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LINEAGE COMMITMENT OF CONDITIONALLY IMMORTALIZED BONE MARROW MESENCHYMAL STROMAL CELLS FROM TETRACYCLINE-REGULATED SV40 LARGE T-ANTIGEN TRANSGENIC MICE

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TRANSGENIC MICE

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Abstract

Adult bone marrow contains a population of mesenchymal stem cells capable to self-renew and to differentiate into haematopoietic-supportive stroma, osteo, adipo- and chondrocytes. However, the identity of mesenchymal stem cells still remains uncertain. The complex population of their descendants, bone marrow mesenchymal stromal cells (BM MSCs), represents a model to study the principles of differentiation and commitment into mesodermal lineages. The experiments using BM MSCs are often hampered by their low proliferative capacity *in vitro*. In the present study, we established conditionally immortalized BM MSCs from tetracycline-regulated SV40 Large T-antigen transgenic mice. The identity of the conditionally immortalized BM MSCs was confirmed by marker expression, ability to support haematopoiesis and differentiation potential. The advantages of the conditional immortalization are encompassed in (1) indefinite expansion of cell populations, (2) possibility to perform cellular cloning and (3) prevention from spontaneous differentiation.

We demonstrated the heterogeneity of BM MSCs and identified at least 6 types of progenitors within BM MSCs population based on their differentiation potential (“OAC”, “OA”, “OC”, “AC”, “O”, “A”). A hypothetical model of BM MSC hierarchy and the relationships between the progenitors has been proposed.

We observed that the Wnt/β-catenin signaling pathway and GSK3 activity could modulate the efficiency of osteo- and adipogenic differentiation pathways, but we didn’t find evidence that the lineage commitment of BM MSCs is determined by Wnt.

We elucidated the mechanism of transcriptional regulation of the adipogenic induction of BM MSCs *in vitro*. Our data revealed the key regulatory role of PPARγ1 during adipogenesis in BM MSCs. Furthermore, we assume that PPARγ1 is a potential trigger of the adipogenic commitment of the BM MSCs progenitors. Finally, the non-adipogenic BM MSCs progenitors were converted into the adipogenic lineage using ectopical expression of the transcription factors C/EBPα, C/EBPβ and C/EBPδ. Our findings provide a novel insight into the molecular mechanisms of BM MSCs lineage commitment.

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1. Introduction

Bone marrow represents a complex structure combined of two interacting tissues, haematopoietic and stromal. Haematopoietic tissue within the bone marrow generates all blood lineages, and in mammals serves as the only permanent source of blood cells in the adults. Bone marrow stroma plays an integral role providing a mechanical support for haematopoietic tissue and regulatory signals supporting the blood homeostasis. At the same time stromal cells have the function on their own, which is to form the skeletal tissues and differentiate into the cells of mesodermal origin: osteocytes, adipocytes, chondrocytes. Those two compartments, haematopoietic and stromal, are distinct and interdependent, therefore often considered in parallel. Haematopoietic system has one of the fastest turnovers in the organism. Little is known about the cycling of the stroma itself, but the skeleton as a derivative of stromal progenitors is renewed about 3-5 times during the life. Haematopoietic tissue is formed by haematopoietic stem cells, which are well defined phenotypically and physiologically. The existence of self-renewing stem cells within the stroma was proven in the experiments maintaining the intact bone marrow tissue, but for a long time those cells have not been identified *in situ* and have not been obtained as a pure cell population *in vitro*. The main obstacle for the experiments in cell culture conditions was the low proliferation capacity of stromal cells. At the same time, the low migratory capacity and engraftment potential restrict *in vivo* transplantation assays, which are the only ones rigorously proving the self-renewal and hence stemness. As a result, till latest years the stem cells within bone marrow stroma were actually defined neither by marker expression nor by physiological properties. According to the current nomenclature, the cell populations from stroma should be called Bone Marrow Mesenchymal Stromal Cells (BM MSCs), and Mesenchymal Stem Cells might exist as a subset of this cell population.

Although BM MSCs *in vitro* represent a mixed cell population of the mesenchymal stem cells progeny, they still can serve as a relevant model to study stroma functions, origin and homeostasis. Those cells support haematopoiesis *in vitro* and differentiate into mesenchymal lineages. Additionally to application in fundamental studies, BM MSC properties *in vitro* promoted them in the category of cells that have an invaluable therapeutic potential.

In this literature review we aimed to describe the original studies from the past, which formed understanding of the main principles of bone marrow stroma biology, and to present the current state of the art in this field. Particularly, we will

concentrate on the mechanisms of differentiation of BM MSCs. Special attention will be paid to the heterogeneity of BM MSCs, as a mixed population of the cells with different differentiation potential.

The second part of the introduction will be dedicated to the strategies efficiently used to study cell populations. In particular, we will consider the cellular immortalization method as a powerful tool for cell expansion.

1.1 Bone marrow mesenchymal stromal cells

1.1.1. Bone marrow stroma structure and developmental origin

Bone marrow stroma is located in the cavity of the bones between the sinusoidal walls and endosteum, and includes the cells, which do not belong to haematopoietic lineage, Fig. 1 (Krebsbach, Kuznetsov et al. 1999; Bianco and Gehron Robey 2000). The bone marrow stroma is a heterogeneous tissue composed of the different cell types and extracellular matrix, which form a complex three-dimensional network. The cellular elements of the bone marrow include reticular cells (or adventitial cells, or Westen-Bainton cells), marrow adipocytes, osteoblastic cells, fibroblasts, and other cell types. The cellular composition of stromal tissue is dynamic and strongly depends on the age and physiological state of the organism. The reticular cells are important myelosupportive elements of haematopoietic microenvironment. They are located along the sinusoidal walls and phenotypically represent pericytes. These cells are able to convert into osteoblasts and adipocytes *in vivo* (Bianco, Costantini et al. 1988) and probably contain the population of marrow stem cells.

The osteoblasts form the bone tissue and possibly build the niche for the haematopoietic stem cells (Zhang, Niu et al. 2003). Bone marrow adipocytes differ from the adipocytes of the other sites of the body. It is known that these adipocytes do not supply a reserve of energy and do not loose the fat inclusions during starvation (Tavassoli 1974). Recently it was shown that marrow adipocytes contribute to the negative regulation of haematopoiesis by repressing the proliferation of haematopoietic progenitors (Naveiras, Nardi et al. 2009). Possibly, the balance between the osteoblastic and adipocytic differentiation of marrow stem cells provides the regulation of haematopoiesis in the bone marrow.

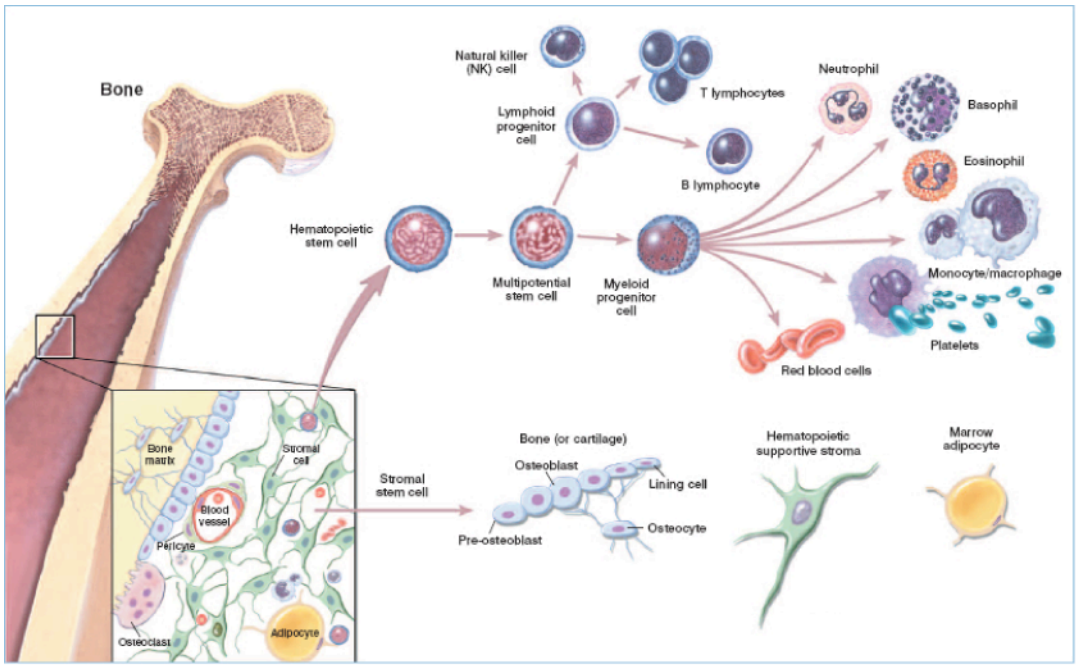


Figure 1. Bone marrow is composed of haematopoietic tissue and stroma. The stromal components are comprised of reticular cells (pericytes, adventitial cells), adipocytes, osteoblasts, fibroblasts. Modified from NIH Stem Cell Reports, http://stemcells.nih.gov/index.asp.

During embryonic development the stroma is established after the bone rudiment has formed. The precursors of stromal cells are located outside in the periosteum, and have characteristics of osteoblasts. The osteoclasts erode the bone tissue to form the bone cavity allowing the vessels invasion, which guides the migration of the osteogenic cells, and those cells have perivascular location (as pericytes). When the primitive haematopoietic microenvironment is established inside the bone, the blood-borne haematopoietic stem cells migrate to complete the bone marrow formation (Bianco and Gehron Robey 2000).

The ontogeny of the stem cells within bone marrow stroma is much less understood. The development of primitive embryonic bone marrow stromal tissue from the periosteum suggests that the stromal stem cells might evolve from the osteogenic cell population. It was shown that different cells in the bone marrow stroma express markers implicated in the early bone development (runt-related transcription factor 2, RUNX2), independently of their osteogenic properties (Satomura, Krebsbach et al. 2000). Hence, this might indicate that the stromal stem cells arise from the cells that have been committed to the osteogenic lineage in the embryo and maintain the expression of RUNX2 as an “imprint” of their origin. But the mechanism of stromal stem cells development might be more complex. Using the cell fate mapping approach in mouse embryonic stem cells and mice, it was

demonstrated that stromal stem cells originate from different sources, and the earliest wave of those cells in the embryo rises from neuroepithelium and neural crest (Takashima, Era et al. 2007; Morikawa, Mabuchi et al. 2009). However, the presence of neural crest-originated stromal stem cells in the adults remains controversial. The fact that stromal stem cells have multiple origins may explain the heterogeneity of stromal cell population and phenomenon of plasticity, which we will discuss in the further chapters.

1.1.2. Identity of bone marrow mesenchymal stromal cells

The evidence for the osteogenic potential of bone marrow was known long time ago. However, the particular elements responsible for the formation of bone tissue were not identified. The important information about non-haematopoietic cells with the properties of stemness appeared in the 1960-70s. It was shown that ectopical transplantation of the bone marrow cylinder subcutaneously or under the renal capsule leads to the formation of bone structure with fully functioning bone marrow (the terms usually used for this structure are “ossicle” or “haematopoietic foci”) (Friedenstein, Piatetzky et al. 1966; Friedenstein, Petrakova et al. 1968; Tavassoli and Crosby 1968). The cellular components of haematopoietic tissue were identical to the bone marrow in the regular sites, but the interesting finding was that it was originating from the cells of the host. On the contrary, the stromal tissue and the bone were formed from the cells of donor transplant. The formed foci were present in the site of transplantation indefinitely long during the life of the host. The descendants of the transplanted cells could not be found in the other tissues. Finally, the marrow from the ossicle could be passaged through the series of hosts virtually indefinitely. The conclusions from the series of those pioneer works were (1) the existence of elements in the bone marrow, which can differentiate into the bone tissue, (2) the existence of components, which attract HSCs from the host and support haematopoiesis, (3) the cells with osteogenic differentiation potential and the cells with haematopoietic supportive function can self-renew presuming stemness, and (4) it was most likely that the non-haematopoietic stem cells cannot migrate and be recruited to the host tissues. At the same time whether those properties are possessed by the same cell and which kind of cell was not known. The breakthrough was done in the studies of Friedenstein (Friedenstein, Deriglasova et al. 1974). It was found that plating of the bone marrow in the low density leads to the formation of single-cell derived clones of fibroblastic cells (colony forming units-fibroblasts, CFU-F’s). Those cells could induce the formation of heamatopoietic foci in the ossicle

upon transplantation. Importantly, single-cell derived clone could establish the bone tissue and induce the marrow, connecting those two functions in one cell type. Hence, the existence of stem cells supporting haematopoiesis and differentiating into the bone tissue was proved. The clonogenic presursors of CFU-F's within bone marrow were termed "stromal stem cells" or "osteogenic stem cells" (Owen and Friedenstein 1988).

Since that time a number of studies concerning stromal stem cells appeared, attempting to improve methods for isolation, to find a set of surface markers (Gronthos, Graves et al. 1994), to characterize differentiation potential (Ashton, Allen et al. 1980; Bennett, Joyner et al. 1991) and to elucidate the role in regeneration (Haynesworth, Goshima et al. 1992). The evidence grew that stromal stem cells can differentiate into multiple lineages including osteocytes, adipocytes, chondrocytes, fibroblastic tissues, myocytes. Summarizing all accumulating information, Caplan proposed the concept of "mesenchymal stem cell" as a cell from adult bone marrow capable for multilineage differentiation (Caplan 1991; Caplan 1994), and this was the moment when the "mesenchymal stem cell" term was applied for stromal stem cells. Some years later Pittenger and colleagues reported the isolation of homogeneous population of mesenchymal stem cells from human bone marrow and hence, the validation of the concept (Pittenger, Mackay et al. 1999). They described plastic-adherent cells from the bone marrow stroma, which proliferated extensively, were characterized by the expression of the markers SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a, CD124. These cells were able to form osteocytes, adipocytes and chondrocytes *in vitro*. Remarkably, single-cell derived lines were also obtained and tested for the differentiation, and around 1/3 (2 out of 6) showed tri-potential differentiation. However, self-renewal, an essential property of stem cells, has not been demonstrated. Hence, the actual stemness of "mesenchymal stem cells" was not completely proven.

This work was a milestone of the whole stromal stem cell field and was followed by a huge increase of the interest to those studies. Despite the historical and scientific significance, some confusion was generated and for a long time the term "mesenchymal stem cell" was not subjected to doubts and widely and often inappropriately used (Bianco, Robey et al. 2008). The methods for isolation were not standardized and the criteria to define the population as "mesenchymal stem cell" were not considered. As a result, a lot of discrepancies appeared in the literature. In 2006 the scientific community postulated that the cell populations isolated from bone marrow stroma should be called "Mesenchymal Stromal Cells" (MSCs) and not "stem

cells" until stemness at the single-cell level is proved, and defined minimal criteria for characterization of MSCs (Dominici, Le Blanc et al. 2006).

Here we will use the term "MSC" for "mesenchymal stromal cells", and in turn "mesenchymal stem cells" will be specially noted, when appropriate. We will give a brief review on some basic properties of MSCs *in vitro*, without aiming to include all vast information circulating in the literature. Despite undoubted importance of the therapeutic potential and application of MSCs (Horwitz, Prockop et al. 1999; Le Blanc, Frassoni et al. 2008), those topics are not included since they are not relevant to the object of our study and have been described in detail elsewhere.

1.1.3. Sources of mesenchymal stromal cells

Mesenchymal stromal cells were identified first in the bone marrow (BM MSCs); nevertheless a large number of studies were dedicated to isolation of MSCs from the other tissues of mesodermal origin. Cells with properties of MSCs were obtained from human lipoaspirates (Zuk, Zhu et al. 2001; Zuk, Zhu et al. 2002). The comparison of those cells with BM MSCs revealed many similarities in the potential and marker expression (De Ugarte, Morizono et al. 2003). For the moment it is not exactly known whether those cells are identical to BM MSCs by the function in the organism and how close they are in their developmental origin. This reasoned to name MSC-like cells from adipose tissue as "adipose-derived stromal cells" (ADSC).

The cells similar to BM MSCs and ADSCs were also isolated from bone (Nakahara, Goldberg et al. 1991), tendon (Bi, Ehirchiou et al. 2007), umbilical cord blood (Erices, Conget et al. 2000), umbilical cord (Romanov, Svintsitskaya et al. 2003), placenta (In 't Anker, Scherjon et al. 2004) and other tissues. At the same time, the comprehensive analysis and comparison of those cells with similar properties has never been performed. It cannot be excluded that the related or even identical progenitor cells reside in many mesodermal tissues maintaining the homeostasis and regeneration of the hosting tissue (Caplan 2008; Ratajczak, Zuba-Surma et al. 2008). On the other hand, the similarity in properties can be a result of cell culture methods artifacts. However, the origin and the properties of those cells have to be thoroughly studied to make a conclusion.

1.1.4. Isolation of bone marrow mesenchymal stromal cells

Different isolation procedures are applied to derive MSCs from the bone marrow. The basic property of stromal cells in the bone marrow is plastic adherence (despite other cell types like macrophages can also attach). The bone marrow can be plated as a whole cell suspension or after centrifugation in the Percoll or Ficoll gradient to isolate mononuclear fraction (Haynesworth, Goshima et al. 1992; Pittenger, Mackay et al. 1999). The negative selection to deplete haematopoietic cells from the leukocyte ($CD45^+$) and erythrocyte lineage ($TER199^+$ or $GlyA^+$) is widely used. The positive selection for different markers can enrich the MSC population (CD90, CD105), but specifically might be applied for isolation of the specific subsets of cells, described below in details.

The cell density of the primary culture has a significant influence to the properties of MSC culture. As initially was discovered, MSC represent population of clonogenic cells, hence, the bone marrow should be plated at a low density (10^5-10^6 cells per 10cm dish) to select for the cells capable to grow in the density-independent manner. Plating of the bone marrow with higher density enables more committed cells from stroma to survive and grow in the monolayer (Sacchetti, Funari et al. 2007). Hence, the population from high-density cultures is more heterogeneous and might contain more progenitors than stem cells.

It should be noted that most of the methods were developed for the cells from human bone marrow, and work with the mouse tissues is still obstructed with many difficulties (Sun, Guo et al. 2003; Peister, Mellad et al. 2004). The cells have much less proliferative potential and stop growing very fast. One of the possible explanations was proposed that mouse bone marrow cultures are contaminated with macrophages and other blood cell types more substantially than the human ones (Krebsbach, Kuznetsov et al. 1999; Tropel, Noel et al. 2004). Another possibility could be that murine equivalents of the human BM MSCs require the presence of unknown growth factors.

1.1.5. Markers of bone marrow mesenchymal stromal cells

The absence of the specific markers defining BM MSCs was always a serious obstacle for the isolation, studies *in vitro* and search for BM MSCs *in situ*. BM MSCs can be characterized by a set of surface molecules, but none of them can specifically recognize BM MSCs from the other cells types. According to the accepted rules

(Dominici, Le Blanc et al. 2006), populations of BM MSCs should express CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. Specific subpopulations of BM MSCs defined by the markers will be described in the other chapters.

1.1.6. Differentiation of bone marrow mesenchymal stromal cells

One of the basic properties of BM MSCs is the ability to differentiate *in vitro* into the cells of mesodermal origin, at least to osteocytes, chondrocytes and adipocytes. Most of the protocols of *in vitro* differentiation were established for the human BM MSCs, but from our experience can be translated to mouse cells (Anastassiadis, Rostovskaya et al. 2010). The fundamental mechanisms of differentiation can be studied using an *in vitro* model. The next chapter will be dedicated to the differentiation of BM MSCs, the molecular control and mechanisms of *in vitro* induction. Most of the information in the literature is devoted to the osteo- and adipogenesis, thus those pathways will be discussed more detailed.

1.1.6.1. Adipogenic induction and molecular control of adipogenesis

The mechanism of adipogenic induction *in vitro* is probably the best understood amongst the lineages of MSC differentiation. For a long time it was intensively studied on the alternative model, adipogenic clones of 3T3 fibroblasts L1 and F442A, which provided a lot of information on the adipogenic induction *in vitro* (Green and Meuth 1974). It has to be taken into consideration that those clones of fibroblasts were selected specifically for their ability to generate adipocytes, and the exact identity of these cells and the relevance to the adipogenic differentiation of MSCs is not obvious. The protocols for adipocyte induction of L1 and F442A are not identical to the one for MSCs and also differ from each other (Wu, Xie et al. 1995).

The regulation of adipogenic differentiation involves numerous positive and negative signals, which interact to keep a controlled balance (Fig. 2). The activation of adipogenic program involves the cooperation of transcription factors, PPARγ (peroxisome proliferator-activated receptors) and C/EBP family (CCAAT-enhancer binding proteins, (Wu, Bucher et al. 1996; Wu, Rosen et al. 1999)). PPARγ is a known master gene for adipogenesis (Tontonoz, Hu et al. 1994). There are 2 isoforms, PPARγ1 and PPARγ2. PPARγ1 is widely expressed in the tissues, whereas

PPARγ2 is restricted to the adipose tissue. Those 2 isoforms perform the same function during adipogenesis, but γ2 was found to be more active (Mueller, Drori et al. 2002). PPARγ forms heterodimers with RXRα (retinoid X receptor α) and in the presence of activators regulates the transcription of genes responsible for lipid methabolism and adipocyte development. PPARγ is an orphan receptor and natural ligands for it were not identified, although there is an evidence of their existence (Kim, Wright et al. 1998). It was shown that prostaglandins could activate PPARγ, although in the concentrations that are non-physiological. The synthetical activators are thiazolidinediones (TZDs, rosiglitazone, troglitazone).

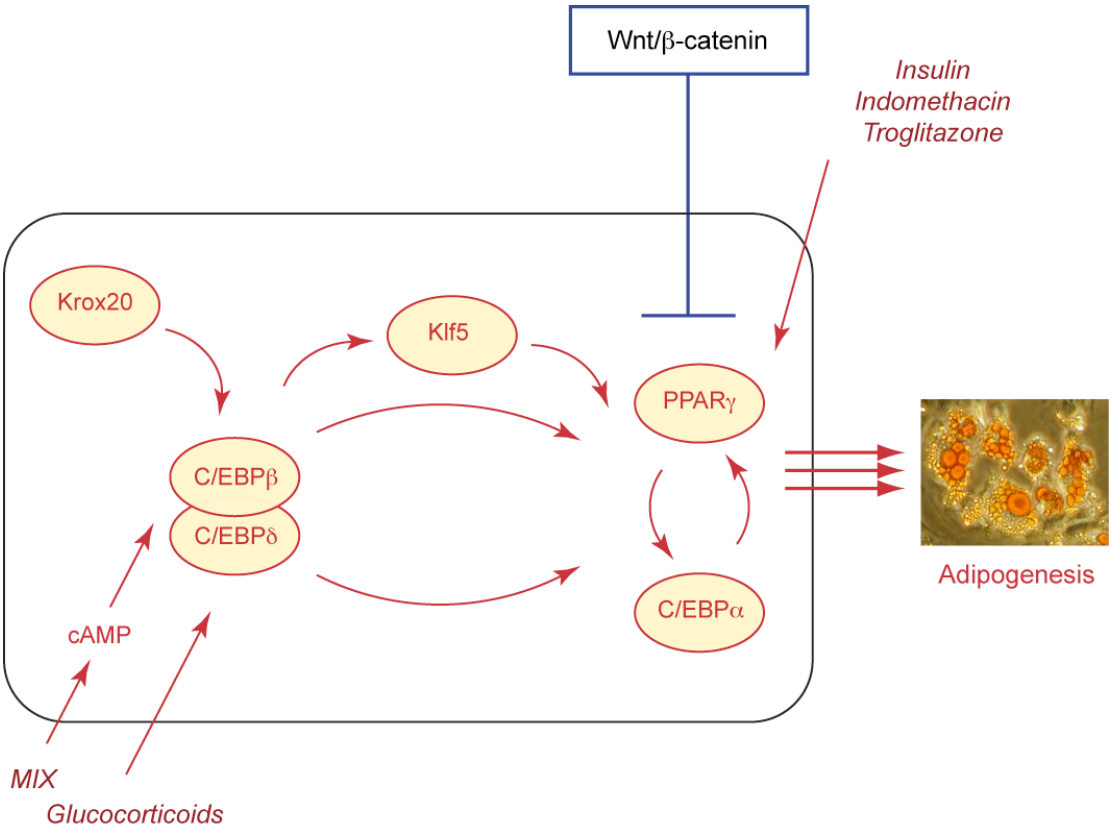


Figure 2. Molecular regulatory pathways of adipogenesis and adipogenic induction *in vitro*. In red - activatory signals, in blue - inhibitory signals.
Krox20 is the earliest regulator that activates the expression of C/EBPβ. The complex of C/EBPβ and C/EBPδ induces expression of C/EBPα and PPARγ, which maintain the expression of each other. Klf5 is induced by C/EBPβ and functions as co-activator to induce PPARγ. C/EBPα and PPARγ start the whole adipogenic programm. Wnt/β-catenin pathway inhibits adipogenesis.
Exposure to MIX activates C/EBPβ through cAMP signaling; glucocorticoids promotes C/EBPδ; insulin, indomethacin, troglitazone stimulate PPARγ.

Krox20 - early growth response-2, Egr-2; C/EBP - CCAAT - enhancer binding proteins; PPAR - peroxisome proliferator-activated receptor; Klf5 - Krueppel-like factor 5; Wnt - wingless-type MMTV integration site; MIX - methyl-isobutyl-xanthine.

C/EBP's are a family of transcription factors that contain basic-leucine zipper domain (bZIP) that is involved in dimerization and DNA binding. The expression of different C/EBP's is not restricted to the particular tissues, but the combination of them might be tissue-specific. C/EBPα plays an important role in terminal adipocyte differentiation. C/EBPα activates the expression of some genes involved in adipogenesis in cooperation with PPARγ, in turn C/EBPα and PPARγ cross-activate each other. Ectopical expression of C/EBPα and PPARγ is enough to stimulate the adipogenesis in fibroblasts even in the absence of PPARγ ligands (Tontonoz, Hu et al. 1994). At the same time the role of C/EBPα and PPARγ is not redundant, and PPARγ is believed to be the main activator of adipogenesis in fibroblasts. This was demonstrated in the fibroblasts lacking one of those factors. Both C/EBPα$^{-/-}$ and PPARγ$^{-/-}$ cells fail to undergo adipogenesis. Adipogenic properties of C/EBPα$^{-/-}$ fibroblasts can be rescued by ectopical expression of PPARγ, but not the other way around indicating that adipogenesis can occur in the absence of C/EBPα, but PPARγ is essential (Rosen, Hsu et al. 2002).

C/EBPα is expressed in two isoforms, 42kDa and 30kDa. This is one exon gene and the alternative splicing cannot take place, the generation of the two isoforms occurs through the alternative ATG. Presumably, 30kDa isoform can act as a dominant-negative regulator of 42kDa (Ramji and Foka 2002).

C/EBPβ and C/EBPδ stand upstream of C/EBPα and PPARγ. It was shown that glucocorticoids could induce C/EBPδ, which forms heterodimers with C/EBPβ, and this was enough for initiation of PPARγ and C/EBPα expression (Wu, Bucher et al. 1996). Conditional ectopical expression of C/EBPβ in non-adipogenic cells (fibroblasts and myoblasts) can induce adipogenesis, whereas C/EBPδ was not efficient (Wu, Xie et al. 1995).

Several isoforms were shown for C/EBPβ, larger 38kDa and 35kDa (liver-activated protein, LAP), and smaller 21kDa (liver inhibitory protein, LIP) and 14kDa. The smaller isoforms are lacking the transactivation domain and cannot activate transcription of downstream genes. Those isoforms can form complexes with C/EBPδ, but stay inactive and serve as dominant-negative forms. Particularly, it is known that expression of LIP blocks adipogenesis. It is interesting, that the gene of C/EBPβ has one exon, and the formation of isoforms occurs similarly to C/EBPα, through the alternative ATG usage (Ramji and Foka 2002; Farmer 2005).

Later two other factors were suggested to participate in the cascade of adipogenic induction, Krox20 and Klf5. Zinc finger-containing transcription factor Krox20 (early growth response-2, Egr-2) was induced during the first minutes of adipogenic stimulation *in vitro*, and presumably was responsible for activation of C/EBPβ expression (Chen, Torrens et al. 2005). Klf5 (krueppel-like factor 5) was upregulated by C/EBPβ and C/EBPδ and in concert with C/EBPβ;C/EBPδ complex could activate the expression of PPARγ (Oishi, Manabe et al. 2005). Taken together, the complex system of transcriptional factors activating expression of adipogenic genes and each other is involved in the induction of the differentiation.

The negative regulation of adipogenesis particularly involves Wnt/β-catenin signaling (wingless-type MMTV integration site), Fig. 3. Wnt's are the family of secreted paracrine and autocrine factors that regulate cell growth and cell differentiation. The canonical and non-canonical Wnt pathways are described, and canonical Wnt/β-catenin pathway is believed to play an important role in the regulation of adipogenesis (Prestwich and Macdougald 2007). Wnt signal is transmitted through Dishevelled proteins that inhibits the kinetic activity of the complex containing glycogen synthase kinase 3 (GSK-3), axin, adenomatosis poliposis coli (APC) and other components. In the absence of Wnt signaling, GSK-3 complex phosphorylates β-catenin targeting it to the proteasomal degradation. When Wnt binds to its receptor Frizzled (Fz) and the kinetic activity of GSK-3 is inhibited, unphosphorylated β-catenin is stabilized, translocates to the nucleus and forms the complex with T-cell factor/lymphoid-enchancing factors family (TCF/LEF's) activating the trascription of downstream genes (Nelson and Nusse 2004).

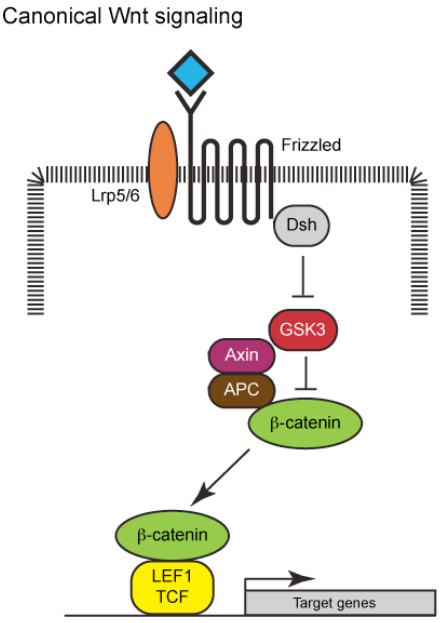


Figure 3. Canonical Wnt/β-catenin signaling pathway. Extracellular Wnt binds to the Frizzled/LRP receptor, Dishevelled transmits the signal and represses GSK-3 complex. Stabilized β-catenin translocates to the nucleus and in complex with TCF/LEF activates transcription of target genes.

LRP - low-density lipoprotein receptor-related protein; Dsh - Dishevelled; GSK-3 - serine/threonine kinase, glycogen synthase kinase-3; APC - adenomatosis poliposis coli; LEF1 - lymphocyte enhancer factor 1; TCF - T-cell factor .

Pre-adipocytic fibroblast lines L1 and F442A express Wnt10b and Wnt receptors, Fz-1, 2 and 5. It was suggested, that active endogenous canonical Wnt pathway prevents 3T3-L1 cells from adipogenic differentiation. Ectopical expression of the canonical Wnt1 blocks adipogenic properties of L1 cells. The expression of C/EBPβ and C/EBPδ was not affected, but C/EBPα and PPARγ were largely repressed. Additional ectopical expression of C/EBPα and PPARγ could rescue the differentiation potential of L1-Wnt1 cells (Ross, Hemati et al. 2000). These data were confirmed by activation of Wnt/β-catenin pathway using GSK3 inhibitors such as lithium chloride and CT99021 (Bennett, Ross et al. 2002). The differentiation of L1 cells was sensitive to the inhibition of GSK3 on the early stages (1-3 days of induction), but was not susceptible later anymore. This was correspondent to the time of initiation of C/EBPα and PPARγ expression. The authors concluded that Wnt blocks the establishment of C/EBPα and PPARγ expression, but once the expression is established, then it is maintained. Conversely, during the adipogenic induction of 3T3-L1 cells PPARγ suppresses Wnt/β-catenin signaling allowing differentiation to proceed (Moldes, Zuo et al. 2003). Hence, there is a reciprocal relationship between PPARγ and Wnt in the maintenance of balance between differentiated/undifferentiated state.

Inactivation of Wnt/β-catenin pathway by the expression of dominant-negative form of TCF4 in myogenic cell line C2C12 caused the spontaneous differentiation of myoblasts into adipocytes (Ross, Hemati et al. 2000). Those data suggested that canonical Wnt pathway might function as the main adipogenic switch in the cells.

Adipogenic differentiation of BM MSCs can be induced by hormonal coctail of dexamethasone, methyl-isobutyl-xanthine (MIX), indomethacin and insulin (Sekiya, Larson et al. 2004), Fig. 2. The role of dexamethasone is to enhance the expression of C/EBPδ. MIX is a phosphodiesterase inhibitor, which inhibits cAMP decay. This results in the activation of protein kinase A (pkA)-dependent and pkA-independent signaling (Martini, Plaza et al. 2009). The pkA-dependent mechanism leads to the translocation of CREB (cAMP-responsive element binding protein) to the nucleus and hence, to the activation of C/EBPβ expression. Another effect is the production of PPARγ cellular ligands, which are not identified yet (Farmer 2005). Indomethacin itself can also serve as a ligand for PPARγ (Lehmann, Lenhard et al. 1997). Alternative protocol suggests the use of dexamethasone, troglitazone and insulin for the differentiation. Troglitazone is a synthetic agonist of PPARγ, which can substitute MIX and indomethacin. The main morphological sign of the differentiation is the

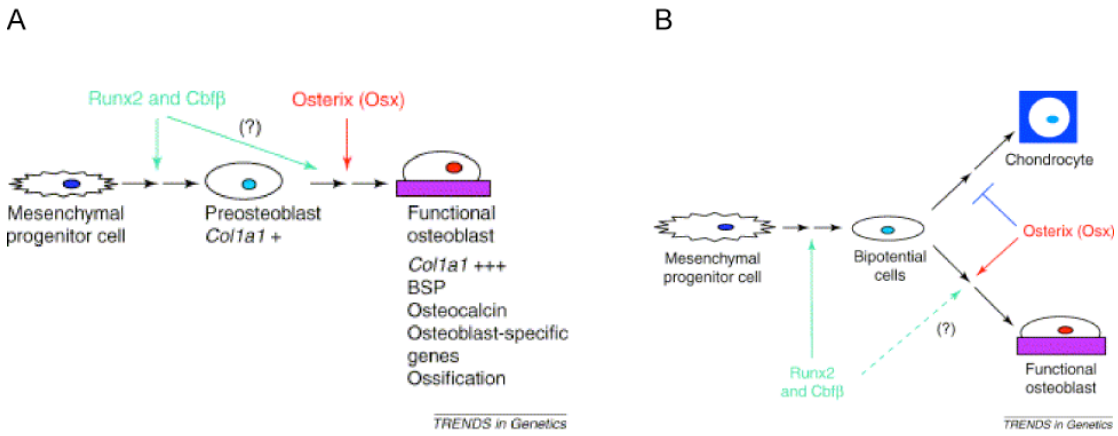
accumulation of oil drops in the cytoplasm, which can be visualized by Oil Red staining. The main markers used to verify adipocytes are adipsin, adiponectin, AP2 (adipocyte protein 2), PEPCK (phosphoenolpyruvate carboxylase), LPL (lipoprotein lipase).

1.1.6.2. Osteogenic induction and molecular control of osteogenesis

Most of the information about the transcriptional control of osteogenesis rises from the studies of the phenotypes of mutant mice. However, most of the effects are associated with osteogenesis in the embryo, and this makes it difficult to separate the role of those factors during the osteogenesis from adult BM MSCs. Nevertheless, the expression of main transcription factors and markers during osteogenic differentiation of BM MSCs *in vitro* reproduces the ones during embryonic bone formation, suggesting that the main principles may be translated (Fig. 4A).

The key regulator of osteoblast development *in vivo* is Runt-related transcription factor 2 (RUNX2, also known as Cbfa1, AML3 or PEBP2αB). RUNX2 is expressed in chondrogenic mesenchyme in the embryo, chondrocytes, hypertrophic chondrocytes and the whole osteoblastic lineage. RUNX2 forms heterodimers with Cbfb and activates transcription of osteoblast-specific genes. The RUNX2 null-mutant mice die just after birth and lack completely bone formation (Komori, Yagi et al. 1997). The skeleton of $RUNX^{-/-}$ mice is built exclusively from the mesenchymal tissue, which is not maturated into the cartilage, and hypertrophic chondrocyte differentiation was inhibited. Similar phenotype was observed in mice deficient for Osterix (Osx), which is a zinc-finger transcription factor expressed specifically in osteoblasts (Nakashima, Zhou et al. 2002). The skeletal tissue of these mutants was structured to chondrogenic phenotype. In Osx-null mutants the level of RUNX2 was comparable to the wild type, whereas Osx was not detected in $RUNX^{-/-}$ mice indicating that these factors belong to one pathway, but Osx is downstream of RUNX2. It was suggested that the osteoblasts in Osx-mutants are arrested in the development and acquire the phenotype of chondrocytes expressing Sox9, Sox5 and Collagen II. The formation of hypertrophic cartilage was not affected in Osx-null mice. These data allowed to propose a model of the embryonic bone development (Nakashima and de Crombrugghe 2003). Osteoblast progenitors differentiate into pre-osteoblasts and RUNX2 plays an essential role in this process (Fig. 4B). Those pre-osteoblasts are bi-potential and could develop into both osteo- and chondrocytes. Osterix is required to commit the cells into mature osteoblasts and to

restrict from chondrocyte phenotype. In the absence of RUNX2 osteoblasts and chondrocytes cannot be formed, and knockout of Osterix blocks the specification to osteoblasts and the cells adopt the phenotype of chondrocytes.



Nakashima, Crombrugghe, 2003.

Figure 4. Osteogenic differentiation of MSCs. (A) Multipotential mesenchymal progenitor cells differentiate into preosteoblasts and RUNX2 and Cbfb are essential for this step. Preosteoblasts differentiate further into functional osteoblasts in a process that requires Osterix. Osteoblasts express collagen Ia, bone sialoprotein, osteocalcin and produce mineralized extracellular matrix. (B) A hypothetical role of Osterix in segregating osteoblasts and chondrocytes from a common osteochondroprogenitor. Precursor cells that express Runx2 are bipotential and can differentiate into either osteoblasts or chondrocytes. Osx is a negative regulator of Sox9 that commits precursors to osteoblast lineage and inhibits chondrocyte formation. From Nakashima and Crombrugghe, 2003.

The signaling pathways modulating osteogenesis are far from clear understanding. Mitogen-activated protein kinase family members, MAPK, were shown to enhance the osteogenic potential of pre-osteoblasts *in vitro* by the positive regulation of RUNX2 (Xiao, Jiang et al. 2000). Wnt has an evident role in the regulation of osteogenesis, however the reported effects of Wnt's are often controversial, depending on the model used in the study. Ectopic expression of the canonical Wnt10b in bi-potential ST2 cells led to the inhibition of adipogenic potential and enhancement of osteogenic properties (Kang, Bennett et al. 2007). This is in agreement with the observation that mice mutant for Lrp5, a co-receptor of Wnt, have impaired bone development (Krishnan, Bryant et al. 2006). The other studies on primary BM MSCs suggested an inhibitory effect of exogenous Wnt1 and Wnt3a on the osteogenic differentiation (Baksh, Boland et al. 2007; Liu, Vijayakumar et al. 2009). The reason for such discrepancy is unclear. In the context of different models, cell lines and cell culture conditions, it is difficult to compare the results of the experiments. It cannot be excluded that the effect of Wnt on the osteogenesis might

be stage-specific or concentration-dependent (De Boer, Wang et al. 2004). In this case it is not possible to make a clear conclusion using constitutive overexpression assays or exogenous factors.

Osteogenic differentiation of BM MSCs can be induced in subconfluent cultures by adding dexamethasone, ascorbic acid and β–glycerophosphate. The cells undergo the stage of transient proliferation until they reach monolayer, and then stop proliferating and differentiate terminally after 3-4 weeks. The differentiation is usually accompanied by the synthesis of extracellular matrix composed of collagen I and calcification of the matrix. Calcification of the matrix visualized by histological staining (Alizarin Red or Von Kossa) serves as the most often used validation of differentiation. It is unlikely that differentiation conditions *in vitro* reflect physiological signals *in vivo*. According to some studies, bone morphogenic proteins promote the bone growth. It was shown that BMP-2 and BMP-6 (bone morphogenic proteins 2 and 6) increase the expression of osteogenic markers in BM MSCs and can induce osteogenic differentiation (Hanada, Dennis et al. 1997; Sammons, Ahmed et al. 2004; Friedman, Long et al. 2006). Interestingly, parathyroid hormone and vitamin D3 increase the efficiency of osteogenic differentiation of MSCs, probably reproducing the development of the bone tissue (Sammons, Ahmed et al. 2004). At the same time the differentiation of BM MSCs by the classical protocol using dexamethasone reproduces the changes of the markers and transcription factors during osteoblast development. Particularly, RUNX2, osterix, Msx2 (msh homeobox-2), osteopontin, osteocalcin and osteonectin are upregulated during the osteogenic differentiation *in vitro*.

1.1.6.3. Chondrogenic induction and molecular control of chondrogenesis

The regulation of chondrogenic differentiation is less described in the literature than the other pathways. Chondrogenesis in BM MSCs can be induced by the cell aggregation and adding TGFβ (transforming growth factor β) and dexamethasone for 21 days (Johnstone, Hering et al. 1998). The cell aggregation can be achieved by centrifugation, and then the cells are cultured in the dense pellet. It was observed, that some MSC and ADSC lines express low level of TGFβ-receptor, in this case BMP-6 can induce TGFβR expression and make the cells permissive to TGF signaling (Sekiya, Colter et al. 2001). The detailed mechanism of

chondrogenic induction is not described. The microarray studies characterized the progression of the events during the chondrogenic differentiation (Sekiya, Vuoristo et al. 2002). The authors found that chondrogenesis occurs through the particular stages, and the expression of the transcription factors and markers changes sequentially. Differentiation was accompanied with a large increase in the expression of matrix proteins (in case of collagen II more than 500-fold upregulation). The early stages were connected with the increase in collagen II and XI, COMP (cartilage oligomeric matrix protein), dermatopontin, aggrecan. Later on, the components present in hypertrophic cartilage appeared, such as collagen X, fibromobulin and osteomodulin. The transcription factors were expressed also stepwise according to the stage of the differentiation. The earliest detected event was the upregulation of Sox4 (on the 1 day of induction, and then downregulation on the day 14). Sox5, Sox9 and HoxA7 were upregulated from day 7 till 21. BMP-2 and TGFβ3 were elevated during the whole course of differentiation.

