain et al., 2005]. Subsequently, this studies challenge the statement whether transcriptional regulators of the pluripotent state, expressed in pluripotent ESCs, are in fact expressed in postnatal mesenchymal progenitors and whether the expression of the markers do predict stem cell activity. Due to the lowest number of pseudogenes for POU5F1 as well as a known gene for the regulation of the pluripotent state in ESC and additionally often used in many publications for definition of the undifferentiated state, POU5F1 is used as a model “stemness” gene.

In the present study, I have used two different sources of mesenchymal progenitor cells: BM-MSC and UVSC. UVSC were isolated as subendothelial umbilical vein cells in a two-phase collagenase treatment. Characterization of the BM-MSC used in this study has been described in detail elsewhere [Delorme et al., 2008]. UVSC have been shown to be multipotent, and are considered an alternative for bone marrow derived MSC [Romanov et al., 2003; Panepucci et al., 2004; Sarugaser et al., 2005]. In our hands, in detailed flowcytometric profiles, UVSC (Figure 12) are very similar to BM-MSC.

2.2.1 Expression of “stemness” Markers in BM-MSC and UVSC

The genes involved in stem cell properties of mesenchymal stromal cells have to date not been defined. Several authors have suggested that the so-called “stemness” factors, might be involved in stem cell-like behavior of BM-MSC [Pochampally et al., 2004; Jiang et al., 2002; Tondreau et al., 2005].

Moreover, some studies also reported the expression of embryonic stem cell marker POU5F1 in cells of the umbilical cord [Carlin et al., 2006; La Roca et al., 2008].

The "stemness" profile in ES cells is still elusive. But, a transcriptional network governing self-renewal and pluripotency involving POU5F1, NANOG, SOX2, and their up- and downstream partners is emerging [Ivanova et al., 2006]. Considering the differences between BM-MSC and UVSC in *in- vivo* stem cell-like behavior, I speculate whether this difference was correlated to differences in expression of “stemness” markers.

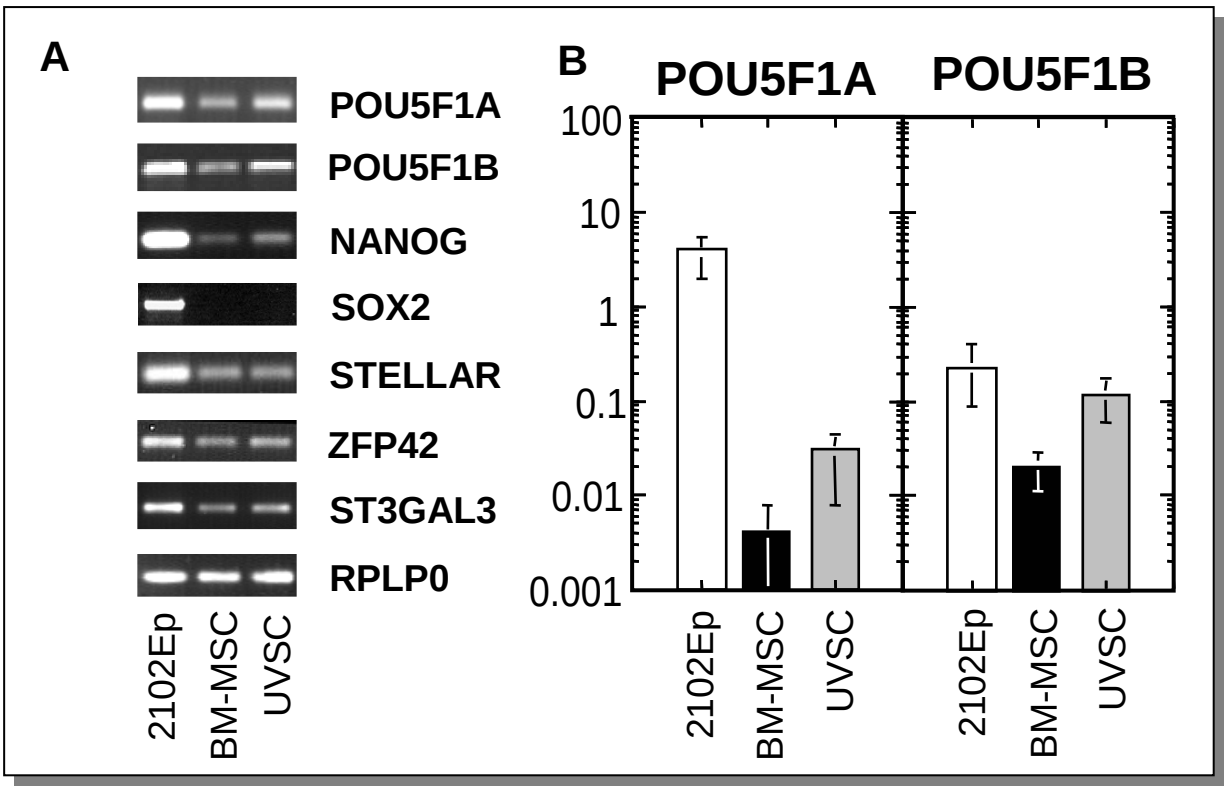


Figure 16: Expression of „stemness“ markers in BM-MSC and UVSC

In (A) PCR analysis were performed for detection different “stemness” transcripts in 2102Ep embryonic carcinoma cells, BM-MSC, and UVSC. POU5F1A and POU5F1B transcripts were quantitative determined by Real time PCR analysis (B) in normalization to RPLP0 housekeeping gene. 2102Ep embryonic carcinoma cells were used as positive controls.

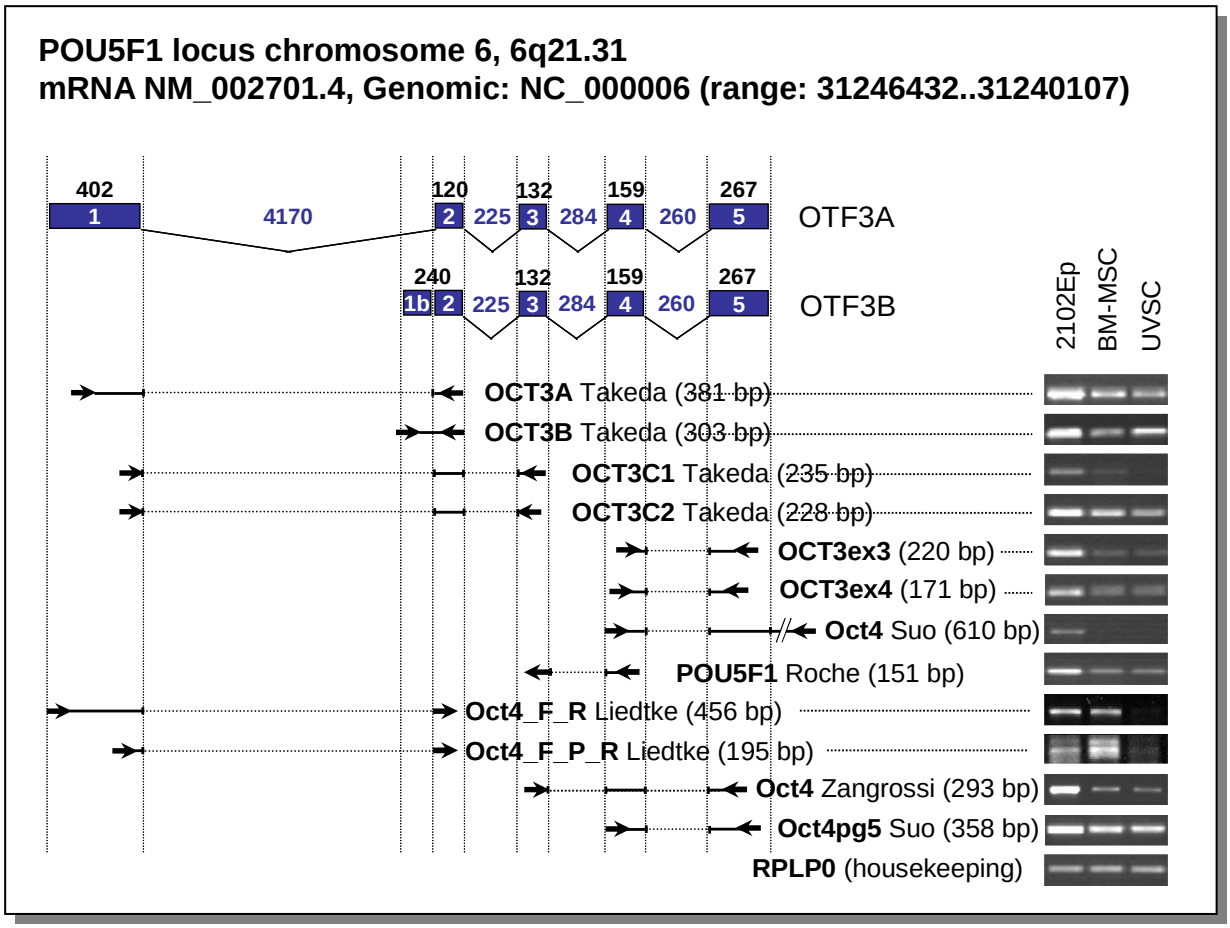


Figure 17: Amplification of POU5F1 transcripts and localization of primers in the POU5F1 locus

The primer binding sites for all POU5F1 primers used in this study are shown. The demonstrated primers span almost over the entire POU5F1 locus by ligating at different Exons. Primer of the two isoforms (OCT3A/B or POU5F1A/B) and pseudogene (OTF1/2) are the only primers binding at Exon 1 to Exon 2. Further primers for Exon 3 and 4 [all published by Takeda et al., 1992], OCT4, POU5F1 pseudogene 5 as well as primers of other publications ligate at Exon3 and 4 or Exon 4 and 5.The reverse primer of the Oct4pg5 pseudogene maps outside the coding region of POU5F1 [Liedtke et al., 2007]. The primer pairs described by [Roche et al., 2007] do not distinguish POU5F1 from its pseudogenes [Zangrossi et al., 2007]. Apparently, the OCT4 primers from Suo and coworkers, are the only ones distinguishing true POU5F1 from pseudogenes.

In particular, I was interested in the expression of POU5F1. RT-PCR experiments show, that BM-MSC are remarkably similar to UVSC in their expression of POU5F1, NANOG, STELLAR, ZFP42 (REX1), and the SSEA-4 synthase ST3GAL3 [Saito et al., 2003] (Figure 16). As a positive control, I used the pluripotent 2102Ep embryonic carcinoma cells [Andrews et al., 1982].

When human POU5F1 was first described, two transcript variants were presented: OTF3A and OTF3B [Takeda et al., 1992].

The B isoform, is formed through an alternative starting point of transcription in the intron between exons 1 and 2. Though both isoforms contain both parts of the DNA-binding POU-domain, the protein product of OTF3B, POU5F1B, lacks the N-terminal proline-rich transactivation domain of POU5F1 which is encoded in exon 1 (Figure

17, [Takeda et al., 1992]). Using reported POU5F1 primer pairs I amplified PCR products from both BM-MSC and UVSC (Figure 16 and 17 for localization of primers in the POU5F1 locus). In further studies quantitative Real-Time analysis were performed using the same primer pairs. The results showed that both POU5F1 transcript variants 1 (OTF3A) and 2 (OTF3B) are expressed by the embryonic carcinoma cells 2102Ep, BM-MSC and UVSC (Figure 16 A/B).

The experiments revealed that variant 1 (POU5F1A) is expressed at 970-fold higher in ECC in comparison to BM-MSC 205-fold higher level than UVSC (Figure 16).

In contrast, variant 2 (POU5F1B) is expressed in 34-fold lower level in comparison to variant 1 in 2102Ep cells. However, in UVSC and BM-MSC the OTF3B variant was expressed at a consistently higher level than OTF3A. On the contrary, in UVSC and BM-MSC, OTF3B was expressed at a consistently higher level than OTF3A (5.7- and 4.8-fold, Figure 16).

2.2.2 Analysis of the POU5F1 Transcripts

Takeda and coworkers did not only predict two transcription variants, they also described the POU5F1P1 pseudogene (OTF3C, [Takeda et al., 1992]). Since then, it has become clear that the “stemness” genes POU5F1, NANOG and STELLAR all have a number known pseudogenes [Pain et al., 2005]. The identity of POU5F1 transcripts were analysed in more detail. Different published primer pairs used for identification of POU5F1 and its pseudogenes were analysed for detection in BM-MSC, UVSC and ECC. In Figure 17 the results are demonstrated together with their binding in the POU5F1 gene.

Therefore, I looked more closely at the identity of the POU5F1 transcripts. As described above, POU5F1B lacks most of the N-terminal transactivation domain. Most known pseudogenes also lack this exon, and some only show similarity with the POU5F1 locus outside the POU5F1 coding region [Pain et al., 2005] (see Figure 17 and also Appendix Table1 and 2). Since most of the published primer pairs [Suo et al., 2005 and Roche et al., 2007] do not distinguish between the POU5F1 and its pseudogenes, I decided to determine the sequence of the PCR products from the POU5F1A and POU5F1B primer pairs [Takeda et al., 1992].

A **Sequencing of the PCR products from BM-MSC, UVSC, and Ep2102**

Cell type		Number of clones	%		Cell type		Number of clones	%
BM-MSC	**POU5F1**	3	11		**UVSC**	**POU5F1**	1	5
BM-MSC	**POU5F1P1**	7	26		**UVSC**	**POU5F1P1**	7	33
BM-MSC	**POU5F1P3**	10	37		**UVSC**	**POU5F1P3**	8	38
BM-MSC	**POU5F1P4**	7	26		**UVSC**	**POU5F1P4**	5	24
	Total	**27**	**100**			**Total**	**21**	**100**

Cell type		Number of clones	%
2102Ep	**POU5F1**	14	100
2102Ep	**POU5F1P1**	0	0
2102Ep	**POU5F1P3**	0	0
2102Ep	**POU5F1P4**	0	0
	Total	**14**	**100**

B **Summary of PCR products sequencing**

POU5F1	Chromosomal Location	Gene ID	Gene Bank Accession mRNA	Identity with POU5F1 exon 1
-	**6q21.31**	5460	NM_002701.4	381/381
P1	**8q24.21**	5462	NR_002304.1	371/381
P2	**8q22.2**	100009665	inferred	none
P3	**12p13.31**	642559	AF268617.1	371/381
P4	**1q22**	645682	AF268613	367/381
P5	**10q21.3**	100009667	Inferred	None
P6	**3q21.3**	100009668	Inferred	None
P7	**3q11.2**	100009669	Inferred	None
P8	**17q25**	100009670	inferred	None

Table 3: Sequencing of PCR products

(A) PCR-products shows the presence of POU5F1 in 2102Ep cells and pseudogenes in BM-MSC and UVSC. The different PCR products of POU5F1 and his pseudogenes are summarized in (B). Detailed analysis and alignments are given in Appendix Table1-3. 2102Ep were used as positive control.
Pseudogenes P5, P6, and P7 start their homology at the end of exon 2 of POU5F1
Pseudogenes P2 and P8 share their homology with POU5F1 outside its coding region

For this purpose, 62 POU5F1A PCR products were isolated from the electrophoresis gel, cloned and finally sequenced as described in chapter 5.3.6-5.3.8. From the 21 UVSC-derived clones, I found that one clone (1/25 is about 5% of the total) represented the parent POU5F1 gene.

The other clones represented the pseudogenes, with roughly equal distribution amongst P1, P3 and P4 (Table3 and in Appendix Table1 and Table2 and Figure 1). In the 27 clones derived from BM-MSC, three (11% of the total) represented the parent POU5F1 gene (Table3). Again, the remaining sequences were equally divided over P1, P3, and P4 POU5F1 pseudogenes. In contrast, the 14 POU5F1A amplicon clones from the ECC line 2102Ep all corresponded to the parent POU5F1 gene (Table3). Sequencing of the POU5F1B amplification products showed consistent amplification of products with identity ranging from 98 to 100% with POU5F1 transcript variant 2. In the OTF3B-like sequence both deletions and insertions were found, regardless of the cell source (Alignments in Appendix Table2).

Since transcriptional variants of POU5F1 pseudogenes have, to date, not been described, I could, unfortunately, not determine if, and when, to which pseudogenes our POU5F1B amplicons might map.

The results so far demonstrate that both splice variants were expressed in BM-MSC, UVSC and 2102Ep. However, the transcript variant1 shows in comparison to variant2 low expression level and more importantly reveal also the expression of pseudogenes in POU5F1A PCR clones.The analysis presents further on that most of the available primers used for identification of POU5F1 transcripts in adult stem cells, mainly detect its pseudogenes. In conclusion, the detection of POU5F1 will not predict the expression of the real OCT4 gene.

2.2.3 Detection of POU5F1 Protein

The sequencing results suggested that the majority of the putative functional POU5F1A transcripts from BM-MSC and UVSC corresponded to pseudogene transcripts. Thus, transcript level of POU5F1 might not necessarily correlate to the level of functional protein. To find out whether the POU5F1 protein was expressed by BM-MSC and UVSC, Western blot analyses were performed with monoclonal as well as polyclonal antibodies on 50 µg of nuclear or cytoplasmic protein fractions. For these analyses, I used antibodies raised to the N-terminal sequence of POU5F1 corresponding to Exon 1, which recognize the POU5F1A isoform, but not POU5F1B. In these experiments, I detected a protein corresponding to the expected size for POU5F1 in UVSC and BM-MSC (50 kDa, Figure 18A). This protein band was present at a level of 34% and 20% of that of the ECC cells, whereas there was no band present above the background levels in UVSC and BM-MSC respectively (Figure 18 A/B). In protein databases, putative sequences for protein products of POU5F1P3 (20 kDa) and POU5F1P4 (32 kDa) can be found (Appendix Table3A/B). Although these proteins contain N-terminal domains similar to that of POU5F1, such proteins were not detected.

Since the POU5F1 concentration was low or undetectable in these experiments, I wondered whether I could enrich for POU5F1 from nuclear extracts and cytoplasmic fractions of BM-MSC and UVSC.

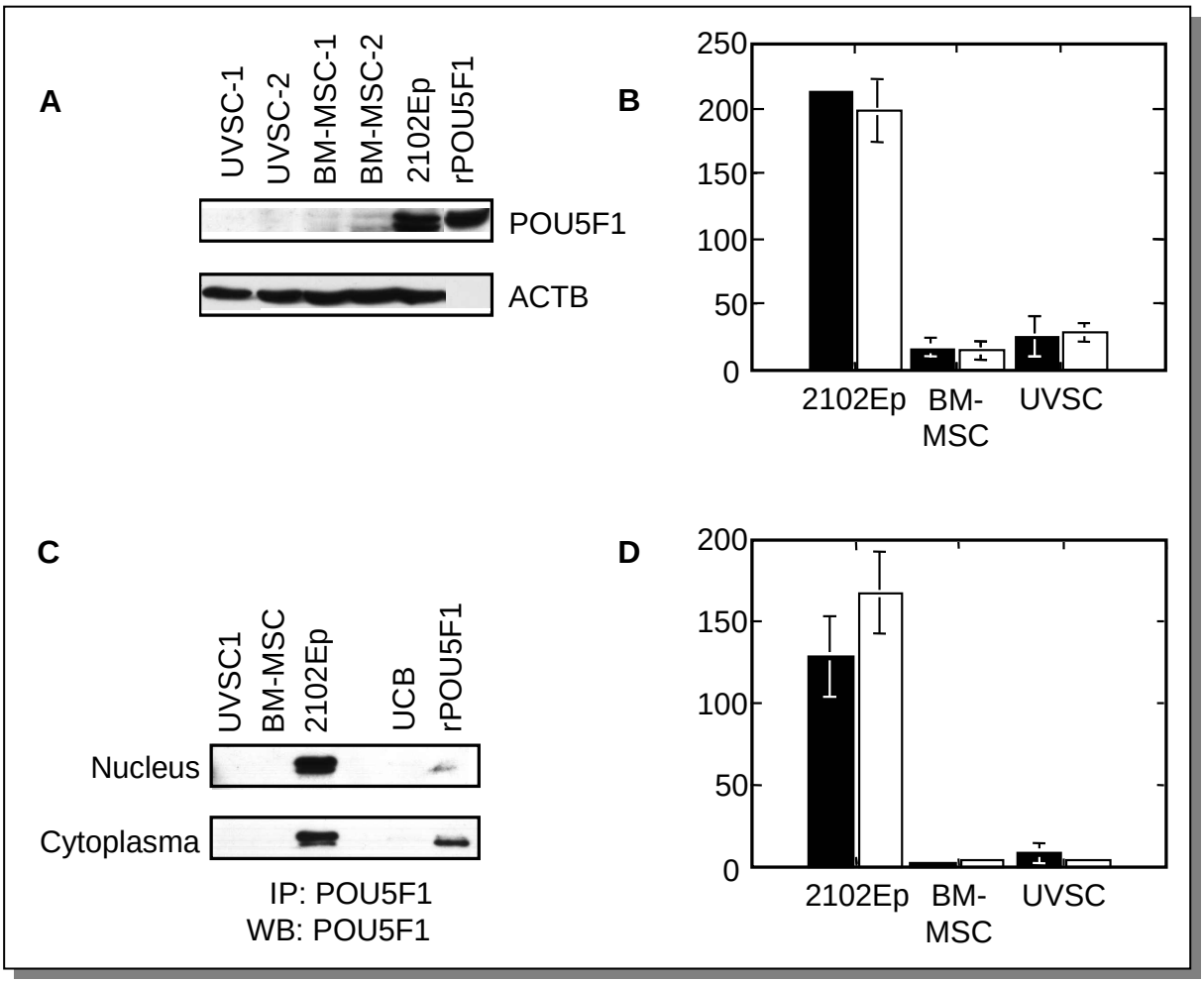


Figure 18: Expression of POU5F1 protein in BM-MSC, UVSC, and 2102Ep cells

Detection of the nuclear and cytoplasmatic POU5F1-protein was performed by Western Blot (A). The pixel quantitation of the protein band was examined in (B). Normalization of the Western Blots was performed relative to β-actin. For enrichment of POU5F1 protein of cytoplasmatic and nuclear fractions, IP (C) was performed, and pixels were quantitated as described following (D). In (D), no normalization was performed, and quantitation is shown as absolute pixels minus background for each sample (n=3-5 per sample), Solid bars represent WB of cytoplasmatic protein fraction and the open bars represent nuclear fractions.

For immunoprecipitation I used a polyclonal antibody (Figure 18), and the detection antibody was the same as used in the western blot analyses (WB) above. In the immunoprecipitation (IP) of 400 µg lysates, I did not find the slightly smaller POU5F1 reactive bands found in the western blot experiments and only detected POU5F1 protein in the ECC cells (Figure 18C), suggesting that POU5F1 protein cannot be enriched through this procedure.

These experiments indicate that POU5F1 is not present at detectable quantities in either BM-MSC or UVSC (Figure 18D).

2.3 Novel Markers for Isolation of mesenchymal Progenitors

Cultured MSC contain multipotent cells. However, it is unclear how many of these cells remain in culture. Moreover, the MSC derived from different tissue contain also a heterogeneous population as presented in chapter “Isolation of BM-MSC and UVSC”. Due to the lack of clear markers distinguish between different stages in MSC committment; the study of differentiation of mesenchymal mechanism has not given definitive answers. In the previous chapter, I tested the hypothesis that the expression of “stemness” markers might predict multipotentiality and *in vivo* transplantability of MSC. However, I found that though “stemness” transcripts can be amplified from BM-MSC and UVSC, their expression does not correlate to functional activity of these cells. In addition, the amplified transcripts did not correspond to “stemness” markers, but, rather, to their pseudogenes. Thus, using the primers and western blot techniques I used, I conclude that “stemness” markers are not predictive of multipotent activity.

To approach this problem, in the present study I compared gene expression patterns of BM-MSC and UVSC to define markers, which could distinguish multipotent, bi-potent cells from uni-potent progenitors. The gene expression studies included previously published gene expression studies from other mesenchymal populations periosteal cells (POC) and synovial fibroblasts (SFb), as well as gene expression of unrelated hematopoietic populations ([Delorme et al., 2008], Microarray data presented at Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE9894 and data for SFb available under PMID:16277684).

The strategy I pursued in my work was to combine the definition of surface markers using gene expression studies with flow cytometry, cell sorting and functional validation of cellular function of differentiation assay in vitro. Based on gene expression data of a more limited set of microarray data, markers to isolate CFU-F, putative mesenchymal cells with stem cell activity were proposed and validated in vitro [Delorme et al., 2008]. My studies described in the previous chapter showed that UVSC were of mesenchymal origin, but did not represent stem cell like cells.

Thus, including UVSC in the gene expression analysis as an additional population of differentiated cells to define a more limited number of candidate genes expressing cell surface markers for the isolation of stem cell-like CFU-F.

2.3.1 Microarray Analysis

2.3.1.1 Hierachical Clustering

The microarray analysis presents a comprehensive overview of gene expression data. This analysis was intended as preliminary screen to identify useful antigens for the characterization of MSC populations in BM-MSC and UVSC.

The genomewide microarray analysis here was processed using Affymetrix Human Genome-U133 Plus 2.0 *GeneChips.* This GeneChip Array analyzes gene expression across the entire human genome for one sample with 54.000 Probe sets and 47.000 transcripts and 38.500 genes. In BM-MSC and UVSC an average of 23.000 “present” detected signals are available. The decision of a detected gene expression was classified regarding to the measured fluorescence intensity. The evaluation was performed with GCOS 1.2 software and predicate the detection modus of a probe set (known as call) with absent, marginal or present.

Two different experiments were performed. One experiment in which six BM-MSC samples were compared to five UVSC samples, and a second experiment in which two BM-MSC samples were compared to three UVSC samples. The goal of the second experiment was to increase the total number of sample tested (for statistical relevance testing) as well as to independently confirm the results from the first experiment.

Gene expression data from an external database was transferred into *File marker Pro* 7.0 software to construct a searchable database of all array results. The data sets of various donors and markers were averaged and compared between BM and UVSC. For representation of reproducibility and similar gene expression profile, the microarray analyses were performed with BM-MSC and UVSC donors in two independent microarray analyses. The first analysis we performed was hierachical clustering using a previously established set of possible surface markers and which also included the analysis of periosteal cells (POC), synovial fibroblasts (SFb) and hematopoietic leukocytes (CD45+ and CD11b+) as well as erythrocytic (GlyA) cells.

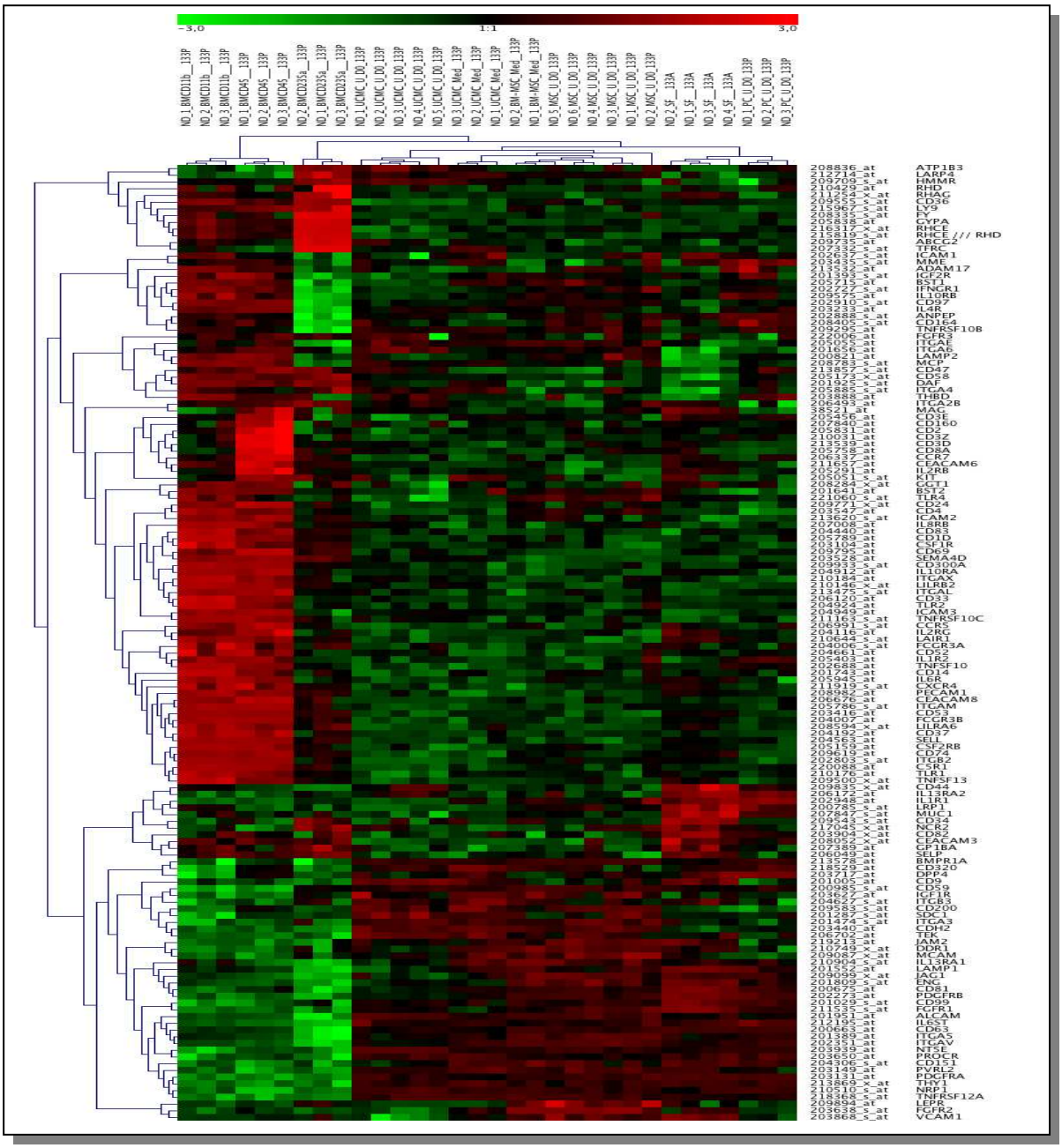


Figure 19: Hierachical Clustering of Microarray-Analysis

Microarray was performed in two independent experiments with BM-MSC and UVSC samples. Hierarchical analysis was performed to find candidate differentially expressed genes. The dendrogramm above shows the expression of several markers based on a fluorescence ratio of green and red. The detection of different colour intensities represents the quantity of the expression for repressed (green) or induced (red) in comparison to the baseline amount of expression.

The clustering (Figure 19) is plotted as a dendrogram that presents the relations between the clusters bringing together pairs of similar genes with further equal gene-pairs. The expression quantity of the several markers is determined in use the fluorescence signal intensity of green, for repression, and red for induction.

This clustering shows that all UVSC and BM-MSC samples (from both experiments) cluster together but separate from each other, showing they are different cell types. Also, the UVSC and BM-MSC cluster away from POC, SFb and hematopoietic cells.

For evaluating the gene expression data only detection signals that were demonstrated in the call with “present”, are used in this study (the data aggregate to 90-95%).

2.3.1.2 Principal Component Analysis

The principle components analysis (PCA) is used as mathematical model for multivariant statistical analysis. At this, a high number of data with multiple variables will be simplified illustrated in a graphic containing a low number of uncorrelated variables (principal components). The PCA is performed using only membrane associated molecules. The distribution is linked to the different samples used in this analysis whereas each data point represents a single profile. For the presentation of the results different components were chosen and graphically demonstrated. The different components, consisting of the different hematopoietic cells (GlyA; CD11c/CD45) as well as different cells of mesenchymal origin like skin fibroblast (SFb), peristeal cells (POC) and finally BM-MSC and UVSC.

At this, the huge number of data from the different samples appears as different dot clouds in the coordination system. For setting of the axes in the three-dimensional system, a trend-line was position into the dot cloud whereas the line represent the closest approximation to each data point in the dot cloud. Based on an algorithmic model, the variances of the different data points along an axis will be calculated and justified by vectors, which subdivide the data cloud in different dimensions. According to the criteria and the multiple components (different molecules, etc.) the dimension can be extended.

The calculation and the three-dimensional illustration is performed by Thomas Häupl (Charité, Berlin) using Genesis software (http://www.genesissoftwareonline.com/).

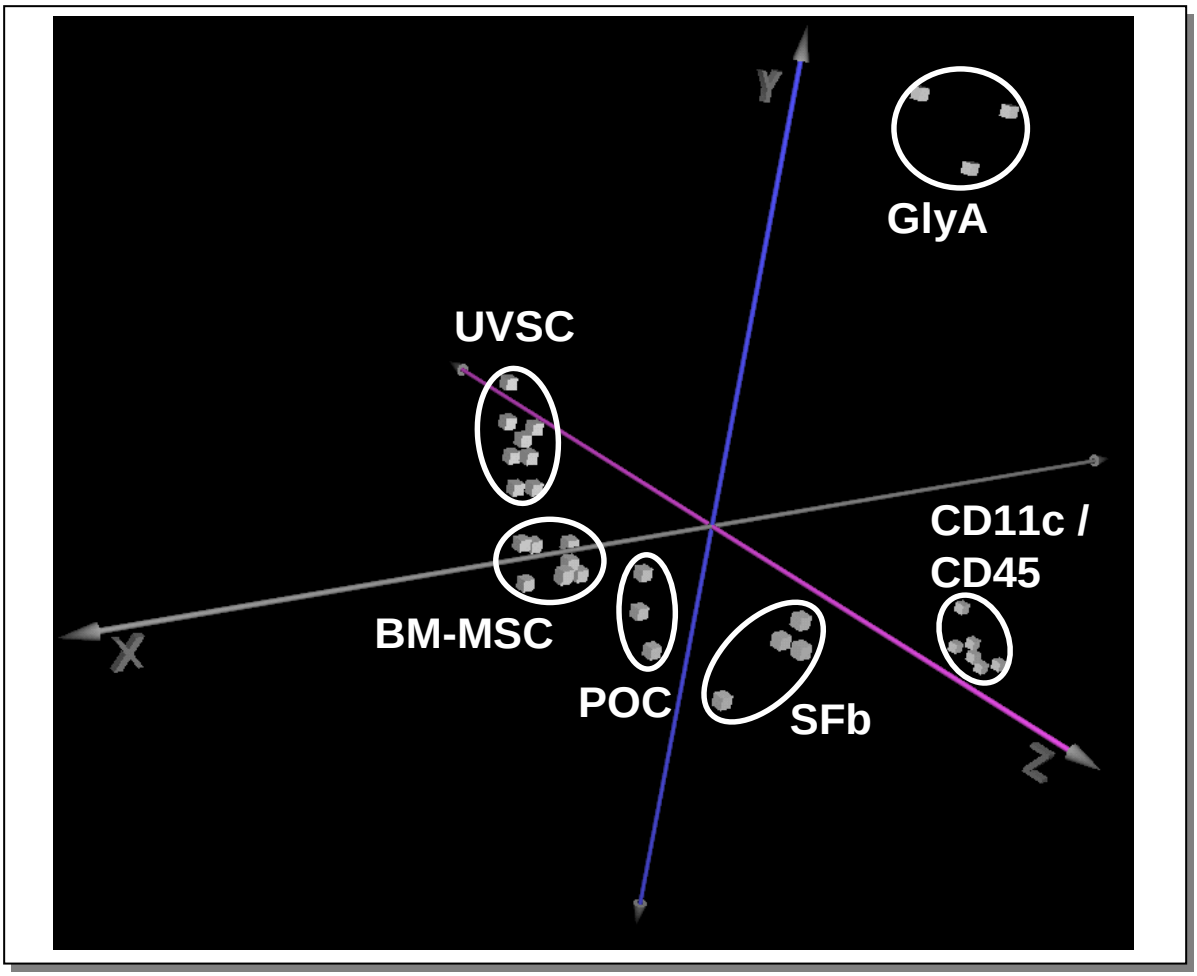


Figure 20: Principal Component Analysis

Array data gathered from 6 BM-MSC and 5 UVSC samples were analysed and compared. K-means cluster analysis was performed to find differentially expressed genes. A second experiment with 2 BM-MSC and 3 UVSC was performed to confirm these results. BM-MSC and UVSC clusters were compared to hematopoietic lineages (CD45/CD11c, CD235a (GlyA)), fibroblast (SFb) and periosteal cells (POC).

The Principal Component Analysis (PCA) here was performed of selected markers for BM-MSC and UVSC (Figure 20). Both cell types were compared to each other, but also to hematopoietic lineages CD45+/CD11b+, CD235a+ (GlyA), synovial fibroblast (SFb) and periosteal cell (POC) population as described in our previous study [Delorme et al., 2008]. The UVSC clustered very closely to the BM-MSC, but different from the other cell populations (Figure 20 and Appendix Table8 and 9). The distance to the other clusters demonstrate that UVSC are different from CD45/CD11b, GlyA, SFb, POC populations (Figure 20 and Appendix Table8 and 9). The PCA was performed in cooperation of GENOSTEM partners and combined with published data presented at Delorme et al. [Delorme et al., 2008].

2.3.1.3 Evaluation of Microarray-Data

For initial analysis of the microarray results, the normalized data was imported into *File marker Pro* 7.0 software. At this BM-MSC and UVSC of both microarray analysis experiments are pictured next to each other (Figure 21) for direct comparison of both analysis and for specification of possible candidate genes. The databank surface shows information about the particular gene (Figure 21A), the gene description (D) and the expression of each tag in each of the eight different BM-MSC and UVSC samples (B, C).

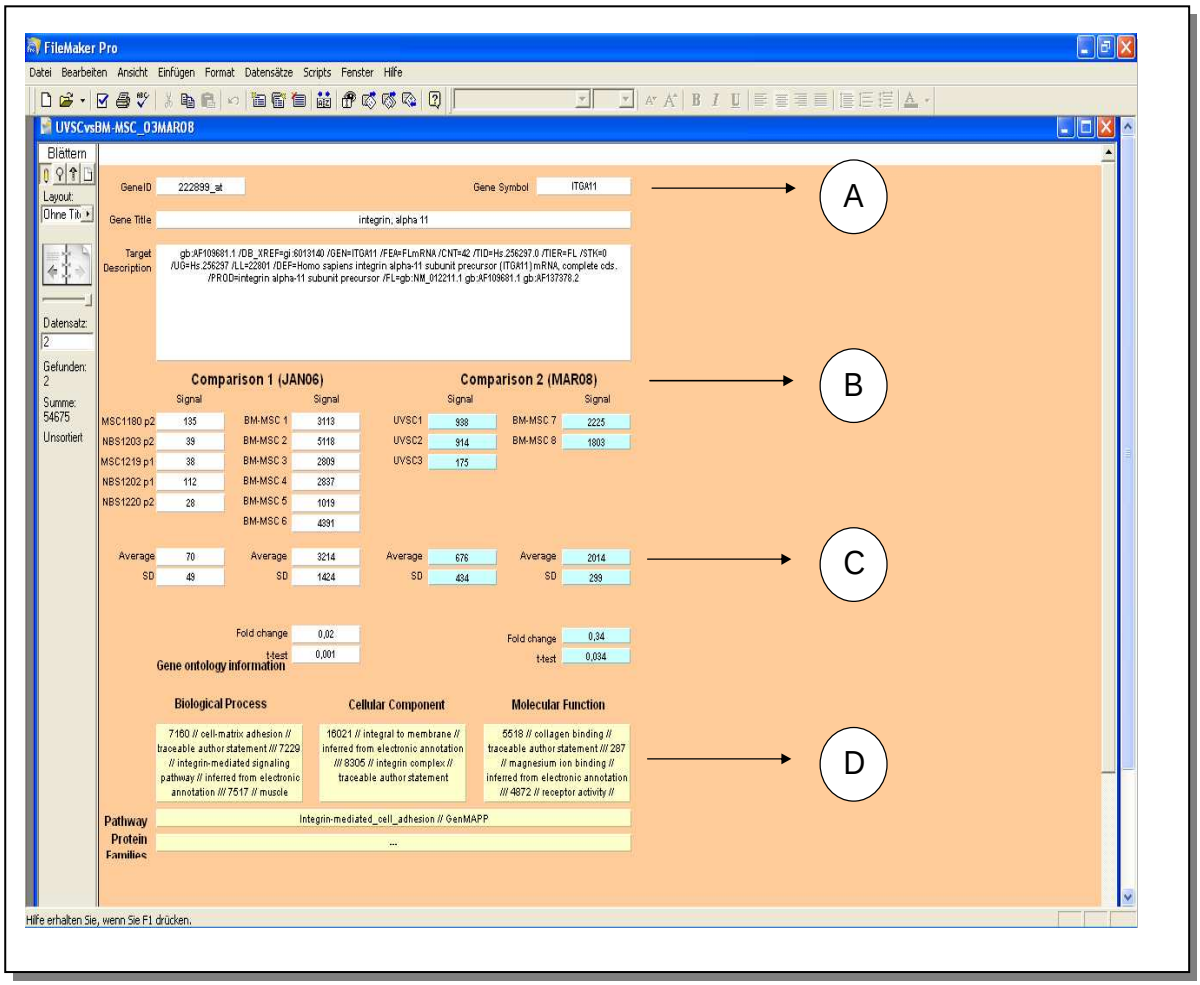


Figure 21: *File maker Pro7* archive software for representation and studying of gene expression data of SIPAGENE Database

Two separately performed microarray analyses (B) were performed to guarantee precise data of differences and commonalities in gene expression in both cell types. Gene expression dates were averaged (C) and compared between BM-MSC and UVSC. The Fold Change is calculated of averaged signal of BM-MSC and UVSC (D). The particular gene is termed in Gene Symbol and Gene ID access number (A) as well as a short description of their functional properties.

