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Martin Fröhlich

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1. Introduction

1.1. Fundamental properties of stem cells

Stem cells represent immature progenitor cells which possess the ability to self-renew and are able to differentiate into multiple functional cell types. Stem cells can be dived into different subtypes corresponding to their differentiation potential or potency (Krampera, Franchini et al. 2007). Cells isolated from the morula, an early stage of embryonic development, are considered totipotent. They possess the ability to differentiate into all embryonic tissues and the extra-embryonic tissues of the placenta (Mitalipov and Wolf 2009). Pluripotent stem cells can be isolated from the inner cell mass of the blastocyst, and these cells are usually called embryonic stem cells. These cells are able to differentiate into cells of all three germ layers (ectoderm, mesoderm and endoderm), but are no longer able to form extra-embryonic tissues. Stem cells which are able to differentiate into cells of only one embryonic germ layer are called multipotent. Embryonic cells from later embryonic stages and adult stem cells such as hematopoietic stem cells, which give rise to all cell types of the hematopoietic system, or mesenchymal stem cells, are considered multipotent (Krampera, Franchini et al. 2007).

The second hallmark of stem cells is their ability of self-renewal. Self-renewal describes the process by which stem cells divide, symmetrically or asymmetrically, to generate one or two daughter cells that possess the same stem cell properties as the mother cell. For adult stem cells, e.g. hematopoietic stem cells (HSC) or mesenchymal stem cells (MSC), this means to produce lineage committed progeny while maintaining multipotency. The ability to self-renew is important for stem cells to expand during embryonic development or to maintain a stem cell population throughout the lifetime of an individual. Stem cell populations are required for the regeneration of tissues after injury or for the maintenance of the hematopoietic system. Deficiencies in stem cell self-renewal mechanisms can lead to defects in development, premature aging or cancer. Although all stem cells possess the ability of self-renewal, stem cells differ in their mechanisms and regulation of self-renewal. Embryonic stem cells have an unlimited self-renewal potential and are pluripotent in contrast to adult stem cells. Therefore, self-renewal in embryonic stem cells is regulated via unique regulatory networks. Adult stem cells are limited in their self-renewal potential compared to embryonic stem cells and are multipotent. In contrast to embryonic stem cells, the regulation of self-renewal in adult stem cells is more complex. Adult stem cells often persist throughout life and must be able to dynamically respond to different demands in their tissue. These stem cells must be able to stay quiescent over long periods, retaining their potency, and must also be able to proliferate when required. Therefore, self-renewal in adult stem cells is regulated by mechanisms that are more complex than in embryonic stem cells, and regulation also occurs through more external signals than in embryonic stem cells (He, Nakada et al. 2009).

Strategies for self-renewal of stem cells in adult tissues are restricted by tissue homeostasis, because the tissue must not change over time. Consequently, there are only two possible mechanisms of self-renewal for stem cells, either

asymmetric cell division or population asymmetry. Together with the possibility of internal or external regulation there are four different strategies of self-renewal in tissue stem cells to maintain long-term self-renewal and tissue homeostasis (Figure 1). The first mechanism is asymmetric cell division which gives rise to one daughter cell that differentiates and one daughter cell that resides as a stem cell. This mechanism can be controlled by internal signals in individual stem cells or is regulated via external signals when one daughter cell stays in contact with the niche and the other cell is displaced from the niche, which induces differentiation. The second mechanism is population asymmetry, where the stem cell population as a whole remains constant over time, but individual stem cells are lost. Population asymmetry can be regulated autonomously by individual stem cells or via external regulation, when the loss of stem cells from the niche is compensated by symmetric division of other stem cells in the niche (Simons and Clevers 2011).

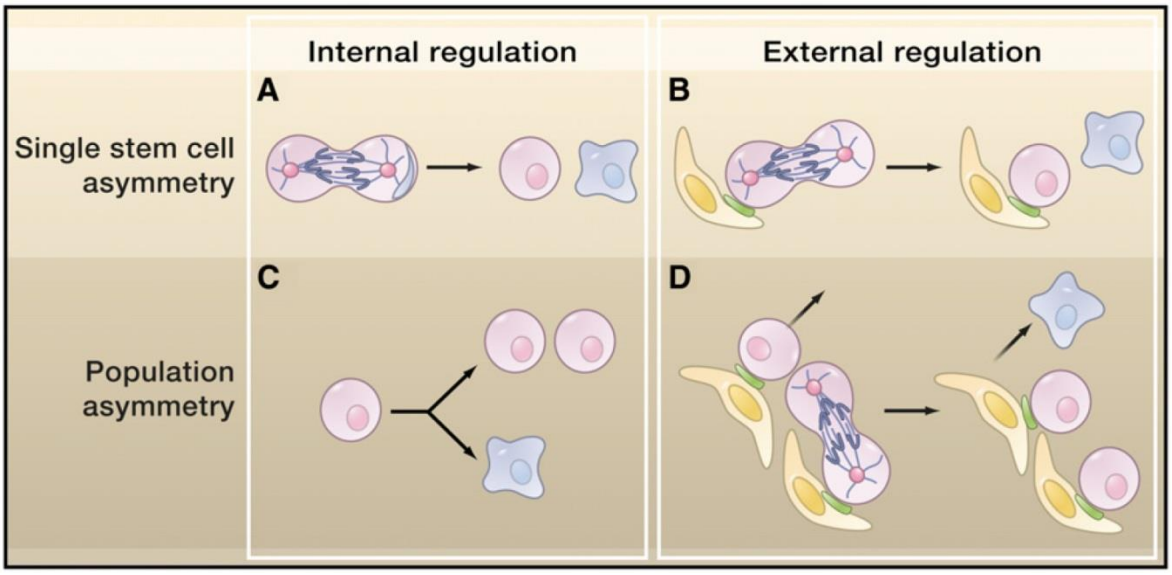


Figure 1 | Different strategies of stem cell self-renewal.

A | Asymmetric division of a single stem cell leads to one cell which differentiates and one cell that retains the stem cell phenotype. **B** | Cell division leads to the displacement of the progenitor cell from the niche and commitment, while the other cell stays within the stem cell compartment. **C** | The regulation between proliferation and differentiation of individual stem cells is independently controlled by the stem cells. **D** | To maintain the stem cell population the loss of stem cells, which follow a differentiation pathway, is compensated by symmetric division of other stem cells in the niche (Simons and Clevers 2011). Figure taken from (Simons and Clevers 2011).

1.2. Self-renewal of mesenchymal stem cells

Mesenchymal stem cells are capable of self-renewal and different studies were able to identify signal molecules and genes which are involved in the self-renewal process (Figure 2). Most of the signal molecules that promote self-renewal in MSCs also promote self-renewal in other stem cells. One example is leukemia inhibitory factor (LIF) which plays an important role in maintenance of pluripotency

in mouse embryonic stem cells (Metcalf 2003) (Kolf, Cho et al. 2007). Other examples are various types of growth factors such as fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or hepatocyte growth factor (HGF). It was shown that FGF2 maintains the stem cell state of mesenchymal stem cells (Kolf, Cho et al. 2007). In a study, Zaragosi et al. demonstrated that FGF2 plays an important role in the maintenance of self-renewal in human adipose tissue-derived stem cells (Zaragosi, Ailhaud et al. 2006).

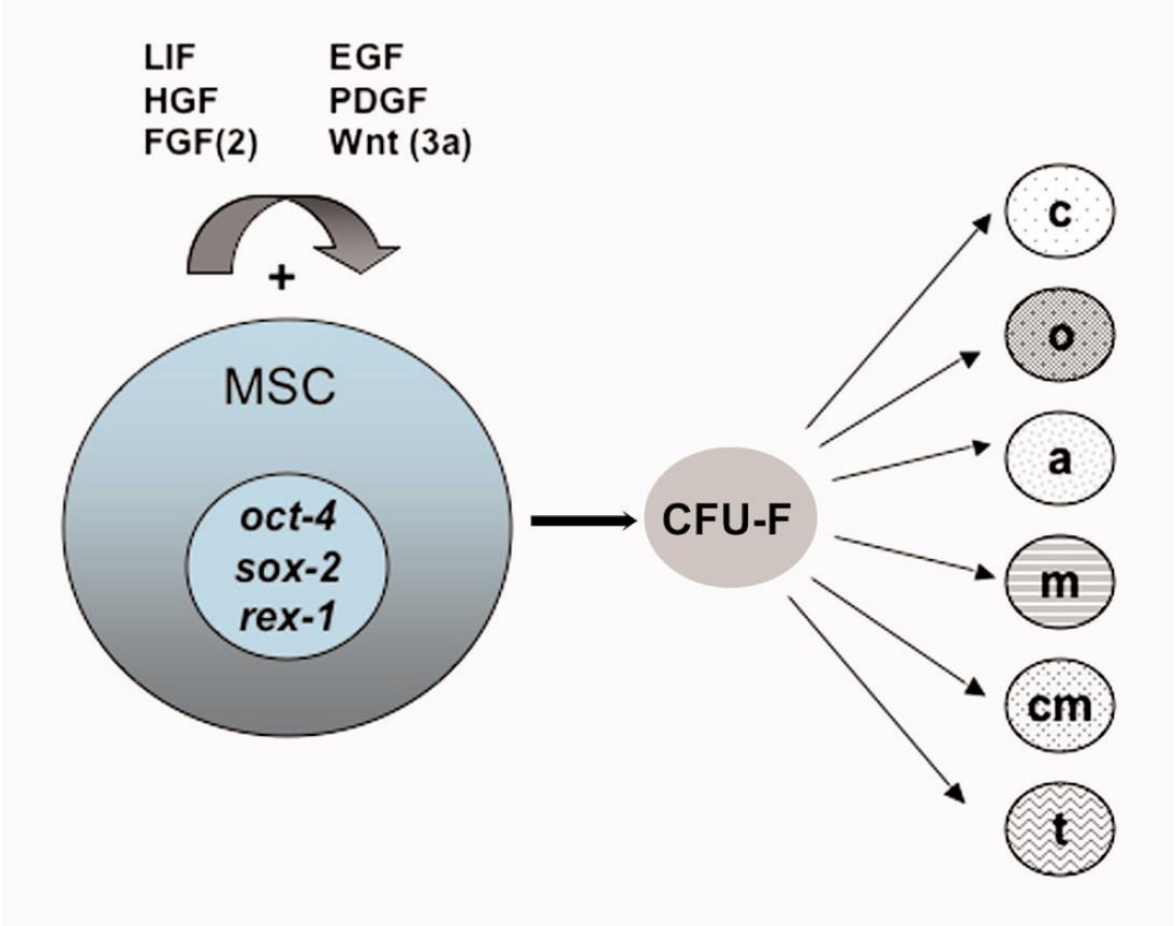


Figure 2 | Self-renewal of mesenchymal stem cells.

Signal molecules that play a role in the self-renewal of mesenchymal stem cells include leukemia inhibitory factor (LIF), various fibroblast growth factors (FGF2, EGF, HGF, PDGF), and proteins of the Wnt gene family. The transcription factors Oct-4, Sox-2, and Rex-1 that are expressed in embryonic stem cells are also expressed in MSCs. MSCs are able to differentiate into various mesenchymal cell types such as chondrocytes (c), osteoblasts (o), adipocytes (a), myocytes (m) and cardiomyocytes (cm), or tenocytes (t) (Izadpanah, Trygg et al. 2006; Kolf, Cho et al. 2007). Figure taken from (Kolf, Cho et al. 2007).

Although a number of signaling molecules have been identified that promote self-renewal and long-term culture expansion of mesenchymal stem cells, the underlying molecular mechanism are still largely unknown (Kolf, Cho et al. 2007).

In one study, Izadpanah et al. analyzed the expression of a set of transcription factors, which are essential in embryonic stem cells for maintenance of pluripotency and self-renewal, in mesenchymal stem cells from various tissues. They found that Oct-4, Sox-2, and Rex-1 are expressed in mesenchymal stem cells isolated from human bone marrow and adipose tissues as well as in MSCs from rhesus monkeys. They could show that these embryonic stem cell markers are expressed over several passages in all analyzed MSC populations, but expression decreases over time (Izadpanah, Trygg et al. 2006).

1.3. Discovery of mesenchymal stem cells

Bone marrow is the source of hematopoietic stem cells (HSC), which are required for the long-term maintenance of the hematopoietic system. The first hint that the bone marrow contained non-hematopoietic stem cells came from the german pathologist Cohnheim in 1867. Cohnheim studied wound healing and was interested in cells that played a role in this process. Therefore, he injected insoluble dye and analyzed which cells appeared at the site of injury. Among other cells he identified cells with a fibroblast-like morphology. He concluded that these cells came from the bloodstream and therefore originated from the bone marrow (Prockop 1997).

The first substantial evidence that bone marrow also contained non-hematopoietic stem cells came from the seminal work of Friedenstein and his colleagues in the 1960s and 70s. Friedenstein prepared bone marrow cell suspensions and small pieces of bone marrow from the femur of mice and placed pieces of bone marrow or bone marrow cell suspension in diffusion chambers. The diffusion chambers were transplanted into adult mice and osteogenesis was analyzed after transplantation by alkaline phosphatase activity. A few days after transplantation, the filters of the chambers were covered with cells that had a fibroblast-like appearance. Alkaline phosphatase activity could be detected in osteogenic foci that were present in the cell layer on the filters. Similar results were obtained in transplanted diffusion chambers that contained bone marrow cell suspensions. No alkaline phosphatase activity could be detected in the initial cell population. Three days after transplantation, the filters were covered with fibroblast-like cells, but no alkaline phosphatase could be detected in these cells. However, after 7-12 days, osteogenic foci were visible, which were positive for alkaline phosphatase activity and showed signs of deposited bone matrix (Friedenstein, Piatetzky et al. 1966).

In later studies, Friedenstein and his colleagues isolated fibroblast-like cells from bone marrow cell suspensions through their adherence to tissue culture plastic. They transferred whole bone marrow aspirates into tissue culture plastic dishes and non-adherent cells were discarded after 4 hours, when the remaining cell suspension was removed. The adherent cell fraction began to divide rapidly after a few days in culture. After a few passages, the cultured cells appeared as a homogenous cell population with fibroblast-like morphology. These cells were termed colony forming unit-fibroblasts (CFU-F) and formed osteogenic foci that

deposited small amounts of bone (Friedenstein, Chailakhjan et al. 1970; Chamberlain, Fox et al. 2007).

In the 1970s and 80s, other groups showed that cells isolated by Friedenstein's method could not only differentiate into osteoblast, but also into adipocytes, chondrocytes and myoblasts. These findings were confirmed by Pittenger and his colleagues. In their studies, they demonstrated that cells derived from single colony forming unit-fibroblasts can be differentiated into adipocytes, chondrocytes, and osteoblasts *in vitro* and therefore are multipotent. These cells are currently referred to as mesenchymal stem cells or mesenchymal stromal cells (Prockop 1997; Chamberlain, Fox et al. 2007; Nombela-Arrieta, Ritz et al. 2011).

1.4. Mesenchymal stem cells *in vivo* and the MSC niche

The concept of a stem cell niche was proposed by Schofield in 1978 as a regulatory unit for HSCs. According to Schofield, the niche consists of cellular, soluble, and extracellular matrix components that directly surround the stem cells in their naïve state. The components of the niche maintain the undifferentiated state of stem cells and control their self-renewal and differentiation (Kolf, Cho et al. 2007). This concept was later supported by studies of germ-line stem cells in C. elegans and Drosophila, where specific niche cells exist that maintain the stem cells (Frenette, Pinho et al. 2013).

Mesenchymal stromal cells can be isolated from various adult tissues such as bone marrow and adipose tissue. Recent studies (Sacchetti, Funari et al. 2007; Morikawa, Mabuchi et al. 2009; Corselli, Chen et al. 2010), which tried to identify tissue-resident MSCs in different tissues, suggested that MSCs are located near blood vessels (Nombela-Arrieta, Ritz et al. 2011). Another study by Crisan et al. identified a population of perivascular cells that expressed NG2, CD146, and PDGF-Rβ, but no endothelial or hematopoietic markers, in various tissues such as skeletal muscle, adipose tissue, and placenta. In addition, these cells also expressed the MSC markers CD44, CD73, CD90, and CD105. Further they could show that these cells possess osteogenic, adipogenic, and chondrogenic potential *in vitro*. These findings support the general acceptance that precursors of MSCs are closely associated with blood vessels and may belong to a subset of perivascular cells (Crisan, Yap et al. 2008). This would be in line with other multipotent stem/progenitor cells which were found near blood vessels in their respective tissues such as HSCs, white fat progenitor cells, and skeletal muscle cells (Nombela-Arrieta, Ritz et al. 2011).

The bone marrow is the source of two different stem/progenitor cell populations, HSCs and MSCs. MSCs were first isolated from bone marrow by Friedenstein, and bone-marrow derived MSCs are the best studied MSCs. They are located in the same tissue as HSCs and they have long been implicated in the regulation of HSCs. This is supported by findings that osteoblasts and adipocytes, both progeny of MSCs, play a role in the regulation of hematopoiesis (Figure 3) (Nombela-Arrieta, Ritz et al. 2011). *In vitro* studies demonstrated that osteoblasts are able to support short-term HSC expansion and that they secrete factors that can regulate

HSCs. Furthermore, studies in mice revealed that higher numbers of osteoblasts correlate with higher numbers of HSCs. Similar results were obtained when mice were treated with parathyroid hormone (Frenette, Pinho et al. 2013). A study by Visnjic et al. revealed that depletion of osteoblasts in the bone marrow of mice leads to dramatic alterations in hematopoiesis. Upon the depletion of osteoblasts, the number of lymphoid, erythroid, and myeloid progenitors declined in the bone marrow, as did the number of HSCs (Visnjic, Kalajzic et al. 2004). These findings suggest that osteoblasts play a role in the regulation of hematopoiesis. Contrary to osteoblasts, adipocytes seem to act as negative regulators of hematopoiesis. Early studies showed that adipocyte-rich primary stromal cultures do not support HSCs and that adipocytes secrete adiponectin, which decreased the proliferation of hematopoietic precursors. A recent study in mice showed that adipocyte-rich bones harbor less HSCs compared to bones with less adipocytes (Frenette, Pinho et al. 2013). MSCs give rise to both cell types implicated in the regulation of hematopoiesis, and therefore, MSCs could regulate HSCs by regulating their differentiation and lineage commitment. The underlying molecular mechanisms remain unclear (Nombela-Arrieta, Ritz et al. 2011).

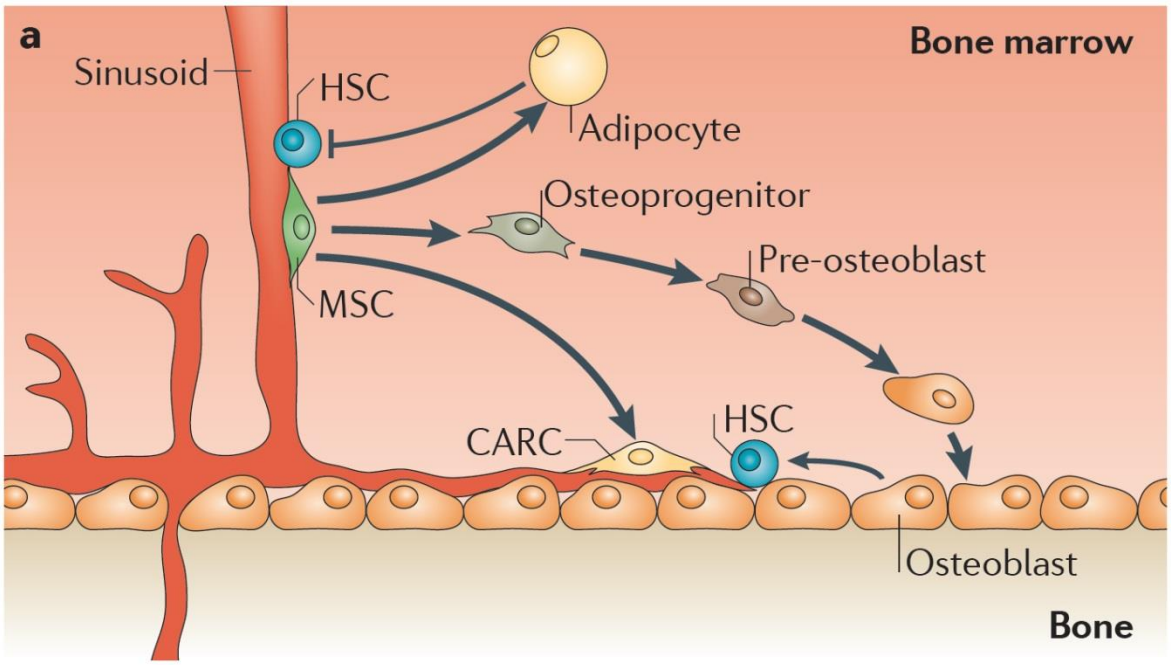


Figure 3 | Functions of BM-MSCs *in vivo.*

BM-MSCs give rise to osteoblasts, adipocytes, and CXCL12-abundant reticular cells (CARCs). Several studies demonstrated that these cell types play a role in the regulation of hematopoiesis and the HSC niche. Osteoblasts act as positive regulators of HSCs, whereas adipocytes negatively regulate HSCs activities. Other studies showed that HSCs co-localize with CARCs, which represent a poorly characterized population of cells with osteogenic and adipogenic potential. In a recent study, nestin^{+} MSCs were identified that co-localize with HSCs in a perivascular dual stem cell niche, which also regulate HSC maintenance (Izadpanah, Trygg et al. 2006; Mendez-Ferrer, Michurina et al. 2010; Nombela-Arrieta, Ritz et al. 2011). Figure taken from (Nombela-Arrieta, Ritz et al. 2011).

There is also evidence that immature MSCs play a role in the HSC niche in the bone marrow. This notion was based on experiments that demonstrated a positive effect of mixed cultures of MSCs and HSCs on the survival and proliferation of HSCs. The concept of a dual stem cell niche is supported by two studies that demonstrated that HSCs co-localize with a poorly characterized population of fibroblastic reticular cells. It was shown that these cells express high levels of CXC chemokine ligand 12 and therefore are termed CXCL12-abundant reticular cells (CARCs). Further, it was demonstrated that these cells possess adipogenic and osteogenic potential, and therefore, it was assumed that these cells could be derived from MSCs (Nombela-Arrieta, Ritz et al. 2011).

Another study by Méndez-Ferrer et al. identified a population of nestin$^+$ MSCs in the bone marrow and demonstrated that these cells play a role in the HSC niche. Nestin$^+$ MSCs are functionally similar to MSCs when tested for their differentiation capacity, self-renewal, and in *in vivo* transplantation assays. The authors demonstrated that depletion of nestin$^+$ MSCs has a direct impact on HSCs. Deletion of nestin$^+$ MSCs *in vivo* resulted in a decline in the number of HSCs in the bone marrow and impaired the homing of hematopoietic progenitors into the bone marrow (Mendez-Ferrer, Michurina et al. 2010).

Taken together, these findings reveal that MSCs seem to play an important role in the regulation of hematopoiesis and in the HSC niche. Furthermore, these studies promote the model of a dual stem cell niche for MSCs and HSCs in the bone marrow, but the exact mechanisms how MSCs and HSCs interact with each other in the niche are still unclear. BM-MSCs are the most studied and best characterized MSCs, and much less is known about MSCs and their niche in other tissues from which these cells were isolated (Nombela-Arrieta, Ritz et al. 2011).

1.5. Sources and isolation of mesenchymal stem cells

Mesenchymal stem cells were first isolated by Friedenstein and his colleagues in the 1960s and 70s from bone marrow aspirates. Bone marrow is still a major source for MSCs. Since the first isolation of MSCs from bone marrow, MSCs were found in various other adult tissues e.g., adipose tissue, the lung, and peripheral blood (Figure 4) (Hass, Kasper et al. 2011).

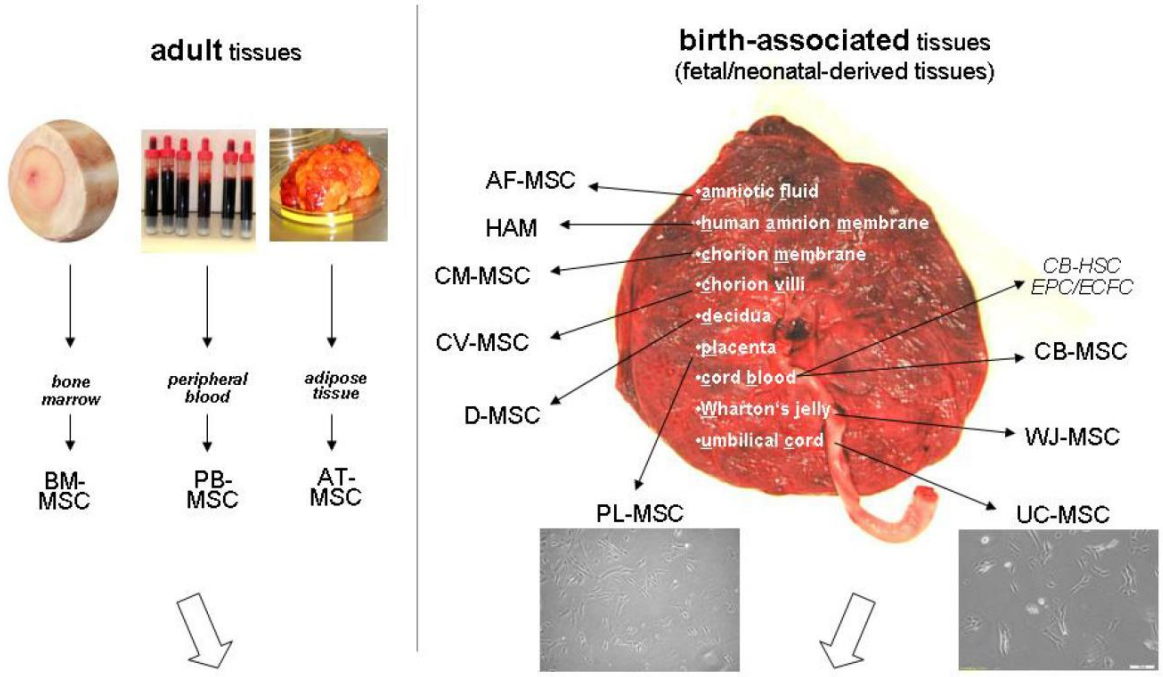


Figure 4 | Major sources of mesenchymal stem cells in human tissues.

MSCs are located in many adult tissues like bone marrow, peripheral blood, and adipose tissue. Birth-associated tissues represent a rich source for human MSCs. The most common used MSCs are BM-MSCs and AT-MSCs from adult tissues and UC-MSCs or WJ-MSCs from birth-associated tissues (Hass, Kasper et al. 2011). Figure modified from (Hass, Kasper et al. 2011).

MSCs isolated from these tissues possess similar properties in regards to proliferation potential and differentiation capacity as BM-MSCs. Several methods are used for the isolation of MSCs depending on the tissue. BM-MSCs are usually isolated from bone marrow aspirates by the following two methods. The bone marrow aspirates are fractionated by density gradient centrifugation and the mononuclear cell fraction, containing BM-MSCs, is seeded in tissue culture dishes. The other method, originally used by Friedenstein, relies on the attachment of BM-MSCs to cell culture plastic, and the whole bone marrow aspirate is transferred into a culture dish. After several hours, the non-adherent cells are removed with a medium change and the adherent cells represent BM-MSCs. In either case, the isolated MSCs attach to the tissue culture plastic and can be expanded for several passages (Pittenger, Mackay et al. 1999; Hass, Kasper et al. 2011). Density gradient centrifugation is also used for the isolation of peripheral blood-MSCs (PB-

MSCs). MSCs can be isolated from tissues, e.g. adipose tissue, through enzymatic digestion of the tissue, and the MSCs are then purified from the resulting cell suspension. The use of bacterial-derived products and the risk of contamination with xeno-antigens are drawbacks of this method and may not be appropriate for the isolation of MSCs for clinical applications (Priya, Sarcar et al. 2012). The frequency with which MSCs can be isolated from the different adult tissues varies to a great degree. Pittenger and colleagues estimated in their studies that only 0.01 to 0.001% of cells present in the mononuclear cell fraction after density gradient centrifugation are BM-MSCs. In comparison, 5x10^3 MSCs can be isolated from 1g of adipose tissue, which are 500 times more cells as in the same amount of bone marrow. PB-MSCs only make up a tiny fraction of cells in the peripheral blood and account for 0.0001 to 0.001% of mononuclear cells (Hass, Kasper et al. 2011). Generally, for the isolation of MSCs from different adult tissues, invasive procedures are necessary. These procedures are painful for the donor and bear the risk for infection. Another aspect of MSCs derived from adult tissues is that the frequency, proliferation potential, and differentiation capacity decreases with the age of the donor (Chen, Lie et al. 2009).

Neonatal tissues were discovered as an alternative source for human MSCs that have similar properties as adult tissue-derived MSCs. Birth-associated tissues as source for MSCs have advantages compared to adult tissues. Birth-associated tissues represent a large, readily available tissue source, the collection is painless, and requires no invasive procedures. In recent years, MSC-like cells have been isolated from different birth-associated tissues (Figure 4). Several MSC populations can be isolated from different regions of the placenta, e.g. chorion membrane (CM-MSCs), chorionic villi (CV-MSCs), and amnion membrane (HAM; human amniotic membrane). It was reported that MSCs isolated from the amniotic fluid (AF-MSCs) can be differentiated into cell types of all three germ layers, a property of pluripotent stem cells (De Coppi, Bartsch et al. 2007). Three tissue parts of the umbilical cord can be used for the isolation of MSCs, the whole umbilical cord (UC-MSCs), the Wharton's jelly (WJ-MSCs), and the umbilical cord blood (CB-MSCs), which also contains hematopoietic stem cells (HSCs). Several studies showed that MSCs, derived from the umbilical cord or Wharton's jelly, have several advantages compared to MSCs isolated from adult tissues. These cells exhibit a higher proliferation capacity than BM-MSCs, have shorter population doubling times over several passages, and can be cultured for longer periods before they show signs of senescence (Hass, Kasper et al. 2011).

The umbilical cord can be divided into four compartments, the umbilical vein subendothelium, the perivascular regions around the arteries, the intervascular space, and the subamnion (Figure 5). Wharton's jelly includes the perivascular, the intervascular, and the subamnion regions (Troyer and Weiss 2008). Mesenchymal stem cells can be isolated from the Wharton's jelly either by enzymatic digestion of the tissue or by explant culture. In a study, performed by Xu and colleagues, they reported that better results were obtained with the explant culture method than with enzymatic digestion.

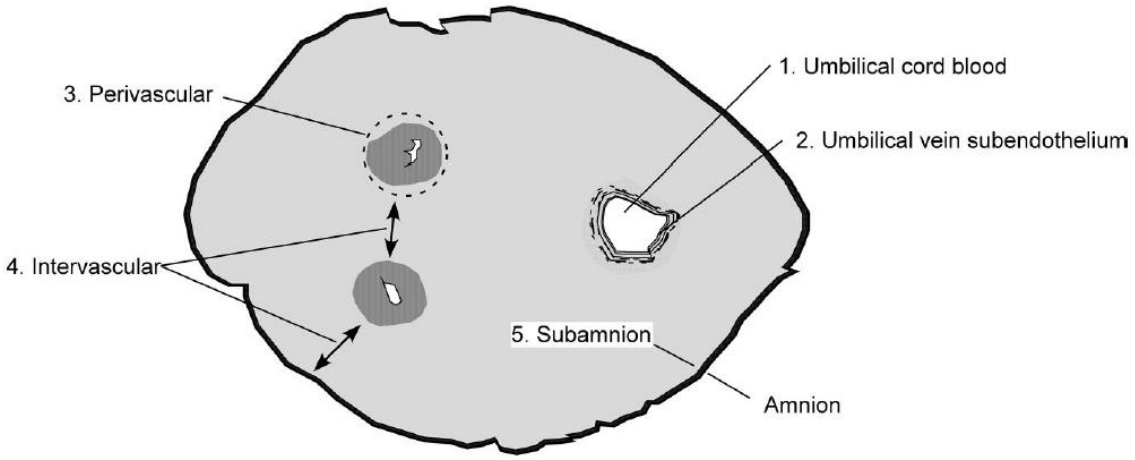


Figure 5 | Compartments of the human umbilical cord.

In a cross-section of the umbilical cord the different compartments can be identified, the umbilical vein subendothelium, the perivascular region around the arteries, the intervascular space, and the subamnion. Wharton's jelly includes the compartments 3 – 5 and is a rich source for MSCs (Troyer and Weiss 2008). Figure taken from (Troyer and Weiss 2008).

UC-MSC cultures, isolated with the explant culture method, had higher culture homogeneity and no endothelial cell contamination compared to cultures obtained with enzymatic digestion. In addition, higher cell yields and lower passage times were observed in cultures derived from cells that were isolated with the explant culture method. UC-MSCs isolated with the explant culture method had a passage time of 4.5 ± 1.5 days, compared to 9.0 ± 2.9 days for UC-MSCs isolated with enzymatic digestion. Another advantage of the explant culture method was that it was possible to isolate a higher number of cells from the same amount of umbilical cord tissue than with enzymatic digestion. According to Xu et al., 5.2 ± 1.7 x10^5 cells were isolated from 0.5 cm cord tissue with the explant culture method, compared to 0.03 ± 0.01 x10^5 cells with enzymatic digestion. Explant cultures were prepared using the strategy outlined in (Figure 6). In the first step, the umbilical cord vein and arteries are removed and the remaining tissue is cut into small explants. The explants are placed into a tissue culture flask with a minimal amount of medium. The explants attach to the flask, and during an incubation period of two weeks, MSCs migrate from the explant onto the culture flask. Finally, the explants are removed and the adherent UC-MSCs are harvested (Xu, Meng et al. 2010).

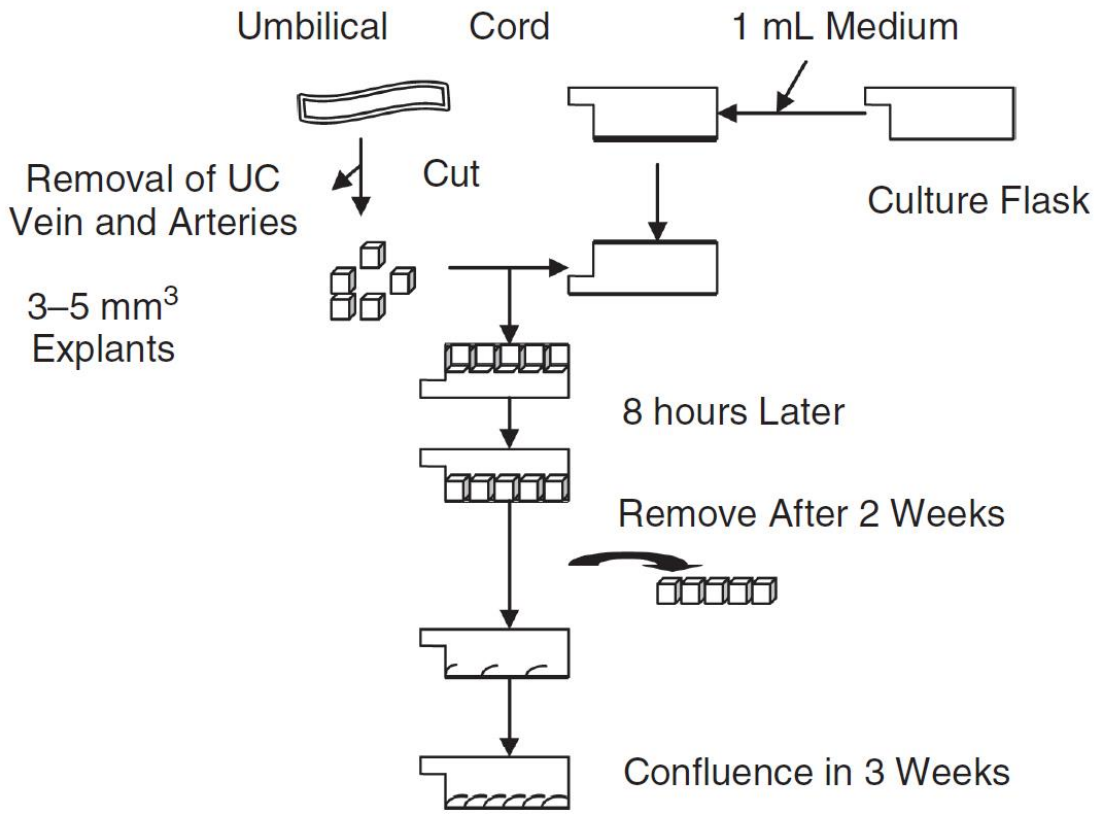


Figure 6 | Explant culture method for the isolation of UC-MSCs.

Figure taken from (Xu, Meng et al. 2010)

1.6. Characterization of mesenchymal stem cells

MSCs harbor a great therapeutic potential that led to an increased usage in biomedical research in recent years. MSCs are isolated from a wide range of adult and birth-associated tissues. Different isolation methods e.g. density gradient centrifugation, enzymatic digestion of tissues, and explant culture are used, depending on the tissue source. The different tissue sources and isolation methods bring up the question if the isolated cells are sufficiently similar, so that experimental outcomes and biological properties can be compared between the different MSC populations. These aspects are especially important concerning the use of these cells in cell therapy. Therefore, it is necessary to define criteria by which the isolated MSCs can be characterized. In 2006, Dominici et al. and the International Society for Cellular Therapy published a position statement in which the minimal criteria for multipotent mesenchymal stromal cells (MSCs) were defined. The first criterion is that MSCs must adhere to tissue culture plastic under standard culture conditions. The second criterion is that ≥ 95% of the MSC population must express the surface markers CD73, CD90, and CD105, measured by flow cytometry. In addition, ≤ 2% of the MSC population must be positive for

CD45, CD34, CD14, or CD11b, CD79α, or CD19, and HLA class II. Finally, MSCs must be able to differentiate into adipocytes, chondrocytes, and osteoblasts, *in vitro*. The differentiation into adipocytes is demonstrated with Oil Red O staining and differentiation into osteoblasts is demonstrated with Alizarin Red S or von Kossa staining. Chondrogenic differentiation is demonstrated with Alcian blue staining or immunohistochemical staining for type II collagen (Dominici, Le Blanc et al. 2006).

However, the surface markers suggested by Dominici et al. are not specific for MSCs. Different groups used additional markers in their studies that defined populations of more immature or "true" MSCs, within the general population of BM-MSCs. Some of these additional markers for BM-MSCs are STRO1 and CD106, SSEA4, CD56, and CD271. These markers, however, are not uniformly expressed by all MSC populations isolated from bone marrow or other tissues (Nombela-Arrieta, Ritz et al. 2011). There is also evidence that the markers expressed on *in vitro* propagated MSCs are not present on freshly isolated MSCs or MSCs *in vivo*. This also hampered the identification of tissue resident MSCs *in vivo* (Frenette, Pinho et al. 2013). The most stringent criterion today is the ability of MSCs to differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro*. The already described isolation methods for MSCs lack specificity, and therefore, bulk populations of MSC-like cells are obtained. Often the tri-lineage differentiations are performed with bulk populations of isolated MSCs rather than with clonal populations. Differentiation assays with non-clonal populations cannot provide definite evidence that all three cell types are derived from a single progenitor cell, and therefore, the possibility remains that tri-lineage differentiation is achieved through a mixed population of precursor cells for each differentiated cell type (Frenette, Pinho et al. 2013). In addition to the limited capabilities to characterize MSCs *in vitro,* there are no universally accepted assays to characterize MSCs *in vivo,* in contrast to hematopoietic stem cells (HSCs). The ability to reconstitute the entire hematopoietic system in lethally irradiated recipients is the gold standard to demonstrate the self-renewal and differentiation potential of single HSCs (Nombela-Arrieta, Ritz et al. 2011).

Despite the weakness to reliably identify MSCs *in vivo* or bona fide MSCs isolated from different tissues, the original criteria defined by the International Society for Cellular Therapy are widely used to characterize isolated MSCs in biomedical research (Nombela-Arrieta, Ritz et al. 2011; Frenette, Pinho et al. 2013).

1.7. MSCs *in vitro*: cultivation and differentiation

MSCs isolated from adult or birth-associated tissues are cultured in media e.g. α-MEM or DMEM, supplemented with 5-15% fetal bovine/calf serum. At low cell densities, adherent cells form proliferating colonies, colony forming unit-fibroblasts (CFU-F), and grow to confluency. When the cells reach confluency, they have to be replated periodically (Krampera, Franchini et al. 2007). MSCs divide rapidly with population doubling times dependent on the MSC population, e.g. UC-MSC about 24 hours, BM-MSCs 61.2 hours, and AT-MSCs 45.2 hours, and the seeding density (Hass, Kasper et al. 2011). MSCs can be expanded over several passages

in vitro and proliferate for up to 40 population doublings without spontaneous differentiation (Krampera, Franchini et al. 2007). Higher population doublings were achieved by the addition of fibroblast growth factor 2 (FGF-2) to the basal cultivation medium. *In vitro* cultivated MSCs display a fibroblast-like morphology with a small cell body and few cell processes. When MSC cultures reach confluency, they grow in swirling patterns (Figure 7) (Menssen, Haupl et al. 2011).

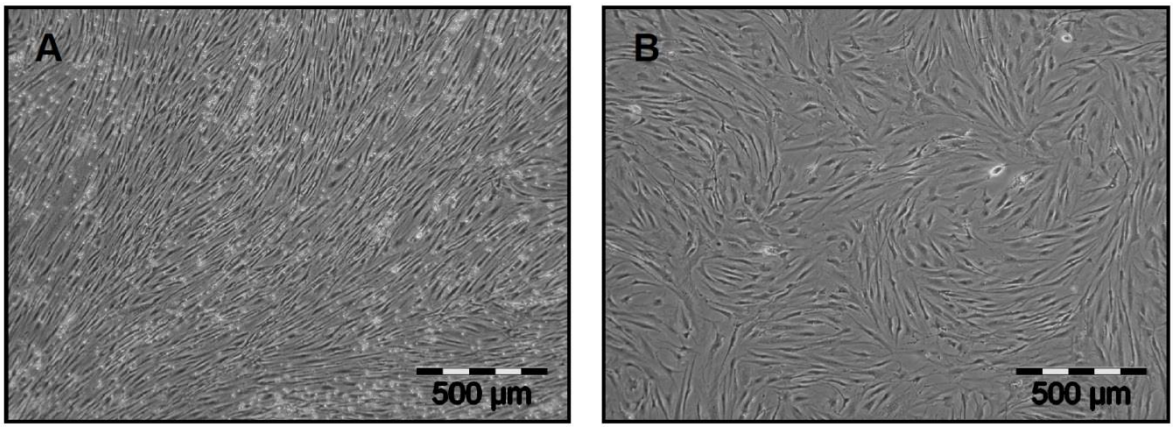


Figure 7 | Morphology of human BM-MSCs during *in vitro* cultivation.