The verification of differentiation usually includes the detection of specific cartilage matrix components, as aggrecan and collagen II using the antibodies, and glycosaminoglycans with Alcian Blue. The important parameter is the structure of the pellet; it reproduces the structure of articular cartilage, where chondrocytes are located in the lacunas of the matrix.

1.1.7. Plasticity of bone marrow mesenchymal stromal cells

The above described pathways are considered as “classical” pathways of BM MSC differentiation. BM MSCs can give rise to osteocytes, adipocytes, chondrocytes, haematopoietic-supportive stroma, and fibroblastic tissue in the *in vitro* and *in vivo* environments. The lack of BM MSC markers makes it impossible to perform lineage tracing experiments, therefore it has never been demonstrated, which cell populations within the stroma develop into those pathways and how they pass through the sequence of intermediate progenitors. However, several observations could suggest the principles of BM MSC development. It was reported that clonal strains of primary bone marrow adipocytes could de-differentiate *in vitro* and in the transplantation assay, and then can be induced to the osteogenic lineage (Bennett, Joyner et al. 1991). Different studies showed that primary hypertrophic chondrocytes could de-differentiate in culture and shift to osteogenic phenotype (Descalzi Cancedda, Gentili et al. 1992). At least similar process takes place in the organism during ossification of cartilage (Galotto, Campanile et al. 1994). Those

studies suggest, that it shouldn't be excluded, that the primary cells from different lineages of BM MSC differentiation can switch between each other. It has been also noted, that differentiated cells derived *in vitro* from BM MSCs can transdifferentiate upon inductive extracellular signals (Song and Tuan 2004; Schilling, Noth et al. 2007; Schilling, Kuffner et al. 2008). It was claimed that switch happens through the de-differentiation step and possible in all the directions between osteo-, adipo- and chondrogenic pathways. The stromal lines applied in those works were not clonal and represented heterogeneous populations, but the authors sorted out the committed osteoblasts based on the expression of osteocalcin reporter and observed the same phenomenon of transdifferentiation.

In some literature sources the property of BM MSC progeny to switch between lineages within the classical context of differentiation (osteo, adipo, chondro) is termed "orthodox plasticity" (Bianco, Riminucci et al. 2001). It is not clear whether and how this phenomenon occurs in the organism, and so far it has not been possible to present the evidence of those events. But it was shown that the key regulator of osteogenic lineage commitment Cbfa1/RUNX2 is expressed in BM MSC capable for adipogenesis (Satomura, Krebsbach et al. 2000). According to our observations, the main regulator of adipogenic commitment, PPARγ, is expressed at the protein level in clonal lines of BM MSC capable for osteogenesis (unpublished data). The model explaining such "orthodox" plasticity implies that undifferentiated BM MSCs can possess "lineage imprinting" at the single-cell level before specialization. This means, that the commitment into osteo-, adipo, and chondrogenic pathways might exist independently in the cells, and maintained after the cell enters into one or another pathway (Satomura, Krebsbach et al. 2000; Bianco, Riminucci et al. 2001). As a consequence, the transdifferentiation can occur. In this case, the classical and apparent logical hierarchical model of progressive restriction of differentiation potential is not applicable. The well-known example of such system is haematopoiesis, where the stem cells undergo multistep and unidirectional commitment and differentiation, and every intermediate stage is described as a specific identity. But for the BM MSCs this model has never been demonstrated and can't be taken without the proof. Taken together, the mechanism of BM MSC commitment has to be investigated with having regard to the uniqueness nature of the system and with unbiased consideration.

1.1.8. Cell fate choice

Bone marrow mesenchymal stromal cells have the potential to differentiate into osteo-, adipo- and chondrogenic lineages. There is accumulated information about the mechanisms regulating one or another differentiation pathway, and the essential regulators are already known. However, the control of the lineage commitment of multipotential cell is not understood. The primary events for the cell fate choice and the signals, which direct the cell to make this decision still remain mysterious. Several mechanisms are currently discussed in the literature, and most of them consider the switch between adipogenic and osteogenic pathways driven in BM MSCs by the key regulators, PPARγ and RUNX2, respectively.

The transcriptional modulation of PPARγ and RUNX2 activity was suggested as a mechanism of osteo/adipogenesis switch (Hong, Hwang et al. 2005), Fig. 5. TAZ (transcriptional coactivator with PDZ-binding motif) contains WW domain that binds Pro-Pro-R-Tyr motif. It was found that PPARγ and RUNX2 contain such motif and physically interact with TAZ. It was shown that interaction of TAZ with RUNX2 stimulates osteogenesis in primary MSC, on the other hand binding of PPARγ inhibits adipogenesis. This was an indication, that TAZ functions upstream of PPARγ and RUNX2 and participates in the lineage switch between osteo and adipogenesis. TAZ was found to be responsive to BMP-2, and BMP-2 was suggested as the main extracellular signal for the cell commitment. Inhibition of TAZ in zebrafish embryos by morpholino injection led to the block of bone formation demonstrating the role of TAZ *in vivo* (Hong, Hwang et al. 2005).

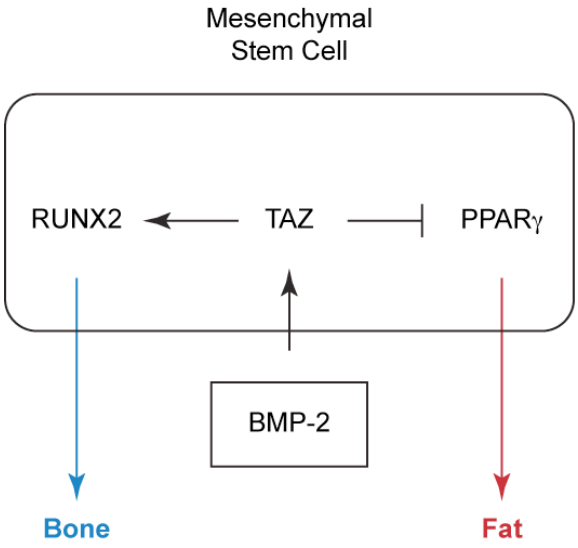


Figure 5. A model for TAZ modulation of mesenchymal stem cell differentiation. BMP-2 stimulates the expression of TAZ, which activates RUNX2 and represses PPARγ function. Hence, the differentiation is driven to osteogenic pathway. Modified from Hong et al., 2005.
TAZ - transcriptional coactivator with PDZ-binding motif; BMP-2 - bone morphogenic protein 2; PPARγ - peroxisome proliferator-activated receptor γ; RUNX2 - runt-related transcription factor 2.

In another study the main role in the cell fate decision was given solely to PPARγ (Takada, Mihara et al. 2007). The authors reasoned that young animals during extensive skeletal growth have suppressed PPARγ function, and PPARγ haploinsufficiency causes the loss of adipocytes and increase in the bone mass. The opposite phenotype was observed in Wnt5a$^{+/-}$ adult mice providing an evidence of negative regulation of PPARγ by non-canonical Wnt signaling. The assumption was that many nuclear receptors function through the formation of complexes including epigenetic modification enzymes. In the inactive state the complex contains epigenetic repressors, such as histone deacetylases (HDAC) and histone 3 lysine 9 methyltransferases (H3K9 HKMT). Ligand binding to the nuclear receptor leads to the remodeling of the complex and recruiting epigenetic activators, such as histone acetylases (HAT) and histone 3 lysine 4 methyltransferases (H3K4 HKMT). Taking this into consideration, the authors proposed the epigenetic regulation of PPARγ function in response to non-canonical Wnt signaling as a mechanism for the lineage switch in MSCs (Fig. 6). The study was done on ST2 cells with osteo/adipogenic potential. The authors demonstrated, that activation of non-canonical Wnt5a favored cells to osteogenic differentiation in expense of adipocyte formation. The analysis of the key players in the chain allowed the authors to suggest a model of activation/repression of PPARγ. In the absence of Wnt5a PPARγ is active and the promoters of target genes have active epigenetic marks with methylated H3K4. Wnt5a activates NLK (Nemo-like kinase), which in turn phosphorylates a histone methyltransferase, SETDB1 (Set domain bifurcated 1), leading to the formation of co-repressor complex that methylates H3K9 of PPARγ target genes and inhibits its transactivation function. The inactivation of PPARγ function results in the switch to osteogenic cell fate.

The role of canonical and non-canonical Wnt signaling in the BM MSC lineage commitment is extensively discussed in the literature. At the same time, the data and the conclusions are non consistent and sometimes opposite. For example, the canonical Wnt pathway was suggested to have a role in the lineage switch in ST2 cells by stimulation of osteoblastogenesis and inhibition of adipogenesis (Kang, Bennett et al. 2007). In another study, it was observed that canonical Wnt3a inhibits osteogenic differentiation and non-canonical Wnt5a pathway enhances osteogenesis (Baksh, Boland et al. 2007). The authors proposed a model of functional antagonism between canonical and non-canonical Wnt pathways. As we mentioned earlier, the application of different models complicates the comparison of the results from different studies and makes it impossible to bring to consistence.

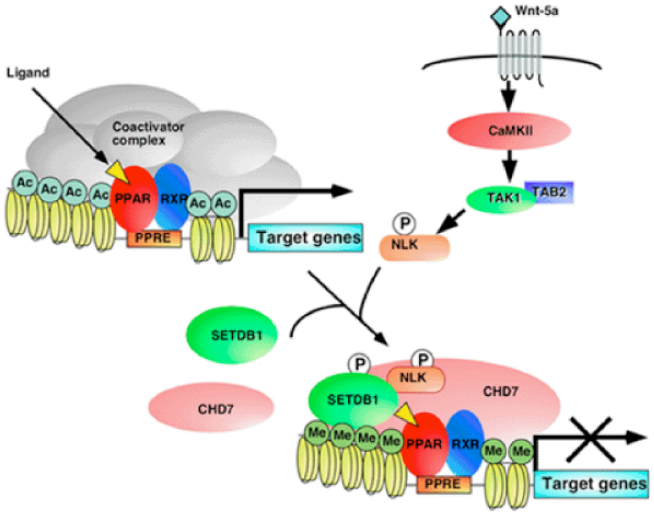


Figure 6. A model for Wnt5a-dependent epigenetic suppression of PPARγ function. In the absence of Wnt5a signaling, PPARγ associates with epigenetic modifiers making active marks on histone tails (HAT, H3K4 methyltransferase) that allow the transcription of target genes in the presence of PPARγ ligands. Wnt5a signaling leads to the remodeling of the complex that contains HDAC and H3K9 methyltransferase and represses PPARγ activity through histone modifications on the promoters of target genes. From Takada et al., 2007.

PPAR - peroxisome proliferator-activated receptor; RXR - retinoid X receptor; PPRE - PPAR resopnsive element; Ac - acetyl; CaMKII - calcium/calmodulin-dependent protein kinase II; TAB2 - TAK1-binding ptotein 2; TAK1 - TGFβ-activated protein kinase 1; NLK - Nemo-like kinase; SETDB1 - H3K9 methyltransferase, SET domain bifurcated 1; CHD7 - chromodomain-helicase-DNA-binding protein 7; Me - methyl.

Indeed, there is a growing evidence that those signaling pathways interact and cross-talk in the cells, providing a balance and fine tuning of the cellular state (Ling, Nurcombe et al. 2009). The utilization of multiply pathways reflects a large responsiveness of the cells to the extracellular stimuli, which is required for the specification of the cells in the complex system of the whole tissue and for the maintenance of the integrity of the organism. However, the dual role of the signals being inductive/repressive for the different processes does not imply the role in the lineage choice, and may stay downstream from the initial events. Taking into account the possible plasticity of the BM MSC descendants between osteo-, adipo- and chondrogenic lineages, which we discussed earlier, the dual function of the signaling can maintain the cell in the particular lineage and prevent from the escape into another pathway. From this point of view, the critical role of the factor in the lineage commitment cannot be proven by the opposite effects to the alternative types of differentiation.

1.1.9. Heterogeneity of bone marrow mesenchymal stromal cells

Bone marrow stroma represents a complex tissue of mesenchymal stem cell descendants. The *in vitro* differentiation potential presumes the presence of intermediate progenitors of different lineages and probably mesenchymal stem cells themselves in the heterogeneous cell population. The first findings connected stromal stem cells with CFU-F's (colony forming unit-fibroblasts), showing that the progeny of single CFU-F can differentiate and self-renew (Friedenstein, Gorskaja et al. 1976). However, only proportion of CFU-F's exhibited such properties. Later, characterization of CFU-F's from rabbit bone marrow revealed differences in the size, morphology and alkaline phosphatase activity in the CFU-F's indicating that they might represent cells on the different stages of development (Owen, Cave et al. 1987). The differences in the osteogenic potential of human CFU-F's was confirmed in the transplantation assays (Kuznetsov, Krebsbach et al. 1997).

The first study summarizing the multilineage differentiation properties of BM MSCs was done by Pittenger and colleagues (Pittenger, Mackay et al. 1999), and since then differentiation potential into osteo-, adipo- and chondrocytes started to be intensively analysed. Already in this work, the authors noted that only 2 out of 6 clonal lines possessed three-lineage potential, the others were bipotent (osteo/adipo) or monopotential (osteo). These observations were extended further in the larger scale in two works from the group of R. Quarto (Banfi, Muraglia et al. 2000; Muraglia, Cancedda et al. 2000). Human CFU-F's were assessed by the differentiation potential (80 CFU-F's in the first study and 185 CFU-F's in the second), and 24-34% of them were tripotential, 61-71% combined osteo and chondrogenic properties, and 4-5% were purely osteogenic. The differentiation was checked on different passages. It was observed, that the clones were loosing their differentiation abilities with passaging, and they displayed at first the loss of adipogenic potential (great reduction after 19 doublings) and then chondrogenic (drop after 22 doublings), at the end becoming osteoblasts. The authors suggested that osteoblastic cell fate as a "default" lineage in BM MSC, and the existence of "linear hierarchy" in the differentiation pathway. They proposed, that BM MSCs proceed differentiation gradually from multipotent state through stepwise diverging of (1) adipogenic and (2) chondrogenic lineages. The heterogeneity of CFU-F's and the loss of potential with passaging was confirmed by the other groups in the analysis of osteo- and adipogenic potential *in vitro* (Digirolamo, Stokes et al. 1999). At the same time their observations could be interpreted that BM MSC heterogeneity is a result of the

influence of cell culture conditions and spontaneous differentiation, which was not taken into account.

A different result was obtained using human BM MSCs immortalized with human telomerase (hTERT) and human papilloma virus E6 and E7 genes (Okamoto, Aoyama et al. 2002). Immortalized clonal lines were established and analysed for differentiation potential. Only 34 out of 100 clones retained the potential to differentiate, and amongst them tripotent (osteo-adipo-chondro), bipotent (osteo-adipo and osteo-chondro), and monopotent (osteo, adipo or chondro) were found. Immortalized BM MSCs did not loose their potential during the passaging. Those data contradicted the "linear hierarchy" proposed by Muraglia et al (Muraglia, Cancedda et al. 2000) indicating that the process is not completely understood and requires more comprehensive analysis.

1.1.10. Subpopulations of BM MSCs and shifting of paradigms

Different subpopulations of BM MSCs were isolated by the surface marker expression, morphology, proliferation capacity, cell cycle state, clonogenicity or other characteristics. The multiple origins of MSCs during the embryonic development can explain the differences in the properties between the described subsets of MSCs (Takashima, Era et al. 2007). It is possible that some of those subpopulations are identical or overlap, but the comprehensive study to consolidate the results of different groups has not been performed. Some of those works raised important questions on fundamental properties and developmental origin of MSCs, and generally adult stem cells. These studies deserve to be specially mentioned.

1.1.10.1. Rapidly Self-renewing (RS) Cells and MSC heterogeneity

The stromal cells change their morphology, population doubling time and differentiation properties during the passaging, causing the discrepancies in the results of the experiments. The morphologically diverse cells in the primary cultures turn into homogeneous populations, which can be explained by the selective proliferation of one cell type or by the conversion of the different cells into one type *in vitro*. The team of D. Prockop concentrated their efforts on study of primary MSCs plated with a low density (3 cells/cm^2) to observe different cell types in culture (Colter, Sekiya et al. 2001; Prockop, Sekiya et al. 2001; Smith, Pochampally et al.

2004). They identified three types of cell: large flattened, spindle-shaped, and small rapidly proliferating cells. The small rapidly proliferating (RS) cells were proposed as more primitive cells state. Those cells had higher differentiation potential (the efficiency of osteo-, adipo- and chondrocyte formation was estimated as 1.4 – 6-fold as more efficient) and expressed some specific markers as Flk-1. At the same time RS cells could not be maintained in culture as pure cell populations as they were rapidly turning into the fibroblastic stromal cells, presumable more mature. Those studies did not intensified furthermore, and whether RS cells represent a specific cell type and resemble any cells in the bone marrow is unclear. However, this was one of the first works attracted attention to the heterogeneity of stromal cells in primary cultures. The effort to characterize the stromal cultures on the single-cell level should be considered on its merits.

1.1.10.2. Multipotent Adult Progenitor Cells (MAPCs) and the plasticity of BM MSCs

One of the first and the most-pronounced reports on the existence of the cells with extended differentiation potential in the bone marrow was done by the group of C. Verfaillie. The cells termed multipotent adult progenitor cells (MAPCs) were isolated form human (Reyes, Lund et al. 2001) and mouse (Jiang, Jahagirdar et al. 2002) bone marrow. MAPCs were identified as a rare subpopulation of cells in the bone marrow, which can be expanded indefinitely in the presence of EGF (epidermal growth factor) and PDGF-BB (platelet-derived growth factor BB), expressed telomerase, Rex-1 and Oct-4 (1000-fold lower than embryonic stem cells according to qPCR). Those cells were found to have the potential to differentiate *in vitro* into neuroectoderm, endothelium and hepatocytes. Additionally, MAPCs could engraft to many organs (intestine, lung, liver, blood, bone marrow) after intravenous injection. The most stricking result was shown that murine MAPCs could contribute to different tissues of embryo and adult progeny after injection into blastocyst. Those tissues included brain, liver, intestine, kidney, spleen, muscle, bone marrow, blood and others. Surprisingly, the germ line transmission was not shown. All those results allowed to the authors to claim the pluripotency of MAPCs isolated from the bone marrow and to suggest similarity to embryonic stem cells. In support, the murine MAPCs required the presence of LIF (leukemia inhibitory factor) in the medium similarly to embryonic stem cells.

Several other publications were released on the MAPC biology (Subramanian, Geraerts et al. ; Jiang, Vaessen et al. 2002; Serafini, Dylla et al. 2007). Nonetheless, the results from the first report could not be reproduced by the other groups. For the moment, the identity of MAPCs is unclear and their existence is questionable. It is possible, that those cells represent cell culture artifact selected by the *in vitro* conditions (Phinney and Prockop 2007), or resulted from the chromosomal aberrations and malignization of cells.

The phenomenon of differentiation into the cells from another germ layer is generally called “plasticity”, or “non-orthodox plasticity” to distinguish from the transdifferentiation events within one germ layer. The results of Verfaillie and colleagues raised the interest of the scientific society to look for the plasticity of bone marrow-derived stem cells (Krause, Theise et al. 2001; Weissman, Anderson et al. 2001; Graf 2002). The *in vitro* differentiation of BM MSCs into neurons (Kohyama, Abe et al. 2001), hepatocytes (Lee, Kuo et al. 2004), insulin-producing cells (Moriscot, de Fraipont et al. 2005) was reported. Many transplantation assays confirmed the plasticity of BM MSCs (Petersen, Bowen et al. 1999; Brazelton, Rossi et al. 2000; Lagasse, Connors et al. 2000; Mezey, Chandross et al. 2000). On the other hand, many of those experiments could be poorly reproduced by the other groups (Wagers, Sherwood et al. 2002). Several alternative explanations can be proposed how the progeny of BM MSCs can be found in multiply tissues after transplantation. According to one point of view, cell fusion can explain the persistence of the cells marked as transplanted cells in the tissues, which are not actually derived from the latter (Terada, Hamazaki et al. 2002; Ying, Nichols et al. 2002). The other opinion states that the response to the external stimuli can adopt the cell phenotype to the hosting tissue, for example, via epigenetic changes (Morshead, Benveniste et al. 2002). At third, the existence of unknown subpopulation of cells within bone marrow can never be excluded.

1.1.10.3. Very Small Embryonic-like Stem Cells (VSELs) and pluripotent lineages in the adults

The plasticity of bone marrow-derived cells observed by some groups reasoned for the search of the rare subpopulation of cells capable for the wide spectrum of differentiation. Kucia et al applied multiparameter sorting of the mouse bone marrow to isolate the side population from $lin^{-}Sca\text{-}1^{+}CD45^{-}$ cells by staining with Hoechst33342 (Kucia, Reca et al. 2006). The cells were small in size (2-4μm), which

might explain why those cells were excluded during the usual sorting procedures. Transmission electron microscopy showed that VSEL contained large nucleus with opened chromatin (euchromatin). The cells expressed the markers usually associated with embryonic stem cells, Oct4, Nanog and SSEA1 at the protein level. The authors named the cells as “very small embryonic-like stem cells”, VSELs. It was not possible to expand those cells, only to maintain them in culture, possibly reflecting quiescence. In the specific conditions VSELs could form spheres in culture, which resemble embryoid bodies. VSELs could differentiate *in vitro* into cardiomyocytes, neural and pancreatic cells, showing multipotency. Additionally, VSELs expressed CXCR4 (SDF-1 receptor) and could migrate by SDF-1 gradient in culture.

Interestingly, in mice the number of VSELs isolated from bone marrow was significantly reducing with the age. It was noted that long-living strains of mice (C57Bl/6) have higher number of VSELs than short-living strains (DBA/2J). The authors suggested that this phenomenon could explain the impairment of the regeneration capacity associated with ageing (Ratajczak, Zuba-Surma et al. 2008; Ratajczak, Zuba-Surma et al. 2008).

The origin of VSEL cells still remains unclear. The authors suggested a relation between VSELs and primordial germ cells (PGCs) based on the expression of some common markers as Stella, Fragilis, Mvh, Oct4. According to the proposed model, the pluripotency is maintained throughout the life of the organism in sequence ICM (inner cell mass) - epiblast – PGCs – gonads. The authors hypothesized, that epiblast contains common precursors for PGCs and tissue-specific stem cells, and they diverge at this stage, but stay in one stream of pluripotency. Those cells express CXCR4 and they migrate throughout the body by the gradient of SDF-1. During this process the naïve pluripotent $Oct4^{+}$ precursor cells could be deposited in the developing tissues undergoing epigenetic erasure of imprinted genes (Kucia, Wysoczynski et al. 2008; Ratajczak, Zuba-Surma et al. 2008; Shin, Zuba-Surma et al. 2009).

Nevertheless, this model stays theoretical and has no practical evidence so far. Despite it captures by the elucidation of stem cell plasticity, which still stays at the position of unexplained experimental artifacts, it is based on speculations and remains as a hypothesis for the moment.

1.1.10.4. Marrow-Isolated Adult Multilineage Inducible (MIAMI) cells and microenvironment

Modeling of niche-specific conditions can provide a new insight on the cell interactions in tissue development. It has been proposed that conditions resembling the bone marrow microenvironment might result in the population of real stem cells identical to their state in the tissue (D'Ippolito, Diabira et al. 2006). The unfractionated bone marrow was plated onto fibronectin-coated dishes in the low-serum (2%) and low-oxygen (3%) conditions. The isolated cells were termed Marrow-Isolated Adult Multilineage Inducible (MIAMI) cells (D'Ippolito, Diabira et al. 2004; D'Ippolito, Howard et al. 2006). MIAMI cells were maintained at the low density and passaged at 100 cells/cm^2. They had the potential to differentiate into osteo-, adipo- and chondrocytes, neurons and pancreatic cells. Expression of Oct4 and Rex1 was shown at mRNA level. MIAMI cells were considered as pluripotent cells, which might serve as a source of cells for regenerative therapy.

MIAMI cells were not studied more intensively and the equivalent of those cells *in situ* was not shown. The expansion in a very low density from few cells might be a feature of spontaneous immortalization of primary cells, but the karyotype of the cells was not tested. But this study called the attention for the natural conditions for tissue-residing stem cells. Hypoxic conditions (3% O_2) could be more physiological and found a wide application in culture of stem cells.

1.1.10.5. Human MCAM-positive cells and pericyte concept.

One of the most extraordinary studies, in which the issue of the identity of mesenchymal stem cells was brought up, was published in 2007 by Sacchetti and colleagues (Sacchetti, Funari et al. 2007). The authors postulated that multipotency of stem cells has to be shown at the single-cells level and self-renewal can be tested only in the transplantation assays (Bianco, Robey et al. 2008). Those stringent rules were applied further for the establishment of the experiments and the result interpretation. Remarkably, the authors excluded the cell culture methods to characterize the differentiation potential of the cells, testing it only in the transplantation assays, aiming to obtain the most “physiologically” relevant data and to avoid cell culture artifacts.

CD146 (MCAM, melanoma-associated cell adhesion molecule) was proposed as a marker for the primitive MSCs in human bone marrow. The authors showed a

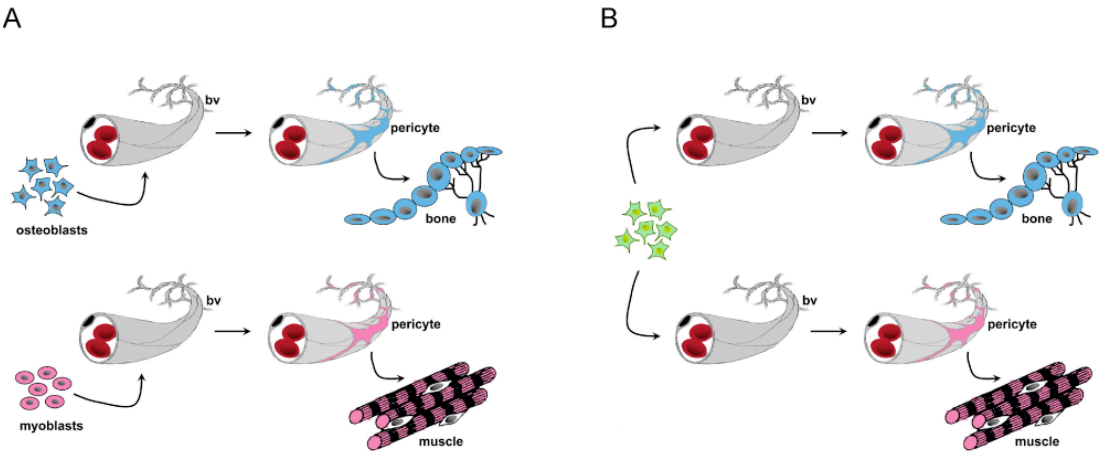
clear difference between the populations of MSCs generated from the high-density bone marrow cultures and those derived from clonogenic cells. All CFU-F generating cells were found in the $CD146^+$ compartment of the bone marrow. Using CD146 as a marker allowed the finding of the *in situ* counterpart of clonogenic CFU-F precursors. The authors revealed that those cells are located in subendothelial layer of sinusoidal walls and correspond to adventitial reticular cells (pericytes). $CD146^+$ cells shared many properties with pericytes, marker expression (PDGFRβ, α-smooth actin) and produced factors regulating HSC homeostasis (CXCL12, SCF, Angiopoeitin-1).

Cell culture-expanded $CD146^+$ cells were tested for their differentiation capacity after subcutaneous transplantation into immunodeficient mice. The progeny of the cells was recognized by the human mitochondria labeling. The transplanted cells formed bone structure, adipocytes and fibroblastic tissue. Remarkably, haematopoietic sites originating from the host were induced in the transplants. Pericytes appeared to be of human origin. Those cells expressed CD146 and demonstrated the same subendothelial position as $CD146^+$ cells observed in the human bone marrow. Importantly, human-originating $CD146^+$ cells could be recovered as CFU-F's from the transplants, suggesting self-renewal of the cells. The authors did not analyse the passaging of those cells through the series of recipients, which could serve as stronger evidence for self-renewal. Nevertheless, this study linked the gap between the *in vitro* cultured BM MSCs and their *in situ* equivalent. It can be considered as a pioneer work to obtain cell culture expanded BM MSCs capable to re-establish marrow environment and self-renew. The question marks, which the authors put on the cell culture artifacts and interpretation of multipotency and self-renewal of adult tissue-specific stem cells, had influenced the concept of Mesenchymal Stem Cell in the whole field.

Another conceptual work should be noted in the regard of $CD146^+$ stem cells. The group of Bruno Peault hypothesized that pericytes in multiple organs represent a harbor for progenitor cells, which might be integral for the bone marrow MSCs and other tissue-specific stem cells (Crisan, Yap et al. 2008). Pericytes were isolated from a list of human tissues including skeletal muscle, heart, pancreas, adipose tissue, placenta, skin, gut, lung, bone marrow, brain, eye, umbilical cord, and those cells were $CD146^+PDGFR\beta^+NG2^+$. Single-cell derived populations from those cells had myogenic potential, they were able to repair the injured muscle, and they also had the properties of MSCs, such as differentiation into osteo-, adipo- and

chondrocytes. At the same time, self-renewal capacity and differentiation in the *in vivo* environment were not analysed in this study.

These two landmark papers together formed the hypothesis of pericytes as stem cells in the adult tissues (Fig. 7). Despite the relations between pericytes and osteoblastic progenitors had been suggested before (Brighton, Lorich et al. 1992), the observation was not developed into the general principal of stem cell localization and homeostasis that time. The model proposes that all MSCs represent pericytes, whereas not all pericytes are MSCs (Caplan 2008; da Silva Meirelles, Caplan et al. 2008). This localization makes it possible to respond to the tissue-generated chemokines and systemic signals as well. The cells can support the tissue homeostasis and be recruited to repair in case of injury. At the same time, there is still a controversy in the interpretation of the identity of those cells. The first group of the authors suggested that stem cells are tissue-specific, and recruited to the vessel walls, where they reside. Therefore, the pericytes in different connective tissues share the anatomic identity, but the differentiation capacity is likely to be specific (Sacchetti, Funari et al. 2007; Bianco, Robey et al. 2008). The second group proposed that a common precursor of MSCs in different tissues reside around the vasculature and those cells are identical (Crisan, Yap et al. 2008; Crisan, Chen et al. 2009). Currently, there is no solid evidence toward one or another model. The developmental origin of MSCs and the relations between MSCs in different tissues are far from the clear understanding.



Modified from Bianco et al., 2008.

Figure 7. Two alternative models of pericytes as stem cells. (A) Local tissue progenitors are recruited to the blood vessels (bv) where they reside as pericytes. Hence, pericytes are tissue-specific, they arise independently by a common developmental pathway and share only anatomical identity. (B) Common multipotential progenitors migrate to the different connective tissues and locate as subendothelial mural pericytes. Hence, the mesenchymal progenitors from different tissues are identical and share the differentiation potential. Modified from Bianco et al., 2008.

1.1.10.6. PDGFRα$^+$Sca-1$^+$ (PαS) cells from mouse bone marrow and self-renewal

One of the remarkable works on the isolation of *bona fide* mesenchymal stem cells capable for self-renewal was published in 2009 by Morikawa et al. (Morikawa, Mabuchi et al. 2009). Several markers were used for the prospective isolation of MSCs from mouse bone marrow. The authors applied negative selection of the bone marrow mononuclear cells (BMMNCs) to sort out the haemopoeitic cells (CD45$^+$TER199$^+$ fraction), and then from the resulted cells they analysed subpopulations separated by the expression PDGFRα (platelet-derived growth factor receptor α) and Sca-1 (stem cell antigen-1). PDGFRα is a known early mesodermal marker and Sca-1 marks different types of stem cells in mouse and human. Only the cells positive for both markers showed fibroblastic morphology, proliferated without signs of senescence and differentiated efficiently into osteocytes, adipocytes and chondrocytes. Moreover, the cells of PDGFRα$^+$Sca-1$^+$ population, named PαS, were highly clonogenic in CFU-F assay. At the same time the population of PαS was not homogeneous in terms of differentiation potential, and only the subset of PαS clonally derived lines were able to differentiate into three lineages, and additionally to endothelium. The cells with phenotype PαS were identified *in situ* in the perivascular space. The most rigorous test for the stemness used in this work was the systemic transplantation assay. The freshly sorted PαS cells from the bone marrow of CAG-GFP transgenic mice were injected intravenously into lethally irradiated recipients, together with sorted HSCs to reconstitute haematopoiesis. The progeny of GFP$^+$ cells was found 16 weeks later in bone marrow stroma, bone and adipose tissue. The engrafted cells gave rise to mature adipocytes, osteocytes and reticulocytes. The authors were able to isolate GFP$^+$PαS using flow cytometry and 2% of those cells generated colonies with the same properties as transplanted PαS cells. Hence, the self-renewal of PαS cells was confirmed. However, the authors did not show the cells with phenotype PαS originated from the transplanted cells *in situ*, probably because of the rareness of those cells. Also, the cell fusion has not been excluded in the experiment and the cell tracing was performed only by GFP expression. Another interesting note was that PαS had lost their ability to engraft after culturing and were eliminated form the recipient after 7 days post-transplantaition. This fact can lead to the conclusion that cell culture conditions can influence the properties of the cells. On the other hand, it's not clear whether the subpopulation of repopulating PαS cells was actually present in the cultured cells. It was found in this work that 70% of PαS cells in bone marrow are in G_0 phase, presuming quiescence. Hence, it is possible

that the real primitive self-renewing PαS cells were not proliferating in the culture conditions.

Nevertheless, PDGFRα and Sca-1 could be considered as the potential markers for murine mesenchymal stem cells. The physiological test applied in the transplantation experiments attributes a special importance to the work.

1.2. Cellular immortalization

For a long time it was believed that all cells in the organism have unlimited growth capacity and restricted only by insufficient space in the body and lack of nutrients. The confusion was generated by the fact that the attempts to obtain the somatic cell lines that could grow in the cell culture conditions led to the selective propagation of rare spontaneously immortalized cells. Only in 1961 it was demonstrated that cultured normal cells are mortal and undergo a limited number of cell divisions before reaching unproliferative state called replicative senescence (Hayflick and Moorhead 1961; Hahn 2002). Only certain cell types harbor “natural” immortality enabling to expand them without alterations virtually indefinitely *in vitro*, amongst them embryonic stem cells (ESC) and neural stem cells (NSC), (Evans and Kaufman 1981; Martin 1981; Conti, Pollard et al. 2005). But for most of the cell types the aim to obtain a considerable amount of primary cells for biological studies represents practically an impossible task. Cellular immortalization is a phenomenon to overcome the replicative senescence, and happens *in vitro* and *in vivo* during carcinogenesis. Thus, the methods used for the directed cell immortalization often reproduce the oncogenic transformation events, and conversely the immortalization *in vitro* is often considered as a model to study the cancer development.

This chapter is dedicated to the methods of cellular immortalization, in particularly using simian virus 40 Large T-antigen; additionally the conditional immortalization of primary cells from the Large T-antigen transgenic mice used in this project will be described.

1.2.1. Cellular senescence of human and mouse cells

Cellular senescence in human fibroblasts was described as two phases, mortality stage 1 and 2, M1 and M2 (Wright and Shay 1992). During the M1 stage the cells are arrested in G1 phase of the cell cycle and do not respond to the mitogen signals. This stage is correspondent to the replicative senescence. The replicative senescence is not a simple unproliferative situation, but a specific cellular state characterized by certain markers (such as increased β–galactosidase activity, upregulation of $p21^{cip1}$, $p16^{INK4A}$) and biochemical processes. The cells are metabolically active and can survive in the cell culture conditions for relatively long time. The cell cycle is blocked by the factors responsible for DNA damage checkpoint, such as p53 and $p16^{INK4A}$. If the cells escape from the checkpoint by the mutations or other events, the cell divisions continue until M2 stage. In the M2 the cells proliferate, but the cell population undergo crisis characterized by the significant cell death. It is likely that M2 is a consequence of the extensive chromosome breakages and end-to-end fusions, which lead to the apoptosis. With a very low frequency the cells can overcome the crisis and establish the stably proliferating cell line (for the human cells the frequency is approximately 10^{-7}). In all the cases spontaneous bypass of the crisis is associated with mutations.

The mechanisms for M1 and M2 are distinct. Therefore, to overcome the senescence the cells should inactivate both thresholds of mortality and the probability of such an event is extremely low, at least for the human cells. If that happens, the cells are considered to have undergone “establishment”, and the derived population is termed “the established cell line” (Hahn 2002).

Similar processes occur in cultures of the mouse cells. Nevertheless, it is important to note a great difference between the human and the mouse cells in terms of proliferation. It is known that the frequency of immortalization of mouse cells, spontaneous and induced, is much higher than of human, and according to some data reaches a difference up to seven orders of magnitude (10^7), (Wright and Shay 2000). The events leading to the immortalization of mouse cells are often inefficient for the immortalization of human cells, for example, expression of catalytic subunit of telomerase TERT, or early region of simian virus 40. It has to be considered that the human and rodent cells exhibit a substantial difference in the growth control, so that the interpretation of data should be done very carefully (Wright and Shay 2000). We will describe some of those differences between mouse and human cells in more details further.

1.2.2. Molecular regulators of cellular senescence

The phenomenon of cellular senescence is characterized by the irreversible proliferative arrest with continued metabolic activity and occurs both *in vitro* and *in vivo*. Its function in the organism is likely to prevent tumorigenesis. Several lines of evidence show that at least three major mechanisms are involved in the regulation of cellular growth and senescence – telomere maintenance, Retinoblastoma pathway and p53 pathway. Those pathways interact and work in concert to provide a tight control for the cell proliferation.

1.2.2.1. Telomere maintenance

The induction of replicative senescence is associated most of all with the length of telomeres (Vaziri and Benchimol 1996). Telomeres are highly conserved nucleoprotein structures at the end of chromosomes essential for the chromosomal stability. The telomeres protect the ends of linear chromosomes from exonucleases and illegitimate recombination. DNA of telomeres consists of the tandem repeats, which are in case of vertebrates TTAGGG/CCCTAA (Blackburn 1991). Telomeres cannot be fully replicated due to the linear nature of eucaryotic chromosomes, hence, decreasing their length during each round of replication. The repeats can be synthesized *de novo* by the ribonucleoprotein enzyme telomerase consisting of the RNA component, TERC, and the catalytic subunit possessing the reverse transcriptase activity, TERT, (Greider and Blackburn 1985; Weinrich, Pruzan et al. 1997). The telomerase is expressed in germline cells, embryonic stem cells and many cancer cells, but is absent in most of the somatic cells. Thus, the telomere length is a result of constant shortening and elongation during the cell divisions. Somatic cells lacking telomerase activity show shortening of telomeres for about 50-100 nucleotides per one cell cycle (Harley, Futcher et al. 1990). The telomere hypothesis of cellular ageing suggests that critically short telomeres serve as a “mitotic clock” to signal to the cell to undergo senescence *in vitro* and possibly *in vivo* (Olovnikov 1971; Vaziri and Benchimol 1996). The M1 stage is induced when the shortened telomeres are recognized by the double strand DNA break repair system, and as a consequence the cell is arrested in DNA damage checkpoint. If the cells bypassed the M1 stage and continue proliferating, the length of telomeres is decreasing further leading to the extensive chromosomal aberrations and end-to-end

fusions, resulting in the apoptotic death. The ectopical expression of human catalytic subunit of telomerase hTERT resulted in the extension of life span in human fibroblasts (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998; Vaziri, Squire et al. 1999). The cells could pass the Hayflick limit, they had normal karyotype, extended telomeres and did not show the signs of senescence. These data provided an evidence for the telomere hypothesis.

The mechanism of telomere maintenance might be greatly different between mouse and human cells. Indeed, mouse cells were shown to have extremely long telomeres, approximately 150 kilobases compared to human, which are around 10 kilobases long. Literally, the shortest mouse telomere is longer than the longest human one (Kipling and Cooke 1990). The knockout mice for RNA component of telomerase ($mTR^{-/-}$) were viable at least for 6 inbred generations, reducing the telomere length each generation, demonstrating that the telomerase is essential for the telomere maintenance, but not for the viability of mice (Blasco, Lee et al. 1997). Importantly, $mTR^{-/-}$ cells from different generations of those mice were similar in the ability to proliferate in culture despite the difference in the telomere length. Those cells undergo senescence/crisis and spontaneously immortalize with similar dynamics and frequency indicating that (1) the senescence of mouse cells is not caused by the telomere shortening, and (2) active telomerase is not required for the mouse cell line establishment and oncogenic transformation. These observations suggest that there is a substantial difference in the role of telomere length in replicative senescence, immortalization and carcinogenesis of human and mouse cells.

1.2.2.2. Retinoblastoma pathway

Retinoblastoma protein (pRb) and two related proteins, p130 and p107 belong to the family of pocket proteins, and function as negative regulators of the cell proliferation. pRb binds and represses the E2F transcription factors, which stimulate the expression of genes involved in replication and cell cycle progression (Hurford, Cobrinik et al. 1997). pRb binds specifically to the activating E2F1, E2F2 and E2F3. p130 and p107 bind to E2F4 and E2F5 and occupy the promoters of E2F-dependent genes as a repression complex. In the late G1 stage the positive cell cycle regulators, such as complexes CDK2/Cyclin E, CDK2/Cyclin A, CDK4/Cyclin D, phosphorylate pRb leading to its inactivation. The E2F1-3 release permits to enter to the S-phase.

pRb remains active in the senescent and non-dividing cells blocking the enter to the cell cycle (Stein, Drullinger et al. 1999). The cell cycle inhibitors $p16^{INK4a}$ and $p21^{cip1}$ bind to the CDK's suppressing the formation of complexes with Cyclins and phosphorylation of pRb. Thus, senescent cells exhibit elevated level of p16 and p21, and the spontaneous immortalization of cells is often associated with the loss of p16. pRb function is disrupted in nearly every human cancer directly, or indirectly by p16 inactivation (Duncan, Wadhwa et al. 2000). Mouse embryonic fibroblasts with triple knockout of pRb, p130, p107 are immortal (Sage, Mulligan et al. 2000).

1.2.2.3. p53 tumor suppressor

p53 is one of the main tumor suppressors in the cell (Lane and Crawford 1979; Linzer and Levine 1979). p53 is a transcription factor that activates the expression of genes inducing the cell cycle arrest and apoptosis in response to the cellular stress and DNA damage. The level of p53 in the cells is relatively low due to the short half-life time of the protein (around 20 minutes), which makes it more versatile in cellular regulation (Donehower, Harvey et al. 1992). The expression of p53 is regulated by a negative feedback loop with Mdm2 (Murine Double Minute 2). p53 activates the transcription of Mdm2, which functions as an E3 ubiquitin ligase. Mdm2 ubiquitinates p53 and targets it to the proteasomal degradation. In case of DNA breaks, UV radiation, stress or oncogene activation, the positive regulators of p53, such as protein kinases $p14^{ARF}$ (alternative reading frame), ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related) inhibit Mdm2 and stabilize p53.

p53-mediated growth arrest occurs through the induction of $p21^{cip1}$. p21 binds CDK's inhibiting the formation of CDK/cyclin complexes and therefore pRb remains hypophosphorylated and active. As a result, the cell cycle progression is blocked. Additionally, p21 might inactivate proliferating cell nuclear antigen, PCNA, a factor essential for replication (Stein, Drullinger et al. 1999). In the senescence the level of p53 might not be increased but the DNA binding activity is enhanced. It is possible that the mechanism of M1 and M2 include activation of p53 in response to short telomeres, which are recognized as DNA damage (Duncan, Wadhwa et al. 2000).