When we took a threshold of a two-fold difference between BM-MSC and UVSC in either experiment, we found that 473 tags were significantly overrepresented more in BM-MSC in both experiments. Of these, 102 correspond to 89 membrane-associated gene products (Appendix Table4). Differentially expressed genes include known markers of multipotent cells, such as VCAM1 [Short et al., 2001] and ALPL [Gronthos et al., 2007]. In addition, many molecules involved in extracellular matrix were expressed by BM-MSC, including HAS1, GALNT5, CSPG2, COL14A1, and POSTN.

In contrast, 182 tags were significantly overrepresented more than 2-fold in UVSC in both experiments. Of which 43 tags corresponded to 29 membrane-associated gene products (Appendix Table5). These molecules included surface markers such as the endothelial marker FLT1 and the myogenic precursor marker NCAM1 (CD56).

2.3.2 Novel transmembran Molecules derived from Microarray-Analysis

In the literature, some molecules had been described, which have been used to enrich CFU-F (Table2). The evaluation between the BM-MSC and UVSC were performed of two independent microarray analyses.

The analysis is limited to the outer plasma membrane proteins of and included all membrane proteins. Differentially expressed genes include known markers of multipotent cells, such as VCAM1 [Short et al., 2001] and ALPL [Gronthos et al., 2007]. In addition, many molecules involved in extracellular matrix were expressed by BM-MSC, including HAS1, GALNT5, CSPG2, COL14A1, and POSTN. There were 182 tags significantly overrepresented more than 2-fold in UVSC of both experiments, of which 43 tags corresponded to 29 membrane-associated gene products (Appendix Table5).

The gene expression analysis reveal characteristic expression of typical MSC transmembran markers in BM-MSC and UVSC (CD105 (ENDOGLIN), VCAM-1; CD44, CD146 (MCAM), CD73 (NT5E), CD90 (THY1), CD71 (TFRC), CD166 (ALCAM), CD29 (ITGB1), HLA-A/B/C). In contrast, hematopoietic markers CD45, CD34, CD14 and CD11 are absent. The expression of costimulatory molecules like CD80, CD86 or CD40, the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule PCAM-1) a marker expressed on endothelial cells [Mamdouh et al., 2003] as well as CD18 (leucocyte function-associated antigen-1 LFA-1) are also not present in BM-MSC or UVSC. The myogenic precursor marker CD56 (NCAM-1),

predominantly expressed in UVSC, is controversially discussed in literature. Latest studies show that a MSCA-1^{+} CD56^{+} subpopulation has a strong chondrogenic differentiation potential [Battula et al., 2009] However, the expression of CD56 is mainly identified on natural killer cells, neural and muscle cells, but the expression of this marker is contentious in MSCs.

UVSC show expression of members of teraspanin family like TM4SF or CD9. The genes are involved in cell motility, osteoclastogenesis, metastasis, neurite outgrowth and myotube formation. Beyond CD9 promote juxtacrine signalling and is involved in proliferation and attachment [Kim et al., 2007]. Another member of this group CD63 is highly expressed in both BM-MSC and UVSC. The antibody is expressed in CFU-F populations of bone marrow [Joyner et al., 1997] and used for isolation and enrichment.

BM-MSC and UVSC both show the detection of members of the integrin-alpha family. Some of them are known to be good markers for CFU-enrichment (Table2). While BM-MSC overexpress the markers CD49e (ITGA5) and ITGA11 the UVSC mainly show expression of CD49b (ITGA2) and CD49d (ITGA4).

ITGA11 is strongly expressed in BM-MSC and downregulated during adipogenic and chondrogenic differentiation. Velling et al. described the expression of ITGA11 is highly leveled in the human adult uterus, heart and intermediate in skeletal muscle [Velling et al., 1999]. To elucidate further properties of ITGA11 the marker was used for this study. Equally, a member of the NOTCH family NOTCH3 is exclusively expressed in BM-MSC. Mutations in NOTCH3 have been identified as cause of CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). However, the NOTCH interactions with its cell-bound ligands show multiple functions like in cell-cell communication, regulation of cell differentiation processes during the embryonic and adult development and is part of the non-canonical WNT pathway. The NOTCH3 promote the expression of his ligand JAGGED-1 (JAG1) that is also expressed in this analysis.

Additionally, the analysis represent the expression of FGFR2, which is receptor for multiple FGFs. Growth factors like FGF2 are used in many laboratories to improve the cell proliferation [Delorme et al., 2009]. Further analysis show the expression of members of the PDGF-family (PDGFC, PDGFA, PDGFRA, PDGFRB) and VEGF-family (vascular endothelial growth factor) like the endothelial marker FLT1 that is

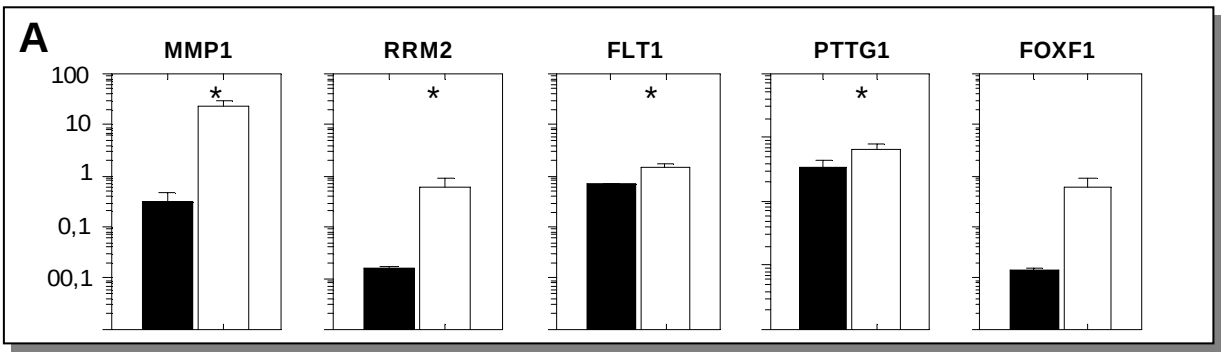
strongly expressed in UVSC. In addition, ANGPT1 (Angiopoietin-1) was detected that is a known regulator in angiogenesis for vascular remodelling [Suri et al., 1996] and of the HSC niche [Arai et al., 2004] as well as are expressed by CD146 positive stromal progenitors [Sacchetti et al., 2007].

2.3.2.1 Real-Time Analysis for Confirmation of selected Markers from Microarray-Analysis

The Real time reverse transcriptase PCR represents a sensitive system for quantification of an initial amount of cDNA template by detection of emitted fluorescence. At this, the fluorescence dye is used as reporter. The signal intensity increases proportional with the amount of PCR product [Freeman et al., 1999, Raeymaekers et al., 2000]. The quantification of mRNA was normalized to the endogenous control as reference. The primer to the selected candidate genes of microarray analysis were designed and controlled based on the corresponding NM_reference number.

A selected number of transcripts was chosen from the above microarray analysis to show expression in an independent assay for gene expression - realtime PCR. The genes chosen for this analysis are expressed at the cell surface, represent extracellular matrix molecules, or are molecules commonly associated with MSC. The expression profile was then compared between markers overexpressed in BM-MSC or UVSC.

For these confirmation experiments, total RNA was extracted from subconfluent BM-MSC and UVSC. The RNA isolation was performed with DNAse digestion to avoid contamination with genomic DNA. The concentration of the extracted RNA was measured and controlled for purification. Finally, the RNA was amplified in cDNA synthesis (see chapter 5.3.1 and 5.3.2). Each marker was tested at least three times in duplicates using various donors.



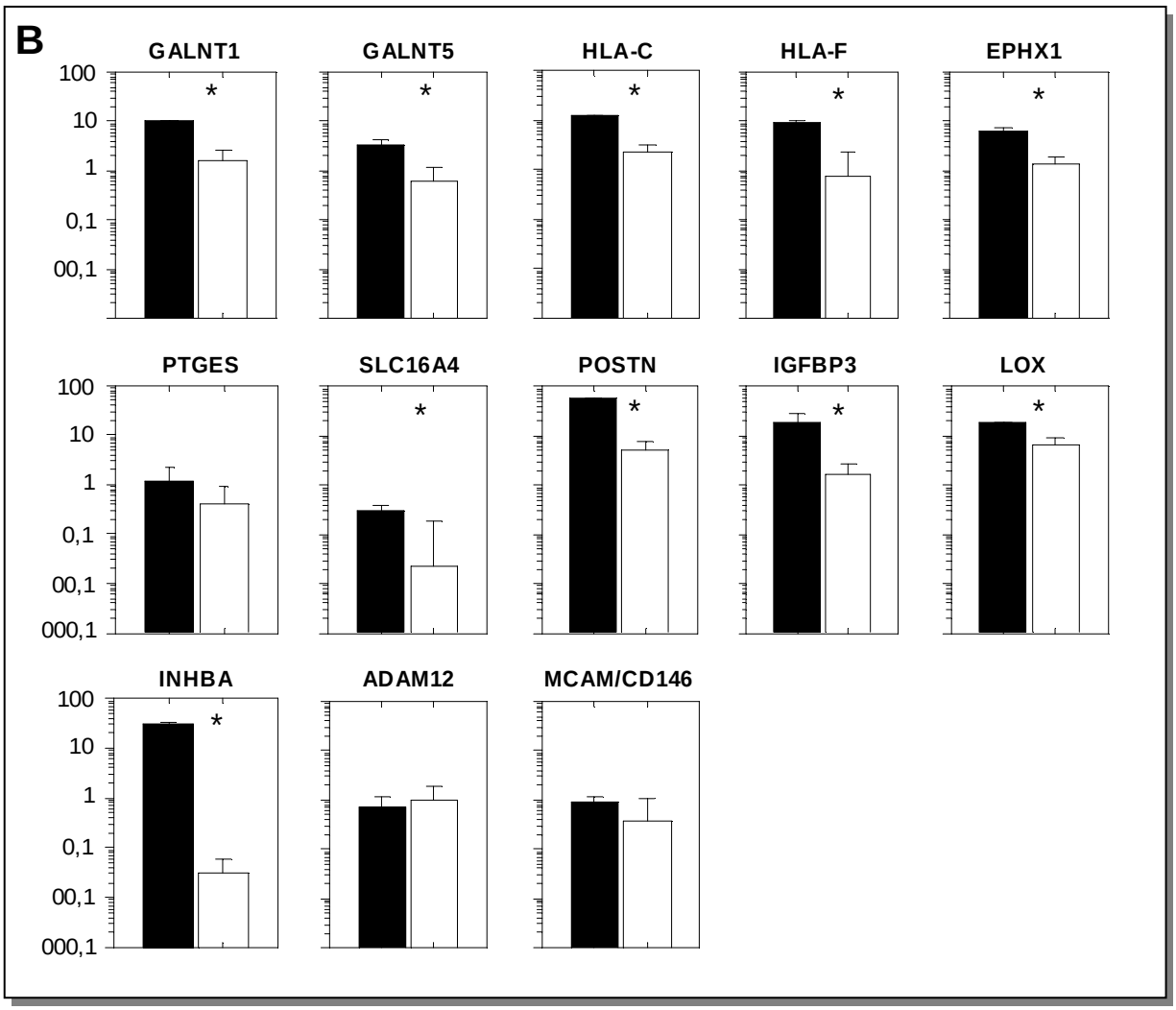


Figure 22: Real-Time Analysis of selected surface markers and extracellular matrix molecules

Real time analysis was performed as described in Material and Methods. (A) Shows markers overexpressed in UVSC. In (B) further markers are demonstrated overrepresent in BM-MSC. The last panel shows the marker MCAM or CD146 which is expressed in both cell types at similar level.

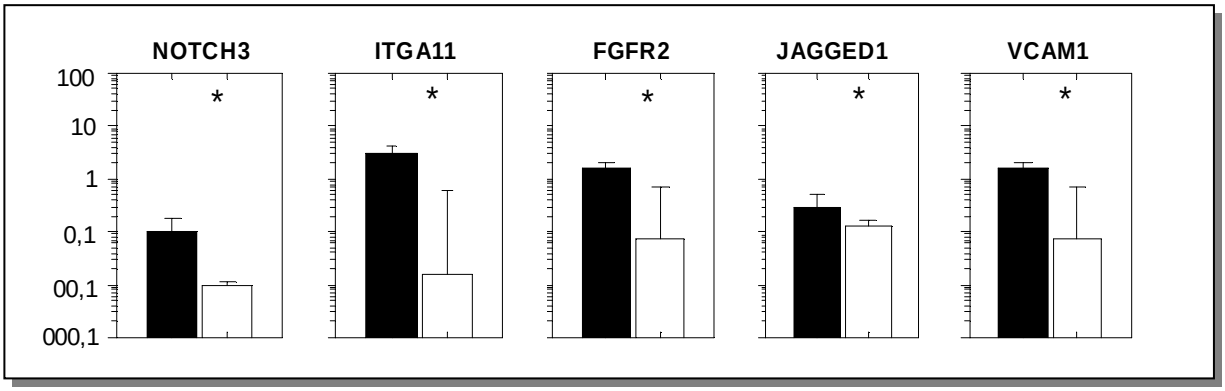


Figure 23: Real-Time Analysis of selected candidate genes

Candidates markers overrepresent in BM-MSC (black columns) are shown in comparison to UVSC (white columns). Real-time PCR analysis was performed on independent samples. The signal of the BM-MSC and UVSC is represented relative to the housekeeping gene RPLP0.

Realtime PCR analysis of differentially expressed genes showed that of the 23 molecules I checked (Figure 22 and 23), 19 were indeed differentially expressed between BM-MSC and UVSC.

The high fold change found in selected markers like NOTCH3, ITGA11, VCAM-1 as well as FGFR2 and JAG1 (Figure 23 and Appendix Table4) could be confirmed in Real-Time analysis. MCAM (CD146) show comparable fold change in both analyses.

MCAM is used later for double staining in FACS-Analysis (Figure 25). The expression of markers predicted to be overexpressed in UVSC in the microarray analysis (for instance, MMP-1, FLT-1, PTTG-1, FOXF-1) (Figure 21A), was also confirmed by the Real-time PCR analysis.

2.3.3 FACS-Analysis and Isolation of Subpopulations using novel surface Markers

To find out whether the differentially expressed molecules could be useful as markers to prospectively isolate CFU-F, we sorted cultured BM-MSC and UVSC for the presentation of these markers. Our array analysis confirmed the differential expression between BM-MSC and UVSC of some surface markers we had previously investigated, including CD49a (ITGA2), CD49e (ITGA5), CD61 (ITGB3), CD105 (ENG), and CD106 (VCAM1) [Kaltz et al., 2008] as described before in chapter 2.1. In the present study, however, the focus was on novel and previously not investigated non-CD classified markers. Stro-1 [Simmons et al., 1991], CD146 [Sacchetti et al., 2007] and MSCA1 (W8B2) [Battula et al., 2009] were used as controls. Flow cytometric analysis showed that the control markers (Figure 24) and the markers defined by the gene expression analysis (Figure 25) were expressed higher in BM-MSC than in UVSC.

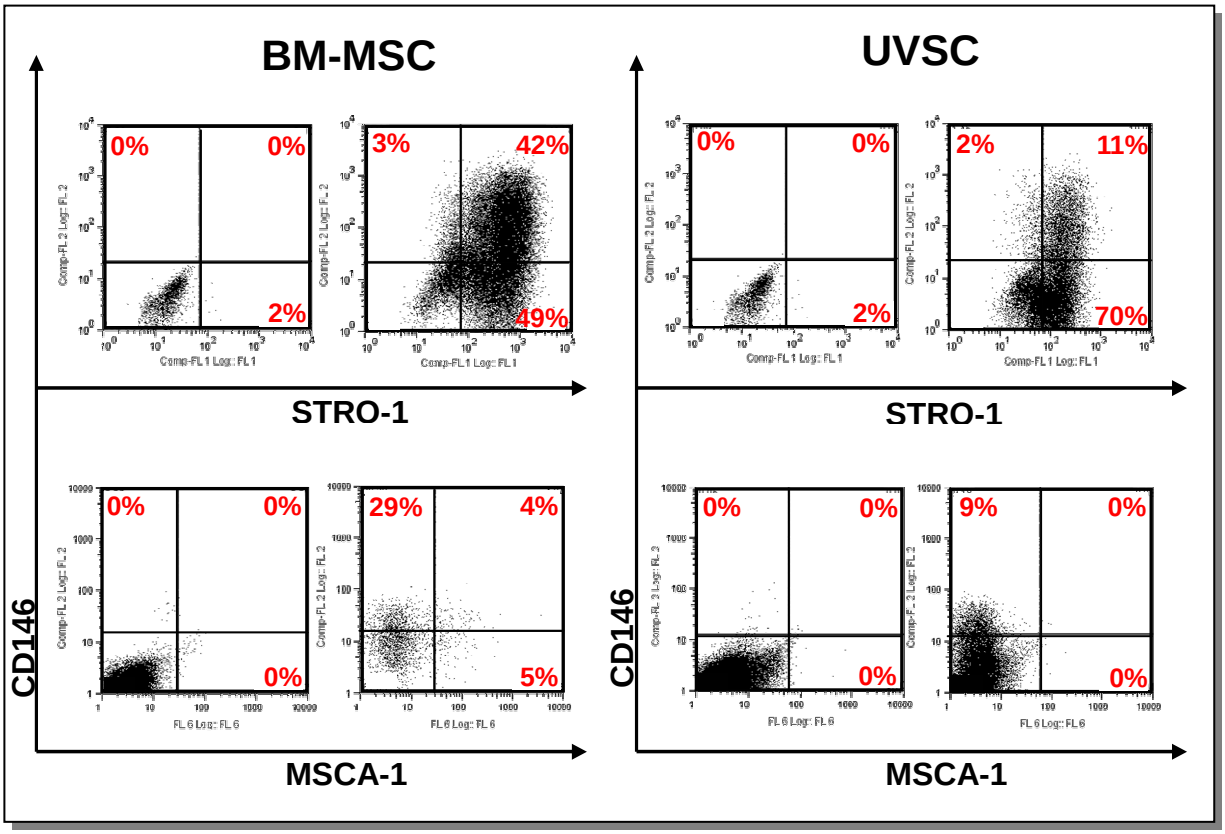


Figure 24: FACS-Analysis of Control Markers

For internal control of MSC the markers STRO-1 and MSCA-1 was used in double staining with CD146. The stromal cells were trypsinized and prepared for FACS analysis (see Methods 5.1.7). Both markers show in the double positive subpopulation a higher expression in BM-MSC in comparison to UVSC.

For representation of the expression of prospective candidates, BM-MSC and UVSC were cultured until 70% confluency. After deattchment of the stromal cells, BM-MSC and UVSC were stained as described in Material and Methods. For each antibody the corresponding isotype control were used for detection of the correct subpopulation and exclusion of false positive cells. After performing the settings at the flow cytometer the chosen candidates of the microarray analysis (ITGA11, NOTCH3, JAG1, FGFR2) of both BM-MSC or UVSC were measured in FACS-Analysis.

The FACS-Analysis was performed in a two-colour staining with CD146 (MCAM) and another antibody. Figure 25 shows the results of flow cytometry in dot plots of representative stains of the markers NOTCH3, ITGA11, JAG1 and FGFR2 each with CD146. The different subpopulation and the percentage distribution of various populations are demonstrated. Here, the expression of each marker is gated to the corresponding Isotyp control as well as pictured in comparison between BM-MSC and UVSC. The results show that the selected candidate markers were more expressed in BM-MSC in comparison to UVSC. Strong expression differences in comparison of both cell types are particularly presented in NOTCH3 (2-fold) and ITGA11 (3-fold) double positive population in BM-MSC. Furthermore, the study shows the successful isolation of in vitro expanded human stromal cells with novel markers selected from gene expression analysis.

In Sorting-Analysis, the different subpopulations of single and double positive subpopulations were precisely isolated in accordance to the sort scheme described in chapter 5.1.7 in Methods. After setting up the flow cytometric cell sorter the various subsets of BM-MSC or UVSC were collected in culture medium supplemented with 20% FCS (according to [Sacchetti et al., 2007]) and recultured immediately for each subpopulation sorted.

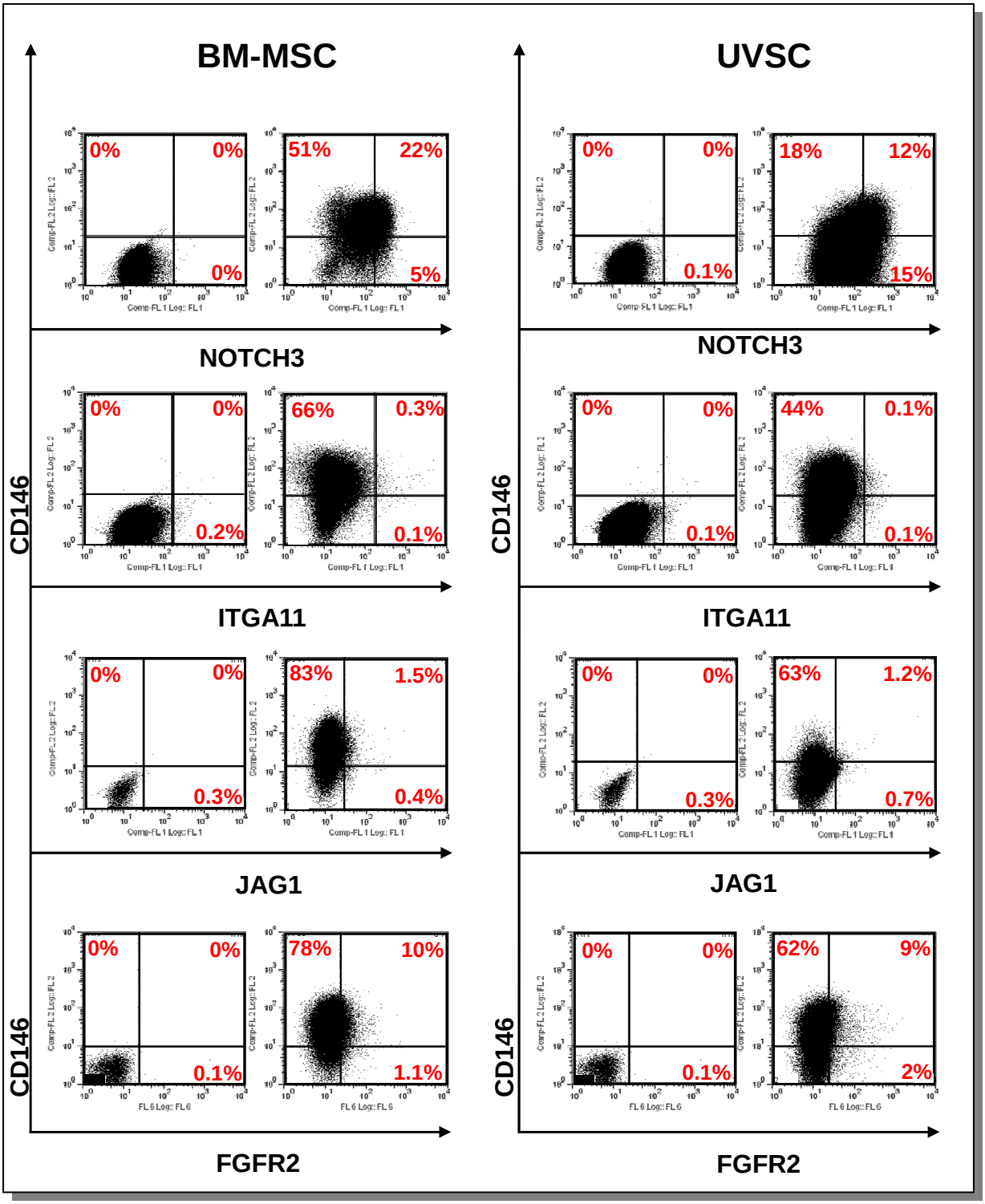


Figure 25: FACS-Analysis of novel Marker

Novel surface markers from microarray analysis are used for detection and isolation in BM-MSC and UVSC. All markers were overrepresented in gene expression analysis in BM-MSC and show also here increased detection in BM-MSC. The Dot Plots are subdivided in quadrants to demonstrate the percentage of the several subpopulations for each marker. The quadrants as well as the gating of these cells were set to the corresponding Isotyp control. The isolation of the different subpopulations was performed as described in Methods.

2.3.4 CFU-F-Assay of sorted Subpopulations

Friedenstein et al. and Owen et al. first described that CFU-F (Colony Forming Units) of bone marrow precursors give rise to various connective tissue of mesenchymal lineage including osteogenic, adipogenic and chondrogenic differentiation potential [Friedenstein et al., 1974 and Owen et al., 1988]. Further studies of Friedenstein et al. and Ashton et al. demonstrate that transplantation of multi-colony derived cell strain form ectopic ossicles including adipocytes, cartilage and support of myelopoiesis [Friedenstein et al., 1982; Ashton et al., 1980].

However, not all CFU-F are multipotent [Sacchetti et al., 2007; Pittenger et al., 1999]. The differences in colonies based on the heterogenity of the population. At this, the cell morphology as well as proliferation rate and the expression of distinct differentiation markers can vary.

The CFU-F assay is performed to determine the clonogenic potential and morphological appearance of sorted subsets. The present study will show the enrichment and recovery of sorted subpopulations using novel antibodies for prospective isolation of CFU-F.

2.3.4.1 Enrichment and Recovery of isolated Subpopulations

The different sorted subpopulations were replated in a density of 100cells/cm^2 in 6-well plates and grown ca. two weeks.

To calculate CFU-F enrichment the number of colonies found from single and double positive fractions were normalized to 5000cells/ cm^2. The CFU-Fs of the different subpopulations were then compared with unfractioned BM-MSC and UVSC (as well as CD146 and MSCA1 single-positive populations).

CD146 is expressed by MSC forming bone and adipose cells *in vivo* [Sacchetti et al., 2007]. Therefore, in this study also the possibility was explored whether the novel markers would enrich CFU-F above and beyond CD146 as a single marker.

All of the single marker-sorted subpopulations (Figure 26A and Table4) show significant enrichment in comparison to unsorted cells. CD146, MSCA1, and anti-FGFR2 increase the frequency of CFU-F up to 10-fold, NOTCH3 and ITGA11 more than 20-fold, and anti-JAG1 90-fold (Table4). The number of colony-forming cells was

more enriched 2- to 8-fold in cells sorted from BM-MSC compared to those sorted from UVSC in all tested markers, except ITGA11 (Table4).

The enrichment differences in single positive subpopulations (NOTCH3, ITGA11, JAG1 and FGFR2) of mesenchymal stromal cell from the bone marrow show in comparison to UVSC an enrichment difference of 2.3, 7.3, 3.99, 8.77 fold. Only the ITGA11 cultivated subpopulation show no significant difference in CFU-F enrichment between UVSC (24.35 ± 18.85) and BM-MSC (21.8 ± 9.7). At this, the markers NOTCH3, ITGA11 and JAG1 show the highest enrichment factor in BM-MSC in comparison to unfractioned BM-MSC and UVSC.

Compared to BM-MSC-derived subpopulations, the cultured subpopulations show for CD146+/JAG1+ (5.6 ± 0.95) 11,2 fold, CD146+/ITGA11+ (37 ± 27) 19.27 fold and in particular for CD146+/NOTCH3+ (27.3 ± 6.9) 33 fold higher difference to UVSC.

Moreover, the experiments here presents that only CD146+ NOTCH3+ and CD146+ ITGA11+ double-positive sorted cells show a higher enrichment in CFU-F than cells sorted by CD146+ alone (Figure 26B and Table4).

	ENRICHMENT		**RECOVERY**	
	BM-MSC	**UVSC**	**BM-MSC**	**UVSC**
CD146	5,2 ± 1,3	1,3 ± 0,65	3,2 ± 1,8	12,1 ± 10,3
MSCA1	10,6 ± 8,9	-	1,235 ± 1,1	-
NOTCH3	21,3 ± 2,5	9,1 ± 4,1	3,6 ± 0,2	0,35 ± 0,35
ITGA11	21,8 ± 9,7	24,35 ± 18,85	6,97 ± 2,4	33,79 ± 21,79
JAG1	90,7 ± 79,015	22,7 ± 22,7	40,4 ± 35	6,3 ± 6,3
FGFR2	4,3 ± 1,1	0,49 ± 0,49	1,73 ± 0,64	0,135 ± 0,135
CD146+ MSCA1+	2,8 ± 2,8	-	0,35 ± 0,35	-
CD146+ NOTCH3+	27,3 ±6,19	0,82 ± 0,82	8,36 ± 2,94	0,15 ± 0,15
CD146+ ITGA11+	37 ± 27	1,92 ± 1,69	21,7 ± 15	0,82 ± 0,58
CD146+ JAG1+	5,6 ± 0,95	0,5 ± 0,5	2,52 ± 0,39	0,16 ± 0,16

Table 4: Enrichment and Recovery of CFU-F from sorted Subpopulations

Cultured BM-MSC and UVSC were harvested by trypsin digestion and stained with antibodies against the markers mentioned above (see Figure 24). Afterwards, cells were sorted based on the marker alone, or as part of the CD146+ population. Sorted and unsorted cells were seeded as 100cells/cm^2. Two weeks later, the number of fibroblastoid colonies was counted. Enrichment was calculated as CFU-F_{marker}/CFU-$F_{unsorted}$ for each donor. The recovery (($Cells_{marker}$ x CFU-F $frequency_{marker}$/ $Cells_{unsorted}$ x CFU-F $frequency_{unsorted}$) x 100) is demonstrated in percentage of the sorted population for each marker in comparison to the unsorted control. The above numbers represent the average ± standard deviation of three to five independent experiments (except the experiments with JAG1, which were performed only twice).

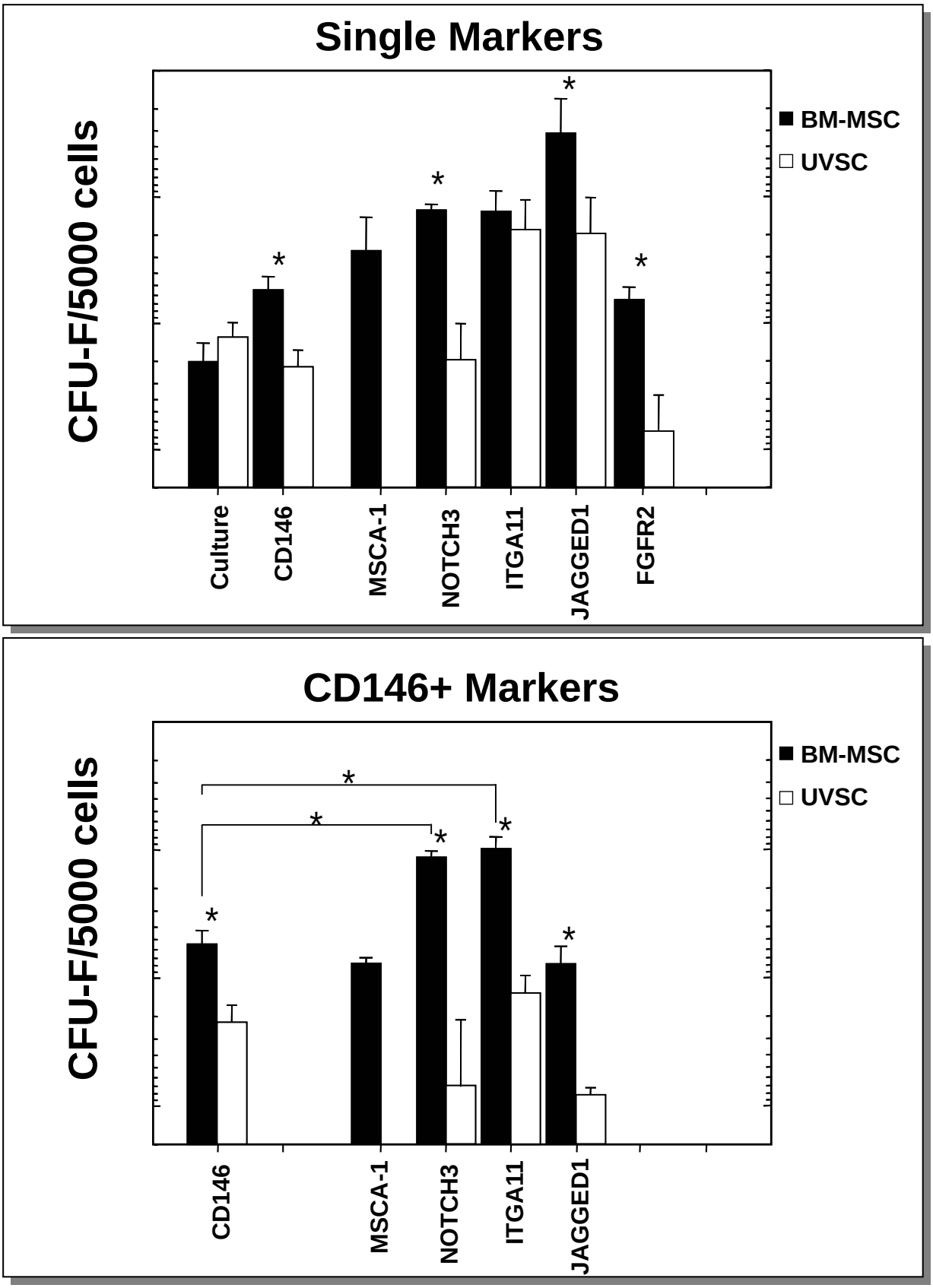


Figure 26: CFU-F of sorted Populations

The isolated subpopulations of single positive and CD146+ double positive cells were cultivated in a density of 10-100 cells per cm^2 in 6-well plates. After two weeks, the number of CFU-F was counted and normalized to 5000 seeded cells. (A) Shows CFU-F of sorted single positive populations while (B) presents the double positive subfraction with CD146 and other markers. BM-MSCs (black columns) are presented in comparison to UVSC (white columns). The CFU-F of the novel candidate markers are shown with the culture control as well as CD146 and MSCA-1. (SEM ± ; p-value < 0,05)

Taken together the findings demonstrate a significant enrichment of CFU-F in CD146+ supopulations and a further enrichment when using NOTCH3 and ITGA11 as additional markers. Hence, the markers used here do not only identify a certain subpopulation of stromal cells in the bone marrow but also can be used for CFU-F enrichment. In addition, the use of such markers may be useful to isolate much smaller populations of multipotent cells from other tissues, like UVSC.

2.3.5 Functional Analysis

MSC can differentiate in at least three lineages (adipogenic, osteogenic, chondrogenic). For validation of the cellular function in vitro, the differentiation assay is indispensable. Adipogenic cells will show fatty vesicles while osteogenic induced cells will present the formation of a mineralized matrix. The osteogenic differentiation is a step-wise developmental process from undifferentiated precursor to secretory osteoblast and at last terminally differentiated osteocyte [Jaiswal et al., 1997].

In the present study, the already mentioned markers above will be used to refine gene expression data of possible multipotent cells. To find out whether the progeny of the sorted CFU-F show adipogenic or osteogenic potential, we performed directed differentiation studies. The idea of these experiments was, that the CFU-F-enriching antibodies could also predict uni-, bi-, and/or tripotent capacity.

2.3.5.1 Differentiation of sorted Subpopulations

The in vitro differentiation was performed in use of standard protocols (see Methods 5.2). The detection of adipogenic differentiated stromal cells was performed with Oil Red O. In contrast, the osteogenically differentiated cells were stained with von Kossa in both BM-MSC and UVSC.

The experiments above, had already shown that in comparison to unfractionated BM-MSC, the UVSC show less pronounced adipogenic and osteogenic differentiation potential (Figure 14). Thus, in this first series of experiments, we focused on differentiation of BM-MSC-derived cell fractions. Detailed differentiation studies with UVSC were not performed.

Subpopulations of BM-MSC isolated with CD146 and MSCA-1 both show adipogenic potential (Figure 27). The appearance of fatty vesicles in MSCA-1 positive subpopulations is confirmed to the results of Battula et al. [Battula et al., 2009]. To find out whether CD146 is expressed by both adipogenic and osteogenic progenitors, the cells positive for both CD146 and other markers were re-cultured. However, the BM-MSC grown from CD146+ with another marker primarily shows osteogenic differentiation and apparently reduced adipogenic differentiation capacity. Robust osteogenic differentiation is found in monolayers grown from NOTCH3+, FGFR2+ and ITGA11+ positive BM-MSC. Of these cells, only NOTCH3+ and FGFR2+ cells show robust adipogenic differentiation. ITGA11+ single positive BM-MSC-derived cells show only limited adipogenic potential (Figure 27).

Differentiation was also studied using PCR analysis of the cell populations before and after either adipogenic or osteogenic induction. In these experiments we found that osteogenic differentiation, both osteogenic factors RUNX2, and ALPL were expressed. We further showed that adipogenic PPAR-γ and LPL also showed up after osteogenic differentiation of either CD146+ or NOTCH3+ cells. This finding suggests that the standard osteogenic induction protocol may, aberrantly also stimulate the adipogenic differentiation pathway. Similarly, we found that the osteogenic transcription factor SP7 (osterix) was expressed after adipogenic differentiation, as well as in adipogenic and cultured NOTCH3+ cells (Figure 28). These experiments demonstrate that the “standard” differentiation protocols (see chapter 5.2), do not result in pure differentiated cell populations and they should, therefore, be improved.

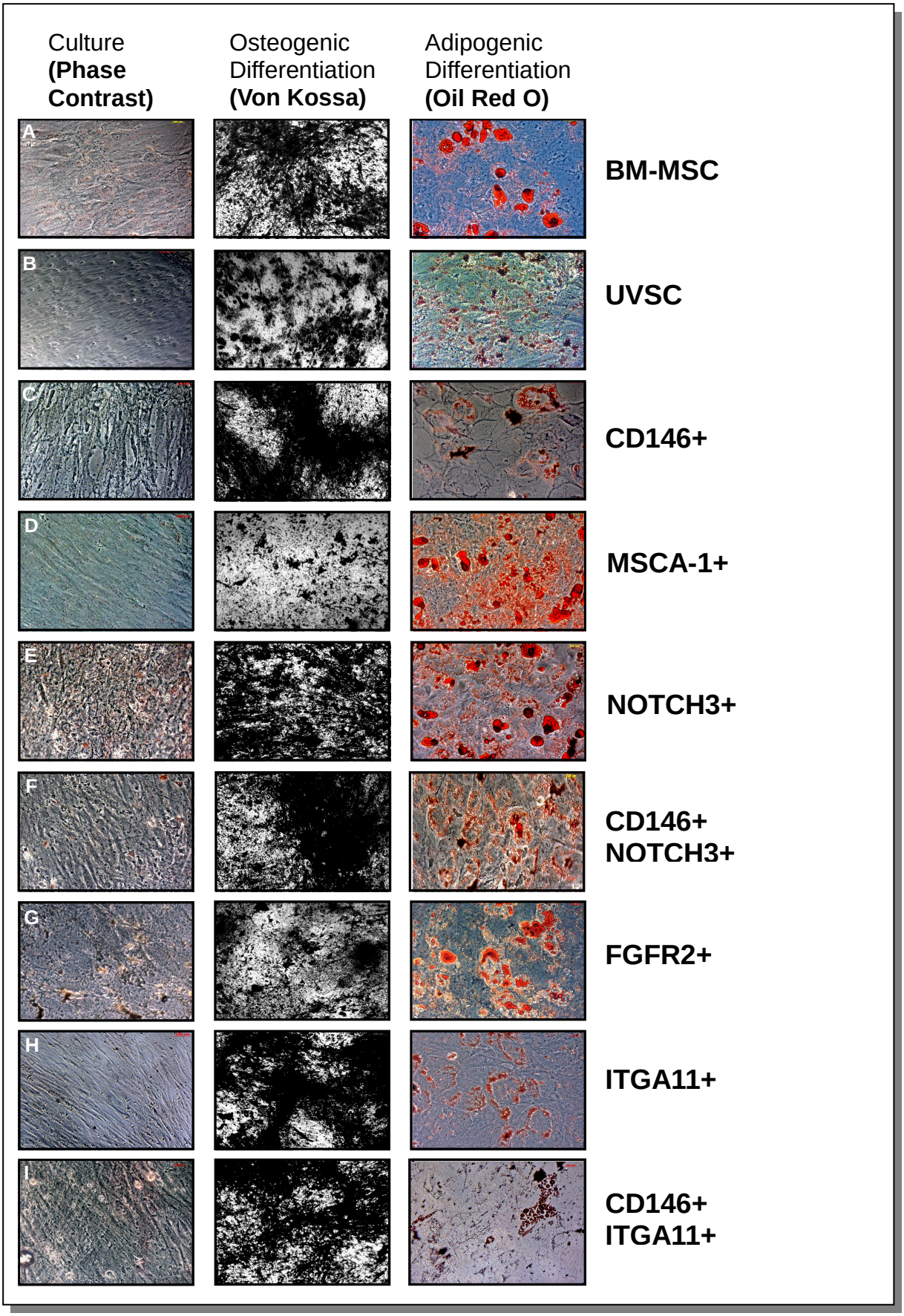


Figure 27: Differentiation of sorted Subpopulations

BM-MSCs and UVSC were treated for differentiation as described in chapter 5.2. The Figure shows phase contrast images of confluent non-differentiated cells (left panels) osteogenic differentiation (middle panels) and adipogenic differentiation (right panels). Picture A and B show culture control, C and D reference controls, E to I differentiation of novel marker. Magnification: Culture and adipogenic differentiation in 32x, osteogenic differentiation in 10x.