A | Primary culture of human MSCs after the isolation from bone marrow aspirates. **B** | BM-MSCs display a typical fibroblast-like morphology and grow in a swirling pattern (Passage 3) (Menssen, Haupl et al. 2011). Figure taken from (Menssen, Haupl et al. 2011).

In a study conducted by Haack-Sørensen and colleagues, the authors found that BM-MSCs maintained their differentiation potential during culture expansion independently of the cell density (Haack-Sorensen, Hansen et al. 2012).

One of the defining biological properties of MSCs is their capacity to differentiate into cell types of mesenchymal origin e.g. adipocytes, chondrocytes, and osteoblasts, *in vitro*. Osteogenic differentiation of MSCs is achieved by subjecting a confluent layer of MSCs to osteogenic differentiation medium. Basal osteogenic differentiation medium consists of a basic medium e.g. α-MEM or DMEM, supplemented with 10% fetal bovine serum (FBS), 0.2 mM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone (Lee, Kuo et al. 2004). During osteogenic differentiation, MSCs increase their expression of alkaline phosphatase, and at later stages, a calcified extracellular matrix is formed. Alkaline phosphatase activity is detected after 10 days of differentiation, whereas the calcified extracellular matrix is earliest detected after 21 days. The expression of alkaline phosphatase is visualized by incubating cells with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) after fixation of the cells with formaldehyde. Alkaline phosphatase hydrolyses BCIP to 5-bromo-4-chloro-3-indole, and in the second step of the reaction, 5-bromo-4-chloro-3-indole is oxidized by NBT to an insoluble, dark violet, diformazan precipitate, which labels osteoblasts violet in a bright field microscopy image (Figure 8). The calcified extracellular matrix is detected by the staining of calcium deposits with Alizarin

Red S. Calcium deposits are stained intense red with Alizarin Red S in contrast to cells and non-calcified matrix which appear reddish (Figure 9) (Chamberlain, Fox et al. 2007).

Induction of adipogenic differentiation in MSCs is achieved by incubating subconfluent (90%) cells in adipogenic differentiation medium for 21 days, but longer incubation periods have been reported. Basal adipogenic differentiation medium consists of a basic medium (DMEM-LG) supplemented with 10% FBS, 60 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutyl methyl xanthine, and 5 µg/ml insulin (Karahuseyinoglu, Cinar et al. 2007). During the differentiation towards adipocytes, the cells begin to express the peroxisome proliferator-activated receptor γ2 (PPAR-γ2), lipoprotein lipase, and the fatty acid-binding protein aP2, which are detected with real-time PCR.

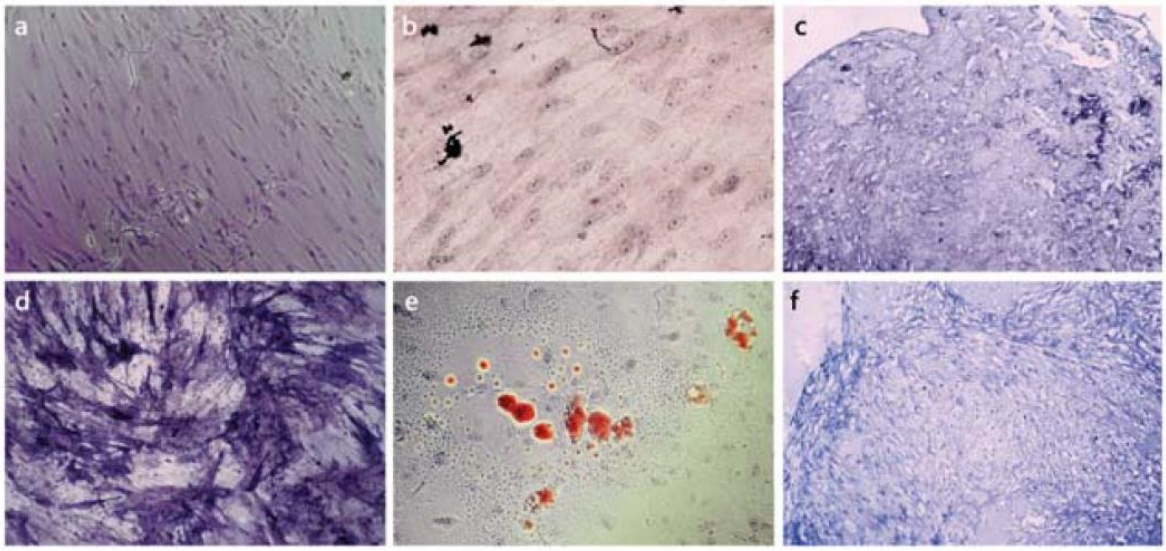


Figure 8 | *In vitro* differentiation of human BM-MSCs.

a,d | Differentiation into osteoblasts. BM-MSCs cultured in osteogenic differentiation medium express high levels of alkaline phosphatase detected with BCIP/NBT (d, violet), compared to cells in control medium (a). **b,e** | Differentiation into adipocytes. After adipogenic induction, BM-MSCs developed lipid-filled vacuoles that are stained intense red with Oil Red O (e), which are absent in control cells (b). **c,f** | Differentiation into chondrocytes. Chondrogenic differentiation was confirmed with toluidine blue staining of the chondrogenic micromass, mucopolysaccharides are stained red/violet (f) and cells cultured in control medium are not specifically stained (c) (Karahuseyinoglu, Cinar et al. 2007). Figure taken from (Carrancio, Blanco et al. 2011).

Another hallmark of adipocytes is the accumulation of lipid-filled vacuoles in the cytoplasm of differentiated cells. These lipid-filled vacuoles are detected with Oil Red O staining. Oil Red O is a lysochrome dye and stains the lipid vacuoles intensely red in a bright field microscopy image (Figure 8) (Chamberlain, Fox et al. 2007).

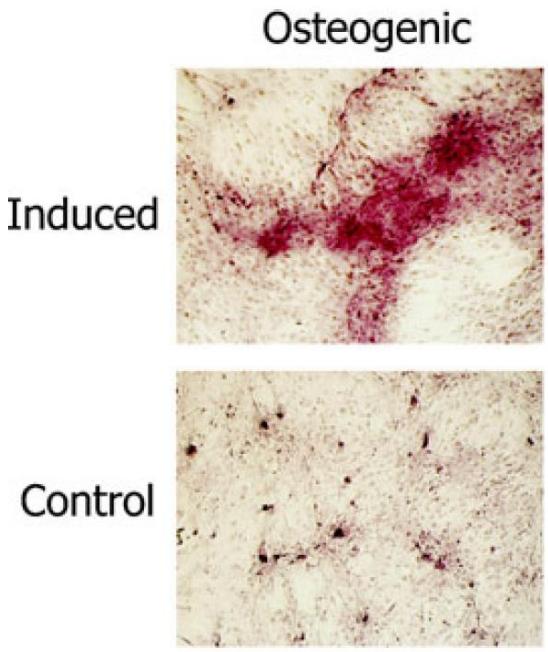


Figure 9 | Osteogenic differentiation of BM-MSCs.

Calcium deposits in the extracellular matrix are stained intense red and indicate osteogenic differentiation. Control cultures are not stained with Alizarin Red S (Chamberlain, Fox et al. 2007). Figure modified from (Haack-Sorensen, Hansen et al. 2012).

Chondrogenic differentiation of MSCs is achieved by pelleting 2x10^5 cells into a dense micromass and subjecting the cells to chondrogenic differentiation medium for 21 days. Chondrogenic differentiation medium contains 10 ng/ml transforming growth factor β (TGF-β), 100 nM dexamethasone, 50 µg/ml ascorbic acid, 1 mM sodium pyruvate, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenous acid, 1.25 mg/ml BSA, and 5.25 mg/ml linoleic acid (Karahuseyinoglu, Cinar et al. 2007). A sign of chondrogenic differentiation is the formation of shiny cell-spheres in the pellet cultures (Hass, Kasper et al. 2011). Chondrogenic differentiation of MSCs is detected with histochemical stains (Toluidine blue, Alcian blue) or immunohistochemistry methods. Toluidine blue staining shows a metachromatic reaction with glycosaminoglycans, which are stained pinkish (Figure 8), and Alcian blue stains glycosaminoglycans blue-greenish. Immunohistochemistry methods focus on the detection of the extracellular matrix proteins type II collagen and aggrecan in sections of the chondrogenic micromass (Chamberlain, Fox et al. 2007; Karahuseyinoglu, Cinar et al. 2007; Hass, Kasper et al. 2011).

1.8. Comparison of WJ-MSCs and BM-MSCs

Both WJ-MSCs and BM-MSCs fulfill the minimal criteria for MSCs defined by the ISCT. However, the two MSC populations differ in their biological properties. It was reported that WJ-MSCs have a higher colony forming unit-fibroblast (CFU-F) frequency than BM-MSCs and, as a result, shorter population doubling times. The shorter population doubling times are a common feature of MSCs derived from fetal or neonatal tissues, and it is thought that this reflects their more primitive nature in comparison to adult MSCs. In addition, WJ-MSCs have greater *in vitro* expansion capabilities and can be passaged more often before they enter senescence. WJ-MSCs produce similar cytokines as BM-MSCs plus the additional cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) (Troyer and Weiss 2008). WJ-MSCs have a poor capacity to differentiate into adipocytes compared to BM-MSCs. Karahuseyinoglu et al. found that adipogenic differentiation of WJ-MSCs takes longer than in BM-MSCs, up to 40 days, and that WJ-MSCs did not produce a mature adipocyte phenotype as BM-MSCs did. WJ-MSCs have a higher

chondrogenic potential than BM-MSCs, which was ascertained through a higher amount of type I and II collagens in the chondrogenic micromass (Karahuseyinoglu, Cinar et al. 2007). Interestingly, when WJ-MSCs were seeded onto poly(lactic-co-glycolic acid) scaffolds and cultured in chondrogenic differentiation medium, they produced higher amounts of type I and II collagen, and glycosaminoglycans than cartilage cells isolated from condylar cartilage from the temporomandibular joint (Troyer and Weiss 2008).

1.9. Regulation of MSC differentiation

The capacity of MSCs to differentiate into cell types of mesenchymal origin is well established and has been demonstrated for many MSC populations. It is crucial to understand how the differentiation of MSCs into functional cell types is regulated, especially if the cells should be used for regenerative medicine. The osteogenic and chondrogenic potential of MSCs steers a lot of interest to develop treatments for degenerative bone diseases and regeneration of cartilage (Smink and Leutz 2012). Over the years, several signaling pathways and transcription factors have been identified that are necessary to drive the differentiation of MSCs. The Wnt/β-catenin and the TGF-β superfamily pathways play a key role in chondrogenic and osteogenic differentiation (Augello and De Bari 2010). The Wnt signaling proteins bind to a frizzled family receptor and a co-receptor, usually Lrp5/6, and activate the canonical Wnt signaling pathway. Receptor activation leads to the disruption of the cytoplasmic β-catenin degradation complex. As a result, β-catenin accumulates in the cytoplasm and finally translocates to the nucleus, where it interacts with members of the Lef1/Tcf family of nuclear proteins. Subsequently, the expression of specific target genes is activated (Karsenty, Kronenberg et al. 2009; Augello and De Bari 2010).

The effects of Wnt/β-catenin signaling on osteogenic and chondrogenic differentiation were demonstrated in *ex vivo* studies. Mesenchymal cells were isolated from limb buds of chicken and mouse embryos and the influence of different Wnt proteins on chondrogenic differentiation was tested. It was found that the signaling proteins Wnt 1, 3a, 4, 7a, 9a, and 11, inhibit chondrogenic differentiation in these cells. A similar effect was observed for β-catenin, which led to the assumption that most of the Wnt proteins inhibit chondrocyte differentiation. The results were confirmed in *in vivo* studies where the inactivation of β-catenin caused abnormal chondrocyte differentiation. In contrast to these findings, two *in vivo* experiments showed that chondrocyte-specific β-catenin knockout decreases chondrocyte proliferation and that overexpression of Wnt5 in chondrocytes, which leads to β-catenin degradation, inhibits chondrogenic differentiation of mesenchymal cells (Karsenty, Kronenberg et al. 2009).

Wnt/β-catenin signaling also plays an important role during osteogenic differentiation. Proteins of the Wnt family control the differentiation of osteoblast progenitors into mature osteoblasts, and Wnt proteins are expressed in mesenchymal cells. The disruption of β-catenin in early stages of osteogenic differentiation leads to chondrogenic differentiation of these cells and inhibits osteogenic differentiation (Karsenty, Kronenberg et al. 2009). A study performed

by Quarto and colleagues (2010) revealed that Wnt proteins, specifically Wnt3a, have different effects in osteogenic differentiation, depending on the differentiation stage of the cells. They treated calvarial osteoblasts from juvenile mice and adult mice with high doses of Wnt3a and found that osteogenesis was inhibited in juvenile calvarial osteoblast, but increased in cells from adult mice. These findings were supported by *in vivo* results where only low doses of Wnt3a promote healing of calvarial defects in young mice and that high does enhance bone regeneration in adult mice (Quarto, Behr et al. 2010).

The TGF-β superfamily of signaling molecules includes different growth factors and morphogens e.g. bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), and TGF-β, that play roles in bone development and bone homeostasis. TGF-β and BMPs both relay their signal through the SMAD signaling pathway. After the binding of TGF-β and BMPs to their target receptors, receptor-associated SMADs are phosphorylated, and form a complex with SMAD4. This complex translocates to the nucleus, where it interacts with transcription factors to control gene expression (Augello and De Bari 2010). BMPs play a role in the first step of bone formation, the mesenchymal condensation. Mesenchymal cells within these condensations then differentiate into chondrocytes (Karsenty, Kronenberg et al. 2009). *In vitro* studies by Denker and colleagues, performed with micromass cultures of C3H10T1/2 cells, showed that BMP-2 enhanced the appearance of chondrocytes in those cultures (Denker, Haas et al. 1999). Positive effects on chondrogenic micromass cultures of human BM-MSCs were also reported for BMP-6. TGF-β is another signaling molecule of the TGF-β superfamily that promotes chondrogenic differentiation and is widely used for *in vitro* differentiation of MSCs (Augello and De Bari 2010).

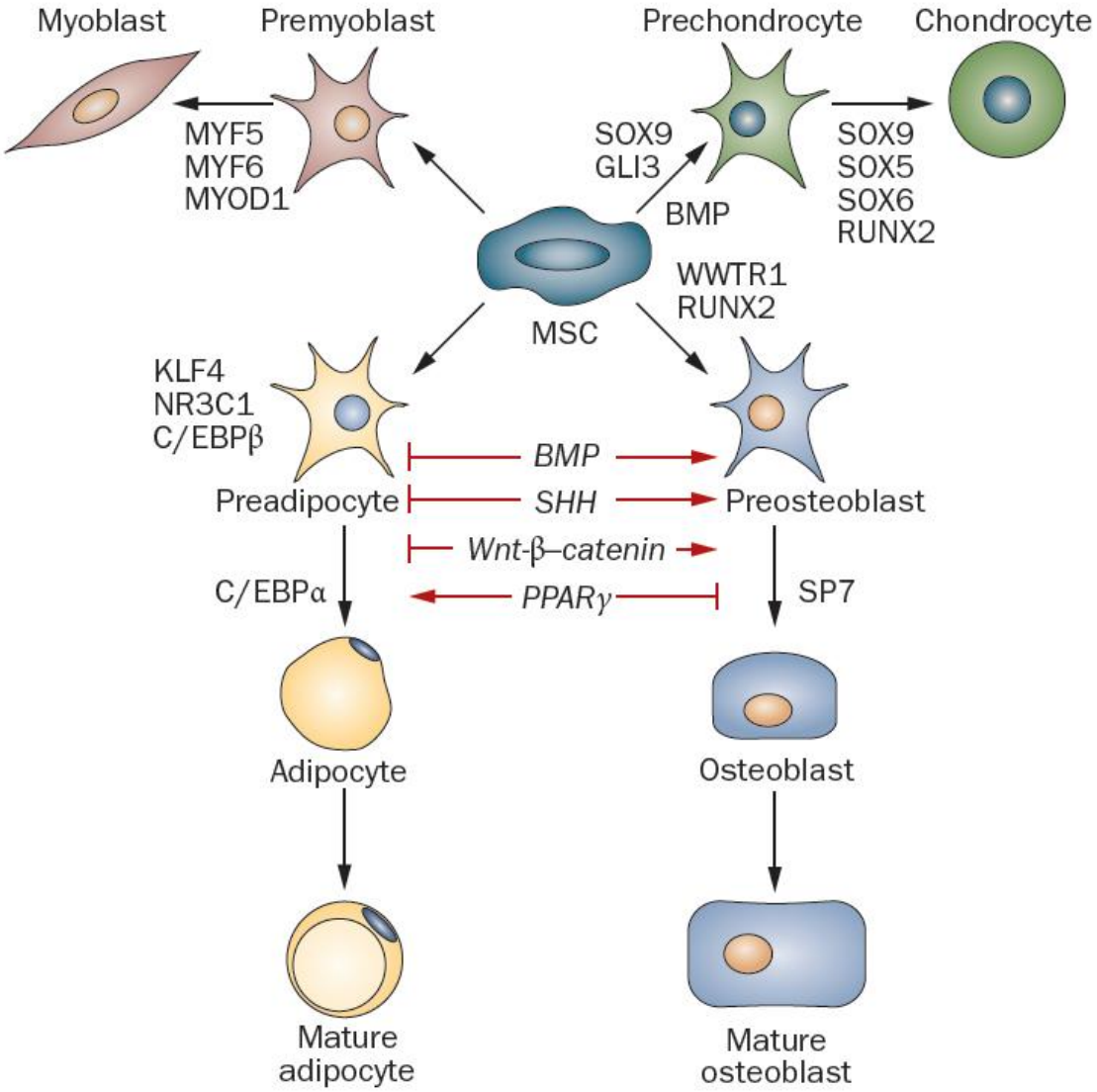


Figure 10 | Regulation of mesenchymal stem cell differentiation.

The differentiation of MSCs into distinct lineages is controlled by cues from signaling molecules like Wnt ligands, BMPs, and TGF-β. Transcription factors also play a critical role in lineage decisions of MSCs during differentiation (Takada, Kouzmenko et al. 2009). Abbreviations: BMP, bone morphogenetic protein; C/EBP, CCAAT/enhancer binding protein; KLF, Kruppel-like factor; PPARγ, peroxisome proliferator-activated receptor γ; RUNX2, runt-related transcription factor 2; SOX, sex determining region Y-box; SHH, sonic hedgehog homolog; SP7, sp7 transcription factor (formerly known as Osterix); TGF-β, transforming growth factor β. Figure taken from (Takada, Kouzmenko et al. 2009)

In addition to chondrogenic differentiation, BMPs are involved in the regulation of osteogenic differentiation. The important role of BMP-2 and BMP-4 was revealed in genetic studies where BMP-2 and BMP-4 enhanced osteogenic differentiation. Knockout studies demonstrated that a sufficient level of BMP-2 and BMP-4 signaling is required for the differentiation of mature osteoblasts. BMP-2 also plays

a crucial role in postnatal bone development, which was demonstrated with studies in mice that lacked BMP-2 in the limb mesenchyme. These mice showed a normal bone development during embryogenesis, but had a reduced bone mineral density after birth (Long 2012). *In vitro* studies performed with rat BM-MSCs also demonstrated a positive effect of BMP-2 on osteogenic differentiation (Augello and De Bari 2010). BMPs may not only exert a stimulating effect on osteogenic differentiation, as knockout studies of BMP-3 in mice revealed. After the knockout of BMP-3, mice had more trabecular bone than wild-type mice. Therefore, BMP-3 seems to impair the activities of BMP-2 and BMP-4 to regulate the amount of bone *in vivo*. BMP-3 is expressed in osteoblasts and osteocytes in the postnatal skeleton and seems to inhibit osteoblast differentiation and therefore regulates the number of osteoblasts (Long 2012).

During the developmental stages of differentiation of MSCs into functional cell types, a specific set of transcriptions factors is expressed that regulates this process (Figure 10). Chondrogenic differentiation occurs in several distinct stages. First, mesenchymal cells differentiate into nonhypertrophic chondrocytes, which are still proliferating and express aggrecan and type II collagen. In the second step, nonhypertrophic chondrocytes stop proliferating and become hypertrophic chondrocytes (Karsenty 2008). Hypertrophic chondrocytes can be divided into prehypertrophic chondrocytes that still express type II collagen at low levels and hypertrophic chondrocytes that do not express type II collagen, but type X collagen and vascular endothelial growth factor (VEGF) (Karsenty, Kronenberg et al. 2009). Studies identified the sex determining region Y (SRY)-box 9 (Sox9) as a master regulator of chondrogenic differentiation. The transcription factor Sox9 regulates the expression of aggrecan and type II collagen, and both proteins are characteristic for nonhypertrophic chondrocytes. The importance of Sox9 in chondrogenesis was shown with knockout studies in embryonic stem cells. Sox9 $^{-/-}$ ES cells were excluded form mesenchymal condensations, the first step in bone development, and did not produce nonhypertrophic markers. In addition to Sox9, Sox5 and Sox6 play a role during chondrogenic differentiation. *In vitro* studies showed that Sox5 and Sox6 bind to Sox9 and increase the transactivation function of Sox9. Sox5 and Sox6 have important functions *in vivo,* which were demonstrated with knockout experiments in mice. Double knockout leads to the death of embryos at embryonic day E16.5 and an inability of chondrocyte progenitors to differentiate into hypertrophic chondrocytes. Other transcription factors with important roles in chondrogenic differentiation are Runt-related-2 (Runx2) and Runx3. Runx2 is expressed in prehypertrophic chondrocytes and regulates chondrocyte hypertrophy. The roles of Runx2 and Runx3 in chondrocyte hypertrophy were shown in Runx2/Runx3 double-knockout mice that lacked hypertrophic chondrocytes (Karsenty 2008).

Differentiation of MSCs into functional osteoblasts occurs in several steps. In the first step, MSCs generate osteo/chondroprogenitos, which subsequently progress to preosteoblasts, functional osteoblasts, and finally osteocytes. Several transcription factors are involved in the regulation of these events, and during the different stages of differentiation, the osteogenic marker genes osteopontin

(SPP1), osteocalcin (BGLAP), alkaline phosphatase (ALPL), and type I collagen (α1) (COL1A1), are expressed (Figure 11) (Baksh, Song et al. 2004).
The transcription factor Runx2 is a master regulator of osteogenic differentiation. Runx2 was identified as a factor that bound to an osteoblast-specific cis-acting element in the osteocalcin promoter. Osteocalcin is an osteoblast-specific hormone that is involved in the regulation of the energy metabolism and is only expressed in fully differentiated osteoblasts (Karsenty 2008).

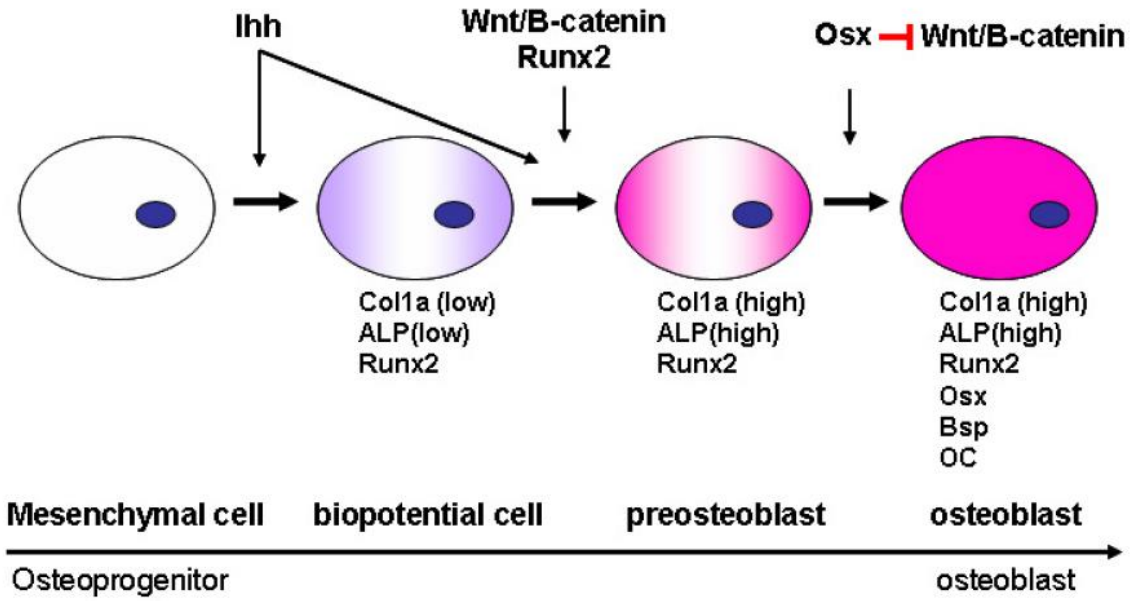


Figure 11 | Stages of osteogenic differentiation in mesenchymal stem cells.

Wnt/β-catenin and the transcription factors Runx2 and osterix (Osx) play important roles in the osteogenic differentiation of MSCs. Each stage of osteogenic differentiation is characterized by the specific expression of bone-related genes. Col1a and ALP are early osteogenic marker genes. Osteocalcin (OC) is only expressed in fully differentiated osteoblasts (Karsenty 2008). Abbreviations: Col1a, type I collagen (α1); ALP, alkaline phosphatase; Bsp, bone sialoprotein; Ihh, Indian hedgehog homolog. Figure taken from (Zhang 2010).

The importance of Runx2 for osteoblast development and skeletogenesis is highlighted by the absence of osteoblasts in the skeleton of Runx2 $^{-/-}$ mice (Karsenty, Kronenberg et al. 2009). The activity and expression of Runx2 is strictly regulated by many factors. One of these factors, Twist-1, was identified as a negative regulator of Runx2 in the human Saethre-Chotzen syndrome, which is caused by haploinsufficiency of the Twist-1 locus, and is characterized by increased bone formation in the skull. Further studies in mice confirmed that Twist-1 is a negative regulator of Runx2 and delays osteogenic differentiation through inhibition of Runx2. Runx2 activity is enhanced by several factors, one of them is the nuclear matrix protein special AT-rich sequence binding protein 2 (SATB2), which was discovered in humans with cleft palate. Genetic studies revealed that SATB2 plays key roles in craniofacial development, skeletal pattering, and

osteoblast differentiation. Further, it was found that SATB2 interacts with another transcription factor that is involved in osteoblast differentiation, activating transcription factor 4 (ATF4). Besides Runx2, Osterix (Osx/SP7) is the second transcription factor that is essential for osteogenic differentiation. Expression of Osterix was found in osteoblasts of all skeletal elements. Studies in mice revealed that Osterix acts downstream of Runx2, as it was not expressed in Runx2 $^{-/-}$ mice, but Runx2 was expressed in Osx $^{-/-}$ mice. In contrast to Runx2, Osx does not seem to be involved in chondrogenic differentiation and is strictly required for osteoblast differentiation. This was the result of studies in Osx knockout mice, which lacked a mineralized matrix in bones formed by intramembranous ossification, but still contained some mineralized matrix in bones that formed by endochondral ossification. The transcription factor ATF4 is required for osteoblast differentiation and proper function of mature osteoblasts. It was shown that ATF4 regulates the transcription of osteocalcin and the production of type I collagen. The lack of ATF4 in mice produced a delay in skeletal development and caused a low-bone-mass phenotype, which resulted from a decreased bone formation (Karsenty 2008).

The transcription factors CCAAT/enhancer binding protein α, β (C/EBPα, C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ), and Kruppel-like factors (KLFs), play key roles in adipogenic differentiation of MSCs (Figure 10). C/EBPβ is expressed during early stages of adipogenic differentiation. Knockout of C/EBPβ in mouse embryonic fibroblasts (MEFs) reduced their adipogenic differentiation potential, demonstrating its function in adipogenic differentiation. Studies have shown that the expression of C/EBPβ is regulated by KLFs as well as Krox20 and cAMP activated cAMP response element-binding (CREB) protein. Both, PPARγ and C/EBPα are expressed at later stages of adipogenic differentiation and are required for terminal differentiation of adipocytes (Smink and Leutz 2012). In addition to promoting adipogenic differentiation, PPARγ inhibits the osteogenic differentiation of MSCs (Kolf, Cho et al. 2007). Hedgehog signaling affects the early stages of adipocyte differentiation and promotes osteogenic differentiation by interfering with C/EBPα and PPARγ expression (Fontaine, Cousin et al. 2008).

1.10. Mesenchymal stem cells for regenerative medicine

The proposed biological properties of MSCs like immunomodulation, capacity to differentiate into cell types of mesenchymal origin, and their great *in vitro* expansion potential, make them ideal candidates for cell therapy. Another important point in respect to their use in regenerative medicine is that MSCs can easily be obtained from various adult and birth-associated tissues. The increasing knowledge about MSCs led to an increasing number of clinical trials that use MSCs in the treatment of various diseases. Clinical trials with MSCs are carried out in the fields of immunomodulation, tissue protection, graft enhancement, and regenerative medicine (Figure 12). In clinical trials, in the area of immunomodulation, MSCs are most commonly used to treat multiple sclerosis/arteriosclerosis, type I diabetes, and Crohn's disease. For tissue protection, the use of MSCs is focused on myocardial infarction and liver cirrhosis. MSCs are of particular interest in regenerative medicine for the treatment of osteoarthritis/osteogenesis imperfecta and bone and cartilage repair. Many clinical

Indication	Number of studies
Immunomodulation	**48**
Multiple sclerosis/atherosclerosis	12
Type 1 diabetes	12
Crohn's disease	10
Systemic lupus erythematosus/colitis	4
Rheumatoid arthritis/Sjögren's syndrome	3
Buerger's disease/sickle cell disease	2
HIV	1
Limbus corneae insufficiency syndrome	1
Periodontitis	1
Progressive hemifacial atrophy	1
Retinitis pigmentosa	1
Tissue protection	**76**
Myocardial infarction/stroke/ischemia	34
Liver cirrhosis	20
Alzheimer's/Parkinson's disease	4
Amyotrophic lateral sclerosis	4
Fibrosis/emphysema	4
Necrosis	4
Acute kidney injury	2
Bronchopulmonary dysplasia	2
Multiple system atrophy/multiple trauma	2
Regenerative medicine	**69**
Osteoarthritis/osteogenesis imperfecta	22
Bone/cartilage repair	18
Spinal cord injury/neuroblastoma	8
Anemia	4
Type 2 diabetes	4
Dilated cardiomyopathy	4
Wound healing/umbilical cord varices	3
Ataxia	2
Autism	1
Epidermolysis bullosa	1
Erectile dysfunction	1
Wilson's disease	1
Graft enhancement	**27**
GvHD	23
Hematopoietic malignancies	4

Figure 12 | Clinical trials using mesenchymal stem cells retrieved June 2, 2012.

Figure taken from (Frenette, Pinho et al. 2013)

trials try to use the immunomodulatory properties of MSCs to treat Graft vs. Host Disease (GvHD) (Figure 12).

MSCs have an immunosuppressive capacity, which led to clinical trials with promising results. The immunosuppressive effects seem to be mediated by the suppression of T cell proliferation, but MSCs also affect other types of immune cells, such as natural killer cells, dendritic cells, and macrophages. The exact mechanisms, by which MSCs act immunosuppressive, are not clear. Proposed mechanisms are that MSCs exert their effect through close proximity or direct contact with the target cells. These interactions could be mediated through several cell adhesion molecules e.g. CD90, CD106, and integrins, that are expressed by MSCs. The immunosuppression could also be mediated through soluble factors that are secreted by MSCs e.g. nitric oxide (NO), TGF-β1, IL-6, and leukemia inhibitory factor (Frenette, Pinho et al. 2013).

The ability to differentiate into bone forming cells (osteoblasts) and cartilage cells (chondrocytes) was among the first biological properties of MSCs that were identified. This ability makes MSCs ideal candidates in regenerative medicine for the treatment of bone and cartilage defects. MSCs have been used in mouse models of osteogenesis imperfecta, a disease characterized by defective bone formation. When BM-MSCs were infused into irradiated mice they were able to form functioning bone and cartilage tissues. In addition to the systemic administration the seeding of MSCs

on porous biomaterials such as hydroxyapatite and tricalcium phosphate was very effective to induce local bone and cartilage regeneration (Krampera, Franchini et al. 2007). Biomaterials seeded with MSCs have been implanted into rats with critical-sized segmental bone defects, and it was found that bone regeneration was higher in bones with MSC-loaded implants than in bones with cell-free implants (Bruder, Kurth et al. 1998). Positive therapeutic effects of MSCs have also been observed in animal models and first clinical trials for cartilage regeneration. Several studies have used BM-MSCs to treat partial or complete cartilage defects in animal models and demonstrated that MSCs had beneficial effects (Krampera, Franchini et al. 2007). Osteoarthritis is the most common joint disease in adults, and first studies with the local administration of MSCs in animal models and clinical trials have produced promising results (Frenette, Pinho et al. 2013).

MSCs have been used in several animal disease models and initial clinical trials, and some studies produced promising results. However, there are still many unresolved obstacles for cell therapy with MSCs, such as risk of immune rejection of allogeneic cells, risk of pathogen contamination, cell delivery, *in vivo* behavior and safety of transplanted cells, and high costs. BM-MSCs show a very low expression of MHC class I and a complete absence of MHC class II, combined with the lack of the co-stimulatory molecules B7 and CD40 ligand, and therefore they are considered to be immune privileged. It was shown that transplanted MSCs can survive for several months in recipients. On the contrary, there were reports that observed T cell mediated apoptosis, and a study in rats found that MSCs accelerate graft rejection of allogeneic skin grafts without an additional immunosuppressive therapy (Sbano, Cuccia et al. 2008; Frenette, Pinho et al. 2013). Another challenge on the way to an effective cell therapy is the delivery of MSCs to the sites within the body, where they are needed. One common delivery method is intra-venous administration, but MSCs were also injected locally. However, no well-defined homing mechanism exists for MSCs *in vivo*, which makes it hard to track homing events after the infusion of MSCs. Whether MSCs use leukocyte-like cell-adhesion or transmigration mechanisms to actively home to injured tissues or simply become trapped in small blood vessels is not clear. The methods to track infused MSCs are limited, and therefore, it is not clear if the observed effects are from infused MSCs that engrafted in the desired region and replaced the injured cells with their differentiated progeny. Homing of MSCs to sites of tissue injury or inflammation is well established, but the homing efficiency is low compared to leukocytes and HSCs. (Karp and Leng Teo 2009).

1.11. Aim of this work

The Medical University of Vienna was one of seven partners of the EU project HYPERLAB, which stands for High Yield and Performance Stem Cell Lab. The goal of the HYPERLAB project was to develop new, innovative, microfluidics-based technologies adapted for the cultivation of stem cells for clinical use. Project partner IBMT (Fraunhofer Institut für Biomedizinische Technik, IBMT, Saarbrücken, Germany) installed a TECAN robotics system, in which mesenchymal stem cells could be cultivated automatically in 96 well plates to screen for factors that promote osteogenic differentiation of MSCs. The automated screening for compounds that enhance osteogenic differentiation of mesenchymal stem cells should be performed with an osteogenic MSC reporter cell line in 2D culture and a modified hanging droplet method for 3D culture. The osteogenic differentiation of MSCs and the effect of different compounds should be analyzed through the detection of GFP expression with flow cytometry.

The aim of this study was to generate an osteogenic MSC reporter line that should be used for the screening of compounds which enhance osteogenic differentiation with the TECAN robotics system. The study was structured into several parts to accomplish this aim. In the first part of this study, protocols for the isolation, expansion, and differentiation of MSCs from umbilical cord Wharton's jelly were established. After the characterization of the cells according to the ISCT criteria, the obtained cells were used for the generation of the reporter cells and the screening experiments.

The second part of this study was the generation of the reporter constructs. Therefore, the promoter regions of five bone-related marker genes COL1A1, SPP1, ALPL, BGLAP, and SP7, were amplified from genomic DNA with PCR and ligated into the pGreenFire lentiviral pathway reporter vector. The pGreenFire vector contains a dual reporter system that enables the simultaneous expression of GFP and luciferase.

The final part of the project was the generation of the MSC reporter line and the initial testing of the obtained reporter cells. The generated reporter constructs COL1A1, SPP1, ALPL, BGLAP, and SP7, as well as a positive control with the CMV promoter were packed into lentiviral particles and transduced into WJ-MSCs. The stably transduced cells were selected with puromycin and subsequently differentiated into osteoblasts. The generated reporter cells were then tested for GFP expression during osteogenic differentiation with flow cytometry.

2. Materials and Methods

2.1. Cell Culture

2.1.1. Collection of umbilical cord samples

Umbilical cords were collected from the placenta of full terms, after caesarian delivery, from General Hospital Vienna, Medical University of Vienna, Department of Obstetrics and Gynaecology, with informed consent of the mother. Sample collection was approved by the local institutional review board. After harvesting of the umbilical cord blood, a piece of umbilical cord (10-15 cm) was cut off with sterilized scissors and placed in a 50 ml falcon tube containing Dulbecco's Phosphate Buffered Saline (DPBS) with penicillin/streptomycin for transportation. The umbilical cord sample was processed within 2 hours. A total of three umbilical cord samples were collected which were termed UCXIX, UCXXII, and UCXXIII.

2.1.2. Explant cultures from umbilical cord

MSCs were isolated from umbilical cord Wharton's Jelly by explant culture. The collected umbilical cord was transferred to a tissue culture hood and all steps of the isolation were performed under sterile conditions:

- The umbilical cord piece was washed with DPBS with penicillin/streptomycin by inverting the falcon tube several times to remove most of the blood.
- After the first washing step, the umbilical cord piece was transferred to a 15 cm cell culture dish filled with DPBS with penicillin/streptomycin.
- The umbilical cord was cut into 2-5 mm slices with sterile scalpels and sterile forceps to fix the umbilical cord.
- The pieces of umbilical cord were transferred to a 50 ml falcon tube containing DPBS with penicillin/streptomycin and washed by inverting the tube several times to remove the remaining blood.
- Pieces of umbilical cord were transferred to a fresh 15 cm cell culture dish filled with DPBS with penicillin/streptomycin and tissue biopsies were taken from the Wharton's jelly with a sterile 2 mm biopsy punch and sterile forceps to hold the umbilical cord (Figure 13A).
- From one slice of umbilical cord an average of three biopsies were taken.
- The tissue biopsies were flushed out of the biopsy punch with a 1000 µl Eppendorf pipet using 200 µl DPBS with penicillin/streptomycin and collected in a fresh 10 cm tissue culture dish.
- The tissue biopsies were aspirated with a 1000 µl pipet tip and transferred to a T25 tissue culture flask which contained 2 ml NH Expansion medium with Cytomix (Figure 13B). Care was taken to transfer a minimum of DPBS to the tissue culture flask with the tissue biopsies.

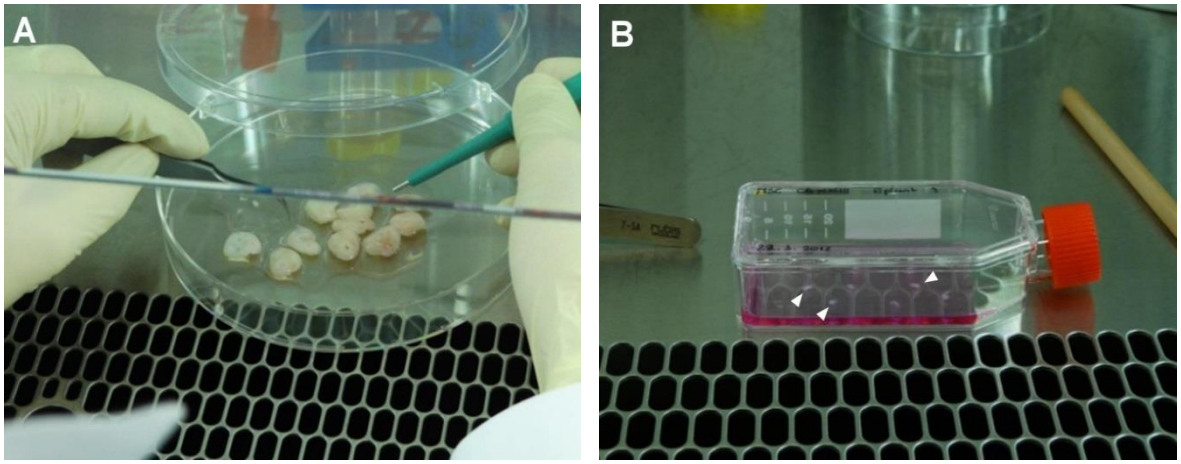


Figure 13 | Preparation of explant cultures from umbilical cord Wharton's jelly.

A | Prepared slices of umbilical cord for harvesting of tissue biopsies. **B** | Explant culture in a T25 tissue culture flask for the isolation of UC-MSCs. Tissue biopsies are indicated by arrow heads.

- About 8-12 tissue biopsies were transferred to one T25 tissue culture flask and three to four flasks were prepared from each umbilical cord sample.
- The T25 tissue culture flasks were incubated at 37°C, 95% humidity and 5% CO_2 for up to 14 days.
- During the first incubation period the tissue culture flasks were handled with extreme care so that the tissue biopsies could adhere to the surface of the tissue culture flask, which is required for the outgrowth of MSCs.

2.1.3. Passage one of isolated WJ-MSCs

To generate a sufficient number of MSCs for future experiments, the isolated MSCs were expanded after the outgrown cells reached a critical density around the tissue biopsies:

- To remove the tissue biopsies from the tissue culture flasks 3 ml NH Expansion medium were added to a T25 tissue culture flask.
- The tissue biopsies were detached by pipetting medium directly onto the tissue biopsies with a 1000 µl Eppendorf pipet.
- The detached tissue biopsies were aspirated with a 1000 µl pipet tip and were removed from the T25 tissue culture flask and discarded as biomedical waste.
- NOTE: The tissue biopsies can be transferred to a fresh tissue culture flask with NH Expansion medium with Cytomix for an additional isolation step.
- After the tissue biopsies have been removed the remaining NH Expansion medium with Cytomix was aspirated.
- The tissue culture flask was washed once with 5 ml sterile DPBS.

- Cells were incubated with 1000 µl Trypsin/EDTA solution for 5-10 minutes at 37°C, 95% humidity and 5% CO_2 in an incubator.
- For detachment of the cells the tissue culture flask was gently tapped several times and detachment of the cells was checked with a phase-contrast microscope.
- Trypsinization was stopped with 4 ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and penicillin/streptomycin.
- The flask was rinsed several times with the cell suspension and the cell suspensions from all explant cultures were transferred and combined in one 50 ml falcon tube.
- The cell suspension was centrifuged at 1000 rpm (220xg) for 3 minutes.
- The supernatant was aspirated and the cell pellet was resuspended in 2 ml NH Expansion medium with Cytomix.
- From this cell suspension, a 20 µl aliquot was taken for cell counting with a CASY cell analyzer (Roche-Innovatis, Germany).
- After cell counting the cells were resuspended in an appropriate amount of NH Expansion medium with Cytomix and seeded in fresh tissue culture flasks with a density of 4000 cells/cm².
- Cells were incubated at 37°C, 95% humidity and 5% CO_2 until they reached confluency (about 4 days) with complete medium change every 2 days.