Despite obvious important function of p53, the knockout for p53 mice are viable, but sensitive to the tumor formation (Donehower, Harvey et al. 1992). The fibroblasts isolated from those mice are immortalized. This data indicates that p53 is

not essential for the mouse development but predisposes to neoplastic transformation.

1.2.3. Methods of cellular immortalization

Immortalization is a phenomenon to bypass the replicative senescence and break the growth arrest. The three mechanisms involved in the cell growth control have to be perturbed to overcome senescence – the telomere shortening, pRb pathway and p53 pathway. The immortalization can occur spontaneously, be indirectly induced or directly induced.

Spontaneous immortalization is a capacity of the cells to proliferate indefinitely in the cell culture conditions without adding of any exogenous agents. The criteria for defining the immortalized state in this case is quite empirical, the cultures proceeded more than 150 doublings can be considered as immortal (Shay, Wright et al. 1991). In most of the cases the spontaneous immortalization is connected to the genetic or epigenetic changes in tumor suppressor genes, namely p53, $p16^{INK4a}$, $p14^{ARF}$. In addition, in almost 100% of spontaneously immortalized human cells telomeres are maintained by the telomerase or other alternative mechanism (ALT, alternative lengthening mechanism), (Hahn 2002).

Most of the mouse cells have a capacity to establish an immortal line spontaneously, while for the human cells this event is extremely rare. The example of a spontaneously immortalized mouse line is NIH 3T3, which were established from primary mouse embryonic fibroblasts (MEFs) using so-called “3T3 protocol – 3 day transfer $3x10^5$ inoculum” (Todaro and Green 1963). Frequent passaging of the cells in a low density regardless of their growth rate is selective for the cells capable for indefinite growth. This protocol can be reproduced and results in the establishment of spontaneously immortalized line after 20-30 generations.

Indirect methods to induce immortalization implicate the increase of unspecific chromosomal abnormalities rate and the probability of the mutations leading to the immortalized state. Irradiation with UV, X-ray or γ-rays, and chemical treatment with carcinogenic agents such as benzoapyrene (BaP) have been used widely to immortalize breast epithelial cells (Gudjonsson, Villadsen et al. 2004).

Directed immortalization can be achieved by the genetic modification of the cells using expression of oncogenes or viral oncoproteins. The combination of the

immortalizing agents is highly specific on the cell type and the species, as was already discussed. In almost all of the cases, this combination should provide disabling of p53 and pRb functions, which is sufficient to immortalize mouse cells. The human cells need additionally to activate telomerase and in some cases, to express oncogenes (such as Myc or Ras) for immortalization.

Many DNA-containing viruses utilize the mechanism to stimulate DNA replication in the host to reproduce themselves. The regulators driving the proliferation of the cells are often used for the directed cellular immortalization (Fig. 8).

Human Papilloma Viruses are small DNA viruses inducing epithelial proliferation. HPV's 16, 18, 31, 33 are found in 80% of cervical tumors. Papilloma Virus proteins E6 and E7 are used for immortalization of many epithelial primary cells. E6 protein binds and inactivates p53 and E7 directly inhibits pRb (Munger and Howley 2002).

Adenoviral oncoproteins can also be used for the cellular immortalization. Adenoviral protein E1A and E1B bind pRb and p53, respectively. The expression of E1A region alone is enough to immortalize rodent cells. Probably, the most famous example of adenovirus-immortalized cell line is 293 rising from the human embryonic kidney cells transformed with sheared DNA of adenovirus 5a (Graham, Smiley et al. 1977).

One of the most well studied and probably most often applied oncogenic viruses is simian virus 40 (SV40). The high transforming activity of the early region made it an attractive tool for cellular immortalization. We dedicate a special chapter to the mechanism utilized by SV40.

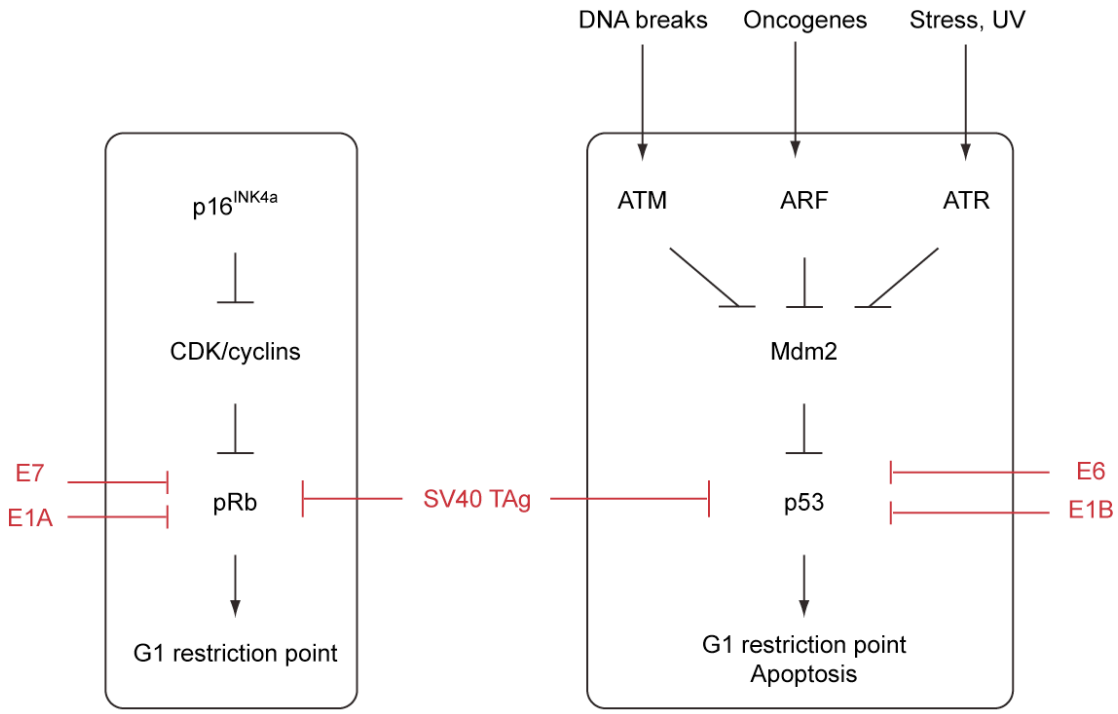


Figure 8. The targets of viral oncoproteins in cellular immortalization. pRb (Retinoblastoma) and p53 induce the growth arrest in G1 stage or apoptosis. DNA viruses inhbit both pathways to promote DNA replication and cell division. Simian virus 40 Large T-antigen (SV40 TAg) binds and incativates both pRb and p53. Human papilloma virus protein E7 and E6 bind pRb and p53, respectively. Adenoviral E1A and E1B directly repress pRb and p53. Modified from Hahn, 2002.

1.2.4. Cellular immortalization by Simian Virus 40

Simian virus 40 (SV40) belongs to Polyoma viruses (Sweet and Hilleman 1960). The genome of SV40 is about 5 kilobases, and the compact structure of regulatory sequences, such as origin, expression promoter, polyA signal, splicing sites, made it widely used for the genetic engineering. Another wide application of SV40 is due to the high immortalization potential. SV40 causes lytic infection in monkey cells, and human and rodent cells stay permissive. The immortalization properties of SV40 are associated with the expression of the genes of the early region encoding Large T-antigen, Small t-antigen, and 17k T-antigen. Three proteins are generated through the alternative splicing of early mRNA. The Large T provides most of the transforming activity of SV40 by the simultaneous inactivation of the main control systems for the cellular growth, pRb and p53. Large T has a distinctive domain organization and the domains possess a specific activity for the transforming of the host cell (Fig. 9).

The N-terminal part of the Large T forms the J domain. This part of the protein targets Hsc70 (heat shock cognate protein 70kDa), which acts as ATPase and causes the release of E2F's from the complexes with p130 and p107.

LXCXE domain of the Large T-antigen binds directly to pRb, p130 and p107 and inactivates pRb-dependent block of the cell cycle. Large T binds preferentially unphosphorylated form of pRb causing the release of E2F's and promoting the progression from G1 to S phase. For the immortalization it is required to inactivate the functions of all three pocket proteins, pRb, p130 and p107 (Ahuja, Saenz-Robles et al. 2005; Cheng, DeCaprio et al. 2009).

The helicase domain of the Large T-antigen binds directly to the DNA-binding domain of p53 resulting in its inactivation. Consequently, the expression of Mdm2, the negative regulator of p53, is also reduced and non-ubiquitinated p53 is accumulating. The bound to the Large T p53 is greatly stabilized, half-life time of p53 is reaching around 24 hours. Inactivation of p53 by Large T is essential for the cell transformation (Hahn, Dessain et al. 2002).

The role of Small t-antigen is less studied. Small t-antigen cannot immortalize the cells alone, but contributes to the Large T-promoted immortalization. The main target of the Small t is serine-threonine phosphatase A (PP2A), interaction with which leads to the activation of several signaling pathways, stabilization of Myc, and other effects.

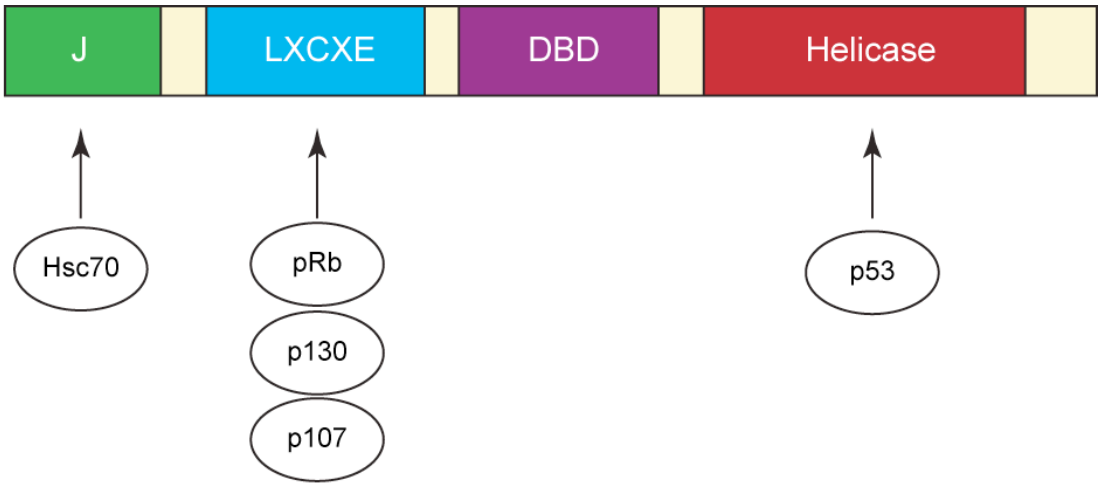


Figure 9. Domain structure and protein targets of SV40 Large T-antigen. T-antigen contains several domains: J - J domain, LXCXE motif, DBD - DNA-binding domain, Helicase domain. The main cellular interactors of the T-antigen are Hsc70 - Heat Shock Cognate 70, pRb - Retinoblastoma protein, p130, p107, p53.

The mouse cells are readily immortalized by SV40, the block of pRb and p53 by the Large T is enough for cellular immortalization of murine cells. At the same time, infection of human fibroblasts by SV40 extends the lifespan for about 20-30 doublings and then the cells undergo crisis. The cooperation of SV40 Large T-antigen with hTERT in the human cells can efficiently immortalize them (Cheng, DeCaprio et al. 2009).

However, any type of immortalization might interfere with the cellular processes and affect the signaling pathways. The reversible immortalized state would have a great advantage to avoid this interference. There are several attempts to obtain an inducible SV40 Large T-antigen that discussed in the literature. Wright et al. generated a steroid inducible mouse mammary tumor virus (MMTV)-driven Large T (Wright, Pereira-Smith et al. 1989) and transfected it to human fibroblasts. The cells expressed T-antigen and proliferated only in the presence of Dexamethasone (Dex). The cells passed through the crisis yielding the immortal cell line. However, the growth was still Dex-dependent and Dex withdrawal blocked the cell proliferation of those cells. It was suggested that M1 (replicative senescence) and M2 (crisis) have distinct mechanisms, and Large T-antigen can inactivate the M1 mechanism but does not eliminate it. Upon deinduction of T-antigen the cells turned into M1 stage, which was not abolished by T-antigen but bypassed.

A temperature-sensitive version of Large T-antigen, named tsA58, was found by the screening of mutated forms of SV40 (Tegtmeyer 1975). tsA58 exhibited immortalization activity only at the permissive temperature of 33°C, and the temperature shift to 39°C inactivated it (in some studies permissive temperature was 35°C). tsA58 was successfully used for generation of the inducible immortalized cell lines of human and mouse fibroblasts (Radna, Caton et al. 1989; Ikram, Norton et al. 1994). The growth of the stable cell lines was observed only at the permissive temperature. The temperature shift induced the cellular senescent state in concordance to the observations used MMTV-controlled T-antigen.

Following the success in generating the conditionally immortalized cell lines using temperature-sensitive T-antigen, the attempt to establish the transgenic mouse carrying tsA58 was performed (Yanai, Suzuki et al. 1991). The transgenic mice consistently developed tumors at the age 5-6 weeks showing leakiness of the system. Nevertheless, the mouse was successfully used to establish several lines of immortalized primary cells (Okuyama, Yanai et al. 1995).

To perform the additional control on the Large T-antigen expression, tsA58 was placed under control of H-2K^b promoter, and used to generate the transgenic mouse line (Jat, Noble et al. 1991). Histocompability complex H-2K^b is widely expressed and can be further upregulated by interferon treatment. The hybrid construct of tsA58 under control of H-2K^b promoter was injected into fertilized oocytes of the mice, and the transgenic mouse line was established ("immortomouse"). The conditionally immortalized fibroblasts were obtained from those mice. The growth of conditionally immortalized cells was temperature-dependent, and colony-forming efficiency and T-antigen expression was enhanced upon interferon treatment. The transgenic mice were showing hyperplasia's in thymus, but did not develop the tumors. Subsequently, different conditionally immortalized primary cell lines were successfully established from the "immortomouse" providing experimental systems to study cell functions (Whitehead, VanEeden et al. 1993; Okuyama, Yanai et al. 1995).

1.2.5. Conditional immortalization of mouse cells using tetracycline-regulated SV40 Large T-antigen

Recently our group has developed a system for the precise conditional immortalization of cells using SV40 Large T-antigen (Anastassiadis, Rostovskaya et al. 2010). We reasoned that the transcriptional control of the transgene expression is preferable for the tight and reversible regulation. The most commonly used system for the transcriptional regulation is a tetracycline-inducible system. The system consists of two components – a tetracycline regulator and a regulatable transgene under control of tetracycline operator and minimal promoter. The tetracycline-inducible system exists in two versions, TET-OFF and TET-ON. In case of TET-OFF system, the tetracycline transactivator (tTA) is constantly active, and adding of Doxycycline (Dox) removes tTA from the tet-operator and represses transgene expression. In case of TET-ON, reverse tetracycline transactivator (rtTA) is active only in the presence of a ligand, Dox, to induce the transcription of transgene. We used the TET-ON strategy to generate a system for tightly controlled transgene expression.

The idea to utilize tet-regulated system for Large T-antigen expression was already successfully applied by the others (May, Hauser et al. 2004). The tet-regulated Large T (TET-ON) was established and used for the conditional immortalization of MEFs. However, the attempt to generate a tet-regulated T-Antigen

transgenic mouse using TET-OFF or TET-ON faced with the following difficulties (Bendiksen, Van Ghelue et al. 2004). The TET-OFF system tightly controlled the expression of T-antigen in the mice and the cells from those mice, but the mice needed constantly administration of Dox during the entire life to repress the T-antigen expression. The TET-ON system was found to be not efficient and the Large T was not expressed in the mouse tissues and in the cells from those mice after the Dox treatment.

Considering these problems, we aimed to improve the system for conditional expression of Large T-antigen and subsequently generate a transgenic mouse (Fig. 10). Previously, the improved version of rtTA by codon optimizing was reported, irtTA (Anastassiadis, Kim et al. 2002). The irtTA was placed under control of constitutively expressed CAGGs promoter, and could be induced by the Dox treatment. The second level of control was added by the fusion of irtTA with a mutated Glucocorticoid Binding Domain (GBD*) capable for binding of Dexamethasone. In the uninduced state the fusion protein irtTA-GBD* is bound to heat-shock protein (Hsp90) in cytoplasm and the system stays inactive. The adding of Dexamethasone (Dex) releases the irtTA-GBD* and makes it available for the induction with Dox. In the presence of both ligands, the irtTA is active and stimulates the expression of the transgene under the tet-operator. For the experiments shown here we used both versions, the original irtTA (Dex-independent) and irtTA-GBD* (Dex-dependent).

The Large T-antigen was placed under control of the tet-tk minimal promoter. The double stable colonies of mouse ES cells E14TG2a were established by the sequential electroporation of CAGGs-irtTA(-GBD*) and tet-tk-TAg, and antibiotic selection. The transgenic mice were generated either by pronucleus co-injection of DNA constructs or by injection of mES into the blastocysts. The mice had normal phenotype and did not show abnormalities or tumor formations. The expression of Large T in the mice was tested with Western blot before and after the intraperitoneal administration of ligands (Dox or Dex/Dox). In case of irtTA-GBD*-TAg mice the Large T was expressed only in the presence of both ligands in all the examined tissues. In case of irtTA-TAg mice, only 1 founder line out of 17 tested expressed the irtTA at very low level. Hence, we confirmed the advantage of the irtTA-GBD*-TAg system. We showed that the MEFs isolated from the transgenic irtTA(-GBD*)-TAg mice could be conditionally immortalized and expanded practically indefinitely. Finally, we isolated conditionally immortalized stromal lines from the bone marrow and confirmed their identity by the marker expression and differentiation potential.

Thus, we generated a system for the conditional immortalization of primary mouse cells with excellent properties for regulation.

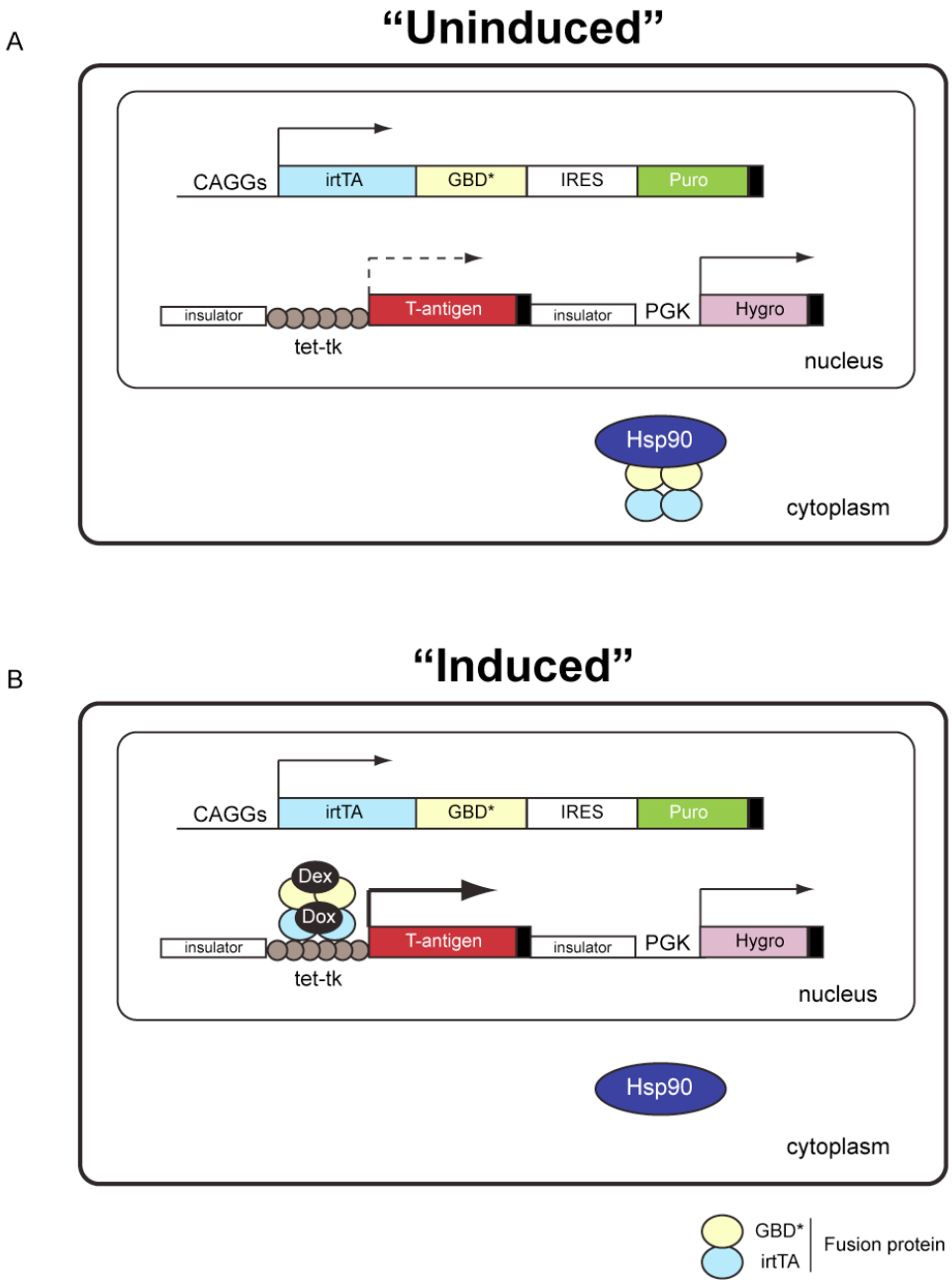


Figure 10. Modified tetracycline-regulated system for the conditional expression of Large T-antigen. (A) In the uninduced state the irtTA-GBD* fusion is bound to Hsp90 in cytoplasm and the system is inactive. (B) Upon induction with Dexamethasone (Dex) the irtTA-GBD* is released from the complex with Hsp90, and in the presence of Dox binds to tet-operator and induces T-antigen transcription.
CAGGs and PGK - constitutively expressed promoters, irtTA - improved reversed tetracycline transactivator, GBD* - mutated glucocorticoid binding domain, IRES - internal ribosome binding site, Puro and Hygro - puromycin and hygromycin resistance genes, tet tk - tetracycline operator and minimal promoter, Dex - Dexamethasone, Dox - Doxycycline, Hsp90 - heat shock protein 90.

1.2.6. Immortalization of bone marrow mesenchymal stromal cells

The cell culture model represents an important tool to study the biological properties of the cells. Bone marrow mesenchymal stromal cells have a limited proliferation potential, therefore immortalized cells are often used as a model to study bone marrow mesenchymal stromal cells and the processes of differentiation into mesodermal tissues. In this chapter we will describe some examples of the generation of immortalized cell lines with BM MSC properties.

1.2.6.1. Spontaneously immortalized stromal lines.

Several spontaneously immortalized stromal cell lines are known from the literature. C3H10T1/2 cells were established from the mouse embryos using the modified "3T3" protocol, seeding $0{,}5x10^5$ cells into 60mm dishes every 10 days (Reznikoff, Brankow et al. 1973). Later it was found that the cells of this line can be induced into the bone, fat, cartilage and muscle cells by the 5-azacytidine treatment (Pinney and Emerson 1989).

ST2 clonal stromal line was established from the bone marrow of adult mice by spontaneous immortalization (Ogawa, Nishikawa et al. 1988). The cells were supporting haematopoiesis and were used to study B-cell development. The cells were able to differentiate into osteo- and adipocytes, were applied as a model for multipotential mesenchymal stromal cells.

The subclones of NIH 3T3 fibroblasts with adipogenic potential are widely used to study the regulation of adipogenic differentiation. 3T3-L1 and F442A clones were selected by the screening of clones for the adipogenic potential. The L1 and F442A cells might represent a faithful model of adipogenic induction, however, the clones need different stimulation to convert into adipocytes that questions the identity of those cells (Cornelius, MacDougald et al. 1994; Wu, Xie et al. 1995).

1.2.6.2. Induced immortalization in stromal lines

Despite the utility of the described above spontaneously immortalized cell lines, the undefined identity of those cells and the unknown genetic alterations might interfere with the interpretation of the results obtained using those cells. Therefore, the directly induced immortalization could be advantageous to establish stably proliferating stromal lines.

Human BM MSCs were immortalized by the combination of hTERT, and papillomavirus proteins E6 and E7 (Okamoto, Aoyama et al. 2002). The clonally derived lines were examined for the differentiation potential into osteo-, adipo- and chondrocytes. The properties of the clones varied considerably, and the clones with potentials “OAC”, “AO”, “OC”, “O”, “A”, “C” were established (“O” – osteo, “A” – adipo, “C” - chondro). The authors suggested the heterogeneity of stromal cell populations.

1.2.6.3. Conditionally immortalized stromal lines from transgenic mice.

There are several known examples of the establishment of conditionally immortalized BM MSCs from the transgenic mice in the literature.

Mesenchymal stromal cell lines were established from temperature-sensitive Large T-antigen transgenic mice (Okuyama, Yanai et al. 1995). The cells were grown at the permissive temperature 33°C. A variety of clonally derived lines were obtained and characterized. Amongst them different cell types were found, such as endothelial cells, fibroblasts, pre-adipocytes, osteoblasts. One of the clones from this study, TBR31-2, had a potential for multilineage differentiation (osteo-, adipo-, chondro- and myogenic) and was reported separately (Negishi, Kudo et al. 2000).

Conditionally immortalized BM MSCs were isolated from the “immortomouse” harboring tsA58 T-antigen in the H-2K^{b} locus, described before (Jat, Noble et al. 1991; Dennis, Merriam et al. 1999). The cells were growing at the permissive conditions (temperature 33°C and adding of interferon) upon the induction of Large T-antigen. One of the clonally derived lines was able for quadripotential differentiation into osteocytes, adipocytes and chondrocytes (clone BMC9). Thus, the authors reported the isolation of multipotential early mesodermal progenitor, and this was one of the first confirmations that multipotent BM MSC exist in the adult bone marrow, which appeared at the same time with (Pittenger, Mackay et al. 1999).

2. Materials and methods

2.1. General materials

2.1.1. Reagents

2.1.1.1. Cell culture media and supplements

Reagent	Company producer	Cat. number
DMEM - Low Glucose (1000mg/l) with GlutaMax	Gibco Invitrogen	21885-025
DMEM-High Glucose (4500mg/l) with GlutaMax	Gibco Invitrogen	61965-026
DMSO Hybri-Max	Sigma	D2650
Fetal Calf Serum	PAA	A15-101
L-Glutamine	Gibco Invitrogen	25030-024
OPTI MEM with GlutaMax	Gibco Invitrogen	51985-026
Penicillin/Streptomycin	Gibco Invitrogen	15140-122
Trypsin/EDTA 1x	Gibco Invitrogen	25300-054
TGFβ1	Peprotech	100-21

2.1.1.2. Chemicals and dyes

Reagent	Company producer	Cat. number
Acrylamide/bis-acrylamide 30%	Severn Biotech Ltd.	20-2100-10
Alizarin Red	Sigma	A5533
Alcian blue	Sigma	A3157
Apo-transferrin	Sigma	T1147
Ascorbate-2-phosphate	Fluka	49752
Ascorbic Acid	Sigma	A4544
BIO	Sigma	B1686
β-glycerophosphate	Sigma	G9891
BSA	Sigma	A7906
CT99021	ABCR	AB253776
DAB Substrate	Vector Lab.	SK-4100
Dexamethasone	Sigma	D4902
Doxycycline	Sigma	D9891
Formaldehyde 37%	Merck	1.040003.100
Glycogen	Calbiochem	361507
Guanidine Hydrochloride	Sigma	G3272
Indomethacin	Sigma	I7378
Insulin	Sigma	I6634
Isobutyl-methylxantine	Sigma	I5879
Lithium Chloride	Sigma	L4408
Nuclear Fast Red	Fluka	60700
Oil Red	Sigma	O0625
TEMED	Sigma	T9281
TRI reagent	Invitrogen	T9424
Troglitazone	Sigma	T2573
TWEEN-20	Sigma	P7949

2.1.1.3. Other reagents (markers, inhibitors etc)

Reagent	Company producer	Cat. number
Eukitt Quick Hardening Mounting Medium	Fluka	03989
NuPAGE 4x loading buffer	Invitrogen	NP0007
Prestained Protein Marker, broad range (7-175 kDa)	New England Biolabs	P7708S
Protease Inhibitors Cocktail	Sigma	P8340
TriDye 100bp DNA ladder	New England Biolabs	N3271S

2.1.1.4. Buffers and solutions

1x PBS:

NaCl	171 mM
KCl	3.4 mM
Na2HPO4	10 mM
KH2PO4	1.9 mM

1x TBE:

Tris	0.9 M
Boric Acid	0.9 M
EDTA pH 8.0	1 mM

10xSDS Running buffer for Western blot:

Tris	0.25 M	30.2 g
Glycin	2.5 M	188 g
SDS (20% stock solution)	1%	50 ml
H_2O		up to 1L

Transfer buffer for Western blot (pH 8.3):

Tris	48 mM	5.8 g
Glycin	39 mM	2.9 g
SDS (20% stock solution)	0.037%	1.85 ml
Methanol	20%	200 ml
H_2O		up to 1L

Running gel for Western blot:

	Stock solution	Final concentration	Volume
Acrylamide/bis-acrilamide	30%	10%	4 ml
TRIS	1.5M pH 8.8	0.375 M	3 ml
SDS	20%	0.1%	60 μl
APS	10%	0.033%	40 μl
TEMED		0.067%	8 μl
H_2O			5 ml

Stack gel for Western blot:

	Stock solution	Final concentration	Volume
Acrylamide/bis-acrilamide	30%	4.5%	1.5 ml
TRIS	1.0M pH 6.8	0.25 M	2.5 ml
SDS	20%	0.1%	50 μl
APS	10%	0.033%	30 μl
TEMED		0.15%	15 μl
H_2O			6 ml

2.1.1.5. Antibodies

2.1.1.5.1. Primary antibodies

Antibody	Company producer	Cat. number
anti-CD9	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2a 553758
anti-CD34-PE	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2a 551387
anti-CD44-PE	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2b 553134
anti-CD45-PE	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2b 553081
anti-CD49e-PE	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2a 557447
anti-CD117-PE (c-Kit)	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2b 553355

Anti-CD146-FITC (MCAM)	GenTex	Mouse monoclonal IgG1 GTX18166
anti-CD184-PE (CXCR4)	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2b 551966
anti-Flk1-PE (VEGF-R2)	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2a 555308
anti-Sca1-FITC	BD Pharmingen, USA	Rat anti-mouse monoclonal IgG2a 557405
Anti-CD271 (p75, NGFR)	Abcam	Rat anti-mouse IgG2b monoclonal Ab27007
IgG2a-FITC	BD Pharmingen, USA	FITC-conjugated rat IgG2b monoclonal isotype control 553929
IgG2b-PE	BD Pharmingen, USA	PE-conjugated rat IgG2b monoclonal isotype control 553989
anti-CD140b-PE (PDGFRb)	eBioscience Inc, USA	Rat anti-mouse monoclonal IgG2a, 12-1402
anti-collagen II	Chemicon	Mouse anti-chicken monoclonal IgG1, MAB8887
anti-aggrecan	Chemicon	Rabbit anti-mouse polyclonal antibody, AB1031
anti-T-antigen	Santa Cruz	Mouse monoclonal IgG2a, sc-148
anti-p53	Santa Cruz	Rabbit polyclonal IgG, sc-6243
anti-cyclin D1	Cell Signaling	Mouse monoclonal IgG, 2926
anti-p27	Cell Signaling	Rabbit polyclonal IgG, 2552
anti-β-actin	Sigma	Mouse monoclonal, ascites fluid, A5441
anti-C/EBPα	Santa Cruz	Rabbit polyclonal, 14AA, sc-61
anti-C/EBPβ	Santa Cruz	Rabbit polyclonal, C-19, sc-150
anti-C/EBPδ	Santa Cruz	Rabbit polyclonal, C-22, sc-151
anti-PPARγ1,2	Santa Cruz	Rabbit polyclonal, E-8, sc-7273
anti-PPARγ1	Abcam	Mouse monoclonal, ab41928

2.1.1.5.2. Secondary antibodies

Antibody	**Company producer**	**Cat. number**
FITC-conjugated goat anti-rat IgG	Jackson ImmunoResearch laboratories, USA	112-045-107

TRITC-conjugated goat anti-mouse IgG, IgM	Jackson ImmunoResearch laboratories, USA	115-025-068
Horseradish peroxidase-conjugated mouse anti-rabbit IgG	Pierce, Thermo Scientific	31464
Horseradish peroxidase-conjugated goat anti-mouse IgG	Pierce, Thermo Scientific	31430

2.1.1.6. Kits and enzymes

Name	Company Producer	Cat. number
Chondroitinase ABC	Sigma	C2905
DNase I, Amplification Grade	Invitrogen	18068-015
Dual-Luciferase Reporter Assay System	Promega	E1910
Lipofectamine LTX	Invitrogen	15338-100
SuperScript III First-Strand Synthesis System	Invitrogen	18080-051
SuperSignal West Femto Substrate kit	Thermo Scientific	34096
Taq DNA polymerase	5PRIME	2200010
Vectastain ABC RTU kit	Vector Laboratories	PK-7200

2.1.1.7. Primer sequences

	Product size (bp)	Forward (5' – 3')	Reverse (5' – 3')
Msx2	107	AATTCCGAAGACGGAGCAC	CGGTTGGTCTTGTGTTTCCT
Osterix	191	TCTCCATCTGCCTGACTCCT	AGCGTATGGCTTCTTTGTGC
RUNX2	105	CCTCTGACTTCTGCCTCTGG	ATGAAATGCTTGGGAACTGC
Osteopontin	170	TGTGATGAGACCGTCACTGC	AGGTCCTCATCTGTGGCATC
Osteocalcin	175	TGACAAAGCCTTCATGTCCA	TTTGTAGGCGGTCTTCAAGC
PPARγ1	91	TGAAAGAAGCGGTGAACCACTG	TGGCATCTCTGTGTCAACCATG
PPARγ2	153	CTCCTGTTGACCCAGAGCAT	AATGCGAGTGGTCTTCCATC
C/EBPα	198	GCAGTGTGCACGTCTATGCT	AGCCCACTTCATTTCATTGG
C/EBPβ	156	CAAGCTGAGCGACGAGTACA	AGCTGCTCCACCTTCTTCTG
C/EBPδ	177	ATCGCTGCAGCTTCCTATGT	AGTCATGCTTTCCCGTGTTC
Klf-5	199	GCTCACCTGAGGACTCATACG	AGTTCTGGTGGCGCTTCAT

Krox20 (Egr2)	148	CTCTACCCGGTGGAAGACCT	GGAGATCCAGGGGTCTCTTC
Adipsin	169	CGTACCATGACGGGGTAGTC	GTATAGACGCCCGGCTTTTT
Adiponectin	170	GGAACTTGTGCAGGTTGGAT	CCTTCAGCTCCTGTCATTCC
PEPCK	194	TTGTAGGAGCAGCCATGAGA	CTTCCGGAACCAGTTGACAT
UCP-1	182	TTCAAAGGGTTTGTGGCTTC	ATTCGTGGTCTCCCAGCATA
Aggrecan	150	GGTCACTGTTACCGCCACTT	CCCCTTCGATAGTCCTGTCA
Collagen II	200	AGAGACCTGAACTGGGCAGA	GCACCATTGTGTAGGACACG
β-actin	440	GGCCCAGAGCAAGAGAGGTATCC	ACGCACGATTTCCCTCTCAGC

2.1.2. Materials

Tissue culture plastic	Falcon (BD Biosciences) or NUNC
Nitrocellulose Membrane for protein transfer PROTRAN	Schleicher +Schuell (Whatman)

2.1.3. Plasmids and constructs

The luciferase reporter assay was done using pGL3 BAR containing Firefly Luciferase reporter gene under control of twelve TCF/LEF tandem elements, Promega (Biechele and Moon 2008). pGL4.73hRluc/SV40 containing constitutively expressed Renilla luciferase gene was used for the transfection efficiency control. The plasmids were kindly gifted by G. Weidinger (CRTD, Dresden).

The following expression constructs were obtained from different sources as listed below: PPARγ1 and PPARγ2 from Addgene kindly provided by Bruce Spiegelman group, C/EBPβ, LIP and C/EBPδ from Addgene kindly provided by Peter Johnson group, and C/EBPα and Klf5 from the commercial source (Geneservice). C/EBPβ, LIP and C/EBPδ cDNAs were cloned in pcDNA3.1(-) plasmid backbone under CMV promoter and carried neomycin resistance gene. PPARγ1 and PPARγ2 were in pSV Sport plasmid backbone under control of SV40 promoter, and C/EBPα and Klf5 were in pCMV Sport6 under control of CMV promoter, all without eukaryotic resistance genes. Those plasmids were modified by exchange of the ampicillin resistance gene to the PGK-neo-pA or PGK-BSD-pA cassettes using recombineering (not shown in the details).

The control plasimds in different experiments were CAGGs-GFP-IRES-puro/neo-pA, constitutively expressing GFP.

2.2. Transgenic mice

Double-transgenic mice with tetracycline-regulated SV40 Large T-antigen (irtTA/T-Ag and irtTA-GBD*/T-Ag) were generated in our group as was described in details before (Anastassiadis, Rostovskaya et al. 2010). The tetracycline activator plasmids CAGGs-irtTA-IRES-Puro and CAGGs-irtTA-GBD*-IRES-Puro were constructed by ligating the irtTA and irtTA-GBD* fusion as *Eco*RI fragments in the *Eco*RI site of the CAGGs-IRES-puro construct. The ins-Hyg-TAg construct was generated by first cloning the Large T between the tet-tk minimal promoter and SV40 polyA in the pKS-tet-tk (Anastassiadis, Kim et al. 2002) and then by inserting the tet-tk-Large-T-pA fragment between the pair of insulators in the pins-Hyg-Ad2, a construct that contains a multi-cloning site between the insulator sequences.

The obtained constructs were sequentially electroporated into mouse embryonic stem cells (mES), E14Tg2a, and the double-stable cells (CAGGs-irtTA-IRES-puro/ins-Hyg-TAg and CAGGs-irtTA-GBD*-IRES-puro/ins-Hyg-TAg) were selected using 200 μg/ml Hygromycin (Roche) and 1 μg/ml puromycin (Sigma). Transgenic mice were generated either by injection of those double-stable E14Tg2a mES cells (CAGGs-irtTA-IRES-puro/ins-Hyg-TAg and CAGGs-irtTA-GBD*-IRES-puro/ins-Hyg-TAg) into blastocysts or by pronucleus co-injection of the same combinations of constructs. All manipulations were done in the Transgenic Core Facility (TCF) of the MPI-CBG, Dresden.

2.3. Cell culture methods

2.3.1. Isolation of bone marrow mesenchymal stromal cells (BM MSCs)

For the isolation of bone marrow mesenchymal stromal cells (BM MSCs) femurs and tibiae from mice were used. The bones were collected and cleaned carefully from the muscle fibers. The bone marrow was flashed out from the bones with 5 ml of DMEM-low glucose supplemented with 10% FCS (MSC growth medium) using 2 ml syringe with 24G needle. The suspension of bone marrow was centrifuged for 5 min at 200g. The pellet was resuspended in 3 ml of the MSC growth medium and plated into a well of 6-well tissue culture plate. The medium was changed in 24 hours. Usually the cells reached the confluence after 10-14 days.

2.3.2. Basic procedures with BM MSCs

For the passaging the confluent monolayer of cells was washed with PBS, then treated with trypsin (0.5ml/well of 6-well plate) for 5 minutes at 37°C. The cells were pipetted up and down in the MSC growth medium and centrifuged for 5 minutes at 200g. The cell pellet was resuspended in the MSC growth medium and distributed to the dishes.

For the cryopreservation of BM MSCs the cells were trypsinized and pelleted as described above, then resuspended in the MSC growth medium. Ice-cold freezing medium was added in the ratio 1:1 to the cells, and then the suspension was distributed to the cryovials (1ml/vial) and quickly transferred to -80°C. After 1-2 days the frozen cells were moved to the liquid nitrogen for the long-term storage.

Freezing medium:

		For 10 ml
DMEM-low glucose (1g/L)	50%	5 ml
FCS	30%	3 ml
DMSO	20%	2 ml

2.3.3. Induction of immortalization of the bone marrow mesenchymal stem cells

The immortalization of primary cells was induced by the conditional expression of Large T-Antigen of simian virus 40 (SV40). Primary BM MSCs were grown till confluence (passage 0, P0). During the first passage the cells were diluted in the ratio 1:2. One half of the cells was plated in the MSC growth medium, whereas another half was cultured in the MSC growth medium supplemented with inductors of large T-antigen. In case of the cells from irtTA-GBD*/TAg mice the combination of 10^{-7}M Dexamethasone (Dex) and 1μg/ml Doxycycline (Dox) was used for the induction. In case of the cells from irtTA/TAg mice 1μg/ml of Dox alone was added. The growth of the immortalized cells was observed after 7-10 days. All the procedures with immortalized cells were held routinely as described above. The medium was changed every other day. The cells were passaged every 3-7 days in the dilution 1:5-1:20.

2.3.4. Colony-forming units fibroblasts (CFU-F) assay

For the colony-forming units fibroblasts (CFU-F) assay the bone marrow cells were isolated from femurs and tibiae of 5 mice as described above. The cells in the suspension were counted and plated in the concentration $5x10^5$ and $5x10^6$ cells per 10cm tissue culture dish in the MSC growth medium. The rest of the cells were plated in the well of 6-well plate to derive the stromal cell lines from the same mice. After 2 weeks the growing colonies were counted. At this time point the medium was supplemented with Dex and Dox to induce Large T-antigen expression. 2 weeks later the single colonies were picked using the cloning cylinders and then expanded.

2.3.5. Cell proliferation assay

Cell proliferation assay was performed with conditionally immortalized BM MSCs. The cells were plated into 6-well plates in the concentration $5x10^3$ cells per well. After 48 hours the medium was exchanged to the MSC growth medium supplemented with either Dex (10^{-7}M) and Dox (1μg/ml), or with Dex alone, or Dox alone, or without the drugs (day 0 of the experiment). The cells were counted in triplicates using heamatocytometer every day from the day 0 to the day 5 of the experiment in all the conditions described above. At the same time points the cell pellets were collected for protein extraction. On the day 5 Dex and Dox were added again to the cell culture medium to the deinduced cells in order to re-induce the expression of Large T-antigen again. The cells were counted 3 days later (day 8 of the experiment) and the cell pellets for the protein extraction were collected.