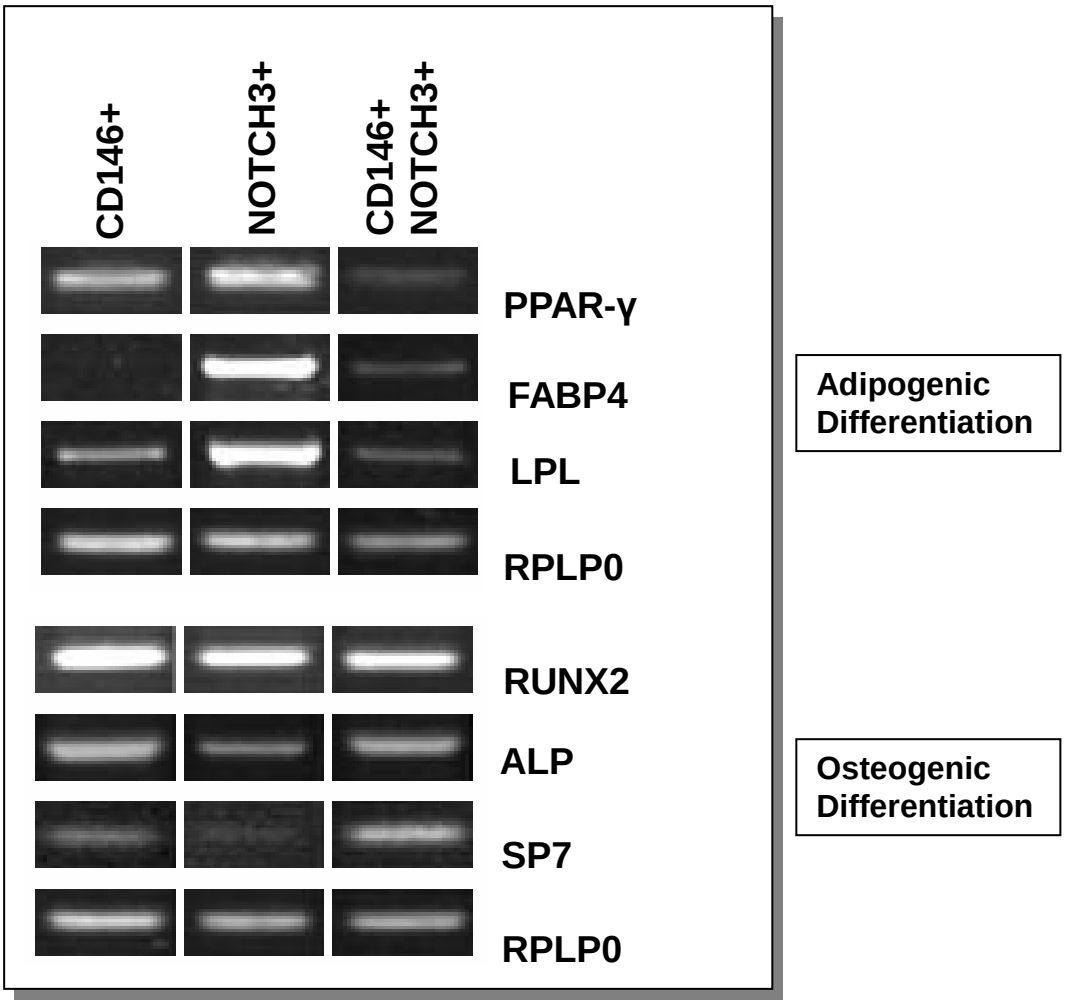


Figure 28: PCR-Analysis of sorted Subpopulation after Differentiation

Subfractions of CD146/NOTCH3 stained BM-MSC were isolated as CD146+, NOTCH3+ and CD146+/NOTCH3+ populations. The different fractions were cultured and differentiation was induced as described in Material and Method. PCR was performed using several adipogenic (PPAR-γ, FABP4, LPL), osteogenic markers (RUNX2, ALP, SP7).

Furthermore, the above results demonstrate that NOTCH3+ and FGFR2+ single positive populations include adipo- and osteo(bi)potent cells, but also showed that cells double positive for both CD146 and ITGA11 present strong osteogenic, but limited adipogenic potential. In addition, we confirm literature reports that CD146 is expressed by osteogenic progenitors [Sacchetti et al., 2007].

PCR analysis further showed that the CD146+ NOTCH3+ population appear to be more homogeneous with regard to adipogenic as well as osteogenic differentiation, with less leaking into other populations (Figure 27 and 28).

2.4 Future Directions and preliminary Studies

2.4.1 Isolation of CD146/NOTCH3 double positive Subpopulation of primary uncultured BM-MSC

The studies above suggest that NOTCH3 and CD146 are markers of multipotent BM-MSC. Due to limited access to normal primary bone marrow samples, all of the studies above (microarray, realtime PCR, flowcytometry, sorting) were performed using cultured CFU-F or their derived progeny. To find out whether the markers possibly identifying multipotent cells in cultured MSC preparations, would also enrich for CFU-F and multipotent cells from primary cell sources, uncultured, frozen, primary stromal cells of bone marrow were used for isolation of CD146+NOTCH3+ populations. The analysis above shows that NOTCH3 may be a marker of multipotent MSC, whereas ITGA11 appears to be expressed primarily on osteogenic progenitors. Thus, we isolated mononuclear bone marrow cells as described in Materials and Methods. The fresh isolated primary bone marrow cells were then stained for markers of endothelial (CD31) and hematopoietic cells (CD45) as well as NOTCH3 and CD146. Figure 29 shows that 15% of the CD31-negative, CD45-negative primary bone marrow cells are double positive for CD146 and NOTCH3 in contrast to the 22% of in vitro expanded BM-MSC.

The results indicate the presence of a CD146/NOTCH3 double-positive subpopulation within primary uncultured, human bone marrow for the first time. Whether this subpopulation is enriched in CFU-F, and if so, if it is enriched above and beyond the enrichment based on CD146 only remains to be established in future studies.

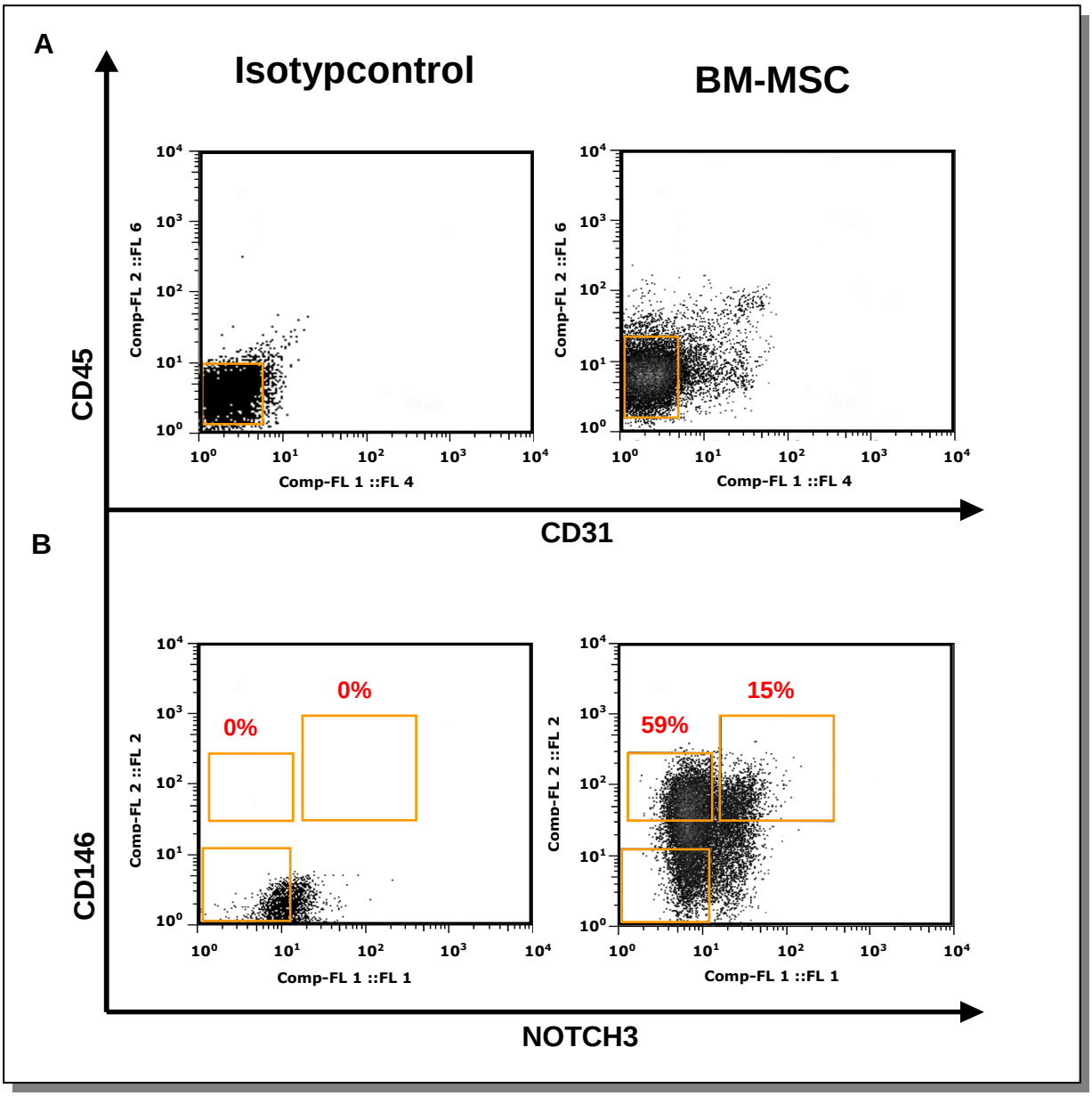


Figure 29: Isolation of CD146+/NOTCH3+ double positive subpopulations of primary unexpanded BM-MSC:

Primary BM-MSC were freshly collected and mixed of various donors to increase cell number for sorting of double positive subfraction. The fresh BM-MSCs were directly used for FACS analysis without previous cultivation. The heterogeneous stromal population were gated from hematopoietic and endothelial cells (A) and finally sorted for CD146PE and NOTCH3FITC single and double positive cells (B). For correct gating and isolation of the CD146+NOTCH3+ double positive subpopulation the sample were compared to the corresponding isotyp control.

2.4.2 Bipotent Differentiationpotential of in vitro expanded BM-MSC in the same Culture

In the present study, we observed that despite osteogenic or adipogenic differentiation stimuli, cells belonging to a different lineage were present (Figure 29). In a pilot experiment, we asked ourselves whether it would be possible to demonstrated both adipogenic and osteogenic differentiation in a single culture. Thus, the sorted subpopulations, here NOTCH3 single-positive BM-MSC, were induced to differentiate into the osteogenic lineage for 10 days. Subsequently, the osteogenic induction medium was removed and substituted with adipogenic differentiation media for further 10 to 15 days. After formation of fatty vesicles, the differentiated BM-MSCs were first stained with Oil red O and than with von Kossa staining. The decision in using the adipogenic staining in the first line was because of the invasive staining process of the von Kossa staining via chemically reduction. It was presumed that the fatty vesicles could collapse and being ineffective to adipogenic staining when performing osteogenic staining first. Due to this fact, the osteogenic procedure was shortened in the last incubation step (see in comparison chapter 5.2.3) and is demonstrated in a weaken colouration in comparison to cells stained with von Kossa only.

In Figure 30 is clearly shown that both adipogenic and osteogenic differentiated cells can be detected simultaneously. In all pictures, adipogenic as well as osteogenic differentiated BM-MSC are presented. Importantly, if the staining I performed on colonies could be a procedure which can be used to show both differentiated lineages in the same colonies.

To prove multilineage differentiation of single CFU-F, clonal differentiation experiments will have to be performed for confirmation of bipotent stromal cell subpopulation in the bone marrow in use of these novel markers.

The possible advantage of this method is that it enables the study of multilineage differentiation in single colonies without the need of dividing the colonies in three fractions and perform separate differentiation experiments. Further development and establishment of its use in MSC studies should be investigated in future studies.

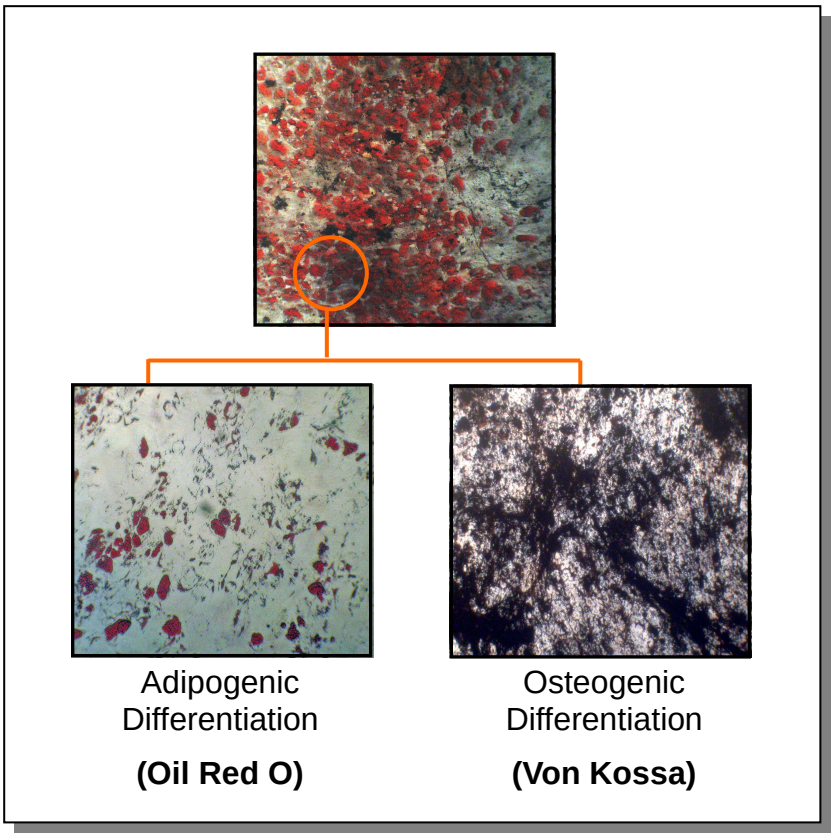


Figure 30: Differentiation of NOTCH3 subpopulations

Osteogenic differentiation was first induced for 10 days and afterwards substitutes with adipogenic media for further 10 to 15 days. For detection of the mineralized matrix of osteogenic differentiated cells von Kossa staining was used. The stromal cells appear in black. In contrast, the fatty vesicle of adipogenic differentiated cells are stained in red by Oil red O. In picture (A) NOTCH3 positive colonies show both detection of adipogenic and osteogenic differentiated BM-MSC. NOTCH3 positive BM-MSCs were also seperatly induced and detected with adipogenic (B) and osteogenic (C) media.

Chapter 3: Discussion

The interest of the scientific community in multipotent stromal cells (MSC) and their possible therapeutic use in regenerative medicine urge a better understanding of the basic biology of these cells. In particular, there is a need to understand early events in mesenchymal differentiation and a proper definition of MSC.

One of the main problems in stem cell biology in general is that in different stem cell systems, the stem cell forms the top of a hierarchy of proliferating and differentiating cells. In any isolation procedure, it is therefore unlikely to isolate many stem cells. It has been shown by many investigators that MSC consist of heterogeneous mixtures of cells, and that the frequency of cells capable of forming mesenchymal colonies is very low [Castro-Malaspina et al., 1980; Owen et al., 1988]. Also, like in many other stem cell systems, colony-forming ability is gradually lost upon extended culture. Thus, considering these analogies to other stem cell systems, the existence of a mesenchymal hierarchy is likely. However, the existence of such a hierarchy has not been definitively demonstrated. In most hierachycal systems MSCs are demonstrated as a population of cells with mixed differentiation potential without showing a clear definition of markers for identification of uncommitted MSC. Hence, there is a search going for markers which would enable prospective isolation of the cell which would form the top of the MSC hierarchy, and then study their ability to self renew and to differentiate.

When the work on this thesis was started, no set of markers was defined which would enable the isolation of multipotent MSC or describe the pathways involved in their self-renewal and differentiation. But also, the use of another mesenchymal source in direct comparison to bone marrow-derived MSC is rarely found. Although the use of two different mesenchymal cell sources would help to give a closer insight of different mesenchymal expression potential and contract the number of possible marker to distinguish between committed and uncommitted progenitors.

Many investigators have found transcripts for genes involved in the regulation of ES cells in MSC. In particular, transcripts for “stemness”-associated genes POU5F1, REX1 (ZFP42), NANOG and STELLA (DPPA3) have all been detected in MSC of the umbilical cord blood or respectively in the bone marrow. At this different embryonic stem cells markers were expressed in rare populations within the bone marrow cells

described as VSEL, MAPC and MAIMI cells [Jiang et al., 2002; Pochampally et al., 2004; D'Ippolito et al., 2004], which suggested that a transcriptional network governing multipotency might be in place in MSC. However, Ratajczak et al. supposes that all research studies focus on the same populations but used different isolation methods [Ratajczak et al., 2007].

Therefore, one subject of the present thesis is whether transcriptional regulators of the pluripotent state, expressed in pluripotent ESCs, are in fact expressed in postnatal mesenchymal progenitors. At this point, I used for comparison different mesenchymal sources, UVSC and BM-MSC and chose the key regulator of the pluripotent state in ESC - POU5F1. An advantage of POU5F1 is not only that this gene is well studied but also that POU5F1 posses in contrast to further known genes of ESC a lower number of pseudogenes.

In a second series of experiments, I set out of novel markers for prospective isolation of uncommitted MSC and also the distinction to more committed progenitors on an antibody-based methode. For this purpose, I compare the gene expression profile BM-MSC and UVSC to determine novel surface markers for isolation of MSC subpopulations and performance of functional studies.

3.1 Osteoprogenitor Potency in comparison of different Stromal Tissues

It has been a long-standing issues of speculation that CFU-F and its derived multipotent progeny, can be isolated from other tissues than the bone marrow. The concept of MSC originated from studies conducted on bone marrow-derived stromal cells [Owen et al., 1988; Caplan et al., 1991]. A number of studies have claimed that multipotent progenitors similar to those found in the bone marrow can also be isolated from a variety of other sources, including the synovial membrane, periosteum, skeletal muscle, fatty tissue, as well as major blood vessels.

The latter also includes the possibility of the existence of multipotent MSC in the blood vessels and surrounding tissue of the umbilical cord. Romanov et al. and Covas et al. described first methodically the isolations and further on the characterization of stromal cells of the subendothelial layer in the umbilical cord vein [Romanov et al., 2003; Covas et al., 2003]. Both authors found that such cells are very similar to bone marrow-derived MSC and the authors proposed the use of these stromal cells in clinical applications. The evidence for the similarity between BM-MSC and UVSC is the similar morphological appearance in culture as demonstrated by a fibroblast-like shape with the ability to adhere on plastic surface as well as expression of the so-called “classical” mesenchymal surface markers STRO-1, CD73, CD90, and CD105, as well as the lack of hematopoietic and endothelial markers. Our own studies described in this thesis, confirm these morphological and phenotypical findings.

The heterogeneic nature of MSC results in several kinds of colonies that show variable differentiation potential [Friedenstein et al., 1982, Herbertson et al., 1997 Berry et al., 1992]. Morphologically, stromal cells present with a typical fibroblastic shape, but also as large flattened cells. Yet, we found that the morphology of UVSC appears more heterogeneous than BM-MSC.

Various studies suggest that UVSC can differentiate in all three AOC mesenchymal lineages [Baksh et al., 2007, Panepucci et al., 2004, Ishige et al., 2009]. Moreover the proliferative potential as well as enhanced osteogenic [Baksh et al., 2007] and adipogenic, chondrogenic differentiation potential claim the stromal cells of the umbilical cord as a better source for mesenchymal stem cells. In the present study,

we cannot confirm the superior differentiation capacity of UVSC suggesting that UVSC and BM-MSC may have different capacities to differentiate, or that the mixture of different progenitor types differs between UVSC and BM-MSC.

Our study is the first in which it was attempted to show MSC activity in an *in vivo* assay. In many other stem cell systems, the ability of stem cells to repopulate the tissue of which the stem cell was derived *in vivo* was not demonstrated in multiple publications. The best example of such a system is the hematopoietic system. However, such *in vivo* transplantation has, so far, not extensively been studied with MSC. Our *in vivo* differentiation experiments revealed substantial differences between the two populations of cells we studied: whereas BM-MSCs were able to generate a complete heterotopic ossicle, as previously reported [Sacchetti et al., 2007], UVSC did not generate bone, adipocytes, or recruit the hematopoietic-microenvironment *in vivo*.

Taken together, the comparison of the stem cell properties of BM-MSC and UVSC *in vivo* shows that the former may be considered to be stem cell-like cells, whereas the latter cannot. These data highlight the importance of assessing multipotency of stromal progenitors *in vivo* and the relatively poor stringency of analyses of phenotype and differentiation conducted in vitro [Sacchetti et al., 2007].

3.2 Misleading Interpretation of “Stemness” Transcripts as Markers for undifferentiated Multipotent Stromal Cells

At the start of this thesis, there were no descriptions that could reliably be used to isolate multipotent CFU-F from primary cell samples of cultures of CFU-F-derived cells. Some investigators, had noticed, however, that transcripts for factors involved in self-renewal of embryonic stem cells, so-called “stemness” markers were expressed by a variety of human cell lines as well as MSC. The present study confirmed that both BM-MSC and UVSC types of putative mesenchymal progenitors expressed PCR-detectable “stemness” transcripts, including POU5F1, NANOG, and others (Figure 16). It has been argued, that the detection of such transcripts may support a genuine pluripotency of postnatal mesenchymal progenitors [Jiang et al., 2002, Pochampally et al, 2004]. To find out whether these views held any truth, we focused on the expression of POU5F1 (also known as OTF3, OCT3 or OCT4), a pivotal transcriptional regulator of the pluripotent state in embryonic stem cells and hypothesized that detection of POU5F1 transcripts would predict the presence of multipotent cells.

POU5F1 is a key regulator of pluripotency in initiation, maintenance and differentiation of pluripotent cells. Niwa et al. demonstrate that not only the presence, but also the dose of POU5F1 (OCT4) determines the balance between stem-cell renewal or differentiation into endoderm, mesoderm and trophoectoderm in ES-cells [Niwa et al., 2000]. The expression of POU5F1 is remarkably dependent on cell density [Jiang et al., 2002] and FCS content [Pochampally et al., 2004], suggesting tight regulation of this “stemness” gene in MSC. These results lend support to the notion that monitoring of “stemness” transcripts could identify undifferentiated multipotent MSC.

The lack of *in vivo* multipotency of umbilical-cord-derived UVSC, would predict that these cells would not express functional levels of either POU5F1 transcripts or protein. Nevertheless, PCR and real-time PCR analysis demonstrate the presence of POU5F1 transcripts in both BM-MSC and UVSC as well as internal control in 2102Ep embryonic carcinoma cells.

The problem with this and other studies is that most primer pairs used to amplify POU5F1 transcripts do not distinguish between a) the POU5F1A and POU5F1B transcript variants or b) POU5F1 and its pseudogenes. Therefore, the detection of pseudogenes may lead to false predication of “stemness”-related markers in the corresponding cell systems. The extent of this problem becomes clear when it is noted that the majority of investigators relies solely on the detection of POU5F1 by PCR and confirmation that a protein product is, in fact, translated from such transcripts is usually not performed.

Indeed, when we sequenced the POU5F1A and B PCR products, we found identifiable PCR products from both transcript variants in both BM-MSC and UVSC. More importantly, we unequivocally demonstrated that the vast majority of amplified transcripts, could in fact be attributed to POU5F1 pseudogene transcripts. Conformation of the lack of expression of the POU5F1 transcription factor was that immunoprecipitation experiments indicated that POU5F1 protein was not detectable, neither in BM-MSC nor in UVSC. Taken together, our results indicate that the presence of POU5F1 transcripts cannot have a dominant regulatory role in MSC multipotency and engraftment.

During the course of this thesis, the support for POU5F1 as an important factor regulating multipotency of adult stem cells has been waning. Primer pairs designed to amplify only true POU5F1A and not its pseudogenes confirmed the expression of POU5F1A in embryonic carcinoma cells but failed to amplify PCR products from peripheral blood mononuclear cells (PBMNC), suggesting POU5F1A is not transcribed in these cells [Liedtke et al., 2007]. Indeed, the present study demonstrate that like human PBMNC [Zangrossi et al., 2007], human tumor cell lines [Cantz et al., 2008], and murine mesenchymal stromal cells [Lengner et al., 2007], neither the multipotent BM-MSC nor non-UVSC in fact expressed detectable levels of POU5F1A protein (Figure 18).

Besides describing unequivocal expression of POU5F1 pseudogenes in BM-MSC and UVSC, this study is also the first to demonstrate clear expression of simultaneous both POU5F1A and B transcripts in these cell types (Table3 and Appendix Table1 and 3). Most primer pairs used in the literature, however, fail to distinguish between the two isoforms, including some I have used [Suo et al., 2005; Roche et al., 2007] and, thus, do not distinguish between POU5F1 isoforms which

contain the N-terminal domain and those that do not. In this study also various published primers for detection of POU5F1 are tested (Figure 17). The analysis reveals that most of the primers used in literature reports, are designed to amplify transcripts containing messages from exons 3 to 5 which are shared between both POU5F1A and B variants as well as most of the known pseudogenes. In our hands, using the transcript variant specific primers described originally by Takeda et al., we can readily amplify both POU5F1 transcript variants from 2102Ep cells, UVSC, and BM-MSC [Takeda et al., 1992]. Thus, our study clearly shows that transcripts for both POU5F1 transcript variants are expressed in BM-MSC, UVSC, and embryonic carcinoma cells.

The abundant number of pseudogenes for “stemness” factors have led to speculations about the possible roles of these genes. However, the relevance of pseudogene expression has remained unclear. Indeed, the existence of pseudogenes is a common phenomenon for homeobox genes involved in early embryogenesis [Holland et al., 2007]. For instance, eleven human NANOG pseudogenes have been described to date [Holland et al., 2007]. One of these pseudogenes, NANOGP8 appears to give rise to a protein product which promotes proliferation in NIH3T3 cells [Zhang et al., 2006]. The murine embryonal carcinoma line P19 expresses a protein which is closely related to POU5F1 and was called Oct4P1. This protein contains most of the N-terminal transactivation domain and part of the DNA-binding POU specific A domain. Like NANOGP8, Oct4P1 promotes proliferation. Perhaps more interestingly with regard to the present study is the observation that Oct4P1 reduces osteogenic differentiation of porcine MSC [Lin et al., 2007], suggesting that the N-terminal transactivation domain of POU5F1 may play a role in the regulation of mesenchymal differentiation.

This highly interesting finding suggests that POU5F1 pseudogene products containing the N-terminal domain, like the POU5F1P1, -P3, and -P4, might support multipotency of MSC. However, whether protein products from these pseudogenes exist or not, is unclear. In fact, sequences for putative proteins from POU5F1P1 [Takeda et al., 1992], -P3 (20 kDa), or -P4 (32 kDa), all expressed by UVSC, have been reported or listed in protein databanks (Appendix Table3).

For detection of POU5F1 protein, we used both monoclonal as well as polyclonal antibodies. The antibodies which detected POU5F1 in 2102Ep cells, recognized epitopes spanning of amino acids 1-134 of the POU5F1 protein which correspond to Exon1 of the POU5F1A variant. Thus, these antibodies will not recognize the POU5F1B variant which lacks the amino acids encoded by exon 1 of the POU5F1 gene. While the monoclonal antibody is highly specific for a single epitope at the N-terminal residue, the polyclonal antibody may possibly bind more different epitopes. Since the pseudogenes P1, P3 and P4 actually contain sequences similar to the exon 1 of the POU5F1 gene, one could speculate, that the antibodies we used, might in fact, detect pseudogene protein products, if any were produced. However, the lack of consistent detection of any protein in Western Blot analysis or Immunoprecipitation using the POU5F1 antibodies (Figure 18) indicates that the POU5F1A transcripts do not give rise to sufficient protein to be detectable. In addition, the fact that we never saw consistent bands with a different size than the ones expected for “true” POU5F1, suggests that the antibodies we have used do either not react with the putative pseudogene proteins or that these pseudogenes do not give rise to a protein product. Since there are no antibodies available which specifically distinguish between these proteins, we cannot exclude the possibility that proteins derived from pseudogenes are expressed or respectively functional active in UVSC or BM-MSC.

Despite the fact that our results show that more than 90% of all POU5F1 amplification products we have sequenced, our results also clearly demonstrate that transcripts encoding the true POU5F1 is, in fact, transcribed in both BM-MSC and UVSC (Table3). When we combine our realtime PCR results with these sequencing results, we can calculate that the level of true POU5F1 transcripts is extremely low and corresponds to less than 8300- (BM-MSC) or 4500-fold (UVSC) the expression we find in pluripotent 2102Ep cells (Figure 16). Thus, we cannot exclude the possibility that a very low level of “real” POU5F1 protein is present at levels undetectable by western blotting or immunoprecipitation. Conversely, we only find POU5F1 transcripts in the 2102Ep cells. However, due to the enormous difference in the level of POU5F1 transcripts compared to BM-MSC and UVSC, we cannot exclude the possibility that pseudogene transcripts are also present in these pluripotent embryonic carcinoma cells.

Given the published finding that high POU5F1 levels are important for its ability to maintain pluripotency and that even the slightest fluctuation in POU5F1 levels may promote differentiation [Niwa et al., 2000], it seems unlikely that the very low level of POU5F1 we detect, plays a major role in “stemness” of BM-MSC. However, since we did not perform our experiments on a single cell level, our experiments leave open the possibility that single cells exist, which may express a high level of POU5F1. If such cells exist, these may be responsible for all true POU5F1 transcripts we find. Nevertheless, based on our results, we predict that such cells would have a frequency of less than 1 in 8,000 BM-MSC or 10,000 UVSC. Therefore, without technology to specifically enrich for such cells, MSC expressing true POU5F1 do not form a relevant proportion of BM-MSC.

3.3 Gene expression Analysis of BM-MSC and UVSC define novel Markers for mesenchymal Progenitor Cells

The previous part has shown that although many investigators have used so-called “stemness” markers (POU5F1, NANOG, etc.) to predict the presence of stem cell-like MSC, these transcripts mostly correspond to pseudogenes [Kaltz et al., 2008]. Thus, the expression of “stemness” transcripts is not a predictor of MSC stem cell-like activity. This conclusion left us with the realization that there is no marker that can uniformally detect or enrich for multipotent MSC or specifically identifying non-differentiated MSC from their differentiated progeny.

Thus, at this point in my studies, I have not come closer to resolve the problem that heterogeneous populations have to be used in situations where only single lineage would be required. In fact, the isolation of homogeneous CFU-F-derived cell populations with defined attributes is still incomplete. The prospective molecules, which could be used for separation of certain well-defined populations remains elusive.

To solve this problem, many research groups tried define more primitive subpopulations. Primitiveness was defined as high proliferative potential combined with a wide spectrum of differentiation potentials. Here, Smith et al. show that a subpopulation of nongranulated small cells (FSC^{low}/SCC^{low}) bone marrow derived cells have high clonogenicity and the potential to differentiate in osteogenic and adipogenic lineages [Smith et al., 2004]. Colter et al. described these fast growing cell type first and named them RS-1 cells (rapidly self-renewing cells) [Colter et al., 2001]. Similarly, the research group around Catherine Verfaillie show the existence of a small, highly proliferative cell population they dubbed MAPC which have the ability to differentiate to cells from different germ layers. Jiang et al. give rise to the most somatic cell types after injection into an early blastocyst [Jiang et al., 2002]. Also, similar to this cell type is the MIAMI cell which shows a high population doubling as well as the differentiation into mesodermal lineages [D'Ippolito et al., 2004]. The MIAMI cells distinguish from the RS-1 cells and MAPC in their expression of surface molecules.

Our own solution to the problem of identifying multipotent MSC was to take the hematopoietic stem cell hierarchy as a model. In hematopoiesis, many different

markers form part of the set of markers known as lineage markers, as well as markers, which are only present or absent from true repopulating stem cells [Shizuru et al., 2005; Kent et al., 2009]. To identify markers that could be used for prospective isolation of multipotent MSC, we performed extensive gene expression studies of populations with known multipotentiality in vitro and *in vivo* (BM-MSC) and compared those with cells without *in vivo* multipotency (UVSC) or limited differentiation potential in general (SFb, POC), or belonging to a different lineage altogether (CD45, CD11b, GlyA). Prior to our study, several surface markers associated with CFU-F (Table2), [Delorme et al., 2008] and bone-forming ability *in vivo* [Sacchetti et al., 2007; Delorme et al., 2008]. Our analysis of gene expression also included non bone-forming cells from the umbilical cord vein [Kaltz et al., 2008]. The idea of this analysis was to include many non-MSC cell types, so that the analysis would result in the identification of a much smaller number of candidate molecules, which would possibly describe uncommitted MSC.

We here refine previously published gene expression comparisons, by comparing BM-MSC, skin fibroblasts (SFb), periosteal cells (POC), hematopoietic lineages (CD45, CD11b, CD235a (GlyA)) as well as UVSC. Our experiments had shown that UVSC did not posess bone-forming ability *in vivo* (Figure 15).

On the basis of this complex comparison, we constructed a list of molecules possibly preferentially expressed on multipotent BM-MSC (Appendix Table4). We then attempted enrichment for CFU-F using antibodies directed against some of these molecules (Figure 25 and 36, Appendix Table4).

The gene expression analysis of the study presented in this thesis indicates that differences between BM-MSC and UVSC exist (Appendix Table4 and 5) which are comparable to a previously published SAGE analysis of these two cell types [Panepucci et al., 2004]. For instance, UVSC express more MMP1, ACTG2, and KRT8 and BM-MSC seem to show increased expression of osteogenesis-associated genes like POSTN and IGFBP3.

Interestingly, the gene expression pattern of our BM-MSC show remarkable overlap with that of the D7 MSC published by Larson and coworkers [Larson et al, 2008]. In fact, 122 tags which are expressed more than two-fold in D7 MSC compared to D2 MSC are also expressed more than two-fold in BM-MSC (26%), and the proportion is even slightly higher in the membrane-associated tags (an overlap of 31 overlapping

tags of 102, 31%, Appendix Table6 and 7), which include NOTCH3 and FGFR2.

As part of this thesis, I identified possible new markers for identification and prospective isolation of MSC and their progenitors. For practical reasons, due to the availability of specific antibodies, the eventual follow-up study of MSC function was limited to molecules to which antibodies were available which stained sub-populations of BM-MSC in flow cytometric analyses. My study shows that NOTCH3 and FGFR2 are expressed on multipotential MSC. Whether these molecules are involved in maintaining MSC multipotency remains to be established in future studies.

3.3.1 NOTCH3 and JAG1 as a novel Markers of multipotent MSC

NOTCH3 is only identified in mammals and therefore it is thought that its phylogenetic origin is relatively recent [Faux et al., 2001]. NOTCH3 is important for vascular formation, and appears to be involved in regulating proliferation and migration of endothelial cells and vascular remodeling processes. Iso et al. and Domenga et al. reported a role for NOTCH3 in maturation of vascular smooth muscle and suggested its role in smooth muscle differentiation [Iso et al., 2003; Domenga et al., 2004]. Therefore, it is not surprisingly that the mutation of NOTCH3 cause vascular degenerative disease named as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy).

As the name already implies NOTCH3 is member of the NOTCH-family that is known to be involved in the embryonic development and in adults for the maintenance of the homeostasis including proliferation, apoptosis and cell-fate decision [Artavanis-Tsakonas et al., 1999]. The regulation of self-renewal together with other signal pathways such as the WNT-signaling and hedgehog [Molofsky et al., 2004] belong equally to the role of NOTCH-signaling. Calvi et al. demonstrate regulatory effect of the HSC niche *in vivo* by osteoblastic cells [Calvi et al., 2003].

Depending on the context, the activity of NOTCH signaling reacts differentially. While on the one hand NOTCH inhibits differentiation for maintenance of an undifferentiated state like in stem cell, on the other hand NOTCH can also induce differentiation for terminally differentiation. Due to the importance of NOTCH1 in developing stem cells in the fetal hematopoiesis NOTCH function in inhibitory way in adult bone marrow. Calvi et al. demonstrate regulatory effect of the HSC niche *in vivo* by osteoblastic cells with increased expression PTH (parathyroid hormone) [Calvi et

al., 2003]. In our hands, NOTCH3 antibodies strongly stain BM-MSC with the polyclonal antibody we used. The NOTCH3+ and NOTCH3+ CD146+ subpopulations we isolated contain CFU-F which reveal a strong adipogenic and osteogenic potential. Thus, NOTCH3 is expressed by multipotent CFU-F and can be used for prospective isolation and enumeration of CFU-F.

Another molecule, which is a known ligand of the NOTCH3 receptor, is JAG1 [Liu et al., 2009; Chiba et al., 2006]. Our microarray analysis and subsequent functional studies reveal that JAG1 is expressed by a small subset of CFU-F with a very high cloning efficiency. Thus JAG1 is demonstrated as novel molecule for isolation and enumeration of prospective MSC subpopulations. However, since JAG1 is trypsin sensitive, in order to detect the JAG1 molecule, the staining procedure required a brief culture period to re-express the antigen. Since we also did not detect a good expression after collagenase treatment, the required culture prior to detection somewhat limits the use of JAG1 as a marker for multipotent BM-MSC. Further on, we did not analyze this molecule.

The prominent expression of both NOTCH3 and JAG1 in BM-MSC strongly suggests that NOTCH signaling may be functionally active in MSC. What exact role NOTCH3 and JAG1 may play in MSC biology is a matter of speculation. NOTCH3 is known to be expressed by perivascular cells and its expression is upregulated by JAG1 [Liu et al., 2009]. Since NOTCH3 is involved in proliferation and differentiation of vascular smooth muscle cells [Wang et al. 2002; Domenga et al., 2004; Wang et al., 2008], BM-MSC could be similarly affected. Indeed, there is experimental evidence that NOTCH stimulation may inhibit differentiation of MSC [Buas et al., 2009]. Hence, an autocrine NOTCH3/JAG1 loop may serve to keep MSC in an undifferentiated state, a hypothesis which needs to be investigated in future studies.

3.3.2 FGFR2 as Marker of uncommitted MSC

A similar differentiation potential to that of NOTCH3 was detected in FGFR2 positive subpopulations. FGFR2 is a receptor for multiple FGFs. Since it has been reported that FGF2 improves the proliferation of MSC [Hanada et al., 1997; Delorme et al., 2009], it may will be that FGFR2 is involved in MSC proliferation. The fibroblast growth factor receptors are known to be expressed in multiple tissues throughout the vertebrate development and play important roles in skeletal differentiation and

growth. Mutations along with FGFR genes result in craniosynostosis syndromes [Wilkie et al., 1997, Muenke et al., 1994]. The mutation in the isoform (region IgIIIc) of FGFR2 causes next to Crouzon the Pfeiffer syndrome characteristics in craniosynostosis causing facial bones and facial structure or respectively broad thumbs and toes, flat occipitus, and cloverleaf skull.

It is therefore not surprisingly that FGFR2 is expressed in osteoprogenitors [Iseki et al., 1997] or respectively some of the isoforms of FGFR2 (IIIb) are detected in differentiating osteoblasts [Moerloze et al., 2000].

We here show for the first time that FGFR2 positive subpopulations from mesenchymal stromal cells of the human bone marrow differentiate into adipogenic cells. Involvement of FGFR2 in bone formation was already known. It has been demonstrated that both FGFR1 and FGFR2 are imporatant in skeletogenesis [Iseki et al.,1997]. In mice, FGFR2 appears to be mainly expressed in proliferating osteoprogenitors. This study also suggested that signaling through FGFR2 regulates MSC proliferation [Iseki et al., 1999].

3. 3.2 ITGA11 as a novel Marker of osteogenically inclined MSC

A third marker, which was overrepresented in BM-MSC in comparision to UVSC, SFb, POC and hematopoietic lineages was the integrin alpha-11. Sorting of ITGA11-positive subpopulations show a strong enrichment of CFU-F from BM-MSC and ITGA11+ CD146+ double positive show a significant higher enumeration of colonies compared to CD146 positive subpopulations alone. In differentiation studies, cells expanded from ITGA11+ BM-MSC appear to have only limited adipogenic potential and differentiate much more strongly into osteogenic cells.

Since ITGA11 expression was previously noted predominantly in bone, cartilage, cardiac muscle as well as skeletal muscle [Lehnert et al., 1999], its preferential expression on osteoprogenitors is not a surprise. ITGA11 belongs to the integrin family that is known to be involved in processes of proliferation, migration, and differentiation [reviewed Adams et al., 1993]. Tormin et al. shows a high expression of ITGA11 in bone marrow-derived MSCs in a population with SD/ND (slowly/non-dividing) cells in comparison to the RD (rapidly dividing) cells [Tormin et al., 2009].