2.1.4. Passaging of WJ-MSCs

- The old NH Expansion medium with Cytomix was aspirated from the tissue culture flask.
- The tissue culture flask was washed once with the appropriate volume of sterile DPBS (5 ml for T25 tissue culture flasks, 13 ml for T75, T175, T182.5 tissue culture flasks).
- Cells were incubated with 1000 µl (T25 tissue culture flasks) or 2000 µl (T75, T175, and T182.5 tissue culture flasks) Trypsin/EDTA solution for 3-5 minutes at room temperature.
- For detachment of the cells, the tissue culture flask was gently tapped several times and detachment of the cells was checked with a phase-contrast microscope.
- Trypsinization was stopped with an appropriate amount of Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and penicillin/streptomycin (4 ml for T25, 8 ml for T75, T175 and T182.5 tissue culture flasks).
- The flask was rinsed several times with the cell suspension and the cell suspension was then transferred to a 50 ml falcon tube.
- From this cell suspension a 50 µl aliquot was taken for cell counting with a CASY cell analyzer (Roche-Innovatis, Germany).

- After cell counting the cell suspension was centrifuged at 1000 rpm (220xg) for 3 minutes.
- The supernatant was aspirated and the cell pellet was resuspended in an appropriate amount of NH Expansion medium with Cytomix and seeded in fresh tissue culture flasks with a density of 4000 cells/cm².
- Cells were incubated at 37°C, 95% humidity and 5% CO_2 until they reached confluency (about 4 days) with complete medium change every 2-3 days.

2.1.5. Freezing of MSCs for long-term storage

As the isolation and expansion of MSCs takes several weeks, it was necessary to freeze and store the isolated MSCs at a low passage number to ensure that cells are readily available for future experiments:

- After trypsinization and cell counting the cell suspension was centrifuged at 1000 rpm (220xg) for 3 minutes.
- The supernatant was aspirated and the cell pellet was carefully resuspended in cold (4°C) CryoSure MSC freezing medium or FBS with 10% DMSO to achieve a final cell concentration of about 2.0x10^6 cells/ml.
- MSCs were frozen in aliquots of 2.0x10^6 cells.
- 1 ml cell suspension was transferred to 1.8 ml Nunc CryoTubes and the cryotubes were placed in a Mr. Frosty freezing box, filled with 250 ml isopropanol, which provides a freezing rate of about -1°C/min.
- The freezing box was transferred to a -80°C freezer overnight.
- On the next day the cryotubes were transferred to a liquid nitrogen storage tank for long-term storage.

NOTE: The same freezing protocol was used for MSCs which were transduced with pGF reporter constructs but the final cell concentrations in freezing medium were between ~1.0x10^5 – 1.0x10^6 cells/ml.

2.1.6. Thawing of frozen WJ-MSCs

- Frozen aliquots of WJ-MSCs or BM-MSCs were quickly thawed by placing the tubes in a 37°C water bath with constant agitation until the cells were just thawed (< 5 minutes).
- The cryotubes were thoroughly rinsed with 70% ethanol or Mikrozid® AF liquid (Schuelke&Mayr) to avoid microbial contamination and transferred to a tissue culture hood.
- The thawed cell suspension was transferred to a 50 ml falcon tube and 9 ml of pre-warmed DMEM medium with 10% FBS were slowly added under gentle agitation of the falcon tube.

- Next, the cell suspension was centrifuged at 1000 rpm (220xg) for 3 minutes to remove DMSO which has cytotoxic effects at higher concentrations.
- Therefore, the supernatant was aspirated and the cell pellet was resuspended in an appropriate amount of NH Expansion medium with Cytomix and seeded in a tissue culture flask of appropriate size to achieve a density of about 4.0x10^3 – 6.0x10^3 cells/cm². Aliquots of 2.0x10^6 cells were transferred to T175 tissue culture flasks with a cell density of 1.1x10^4 cells/cm².
- The thawed WJ-MSCs were incubated at 37°C, 95% humidity, and 5% CO_2.

2.1.7. Determination of cell number with a CASY® cell analyzer

For the determination of cell numbers and cell size a CASY® cell analyzer (Roche-Innovatis, Germany) was used.

- After trypsinization the cell suspension was transferred to a 50 ml falcon tube and an aliquot of 20 µl or 50 µl was transferred to a CASY® tube.
- The cell suspension was diluted with 5 ml or 10 ml CASY®ton (Roche-Innovatis, Germany) resulting in a dilution between 1:100 and 1:250.
- The measurement was performed with the 150 µm capillary and the following settings:
    - Range: 0-40 µm
    - Dilution: 1:100, 1:200 or 1:250
    - Measurement mode: 2x 200 µl
- Immediately before the measurement the diluted cell suspension was mixed by inverting the CASY® tube several times and the tubes were fitted over the capillary.
- The measurement was started and two 200 µl aliquots of cell suspension were analyzed.
- The average number of cells was determined in range between 11 and 40 µm. Events measured below 11 µm were considered as dead cells or cell debris and were excluded from the measurement.
- The result of the measurement was displayed in “number of cells/ml”. The total number of cells was calculated by multiplying the number of cells per ml with the volume of the cell suspension.

2.1.8. Immunophenotyping of MSCs

After the initial expansion of MSCs, the expression of mesenchymal markers was analyzed by flow cytometry. Flow cytometry was carried out on a BD FACScan flow cytometer.

- After trypsinization about 1.5x10^5 cells per staining were transferred to FACS tubes and 1 ml of cold (4°C) MACS buffer was added to the cells.
- Cells were centrifuged at 220xg for 3 minutes and the supernatant was aspirated.
- The cell pellet was resuspended in 1 ml cold MACS buffer.
- After centrifugation at 220xg for 3 minutes, the supernatant was aspirated and 50 µl cold MACS buffer were added to each FACS tube.
- The following amounts of antibody solution were added to the correspondingly labeled FACS tubes:

Staining	**Antibody**	**Volume of antibody solution**
Unstained	-	-
Isotype control FITC	IgG1-FITC	0,5 µl
Isotype control PE	IgG1-PE	5 µl
CD90	CD90-FITC	1,0 µl
CD105	CD105-FITC	5 µl
CD73	CD73-PE	5 µl
CD44	CD44-PE	5 µl
CD45	CD45-FITC	5 µl
CD34	CD34-FITC	5 µl

- Cells were incubated for 30 minutes at 4°C in the dark.
- Then, 1 ml cold MACS buffer was added, the cells were briefly vortexed and centrifuged at 220xg for 3 minutes.
- The supernatant was aspirated and the cell pellet was resuspended in 500µl cold MACS buffer.
- Immediately before the measurement, the cells were briefly vortexed and the FACS tube was attached to the intake nozzle.
- For each staining condition, 30000 events were measured and the data was exported in FCS2.0 format and analyzed with FlowJo software.

2.1.9. Osteogenic differentiation of WJ-MSCs

Osteogenic differentiation of cells, isolated from Wharton's jelly, was performed with NH OsteoDiff medium according to the manufacturer's instructions.

- Before osteogenic differentiation, WJ-MSCs were cultured and trypsinized according to the protocols described above.
- From the cell suspension, the required amount of cells (e.g. 5.0x10^4 cells for a 35 mm cell culture dish) was transferred to a falcon tube and centrifuged at 220xg for 3 minutes.
- After centrifugation the supernatant was aspirated and the cells were suspended in an appropriate volume of osteogenic differentiation medium.
- The WJ-MSCs were seeded with a density of 5000 cells/cm² in an appropriate volume of NH OsteoDiff medium.
- As a control, WJ-MSCs were seeded with 4000 cells/cm² in an appropriate amount of NH Expansion medium or DMEM with 4% FBS.
- Osteogenic and control cultures were incubated at 37°C, 95% humidity, and 5% CO_2.
- A total medium change was performed twice a week and the cells were cultured for 10-21 days in osteogenic differentiation or control medium.

2.1.10. Chondrogenic differentiation of WJ-MSCs

Chondrogenic differentiation of cells, isolated from Wharton's jelly, was performed with NH ChondroDiff medium according to the manufacturer's instructions.

- Before chondrogenic differentiation, WJ-MSCs were cultured and trypsinized according to the protocols described above.
- From the cell suspension 2.5x10^5 cells were transferred to a 15 ml falcon tube and centrifuged at 150xg for 3 minutes.
- The supernatant was aspirated and the cell pellet was resuspended in 1 ml NH ChondroDiff medium.
- For chondrogenic differentiation the cells were aggregated by centrifugation of the cell suspension at 150xg for 3 minutes.
- After centrifugation the cap of the 15 ml falcon tube was replaced with a filter cap from a T25 tissue culture flask, which allowed gas exchange.
- Then the falcon tubes were carefully placed in the incubator to avoid resuspension of the aggregated cells.
- Chondrogenic cultures were incubated at 37°C, 95% humidity, and 5% CO_2.
- A total medium change was performed twice a week and the cells were cultured for 24 days in NH ChondroDiff medium.

2.1.11. Adipogenic differentiation of WJ-MSCs

Adipogenic differentiation of cells, isolated from Wharton's jelly, was performed with NH AdipoDiff medium.

- Before adipogenic differentiation, WJ-MSCs were cultured and trypsinized according to the protocols described above.
- From the cell suspension the required amount of cells (e.g. 5.0x10^4 cells for a 35 mm cell culture dish) was transferred to a 15 ml falcon tube and centrifuged at 220xg for 3 minutes.
- The cell pellet was suspended in an appropriate volume of NH Expansion medium and the cells were seeded with a density of 5000 cells/cm² in an appropriate volume of NH Expansion medium.
- The cells were incubated at 37°C, 95% humidity, and 5% CO_2 until they reached confluency.
- When the cells reached confluency the medium was switched from NH Expansion medium to NH AdipoDiff medium.
- As a control, WJ-MSCs were seeded with 4000 cells/cm² in an appropriate amount of NH Expansion medium or DMEM with 4% FBS.
- Adipogenic and control cultures were incubated at 37°C, 95% humidity, and 5% CO_2.
- A total medium change was performed twice a week and the cells were cultured for 21 days in adipogenic differentiation or control medium.

2.1.12. Osteogenic differentiation of WJ-MSCs (mineralization)

For the formation of a mineralized extracellular matrix from WJ-MSCs, a different osteogenic differentiation protocol was used.

- Before osteogenic differentiation, WJ-MSCs were cultured and trypsinized according to the protocols described above.
- From the cell suspension, the required amount of cells, 6.0x10^4 cells for a well of a 4 well or 24 well plate, was transferred to a falcon tube and centrifuged at 220xg for 3 minutes.
- After centrifugation the supernatant was aspirated and the cells were suspended in an appropriate volume of osteogenic differentiation medium to achieve a final cell concentration of 1.2.10^5 cells/ml.
- The WJ-MSCs were seeded with a density of 3.0x10^4 cells/cm² in a well of a 4 or 24 well plate in 0.5 ml NH OsteoDiff medium.
- As a control, WJ-MSCs were seeded with 5000 cells/cm² in an appropriate amount of NH Expansion medium or DMEM with 4% FBS.
- Osteogenic and control cultures were incubated at 37°C, 95% humidity, and 5% CO_2.

- A total medium change was performed twice a week and the cells were cultured for 18-21 days in osteogenic differentiation or control medium.

2.1.13. Chondrogenic differentiation of WJ-MSCs in 96 well plates

- WJ-MSCs were harvested by trypsinization and the cell number was determined with a CASY® cell analyzer.
- For chondrogenic differentiation of WJ-MSCs 8.0x10^4 cells were used per well.
- For control cultures, 5.0x10^3 cells were used per well.
- The required amount of cell suspension was transferred to a 15ml falcon tube and cells were centrifuged at 220xg for 3 minutes.
- The supernatant was aspirated and the cells were resuspended in an appropriate volume of medium. The final cell concentration for chondrogenic differentiation was 1.6x10^7 cells/ml and for control cultures 2.5x10^4 cells/ml.
- From this cell suspension, a 5 µl droplet, containing 8.0x10^4 cells, was placed in the center of a well of a 96 well plate.
- For control cultures, 200 µl cell suspension containing 5.0x10^3 cells were transferred to the according wells. DMEM with 4% FBS and 2x penicillin/streptomycin was used as control medium.
- For aggregation of the cells, the plates were incubated for 2 hours at 37°C, 5% CO_2, and >95% humidity.
- After 2 hours, 200 µl of NH ChondroDiff were carefully added to the droplets and the plate was incubated at 37°C, 5% CO_2 and >95% humidity.
- A Medium change was performed every 3 days and after 15 days the chondrogenic differentiation was assessed by toluidine blue staining.

2.1.14. Production of lentiviral particles

For the production of stable osteogenic reporter cell lines it was necessary to integrate the cloned reporter constructs into the genome of the cells. Therefore a lentiviral gene delivery system was used.

- On the first day, HEK-293T cells were seeded in 6 well plates with 3.0x10^5 cells/well in 3 ml DMEM with 10% FBS without penicillin and streptomycin.
- HEK-293T cells were incubated overnight at 37°C, 5% CO_2, and 95% humidity.

- In the afternoon of day 2, plasmid mixes were prepared according to the following scheme (in polypropylene tubes):
    - 1 µg of the pGreenFire Pathway Reporter Lentivector
    - 750 ng psPAX2 packaging plasmid
    - 250 ng pMD2.G envelope plasmid
    - Serum-free OPTI-MEM medium to a final volume of 20 µl
- For the FuGENE® master mix the according volume of serum-free OPTI-MEM was transferred to a 1.5 ml microfuge tube (in a polypropylene tube) and then FuGENE® was added directly into the OPTI-MEM without touching the walls of the tube.
    - 1x master mix: 74 µl OPTI-MEM plus 6 µl FuGENE®
- The FuGENE® master mix was gently mixed by flicking the tube several times and incubated for 5 minutes at room temperature.
- Next, 80 µl of FuGENE® master mix were added to each plasmid mix without touching the walls of the tube. The solution was gently mixed by flicking the tube several times.
- The transfection mixture was incubated for 25 minutes at room temperature.
- Without touching the sides of the well, 100 µl of the transfection mix were carefully added (drop wise) onto the HEK-293T cells. The mixture was dispersed by gentle agitation of the 6 well plate, so that the HEK-293T cells were not detached.
- The transfected cells were incubated overnight at 37°C, 5% CO_2, and 95% humidity.
- In the morning of day three, the medium of the transfected cells was changed to fresh DMEM with 10% FBS and 1x penicillin and streptomycin. Cells were incubated at 37°C, 5% CO_2, and 95% humidity.
- On day five, the supernatant of each well, containing the lentiviral particles, was aspirated with 1250 µl SafeSeal pipette tips and transferred to a 15 ml falcon tube.
- The viral supernatants were centrifuged at 1900xg for 10 minutes to pellet any HEK-293T cells that were transferred with the supernatant.
- After centrifugation, 500 µl of the cleared supernatant were directly used for infection of WJ-MSCs.
- From the remaining supernatant 500 µl aliquots were transferred to 1.5 ml screw cap microfuge tubes and stored at -80°C.

2.1.15. Infection of WJ-MSCs with lentiviral particles

The infection of WJ-MSCs with lentiviral particles was performed in a laminar flow hood which was designated for the handling of viral particles and under special safety measures. Applied safety measures included eye protection, lab coat, and a double pair of latex gloves. Tips and cell culture materials that came in contact with lentiviral particles were rinsed 3 times with hypochlorite solution and discarded in autoclave bags. Contaminated culture medium, supernatants and wash solutions (from trypsinization) were discarded in a viral waste bottle that contained hypochlorite. The viral waste was autoclaved for at least 15 minutes at 140°C before it was discarded into the 'standard' biohazard waste.

- Before the infection of WJ-MSCs with lentiviral particles, 1.0x10^5 MSCs were seeded in T25 tissue culture flasks in 3 ml DMEM with 10% FBS and 1x penicillin/streptomycin. Cells were incubated at 37°C, 5% CO_2, and 95% humidity.
- After 24-48 hours, a complete medium change was performed and 4.5 ml fresh DMEM with 10% FBS and 1x penicillin/streptomycin were added to each flask.
- The infection of cells with the lentivirus was increased by the addition of 5 µl Polybrene (final concentration: 10 µg/ml) to each flask.
- Next, 500 µl of viral supernatant were added to the culture medium and mixed by gentle agitation of the flask.
- The cells were incubated for 2 days at 37°C, 5% CO_2, and 95% humidity.

2.1.16. Puromycin selection of transduced WJ-MSCs

- Stably transduced cells were selected by the addition of puromycin to the culture medium.
- 48 hours after the WJ-MSCs had been infected with lentiviral particles puromycin was added to the medium of transduced cells (final concentration 0.5 µg puromycin/ml).
- A complete medium change was performed if the cells had not reached confluency. Therefore the old medium was aspirated and 3 ml of fresh DMEM with 10% FBS, 1x penicillin/streptomycin and 0.5 µg/ml puromycin were added.
- When the transduced cells reached confluency, infected WJ-MSCs were trypsinized according to protocol 2.1.4. and transferred to T75 tissue culture flasks in 13 ml DMEM with 10% FBS, 1x penicillin/streptomycin, and 0.5 µg/ml puromycin.
- The transduced cells were cultured in puromycin containing culture medium for up to 14 days.

- After puromycin selection the cells were typsinized and were used for osteogenic reporter cell assays or frozen for long-term storage according to protocol 2.1.5.

2.1.17. Preparation of cell lysates

For the determination of protein concentrations and luciferase activity, cell lysates were prepared from osteogenic, adipogenic, and chondrogenic micromass cultures of WJ-MSCs.

- The old medium was aspirated and the cells were washed once with DPBS.
- Next, 400 µl of Trypsin/EDTA were added to each well and the cells were incubated for 10 minutes at 37°C. Detachment of the cells was checked after 10 minutes with a phase contrast microscope.
- Trypsinization was stopped with 400 µl DMEM with 10% FBS and the cell suspension was transferred to 1.5 ml microfuge tubes.
- The cells were centrifuged at 400xg for 3 minutes, the supernatant was aspirated, and the cell pellet was resuspended in 1000 µl DPBS.
- After centrifugation for 3 minutes at 400xg the supernatant was aspirated and the cell pellet was resuspended in 70 µl lysis buffer.
- Optional: Cell pellets resuspended in lysis buffer were stored at -80°C.
- For lysis of the cells, the resuspended cells were incubated on wet ice for 20 minutes and were vortexed every 5 minutes.
- After the lysis, cell debris and insoluble extra cellular matrix components were pelleted by centrifugation of the cell lysate at 16100xg for 3 minutes.
- The clear supernatant was used for BCA protein assays and luciferase assays.

2.1.18. Passaging of HEK-293T cells

- Frozen aliquots of HEK-239T cells (2.0x10^6 cells) were quickly thawed by placing the tubes in a 37°C water bath with constant agitation until the cells were just thawed (< 5 minutes).
- The cryotubes were thoroughly rinsed with 70% ethanol or Mikrozid® AF liquid (Schuelke&Mayr) to avoid microbial contamination and transferred to a tissue culture hood.
- The thawed cell suspension was transferred to a 50 ml falcon tube and 9 ml of pre-warmed DMEM medium with 10% FBS were slowly added under gentle agitation of the falcon tube.
- Next, the cell suspension was centrifuged at 1000 rpm (220xg) for 3 minutes to remove DMSO which has cytotoxic effects at higher concentrations.

- Therefore, the supernatant was aspirated and the cell pellet was resuspended in an appropriate amount of DMEM with 10% FBS and seeded in a T175 tissue culture flask with a cell density of 1.1x10^4 cells/cm².
- HEK-293T cells were incubated at 37°C, 5% CO_2, and >95% humidity. The medium was changed every two to three days.
- When HEK-293T cells reached confluency, the old medium was aspirated and the cells were washed once with 13 ml DPBS.
- Next, cells were incubated with 2 ml Trypsin/EDTA solution for 3-5 minutes at room temperature.
- For detachment of the cells, the tissue culture flask was gently tapped several times and detachment of the cells was checked with a phase-contrast microscope.
- Trypsinization was stopped with 8 ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and penicillin/streptomycin (T175 and T182.5 tissue culture flasks).
- The flask was rinsed several times with the cell suspension and the cell suspension was then transferred to a 50 ml falcon tube.
- From this cell suspension a 50 µl aliquot was taken for cell counting with a CASY cell analyzer (Roche-Innovatis, Germany).
- For the production of lentiviral particles, the appropriate amount of cell suspension (3.0x10^5 cells per plasmid) was transferred to a 50 ml falcon tube.
- OPTIONAL: From the remaining cell suspension, aliquots with 2.0x10^6 HEK-293T cells were frozen in FBS with 10% DMSO.
- The cells were centrifuged at 1000 rpm (220xg) for 3 minutes, the supernatant was aspirated and the cell pellet was resuspended in the appropriate volume of DMEM with 10% FBS (without P/S) (final cell concentration 1.0x10^5 cells/ml).
- For each plasmid, 3 ml of cell suspension were transferred to a well of a 6 well plate.

2.1.19. Measurement of GFP expression in WJ-MSCs

The GFP expression in pGreenFire transduced WJ-MSCs after osteogenic differentiation was measured by flow cytometry. Flow cytometry was carried out on a BD FACScan cytometer.

- For analysis of the GFP expression, cells from one well of a 4 well plate, were used for each reporter construct.
- The old medium was aspirated and the cells were washed once with 500 µl DPBS.

- 400 µl Trypsin/EDTA solution was added to each well and the cells were incubated for 10 minutes at 37°C.
- Trypsinization was stopped with 400 µl DMEM with 10% FBS and the cell suspension was transferred to FACS tubes.
- Next, 1 ml of DPBS was added to each cell suspension and the cell suspension was centrifuged at 220xg for 3 minutes.
- The supernatant was aspirated and the cell pellet was resuspended in 500µl DPBS.
- Immediately before the measurement, the cells were briefly vortexed and the FACS tube was attached to the intake nozzle.
- For each staining condition, 10000 events were measured and the data was exported in FCS2.0 format and analyzed with FlowJo software.

2.1.20. Puromycin titration of WJ-MSCs

For the selection of pGreenFire transduced WJ-MSCs the optimal puromycin concentration was determined by puromycin titration. The optimal puromycin concentration was defined by 100% dead cells after 4-5 days of selection.

- Puromycin concentrations of 0, 0.1, 0.25, 0.5, 0.75, and 1.0 µg puromycin/ml culture medium were tested.
- WJ-MSCs were harvested by trypsinization and the cell number was determined with a CASY® cell analyzer.
- For each puromycin concentration, 1.9x10^4 cells were seeded in a well of a 12 well plate.
- The cells were incubated overnight at 37°C, 5% CO_2, and >95% humidity.
- On the next day, the old medium was aspirated and replaced with puromycin containing medium.
- The puromycin stock solution (1 mg/ml) was diluted 1:10 in NH Expansion medium to a final concentration of 100 µg/ml.
- The puromycin containing medium was prepared according to the following table:

µg Puromycin/ml	**µl Puromycin stock (100 µg/ml)**	**NH Expansion medium**
0	-	1.5 ml
0.1	1.5	1.5 ml
0.25	3.75	1.5 ml
0.5	7.5	1.5 ml
0.75	11.25	1.5 ml
1.0	1.5 µl with 1mg/ml	1.5 ml

- The viability of the cells was checked every day with a phase contrast microscope.

- After two days the old medium was aspirated and replaced with fresh puromycin containing medium.
- After 5 days of puromycin selection, the cells were fixed and stained with crystal violet.

2.1.21. Passaging of human osteoblasts

- Frozen human osteoblasts were thawed according to protocol 2.1.6. and were seeded in osteoblast growth medium with the recommended density of 10000 cells/cm².
- In subsequent passages, the old osteoblast growth medium was aspirated and the tissue culture flask was washed once with the appropriate volume of sterile DPBS (5 ml for T25 tissue culture flasks, 13 ml for T75, T175 tissue culture flasks).
- Cells were incubated with 1000 µl (T25 tissue culture flasks) or 2000 µl (T75, T175, and T182.5 tissue culture flasks) Trypsin/EDTA solution for 3-5 minutes at room temperature.
- For detachment of the cells, the tissue culture flask was gently tapped several times and detachment of the cells was checked with a phase-contrast microscope.
- Trypsinization was stopped with an appropriate amount of Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and penicillin/streptomycin (4 ml for T25, 8 ml for T75, T175 and T182.5 tissue culture flasks).
- The flask was rinsed several times with the cell suspension and the cell suspension was then transferred to a 50 ml falcon tube.
- From this cell suspension a 50 µl aliquot was taken for cell counting with a CASY cell analyzer (Roche-Innovatis, Germany).
- After cell counting the cell suspension was centrifuged at 1000 rpm (220xg) for 3 minutes
- The supernatant was aspirated and the cell pellet was resuspended in an appropriate amount of osteoblast growth medium and seeded in fresh tissue culture flasks with a density of 10000 – 20000 cells/cm².
- Optional: Human osteoblast were frozen in aliquots of 8.0x10^5 cells in CryoSure MSC freezing medium according to protocol 2.1.5.
- Cells were incubated at 37°C, 95% humidity and 5% CO_2 until they reached confluency (about 4 days) with complete medium change every 2-3 days.

2.1.22. Induction of mineralization in human osteoblasts

Mineralization of human osteoblasts was performed with osteoblast mineralization medium according to the manufacturer's instructions.

- Before mineralization human osteoblasts were cultured and trypsinized according to protocol 2.1.21.
- From this cell suspension the required amount of cells (e.g. 6.0x10^4 cells for a well of a 4 well plate) was transferred to a falcon tube and centrifuged at 220xg for 3 minutes.
- After centrifugation the supernatant was aspirated and the cells were resuspended in an appropriate volume of osteoblast growth medium.
- Human osteoblasts were seeded with a density of 30000 cells/cm² per well in a 4 well plate in osteoblast growth medium.
- The cells were incubated at 37°C, 95% humidity, and 5% CO_2.
- After two days, the osteoblast growth medium in two wells (of 4) was changed to osteoblast mineralization medium to induce mineralization. The osteoblasts in the other two wells were cultured in osteoblast growth medium as a control.
- A total medium change was performed twice a week and the cells were cultured for 21 days.

2.1.23. Osteogenic time-course differentiation of MSCs

- The expression of osteogenic marker genes was analyzed at 6 different time points during 30 days of osteogenic differentiation.
- Before osteogenic differentiation, WJ-MSCs were cultured and trypsinized according to protocol 2.1.4.
- From this cell suspension the required amounts of cells, 1.2x10^5 cells for 6 differentiation cultures and 1.2x10^5 cells for 6 control cultures, were transferred to falcon tubes and centrifuged at 220xg for 3 minutes.
- After centrifugation the supernatant was aspirated and the cells were suspended in an appropriate volume of NH OsteoDiff medium or NH Expansion medium (control).
- From each cell suspension, 2.0x10^4 cells were pipetted into the according wells of a 12 well plate.
- Osteogenic and control cultures were incubated at 37°C, 95% humidity, and 5% CO_2.
- A total medium change was performed twice a week and the cells were cultured for 30 days in osteogenic differentiation or control medium.
- Cell pellets from osteogenic/control cultures were prepared at 6 different time points during osteogenic differentiation.

- The old medium was aspirated and the cells were washed once with DPBS.
- Next, 500 µl of Trypsin/EDTA were added to each well and the cells were incubated for 10 min at 37°C. Detachment of the cells was checked after 10 minutes with a phase contrast microscope.
- Trypsinization was stopped with 500 µl DMEM with 10% FBS and the cell suspension was transferred to 1.5 ml microfuge tubes.
- The cells were centrifuged at 2000 rpm for 5 minutes, the supernatant was aspirated, and the cell pellet was washed with 1000 µl DPBS.
- After centrifugation at 2000 rpm for 5 minutes, the supernatant was aspirated and the cell pellet was snap-frozen in liquid nitrogen.
- The frozen cell pellet was stored at -80°C.

2.1.24. Cell culture materials

Commercial Cells:

Human osteoblasts: Promocell #C-12720
hMSC-UC: (human MSCs from umbilical cord matrix) Promocell #C-12971
hMSC-BM: (human MSCs from bone marrow) Promocell #C-12974

Media and solutions:

DPBS: DPBS (1x) no calcium, no magnesium: Invitrogen #14190-094
DMEM high glucose: 50U/mL penicillin, 50ug/mL streptomycin (PAA #P11-010, 100x), 10% FBS (PAA #A15-108), DMEM (1x) + GlutaMAX™-I (Invitrogen #31966-021)
Trypsin/EDTA solution 1x: Sigma #T3924
Penicillin/Streptomycin (100x): PAA #P11-010
CryoSure® MSC freezing Medium: WAK – Chemie Medical GmbH (product sample)
NH Expansion Medium: Miltenyi Biotec #130-091-680
CytoMix – MSC, human: Miltenyi Biotec #130-093-552
NH AdipDiff Medium: Miltenyi Biotec #130-091-677
NH ChondroDiff Medium: Miltenyi Biotec #130-091-679
NH OsteoDiff Medium: Miltenyi Biotec #130-091-678
Osteblast Growth Medium: Promocell #C-27010
Osteoblast Mineralization Medium: Promocell #C-27020
MACS buffer: autoMACS® Rinsing Solution (Miltenyi Biotec# 130-091-222) with 0.5% BSA (MACS® BSA Stock Solution (20x) Miltenyi Biotec # 130-091-376)
BSA: MACS® BSA Stock Solution (20x) Miltenyi Biotec # 130-091-376
Fetal Bovine Serum: PAA #A15-108
Opti-MEM®: Invitrogen #31985
FuGENE® 6: Roche Applied Biosciences #11815091001
CryoSure-DMSO: WAK – Chemie Medical GmbH #WAK-DMSO-10
Puromycin: Sigma #P8833
Polybrene®: Santa Cruz Biotechnology #sc-134220

Antibodies:

CD90-FITC: 50 µg/ml BioLegend #328107
CD105-FITC: 100 µg/ml BioLegend #323204
CD44-PE: BD Pharmingen #555446
CD34-FITC: Immunotech #IM1870
CD45-FITC: BD Pharmingen #555482
CD73-PE: 100 µg/ml BioLegend #344004
ITC IgG6-FITC: BD Pharmingen #555742
ITC IgG6-PE: 0.2 mg/ml BioLegend #400112

Plasmids:

psPAX2: Addgene #12260
pMD2.G: Addgene #12259
pGreenFire1-mCMV Plasmid (pTRH1 mCMV dscGFP T2A Fluc, negative control)+ EF1-Puro: System Biosciences # TR010PA-P

2.2. Molecular Cloning

2.2.1. Plasmid Midipreps

For the extraction of plasmid DNA from 100 ml overnight cultures of transformed bacteria the Wizard® *Plus* Midipreps DNA purification system was used.

- About 90 ml of LB culture were transferred to 500 ml Sorvall centrifuge bottles and the bacteria were harvested at 5000xg for 10 minutes in a Sorvall RC5C centrifuge in a GSA rotor.
- The supernatant was discarded into the bacterial waste and the bacterial pellet was resuspended in 3 ml Cell Resuspension Solution.
- To lyse the cells, 3 ml Cell Lysis Solution was added, thoroughly mixed without shaking and incubated for 3 minutes at room temperature.
- Next, 5 ml Neutralization Solution were added, thoroughly mixed without shaking and incubated for 3 minutes at room temperature.
- Precipitated proteins, RNA, and genomic DNA, were pelleted by centrifugation at 15000xg for 10 minutes in a Sorvall RC5C centrifuge with a GSA rotor.
- In the meantime a blue clearing column was attached on a white binding column and the column assembly was attached to a vacuum station.
- After centrifugation, the supernatant was applied on the blue clearing column and the complete solution was drawn through both columns by applying vacuum. The blue clearing column was discarded.
- Vacuum was applied and 5 ml Endotoxin Removal Wash was drawn through the white binding column. The vacuum was released after the Endotoxin Removal Wash had passed through the column.
- Next, 20 ml Column Wash Solution was applied on the white binding column, vacuum was applied and the solution was drawn through the column. The vacuum was released after the Column Wash Solution had passed the column.
- The binding column was transferred to a 50 ml falcon tube and 5 ml Buffer QF elution buffer was applied on the column.
- The column was centrifuged at 2000xg for 5 minutes in a Hettich - Rotanta TRC centrifuge with a swing-out rotor.
- For precipitation of the plasmid DNA, the QF buffer was transferred to Schott-Duran glass centrifuge tubes and mixed with 3.5 ml room temperature isopropyl alcohol.
- This solution was centrifuged at 15000xg for 30 minutes at 4°C in a Sorvall RC5C centrifuge in a HB-4 swing-out rotor. Plastic adapters were used to fit the glass tubes into the HB-4 centrifuge buckets.

- After centrifugation, the supernatant was carefully decanted and 2 ml of 70% ethanol (room temperature) were added to the DNA pellet.
- The DNA pellet was centrifuged at 15000xg for 10 minutes at room temperature.
- The supernatant was carefully decanted and the DNA pellet was air-dried in a fume hood.
- The plasmid DNA was dissolved in 300-600 µl nuclease free water (for large plasmids > 7 kb: pre-warmed to 60°C) for 10 min at room temperature.
- The plasmid DNA solution was then transferred to a 1.5 ml microfuge tube, the DNA concentration was determined on a Nanodrop spectrophotometer, and stored at -20°C.

2.2.2. Plasmid Minipreps

Overnight cultures (with 4 ml LB AMP medium) were inoculated with bacterial colonies of transformed bacteria. The plasmid DNA from these overnight cultures was extracted with the Wizard® *Plus* SV Minipreps DNA purification system according to the manufacturer's instructions.

- Two milliliters of the overnight culture was transferred to a 2 ml microfuge tube and the bacteria were harvested by centrifugation at 1700xg for 5 minutes in an Eppendorf MiniSpin centrifuge.
- The supernatant was aspirated with a pump and the cell pellet was resuspended in 250 µl Cell Resuspension Solution.
- For cell lysis, 250 µl Cell Lysis Solution was added and the lysate was mixed by inverting the tube 4 times.
- Next, 10 µl alkaline protease solution was added, the solution was mixed by inverting the tube 4 times, and incubated for 5 minutes at room temperature.
- The solution was neutralized by the addition of 350 µl Neutralization Solution and mixed by inverting the tube several times.
- Precipitated proteins, RNA, and genomic DNA, were pelleted by centrifugation at 12100xg for 10 minutes.
- In the meantime, vacuum adapters were attached to the manifold port of the vacuum station and labeled spin columns (binding column) were inserted into the adapters.
- The clear supernatant was transferred onto the spin column and vacuum was applied until all liquid passed through the column.
- The vacuum was released, 750 µl Column Wash Solution was added onto the column and vacuum was applied until all liquid passed through the column.

- The vacuum was released and the previous step was repeated with 250 µl Column Wash Solution and vacuum was applied for 10 minutes.
- Remaining amounts of ethanol were removed by centrifugation. Therefore, the spin column was transferred to a 2 ml collection tube and centrifuged at >12100xg for 2 minutes.
- The spin column was transferred to a clean 1.5 ml microfuge tube and 50 - 100 µl of nuclease free water was added onto the column.
- After one minute incubation, the spin column was centrifuged at 12100xg for one minute.
- The DNA concentration was determined on a Nanodrop spectrophotometer and the plasmid DNA solution was stored at -20°C.

2.2.3. Transformation of E. coli JM109 competent cells

For transformation of the ligation reaction, E. coli JM109 high efficiency chemical competent (>10^8 CFU/µg) cells were used.

- Sterile 1.5 ml microfuge tubes were chilled on ice.
- Frozen competent cells were thawed on wet ice until just thawed.
- The thawed competent cells were gently mixed by flicking the tube several times and 50 µl of competent cells were transferred to a chilled 1.5 microfuge tube.
- OPTIONAL: Remaining competent cells were frozen in an isopropyl alcohol/ dry ice bath for 5 minutes and stored at -80°C
- To 50 µl competent cells, 1 to 5 µl ligation reaction was added; the tube was flicked several times, and immediately returned on ice. For the transformation control, 1 µl (0.1 ng) competent cells control DNA was added to 50 µl competent cells.
- After 10 minutes incubation on ice, the tubes were transferred to a 42°C water bath or a 42°C Eppendorf ThermoStat plus for 45 to 50 seconds to heat-shock the competent cells.
- The tubes were immediately placed on ice for another 2 minutes.
- For recovery, 950 µl cold (4°C) SOC medium was added to the competent cells and the cells were incubated for 60 minutes at 37°C in a Thermomixer R shaking at 400 rpm.
- From this transformation reaction 100 µl were plated out on LB agar plates which contained 50 µg/ml ampicillin.
- Another 300 µl of transformation reaction was transferred to a 1.5 ml microfuge tube, cells were pelleted at 1700xg for 5 minutes, the cell pellet was resuspended in 100 µl SOC medium and cells were plated out on LB agar plates with 50 µg/ml ampicillin.

- For the control, 100 µl of a 1:10 dilution in SOC medium was prepared and plated out on LB plates supplemented with 50 µg/ml ampicillin.
- The remaining transformation reactions were stored at 4°C and could be used to plate out additional cells on the next day.
- LB plates were incubated at 37°C overnight.

2.2.4. Isolation of DNA from agarose gels

For the isolation of PCR fragments and digested vectors from agarose gels, the Wizard® SV Gel and PCR Clean-Up System was used.

- After electrophoresis, a picture of the gel was taken and the gel was transferred onto an UV transluminator.
- With a clean, sharp scalpel, the desired band was cut from the gel and transferred to a weighted microfuge tube.
- The weight of the gel piece was determined and per 10 mg of gel, 10 µl of MEM binding solution was added, e.g. 120 µl MEM binding solution was added to a 120 mg gel slice.
- The gel piece was incubated in MEM binding solution at 55°C until the gel piece was completely dissolved.
- In the meantime, the spin column was labeled and inserted onto a 2 ml collection tube.
- The solution was mixed by vortexing, drops were collected by brief centrifugation and up to 700 µl of the mixture was transferred onto a spin column. If the volume exceeded 700 µl the remaining solution was added onto the same column after a first centrifugation step.
- The column with the collection tube was transferred to an Eppendorf centrifuge 5417 R and centrifuged at 16100xg for 1 minute at room temperature.
- The flow-through was discarded and 700 µl column wash solution was added onto the spin column.
- The spin column was centrifuged at 16100xg for 1 minute at room temperature, the flow-through was discarded and 500 µl of column wash solution was added onto the spin column.
- The spin column was centrifuged at 16100xg for 5 minutes to dry the column and the flow-through was discarded.
- To remove any remaining ethanol the spin column was reinserted into the empty collection tube and centrifuge at 16100xg for 1 minute without the rotor lid.
- The spin column was transferred to a clean 1.5 ml microfuge tube and 30-50 µl nuclease free water was added onto the spin column.

- After 1 minute of incubation at room temperature the column assembly was transferred to the centrifuge and centrifuged at 16100xg for 1 minute.
- The spin column was discarded and the eluted DNA was stored at -20°C.

2.2.5. Optimized ligation protocol

The vector:insert ratio for ligation of promoter fragments into pGreenFire vectors was 1:1, 1:2, or 1:3. The required amount of insert was calculated by using the following formula:

$$\frac{ng\ vector * size\ of\ insert\ in\ bp}{size\ of\ vector\ in\ bp} * molar\ ratio\ of\ insert{:}\ vector = ng\ insert$$

- Ligation reactions in 10 µl volume were prepared according to the following table:

Component	**Volume**
10x T4 Ligase Buffer	1 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Insert (x ng)	x µl
Optional: 5% PEG 6000	2.08 µl
Water to 10 µl	x µl
Total	10 µl

- Ligation reactions in 20 µl volume were prepared according to the following table:

Component	**Volume**
10x T4 Ligase Buffer	2 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Insert (x ng)	x µl
Optional: 5% PEG 6000	4.17 µl
Water to 20 µl	x µl
Total	20 µl

- Control ligations (Vector only controls) in 10 µl volume were prepared according to the following table:

Component	Volume
10x T4 Ligase Buffer	1 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Optional: 5% PEG 6000	2.08 µl
Water to 10 µl	x µl
Total	10 µl

- Control ligations in 20 µl volume were prepared according to the following table:

Component	Volume
10x T4 Ligase Buffer	2 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Optional: 5% PEG 6000	4.17 µl
Water to 20 µl	x µl
Total	20 µl

- The prepared ligation reactions were mixed by gently flicking the tube, centrifuged and incubated at room temperature between 1 (with PEG 6000) and 3.5 (no PEG 6000) hours.
- To facilitate the circularization of the vector, the volume of the reaction was increased to 250 µl and the following components were added:
    - 24 µl 10x T4 Ligation Buffer
    - 2 µl T4 DNA Ligase
    - 214 µl nuclease free water
- The ligation reaction was mixed by flicking the tube several times, centrifuged and split to two 200 µl PCR reaction tubes.
- The PCR reaction tubes were placed in a PTC-200 peltier thermocycler (MJ Research) and incubated overnight at 15°C.
- On the next day, the ligation reactions were combined in one 1.5 ml microfuge tube.
- The ligated vectors were precipitated by addition of the following components:
    - 25 µl 3M $NaCH_3COO$
    - 1 µl 20mg/ml glycogen
    - 550 µl 70% ethanol (4°C)
- The solution was mixed by flicking the tube several times, briefly centrifuged and incubated at -20°C for 30 minutes.

- After incubation the microfuge tubes were centrifuged at 20800xg for 10 minutes at 4°C to pellet the vector DNA.
- The supernatant was aspirated, 200 µl 70% ethanol (4°C) was added to the DNA pellet and the microfuge tubes were again centrifuged with 20800xg for 10 minutes at 4°C.
- The previous washing step was repeated.
- After the last washing step, the supernatant was removed and the DNA pellet was dried in a vacuum concentrator at 30°C until remnants of ethanol were completely removed.
- The dried DNA pellet was dissolved in 10 µl nuclease free water.

2.2.6. Restriction digests of PCR products and plasmids

For restriction digests, enzymes from New England Biolabs were used in the recommended restriction enzyme buffer for single and double digest. If a double digest was not possible, sequential digests were performed.