2.3.6. *In vitro* differentiation assays

Conditionally immortalized BM MSCs were assayed for the differentiation into osteo, adipo- and chondrogenic pathways. The expression of Large T-Antigen was deinduced prior differentiation by withdrawal of Dex and Dox for at least 3 days before starting differentiation.

2.3.6.1. Osteogenic differentiation

For the osteogenic differentiation the cells were plated at $2\text{-}5x10^4$ cells/cm^2 in 6-well plate in the osteogenic medium (DMEM-low glucose, 10% FCS, 10^{-8} M dexamethasone, 300

μM ascorbic acid, 10 mM β-glycerophosphate). The medium was replaced every 2-3 days during 28 days. Then the cells were washed with PBS and fixed with methanol for 10 minutes at –20°C. The staining for the calcification of extracellular matrix was done with 2% (w/v) Alizarin Red solution in PBS at pH 4.3 for 10 minutes, followed by washing with water and drying.

For the quantification of the calcification of extracellular matrix Alizarin Red was extracted from the stained cells using 4M guanidine chloride solution at 37°C overnight (4ml/well of 6-well plate). The optical density of the extract was measured at 490 nm using spectrophotometer Ultrospec 2100pro (Amersham Biosciences).

2.3.6.2. Adipogenic differentiation

Adipogenic differentiation was induced with the cells at 50-70% of confluency in 6-well plates with the adipogenic medium. The composition of adipogenic medium: (1) DMEM-low glucose, 10% FCS, 10^{-7} M dexamethasone, 5μg/ml insulin, 0.2mM indomethacin, 0.5mM isobutyl-methylxantine; or (2) DMEM-low glucose, 10% FCS, 10^{-7}M dexamethasone, 5μg/ml insulin, 5μg/ml Troglitazone.

The medium was replaced every 2-3 days during 10 days. Then the cells were washed with PBS and fixed with formaldehyde for 1 hour at room temperature.

Oil Red solution was prepared freshly (0.3% stock solution of Oil Red in isopropanol mixed 3:2 with water) and filtered through 0.22μm filter. Cells were quickly washed with 60% isopropanol and stained with Oil Red for 5 minutes followed by extensive wash with water.

Oil accumulation in the differentiated cells was assessed by the extraction of Oil Red dye after the staining. The cells were air-dried and the dye was extracted with isopropanol (1ml/well of 6-well plate) by gentle pipetting up and down several times. Optical density of the extract was measured at 510 nm using spectrophotometer Ultrospec 2100pro (Amersham Biosciences).

2.3.6.3. Chondrogenic differentiation

2.3.6.3.1. Differentiation *in vitro*

Chondrogenic differentiation was performed using the micromass culture technique or by aggregation in the pellet.

Briefly, 10μl of concentrated BM MSCs suspension (10^6 cells) was plated in the well of 4-well plate and allowed to aggregate at 37°C in CO_2 incubator. Chondrogenic medium (DMEM high glucose, 1% FSC, 10ng/ml TGF-β1, 10^{-7}M dexamethasone, 6,25μg/ml apo-transferrin, 6,25μg/ml insulin, 50μg/ml ascorbate-2-phosphate) was gently overlaid after 4 hours. After less than 24 hours the cells formed dense nodules and detached from plastic.

Alternatively, 10^6 cells were resuspended in 1ml of the chondrogenic medium described above and spun down in 15ml Falcon tubes. The lids were half-unscrewed. Then the tubes were carefully placed into CO_2 incubator at 37°C without disturbing the pellet. After 1-2 days the cells formed dense nodules on the bottom of the tube.

The medium was carefully changed every 2-3 days. After 4 weeks of the differentiation the aggregates were fixed in 4% formaldehyde for 10 minutes at room temperature.

2.3.6.3.2. Paraffin sectioning

Fixed nodules were washed with PBS 3 times, then once with physiological solution (0.08% NaCl and 0.002% KCl), each wash for 10 minutes. The dehydration was performed by sequential incubation in the solutions:

1) 70% ethanol (2 times for 10 minutes),
2) 80% ethanol (one time for 10 minutes),
3) 95% ethanol (2 times for 10 minutes),
4) 100% ethanol (3 times for 10 minutes),
5) xylene (3 times for 10 minutes).

Paraffinization of the samples was done by treatment with paraffin 3 times for 15 minutes using the Paraffin Embedding Center EG1160 Leica. 5μm sections were prepared using microtom (Microm).

For deparaffinization and rehydration the sections were incubated for 15 minutes at 55°C and proceeded in the next solutions (5 minutes each step):

1) xylene,
2) 100% ethanol,
3) 96% ethanol,
4) 90% ethanol,
5) 70% ethanol,
6) water.

For dehydration the sections were incubated in the same solutions in the opposite order, 30 seconds each step. The slides were mounted using Eukitt Quick Hardening Mounting Medium.

The differentiation was confirmed using the histological stain Alcian blue or with immunostaining for aggrecan.

2.3.6.3.3. Alcian Blue staining

Rehydrated sections were washed with 0.1 N HCl for 5 minutes, then stained with 1% solution of Alcian Blue in 0.1N HCl (pH 1.0) for 5 hours at room temperature. Excess of the staining was removed with 0.1 N HCl. Counter staining was done with Nuclear Fast Red (30 seconds).

2.3.6.3.4. Immunohistological staining

Rehydrated sections were treated with 0.1 U/ml chondroitinase ABC for 1 hour at 37°C to facilitate the antibodies access. Endogenous peroxidase activity was quenched by incubating in 0.3% solution H_2O_2 for 30 minutes at room temperature. Non-specific sites were blocked by incubation in 2.5% solution of horse serum for 20 minutes at room temperature. The sections were subsequently incubated with primary antibodies to aggrecan (1:100) for 1 hour at 37°C, and following the washing, treated with a kit for detection of primary antibodies

according to manufacturer protocol (Vectastain ABC RTU kit, Vector Labs Inc.) Counterstaining was performed using hematoxylin.

2.3.7. Cobblestone-area forming assay (CAFC)

The cobblestone-area forming assay was performed in collaboration with Martin Bornhaeuser group (Uniklinikim, Dresden), Dr. Manja Wobus.

Conditionally immortalized BM MSCs were deinduced by Dex/Dox withdrawal for at least 3 days and plated to 24-well plates in the concentrations 10^4 and $5x10^4$ cells/well.

Mobilized peripheral blood was collected from healthy donors after treatment with 7.5μg/kg granulocyte colony-stimulating factor (G-CSF) for 5 days. Haematopoietic Stem Cells (HSC) were purified from leukapheresis samples using CD34 antibody conjugated magnetic beads according to manufacturer's instruction (Myltenyi Biotec). $CD34^+$ suspension was plated at a density of 10^4 cells/cm^2 onto BM MSCs layer. The cobblestone formation was assessed visually after 8 days.

2.3.8. Transfection of conditionally immortalized MSCs

Conditionally immortalized BM MSCs were transfected either by electroporation or lipofection.

2.3.8.1. Electroporation of conditionally immortalized BM MSCs

Conditionally immortalized BM MSCs were trypsinized, resuspended in the growth medium and centrifuged at 200g for 5 minutes. $3x10^6$ cells were resuspended in 900μl of cold PBS, mixed with appropriate amount of DNA and transferred into the cuvette with 4mm gap. Electroporation was done using Biorad Gene Pulser with parameters 250V and 500μF. The cuvette was placed on ice, and then the cells were plated to the 10cm tissue culture dish.

2.3.8.2. Lipofection of conditionally immortalized BM MSCs

The lipofection of conditionally immortalized BM MSCs was done using Lipofectamine LTX (Invitrogen) according to the manufacturer protocol. The cells were plated in the concentration $5x10^4/cm^2$ one day before transfection. The DNA for one well of 6-well plate was thoroughly mixed with 500μl of Opti-MEM medium and 2.5μl of PLUS reagent and incubated for 5 minutes at room temperature. 6.25μl of Lipofectamin 2000 was added to the mix for the further incubation for 30 minutes. The mix was distributed dropwise to the cells. After 24 hours the medium was changed.

For the transfection of the cells in the other size of tissue culture plates all the volumes of the reagents could be scaled.

2.3.9. Wnt-reporter assay

Wnt/β-catenin pathway activation was measured using transient transfection of the cells with β-catenin-activated reporter (pBAR), which represents twelve tandem TCF/LEF DNA-binding sites controlling firefly luciferase reporter gene in the pGL3 plasmid backbone

(Promega). The plasmid was co-transfected with pGL4.73hRluc/SV40 containing constitutively expressed Renilla luciferase gene to control the transfection efficiency.

Conditionally immortalized BM MSCs were plated in 96-well plates one day before transfection. 293 cells were used as a positive control of Wnt/β-catenin pathway induction. The cells were lipofected with 30ng of pBAR and 5ng of hRluc per one well. At the same time different activators of Wnt pathway were added to the half of the wells (LiCl, BIO or CT99021). After 24 hours the medium was changed. On the second day after transfection the cells were lysed in 25μl Passive Lysis Buffer (Promega) for 15 minutes at room temperature, and then an extra portion of 15μl of Passive Lysis Buffer was added and carefully mixed with multichannel pipette for the complete lysis. The activity of luciferase was measured using Dual-Luciferase Reporter Assay (Promega) with 96-well Microplate Luminometer GloMax (Promega). 12μl of lysate from each well was transferred to the reader plate. The activity of firefly luciferase was measured in the reaction with 50μl of LARII substrate (integration time 6s, delay 2s), then renilla luciferase was measured with 50μl of Stop&Glo reagent (integration time 3s, delay 2s).

The activity of Wnt/β-catenin pathway was calculated as firefly activity normalized to the transfection efficiency (evaluated as renilla luciferase activity) and expressed in Relative Luciferase Units (RLU). All the measurements were performed in triplicates.

2.3.10. Establishment of the clonally derived conditionally immortalized BM MSCs

2.3.10.1. Cellular cloning of conditionally immortalized BM MSCs

Cellular cloning of conditionally immortalized BM MSCs was done (a) by limiting dilutions or (b) using FACS.

(a) The cells were trypsinized, washed with the MSC growth medium and counted. The dilutions of 1, 5 and 10 cells/100μl were prepared, and each cell suspension was plated into 96-well plate (100μl/well) using multi-channel pipette in the MSC growth medium supplemented with Dex and Dox.

(b) Conditionally immortalized BM MSCs were grown till confluence. The medium from the cells was collected and filtered through 0.22μm filter (conditioned medium). The cells were trypsinized, washed with the MSC growth medium, counted and resuspended in DMEM-low glucose with 2% FCS in the concentration 10^6/ml. The cells were plated into 96-well plates with conditioned medium prepared before using FACS sorter (Becton Dickinson) in the concentration 1, 5 or 10 cells per well. The plates were centrifuged for 2 minutes at 700 rpm. After 2 days the medium was exchanged for the usual MSC growth medium supplemented with Dex and Dox.

In both cases after 2 weeks the plates were assessed for the clone growth. The wells with only 1 single-cell derived clone were marked. Single-cell derived clones were collected into one 96-well plate.

2.3.10.2. Manipulations with the clonally derived cells in the 96-well plates

The clonally derived conditionally immortalized cells were grown in 96-well plates, passaged and frozen using multichannel pipette following standard protocols. Moreover, the cells could be deinduced, differentiated into osteocytes and adipocytes, and stained with appropriate protocols scaling down the volumes of the reagents. For the chondrogenic

differentiation the clones of interest were expanded till the needed number of cells and proceeded with the standard protocol described above.

2.4. Flow cytometry analysis

Conditionally immortalized and deinduced for 3 days BM MSCs were analyzed by flow cytometry analysis. The following antibodies were used for the staining: primary anti-CD9, CD34-PE, CD44-PE, CD45-PE, CD49e-PE, CD117-PE, CD184-PE, Flk1-PE and Sca1-FITC (all from BD Pharmingen, USA) and CD140b-PE (eBioscience Inc, USA), and secondary goat anti-rat FITC-coupled antibodies (Jackson ImmunoResearch laboratories, USA). Isotype controls were FITC-conjugated IgG2a or PE-conjugated IgG2b (both from BD Pharmingen, USA).

Cells were trypsinized and washed with 1x PBS with 2% FCS, then incubated with primary antibodies (dilution 1:100 in PBS with 2% FCS) for 30 minutes at +4°C followed by washing. For indirect labeling the cells were then incubated with the secondary antibodies (dilution 1:200 in PBS with 2% FCS) for 30 minutes at +4°C and washed again. Cells were fixed with 4% formaldehyde in PBS. Measurement was performed with flow cytometer LSR II (Becton Dickinson) and the data was processed using FloJo software.

2.5. Immunostaining

For the immunostaining the cells were plated into 24-well plates with gelatinized glass cover slips 12mm in diameter, in the concentration 10^5 cells/well. The cells were fixed with 4% formaldehyde solution in PBS for 10 minutes at room temperature and washed twice with PBS. The permeabilization was done with 0.5% Triton X-100 in PBS, for 15 minutes at room temperature. After the wash with PBS, the cells were blocked in 3% BSA solution in PBS+0.1% Tween-20, for 30 minutes at room temperature. The primary antibody (anti-T-antigen) was diluted 1:100 in the blocking solution and the cells were incubated with the antibody for 1 hour at room temperature in a wet chamber. After the wash in PBS, the cells were incubated with the secondary antibody (TRITC-conjugated goat anti-mouse, 1:500 in blocking solution) for 1 hour at room temperature in a wet chamber. Then the cells were washed again and stained with DAPI (100ng/ml), for 3 minutes. After sequential wash in PBS and water, the slides were mounted using Mowiol.

2.6. Molecular biological methods

2.6.1. RNA isolation and RT PCR

2.6.1.1. Total RNA isolation

Total RNA isolation was done using TRI reagent (Sigma). Cells were lysed directly on the tissue culture dish (1 ml of TRI reagent per 10cm dish) and transferred to the Eppendorf tube. Alternatively, frozen or fresh cell pellets were resuspended in TRI reagent by pipetting several times. Samples were incubated for 5 minutes at room temperature. Then 1/5 volume of chloroform was added, samples were vortexed and allowed to stand additionally 10 minutes at room temperature. The mixture was centrifuged at 16000g for 15 minutes at +4°C. The aqueous phase was transferred to the fresh tube. 0.7 volume of isopropanol was added (and 2 μl of glycogen as carrier in case of small amount of RNA). After incubation for 30 minutes on ice, RNA was precipitated by centrifugation at 16000g for 15 minutes at +4°C. RNA pellet was washed twice with 75% ethanol, then air-dried and dissolved in DEPC-treated water.

2.6.1.2. DNase treatment of total RNA

Elimination of genomic DNA from RNA samples was performed by DNaseI treatment (Invitrogen) according to the manufacturer protocol. Up to 1μg of total RNA was used in 10μl reaction mix containing 1U of DNase in the DNase buffer. The tubes were incubated for 15 minutes at room temperature. Inactivation of DNase was achieved by addition of 1μl of 25mM EDTA to the reaction mix and heating up for 10 minutes at 65°C.

2.6.1.3. Reverse transcription

DNase I treated samples of RNA were used for the reaction of cDNA synthesis with SuperScript III First-Strand Synthesis System. Up to 1μg of total RNA were incubated in DEPC treated water with 5μM oligo$(dT)_{20}$ primers and 1mM dNTP mix in a total volume of 10μl at 65°C for 5 minutes to allow annealing and placed on ice. 10μl of cDNA synthesis mix were added to RNA to the final concentrations of 5mM $MgCl_2$, 10mM DTT, 2U/μl RNase OUT, 10U/μl SuperScript III RT in the buffer contained 20mM TRIS-HCl (pH 8.4) and 50mM KCl. The synthesis was performed at 50°C for 50 minutes, and then the reaction was terminated at 85°C for 5 minutes. The samples were treated with 2U RNaseH to remove the RNA template from heteroduplexes at 37°C for 20 minutes.

2.6.1.4. PCR analysis

For the PCR analysis 0.5μl of the reverse transcription reaction was used. The 50 μl of PCR mix contained 1x buffer including 1.5mM $MgCl_2$ (5 Prime), 0.2 mM of each dNTP, 0.4μM forward and reverse primers, DNA template and 1U Taq polymerase (5 Prime). The elongation step for 5 Prime Taq has to be performed at 68°C.

The PCR programm was 94°C – 2min; 35 cycles x (94°C – 30sec, 60°C – 1min, 68°C – 1min); 72°C – 3min.

10μl of the mix were checked by electropheresis in 1% agarose gel, visualizing using the gel documentation system G:box (Syngene).

2.6.2. Protein methods

2.6.2.1. Protein extraction

For protein extraction the cells from 10cm tissue culture dish were washed twice with cold PBS, scraped and spun down at 1500g for 4 minutes at +4°C. The cell pellets were resuspended in 50μl of the extraction buffer containing 20mM Hepes pH 8.0, 350mM NaCl, 10% glycerol, 0.1% Tween-20, 2mM EDTA, 1mM DTT, 1mM PMSF, 1% Protease Inhibitors Cocktail (Sigma). Cell suspension was repeatedly frozen in the liquid nitrogen and thawed in hot water, 3 times. The cellular fragments were pelleted in mini-centrifuge at 13000 rpm for 15 minutes at +4°C and discarded. The protein extracts was stored at -80°C.

2.6.2.2. Immunoprecipitation (IP)

For protein extraction for the IP the cells from 10cm tissue culture dish were washed twice with cold PBS, scraped and spun down at 1500g for 4 minutes at +4°C. The cell pellets were resuspended in 1ml of the homogenization buffer containing 20mM Hepes pH 8.0, 150mM KCl, 1.5mM $MgCl_2$ 10% glycerol, 0.05% Tween-20, 2mM EDTA, 1mM DTT, 1% Protease Inhibitors Cocktail, PIC (Roche). The cells were spun down and washed again with 1 ml of the homogenization buffer. The pellet was resuspended in 1ml of the homogenization buffer and sonicated 1 cycle, 30 sec for 10 min. The homogenate was centrifuged for 10 min at 12000g at +4°C to pellet the cellular fragments. The supernatant was stored at -80°C. An aliquote from the homogenate could be used as an "Input" for the Western blot.

The sepharose beads for IP were washed two times with PBS. 500μl of the protein extracts were incubated with 5μg of the antibody (anti-T-antigen or anti-p53) and 100μl of the beads, in a total volume of 1ml. The incubation was done for 4 hours, at +4°C rotating the tubes. After that the beads with bound antibody complexes were centrifuged at 6000rpm for 10 min. The beads pellet was quickly washed with homogenization buffer 3 times. Then the beads were resuspended in 20μl of water and 20μl of protein loading buffer (NuPage). The samples were boiled for 10 min and cooled down to the room temperature. The samples were used for Western blot.

2.6.2.3. Western blot

Western blot analysis was done with 20μg of protein extracts, or 15μl of IP samples. Extracts were mixed with the 4x NuPage loading buffer containing 4% β-mercaptoethanol, denatured at 70°C for 10 minutes and cooled down. The samples were fractionated using PAGE-electrophoresis (4.5% stack gel and 10% running gel) at 70V. The proteins were transferred to the nitrocellulose membrane PROTRAN using semidry electroblotting at 15V for 60 minutes (TransBlot SD, BioRad). The membranes were blocked in 5% milk powder in 0.1% Tween-20 in PBS overnight at +4°C. The incubation with the primary antibodies was for 1 hour at room temperature in the blocking solution (dilution 1:250-1:1000) followed by 3 times washing in 0.1% Tween-20 in PBS. The membranes were incubated with correspondent secondary horseradish peroxidase-conjugated antibodies (1:2000 in blocking solution) for 1 hour at room temperature and washed again 3 times. The detection was performed with SuperSignal West Femto Substrate kit. Visualization has been done using FujiFilm LAS-3000 luminiscent imager and AIDA software 3.20 (Raytest). Alternatively, the signals were detected using autoradiographic Kodak films.

3. Results

3.1. Establishment of conditionally immortalized bone marrow mesenchymal stromal cells (BM MSCs)

3.1.1. Isolation of mouse BM MSCs

Bone marrow mesenchymal stromal cells (BM MSCs) represent a plastic-adherent fraction of the bone marrow cells (Friedenstein, Chailakhjan et al. 1970; Pittenger, Mackay et al. 1999). The bone marrow was flushed out from the long bones (tibia and femurs) using the growth medium, spun down to clear from the cell debris and matrix fibers, and plated in DMEM with low concentration of glucose (1000mg/L) supplemented with 10% FCS. The number of cells pooled from tibia and femurs of one mouse ranged from 4.4 to $6.7x10^7$. The cells from each animal were plated into one well of 6-well plate. After 8-12 days the cells reached confluency and were passaged in the dilution of 1:2 (Fig. 11A). Totally 44 lines were established from the bone marrow of the mice from the strains C57Bl/6, CD1 or mixed 129/C57Bl/6.

The cells were examined for the expression of the surface markers by flow cytometry (Fig. 11E). Primary lines from bone marrow were positive for CD44, negative for the haematopoietic marker CD34, and heterogeneously expressed Sca-1, resembling the known phenotype of BM MSCs. The primary lines from bone marrow could be differentiated into osteocytes (result not shown), supporting the evidence that the cells represented BM MSCs. Nevertheless, in all the cases after 2-3 passages the cells stopped growing showing senescent phenotype (Fig. 11B). The low proliferation capacity of mouse BM MSCs is generally discussed in the literature and also concords to the experience of many others (Peister, Mellad et al. 2004; Morikawa, Mabuchi et al. 2009; Prockop 2009). According to our observations, the poor expansion ability of mouse BM MSCs was independent of the age, sex, or strain of the mice.

3.1.2. Conditional immortalization of BM MSC

3.1.2.1. Transgenic tetracycline-inducible Large T-antigen mice

The tetracycline-inducible SV40 (simian virus 40) Large T-antigen transgenic mice were established before as described in the Introduction (Anastassiadis, Rostovskaya et al. 2010). The system used for the regulation of the transgene

expression was TET-ON, where the transgene placed under the control of tetracycline operator and minimal promoter is not transcribed unless the inductors are added.

Two variations of the inducible system were used (Fig. 11D). In one case, the improved reverse tetracycline transactivator (irtTA) was regulating Large T expression in Doxycycline (Dox)-dependent fashion. Otherwise, a modified irtTA by fusion with a mutated version of glucocorticoid-binding domain (irtTA-GBD*) was introduced, and it required the presence of two inductors, Dexamethasone and Doxycycline (Dex/Dox) for the activation of the transgene expression.

Large T-Antigen was expressed in the mouse tissues after intra-peritoneal administration of the inductors (Dox for irtTA-TAg mice or Dex/Dox for irtTA-GBD*-TAg mice) and was very tightly controlled (Anastassiadis, Rostovskaya et al. 2010). Also the cells isolated from these mice could be induced for the expression of Large T-antigen when the appropriate ligands are added to the cell culture medium. Both transgenic mouse lines (irtTA-TAg and irtTA-GBD*-TAg) were used in this work.

3.1.2.2. Large T-antigen can be induced in BM MSCs

BM MSCs were established from the transgenic tet-regulated T-antigen mice. During the first passage the cells were split 1:2, and half of the cells was plated in the usual MSC Growth Medium, whereas another half was induced by adding of the inductors of the tet-system (Dox or Dex/Dox). The cells without induction stopped growing and exhibited senescent phenotype during next 1-2 passages. At the same time the cells in the induction conditions showed increased proliferation and could be passaged indefinitely long, for at least 40-50 passages (Fig. 11B, 11C). SV40 Large T-antigen is known to stimulate cell proliferation and inhibit apoptosis. Western blot analysis confirmed that primary BM MSCs started expressing Large T-antigen after induction with Dox or Dex/Dox (Fig. 11F). After withdrawal of tet-inducers (Dox or Dex/Dox) the cells were not expressing Large T anymore and stopped proliferating, showing that the induction of large T-antigen in BM MSCs was reversible.

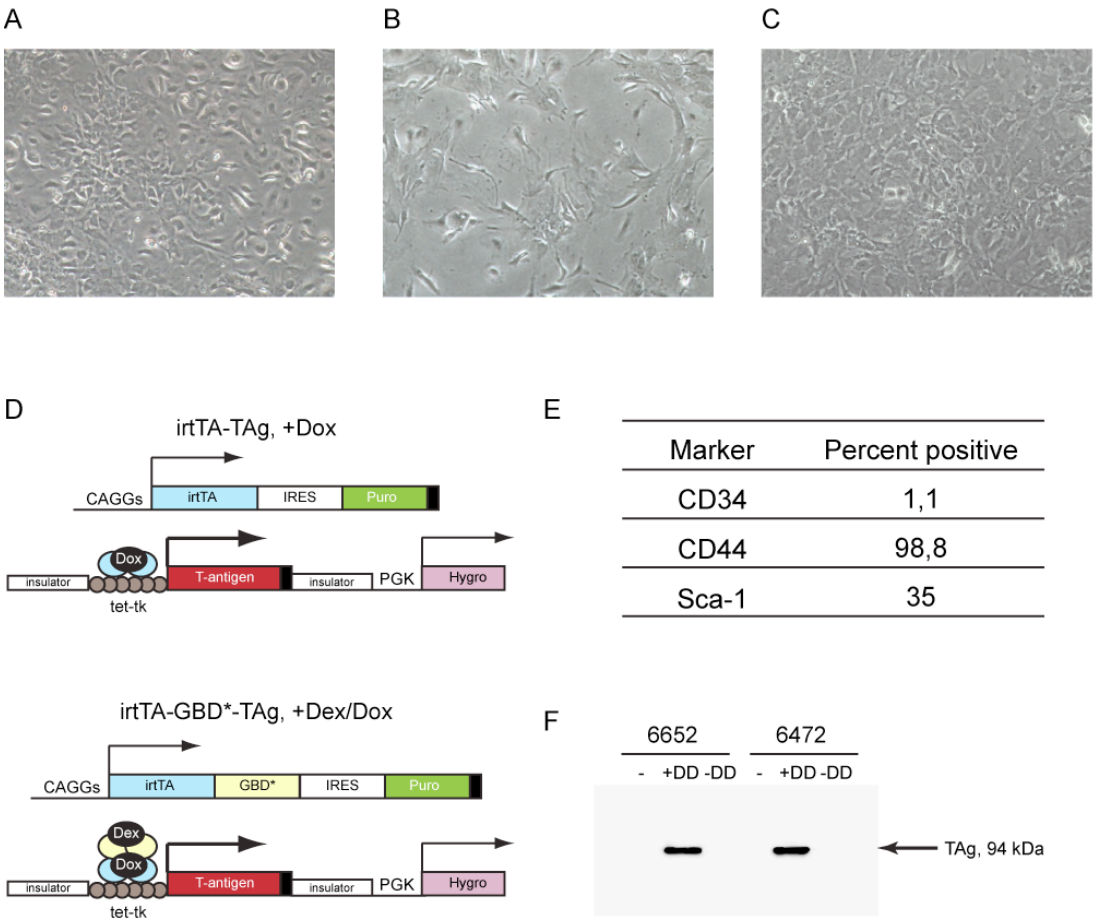


Figure 11. Establishment of conditionally immortalized murine BM MSCs. (A) Primary culture of bone marrow stromal cells (passage 0). (B) Murine BM MSCs stop growing and exhibit senescent phenotype during the passaging (passage 2). (C) BM MSCs start proliferating after induction of Large T-antigen. (D) Two systems for the conditional immortalization were used, irtTA-TAg, Dox-inducible, and irtTA-GBD*-TAg, Dex/Dox-inducible. (E) Immunophenotype of primary BM MSCs. (F) Western blot showing conditional induction of T-antigen expression in BM MSCs from two mice, 6652 and 6472. ("-" primary cells before induction, "+DD" induction with Dex/Dox, "-DD" Dex/Dox withdrawal from the induced cells.)

Since the cells were proliferating as stable lines in T-antigen-dependent manner without signs of senescence, and this effect was reversible, we termed this phenomenon as "conditional immortalization" and the cells as "conditionally immortalized BM MSCs. Interestingly, immortalization could be switched "on" and "off" many times for those cells.

The time and the efficiency of induction of primary cells were significantly different for the two systems of irtTA activation. The proliferation of BM MSCs from irtTA-TAg mice was usually observed 12-35 days after the start of induction. Notably, only 8 lines out of 25 were established resulting from the uniform proliferation of the stromal cells within 12 days. In 4 isolates only few cells were proliferating, giving rise to the stromal line. Also, the cells from 13 irtTA-TAg mice could not be induced completely. These observations are in accordance to the known mosaicism in the

irtTA mice, reported before (Anastassiadis, Rostovskaya et al. 2010). The level of the induction in those mice is often poor because the system is probably getting silenced and can be activated only in some of the cells.

The BM MSCs from irtTA-GBD*-TAg mice showed rapid growth after 6-8 days of Dex/Dox induction (11/11 lines, 100%). In all cases the majority of the cells were uniformly proliferating suggesting the high efficiency of the induction. The fusion with GBD* domain gives an additional level of regulation for irtTA, making the expression more tightly controlled and efficient.

3.1.2.3. Proliferation of conditionally immortalized BM MSCs

BM MSCs isolated from tet-regulated Large T-antigen mice were conditionally immortalized upon the induction of the Large T. In order to check whether proliferation of the immortalized cells is T-antigen-dependent, the following experiment was performed. The cells from irtTA-GBD*-TAg mice were seeded into 6-well plates in a low density and different conditions were applied – (1) with inductors (Dex/Dox), (2) withdrawing one or the other ligand (Dex or Dox) or (3) withdrawing both ligands (deinduction). The number of cells per well was counted for 13 consecutive days (Fig. 12A). The cells induced with Dex/Dox were gradually increasing the number until they reached confluency at day 6. In the absence of one or both inductors the cells slowed down the proliferation and did not increase their numbers from day 3 after withdrawal. On day 6 of the experiment the deinduced cells were induced again by adding Dex/Dox into the medium. The cells started proliferating from day 9, onwards reaching similar growth speed as the original induced cells.

These results strongly show that proliferation of the conditionally immortalized BM MSCs from irtTA-GBD*-TAg mice depends on the presence of both inductors of tet-system (Dex and Dox). Similarly, we observed in our experiments that proliferation of the cells from irtTA-TAg mice (Dox) is ligand-dependent and tightly regulated (results not shown).

Additionally, the immortalization of BM MSCs can be reversibly induced and deinduced. We have never observed the proliferation of the cells after deinduction of conditionally immortalized BM MSCs, suggesting that the cells do not become spontaneously transformed after expansion in the immortalization conditions.

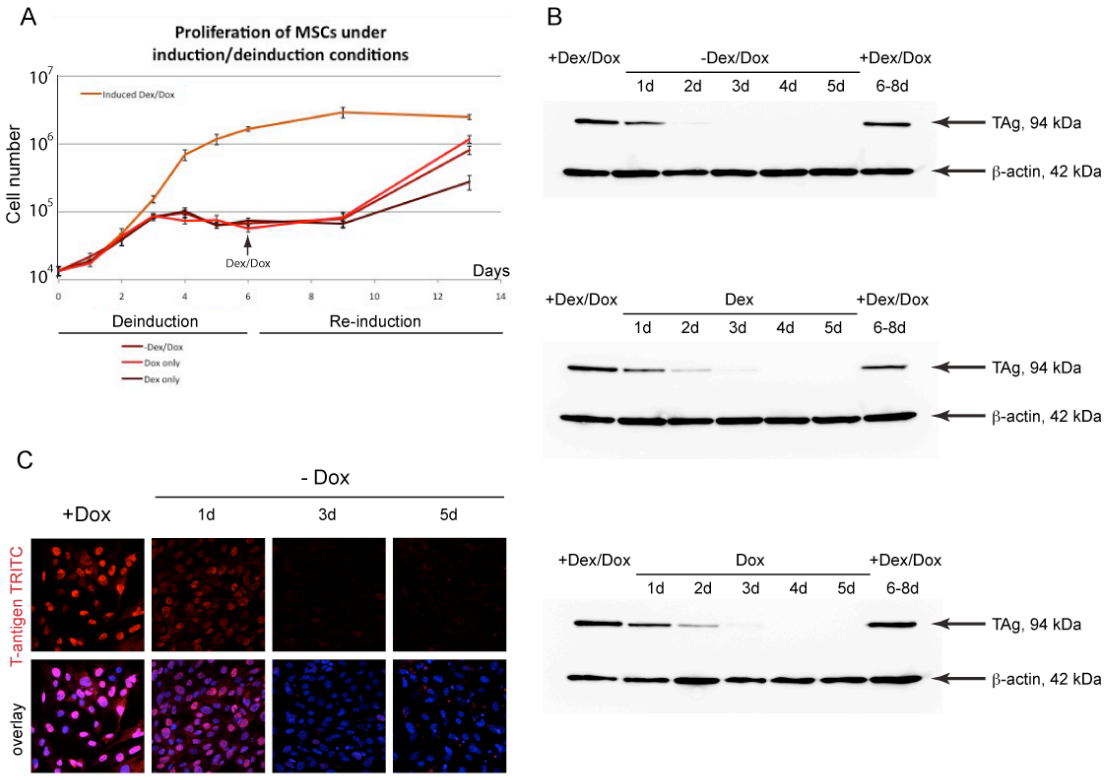


Figure 12. Proliferation of conditionally immortalized BM MSCs. (A) Cell proliferation during induction, deinduction and re-induction of the cells. Cells stopped proliferating after 3 days of Dex/Dox withdrawal. Similar kinetics was observed for the cells kept with Dex or Dox only. Dex/Dox were added again on day 6 of the experiment and the cells started growing again 3 days after re-induction. (B) Western blot showing the expression of Large T after deinduction and following re-induction. Large T was not detected after 3 days of withdrawal of the drugs. β-actin was used as a loading control. The cells were induced again from day 6 of the experiment and expression was analyzed again at day 8. (C) Immunostaining for Large T-antigen during the deinduction of irtTA-TAg BM MSCs by Dox withdrawal (from left to the right: induced cells, days 1, 3 and 5 of deinduction). T-antigen-TRITC, overlay with DAPI, 60x.

3.1.2.4. Expression of Large T is tightly regulated

The previous experiment demonstrated that conditionally immortalized BM MSCs from irtTA-GBD*-TAg mice stop proliferating in the absence of at least one of the ligands for the tet-system induction (Dex, Dox, Dex/Dox). The expression of SV40 Large T-antigen in different conditions was monitored by Western blot analysis. The Large T-antigen expression in the cells was gradually decreasing and could not be detected from 3-4 days after deinduction (Fig. 12B). The expression of Large T was fully reversed after adding of the inductors to the cells again. The level of T-antigen expression was in a strong agreement with the proliferative activity of the cells.

The similar dynamics of T-antigen deinduction was observed in the conditionally immortalized BM MSCs from irtTA-TAg mice upon Dox withdrawal. Here, the downregulation of T-antigen was also showed on the cellular level with

immunostaining for Large T-antigen (Fig. 12C). In the induction conditions all of the cells were expressing Large T-antigen, which localized in the nucleus. The cells were uniformly loosing T-antigen after Dox removal and were completely negative for the immunostaining after 3 days.

Altogether, these results demonstrated that the expression of large T-antigen completely depends on the presence of the inductors and is fully reversible. The transgene expression and subsequently cell proliferation were tightly controlled and thus, the conditional immortalization system can be characterized as excellently regulated.

3.1.2.5. The effects of Large T-antigen on the cells are reversible

The mechanism by which SV40 Large T-antigen contributes to the cellular transformation involves importantly the interaction with the main tumor suppressors, retinoblastoma protein (pRb) and p53, and their inactivation (Jiang, Srinivasan et al. 1993; Ahuja, Saenz-Robles et al. 2005). These events cause large changes in the protein interaction networks regulating the cell cycle and apoptosis. The expression of some of the components playing role in the cell cycle regulation of the conditionally immortalized BM MCSs was examined by Western blot.

Conditionally immortalized BM MSCs from irtTA-TAg mice were expanded in the induction conditions (+Dox) and deinduced by Dox withdrawal for 5 days. Dox was added again to the cells for 3 days. Cell pellets were collected every day during the course of deinduction and after re-induction again. Western blot analysis showed gradual decrease of the Large T after deinduction and from the 3 day T-antigen was not detected in the cells. The expression of Large T was reversed after the re-induction (Fig. 13A).

p53, p27 and cyclin D1 were detected on the same membrane using the appropriate antibodies. The basal level of p53 is usually reported as low in the cells (Coutts and La Thangue 2005). According to our observations Large T-antigen binds p53 leading to the accumulation of p53 in the cells. High level of p53 expression was detected in the induced conditionally immortalized BM MSCs. p53 was downregulated after the deinduction of the cells and in an absolute agreement with Large T level. p53 expression was re-gained after the cells were re-induced with Dox again (Fig. 13A). To support those data, p53 and Large T-antigen were co-immunoprecipitated from the induced conditionally immortalized BM MSCs and detected by Western blot (Fig. 13B). This result shows the direct interaction between

T-antigen and p53 proteins. Similar intensity of the signal for Large T in the input and anti-p53 IP suggests that a large proportion of T-antigen is in the unbound state. At the same time p53 was highly enriched in anti-Large T IP showing that the most of p53 is bound to the T-antigen. Upon deinduction both of the components of the complex were not detected in the cells.

p27 is a negative regulator of the cell cycle and it forms a complex with cyclins D and E upholding the cells in G1 check-point. The p27 expression is usually elevated in the senescent cells (Kato, Matsuoka et al. 1994; Polyak, Lee et al. 1994). Western blot analysis showed the increase of p27 level in the deinduced BM MSCs and its downregulation after re-induction. At the same time cyclin D1 was accumulating in the non-proliferating deinduced cells, reflecting the formation of the inhibitory complex p27-cyclins. Nevertheless, after re-induction of the cells the expression of D1 returned to the same level as in the initial induced state (Fig. 13A).

Taken together, we have observed the main changes in the cell cycle regulators mediating Large T-antigen-induced immortalization (Ahuja, Saenz-Robles et al. 2005). Importantly, those changes were reversed after T-antigen deinduction in a strong concordance with the cell proliferation and the expression of T-antigen itself. Re-induction led to the “immortal” state of the cell cycle machinery again. All those data reveal that conditional immortalization of cells is functioning according to the well-described classical T-antigen mechanism. The advantage of the conditional system makes it possible to deinduce immortalization and revert the expanded cells to their initial state at least without obvious alterations associated with cell cycle progression.

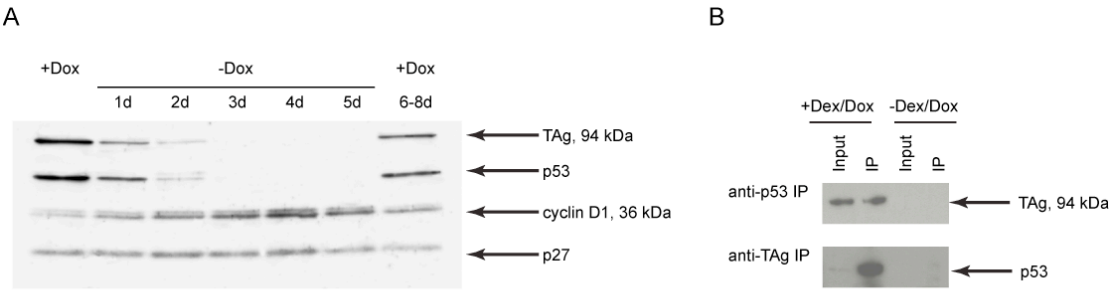


Figure 13. Expression of the cell cycle regulators in conditionally immortalized BM MSCs. (A) Western blot showing the expression of the cell cycle regulators during the deinduction of T-antigen. The conditionally immortalized MSCs (irtTA-TAg) were deinduced for 5 days and then induced with Dox again, and the protein lysates were prepared from the induced cells, deinduced cells on every day of the experiment, and from re-induced cells. (B) Immunoprecipitation was done from the whole cell lysates of BM MSCs from irtTA-GBD*-TAg mice under induction (+Dex/Dox) and deinduction (-Dex/Dox) using anti-TAg or anti-p53 antibodies. Western blot shows the formation of TAg-p53 complex in the induced cells.

3.2. Properties of conditionally immortalized BM MSCs

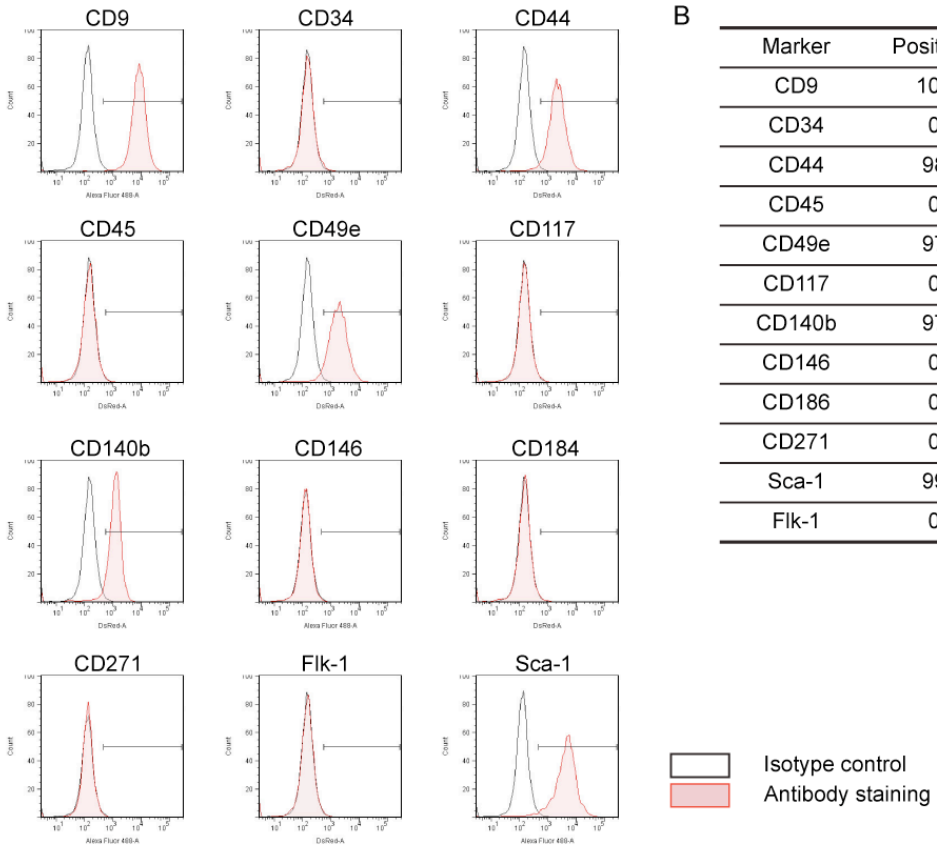
Conditionally immortalized BM MSCs were established from transgenic irtTA-GBD*-TAg mice by induction with Dex/Dox, or alternatively from irtTA-TAg mice by Dox induction. The cells were proliferating as stable lines maintaining the properties typical for BM MSCs. To verify the identity of the cell lines we analysed the expression of surface antigens by flow cytometry, their ability to support haematopoietic stem cells and their differentiation potential *in vitro*.