The ND cell population was found to represent a large and cuboid-shaped morphology and show osteogenic differentiation. Among these ND cells also rare population of small spindle-shaped cells are found with full differentiation potential. These cells within the ND cell population are suggested to act as an early progenitor with high expression of ITGA11.

Based on the findings presented in this thesis, it can be postulated that ITGA11 represents a marker of early osteoprogenitors in a small population of non-dividing cells. Further studies have to be performed to test this hypothesis with respect to clonality and osteogenic differentiation in certain MSC subpopulations in the bone marrow.

To test the expression of ITGA11 in osteogenesis-committed cells, staining could be combined with other markers already shown to be preferentially expressed in osteogenic cells, such as CD63 or HOP-26 [Joyner et al., 1997; Zannettino et al., 2003; Steward et al., 2003]. This glycoprotein belongs to the tetraspanin family (TMS4) which is involved in migration, adhesion, proliferation as well as differentiation and, interestingly, CD63 is part of integrin-assembled complexes [Berditchevski et al., 2001]. Tetraspanins like CD63 have been described to modulate integrin signaling and compartmentalize integrins on the cell surface [Berditchevski et al., 2001]. Whether both molecules, CD63 and ITGA11, are involved in the same protein complex in early osteoprogenitors is unknown.

3.4 Hypothetical hierachical Model based on an antibody-mediated Classification

As discussed in the introduction to this thesis, one of the goals of the work performed was to find out whether a mesenchymal hierarchy, containing early multipotent cells as well as more mature bi- and uni-lineage-restricted cells, exists.

Reviews of Minguell et al. and Baksh et al. represent a highly similar delineation by distinguish between committed compartment with unipotent until terminally differentiated MSC and stem cell compartment including multipotent MSCs that through asymmetric and symmetric division tri- or bipotent precursors arise to more commited MSC [Minguell et al., 2001; Baksh et al., 2004]. In most hypotheses, the tripotent MSC type represents an early mesenchymal progenitor, which shows a sequential loss of lineage potential [Muraglia et al., 2000]. Dennis et al. distinguishes between MSC compartment and differentiation compartment and proposes that the MSC compartment consist of uncommitted and committed progenitors with the ability to tri-, bi-, and mono-lineage differentiation [Dennis et al., 2002]. These authors go a step further and also propose that the lineage relationships between the early and more mature progenitors depends on stochastic repression/activation processes. Between them existing also MSC phenotypes with various mixed gene set which not belong to the classical adipogenic, osteogenic and chondrogenic mesenchymal differentiation way. This model allows a high plasticity beyond different MSCs induced by preceding epigenetic events.

Therefore, lineage-specific epigenetic modification like methylation or demethylation will arise to alterations in lineage commitment. It can be propose that modulation and diversification will restrict or allow differentiation processes by imprinting effects like in osteogenic differentiation of stromal cells [Satomura et al., 2000]. The mechanism how these processes function is not completely understood as well as how these processes will distinguish between MSC compartment and differentiation compartment [Dennis et al., 2002] and pursues that the MSC compartment consist of uncommitted and be influenced by possible intrinsic, extrinsic factors, or effects still needs to be elucidated.

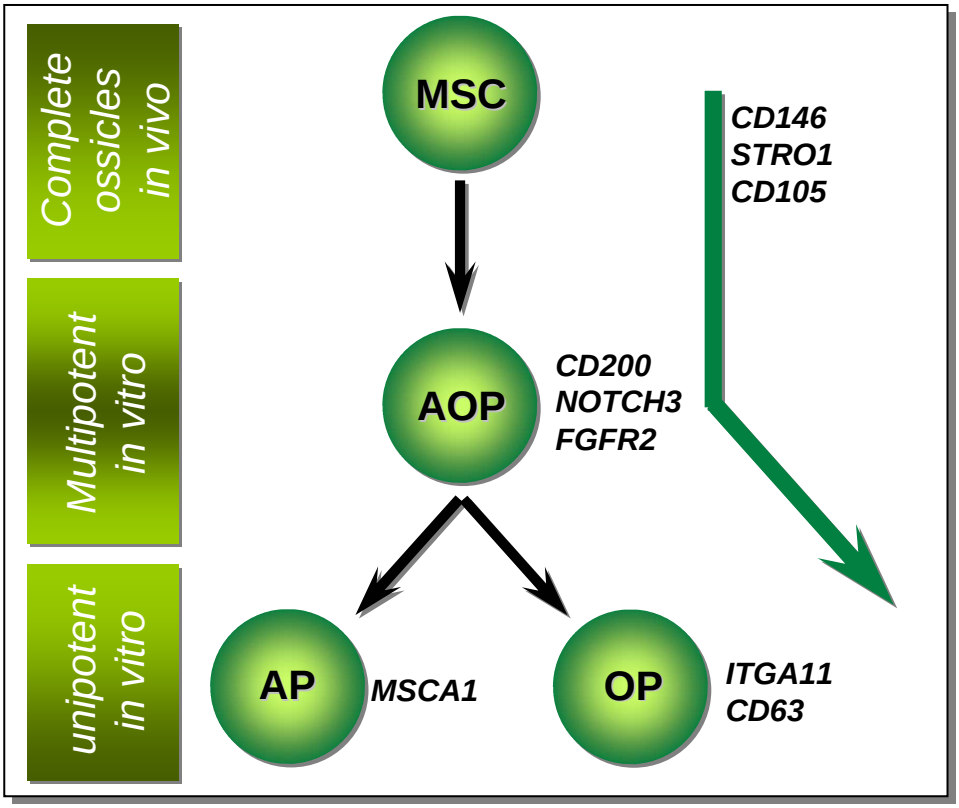


Figure 31: Hypothetical model of selected cell surface markers for representation of the mesenchymal hierachy

The expression of the genes appears on MSCs with different lineage potential. MSCs with the ability of in vivo bone formation and HME support expressing STRO1 and CD146, which is also detected throughout all delineated potentials. Additionally, multipotent MSCs could be detected with the expression of CD200, NOTCH3 and FGFR2 *in vitro* while unipotent stromal cells demonstrate markers like MSCA-1 for adipogenic lineage and ITGA11, CD63 for osteogenic lineage.

The hierachical model presented in Figure 31 distinguishes multipotent MSC *in vivo* and multipotent MSC *in vitro* as well as unipotent progenitors. As mentioned before, the tripotent CFU-F or CFU-F-derived MSC with tri-lineage differentiation potential represent a more uncommitted mesenchymal cell type.

We propose that *in vivo* transplantation of possible multipotent MSC is an important part of the model. As we have shown, cells which may be very similar *in vitro* may have strongly diverging *in vivo* repopulating abilities (Figure 14). Thus, sorting of cells for surface markers which allow the formation of ossicles including the establishment of the hematopoietic niche, like CD146 [Sacchetti et al., 2007] may mark earlier cell populations than markers expressed by *in vitro* the multipotent cells which only form bone (but no HME recruitment) *in vivo*, like CD200 [Delorme et al., 2008].

Thus, CD200 expression is probably acquired after cell fate decision towards *in vivo* osteogenesis-regenerating cells.

In the study presented in this thesis, we followed the idea that for clinical applications, it might be more useful to isolate clinically relevant MSC subpopulations after culture expansion, rather than isolate those cells from primary, uncultured marrow samples. Our data support the view that FGFR2 and NOTCH3 expressing MSC represents more primitive mesenchymal progenitors *in vitro*. Very likely, these cell populations overlap in differentiation potential with CD200-positive cell fraction described in the literature [Delorme et al., 2008]. Also very likely, ITGA11- and MSCA-1-positive subpopulations may constitute a more committed cell type with lineage specific differentiation potential. Whereas ITGA11 represents a novel marker for osteogenic lineage and can be categorized with CD63 as marker for osteoblasts lineage, MSCA-1 demonstrates a representative marker for adipogenic lineage.

Taken together, the novel surface markers identified in the latter part of this thesis lend support to a new hypothetical, hierachical model which distinguishes between multipotent and unipotent mesenchymal stromal cells of the bone marrow.

Chapter 4: Future Aspects – Trouble shooting to improve the Study of MSC Biology

The presented study employs a genomics approach for identification of novel markers that can be used to enrich for subpopulations of uncommitted and mature MSC. To prove whether the model proposed in hierarchical model presented in Figure 31 is correct, more in-depth studies will have to be performed.

- One very important aspect which hasn't been addressed in this thesis is the study of differentiation on a clonal level. Such experiments should confirm the multipotent capacity of the isolated subpopulations of NOTCH3 and FGFR2. Clonal studies on isolated CFU-F are absolutely required to nail down the proposed hierarchy as well as definitively proving that single CFU-F with tri-lineage AOC potential exist, as well as what their frequency is in primary BM or cultured BM-MSC.
- Additionally, it has to be demonstrated that chondrogenic differentiation is also present in the sorted subpopulations. Due to the screening procedures we used, it was not possible to do such cultures in the expensive and very labor-intensive micropellet cultures. However, now that some possible CFU-F markers have been established, some selected subpopulations should be studied for chondrogenic potential.
- Based on the differentiation, it is also important to consider stringent cell culture conditions to optimize CFU-F density and optimal expression of CD146 as described at Sacchetti et al. [Sacchetti et al., 2007]. In our studies, we mostly used MSC at a density of around 60-70% confluence. It may well be that, like recently shown for expression of PODXL on BM-MSC, that NOTCH3, ITGA11, FGFR2 or JAG1 expression are affected by cell density.
- Also, we used commercially available antibodies to detect NOTCH3, ITGA11, FGFR2 and JAG1 which were optimized for detection of these antigens in western blot. Thus, the specificity and detection of these antigens may be greatly improved by establishing antibodies specifically detecting unreduced surface-expressed antigens.

- Another important factor for future studies is to validate the robustness of the differentiation. It will be very helpful to be able to measure and normalize AOC differentiation to cell number. Furthermore, for assessment of the differentiation studies shall be performed for quantification of mineralized cells by densitometry [Chateauvieux et al., 2007]. Adipogenic differentiation should be assessed by histochemical observation as described in Baksh et al. [Baksh et al., 2007].
- Importantly, since we have shown that “classical” protocols for adipo- and osteogenic differentiation induction of MSC may give rise to expression of markers for the “wrong” lineage, optimation of induction protocols is required. The studies with regard to surface marker expression of MSCA-1 (adipogenic marker) and ITGA11 and CD63 (osteogenic markers) could in fact, be used in such experiments. We propose that it might be useful to include PPAR-γ antagonists to improve osteogenic differentiation, and conversely, PPAR-γ agonists in adipogenic induction. Also, since Wnt signaling has been shown to be involved in osteogensis, Wnt activators should be helpful in increasing osteogenic potential.
- Finally, the MSC field suffers from the lack of an assay which unequivocally demonstrates possible stem-cell like behaviour *in vivo*. For this purpose, an *in vivo* assay which allows the complete regeneration of mesenchymal tissues (bone, cartilage and fat, as well as bone marrow microenvironment recruitment) has to be established. The transplantion of MSC *in vivo* and harvests based on protocols of Krebsbach et al. and Sacchetti et al. may greatly enhance the field [Krebsbach et al., 1997 and Sacchetti et al., 2007]. The determination of the frequency of stem cell-like MSC will be a major challenge. In hematopoiesis, such frequencies are determined using limiting-dilution analyses. However, since the activity of MSC may depend on the dose of cells transplanted [Mankani et al. 2007] such an analysis may prove challenging.

Chapter 5: Methods

5.1 Cell Culture Methods

5.1.1 Isolation of primary human Stromal Cells from Umbilical Cord Vein (UVSC)

Human umbilical cord was obtained after parental consent from clinically normal pregnancies and caesarean sections. All clinical procedures were approved by the Committee on Medical Ethics of the Klinikum rechts der Isar.

Fresh umbilical cords were stored in cold (4℃) in HF/1+ (see below) until further preparation. Prior to cell isolation, umbilical cords washed in 1xPBS (Gibco, Invitrogen, Germany) and then sterilized in 70% ethanol. To obtain the umbilical vein stromal cells (UVSC), the umbilical vein was flushed out by attaching the vein to a knobbed canula at both ends of the cord. The canulas were linked to a faucet (Discofix, BBraun, Germany). Subsequently, the umbilical vein was washed twice with cold 1xPBS and one time with cold HF/2+ buffer (see below) to wash out the remaining blood and for preparation of the digestion.

UVSC were isolated from the umbilical vein in a two-step procedure. First, after the last wash with HF/2+, the vein was filled with 0.5 mg/ml collagenase type 2 (205 Unit/mg CLS2, Worthington Biochemical Corporation, USA, distributor CellSystems GmbH, Germany) and incubated for 40 min at 37℃ to digest the inner endothelium wall. Thereafter, the vein was washed twice with HF/2+ buffer and once with 1x PBS.

HF/1+ Buffer* (Total Volume 500ml)

Components	Volume/Concentration	Company
Hank's Buffered Salt Solution (HBSS) 1x		Gibco, Invitrogen, Germany
Heat-inactivated Fetal calf serum (FCS)	1%	PAA,Germany
Penicillin/Streptomycin (P/S) 1x	1%	Gibco, Invitrogen, Germany
Hepes	1M	Gibco, Invitrogen, Germany

* HF/2+ buffer is supplemented with 2% FCS

The first flow through contain endothelial cells (human umbilical vein endothelial cells, also known as Huvec). In the second step, the umbilical vein was filled with collagenase for a second time and again incubated for 40 min. at 37℃. The wash-out of this second incubation contains the subendothelial cells, most of which are stromal cells (UVSC). The subendothelial cells were washed out by HF/2+ and collected in a separated tube. The cell number was determined and finally the UVSC were plated out and cultured described below.

5.1.2 Isolation of primary human Stromal Cells from Bone marrow

Bone marrow was obtained from iliac crests from diagnostic samples after informed consent and were approved by the Committee on Medical Ethics of the Klinikum rechts der Isar.

The BM-MSCs were cultured from bony pieces and the fatty layer of diagnostic bone marrow donor samples. The marrow samples were shaked vigorously in HF2+ to release the cells from the fat and bony pieces. Bone marrow cells were then isolated using Ficoll-gradient centrifugation. At this, the samples were diluted 1:1 with 1x PBS and slowly covered on the Ficoll (density is 1.077 g/ml; Biocoll Biochrome AG, Germany) layer. Diluted samples were centrifuged (2000 rpm for 20 min. without brake) to separate the mononuclear cells from blood, death cells and further residues. The mononuclear bone marrows cells were collected from the interphase and washed twice in HF/2+ buffer. After determination of the cell number, the bone marrow cells were cultured in MSC culture medium (see below).

For the experiments, only cells from donors without hematopoietic abnormalities were used.

Cell Culture Medium (Total Volume 500ml)

Components	Volume/Concentration	Company
Alpha Minimal Essential medium (MEM) GlutaMax	(445ml)	Gibco, Invitrogen, Germany
Heat-inactivated Fetal calf serum (FCS)	10% (50ml)	PAN, Germany
Penicillin/Streptomycin (P/S) 1x	1% (5ml)	Gibco, Invitrogen, Germany

5.1.3 Isolation of Umbilical Cord Blood

For mononuclear cell separation, umbilical cord blood (UCB) was diluted 1:1 with PBS and layered on a Ficoll-gradient and centrifuged as described above. After centrifugation, the interphase (buffy coat) was isolated and washed twice in 1x HF/2+ buffer. The cell number was determined using hematocytometer (slides) (Neubauer counting chamber; Marienfeld GmbH, Germany). Finally, the UCB were resuspended in FCS (PAA, Germany) with 11% DMSO and frozen in liquid nitrogen for storage.

5.1.4 Culture of Stromal Cells and embryonic Carcinoma Cells Ep2102

Freshly isolated UVSC (second collagenase treatment flow-through of the umbilical cord) were cultured at 10.000 cells/cm^2. Contaminating non-adherent cells were removed after medium change a day later. Both bone marrow-derived MSC (BM-MSC) and UVSC were cultured in Cell Culture Medium [described also in Delorme et al., 2008] and incubated at 37℃ with 5% CO^2. After two to three weeks, the formation of fibroblastoid colonies was visible. For passaging, the these CFU-F-derived colonies were detached by trypsin digestion (0,1% trypsin/EDTA).

For enumeration studies, primary CFU-F cultures were established at lower cell densities: 0.1 -10 x 10^3 cells per cm^2 for BM-MSC or 0.5–20 x 10^3 for UVSC. The number of colonies was then determined prior to detachment. After enumeration, CFU-F-derived colonies were harvested using trypsin, pooled to generate multiclonal strains, and replated for additional studies. In UVSC and BM-MSC cultures, the medium was replaced every three days. In subsequent culture, adherent cells were passaged at 60 -70% confluency.

Embryonic carcinoma cells (ECC) (h2102Ep Clone 2/Ab, [Andrews et al., 1982]) were a friendly gift of Peter W. Andrews (University of Sheffield, UK). The EC cells were cultured in EC-medium (see below). The medium was changed every 3-4 days. The 2102Ep cells were passaged with 80-90% confluency and reseeded at 100.000 cells/cm^2.

Embryonic Carcinoma Medium (Total Volume: 500ml)

Components	Volume/Concentration	Company
Dulbecco's Modified Eagle Medium (DMEM) (without Na-pyruvate)	(440ml)	Gibco, Invitrogen, Germany
Heat-inactivated Fetal calf serum (FCS)	10% (50ml)	PAN, Germany
L-Glutamin (200mM)	1% (5ml)	Gibco, Invitrogen, Germany
Penicillin/Streptomycin (P/S) 1x	1% (5ml)	Gibco, Invitrogen, Germany

5.1.5 Determination of the Cell Number and Viability

The MSCs were expanded until the flattened cells are just starting to touch each other (60 - 70%) confluency and detached using 1ml 0,1% Trypsin/EDTA (GIBCO) diluted in 1xPBS. This trypsination procedure described below has been performed to all other adherent cells used in this study. After 3-5 min. incubation, detachment is assessed under a phase-contrast inverted microscope. When appropriate, the trypsin/EDTA reaction will be stopped by adding serum-containing washing medium (see below). The detached cells will be collected and washed by several centrifugation steps (5 min, 500g (1500 rpm) and finally resuspended in 1ml DMEM. Depending on the size of the pellet, the resuspension volume can vary and was considered later after counting of the cells.

The number of viable cells was determined using the trypan blue exclusion method. For this purpose, cells were mixed with trypan-blue solution (Gibco, Invitrogen, Germany) and counted in a hematocytometer (Marienfeld GmbH, Germany). The assay is based on the observation that viable cells have an intact cell membrane, which trypan blue cannot infiltrate (trypan blue is excluded) whereas a damaged cell membrane allows blue staining of the cell which marks them as not viable.

Washing Medium (Total Volume: 500ml)

Components	Volume/Concentration	Company
Dulbecco's Modified Eagle Medium (DMEM) (without Na-pyruvate)	(440ml)	Gibco, Invitrogen, Germany
Heat-inactivated Fetal calf serum (FCS)	10% (50ml)	PAA, Germany
Penicillin/Streptomycin (P/S) 1x	1% (5ml)	Gibco, Invitrogen, Germany

5.1.6 Freezing and Thawing of the Cells

Cells were washed twice in 1xPBS to remove media and in particular FCS. Afterwards, cells were detached and collected with washing media. After centrifugation, the cells were resuspended in 1ml washing medium for determination of the cell number.

UVSC (>1x10^6) were resuspended in 500µl basic-freezing media. After 15 min. incubation at 4℃ 500µl Freezing-medium was added a nd finally stored at -80℃ over night in a freezing container ("Mr. Frosty", Nunc-Nalgene USA, distributor Thermo Fisher Scientific, Germany) containing isopropanol. The following day the cells were transfered to liquid nitrogen.

BM-MSC (>1x10^6) were frozen in FCS, 11% DMSO and stored like described before. The same freezing method was also used for for UCB, HUVEC as well as for Ep2102 embryonic carcinoma cells.

UVSC and BM-MSC were thawed using a step-wise thawing whereas all two minutes the double amount of thawing media were added for 10 min. Afterwards the cells were washed by centrifugation at room temperature at 1400rpm for 5 min. The supernatant was removed and the cell pellet was resuspended, depending on the size of the pellet, in 1 to 10 ml washing medium. Subsequently, the cells were cultured as described above.

UCB, and 2102Ep were thawed slowly by adding of washing medium until the cells are completely fluid. The cells were centrifuged, counted, and cultured as mentioned above.

Basic Freezing Media

Components	Volume/Concentration	Company
Iscove's Modified Dulbecco's Media (IMDM)		Gibco, Invitrogen, Germany
Heat-inactivated Fetal calf serum (FCS)	25%	PAA, Germany
Penicillin/Streptomycin (P/S) 1x	1%	Gibco, Invitrogen, Germany

Freezing Medium

Components	Volume/Concentration	Company
Basic Freezing Media		
DMSO	11%	Serva, Germany

Thawing Medium

Components	Volume/Concentration	Company
Hank's buffered salt solution (HBSS) 1x		Gibco, Invitrogen, Germany
FCS	10%	PAA, Germany
P/S	1%	Gibco, Invitrogen, Germany
DNase (3000u/ml)	1%	

5.1.7 Flow Cytometric Analysis and Sorting of UVSC and BM-MSC

Flourescence activated cell sorting (FACS-Analysis) is used for fast and quantitative analysis of single cell suspensions. Various cell properties can be analysed simultaneously, such as cell size (forward scatter) and granularity (side scatter) and this can be combined with the detection of surface markers labelled with different fluorescent dyes, or other properties (for instance enzyme activity) which emit at certain wavelengths. In this thesis, the FACS-analysis is performed for determination of the surface markers in MSC derived from bone marrow and umbilical vein and for sorting experiments to isolate certain subpopulations.

5.1.7.1 Performance of the FACS-Analysis

Stromal cells derived from umbilical cord and bone marrow were cultured as described above. Adherent BM-MSC and UVSC were enzymatically detached using Collagenase (0.5 mg/ml collagenase in HF/2+) Type II or 0,1% trypsin/EDTA following manuals instruction of the used antibody. After culture, the detached cells were washed and pelleted (600g, 5 min). Cells which receptors are sensitive to trypsin digestion were incubated in MSC medium to allow regeneration of receptor formation for at least 2 hours under vigorously rocking movement following the manuals instruction.

For the staining procedure with the antibodies indicated (see List of Antibodies), the cells were labelled with unconjugated or conjugated primary antibodies for 20 min. on ice. For each specific antigen-binding antibody sample, an additional sample was stained with the corresponding isotype controls for FACS-Analysis and sorting experiments. After washing (600g, 5 min) once in FACS buffer, the cells were resuspended and labelled with secondary antibodies or resuspended in FACS buffer directly for analysis. Before the FACS-analysis started, each sample was supplemented with 1 µg/ml Propidiumiodid (PI) (Invitrogen) in 1x PBS (Carl Roth, Germany). The propidium iodide is used for staining and selecting the dead cells from the viable cells in the analysis. Like the trypan blue exclusion described above, PI only penetrates the perforated membrane of dead cells and can be detected in fluorescence channel (FL)3.

The samples were analysed on an EPICS XL flow cytometer (Beckman Coulter) or were sorted on a MoFlo high performance cell sorter (Beckmann-Coulter). The FCS data was analysed using Flowjo Software (Tree Star Inc.,Ashland, USA).

FACS-Buffer

Components	Volume/Concentration	Company
1x PBS		Gibco, Invitrogen, Germany
Bovine Serum Albumin (BSA) (Albumin fraction V)	0,5%	Carl Roth GmbH & Co KG, Germany
P/S	1%	Gibco, Invitrogen, Germany

5.1.7.2 Testing of Antibodies for optimised Use

In order to optimize the FACS-Analysis and especially to perform sorting experiments the choice of the correct antibody concentration is important. Too much antibody results in unnecessary background staining; less amount of antibody may result in weak staining. For this purpose, newly acquired antibodies were tested for binding efficiency and optimized use by titration of different concentrations. At this, the stromal cells of both BM-MSC and UVSC were prepared as described above with different antibody concentration as shown in Figure 1.

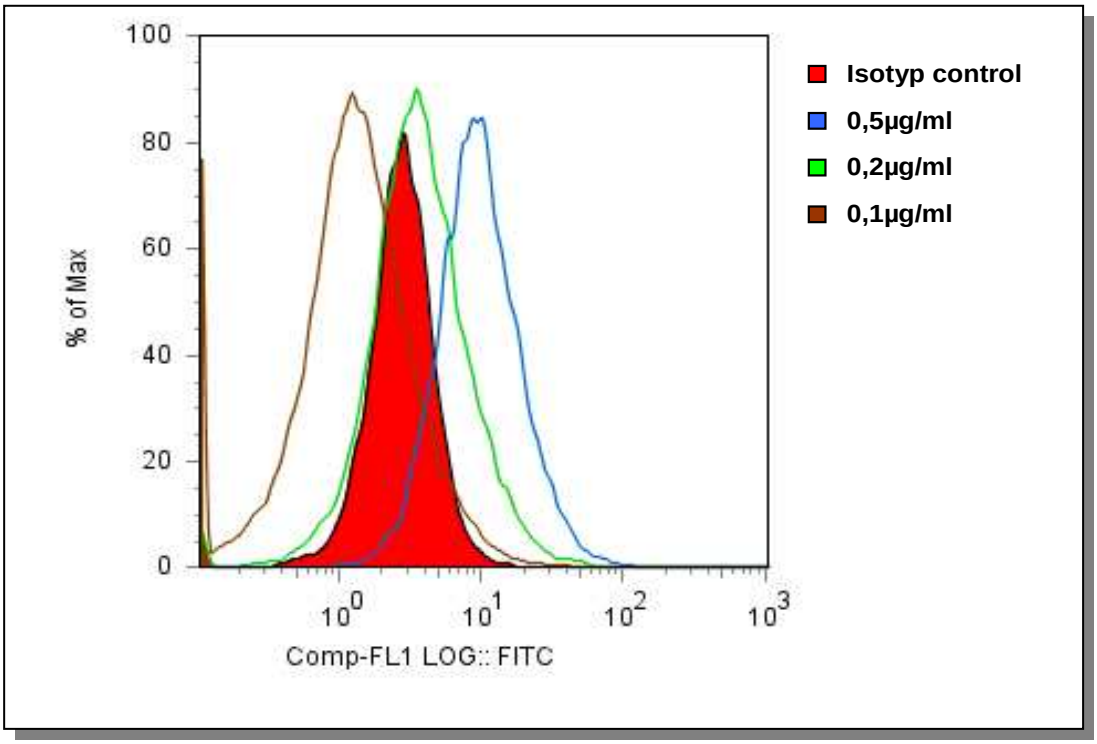


Figure 32.: Titration of used antibodies for optimal concentration

Shown is titration of Alexa Fluor 488 labelled NOTCH3 antibody. The shifts of the different concentrations are clearly demonstrated. The negative fraction is presented in red filled peak.

5.1.7.3 Gating strategy and isolation of single and double positive subpopulations in FACS-Analysis

The antibodies used in this thesis are either directly labelled with fluorescent dye or biotin or alternatively will be stained indirectly by using a fluorescent labelled secondary antibody or streptavidin. The staining of the cells with certain antibodies, which are known to recognize epitopes of pre-determined antigens, can be used to separate the heterogeneous population in distinct subpopulations.

To distinguish between dead and viable cells, I additionally stained cells in the last washing step with the DNA-binding dye propidium iodide (PI). In the present work most of the cells were stained using a two-colour method including FITC, PE, and PE-Cy5 (see List of Antibodies below). After gating of the viable cell populations, the cells were analysed for surface characteristics positive or negative of the used antibody.

Procedure of gating and Isolation of distinct subpopulation

The MSCs were washed and stained as described before. For isolation of the single and double positive cell populations different steps were performed to guarantee the sorting of purified subfraction:

- Set up of FSC/SSC with unstained MSCs of both cell types BM-MSC and UVSC to separate the cells from contaminating and disturbing material.
- Setting of all detectors (on unstained cells), like FL3 for exclusion of dead cells stained with PI as well as FL1 (FITC), FL2 (PE) and FL4 (PE-Cy5) staining.
- Compensation: this requires the recording of positive controls for each single color (like CD13 for stromal cells or CD45 for hematopoietic cells). Using single stained controls like FITC, PE, PE-Cy5, compensation parameters for precise determination of the compensation matrix can be calculated.
- Isotype control: to each antibody in the sort analysis, the appropriate antibody control is used for exclusion of false positive cells.

Cell sorting was performed on a MoFlo sorter in collaboration with Dr. Matthias Schiemann (Institute of Microbiology, Hygiene, and Immunology, TU Munich). The Moflo cell sorter allows detection of up to 13 different fluorescence labels and sorting

of up to eight. Thus, additional fluorescence labels (PE-Cy5, PE-Cy5.5, PE-Cy7, Pacfic Blue, APC, APC-Cy7) could be used. In essence, the FACS procedure for the MoFlo is the same as on the Epics XL with only four detectors. For optimization of the isolation of highly purified subpopulations, MoFlo detectors were set using single fluorescent dye controls first. For each sample, the corresponding isotype controls were then measured to distinguish between false-positive populations and to finally determine the correct subfraction (see Figure 33).

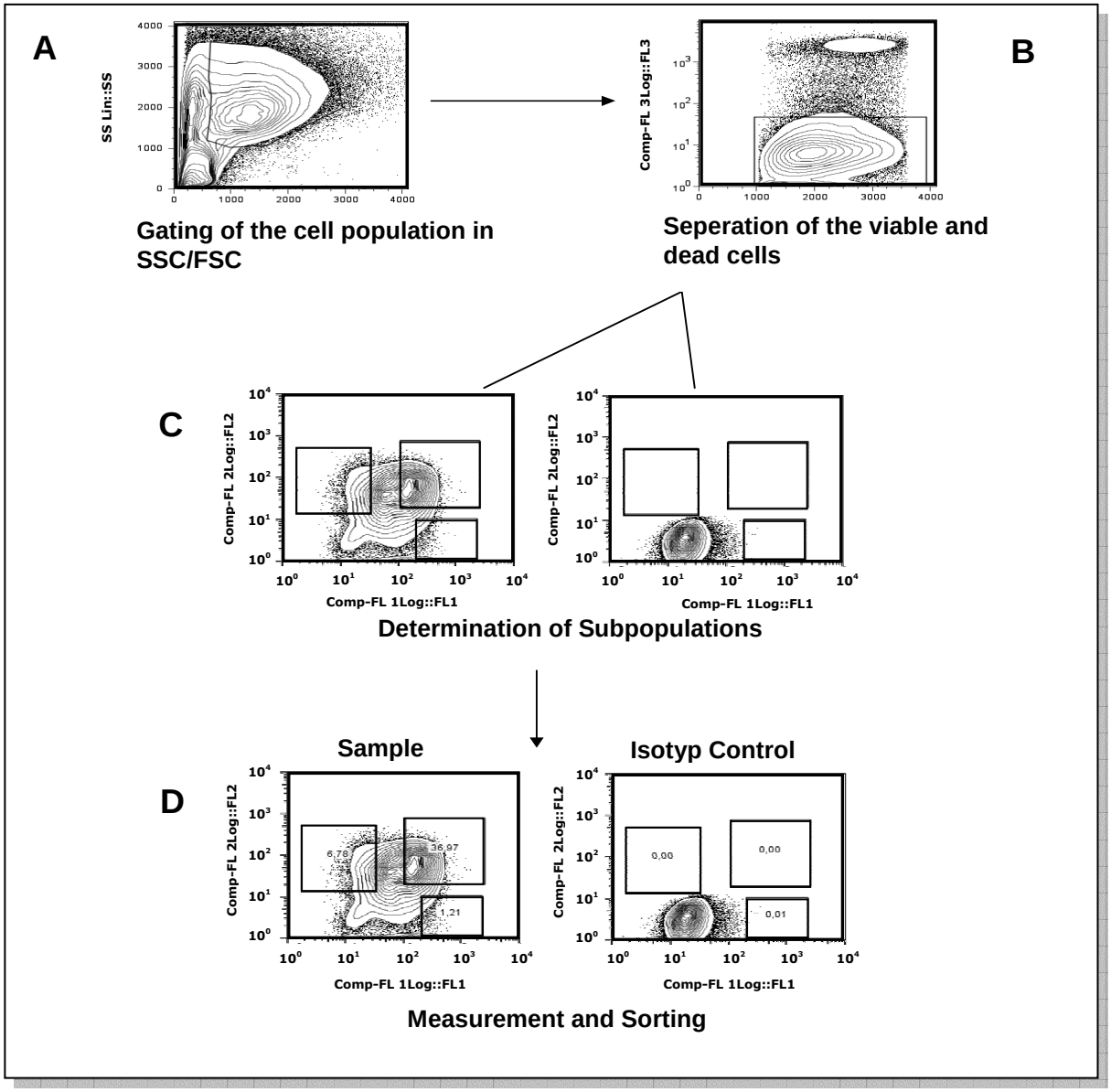


Figure 33: Performance of FACS-Analysis and Sort

Labelled MSCs will be isolated in FSC/SSC (A) followed by the separation of viable (Gate) and dead cells in FL3 (B). For exact determination of viable cells it is necessary to compensate the labelled cells in the particular fluorescence channel (for example for FITC (FL1) or PE (FL2)) together with FL3. The viable cells will be gated in FL3/SSC (B) and presented in the different FL-channel (FL1/FL2) (C) corresponding to the staining. Furthermore, for determination of distinct subpopulations positive for the used markers and to avoid the sorting of false positive cell populations the corresponding isotyp control (C,D).

List of Antibodies

Antigen	Gene name	Alternative Clone	Conjugate	Isotype	Company Cat.Nr.	Company
CD105	ENG, SH4	SN6	APC	Mouse IgG1	Caltag MHCD1054	Invitrogen
CD106	VCAM1	51-10C9	PE	Mouse IgG1	BD555647	BD Bioscience
CD117	KIT	YB5.B8	PE	Mouse IgG1	BD555714	BD Bioscience
CD120a	TNFRSF1A	H398	PE	Mouse IgG2a	MCA 1340	Serotec
CD120b	TNFRSF1B	MR2-1	PE	Mouse IgG1	MCA 1944	Serotec
CD13	ANPEP	WM15	PE	Mouse IgG1	BD555394	BD Bioscience
CD13	ANPEP	WM-47	FITC	Mouse IgG1	F0831	DAKO
CD13	ANPEP	SJ1D1	PE	Mouse IgG1	IM0778U	Beckmann-Coulter
CD13	ANPEP	SJ1D1	FITC	Mouse IgG1	A07762	Beckmann-Coulter
CD13	ANPEP	Immu103.44	PC5	Mouse IgG1	IM2639U	Beckmann-Coulter
CD140a	PDGFRB	alphaR2	PE	Mouse IgG2a	BD556003	BD Bioscience
CD140b	PDGFRB	28D4	PE	Mouse IgG2a	BD558821	BD Bioscience
CD146	CD146, MCAM	S-Endo-1	PE	Mouse IgG1	5050-PE100T	Biocytex
CD146	CD146, MCAM	S-Endo-1	FITC	Mouse IgG1	5050-F100T	Biocytex
CD164	CD164, Endosalin	N6B6.2	PE	Mouse IgG2a	BD551298	BD Bioscience
CD166	ALCAM	3A6	PE	Mouse IgG1	BD559263	BD Bioscience
CD18	ITGB2	6.7	PE	Mouse IgG1	BD555924	BD Bioscience
CD18	ITGB2	6.7	FITC	Mouse IgG1	BD555923	BD Bioscience
CD18	ITGB2	HIB19	FITC	Mouse IgG1	BD555412	BD Bioscience
CD26	DPP4	M-A261	PE	Mouse IgG1	BD555437	BD Bioscience
CD271	NGFR	ME20.4-1.H4	APC	Mouse IgG1	130-091-0	Miltenyi-Biotech
CD29	ITGAB1	MAR4	PE	Mouse IgG1	BD555443	BD Bioscience
CD31	PECAM1	WM59	PE	Mouse IgG1	BD555446	BD Bioscience
CD31	PECAM1	WM59	FITC	Mouse IgG1	BD555445	BD Bioscience
CD32	FCGR2A	FLI8.26	FITC	Mouse IgG2b	BD555448	BD Bioscience

Continuing List of Antibodies

Antigen	Gene name	Alternative Clone	Conjugate	Isotype	Company Cat.Nr.	Company
CD338	ABCG2, BCRP1	5D3	PE	Mouse IgG2b	FAB995P	R&D Systems
CD34	CD34	8G12	PE	Mouse IgG1	BD345802	BD Bioscience
CD34	CD34	8G12	FITC	Mouse IgG1	BD346801	BD Bioscience
CD38	CD38	HIT2	PE	Mouse IgG1	BD555460	BD Bioscience
CD38	CD38	HIT2	FITC	Mouse IgG1	BD555459	BD Bioscience
CD44	CD44, HCAM	G44-26	PE	Mouse IgG2b	BD555479	BD Bioscience
CD45	PTPRC	HI-30	PE	Mouse IgG1	BD555483	BD Bioscience
CD45	PTPRC	HI-30	FITC	Mouse IgG1	BD555482	BD Bioscience
CD47	CD47, IAP	B6H12	PE	Mouse IgG1	BD556046	BD Bioscience
CD49a	ITGA1	SR84	PE	Mouse IgG2a	BD559596	BD Bioscience
CD49b	ITGA2	12F1-H6	PE	Mouse IgG1	BD555669	BD Bioscience
CD49c	ITGA3	C3II.1	PE	Mouse IgG1	BD556025	BD Bioscience
CD49d	ITGA4	gF10	PE	Mouse IgG1	BD555503	BD Bioscience
CD49d	ITGA4	HP2/1	FITC	Mouse IgG1	IM1404	Beckman-Coulter
CD49e	ITGA5	IIA1	PE	Mouse IgG1	BD555617	BD Bioscience
CD49f	ITGA6	450-30A	PE	Mouse IgG1	MCA1457	Serotec
CD51	ITGAV	P2W7	PE	Mouse IgG1	FAB1219P	R&D Systems
CD54	ICAM1	HA58	PE	Mouse IgG1	BD555511	BD Bioscience
CD54	ICAM1	6.5B5	FITC	Mouse IgG1	F7143	DAKO
CD56	NCAM1	N901	PC5	Mouse IgG1	IM2654U	Beckmann-Coulter
CD61	ITGB3	VI-PL2	PE	Mouse IgG1	BD556020	BD Bioscience
CD63	TSPAN30	H5C6	PE	Mouse IgG1	BD550257	BD Bioscience
CD73	NT5E, SH3	AD2	PE	Mouse IgG1	BD555596	BD Bioscience
CD9	CD9	M-L13	PE	Mouse IgG1	BD341647	BD Bioscience
CD90	THY1	5E10	APC	Mouse IgG1	BD559869	BD Bioscience

Continuing List of Antibodies

Antigen	Gene name	Alternative Clone	Conjugate	Isotype	Company Cat.Nr.	Company
CD90	THY1	5E10	FITC	Mouse IgG1	BD555595	BD Bioscience
FGFR2	FGFR2	98725	APC	Mouse IgG1	FAB684A	R&D Systems
HLA-ABC	HLA-ABC	B9.12.1	FITC	Mouse IgG1	IM1838U	R&D Systems
ITGA11	ITGA11	396214	unconjugated	Rat IgG1	MAB4235	R&D Systems
JAGGED1	JAGGED1	188331	FITC	Mouse IgG2b	FAB1277F	R&D Systems
NOTCH3	NOTCH3		biotinylated	Sheep IgG	BAF1559	R&D Systems
PLEXIND1	PLXND1		biotinylated	Goat IgG	BAF4160	R&D Systems
SSEA4		MC-813-70	PE	Mouse IgG3	FAB1435P	R&D Systems
SSEA4	SSEA-4	MC-813-70	APC	Mouse IgG3	FAB1435A	R&D Systems
STRO-1	Stro-1			Mouse IgM	MAB1038	R&D Systems
W8B2	MSCA-1	W8B2B10	APC	Mouse IgG1	130-093-589	Miltenyi

List of Secondary Antibodies

Secondary antibodies		Clone	Conjugate	Isotype	Company Cat.Nr.	Company
IgG		Goat-anti rat IgG	Alexa Fluor 594	Goat IgG	A11007	Invitrogen
IgG		Donkey anti-sheep IgG	Alexa Fluor 488	Donkey IgG	A11015	Invitrogen
IgG		Streptavidin	PE		554061	BD Bioscience
IgG1		Streptavidin	Alexa Fluor 488	IgG1	S32354	Invitrogen

List of Isotype controls

Isotyp control		Clone	Conjugate	Isotype	Company Cat.Nr.	Company
IgG control		polyclonal	PE	Goat-anti mouse IgG	BD550589	BD Bioscience
IgG control			FITC	Goat-anti rat IgG	R40101	CALTAG
IgG1 control		679.1Mc7	FITC	Mouse IgG1	A07795	Beckman-Coulter
IgG1 control		679.1Mc7	PE	Mouse IgG1	A07796	Beckman-Coulter
IgG1 control		G17.1	Biotin	Mouse IgG2b	BD555869	BD Bioscience
IgG1 control		MOPC-21	APC	Mouse IgG1	BD555751	BD Bioscience
IgG1 control		MOPC-21	FITC	Mouse IgG1	BD555748	BD Bioscience
IgG1 control		MOPC-21	PE	Mouse IgG1	BD555749	BD Bioscience
IgG2a control		G155-178	APC	Mouse IgG2a	BD555576	BD Bioscience
IgG2a control		DAK-GO5	FITC	Mouse IgG2a	X0933	DAKO
IgG2a control		G155-178	PE	Mouse IgG2a	BD555574	BD Bioscience
IgG2a control		7T41F5	FITC	Mouse IgG2a	IM0640	Beckman-Coulter
IgG2b control		27-35	APC	Mouse IgG2b	BD555745	BD Bioscience
IgG2b control		27-35	FITC	Mouse IgG2b	BD555742	BD Bioscience
IgG2b control		27-35	PE	Mouse IgG2b	BD555743	BD Bioscience
IgG2b control		A95-1	PE	Rat IgG2b	BD553989	BD Bioscience
IgG2a+b control		X57	PE	Mouse IgG2a+b	BD340269	BD Bioscience
IgM control		G155-228	FITC	Mouse IgM	BD555583	BD Bioscience
IgM control		G155-228	PE	Mouse IgM	BD555584	BD Bioscience

Antibodies used for detection of osteogenic differentiation

Antibodies		Clone	Conjugate	Isotype	Company Cat.Nr.	Company
Anti human/mouse alkaline phosphatase		B4-78	purified	Mouse IgG1	MAB1448	R&D Systems
IgG			Alkaline Phosphatase	Goat IgG	A3688	Sigma-Aldrich

5.1.8 Re-cultivation of sorted Subpopulations and CFU-F - Assay

The CFU-F assay is performed to measure the clonal potential of the isolated cells. Different studies have shown that CFU-F-derived stromal cells are multipotent in vitro and can form in vivo ectopic bone with establishment of the hematopoietic niche (see also: Introduction, section 2.5) [Delorme et al., 2008; Gronthos et al., 2003; Sacchetti et al., 2007]. Due to these results, the CFU-F assay is used as a tool for first predictions of possible enrichment of multipotent MSC.