- Preparative restriction digests in 50 µl volume were prepared according to the following table:

Component	**Volume**
Enzyme 1	1 µl
Enzyme 2	1 µl
Vector/Insert DNA	x µl
10x NEBuffer 1,2,3,4, or EcoRI Buffer	5 µ
10x BSA (if required)	5 µl
Water to 50 µl	
Total	50 µl

- Preparative restriction digests in 20 µl volume were prepared according to the following table:

Component	**Volume**
Enzyme 1	1 µl
Enzyme 2	1 µl
Vector/Insert DNA	x µl
10x NEBuffer 1,2,3,4, or EcoRI Buffer	2 µ
10x BSA (if required)	2 µl
Water to 20 µl	
Total	20 µl

- Control restriction digest plasmids were prepared according to the following table:

Component	Volume
Enzyme 1	0.2 µl
Enzyme 2	0.2 µl
Plasmid DNA	X µl
10x NEBuffer 1,2,3,4, or EcoRI Buffer	1 µl
10x BSA (if required)	1 µl
Water to 10 µl	x µl
Total	10 µl

- The prepared restriction digest was mixed by gently flicking the tube several times and drops were collected by brief centrifugation.
- The restriction digest was incubated at the recommended temperature for at least 2 hours or overnight and analyzed/purified by gel electrophoresis according to protocols 2.2.4./2.2.7.

2.2.7. Agarose gel electrophoresis

Agarose gel electrophoresis was used to check restriction digests, screen plasmids for the correct promoter fragment or purification of PCR products and vectors.

- Low melt agarose was weighted-out in an Erlenmeyer flask to achieve a final concentration of about 0.7-1 % agarose in the desired volume of TAE buffer.
- For small gels, about 65 ml and for large gels about 160 ml TAE buffer was used.
- A magnetic stir bar was added to the Erlenmeyer flask and the solution was boiled in a microwave oven until the agarose was completely melted.
- The Erlenmeyer flask was placed on a magnetic stirrer until the solution reached pouring temperature (approx. 60°C).
- In the meantime, an appropriate mold with a comb was prepared.
- Right before the gel was poured, GelRed nucleic acid stain was diluted 1:10000 in the gel solution.
- The gel was poured into the mold and was allowed to solidify at room temperature.
- The comb was removed from the solid gel and it was inserted into the electrophoresis chamber and, if necessary, TAE buffer was added until the gel was completely covered with buffer.
- The pockets of the gel were flushed with TAE buffer before the samples were loaded.

- One fifth of the sample volume of 6x loading dye was added to each sample, mixed by pipetting, and pipetted into the pockets of the gel. Next, 5 µl Thermo Scientific MassRuler DNA Ladder Mix were pipetted into one pocket of the gel.
- The lid of the electrophoresis chamber was closed and the electrical leads were connected to the chamber.
- Depending on the size of the gel, the gel was run with voltages between 60 V and 180 V. Small gels were run with 60-80 V for 45-60 minutes depending on the sample. Large gels were run with 120-180Vfor 45-60 minutes depending on the sample.
- After electrophoresis, a picture of the gel was taken in an EpiChemi II Darkroom equipped with a Benchtop UV transilluminator and a CCD camera. As imaging software, LabWorks 4.5 was used.

2.2.8. Dephosphorylation of vector DNA

Vectors were dephosphorylated after the restriction digest to prevent self-ligation.

- After the restriction digest the following components were added to the restriction digest:
    - One tenth of the final volume of 10x dephosphorylation buffer
    - 1 µl alkaline phosphatase (shrimp)
- The reaction mixture was mixed by flicking the tube several times and drops were collected by brief centrifugation.
- The reaction was incubated for 30 minutes at 37°C in an Eppendorf ThermoStat plus.
- After 30 minutes incubation, another 1 µl of alkaline phosphatase (shrimp) was added and the reaction mixture was mixed by flicking the tube several times.
- The drops were collected by brief centrifugation and the reaction was incubated for another 30 minutes at 37°C.
- Alkaline phosphatase (shrimp) was heat inactivated for 15 minutes at 65°C.
- After the heat inactivation the tubes were chilled on ice and drops were collected by brief centrifugation.
- The dephosphorylated vector was purified by agarose gel electrophoresis.

2.2.9. A-tailing of blunt-ended PCR products for T-vector cloning

For the ligation of blunt-ended PCR products into the pGEM®T Easy vector, it was necessary to add A (adenine) overhangs to the PCR products.

- The A-tailing reaction was prepared according to the following table in the indicated order:

Component	Volume
Purified PCR product	5 µl
Taq DNA Polymerase 10x reaction buffer without $MgCl_2$	1 µl
2 mM dATP (final conc. 0.2 mM)	1 µl
Taq DNA Polymerase (5 units)	1 µl
50 mM $MgCl_2$ (final conc. 3 mM)	0.6 µl
Nuclease free water	1.4 µl
Total	10 µl

- The prepared reaction was mixed by flicking the tube several times and drops were collected by brief centrifugation.
- The reaction mixture was incubated at 70°C for 30 minutes in an Eppendorf ThermoStat plus.
- After the incubation the microfuge tube was chilled on ice and drops were collected by brief centrifugation.
- The A-tailed PCR products were subsequently used for T-vector cloning. A-tailed PCR products are not suited for long-term storage.

2.2.10. T-vector cloning

- The ligation reaction was prepared according to the following table in the indicated order:

Component	Volume
2x Rapid Ligation Buffer	5 µl
pGEM®T Easy vector (50 ng)	1 µl
A-tailing reaction	2 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Nuclease free water	1 µl
Total	10 µl

- Rapid Ligation Buffer (2x) was vortexed before each use.
- As control, 2 µl of the provided control insert DNA were used instead of the A-tailing reaction.
- The reaction was mixed by gently flicking the tube several times and drops were collected by brief centrifugation.
- The ligation reaction was incubated for one hour at room temperature and 2 µl were used for the transformation of competent cells.

2.2.11. Standard ligation protocol

The vector:insert ratio for ligation of promoter fragments into pGreenFire vectors was 1:1, 1:2, or 1:3. The required amount of insert was calculated by the following formula:

$$\frac{ng\ vector * size\ of\ insert\ in\ bp}{size\ of\ vector\ in\ bp} * molar\ ratio\ of\ insert{:}\ vector = ng\ insert$$

- Ligation reactions in 10 µl volume were prepared according to the following table:

Component	**Volume**
10x T4 Ligase Buffer	1 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Insert (x ng)	x µl
Water to 10 µl	x µl
Total	10 µl

- Ligation reactions in 20 µl volume were prepared according to the following table:

Component	**Volume**
10x T4 Ligase Buffer	2 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Insert (x ng)	x µl
Water to 20 µl	x µl
Total	20 µl

- Control ligations in 10 µl volume were prepared according to the following table:

Component	Volume
10x T4 Ligase Buffer	1 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Water to 10 µl	x µl
Total	10 µl

- Control ligations in 20 µl volume were prepared according to the following table:

Component	Volume
10x T4 Ligase Buffer	2 µl
1:10 dilution of T4 Ligase	1 µl
Vector (100 ng)	x µl
Water to 20 µl	x µl
Total	20 µl

- The prepared ligation reactions were mixed by gently flicking the tube, centrifuged and incubated between 2 and 4 hours or overnight at room temperature.
- 1 or 2 µl of the ligation reaction was used for the transformation of competent cells.

2.2.12. Preparation of LB medium and LB agar

- First, 25g of LB Broth (high salt) were weighed out in a 1L borosilicate bottle and dissolved in 1L M.Q. water with a magnetic stirrer.
- When the powder was completely dissolved, the LB medium was split to four 500 ml borosilicate bottles (250 ml each).
- Optional: For the preparation of 250 mL LB agar, 3,75g of agar-agar was weighed out in 500 ml borosilicate bottles and mixed with 250 ml LB medium.
- Next, the bottles were autoclaved at 121°C for 15 minutes.
- Optional: The autoclaved LB medium and LB agar can be stored at room temperature for several weeks (ampicillin/other antibiotics are added before use). LB agar was melted in a microwave oven and was allowed to cool too approximately <60°C before ampicillin (or another antibiotic) was added.
- After the LB medium and LB agar was autoclaved, the bottles were allowed to cool to about 45-55°C and the appropriate amount of ampicillin stock was added. The final ampicillin concentration was 50 µg/ml for LB medium and LB agar.

- The melted LB agar was subsequently used for pouring of LB AMP plates (15-20 mL LB agar/plate). The plates were stored in the fridge at 4°C until use (for a maximum of 4 weeks).

2.2.13. Cloning materials

Kits:

Wizard® SV Gel and PCR Clean-Up System: Promega # A9281
Wizard® Plus SV Minipreps DNA Pruification Systems: Promega # A1470
Wizard® Plus SV Midipreps DNA Purification Systems: Promega # A7640

Vectors:

pGEM®-T Easy Vector System I: Promega # A1360
pGEM®4Z Vector: Promega # P2161
pGreenFire™ Pathway Reporter Lentivector: System Biosciences #TR0XX Series

Ligase and Buffers:

T4 DNA Ligase: New England Biolabs # M0202L
10x T4 Ligase reaction buffer: New England Biolabs # B0202S
24% (w/v) polyethylene glycol 6000: Thermo Scientific
NEBuffer 1: New England Biolabs # B7001S
NEBuffer 2: New England Biolabs # B7002S
NEBuffer 3: New England Biolabs # B7003S
NEBuffer 4: New England Biolabs # B7004S
NEBuffer EcoRI: New England Biolabs # B0101S
BSA: New England Biolabs # B9001S
Taq DNA Polymerase, recombinant: Invitrogen # 10342-020, 10x PCR buffer and 50 mM $MgCl_2$ included

Restriction enzymes:

BamHI: New England Biolabs # R0136S
HindIII: New England Biolabs # R0104S
EcoRI: New England Biolabs # R0101S
SpeI: New England Biolabs # R0133S
ClaI: New England Biolabs # R0197S
MfeI: New England Biolabs # R0589S
BclI: New England Biolabs # R0160S
NotI: New England Biolabs #R0189S

Miscellaneous materials:

GelRed™ Nucleic Acid Gel Stain: Biotium #41002
MassRuler DNA Ladder Mix: Thermo Scientific #SM0403
6x DNA loading dye (Fermentas): Thermo Scientific # R0611
E. coli JM109: Promega # P9751
LB Broth (high salt): Lab M Limited # LAB191
Agar-Agar: Carl Roth # 5210.3
LE Agarose: Biozym # 840004
Buffer QF: Qiagen # 19056
Ampicillin stock: 50mg ampicillin (Sigma # A0166) per ml
Alkaline phosphatase (shrimp): Roche Applied Science # 11 758 250 001, 10x Dephosphorylation buffer included

2.3. PCR and Q-PCR

2.3.1. RNA isolation from MSCs

Total RNA was isolated from MSCs with the Qiagen RNeasy Mini Kit to analyze the expression of osteogenic marker genes by q-PCR. RNA was isolated from frozen cell pellets which were stored at -80°C.

- Cell pellets were thawed on wet ice and the tubes were flicked several times to disrupt the cell pellet.
- To lyse the cells, 350 µl Buffer RLT with 2-mercaptoethanol was added to the cell pellet and the solution was homogenized by passing the liquid through a 22G (0,7x30 mm) needle-10 times.
- One volume of 70% ethanol was added to the sample, mixed by pipetting and 700 µl of this solution were transferred onto an RNeasy Column.
- The lid of the RNeasy column was gently closed and the column was centrifuged for 15 seconds at >8000xg.
- The flow-through was discarded; the column was re-inserted into the collection tube and 700 µl Buffer RW1 was added onto the column.
- The lid of the RNeasy column was gently closed and the column was centrifuged for 15 seconds at >8000xg.
- The flow-through was discarded; the column was re-inserted into the collection tube and 500 µl Buffer RPE was added onto the column.
- The lid of the RNeasy column was gently closed and the column was centrifuged for 15 seconds at >8000xg.
- The flow-through was discarded; the column was re-inserted into the collection tube and 500 µl Buffer RPE was added onto the column.
- The lid of the RNeasy column was gently closed and the column was centrifuged for 2 minutes at >8000xg to dry the column.
- The RNeasy column was transferred to a new 2 ml collection tube and centrifuged at 20800xg for one minute to completely remove last amounts of ethanol and Buffer RPE.
- After the last step, the column was transferred to a 1.5 ml microfuge tube and 30-50 µl nuclease free water was directly added onto the column.
- The column was centrifuged at >8000xg for one minute to elute the RNA. The RNeasy column was discarded and the RNA was stored at -80°C.

2.3.2. cDNA synthesis from isolated RNA

To analyze the expression levels of different osteogenic marker genes in differentiated MSCs, it is necessary to reverse-transcribe the mRNA into cDNA. For this procedure, the Thermo Scientific First Strand cDNA Synthesis Kit was used.

- For cDNA synthesis, 100 to 500 ng of RNA can be used. If enough RNA was isolated from the samples, 250 ng total RNA was used, otherwise cDNA synthesis was performed with 100 ng total RNA.
- OPTIONAL: If the RNA concentration was so low that the volume for 100 ng or 250 ng of RNA exceeded 10 µl, the RNA sample was concentrated in a vacuum concentrator (SpeedVac).
- Therefore, the microfuge tube containing the RNA sample was placed in the vacuum concentrator and the solution was either reduced to a smaller volume or completely vaporized. The remaining RNA pellet was then dissolved in an appropriate volume of RNase free water.
- After the concentration step the new RNA concentration was determined on a Nanodrop® spectrophotometer.
- The reaction mixture was prepared according to the following scheme in a 1.5 ml microfuge tube:

Component	**Volume**
250 or 100 ng RNA	x µl
Random Hexamer Primer	1 µl
DEPC treated water	to 11 µl
Total volume	11 µl

- The prepared reaction mixture was mixed by flicking the tube several times and drops were collected by brief centrifugation.
- The reaction mixture was incubated at 70°C for 5 minutes, the microfuge tube was chilled on wet ice, and drops were collected by brief centrifugation.
- The microfuge tube was placed on ice and the following components were added in the indicated order:

Component	**Volume**
5x Reaction Buffer	4 µl
RNase Inhibitor	1 µl
dNTP mix	2 µl
Total volume	18 µl

- The reaction mixture was mixed by flicking the tube several times and drops were collected by brief centrifugation.

- The reaction mixture was incubated at room temperature for 5 minutes.
- Next, 2 µl of M-MuLV Reverse Transcriptase were added to the reaction mixture.
- The solution was mixed by gently flicking the tubes several times, drops were collected by brief centrifugation and the mixture was incubated at room temperature for 10 minutes.
- The reaction mixture was transferred to an Eppendorf ThermoStat plus and incubated for 60 minutes at 37°C.
- The reaction was stopped by heat inactivation at 70°C for 10 minutes.
- The reaction mixture was chilled on ice and drops were collected by brief centrifugation.
- The prepared cDNA solution was diluted with 40 µl nuclease free water (for 250 ng RNA) or 20 µl nuclease free water (for 100 ng RNA) and stored at -20°C.

2.3.3. TaqMan® real-time PCR

TaqMan® real-time PCR was used to analyze the expression of osteogenic marker mRNAs during osteogenic differentiation of MSCs.

- For each osteogenic marker, a TaqMan® master mix was prepared according to the following scheme:

Component	**Volume per well**
2x TaqMan® Fast Master Mix	5 µl
TaqMan® probe	0.5 µl
Water	2.5 µl
Total	8 µl

- The required volume of master mix was calculated by multiplying the volume per well with the number of wells plus two.
- The calculated volumes were pipetted into 1.5 ml microfuge tubes, briefly vortexed, centrifuged and stored on ice until use.
- The TaqMan® real-time PCR was performed in duplicates in 96 well-plates in a total reaction volume of 10 µl. To each well, 8 µl of the according master mix and 2 µl of sample cDNA were added.
- The 96 well-plate was sealed and briefly centrifuged before analysis.

- The following conditions were used to perform the RT PCR on an Applied Biosystems 7500 Fast Real-Time PCR system:

Thermal Cycler Profile				
Stage	Repetitions	Temperature	Time (sec)	Ramp Rate
1	1	95.0 °C	00:20	Auto
2	40	95.0 °C	00:03	Auto
		60.0 °C	00:30	Auto

2.3.4 Advantage®-GC 2 PCR Kit

Since it was not possible to amplify the promoter regions of COL1A1, SPP1, and ALPL with the standard PCR protocol (2.3.6.), the Advantage®-GC 2 PCR Kit (Clontech) was used for the amplification. The promoter regions of these genes contain GC rich regions which are difficult to amplify with standard PCR protocols.

- The components of the Advantage®-GC 2 PCR Kit (Clontech) were thawed at room temperature, briefly vortexed and centrifuged before use.
- PCR reactions were prepared according to the following scheme:

Component	**Amount in µl**
PCR-grade water	30
5x GC 2 PCR Buffer	10
GC Melt (5M)	5
DNA template (70 ng/µl)	1
FW Primer (10 µM)	1
RW Primer (10 µM)	1
50x dNTP mix (10 mM each)	1
Advantage GC 2 Pol. Mix (50x)	1
Total volume	50

- The prepared reaction mixtures were mixed by flicking the tube several times and drops were collected by brief centrifugation.

- The following basic PCR program was used for the amplification of promoter fragments:

Step 1	94°C for 3 min	1 Cycle
Step 2	94°C for 30 sec	35 Cycles
	X°C for 30 sec (annealing)	
	68°C for 3 min (extension)	
Step 3	68°C for 3 min	1 Cycle
Step 4	8°C forever	

- NOTE: If possible, a two-step PCR protocol was used, with annealing and extension at 68°C for 3 minutes. If the annealing temperatures of primers were lower than 68°C, a three step protocol was used instead. The optimum annealing temperature for these primers was experimentally determined and set accordingly.

2.3.5. Single-colony PCR with JumpStart™ REDTaq® ReadyMix™

Single-colony PCR was used to screen large numbers of colonies from transformed bacteria for the correct plasmid.

- For the screening of bacterial colonies with PCR, a single-colony was picked with a yellow tip and streaked on a LB AMP plate to maintain the colonies for DNA preparation. The plate was incubated overnight at 37°C.
- The streak was marked on the back of the plate and the rest of the colony was suspended in 50 µl PCR grade water by pipetting up and down several times.
- The bacteria were lysed by heating the microfuge tube to 95°C for 5 minutes.
- The chilled microfuge tubes were centrifuged at 20800g for 1 min to pellet cell debris and 5 µl of the supernatant were used as PCR template.
- PCR reactions were prepared according to the following scheme:

Component	**Amount in µl**
2x JumpStart REDTaq ReadyMix	10
FW Primer 10 µM	1
RW Primer 10 µM	1
Bacterial supernatant	5
PCR grade water	3
Total volume	20

- The prepared reaction mixture was mixed by flicking the tube several times and drops were collected by brief centrifugation.
- The following PCR program was used for single-colony PCR screening:

Step 1	95°C for 3 min	1 Cycle
Step 2	95°C for 30 sec	30 Cycles
	60°C for 30 sec (Annealing)	
	72°C for 2 min (Extension)	
Step 3	72°C for 5 min	1 Cycle
Step 4	4°C forever	

2.3.6. Polymerase Chain Reaction (PCR) with Pfu Polymerase

The initial PCR reactions for the amplification of osteogenic promoter regions were performed with Pfu Polymerase.

- Reaction components were thawed on ice (if necessary), briefly vortexed and briefly centrifuged before use.
- PCR reactions were prepared according to the following scheme:

Component	**Amount in µl**
PCR grade Water	36.5
10x Pfu Buffer with $MgSO_4$	5
FW Primer 10 µM	1
RW Primer 10 µM	1
Genomic DNA 100ng/µl	1
Pfu Polymerase	0.5
dNTPs 2 mM	5
Total volume	50

- The prepared reaction mixture was mixed by flicking the tube several times and drops were collected by brief centrifugation.

- The following basic PCR program was used but annealing temperatures were changed according to the Tm temperatures of the primers for each promoter fragment:

Step 1	95°C for 3 min	1 Cycle
Step 2	95°C for 90 sec	40 Cycles
	60°C for 90 sec (Annealing)	
	72°C for 3 min (Extension)	
Step 3	72°C for 5 min	1 Cylce
Step 4	4°C forever	

2.3.7. PCR and RT-PCR materials

TaqMan RT-PCR probes:

ALPL FAM: Applied Biosystems #Hs00758162_m1
Runx2 FAM: Applied Biosystems #Hs00231692_m1
BGLAP FAM: Applied Biosystems #Hs01587813_g1
SP7 FAM: Applied Biosystems #Hs01866874_s1
Col1a1 FAM: Applied Biosystems #Hs00164004_m1
SPP1 FAM: Applied Biosystems #Hs00167093_m1

Kits:

Advantage® GC 2 PCR Kit: Clontech #639119
RNeasy Mini Kit: Qiagen # 74106
First Strand cDNA Synthesis Kit: Thermo Scientific # K1612

PCR primers:

PCR primers were designed using the Primer-BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were ordered from Sigma Life Sciences.

Primer name	**Primer sequence 5' to 3'**
AP fwd P1	AGT GCT GGG TGC TTT CAC CTG C
AP rev P1	GGT CCG TCC AGG CAC AAG CG
AP fwd P2	CCGG CAA TTG AAA AGG GAA CCT ATG TCA CGC C
AP rev P2	GGCC TGA TCA GAG TGG CGG GAG CGC AA
SPP1 fwd P1	CCGG CAA TTG GCA AAA GGA AGC TGA CAC TTT AGG AC
SPP1 rev P1	TGG CTG AGA AGG CTG CAA CTG G
SPP1 rev P2	GGCC TGA TCA CTC CTC CTG CTG CTG CTG ACA AC
COL1A1_NEU_f_P1	AAG ACA CAT CTT CAG CCT GGG CAC C
COL1A1_NEU_r_P1	TCA TCC ACG TCT CGT TTT AAG CCG C
COL1A1_NEU_f_P2	AGAGAG ATCGAT TTAAGTCGAAGAGTGGCAGGGGAGG
COL1A1_NEU_r_P2	GAGAGA ACTAGT GTAGACTCTTTGTGGCTGGGGAGGG
CMV fwd	CCGG GAA TTC ATA GTA ATC AAT TAC GGG GTC
CMV NEU rev	GGCC GGA TCC GCT AGC GGA TCT GAC GG
Osteocalcin fwd P1:	GGC AGG ATG GGT GCT TCC CG
Osteocalcin rev P1:	GCG ATG CAA AGT GCG GCC AG
Osteocalcin fwd P2:	CCGG GAA TTC GGG GTT GGG CTG GTA TGG GG
Osteocalcin rev P2:	GGCC GGA TCC CAT GGT GTC TCG GTG GCT GCG
Osterix: fwd P1:	CGC CAC CCT CAC TCC TGC TTG
Osterix rev P1:	TCC ACT GCC CTC TGA CCC CTG
Osterix fwd P2:	CCGG GAA TTC GGC ACT GGG AGG AAG ACT GAG GG
Osterix rev P2:	CCGG ACT AGT AGG AGG TGG GTG GGC AAG GAC

Sequencing Primer:

The sequencing primers for the pGreenFire reporter constructs were designed using the Primer-BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized by Microsynth Austria for sequencing.

Primer name	Primer sequence 5' to 3'	Primer start
CMV FW	ACAGTGCAGGGGAAAGAATAGT	1833
Osterix/SP7 FW	ACAGTGCAGGGGAAAGAATAGT	1833
Osterix/SP7 RW	TCGGTCCCGGTGTCTTCTAT	3428
Osteocalcin/BGLAP FW	ACAGTGCAGGGGAAAGAATAGT	1833
Osteocalcin/BGLAP RW	CGCCTTTGGTGCTCTTCATC	3425
SPP1 FW	ACAGTGCAGGGGAAAGAATAG	1833
SPP1 RW	CGCCTTTGGTGCTCTTCATC	3514
Col1a1 FW	ACAGTGCAGGGGAAAGAATAGT	1833
Col1a1 FW2	CTGCAGTCTCCCTCTGCTG	2616
Col1a1 FW3	CCAATCCCCACCTCTGTGTC	3507
Col1a1 RW	TCGGTCCCGGTGTCTTCTAT	4598

Miscellaneous materials:

Pfu Polymerase: Thermo Scientific # EP0502, 10x Pfu Buffer with $MgSO_4$ included
JumpStart™ REDTaq® ReadyMix™ Reaction Mix: Sigma #P1107
TaqMan Fast Universal PCR Master Mix 2x: Applied Biosystems # 4352042
Fast Optical 96-well reaction plate with Barcode (0.1 ml): Applied Biosystems #4346906

2.4. Histochemistry and immunohistochemistry

2.4.1. Detection of alkaline phosphatase activity

Alkaline phosphatase activity is a hallmark of osteoblasts and is therefore used to visualize the amount of osteogenic differentiation in MSCs which have been induced with osteogenic differentiation medium. Alkaline phosphatase hydrolyzes BCIP and the product of this reaction reacts with NBT to yield an insoluble indigo pigment.

- First, the medium was aspirated and the cells were washed once with DPBS.
- Cells were fixed for one minute with 4% formaldehyde in DPBS at room temperature. Longer fixation leads to inactivation of alkaline phosphatase.
- After fixation, the formaldehyde solution was aspirated, discarded into the formaldehyde waste, and the cells were washed once with DPBS.
- After the washing step, the cells were incubated with BCIP/NBT substrate solution for up to 2 hours at room temperature in the dark. The required incubation time varied a lot between different differentiation experiments and was checked every 30 min.
- The reaction was stopped by aspirating the BCIP/NBT substrate solution, and cells were washed once with DPBS.
- After the last washing step the cell layer was covered with DPBS for the evaluation of the staining results.
- Stained cell can be stored for a few days at 4°C without much alteration of the staining.

2.4.2. Detection of lipid vacuoles with Oil Red O

MSCs which were differentiated into adipocytes form a large number of lipid vacuoles / droplets which can be detected by Oil Red O staining. Oil Red O is a lysochrome which stains lipids. Lipid filled organelles appear bright red under the microscope.

- The medium was carefully aspirated from the 35 mm cell culture dish and the cell culture dish was gently rinsed with 2 ml DPBS. Adipocytes are fragile and must be handled with care.
- The DPBS was aspirated from the cell culture dish, and the cells were fixed with 2 ml 4% formaldehyde in DPBS for 60 minutes at room temperature.
- The formaldehyde solution was aspirated and discarded into the formaldehyde waste.
- The dish was gently washed once with 2 ml M.Q. water. The wash solution was discarded into the formaldehyde waste.

- Next, 2 ml 60% isopropanol were added to the dish and incubated for 5 minutes at room temperature. This step is required because Oil Red O is dissolved in isopropanol.
- The isopropanol was aspirated and 2 ml of freshly prepared Oil Red O staining solution was added to each cell culture dish. Cells were incubated for 10 minutes at room temperature with Oil Red O staining solution.
- Oil Red O staining solution was aspirated and discarded into the staining solution waste.
- The cell culture dish was placed over a beaker and gently rinsed with a 1000 µl Eppendorf pipette with room temperature tap water until the water rinsed off clear.
- The remaining tap water was aspirated and 2 ml fresh tap water was added to the cell culture dish to keep the cell moist.

2.4.3. Preparation of tissue sections from chondrocyte nodules

- The supernatant from the micro mass cultures was carefully removed with a 1000 µl Eppendorf pipette so that the micro mass culture was not damaged.
- Carefully, 5 ml DPBS was added into the 15 ml falcon tube and the chondrocyte nodule was incubated for 5 minutes in DPBS.
- Next, about 4 ml of DPBS was aspirated and the chondrocyte nodule was transferred onto a strip of filter paper with a disposable pipette. If necessary the tip of the disposable pipette was cut off.
- The chondrocyte nodule was stained with ink, permitting that the nodule can easily be seen in the paraffin block after embedding.
- The chondrocyte nodule was carefully wrapped in a sheet of filter paper and transferred to a tissue embedding and processing cassette.
- The embedding cassette was submerged in 4% formaldehyde in DPBS and the chondrocyte nodule was fixed overnight at room temperature.
- On the next day the embedding cassette with the fixed chondrocyte nodule was dehydrated by applying ethanol in increasing concentrations (70%, 80%, and 90%).
- After dehydration, the embedding cassette with the sample was incubated two times for 30 min in Roti-Histol at 60°C.
- Next, the embedding cassette was incubated three times for 30 min in 58°C paraffin.
- The chondrocyte nodule was removed from the embedding cassette, unwrapped and placed into a tempered embedding mold.
- The embedding mold was placed on an embedding station and filled with hot, molten paraffin.

- The lid was removed from the embedding cassette, the embedding cassette was pressed into the paraffin and additional paraffin was added onto the embedding mold if needed to embed the cassette. The embedding cassette is required to fix the paraffin block in the microtome.
- The embedding mold was placed on a cooling plate with -20°C for at least 40 minutes until the paraffin was solid.
- The paraffin block was broken out of the embedding mold and stored at room temperature until sectioning.
- From the paraffin blocks, 4 µm thin tissue sections were generated using a microtome.
- The cut sections were transferred to a 40°C water bath with a wet fine brush and were mounted onto Histobond slides.
- Slides were cooled to room temperature and stored protected from light at 4°C.

2.4.4. Detection of aggrecan by immunofluorescence staining

To verify that cells isolated from umbilical cord are capable of differentiating into chondrocytes, the presence of cartilage-specific proteoglycan core protein, also known as aggrecan, was detected by immunofluorescence with an aggrecan specific antibody.

- Prior to the deparaffinization the slides were heated to 60°C for 20 minutes in a dry oven.
- The prepared tissue sections were deparaffinized by washing the slides in a descending ethanol series starting with xylol, 100% ethanol, 96% ethanol, and finally 70% ethanol.
- Next, the slides were briefly transferred into M.Q. water, excess amounts of water were removed and the tissue section was marked with a hydrophobic pen.
- The marked tissue section was incubated with 60 µl PBS for 5 minutes at room temperature.
- The PBS was aspirated with a pipette and the tissue section was washed once with 60 µl permeabilization buffer. The tissue section was incubated with permeabilization buffer for 45 min at room temperature in a humidity chamber.
- In the meantime a 1:100 dilution of the primary antibody (mouse-anti-human aggrecan) in blocking buffer was prepared. Final antibody concentration was 10 µg/ml.
- The permeabilization buffer was removed and the tissue section was incubated with 60 µl of the primary antibody solution. Incubation was done overnight in a humidity chamber at 2-8°C.

- On the next day, the tissue section was washed three times for 5 minutes with washing buffer.
- The secondary antibody (goat-anti-mouse AlexaFluor 546) was diluted 1:800 in washing buffer and 60 µl of the secondary antibody solution were added on the tissue section. Final concentration of the secondary antibody was 2.5 µg/ml.
- The secondary antibody was incubated for 60 minutes at room temperature in a humidity chamber in the dark.
- The secondary antibody solution was aspirated and 60 µl of DAPI staining solution (1 µg/ml) were added on the tissue section. DAPI stains the DNA in the nucleus bright blue.
- The section was incubated with DAPI for 15 minutes at room temperature in a humidity chamber in the dark.
- Next, the tissue section was washed two times for 5 min with 60 µl washing buffer in the dark.
- The slides were briefly rinsed with M.Q. water and excess liquid was dabbed off.
- The tissue section was mounted by applying 50-60 µl mounting medium on each tissue section for a 24x32 mm coverslip. The coverslip was gently pushed down and excess mounting medium was removed with a white paper towel.
- The mounting medium was dried for 10 min at room temperature and the coverslip was fixed with nail polish.

2.4.5. Detection of calcium deposits with Alizarin Red S

In addition to alkaline phosphatase activity, the osteogenic differentiation of MSCs can be detected by staining of calcified extra cellular matrix with Alizarin Red S. Alizarin Red S forms a chelate complex in the presence of calcium which is insoluble in water and DPBS. Stained calcium deposits appear bright red under the microscope.

- The medium was aspirated from the cells and the cells were washed once with DPBS without Ca^{2+} or Mg^{2+}.
- Next, the cells were fixed for at least 30 minutes with 4% formaldehyde in DPBS at room temperature.
- After the fixation, the formaldehyde was aspirated, discarded into the formaldehyde waste, and the cells were washed once with M.Q. water.
- Next, the cells were incubated in Alizarin Red S solution for 45 minutes at room temperature, in the dark.
- The Alizarin Red S staining solution was aspirated and the cells were washed with M.Q. water until the washing solution was colorless or slightly

reddish. The number of washing steps varied with the amount of calcium deposits.
- The last washing step was performed with washing buffer. After this washing step, the cells were covered with DPBS without Ca^{2+} and Mg^{2+} for evaluation.
- Stained cells can be stored for a few days at 4°C without much alteration of the staining or dried and stored at room temperature.

2.4.6. Detection of chondrocytes with toluidine blue

Chondrogenic differentiation in tissue sections of chondrocyte nodules was also detected with toluidine blue staining, which stained extracellular proteoglycans purplish.

- Prior to the deparaffinization, the slides were heated to 60°C for 20 minutes in a dry oven.
- The prepared tissue sections were deparaffinized by washing the slides in a descending ethanol series starting with xylene, 100% ethanol, 96% ethanol, and finally 70% ethanol.
- Next, the slides were briefly transferred into M.Q. water, excess amounts of water were removed and the tissue sections were transferred into a staining chamber with toluidine blue working solution for 2 – 3 minutes.
- The slides were then washed three times with M.Q. water to remove excess amounts of toluidine blue.
- The tissue sections were quickly dehydrated by washing the slides in an ascending ethanol series starting with two times 95% ethanol (10 dips), one times 100% ethanol (10 dips), and finally two times 3 minutes in xylene.
- The tissue sections were mounted by applying Entellan mounting medium on the tissue section. The coverslip was gently pushed down and excess mounting medium was removed.

2.4.7. Von Kossa staining of calcium deposits

Calcium phosphate deposits in the extracellular matrix of osteogenic differentiated MSCs were stained intense black. In contrast to Alizarin Red S, the silver ions react with phosphate instead of calcium.

- The medium was aspirated from the cells and the cells were washed once with DPBS without Ca^{2+} or Mg^{2+}.
- The cells were fixed for 30 minutes with 4% formaldehyde in DPBS at room temperature.
- The formaldehyde was aspirated, discarded into the formaldehyde waste, and the cells were washed three times with M.Q. water.

- The cells were incubated with a 5% $AgNO_3$ solution for 30 minutes in the dark.
- The $AgNO_3$ solution was aspirated and the cells were washed three times with M.Q. water.
- Afterwards, the cells were incubated with 5% Na_2CO_3 in 25% formaldehyde for 5 minutes.
- The Na_2CO_3 solution was aspirated and the cells were washed three times with M.Q. water.
- The staining was fixed with a 5% $Na_2S_2O_3$ solution for 2 minutes
- After the fixation the cells were washed 3 times with M.Q. water and were covered with M.Q. water during the evaluation.

2.4.8. Crystal violet staining

To visualize the effect of puromycin on MSCs, the cells were stained with crystal violet after 5 days of selection.

- The medium was aspirated and the cells were washed once with 1 ml DPBS.
- After the washing step, the cells were incubated in 1 ml 50% methanol in DPBS for 2 minutes at room temperature.
- Next, the 50% methanol was aspirated and 1 ml of pure methanol was added to the cells.
- The cells were incubated for 10 minutes at room temperature, the methanol was aspirated, and the cells were air-dried.
- Next, 1 ml of a 0.1% crystal violet staining solution was added to the cells and incubated for 10 minutes at room temperature.
- The crystal violet solution was discarded into the staining waste and the cells were washed several times with M.Q. water until the washing solution was colorless.
- The cells were air-dried before evaluation.

2.4.9. Histochemistry and immunohistochemistry materials

Solutions and buffers:

BCIP/NBT solution: 1 tablet of BCIP/NBT (SIGMA *FAST*™ BCIP/NBT Sigma #B5655) was dissolved in 10 ml M.Q. water.
40 mM Alizarin red S: 1g Alizarin Red S (Sigma #A5533) was dissolved in 50 ml M.Q. water, the pH was adjusted to 4.2, and passed through a pleated filter.
0.3% Oil red O stock solution: 0.15g Oil Red O (Sigma #O0625) was dissolved in 50 ml isopropyl alcohol.

Oil Red O working solution: Oil Red O stock was diluted 3:2 (ratio) in M.Q: water, incubated for 10 min at room temperature, and passed through a pleated filter.
DAPI: Sigma #D9542
Permeabilization buffer: Blocking buffer with 10% Triton X-100
Washing buffer: PBS with 1%BSA
Blocking buffer: Washing buffer with 10% FCS
BSA: MACS® BSA Stock Solution(20x) Miltenyi Biotec # 130-091-376
Triton X-100: Sigma #T8787
DPBS: DPBS (1x) no calcium, no magnesium: Gibco #14190-094
25% formaldehyde in PBS: 25 ml formaldehyde (36,5%) + 11,5 ml PBS (1x w/o Ca/Mg).
4% formaldehyde in PBS: 4 ml formaldehyde (36,5%) was diluted with 32.5 ml PBS (1x w/o Ca/Mg).
5% silver nitrate solution: 0.5497g $AgNO_3$ (Sigma #6506-25G) was weighed out and dissolved in 11.0 ml M.Q. water (stored protected from light).
5% sodium carbonate solution: 0.5435g Na_2CO_3 (Sigma #S7795) were weighed out and dissolved in 10.9 ml 25% formaldehyde in PBS.
5% sodium thiosulfate solution: 0.8990g $Na_2S_2O_3$ (Sigma #217263-25G) was weighed out and dissolved in 11.5 ml M.Q. water.
Toluidine blue stock solution: 0.4g Toluidine blue (Fluka #89640-25g) was dissolved in 40 ml of 70% ethanol.
Toluidine blue working solution: Toluidine blue stock solution was diluted 1:10 in a 1% sodium chloride solution (pH = 2.25).
1% Sodium chloride solution: 0.5 g sodium chloride (Sigma) was dissolved in 50 ml M.Q. water and the pH was adjusted to 2.25 with hydrochloric acid.
10% formaldehyde in PBS: 2.8 ml 36.5% formaldehyde (Sigma #33220) was diluted with 7.2 ml PBS (1x w/o Ca/Mg).

Miscellaneous materials:

Mouse-anti-human aggrecan: 1.0 mg/ml Millipore #MAB19310
Goat-anti-mouse AlexaFluor 546-Konjugat (Fab fragment): 2 mg/mL Molecular Probes, Prod. Nr. A11018
Fluromount-G™: Southern Biotech #0100-01
Entellan: Merck #1079610100

2.5. Biochemical methods

2.5.1. BCA protein assay

For the determination of the protein concentration in cell lysates the Pierce BCA Protein assay kit was used.

- First, the assay reagent was prepared by adding one part of reagent B to 50 parts of reagent A and the mixture was briefly vortexed. The prepared assay reagent had a light green color.
- For determination of the protein concentration, 10 µl of the cell lysate was pipetted into a well of a clear 96 well plate. Measurements were performed in duplicates.
- Next, 200 µl of the prepared assay reagent was added to each well with a multi-pipette and the plate was placed on a plate shaker for at least 30 sec at 300 rpm.
- The prepared plate was incubated at 37°C for 30 min and was allowed to cool to room temperature before the measurement.
- After the plate was equilibrated, the absorbance of each well was measured at 584 nm in a FLUOstar Optima microplate reader (BMG LABTECH) with an automated measurement routine.
- From each well 20 absorbance measurements were taken and combined to one measurement.

2.5.2. Luciferase assay

Luciferase activity in cell lysates was measured on a FLUOstar Optima microplate reader (BMG LABTECH) using the Luciferase Assay System (Promega).

- The luciferase assay reagent was stored at -80° and was equilibrated to room temperature before the measurement.
- In the meantime, 10 µl of the cell lysate was pipetted into a well of a 96 well plate. Measurements were performed in duplicates.
- After the plate was prepared, the pipetted layout was programmed on the FLUOstar Optima microplate reader and the luciferase assay reagent was loaded into the injection system.
- The plate was inserted into the FLUOstar Optima microplate reader and luciferase activity was measured with the following automated measurement routine:
    1. 50 µl of luciferase assay reagent were injected into the according well
    2. After the injection, the plate was shaken for 5 seconds.
    3. After 10 seconds the luminescence was measured in a 10 second time interval.

4. After the readout the next well was measured.

- After the measurement, the remaining luciferase assay reagent was collected and stored at -80°C for the next measurement. The injection system of the FLUOstar OPTIMA was flushed with M.Q. water until the remaining assay reagent was removed.

2.5.3. Assay materials

BCA Protein Assay Kit: Pierce #23227
Luciferase Assay System: Promega #E1500
96-well FluoroNunc Plate MaxiSorp Surface (flat bottom, white):
NALGE-NUNC # 436110
96-well Microtest Plate (flat bottom, clear): Sarstedt #82.1581

2.6. Data evaluation

2.6.1. Evaluation of q-PCR data

- The q-PCR reactions were performed in duplicates for each time point and analyzed gene.
- For each time point and gene, the cycle threshold (Ct) was recorded (Figure 14).
- In the first step, the mean value (MW) of the two obtained cycle thresholds was calculated for each osteoblast-specific and the housekeeping gene.
- Next, the mean value of the house keeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1), determined at the same time point, was subtracted from the mean value of the osteoblast-specific genes e.g. COL1A1. The result was the delta CT value (dCT).
- The calculated dCT value of each gene, in the control cultures at day 6 (K6), was subtracted from the dCT values of the corresponding gene in the differentiation cultures. The result was the delta delta CT value (ddCT).
- The ddCT value equaled the difference in amplification cycles between the control culture and the differentiation culture. A negative value corresponds to a higher expression and a positive value to a lower expression of the gene, compared to the control culture.
- The ddCT value was converted into an n-fold change of expression by raising 2 to the power of ddCT (2^ddCT).

Well	Sample Name	Detector	Task	Ct	StdDev Ct	MW	dCT	ddCT	2^-ddCT	Time Point
E3	D10	COL1A1	Unknown	17,65	0,034	17,625	-11,72	-3,485	11,2	Day 10
E4	D10	COL1A1	Unknown	17,6	0,034					

Well	Sample Name	Detector	Task	Ct	StdDev Ct	MW	dCT
F3	K6	COL1A1	Unknown	20,99	0,062	20,945	-8,235
F4	K6	COL1A1	Unknown	20,9	0,062		

Well	Sample Name	Detector	Task	Ct	StdDev Ct	MW
G3	D10	hPRT1 Taqman	Unknown	29,26	0,116	29,345
G4	D10	hPRT1 Taqman	Unknown	29,43	0,116	

Figure 14 | Evaluation of q-PCR data.

The cycle threshold and StdDevCt was determined with the Applied Biosystems 7500 Fast Real-Time PCR system. With these results, the change in gene expression in relation to the control cultures was calculated.

2.6.2. Evaluation of luciferase expression

The luciferase activity was determined with a luciferase assay kit and the luminescence was recorded with a FLUOstar Optima microplate reader. In addition to the luciferase activity, the relative protein concentration was determined for each sample. Each measurement was performed in duplicates. First, the mean value of two measurements for the luminescence and the protein concentration were calculated. From these values the luminescence/protein ratio was calculated by division of the luminescence with the relative protein concentration. This allowed the comparison of the luciferase activity between cell lysates from different time points and with different total protein concentrations.