3.2.1. Expression of surface antigens

Conditionally immortalized BM MSCs were analyzed for the expression of surface markers by flow cytometry (Fig. 14A, B). Antigens with different cellular functions and with known pattern for the expression in human BM MCSs were chosen for the analysis. The cells were found to be homogeneously positive for the proteins involved in the adhesion of stromal cells CD9 (p24), CD44 (HCAM, hyaluronan receptor), CD49e (α5-integrin). Conditionally immortalized MSCs were negative for the markers responsible for adhesion and migration of haematopoietic and endothelial cells CD34 (sialomucin) and CD146 (MCAM). Conditionally immortalized MSCs expressed receptors for growth factors CD140b (PDGF receptor β) and Sca-1 (stem cell antigen). The cells were negative for the signal transduction molecules and chemokine receptors of haematopoietic cells CD45 (leukocyte common antigen, LCA), CD117 (c-kit, receptor for stem cell factor SCF), CD184 (SDF1-receptor, CXCR4), CD271 (p75, NGFR), Flk-1 (VEGF-R2).

Murine BM MSCs are generally poorly characterized for the marker expression. We found that the profile of the surface markers was similar to the known markers for human BM MSCs with some minor changes. For example, CD146 (MCAM) is often used as a marker for multipotent human BM MSCs (Sacchetti, Funari et al. 2007), despite murine cells appeared to be negative. At the same time, it is often reported that similar stem cell types from different species are different in marker expression. For example, mouse embryonic stem cells are different in the expression of SSEA1 and SSEA4 from their human counterparts (Henderson, Draper et al. 2002). The commonly accepted markers for murine haematopoietic stem cells are $CD34^-/CD38^+$ whereas human HSCs have phenotype $CD34^+/CD38^-$ (Spangrude, Heimfeld et al. 1988; Baum, Weissman et al. 1992).

A



B

Marker	Positive %
CD9	100,0
CD34	0,0
CD44	98,7
CD45	0,0
CD49e	97,3
CD117	0,0
CD140b	97,4
CD146	0,0
CD186	0,0
CD271	0,0
Sca-1	99,3
Flk-1	0,1

C

Cells	1556 P+9		1556 P+21	4190 P+5	
	Induced +Dex/Dox	Deinduced -Dex/Dox	Induced +Dex/Dox	Induced +Dox	Deinduced -Dox
CD9	100,0	99,9	99,9	100,0	100,0
CD34	0,0	0,4	0,2	11,1	12,6
CD44	98,7	98,4	99,2	99,6	99,6
CD45	0,0	0,2	0,2	0,5	1,0
CD49e	97,3	91,5	96,0	97,9	99,7
CD117	0,0	0,2	0,2	0,4	0,7
CD140b	97,4	87,7	94,0	96,2	91,1
CD146	0,0	0,0	0,1	0,5	0,3
CD184	0,0	0,4	0,3	0,7	0,9
CD271	0,0	0,0	0,1	0,5	0,4
Sca-1	99,3	99,6	99,6	42,0	81,9
Flk-1	0,1	0,5	0,4	0,3	0,6

Figure 14. Expression of surface markers in conditionally immortalized BM MSCs. Flow cytometry analysis of conditionally immortalized BM MSCs induced with Dex/Dox, (A) histograms and (B) percents of positive. (C) Comparison of the marker expression: BM MSCs induced vs deinduced, early passage (P+9) vs late passage (P+21), Dex/Dox induction vs Dox induction.

The expression of markers was similar in the cells induced with Dex/Dox (n=7) and for the Dox-induced cells (n=6) indicating that dexamethasone did not have influence on the antigen expression (Fig. 14C). The markers were also not changed when the cells were deinduced for 3 days, showing that Dox and

combination of Dex/Dox, and the expression of Large T-antigen itself do not change this property of BM MSCs. Importantly, the expression of surface antigens was not altered during long-term passaging (passage 9 versus passage 21).

Taken together, our data suggest that conditional immortalization does not influence the marker expression, and conditionally immortalized BM MSCs can be used as a relevant model for surface marker characterization of murine stromal cells.

3.2.2. Support of haematopoietic stem cells *in vitro*

An important systemic function of bone marrow stroma is to support haematopoiesis *in vivo* (Dexter 1982; Friedenstein, Latzinik et al. 1982; Patt, Maloney et al. 1982; Bianco, Robey et al. 2008). As a reflection of this property BM MSCs can re-create the haematopoietic niches also in *in vitro* conditions, thus supporting haematopoietic stem cells (HSCs) and their repopulation potential, and expansion/differentiation of the committed progenitors in co-culture.

One of the functional quantitative tests for the interaction of the BM MSCs with HSCs is Cobblestone-Area Forming Cells assay (CAFC). HSCs can settle between the layer of stromal cells and the tissue culture dish forming phase-dark clusters of cells that can be visualized under the microscope and counted (Olesen, Tonder et al. 2001; van Os, Dethmers-Ausema et al. 2008).

Conditionally immortalized BM MSCs from two irtTA-GBD*-TAg mice (induction with Dex/Dox) were checked for their ability to support HSCs in CAFC assay (Fig. 15). The experiment was performed in collaboration with Manja Wobus, Prof. Bornhauser group, Uniklinikum, Dresden. BM MSCs were expanded upon induction of Large T-antigen and then deinduced for at least 3 days before the assay to stop proliferation. $CD34^+$ HSCs were sorted form the human mobilized peripheral blood using CD34-conjugated magnetic beads and seeded onto the confluent layer of BM MSCs. The CAFC areas were counted after 8 days (endpoint of the experiment). 2-3 areas of CAFC per well of 24-well plate for both BM MSC lines were observed. Thus, conditionally immortalized BM MSCs can support HSCs *in vitro* and maintain this property after induction of Large T-antigen.

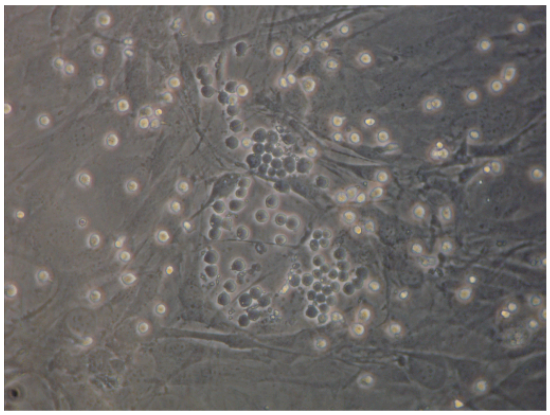


Figure 15. CAFC assay of conditionally immortalized BM MSCs. BM MSCs were expanded in the immortalization conditions and then deinduced for 3 days. CD34-positive human HSCs were seeded on the confluent layer of MSCs. CAFCs were observed and counted after 8 days.

3.2.3. *In vitro* differentiation potential of conditionally immortalized BM MSCs

One of the characteristic properties of BM MSCs *in vitro* is their ability to differentiate into the cells of mesenchymal lineages: osteocytes, adipocytes and chondrocytes (Prockop 1997; Pittenger, Mackay et al. 1999; Colter, Sekiya et al. 2001). *In vitro* differentiation of BM MSCs is believed to reproduce closely those processes taking place *in vivo* and can be used as an appropriate model to study their mechanisms. Conditionally immortalized BM MSCs were tested for their differentiation potential. The cells were expanded upon induction of Large T-antigen, deinduced to stop the proliferation, and then differentiated using the known protocols for human BM MSCs. Conditionally immortalized BM MSCs from both transgenic mouse lines irtTA-GBD*-TAg (Dex/Dox induction) and irtTA-TAg (Dox induction) were used in the experiments to exclude the influence of Dex during the induction period.

3.2.3.1. Osteogenic differentiation

Conditionally immortalized BM MSCs were differentiated to the osteogenic lineage using the medium containing Dex, β-glycerophosphate and ascorbic acid. The cells were proliferating during the first 5-7 days (the expansion of progenitors phase) and then stopped divisions (the differentiation phase). The cells secreted dense protein matrix and started mineralization after 7 days of differentiation. The accumulation of mineralization was monitored using Alizarin Red staining (Fig. 16A, B) and quantified by extraction of the dye with guanidine chloride solution and measurement of optical density at 490 nm. The maximum of mineralization was reached between 21-28 days of the differentiation (terminal stage). The expression of

osteocyte markers such as Msx2, osterix, RUNX2, osteopontin and osteocalcin was detected by RT-PCR from day 14 (Fig. 16C).

The osteogenic potential was compared for the cells of the same line on the earlier passage (p11) and after the long-term culturing (p23). The kinetics of mineralization and osteocyte marker expression was similar between the samples. Hence, the osteogenic potential was not changed after expansion of the cells in culture indicating that conditional immortalization does not influence the differentiation properties of those cells and the cells do not undergo spontaneous differentiation during passaging.

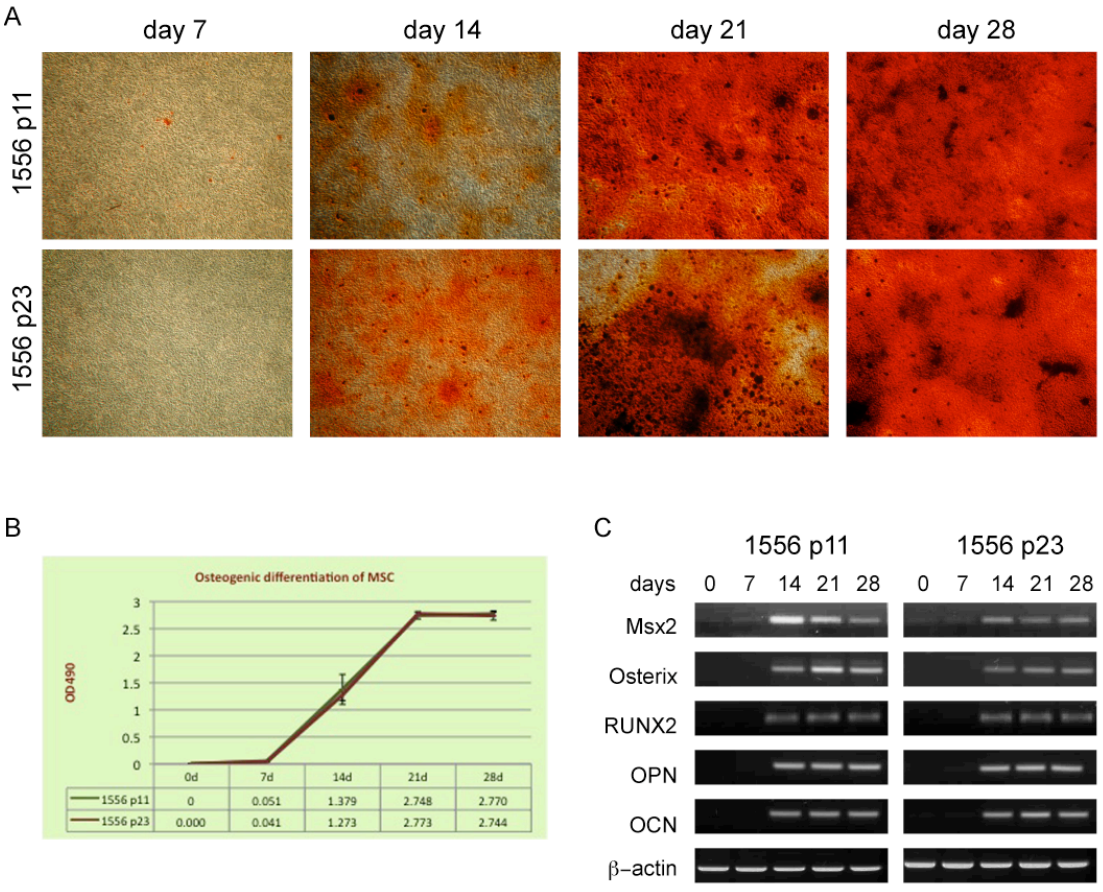


Figure 16. Osteogenic differentiation of conditionally immortalized BM MSCs. (A) Alizarin Red staining of the cells after 7, 14, 21 and 28 days of differentiation (4x). Upper panel - the cells were after 11 passages, lower panel - after 23 passages in culture. (B) Kinetics of Alizarin Red accumulation during osteogenic differentiation of cells on passage 11 and passage 23. The cells were fixed and stained with Alizarin Red, then the dye was extracted with Guanidine Chloride and the content of the dye was evaluated spectrophotometrically at OD490. (C) RT-PCR for the markers of osteogenesis before and after 7, 14, 21 and 28 days of differentiation. PCR for β-actin served as a control.

3.2.3.2. Adipogenic differentiation

Conditionally immortalized BM MSCs were differentiated to adipogenic lineage using the medium containing Dex, insulin, methyl-isobutyl-xanthine (MIX) and indomethacin. The cells changed the morphology to flat large polygonal shape and started accumulating the oil drops visible from day 3-4 (Fig. 17A). Adipocytes were visualized using Oil Red staining. The efficiency of adipogenic differentiation was expressed as a percent of mature adipocytes from the total cell number, or as an optical density of Oil Red extracted with isopropanol from the stained samples. The differentiation proceeded till day 10, when it reached its maximum (the result after 10 days is not shown). The expression of adipocyte markers was monitored by RT-PCR before and after 4, 7 and 10 days of differentiation. The transcript level of important regulators of adipogenesis such as PPARγ2, C/EBPβ and C/EBPδ was increasing during the course of differentiation. C/EBPα was found already in undifferentiated cells and then during differentiation as well. The markers of mature adipocytes such as adiponectin and adipsin were expressed from day 4, PEPCK was detected later, from day 7. *In vitro*-derived adipocytes belonged to the white and not brown adipose tissue-type indicated by the absence of UCP-1 expression.

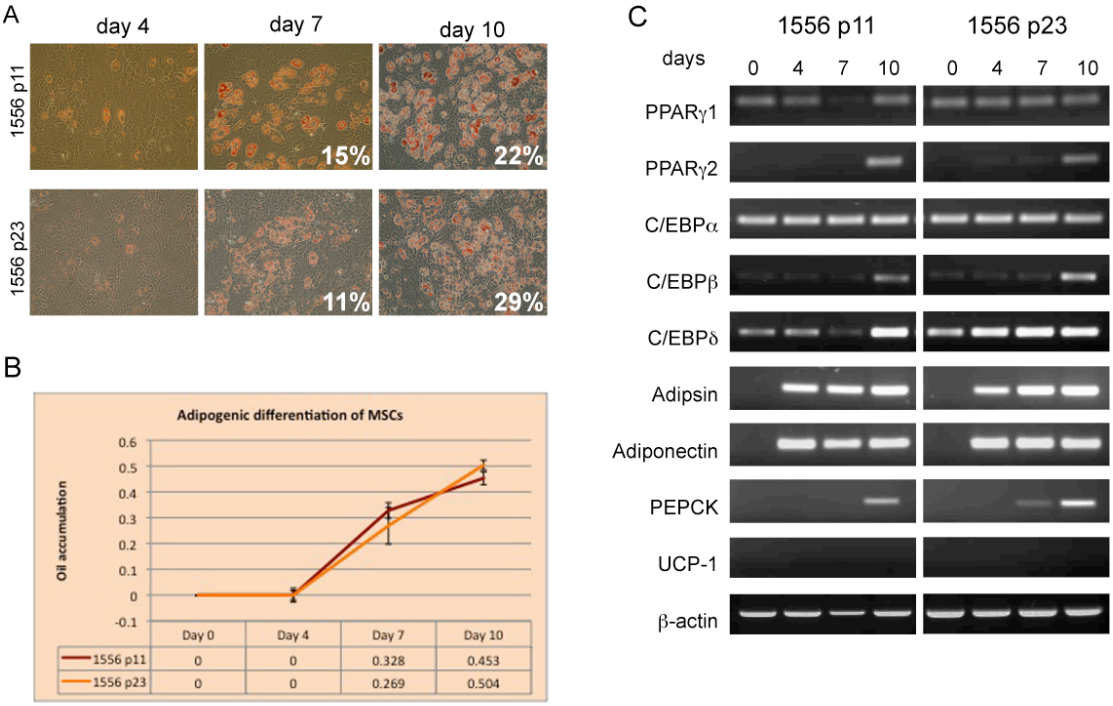


Figure 17. Adipogenic differentiation of conditionally immortalized BM MSCs. (A) Oil Red staining of the cells after 4, 7 and 10 days of differentiation (20x). Upper panel - cells after 11 passages in proliferation conditions, lower panel - after 23 passages. The adipocytes were counted and percents from the total cell number are shown on the correspondent image. (B) Kinetics of oil drops accumulation during differentiation of the cells on passage 11 and 23. The cells were fixed and stained with Oil Red, then the dye was extracted with isopropanol and the content of the dye was evaluated spectrophotometrically at OD510. (C) RT-PCR for the markers of adipogenesis before and after 4, 7 and 10 days of differentiation. PCR for β-actin served as a control.

Similarly to the osteogenic differentiation, adipogenic potential of BM MSCs was compared for the cells that have proceeded different number of passages (Fig. 17A-C). The efficiency and the kinetics of differentiation were very close between the cells on the passage 11 and passage 23. The expression of the markers between samples was also almost not different. This shows that conditional immortalization does not influence the differentiation capacity of those cells and the cells do not loose adipogenic potential spontaneously during passaging.

3.2.3.3. Chondrogenic differentiation

Conditionally immortalized BM MSCs were differentiated to chondrocytes by forming aggregates in low serum conditions (1%) and in the presence of TGF-β1, Dex, ascorbic acid and insulin. The starting numbers of the cells were $5x10^4$, 10^5, $5x10^5$ or 10^6. The aggregation of cells was achieved by micromass or cell pellet method (details explained in the Material and Methods section). The cells formed dense nodules, which were increasing in size only in the presence of TGF-β1. The differentiation was tested after 10 and 23 days for the expression of the cartilage matrix components aggrecan and collagen II by RT-PCR and immunostaining (Fig. 18). The cells were positive for those markers from the day 10 of differentiation, increasing the level of transcript and the area and intensity of the antibody staining at the later stages. The aggregates showed lacunal structure typical for cartilage on the histological sections. The extracellular matrix contained proteoglycans characteristic for cartilage as shown by alcian blue staining. The efficiency of differentiation was dependent on the starting cell number according to the transcripts level and the intensity of the antibody staining. The chondrocyte formation was more efficient starting with 10^6 cells comparing to $5x10^4$, 10^5 or $5x10^5$ cells (Fig. 18B, RT-PCR).

Summarizing the results described above, conditionally immortalized stromal lines established from the bone marrow maintain the properties to differentiate *in vitro* into the cells of mesodermal origin providing the evidence for their identity of BM MSCs.

We could differentiate BM MSCs from both transgenic mouse lines irtTA-GBD*-TAg and irtTA-TAg into osteo-, adipo- and chondrocytes indicating that the presence of Dex during induction of immortalization does not change differentiation capacity (result not shown).

Importantly, the differentiation capacity (at least toward osteocytes and adipocytes) was not changed during the long passaging in the cell culture conditions showing the stability of properties of the conditionally immortalized BM MSCs lines.

Interestingly, we have observed variation between isolates from different mice in terms of the efficiency of differentiation. In many cases the cells from males were more efficient for osteogenesis and less efficient for adipogenesis *in vitro* than the cells from females (result not shown).

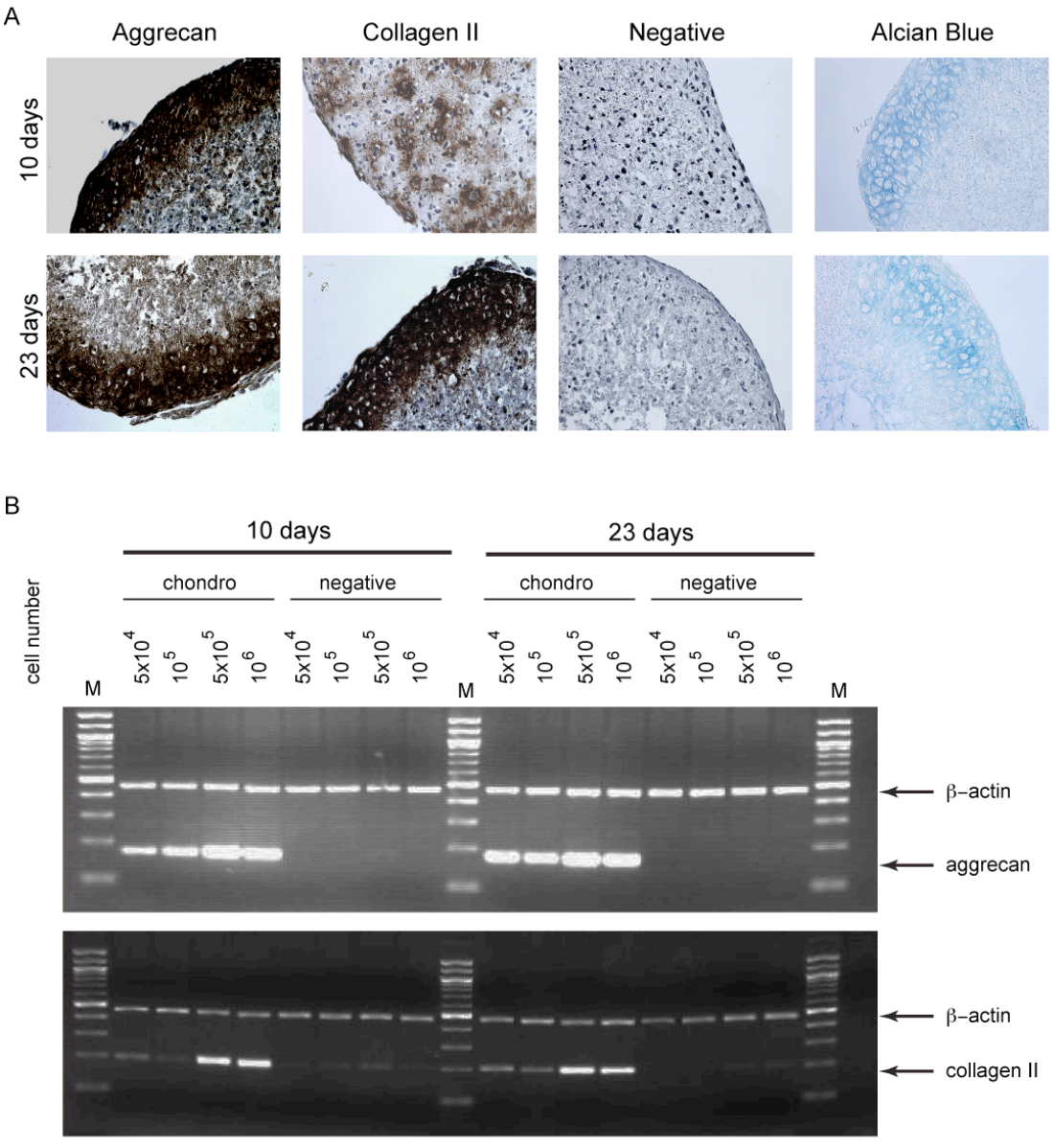


Figure 18. Chondrogenic differentiation of conditionally immortalized BM MSCs. (A) The cells were fixed after 10 and 23 days of differentiation into chondrocytes. The staining of histological sections (from the left to the right): anti-aggrecan, anti-collagen II, only secondary antibodies (negative control), alcian blue, 40x. The result is shown for a starting number of 10^6 cells. (B) Multiplex RT-PCR for the markers of chondrogenic differentiation. Chondrogenic differentiation was started with different cell numbers and analyzed on the day 10 and 23. "Chondro" - differentiated samples, "negative" - cells cultured in DMEM+10%FCS, negative control for differentiation. Upper gel - RT-PCR for aggrecan, lower gel - RT-PCR for collagen II. β-actin was used as an internal loading control.

3.2.4. Genetic modifications of conditionally immortalized BM MSCs

Genetic manipulation of primary cells is often inefficient and represents an obstacle for generating reporter lines that can be used for gene function studies. Due to the low proliferative potential the establishment of stably transduced somatic cells is almost an impossible task. One of the advantages of conditional immortalization is an easy expansion of stably transfected clones after their establishment. Additionally, DNA integration is usually more efficient in proliferating cells.

Conditionally immortalized BM MSCs were checked for the transfection ability using lipofection and electroporation. Both induced (+Dex/Dox) and deinduced (-Dex/Dox for 3 days) cells were tested in the experiment. The cells were transfected with different amount of CAGGs-GFP-IRES-puro-pA construct and checked for GFP expression by flow cytometry after 48 hours (Fig. 19A, B). BM MSCs under induction showed high transfection efficiency using both methods (up to 30-40% of GFP-positive cells). At the same time deinduced non-proliferative cells showed maximum 10% of transfected cells using lipofection. Deinduced BM MSCs had very low survival after electroporation and only few cells were found to be GFP-positive (up to 3%).

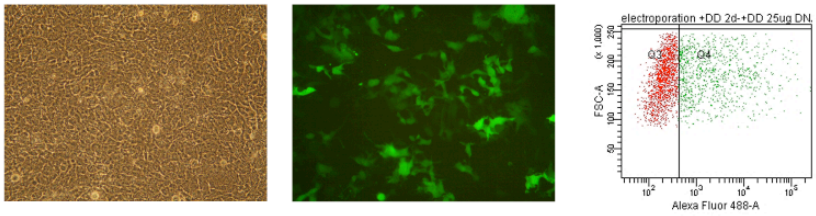
We were able to select conditionally immortalized BM MSCs electroporated with CAGGs-GFP-IRES-neo construct and to expand stably transfected GFP-positive clones (Fig. 19C). The frequency of the transgene integration was estimated as 1 in $5x10^3$ – $5x10^4$ electroporated cells.

Notably, we were able to transfect stably bacterial artificial chromosomes (BACs) in conditionally immortalized BM MSCs using lipofection (result not shown). Taken together, conditional immortalization provides a useful tool for genetic manipulation of the somatic cells.

A

DNA per 10 cm dish	Lipofection		Electroporation	
	Induction +Dex/Dox	Deinduction -Dex/Dox	Induction +Dex/Dox	Deinduction -Dex/Dox
3 μg	3,1%	0,9%	17,1%	1,6%
10 μg	16,9%	10,0%	23,2%	2,4%
25 μg	31,0%	6,5%	38%	2,3%
40 μg	14,7%	4,0%	34%	3,0%

B



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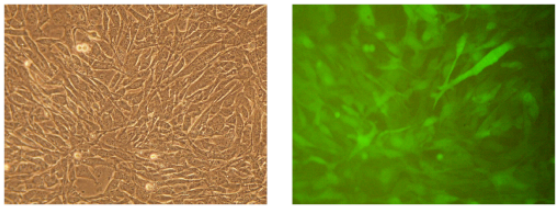


Figure 19. Transfection efficiency of conditionally immortalized BM MSCs. Induced (+Dex/Dox) or deinduced (-Dex/Dox) cells were transiently transfected by lipofection or electroporation using CAGGs-GFP-IRES-puro-pA construct. Efficiency of transfection was checked after 48 hours by flow cytometry. (A) Percents of positive cells after transfection using different conditions. (B) Conditionally immortalized BM MSCs (+Dex/Dox) were electroporated with 25 μg of plasmid. Phase contrast, fluorescence (20x), dot plot of flow cytometry analysis. (C) Conditionally immortalized BM MSCs were electroporated with linearized CAGGs-GFP-IRES-neo-pA construct and selected with G418. Cells stably expressing GFP, phase contrast and fluorescence (20x).

3.3. Establishment of conditionally immortalized populations of CFU-F's from bone marrow

The existence of clonogenic cells (colony forming unit-fibroblasts, CFU-F's) in the bone marrow which can re-build bone and haematopoietic tissue after ectopical transplantation has been shown years ago (Friedenstein, Chailakhyan et al. 1974; Friedenstein, Gorskaja et al. 1976; Patt, Maloney et al. 1982; Owen and Friedenstein 1988). The transplant could be passaged through the series of hosts, thus those cells possess self-renewal and differentiation properties. The size of new-forming marrow tissue correlated to the number of CFU-F's. This brought up the idea that CFU-F's

are *bona fide* self-renewing mesenchymal stem cells or at least the closest descendants of them.

Most of the works on CFU-F's were performed on the cells from human bone marrow; at the same time isolation of their mouse counterparts is problematic due to the low proliferation capacity (Sacchetti, Funari et al. 2007; Morikawa, Mabuchi et al. 2009). The aim of this chapter was to obtain conditionally immortalized populations of murine CFU-F's and explore their differentiation potential.

3.3.1. Establishment of CFU-F's

Bone marrow was flushed out from femurs and tibia of 5 transgenic irtTA-GBD*-TAg mice and plated to the tissue culture dishes at high density (~$5x10^6/cm^2$) and at low density ($10^4/cm^2$ and $10^5/cm^2$), separately for each mouse. The cells at the high density (bulk population) were induced with Dex/Dox for Large T-antigen expression and they proliferated as stable immortalized lines.

Plating of the bone marrow at the low density resulted in appearance of the cell colonies; at least most of them were single-cell derived (colony-forming units-fibroblasts, CFU-Fs). We counted from 78 to 360 CFU-F's on the 10cm dish for bone marrow from different mice when plated at $10^5/cm^2$. At the lowest density ($10^4/cm^2$) only 6-21 CFU-F's per 10cm dish grew. The frequency of CFU-F's in the bone marrow varied between mice and was not due to the sex or age, and was estimated as from 10^{-5} to $7x10^{-5}$ in the total bone marrow (Table 1).

Mouse	Sex	Age	Number of CFU-F's per dish	
			$5x10^5$	$5x10^4$
6427	female	21 weeks	103	11
6472	male	20 weeks	259	21
6652	male	17 weeks	360	16
6423	male	21 weeks	78	12
6421	male	21 weeks	79	6

Table 1. Efficiency of CFU-F formation from the mouse bone marrow. Bone marrow from 5 mice was plated at $5x10^4$ or $5x10^5$ cells per 10 cm dish. Number of CFU-F per dish is shown for each mouse.

Nevertheless, it was not possible to expand the CFU-F's further since they showed senescent phenotype and stopped growing. Dex/Dox were added to the medium to induce Large T expression. The proliferation of the cells was observed, and after 14 days the CFU-F's were picked to 96-well plates (from 5 to 8 CFU-F's from each mouse). CFU-F's were picked from the dishes with the lowest density, presuming that they were derived from single cells and represent clones. Immortalized cell lines were established from all picked CFU-F's.

3.3.2. Differentiation assay of CFU-F's

Conditionally immortalized stromal lines of MCSs were derived from the bulk populations of bone marrow cells from 5 mice (further called as "stromal lines" or "BM MSCs"). Alternatively, populations from clonogenic cells (CFU-F's) were established from the same mice. The single-cell derived clones were individually picked and expanded by induction of Large T-antigen in the presence of Dex/Dox (those CFU-F's descendants are mentioned further in the text as "immortalized CFU-F's"). The stromal lines (n=5) and immortalized CFU-F's (totally n=27 from 5 mice) were tested for their differentiation into osteogenic and adipogenic pathways (Fig. 20), chondrogenic differentiation was not analyzed. The differentiation was performed and evaluated as described in the previous section.

The analysis of BM MSCs showed that 4 out of 5 lines exhibited both osteo- and adipogenic potentials ("OA"). One line was not efficient to differentiate to adipocytes and could be differentiated only to osteocytes ("O"). We found diversity in differentiation potential between the immortalized CFU-F's. All possible combinations of two differentiation pathways were found: bipotential osteo-adipogenic populations ("OA", 8/27 clones), and monopotential osteogenic ("O", 17/27 clones) and adipogenic populations ("A", 2/27 clones).

We observed that the correlation between the properties of the stromal line and CFU-F's derived from one mouse was not always strong. "OA" lines were correspondent to "OA", "O" and "A" CFU-F's.

At the same time, although the stromal line derived from mouse 6423 was osteogenic ("O"), we identified one adipogenic CFU-F ("A"). This might reflect the diversity of the cells within the bone marrow, and even in case of the dominating of osteogenic progenitors in the stromal bulk population, adipogenic cells are still present.

Our results revealed heterogeneity within CFU-F's compartment in terms of multipotentiality. These data suggests possible hierarchy in CFU-F's organization, as

only a fraction of CFU-F's is multipotent. Still, growth in density-insensitive mode can be an attribute for the stem cells, but not sufficient ground of stemness. Our *in vitro* observations are confirmed by some recent literature data showing that not all CFU-F's are multipotent in transplantation assays (Kuznetsov, Krebsbach et al. 1997; Sacchetti, Funari et al. 2007; Nifontova, Svinareva et al. 2008).

A

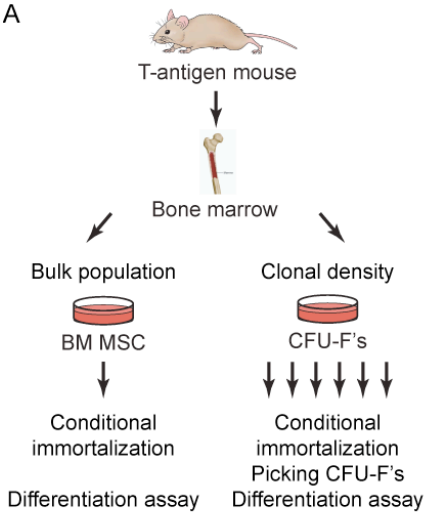
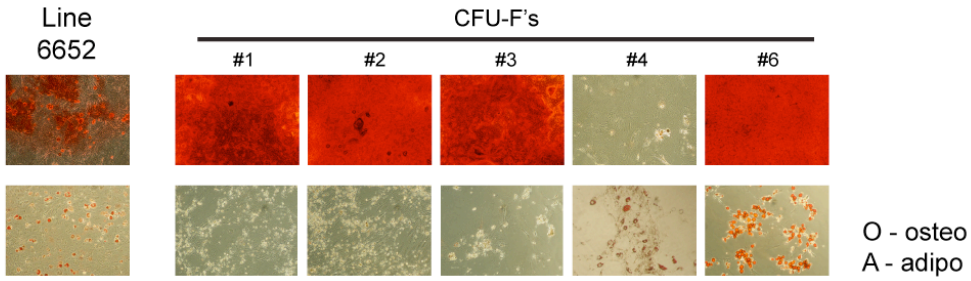


Figure 20. Heterogeneity of CFU-F's from the mouse bone marrow. (A) Bone marrow from each mouse was plated at high density to derive bulk population of stromal cells and at clonal density to establish CFU-F's. Stromal lines and individual CFU-F's were expanded under immortalization conditions, and then tested for differentiation into osteo- and adipogenic lineages. (B) Differentiation of the stromal line from one mouse (6652) and CFU-F's from the same mouse, representative example. Efficiency of osteogenic differentiation was estimated by Alizarin Red staining and adipogenic differentiation by Oil Red staining. The stromal line showed bipotential ("OA") properties, whereas the CFU-F's exhibited differences in the potential ("O", "A", "OA"). (C) Analysis of CFU-F's from 5 mice revealed heterogeneity in differentiation potential. Summary showing properties of the stromal lines and CFU-F's from the same mice. "O" - osteogenic, "A" - adipogenic.

B



C

CFU-F's potential

	Line		#1	#2	#3	#4	#5	#6	#7	#8
6421	OA		OA	O	O	O	OA	O	OA	
6423	O		O	A	O	O				
6427	OA		O	OA	O	OA				
6652	OA		O	O	O	A	OA			
6472	OA		O	O	OA	O	OA	O	O	

O - osteo
A - adipo

3.4. Heterogeneity of bone marrow stromal cells

Conditionally immortalized BM MSCs were established from transgenic irtTA-GBD*-TAg or irtTA-TAg by induction of SV40 Large T-antigen, and subsequently characterized. We showed that conditionally immortalized BM MSCs maintain the properties of primary stromal lines. Even though the culture conditions are not identical to the “niche” environment, the cultured cells represent the most available model to study their biological properties. We applied conditionally immortalized lines as a system to study the bone marrow stroma.

We also obtained and immortalized CFU-F’s (Colony Forming Units-Fibroblasts) from mouse bone marrow, and found heterogeneity within CFU-F’s population. This showed the existence of different types of mesenchymal progenitors in the bone marrow. At the same time, bone marrow stromal lines varied in differentiation potential indirectly showing that they might represent a mixture of progenitors with different ratio between different types.

For comprehensive analysis of bone marrow stromal cells we tried to dissect the population of BM MSCs at the single-cell level, to establish single-cell derived clones from immortalized lines and to compare their properties.

3.4.1. Establishment of the single-cell derived clones of BM MSCs

Conditionally immortalized BM MSCs were cloned by manual dilutions (line 1556, irtTA-GBD*-TAg, Dex/Dox induction). The cells were seeded to 96-well plates in the dilutions 1, 5 and 10 cells/well. The growth of clones was observed from the 4th day after plating. The single-cell origin of the clone was considered visually as a single colony of the regular round shape in the well. Totally, 66 out of 288 seeded wells contained clones after plating 1 cell/well (22.9% efficiency), most of them raised from single cells. The plating of 5 and 10 cells per well resulted in higher efficiency (61.4% and 82.3% respectively), at the same time the majority of the wells contained more than one clone. Only the clones derived from single cells were picked and expanded.

Alternatively, we performed cellular cloning using FACS with the line 4190 (irtTA-TAg, Dox induction). 1 cell was introduced to each well of 96-well plate. To improve cell survival after the procedure, the medium used for cell sorting had been conditioned by the same cells and the plates after the sorting were gently centrifuged to let the cells to settle down. In parallel we performed cellular cloning by manual

dilutions. The number of clones was analyzed after 12 days. 34,8% of the wells seeded by FACS contained clones, visually raised from single-cells. The manual dilutions resulted in 39,8% of wells with the clones, and a small proportion of them contained more than one clone (around 1/10). The clones from FACS sorting were picked and expanded.

Hence, single-cell derived clones of stromal cells can be established from conditionally immortalized BM MSCs.

3.4.2. Differentiation assay of the single-cell derived clones of BM MSCs

Single-cell derived clones obtained from the line 1556 (irtTA-GBD*-TAg, Dex/Dox induction) were expanded and assayed for the differentiation potential to osteogenic, adipogenic and chondrogenic lineages (Fig. 21). The cells were cultured in 96-well plates, split using multi-channel pipette, and the replicates were frozen directly in 96-well plates. The osteogenic and adipogenic differentiation were performed in 96-well format. For chondrogenic differentiation the cells were expanded till the required number. The differentiation was carried out using the protocols for the stromal lines described in the previous section. Totally 30 clones were tested.

Osteogenic differentiation was done at least in triplicates. The cells were stained with Alizarin Red (Fig. 21B), and the efficiency of differentiation was evaluated visually (positive/negative staining). The final potency of the clone (efficient/non-efficient for the given pathway) was considered only if the differentiation was reproducible in all the attempts, without variation. We found 18 out of 30 clones to have osteogenic potential. 6 clones were non-osteogenic, and 6 clones gave non-reproducible results.

Adipogenic differentiation was performed in tri- or quadruplicates as well. Adipocytes were stained with Oil Red and assessed visually. 11 clones out of 30 were found to be adipogenic and 15 – non-adipogenic. For 4 clones the result was poorly reproducible. To confirm the result, total RNA was extracted from the differentiated cells and checked by PCR with primers for markers of adipocytes, adiponectin and adipsin, after reverse transcription reaction (Fig. 21C).

Chondrogenic differentiation was performed once, and those 18 clones that had defined osteo-adipogenic potential were taken into analysis. The aggregates after differentiation were sectioned and stained with antibodies for aggrecan and collagen II, and with Alcian Blue dye. The efficiency was estimated by observing the area of positive staining. All samples with positive staining acquired cartilage-specific

lacunal structure. Additionally, RNA was extracted from differentiated cells and RT-PCR was performed for aggrecan expression (Fig. 21D). The results were consistent between the section staining and PCR. Totally, 12 clones were found to be efficient for chondrocyte differentiation and 6 clones were negative.

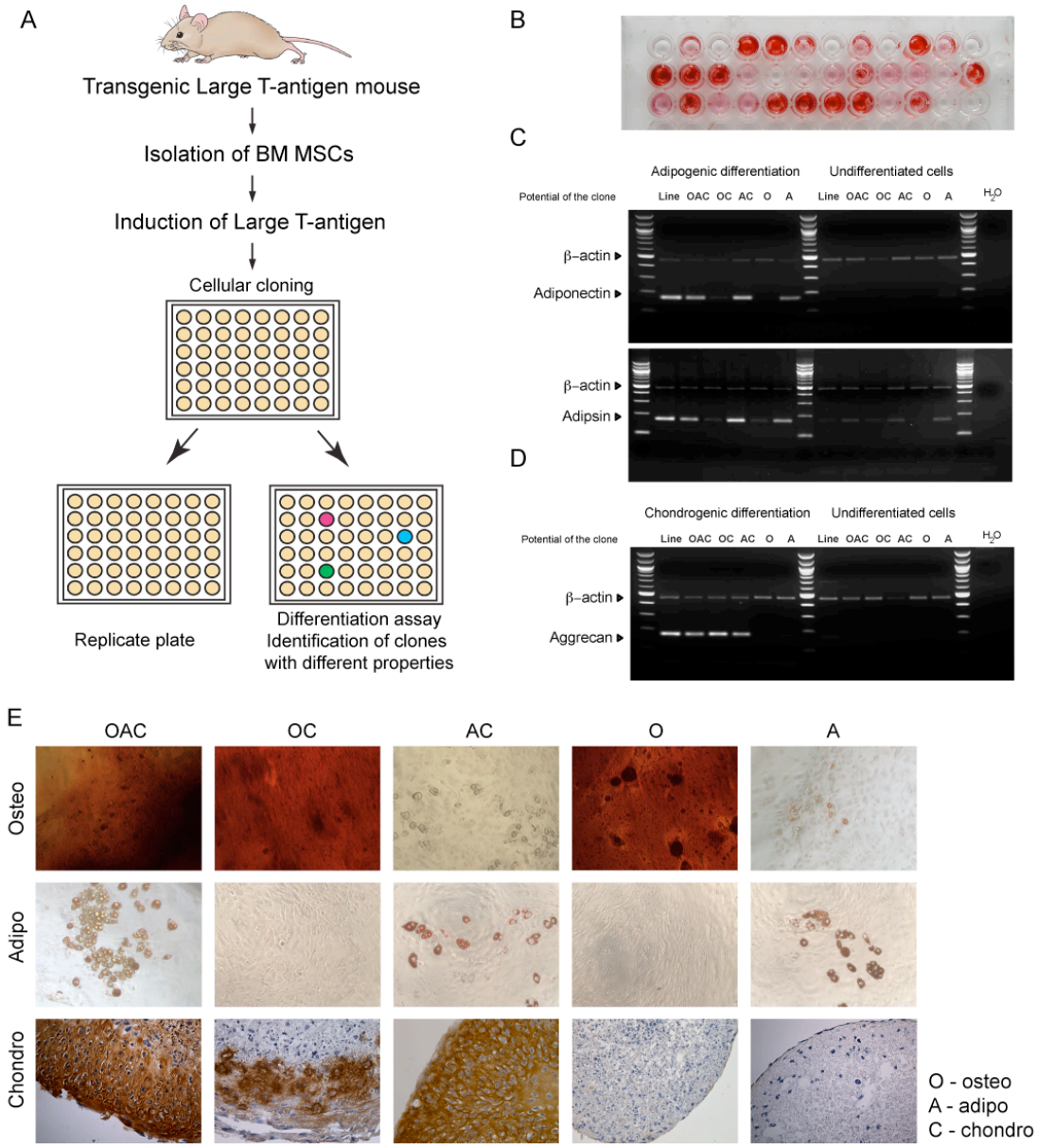


Figure 21. Heterogeneity of bone marrow stromal cells. (A) Bone marrow stromal lines were established from transgenic large T-Antigen mice and conditionally immortalized. Subsequently, populations from single-cells were derived by manual dilutions. The cells were passaged in 96-well plates and assayed for differentiation. (B) Osteogenic differentiation of single-cell derived clones of BM MSCs in 96-well plate, Alizarin Red staining, macroscopic view. (C) Single-cell derived clones and the original line of BM MSCs were differentiated into adipocytes and checked for the expression of adipogenic markers adiponectin and adipsin, by RT-PCR. Primers for β-actin were used as internal control. (D) Single-cell derived clones and the original line of BM MSCs were differentiated into chondrocytes and checked for the expression of chondrogenic marker aggrecan, by RT-PCR. Primers for β-actin were used as internal control. (E) Summary of differentiation potential of single-cell derived populations of BM MSCs. Osteogenic differentiation (”O”) was evaluated by Alizarin Red staining, adipogenic differentation (”A”) was assayed with Oil Red staining, chondrogenic differentiation (”C”) was confirmed by immunostaining for aggrecan of histological sections. The clones were found to have “OAC”, “OC”, “AC”, “O”, “A” potential.