Different subpopulations of cultured and unsorted or stringently sorted (single- or double-marker positive) BM-MSC and UVSC were collected in Alpha-MEM supplemented with 20% FCS and antibiotics. The cells were washed, counted, and seeded as 10-100 sorted cells cm^2 in 6-well plates in re-culture medium, culture medium supplemented with 20% FCS, as described elsewhere [Sacchetti et al., 2007]. 24hours after re-culture of the cells the medium was completely changed to avoid contamination with non-adherent cells and to remove death cells. Afterwards, 50% the medium was replaced by new medium every third day. After two weeks, fibroblastoid colonies (CFU-F-derived) appear.

For enumeration studies, colonies were visualized using staining with Giemsa-Solution. For this purpose, the stromal cells were washed three times with 1xPBS to remove medium and FCS and then fixed for 10 minutes in 5ml ice-cold concentrated Methanol at room temperature. After this, the methanol was removed and the cells as well as CFU-F were air-dried for a few minutes. Finally, the Giemsa-solution (Sigma-Aldrich, Germany) (3ml) was added following by an incubation of further 10 min. Afterwards, the solution was removed and the cells were washed with distilled water until the blue coloured CFU-F remained.

For comparisons, the number of CFU-F was counted and normalized as number of colonies per 5000 cells seeded. For evaluation of the enrichment of CFU-F, first the frequency was determined, which is calculated from the number of CFU-F received by the number of seeded cells. The enrichment factor is calculated by $CFU\text{-}F_{frequency}$ other marker/$CFU\text{-}F_{frequency}$ culture control. The recovery is expressed in percentage. Hence, (Sorted cells x $CFU\text{-}F_{frequency}$) / (Unsorted cells x $CFU\text{-}F_{frequency}$) x 100.

Re-Culture Medium

Components	Volume/Concentration	Company
AlphaMEM GlutaMax		Gibco, Invitrogen, Germany
FCS	20%	PAN, Germany
P/S	1%	Gibco, Invitrogen, Germany

5.2 MSC Differentiation

To test whether isolated and/or sorted MSC populations show the capacity for multipotential differentiation in vitro, seeded MSCs were examined of their functionality to differentiate in mesenchymal lineages. In previous work [Friedenstein et al., 1974; Friedenstein et al., 1987; Caplan et al., 1991; Pittenger et al., 1999], three main differentiation pathways of the mesenchymal stem cells are determined in vitro and in vivo. In vitro, true MSCs should have the potential to differentiate into adipogenic, chondrogenic and osteogenic lineage. For this purpose, the cells need to grow confluent to monolayer cultures for induction of adipogenic and osteogenic differentiation in vitro. The chondrogenic differentiation is performed by formation of densed micropellet cultures. All differentiation lineages in vitro need until terminal differentiation between 21-25 days with constant change of the differentiation media. More importantly, in vivo, the formation of bone and the recruitment of the hematopoietic niche in heterotopic transplantation should be detectable.

In this thesis, I performed experiments to determine the differentiation potential of BM-MSC and UVSC. Adipogenic and osteogenic differentiation was induced after the MSCs had grown a confluent monolayer. Chondrogenic differentiation was, however, performed in micromass culture and high-density micropellet culures.

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5.2.1 Adipogenic Differentiation

Adipogenic differentiation was performed following the protocol of Pittenger et al. [Pittenger et al, 1999].

The adipogenic differentiation procedure consists of a cycled process by changing induction medium with maintenance medium. First, the cells were grown to confluent monolayer cultures. Subsequently, cell culture medium was removed and replaced with induction media. The confluent cells were then cultured three days in induction medium, after which the medium was removed and the cells were cultured for another two days in maintenance medium. The process of induction and maintenance media was repeated three times. Finally, the cells will be incubated for another week in maintenance media.

Adipogenic Induction medium

Components	Concentration	Company
α-MEM plus GlutaMAX		Gibco, Invitrogen, Germany
Human recombinant insulin	10µg/ml	Roche, Germany
3-isobutyl-1-methylxanthine (IBMX)	0.5mM	Calbiochem, Merck KgaA, Germany
Dexamethasone	1 µM	Sigma-Aldrich, Germany
FCS	20%	PAA, Germany
Cibrobay antibiotic	1:200	Bayer, Germany

Adipogenic Maintenance Medium

Components	Concentration	Company
α-MEM plus GlutaMAX		Gibco, Invitrogen, Germany
Human recombinant insulin	10µg/ml	Roche, Germany
FCS	20%	PAA, Germany
Cibrobay antibiotic	1:200	Bayer, Germany

5.2.2 Histological Detection of adipogenic Differentiation

After a total of 20 days (3 times induction/maintenance), adipogenic differentiation is visible by expansion of the cytoplasmatic lumen and appearance of fatty vesicles of different sizes. For colouration of the lipid vesicles, Oil Red O staining was performed. Oil Red O was incorporated in the fatty vesicles and appears red.

Oil Red O Stock Solution

Components	Volume/Concentration	Company
Oil Red O	0,5g	Sigma-Aldrich, Germany
Isopropanol	100ml	drug store of the clinic, TUM Germany

To prepare the Oil Red O staining solution, six parts of Oil Red O stock solution have to be diluted with four parts distilled water. The solution should be mixed well and incubated for 24h at room temperature. The following day the solution will be filtered (Rotrand 0,45µm Celluloseacetat, Whatman) to remove disturbing unsolved particles. Now, the solution is ready-to-use for staining.

After removing the maintenance media, BM-MSC and UVSC were washed carefully with 1xPBS. Thereafter, the differentiated cells were fixed with 2% Paraformaldehyd (PFA) in 1xPBS for 30 min. at 4℃. After fixaion, the PFA was discarded and the differentiated cells incubated for 30 min. with Oil Red O staining solution at room temperature. Finally, the cells were carefully washed several times with distilled water until only the red staining of the fatty vesicles is visible. The staining is stable for only a few days.

5.2.3 Osteogenic Differentiation

Osteogenic differentiated cells will change their morphological appearance into cuboidal phenotype and deposit calcium, which can be detected by staining of mineralized osteogenic membrane. To detect this calcium deposition, I used the so-called von Kossa procedure. The induction of the osteogenic differentiation is performed as described at Jaiswal et al. [Jaiswal et al., 1997]. Potent inducers for this osteogenic lineage are Dexamethasone, β-glycerolphosphat and ascorbic acid.

Osteogenic differentiation media

Components	Concentration	Company
α-MEM plus GlutaMAX		Gibco, Invitrogen, Germany
Ascorbic acid-2-phosphat	0.05mM	Sigma-Aldrich, Germany
β-glycerolphosphat	10mM	Sigma-Aldrich GmbH, Germany
Dexamethasone	100nM	Sigma-Aldrich GmbH, Germany
Human recombinant insulin	10µg/ml	Roche, Germany
Heat-inactivated FCS	20%	PAA, Germany
Cibrobay	1:200	Bayer, Germany

After formation of a confluent monolayer culture, MSC culture medium was replaced with osteogenic differentiation media. The differentiation medium was changed every 3. day for a total of three weeks. In the culture, dense brown-coloured areas appear, which represent calcium accumulations. The mineralized matrix of osteogenic differentiated cells was visualized with von Kossa staining as described below.

5.2.4 Histological Detection of osteogenic Differentiated cells with von Kossa staining and Alkaline phosphatase

For detection of calcium deposits at the membrane of osteogenic differentiated cells, the von Kossa staining represents the optimal procedure. The basis of this procedure is that the calcium salts (phosphate, carbonate) of the mineralized matrix will be reveal by substitution to metallic cations from the silver nitrate. The silver nitrate will be visualized after reduction into metallic silver (black stain). These black staining indicates the mineralization of the bone matrix.

The osteogenic differentiated cells were fixed with ice-cold methanol for 20 min. on ice. Afterwards, the cells were washed and covered with silver nitrate and incubated for 30 min. at room temperature in full darkness. The silver nitrate will be bind to the calcium and will mark the osteogenic differentiated cells. The reaction will be stopped by washing the cells several times with 1x PBS after removing silver nitrate. Finally, for visualization of the calcium deposits, sodium carbonate was added. After incubation of 5-10min the silver nitrate will be reduced and appeared in black. The calcium-incorporated cells were washed and stored in distilled water until photography.

Silver Nitrate Solution

Components	Volume/Concentration	Company
$AgNO_3$	5% (1g)	Sigma-Aldrich GmbH, Germany
Aqua dest.	20ml	

Sodium Carbonate Solution

Components	Volume/Concentration	Company
Na_2CO_3	5g	Sigma-Aldrich GmbH, Germany
Formaldehyde (37%)	200µl	Sigma-Aldrich, Germany
Aqua dest.	100ml	

Detection with Alkaline phosphatase

An alternative method for detection of osteogenic differentiated cells is the detection of the bone isoform of the enzme alkaline phosphatase. The method is based on the visualization of alkaline phosphatase using specific antibodies. Antibodies are then detected by second-step reagents and an enzymatic reaction.

The differentiated cells were prepared for staining by fixation with ice-cold methanol as described above. Further on, the unspecific binding sites of the cells were saturated with blocking buffer for 30 min. at room temperature and subsequently incubated with primary antibody anti-alkaline phosphatase (anti Human/Mouse alkaline phosphatase purified mouse monoclonal antibody IgG1 clone B4-78, 500µg/ml, R&D Systems, Minneapolis, MN, USA) for 1h at 4℃. Afterwards, the cells were washed and the secondary polyclonal goat anti-mouse antibody which was conjugated to intestinal alkaline phosphatase (1:400, Sigma-Aldrich) and incubated for 1h at 4℃. Finally, the cells were washed in bl ocking buffer and one time in 1xPBS and then the antibody was visualized using SIGMA FAST^{TM} (Fast Red TR/Naphtol AS-MX Alkaline Phosphatase Substrate Tablets Set, Sigma-Aldrich GmbH, Steinheim, Germany). In the final step, the surplus of the staining solution is removed and the red coloured osteogenic cells remain.

Stained MSCs were photographed with an Axio Vision microscope (Carl Zeiss, Germany) attached to a computer equipped with Axiovert 135 software (Carl Zeiss, Germany).

Blocking Buffer

Components	Volume/Concentration	Company
PBS	1x	Gibco, Invitrogen Germany
Bovine Serum Albumin (BSA) (Albumin fraction V)	1%	Carl Roth GmbH & Co KG Germany

5.2.5 Chondrogenic Differentiation

For induction of the chondrogenic differentiation, stromal cells from bone marrow and umbilical cord were cultured to monolayer cultures in 6-well plates. The culture conditions were chosen as described under section 5.1.4.

The chondrogenic differentiation can be induced using two different methods, the monolayer culture [Denker et al., 1999], or the micropellet culture, a highly densed cell mass [Johnstone et al, 1998]. For both methods, the same chondrogenic mix supplemented with TGF-β3 was used.

The micromass cultured was modified based on the protocol of Denker et al. and Ahrens et al. [Denker et al., 1999 and Ahrens et al, 1977]. The cells were grown to monolayer culture. Afterwards, the culture medium was removed and the cells were washed twice in 1x PBS. The cells were incubated in chondrogenic differentiation medium supplemented with hTGF-β3 (10ng/ml, R&D Systems, Wiesbaden, Germany). The medium was changed all three days. After 21 days the differentiated cells were prepared for chondrogenic staining alcian blue (see histological staining below).

For performance of the micropellet cultures, the subconfluent grown stromal cells were trypsinized and washed for removal of the culture medium and another time with incomplete chondrogenic medium (without hTGF-β3) for preparation of the chondrogenic induction. Afterwards, the cells were resupended in complete chondrogenic medium and cell number was determined. For each micropellet culture per tube 2.5 x 10^5 cells/tube were pelleted by centrifugation (5min at 350g) in 15ml polypropylene tube (BD Falcon tubes Becton Dickinson, Heidelberg, Germany). The micropellet cultures were incubated at 37℃ with 5% CO_2. The medium was changed in complete chondrogenic differentiation medium supplemented with hTGF-β3 (10ng/ml).

After day1, the tubes were checked by flicking that the pellets do not attach at the tube or split. As in the monolayer technique above, the medium was changed every 2-3 days until day 21. The detection of the chondrogenic differentiated cells were analyzed with molecular biological using PCR. For immunhistochemical methods using safranin O staining or respectively alcain O staining the micropellets were first washed with 1x PBS followed by fixation in 4% formaldehyde in 1x PBS for 1h. Finally, 7 µm histological slices were performed from the micropellets.

Chondrogenic Differentiation medium [Johnstone et al, 1998]

Incomplete Medium

Components	Volume/Concentration	Company
Penicillin/Streptomycin	1%	Gibco, Invitrogen Germany, PAN Germany
Ascorbate-2-phosphateP/S	50µg/ml	Gibco, Invitrogen Germany
Dexamethasone	100nM	Sigma-Aldrich, Germany
Sodium Pyruvat	100µg/ml	Sigma-Aldrich, Germany
Proline	40µg/ml	Sigma-Aldrich, Germany
BSA	1,25mg/ml	Sigma-Aldrich, Germany
Linoleic acid	5,33µg/ml	Sigma-Aldrich, Germany
Seleneous acid	6,25µg/ml	Sigma-Aldrich, Germany
Transferrin	6,25µg/ml	Sigma-Aldrich, Germany
Insulin	6,25µg/ml	Roche, Germany
AlphaMEM GlutaMax	Add to total volume of 200ml	Gibco, Invitrogen Germany

Chondrogenic differentiation Medium

Compete medium

Components	Volume/Concentration	Company
Incomplete Medium		
hTGF-β3	10ng/ml	R&D Systems, Germany

5.2.6 Detection of chondrogenic Differentiation by PCR-Analysis

At least, 10 pellets have to be collected to obtain up to 5 µg of RNA for the PCR analysis. After chondrogenic differentiation the pellets were washed twice in 1x PBS and were immediately used or freeze as dry pellets at -80C° until isolation of the RNA.

Finally, the pellets were suspended in lysis buffer (ca. 200µl) and disaggregated using Ultraturax. The RNA isolation will be processed using classical methods for total RNA extraction as described in section 5.3.1**.**

The PCR analysis is performed on 500ng or 1µg total RNA (20µl final volume). The PCR analysis is performed using markers Collagen II, Aggrecan, and the housekeeping gene GAPDH.

5.2.7 Immunhistochemical Detection of chondrogenic Differentiation

Finally, the micropellets were carefully washed twice with 1x PBS and fixed in 4% paraformaldehyde for maximal 24 h for histochemical and immunhistochemical analysis. Afterwards, the pellets were embedded in 50µl of 1.5% low melting agarose and then embedded in paraffin. The embedded pellets were sliced in thin layer (Average size of 0.2-0.5 mm) by microtom and placed on an object slide for staining with safranin O or respectively alkaline blue.

5.2.7 Histological Staining of Chondrogenic Differentiation

Safranin O Staining

Safranin O stains proteoglycans and glycosaminoglycans. This protocol is for paraffin embedded sections of tissues on slides:

In the first step, the chondrogenic differentiated cell section will be deparaffinized and rehydrated using a step-wise declining concentrations of alcohol. (100%-90%-70%). After washing the slices were stained with safranin O staining (0,02% Fast Green 3min; 1% Acetic Acid 30 sec; 0,1% Safranin O for 5 min.).

For dehydration the slices were treated with an increasing concentration of alcohol (70%, 90%, and 100%). The alcohol was removed using xylol and at least the slices were mounted with coverslips. The slides are ready for documentation.

Alcian blue Staining

Similar to the Safranin O staining, alcaine blue is also a cationic dye and mark glycosaminoglycane as well as mucopolysaccharides in bluish-green. It can be combined with Hematoxylin-Eosin staining.

For alkaline blue staining the paraffined slices were pre-treated in a oven for 50min at 56℃ for supporting better antibody staining and re ducing background. Afterwards the slides were de-paraffinized using Xylol for 5min and rehydrate with alcohol (96%; 80%, 70%). Then, the slides were washed with water and stained with Hematoxylin Harris solution for 20min. After another washing step, the cells were dipped in 1%HCL in 70% ethanol, washed immediately in water and finally the slides were incubated with alcian blue solution (3% Alcian Solution mixed 1:1 (v/v) with 3% methanol) for 10min. After dehydration with increasing EtOH concentration (96%-100%) and removal of the alcohol by xylol the slides were mounted with coverslips and are ready for documentation.

Safranin O Stain (0,1%)

Components	Volume/Concentration	Company
Safranin O powder	0,1g	Sigma-Aldrich, Germany
Water	100ml	

Alcian Blue Stain (3%)

Components	Volume/Concentration	Company
Alcian Blue 8GX	3g	Sigma-Aldrich, Germany
aqua dest.	100ml	

5.3 Molecular Biological Methods

5.3.1 RNA-Isolation and Measurement of the Concentration

To isolate total cellular RNA, the cell culture medium was removed from appropriate cultures and the cells were washed twice in 1xPBS to remove residual culture medium. The cells were then detached by scraping and counted before total RNA was extracted from 10^5 to 10^7 cells using RNeasy (RNeasy Mini Kit, Qiagen GmbH Germany). The RNA was isolated with a silica-membrane based technology using a spin column. A high-salt buffer system containing guanidine salt allows the binding of the RNA on the membrane only and the removing of contaminants.

BM-MSC, UVSC, and 2102Ep were lysed using guanidine-thiocyanate-containing (GITC) RLT buffer; the GITC provides the inactivation of the RNase and guarantees the purification of the intact RNA. The addition of ethanol ensures appropriated binding conditions of RNA to the RNeasy spin column.

After lysis with RLT buffer the cells were homogenized in a QIAshredder spin column for 1min at full speed at room temperature. The homogenized flow through were supplemented with 70% ethanol at a total volume of 700µl.

The sample were transferred to an RNeasy spin column and centrifuged for 15s at 8.000g at room temperature. The flow through were discarded. For guarantee a high RNA purity additionally DNase digestion was performed using DNase I enzyme (RNAse-free DNAse I, Invitrogen GmbH, Germany). The bound RNA on the silica membrane was incubated with DNase I solution (10µl) with RDD-buffer (70µl) at 20-30 ℃ for 15min. at room temperature.

Afterwards, 350µl RW1 buffer (containing guanidine salt) were carefully added and centrifuged for 15s at 8.000g at room temperature. The flow through were discarded and the column were transferred in a new collection tube to avoid remaining contaminants. Following 500µl RPE buffer (containing ethanol) were carefully pipetted on the RNA containing silica-membran and centrifuged at 8.000g for 15s at room temperature. After discarding the flow through, this step were repeated with 2min centrifugation at 8.000g. To ensure the complete remove of the RPE buffer from the silica membrane, the spin column were placed in a new collection tube and were centrifuged another minute at full speed. Finally, 30-50µl RNase-free water was

added on the silica-membran to elute the RNA at 8.000g for 1min at room temperature. The amount of RNA were photometrically determined and additionally controlled for purity by agarose gel electrophoresis.

The concentration of the RNA was determined using an optical densitometer (SmartSpec Plus, BioRad Laboratories GmbH)

For this purpose, the samples were prepared by 1:50 dilution in water in an end volume of 100µl. The purity was determined at a wavelength A260/A280 and was defined as pure in the range between factors 1.7 to 1.8. Furthermore, the RNA was tested in gel electrophoresis for correct size and DNA contamination (see Fig.34) The samples were frozen at –80℃ until use or used dire ctly for cDNA synthesis.

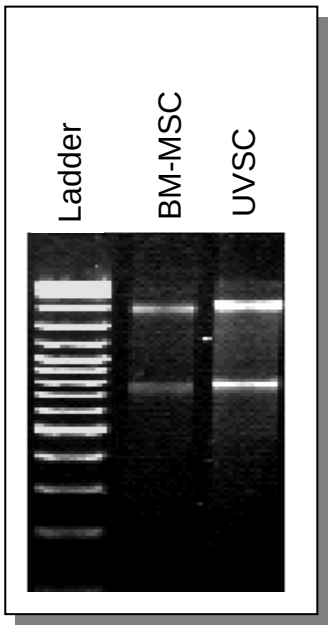

Figure 34: Testing of RNA purity in Gel-Electrophoresis

cDNA synthesis is performed based on Omniscript Reverse Transcriptase system (Qiagen,Germany). Before starting the cDNA synthesis the cells were proved for DNA contamination. Shown are RNA of MSCs derived from umbilical vein and bone marrow separated in 1,5% Agarose Gel

5.3.2 cDNA-Synthesis

The RNA was copied into DNA (cDNA synthesis) using reverse transcription primed with oligo-dT primers (QIAGEN GmbH, Germany). Therefore, the formation of cDNA based on the activity of reverse transcriptase after binding of the oligo-dT primer at the 3' poly-A-tail. The process was performed at 37℃ for 1h in a thermo cycler (PTC-100 Peltier Thermal Cycler, MJ Research BioRad GmbH, Germany). The cDNA were stored at –20℃ until use.

The scheme below shows the constitution of the Reverse Transcription reaction.

Master mix (Omniscript Reverse Transcriptase Qiagen, Germany)

Component	Volume/reaction	Final Concentration
10x Buffer RT	2µl	1x
dNTP Mix (5mM each dNTP)	2µl	0.5mM each dNTP
Oligo-dT primer (10µM)	2µl	1µM
RNase Inhibitor (10 untits/µl)	1µl	10 units
Omniscript Reverse Transcriptase	1µl	4 units
RNase-free water	variable	
Template RNA	variable	0.5-1µg
Total volume	20µl	

5.3.3 Microarray-Analysis

The microarray analysis can be used for detection of gene variations in gene sequences or expression as well as for identification of novel genes, or binding sites of transcription factors, and mutation in genes of diseases. For this purpose, nucleic acid sample were hybridized to a high number of preset oligonucleotide probes. These probes are attached to a solid substrate, like glass ("chips"). We used chips manufactured by Affymetrix (Santa Clara, CA, USA).

In the microarray analysis using Affymetrix GeneChip Probe Assay (Affymetrix Human Genome U133 Plus 2.0 Chips) each oligonucleotide probe is allocated in a specific area - the Probe Cell. Each of these Probe Cells contains up to millions of copies of a given oligonucleotide.

The gene chip comprises over 54,000 probes containing more than 47,000 transcripts including over 38,500 known genes and expressed sequence tags selected from sequences in GenBank®, UniGene, dbEST and RefSeq. These high amounts of probes enable large scale analysis of expression patterns of cells. For performance of the microarray analysis, 1µg total RNA was reverse transcribed in cDNA using T7- Oligo (dT) primer. Following in the in vitro transcription (IVT) reaction with T7 polymerase and a biotinylated nucleotide analog/ribunucleotide (UTP, CTP) mix the complementary RNA (cRNA) were amplificated and labelled with biotin (see Figure 35). The cRNA refer as “target” and were subsequently prepared for fragmentation to allow optimal assay sensitivity. Finally the cRNA were hybridized to Affymetrix Human Genome U133 Plus 2.0 Chips. The binding of the RNA to the sequences will be represented in spots that can be quantified by different signal intensity.

These spots results from the streptavidin-PE labelling of the cRNA-biotin complex, which will be exciting (488nm) and scanned. The emitted light (570nm) is proportional to the bound amount on the cRNA. The results were analyszed with Affymetrix GeneChip Operating Software (GCOS) 1.2 software [http://www.affymetrix.com/products_services/software/specific/_gcos.affx#1_2). The scanning of each Probe Cell is represented in a fluorescence pixel image containing different signal intensities. In consideration of the background signal, the intensities will be corrected and the signal value of the probe cell will be calculated.

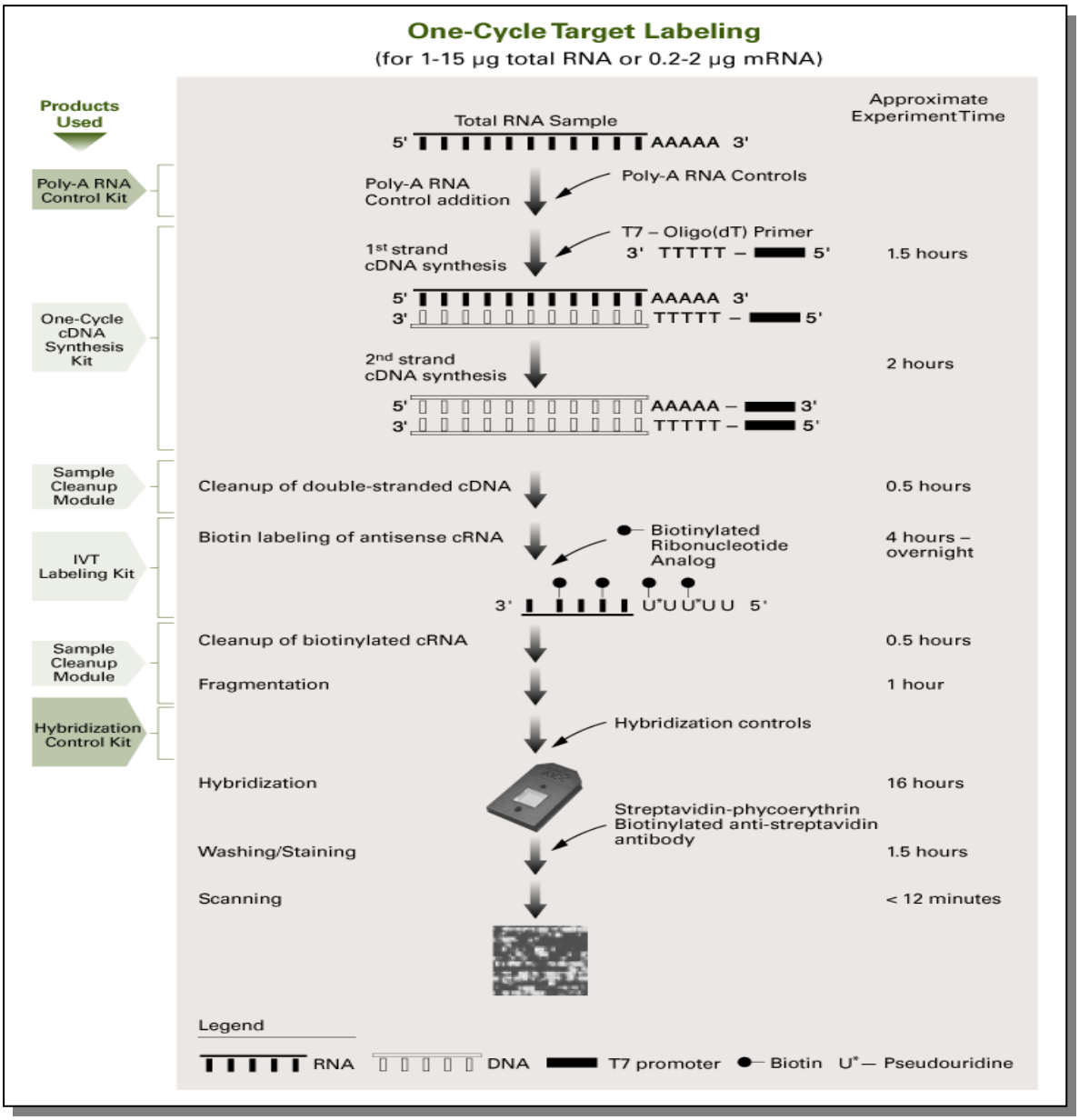

Figure 35: GeneChip Labelling Assay for Gene Expression Analysis

Schematic presentation of the One-cycle target labelling process (http://www.affymetrix.de)

For each Probe Pair the differences were formed. Each variation is defined in so called “Discrimination score”, which will be compared to a defined threshold value. From this comparison the p-value is formed. It can be concluded like this: as high as the Discrimination score is over the defined threshold, the smaller is the p-value and more likely the transcript can be defined as “Present”. The strength of the signal intensity represents the expression potential of the detected transcript (for more informations, and manual see http://www.affymetrix.com)

The data were transferred to the searchable expression database File Maker Pro software 7.0.

In the present work, the analysis was focused primarily on the molecules of the Gene Ontology (GO) class: extracellular (GO:0005575 cellular component; is_a GO: 0005576 extracellular region)

Two separate global gene expression experiments were performed. One in which five p1/p2 UVSC samples were compared to six p1 BM-MSC samples, and a second (confirmative) experiment in which three p2 UVSC samples were compared to two p2 BM-MSC samples. Hybridization on HG-U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA) was performed as described elsewhere [Delorme et al., 2008]. Hierarchical and k-means clustering as well as principal components analyses were performed using Genesis software (http://www.genesis-softwareonline.com/).

Microarray data are available in the Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE9894.

5.3.4 PCR Analysis and quantitative Real-Time Analysis

The polymerase chain reaction (PCR) was first described and performed by Saiki et al. [Saiki et al., 1985]. The PCR based on the analysis of the expression of genes using an amplification process of DNA or double-strand RNA (cDNA). Oligonucleotides (primers) identify the sequence of the gene of interest and replicate the region with the activity of DNA-polymerase (Taq-polymerase). The reaction is subdivided in different thermo-directed steps, which include the denaturation of the double-strand DNA into single-strands followed hybridisation (annealing) of the primers flanking at 3’ and 5’ end of the region of interest and the synthesis of a new complementary strand. This procedure corresponds to one PCR cycle and will be repeated several times representing an exponential amplification.

The PCR reaction is performed using a reaction mix called PCR Master Mix (PCR-Master-Mix Y 2x concentrated (peqlab Biotechnology GmbH, Germany, constitution see table below). The Master Mix is available as ready-to-use mixture containing all necessary ingredients for the PCR reaction. These PCR Master Mixes contain the known nucleotides dATP, dGTP, dTTP, and dCTP, which were concluded as dNTPs and are needed for the reaction to work. The addition of Tris-HCL buffer support the maintaining of the pH and guarantee optimal primer binding as well as proceeding of the PCR reaction. Furthermore MgCl is added to the reaction to allow correct function

of the Taq-polymerase. The Mg^{2+} ions act as cofactor to the enzyme. The concentration of the MgCl is usually between 0.5 to 5.0 mM. Using a too low MgCl concentration the reaction will results in a low or even no yield while an high concentration will lead to a mispriming.

Furthermore, in many commercial available PCR Master Mixes also enhancers are added to the known ingredients above. Here, often $(NH_4)_2SO_4$ is used, which promotes the specific primer binding during the annealing step of each PCR cycle. Tween 20 is a detergent for maintaining the pH but also facilitate the reading through G/C rich regions. In other PCR mixes also the use of DMSO (Dimethyl sulfoxide, $(CH_3)_2SO_4$) is added, which is used for reduction of secondary structures in G/C rich templates, but can also reduce the activity of the Taq polymerase and increase the mutation rate in PCR. Therefore, the use of DMSO in PCR analysis seems to be doubtful.

The PCR reaction constitutes of a ready-to-use Master Mix (PCR-Master-Mix Y, peq-Lab Biotechnology GmbH, Germany) as described below. The concentration of the RNA can vary and was diluted for the use 0f 0.5-1µg/µl. Due to the high risk of imprecise pipetting by technical variation and to guarantee equal conditions for each reaction a Master Mix solution was chosen including all ingredients except primer, cDNA, and water.

PCR-Master-Mix Y 2x concentrated (peqlab Biotechnology GmbH, Germany)

Components	Concentrate
Taq-Polymerase	1.25 units
dNTPs	0.4mM
Tris-HCL	40mM(pH 8.55)
$(NH_4)_2SO_4$	32mM
Tween 20	0,02%
$MgCl_2$	4mM

Reaction Mix

Component	Volume
PCR-Master-Mix Y (peqlab)	10µl
Pimer sense (100µM)	0.5µl
Primer antisense (100µM)	0.5µl
cDNA	variable
Water	variable
Total volume	20µl

The settings of the PCR run was either performed for 30 cycles at 95℃ 20s for denaturation followed by hybridisation of the primers at oligonucletide specific temperature as demonstrated for POU5F1 at 63℃ for 30 s; and the synthesis reaction at 70℃ for 2 min. with a PTC100 thermocyc ler (BioRad, Germany).

5.3.4.1 Gradient PCR

Before performing the PCR reaction on samples, the primers were tested in Gradient-PCR for optimization of the correct annealing temperature of the primers. The PCR reaction was composed as described above. As an example, the Gradient-PCR of POU5F1 is demonstrated (see Fig.36). The annealing temperature range between 65 to 62℃ showed the strongest detection i n both splice variants whereas 63,8℃ show the best expression in POU5F1B. Due to the good detection of both isoforms at 63℃, this temperature was chosen for f urther PCR analysis.

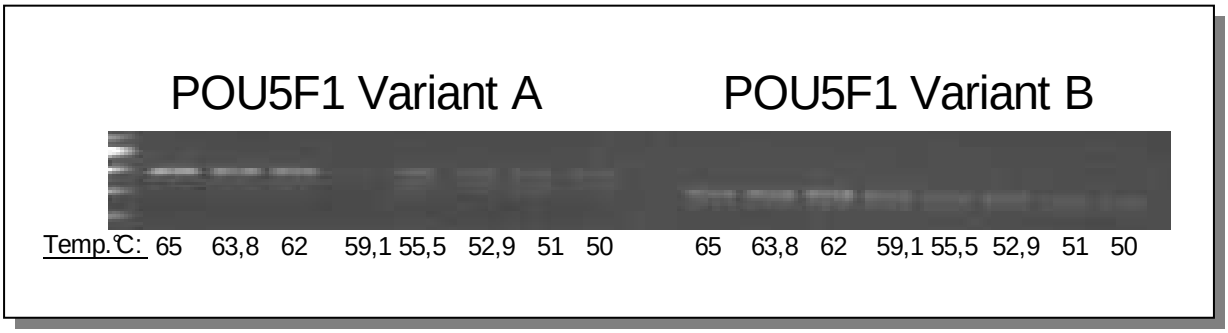

Figure 36.: Gradient-PCR of POU5F1 transcript variants A and B:

Shown are PCR of POU5F1A and POU5F1B. The temperature range is between 65℃ (beginning left to right) to 50 ℃. The best results are given in the a nnealing temperature of 65-62℃ in both isoforms.

5.3.4.2 Quantitative Real-Time Analysis

In contrast to the common PCR technique, Real-Time analysis is used for quantification of an amplified product by measuring the emission of a fluorescence reporter at the extension phase of the Real-Time process. The fluorescence reporter molecule is linked to the amplification cycles and subsequently the emitted signal will increases proportional to the number of PCR products. The fluorescence reporter molecule used in this work is SYBR-green. The dye molecule intercalate unspecifically in the minor groove of the dsDNA [Morrison et al., 1998]. The fluorescent intensity of the SYBR Green will be increased with the amount produced double stranded amplicons. In the extension phase, the SYBR Green bind to the double stranded DNA after the primer annealing. The fluorescence emission will be measured after complete formations of double stranded amplicons.

The important parameter for quantitative real-time analysis is the C_T-value. This parameter is defined by an increase of the amplified products and therefore an accumulation of fluorescence signals over a certain threshold, which differs from the background level. The rises of the C_T-value correspond to the initial amount of DNA that means as higher as the initial amount of DNA the earlier the C_T-value. The setting of the baseline for constitution of the threshold should be placed at the exponential increase.

In additional, to correct inter-experiment variation, the quantitative analysis of a gene of interest was normalized by an endogenous control. This control can be GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin. In this thesis, the relative expression of target cDNA was determined by normalisation with the housekeeping genes RPLP0, and a housekeeping gene chosen from the microarray analysis: EIF3SF12. In Real-time results, the normalization is shown relative to RPLP0.

For all primer pair amplifications in the real-time process and quantification of the initial amount of the cDNA, standard curves were prepared in each experiment to determine correct amplification of the PCR product. Also, at all times, for each amplification, melting curves were recorded to determine whether the correct amplification product forms and whether primer-dimer amplifications occurred. Primer pairs were chosen in such a way that primer dimer formation was negligable.

Components and run parameters of quantitative Real-Time reaction:

Real Time Master Mix

Component	Volume
Power SyberGreen Master Mix (Applied Biosystems)	10µl
Pimer sense (100µM)	0.5µl
Primer antisense /100µM)	0.5µl
cDNA	variable
Water	variable
Total volume	20µl

For the run of the analysis following parameters were chosen 40 cycles (95℃ 15 s; 60℃ 1 min.) in Power SyberGreen Master Mix (Applie d Biosystems) with an Applied Biosystems 7900HT Sequence Detection System (Appelera, Darmstadt). The corresponding software SDS 2.2 (Applied Biosystems) was used for detection and for evaluation.