2.6.3. Calculation of the population doubling time (PDT) from WJ-MSCs

The population doubling time of WJ-MSCs was calculated between passages two and nine according to the following formula:

$$PDT = \frac{t * \log(2)}{\log(Nt) - \log(N0)}$$

For the calculation, the initial cell number (N0), the number of harvested cells (Nt), and the time between passages in hours (t), were recorded for each passage (Xu, Meng et al. 2010). From these data, the population doubling times for the three WJ-MSCs populations XIX, XXII, and XXIII, were calculated.

3. Results

3.1. Isolation and expansion of WJ-MSCs

3.1.1. Isolation of mesenchymal stromal cells from Wharton's jelly

Mesenchymal stromal cells were isolated from umbilical cord Wharton's jelly, from three different umbilical cord samples, using the explant culture method as described in Materials & Methods. The outgrowth of single cells around the tissue biopsies was visible after three days of incubation. After six days of incubation, the outgrowing cells around the tissue biopsies were easily detected with a phase contrast microscope (Figure 15A and 15B).

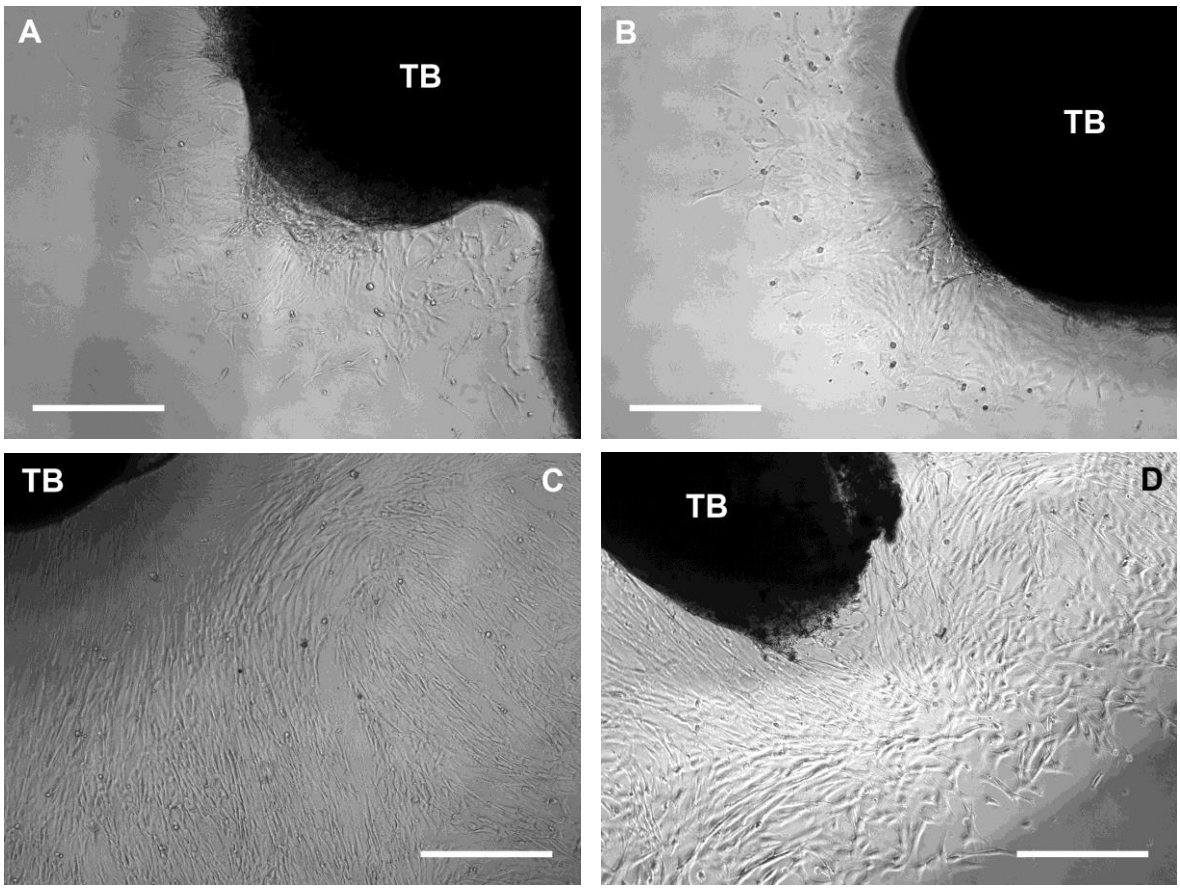


Figure 15 | Phase-contrast images of outgrowing WJ-MSCs from Wharton's jelly biopsies.

A, B | Images were taken from tissue biopsies (TB) in explant culture at day 6. Outgrowing WJ-MSCs were seen around the tissue biopsy. **C, D** | Until day 11, a massive outgrowth of UC-MSCs occurred around the tissue biopsies. Pictures taken from WJ-MSC XIX isolation (Scale bar: 500 µm)

The tissue biopsies were incubated for up to 13 days. Until day 13 of the explant culture, a profuse outgrowth of cells occurred around the tissue biopsies (Figure 15C and 15D). The outgrown cells were passaged when they reached a critical density around the tissue biopsies. Therefore the tissue biopsies were removed and the isolated cells were counted after trypsinization. WJ-MSCs were successfully isolated from each of the three different umbilical cord samples (Table 1).

Umbilical cord sample	Total number of isolated cells	Duration of explant culture
XIX	1.062x10^5	12 days
XXII	1.175x10^5	13 days
XXIII	3.94x10^5	11 days

Table 1 | Number of cells isolated from three different umbilical cord samples at passage 1.

The number of isolated cells was determined at the end of the explant culture with a CASY cell analyzer.

The highest number of cells was obtained from umbilical cord sample XXIII and was about 4 times higher as in previous isolations. The higher cell numbers were obtained through a higher number tissue biopsies that were incubated in each flask compared to the previous explant cultures prepared from umbilical cord XIX and XXII. In addition to the higher number of tissue biopsies the tissue biopsies from umbilical cord XXIII showed a higher outgrowth rate of mesenchymal stromal cells.

3.1.2. Expansion of WJ-MSCs from Promocell

A frozen aliquot of mesenchymal stromal cells isolated from the umbilical cord matrix (Wharton's jelly) was purchased from Promocell. The cells were thawed as described and expanded according to the manufacturer's instructions. The WJ-MSCs possessed a high proliferation rate and reached 70-90% confluency within four days of passaging with an initial seeding density of 4000 cells/cm² (Figure 16). WJ-MSCs from Promocell were expanded over several passages and cell aliquots were frozen from passage 4 to passage 7. After passage 7 a total of 1.24x10^8 cells were obtained and stored in liquid nitrogen (Table 2).

Label	Passage number	Cells per aliquot	Number of Aliquots	Date
WJ-MSC Promocell	4	1 T25 flask	1	16/01/2012
WJ-MSC Promocell	5	2.0x10^6	16	19/01/2012
WJ-MSC Promocell	6	2.0x10^6	16	22/01/2012
WJ-MSC Promocell	7	1.5x10^6	40	25/01/2012

Table 2 | Overview of frozen aliquots of WJ-MSCs from Promocell.

The WJ-MSCs were frozen in CryoSure MSC freezing medium and stored in liquid nitrogen.

A small population of WJ-MSCs from Promocell was kept in culture for the evaluation of the long-term proliferation potential. The high proliferation rate of the cells remained constant during long-term culture over several more passages and slowly declined after passage 10. After 24 passages, the proliferation rate of the cells had decreased dramatically and the long-term culture was stopped at passage 25.

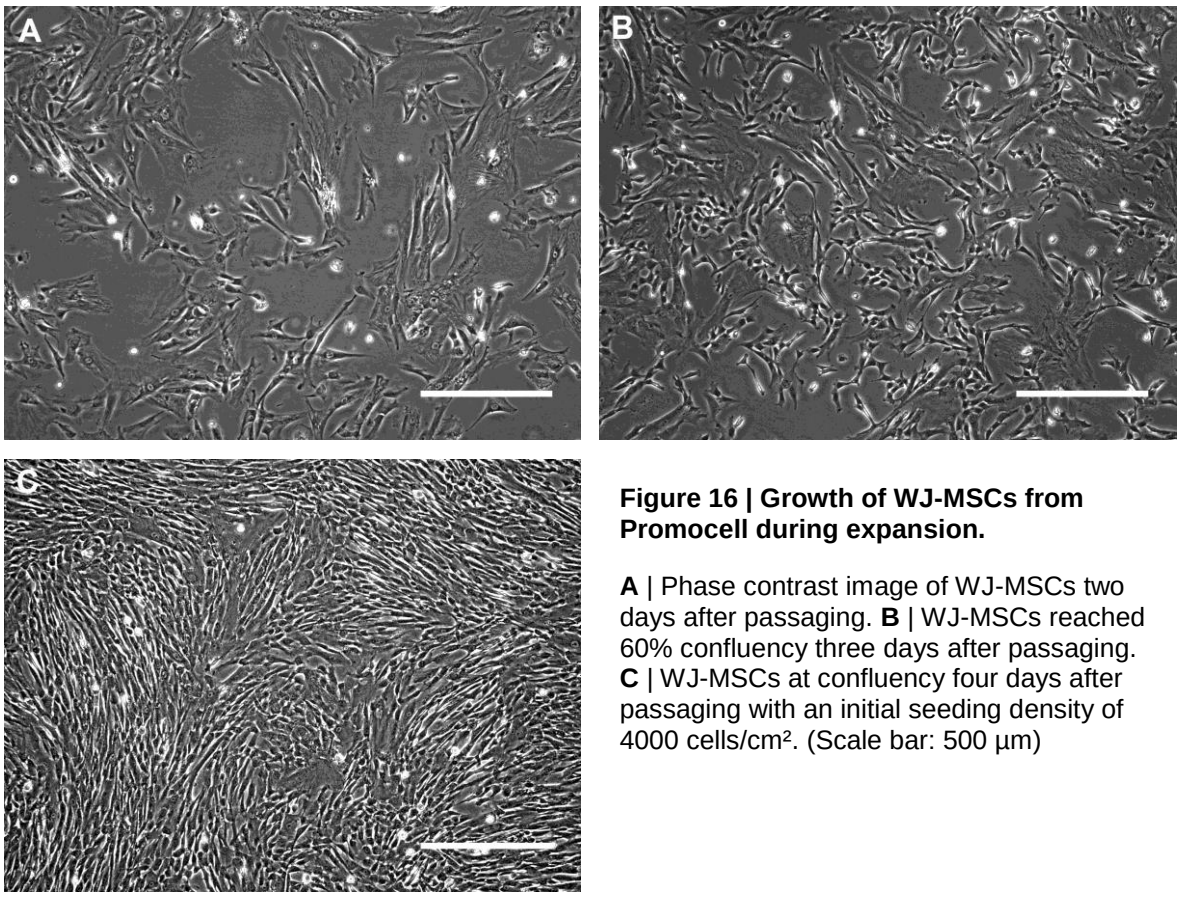


Figure 16 | Growth of WJ-MSCs from Promocell during expansion.

A | Phase contrast image of WJ-MSCs two days after passaging. **B** | WJ-MSCs reached 60% confluency three days after passaging. **C** | WJ-MSCs at confluency four days after passaging with an initial seeding density of 4000 cells/cm². (Scale bar: 500 µm)

During long-term cultivation the morphology of the cells clearly changed. At passage 4 most of the cells were small, compact, and had a clear defined cell shape (Figure 17A). After passage 10, a population of larger cells appeared that displayed completely different cell morphology. These cells were considerably

larger and flattened out than cells at lower passages. These cells showed signs of replicative senescence (Figure 17B).

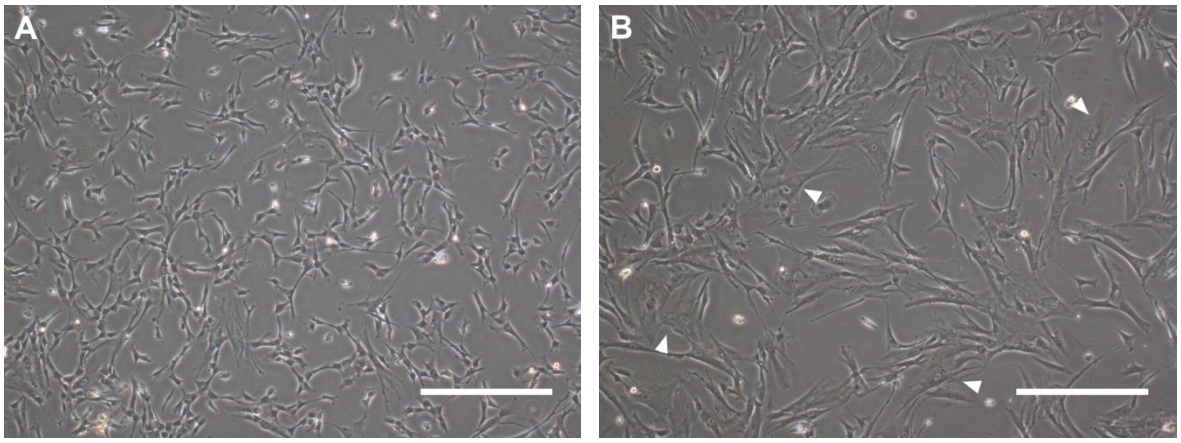


Figure 17 | Cell morphological changes in WJ-MSCs during long-term culture.

A | WJ-MSCs at passage four had a characteristic small cell body with a few cell processes. **B** | During long-term culture cell morphology changed and the cells became larger, flattened out with a less defined cell body. Cells also displayed signs of replicative senescence (arrowheads). Pictures taken from WJ-MSC XIX long-term culture (Scale bar: 200 µm)

3.1.3. Expansion of MSCs isolated from Wharton's jelly

Mesenchymal stromal cells were isolated from Wharton's jelly from three umbilical cord samples with the explant culture method. After the explant culture, the isolated WJ-MSCs were harvested and expanded. The isolated WJ-MSCs possessed a high proliferation rate and reached confluency four days after passaging with an initial cell density of 4000 cells/cm² (Figure 18). WJ-MSCs isolated from different umbilical cord samples possessed similar proliferation rate and cell morphology. The WJ-MSCs displayed a fibroblast-like morphology similar to the WJ-MSCs obtained from Promocell. WJ-MSCs from all three umbilical cord samples were expanded and cell aliquots were frozen at passages two and three. A total of 6.6x10^7 cells, 5.94x10^7 cells, and 2.8x10^7 cells, were obtained from umbilical cord XIX, XXII, and XXIII, respectively (Table 3). The cells were frozen as described and stored in liquid nitrogen. The isolated cells were labeled as WJ-MSC XIX, XXII, and XXIII.

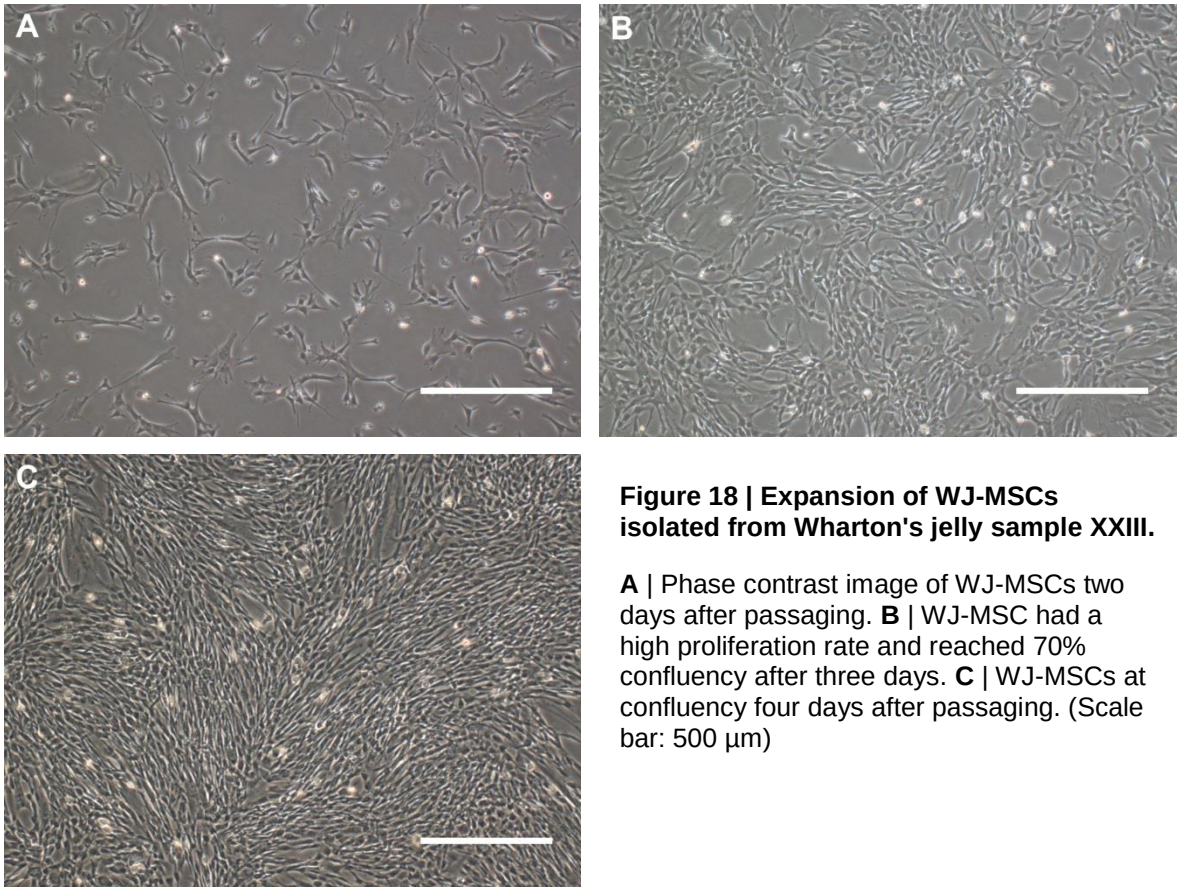


Figure 18 | Expansion of WJ-MSCs isolated from Wharton's jelly sample XXIII.

A | Phase contrast image of WJ-MSCs two days after passaging. **B** | WJ-MSC had a high proliferation rate and reached 70% confluency after three days. **C** | WJ-MSCs at confluency four days after passaging. (Scale bar: 500 µm)

Label	Passage number	Cells per aliquot	Number of Aliquots	Date
WJ-MSC XIX	2	1.0x10^6	2	13/02/2012
WJ-MSC XIX	3	2.0x10^6	18	17/02/2012
WJ-MSC XIX	3	4.0x10^6	7	17/02/2012
WJ-MSC XXII	3	2.0x10^6	9	16/03/2012
WJ-MSC XXII	4	2.3x10^6	18	20/03/2012
WJ-MSC XXIII	2	2.0x10^6	2	06/04/2012
WJ-MSC XXIII	3	2.0x10^6	6	10/04/2012
WJ-MSC XXIII	3	2.0x10^6	6	11/04/2012

Table 3 | Overview of frozen MSC aliquots isolated from Wharton's jelly.

Cells isolated from umbilical cord XIX, XXII, XXIII, were labeled as WJ-MSC XIX, XXII, and XXIII, respectively.

3.1.4. Population dynamics of WJ-MSCs

The population doubling time and total population doublings of the three isolated WJ-MSC populations were determined during passages 2 to 9. The population doubling time was calculated from the cell numbers that were determined for each passage and the initial cell number as described in Materials & Methods. The population doubling time was calculated in hours for each passage and WJ-MSC population (Figure 19). The mean population doubling time for WJ-MSCs XIX until passage 9 was 29.0 ± 5.9 hours, for WJ-MSCs XXII 31.1 ± 12.4 hours, and for WJ-MSCs XXIII 26.6 ± 2.7 hours. In addition to the population doubling time the number of population doublings, from passage 2 to 9, were calculated as described in Materials & Methods. The population doublings for WJ-MSC XIX, XXII, and XXIII were 33, 30, and 31, respectively. The three WJ-MSC populations derived from different umbilical cord samples had similar population doubling times and proliferation potential.

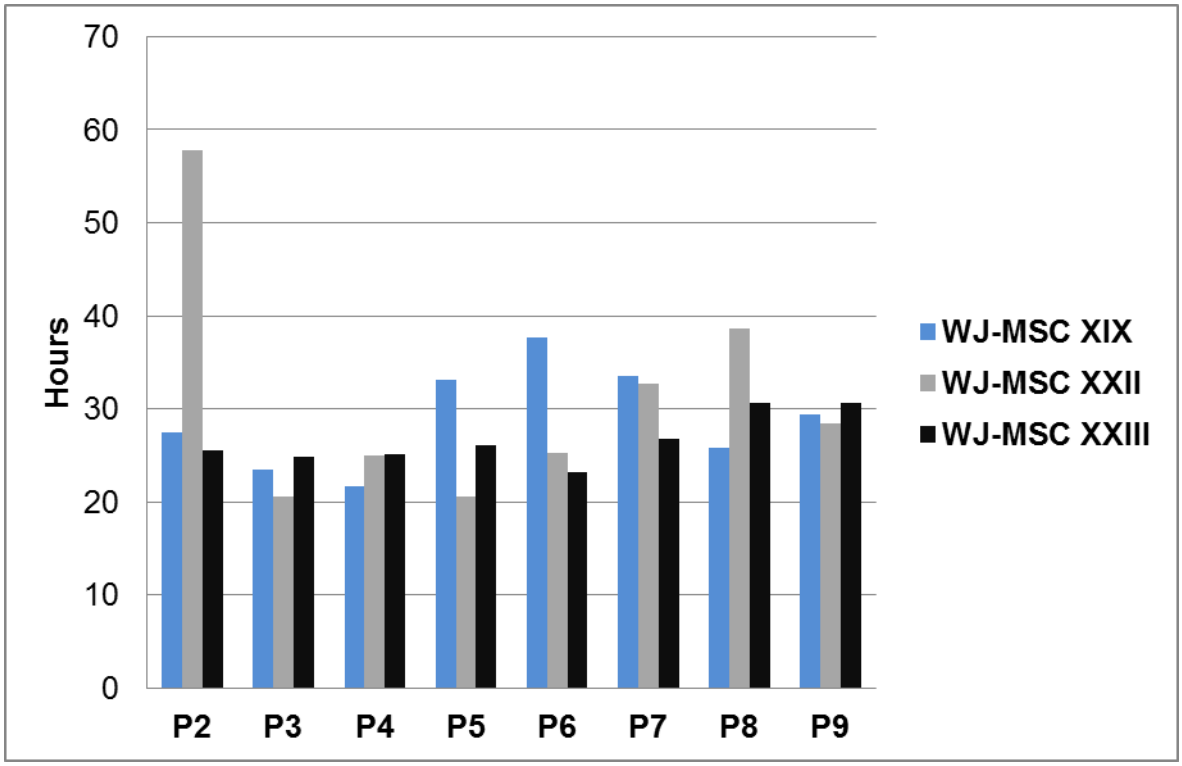


Figure 19 | Population doubling times of the WJ-MSC populations XIX, XXII, and XXIII.

The three WJ-MSC populations had a similar population doubling time over 8 passages. WJ-MSC XXII had a very high population doubling time until passage two but reached normal population doubling times at later passages.

3.2. Characterization of WJ-MSC

3.2.1. Immunophenotyping of WJ-MSCs

The expression of the mesenchymal markers CD44, CD73, CD90, CD105, and the absence of the hematopoietic markers CD34 and CD45 was determined by flow cytometry. In addition to these four common MSC markers the expression of CD271 was also examined, which was shown to be expressed in some MSC populations and can be used for MSC isolation from bone marrow. The staining of WJ-MSCs was performed as described in Materials & Methods and from each staining 3.0x10^4 events were recorded on a BD FACScan. The recorded data were evaluated with FlowJo (Figure 20-23).

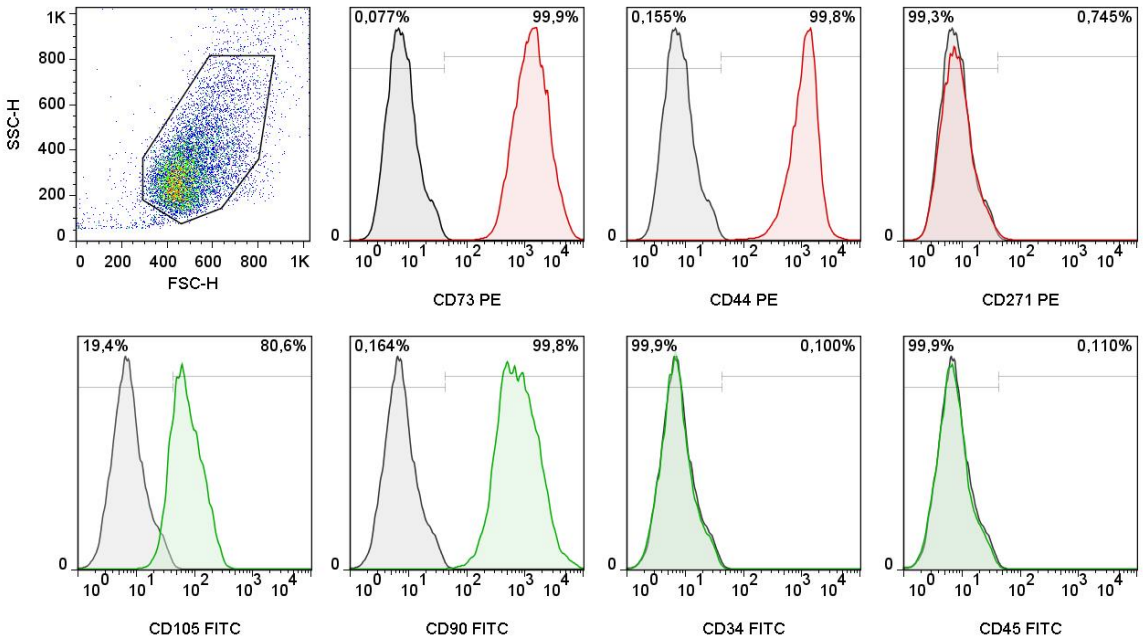


Figure 20 | Flow cytometric immunophenotyping of WJ-MSCs Promocell.

In the dot plot, each event is represented as a single dot with its corresponding SSC and FSC value. With the indicated gate cell debris was excluded and WJ-MSCs were selected for analysis. WJ-MSCs Promocell expressed the mesenchymal markers CD44, CD73, CD90, and CD105. The absence of the hematopoietic markers CD34 and CD45 was confirmed. CD271 was not expressed by these cells.

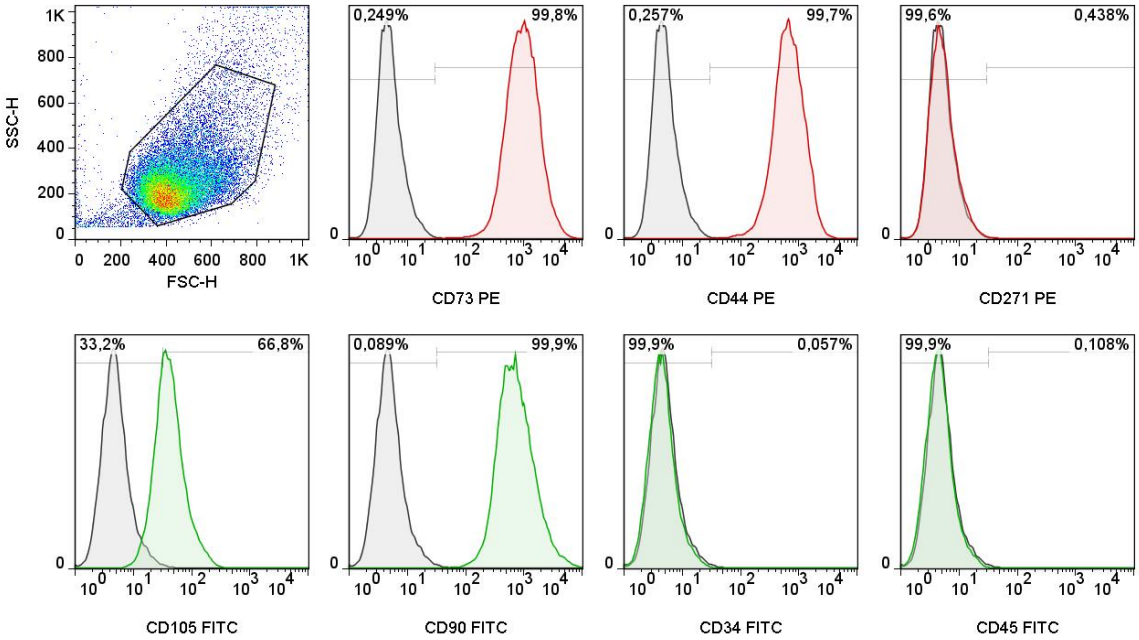


Figure 21 | Flow cytometric immunophenotyping of WJ-MSCs XIX.

In the dot plot each event is represented as a single dot with its corresponding SSC and FSC value. With the indicated gate cell debris was excluded and cells were selected for analysis. WJ-MSCs XIX expressed the mesenchymal markers CD44, CD73, CD90, and CD105. The absence of the hematopoietic markers CD34 and CD45 was confirmed. CD271 was not expressed by these cells.

Flow cytometric analysis of WJ-MSCs from Promocell was performed with cells harvested at passage 7 (Figure 20). The analysis confirmed the expression of the mesenchymal markers CD44, CD73, CD90, and CD105. The staining for CD105 resulted in lower intensities than the other 3 markers. The staining intensity was lower so that the peaks from the isotype control and the stained cells were not completely separated. CD271, which is expressed by some MSC populations, was not detected. WJ-MSCs from Promocell were negative for the hematopoietic markers CD34 and CD45. The flow cytometric analysis showed that the WJ-MSCs from Promocell were a homogenous cell population with no hematopoietic contamination.

WJ-MSCs XIX were harvested at passage 3 and the expression of mesenchymal markers was analyzed by flow cytometry (Figure 21). The cells expressed the mesenchymal markers CD44, CD73, CD90, and CD105. Similar to WJ-MSCs from Promocell, the staining for CD105 resulted in lower intensities than the other three markers. In addition, the hematopoietic markers CD34 and CD45 were not expressed on WJ-MSCs XIX.

A similar result was obtained from the analysis of WJ-MSCs XXII cells, which were harvested at passage 3 (Figure 22). The four mesenchymal markers were expressed by all cells and the hematopoietic markers CD34 and CD45 were absent. In the FITC isotype control staining a small population of stained cells was detected. This cell population was not detected in the CD34 and CD45 staining and resulted from an unspecific staining.

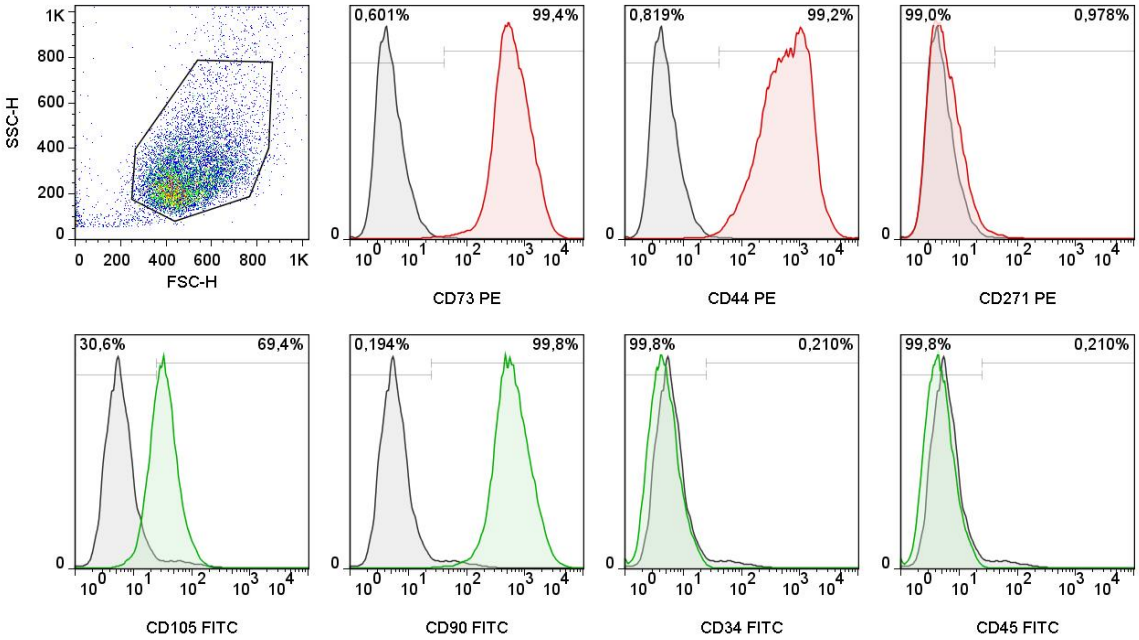


Figure 22 | Flow cytometric immunophenotyping of WJ-MSCs XXII.

In the dot plot each event is represented as a single dot with its corresponding SSC and FSC value. With the indicated gate cell debris was excluded and cells were selected for analysis. WJ-MSCs XXII expressed the mesenchymal markers CD44, CD73, CD90, and CD105. The absence of the hematopoietic markers CD34 and CD45 was confirmed. CD271 was not expressed by these cells.

Immunophenotyping of WJ-MSCs XXIII was performed at passage 3 (Figure 23). WJ-MSCs XXIII were positive for the mesenchymal markers CD44, CD73, CD90, CD105, and negative for the hematopoietic markers CD34 and CD45.

In all three WJ-MSC populations, isolated from umbilical cord Wharton's jelly, a similar expression profile for the four different mesenchymal markers was detected. Staining for CD44, CD73, and CD90, resulted in high staining intensities in all three cell populations. The staining for CD105 resulted in lower staining intensities. The isolation of WJ-MSCs from umbilical cord Wharton's jelly, via the explant culture method, resulted in a homogenous cell population with no detectable hematopoietic contamination. In the three different WJ-MSC populations XIX, XXII, and XXIII, the flow cytometric analysis confirmed the expression of the four mesenchymal markers and the absence of hematopoietic

markers that were proposed by the ISCT. After we had demonstrated that the isolated cells fulfilled this criterion, the multipotency of these cells was tested by tri-lineage differentiation.

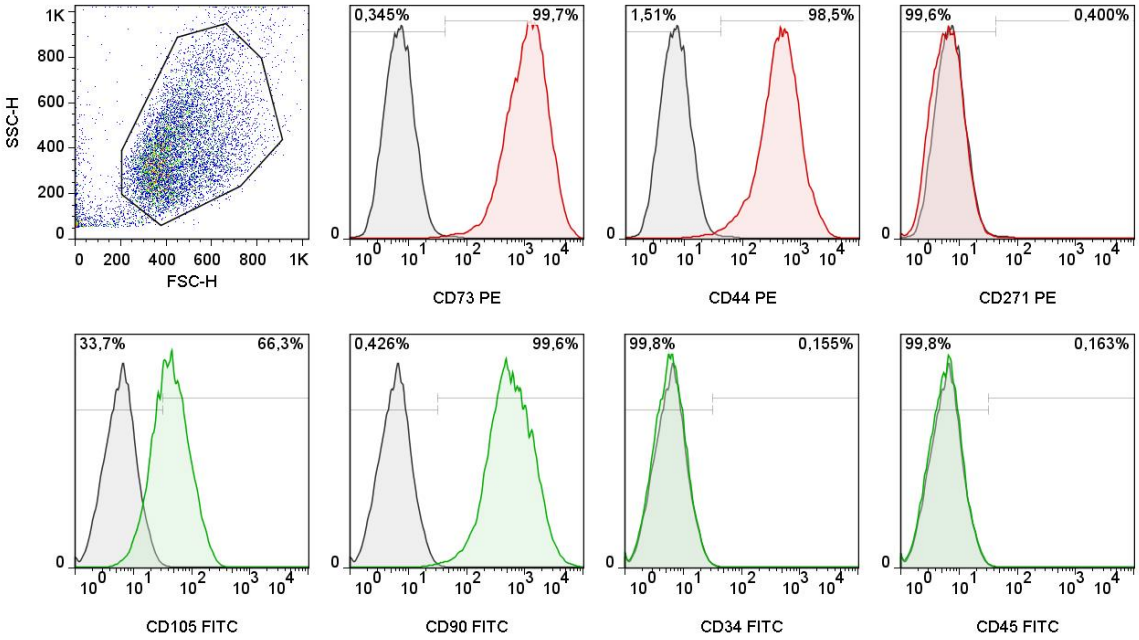


Figure 23 | Flow cytometric immunophenotyping of WJ-MSCs XXIII.

In the dot plot each event is represented as a single dot with its corresponding SSC and FSC value. With the indicated gate cell debris was excluded and WJ-MSCs were selected for analysis. WJ-MSCs XXIII expressed the mesenchymal markers CD44, CD73, CD90, and CD105. The absence of the hematopoietic markers CD34 and CD45 was confirmed. CD271 was not expressed by these cells.

3.2.2. Tri-lineage differentiation of WJ-MSCs

The tri-lineage potential of WJ-MSCs was tested by differentiation of these cells into adipocytes, chondrocytes, and osteoblasts. WJ-MSCs were cultured for 21 days in adipogenic differentiation medium and adipogenic differentiation was detected with Oil Red O, as described in Materials & Methods, which stained lipid droplets intensely red. Similar results were obtained with the four different populations of WJ-MSCs (WJ-MSCs Promocell, WJ-MSCs XIX, XXII, and XXIII) and all WJ-MSC populations were successfully differentiated into adipocytes (Figure 24). The lipid droplets within the cytoplasm of different cells varied in size. Large lipid droplets, the hallmark of adipocytes, were only detected in differentiated cells (Figure 24C), but not in the control cultures. In the control cultures very small, weakly stained, droplets were detected that were not specific for adipocytes (Figure 24D) when compared to cells that were cultured in adipogenic differentiation medium (Figure 24B).

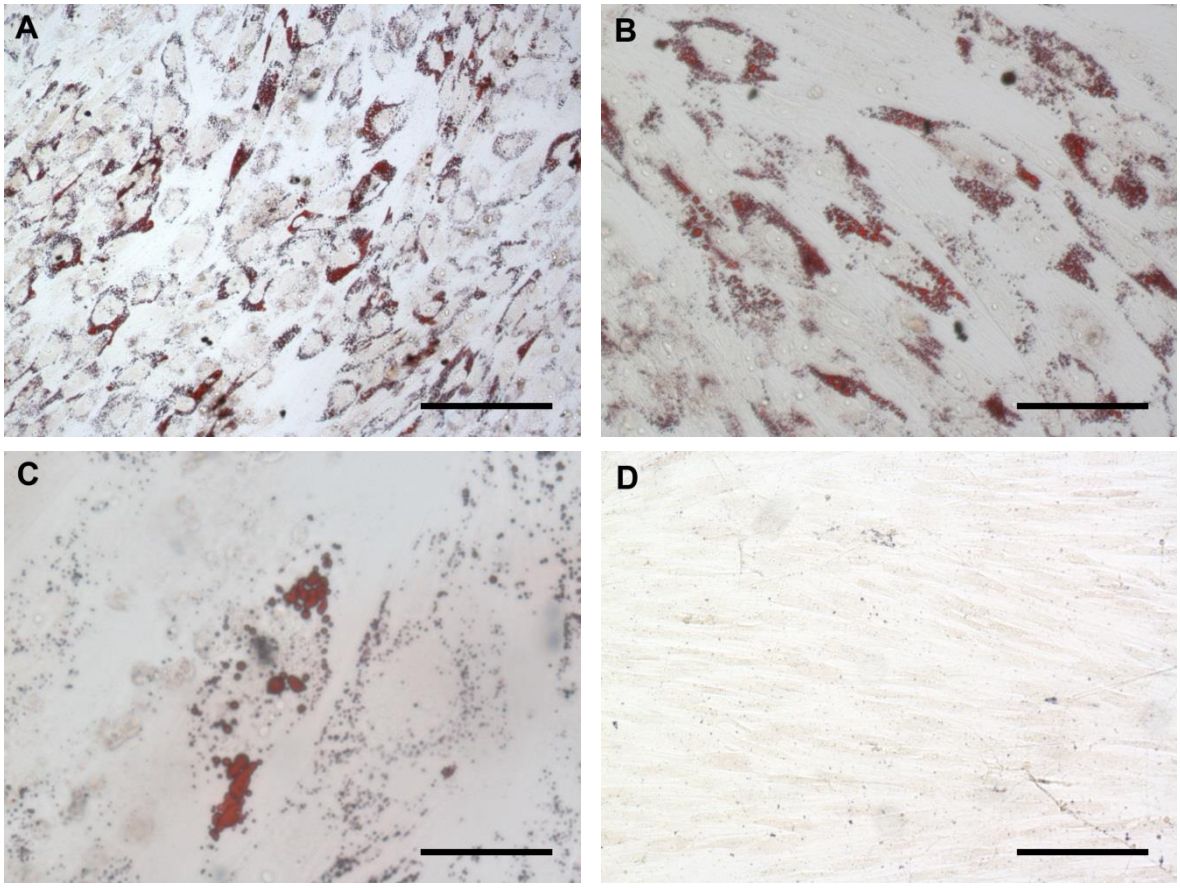


Figure 24 | Detection of adipogenic differentiation in WJ-MSCs.

A | Lipid filled droplets were detected with Oil Red O after 21 days of adipogenic differentiation (Scale bar: 200 µm). **B** | Higher magnifications of stained cells showed different amounts of lipid droplets in the cytoplasm of cells (Scale bar: 100 µm). **C** | Different sizes of lipid droplets within the cytoplasm of cells were detected. Small lipid droplets were detected in many cells. Large lipid droplets a hallmark of adipocytes were detected in a small number of cells (Scale bar: 50 µm). **D** | In the control cultures no large lipid droplets were detected that indicated adipocytes or adipogenic differentiation (Scale bar: 100 µm). (Pictures A, B were taken from WJ-MSCs XXII and Pictures C, D were taken from WJ-MSCs XXIII)

Adipogenic differentiation was also performed with BM-MSCs, as described in Materials & Methods, as reference for the results obtained with WJ-MSCs. Surprisingly, already after 14 days of adipogenic differentiation, a population of cells with large droplets that appeared bright white in the phase contrast microscope was detected. These cells were rated as differentiated adipocytes (Figure 25A). The droplet-filled cells were readily identified at day 21 of adipogenic differentiation by Oil Red O staining. The large lipid droplets appeared intensely red under bright field illumination (Figure 25B and 25C). Compared to adipogenic differentiated WJ-MSCs these cells had much larger lipid droplets that completely filled the innards of the cells in contrast to WJ-MSCs, where the lipid droplets only filled large parts of the cytoplasm. Among the Oil Red O stained cells, there was a rare population of cells that looked similar to adipogenic differentiated WJ-MSCs (Figure 25C, arrowhead). Large parts of the cytoplasm of these cells were filled

with small lipid droplets similar to those detected in differentiated WJ-MSCs (Figure 24B and 25C, same magnification). Despite a population of terminally differentiated adipocytes in BM-MSCs that was not detected in WJ-MSCs, a greater number of adipogenic differentiated cells was present in WJ-MSC cultures. In BM-MSC control cultures no adipogenic differentiated cells were detected (Figure 25D).