Taken together the results of differentiation into 3 lineages, we have identified the single-cell derived clones of BM MSCs with different properties (Fig. 21E): tri-potential (“OAC”, 8 clones), bipotential osteo-chondrogenic (“OC”, 2 clones) and adipo-chondrogenic (“AC”, 2 clones), monopotential osteogenic (“O”, 3 clones) and adipogenic (“A”, 1 clone), and clones that did not exhibit differentiation capacity (2 clones).

Thus, single-cell derived clones of stromal cells vary in differentiation potential and might represent distinct populations of progenitor/stem cells. These results confirm heterogeneity of the bone marrow stromal cells.

3.4.3. Subcloning of single-cell derived populations of BM MSCs and differentiation assay

Conditionally immortalized subpopulations of BM MSCs were obtained by manual dilutions and expansion of the clones. These subpopulations showed distinct differentiation potential suggesting that they represent different types of progenitors or intermediates from bone marrow stroma. Single-cell origin implies homogeneity of the subpopulations, which is required for the analysis of those cells. On the other hand, expansion of the cells *in vitro* might cause spontaneous differentiation and variation in characteristics. In order to check the homogeneity of the cells populations, the clones of conditionally immortalized BM MSCs were subjected to the second round of cloning and analyzed for the differentiation potential (Fig. 22).

Six clones of BM MSCs with defined differentiation potential were used for the second round of cellular cloning by manual dilutions. At first we concentrated on the analysis of osteogenic and adipogenic pathways, which are easier to perform in the large scale. In terms of osteogenic (“O”) and adipogenic (“A”) properties, the clones were: “OA” (2 clones), “O” (2 clones) and “A” (2 clones). The resulted cell populations will be referred as “subclones” further in the text. Up to 24 subclones of each clone were picked into the 96-well plate, expanded and analyzed for osteogenic and adipogenic differentiation (Fig. 22B, C). The differentiation was analyzed visually using color staining with Alizarin Red and Oil Red, respectively. A large proportion of the subclones reproduced the differentiation properties of the parental clone (up to 96%, clone 1C, 23/24 subclones). Hence, the clones of BM MSCs subjected for the subcloning represented homogeneous populations with stable differentiation potential.

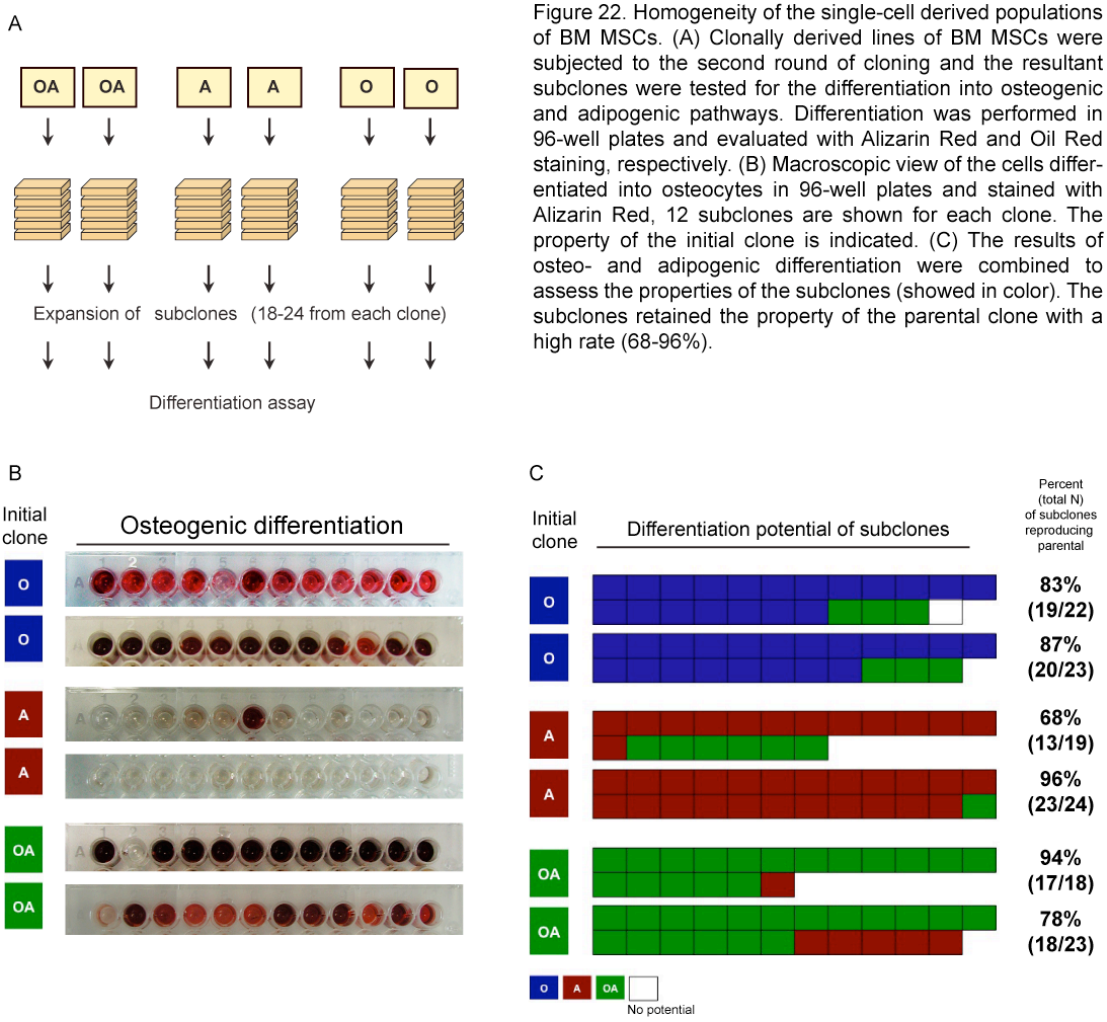


Figure 22. Homogeneity of the single-cell derived populations of BM MSCs. (A) Clonally derived lines of BM MSCs were subjected to the second round of cloning and the resultant subclones were tested for the differentiation into osteogenic and adipogenic pathways. Differentiation was performed in 96-well plates and evaluated with Alizarin Red and Oil Red staining, respectively. (B) Macroscopic view of the cells differentiated into osteocytes in 96-well plates and stained with Alizarin Red, 12 subclones are shown for each clone. The property of the initial clone is indicated. (C) The results of osteo- and adipogenic differentiation were combined to assess the properties of the subclones (showed in color). The subclones retained the property of the parental clone with a high rate (68-96%).

To make the analysis of differentiation more comprehensive we tested chondrogenic potential ("C") for the limited number of the subclones (in total n=51). In terms of differentiation into three lineages, the initial clones were "OAC" (22 subclones tested), "OC" (12 subclones), "AC" (8 subclones) and "O" (9 subclones). The cells were expanded till the required amount and differentiated using the pellet culture (more detailed in Materials and Methods). The resulted three-dimensional structures were sectioned, stained with Alcian Blue, and the analysis was done visually by the intensity of staining. We found that many of the subclones had the same potential as the parental clone. The summary of differentiation of the subclones into three lineages is presented on Fig. 23A. The results for "O", "A" and "C" lineages were calculated separately. We counted the number of the subclones, which had the given potential ("+") and which are not efficient for it ("-"). We calculated the rate of the subclones reproducing the property of parental clone, which means the percent of the efficient subclones ("+") if the initial clone was capable for this pathway, or alternatively the percent of inefficient subclones ("-") if the initial clone was not

capable for this pathway. This percent reflects how stable this property is in culture and how homogeneous the clone is. We found osteogenic and adipogenic differentiation to be highly reproducible (90.2% and 92.2%, respectively). The chondrogenic differentiation was less reproducible; from the total number of subclones 74.5% had the property of the parental clone. As a result, we identified the subclone with new bipotential “OA” property, which were not presented within the initial clones.

Taken together, the second round of cloning showed that the clonal lines of BM MSCs represent homogeneous populations of cells with inherited stable differentiation potential. We observe some variability between the subclones, mostly in chondrogenic capacity. There could be different explanations for the reduced stability of this property. First of all, chondrogenic differentiation is difficult to perform and the efficiency can vary between the experiments. Moreover, there is lack of efficient protocols for the mouse BM MSCs and those ones for human might not give the reliable result. Second, the chondrogenic differentiation occurs at the low level of serum and is more efficient in hypoxic conditions, whereas the normal culturing environment (10% FCS, 21% oxygen) might influence the chondrogenic properties of the cells.

Nevertheless, the high level of reproducibility of osteogenic and adipogenic potentials indicates homogeneity of the clones and proves their single-cell origin. We conclude that the isolated populations represent conditionally immortalized progenitors/stem cells with defined potential. Since the subclones often showed high efficiency of differentiation (when it is positive) and might be considered as more “pure” populations with higher confidence of differentiation properties, we decided to use populations of the subclones for the further experiments. The final panel of the subclones with defined properties is represented on Fig. 23B.

A

Differentiation potential of the parental clone	Differentiation assay for the subclones	Result of the differentiation assay		Total number	Reproducibility
		+	-		
OAC	O	18	4	22	81,8%
	A	22	0		100,0%
	C	15	7		68,2%
OC	O	12	0	12	100,0%
	A	2	10		83,3%
	C	10	2		83,3%
AC	O	1	7	8	87,5%
	A	8	0		100,0%
	C	6	2		75,0%
O	O	9	0	9	100,0%
	A	2	7		77,8%
	C	2	7		77,8%
Total	O			51	90,2%
	A				92,2%
	C				74,5%

B

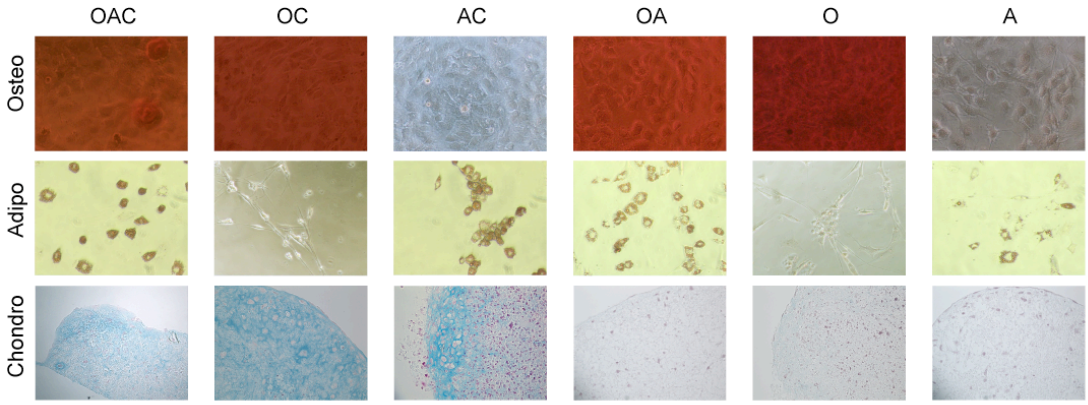


Figure 23. Differentiation of the subclones of BM MSCs into three lineages. Clonally derived lines of BM MSCs were subjected to the second round of cloning and the resultant subclones were analysed for osteo- and adipogenic potential as described in the previous experiment. Chondrogenic differentiation was performed with a subset of the subclones and checked with Alcian blue staining of histological sections. (A) The table summarizes the results of differentiation into 3 lineages (O, A, C). The numbers of subclones, which were efficient (”+”) or inefficient (”-”) for the given pathway are shown. In red color - the number of subclones, which have the same potential as the parental clone (”+” or “-”). Reproducibility - % of those subclones from the total number (see also explanation in the text). Chondrogenic lineage was found to be less reproducible compared to osteo- and adipogenic (74.5% to 90.2% and 92.2%, respectively). Nevertheless, the majority of the subclones retain the potential of initial clone, indicating homegeneity of the established populations. (B) Differentiation potential of the resultant subclones.

3.4.4. Expression of the surface markers in different subpopulations of BM MSCs progenitors

We established conditionally immortalized single-cell derived subpopulations of BM MSCs representing different types of progenitors/stem cells. These subpopulations were identified by the screening of differentiation potential of the large number of clones. Direct isolation or sorting of particular progenitor types cannot be achieved because the specific markers for BM MSCs and/or their subgroups are not known. The subclones of conditionally immortalized BM MSCs were tested for the expression of the surface markers for characterization and comparison.

Flow cytometry analysis for 12 markers was performed with 6 different subclones of conditionally immortalized BM MSCs. The differentiation properties of the subclones were: “OAC” (clone 8C-12A), “OA” (clone 8C-2B), “AC” (clone 1C-1A), “OC” (clone 5A-12A), “O” (2B-3A) and “A” (clone 1C-3A). The subclones showed similar pattern of the surface markers expression between each other, and not different from the initial stromal line of BM MSCs from which they were derived (Table 2). This result confirms that most of the known markers are homogeneously expressed in BM MSCs despite the presence of different subsets of the cells and cannot be used to enrich the population for individual cell types. The specific markers of bone marrow progenitors need to be further investigated.

	line	OAC	OA	OC	O	AC	A
	1565	8C-12A	8C-2B	5A-12A	2B-3A	1C-1A	1C-3A
CD9	100,0	100,0	98,9	100,0	100,0	99,9	100,0
CD34	0,0	0,0	0,1	0,0	0,0	0,1	0,0
CD44	98,7	99,8	99,9	100,0	100,0	100,0	100,0
CD45	0,0	0,1	0,1	0,0	0,0	0,0	0,0
CD49e	97,3	96,8	90,4	99,9	99,9	98,3	94,2
CD117	0,0	0,2	0,1	0,0	0,1	0,1	0,0
CD140b	97,4	97,2	96.9	61,2	78,1	74,0	82,9
CD146	0,0	0,2	0,1	0,0	0,2	0,0	0,0
CD184	0,0	0,2	0,0	0,1	0,2	0,1	0,1
CD271	0,0	0,1	0,1	0,1	0,3	0,0	0,0
Sca-1	99,3	99,5	98,3	98,3	86,7	99,7	100,0
Flk-1	0,1	1,6	0,5	0,5	0,1	0,6	0,2

Table 2. Expression of the surface markers by subpopulations of BM MSCs established from 2 rounds of cloning and the initial stromal line 1556. The differentiation potential is shown in the first row, positive markers are outlined in red. The subclones did not show the difference in the expression of surface markers between each other and from the initial stromal line.

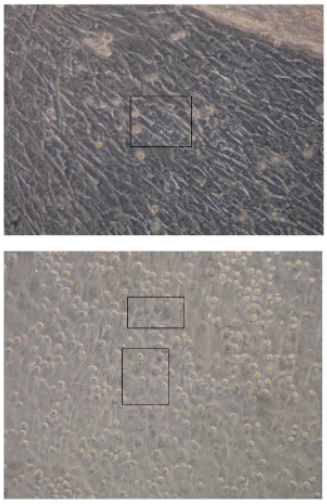
3.4.5. Different subpopulations of BM MSC progenitors support HSCs *in vitro*

Bone marrow represents a complex system consisting of closely interacting haematopoietic cells and supportive stroma (Dexter 1982; Krebsbach, Kuznetsov et al. 1999; Gregory, Prockop et al. 2005). One of the main functions of bone marrow is haematopoiesis whereby HSCs provide the source for all blood cell lineages and MSCs supply the signals for regulation and maintenance of haematopoiesis. Currently it is not exactly known which components of stroma support haematopoiesis. Different models of the niche of HSCs have been proposed, consisting of vascular cells or osteoblasts (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003; Arai, Hirao et al. 2004; Avecilla, Hattori et al. 2004; Kiel, Yilmaz et al. 2005). Also the role of bone marrow adipocytes in the regulation of haematopoietic differentiation was discussed in the literature (Naveiras, Nardi et al. 2009). Hense, it is not excluded that different cell types within bone marrow stroma regulate different processes of haematopoiesis providing homeostasis of the blood system.

CAFC assay (described in the section 2.2) was done with the clonally established lines of different progenitors from the bone marrow (Fig. 24). The experiment was performed in collaboration with Manja Wobus, Prof. Bornhauser group, Uniklinikum, Dresden. The differentiation properties of the progenitors were “OAC”, “OA”, “OC”, “AC”, “O”, “A” (“O” - osteogenic, “A” - adipogenic, “C” - chondrogenic). The initial stromal line was also included in the experiment. Human CD34-positive HSCs isolated from mobilized peripheral blood were seeded onto the confluent layer of BM MSCs and cobblestone areas were counted after 8 days, as summarized in the table. The number of CAFC’s was relatively low in all the cases, which might be explained by the differences in the inter-species interactions of mouse stromal cells and human HSCs. Nevertheless, many clones supported HSCs *in vitro* (“OAC”, “OA”, “AC”, “A”), with slightly higher number for multipotent clones. Surprisingly, only the clones retaining the adipogenic potential supported CAFC formation, and clones “OC” and “O” did not show any cobblestone formation, although their ability to support CAFC might not be completely excluded because of the relatively low statistics in this experiment. Another possibility might be that osteoblastic components have different function in haematopoiesis regulation, and promote quiescent state of HSCs rather than proliferation, or lineage differentiation rather than maintenance.

Taken together, different components of bone marrow stroma can support HSCs *in vitro* and their proliferation. The differences in the progenitor functions can be further investigated.

A



B

Clone	Potential	CAFC/well
8C-12A	OAC	4
8C-2B	OA	3
5A-12A	OC	0
1C-1A	AC	3
2B-3A	O	0
1C-3A	A	1
1556	(initial line)	3

Figure 24. CAFC assay of single-cell derived subpopulations of BM MSCs. The cells were derived after 2 rounds of cellular cloning of conditionally immortalized stromal line 1556. The clones with different potential for differentiation were seeded in 24-well plates and T-antigen was deinduced for 3 days. $CD34^+$ human HSC were plated on the confluent layer of MSCs. CAFCs were counted after 8 days. (A) Morphology of CAFC (co-culture with clone 1C-3A). (B) Number of CAFC per well (average from two independent experiments) formed on the layer of clones with indicated potential. In collaboration with Manja Wobus (Prof. Bornhaeuser group, Uniklinikum, Dresden)

3.5. Regulation of differentiation of mesenchymal stem/progenitor cells by GSK3 and Wnt/β-catenin pathway

Our studies confirmed the heterogeneity of progenitors in the bone marrow. The regulatory pathways of differentiation of stromal cells have been extensively studied, however little is known about the mechanisms of lineage priming or cell fate choice. Essentially canonical Wnt signaling is involved in the development of skeletal tissues and possibly has a role in the molecular switch between osteo/adipogenesis (Davis and Zur Nieden 2008; Ling, Nurcombe et al. 2009; Muruganandan, Roman et al. 2009). Therefore it was particularly interesting to explore and compare the function of Wnt/β-catenin pathway and its central regulator GSK3 in the progenitors with distinct differentiation potential.

3.5.1. Functional inhibition of GSK3 in BM MSCs

GSK3 is the central regulator of canonical Wnt signaling and at the same time it is involved in the number of different cellular regulation pathways (Nelson and Nusse 2004). The studies of GSK3 function often include the application of inhibitors, which might exhibit different activity, specificity and toxicity for the cells (Wagman, Johnson et al. 2004). One of the most often applied GSK3 inhibitors is lithium chloride (LiCl). However lithium is not highly potent or selective since it might inhibit other kinases such as casein kinase 2 (CK2) and MAPK-activated protein kinase 2. Pharmacological agent BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime) is another synthetic GSK3 inhibitor, but might be toxic for the cells from certain concentrations. Relatively recently another small molecule, Chiron inhibitor (CT99021), started being widely used because of its high selectivity to GSK3. We applied these 3 inhibitors to the cells in the further experiments in order to specify the effect to GSK3 function.

To determine the optimal conditions for GSK3 inhibition and activation of Wnt downstream genes we performed luciferase reporter assay using pGL3 BAR construct containing Firefly Luciferase reporter gene under control of twelve TCF/LEF tandem elements (Biechele and Moon 2008), Fig. 25A. Conditionally immortalized BM MSCs and 293 control cells were seeded in 96-well plates and co-lipofected with pGL3 BAR (30ng/well) and Renilla luciferase expression construct as a control of transfection efficiency (5ng/well). The visualized control of transfection was CAGGs-GFP expression plasmid lipofected in the separate well. At the moment of transfection different conditions were applied to the cells to inhibit GSK3: LiCl (0, 1, 5, 10, 20, 30mM), BIO (0, 1, 3, 5, 7, 10μM) and Chiron inhibitor, CT99021 (0, 0.5, 1, 3, 5, 10μM). Firefly and Renilla luciferase activity were measured in the cell lysates prepared after 48 hours of transfection and GSK3 inhibition. The activity of TCF/LEF elements was calculated as Firefly activity normalized to transfection efficiency represented by activity of Renilla (Fig. 25C).

All tested GSK3 inhibitors activated TCF/LEF elements in 293 and BM MSCs in concentration-dependent manner. The maximum response of BM MSCs to LiCl was detected at the concentration of 10mM, further increase of the concentration caused the drop of the activity correspondent to the observed toxicity to the cells. BIO showed maximum of activation at 5μM, higher concentrations were found to be very toxic for the cells. Chiron inhibitor was the less toxic for the cells and caused the highest response compared to the other GSK3 inhibitors in the tested range of concentrations.

Hence, downstream Wnt/β-catenin targets can be induced by application of GSK3 inhibitors in BM MSCs, and the optimal concentrations were determined as 10mM for LiCl, 5μM for BIO and 10μM for Chiron inhibitor.

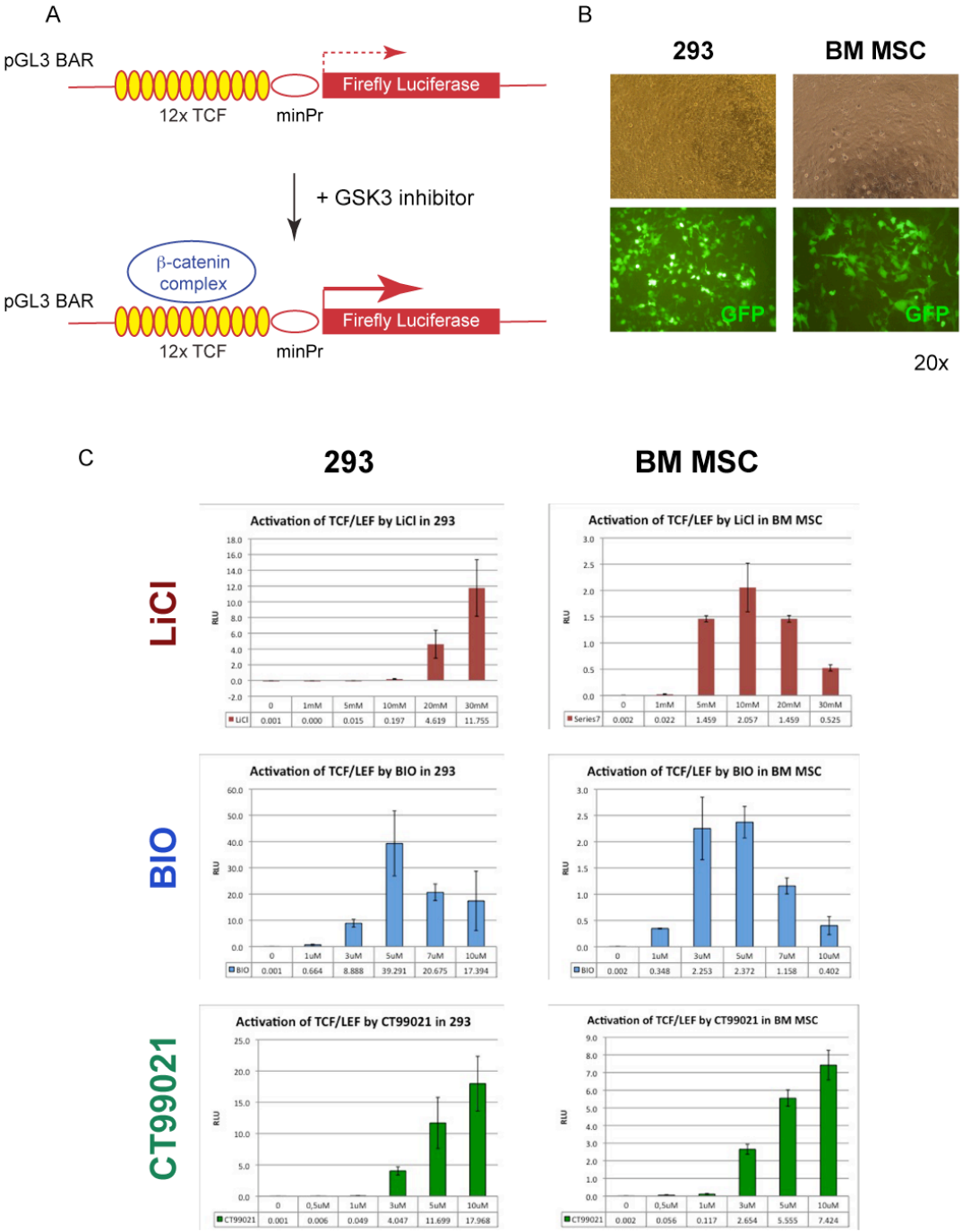


Figure 25. Activation of TCF/LEF reporters by GSK3 inhibitors. (A) Conditionally immortalized BM MSCs and 293 control cells were transfected with luciferase reporter construct pGL3 BAR. minPr - minimal promoter. (B) Transfection efficiency was controlled by lipofection of GFP-expression construct. (C) GSK3 inhibitors (LiCl, BIO, CT99021) were applied at the moment of transfection, the range of concentrations is indicated. 48 hours after transfection luciferase activity was measured. Activation of TCF/LEF promoters was calculated as reporter activity normalized to the transfection efficiency (RLU). The experiment was done in triplicate and standard deviations are indicated.

3.5.2. Activation of Wnt/β-catenin target promoters by inhibition of GSK-3 in conditionally immortalized BM MSCs

Different lines of conditionally immortalized BM MSCs were tested for the ability to activate Wnt downstream targets. BM MSCs used for the experiment were: line 4190 irtTA-TAg (Dox induction), 6472 line irtTA-GBD*-TAg (Dex/Dox induction), and 1556 line irtTA-GBD*-TAg (Dex/Dox induction) on the earlier passage (p11) and on the later passage (p23). The assay was performed as described before, using the concentrations of inhibitors determined in the previous section.

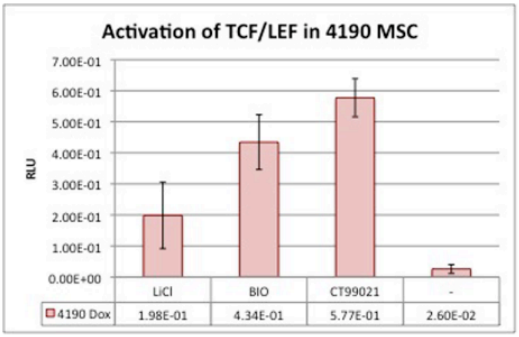
All of the checked BM MSC lines were able to activate Wnt/β-catenin downstream targets upon inhibition of GSK3 (Fig. 26A). The cells on the different passages did not exhibit significant difference in the response to inhibitors. Notably, the basal activity of TCF/LEF binding sites in the cells was virtually absent.

3.5.3. Activation of Wnt/β-catenin target promoters by inhibition of GSK-3 in conditionally immortalized clonal progenitors/stem cells from BM MSCs

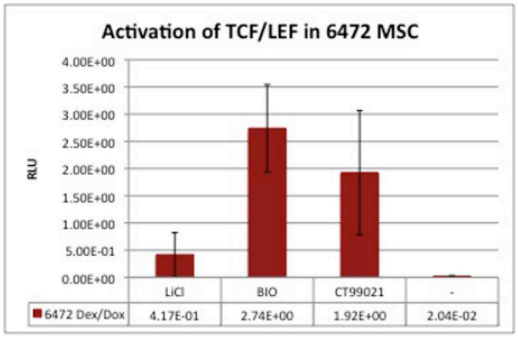
The subpopulations of progenitors/stem cells from BM MSCs were established from single cells and purified by two rounds of cellular cloning. As was described before, by screening of differentiation *in vitro* into osteo-, adipo-, chondrocytes we identified populations capable for “OAC”, “OA”, “AC”, “OC”, “O” and “A” lineages. Since Wnt/β-catenin pathway is involved in the regulation of differentiation of BM MSCs and often suggested as the mechanism for the lineage switches, it was particularly interesting to compare the clones for the ability to activate TCF/LEF promoter elements.

The experiment was performed with one clone of each differentiation property and the initial stromal line of BM MSCs (1556, irtTA-GBD*-TAg). The reporter assay was performed as described before, and GSK3 was inhibited by LiCl. The activity of TCF/LEF binding sites was represented as the reporter activity (Firefly luciferase) normalized to the transfection efficiency (Renilla luciferase), Fig. 26B. All the populations of progenitors/stem cells showed ability to activate Wnt targets, with slight variation in the efficiency. Nevertheless, there was no significant difference in the rate of response to LiCl, neither correlation with the differentiation properties of the cells. Similar result was obtained in the independent experiment using CT99021 (result not shown). Also all the clones did not exhibit the basal activity of Wnt/β-catenin pathway.

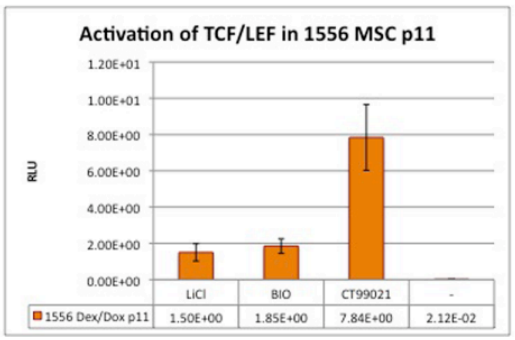
A



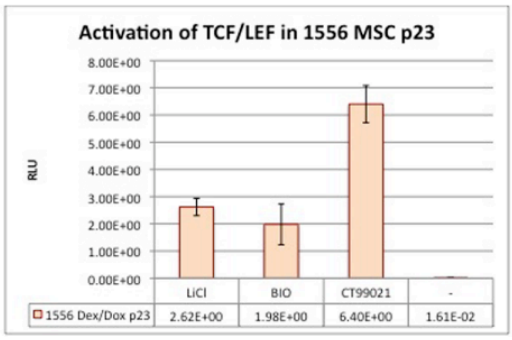
irtTA-TAg, Dox



irtTA-GBD*-TAg, Dex/Dox



irtTA-GBD*-TAg, Dex/Dox



irtTA-GBD*-TAg, Dex/Dox

B

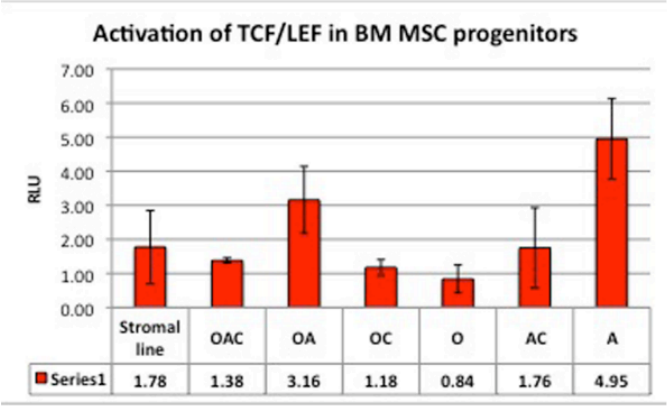


Figure 26. Activation of Wnt/β-catenin target promoter by GSK3 inhibitors in conditionally immortalized BM MSCs. (A) Conditionally immortalized stromal lines from irtTA-TAg (4190, Dox induction), irtTA-GBD*-TAg (6472 and 1556, Dex/Dox induction) mice were tested with TCF/LEF reporter assay using LiCl, BIO and CT99021 inhibitors of GSK3. All of the cell lines showed inducible activity of downstream Wnt promoters. This property of the cells was not changed after passaging of the cells (compared p11 and p23 of 1556 cells). (B) Clonally established progenitors and the initial stromal line were tested for activation of TCF/LEF binding sites using LiCl treatment. The clones with "OAC", "OA", "OC", "O", "AC" and "A" properties did not show significant difference in the ability to activate downstream Wnt genes. The experiments were done in triplicate and standard deviations are indicated.

3.5.4. Role of GSK3 during adipogenic differentiation of BM MSCs

The negative regulation of adipogenesis by Wnt/β-catenin signaling is extensively explored using different models, such as 3T3-L1 adipogenic fibroblasts or mice (Ross, Hemati et al. 2000; Bennett, Ross et al. 2002). We tried to investigate the role of canonical Wnt and GSK3 in BM MSCs and different types of progenitors from bone marrow during adipogenic differentiation using GSK3 inhibitors. We considered BM MSCs as more biological relevant model than fibroblasts to study adipogenesis. Furthermore, cultured cells allow attributing the effects to the particular cellular processes and states, which is more difficult to conclude using mice.

An important note is that it is not possible to distinguish different functions of GSK3 by application of the inhibitors, and we cannot assign the observations solely to the activation of Wnt/β-catenin pathway. We could not apply Wnt3a conditioned medium in our experiments because it caused significant cell death. The role of GSK3 in the other pathways during adipogenesis has to be considered.

3.5.4.1. Block of adipogenesis by GSK3 inhibitors

Adipogenic differentiation was performed in conditionally immortalized stromal line from irtTA-GBD*-TAg mouse (Dex/Dox induction). Inhibitor of GSK3 LiCl was applied during differentiation (Fig. 27A). The cells without GSK3 inhibition formed efficiently adipocytes, in contrast LiCl blocked completely differentiation even during prolonged adipogenic stimulation (up to 21 days). However, adipocytes appeared in the differentiated culture after withdrawal of LiCl showing that the block of differentiation was reversible.

To determine the role of GSK3 in different types of progenitors the inhibitors LiCl, BIO and CT99021 were applied during adipogenic differentiation. The clonal lines capable to adipogenesis were used in the experiment (“OAC”, “OA”, “AC” and “A”). After 10 days adipocytes were readily formed in the differentiation conditions. Presence of any of GSK3 inhibitors completely blocked the differentiation (Fig. 27B). This shows that the effect was attributed to GSK3 function. Hence, active GSK3 is needed for the adipogenic induction in the progenitors with different potential.

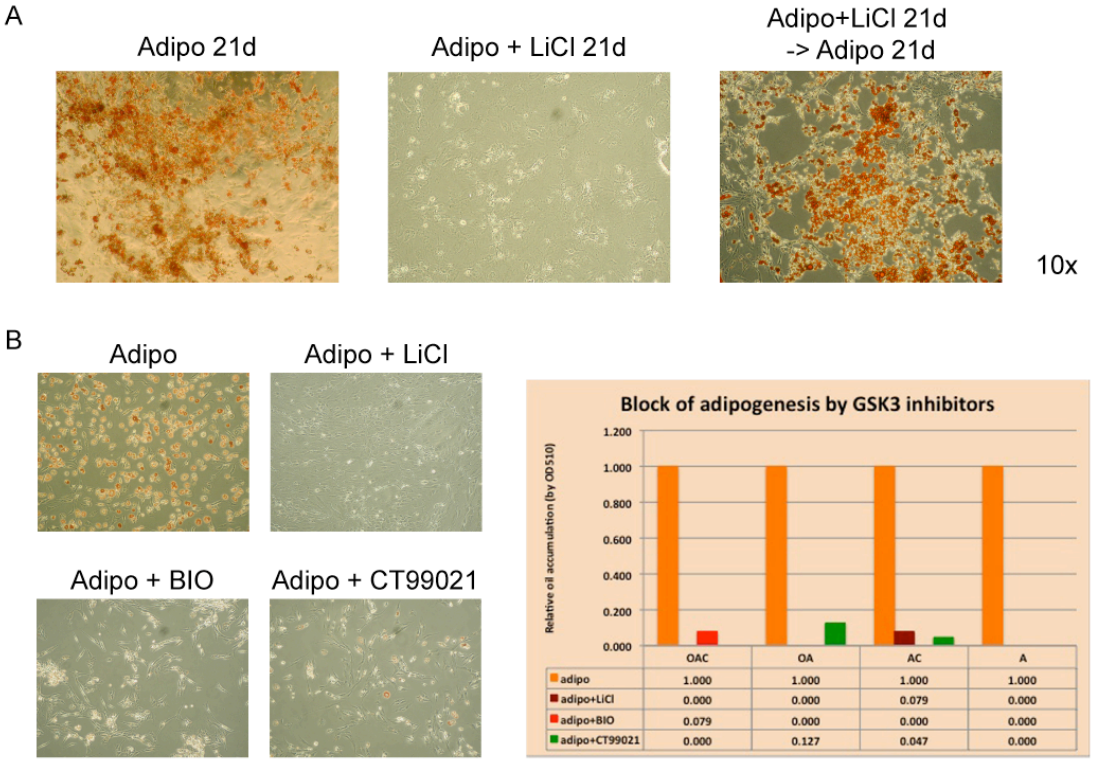


Figure 27. Adipogenesis is blocked by GSK3 inhibitors. (A) Conditionally immortalized bone marrow stromal line was differentiated into adipocytes. In the presence of LiCl adipogenesis was completely blocked. After withdrawal of LiCl the cells could be differentiated to adipocytes with similar efficiency as before LiCl treatment. Oil red staining, 10x. (B) The clonally established lines of adipogenic progenitor cells (types “OAC”, “OA”, “AC”, “A”) were differentiated into adipocytes. In all of the clones adipocyte formation was blocked when inhibitors of GSK3 (LiCl, BIO, CT99021) were applied to the cells as monitored by Oil Red staining (10x). Representative example for one of the clones is shown. Quantification was done by extraction of Oil Red and measurement at OD510. The oil accumulation relatively to the cells differentiated without inhibitors is shown on the graph.

3.5.4.2. Requirement of GSK3 activity during adipogenic differentiation

The differentiation *in vitro* proceeds through the steps of the lineage commitment of the cells and terminal differentiation, when the cells irreversibly acquire the specialized phenotype. To determine the stage of adipogenic differentiation when GSK3 is essential, the time course experiment was performed (Fig. 28). The single-cell derived lines of progenitors with potential for adipogenesis were used and the properties of the cells were “OAC”, “OA”, “AC” and “A”. Adipogenic differentiation was induced for 10 days. The cells were fixed and stained with Oil Red on the days 0, 3, 5, 7 and 10 to observe the dynamics of differentiation. The efficiency of differentiation was estimated by the optical density of the dye extracted from the stained samples. The inhibitors of GSK3 LiCl, BIO and CT99021 were applied on the different days of stimulation: day 0, 3, 5 or 7. On the day 10 of the experiment the cells were fixed and stained with Oil Red (Fig. 28A).

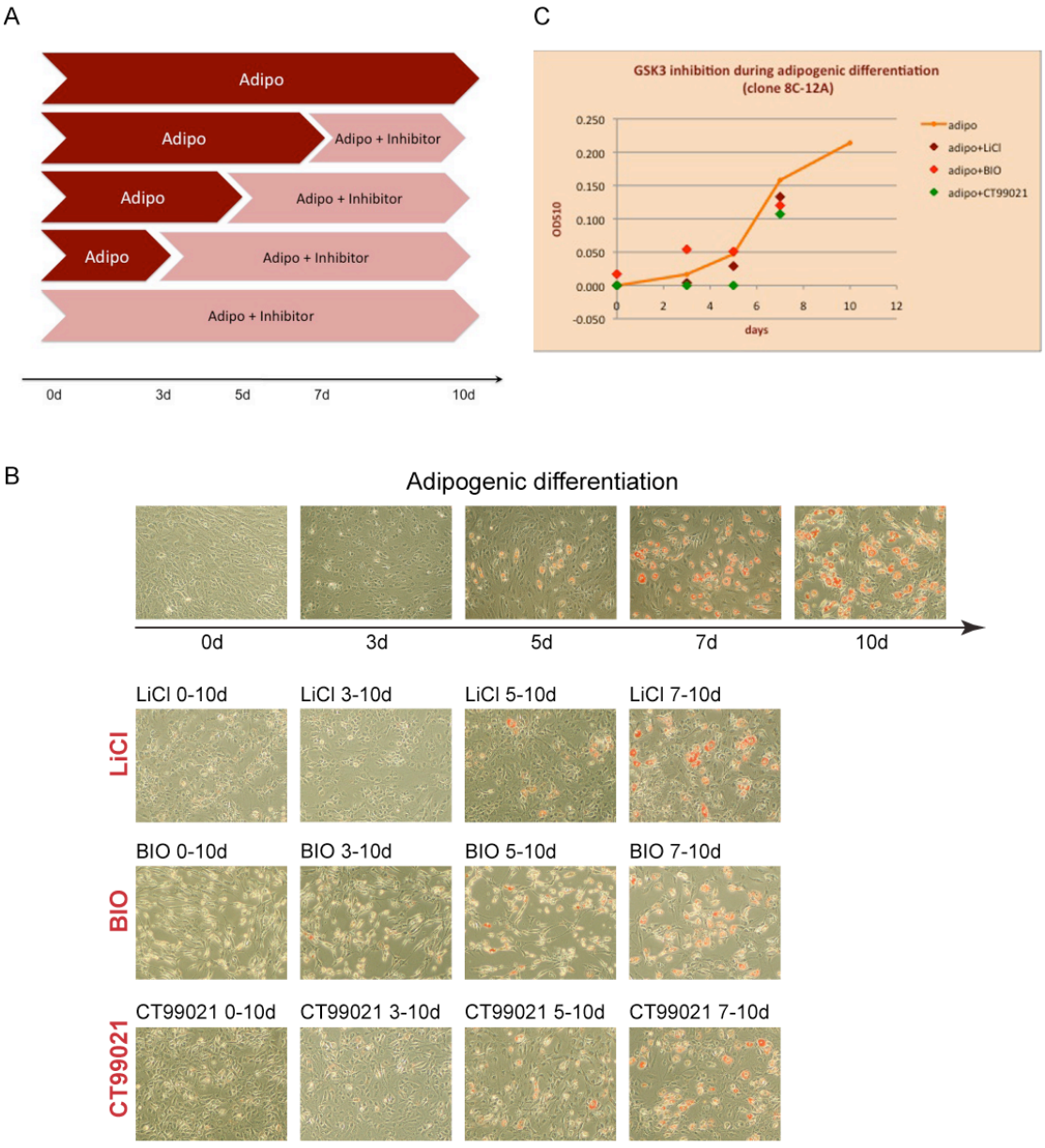


Figure 28. Requirement of GSK3 during adipogenic differentiation. (A) Adipogenic differentiation was induced in the adipogenic clones (”OAC”, “OA”, “AC”, “A”) and monitored on days 0, 3, 5, 7 and 10 (endpoint) by imaging and quantification of oil accumulation. GSK3 inhibitors (LiCl, BIO, CT99021) were added on days 0, 3, 5, 7 and maintained till the endpoint. (B) On day 10 the cells were fixed and stained with Oil Red (20x). Upper row - progression of adipogenic differentiation from d0 to d10. Rows below - staining of the cells at the endpoint of differentiation where the inhibitors were added on different days as indicated. The number of adipocytes in the presence of inhibitors was correspondent to the day when they were added in the cells without inhibition. Hence, the presence of GSK3 inhibitors blocked differentiation at the stage when they were added. (C) Quantification of Oil Red extracted from the cells confirms the observation. Orange line - dynamics of oil accumulation during differentiation. Dots show the amount of oil on the day 10 in the presence of inhibitors, and the dots are placed to the day when the inhibitors were added. This graph illustrates that oil amount was not increasing anymore if the inhibitor is applied.