List of Primers used for PCR and Real-Time Analysis

Primer Name		Forward	Reverse	
Primer for housekeeping				
RPLP0	189	CTGGAGGGTGTCCGCAATGT	AGCAGCCACAAAGGCAGATGGA	Housekeeping
GAPDH	226	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	Housekeeping
EIFSK	150	TGTCGGACAGCCAGCTAAAG	CCATGATGCTGGACACACTG	Housekeeping
Conventional RT-PCR				
GAPDH	108	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA	NM_002046
COL2A1 Collagen II	106	GCCAGGATGTCCGGCAACCAG	TCCCCTCTGGGTCCTTGTTC	NM_033150
ACAN	98	CTGCTTCCGAGGCATTTCAG	CTTGGGTCACGATCCACTCC	NM_013227 NM_001135
"Stemness" primers				
OTF3A POU5F1, variant 1	381	CTCCTGGAGGGCCAGGAATC	CCACATCGGCCTGTGTATAT	NM_002701 [Takeda et al., 1992]
OTF3B POU5F1, variant 2	303	ATGCATGAGTCAGTGAACAG	CCACATCGGCCTGTGTATAT	NM_203289 [Takeda et al., 1992]
OTF3C1 POU5F1P1	215	TGAAGAATCTAGCATCATGGTATG	TGAATGTGTTAGGAACTCTTCAAAA	[Takeda et al., 1992]
OTF3C2 POU5F1P1	228	GTCAGGGTGGAGAGCAACTC	AACACCTTCCCAAATAGAACC	[Takeda et al., 1992]
OTF3 exon3	220	AGAGGCAACCTGGAGAATTT	CCAGAGGAAAGGACACTGG	NM_203289 [Takeda et al., 1992]
OTF3 exon4	171	GAACCGAGTGAGAGGCAACC	CGTTGTGCATAGTCGCTGCT	NM_002701 [Takeda et al., 1992]
Oct4_FR POU5F1	456	AGCCCTCATTTCACCAGGCC	CAAAACCCGGAGGAGTCCCA	[Liedtke et al., 2007]
Oct4_FPR POU5F1	195	GATGGCGTACTGTGGGCCC	CAAAACCCGGAGGAGTCCCA	[Liedtke et al., 2007]
POU5F1	293	ACATGTGTAAGCTGCGGCC	GTTGTGCATAGTCGCTGCTTG	[Zangrossi et al., 2007]
POU5F1	611	TTGCTGCAGAAGTGGGTGGAGGAAG	CCCCAGCCTTTGTGTTCCCAATTCC	[Suo et al., 2005]
Oct4pg5	358	TTGCTGCAGAAGTGGGTGGAGGAAG	GTACCAAAATGGGAGCCTGGGGC	[Suo et al., 2005]
Primers for Identification of novel markers				
STELLAR	294	CCCAGGGTCTCCACAAATGC	GTACGAACTCCGCCCAGTAA	NM_199286
SOX2	437	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCCATTT	NM_003106
ST3GAL3 SSEA-4 synthase	225	TTGGCCTGCCCTTCAACAAT	GTGGTGCCTGGGCCTCTATG	NM_174971
ZFP42 REX1	554	TGAAAGCCCACATCCTAACG	CAAGCTATCCTCCTGCTTTGG	NM_174900
TERT	233	CGGAAGAGTGTCTGGAGCAA	GGATGAAGCGGAGTCTGGA	NM_198255

Continuing List

Primer Name		Forward	Reverse	
CDX2	435	ACTCCGGGAGGACTGGAATG	GCTTTCCTCCGGATGGTGAT	NM_001265
B3GALT2	200	GTTGCAACCCAGTTCATGC	TGGGGCAATGAAAGTCTAGC	NM_003783
EPHX1	179	AAAAGGGAAGGCAGAGAAGC	GCCTGGAAAGGAAGTTCTCC	NM_000120
FGFR2	152	ACACTGCCGTTTATGTGTGG	TACTTGGACCTCAGCCAACC	NM_000141
FLT1	414	GTCACAGAAGAGGATGAAGGTGTC	CACAGTCCGGCACGTAGGTGATT	NM_002019
FOXF1	83	GAGCAGCCGTATCTGCACCA	CTGGGCGACTGCGAGTGATA	NM_001451
GALNT1	163	ACTGGGTTTCCCCATTTCTCC	TAGCCACCTCCTTGATTTGG	NM_020474
GALNT5	155	ATGTGGCTTTGGGTAAATGC	GGCTCCTTTATCAGGGATGG	NM_014568
HLA-C	181	GCTAGGACAACCAGGACAGC	ATGGAACCTTCCAGAAGTGG	NM_002117
HLA-F	164	GAGACTGCTCCCATCTCAGG	ACAGACCCAGGACACAGAGC	NM_018950
IGFBP3	279	CCTCCATTCAAAGATAATC	TCCACACACCAGCAGAAG	NM_000598
INHBA	248	CCTCCCAAAGGATGTACCCAA	GTGATGATCTCCGAGGTCTGC	NM_002192
ITGA11	170	ACTCATGCACCACATCTTCG	AAGGTGCCAGACCTACAGG	NM_001004439
JAGGED1	155	TCCAGCTGACAGAGGTTTCC	ATCTGCCACCTGGCTATGC	NM_00214
MCAM	193	ACCCTGAATGTCCTCGTGACC	AGGTGCTGTTGGCTCTGGTATG	NM_006500
MMP1	128	CGGTTTTTCAAAGGGAATAAGTACT	TCAGAAAGAGCAGCATCGATATG	NM_002421
NOTCH3	186	TTACTACCGAGCCGATCACC	CAGAGGAGCTACTGCGTTCC	NM_000435
POSTN	219	GATGGAGTGCCTGTGGAAT	TCCACACACCAGCAGAAG	NM_006475
PTGES	171	GCCAGGGTGTAGGTCACG	GGAGACCATCTACCCCTTCC	NM_004878
PTTG1	113	GAGAGAGCTTGAAAAGCTGT	TCCAGGGTCGACAGAATGCT	NM_004219
PLXND1	181	CCAGCCCAAAACTACACTCC	TGTGTTTCCAACCAGTCTCG	NM_15103
RRM2	51	GCAGCAAGCGATGGCATAGT	GGGCTTCTGTGTAATCTGAACTTC	NM_001034
SLC16A4	173	GCAAAGAAACTGGCAAGTCC	GGCATCACTAACCTGCTTGC	NM_004696
VCAM1	212	ACCTTCTTGCAGCTTTGTGG	ACAGGAAGTCCCTGGAAACC	NM_001078

5.3.5 Gel-Electrophoresis of RNA and DNA

The agarose gel electrophoresis is used for detection of the PCR products and to determine RNA purity. For this purpose, DNA or RNA fragments will be separated corresponding to their size (bands). The agarose Gel was performed with 1.5% Agarose (NEEO Ultra Roti Garose, Carl Roth GmbH & Co KG, Germany) in Sodium-boric-buffer (Sodium Tetraborate decahydrate, Sigma-Aldrich GmbH, Germany).

For visualization of the different bands, ethidium bromide (1% ethidium bromide solution (10mg/ml), Carl Roth GmbH & Co. KG, Germany) was used, which intercalate in the DNA and was detected with UV-light (GelDoc EQ, BioRad GmbH, Germany). The concentration of the agarose gel decides about the size of the pores and separating attributes. Furthermore, the migration of the DNA in the electric field will be influenced by the size and the conformation of the molecules.

The buffer system was optimized in order for faster separation of DNA [Wasta et al., 2004] using Sodium boric acid (pH8) (Sodium Tetraborate decahydrate, Sigma-Aldrich GmbH, Germany). The buffer allows the running of the DNA under high voltage. The run was performed at 180-250V (Ampere current) for 15-25min.

Sodium-Boric Buffer (pH8)

Components	Volume
Sodium Tetraborate decahydrate	19g
Aqua dest.	10l

Agarose-Gel

Components	Volume
Agarose	4,5g (1,5%)
Sodium Tetraborate decahydrate	300ml

5.3.6 Sequencing Analysis of POU5F1 Transcripts

The POU5F1 primer pairs do not only amplify mRNA derived from the POU5F1 gene, but also mRNA derived from the POU5F1 pseudogenes P1, P3 and P4. POU5F1 transcripts were, therefore, analysed more closely in BM-MSC, UVSC, as well as 2102Ep embryonic carcinoma cells as reference. The PCR was performed using primers of Takeda et al. [Takeda et al., 1992], see “List of Primers used for PCR and Real-Time Analysis” above and agarose gel electrophoresis.

To isolate the amplification products, gel pieces were excise and DNA was eluted out of the gel using QIAquick PCR purification Kit (Qiagen, Germany). The isolated amplification products were that inserted into TOPO vectors cloning in TOP10 bacteria (TOPO TA cloning Kit Invitrogen, Germany) and plated on agar selective media plates. The grown clones were picked and expanded. Plasmid-DNA were isolated, controlled and sent for sequencing analysis.

5.3.7 Extraction and Purification of the PCR Products

The PCR fragments of the POU5F1 splicevariants were enzymatically cleaned using the QIAquick PCR purification Kit (Qiagen, Germany). The system based on a silica-membran technology. The high salt buffer system and the optimal pH allow the optimal absorption of the DNA with an amount up to 10µg to the silica membrane and the removal of contaminants using different washing steps. The absorption of the DNA to the silica membrane is performed in presence of chaotropic salts (guanidine hydrochloride), which modify the structure of water. For the control of the right pH and therefore to guarantee the optimal binding conditions of the nucleic acid to the silica membrane, additional indicator are used in the buffer, which appears in yellow for an correct use at a pH at ≤ 7.5. Finally, after washing the DNA will be eluted in TE-buffer and ready for determination of the concentration.

In detail, the correct bands (POU5F1A, 381bp; POU5F1B 303bp) were excised and weighed after gel-electrophoresis. According to the weight of the gel slice triple the amount of QG buffer is added (100mg gel slice = 300µl QG buffer). To dissolve the agarose gel the reaction is heated to 50°C for 10min and vortexed every 2-3 min. The pH indicator in the QG buffer will show whether the right pH is present in the solution (yellow = correct pH at ≤ 7.5). Afterwards, isopropanol is added to the solution for increasing the DNA yield. The reaction (700µl) is transferred to a silica-membran containing spin column with collection tube below and centrifuged at full speed (x17.900g) for 1min at room temperature. After removing the flow through, the bound DNA is washed with 750µl PE buffer (containing 96-100% ethanol) for 1min at full speed. To remove all PE buffer, the spin column were placed in a new collection tube and centrifuged for another minute at full speed. Finally, the spin column is placed to eppendorf tube and the DNA is eluted with 30-50µl water (pH 7.0-8.5) for 1min at full speed at room temperature. Now, the concentration of purified DNA can be determined.

5.3.8 POU5F1 Transcripts for Transfection into TOP 10 Bacteria

The purified POU5F1 transcripts were used for cloning studies in preparation for sequencing analysis.

The cloning is performed using TOPO TA Cloning (Invitrogen GmbH, Germany). The method based on the topoisomerase I linked ligation reaction of the purified PCR fragment containing a desoxyadenosin overlap with the deoxythymidin overlap of the linearized plasmid vector pCR®2.1-TOPO, which allows the optimal binding of the PCR insert into the vector. Additionally, the pCR®2.1-TOPO contains an ampicillin as well as kanamycin-resistance gene for selectively growing of clones.

The Topoisomerase I bind to the double strand DNA and cleave the phosphodiester backbone after 5'-CCCTT in one strand. This lead to a covalent binding structure between the 3' phosphate of the cleaved strand and the tyrosyl residue (Tyr-274) of the topoisomerase I (see Figure 37 and 38). Finally, topoisomerase I will be released by binding of the 5'hydroxyl to the phospho-tyrosyl bond between the DNA and the topoisomerase I

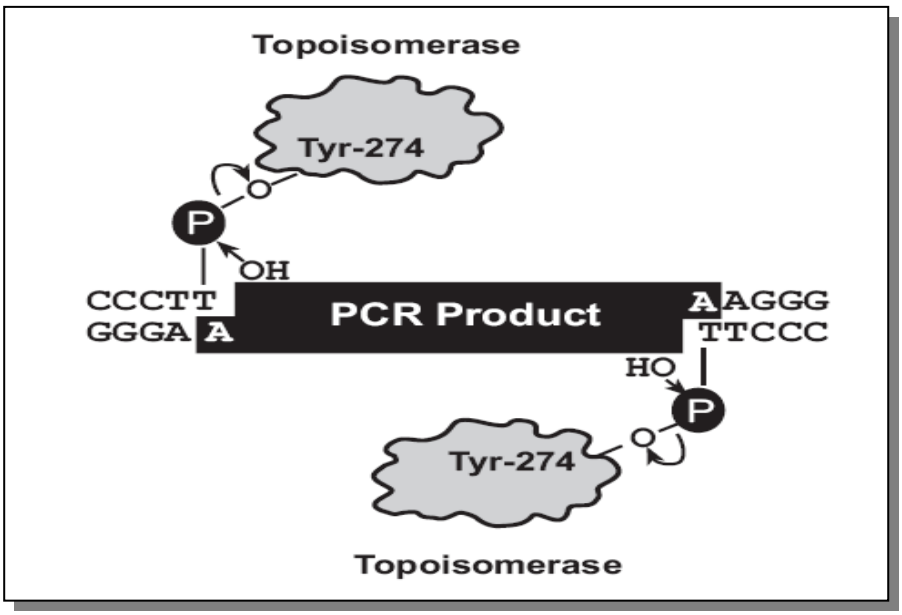

Figure 37: Insertion site of the PCR product in pCR®2.1-TOPO vector

Shematic illustration of the insertion process of the PCR product into the pCR®2.1-TOPO vector using Topoisomerase I.

TOPO-Cloning Reaction Mix

Components	Volume
Fresh PCR product	0.5-4µl
Salt Solution	1µl
Water	Add to a volume of 5µl
TOPO-Vector	1µl
Final Volume	**6µl**

For the insertion of the PCR product into the pCR®2.1-TOPO vector additionally salt solution (200mM NaCl, 10mM $MgCl_2$) is used, which increased the number of transformants 2- to 3-fold.

The TOPO-Cloning reaction, as shown above, was carefully mixed and incubated for 5min at room temperature. Afterwards the reaction mix was stored on ice before use continuing with the One Shot® Chemical Transformation of TOP10 bacteria.

The TOP10 bacteria were slowly thawed on ice and than supplemented with 2µl of the TOPO-Cloning reaction mix containing the PCR insert. The bacteria together with the vector were incubated for 5-30min on ice and than heat-shocked in a pre-warmed 42℃ waterbath for 30s. Finally, 250µl SOC medium were added to the TOP10 bacteria and the shaked for 1h at 37℃. 20-50µl of the SOC-TOP10-Mix were spread out on a pre-warmed Kanamycin or respectively Ampinicilin-selective LB-plates (both resistence genes included in the pCR®2.1-TOPO vector, see Figure 38) and incubated for at least 24h - until maximal 48h at 37℃.

The ampicillin resistance ensures the growing of the bacteria on the ampicillin-selective plates. At this, the enzyme beta-lactamase is formed, which has a neutralizing effect on ampicillin. In contrast to ampicillin, which belongs to the beta-lactam antibiotic like penicillin, kanamycin can also be used for selection of bacteria with or negatively without insert. However, kanamycin is an oligosaccharide, which belongs to a different group of antibiotica in comparison to ampicillin, the aminoglycosid antibiotica.

Individual clones were picked after 24-48h under sterile conditions and incubated for expansion in 15ml tubes with LB-media (5ml) supplemented with Kanamycin (1:1000, Biochrome, Germany) at 37℃ on a rocker plate under vigorous shaking overnight.

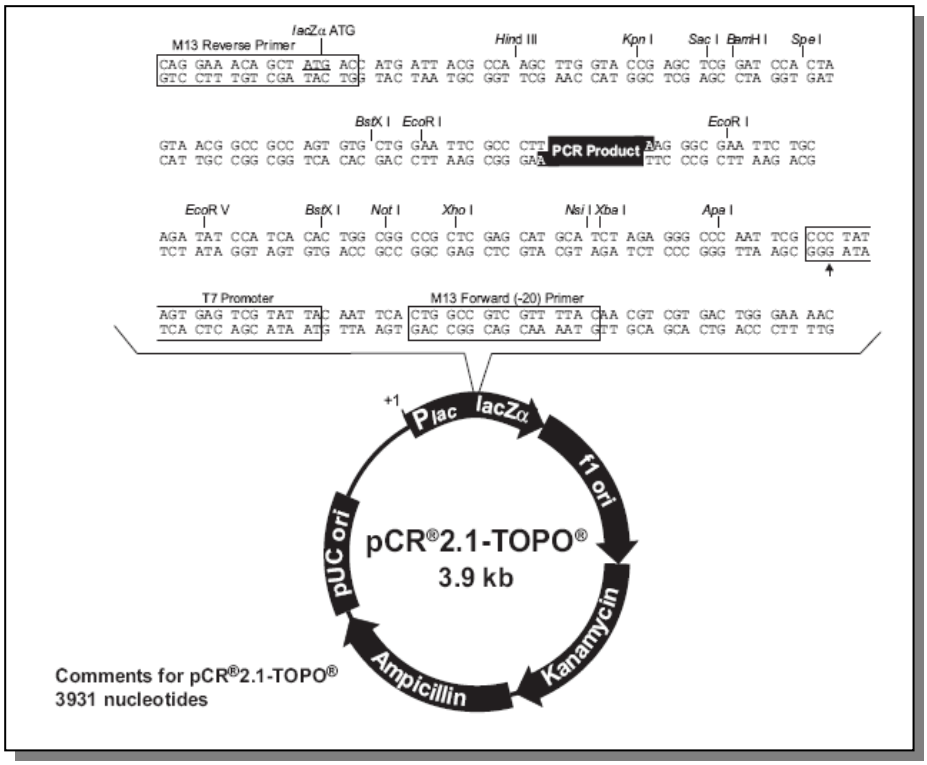

Figure 38: Map of the pCR®2.1-TOPO cloning vector

pCR®2.1-TOPO vector includes insertion site of the PCR product localized between different restriction sites and transcription start of the T7 polymerase (arrow). The vector contains an ampicillin as well as kanamycin resistance ORF.

Afterwards the plasmid-DNA was isolated based on alkaline lysis method [Birnboim and Doly 1979] followed by silica-based membrane technology of Qiagen (QIAprep Spin Miniprep Kit; Qiagen GmbH, Germany). The high-salt buffer system of the QIAprep Spin Kit allows the neutralization of the alkaline conditions after lysis of the bacteria. In different washing steps the isolated plasmid-DNA bound on the silica membrane and will be purified from contaminants.

The overnight grown POU5F1A/B clones in LB-medium were centrifuged at x5400g for 10min at 4℃. The LB-medium was complete remove d and the pelleted bacteria were resuspended with 250µl P1 buffer (containing RNase A and sodium hydroxid). Afterwards, buffer P1 were added to the reaction. The LyseBlue reagent in buffer P1 effects a discoloration in blue. There should be no clumps inside the tube but homogenously blue coloured suspension. After adding N3 buffer (containing guanidine hydrochloride), the suspension show a slurry precipitation, which should be homogenously colourless after inverting the tube repeating times. After centrifugation of 10min at full speed (ca 17,000g) the supernatant will be transferred into a QIAprep spin column. The bound plasmid-DNA was washed with (500µl) PB buffer (containing guanidine hydrochloride and isopropanol) and following with

(750µl) PE buffer (containing 96-100% ethanol). Each step was accompanied with 1min centrifugation at full speed at room temperature. Finally, the spin column was placed in a new collection tube to guarantee the removal of all buffer and eluted in a 1.5-2ml microcentrifuge tube with 50µl water for 1min at full speed. The isolated plasmid-DNA with the respective POU5F1 A or B insert was control-digested using *EcoRI* restriction enzyme (Fermentas GmbH, Germany).

For verification of the correct isolation of the POU5F1 fragments the eluted plasmid-DNA was control digested with *Eco RI* restriction enzyme. According to Figure 38. the PCR insert is integrated between *EcoRI* restriction sites. The control digestion of the plasmid-DNA at 37℃ for 1h will represent two fragments, the plasmid-DNA and the cleaved PCR-insert of the POU5F1 fragment. The results are shown in gel-electrophoresis (1.5% agarose gel in NaB-buffer). In Figure 38 is remarkably presented the POU5F1 fragment with ca 381bp and plasmid-DNA leftover at the top of the agarose gel (of the 1kb ladder).

Plasmids with an POU5F1-insert with the correct size were sequenced (GATC, Germany).

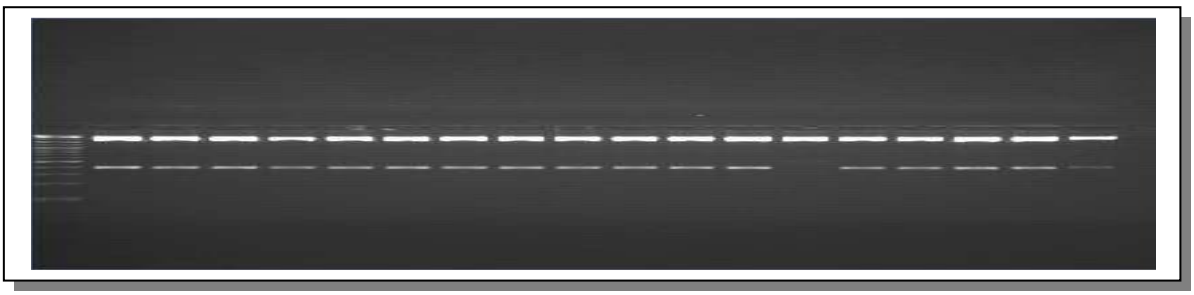

Figure 38: Control Digestion with *Eco RI*

Picture shows the results of the control digestion with *Eco RI* for detection of POU5F1A transcript.

EcoRI Digestion Master Mix

Components	Volume	Company
EcoRI buffer 10x	2µl	Fermentas, Germany
EcoRI enzyme	0.5µl	Fermentas, Germany
Plasmid-DNA (0.5-1µg/µl)	1µl	
Nuclease-free water	16µl	

LB-Medium

Components	Volume	Company
Tryptone	10g	MoBio Laboratories, Germany
Yeast Extract	5g	Sigma-Aldrich, Germany
Sodium Chloride (NaCl)	10g	Sigma-Aldrich, Germany
Aqua dest	To 1l	

The components have to be dissolved in water to 1l volume. Afterwards the LB-media will be autoclaved at 121°for 15 min.

LB plates

Add 20g agar agar to 1l LB media only dissolves during autoclaving and supplemented with antibiotic after cooling down.

Ampicillin to 50 µg/ml

Kanamycin to 30 µg/ml

For finishing the plates, 20 ml per 10cm dish were used and dried under sterile conditions. The plates were stored up side down at 4℃ for later use.

For over-night expansion of picked clones in LB-media or respectively in SOC-media

SOC-Medium

Components	Volume	Company
Tryptone	20g (2%)	
Yeast Extract	5g (0.5%)	Sigma-Aldrich, Germany
Sodium Chloride (NaCl)	0,58g (10 mM)	Carl Roth, Germany
$MgCl_2$	2,5ml (2,5 mM)	Carl Roth, Germany
$MgSO_4$	10ml (10 mM)	Carl Roth, Germany
Glucose	3.4g (20 mM)	Sigma-Aldrich, Germany
Aqua dest	add. to 1L	

5.4 Western Blot Analysis and Immunprecipitation

5.4.1 Protein Isolation and Measurement

For various assays in this thesis, we isolated protein from BM-MSC, UVSC or other cells. For this purpose, cultured UVSC and BM-MSC were trypsinized, washed in ice cold PBS with complete protein inhibitor coktail (cOmplete mini tablets, Roche Applied Science, Mannheim, Germany) and pelleted. The cell pellet was shock frozen in liquid nitrogen and stored at -80℃ until further use. Cytoplasmatic and nuclear fraction of BM-MSCs and UVSCs were separated using established protocols using buffers A and B [Andrews et al., 1991]. The separation of cytoplasmatic and nuclear fraction based on a salt-buffered system. After lysing the cells in buffer A and collecting the cytoplasmatic proteins, the pelleted nuclei were sonicated (10s at duty cyle: 40 and output control: 4, the process will be repeated 3 times) in buffer B to release the nuclear proteins.

For determination of the protein concentration, I used the colorimetric assay based on the method of Bradford et al. [Bradford et al., 1976]. The assay based on a photometric analysis by measuring the absorption maximum of acidic solution of Coomassie Brilliant Blue G-250 from 465nm to 595 nm after binding of proteins. At this, the acidic solution, which appear in brownish-red colour will form a complex with cationic and non-polar region of the protein and turn the colour of the Bradford solution in the blue anionic form of coomassie dye. The absorption spectrum is measured at 595nm.

Total, nuclear or cytoplasmic protein concentration was determined using the Bradford colorimetric assay (BioRad GmbH, Germany). The protein were used in a dilution of 1:1000 in Bradford solution and the absorption spectrum was detected in photometer (SmartSpec Plus, BioRad GmbH, Germany) after defining a standard curve. Afterwards the different protein fractions were frozen at -80℃ for later use.

Protein-Isolation Buffer A

Components	Volume/Concentration	Company
Hepes, pH7,9	100µl (1M)	Sigma-Aldrich, Germany
KCL	100µl (1M)	Carl Roth, Germany
Succrose	3ml (1M)	Sigma-Aldrich, Germany
$MgCl_2$	15µl (1M)	Carl Roth, Germany
DTT	5µl (1M)	Sigma-Aldrich, Germany
NP-40 (10%)	100µl	Sigma-Aldrich, Germany
PMSF	50µl (0.1M)	Sigma-Aldrich, Germany
cOmplete mini tablete	1	Roche
Aqua dest	6630µl	
Total Volume	10ml	

Protein-Isolation Buffer B

Components	Volume/Concentration	Company
Hepes, pH7,9	200µl (1M)	Sigma-Aldrich, Germany
KCL	1000µl (1M)	Carl Roth, Germany
NaCl	200µl (1M)	Carl Roth, Germany
DTT	5µl (1M)	Sigma-Aldrich, Germany
PMSF	50µl (0.1M)	Sigma-Aldrich, Germany
Glycerol	4000µl 50%	Sigma-Aldrich, Germany
cOmplete mini tablet*	1x	Roche, Germany
Aqua dest	4545µl	

* cOmplete mini tablet inhibit serin and cystein proteases like chymotrypsin, trypsin, papain, thermolysin and pancreas-extract (cOmplete, Mini, EDTA-free, protease inhibitir cocktail tablets, Roche Germany)

5.4.2 Western Blot Analysis

The Western Blot Analysis as well as the immunprecipitation represents an analytical method on an antibody-based detection of specific proteins in a sample. Therefore, the protein mixture of cells will be separated by the length of differentially conformed proteins in a polyacryamid gel. Afterwards, the proteins of various sizes will be transferred on a nitrocellulose membrane or PVDF for labelling of the specific proteins with antibody. The detection of the labelled proteins will be performed by chemoluminescence sensitive reaction.

Performance of Western Blot Analysis

For Western blotting, cytoplasmic and nuclear cell extracts (a maximum of 50 µg/lane) were mixed with Laemmli Buffer (2x Loading buffer, see formula below). Laemmli buffer contain β-mercapthoethanol, which reduces the intra and inter-molecular disulfide bond of proteins. Due to the important role of these bonds in folding of secondary and tertiary structures as well as in stabilization of the protein, the addition of Laemmli buffer effect a linearization of the protein and therefore an optimal run in the fragmentation in the SDS-gel. In many formula also Dithiothreitol (DTT) is used, which have a helping effect on reducing the disulphide bonds. Further on, an additional denaturing step for the protein is used in many protocols by heating the sample for 5min at 95℃. The laemmli buffer con tain also bromphenol blue used as indicator dye, to observe the migration of the protein through the SDS gel and guarantee an optimal separation of the protein and glycerol to increase the density of the sample and improve the pipetting and placing of the protein into the pockets of the SDS-Gel (10%).

The SDS shortens for sodium dodecylsulfate polyacrylamide gel is used for analysis of proteins in electrophoresis. The SDS is an anionic detergent, which overlay the protein with a negatively charge and allow the migration through the gel in the electric field.

The protein isolated from BM-MSC, UVSC and 2102Ep carcinoma cells (a maximum of 50 µg/lane) will be supplemented with Laemmli buffer (Loading buffer) to a total volume of 20-25µl. The protein sample will be loaded to the pockets of the SDS-gel in 200-300ml 1x Running buffer (volume depending on the size of the chamber, Mini Protean, BioRad laboratories; or optimal Multigel Long G47 Biometra, Germany). The protein will first run in the collection gel of the SDS gel with 60V and afterwards run with 120V for 1.5 to 2h.

Finally, the gel with the separated protein will be transferred to a 45 µm PVDF membrane (Whatman, Germany) in semi-dry transfer. The semi-dry run with 0.1 Ampere and 25V for 15-25min.

For detection of the POU5F1 protein on the bound PVDF-membran, the protein was incubated with blocking buffer for saturation of unspecific binding sites for 1h at room temperature.

After washing three times with washing buffer the membran was incubated with anti-POU5F1 antibodies (sc-5279, monoclonal mIgG2b, or sc-9081, rabbit polyclonal, both from Santa Cruz Biotechnology Biotechnology, Inc. Germany). Both antibodies were directed against the first 134 aa of the POU5F1 protein, which is only expressed in the POU5F1A isoform. The antibodies were used at a concentration of 100ng/ml and incubated for 45 min. at room temperature or at 4℃ over night at a rocking plate. Goat Anti-mouse or -rabbit HRP-conjugated secondary antibody was used at a concentration of 1ng/ml for 30 to 45 min. at room temperature. The recombinant POU5F1 1-134 protein (42 kDa) was used as positive control (sc-4420, Santa Cruz Biotechnology Inc., Germany). Reactive protein bands were detected using chemoluminescence (Super Signal West Dura or Femto Luminol, Pierce, Thermo Scientific GmbH) and recorded on KODAK film using the Amersham Life Science Hyperprocessor (Automatic Film Processor, Amersham Life Science GE Healthcare, Germany).

5.4.3 Immunprecipitation

For immunoprecipitation, 400µg of either nuclear or cytosolic protein extract was used.

The protein was first treated with a preclearing step (agarose conjugated rabbit IgG-AC antibody (sc-2345, 1 µg/ml, Santa Cruz Biotechnology, Germany) at 4℃ for 30 min) to lower the amount of non-specific contaminants in the lysate and to remove proteins with high affinity for A/G protein.

This step is used optimization and specifically enrichment of the protein lysate for POU5F1 detection.

The pre-cleared protein lysate was incubated overnight at 4℃ with polyclonal rabbit anti-POU5F1 antibody (sc- 9081, Santa Cruz, Germany) at a concentration of 0,2µg/ml. Subsequently, POU5F1 was precipitated using Protein A/G PLUS-Agarose The agarose immunprecipitation reagent consists of highly porous agarose beads with the capacity to bind to the antibody of the antibody-target protein complex. Based on this method high quantities of target protein can be captured. The immunprecipitation reagent (sc-2003 Santa Cruz Biotechnology, Germany) for 1h or optional at 4℃ over night on a rocker plate.

The protein was resuspended in Laemmli buffer (see Loading Buffer) and at 95℃ for 5 min. POU5F1 protein was electrophoresed, blotted onto nylon PVDF membrane, and detected with monoclonal mouse anti-POU5F1 and polyclonal anti-mouse (HRP-conjugated) as described in the previous paragraph. In contrast to the Western Blot and staining protocol above different buffers were used for immunprecipitation.

To avoid non-specific contaminants and optimization of the immunprecipitation blocking buffer was produced according to Santa Cruz protocol (description for TBS BlottoA; http://www.scbt.com/protocol_general_solution.html).

Since precipitating and detecting antibodies are from different species, the precipitating antibody is not detected in the blotted material.

Tris-buffer (1M), pH 6,8

Components	Volume/Concentration	Company
Water	1ml	
Tris-HCL	12,1g	Carl Roth GmbH, Germany

Loading Buffer (2x)

Components	Volume/ Concentration	Company
Dithiothreitol (DTT)	1,55g solved in 12,5 ml water 25%	Sigma-Aldrich, Germany
Tris-buffer	5ml 10%	Carl Roth GmbH, Germany
10%SDS	20ml 40%	Carl Roth GmbH, Germany
1% Bromphenol blue	2,5ml 5%	Gibco GmbH, Germany
Glycerol	10ml 20%	Sigma-Aldrich, Germany

Running Buffer (10x)

Components	Volume/ Concentration	Company
Glycin	288g	Carl Roth GmbH, Germany
Tris base	60g	Carl Roth GmbH, Germany
SDS	20g	Carl Roth GmbH, Germany
Filled with Aqua dest to total volume	2l	

Gel Seperation buffer pH8,8

Components	Volume/ Concentration	Company
Tris	91g	Carl Roth GmbH, Germany
SDS	2g	Carl Roth GmbH, Germany
Aqua dest	Solve in 500ml	

Gel Collection Buffer (4x) pH 6,8

Components	Volume/ Concentration	Company
Tris	6,05g	Carl Roth GmbH, Germany
SDS	0,4g	Carl Roth GmbH, Germany
Aqua dest	To 100ml	

Transfer-Buffer

Components	Volume/ Concentration	Company
Methanol	1l 20%	Merck KGaA, Germany
Running Buffer 10x	0,5l 10%	
Aqua dest	3,5l 70%	

Blocking Buffer

Components	Volume/ Concentration	Company
PBS Tween	0,1%	Carl Roth GmbH, Germany
Milk powder	10%	Fluaka, Sigma-Aldrich, Germay
Total volume	20ml	

TBS-Buffer

Components	Volume/ Concentration	Company
Tris-HCL	10mM (pH7,4)	Carl Roth GmbH, Germany
NaCl	150mM	Carl Roth GmbH, Germany
Water	Add to 1l	

Immunprecipitation Blocking Buffer (refer to the ingredients of TBS BlottoA from Santa Cruz Biotechnology)

Components	Volume/ Concentration	Company
Tween 20	0,05%	Carl Roth GmbH, Germany
Milk powder	5%	Fluaka, Sigma-Aldrich, Germay
1x TBS	Add to 1l	

SDS-Polyacrylamid Separation Gel

Components	Volume/ Concentration	Company
Acryl/Bi-phosphat	1,33%	Carl Roth GmbH, Germany
4x Tris/HCL pH8,8	1%	Carl Roth GmbH, Germany
Aqua dest	2,34%	
Ammonium persulfat	0,017%	Carl Roth GmbH, Germany
TEMED	0,003%	Carl Roth GmbH, Germany

SDS-Polyacrylamid Collection Gel

Components	Volume/ Concentration	Company
Acryl/Bi-phosphat pH 6,8	13%	Carl Roth GmbH, Germany
4x Tris/HCL pH8,8	25,25%	Carl Roth GmbH, Germany
Aqua dest	61,75%	
Ammonium persulfat 10%	0,5%	Carl Roth GmbH, Germany
TEMED	0,1%	Carl Roth GmbH, Germany

5.5 Ossicle Formation *in-vivo*

In-vivo transplantation of UVSC and BM-MSC was performed as reported in Krebsbach et al., Bianco et al., Sacchetti et al. [Krebsbach et al., 1997; Bianco et al., 1998; Sacchetti et al., 2007]. All animal procedures were approved by the relevant institutional committee (Biomedical Science Park, University La Sapienza, Rome). For BM-MSC, cells from three different donors (ages 35–50) were used to generate 12 transplants in six mice. For UVSC, cells from three donors were used to generate 14 transplants in seven mice. MSC from BM-MSC and UVSC were cultured at passages 2-4 with 2 x 10^6 cells. The MSCs were detached after trypsin treatment and resuspended in 30-100µl culture medium (α-MEM supplemented with 10% FCS and antibiotics) after pelleting. Finally, the cells were allowed to attach on vehicles of hydroxyapatite/tricalcium phosphate particles (40 mg powder, 100–200 mm; Zimmer, Warsaw, IN, USA). Cells attached to the carriers were used for transplantation subcutaneously into the back of 8- to 15-weekold female nih/nu/xid/bg (BNX) mice (Harlan-Sprague Dawley, Inc., Indianapolis, IN). As control, carrier alone was also transplanted. The transplantation of the cell-carrier construct was performed in small skin pockets of about 1cm in length on the dorsal surface of each mouse. A single transplant was injected into each pocket, with up to four transplants per animal. The incisions on the back of the mouse were closed with surgical staples.

After 8 weeks, the bony structures called “ossicles” were harvested and fixed as well as decalcified in Bouin’s solution (30% picric acid, 10% formalin, 5% acetic acid) (Sigma-Aldrich, Germany) for 2 days. The ossicles were stored in 70% ethanol until they were embedded in paraffin.

For histological examination, sections were perfomed and were deparaffinized, hydrated, as well as stained with hematoxylin. Due to the osteotropic capacity the fluorochrome eosin is used for representation of the bone formation.

For estimation of bone formation and hematopoiesis, the stained sections were examined histologically, and the extent of bone within each transplant was scored on a semiquantitative scale by 2 independent, blinded, and trained investigators [Sacchetti et al., 2007]. Additionally, each transplant was assessed histologically for either the presence or absence of hematopoiesis.

Chapter 6: Equipment and Materials

6.1 General used Instruments

6.1.1 Scales

Instrument	Company
770	Kern & Sohn GmbH, Germany
PLJ 2100-2M	Kern & Sohn GmbH, Germany

6.1.2 Incubator and Steril Hoot

Instrument	Company
BD53	Binder GmbH, Germany
Hera Cell 240	Heraeus, Kendro Laboratory products GmbH, Germany
Minitron	HT Infors, GmbH, Germany
Biohit	Antares, Germany

6.1.3 Centrifuge and Rocker Plates

Instrument	Company
Biofuge fresco	Heraeus, Thermo electron Cooperation, Germany
Megafuge 3.0 RS	Heraeus Instruments, Thermo Scientific GmbH, Germany
Multifuge 3s	Heraeus Instruments, Thermo Scientific GmbH, Germany
miniSpin	Eppendorf GmbH, Germany
Reax top	Heidolph Elektro GmbH & Co. KG, Germany
RM5	CAT M.Zipperer GmbH, Germany
WT12	Biometra biomedizinische Analytik GmbH, Germany

6.1.4 PCR Machine

Instrument	Company
DNA-Engine	BioRad Laboratories GmbH, Germany
PTC-100 Peltier Thermal Cycler	MJ Research, BioRad Laboratories GmbH, Germany

6.2 Conventional used Instruments

Instrument	Company	Use
Chronos Cryogenic tank	Messer GmbH, Germany	Cryo-conservation of cells
Freezer Premium, -20℃	Liebherr GmbH, Germany	Stora ge of materials for molecular biology
GelDoc EQ	BioRad Laboratories GmbH, Germany	Visualization and documentation of agarose gels
IBS pipetboy acu	Integra Bioscience GmbH, Germany	Individual used for pipetting in cell culture and molecular biology
Mini Protean	BioRad Laboratories GmbH, Germany	Western Blot glass plates and chamber (small)
Multigel Long G47	Biometra biomedizinische Analytik GmbH, Germany	Western Blot glass plates and chamber (large)
Microwave MWS 2820	Bauknecht GmbH, Germany	Melting and homogenizing of agarose gel
Polymax 1040	Heidolph Elektro GmbH & Co. KG, Germany	Power supply for Western Blot run
Power Pac 300 Energy supply	BioRad Laboratories GmbH, Germany	Power supply for agarose gel
Power Pac 3000 Energy supply	BioRad Laboratories GmbH, Germany	Power supply for Western Blot run/Semi-dry transfer
Power Pack P25T Energy supply	Biometra biomedizinische Analytik GmbH, Germany	Power supply for Western Blot run/Semi-dry transfer
PowerShot A620	Canon, Germany	Camera for documentation
Research or Reference in 10/50/100/200/1000µl	Eppendorf GmbH, Germany	Pipette
Sonoplus HD2070	Bandelin electronic GmbH & Co. KG, Germany	Isolation of cytoplasmatic/nuclear protein
Sub-Cell GT	BioRad Laboratories GmbH, Germany	Agarose-Gel electrophoresis
Thermomixer comfort	Eppendorf GmbH, Germany	Preparation and performance in molecular methods
Trans-Blot SD Semi-Dry Transfer Cell	BioRad Laboratories GmbH, Germany	Semi-dry transfer blotting chamber
Ultra low VIP-Series -86℃	Sanyo Sales & Marketing Europe GmbH, Germany	Conservation of RNA, protein extracts, and bacteria
UW 2070	Bandelin electronic GmbH & Co. KG, Germany	Ultrasonic converter and homogenizer
Waterbath	Grant instruments Ltd, UK	Thawing of media and heat-shock transformation

6.3 Plastic Material

Product	Company
24-well culture Plate	Cellstar, Greiner bio-one GmbH, Germany
Cell culture flask 150cm^2/ 100x20mm	TPP, Switzerland
Cell culture test plate 6-well	TPP, Switzerland
Cell Scraper	Sarstedt AG & Co, Germany
Cell strainer 40/70µm	Beckton Dickinson GmbH, Germany
Cryogenic Vial round bottom	Corning Inc. , Germany
Immoblion-psq Transfer membran PVDF 0,2µm	Millipore GmbH, Germany
MicroAmp Optical 96-well Reaction Plate	Applied Biosystems GmbH, Germany
Multi-Guard Barrier Tips	Sorenson Bioscience Inc, Carl Roth GmbH & Co KG, Germany
PCR softstrips 0,2ml	Biozym Scientific GmbH, Germany
Pipette 2/5/10/25/50ml	Greiner bio-one Cellstar, Germany
Pipette-Tips 1-1000µl	Sarstedt AG & Co, Germany
Polystyrene Round-bottom tube	Beckton Dickinson GmbH, Germany
PP-Test tube 15ml/50ml	Greiner bio-one GmbH, Germany
Qiashredder	Qiagen GmbH, Germany
Raucodrape, OR drape system	Lohmann & Rauscher GmbH & Co. KG, Germany
Rotrand 0,2 and 0,45µm Celluloseacetat FP 30/0,2 or o,45 CA-S	Whatman, Switzerland
Steritop GP Express Plus Membran 0,22µm	Millipore GmbH, Germany
Syringe Omnifix 10ml Luer Lock Solo	B/Braun AG, Germany
TipOne Filter Tips 101-1000µl, 0,2-5µl	STARLAB GmbH, Germany
Tissue Culture Flask 75cm^2	Cellstar, Greiner bio-one GmbH, Germany

6.4 Software

Software	Company
QuantityOne 4.5.0	BioRad Laboratories GmbH, Germany
Zoom Browser EX	Canon, Germany
SDS2.2	Applied Biosystems, Germany
Axio Vision	Carl Zeiss, Germany

6.5 Chemicals

Chemical	Company
2-mercapthoethanol	Sigma-Aldrich Inc. , Germany
Basic FGF2	R&D Systems GmbH, Germany
Eco RI + EcoRI 10x buffer with BSA	Fermentas GmbH, Germany
Ethanol 99,8%	Apotheke Klinikum Rechts der Isar, Germany
Germany Methanol 99,8%	Merck KGaA, Germany
NEEO Ultra Roti Garose	Carl Roth GmbH & Co KG, Germany
Platinum Sybr Green qPCR SuperMix-UDG	Invitrogen GmbH, Germany
Protein A Sepharose Beads CL-4B	Amershan Pharmacia Biotech AB, Sweden
Skim Milk Powder	Fluaka, Sigma-Aldrich Inc. , Germany

6.6 Kits used for molecular Biology

Kit	Company
RNeasy Mini Kit	Qiagen, Germany
QIAquick Gel Extraction Kit	Qiagen, Germany
QIAprep Miniprep	Qiagen, Germany
Omniscript Reverse Transcriptase	Qiagen, Germany
TOPO TA Cloning	Invitrogen, Germany

Appendix

Table 1: Alignment of POU5F1P1, -P3, and P4 nucleotide sequences with POU5F1 [Takeda et al., 1992]