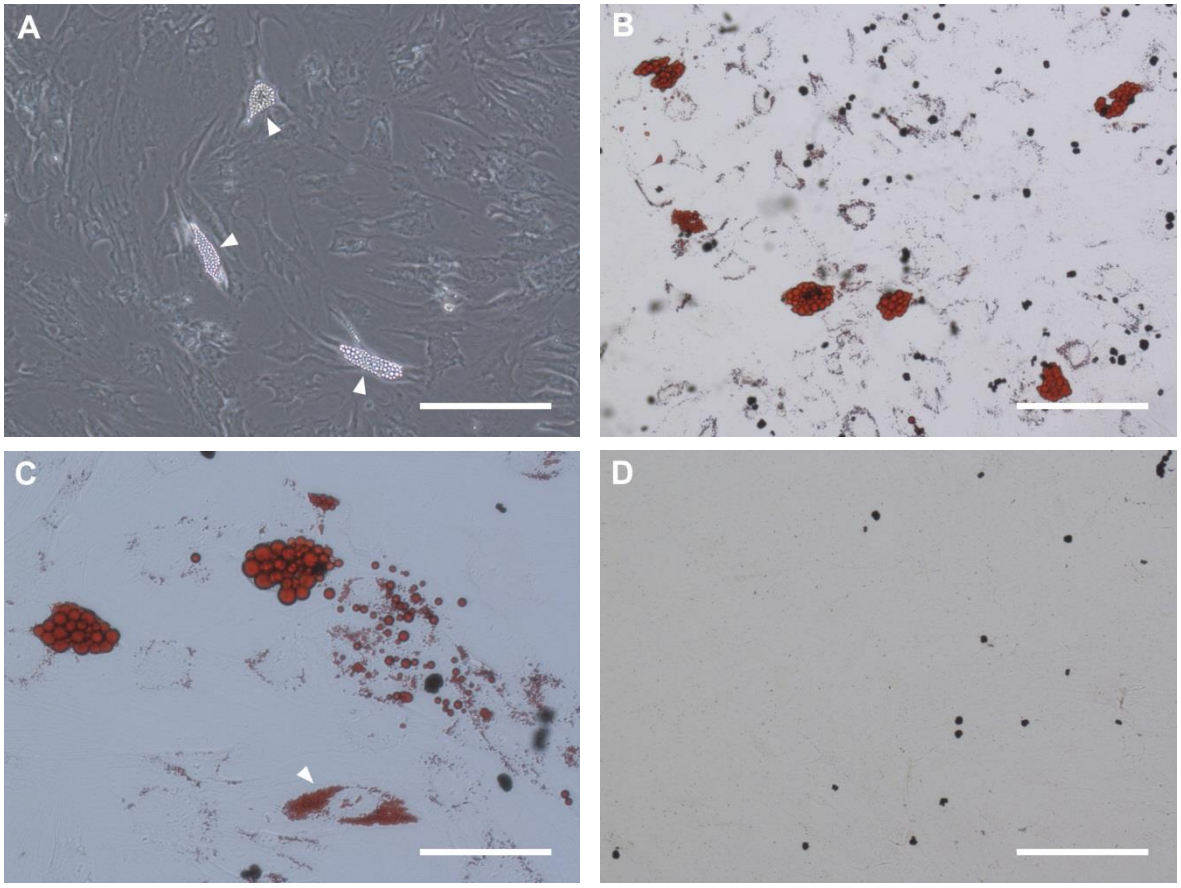


Figure 25 | Detection of adipogenic differentiation in BM-MSCs.

A | Cells with large lipid droplets were detected after 14 days of adipogenic differentiation in BM-MSC cultures (arrowheads). These cells were rated as differentiated adipocytes (Scale bar: 200 µm). **B** | These cells were readily detected, at day 21 of adipogenic differentiation, by Oil Red O staining. The larger lipid droplets were stained red and filled the entire cytoplasm of the cells (Scale bar: 200 µm). **C** | In addition to cells with larger lipid droplets a rare population of cells was detected that had smaller lipid droplets similar to that observed in WJ-MSCs (arrowhead) (Scale bar: 100 µm). **D** | In BM-MSC control cultures no lipid droplets were detected by Oil Red O staining. Black spots are precipitates of Oil Red O dye. (Scale bar: 200 µm).

Chondrogenic differentiation of WJ-MSCs was performed in chondrogenic differentiation medium for 24 days as described in Materials & Methods. Chondral cell aggregates were fixed and embedded in paraffin as described in Materials & Methods. Thin sections were prepared from the chondrogenic micromass and successful chondrogenic differentiation was detected by staining of the extracellular matrix protein aggrecan in tissue sections of the four different WJ-MSC populations (Figure 26A). The prepared tissue sections showed a positive staining for aggrecan and the homogenously stained nuclei indicated that the cells within the micromass were not apoptotic and viable. The specificity of the secondary antibody was tested on tissue sections without primary antibody and no unspecific staining was detected (Figure 26B).

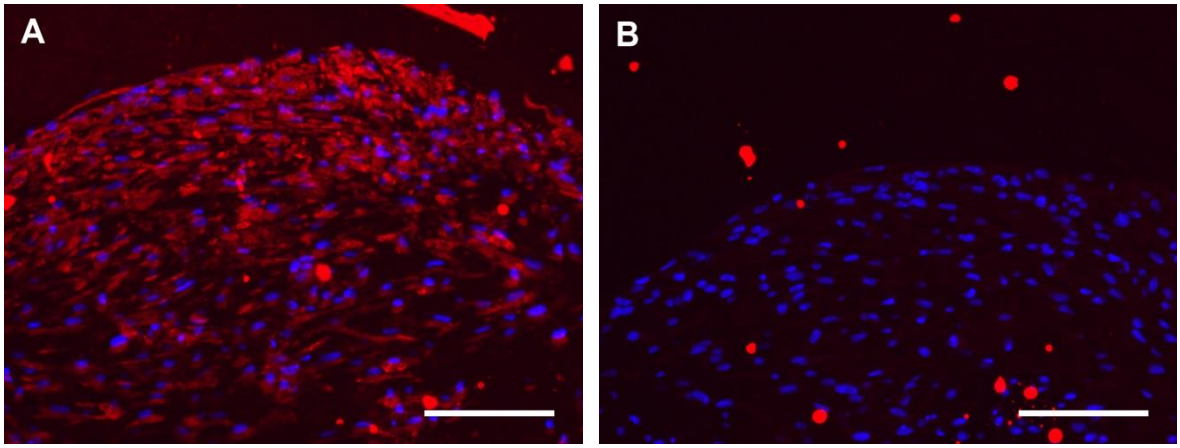


Figure 26 | Detection of chondrogenic differentiation in WJ-MSC micromasses.

A | The extracellular matrix protein aggrecan was detected in tissue sections of chondrogenic differentiated WJ-MSCs by immunocytochemistry (red). The nuclei were visualized with DAPI (blue). (Scale bar: 100 µm). **B** | A tissue section from the same micromass was incubated without the primary antibody as a secondary antibody control. The secondary antibody produced no unspecific staining of the tissue section. Nuclei were stained with DAPI (blue) (Scale bar: 100 µm). Pictures taken from WJ-MSCs XIX.

In addition to the immunohistochemical detection of aggrecan, the chondrogenic differentiation was also assessed by Toluidine blue staining of chondrogenic tissue sections (Figure 27). The stained tissue section revealed groups of cells that were surrounded by vast amounts of extracellular matrix. A metachromatic reaction of Toluidine blue with the matrix was detected that stained large areas of the tissue section purplish. The metachromatic reaction of Toluidine blue is a hallmark of glycosaminoglycans. Glycosaminoglycans represent a major component of the chondrogenic matrix and their presence confirmed the differentiation of WJ-MSCs into chondrocytes.

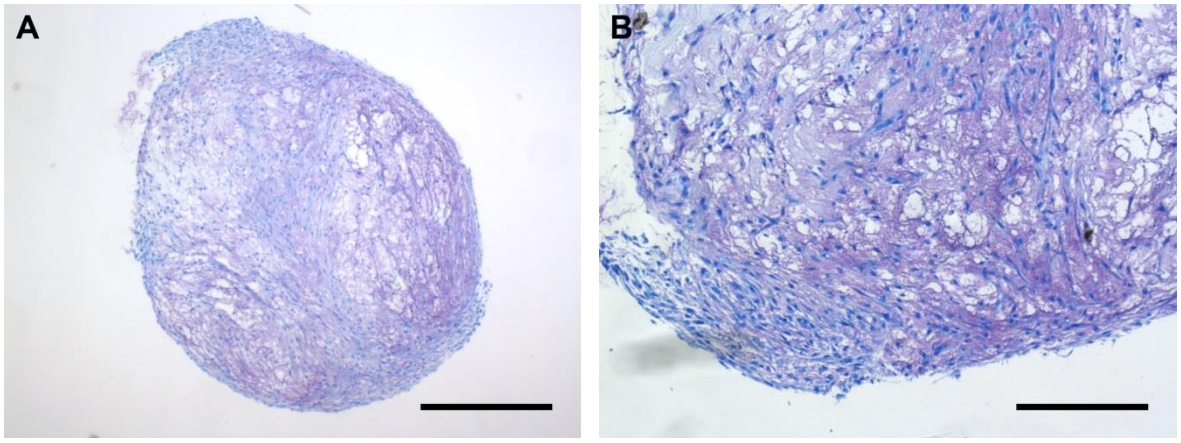


Figure 27 | Toluidine blue staining of chondrogenic micromass sections.

A | A micrograph of a complete tissue section of a chondrogenic micromass, stained with Toluidine blue. Vast amounts of glycosaminoglycans were detected within the chondrogenic matrix that was stained purplish with Toluidine blue. (Scale bar: 500 µm) **B** | Higher magnifications revealed the fine structure of the chondrogenic matrix. Clusters of cells (blue) were surrounded with extracellular matrix, stained purplish. (Scale bar: 200 µm). Pictures taken from WJ-MSCs XIX.

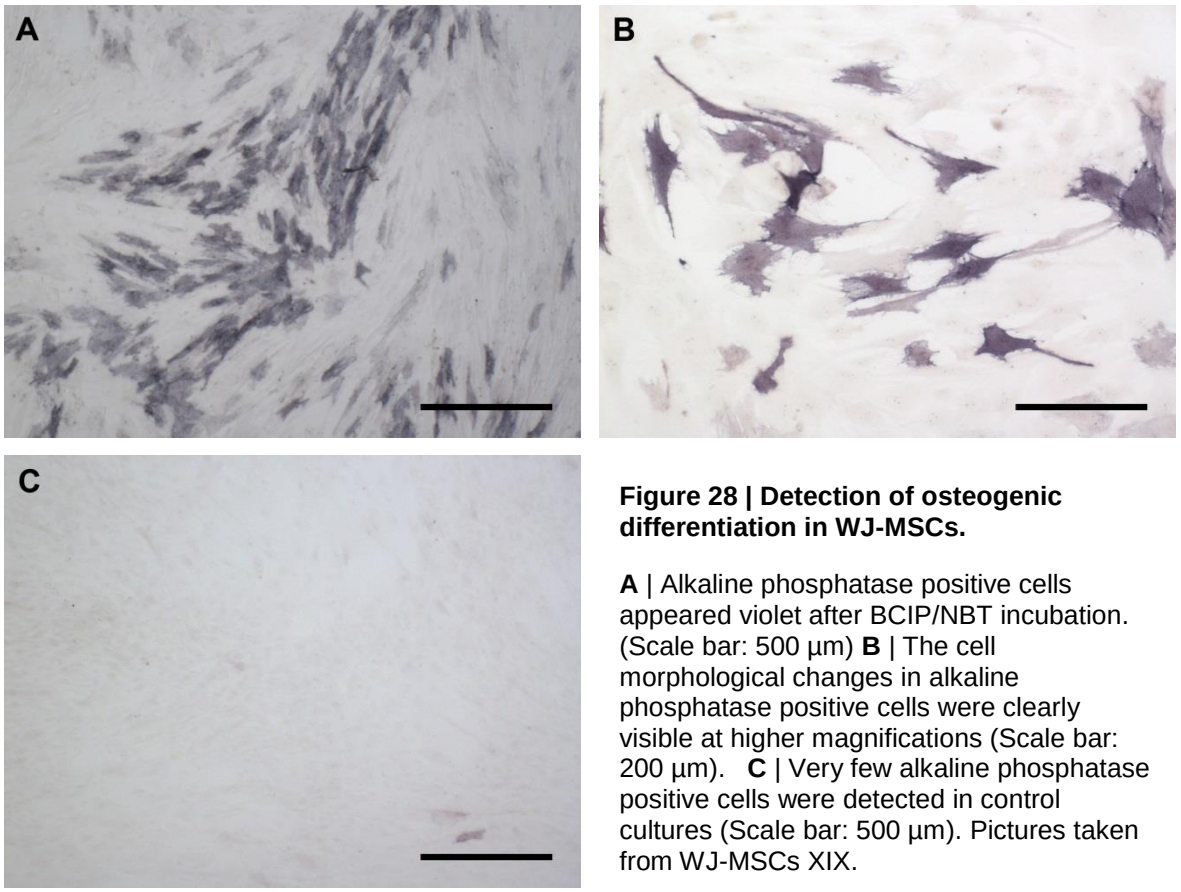
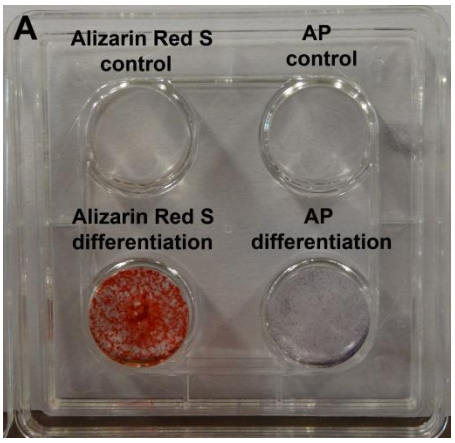


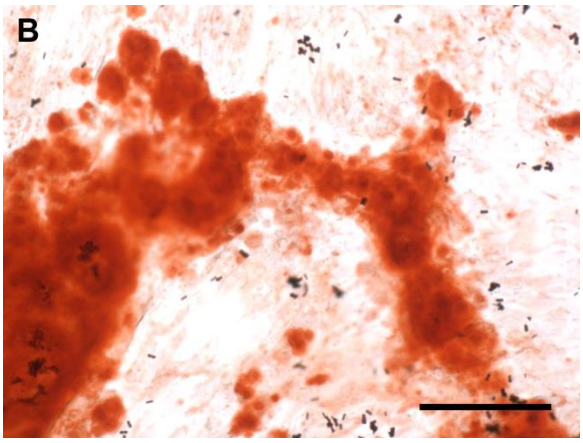
Figure 28 | Detection of osteogenic differentiation in WJ-MSCs.

A | Alkaline phosphatase positive cells appeared violet after BCIP/NBT incubation. (Scale bar: 500 µm) **B** | The cell morphological changes in alkaline phosphatase positive cells were clearly visible at higher magnifications (Scale bar: 200 µm). **C** | Very few alkaline phosphatase positive cells were detected in control cultures (Scale bar: 500 µm). Pictures taken from WJ-MSCs XIX.

WJ-MSCs were cultured in osteogenic differentiation medium for up to 14 days and alkaline phosphatase activity, a hallmark of osteoblasts, was detected as described in Materials & Methods. During osteogenic differentiation, a majority of cells changed their morphology from a spindle-shaped to a cuboidal or polygonal morphology, whereas the cells in control medium retained their spindle-shaped morphology. A high alkaline phosphatase activity was detected in differentiated cells but not in the control cells (Figure 28A). Most of the alkaline phosphatase positive cells had a cuboidal or polygonal morphology (Figure 28B). In the control cultures, alkaline phosphatase activity was detected in a rare population of cells (Figure 28C). The characteristic alkaline phosphatase activity in osteogenic differentiated cells was detected in all four different WJ-MSC cultures.

In addition to the alkaline phosphatase activity, the formation of a calcified extracellular matrix in osteogenic induced cultures was tested by Alizarin Red S staining as described in Materials & Methods.





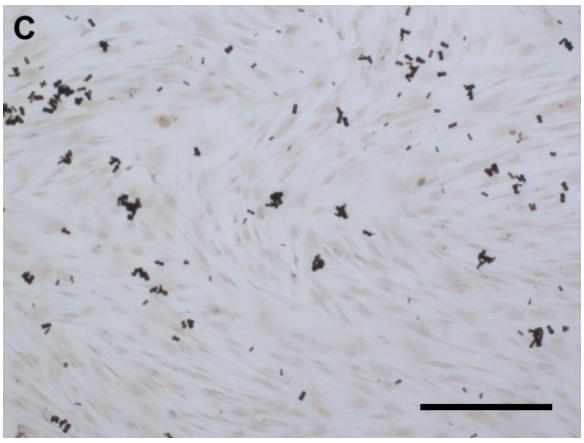


Figure 29 | Detection of a calcified extracellular matrix in osteogenic cultures of WJ-MSCs.

A | Picture of a 4 well plate after the staining for alkaline phosphatase and Alizarin Red S. No extracellular matrix was detected in the control well but massive amounts of calcified extracellular matrix were detected in the differentiation well. A high alkaline phosphatase activity was detected in the second differentiation well and a minor amount was detected in the control cultures. B | The intensely red stained extracellular matrix was clearly distinguished from the slightly reddish cells (Scale bar: 500 µm). **C** | In the control cultures no calcified extra cellular matrix was detected. The black spots were identified as precipitated crystals from the Alizarin Red S staining solution (Scale bar: 500 µm). Pictures taken from WJ-MSCs XXII.

A calcified extracellular matrix could not be detected in osteogenic cultures that were seeded at lower cell densities (according to the standard protocol provided by Miltenyi) after 14 days of differentiation. Therefore, WJ-MSCs were seeded at higher cell densities and differentiated for 21 days as described in Material & Methods. A calcified extracellular matrix was detected in those cultures but not in control cultures. The extracellular matrix was stained intensely red with Alizarin Red S (Figure 29A and 29B).

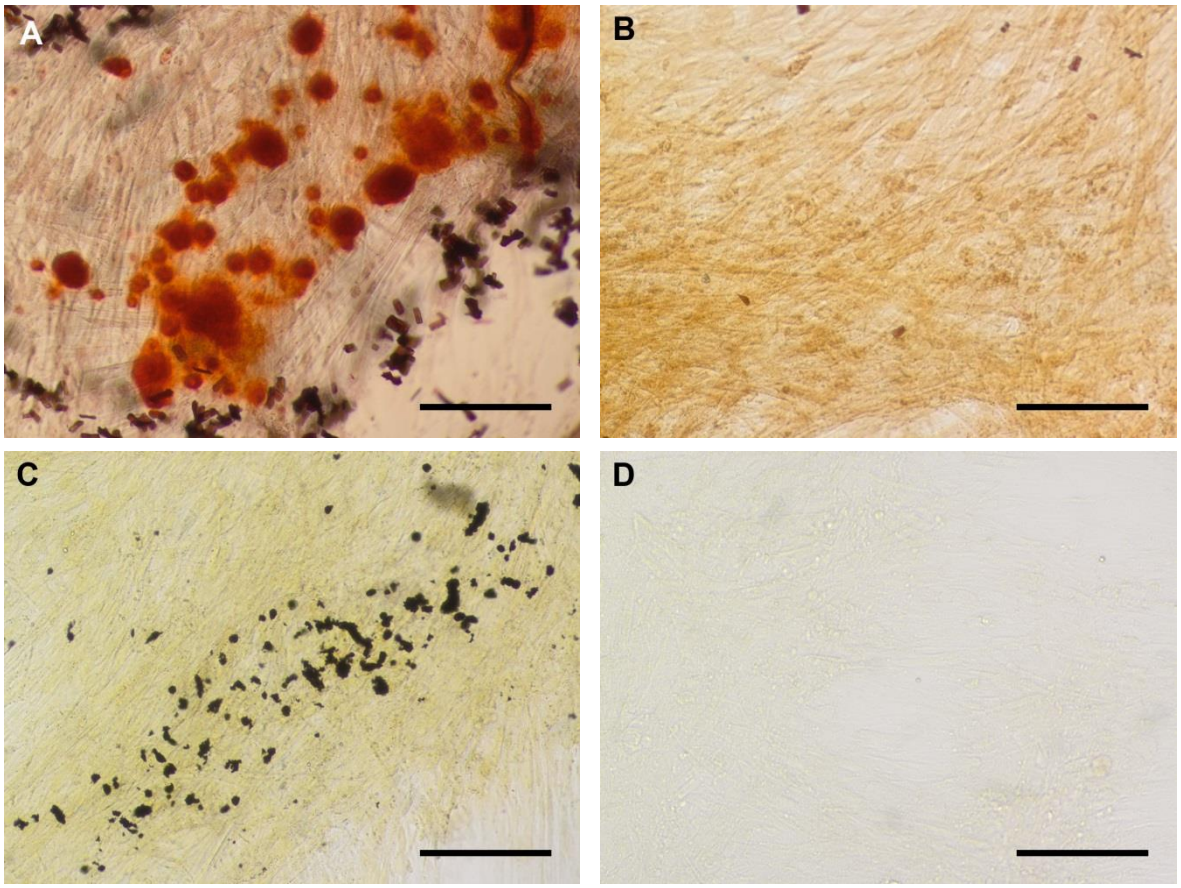


Figure 30 | Alizarin Red S and von Kossa staining of untreated and formic acid treated WJ-MSCs.

A | In untreated osteogenic cultures the calcified extracellular matrix was stained intensely red with Alizarin Red S. The black and red crystals were precipitated Alizarin Red S (Scale bar: 200 µm). **B** | After the formic acid treatment of osteogenic cultures the cells were stained reddish but no calcified extracellular matrix was detected (Scale bar: 200 µm). **C** | Calcium phosphate deposits in the extracellular matrix appeared as black spots after von Kossa staining (Scale bar: 200 µm). **D** | After the formic acid treatment no calcium deposits were detected (Scale bar: 200 µm). Pictures taken from WJ-MSCs XXII.

In addition to Alizarin Red S staining, the calcified extracellular matrix was detected by the von Kossa method, which results in a specific black staining of calcium phosphate deposits in the extracellular matrix. In contrast to Alizarin Red S, the silver ions react with phosphate ions instead of calcium. The specificity of

both staining reactions was verified with formic acid treatment. After the treatment with formic acid the osteogenic cultures were stained with Alizarin Red S or von Kossa as described in Materials & Methods. The formic acid treated cultures showed no Alizarin Red S or von Kossa staining (Figure 30B and 30D). This result demonstrated that the staining was specific for a calcified extracellular matrix, which was dissolved by formic acid treatment.

3.2.3. Time-course analysis of osteogenic differentiation by q-PCR

The expression of the osteogenic marker genes *RUNX2, BGLAP, COL1A1, SPP1, ALPL,* and *SP7,* was analyzed over a period of 30 days in osteogenic differentiated WJ-MSCs. Cell pellets were frozen at different time points and q-PCR was prepared from total isolated RNA from each time point according to the protocol outlined in Materials & Methods. The expression level of each osteogenic marker gene was calculated in relation to the expression of the according gene in WJ-MSCs cultured in control medium at day 6, as described in Materials & Methods (Figure 31).

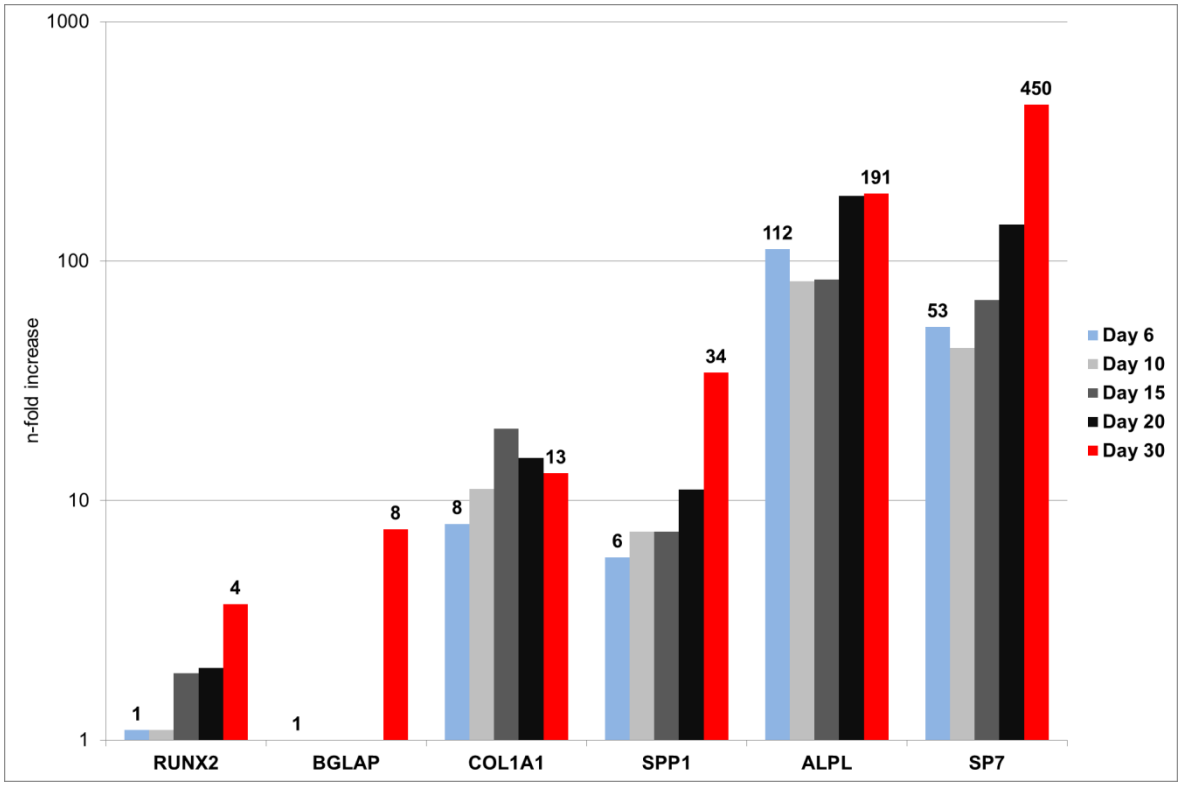


Figure 31 | Expression of osteogenic marker genes during osteogenic differentiation of WJ-MSCs.

The expression of osteogenic marker genes was analyzed at different time points during 30 days of osteogenic differentiation. The increase in expression was calculated in relation to the expression of these marker genes in WJ-MSCs cultured in control medium at day 6. The expression of the different genes was normalized by the housekeeper hPRT1 and was calculated with the ddCT method as described in Materials & Methods.

All osteogenic marker genes analyzed showed an increased expression compared to control cells at day 30. Interestingly, the expression of RUNX2, a master regulator of osteogenic differentiation, only increased after day 15 and showed the least increase at day 30 from all six analyzed osteogenic marker genes.

The expression of BGLAP increased at later stages of differentiation, which was consistent with the literature (Karsenty 2008) where it was demonstrated that BGLAP was expressed at later stages of osteogenic differentiation. The expression of COL1A1 and SPP1, both components of the extracellular matrix, was markedly increased from day 6 onward. The expression of COL1A1 increased slightly from 8-fold at day 6, peaked at day 15 with a 20 fold increased expression and declined afterwards to a 13-fold higher expression at day 30. SPP1 was much stronger expressed at day 30, with a 34 fold up-regulation relative to day 0, compared to day 6 where a 6 fold up-regulation was detected. The strongest increase in expression was detected for the osteogenic markers ALPL and SP7. ALPL was highly expressed in osteogenic differentiated cells at day 6, with a 112-fold increased expression. This was consistent with the detected alkaline phosphatase activity in osteogenic cultures that showed a high AP-activity in differentiated cells and a low AP-activity in control cells. The expression of ALPL was slightly lower at day 10 and 15 but increased afterwards until day 30 with a 191-fold increased expression. For the time-course analysis WJ-MSCs were seeded into 6 wells of a 12 well plate and cultured in osteogenic differentiation medium. At the indicated time points the cells of a single well were harvested and the total RNA was isolated. Despite the fact that the cells were cultured under identical conditions, differences in the osteogenic differentiation between the wells cannot be ruled out, which could explain the decreased ALPL expression at day 10 and 15. The highest increase in expression was detected in SP7, a master regulator in osteogenic differentiation, which was increased 53-fold at day 6 and peaked at 450-fold at day 30.

3.3. Generation of reporter constructs

The upstream promoter regions of five osteogenic marker genes were cloned into the pGreenFire (pGF) lentiviral pathway reporter vector. Therefore, two primer pairs were designed to amplify the promoter region from genomic DNA with nested PCR. Restriction sites were added to the inner primers, which allowed the directional cloning of the promoter regions. The successfully amplified promoter regions were digested and the purified promoter regions were ligated into the pGreenFire lentiviral pathway reporter vector. The sequence of the ligated PCR product was determined by sequencing and the obtained sequence was aligned against the genomic sequence in the Ensembl database.

3.3.1. Cloning of the BGLAP promoter fragment

First, a PCR reaction with the P1 primers and 60°C annealing temperature was performed as described in Materials & Methods. The PCR reaction was analyzed by agarose gel electrophoresis and a PCR product of about 1600 bp was obtained, which was slightly larger than the expected size of 1460 bp (Figure 32A). The reaction mixture of the first PCR reaction was used as a template for the PCR reaction with the P2 primers. The obtained PCR product was digested with EcoRI/BamHI and purified by gel electrophoresis. The digested PCR product ran above the expected 1371 bp at about 1500 bp (Figure 32B).

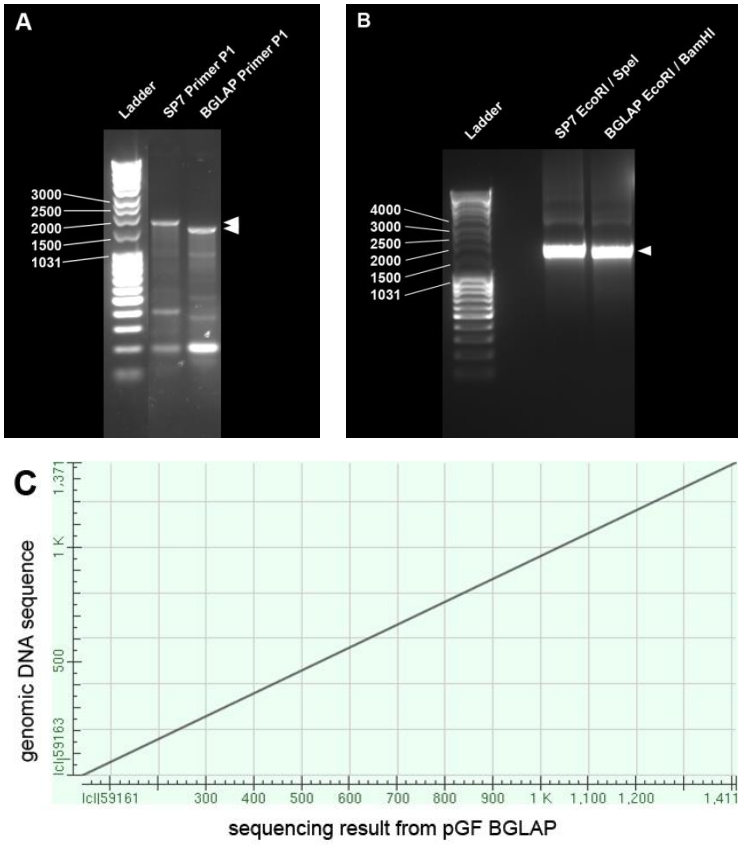


Figure 32 | Cloning of the BGLAP promoter fragment into the pGF vector.

A | Control gel of the BGLAP PCR reaction with primer pair 1. The PCR product had an expected size of 1460 bp and ran slightly above the 1500 bp ladder band. **B** | Preparative gel of the EcoRI/BamHI digested BGLAP promoter fragment. The expected size of the promoter fragment was 1371 bp, but it ran above the 1500 bp band. **C** | Dot matrix representation of the sequence alignment between the genomic sequence of the BGLAP promoter and the sequence of the cloned BGLAP promoter fragment. No major gaps or mismatches were detected in the cloned promoter sequence (processed with Align Sequences Nucleotide BLAST).

The promoter region was ligated into the pGreenFire vector using the standard ligation protocol described in Materials & Methods. The ligated PCR product was sequenced and the obtained sequence was aligned against the genomic sequence of the BGLAP promoter region. The alignment confirmed that the correct sequence was inserted into the pGreenFire vector and that the sequence contained no gaps or a high number of mismatches (Figure 32C).

3.3.2. Cloning of the SP7 promoter fragment

A PCR reaction with Pfu polymerase and the P1 primers, with an annealing temperature of 60°C, was performed as described in Materials & Methods. The PCR reaction was analyzed by gel electrophoresis and a PCR product of about 1800 bp was obtained (Figure 33A).

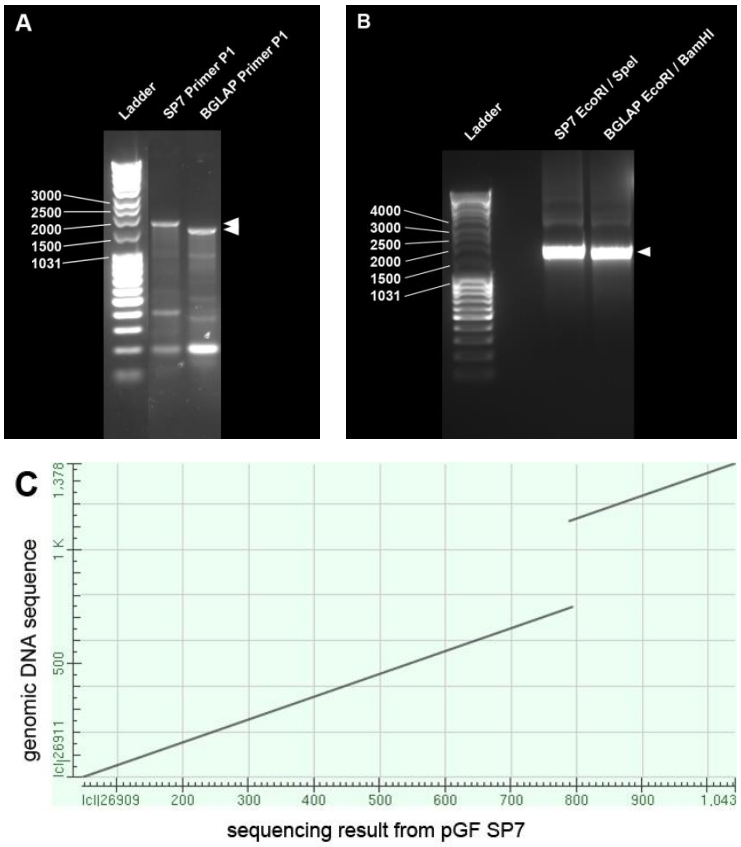


Figure 33 | Cloning of the SP7 promoter fragment into the pGF vector.

A | On the control gel from the SP7 PCR reaction with the P1 primers, a PCR product of about 1800 bp was detected. **B** | The EcoRI/SpeI digested SP7 promoter fragment ran slightly above the BGLAP fragment, which had about the same size. **C** | Dot matrix representation of the sequence alignment between the genomic sequence of the SP7 promoter and the sequence of the cloned SP7 promoter fragment. The gap in the alignment represented the missing 380 bp that were not sequenced, because of a high GC content or secondary structures in the template (processed with Align Sequences Nucleotide BLAST).

The obtained PCR product served as a template for the PCR with the P2 primers. The product of the second PCR reaction was digested with EcoRI/SpeI, analyzed by gel electrophoresis, and purified from gel. The digested SP7 promoter fragment ran slightly above the BGLAP promoter fragment at about 1550 bp and was larger than the expected 1378 bp (Figure 33B). This result was consistent with BGLAP PCR product which also ran about 200 bp higher than expected. After the successful ligation into the vector, the ligated promoter fragment was sequenced.

The sequencing reaction on the reverse strand stopped after about 300 bp, which was a result of the high GC content or prominent secondary structures within the sequence. Therefore, the complete sequence of the insert was not obtained and a gap of 380 bp appeared in the dot matrix plot (Figure 33C). However, the obtained sequences were aligned with the genomic sequence of the SP7 promoter in the Ensembl database and corresponded to the genomic sequence.

3.3.3. Cloning of the SPP1 promoter fragment

The amplification of the SPP1 promoter region was performed with the GC 2 Advantage PCR kit, because the correct PCR product was not obtained with the standard Pfu PCR protocol. A PCR product of the correct size of 1500 bp was obtained with the GC 2 Advantage PCR kit and an annealing temperature of 68°C for the P1 primers (Figure 34A). The first PCR reaction mixture was used as template for the amplification with the P2 primers and the obtained PCR product was purified by gel electrophoresis. The purified product was digested with MfeI for 2 hours at 37°C and with BclI for another 2 hours at 50°C. The digested promoter fragment was gel purified and ran at 1600 bp which was slightly above the expected size of 1440 bp (Figure 34B).

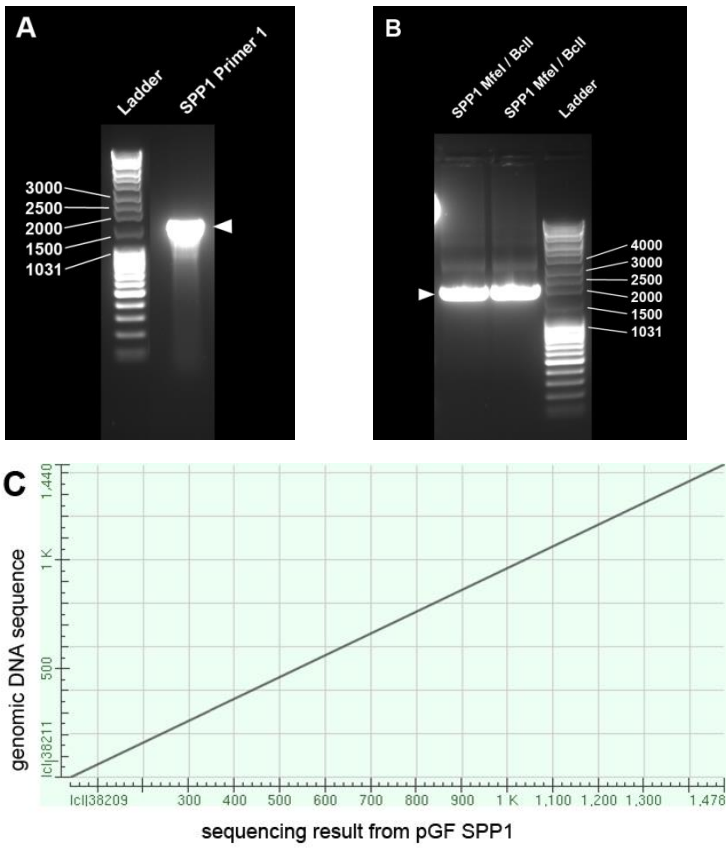


Figure 34 | Cloning of the SPP1 promoter fragment into the pGF vector.

A | On the control gel, of the PCR reaction with the P1 primers, a PCR product of the correct size of 1500 bp was detected. **B** | After the digest with MfeI/BclI the promoter fragment was purified from gel. The promoter fragment ran slightly higher than the expected 1440 bp. **C** | The sequence alignment between the genomic and the cloned sequence of the SPP1 promoter confirmed that the correct sequence was ligated into the pGreenFire vector (processed with Align Sequences Nucleotide BLAST).

The SPP1 promoter fragment was ligated into the pGreenFire vector using the optimized ligation protocol. The ligated PCR product was sequenced and the obtained sequence was aligned against the genomic sequence of the SPP1 promoter in the Ensembl database (Figure 34C). The sequence alignment confirmed that the cloned PCR product was the desired promoter region.

3.3.4. Cloning of the ALPL promoter fragment

The PCR reaction with the P1 primers and an annealing temperature of 64°C yielded a PCR product of the correct size of 1687 bp with less side products than the reaction at an annealing temperature of 60.3°C (Figure 35A).

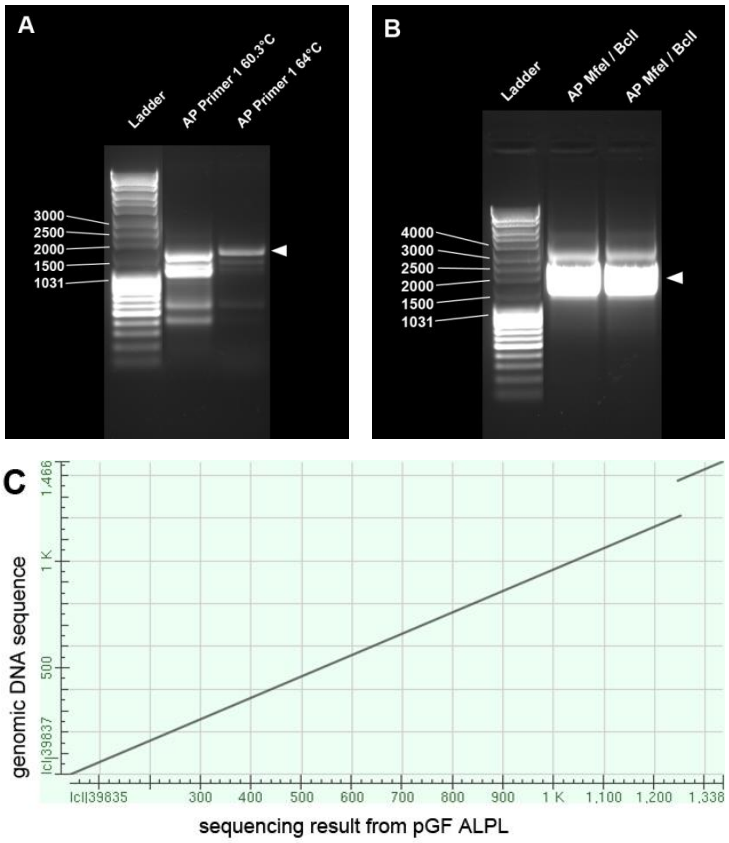


Figure 35 | Cloning of the ALPL promoter fragment into the pGF vector.

A | Analysis of PCR reactions with different annealing temperatures. At 64°C the correct PCR product of 1687 bp, with a minimal amount of byproducts, was obtained. **B** | Preparative gel of the ALPL promoter fragment after the digest with MfeI/BclI. The digested product ran at the correct size of1500 bp. **C** | Dot matrix representation of the sequence alignment between the cloned and the genomic ALPL sequence. The gap in the alignment corresponds to the 162 bp that were not sequenced because of the high GC content and repetitive sequences. The sequence alignment confirmed that the correct sequence was cloned (processed with Align Sequences Nucleotide BLAST).