The cells in the adipogenic conditions were gradually proceeding differentiation, increasing both the number of adipocytes and the size of oil drops till they reached terminal phenotype, as monitored by fat accumulation. In the presence of inhibitors differentiation was completely blocked. Inhibition of GSK3 at the intermediate stages caused stop of differentiation at the state when inhibitors were applied, and the results were reproducible for three reagents (LiCl, BIO, CT99021), Fig. 28B. Measurement of accumulated oil confirmed, that differentiation did not progress anymore as soon as the inhibitor is added (Fig. 28C). Some variations in quantification of the efficiency can be explained by toxicity of the chemicals to the cells because the cell death was detected, but it did not change the main conclusions. Similar results were observed for all the clones included in the experiment.

These results show that active GSK3 is needed for adipogenesis on every stage. The inhibition of GSK3 does not cause the adipocyte death, but prevents further progression in the differentiation.

Another conclusion can be drawn that Wnt/β-catenin pathway and/or GSK3 are rather implicated in negative regulation of adipogenic differentiation than maintaining of pre-adipocytes in the undifferentiated state, otherwise differentiation would proceed in the presence of GSK3 inhibitors once induced. This is in concordance with the previous results that TCF/LEF target sites are inactive in BM MSCs without exogenous stimulation.

3.5.5. Role of GSK3 during osteogenic differentiation of BM MSCs

Studies of mutant mice and phenotypes of human patients indicate that Wnt/β-catenin signaling is critically involved in bone homeostasis, possibly through the regulation of BM MSC differentiation (Krishnan, Bryant et al. 2006; Baksh, Boland et al. 2007; Liu, Vijayakumar et al. 2009). The implication of canonical and non-canonical Wnt's in binary switch between osteo/adipogenic pathways is often discussed. However, the data from mice and *in vitro* cultured cells from different sources are not always in concordance. We addressed a question about the role of GSK3 and Wnt/β-catenin signaling in osteogenic differentiation of bone marrow progenitors/stem cells using conditionally immortalized single-cell derived lines.

Osteogenic differentiation was performed in clonally established BM MSCs progenitors with or without GSK3 inhibitors. We observed large proliferation defect in the cells critically influencing the efficiency of osteogenic differentiation. The effect was especially prominent during the first days of differentiation correspondent to the

stage of progenitor expansion. To avoid this problem we applied inhibitors at the later stages of differentiation. Nevertheless, it was not always possible to achieve similar cell concentration for all of the lines included in the experiment and the conclusions were not always drawn from the whole panel of the cell lines.

The osteogenic differentiation was induced in the clones with osteogenic properties ("OAC" and "OC"). GSK3 inhibitor LiCl was added after 5 and 10 days of differentiation. After 28 days of differentiation the cells were fixed and stained with Alizarin Red, and the efficiency was quantified by measurement of optical density of the extracted dye. We observed that inhibition of GSK3 with LiCl during differentiation of osteogenic clones resulted in the increase of mineralization, 3.4-fold when added on day 10 and 9.3-fold when added on day 5 (Fig. 29A).

To confirm that the effect was due to GSK3 function, different inhibitors (LiCl, BIO, CT99021) were applied to the clone "OAC" after 22 days of osteogenic differentiation. On the day 27 the cells were checked for the efficiency of differentiation. All three reagents, LiCl, BIO and CT99021 resulted in the increase of mineralization 2.2 - 3.7 fold, indicating that indeed inhibition of GSK3 caused enhancement of osteogenic differentiation (Fig. 29B).

Following those results we checked the potential for osteogenesis of "AC" and "A" clones (non-osteogenic in the regular conditions) upon GSK3 inhibition. We applied GSK3 inhibitors (LiCl, BIO or CT99021) from the day of induction of osteogenic differentiation, and observed that inhibition of GSK3 did not change the lineage commitment (Fig. 29C).

Taken together, our results indicate that active GSK3 is not required for osteogenic differentiation of bone marrow progenitors, at least *in vitro*. Activation of canonical Wnt resulted in the enhancement of osteogenic differentiation. At the same time, the role of canonical Wnt might be stage-specific, since we noticed different effects when GSK3 inhibitors were applied from the start or during the course of differentiation. Finally, we haven't observed that Wnt/β-catenin signaling can cause the switch of the lineage fate of non-osteogenic cells.

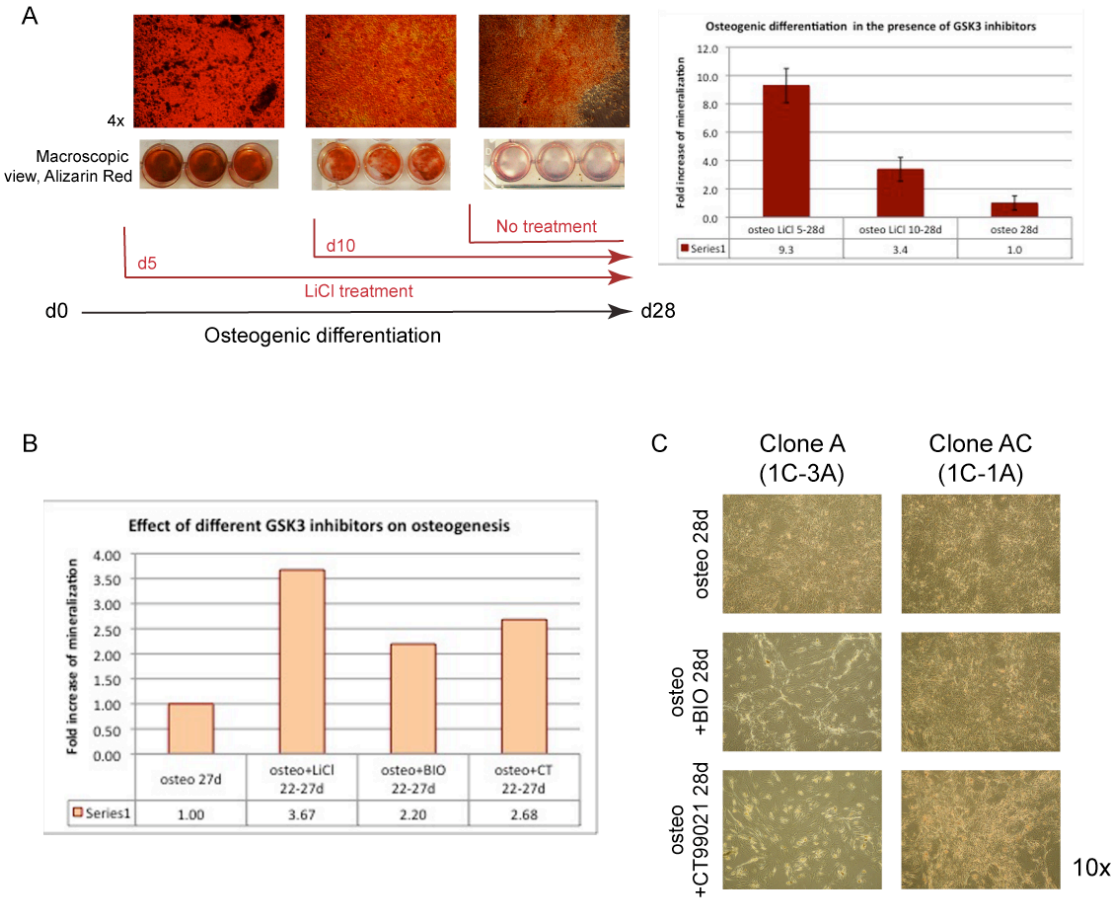


Figure 29. Role of GSK3 during osteogenic differentiation of BM MSCs. (A) Osteogenic differentiation was induced in osteogenic clones and LiCl was added after 5 or 10 days. The cells were fixed after 28 days, stained with Alizarin Red and the rate of mineralization was quantified. Inhibition of GSK3 caused significant increase of the efficiency of osteodifferentiation (3.4 and 9.3-fold, result for "OC" clone). (B) GSK3 inhibitors were added after 22 days of osteogenic differentiation of "OAC" clone. All applied inhibitors, LiCl, BIO and CT99021, caused increase in mineralization assayed on day 27. (C) GSK3 inhibitors (LiCl, BIO, CT99021) were applied during the course of osteogenic induction of the clones without osteogenic potential, from d0 ("A" and "AC"). The cells did not change the potential under these conditions. Alizarin Red staining, 10x.

3.5.6. Activity of Wnt/β-catenin target genes during the early induction of differentiation

In the previously described experiments we observed the effect of GSK3 inhibitors added exogenously during BM MSC differentiation *in vitro*. Inhibition of GSK3 stimulated osteogenesis and blocked adipogenesis. To determine whether this regulation pathway is utilized endogenously in the cell during differentiation and whether it is mediated by Wnt pathway, we monitored TCF/LEF activity in the cells during the differentiation.

The experiment was performed with the single-cell derived clones of progenitors ("OAC", "OA", "OC", "AC", "O", "A"), the initial stromal line 1556 (Dex/Dox induction), and 293 cells were included as a control. The cells were seeded in 96-

well plates, osteogenic and adipogenic differentiations were induced. After 2 days the cells were transfected with reporter construct for TCF/LEF promoter activity, as described before. Differentiation conditions were maintained. For positive control the cells were treated with CT99021. 2 days later the cells were lysed and luciferase activity was measured. In all of the cells under differentiation conditions there was no significant increase of the reporter over basal activity (numbers are not shown).

During this experiment the differentiation was induced for 4 days. At this stage we observe the appearance of adipocytes in the adipogenic cultures, and in the osteogenic conditions the cells might be already committed to the osteogenic lineage. The absence of Wnt activity during the first stage of differentiation leads to several conclusions. First of all, non-adipogenic clones ("OC" and "O") are prevented from adipocyte differentiation not through the activity of Wnt, despite Wnt was shown to be negative regulator of adipogenesis. The block happens through another mechanism. Second, at least on the early stage of osteogenic differentiation the cells ("OAC", "OA", "OC", "O") do not activate Wnt signals. Moreover, as was discussed before, adipogenic progenitors do not activate Wnt to keep undifferentiated. One possible explanation might be that GSK3 is indeed involved in those processes but through different signaling pathways. On the other hand, Wnt pathway might be responsive in those cells and utilized during development of the tissues in response to extracellular signals, but BM MSCs do not utilize Wnt signaling for regulation of differentiation by themselves.

3.6. Regulation of adipogenic commitment in BM MSC progenitors

The adipogenic differentiation is regulated by at least two families of transcription factors, C/EBP (CCAAT/enhancer-binding proteins) and PPAR (peroxisome proliferator-activated receptors). The important knowledge about the mechanism of adipogenic induction is rising from the studies of clones of NIH 3T3 fibroblasts (L1 and F422A) specifically selected by the ability to form adipocytes *in vitro* (Green and Meuth 1974). According to the currently accepted model C/EBPβ and C/EBPδ cooperate together to induce C/EBPα and PPARγ expression. In turn, C/EBPα and PPARγ sustain the expression of each other and control the entire terminal differentiation process, Fig. 2, reviewed by (Rosen, Walkey et al. 2000; Farmer 2006). Additionally, it was shown that Krox20 (Egr2, early growth response 2)

might be upstream of C/EBPβ and could be one of the earliest regulators of adipogenic commitment. Klf5 (Krueppel-like factor 5) was identified as a factor, which is induced by C/EBPβ, and then participates in PPARγ initiation in association with C/EBPβ;C/EBPδ (Chen, Torrens et al. 2005; Oishi, Manabe et al. 2005).

The aim of this chapter was to dissect the process of adipogenic commitment and differentiation of BM MSCs. First, we analysed the factors, which control adipogenic potential of the undifferentiated stromal cells by comparing their expression in non-adipogenic versus adipogenic progenitors. Second, we tried to identify the mechanism of adipogenic commitment of BM MSCs *in vitro*. Third, we attempted to find the factors, which are able to convert non-adipogenic progenitors to adipogenic lineage. The use of BM MSCs to study mechanisms of differentiation and lineage commitment can give a new insight to this field of knowledge.

3.6.1. Expression of adipogenic regulators during differentiation of BM MSCs into adipocytes

Conditionally immortalized BM MSCs were differentiated into adipocytes for 10 days. Expression of adipocyte markers and transcription factors involved in adipogenesis was analysed in undifferentiated cells and after 4, 7 and 10 days of induction by RT-PCR (Fig. 30A). Adipocyte differentiation was confirmed by the expression of adiponectin and adipsin from day 4 of induction. PPARγ1 was present at mRNA level from undifferentiated cells through the whole course of differentiation. Adipose tissue-specific PPARγ2 appeared at detectable level after 7-10 days of differentiation. C/EBPα mRNA was expressed at high level in all the samples. C/EBPβ, C/EBPδ and Klf5 were present as transcripts in undifferentiated cells, increasing their expression during differentiation. Krox20 was shown in undifferentiated cells and terminally differentiated adipocytes as well.

Expression of PPARγ, C/EBPα, C/EBPβ and C/EBPδ at the protein level was analysed by Western blot in undifferentiated cells and after 3 and 10 days of differentiation (Fig. 30B). PPARγ1 was detected at low level in the undifferentiated cells, and it was upregulated during the differentiation. We detected only PPARγ1 isoform, but not PPARγ2 due to the specificity of the antibody (Abcam, ab41928). C/EBPα was found at low level in BM MSCs and the expression highly increased during differentiation. Both isoforms, 42kDa and 30kDa, were detected. C/EBPβ

expression was shown in all the samples at similar level. We could detect the three known isoforms for C/EBPβ - 38kDa, 32kDa (liver-activatory protein, LAP) and 21kDa (liver-inhibitory protein, LIP), (Ramji and Foka 2002). Interestingly, undifferentiated cells expressed mostly the large isoform 38kDa, and during differentiation two other isoforms were detected. C/EBPδ was not expressed at a detectable level in undifferentiated cells (in some independent experiments we could detect a weak band), but its expression was highly increasing during the differentiation.

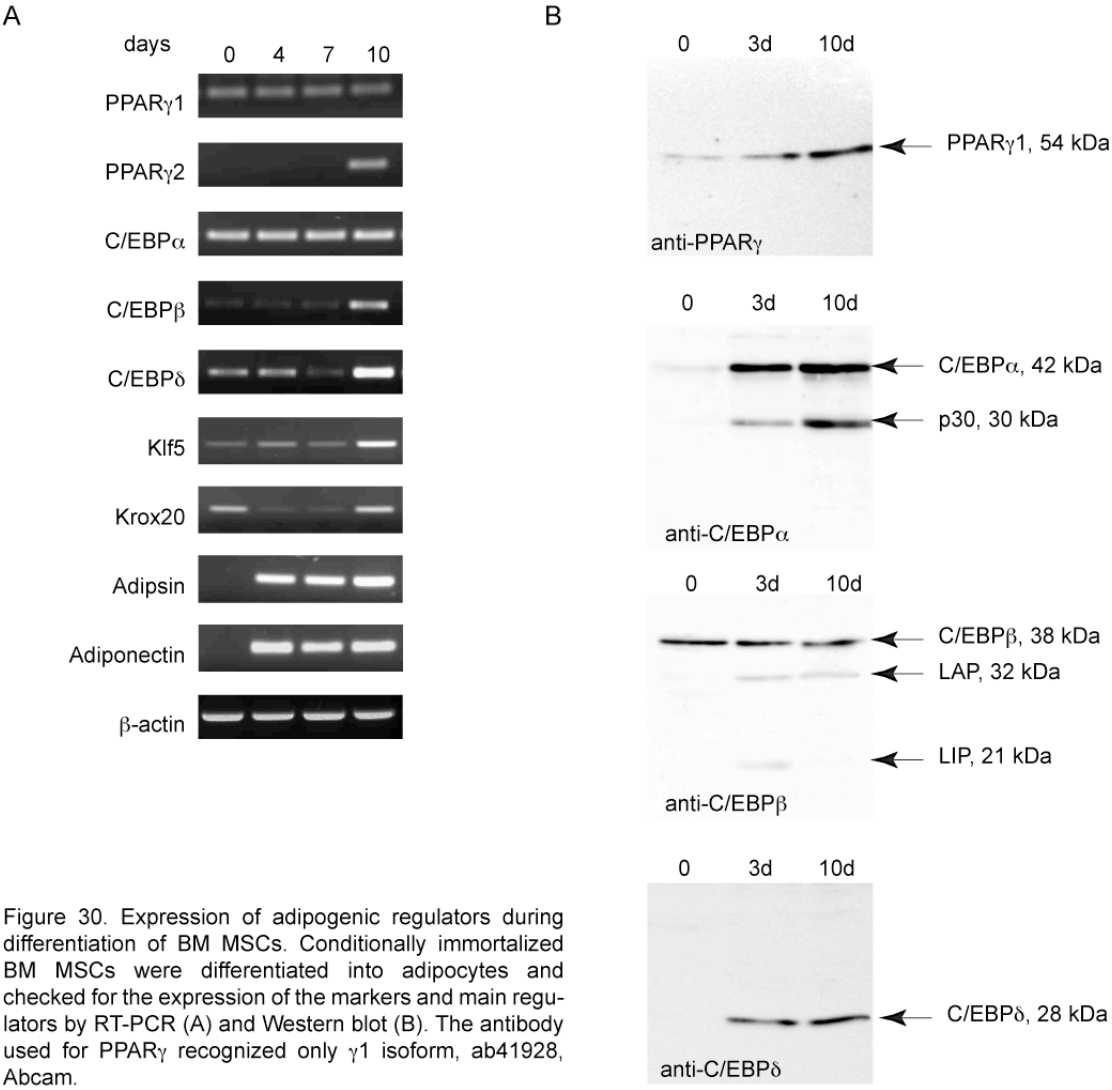


Figure 30. Expression of adipogenic regulators during differentiation of BM MSCs. Conditionally immortalized BM MSCs were differentiated into adipocytes and checked for the expression of the markers and main regulators by RT-PCR (A) and Western blot (B). The antibody used for PPARγ recognized only γ1 isoform, ab41928, Abcam.

We observed some discrepancy in PPARγ1 and C/EBPα expression at the transcript and at the protein levels. mRNAs were present at similar levels in undifferentiated and differentiated cells, whereas the proteins increased many fold during differentiation. This might be an indication of post-transcriptional regulation of PPARγ1 and C/EBPα expression.

3.6.2. Expression of adipogenic regulators in the single-cell derived progenitors from BM MSCs

Single-cell derived clones of BM MSC progenitors were obtained by two rounds of cellular cloning of stromal line from transgenic irtTA-GBD*-TAg mouse (Dex/Dox induction). The clones were tested for differentiation, as described before, and the populations with different potential were found.

In order to identify the factors important for adipogenic commitment of the cells, the clones were tested for the expression of the main adipogenic regulators (Fig. 31). Undifferentiated cells from the initial stromal line 1556 and progenitors with adipogenic potential ("OAC", "OA, "AC" and "A") and without adipogenic potential ("OC" and "O") were deinduced for 3 days by Dex and Dox withdrawal to eliminate the influence of Dex and T-antigen to the cells. Total RNA and proteins were isolated from the cells for the expression test. RT-PCR revealed the presence of transcripts of PPARγ1, C/EBPα, C/EBPβ, C/EBPδ, Klf5 and Krox 20 (Egr2) in all of the cells. PPARγ2 was absent in those conditions.

We performed Western blot to confirm the expression of the factors at the protein level (Fig. 31B). We observed differences from the result of RT-PCR analysis, and between the clones. PPARγ1 was found at the higher level in "AC" and "A" clones and at the lower level in "OAC", "OA" and the stromal line. At the same time we could not detect PPARγ1 in non-adipogenic clones ("OC" and "O") by Western with any of the two antibodies (anti-PPARγ1, γ2, E-8, Santa Cruz, and anti-PPARγ1, ab41928, Abcam). PPARγ2 was absent in all the clones. C/EBPα was expressed in "OAC", "OA", "AC" and "A" clones, and weaker in the stromal line. In non-adipogenic clones ("OC" and "O") C/EBPα was not detectable. We found the expression of C/EBPβ in all the clones at the comparable level. C/EBPδ was not detected by Western blot in this test, although we observed a weak expression in the undifferentiated cells in the independent experiments. In any case we haven't observed the difference in C/EBPδ level between the clones with different potential.

Our results revealed the differential expression of PPARγ1 and C/EBPα in undifferentiated BM MSC progenitors with adipogenic potential compared to non-adipogenic clones. The important role of those factors in the adipogenesis was known before (Tontonoz, Hu et al. 1994; Rosen, Sarraf et al. 1999; Wu, Rosen et al. 1999; Rosen, Hsu et al. 2002). Our observations might be an indication that those transcription factors are involved in the commitment of the cells towards the adipogenic lineage. Taken into account that "OAC" and "OA" clones expressed both factors, it is very possible that PPARγ1 and C/EBPα "direct" the cells into adipogenic fate and not "restrict" from the osteogenic. It is unlikely that C/EBPβ and C/EBPδ play a role in the adipogenic commitment of the cells, because they were expressed similarly in the cells with different properties.

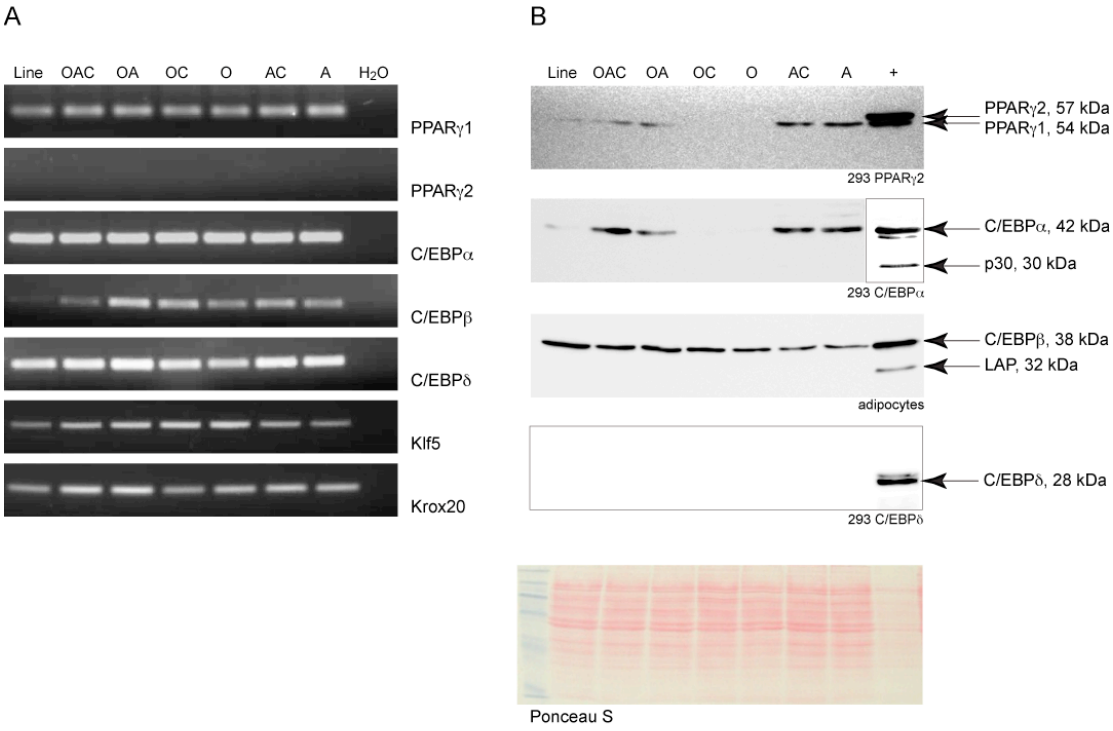


Figure 31. Expression of the main regulators of adipogenesis in clonally derived BM MSC progenitors with defined differentiation potential (O-osteogenic, A - adipogenic, C - chondrogenic). Undifferentiated cells were deinduced for 3 days (Dex/Dox withdrawal) and checked for the expression of indicated markers and regulators by RT-PCR (A) and Western blot (B). The positive controls for Western blot were 293 transfected with PPARγ2, C/EBPα, C/EBPδ expression constructs, or BM MSC-derived adipocytes. The antibody for PPARγ was E-8, Santa Cruz, recognizing γ1 and γ2 isoforms.

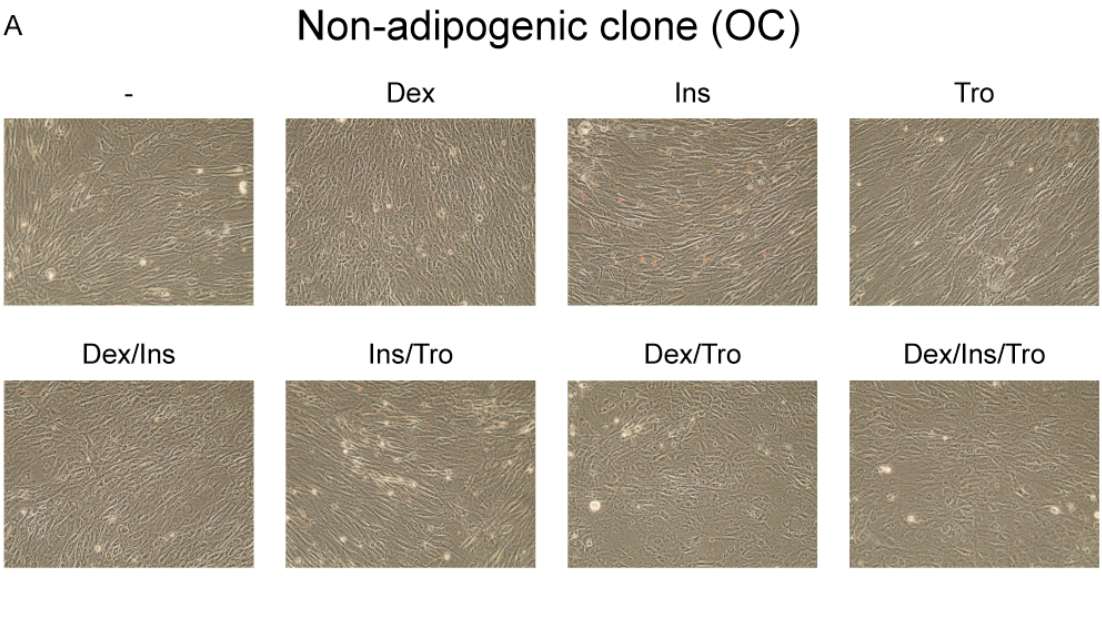
3.6.3. Commitment to adipogenic differentiation in the clonally derived BM MSC Progenitors

In order to find the factors critical for adipogenic commitment in BM MSCs we tried to dissect the mechanism of adipogenic stimulation *in vitro*. The known conditions for adipogenic differentiation of BM MSCs, 3T3-L1 fibroblasts and mES cells include (1) glucocorticoids, which have at least two known functions, to induce the expression of C/EBPδ and to activate the heterodimer complex C/EBPβ;C/EBPδ that stimulates PPARγ and C/EBPα expression; (2) PPARγ agonist troglitazone for its activation and (3) insulin to support the expression of adipogenesis-related genes, Fig. 2 (Tontonoz, Hu et al. 1994). We applied different combinations of dexamethasone (Dex), insulin (Ins) and troglitazone (Tro) to BM MSCs to find minimal conditions capable to induce adipogenic differentiation in the cells.

We used the cells of adipogenic clone ("AC" properties) and non-adipogenic clone ("OC" properties) for the experiment (Fig. 32). The medium containing the single effector (Dex, Ins, Tro), combination of two (Dex/Ins, Ins/Tro, Dex/Tro) or all three compounds (Dex/Ins/Tro) was applied for 10 days to the cells. Adipocyte formation was shown by Oil Red staining and amount of bound Oil Red was quantified spectrophotometrically at OD510. Adipocyte differentiation was observed only in the cells of adipogenic clone. None of the inductors could stimulate adipogenesis in the clone, which was identified as non-adipogenic before.

We found that Tro is necessary for the adipocyte formation in adipogenic clone. We observed very low number of adipocytes in the cells treated with Tro alone or in combination Ins/Tro, and much enchanced in Dex/Tro conditions. Complete adipogenic conditions (Dex/Ins/Tro) led to the most efficient differentiation. Taking into account that non-osteogenic clone did not express PPARγ, we assume that activation of PPARγ is necessary and sufficient to stimulate adipogenesis in BM MSCs.

A



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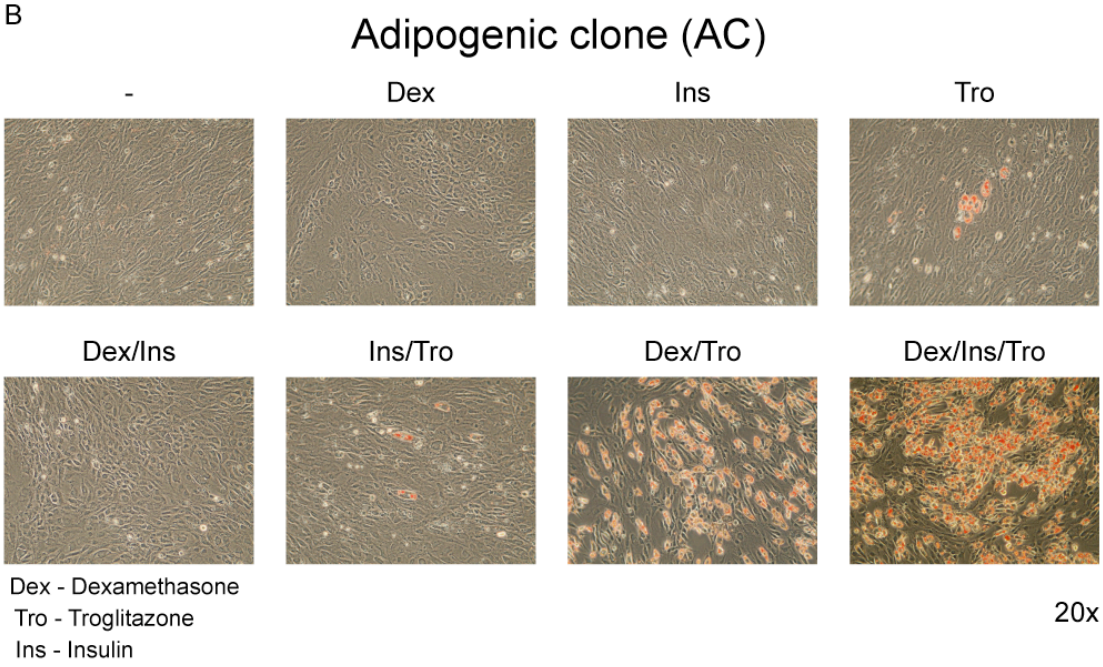

Dex - Dexamethasone
Tro - Troglitazone
Ins - Insulin

C

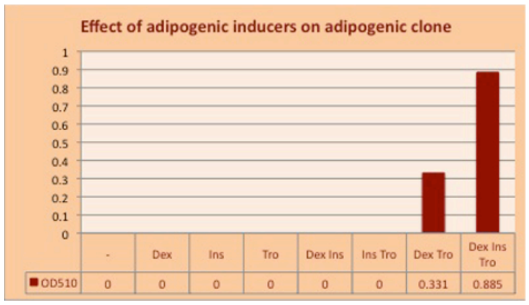

Figure 32. Non-adipogenic (A) and adipogenic (B) clones of BM MSCs were exposed to the different combinations of effectors. After 10 days the cells were fixed and stained with Oil Red. Adipocytes were formed only in the adipogenic clone, when troglitazone was included into the medium composition. Efficiency was estimated by Oil Red extraction and measurement at OD510 (C).

To determine the specific changes induced by Dex, Ins or Tro, we checked the expression of the main adipogenic markers and regulators by RT-PCR and Western blot in the cells cultured in different combinations of those compounds (Fig. 33A). In order to specify, which of the regulators could be triggering for the adipogenic programm, we compared the effects of different conditions on the cells between the clone capable for adipogenesis and non-adipogenic clone.

The expression of adiponectin and adipose-specific PPARγ2 mRNA was found consistently in the conditions where we observed adipocyte formation ("AC" clone - Tro, Ins/Tro, Dex/Tro and Dex/Ins/Tro). At the same time the expression of PPARγ1, C/EBPα and Krox20 was similar in the adipo- and non-adipogenic clones in all conditions, according to RT-PCR. RT-PCR analysis revealed the mRNA expression of C/EBPβ, C/EBPδ and Klf5 in all of the analysed cells, which increased in the conditions including Dex, especially pronounced for C/EBPβ and C/EBPδ.

To verify the protein content, we analysed the expression of PPARγ1 C/EBPα, C/EBPβ and C/EBPδ by Western blot (Fig. 33B). PPARγ1 was present at low level in the adipogenic clone, and increased upon adipocyte formation (slightly in Ins/Tro, and highly in Dex/Tro and Dex/Ins/Tro). Notably, the expression of PPARγ1 was markedly increased during the Dex/Ins treatment, despite no adipocyte formation was observed. At the same time PPARγ1 could not be induced in the non-adipogenic clone after any treatment.

C/EBPα was expressed in the undifferentiated adipogenic cells. The level of expression was increasing, if Tro is present in the medium, and hence PPARγ1 is active (conditions Tro, Ins/Tro, Dex/Tro, Dex/Ins/Tro). The highest content was observed, when adipocytes were efficiently formed (Dex/Tro, Dex/Ins/Tro). Therefore, in the adipogenic cells PPARγ1 upregulation by itself was not sufficient to enhance C/EBPα (conditions Dex/Ins), but the activation of PPARγ1 with Tro stimulated C/EBPα. The osteogenic clone did not express C/EBPα in the undifferentiated state. However, in the presence of Dex C/EBPα was detected at the low level (Dex, Dex/Tro, Dex/Ins/Tro, and very weakly Dex/Ins).

C/EBPβ was expressed in the adipogenic and non-adipogenic clones exposed to different conditions, although at lower level in the non-adipogenic cells. We detected an increase in C/EBPβ level after the Dex treatment.

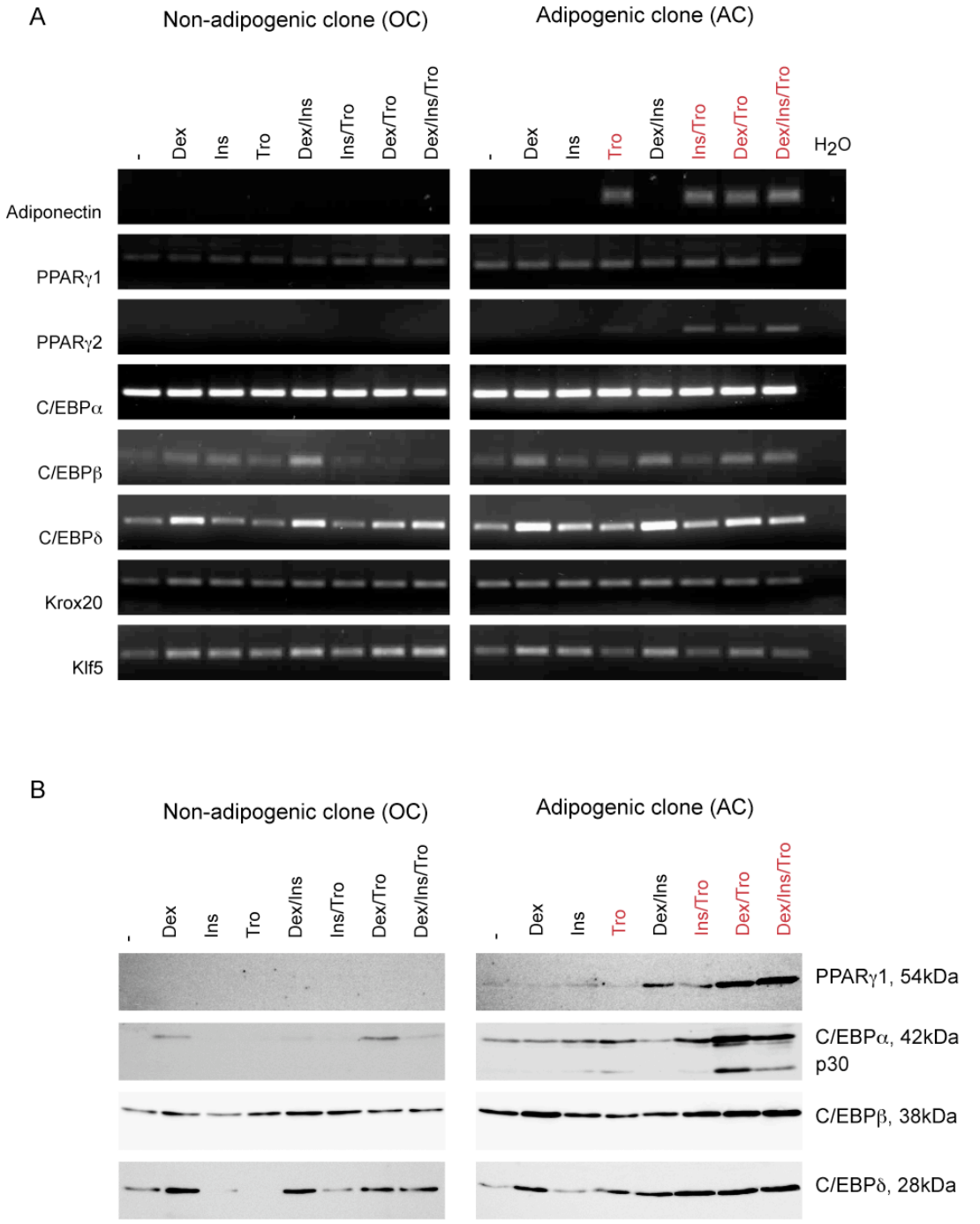

Figure 33. Non-adipogenic and adipogenic clones of BM MSCs were compared for the expression of adipogenic markers and regulators after the treatment with different combinations of inducers. (D) RT-PCR and (E) Western Blot. The conditions sufficient for the adipogenic induction are highlighted in red. The antibody used for PPARγ detection could recognize only γ1 isoform (ab41928, Abcam). PPARγ1 could be induced only in the adipogenic clone. C/EBPα was absent in the undifferentiated osteogenic clone, but appeared in the presence of Dex. The expression of C/EBPβ and C/EBPδ was responsive to the treatment in both types of progenitors in similar conditions.

C/EBPδ was weakly expressed in the untreated adipogenic and non-adipogenic clones. As expected, the content was highly increased after the Dex-containing conditions were applied in both types of progenitors consistent with the increase of the transcript (conditions Dex, Dex/Ins, Dex/Tro, Dex/Ins/Tro). Especially significant was the increase in the efficiently differentiated cells.

Notably, the level of C/EBPβ and C/EBPδ in the adipogenic clone was comparable after the treatment with single Dex (no adipocyte formation) and with Dex/Tro or Dex/Ins/Tro (efficient adipogenesis) indicating that C/EBPβ;C/EBPδ complex cannot trigger the cells into adipocyte conversion. The fact that the cells of non-adipogenic clone were able to upregulate C/EBPβ;C/EBPδ in response to Dex supports our conclusion that C/EBPβ;C/EBPδ does not commit the cells to become adipocytes.

Taken together, we observed that Dexamethasone upregulates C/EBPβ and C/EBPδ, and the complex C/EBPβ;C/EBPδ in the presence of Dex stimulates the expression of C/EBPα. These events can occur in both, adipogenic and non-adipogenic clones, although at lower level. Insulin alone does not influence the levels of expression of the main adipogenic regulators. But in the presence of Dex activating the C/EBPβ;C/EBPδ complex, insulin results in upregulation of PPARγ1 in the adipogenic cells. However, only further activation of PPARγ1 with Troglitazone can promote adipogenesis. Importantly, non-adipogenic clone failed to express PPARγ1 at the protein level in any conditions.

Summarizing our observations with the result of the previous experiment (6.2, Fig. 31), we don't see the evidence that C/EBPβ and C/EBPδ determine the adipogenic commitment of BM MSC progenitors. C/EBPα was expressed only in the undifferentiated adipogenic cells, and not in the non-adipogenic, but it was inducible in both types of progenitors, although at lower level in non-adipogenic clone. PPARγ1 expression was distinctive for the adipogenic clone, which explains the responsiveness of this clone to Troglitazone. We attribute the role of the main adipogenic determinant in BM MSCs to PPARγ, despite the level of C/EBPα might also have an influence.

3.6.4. Converting of osteogenic BM MSC progenitors into adipogenic lineage

The experiments on activation of adipogenesis in non-adipogenic cells by ectopical expression of transcription factors were performed by different groups before (Tontonoz, Hu et al. 1994; Wu, Xie et al. 1995). These studies had a large impact on elucidating the transcriptional network controlling adipogenesis. Nevertheless, the mechanism of the lineage commitment of BM MSC progenitors still has to be unraveled. We tried to overexpress the main factors regulating adipogenesis in the osteogenic cells and checked their ability to differentiate into adipocytes.