Shown is the nucleotide sequence between the two primers (in black) used for PCR amplification (Figure 16, List of Primers in chapter 5.4.3.2). The cloned and sequenced PCR products (Table3) all correspond to one of the sequences shown here. In some clones, additional single nucleotide changes may be present.

```
1          11         21         31         41         51         61
POU5F1   CTCCTGGAGG GCCAGGAATC GGGCCGGGGG TTGGGCCAGG CTCTGAGGTG TGGGGGATTC CCCCATGCCC
POU5F1P1 .......... .......... .......... .......... .......... .......... ....T.....
POU5F1P3 .......... .......... .........T .......... ........A. .......... .......T..
POU5F1P4 .......... .......... ........A. .....T.... .......... .......... ..........

71         81         91         101        111        121        131
POU5F1   CCCGCCGTAT GAGTTCTGTG GGGGGATGGC GTACTGTGGG CCCCAGGTTG GAGTGGGGCT AGTGCCCCAA
POU5F1P1 .......... .....A.... .......... .......... ..T....... .......... ..........
POU5F1P3 .......... ........C. .......... .......... ..T...AC.. .......... ..........
POU5F1P4 .....T.... .......... .......... .......... ..T....... .....C.... ..........

141        151        161        171        181        191        201
POU5F1   GGCGGCTTGG AGACCTCTCA GCCTGAGGGC GAAGCAGGAG TCGGGGTGGA GAGCAACTCC GATGGGGCCT
POU5F1P1 .......... .......... .......A.. .......... .......... .......... A.........
POU5F1P3 .A........ .......... .......... .......... .......... .......... ..........
POU5F1P4 .......... .......... .......... .......... ..A....... .......... ....CA....

211        221        231        241        251        261        271
POU5F1   CCCCGGAGCC CTGCACCGTC ACCCCTGGTG CCGTGAAGCT GGAGAAGGAG AAGCTGGAGC AAAACCCGGA
POU5F1P1 ........A.. .......... C......... .......... .......... .....A.... ..........
POU5F1P3 .......... .......... C..T...... .......... .......... .......... ........A.
POU5F1P1 ...T...... .......... C......... .......A.. .......... .......... ........C.

281        291        301        311        321        331        341
POU5F1   GGAGTCCCAG GACATCAAAG CTCTGCAGAA AGAACTCGAG CAATTTGCCA AGCTCCTGAA GCAGAAGAGG
POU5F1P1 .A........ .......... .......... .......... .......... .......... ..........
POU5F1P3 .......... .......... .......... .......... .......... .......... ..........
POU5F1P4 .......... A......... .......... .........A .......... .......... ..........

351        361        371        381
POU5F1   ATCACCCTGG GATATACACA GGCCGATGTG G
POU5F1P1 .......... .......... .......... .
POU5F1P3 .......... .......... .......... .
POU5F1P4 .......... .......... .......... .
```

Table 2: Alignment of the POU5F1 B transcript in BM-MSC, UVSC, and 2102Ep

The G/T polymorphism shown on position 88 has been described previously as start site for translation of the OTF3B transcript. Since this position would be the ATG startcodon, it has been predicted that only half of the population would, in fact, express the POU5F1B protein isoform [Takeda et al., 1992]. We here identify an additional insertional site between positions 121 and 122. The polymorphism and insertions are found in all three cell types: 2102Ep (ECB1-5), UVSC (BA, BI-III, MSCB5), and BM-MSC (KMB1-4).

```
       1           11          21          31          41          51          61
POU5F1 AATGCATGAGT CAGTGAACAG GGAATGGGTG AATGACATTT GTGGGTAGGT TATTTCTAGA AGTTAGGTGG
ECB2                                               .. .....-.... .-.....-.. ..........
ECB3                                               .. ........-.. .-......... ..........
ECB4                                               .. .......... .......... ..........
ECB5                                               .. .......-.. .-......... ..........
BA     ........... .......... .......... .......... .......... .......... .........
BI     ........... .......... .......... .......... .......... .......... .........
BII    ........... .......... .......... .......... .......... .......... .........
BIII   ........... .......... .......... .......... .......... .......... .........
MSCB5                                              .. .......-.. .-......... ..........
KMB1                                               .. .......-.. .-......... ..........
KMB2                                              ... ......-... .-......... ..........
KMB3                                               .. .....-.... .-......... ..........
KMB4                                               .. .....-.... .-......... ..........
       71          81          91          101         111         121         131
POU5F1 GCAGCTTGGA AGGCAGAGGC ACTTCTACAG ACTATTCCTT GGGGCCACAC G-TAGGTTCTT GAATCCCGAA
ECB2   .......... .......... .......... .......... .......... .-......... ..........
ECB3   .......... .......... .......... .......... .......... .C......... ..........
ECB4   .......... .......... .......... .......... .......... .T......... ..........
ECB5   .........G .......... .......... .......... .......... .-......... ..........
BA     .......... .......... .......... .......... .......... .-......... ..........
BI     .......... .......T.. .......... .......... .......... .-......... ..........
BII    .......... .......... .......... .......... .......... .-......... ..........
BIII   .......... .......... .......... .......... .......... .-......... ..........
MSCB5  .......... .......... .......... .......... .......... .-......... ..........
KMB1   .......... .......... .......... .......... .......... .-......... ..........
KMB2   .......... .......... .......... .......... .......... .-......... ..........
KMB3   .......... .......T.. .......... .......... .......... .-......... ..........
KMB4   .......... .......T.. .......... .......... .......... .T......... ..........
       141         151         161         171         181         191         201
POU5F1 TGGAAAGGGG AGATTGATAA CTGGTGTGTT TATGTTCTTA CAAGTCTTCT GCCTTTTAAA ATCCAGTCCC
ECB2   .......... .......... .......... .......... .......... .......... ..........
ECB3   .......... .......... .......... .......... .......... .......... ..........
ECB4   .......... .......... .......... .......... .......... .......... ..........
ECB5   .......... .......... .......... .......... .......... .......... ..........
BA     .......... .......... .......... .......... .......... .......... ..........
BI     .......... ....C..... .......... .......... .......... .......... ..........
BII    .......... .......... .......... .......... .......... .......... ..........
BIII   .......... .......... .......... .......... .......... .......... ..........
MSCB5  .......... .......... .......... .......... .......... .......... ..........
KMB1   .......... .......... .......... .......... .......... .......... ..........
KMB2   .......... .......... .......... .......... .......... .......... ..........
KMB3   .......... .......... .......... .......... .......... .......... ..........
KMB4   .......... .......... .......... .......... .......... .......... ..........
       211         221         231         241         251         261         271
POU5F1 AGGACATCAA AGCTCTGCAG AAAGAACTCG AGCAATTTGC CAAGCTCCTG AAGCAGAAGA GGATCACCCT
ECB2   .......... .......... .......... .......... .......... .......... ..........
ECB3   .......... .......... .......... .......... .......... .......... ..........
ECB4   .......... .......... .......... .......... .......... .......... ..........
ECB5   .......... .......... .......... .......... .......... .......... ..........
BA     .......... .......... .......... .......... .......... .......... ..........
BI     .......... .......... .......... .......... .......... .......... ..........
BII    .......... .......... .......... .......... .......... .......... ..........
BIII   .......... .......... .......... .......... .......... .......... ..........
MSCB5  .......... .......... .......... .......... .......... .......... ..........
KMB1   .......... .......... .......... .......... .......... .......... ..........
KMB2   .......... .......... .......... .......... .......... .......... ..........
KMB3   .......... .......... .......... .......... .......... .......... ..........
KMB4   .......... .......... .......... .......... .......... .......... ..........
```

Continuing alignment of POU5F1 transcript B

```
       281        291        301   Origin
POU5F1 GGGATATACA CAGGCCGATG TGG
ECB2   .......... .......... ...   2102Ep
ECB3   .......... .......... ...   2102Ep
ECB4   .......... .......... ...   2102Ep
ECB5   .......... .......... ...   2102Ep
BA     .......... .......... ...   UVSC
BI     .......... .......... ...   UVSC
BII    .......... .......... ...   UVSC
BIII   .......... .......... ...   UVSC
MSCB5  .......... .......... ...   UVSC
KMB1   .......... .......... ...   BM-MSC
KMB2   .......... .......... ...   BM-MSC
KMB3   .......... .......... ...   BM-MSC
KMB4   .......... .......... ...   BM-MSC
```

Table 3A: Alignement of POU5F1 with putative POU5F1P1, POU5F1P3, and POU5F1P4 protein products

Corresponding protein sequence to the POU5F1A amplified transcript. The amino acid are illustrated to the inherent codon sequence beneath. The triplet codon specifies a single amino acid.

```
                                        50                                  60
 P   P   G   G   P   G   I   G   P   G   V   G   P   G   S   E   V   W   G   I
CCT CCT GGA GGG CCA GGA ATC GGG CCG GGG GTT GGG CCA GGC TCT GAG GTG TGG GGG ATT

                                        70                                  80
 P   P   C   P   P   P   Y   E   F   C   G   G   M   A   Y   C   G   P   Q   V
CCC CCA TGC CCC CCG CCG TAT GAG TTC TGT GGG GGG ATG GCG TAC TGT GGG CCC CAG GTT

                                        90                                 100
 G   V   G   L   V   P   Q   G   G   L   E   T   S   Q   P   E   G   E   A   G
GGA GTG GGG CTA GTG CCC CAA GGC GGC TTG GAG ACC TCT CAG CCT GAG GGC GAA GCA GGA

                                       110                                 120
 V   G   V   E   S   N   S   D   G   A   S   P   E   P   C   T   V   T   P   G
GTC GGG GTG GAG AGC AAC TCC GAT GGG GCC TCC CCG GAG CCC TGC ACC GTC ACC CCT GGT

                                       130                                 140
 A   V   K   L   E   K   E   K   L   E   Q   N   P   E   E   S   Q   D   I   K
GCC GTG AAG CTG GAG AAG GAG AAG CTG GAG CAA AAC CCG GAG GAG TCC CAG GAC ATC AAA

                                       150                                 160
 A   L   Q   K   E   L   E   Q   F   A   K   L   L   K   Q   K   R   I   T   L
GCT CTG CAG AAA GAA CTC GAG CAA TTT GCC AAG CTC CTG AAG CAG AAG AGG ATC ACC CTG

 G   Y   T   Q   A   D   V   G
GGA TAT ACA CAG GCC GAT GTG GGG
```

Table 3B Protein Alignment

POU5F1A amplified sequence corresponds to protein domains of both POU5F1 and the putative POU5F1P1, POU5F1P3, and POU5F1P4 proteins

```
POU5F1    PPGGPGIGPG VGPGSEVWGI PPCPPPYEFC GGMAYCGPQV GVGLVPQGGL ETSQPEGEAG
POU5F1P1  .......... .......... ........L. .......... .......... ......S...
POU5F1P3  .......... F.....V... .......... .........T .......D.. ..........
POU5F1P4  .......... .......... .....L.... .......... ..R....... ..........

POU5F1    VGVESNSDGA SPEPCTVTPG AVKLEKEKLE QNPEESQDIK ALQKELEQFA KLLKQKRITL
POU5F1P1  .......N.. .......P.. .......... ....K..... .......... ..........
POU5F1P3  .......... .......... .......... ...K...... .......... ..........
POU5F1P4  .........T .L.....P.. .......... ...Q...... .......... ..........

POU5F1    GYTQADVG
POU5F1P1  ........
POU5F1P3  .......A
POU5F1P4  ........
```

References

POU5F1P1 [Takeda et al., 1992]
POU5F1P3 GeneBank: AAG53084 UniProtKB/Swiss-Prot: Q9BZV8
POU5F1P4 UniProtKB/Swiss-Prot: Q9BZW0

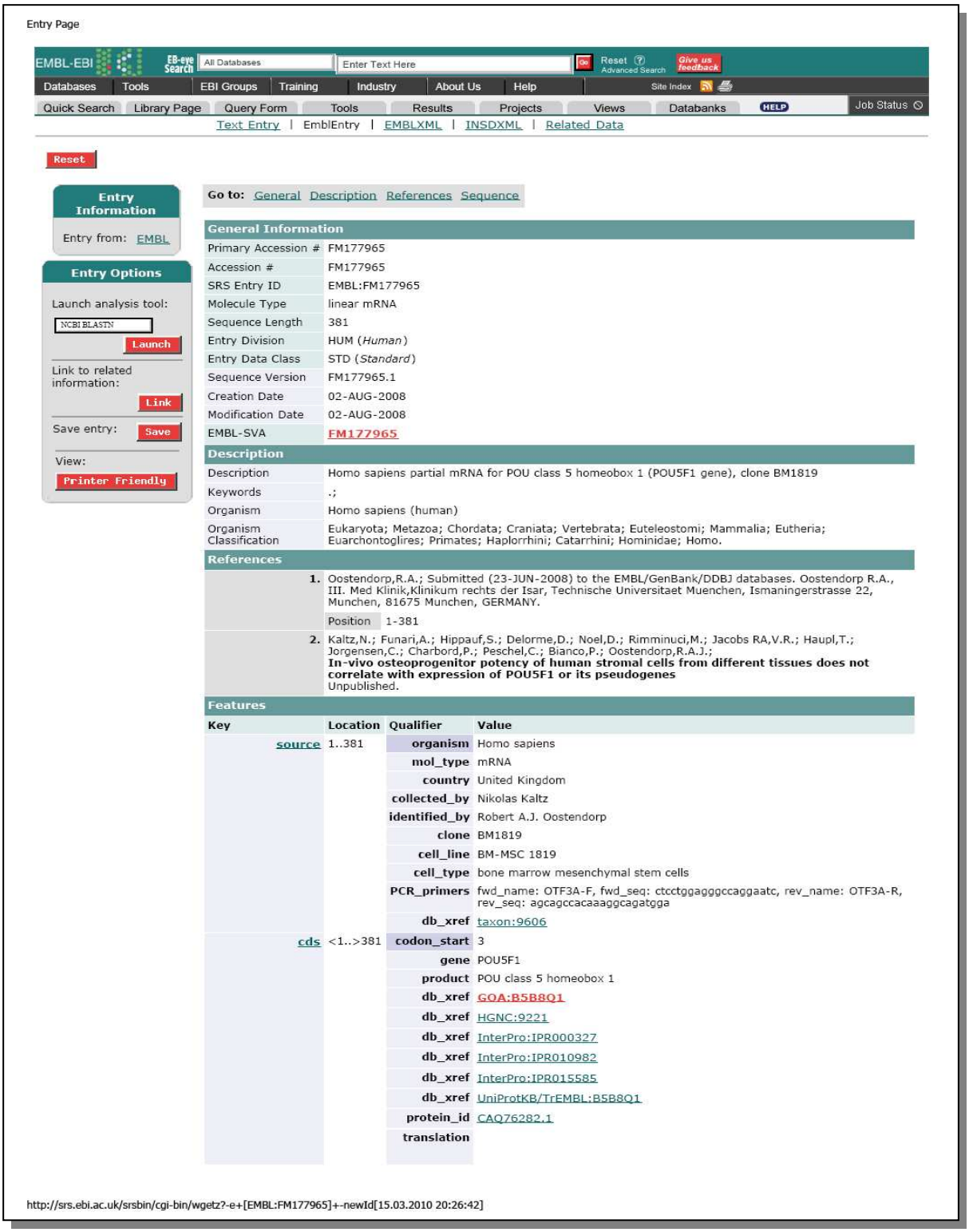

Figure 1: GeneBank Entry of Nucleotide Sequences of POU5F1 Clones

The picture shows an example of the isolated sequences of POU5F1 transcripts derived from one clone (BM1819) analysed in this study. The various sequences are deposed at the Nucleotide Sequence Database EMBL-Bank as shown here. Published sequences from BM-MSC, UVSC, and 2102Ep in the present work correspond to the accession numbers FM177951-17768.

Table 4: Membrane associated genes 2-fold more expressed in BM-MSC compared to UVSC*

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2
205882_x_at	ADD3	2436	1004	***2,42***	2602	765	3,40
201034_at	ADD3	3299	1580	***2,09***	4065	1245	3,26
201752_s_at	ADD3	2537	1217	***2,08***	2754	826	3,33
1557924_s_at	ALPL	104	13	***8,20***	25	5	5,22
223092_at	ANKH	2678	528	***5,08***	2536	1162	2,18
214255_at	ATP10A	484	68	***7,07***	522	109	4,79
212062_at	ATP9A	395	116	***3,40***	378	110	3,44
1563539_at	AZGP1	20	6	***3,50***	18	3	6,73
216627_s_at	B4GALT1	244	54	***4,52***	247	97	2,56
211631_x_at	B4GALT1	196	73	***2,69***	199	73	2,72
203547_at	CD4	166	28	***5,89***	88	25	3,46
209619_at	CD74	109	22	***4,92***	153	14	10,74
202910_s_at	CD97	382	174	***2,20***	419	150	2,79
224794_s_at	CERCAM	775	201	***3,86***	614	191	3,21
32094_at	CHST3	324	104	***3,11***	321	140	2,30
219561_at	COPZ2	1429	256	***5,59***	1977	940	2,10
204570_at	COX7A1	802	142	***5,64***	1298	478	2,72
223454_at	CXCL16	93	44	***2,11***	66	5	13,53
205399_at	DCAMKL1	132	38	***3,49***	101	26	3,82
201809_s_at	ENG	719	218	***3,30***	971	190	5,11
201808_s_at	ENG	280	89	***3,16***	291	109	2,67
203638_s_at	FGFR2	399	8	***51,63***	424	51	8,25
203639_s_at	FGFR2	130	25	***5,16***	109	18	5,99
208228_s_at	FGFR2	284	56	***5,10***	336	61	5,55
211401_s_at	FGFR2	44	20	***2,20***	62	16	3,84
1555523_a_at	FYCO1	97	41	***2,40***	69	28	2,47
203066_at	GALNAC4S-6ST	449	26	***17,40***	447	33	13,72
229555_at	GALNT5	642	69	***9,27***	1025	153	6,70
232110_at	GALNT5	152	57	***2,66***	99	29	3,46
205505_at	GCNT1	316	94	***3,36***	441	172	2,56
239761_at	GCNT1	468	191	***2,45***	445	159	2,80

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2
205498_at	GHR	179	45	***4,00***	146	68	2,14
218805_at	GIMAP5	27	12	***2,35***	35	13	2,64
207316_at	HAS1	966	74	***13,03***	1110	36	30,61
205425_at	HIP1	222	97	***2,28***	179	56	3,19
217478_s_at	HLA-DMA	114	18	***6,45***	181	52	3,48
204773_at	IL11RA	211	66	***3,20***	366	142	2,59
1554819_a_at	ITGA11	892	17	***51,02***	799	241	3,32
222899_at	ITGA11	3214	70	***45,78***	2014	676	2,98
202803_s_at	ITGB2	111	7	***16,71***	97	10	9,37
201124_at	ITGB5	467	137	***3,42***	318	124	2,55
201125_s_at	ITGB5	2798	1234	***2,27***	1987	957	2,08
216268_s_at	JAG1	1150	259	***4,44***	1220	445	2,74
209099_x_at	JAG1	1530	368	***4,16***	1895	589	3,22
209098_s_at	JAG1	203	66	***3,08***	184	78	2,36
209948_at	KCNMB1	145	27	***5,40***	244	37	6,60
200785_s_at	LRP1	349	121	***2,88***	281	120	2,33
206584_at	LY96	579	149	***3,89***	450	131	3,43
235141_at	MARVELD2	123	37	***3,28***	111	38	2,92
205442_at	MFAP3L	262	43	***6,15***	270	55	4,91
206522_at	MGAM	111	13	***8,87***	68	3	22,34
207565_s_at	MR1	162	27	***6,07***	175	77	2,27
210223_s_at	MR1	266	52	***5,08***	218	96	2,27
213438_at	NFASC	346	25	***13,95***	326	1	222,07
203238_s_at	NOTCH3	436	19	***23,50***	450	91	4,95
219790_s_at	NPR3	385	4	***109,24***	341	5	73,10
219789_at	NPR3	1476	82	***18,09***	680	29	23,86
209982_s_at	NRXN2	61	13	***4,76***	36	8	4,63
218589_at	P2RY5	200	74	***2,69***	195	74	2,65
210335_at	PAMCI	60	17	***3,48***	54	15	3,66
223854_at	PCDHB10	90	5	***17,87***	39	6	6,04
231726_at	PCDHB14	93	15	***6,05***	42	8	5,44
231725_at	PCDHB2	95	10	***9,91***	34	4	7,72
240317_at	PCDHB4	70	4	***19,88***	36	8	4,75
239443_at	PCDHB6	33	7	***4,96***	43	7	6,23

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2
231738_at	PCDHB7	60	5	***11,00***	53	14	3,78
202273_at	PDGFRB	598	131	***4,57***	654	244	2,68
205112_at	PLCE1	97	25	***3,92***	55	24	2,35
38671_at	PLXND1	906	246	***3,69***	1120	489	2,29
205880_at	PRKD1	957	203	***4,72***	1194	408	2,93
207733_x_at	PSG9	107	11	***9,51***	107	20	5,29
210367_s_at	PTGES	763	41	***18,47***	1121	306	3,67
207388_s_at	PTGES	209	29	***7,33***	353	133	2,66
204944_at	PTPRG	370	106	***3,49***	610	185	3,30
1552502_s_at	RHBDL2	54	8	***6,94***	60	21	2,91
1553962_s_at	RHOB	258	50	***5,17***	676	281	2,41
212099_at	RHOB	2930	625	***4,69***	3142	1408	2,23
205805_s_at	ROR1	308	63	***4,90***	494	241	2,05
228407_at	SCUBE3	3149	122	***25,81***	3142	368	8,55
230730_at	SGCD	128	32	***4,04***	202	30	6,63
228602_at	SGCD	144	58	***2,48***	120	27	4,54
213543_at	SGCD	85	35	***2,44***	141	28	5,05
205234_at	SLC16A4	543	30	***18,15***	521	92	5,66
213664_at	SLC1A1	1263	343	***3,68***	1031	495	2,08
223194_s_at	SLC22A23	276	27	***10,10***	193	63	3,08
230624_at	SLC25A27	38	5	***6,88***	20	6	3,40
1554161_at	SLC25A27	37	13	***2,78***	33	3	11,07
204204_at	SLC31A2	274	90	***3,06***	209	95	2,19
222783_s_at	SMOC1	154	16	***9,82***	91	9	10,05
208608_s_at	SNTB1	51	8	***6,42***	124	12	10,49
214708_at	SNTB1	67	15	***4,48***	95	17	5,74
226438_at	SNTB1	179	53	***3,34***	338	34	10,02
228072_at	SYT12	83	19	***4,28***	58	17	3,37
211258_s_at	TGFA	27	10	***2,64***	42	12	3,43
221060_s_at	TLR4	214	17	***12,73***	152	26	5,77
209655_s_at	TM4SF10	1300	318	***4,08***	996	356	2,80
209656_s_at	TM4SF10	3160	848	***3,73***	3362	1368	2,46
220021_at	TMC7	60	29	***2,10***	32	7	4,34
204079_at	TPST2	1037	480	***2,16***	1862	486	3,83

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2
224220_x_at	TRPC4	102	26	***3,85***	151	75	2,00
220817_at	TRPC4	110	33	***3,31***	417	194	2,15
227307_at	TSPAN18	148	10	***15,05***	209	25	8,25
206189_at	UNC5C	29	8	***3,47***	58	13	4,54
203868_s_at	VCAM1	1657	51	***32,69***	865	310	2,79
1554062_at	XG	299	26	***11,42***	192	23	8,52

Table 5: Membrane associated genes 2-fold more expressed in UVSC compared to BM-MSC*

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2
225612_s_at	B3GNT5	100	499	***4,98***	51	175	***3,44***
226817_at	DSC2	40	97	***2,43***	16	212	***12,90***
206032_at	DSC3	16	224	***13,91***	11	628	***56,55***
206033_s_at	DSC3	18	122	***6,76***	8	562	***70,23***
217901_at	DSG2	10	887	***91,18***	8	1242	***150,55***
1553105_s_at	DSG2	20	299	***14,62***	3	476	***186,59***
231192_at	EDG7	8	185	***22,58***	1	14	***10,12***
206795_at	F2RL2	21	87	***4,23***	9	72	***8,41***
208963_x_at	FADS1	249	688	***2,76***	166	448	***2,71***
208962_s_at	FADS1	278	613	***2,20***	204	714	***3,50***
208964_s_at	FADS1	422	868	***2,06***	299	790	***2,64***
226498_at	FLT1	5	261	***57,09***	6	793	***135,50***
226497_s_at	FLT1	44	427	***9,78***	19	619	***32,34***
210287_s_at	FLT1	21	144	***6,81***	7	179	***25,72***
207387_s_at	GK	33	143	***4,39***	27	57	***2,08***
217167_x_at	GK	27	74	***2,72***	23	54	***2,33***
205184_at	GNG4	14	35	***2,54***	5	47	***9,37***
1560265_at	GRIK2	15	40	***2,70***	21	53	***2,54***
211796_s_at	IL23A	38	293	***7,71***	10	53	***5,13***
205032_at	ITGA2	173	758	***4,37***	53	154	***2,92***
212843_at	NCAM1	20	152	***7,59***	18	104	***5,79***
232701_at	NRP2	26	90	***3,50***	24	61	***2,59***
229649_at	NRXN3	300	823	***2,74***	195	883	***4,54***
203650_at	PROCR	249	503	***2,02***	294	756	***2,58***
200635_s_at	PTPRF	106	262	***2,48***	93	298	***3,20***
200636_s_at	PTPRF	563	1170	***2,08***	570	1345	***2,36***
200637_s_at	PTPRF	102	207	***2,02***	134	275	***2,04***
200832_s_at	SCD	771	2938	***3,81***	511	1811	***3,54***
222717_at	SDPR	47	109	***2,31***	9	32	***3,72***
220979_s_at	SIAT7E	63	411	***6,55***	27	2065	***76,76***
211576_s_at	SLC19A1	87	287	***3,31***	60	156	***2,58***
201801_s_at	SLC29A1	104	382	***3,69***	63	168	***2,69***

218237_s_at	SLC38A1	336	1248	***3,71***	175	1525	***8,74***
224579_at	SLC38A1	371	1193	***3,22***	254	1700	***6,69***
204087_s_at	SLC5A6	35	188	***5,39***	50	122	***2,44***
213562_s_at	SQLE	108	354	***3,29***	145	309	***2,13***
229744_at	SSFA2	107	242	***2,27***	103	222	***2,14***
235591_at	SSTR1	76	920	***12,09***	23	1556	***66,65***
209890_at	TM4SF9	298	975	***3,27***	93	272	***2,92***
225387_at	TM4SF9	340	1003	***2,95***	238	576	***2,42***
225388_at	TM4SF9	233	494	***2,12***	178	408	***2,29***
205138_s_at	UST	22	69	***3,11***	11	103	***9,62***
205139_s_at	UST	161	332	***2,05***	149	552	***3,71***

*The table represent the 2-fold gene expression differences in comparison between BM-MSC and UVSC of two seperate experiments. The gene MCAM (CD146) is expressed in both cell types equally. MCAM was previously known to be highly expressed in MSC on molecular level and in FACS-Analysis (see Figure 11 and Figure 21).

Table 6: Genes commonly over expressed in BM-MSC compared to UVSC and those overexpressed in D7 BM-MSC compared to D2 BM-MSC [Larson et al., 2008]

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2	L-Mean D2	L-Mean D7	FC D7/D2
239218_at	---	440	11	***41,92***	454	12	***36,89***	1460	6782	***4,65***
226311_at	---	2569	84	***30,44***	3056	452	***6,75***	14836	43127	***2,91***
240460_at	---	187	9	***20,71***	95	7	***13,05***	490	1305	***2,66***
236344_at	---	445	31	***14,49***	650	29	***22,66***	1415	4361	***3,08***
232060_at	---	643	72	***8,88***	643	278	***2,32***	1367	2977	***2,18***
230710_at	---	107	13	***8,21***	115	56	***2,04***	222	1625	***7,32***
213429_at	---	752	151	***4,97***	673	237	***2,84***	3330	8747	***2,63***
213158_at	---	373	89	***4,19***	325	100	***3,25***	1657	3974	***2,40***
227126_at	---	313	107	***2,92***	348	98	***3,53***	2016	5442	***2,70***
235556_at	---	90	36	***2,51***	86	36	***2,40***	382	1109	***2,90***
214454_at	ADAMTS2	657	15	***43,55***	623	89	***7,01***	3268	12411	***3,80***
214535_s_at	ADAMTS2	265	40	***6,58***	283	94	***3,01***	2098	5375	***2,56***
205882_x_at	ADD3	2436	1004	***2,42***	2602	765	***3,40***	17291	36829	***2,13***
201034_at	ADD3	3299	1580	***2,09***	4065	1245	***3,26***	17659	39173	***2,22***
212062_at	ATP9A	395	116	***3,40***	378	110	***3,44***	2548	5416	***2,13***
216627_s_at	B4GALT1	244	54	***4,52***	247	97	***2,56***	2032	4712	***2,32***
238647_at	C14orf28	181	61	***2,97***	204	78	***2,60***	731	1649	***2,26***
225956_at	C50rf28	238	80	***2,96***	231	99	***2,33***	754	1604	***2,13***
219054_at	C5orf10	2127	46	***46,35***	1686	59	***28,37***	11853	41576	***3,51***
227443_at	C9orf150	485	179	***2,71***	789	264	***2,99***	1028	6274	***6,11***
211592_s_at	CACNA1C	38	7	***5,23***	40	8	***4,98***	396	1163	***2,94***
201615_x_at	CALD1	1604	338	***4,75***	2735	1333	***2,05***	32700	81246	***2,48***
231881_at	CALD1	76	32	***2,37***	124	39	***3,15***	1896	4463	***2,35***
224994_at	CAMK2D	530	206	***2,57***	715	300	***2,38***	4530	12271	***2,71***
231793_s_at	CAMK2D	344	161	***2,14***	506	199	***2,55***	3075	10196	***3,32***
225019_at	CAMK2D	1510	722	***2,09***	1874	804	***2,33***	14081	37400	***2,66***
224414_s_at	CARD6	331	75	***4,40***	355	55	***6,48***	1060	2431	***2,29***
224794_s_at	CERCAM	775	201	***3,86***	614	191	***3,21***	3686	9111	***2,47***
200884_at	CKB	214	83	***2,58***	270	74	***3,65***	2167	4833	***2,23***

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2	L-Mean D2	L-Mean D7	FC D7/D2
219561_at	COPZ2	1429	256	***5,59***	1977	940	***2,10***	11726	36795	***3,14***
204570_at	COX7A1	802	142	***5,64***	1298	478	***2,72***	3295	6615	***2,01***
227863_at	CTSD	363	35	***10,44***	293	21	***14,02***	992	3547	***3,58***
200766_at	CTSD	551	73	***7,57***	424	178	***2,39***	2170	6135	***2,83***
233955_x_at	CXXC5	1571	189	***8,33***	1934	686	***2,82***	9293	24318	***2,62***
222996_s_at	CXXC5	824	124	***6,64***	813	325	***2,50***	6175	13254	***2,15***
226632_at	CYGB	218	40	***5,39***	102	24	***4,24***	58	1035	***17,90***
212793_at	DAAM2	279	51	***5,47***	255	62	***4,13***	1334	4812	***3,61***
226573_at	DIRAS1	39	11	***3,55***	70	14	***5,13***	586	1718	***2,93***
221541_at	DKFZP434B044	1065	176	***6,07***	1732	809	***2,14***	3591	14870	***4,14***
227646_at	EBF	539	48	***11,20***	1510	195	***7,74***	2501	6754	***2,70***
232204_at	EBF	309	51	***6,08***	563	95	***5,96***	2091	6255	***2,99***
229487_at	EBF	196	36	***5,42***	383	74	***5,16***	2915	7327	***2,51***
232531_at	EMX2OS	149	51	***2,95***	137	27	***5,12***	552	1973	***3,57***
1554741_s_at	FGF7	3059	56	***54,97***	1029	264	***3,89***	1446	18064	***12,49***
231031_at	FGF7	356	14	***25,21***	200	44	***4,56***	216	2331	***10,82***
1555102_at	FGF7	13	2	***5,27***	9	1	***8,90***	204	422	***2,07***
203638_s_at	FGFR2	399	8	***51,63***	424	51	***8,25***	1431	8494	***5,94***
220327_at	FLJ38507	669	125	***5,37***	828	204	***4,06***	3669	10248	***2,79***
228937_at	FLJ38725	500	140	***3,58***	499	204	***2,45***	962	2379	***2,47***
214701_s_at	FN1	7150	951	***7,52***	7474	2715	***2,75***	54429	154281	***2,83***
214702_at	FN1	1573	271	***5,80***	1657	432	***3,84***	9374	22486	***2,40***
202812_at	GAA	196	15	***13,10***	196	91	***2,14***	659	1568	***2,38***
203066_at	GALNAC4S-6ST	449	26	***17,40***	447	33	***13,72***	678	1946	***2,87***
236129_at	GALNT5	533	51	***10,39***	673	71	***9,53***	1730	8566	***4,95***
229555_at	GALNT5	642	69	***9,27***	1025	153	***6,70***	4119	13786	***3,35***
240509_s_at	GREM2	76	1	***57,30***	161	5	***34,97***	398	1003	***2,52***
220794_at	GREM2	245	6	***39,44***	559	9	***65,49***	1511	8592	***5,68***
235504_at	GREM2	60	14	***4,23***	187	15	***12,89***	675	1878	***2,78***
218934_s_at	HSPB7	247	16	***15,66***	1156	120	***9,60***	560	14865	***26,57***
209291_at	ID4	1900	301	***6,31***	2708	263	***10,28***	18455	40129	***2,17***
226933_s_at	ID4	141	62	***2,27***	52	8	***6,47***	1891	4185	***2,21***
201508_at	IGFBP4	3686	525	***7,03***	4814	2165	***2,22***	35251	115865	***3,29***
203851_at	IGFBP6	1858	240	***7,75***	3175	1065	***2,98***	14889	38995	***2,62***

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2	L-Mean D2	L-Mean D7	FC D7/D2
1557080_s_at	ITGBL1	2788	79	**35,37**	1555	71	**21,94**	6794	15196	**2,24**
231993_at	ITGBL1	1594	46	**34,52**	937	36	**26,28**	5189	12235	**2,36**
205422_s_at	ITGBL1	2688	96	**27,99**	1502	65	**23,17**	8929	18963	**2,12**
212942_s_at	KIAA1199	7004	108	**64,60**	6635	2368	**2,80**	7564	55548	**7,34**
1554685_a_at	KIAA1199	744	12	**61,07**	1173	214	**5,47**	1487	11371	**7,65**
234880_x_at	KRTAP1-3	165	11	**15,08**	971	4	**242,80**	457	6901	**15,09**
233533_at	KRTAP1-5	1514	54	**28,10**	4747	86	**54,90**	805	55911	**69,46**
202202_s_at	LAMA4	1539	39	**39,72**	1809	190	**9,52**	1650	6916	**4,19**
213261_at	LBA1	71	24	**3,01**	77	30	**2,57**	288	1327	**4,60**
224989_at	LOC201895	830	325	**2,55**	657	258	**2,54**	3585	7495	**2,09**
216044_x_at	LOC388650	1012	142	**7,12**	1399	481	**2,91**	4030	13897	**3,45**
1564573_at	LOC402778	305	33	**9,11**	287	25	**11,35**	1217	3081	**2,53**
206584_at	LY96	579	149	**3,89**	450	131	**3,43**	3638	9175	**2,52**
1556047_s_at	MAGEE1	139	68	**2,03**	132	32	**4,09**	772	1577	**2,04**
206522_at	MGAM	111	13	**8,87**	68	3	**22,34**	194	528	**2,73**
224463_s_at	MGC13040	262	97	**2,72**	296	142	**2,09**	1189	2809	**2,36**
204575_s_at	MMP19	265	129	**2,05**	221	101	**2,20**	1257	2655	**2,11**
210223_s_at	MR1	266	52	**5,08**	218	96	**2,27**	2160	4518	**2,09**
1555894_s_at	MTSSL1	140	59	**2,35**	81	34	**2,35**	526	1578	**3,00**
202364_at	MXI1	850	145	**5,86**	632	297	**2,13**	1973	6393	**3,24**
227001_at	NIPAL2	586	162	**3,61**	324	153	**2,12**	1829	3685	**2,01**
203238_s_at	NOTCH3	436	19	**23,50**	450	91	**4,95**	1369	7163	**5,23**
216975_x_at	NPAS1	50	23	**2,18**	62	27	**2,30**	74	1330	**17,98**
219790_s_at	NPR3	385	4	**109,24**	341	5	**73,10**	2489	8360	**3,36**
219789_at	NPR3	1476	82	**18,09**	680	29	**23,86**	7018	22664	**3,23**
209982_s_at	NRXN2	61	13	**4,76**	36	8	**4,63**	405	950	**2,34**
231738_at	PCDHB7	60	5	**11,00**	53	14	**3,78**	235	756	**3,22**
207303_at	PDE1C	197	21	**9,47**	315	15	**20,67**	1332	3885	**2,92**
1553175_s_at	PDE5A	121	13	**9,45**	213	41	**5,20**	508	1712	**3,37**
227088_at	PDE5A	678	74	**9,10**	974	130	**7,49**	188	3356	**17,81**
1562227_at	PDE5A	61	24	**2,57**	70	16	**4,25**	44	493	**11,24**
202273_at	PDGFRB	598	131	**4,57**	654	244	**2,68**	5251	11394	**2,17**
208454_s_at	PGCP	296	85	**3,47**	341	135	**2,54**	1961	4963	**2,53**
227419_x_at	PLAC9	437	62	**7,03**	386	127	**3,05**	1354	3962	**2,93**

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2	L-Mean D2	L-Mean D7	FC D7/D2
219566_at	PLEKHF1	142	31	***4,53***	172	48	***3,55***	899	2494	***2,77***
225373_at	PP2135	499	49	***10,19***	547	167	***3,28***	1387	7712	***5,56***
211741_x_at	PSG3	117	14	***8,50***	83	14	***5,81***	755	1541	***2,04***
208106_x_at	PSG6	177	25	***6,94***	201	30	***6,75***	1598	3625	***2,27***
204944_at	PTPRG	370	106	***3,49***	610	185	***3,30***	1763	5603	***3,18***
1553962_s_at	RHOB	258	50	***5,17***	676	281	***2,41***	6122	15441	***2,52***
212651_at	RHOBTB1	316	143	***2,21***	349	169	***2,06***	2460	5304	***2,16***
213689_x_at	RPL5	930	122	***7,60***	1277	431	***2,96***	3737	14008	***3,75***
228407_at	SCUBE3	3149	122	***25,81***	3142	368	***8,55***	11187	25268	***2,26***
232183_at	SERAC1	518	75	***6,93***	593	230	***2,58***	1940	4180	***2,16***
205234_at	SLC16A4	543	30	***18,15***	521	92	***5,66***	571	2131	***3,73***
230624_at	SLC25A27	38	5	***6,88***	20	6	***3,40***	69	411	***5,97***
208608_s_at	SNTB1	51	8	***6,42***	124	12	***10,49***	701	3162	***4,51***
214708_at	SNTB1	67	15	***4,48***	95	17	***5,74***	507	1347	***2,66***
226438_at	SNTB1	179	53	***3,34***	338	34	***10,02***	1327	5202	***3,92***
210829_s_at	SSBP2	203	73	***2,78***	304	147	***2,06***	2130	5550	***2,61***
209655_s_at	TM4SF10	1300	318	***4,08***	996	356	***2,80***	3002	10681	***3,56***
209656_s_at	TM4SF10	3160	848	***3,73***	3362	1368	***2,46***	8459	26321	***3,11***
224566_at	TncRNA	706	90	***7,81***	2354	992	***2,37***	18946	40414	***2,13***
220817_at	TRPC4	110	33	***3,31***	417	194	***2,15***	287	1190	***4,14***
223253_at	UCC1	1943	143	***13,57***	1430	258	***5,53***	3544	14398	***4,06***
203868_s_at	VCAM1	1657	51	***32,69***	865	310	***2,79***	1296	8482	***6,54***
229816_at	WDR78	52	14	***3,61***	41	15	***2,65***	153	553	***3,61***
222619_at	ZNF281	990	395	***2,50***	1091	515	***2,12***	5691	12431	***2,18***
214761_at	ZNF423	279	18	***15,40***	358	21	***17,25***	647	2837	***4,39***

Table 7: Genes commonly over expressed in UVSC compared to BM-MSC and those overexpressed in D2 BM-MSC compared to D7 BM-MSC [Larson et al, 2008]