The reaction mixture of the first PCR reaction was used as template for the reaction with the P2 primers. The obtained PCR product was purified from gel and digested with BclI for 6 hours at 50°C and MfeI at 37°C overnight. The digested promoter fragment was analyzed and purified by gel electrophoresis and ran at the correct size of 1466 bp (Figure 35B). The purified ALPL promoter fragment was ligated into the pGreenFire vector using the optimized ligation protocol. The sequencing reaction on the reverse strand stopped after 93 bp because of the high GC content of the sequence and 162 bp were not sequenced (Figure 35C).

However, the obtained sequences were aligned with the genomic sequence of the ALPL promoter without gaps and confirmed the successful ligation of the ALPL promoter fragment.

3.3.5. Cloning of the COL1A1 promoter fragment

Analysis of the PCR reaction with the P1 primers with gel electrophoresis revealed two by-products with a size of about 900 bp and 1100 bp (Figure 36A) and a PCR product of about 2900 bp, which matched the expected size of the product with 2800 bp. The 2900 bp band was purified from gel and used as template for the PCR reaction with the P2 primers. The obtained PCR product was digested with ClaI/SpeI at 37°C overnight and purified by gel electrophoresis (Figure 36B). The COL1A1 promoter fragment was ligated into the pGreenFire vector using the optimized ligation protocol and the ligated promoter fragment was sequenced. The obtained sequence was aligned against the genomic sequence of the COL1A1 promoter (Figure 36C). The sequence alignment confirmed that the correct promoter sequence was cloned into the pGreenFire vector and that there were no major mismatches within the sequence.

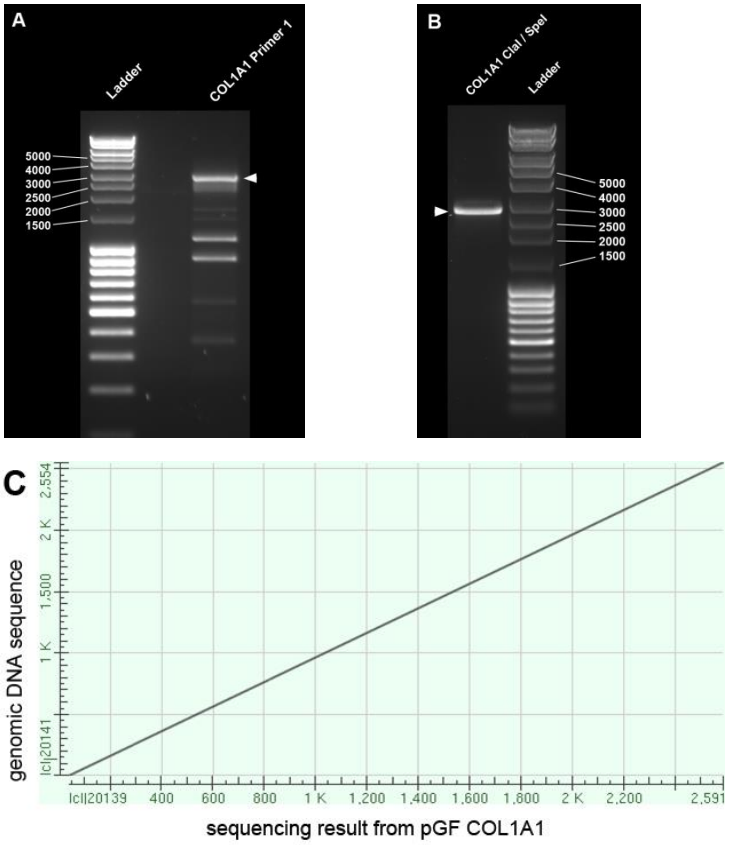


Figure 36 | Cloning of the COL1A1 promoter fragment into the pGF vector.

A | On the control gel of the PCR reaction with the P1 primers a number of byproducts were detected. The desired PCR product ran slightly below the 3000 bp band. **B** | The digested COL1A1 promoter fragment, ran above the expected 2554 bp at ~2900 bp. **C** | Dot matrix representation of the sequence alignment between the cloned and genomic COL1A1 sequence. The alignment confirmed that the correct sequence was cloned into the pGF vector (processed with Align Sequences Nucleotide BLAST).

3.4. Generation of WJ-MSC reporter cells

3.4.1. Puromycin titration for WJ-MSCs

The optimal concentration for the selection of transduced WJ-MSCs was determined by treating the cells with increasing concentrations of puromycin. The optimal puromycin concentration was defined as “the lowest concentration that kills 100% of the cells after five days of incubation”. WJ-MSCs were incubated in NH Expansion medium containing 0, 0.1, 0.25, 0.50, 0.75, 1.0 µg puromycin/ml. After five days the cells were stained with crystal violet, as described in Materials & Methods (Figure 37). At day five, all cells were killed in the wells that contained a higher puromycin concentration than 0.25 µg/ml. The effects of the puromycin treatment were also visible in the wells containing 0.1 and 0.25 µg puromycin per milliliter. In the well containing 0.25 µg puromycin/ml a large amount of cells were killed after five days and only cells in the middle of the well remained attached. Even at a concentration of 0.1 µg/ml WJ-MSCs had a reduced viability compared to WJ-MSCs in control medium. The optimal puromycin concentration for the selection of transduced WJ-MSCs was determined to be 0.5 µg puromycin/ml.

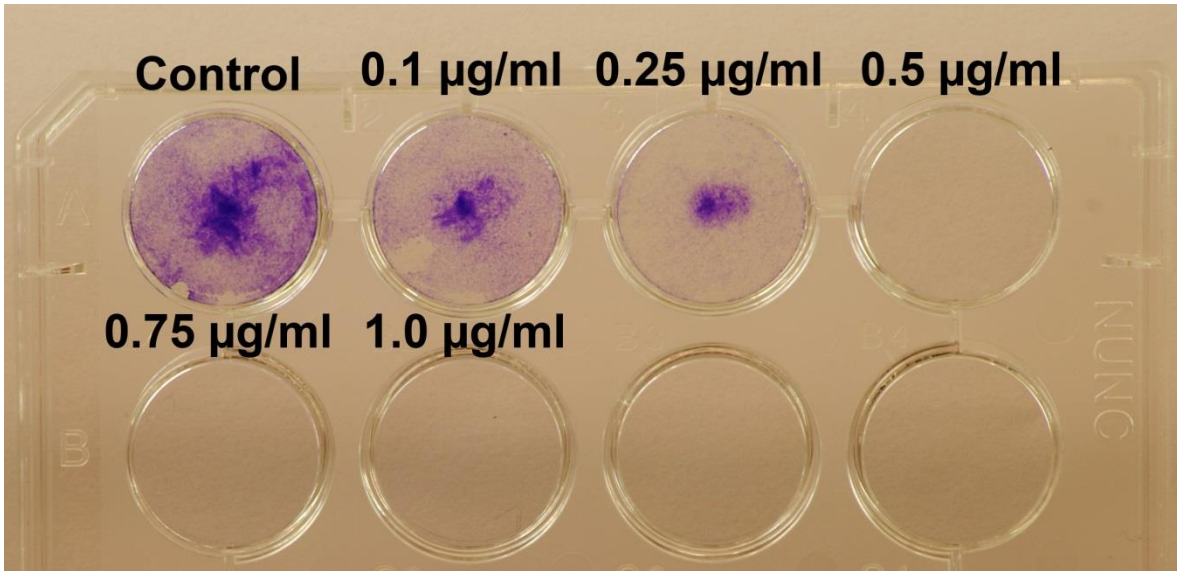


Figure 37 | Crystal violet staining of WJ-MSCs after puromycin selection.

After five days of puromycin treatment the wells were stained with crystal violet for visualization of the remaining WJ-MSCs. In the wells with a puromycin concentration of 0.5, 0.75, and 1.0 µg/ml all cells were killed. In the well with 0.25 µg/ml a large amount of the cells were killed compared to the control well and also in the well with 0.1 µg/ml a reduced viability in WJ-MSCs was visible compared to the control well.

3.4.2. Transduction and selection of WJ-MSC reporter cells

WJ-MSCs XIX were transduced with the pGreenFire lentiviral pathway reporter vectors that contained one of five different promoter regions of osteogenic marker genes, the empty pGreenFire vector (pGF), or as positive control the pGreenFire with a functional CMV promoter, as described in Materials & Methods. After the transduction, stably transduced cells were selected with puromycin as described. After the selection process, the WJ-MSC reporter cells were differentiated into osteoblasts and excess reporter cells were frozen and stored in liquid nitrogen, as described (Table 4).

Label	Cells per aliquot	Number of aliquots	Date
WJ-MSC XIX AP	6.8x10^5	1	30/12/12
WJ-MSC XIX SPP1	1.3x10^6	2	30/12/12
WJ-MSC XIX COL1A1	4.6x10^5	1	30/12/12
WJ-MSC XIX pGF	7.9x10^5	2	30/12/12
WJ-MSC XIX CMV	2.3x10^5	1	30/12/12
WJ-MSC XIX BGLAP	2.9x10^5	1	30/12/12
WJ-MSC XIX SP7	2.3x10^5	1	30/12/12

Table 4 | Overview of frozen aliquots of WJ-MSC XIX reporter cells.

The number of obtained cells after the selection varied dramatically between the different reporter constructs. A possible explanation was that the inserted promoter sequence had an influence on the production of lentiviral particles or on the integration of the reporter construct into the genome of the cells. Another possibility was that the viral titer varied between different virus productions. The highest cell numbers where obtained with the empty pGreenFire vector and the pGreenFire SPP1 vector.

Preliminary results obtained with the WJ-MSC XIX reporter cells indicated that the SPP1 and COL1A1 reporter construct had the highest luciferase expression during osteogenic differentiation and that the other reporter constructs only showed a slightly elevated luciferase expression compared to the empty reporter vector. Therefore, WJ-MSCs from Promocell were only transduced with the pGreenFire, pGreenFire SPP1, and pGreenFire COL1A1 reporter constructs. After the selection the obtained reporter cells were differentiated into adipocytes, chondrocytes, and osteoblasts. The remaining cells were frozen as described and stored in liquid nitrogen (Table 5).

Label	Cells per aliqout	Number of aliquots	Date
WJ-MSC Promocell pGF	1.0x10^6	1	21/01/13
WJ-MSC Promocell SPP1	9.5x10^5	2	21/01/13
WJ-MSC Promocell COL1A1	4.2x10^5	1	21/01/13

Table 5 | Overview of frozen aliqouts of WJ-MSC Promocell reporter cells.

3.5. Analysis of WJ-MSC reporter cells

3.5.1. Detection of GFP expression in reporter cells by fluorescence microscopy

WJ-MSC reporter cells were differentiated for 15 days in osteogenic differentiation medium and the GFP expression was monitored by fluorescence microscopy. In cultures of WJ-MSCs, transduced with the empty pGreenFire vector, a faint fluorescence was detected in osteogenic differentiated cells (Figure 38A). This fluorescence was detected in almost all cells. A characteristic hallmark was that the fluorescence was spread around the nucleus and within the nucleus, no fluorescence was visible. This upregulation in fluorescence was only detectable in cells that were cultured in osteogenic differentiation medium. In WJ-MSCs, cultured in control medium, fluorescence was only detected in a rare population of cells (Figure 38B).

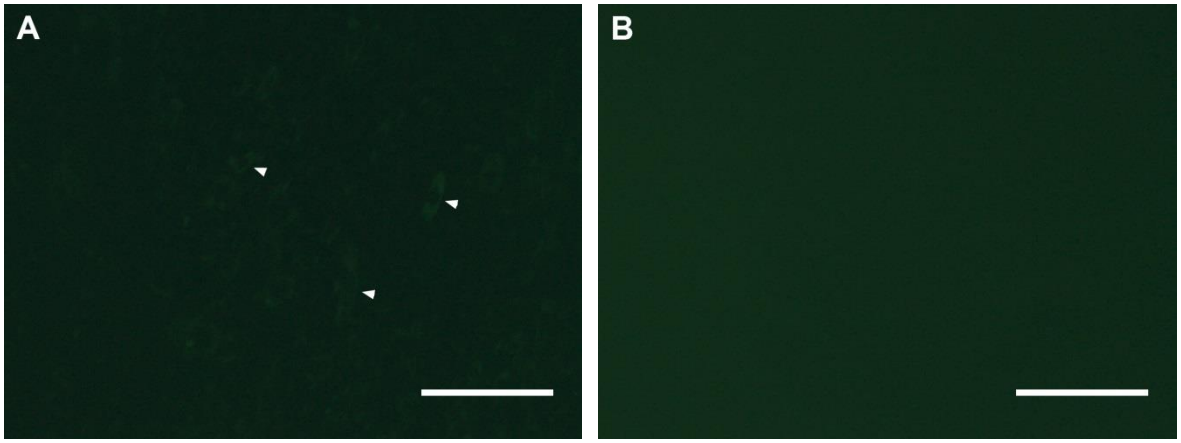


Figure 38 | GFP expression in WJ-MSCs XIX transduced with the empty pGreenFire vector.

A | During osteogenic differentiation of WJ-MSCs, transduced with the empty pGreenFire vector, an increased fluorescence was detectable in cells by fluorescence microscopy. The fluorescence was concentrated around the nucleus but not within the nucleus (arrowheads). (Scale bar: 200 µm). **B** | WJ-MSCs transduced with the empty vector, that were cultured in control medium, did not develop a similar fluorescence as cells that were cultured in differentiation medium (Scale bar: 200 µm).

WJ-MSCs transduced with the pGreenFire vector containing a functional CMV promoter were used as a positive control for the GFP and luciferase expression. In these cells, a strong GFP expression was detected in osteogenic differentiated and control cells (Figure 39A and 39B). The osteogenic differentiated cells contained a population of very bright cells (Figure 39A), compared to cells grown in control medium (Figure 39B). WJ-MSCs transduced with the pGreenFire CMV construct were also used as a control for the effectiveness of the selection process. In most of the pGreenFire CMV transduced cells a strong GFP expression could be detected with a fluorescence microscope, which indicated that the puromycin selection efficiently eliminated the non-transduced cells.

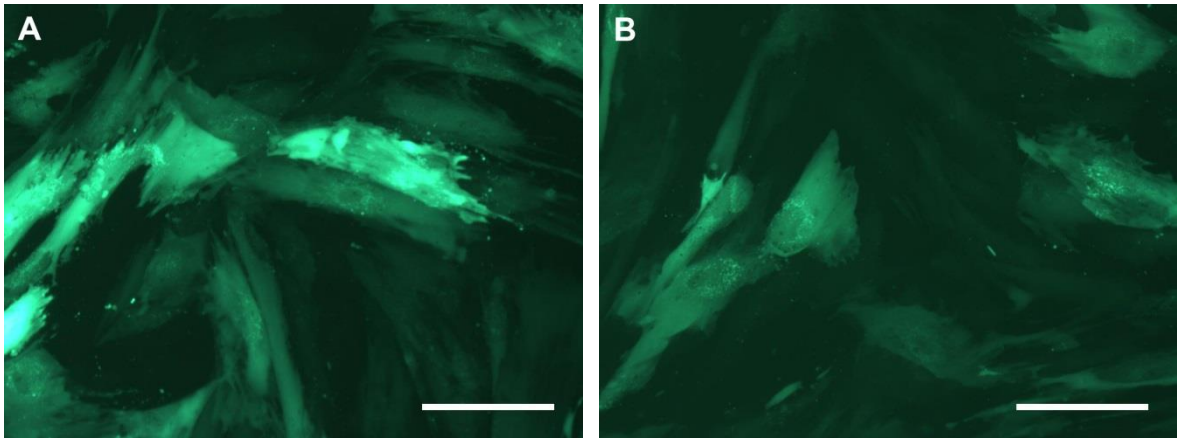


Figure 39 | GFP expression in WJ-MSC XXII CMV reporter cells.

A | WJ-MSCs that were transduced with the pGreenFire CMV construct showed a strong expression of GFP in the majority of cells. (Scale bar: 200 µm). **B** | Cells cultured in control medium appeared slightly dimmer in the fluorescence microscope then cells grown in differentiation medium, but still had a high GFP expression (Scale bar: 200 µm).

WJ-MSCs that were transduced with pGreenFire ALPL, BGLAP, or SP7 constructs had similar fluorescence levels as WJ-MSCs transduced with the empty pGreenFire vector. In WJ-MSC, that were transduced with the ALPL, BGLAP, and SP7 construct, and cultured in control medium, a weak fluorescence was detected in a rare population of cells. Cells that were cultured in osteogenic differentiation medium were much brighter and had a similar fluorescence distribution compared to cells that were transduced with the empty vector, where the fluorescence was concentrated around the nucleus and absent within the nucleus. Most of the cells displayed this pattern of fluorescence, which was considered autofluorescence and indicated that the introduced reporter constructs were not active during osteogenic differentiation of the WJ-MSCs.

GFP expression was detected in osteogenic differentiated WJ-MSCs that were transduced with pGreenFire COL1A1 and SPP1 reporter constructs. In WJ-MSCs transduced with pGreenFire COL1A1, a population of cells that expressed GFP was detected after 15 days of osteogenic differentiation (Figure 40A). The GFP

expression was weak compared to the CMV construct, but the fluorescence was spread throughout the cytoplasm, including the nucleus. These cells were readily distinguished from cells where the fluorescence was concentrated around the nucleus (Figure 40A, arrowheads). WJ-MSC COL1A1 cells that were cultured in control medium for 15 days also showed an increased fluorescence compared to WJ-MSC transduced with the empty pGreenFire vector, which were also cultured in control medium (Figure 40B). A rare population of cells in the control cultures had similar fluorescence levels compared to cells that were cultured in differentiation medium. However, during tri-lineage differentiation of WJ-MSCs, spontaneous osteogenic differentiation was detected in a very small fraction of cells in control cultures through a positive alkaline phosphatase reaction. A possible explanation was that the GFP positive cells resulted from spontaneous osteogenic differentiated WJ-MSCs.

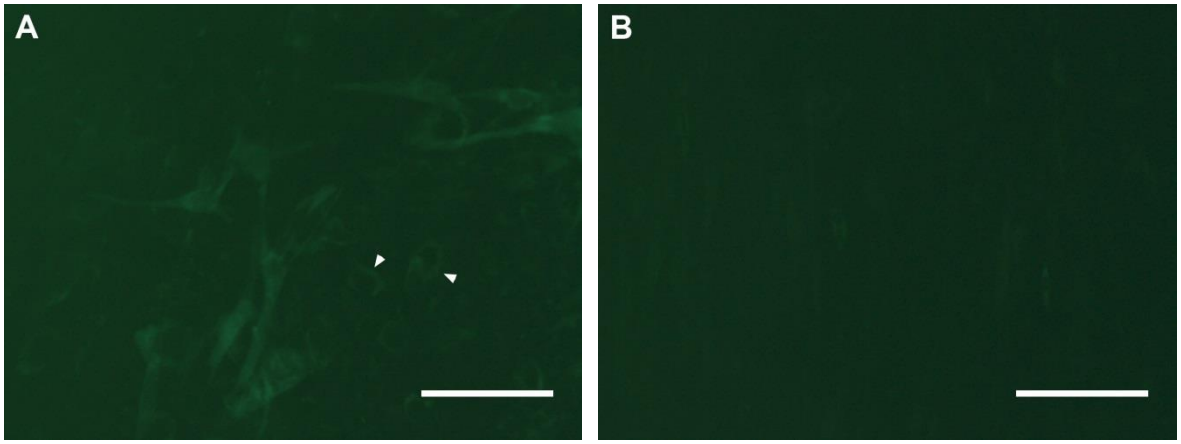


Figure 40 | GFP expression in WJ-MSC XIX COL1A1 reporter cells.

A | During osteogenic differentiation of WJ-MSC COL1A1 reporter cells, a population of GFP positive cells appeared where the fluorescence was spread throughout the cytoplasm compared to cells, where the fluorescence was concentrated around the nucleus.(Scale bar: 200 µm). **B** | To a lesser extent, GFP positive cells were also detected in the control cultures. (Scale bar: 200 µm).

A specific upregulation of the GFP expression during osteogenic differentiation was detected in WJ-MSC transduced with the pGreenFire SPP1 construct. Similar to other WJ-MSCs, there was a high background due to autofluorescence of osteogenic differentiated WJ-MSC (Figure 41A and 41B, arrowheads). A population of cells was detected that had a significantly increased GFP expression compared to the majority of the cells where the fluorescence was concentrated around the nucleus. These cells were also brighter than the GFP positive cells detected in the WJ-MSC COL1A1 cultures (Figure 40A and 40B). WJ-MSC SPP1 cells cultured in control medium had a low autofluorescence with the exception of a very rare population of GFP positive cells, which probably arose from osteogenic differentiated WJ-MSCs.

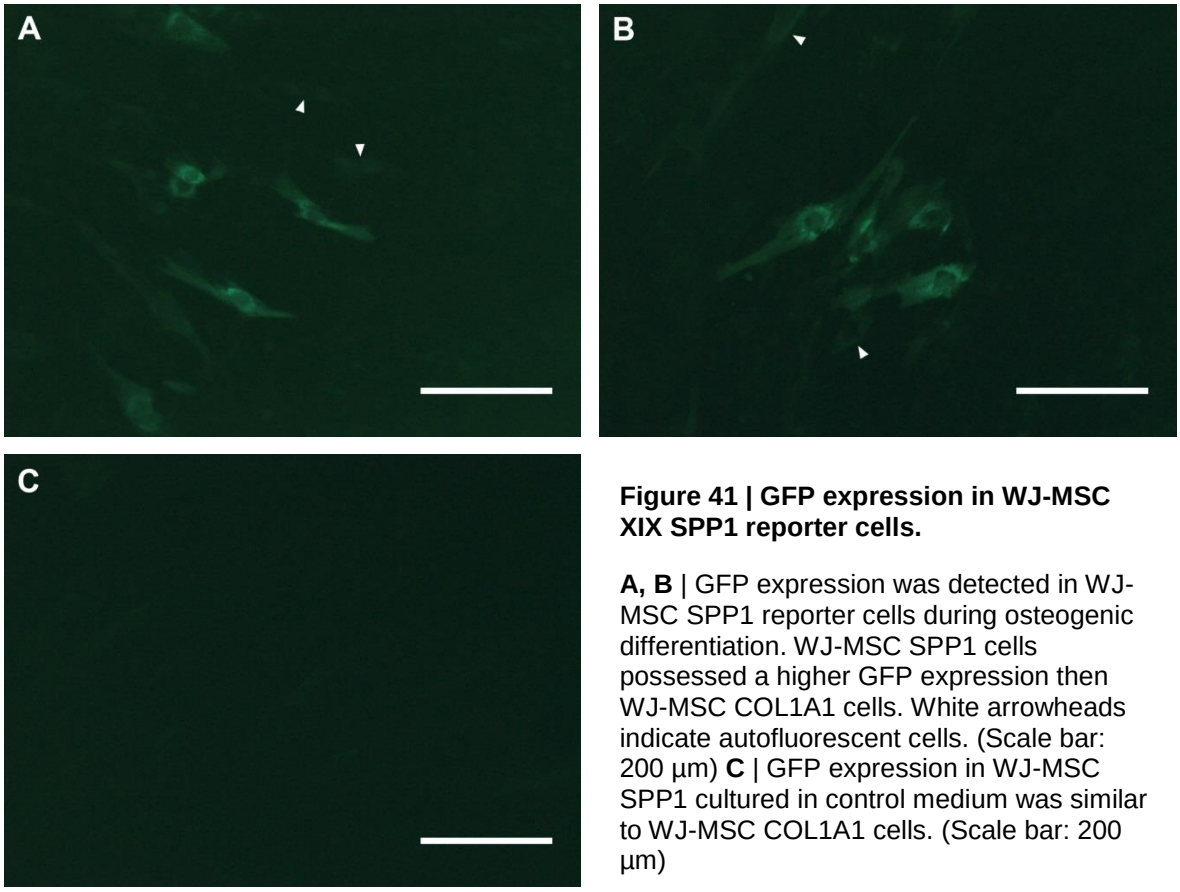


Figure 41 | GFP expression in WJ-MSC XIX SPP1 reporter cells.

A, B | GFP expression was detected in WJ-MSC SPP1 reporter cells during osteogenic differentiation. WJ-MSC SPP1 cells possessed a higher GFP expression then WJ-MSC COL1A1 cells. White arrowheads indicate autofluorescent cells. (Scale bar: 200 µm) **C** | GFP expression in WJ-MSC SPP1 cultured in control medium was similar to WJ-MSC COL1A1 cells. (Scale bar: 200 µm)

3.5.2. Detection of GFP expression by flow cytometry

Analysis of the GFP expression with fluorescence microscopy provided qualitative data for GFP expression during osteogenic differentiation, but was not suitable for the quantification of the extent of osteogenic differentiation. Therefore, the GFP expression was measured with flow cytometry, which provided quantitative data of the GFP expression during osteogenic differentiation of WJ-MSC reporter cells. During osteogenic differentiation WJ-MSC produced an extracellular matrix that prevented the efficient trypsinization of the cells for flow cytometry. Therefore, the GFP expression was measured after 9 days of osteogenic differentiation at a time point were the extracellular matrix was developed to a lesser extent. For each reporter cell type 10000 events were recorded and evaluated with FlowJo as described in Materials & Methods.

The increased fluorescence of osteogenic differentiated WJ-MSCs that was observed with fluorescence microscopy was also detected with flow cytometry (Figure 42). In WJ-MSCs that were transduced with the empty pGreenFire vector a clear shift in the fluorescence intensity between cells that were grown in control medium and cells that were cultured in osteogenic differentiation medium was detected. During osteogenic differentiation about 16% of the cells developed a green fluorescence compared to cells that were cultured in control medium (Figure 42). The high autofluorescence impaired the comparison between control and

differentiated cells and therefore the threshold intensity was determined with differentiated WJ-MSC transduced with the empty pGreenFire vector.

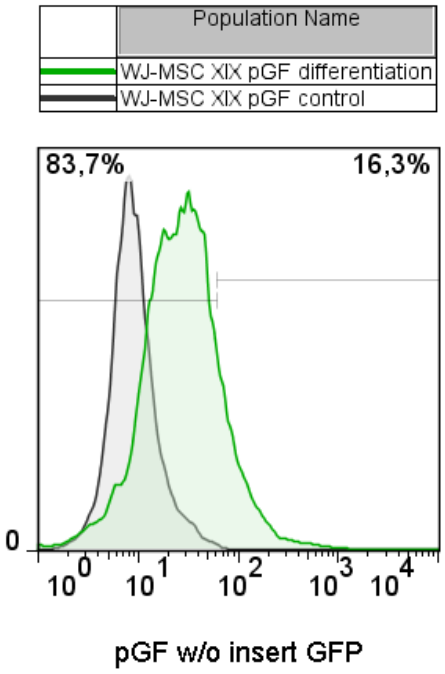


Figure 42 | Increased fluorescence intensity in osteogenically differentiated WJ-MSCs.

An increased fluorescence was detected in WJ-MSCs XIX, transduced with the empty pGreenFire vector, by flow cytometry after osteogenic differentiation. This fluorescence shift was evident when the two peaks were overlapped (Grey: control cells; Green: differentiated cells). After osteogenic differentiation 16% of the cells were positive compared to cell grown in control medium.

The GFP expression was determined for the five different reporter constructs by flow cytometry and compared to the pGreenFire vector without insert (Figure 43). The main peak of fluorescence was nearly identical between the empty vector and the five different reporter constructs. A small peak at slightly higher fluorescence intensities, than the main peak characterized a cell population that expressed more GFP during osteogenic differentiation. The GFP expression was weak so that the peaks were not separated, which impaired the distinction between autofluorescent cells and the GFP positive cells. Flow cytometric analysis confirmed the impression obtained with fluorescence microscopy that, after osteogenic differentiation, only a small fraction of the transduced cells expressed GFP at levels that were high enough for the discrimination of these cells from the background. The highest percentage of GFP positive cells was measured in WJ-MSC COL1A1 and SP7 cells with 6.6 and 6.7%, respectively. Interestingly, only 2.8% of WJ-SPP1 cells were GFP positive despite that WJ-MSC SPP1 cells appeared brighter than WJ-MSC COL1A1 cells in fluorescence microscopy images. WJ-MSC transduced with the pGreenFire CMV positive control vector had a strong GFP expression and reached higher fluorescence intensities. Compared to WJ-MSCs transduced with the empty pGreenFire vector 80% of these cells were clearly GFP positive. The fluorescence intensities of these cells were measured in a range between 10^2 and 10^4.

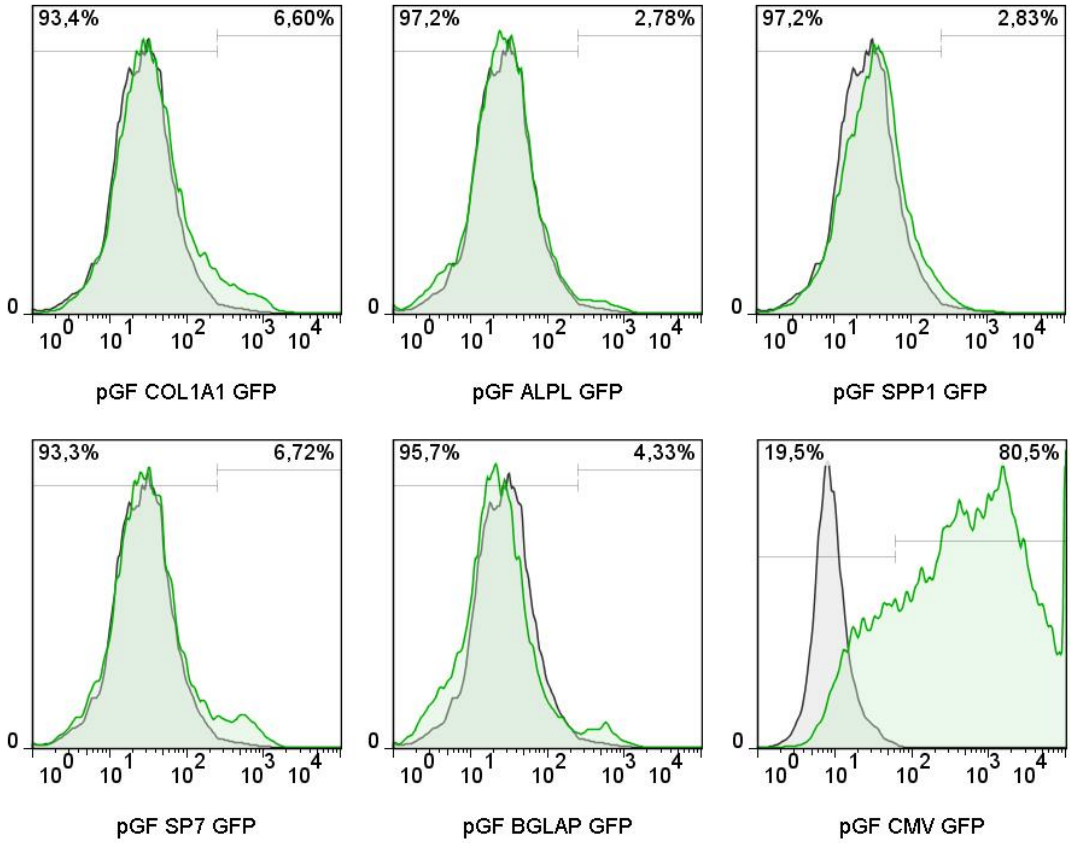


Figure 43 | Flow cytometric analysis of the GFP expression in WJ-MSC XIX reporter cells during osteogenic differentiation.

The majority of cells from the five different reporter cell lines had a similar fluorescence distribution in the flow cytometric analysis compared to cells transduced with the empty pGF vector. GFP positive cells formed a small peak after the main peak. The highest number of GFP positive cells was measured in WJ-MSC SP7 and COL1A1 reporter cells with 6.7% and 6.6%, respectively. 4.3% GFP positive cells were detected in the WJ-MSC BGLAP culture and 2.8% were GFP positive cells were recorded in the WJ-MSC ALPL and SPP1 cultures. Fluorescence intensities in WJ-MSC CMV reporter cells ranged from 10^2 to 10^4 and 80% of these cells were GFP positive.

3.5.3. Detection of luciferase expression in WJ-MSC reporter cells

The high autofluorescence of osteogenically differentiated WJ-MSCs impaired the measurement of the extent of GFP expression during osteogenic differentiation by flow cytometry. Because the pGF vector contains a dual GFP/luciferase reporter, we next tested whether luciferase activity measurements were more informative. Therefore, the luciferase activity was measured in cell lysates of WJ-MSCs after osteogenic differentiation as described in Materials & Methods. The luciferase activity of WJ-MSC XIX reporter cells was measured at three different time points, day 9, day 15, and day 19 (Figure 44).

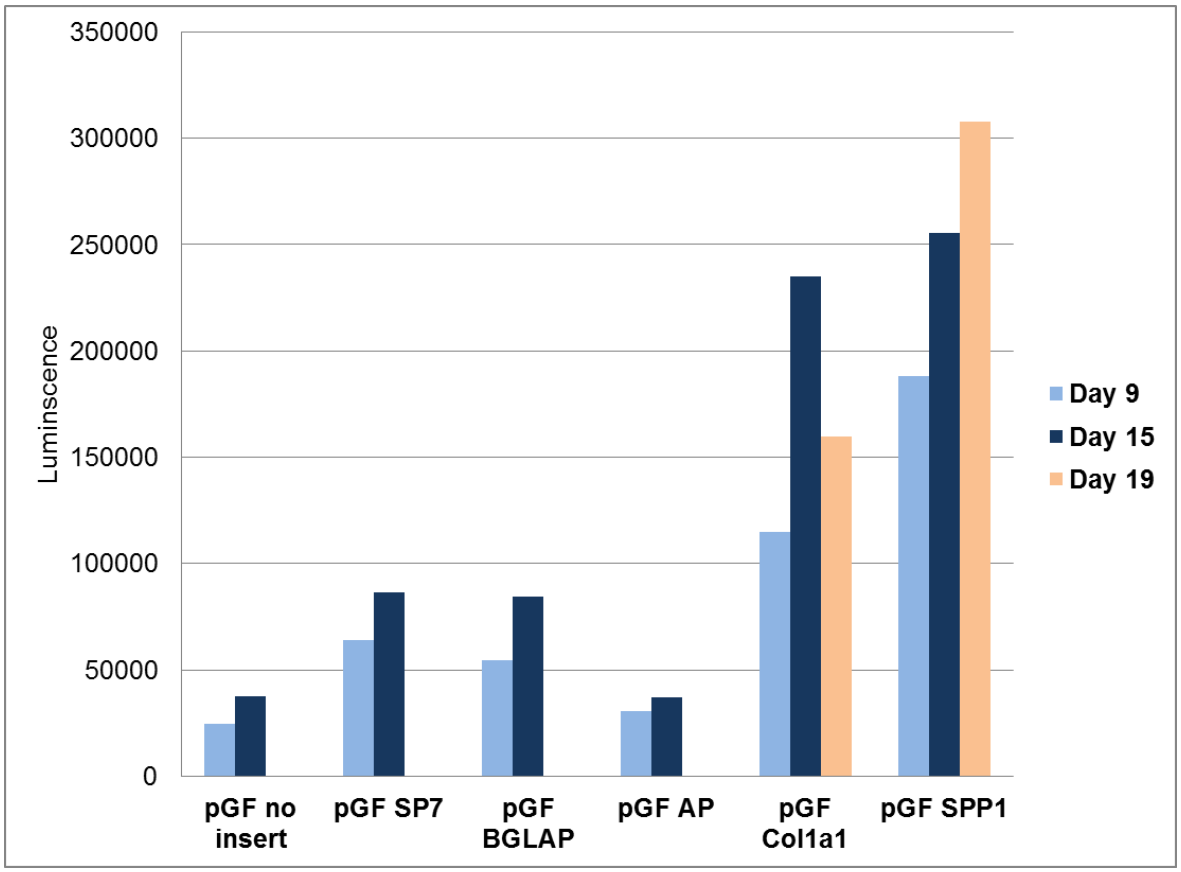


Figure 44 | Luciferase activity of WJ-MSC XIX reporter cells during osteogenic differentiation.

The luciferase expression in the five osteogenic reporter cells was determined and the luminescence was normalized to the protein concentration. The highest luciferase expression was determined in the COL1A1 and SPP1 reporter cells. The luciferase expression in the other three reporter cells was below the 1.0x10^5 units luminescence mark and were not considered for further experiments.

The luminescence was measured in arbitrary units and related to the protein concentration in the cell lysates. After 9 days of osteogenic differentiation, four reporter constructs, SP7, BGLAP, COL1A1, and SPP1, reached a luciferase

activity that was above the level of the empty vector. The highest luminescence was measured in the lysates of the COL1A1 and SPP1 reporter cells with 1.15x10^5 units and 1.88x10^5 units, respectively. At day 15, the luminescence increased in all five reporter constructs. A significant increase compared to day 9 was only measured in the COL1A1 and SPP1 reporter constructs.

The luminescence of the BGLAP and SP7 reporter constructs was below 1.0x10^5 units, which was a 2.29 and 2.23-fold increase compared to the empty pGreenFire. The luciferase activity in the ALPL reporter construct was at the same level as in the empty vector. These results confirmed the impression from the fluorescence microscopy where GFP positive cells were only detected in the COL1A1 and SPP1 reporter cells. Interestingly, the results of the luciferase measurements from the SP7 and SPP1 reporter cells contradicted the results from the flow cytometry. The SP7 cells had a high number (6.7%) and the SPP1 reporter cells a very low number (2.8%) of GFP positive cells. The measurement of the luciferase expression revealed that SPP1 reporter cells had the highest reporter gene expression and SP7 cells a very low expression. Reporter constructs that did not reached a luminescence of 1.0x10^5 units until day 15 were excluded from further studies. Measurements of the luciferase activity at day 19 showed that the luciferase expression further increased in SPP1 reporter cells and dropped in COL1A1 cells. The luciferase expression in pGF SPP1 and pGF COL1A1 transduced cells was lower compared to the luciferase expression in pGF CMV transduced cells that was measured at 4.01x10^6 units. This result was consistent with the previous observations with fluorescence microscopy and flow cytometry where only a small number of cells were GFP positive compared to pGF CMV transduced cells. Based on these results, which showed that the flow cytometric analysis was error-prone and that only the SPP1 and COL1A1 reporter cells had a sufficient level of reporter gene expression, the further experiments were performed with the COL1A1 and SPP1 reporter cells.

The experiments of the first WJ-MSC XIX reporter cells were repeated with new reporter cells that were generated from WJ-MSC Promocell. The luciferase expression was determined at three different time points (Figure 45). The luciferase expression in WJ-MSCs transduced with the empty vector remained roughly the same between day 6 and day 15. The luciferase expression in COL1A1 reporter cells peaked at day 9 at with 1.95x10^5 units. The reporter gene expression in WJ-MSC SPP1 reporter cells was constant at day 6 and 9, and reached its maximum at day 15 with 2.62x10^5 units, which was consistent with the previous result obtained from the WJ-MSC XIX cells that had a luminescence of 2.55x10^5 units at day 15. These results confirmed that WJ-MSCs transduced with the COL1A1 and SPP1 reporter constructs expressed the luciferase reporter gene during osteogenic differentiation and that the reporter gene expression was comparable between different batches of cells under identical differentiation conditions.

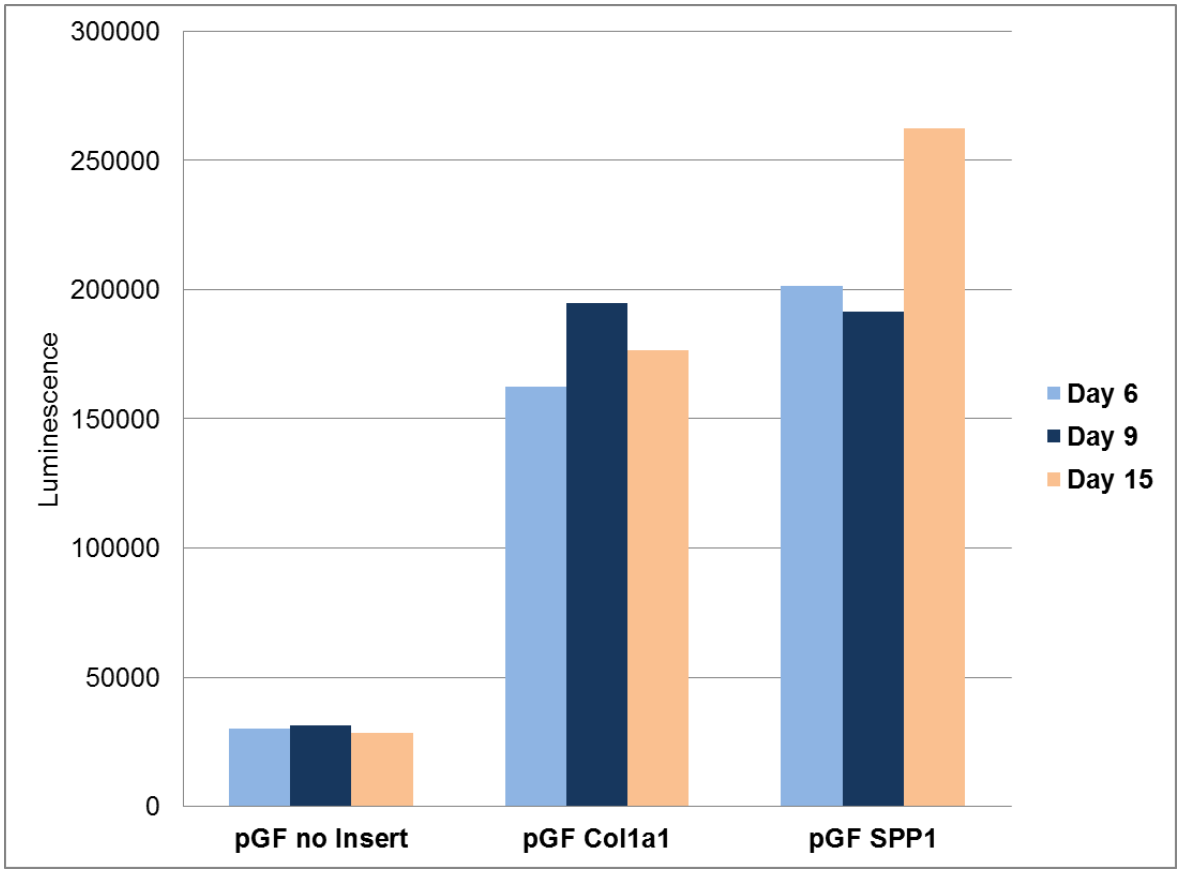


Figure 45 | Luciferase activity of WJ-MSC Promocell reporter cells during osteogenic differentiation.

The luciferase expression in pGF no insert cells remained constant from day 6 to day 15. The luciferase activity in WJ-MSCs transduced with the COL1A1 and SPP1 reporter constructs was comparable with the results obtained in WJ-MSC XIX reporter cells. The highest expression in WJ-MSC Promocell SPP1 cells was measured at day 15 with 2.62x10^5 units and in WJ-MSC Promocell COL1A1 cells at day 9 with 1.95x10^5 units.