Gene ID	Gene Symbol	BM-MSC 1	UVSC 1	FC 1	BM-MSC 2	UVSC 2	FC 2	L-Mean D2	L-Mean D7	FC D2/D7
228570_at	BTBD11	47	528	***11,14***	33	152	***4,67***	1699	221	***10,42***
202345_s_at	FABP5	501	4089	***8,16***	713	2340	***3,28***	43135	10615	***4,11***
212281_s_at	MAC30	135	1068	***7,92***	113	349	***3,10***	8678	2364	***3,97***
212279_at	MAC30	232	1762	***7,58***	150	365	***2,44***	20887	6915	***3,35***
203819_s_at	IGF2BP3	46	346	***7,50***	25	206	***8,17***	1887	371	***6,32***
212282_at	MAC30	167	1180	***7,04***	126	336	***2,66***	10898	2606	***4,47***
204087_s_at	SLC5A6	35	188	***5,39***	50	122	***2,44***	4826	1645	***2,99***
1555812_a_at	ARHGDIB	84	446	***5,31***	81	223	***2,76***	7507	2856	***2,71***
37577_at	ARHGAP19	29	149	***5,19***	41	93	***2,25***	1823	690	***2,74***
205086_s_at	NCAPH2	27	122	***4,55***	39	88	***2,25***	5390	2802	***2,19***
225355_at	NEUR1LB	30	136	***4,54***	38	133	***3,50***	2498	1289	***2,02***
243367_at	---	35	156	***4,46***	30	94	***3,09***	1566	824	***2,08***
241937_s_at	WDR4	97	412	***4,23***	150	354	***2,35***	8386	3385	***2,48***
224610_at	RNU22	322	1289	***4,00***	512	1119	***2,19***	24093	6885	***3,55***
201288_at	ARHGDIB	265	1045	***3,95***	118	433	***3,68***	13481	4365	***3,25***
204702_s_at	NFE2L3	62	240	***3,87***	35	161	***4,64***	863	397	***2,16***
201801_s_at	SLC29A1	104	382	***3,69***	63	168	***2,69***	10107	2550	***3,98***
211576_s_at	SLC19A1	87	287	***3,31***	60	156	***2,58***	4050	1896	***2,21***
219170_at	FSD1	65	202	***3,10***	39	143	***3,70***	2175	684	***3,34***
213610_s_at	KLHL23	32	95	***2,94***	20	72	***3,66***	1040	379	***9,35***
206653_at	POLR3G	69	199	***2,90***	36	118	***3,29***	2012	778	***2,64***
216212_s_at	DKC1	145	391	***2,71***	136	285	***2,10***	10717	4108	***2,69***
231984_at	MTAP	158	421	***2,66***	172	381	***2,21***	5835	2582	***2,30***
220147_s_at	FAM60A	178	420	***2,36***	188	592	***3,15***	8564	4014	***2,14***
200895_s_at	FKBP4	315	741	***2,35***	271	597	***2,20***	16891	7136	***2,46***
204558_at	RAD54L	108	239	***2,21***	21	56	***2,72***	3090	849	***3,74***
236693_at	---	18	37	***2,10***	1	9	***6,60***	530	180	***3,85***
238670_at	FLJ41972	24	49	***2,03***	10	33	***3,22***	1495	538	***2,87***

Table 8: Membran-associated genes, CD selection used to construct clustering and principal component analysis Teil1

UNIQID	NAME	BM-MSC-1-ND1	BM-MSC-1-ND2	BM-MSC-1-ND3	BM-MSC-1-ND4	BM-MSC-1-ND5	BM-MSC-1-ND6	BM-MSC-2-ND1	BM-MSC-2-ND2	UVSC-1-ND1	UVSC-1-ND2	UVSC-1-ND3	UVSC-1-ND4	UVSC-1-ND5	UVSC-2-ND1	UVSC-2-ND2	UVSC-2-ND3
209735_at	ABCG2	47	37	34	18	61	7	33	39	144	183	53	85	11	43	49	89
213532_at	ADAM17	309	446	415	405	303	457	196	226	398	332	229	252	197	271	286	210
201951_at	ALCAM	1065	2331	1136	2391	1209	1587	1445	1404	2427	2162	1800	1720	1726	1044	650	855
202888_s_at	ANPEP	732	1340	310	1334	1377	900	717	734	746	523	718	881	801	121	276	356
208836_at	ATP1B3	1988	3837	3621	1821	3123	2213	2238	1974	3384	5160	5141	3252	4133	2050	2850	2922
213578_at	BMPR1A	487	532	584	477	515	444	448	427	413	497	347	410	411	294	348	363
205715_at	BST1	210	111	248	549	446	1576	679	239	74	48	43	89	48	574	439	495
201641_at	BST2	139	298	134	61	181	130	96	62	39	35	37	18	4	535	44	50
220088_at	C5R1	2	63	29	4	39	36	35	14	5	5	4	9	3	12	3	29
206991_s_at	CCR5	31	10	55	10	5	12	55	46	7	5	8	20	10	21	8	14
206337_at	CCR7	9	8	12	13	24	4	6	33	4	7	7	4	26	10	4	4
201743_at	CD14	5	113	27	72	68	59	10	5	3	3	15	4	3	11	51	61
204306_s_at	CD151	1147	389	338	1012	1128	1495	1757	1056	323	209	371	471	507	1011	958	1268
207840_at	CD160	16	32	48	4	27	58	17	24	78	29	12	10	84	29	32	5
208405_s_at	CD164	3363	4443	4207	3934	4228	3606	3212	2874	4486	3560	3214	2902	3417	3002	3065	2674
205789_at	CD1D	2	5	2	23	32	4	5	25	10	4	6	7	4	27	3	15
205831_at	CD2	71	35	53	17	28	55	22	48	26	5	15	39	21	29	40	56
209583_s_at	CD200	764	598	606	675	378	607	93	231	934	681	270	772	111	362	1300	1458
209771_x_at	CD24	230	102	43	223	15	266	203	141	71	6	40	50	34	23	44	30
209933_s_at	CD300A	86	8	25	16	9	13	6	25	43	41	47	11	35	3	34	22
218529_at	CD320	115	24	29	139	139	163	173	215	73	150	188	189	132	283	400	386
206120_at	CD33	9	22	5	15	6	5	5	5	5	18	7	6	6	3	11	6
209543_s_at	CD34	19	15	15	20	21	18	49	45	8	12	51	6	32	15	16	50
209555_s_at	CD36	44	27	205	2	191	69	42	50	1	4	7	4	4	4	23	3
204192_at	CD37	6	12	8	7	14	3	5	3	6	5	5	6	3	22	3	5
213539_at	CD3D	46	24	37	84	8	22	39	23	38	50	52	24	42	6	13	20
205456_at	CD3E	35	6	29	33	46	34	44	59	29	4	5	21	10	60	43	54
210031_at	CD3Z	12	10	10	12	8	37	13	16	7	4	6	4	11	13	16	9
203547_at	CD4	279	156	119	65	203	152	101	105	55	22	30	26	18	14	62	50

UNIQID	NAME	BM-MSC-1-ND1	BM-MSC-1-ND2	BM-MSC-1-ND3	BM-MSC-1-ND4	BM-MSC-1-ND5	BM-MSC-1-ND6	BM-MSC-2-ND1	BM-MSC-2-ND2	UVSC-1-ND1	UVSC-1-ND2	UVSC-1-ND3	UVSC-1-ND4	UVSC-1-ND5	UVSC-2-ND1	UVSC-2-ND2	UVSC-2-ND3
209835_x_at	CD44	1131	539	714	991	1262	733	923	1063	1232	1215	2532	2514	2429	764	664	1170
213857_s_at	CD47	1415	1674	1678	773	1104	928	1245	1337	2447	2812	2357	1612	2285	977	1034	1153
204661_at	CD52	15	84	12	10	3	15	19	6	20	27	23	16	43	3	4	4
203416_at	CD53	9	6	16	56	61	7	81	41	29	14	56	7	9	52	54	63
205173_x_at	CD58	384	568	810	427	516	416	466	459	677	567	624	592	580	629	555	582
200985_s_at	CD59	5539	5063	3291	5991	6191	6058	6966	5861	3693	3518	6047	4562	5801	4084	3793	4546
200663_at	CD63	9392	11059	8023	9322	9497	8837	10639	10728	8164	6525	7383	8341	8069	6392	7089	7451
209795_at	CD69	2	1	1	2	2	1	2	6	2	12	2	23	12	11	2	2
209619_at	CD74	93	174	41	54	184	91	112	206	33	19	40	8	19	7	18	51
200675_at	CD81	3179	2823	2414	4324	3428	4156	3533	3019	1558	1141	1510	1710	1515	2640	2794	2715
203904_x_at	CD82	114	195	90	57	146	157	35	143	106	53	137	117	76	213	113	110
204440_at	CD83	52	55	72	123	72	54	47	64	72	59	128	63	147	42	48	38
205758_at	CD8A	42	43	77	18	13	34	9	10	48	42	23	37	15	24	14	7
201005_at	CD9	1226	407	406	265	147	472	288	717	1626	1397	1823	1690	2192	780	1074	1709
202910_s_at	CD97	280	392	419	430	312	429	368	422	221	92	203	166	154	182	168	127
201029_s_at	CD99	6272	5701	6239	6525	5379	5539	5753	6492	5674	5789	5097	5563	5968	4277	6525	5071
203440_at	CDH2	2265	1790	4662	2423	836	1887	1346	2616	5512	1856	1642	2704	1636	2884	4236	3626
208052_x_at	CEACAM3	60	84	64	198	72	33	65	57	42	56	53	60	86	53	58	74
211657_at	CEACAM6	17	19	33	5	22	5	61	34	32	40	38	37	22	22	19	24
206676_at	CEACAM8	2	5	4	22	2	1	1	12	3	2	3	2	2	6	3	2
203104_at	CSF1R	14	11	22	67	48	31	57	73	40	33	59	60	31	44	45	37
205159_at	CSF2RB	12	31	18	64	59	104	52	32	10	13	70	15	2	15	17	3
211919_s_at	CXCR4	67	78	65	43	51	74	36	8	23	68	44	80	17	41	35	59
201925_s_at	DAF	162	351	137	43	56	205	156	86	740	353	303	216	241	596	705	949
210749_x_at	DDR1	407	399	368	386	396	458	505	407	301	210	150	210	189	391	364	335
203717_at	DPP4	381	276	562	272	187	439	118	159	787	226	129	259	264	447	849	578
201809_s_at	ENG	898	323	538	1320	560	749	1039	749	115	168	302	244	218	163	165	250
204006_s_at	FCGR3A	5	36	7	6	4	7	18	5	9	9	4	22	41	3	9	3
204007_at	FCGR3B	6	16	7	40	3	10	31	25	3	14	4	9	17	22	29	2
211535_s_at	FGFR1	683	1170	745	937	527	738	395	506	475	485	429	379	485	359	166	226
203638_s_at	FGFR2	557	142	580	344	358	392	409	389	15	12	3	3	7	47	123	36
222006_at	FGFR3	141	126	235	80	114	137	159	131	202	233	212	239	31	171	161	174

UNIQID	NAME	BM-MSC-1-ND1	BM-MSC-1-ND2	BM-MSC-1-ND3	BM-MSC-1-ND4	BM-MSC-1-ND5	BM-MSC-1-ND6	BM-MSC-2-ND1	BM-MSC-2-ND2	UVSC-1-ND1	UVSC-1-ND2	UVSC-1-ND3	UVSC-1-ND4	UVSC-1-ND5	UVSC-2-ND1	UVSC-2-ND2	UVSC-2-ND3
208335_s_at	FY	6	114	7	7	7	10	17	7	6	7	6	10	4	8	6	5
208284_x_at	GGT1	155	57	77	65	67	81	92	70	58	117	66	43	25	99	77	103
207389_at	GP1BA	53	93	51	116	64	63	79	56	109	66	50	49	91	58	33	61
205838_at	GYPA	29	5	32	9	6	14	7	6	35	15	3	8	19	33	8	23
209709_s_at	HMMR	115	24	107	143	252	96	145	266	481	1018	866	667	841	292	328	546
202637_s_at	ICAM1	194	480	257	98	79	92	77	75	156	77	70	8	168	579	203	150
213620_s_at	ICAM2	11	57	75	12	191	130	121	91	46	32	112	46	56	27	43	13
204949_at	ICAM3	254	305	164	45	227	220	186	147	195	183	220	315	323	218	237	280
202727_s_at	IFNGR1	1277	3736	1772	886	1418	1114	1036	796	888	604	560	596	657	935	833	603
203627_at	IGF1R	437	257	387	186	328	291	460	321	594	235	218	246	188	369	375	349
201393_s_at	IGF2R	836	783	966	946	999	780	1059	1029	594	416	707	609	696	1269	891	881
204912_at	IL10RA	7	6	35	34	8	50	4	26	30	28	36	15	29	5	27	44
209575_at	IL10RB	388	370	325	365	425	491	525	399	285	217	218	272	227	307	274	376
210904_s_at	IL13RA1	656	326	301	376	666	393	710	704	171	97	216	220	153	422	620	469
206172_at	IL13RA2	3	18	42	57	754	9	23	71	43	168	380	749	168	5	34	20
202948_at	IL1R1	1207	6074	960	648	1466	692	1808	1166	275	238	350	264	257	288	1278	1352
205403_at	IL1R2	23	280	35	9	19	7	29	28	8	41	7	34	30	13	13	30
205291_at	IL2RB	18	18	37	32	30	8	63	68	64	48	32	84	40	65	80	57
204116_at	IL2RG	16	10	97	99	66	49	38	38	42	17	50	11	34	41	45	40
203233_at	IL4R	118	42	219	231	76	208	166	160	156	111	259	188	220	181	277	182
205945_at	IL6R	24	90	36	34	74	40	32	19	28	8	14	26	17	17	9	35
212195_at	IL6ST	3626	6058	3799	3047	3849	3096	3168	2709	7649	3482	3565	3139	3327	3832	4207	3749
207008_at	IL8RB	38	93	29	50	13	30	1	32	70	42	37	37	3	6	23	1
206493_at	ITGA2B	76	207	61	16	31	19	48	32	54	91	70	134	105	47	40	65
201474_s_at	ITGA3	146	266	237	292	133	257	139	140	151	171	200	250	242	184	221	148
205885_s_at	ITGA4	150	133	325	113	123	115	35	99	247	171	230	154	221	311	201	260
201389_at	ITGA5	1989	2267	2076	2318	1158	1812	2063	1866	923	470	818	872	825	708	1255	1096
201656_at	ITGA6	350	165	164	101	86	236	139	128	349	867	1189	362	747	96	64	67
205055_at	ITGAE	1300	1998	1673	1486	1616	1801	1794	1790	1497	2121	1495	1713	1382	1502	1480	1506
213475_s_at	ITGAL	22	69	21	39	30	76	20	4	74	15	61	39	19	32	39	10
205786_s_at	ITGAM	49	47	8	9	33	6	13	25	34	7	5	7	13	16	15	4
202351_at	ITGAV	5778	4818	7926	5743	4898	5828	5435	5870	2041	911	890	973	1020	3228	3885	2396

UNIQID	NAME	BM-MSC-1-ND1	BM-MSC-1-ND2	BM-MSC-1-ND3	BM-MSC-1-ND4	BM-MSC-1-ND5	BM-MSC-1-ND6	BM-MSC-2-ND1	BM-MSC-2-ND2	UVSC-1-ND1	UVSC-1-ND2	UVSC-1-ND3	UVSC-1-ND4	UVSC-1-ND5	UVSC-2-ND1	UVSC-2-ND2	UVSC-2-ND3
210184_at	ITGAX	60	44	9	31	18	14	15	9	45	57	52	22	44	26	37	28
202803_s_at	ITGB2	92	154	55	56	52	239	135	87	7	7	8	6	6	35	8	16
204627_s_at	ITGB3	84	56	191	61	136	59	149	215	228	252	147	153	97	361	390	218
209099_x_at	JAG1	1565	586	1546	1465	2074	1927	2350	1147	982	185	354	165	98	900	469	262
219213_at	JAM2	272	310	221	248	296	388	90	175	170	54	82	62	109	192	108	45
205051_s_at	KIT	7	10	7	27	45	9	20	21	75	19	6	14	5	62	59	87
210644_s_at	LAIR1	33	12	9	8	54	44	51	34	20	33	51	11	33	24	45	43
201552_at	LAMP1	2629	2157	2162	1422	1784	1869	2810	2677	854	844	1482	1119	1506	2302	2285	1774
200821_at	LAMP2	2065	1712	3384	1257	1756	1322	1580	1175	1904	1197	937	752	1029	1419	1200	1008
212714_at	LARP4	520	583	477	468	487	474	489	462	511	602	565	441	508	596	641	576
209894_at	LEPR	959	5152	1252	779	7472	1990	1562	640	159	103	89	93	93	132	76	78
208594_x_at	LILRA6	13	13	9	8	12	11	11	18	6	6	11	12	10	56	20	6
210146_x_at	LILRB2	22	23	5	2	17	3	18	4	15	30	4	6	46	2	26	28
200785_s_at	LRP1	280	324	195	605	345	354	318	228	96	95	97	159	143	147	153	100
215967_s_at	LY9	29	9	5	15	5	13	28	39	19	10	9	7	12	4	10	8
38521_at	MAG	111	79	87	41	69	62	49	67	74	87	77	85	81	38	37	65
209087_x_at	MCAM	1133	1036	614	554	321	886	586	340	214	160	279	268	154	723	568	278
208783_s_at	MCP	727	2064	1723	1084	1456	1361	1181	1110	2162	1933	1422	1224	1480	1775	1668	1408
203435_s_at	MME	105	1408	215	69	265	11	47	36	634	333	202	262	178	548	727	745
207847_s_at	MUC1	57	60	97	52	86	82	77	88	59	68	33	65	17	61	63	69
217045_x_at	NCR2	172	213	177	168	116	74	87	95	156	184	152	93	82	74	68	104
210510_s_at	NRP1	266	221	154	299	243	196	291	318	259	270	308	445	370	240	260	213
203939_at	NT5E	2024	1390	1096	2429	2399	2201	1275	1278	5035	4520	4786	4599	4324	1338	1129	1290
203131_at	PDGFRA	3184	9745	2295	5392	2500	3533	2817	2859	4528	6258	1286	3787	2065	830	1054	1255
202273_at	PDGFRB	533	687	318	796	596	677	559	659	132	118	122	158	107	303	270	149
208982_at	PECAM1	46	89	39	89	81	22	55	32	42	57	197	40	97	36	9	10
203650_at	PROCR	197	225	355	179	299	206	264	304	520	366	388	734	461	596	700	771
203149_at	PVRL2	426	294	326	352	256	466	303	329	291	266	281	272	273	461	395	381
211254_x_at	RHAG	37	34	50	43	56	13	54	57	64	79	33	44	66	42	61	45
216317_x_at	RHCE	36	6	24	79	26	41	41	50	2	3	23	3	8	13	35	50
215819_s_at	RHCE /// RHD	7	5	4	23	8	28	31	15	7	3	7	5	11	37	31	8

UNIQID	NAME	BM-MSC-1-ND1	BM-MSC-1-ND2	BM-MSC-1-ND3	BM-MSC-1-ND4	BM-MSC-1-ND5	BM-MSC-1-ND6	BM-MSC-2-ND1	BM-MSC-2-ND2	UVSC-1-ND1	UVSC-1-ND2	UVSC-1-ND3	UVSC-1-ND4	UVSC-1-ND5	UVSC-2-ND1	UVSC-2-ND2	UVSC-2-ND3
210429_at	RHD	19	2	7	16	4	3	3	4	7	6	4	21	4	17	2	5
201287_s_at	SDC1	640	364	490	114	97	214	88	370	447	847	424	957	346	374	342	299
204563_at	SELL	14	12	1	3	19	14	2	17	4	13	11	11	7	43	2	21
206049_at	SELP	8	6	51	98	34	59	51	59	43	13	46	34	15	50	46	10
203528_at	SEMA4D	5	6	7	8	4	8	2	5	22	39	8	30	33	20	45	30
206702_at	TEK	200	77	211	361	93	398	214	129	570	417	329	346	376	363	556	256
207332_s_at	TFRC	1119	1997	1685	1865	2378	1612	1551	2118	1793	2182	2106	1869	1929	3742	4513	4330
203888_at	THBD	26	14	38	21	132	8	13	31	26	119	49	151	78	32	31	27
213869_x_at	THY1	1762	1441	772	1400	439	1736	1740	860	1647	1198	836	1500	1227	1862	3039	2423
210176_at	TLR1	3	47	14	32	29	34	26	36	18	4	3	10	3	30	25	36
204924_at	TLR2	15	214	7	14	49	32	37	10	25	9	47	13	60	15	38	9
221060_s_at	TLR4	153	402	162	172	255	127	187	130	15	30	34	5	7	44	47	48
209295_at	TNFRSF10B	803	1235	2366	1137	1419	1298	795	736	1572	1149	1054	996	1330	1480	1443	1136
211163_s_at	TNFRSF10C	13	28	58	15	36	11	7	25	66	14	52	32	40	5	24	40
218368_s_at	TNFRSF12A	2706	917	1218	2077	1846	2490	2258	3244	1418	998	3161	3013	2884	2173	2076	2550
202688_at	TNFSF10	34	171	53	4	17	24	35	20	5	26	25	5	19	10	12	2
209500_x_at	TNFSF13	74	77	75	177	94	115	88	67	67	37	51	103	47	74	79	73
203868_s_at	VCAM1	621	942	1174	2192	5274	746	699	906	154	3	14	23	56	199	444	248

Table 9: Membran-associated genes, CD selection used to construct clustering and principal component analysis Teil2

UNIQID	NAME	BM-CD11b-ND1	BM-CD11b-ND2	BM-CD11b-ND3	BM-CD45-ND1	BM-CD45-ND2	BM-CD45-ND3	BM-CD235a-ND1	BM-CD235a-ND2	BM-CD235a-ND3	SFb-ND1	SFb-ND2	SFb-ND3	SFb-ND4	POC-ND1	POC-ND2	POC-ND3
209735_at	ABCG2	84	163	94	70	46	31	1390	1842	1718	22	51	7	53	24	45	33
213532_at	ADAM17	563	554	534	403	428	443	279	196	229	260	267	210	518	727	388	261
201951_at	ALCAM	116	77	111	103	87	79	4	5	32	561	475	529	1379	354	582	667
202888_s_at	ANPEP	1155	1174	1952	924	717	625	51	6	11	695	1130	1250	2562	2172	2725	1563
208836_at	ATP1B3	1525	2090	2438	939	1707	1331	4608	5160	5311	1558	2915	1540	1549	2778	2788	3171
213578_at	BMPR1A	33	63	3	75	98	126	36	2	53	246	179	193	356	405	493	401
205715_at	BST1	1644	1511	1322	1648	1001	599	47	3	6	82	57	144	200	222	158	114
201641_at	BST2	803	491	679	471	463	436	47	47	90	44	122	24	6	4	29	10
220088_at	C5R1	7046	7603	5659	5550	5141	3270	320	119	26	35	19	16	14	17	35	43
206991_s_at	CCR5	487	357	220	601	1221	751	53	31	11	41	139	26	21	10	20	78
206337_at	CCR7	35	20	51	739	1007	928	41	71	17	38	58	33	9	5	37	38
201743_at	CD14	8042	6966	6727	1102	1519	260	172	9	53	51	15	23	26	57	6	67
204306_s_at	CD151	71	188	157	45	74	100	42	265	109	1032	1483	1041	1381	858	1129	735
207840_at	CD160	36	10	152	503	447	2666	67	4	13	37	48	36	30	21	28	39
208405_s_at	CD164	3861	3410	3934	4520	3543	3690	1918	1751	2067	3499	2656	3006	4320	5478	4261	4047
205789_at	CD1D	1863	1579	1531	478	556	377	41	40	32	11	5	4	17	4	3	26
205831_at	CD2	61	78	129	1349	2487	3349	33	6	57	57	54	21	39	53	46	61
209583_s_at	CD200	16	10	11	150	152	78	25	19	47	38	58	26	7	4	38	14
209771_x_at	CD24	2192	2935	1583	7734	5489	5674	451	71	92	110	53	28	49	7	16	76
209933_s_at	CD300A	2262	2459	1899	1815	1932	1728	95	82	77	15	43	18	16	61	58	28
218529_at	CD320	6	16	8	87	92	113	32	12	20	120	66	148	157	156	288	234
206120_at	CD33	1236	959	1277	322	359	220	11	5	11	6	8	5	7	7	4	7
209543_s_at	CD34	14	7	22	45	10	7	81	140	43	140	174	161	118	18	17	17
209555_s_at	CD36	1874	3371	2608	409	1030	256	4624	7242	4770	119	35	45	43	31	19	20
204192_at	CD37	2812	1051	2429	2146	845	3339	52	3	49	16	6	6	9	5	5	4
213539_at	CD3D	20	40	106	3136	3446	4971	65	40	41	67	86	35	21	12	18	16
205456_at	CD3E	53	40	54	450	415	1266	4	71	17	64	171	77	72	37	78	82
210031_at	CD3Z	8	19	92	1608	1391	2337	3	17	47	13	22	16	39	14	28	9

UNIQID	NAME	BM-CD11b-ND1	BM-CD11b-ND2	BM-CD11b-ND3	BM-CD45-ND1	BM-CD45-ND2	BM-CD45-ND3	BM-CD235a-ND1	BM-CD235a-ND2	BM-CD235a-ND3	SFb-ND1	SFb-ND2	SFb-ND3	SFb-ND4	POC-ND1	POC-ND2	POC-ND3
203547_at	CD4	1270	876	1017	432	434	633	105	43	75	36	39	35	50	5	13	15
209835_x_at	CD44	1529	1435	1362	915	876	751	486	519	509	3242	3607	7552	5277	1687	1887	1450
213857_s_at	CD47	2298	3168	2711	3179	4174	4417	3970	3547	2158	1147	1401	979	1524	1825	3176	2418
204661_at	CD52	2968	171	987	1256	185	588	8	33	11	29	17	28	11	27	38	23
203416_at	CD53	6773	7028	7472	6100	6017	5607	520	321	553	148	173	244	183	38	50	33
205173_x_at	CD58	2144	1671	1688	1836	1317	1255	2098	1613	2718	147	251	99	289	664	752	524
200985_s_at	CD59	303	535	227	777	428	317	3363	3047	2739	3406	4440	2828	2983	3179	5071	3650
200663_at	CD63	3901	4616	4009	4411	3987	3654	720	1228	449	10169	11838	10519	11095	9946	10980	10540
209795_at	CD69	681	208	586	3088	3099	4076	117	33	118	13	2	18	4	2	10	10
209619_at	CD74	7817	7585	9244	6742	10668	11428	476	257	364	203	123	245	602	20	26	27
200675_at	CD81	682	415	660	1207	1175	2215	128	380	251	8402	7639	8158	6178	4271	4151	3114
203904_x_at	CD82	89	136	320	264	150	158	243	54	384	476	385	499	227	213	192	175
204440_at	CD83	1755	1308	1881	517	685	1137	229	197	223	93	13	148	102	82	94	127
205758_at	CD8A	16	12	26	1855	1653	3685	23	124	100	80	43	9	12	7	8	13
201005_at	CD9	126	384	157	228	349	130	209	80	91	1956	1501	1121	2247	80	174	411
202910_s_at	CD97	1932	1884	1453	1381	1848	2297	10	4	17	45	173	38	132	218	148	201
201029_s_at	CD99	1917	2829	2120	2320	3110	3363	5198	4084	1758	9358	9012	8133	7327	6791	7363	6058
203440_at	CDH2	32	6	18	39	10	46	9	7	3	43	123	12	83	140	218	158
208052_x_at	CEACAM3	200	219	114	242	161	146	147	194	260	195	530	228	170	128	99	176
211657_at	CEACAM6	240	172	93	1942	1547	699	100	86	105	56	129	67	76	40	31	7
206676_at	CEACAM8	1415	1293	955	6153	3333	4443	222	28	2	3	9	9	3	4	2	14
203104_at	CSF1R	1637	598	1076	375	210	247	157	122	133	46	37	61	45	43	17	31
205159_at	CSF2RB	3139	3305	3380	2884	3150	1447	125	105	23	80	60	55	46	21	21	10
211919_s_at	CXCR4	2073	441	1742	2925	419	1126	151	153	154	181	217	131	59	40	78	40
201925_s_at	DAF	2820	3176	3204	2050	2391	1325	2473	4156	3222	174	72	188	159	479	818	259
210749_x_at	DDR1	106	97	138	122	102	150	77	289	245	243	328	357	331	166	246	91
203717_at	DPP4	12	51	28	303	376	373	80	99	137	621	248	944	1682	1559	1184	1216
201809_s_at	ENG	166	115	92	84	77	128	43	39	17	993	1064	861	1107	330	562	373
204006_s_at	FCGR3A	713	99	1295	506	43	204	65	10	99	29	42	5	19	15	3	38
204007_at	FCGR3B	6191	7817	9185	7668	7200	6641	372	78	145	46	54	59	35	6	30	28
211535_s_at	FGFR1	84	86	140	102	109	163	156	53	46	1754	1436	1657	1051	625	550	553

UNIQID	NAME	BM-CD11b-ND1	BM-CD11b-ND2	BM-CD11b-ND3	BM-CD45-ND1	BM-CD45-ND2	BM-CD45-ND3	BM-CD235a-ND1	BM-CD235a-ND2	BM-CD235a-ND3	SFb-ND1	SFb-ND2	SFb-ND3	SFb-ND4	POC-ND1	POC-ND2	POC-ND3
203638_s_at	FGFR2	11	3	11	31	10	15	11	4	7	114	12	93	89	15	15	9
222006_at	FGFR3	154	223	211	162	226	257	231	185	170	178	82	120	98	125	51	194
208335_s_at	FY	100	220	12	41	61	4	2485	2829	2572	8	11	6	5	4	26	8
208284_x_at	GGT1	147	188	210	168	185	201	106	42	175	115	114	155	86	104	117	51
207389_at	GP1BA	96	130	160	94	63	122	235	154	134	116	212	215	99	81	87	52
205838_at	GYPA	421	1426	308	134	136	115	11240	11943	9683	23	9	11	7	11	35	6
209709_s_at	HMMR	222	305	252	222	166	161	1858	1433	2683	70	735	93	43	4	103	311
202637_s_at	ICAM1	342	332	425	127	132	136	89	23	20	397	90	404	199	170	95	163
213620_s_at	ICAM2	1064	812	1190	742	1677	1467	173	51	178	37	67	5	6	26	20	35
204949_at	ICAM3	3641	5169	4047	4818	5427	5160	132	120	12	35	34	51	78	190	126	118
202727_s_at	IFNGR1	3693	4126	3596	2418	2916	1725	255	141	125	1118	736	709	1250	1238	1497	872
203627_at	IGF1R	109	147	78	123	131	104	205	243	165	247	117	162	261	185	195	161
201393_s_at	IGF2R	1556	1140	2061	1989	1629	925	299	416	531	896	1075	667	885	1527	1388	565
204912_at	IL10RA	2728	2601	2603	1927	3533	3769	106	35	13	74	80	61	73	33	5	42
209575_at	IL10RB	1219	629	1387	778	446	648	84	70	102	356	289	285	852	579	641	277
210904_s_at	IL13RA1	310	115	313	108	76	59	26	41	39	295	222	419	292	252	436	207
206172_at	IL13RA2	19	2	22	2	15	9	33	110	16	3025	1438	5965	5826	1146	2480	1249
202948_at	IL1R1	200	258	182	343	224	192	507	575	561	4252	2266	4449	6610	5427	6885	5595
205403_at	IL1R2	402	958	626	691	805	230	23	23	20	20	6	26	75	139	220	171
205291_at	IL2RB	65	65	139	1716	1350	2747	66	140	111	190	157	146	122	39	53	72
204116_at	IL2RG	368	376	496	1172	1177	3677	66	20	177	180	489	237	71	69	81	42
203233_at	IL4R	683	568	686	944	912	936	11	6	6	108	130	23	235	110	187	329
205945_at	IL6R	1083	705	1322	1075	569	581	28	10	61	98	86	46	103	28	42	2
212195_at	IL6ST	1188	1001	1250	926	949	839	414	490	336	4154	3400	4849	5493	8023	5563	5525
207008_at	IL8RB	2648	2207	2606	2510	1411	374	74	16	65	4	12	14	3	14	31	8
206493_at	ITGA2B	90	125	118	124	156	201	197	330	206	105	113	93	47	7	80	7
201474_s_at	ITGA3	12	12	49	27	32	51	27	161	19	63	156	60	88	53	24	77
205885_s_at	ITGA4	364	232	225	171	161	110	270	197	646	37	33	22	84	114	207	85
201389_at	ITGA5	508	445	436	332	319	246	32	73	27	545	1318	886	824	1083	1264	1191
201656_at	ITGA6	124	274	187	644	776	799	98	141	5	14	1	1	28	25	51	10
205055_at	ITGAE	1086	1687	1231	1176	1842	1610	607	653	447	1242	1258	650	710	902	889	1258

UNIQID	NAME	BM-CD11b-ND1	BM-CD11b-ND2	BM-CD11b-ND3	BM-CD45-ND1	BM-CD45-ND2	BM-CD45-ND3	BM-CD235a-ND1	BM-CD235a-ND2	BM-CD235a-ND3	SFb-ND1	SFb-ND2	SFb-ND3	SFb-ND4	POC-ND1	POC-ND2	POC-ND3
213475_s_at	ITGAL	1002	763	846	1031	928	1635	50	46	61	14	11	11	29	43	46	42
205786_s_at	ITGAM	5071	7419	6117	6773	7419	6328	383	103	20	43	43	45	32	16	6	57
202351_at	ITGAV	345	291	327	166	274	181	14	29	24	1728	2330	1581	2835	2281	3486	3731
210184_at	ITGAX	880	984	1060	448	654	320	79	67	10	40	27	15	7	15	12	8
202803_s_at	ITGB2	10668	10826	10760	8229	9123	9166	355	33	129	111	127	100	64	17	4	17
204627_s_at	ITGB3	7	23	35	53	15	31	156	118	20	144	121	330	122	40	100	9
209099_x_at	JAG1	212	270	191	136	108	81	36	26	36	1124	1697	921	1020	1221	1209	540
219213_at	JAM2	11	17	19	19	6	19	5	8	64	274	368	122	132	8	58	47
205051_s_at	KIT	15	27	26	140	154	102	36	100	30	62	69	11	12	9	21	8
210644_s_at	LAIR1	1074	523	903	803	459	787	116	61	121	160	197	260	123	59	68	38
201552_at	LAMP1	1418	1190	1497	1398	1112	1138	508	545	393	3331	3154	3359	3120	2982	1930	1978
200821_at	LAMP2	2835	2412	2928	3433	2982	2591	1112	595	985	630	370	537	1075	1320	1457	1312
212714_at	LARP4	261	346	264	257	286	181	1349	1006	908	508	207	308	196	337	356	403
209894_at	LEPR	123	121	57	147	119	67	598	402	184	259	567	41	248	63	163	109
208594_x_at	LILRA6	687	375	660	448	218	346	120	29	208	63	28	44	15	10	22	16
210146_x_at	LILRB2	3142	3428	2594	1863	2376	1155	75	63	22	3	2	6	7	3	8	9
200785_s_at	LRP1	406	341	357	103	124	86	75	240	237	1676	1197	827	2076	1037	572	478
215967_s_at	LY9	66	74	91	158	187	306	374	484	1601	6	12	7	13	4	7	10
38521_at	MAG	19	25	51	166	321	633	23	121	182	196	249	189	126	30	40	21
209087_x_at	MCAM	77	57	100	31	59	79	55	230	152	233	319	142	85	89	57	119
208783_s_at	MCP	3309	3228	3810	3533	3259	2469	683	442	520	426	343	499	577	1313	1067	895
203435_s_at	MME	282	572	724	687	751	369	136	62	19	679	45	570	1634	4292	1321	2182
207847_s_at	MUC1	44	94	137	75	57	88	126	52	49	352	226	278	430	151	131	138
217045_x_at	NCR2	85	96	151	51	64	93	216	311	337	208	305	272	157	155	123	98
210510_s_at	NRP1	23	13	35	5	5	5	17	24	14	112	122	272	316	347	304	235
203939_at	NT5E	19	4	16	117	149	88	28	155	5	977	801	1416	1825	1596	1713	1748
203131_at	PDGFRA	11	46	7	31	17	4	34	24	7	8158	7143	6412	8088	4436	6830	7383
202273_at	PDGFRB	26	61	12	20	44	34	6	6	33	1684	2057	1913	1395	1253	699	676
208982_at	PECAM1	3749	4726	5870	2131	3601	2965	575	384	319	287	306	189	143	112	98	58
203650_at	PROCR	4	17	68	42	55	100	22	29	3	289	581	531	881	359	918	586
203149_at	PVRL2	106	73	118	35	52	84	55	104	49	346	347	332	191	235	249	118

UNIQID	NAME	BM-CD11b-ND1	BM-CD11b-ND2	BM-CD11b-ND3	BM-CD45-ND1	BM-CD45-ND2	BM-CD45-ND3	BM-CD235a-ND1	BM-CD235a-ND2	BM-CD235a-ND3	SFb-ND1	SFb-ND2	SFb-ND3	SFb-ND4	POC-ND1	POC-ND2	POC-ND3
211254_x_at	RHAG	232	210	183	96	98	75	1229	1152	7451	241	185	308	150	13	14	66
216317_x_at	RHCE	270	816	284	124	200	99	7888	9123	7506	9	15	22	10	39	25	54
215819_s_at	RHCE /// RHD	289	1152	308	125	92	138	9821	12318	10457	37	44	31	56	36	13	22
210429_at	RHD	90	3	106	15	1	70	2723	9	2744	18	3	7	3	2	24	31
201287_s_at	SDC1	19	38	11	109	102	98	106	67	90	127	121	134	135	99	117	237
204563_at	SELL	7363	5789	7004	6885	5688	5366	250	52	230	47	68	55	26	32	43	5
206049_at	SELP	75	123	210	155	98	142	243	171	274	248	179	185	139	44	12	53
203528_at	SEMA4D	904	747	1187	1010	1426	1266	345	285	131	8	10	7	4	23	7	9
206702_at	TEK	32	27	36	31	25	18	29	49	5	88	83	104	391	48	40	183
207332_s_at	TFRC	1958	3252	1606	1278	868	1303	16135	16267	16179	1701	1847	1505	1473	2143	976	1674
203888_at	THBD	273	235	239	87	97	98	8	129	10	6	8	12	27	99	110	194
213869_x_at	THY1	40	28	83	23	15	72	71	33	4	1502	2283	1943	1567	1012	2258	1662
210176_at	TLR1	1378	849	997	678	485	282	58	44	45	24	56	35	41	11	26	37
204924_at	TLR2	3425	2680	3323	1019	854	367	46	12	68	27	20	22	31	28	29	52
221060_s_at	TLR4	546	190	457	201	73	53	39	28	114	72	91	77	97	63	37	69
209295_at	TNFRSF10B	1474	1346	1442	716	926	634	77	30	36	518	400	553	682	1575	1910	1343
211163_s_at	TNFRSF10C	635	683	682	782	480	424	45	88	7	6	7	17	6	33	39	6
218368_s_at	TNFRSF12A	30	48	6	39	4	35	22	43	17	1059	1427	881	772	545	992	1196
202688_at	TNFSF10	4111	3659	4330	1971	1644	1150	55	51	27	32	33	28	182	110	64	119
209500_x_at	TNFSF13	1398	1238	1744	576	588	628	126	231	164	148	252	195	80	40	74	109
203868_s_at	VCAM1	105	120	109	107	191	187	881	147	121	2660	4154	1712	2357	193	228	54

List of Abbrevations

APC	Allophycocyanin
BM-MSC	Bone marrow-derived mesenchymal stem cells or Bone marrow-derived multipotent stromal cells
BSA	Bovine Serum Albumin
CFU-F	Fibroblastic Colony Forming Units
Cy (3 or 5)	Cyanin, water soluble fluorescent dye
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EC	Endothelial Cells
ECC	Embryonic Carcinoma Cells
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cells
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FGF2	Basic Fibroblast Growth Factor 2
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GEO	Gene Expression Omnibus
GITC	Guanidine Thiocyanate
GlyA	GlycophorinA
HF (2+ Buffer)	Hank's buffered Salt Solution complemented with FCS
HME	Hematopoietic Microenvironment
IBMX	3-Isobutyl-1-Methylxanthine
IMDM	Iscove's Modified Dulbecco's Medium
IP	Immunprecipitation
MAPC	Multipotent Adult Progenitor Cells
MEM	Minimal Essential Medium
MIAMI	Marrow-isolated Adult Multilineage Inducible
MSC	Multipotent Stromal Cells (Mesenchymal Stem Cells)
P/S	Penicilin/Streptomycin
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
PDGF-BB	Platelet Derived Growth Factor subunit B homodimer
PE	Phycoerythrin
PFA	Paraformaldehyd
PI	Propidium Iodid
PMID	PubMed Identifier
POC	Periosteal cells
PVDF (membrane)	Polyvinylidenfluorid
RNA	Ribonucleic acid
SDS (PAGE)	Sodium Dodecylsulfate (polyacrylamide gel electrophoresis)
SFb	Synovial Fibroblasts
SSC	Side Scatter

TGF (β-3)	Transforming Growth Factor (beta 3)
UCB	Umbilical cord blood
UVSC	Umbilical vein stromal cells
VSEL	Very small embryonic like - stem cells
WB	Western Blot

Amino Acids

A (Ala)	Alanine
C (Cys)	Cytseine
D (Asp)	Aspartic acid
E (Glu)	Glutamic acid
F (Phe)	Phenylalanine
G (Gly)	Glycine
I (Ile)	Isoleucine
K (Lys)	Lysine
L (Leu)	Leucine
M (Met)	Methionine
N (Asn)	Asparagine
P (Pro)	Proline
Q (Glu)	Glutamine
R (Arg)	Arginine
S (Ser)	Serine
T (Thr)	Threonine
V (Val)	Valine
W (Trp)	Tryptophan
Y (Tyr)	Tyrosine

Deoxynucleotides

dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate

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