In addition to the reporter gene expression after osteogenic differentiation, WJ-MSC Promocell reporter cells were differentiated into adipocytes and chondrocytes, and the luciferase expression was determined at day 15 (Figure 46).

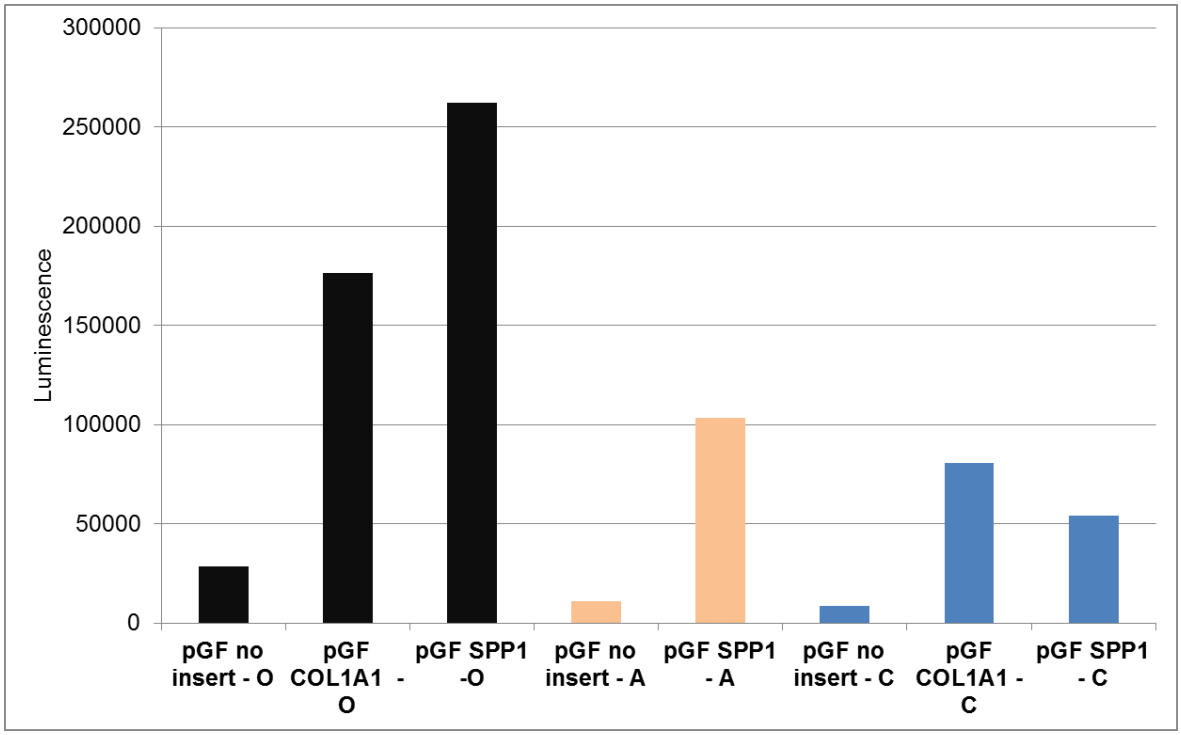


Figure 46 | Expression of luciferase in WJ-MSC Promocell during osteogenic, adipogenic, and chondrogenic differentiation.

The highest expression of luciferase at day 15 was detected in osteogenic differentiated reporter cells (black). The luciferase expression in cells, transduced with the empty vector, was lower during adipogenic and chondrogenic differentiation than osteogenic differentiation. The luciferase expression after chondrogenic differentiation was lower than during osteogenic differentiation with 8.0x10^4 units for COL1A1 and 5.4x10^4 units for SPP1 reporter cells (blue). A higher luciferase activity was determined for SPP1 reporter cells after adipogenic differentiation with 1.0x10^5 units (orange-colored).

Interestingly, the luciferase expression was lower in adipogenic and chondrogenic differentiated cells than in osteogenic differentiated WJ-MSC transduced with the empty vector. The luciferase activity in chondrogenic differentiated WJ-MSC COL1A1 and SPP1 reporter cells was measured at 8.0x10^4 units and 5.4x10^4 units and was significantly lower compared to osteogenic differentiation, with 1.76x10^5 units and 2.62x10^5 units, respectively. The luciferase activity of adipogenic differentiated WJ-MSC SPP1 cells was higher than expected and was measured at 1.0x10^5 units. A possible explanation for such a high luciferase expression was that committed osteochondro progenitor cells were present in the WJ-MSC population and that these cells differentiated towards osteoblasts. Another explanation was that the SPP1 reporter construct was nonspecifically activated during adipogenic differentiation. However, the determined luciferase expression after adipogenic and chondrogenic differentiation was lower in COL1A1 and SPP1 reporter cells than after osteogenic differentiation.

4. Discussion

Mesenchymal stem/stromal cells (MSCs) are multipotent cells that were differentiated into cell types of mesenchymal origin, like adipocytes, chondrocytes, and osteoblasts, and non-mesenchymal origin, e.g. neurons. MSCs were isolated from various adult and birth-associated tissues with different isolation methods (Ishige, Nagamura-Inoue et al. 2009; Hass, Kasper et al. 2011). MSCs isolated from different tissue sources displayed different biological properties in relation to differentiation potential and proliferation capacity. Several studies reported that MSCs isolated from birth-associated tissues have a higher proliferation capacity, reach higher passage numbers before they enter senescence, and have shorter population doubling times than MSCs isolated from adult tissues, e.g. bone marrow (BM-MSCs) or adipose tissue (AT-MSCs) (Hass, Kasper et al. 2011). Another advantage of MSCs derived from birth-associated tissues compared to BM-MSCs was that MSCs from adult tissues showed a decreased proliferation capacity and differentiation potential, depending on the age of the donor (Ishige, Nagamura-Inoue et al. 2009). Depending on the site of isolation, MSCs from birth-associated tissues were divided into several distinct populations that showed different biological properties. MSCs were isolated from the amniotic fluid (AM-MSCs), the placenta (PL-MSCs), the chorion membrane (CM-MSCs), umbilical cord blood (CB-MSCs), and the whole umbilical cord (UC-MSCs) (Hass, Kasper et al. 2011). The umbilical cord is composed of different compartments, from which MSCs were isolated. The umbilical cord is divided into the umbilical vein subendothelium, the perivascular and intervascular regions; and the subamnion regions. Wharton's jelly is the connective tissue that surrounds the umbilical cord vessels and includes the perivascular, intervascular, and subamnion regions (Figure 5) (Troyer and Weiss 2008).

Isolation of MSCs from umbilical cord tissue was achieved with one of the following methods, either enzymatic digestion or explant culture. In the former case, the umbilical cord tissue was cut into small pieces and subsequently incubated with collagenase and trypsin for several hours. After the enzymatic digestion of the tissue, the remaining tissue pieces were removed and the resulting cell suspension was seeded in tissue culture flasks. In the latter case, small pieces of umbilical cord were placed in tissue culture flasks that contained small quantities of culture medium and were incubated for two weeks. During this time, MSCs migrated from the tissue pieces onto the culture plastic where they were harvested. In a study performed by Xu and colleagues, the authors demonstrated that MSCs isolated with the explant culture method were superior compared to MSCs isolated with enzymatic digestion. In contrast to MSCs isolated with the explant culture method, MSCs isolated with enzymatic digestion had a reduced proliferation potential *in vitro* and contained a high percentage of cells that had an endothelial-like morphology. An additional benefit of the explant culture method was that a higher number of MSCs could be isolated from the same amount of umbilical cord tissue as with enzymatic digestion (Xu, Meng et al. 2010).

One objective of this study was to establish a protocol for the isolation and expansion of MSCs derived from neonatal tissues. In the literature it was reported that MSCs isolated from the placenta e.g. amnion membrane or chorion membrane, have a limited proliferation potential (Hass, Kasper et al. 2011). The reports in the literature that MSCs isolated from Wharton's jelly possessed a higher proliferation potential than MSCs isolated from the placenta and that the explant culture method produced better results than the enzymatic digestion, prompted us to isolate MSCs from the Wharton's jelly using the explant culture method. The MSCs were isolated using a modified protocol of the explant culture method reported by Xu and colleagues (described in Materials & Methods). Three umbilical cords were collected from the placenta of full terms, after caesarian delivery, from the General Hospital Vienna, Medical University of Vienna, Department of Obstetrics and Gynecology, with informed consent of the mother. Sample collection was approved by the local ethics-committee (EK357/2006). The three umbilical cord samples were processed within 2 hours of birth, and explant cultures were prepared from Wharton's jelly biopsies. The prepared explant cultures were incubated at standard cell culture conditions, and after 6 days, the first outgrowing, fibroblast-like cells were visible around some tissue biopsies (Figure 15). Until day 10 to 14, a profuse outgrowth of cells was detected around the biopsies, and the outgrowing cells formed a monolayer around the biopsies. WJ-MSCs were successfully isolated from each of the three umbilical cord samples and only small amounts of Wharton's jelly were required to accomplish the isolation. These results were consistent with the results reported by Xu and colleagues that used the same isolation method. They also observed the outgrowth of cells after 6 to 10 days and achieved an isolation frequency of 100% from 9 umbilical cord samples (Xu, Meng et al. 2010). After the outgrown cells reached a critical density around the tissue biopsies, the tissue pieces were removed and the adherent cells were harvested. The harvested cells were further expanded, and cell aliquots were frozen from passage two, three, and four, for long-term storage. Thawed aliquots of WJ-MSCs showed similar cell morphology, proliferation rate, and differentiation potential as freshly isolated cells. Similar observations were made by Karahuseyinoglu and colleagues (Karahuseyinoglu, Cinar et al. 2007).

The isolated cells displayed a fibroblast-like morphology with a small, compact, cell body and few cell processes. When undifferentiated WJ-MSCs reached confluency, they arranged in a swirling-like pattern (Figure 18). The observed cell morphological features and patterns when cells reached confluency matched the reports in the literature, where BM-MSCs and WJ-MSCs were described with a fibroblast-like, spindle-shaped morphology with cytoplasmic extensions (Karahuseyinoglu, Cinar et al. 2007; Ishige, Nagamura-Inoue et al. 2009). The tendency that WJ-MSCs arranged in swirling-like patterns or parallel arrangements when they reached confluency was also reported in the literature (Xu, Meng et al. 2010; Menssen, Haupl et al. 2011). The isolated WJ-MSCs showed a high proliferation potential during the expansion and were passaged every four days when seeded with a seeding density of 4000 cells/cm². This proliferation rate remained almost unchanged during the first 10 passages. Similar growth characteristics were reported by Xu and colleagues for UC-MSCs that were plated

at similar cells densities (2000 – 5000 cells/cm²) and were passaged every 3-6 days (Xu, Meng et al. 2010).

The mean population doubling times, between passage two and nine, of the isolated WJ-MSCs from umbilical cord XIX, XXII, and XXIII, were determined as described by Xu et al. (Xu, Meng et al. 2010). The mean population doubling times were determined with 29.0 ± 5.9h, 31.1 ± 12.4h, and 26.6 ± 2.7h, for WJ-MSCs XIX, XXII, and XXIII, respectively. Our ascertained population doubling times were in the same range of population doubling times reported for UC-MSCs/WJ-MSCs in the literature. Lu and colleagues analyzed the proliferation of UC-MSCs and calculated the mean population doubling time with 24h at passage one, and it remained almost constant until passage 10 (Lu, Liu et al. 2006). Xu et al. reported a population doubling time of 45 hours for MSCs derived from the umbilical cord, which remained almost constant until passage 15 (Xu, Meng et al. 2010). After the initial expansion of the isolated WJ-MSCs, a small fraction of each WJ-MSC population was kept in culture for evaluation of the long-term proliferation potential. WJ-MSCs XIX and XXII were passaged for 20 passages and showed a sufficient proliferation rate up to this passage. The long-term culture of WJ-MSCs XXIII was stopped at passage 15, but these cells behaved similar to the other two WJ-MSC populations. During the long-term culture, a population of cells with altered cell morphology appeared (Figure 17) and their number increased with subsequent passages. These cells were flattened out and a stress-fiber pattern was visible with the microscope in phase contrast. These morphological changes were clear signs of replicative senescence. Several studies showed that UC-MSCs could be passaged for high passage numbers *in vitro* before they reached senescence. In a study performed by Conconi and colleagues, UC-MSCs could be passaged for 16 passages without changes in morphology and signs of replicative senescence (Conconi, Burra et al. 2006). Xu et al. also reported that UC-MSCs were passaged for 15 passages without morphological changes (Xu, Meng et al. 2010). The observed morphological features and proliferation characteristics of our isolated cells matched the reports in the literature and demonstrated that the used isolation and expansion protocol produced satisfying results.

After the expansion of the WJ-MSCs, each cell population needed to be characterized according to the criteria proposed by the International Society for Cellular Therapy, which ensured that the isolated cells were indeed MSCs. MSCs were isolated from different adult tissues like adipose tissue, bone marrow, and peripheral blood, as well as from various birth-associated tissues like the umbilical cord or amniotic fluid (Hass, Kasper et al. 2011). The different tissue sources and isolation methods brought up the question how comparable experimental outcomes with cells isolated from different tissues are. This issue was addressed by the International Society for Cellular Therapy in 2006, when it released a position statement by Dominici et al., where it defined the minimal criteria that MSCs must fulfill. The first criterion was that MSCs must adhere to tissue culture plastic under standard culture conditions. The second criterion was the expression of a specific set of surface antigens. The surface antigens CD105, CD90, and CD73 must be expressed by >95% of all cells, measured with flow cytometry, and in addition, the antigens CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA

class II, must be absent or expressed by fewer than 2% of all cells. The last criterion defined was that MSCs could be differentiated into adipocytes, chondrocytes, and osteoblast under standard *in vitro* tissue culture-differentiation conditions. The successful differentiation of MSCs into osteoblasts should be demonstrated through Alizarin Red S or von Kossa staining of a calcified extracellular matrix. Chondrogenic differentiation should be demonstrated with Alcian blue staining or immunohistochemical staining of extracellular matrix proteins e.g. type II collagen. Adipogenic differentiation of MSCs should be demonstrated by Oil Red O staining of lipid droplets (Dominici, Le Blanc et al. 2006).

Since we isolated our cells with the explant culture method, which relied on the ability that MSCs adhered to tissue culture plastic, our isolated cells fulfilled the first criterion proposed by the International Society for Cellular Therapy. This was also verified during the expansion of the isolated cells, where they showed adherent growth under standard culture conditions. Next, we analyzed the expression of mesenchymal surface antigens with flow cytometry. In each of the four different WJ-MSCs populations, Promocell, WJ-MSCs XIX, WJ-MSCs XXII, and WJ-MSCs XXIII, analyzed, an intense staining of CD73 and CD90 in >99% of all cells was detected (Figure 20 – 23). The staining for CD105 resulted in lower intensities than the other two markers. The staining intensity was low, so that the peaks from the isotype control and the stained cells were not completely separated. Nonetheless, CD105 was expressed by all cells in the four different WJ-MSC populations. In addition to these three markers, the expression of CD44 and CD271 was analyzed. Several studies showed that MSCs expressed CD44, and CD44 was used as an additional MSC marker (Troyer and Weiss 2008; Xu, Meng et al. 2010). CD44 was indeed expressed by >99% of our WJ-MSCs. However, recent studies showed that CD44 was not expressed on primary MSCs and that CD44 expression was acquired during *in vitro* culture and therefore, CD44 expression was not a feature of primary MSCs (Qian, Le Blanc et al. 2012). Other studies with BM-MSCs showed that some MSC populations expressed CD271 and that $CD271^+$ enriched cell populations showed higher CFU-F frequencies. Flow cytometric analysis of our WJ-MSCs showed that CD271 was not expressed. The expression of the hematopoietic markers CD34 and CD45 was also analyzed. In all four WJ-MSCs populations ~99% of the cells were negative for these markers, which confirmed the absence of hematopoietic cells in our WJ-MSCs populations. The results from the flow cytometric analysis of the surface antigens were consistent with reports in the literature. Ishige and colleagues analyzed the immunophenotype of MSCs isolated from different parts of the umbilical cord, and found that WJ-MSCs were positive for the surface antigens CD44, CD73, CD90, and CD105 with similar staining intensities and that CD34, CD45, and CD271 were absent (Ishige, Nagamura-Inoue et al. 2009).

Finally, all four WJ-MSCs populations were tested for their differentiation potential into adipocytes, chondrocytes, and osteoblasts. Adipogenic differentiation was induced in WJ-MSC cultures after they reached confluency with NH AdipoDiff medium. After 21 days of culture, the adipogenic differentiation was detected with Oil Red O staining. Oil Red O stained lipid droplets intensely red, and small lipid

droplets were detected in adipogenic differentiated cells (Figure 24). The adipogenic differentiated WJ-MSCs were compared with adipogenic differentiated BM-MSCs (Figure 25). Despite the fact that, in WJ-MSC cultures, a greater number of cells developed small lipid droplets than in BM-MSCs cultures, no cells with a terminally differentiated adipocyte phenotype (large lipid droplets filling the entire cytoplasm) were detected in WJ-MSC cultures. Such terminally differentiated cells were observed, although at low frequency, in differentiated BM-MSCs. The poor ability of WJ-MSCs or HUCSC (human umbilical cord stroma cells) to differentiate into adipocytes was already described by Karahuseyinoglu and colleagues. They extended the culture period of HUCSCs in adipogenic differentiation medium to up to 40 days. Even after a period of 40 days, they detected no mature adipocyte phenotype in HUCSC cultures, which was readily detectable in BM-MSCs cultures after 21 days. The development of mature adipocytes in BM-MSCs cultures may also have resulted from cells that are committed to form adipocytes *in situ*, which exist within the bone marrow stroma (Karahuseyinoglu, Cinar et al. 2007). In WJ-MSCs that were cultured in control medium for the same period of time, no lipid droplets were detected. Despite the lack of mature adipocytes in WJ-MSCs cultures after 21 days of differentiation, the lack of lipid droplets in the control cultures led us to the conclusion that WJ-MSCs at least showed adipogenic differentiation into preadipocytes. The mature adipocytes in BM-MSCs cultures may also be derived from cells that are committed to form adipocytes *in situ* (Karahuseyinoglu, Cinar et al. 2007).

Two different protocols were used for the osteogenic differentiation of WJ-MSCs. When WJ-MSCs were seeded in osteogenic differentiation medium with a density of 4500 cells/cm², alkaline phosphatase activity was detected after 10 days of culture and further increased by a prolonged incubation with osteogenic differentiation medium. Alkaline phosphatase activity in control cultures was only detected in a rare population of cells (Figure 28). WJ-MSCs seeded at this cell density did not produce a detectable calcified extracellular matrix after 21 days of osteogenic differentiation. Different reports concerning the formation of a calcified extracellular matrix by WJ-MSCs exist in the literature. Ishige and colleagues, which induced osteogenic differentiation in WJ-MSCs cultures at 50-70% confluency, reported that no Alizarin Red S staining was observed after 4 weeks of differentiation (Ishige, Nagamura-Inoue et al. 2009). In contrast, Karahuseyinoglu induced osteogenic differentiation in confluent layers of HUCSCs and detected a mineralized matrix with Alizarin Red S after 4 weeks of differentiation (Karahuseyinoglu, Cinar et al. 2007). These reports suggested that the cell density is a critical parameter for osteogenic differentiation and the production of a calcified extracellular matrix. When we seeded our WJ-MSCs with an initial cell density of 30000 cells/cm², the presence of a calcified extracellular matrix was detectable as dark dots on top of the cells already after 14 days of osteogenic differentiation with a microscope in phase contrast. These WJ-MSCs cultures were intensely stained with Alizarin Red S (Figure 29), which indicated the presence of calcium phosphate deposits. Calcium phosphate deposits were also detected with the von Kossa method. The specificity of the Alizarin Red S and von Kossa staining was tested by treatment of the osteogenic cultures with formic acid that dissolved calcium deposits. In formic acid treated cultures, no Alizarin Red S or

von Kossa staining was detected, which confirmed that the staining in the osteogenic cultures resulted from calcium deposits (Figure 30). Our results confirmed that the initial cell density is a crucial factor for the formation of a calcified extracellular matrix. These results also confirmed that our isolated WJ-MSCs were capable of osteogenic differentiation.

Chondrogenic differentiation of WJ-MSCs was induced in micromass cultures. Therefore, 2.5x10^5 WJ-MSCs were pelleted in 15 ml falcon tubes and incubated in chondrogenic differentiation medium for 24 days. During the culture period, the cells formed a shiny cell sphere, which was a sign of chondrogenic differentiation. Chondrogenic differentiation was confirmed by immunohistochemical detection of aggrecan, a protein of the extracellular matrix in cartilaginous tissues, which is produced by chondrocytes. Aggrecan was detected in tissue sections of chondrogenic differentiated micromasses of WJ-MSCs. In addition to the immunohistochemical detection of aggrecan, the presence of glycosaminoglycans was detected with toluidine blue staining. Toluidine blue shows a metachromatic reaction with glycosaminoglycans, a major component of the chondrogenic matrix. Toluidine blue staining revealed high amounts of metachromatic matrix within the tissue sections that surrounded groups of cells. Our WJ-MSCs showed a high chondrogenic potential that was also reported by Karahuseyinoglu. Karahuseyinoglu and colleagues compared the chondrogenic potential of WJ-MSCs and BM-MSCs and found that WJ-MSCs produced higher amounts of type II collagen, the most common collagen in chondrogenic matrices, than BM-MSCs (Karahuseyinoglu, Cinar et al. 2007). All tested WJ-MSC populations were successfully differentiated into adipocytes, chondrocytes, and osteoblasts. In addition, all WJ-MSC populations expressed the mesenchymal markers CD73, CD90, and CD105, and lacked the expression of the hematopoietic markers CD45 and CD34. Also, WJ-MSCs adhered to tissue culture plastic under standard culture conditions. Taken together, these results confirmed that our isolated cells fulfill the minimal criteria for MSCs proposed by the International Society for Cellular Therapy and were considered as WJ-MSCs.

During osteogenic differentiation of MSCs a number of marker genes e.g. alkaline phosphatase (ALPL), osteopontin (SPP1), type I collagen (COL1A1), and osteocalcin (BGLAP), as well as the transcription factors osterix (SP7) and Runt-related-2 (Runx2), are expressed (Baksh, Song et al. 2004). The expression of these genes involved in osteogenic differentiation was analyzed over a 30 day time period with q-PCR from total RNA extracts, prepared at different time points. The difference in the expression of the osteogenic marker genes was calculated in relation to the expression of these genes in cells cultured in control medium for 6 days (Figure 31). During osteogenic differentiation, the expression of all osteogenic genes analyzed was increased. The highest expression of a bone-related marker gene at day 6 was detected for alkaline phosphatase with a 112-fold increase that further increased until day 30 to 191-fold. The expression of type I collagen was increased 8-fold at day 6, peaked at day 15 (20-fold), and decreased slightly until day 30 to about 16-fold. The expression of osteopontin steadily increased from day 6 (6-fold) until day 30 to 34-fold. An increased expression of osteocalcin was detected after 30 days of osteogenic differentiation.

This was supported by the literature, where it was stated that osteocalcin was only expressed in fully differentiated osteoblasts and therefore only at later stages of osteogenic differentiation (Karsenty 2008). Interestingly, only a modest increase in the expression of the transcription factor Runx2, a master regulator of osteogenic differentiation, was detected. The expression of the transcription factor osterix/SP7 was highly increased from day 6 onwards, and a 450-fold increased expression was detected at day 30. These findings confirmed that the expression of several major osteogenic marker genes was up regulated during osteogenic differentiation of WJ-MSCs. The detected expression of osteocalcin showed that WJ-MSCs developed into fully differentiated osteoblasts.

After the characterization of the isolated WJ-MSCs and the establishment of osteogenic differentiation protocols, the next goal was the generation of the lentiviral reporter constructs. The goal was that the expression of the two reporter molecules, GFP and luciferase, should only occur in WJ-MSCS that were differentiated into the osteogenic lineage. Therefore, the promoter regions of five genes that were up-regulated during osteogenic differentiation were amplified from genomic DNA with nested PCR. The inner primers for the nested PCR were specifically designed to contain restriction sites that allowed the directional ligation of the promoter fragments into the reporter vector. Five different promoter regions from SP7, BGLAP, COL1A1, ALPL, and SPP1, with a size of 1378bp, 1371bp, 2554bp, 1466bp, and 1440bp, respectively, were successfully amplified from genomic DNA (Figure 32 – 36). After the successful ligation of all five promoter regions into the lentiviral reporter vector (pGreenFire lentiviral pathway reporter vector), each promoter fragment was sequenced. Unfortunately, the complete sequence of the cloned promoter region was only obtained from the BGLAP, SPP1, and COL1A1 promoter regions. From the SP7 and ALPL promoter regions, only the flanking regions were sequenced, and for a 380bp and 162bp stretch in the SP7 and ALPL promoter fragment, no sequence data was obtained. This was either the result of the high GC content in the promoter regions or due to prominent secondary structures. The obtained sequences of all five promoter regions were aligned against the published genomic sequence in the Ensembl database. The two sequence parts obtained from the SP7 and ALPL promoter region showed a high degree of identity (99%) with the genomic sequence, apart from the missing regions. The obtained sequences from the other three promoter fragments were largely identical to the genomic sequence in the database and contained only a small number of single nucleotide mismatches, either polymorphisms or sequencing errors (Figure 32 – 36). The sequence alignments confirmed that the desired promoter regions were amplified and ligated into the pGreenFire lentiviral pathway reporter vector (pGF vector). In addition to the five osteogenic reporter regions, the CMV promoter was ligated into the pGF vector as a positive control. Lentiviral vectors possess the ability to stably integrate into the host genome, which allows a permanent activity of the reporter construct once it is introduced. For the transduction of WJ-MSCs with the generated reporter constructs in the pGreenFire vector, the lentiviral vectors were packaged into replication-defective lentiviral particles produced in the HEK293T cell line. Therefore, the reporter constructs together with the psPAX2 and pMD2.G packaging plasmids were transfected into HEK293T cells. The lentiviral particles

produced in HEK293T cells were harvested with the culture medium and after centrifugation of the culture medium, the resulting supernatant containing the lentiviral particles was used for the infection of WJ-MSCs. Excess amounts of supernatant were frozen and stored at -80°C. The pGreenFire vector contained a puromycin resistance gene that was constitutively expressed and allowed the selection of stably transduced WJ-MSCs with puromycin. The optimal puromycin concentration for the selection of WJ-MSCs was determined to be 0.5 µg puromycin per ml culture medium. Puromycin was added 48h after the infection of WJ-MSCs, and the transduced cells were selected for at least seven days. Despite the fact that the same number of cells was used for the infection, there were considerable differences between the cells infected with different reporter constructs during/after the selection. When WJ-MSCs were transduced with the empty pGreenFire vector or the pGF SPP1 reporter construct, a high number of puromycin-resistant cells was obtained. In contrast, WJ-MSCs that were transduced with the pGF COL1A1 reporter construct yielded very low numbers of transduced cells. These differences were probably the result of different numbers of lentiviral particles in the harvested supernatant. The observed differences may be avoided by determination of the number of produced viral particles and the use of the same number of viral particles for each transduction. After the puromycin selection, the stably transduced WJ-MSCs were differentiated towards osteoblasts and excess amounts of cells were frozen.

The main goal of this study was the generation of an osteogenic reporter cell line that could be used for the screening of small molecules that enhanced osteogenic differentiation of MSCs. The pGreenFire reporter vector contained a fusion protein of the two reporter genes GFP and luciferase separated by a self-cleaving peptide, which allowed the simultaneous expression of the two reporter molecules. The initial objective was that the amount of osteogenic differentiation should be determined by measurement of the GFP expression with flow cytometry. During the initial osteogenic differentiations with transduced WJ-MSCs, an increased fluorescence was observed with fluorescence microscopy in WJ-MSCS cultured in osteogenic differentiation medium compared to WJ-MSCs grown in control medium. The increased fluorescence was detected in all WJ-MSC reporter cells, independent of the transduced reporter construct, to a similar extent. Interestingly, the increased fluorescence was also observed in WJ-MSCs that were transduced with the empty pGreenFire vector. The fluorescence within these cells was concentrated around the nucleus but not within the nucleus, which appeared dark (Figure 38). A rare population of GFP positive cells was detected in osteogenic differentiated WJ-MSCs transduced with the COL1A1 and SPP1 reporter constructs with fluorescence microscopy (Figure 40 and 41). These cells were readily distinguished from the autofluorescent cells. The fluorescence in these cells was spread throughout the complete cytoplasm and the nucleus. The increased fluorescence of osteogenic differentiated cells was also detected with flow cytometry and about 16% of the cells, transduced with the empty pGreenFire vector, were measured as GFP positive after osteogenic differentiation compared to cells grown in control medium (Figure 42). This autofluorescence considerably impaired the detection of GFP positive cells. In the initial flow cytometric analysis, only a low percentage of cells were detected above the threshold of

autofluorescent cells. The highest percentage of GFP positive cells was detected in WJ-MSCs transduced with the COL1A1 and SP7 constructs with 6.6% and 6.7%, respectively (Figure 43). These measurements confirmed the observations with the fluorescence microscope that only a small number of reporter cells truly expressed GFP during osteogenic differentiation. The flow cytometric analysis was further impaired by the low GFP expression and the resulting low fluorescence intensities of the cells that were slightly higher than the intensities of autofluorescent cells. A high GFP expression was detected in WJ-MSCs that were transduced with the pGF CMV vector, and 80% of these cells were measured GFP positive compared to cells with the empty vector. The results from the pGF CMV transduced cells demonstrated that the transduction and selection process worked fine and that the non-transduced cells were efficiently eliminated during selection. The low GFP expression, measured in WJ-MSCs transduced with the other reporter constructs, was the result of two factors. First, the GFP used in the pGreenFire vector was a destabilized variant of GFP that is degraded within one hour and therefore required a constant, strong promoter activity to reach sufficient levels. The second factor was that the amplified promoter regions provided a low promoter activity, compared to CMV, and therefore, GFP was only weakly expressed. These two factors combined resulted in the low fluorescence intensities.

The low GFP expression combined with the increased fluorescence of osteogenic differentiated WJ-MSCs raised the question if better results could be obtained with the measurement of the luciferase activity. Luciferase was the second reporter molecule expressed from the reporter vector and, unlike GFP, the luciferase was not destabilized and therefore accumulated to a certain degree. The luciferase activity was determined from total cell lysates of osteogenic differentiated cells at different time points. The detected luminescence from the luciferase reaction was normalized to the total protein concentration in the corresponding cell lysate. WJ-MSCs, transduced with the five osteogenic reporter constructs as well as the empty vector (negative control) and the pGF CMV vector (positive control), were subjected to osteogenic differentiation, and cell lysates were prepared at day 9 and 15. In WJ-MSCs transduced with the empty pGreenFire vector, a luminescence of 2.0x10^4 units and 3.1x10^4 units was measured at day 9 and 15, respectively (Figure 44). The detected luminescence in WJ-MSCs transduced with the SP7, BGLAP, and ALPL constructs, was very low at day 9 and slightly increased until day 15. None of these three constructs was measured above the set threshold that was defined as “three times the luminescence measured in cells transduced with the empty vector”. The measured luminescence in the cell lysates of ALPL reporter cells was on the same level as that of the negative control cells (Figure 44). Considering that ALPL showed the highest upregulation at early time points of osteogenic markers analyzed by q-PCR, the pGF ALPL construct was rated non-functional and was excluded from further studies together with the BGLAP and SP7 constructs. A significant upregulation of luciferase expression was detected in cell lysates of WJ-MSCs transduced with the pGF COL1A1 and SPP1 reporter constructs, where a luminescence of 2.3x10^5 units and 2.5x10^5 units was measured, respectively. The luciferase expression in these cells was still very low compared to cells that were transduced with the pGF CMV vector, where

a luminescence of 4.0x10^6 units was measured at day 15. In addition to the day 9 and 15 measurements, the luciferase activity in WJ-MSCs, transduced with pGF COL1A1 and SPP1 constructs was determined at day 19 to ascertain if longer differentiation periods further increased the luciferase expression. The luciferase activity in SPP1 cells further increased to 3.0x10^5 units, but decreased in COL1A1 cells to 1.6x10^5 units. These findings suggested that longer differentiation periods were beneficial, at least for the SPP1 construct, but the formation of an extracellular matrix in the differentiation cultures became a problem. The extracellular matrix impaired the trypsinization process and the WJ-MSCs could no longer be harvested for the preparation of cell lysates without being ruptured, which reduced the luciferase activity in the lysates. This was the case in the following experiment, where a much lower luminescence was detected after 21 days of differentiation (data not shown). In general, the low luciferase activities compared to the CMV positive control were consistent with previous observations from fluorescence microscopy and flow cytometry, where only a small fraction of cells were identified that were GFP positive. The differentiation experiments were repeated with freshly generated COL1A1 and SPP1 reporter cells, using WJ-MSCs from a different donor. Similar results were obtained with the new SPP1 reporter cells, where a luminescence of 2.6x10^5 units after 15 days of osteogenic differentiation, was measured (Figure 45). The highest luciferase activity in COL1A1 cells was measured at day 9 with 1.9x10^5 units, which was slightly lower than the previous experiment with 2.3x10^5 units. These results confirmed our previous results and demonstrated that the luciferase expression, albeit low, was approximately identical between different cell preparations under the same culture conditions, which is a requirement for a screening system. In addition to osteogenic differentiation, the luciferase activity was determined during adipogenic and chondrogenic differentiation (Figure 46). The luciferase activity was lower in chondrogenic and adipogenic differentiated cells compared to osteogenic differentiated cells. Although the measured luciferase activity of SPP1 cells after adipogenic differentiation reached 1.0x10^5 units, which was higher than expected, the luciferase signal was 2.6 times higher in osteogenic differentiated cells. After 15 days of chondrogenic differentiation, the luciferase activity of COL1A1 and SPP1 cells was measured with 8.0x10^4 units and 5.4x10^4 units, which was considerably lower than in adipogenic differentiated cells. A possible explanation for the higher luciferase activity during adipogenic differentiation was that the SPP1 promoter construct was also activated during adipogenic differentiation or that osteogenic differentiation also occurred in adipogenic cultures.

There are still several problems that need to be addressed. One problem is the low expression of reporter molecules during osteogenic differentiation, which may have several reasons. One reason could be that the lentiviral infection, followed by the long selection process with puromycin, has an impact on the differentiation potential of WJ-MSCs. This could explain why only a small fraction of GFP positive cells was observed with fluorescence microscopy during osteogenic differentiation. The other reason could be that the reporter constructs need further optimization by using shorter or longer versions of the already used promoter fragments. Several problems remained unsolved during this study and further studies are required to

address these problems before these cells can be used as an osteogenic screening cell line for high-throughput screenings. Nonetheless, we were able to successfully establish protocols for the isolation and differentiation of WJ-MSCs and could demonstrate that our cells fulfill the ISCT criteria. We also established a protocol for the ligation of large promoter fragments into the lentiviral reporter vector, and five osteogenic reporter constructs and controls were generated. Further, we demonstrated that two of our generated reporter constructs, SPP1 and COL1A1, showed an increased expression of luciferase during osteogenic differentiation. Initial tests also showed that reporter expression was lower during adipogenic and chondrogenic differentiation, demonstrating the osteogenic specificity of the reporter constructs.

Appendix

Abstract

Mesenchymal stem cells are multipotent cells that were isolated from various adult tissues, e.g. adipose tissue, bone marrow, and from birth associated tissues such as amniotic fluid, umbilical cord blood, and umbilical cord Wharton's jelly. Since the first studies by Friedenstein in the 1970's, several groups achieved differentiation of MSCs into cell types of mesenchymal origin e.g. adipocytes, chondrocytes, and osteoblasts. The successful generation of different cell types from MSCs was evaluated by staining of cell type specific features. The differentiation of MSCs into osteoblasts was detected by staining of a calcified extracellular matrix or the detection of alkaline phosphatase activity.

The Medical University of Vienna was one of seven partners in the EU project HYPERLAB, which stands for High Yield and Performance Stem Cell Lab. The goal of the HYPERLAB project was to develop new innovative, microfluidics-based technologies adapted for the cultivation of stem cells for clinical use. One goal of HYPERLAB was the generation of a reporter cell line for osteogenic differentiation of mesenchymal stem cells, which can be used for automated screening of compounds that promote osteogenic differentiation.

During my diploma thesis I established a protocol for the isolation and expansion of mesenchymal stem cells from umbilical cord Wharton's jelly. The isolated cells were then characterized according to the criteria proposed by the International Society for Cellular Therapy. Therefore, the expression of mesenchymal markers was determined by flow cytometry, and multipotency of the isolated cells was demonstrated by successful differentiation into adipocytes, osteoblasts, and chondrocytes. For the generation of the reporter constructs, I established a method for the ligation of promoter regions into a lentiviral pathway reporter vector. With this method, I was able to ligate the promoter regions of five osteogenic marker genes (ALPL, COL1A1, SPP1, BGLAP, and SP7) that were amplified from genomic DNA into the lentiviral reporter vector.

The reporter constructs were introduced into the genome of MSCs using lentiviral gene transfer. After the transduction, stably transduced cells were selected with puromycin and differentiated into adipocytes, chondrocytes, and osteoblasts. While we couldn't achieve satisfactory results with the measurement of GFP reporter expression because of the high autofluorescence of osteogenically differentiated cells, we were able to detect osteogenic differentiation through measurement of the luciferase activity in cell lysates. The best results were obtained with the SPP1 and COL1A1 promoter constructs. A high expression of luciferase was detected in cells transduced with these reporter constructs already after 6 days of osteogenic differentiation, and the expression further increased until day 15. In addition, we demonstrated that the luciferase expression was higher during osteogenic differentiation than in chondrogenic or adipogenic differentiation.

Zusammenfassung

Mesenchymale Stammzellen (MSCs) sind multipotente Zellen, die aus verschiedenen adulten Geweben, wie z.B. Fettgewebe und Knochenmark sowie aus geburtsassoziierten Geweben wie z.B. Fruchtwasser, Nabelschnurblut, und Wharton-Sulze, isoliert wurden. Seit den ersten Studien von Friedenstein in den 1970ern haben mehrere Autoren MSCs in verschiedene Zelltypen mesenchymalen Ursprungs differenziert, z.B. Adipozyten, Chondrozyten und Osteoblasten. Die erfolgreiche Differenzierung in diese Zellen wurde durch Färbung von zelltypspezifischen Merkmalen evaluiert. Die Differenzierung von MSCs in Osteoblasten wurde durch die Färbung einer kalzifizierten extrazellulären Matrix oder den Nachweis von alkalischer Phosphatase Aktivität detektiert.

Die Medizinische Universität Wien war einer von sieben Partnern im EU Projekt HYPERLAB, die Abkürzung für High Yield and Performance Stem Cell Lab. Das Ziel des HYPERLAB Projekts war es, neue innovative Methoden für die Kultivierung von Stammzellen in kleinen Volumina für klinische Anwendungen zu entwickeln. Ein Ziel von HYPERLAB war die Entwicklung einer Reporterzelllinie für die osteogene Differenzierung von MSCs, die für ein automatisiertes Screening von Molekülen, die die osteogene Differenzierung von MSCs verbessern, verwendet werden kann.

Während meiner Diplomarbeit habe ich ein Protokoll für die Isolierung und Expansion von MSCs aus der Wharton-Sulze etabliert. Die daraus isolierten Zellen wurden anhand der Kriterien der International Society for Cellular Therapy charakterisiert. Hierfür wurde die Expression von mesenchymalen Antigenen mittels Durchflusszytometrie bestimmt und die Multipotenz durch erfolgreiche Differenzierung in Adipozyten, Chondrozyten, und Osteoblasten nachgewiesen. Für die Herstellung der Reportervektoren wurde von mir eine Methode für die Ligation von Promoter-Regionen in einen lentiviralen Signalweg-Reporter-Vektor entwickelt. Mit dieser Methode war es möglich, die Promoter-Regionen von fünf verschiedenen Osteoblasten-spezifischen Genen (ALPL, COL1A1, SPP1, BGLAP und SP7), die aus genomischer DNA amplifiziert wurden, in den lentiviralen Vektor zu ligieren.

Die so erzeugten Reportervektoren wurden mittels lentiviralen Gentransfers in das Genom der MSCs eingeschleust. Nach der Transduktion wurden die stabil transduzierten Zellen mit Puromycin selektioniert und anschließend in Adipozyten, Chondrozyten, und Osteoblasten differenziert. Wir waren nicht in der Lage zufriedenstellende Ergebnisse durch Messung der GFP Expression zu erzielen, jedoch konnte das Ausmaß der osteogenen Differenzierung durch Messung der Luciferase-Aktivität in Zelllysaten bestimmt werden. Die besten Ergebnisse wurden mit den SPP1 und COL1A1 Reportervektoren erzielt. In MSCs, die mit diesen Reportervektoren transduziert wurden, konnte eine hohe Luciferase-Expression schon nach 6 Tagen osteogener Differenzierung nachgewiesen werden, welche bis Tag 15 noch weiter anstieg. Zusätzlich konnten wir zeigen, dass die Luciferase Expression während der osteogenen Differenzierung höher war als während der adipogenen und chondrogenen Differenzierung.

Curriculum Vitae

Martin Fröhlich
Lehnergasse 1a, 3423 St.Andrae-Woerdern, Austria
Nationality: Austria

EDUCATION

2007 – 2013	University of Vienna Diploma studies Molecular Biology Special subjects: cell biology, biochemistry, and molecular medicine
2005 – 2007	Vienna University of Technology Diploma studies Technical Chemistry
1998 – 2003	Polytechnic: Höhere Bundes-Lehr- und Versuchsanstalt für chemische Industrie Rosensteingasse, general qualification for university entrance

CIVILLIAN SERVICE

2004 – 2005	Civilian service (one year) at Freiwillige Feuerwehr Krems a.d. Donau

RESEARCH EXPERIENCE

2012 – 2013	Diploma thesis (13 months) “Establishment of a validated osteogenic MSC reporter line for optimization of osteogenic differentiation” Research Laboratory at the University Clinic for Women Care, Medical University Vienna, Workgroup of Prof. Schneeberger
2011	Wahlbeispiel (2 months) “S-nitrosylation of tubulin and the effect of GSNO on microtubule dynamics” Max F. Perutz Laboratories University of Vienna, Medical University of Vienna, Workgroup of Prof. Propst

SKILLS

Computing	Proficiency in Windows, MS Word, Excel, and PowerPoint. Experience with Adobe Photoshop, Image J, and FlowJo.
Language	German (mother tongue), English (CEFR B2)
Methods	RT-PCR Flow cytometry Isolation and primary culture of MSCs and HSCs Cell culture Lentiviral gene transfer Molecular cloning Immunocytochemistry Fluorescence and deconvolution microscopy DNA and RNA isolation Transformation and plasmid preps Gel electrophoresis BCA protein assay Luciferase assay PCR Western Blot

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