3.6.4.1. Validation of the expression constructs

The expression constructs were obtained from different sources as listed below: PPARγ1 and PPARγ2 from Addgene kindly provided by Bruce Spiegelman group, C/EBPβ, LIP and C/EBPδ from Addgene kindly provided by Peter Johnson group, and C/EBPα and Klf5 from the commercial source (Geneservice). C/EBPβ, LIP and C/EBPδ cDNAs were cloned in pcDNA3.1(-) plasmid backbone under CMV promoter and carried neomycin resistance gene. PPARγ1 and PPARγ2 were in pSV Sport plasmid backbone under control of the SV40 promoter, and C/EBPα and Klf5 were in pCMV Sport6 under control of the CMV promoter, all without eukaryotic resistance genes. In order to include a eukaryotic selection marker, the plasmids were modified by exchange of the ampicillin resistance gene to the PGK-neo-pA or PGK-BSD-pA cassettes using recombineering (not shown in details). Finally, we obtained two expression constructs for each of PPARγ1, PPARγ2, C/EBPα, Klf5 cDNAs, carrying neo or BSD resistance. With these modifications the constructs can be used for generating stable lines.

The constructs were tested in 293 cells for the protein expression (Fig. 34). The cells were lipofected with circular plasmids (3μg/9.5cm^2 well), CAGGs-GFP-IRES-puro-pA was used as a control for transfection. Whole cell extracts were prepared from the cells 48 hours after transfection. Western blot confirmed the expression of given proteins and functionality of the constructs.

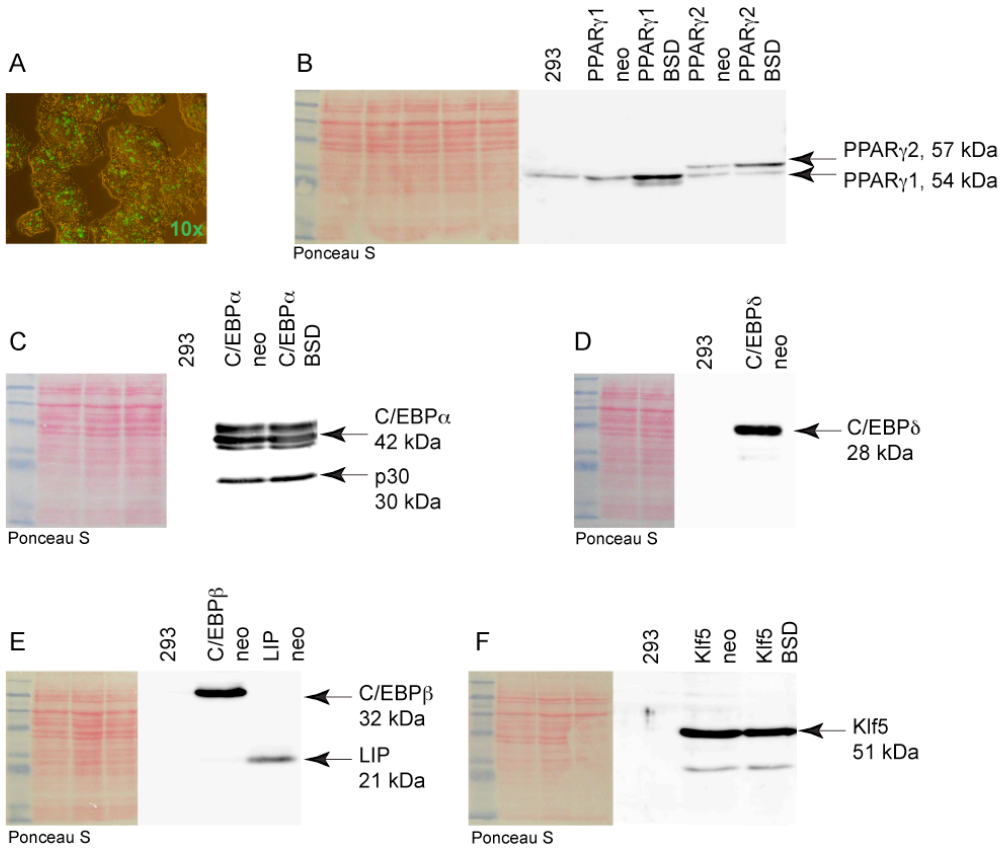

Figure 34. Validation of the expression constructs. 293 cells were lipofected with 3 μg of circular plasmids, CAGGs-GFP-IRES-puro-pA construct served as a control for transfection (A). 48 hours after transfection the cells were harvested for protein extraction. Expression of proteins was detected by Western blot incubated with appropriate antibody (B-F). Membranes were stained with Ponceau S to confirm equal protein loading (shown at left side). The antibody for PPARγ detection recognized γ1 and γ2 isoforms (E-8, Santa Cruz).

3.6.4.2. Transient overexpression of transcription factors during adipogenic differentiation

We have analysed the expression of transcription factors important for adipogenesis in undifferentiated cells and during differentiation in the previous experiments. In order to identify the factors capable of trigerring adipogenesis, we performed transient overexpression of the factors in the cells and induction of adipogenic differentiation. The adipogenic differentiation is visible in the cell culture conditions after 3-4 days, which made it possible to perform the experiment using transient transfection without selection of stable transfectants. We used single factors (PPARγ1, PPARγ2, C/EBPα, C/EBPβ, C/EBPδ and Klf5) and in combinations, which might have cooperative effect: PPARγ2 + C/EBPα; C/EBPα + C/EBPβ; C/EBPβ + C/EBPδ; C/EBPα + Klf5; PPARγ2 + C/EBPα + C/EBPβ + C/EBPδ.

The cells of osteogenic ("OC" properties) and adipogenic clones ("AC") were seeded into 6-well plates and lipofected with 5 μg of circular plasmids (Fig. 35). CAGGs-GFP-IRES-puro-pA was used as a control for the transfection and further for the differentiation. Next day adipogenic conditions were applied. The cells were examined visually for adipocyte appearance 4 days later. At this time point RNA was isolated and checked for the marker expression by RT-PCR.

At the day of analysis, differentiation of the cells in the positive control (adipogenic clone) was clearly recognized. In osteogenic cells transfected with GFP (negative control) adipocytes were not detected. We have observed adipocyte formation in the non-adipogenic cells transfected with C/EBPα, C/EBPβ, C/EBPδ, and consequently in all the combinations that included those factors (Fig. 35C). The efficiency was lower than in the positive control, nevertheless this can be explained that not every cell was transfected and that the levels of overexpressed proteins might vary. We also observed that adipogenic clone was differentiated with similar efficiency in all the transfections, indicating that the increase of those factors did not affect differentiation properties of the cells, neither stimulating nor inhibiting, and also was not toxic (results not shown).

We performed RT-PCR to check the expression of markers at mRNA level (Fig. 35D). We showed the expression of adipocyte markers, such as adiponectin, adipsin and PPARγ2 in the cells overexpressing C/EBPα, C/EBPβ and C/EBPδ, in which we observed morphological adipocytes. This confirms their differentiated status and identity of adipocytes. We detected weak upregulation of adiponectin (but not adipsin) in PPARγ1- and to a less extent in PPARγ2-transfected cells, suggesting that those factors indeed might lead to adipogenic differentiation but less efficiently in our experimental conditions. The transcripts for PPARγ1, C/EBPα, C/EBPβ, C/EBPδ and Klf5 were found in all the samples. In some cases (PPARγ2 and C/EBPβ) we have seen an increase of the transcript in the cells, which were transfected with these constructs, indicating that at this time point the cells are still overexpressing the factors. This was not observed for all of the factors that might reflect different efficiency of transfection, overexpression, or stability of the construct in the cells 5 days after transfection.

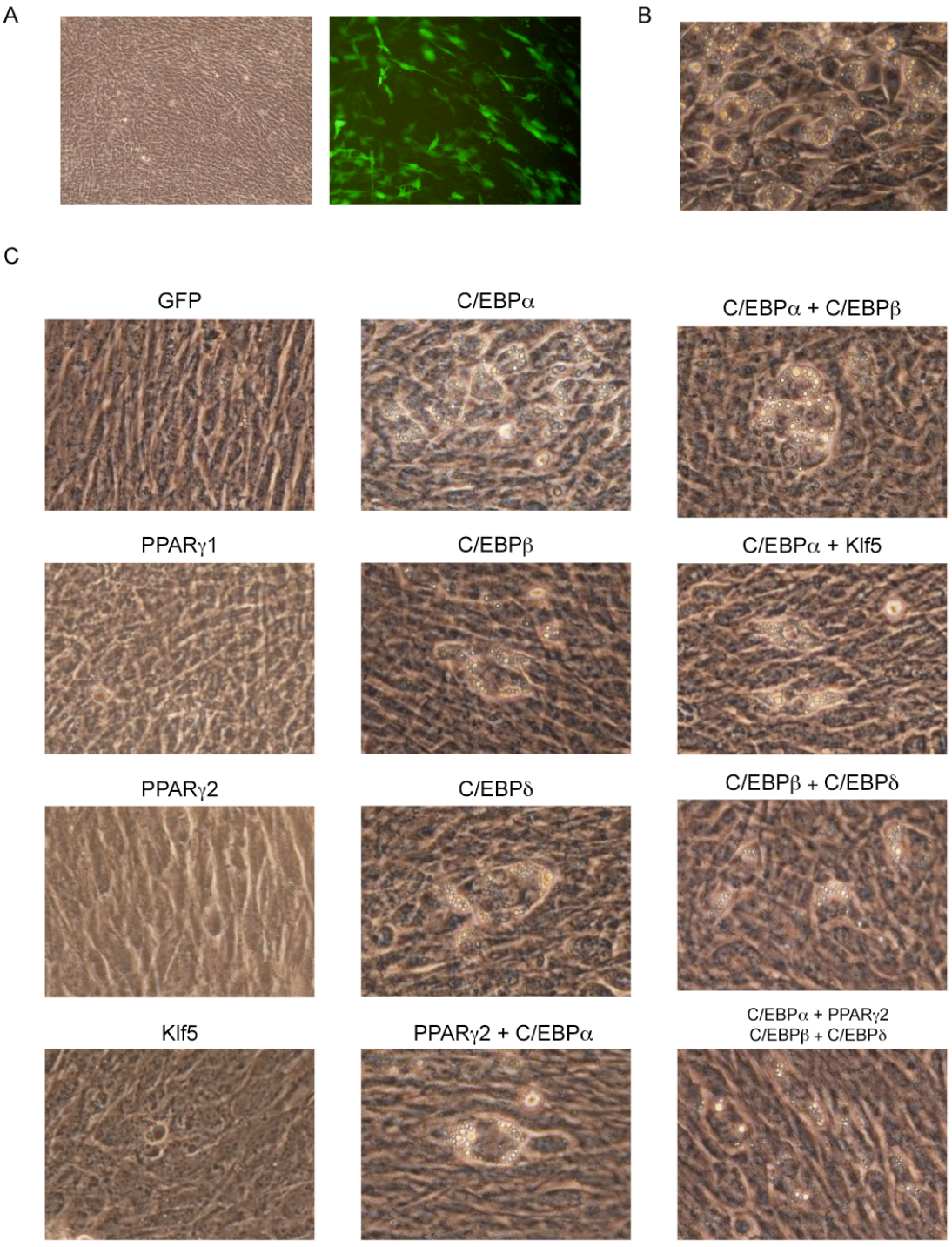

Figure 35. Conversion of non-adpogenic progenitors to the adipogenic lineage. Osteogenic progenitors not capable for adipogenic differentiation (clone 5A-12A, "OC") were transiently lipofected with 5 μg of circular expression plasmids, CAGGs-GFP-IRES-puro-pA construct was used as a control. The cells were induced to adipocyte differentiation next day after transfection for 4 days. (A) Expression of GFP in the control 5 days after transfection (10x). (B) Positive control for differentiation, clone "AC" (40x). (C) Adipocyte appearence in the transfected osteogenic progenitors (unstained, 40x, zoomed).

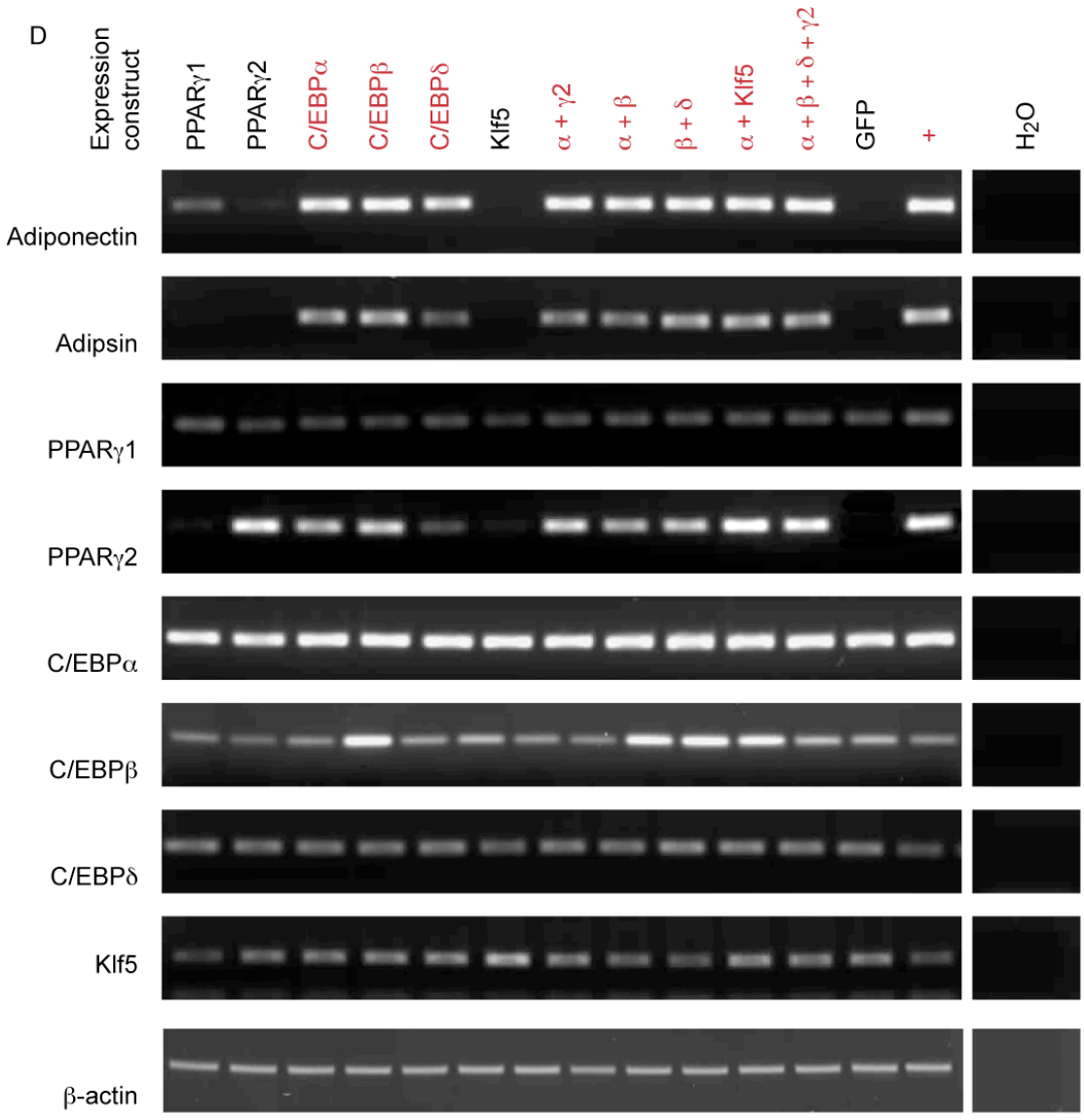

Figure 34 (continue). Expression of markers in the osteogenic clone ("OC") converted to the adipogenic lineage by the ectopic expression of transcription factors after 4 days of differentiation to adipocytes. (D) RT-PCR. β-actin served as a loading control (multiplex PCR). "+" - positive control for PCR, differentiated adipogenic clone. Conditions led to the efficient adipogenic formation are highlighted in red.

We conclude that factors of C/EBP family C/EBPα, C/EBPβ, C/EBPδ can start adipogenic programm in non-adipogenic bone marrow stromal progenitors. Certainly, C/EBPα, C/EBPβ and C/EBPδ play an important role during differentiation (Wu, Xie et al. 1995; Wu, Bucher et al. 1996). Previously (sections 6.2 and 6.3), we observed that non-adipogenic cells can express C/EBPα, C/EBPβ and C/EBPδ, and concluded that those factors are unlikely determinants of adipogenic commitment. Here we showed, that the overexpression of those factors could shift the cells to the adipogenic commitment. We can speculate that the high dose of C/EBP's causes the differentiation of the cells. We also noticed that the C/EBPβ construct we used for the experiment led to the expression of LAP isoform (32kDa), whereas BM MSCs express full-length 38kDa protein and LAP increased only during adipogenic differentiation of BM MSCs. The role of C/EBPβ isoforms in adipogenic commitment should be further investigated.

Unexpectedly, PPARγ1 and PPARγ2 alone did not support adipocyte differentiation of the cells. We have observed quite weak expression of the protein during the test of the constructs, and hence the dosage of overexpressed factor might not be enough for differentiation. Additionally, PPARγ1 and PPARγ2 were controlled by SV40 promoter, whereas all the others by CMV promoter, and the efficiency of the expression in the cells might be different, although we don't have information about this.

Additional experiments have to be done to elucidate the mechanism of the ectopical adipogenic induction, however we present the evidence that non-adipogenic BM MSC progenitors can be converted to the adipogenic cell fate. This shows that non-adipogenic cells are blocked from the adipogenic differentiation not due to the mutations or chromosomal abberations, but via cellular regulatory mechanisms.

4. Discussion

Although bone marrow mesenchymal stem cells were discovered years ago (Friedenstein, Petrakova et al. 1968; Friedenstein, Chailakhyan et al. 1974; Owen and Friedenstein 1988), the identity of those cells is still far from understood. The bone marrow mesenchymal stromal cells, BM MSCs, represent a complex of progeny of mesenchymal stem cells functioning to support haematopoiesis and differentiate into skeletal tissues (Bianco, Robey et al. 2008). In the present study we isolated pure cell populations from the spectrum of BM MSC cell types, and subsequently characterized them. We aimed at elucidating the mechanisms of the lineage commitment and differentiation of BM MSCs and understanding the hierarchy of BM MSCs progeny.

4.1. Generation of transgenic mice for conditional immortalization of primary cells

Amongst more than 200 cell types in the organism only a limited number is characterized due to the rareness, lack of markers or limited ability to expand *ex vivo*. Cellular immortalization provides an important tool for expansion and subsequent characterization of cells. We generated a transgenic mouse for the conditional immortalization of primary cells described detailed in (Anastassiadis, Rostovskaya et al. 2010). The idea of making immortalization inducible was not new. The immortalization of cells is accompanied with the large changes in cell metabolism, which can make it difficult to interpret the results from spontaneously immortalized cells carrying mutations, genetically manipulated cells, or cells boosted with the viral oncoproteins. For those reasons, the advantage of conditionally immortalizing cells and going back to the initial state was taken into consideration in the studies using temperature-sensitive T-antigen, or transcriptionally regulated Large T (Radna, Caton et al. 1989; May, Hauser et al. 2004). The success of the reversible immortalization systems in cells made it possible to go further and produce transgenic mice carrying temperature-sensitive or tetracycline-controlled Large T-antigen. A wide spectrum of conditionally immortalized primary cell lines were isolated from these mice (Jat, Noble et al. 1991; Yanai, Suzuki et al. 1991; Bendiksen, Van Ghelue et al. 2004). However, the leakiness of the system, weak expression of T-antigen and tumorigenicity still represented a problem for using

those models. We aimed to improve the performance of the system in order to make it tightly regulated and reliable (Anastassiadis, Kim et al. 2002). The transcriptional regulation using TET-ON system fulfilled these requirements. The codon optimization improved the expression of the rtTA (irtTA, improved rtTA) and the fusion with GBD* gave an additional level to control the irtTA. We were able to generate transgenic mice in two variations, irtTA-TAg and irtTA-GBD*-TAg. However, Large T expression was weakly induced in the irtTA-TAg mice. The irtTA-GBD*-TAg mice showed excellent properties in the T-antigen regulation and expression proving the advantage of the system (Anastassiadis, Rostovskaya et al. 2010).

4.2. Conditional immortalization of primary bone marrow mesenchymal stromal cells

We have isolated conditionally immortalized bone marrow mesenchymal stromal cells (BM MSCs) from the transgenic mice. Mouse BM MSCs are known to have very limited proliferation potential, which is probably not associated with replicative senescence, but rather with the requirement of the specific extracellular mitogen signals (Peister, Mellad et al. 2004; Prockop 2009). The stromal cells from mouse bone marrow underwent 1-2 cell divisions and then stayed in the arrested state. The induction of Large T-antigen resulted in the extensive cell proliferation, which was practically indefinite (Fig. 11). We could passage the cells for at least 40-50 passages and established large populations from single cells, meaning that the cells were indeed immortalized. We observed the changes in the cell cycle regulators in the immortalized cells (Fig. 13). SV40 T-antigen is unique in its properties to combine all functions required to induce cell proliferation. We have shown the stabilization of p53 and its direct interaction with Large T in the conditionally immortalized cells. We observed the downregulation of negative regulators of the cell cycle, such as p27, after induction of the T-antigen. But the most important fact was that all of the effects of T-antigen on the cell cycle were reversible upon induction/deinduction (Fig. 12). The proliferation of the cells was tightly dependent on the T-antigen expression, and could be switched on and off many times. We have never observed spontaneous transformation of the conditionally immortalized cells. Application of the tet-regulated T-antigen transgenic mice allowed us to establish a large number of the conditionally immortalized lines of BM MSCs, from irtTA-TAg (induction with Dox) and irtTA-GBD*-TAg (induction with Dex/Dox) mice.

4.3. Conditionally immortalized bone marrow mesenchymal stromal cells maintain the identity

The conditionally immortalized bone marrow stromal lines were characterized to confirm their identity. We examined the expression of surface markers and found it similar to the known for human BM MSCs (Fig. 14). We noticed a difference in the expression of CD146, MCAM, a presumable marker for self-renewing stem cells in human bone marrow (Sacchetti, Funari et al. 2007), which was negative in the conditionally immortalized mouse BM MSCs. However, to our knowledge there were no reports about the expression of CD146 in mouse BM MSCs, and we can attribute this fact to the difference between the species. Notably, the expression of markers was not different in the induced and non-induced state, indicating that T-antigen expression did not influence those characteristics of the cells. We did not observe significant differences between the cells from irtTA-TAg (Dox) and irtTA-GBD*-TAg (Dex/Dox) mice, hence, the presence of Dex in the medium did not change the surface marker expression. Another important observation was that markers were not altered after the long-term passaging.

The essential function of BM MSCs *in vivo* is to support haematopoiesis. This can be assessed in the *in vitro* co-culturing assays and after ectopic transplantation (Friedenstein, Chailakhyan et al. 1974; Dexter 1982). Using the cobblestone assay we confirmed the ability of conditionally immortalized BM MSCs to support haematopoietic stem cells *in vitro* (Fig. 15).

The reversibility of immortalization makes it possible to perform differentiation assays with conditionally immortalized BM MSCs, because the differentiation is usually accompanied by the stop of proliferation. We tested differentiation potential of the conditionally immortalized BM MSCs after deinduction of the T-antigen. The stromal lines differentiated into osteocytes, adipocytes and chondrocytes, indicating that the differentiated properties were not affected by the expansion using T-antigen (Fig. 16-18). We didn't observe differences in the differentiation properties between the BM MSC lines from irtTA-TAg (Dox) and irtTA-GBD*-TAg (Dex/Dox) mice. This is an important observation, because Dex is one of inducers of BM MSC differentiation, and Dex is constantly required to induce Large T expression in irtTA-GBD*-TAg cells. However, despite this disadvantage of the system we suggest that the presence of Dex does not change the differentiation state of the conditionally immortalized BM MSCs and the effect of Dex might be reversible after its withdrawal.

It was often discussed in the literature, that the expression of Large T-antigen blocks the adipogenic differentiation of 3T3-L1 and F442A fibroblasts through the inactivation of CBP/p300 that is required for adipogenesis (Cherington, Morgan et al. 1986; Cherington, Brown et al. 1988). According to other data, MEF's expressing T-antigen are able to generate adipocytes, but with the properties of brown adipocytes (Hansen, Jorgensen et al. 2004). In this study, it was suggested that Retinoblastoma protein acts as a regulator of the white type adipocyte development and the inactivation of pRb switches the fate from the white to brown types. The results of (Estervig, Minoo et al. 1990) demonstrate that SV40 T-antigen does not block adipogenesis per se, but makes it more difficult by altering of the cell cycle. In our study we haven't observed the block of adipogenic differentiation of the conditionally immortalized BM MSCs when T-antigen is deinduced. Moreover, we could obtain efficient adipogenic differentiation of the cells induced for T-antigen expression (results are not shown). We conclude that the Large T does not perturb the adipogenic potential of the conditionally immortalized BM MSCs. The discrepancy with the data from 3T3-L1 fibroblasts can be attributed to the differences between the adipogenic induction in those cells and MSCs, considering the undefined identity of the 3T3-L1 cells.

Importantly, the cells maintained differentiation potential after the long-term passaging. It was often noted that human BM MSCs change the differentiation properties spontaneously during the passaging, resulting in the enhancement of alkaline phosphatase expression and osteogenic potential (Bruder, Jaiswal et al. 1997). In concordance to this, the clonally derived lines of human BM MSCs were able for osteo- and adipogenic differentiation on passage 2, but completely lost adipogenic properties at passage 12 and remained osteogenic (Digirolamo, Stokes et al. 1999). Similar observations were done by the group of R. Quarto (Banfi, Muraglia et al. 2000; Muraglia, Cancedda et al. 2000). The clonally derived BM MSCs were tested for the differentiation potential into osteo-, adipo-, chondrocytes on different passages. The majority of the clones had the potential for all three lineages, but lost the ability to differentiate into adipocytes after 22 population doubling (PD) times, and subsequently into chondrocytes after 24 PDs, resulting in the cell population with only osteogenic properties. The authors suggested that the observed phenomenon reflected the differentiation pathway of BM MSCs that the cells underwent during the culturing, and the osteogenesis might be the "default" lineage in BM MSC development (Fig. 35A). Therefore, the population of human BM MSCs is always predisposed toward osteogenic lineage during culturing *in vitro*.

Another explanation to this fact can be viewed from the observation that during osteogenesis BM MSCs undergo the stage of progenitor expansion and extensively proliferate. It was shown that the overexpression of osterix in BM MSCs resulted in the increased cell proliferation (Satija, Gurudutta et al. 2007). On the contrary, adipogenesis is accompanied with the growth cease and one of the major positive regulators of adipogenesis, C/EBPα, possesses an anti-mitogenic activity (Umek, Friedman et al. 1991). As a result, the fibroblasts overexpressing C/EBPα were growth arrested (Umek, Friedman et al. 1991). Hence, the cells with higher osteogenic potential can take an advantage over adipogenic cells during the passaging due to the higher proliferative potential. However, we didn't observe any changes in the efficiency of differentiation after the long-term passaging, at least in adipo- and osteogenic properties. This might imply that (1) conditionally immortalized BM MSCs do not change the differentiation state spontaneously during the culturing, (2) the immortalized progenitors of different types proliferate with comparable rate. Notably, (Okamoto, Aoyama et al. 2002) made similar observations using human BM MSCs immortalized with hTERT and E6/E7 proteins of HPV. These cells proliferated without the loss of differentiation potential, and then non-osteogenic cell subpopulations with single adipogenic or chondrogenic potential were identified. Hence, immortalization might provide a tool to obtain the rare or low-proliferative cell types, and conditionally immortalized cells are likely not biased toward one or another differentiation lineage.

In summary, we confirmed the identity of the conditionally immortalized BM MSCs by the expression of the markers, the ability to support HSCs and the potential for multilineage differentiation. We speculate that the conditional immortalization offers an advantage not only in the expansion of BM MSCs, but also in the prevention of spontaneous differentiation in the cell culture conditions.

4.4. The population of mouse CFU-F's (colony forming units-fibroblasts) is heterogeneous

The main criteria for stemness imply (1) the potential for the differentiation at the single-cell level and (2) the ability for self-renewal. The differentiation of stem cells is commonly assessed in the *in vitro* tests confirmed further by the marker expression. However, the cell culture conditions may generally alter the state of the cells, and the cells become "adapted" to the non-physiological conditions. The most

rigorous examination for the differentiation of stem cells should include not only *in vitro* test, but also the differentiation in the *in vivo* situation, that means the transplantation assay.

The self-renewal characteristic of stem cells means the ability to re-establish the population of identical stem cells. And of course, this is not equal to the proliferation in the cell culture conditions resulting in the population of the same type of cells. Also, an indefinite expansion of the cells *in vitro* cannot prove the stemness. Hence, the self-renewal ability can be tested in the *in vivo* assay (Bianco, Robey et al. 2008; Prockop 2009).

The most firmly ascertained system of stem cells is undoubtedly embryonic stem cells. The contribution to chimeras and germline transmission provide a definite proof of stemness. However, it is more difficult to confirm those principles for the adult stem cells. The “golden standard” for stemness in the adults is a haematopoietic stem cell, which can reconstitute the whole haematopoietic system from a single cell, and be re-transplanted. One of the reasons of this “success” is a high turnover of the haematopoietic system that allowed the establishment of the reconstitution assays.

Originally, BM MSCs were discovered by following those principles (Ashton, Allen et al. 1980; Friedenstein, Latzinik et al. 1982; Patt, Maloney et al. 1982). The plastic adherent fraction of human bone marrow plated with a low density resulted in single-cell derived colonies of fibroblastic cells, which could grow in a cell density-independent manner (colony forming units-fibroblasts, CFU-F’s). The expanded pooled CFU-F’s could be ectopically transplanted under the renal capsule or subcutaneously, and in these conditions they differentiated into skeletal tissues and generated a niche for haematopoiesis, and could be re-established and re-transplanted to the secondary recipients, implying self-renewal capacity. Thus, the population of human CFU-F’s contains “stromal stem cells” with the proven property of stemness. Nevertheless, the progeny of a single human CFU-F had limited potential to transfer the haematopoietic environment, indicating heterogeneity within this population (Friedenstein, Chailakhyan et al. 1987; Kuznetsov, Krebsbach et al. 1997). Thus, CFU-F’s are considered as closest descendants of *bona fide* mesenchymal stem cells but not equal.

Little is known about mouse CFU-F’s. To our knowledge, there is very limited information in the literature about the expanded mouse CFU-F’s obtained without pre-sorting for specific markers. From personal communications, the attempts to

show the self-renewal of mouse CFU-F's so far failed and that is why only seldom discussed (Nifontova, Svinareva et al. 2008). That reasoned the particular relevance to establish expandable populations of CFU-F's from the mice.

Bone marrow from the transgenic irtTA-GBD*-TAg mice was plated with a low density and the appearance of single-cell derived CFU-F's was observed. The immortalization was induced by adding of Dex/Dox to the cell culture medium. The individual CFU-F's were expanded and tested for the differentiation capacity into osteo- and adipogenic pathways (Fig. 20). We found variability in the differentiation properties amongst CFU-F's. The expanded cell populations exhibited bi-potential ("OA") or monopotential ("O" or "A") differentiation. Our observations confirm the heterogeneity of mouse CFU-F's. The presence of CFU-F's with restricted differentiation capacity (at least 70% from the analysis of the two pathways were single "O" or "A") shows that the population of murine CFU-F's contains committed progenitors, which are descendants of mesenchymal stem cells. Unlikely in the other studies using primary bone marrow cells, we presume that the conditional immortalization allows expansion of the cells without the loss of differentiation potential, and our results is probably not a consequence of an *in vitro* artifact. Thus, our observations likely reflect the characteristics of CFU-F's existing within the bone marrow. Mouse bone marrow undoubtedly contains mesenchymal stem cells, which was demonstrated in the transplantation assays of bone marrow plug (Chertkov and Gurevitch 1980). But our data suggest that CFU-F's represent a population of cells with various differentiation potential and only a subset of CFU-F's can possess the properties of stemness. To our knowledge, this is a first report about the large screening of differentiation properties of the expanded mouse CFU-F's. However, additional experiments are required for the more solid conclusion about the mouse CFU-F's identity.

4.5. Hierarchy of progenitors within bone marrow mesenchymal stromal cells

Bone marrow stroma is a complex structure consisting of different cell types functionally united into one tissue. The integrity of the system is provided, firstly, by the functional interactions between the cells and, secondly, the common developmental origin. Mesenchymal stem cells are capable to generate the complete functioning stroma, thus, the stromal cells have a common ancestor. Therefore, the isolated stromal lines represent the whole spectrum of mesenchymal stem cells

descendants, on the different stages of differentiation and from different lineages. The heterogeneity of BM MSCs was reported before, and the progenitors of different types were found within the isolated BM MSCs (Pittenger, Mackay et al. 1999; Banfi, Muraglia et al. 2000; Muraglia, Cancedda et al. 2000; Okamoto, Aoyama et al. 2002). However, the molecular characterization of those cell types is far from comprehensive, and the main obstacle is in the inability to obtain reasonable amounts of pure cell populations for biochemical analysis.

We isolated conditionally immortalized BM MSCs from irtTA-GBD*-TAg mice and expanded upon the induction of Large T-antigen. We performed cellular cloning and established the single-cell derived populations. The screening of differentiation into osteocytes ("O"), adipocytes ("A") and chondrocytes ("C") revealed that the clones had different properties for differentiation. We identified six types of clones that were "OAC", "OA", "OC", "AC", "O" and "A". Hence, we confirmed the heterogeneity of the BM MSCs (Fig. 21).

The heterogeneity of human BM MSCs was shown by many others (Pittenger, Mackay et al. 1999; Banfi, Muraglia et al. 2000; Muraglia, Cancedda et al. 2000; Okamoto, Aoyama et al. 2002). The differentiation assays of clonally derived populations showed the presence of different types of progenitors. However, different sets of the progenitors were obtained in the studies of different groups. For example, the group of R. Quarto identified the clones with types "OAC", "OC" and "O" (Banfi, Muraglia et al. 2000; Muraglia, Cancedda et al. 2000). The authors suggested the "linear" model of hierarchy in BM MSCs, in which the osteogenic pathway is the "default", and adipogenic lineage segregates firstly, and the chondrogenic lineage separates downstream (Fig. 36A). In support to this model, it was observed that the cells are loosing the multipotentiality during the passaging, at first, adipogenic capacity, and secondly, chondrogenic (as we already discussed in the previous section). Similar loss of multipotentiality of BM MSCs *in vitro* during the passaging was shown by (Digirolamo, Stokes et al. 1999). However, the instability of differentiation properties of primary BM MSCs during the passaging questions the relevance of the differentiation assays of the clones. Indeed, even if the initial population was homogeneous, but stochastically some cells were loosing the differentiation capacity faster than the others, then the diverse clones would be isolated. The heterogeneity of the clones might reflect the *in vitro* artifact of spontaneous differentiation. To exclude this problem, the second round of cellular cloning should have been done, but the proliferation limitation did not allow performing of this experiment.

The conditional immortalization made it possible to expand the cells practically indefinitely. In order to check the homogeneity and the stability of the differentiation properties of the single-cell derived clones we performed the second round of cellular cloning and analysed the differentiation of the resulted “subclones” (Fig. 22 and 23). We tested more than 50 subclones for osteo-, adipo- and chondrogenic differentiation. The comparison of the properties of subclones with the parental clones showed a high rate of the maintainance of the differentiation potential, meaning that a large proportion of the subclones had the same potential as the initial clone. These results indicate that (1) the single-cell derived clones of conditionally immortalized BM MSCs represent homogeneous populations of cells, and (2) the differentiation potential of the single-cell derived clones of conditionally immortalized BM MSCs is stable after expansion *in vitro*. We assume that the populations identified in our study indeed reflect heterogeneity of BM MSCs in the bone marrow *in vivo*. The variation in the differentiation properties of CFU-F’s discussed above supports our conclusion.

Those facts made it possible to apply the clonally derived populations of BM MSCs to study lineage commitment and differentiation pathways. We used the populations derived from the second round of cellular cloning for the next experiments. The multilineage potential for the differentiation allows us to name those cells as “progenitors” or “progenitors/stem cells”, however, considering that stemness of those cells is not proven in fact.

Remarkably, similar results to ours were obtained using immortalized human BM MSCs (Okamoto, Aoyama et al. 2002). Human BM MSCs were immortalized by expression of hTERT, E6 and E7 HPV proteins and subsequently clonal populations were obtained. The differentiation capacities of the clones were “OAC”, “OA”, “OC”, “A”, “O” and “C”. “AC” clone was not identified in this study, presumably, by statistical reasons. These data support our observations since similar combinations of properties were found.

During the preparation of this thesis, a similar approach to that we used was published (Russell, Phinney et al. 2010). In this study clonally derived cell populations were obtained from primary human BM MSCs. The authors found all possible combinations of differentiation properties: “OAC”, “OA”, “AC”, “OC”, “O”, “A” and “C”. These data perfectly agree with our results. Unfortunately, the characteristic of the clones did not go further and the study was presented as a developing of the differentiation assay and cryopreservation of the clones in 96-well plate format as a

success-providing unique technique, which was discovered in our group independently.

It should be noted that we did not identify all the possible combinations of differentiation properties amongst the clones and subclones. We never observed “C” type of cells, but we explain it by the statistical reasons. However, the existence of all combinations of bi-potential progenitors (“OA”, “AC”, “OC”) shows that the hierarchy of BM MSC lineages can be more complicated than simple linear deterministic or divergence diagram (Fig. 36B). The data from immortalized and non-immortalized human BM MSCs support our suggestion (Okamoto, Aoyama et al. 2002; Russell, Phinney et al. 2010). For instance, the classical scheme of haematopoiesis implies that the HSCs proceed the differentiation step by step, segregating the lineages, and there is no way back or across the lineages. But this logic of commitment has never been shown for BM MSCs. The presence of “OAC”, “OA”, “OC”, “AC” clones suggests that osteo-, adipo- and chondrocyte lineages might exist independently in the cells. It is possible that during the maturation the undifferentiated progenitors eliminate the other possible pathways, leaving the only one possibility at the end, and then proceed the differentiation. In this case the way from “OAC” to “A” can happen through “OA” and “AC”, and the way from “OAC” to “O” can happen through “OA” and “OC”. Thus, the molecular signature for the osteogenic commitment in the “OAC”, “OA”, “OC” and “O” clones might be similar. However, this model of commitment is empirical and hypothetical and needs to be further proven.

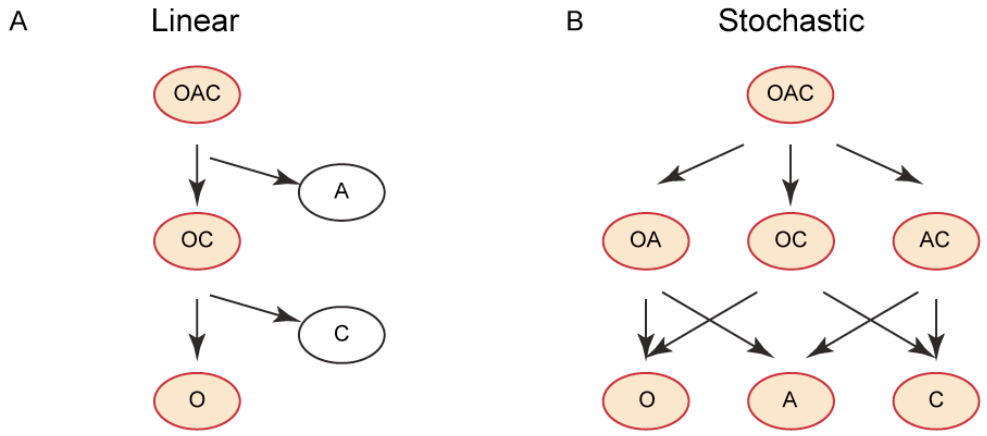

Figure 36. Models of BM MSC hierarchy. (A) Linear model supposes a sequential loss of adipogenic and chondrogenic potentials, and only one possibility for the lineage commitment. (B) Stochastic model presumes the existence of all types of combinations between “O”, “A” and “C” properties, and the differentiation can proceed through different alternative pathways.

4.6. The role of GSK3 and Wnt/β-catenin canonical pathway in BM MSC differentiation and lineage commitment

Although the data on the mechanisms of BM MSC differentiation *in vitro* and *in vivo* is rapidly accumulating, there is no commonly accepted model about the regulation of those processes. Multiple signaling pathways are considered to have an influence to the differentiation, amongst them canonical and non-canonical Wnt, BMP, MAPK, TGFβ. It is very likely that these pathways are interacting and working in concert. Wnt/β-catenin pathway plays an important role in the regulation of adipogenic and osteogenic differentiation and might be involved in the lineage choice in multipotential progenitors. We attempted to elucidate a role of Wnt and its central player GSK3 during the differentiation of BM MSC progenitors. The comparison of the different types of progenitors might provide information about the function of Wnt and GSK3 in lineage commitment.

The main approach we used was the inhibition of GSK3 and monitoring of Wnt activity using the Luciferase reporter pBAR. The application of different GSK3 inhibitors, such as LiCl, BIO, CT99021, in parallel allowed to exclude the side effects on the other signaling pathways. We showed that BM MSC lines had minimal basal activity of the canonical Wnt, but inhibition of GSK3 activated the Wnt downstream target promoters many fold in a concentration-dependent manner (Fig. 25, 26). Similarly, the progenitors of different types did not exhibit Wnt activity, but could activate Wnt independently of the differentiation properties of the cells.

Next we tested the influence of GSK3 inhibition on the differentiation into adipocytes and osteocytes. The application of GSK3 inhibitors during adipogenesis blocked the proceeding of the differentiation at any stage when the inhibitor was added (Fig. 27, 28). These results strongly show that active GSK3 and/or inactive Wnt are required to proceed the adipogenic differentiation. This is in discordance with the data from adipogenic fibroblasts 3T3-L1 and F442A (Ross, Hemati et al. 2000; Bennett, Ross et al. 2002). Adipogenic fibroblasts show high Wnt basal activity without exogenous stimulation. It is assumed that Wnt/β-catenin pathway inhibits the initiation of PPARγ and C/EBPα expression and therefore maintains 3T3 fibroblasts in the undifferentiated state. Repression of Wnt activity promotes PPARγ and C/EBPα expression and the adipogenic differentiation in fibroblasts, and once PPARγ and C/EBPα are established, the differentiation cannot be blocked anymore. However, according to our data this is not the case in the BM MSCs. The undifferentiated state of BM MSCs is not maintained by Wnt/β-catenin and the

differentiation is sensitive to GSK3 inhibition at any stage. Interestingly, all types of the adipogenic progenitors (“OAC”, “OA”, “AC”, “A”) showed the same result. We assume that 3T3 adipogenic fibroblasts represent arrested preadipocytes on the late stage of commitment, or utilize different mechanism of adipogenic induction from BM MSCs due to the uncertain identity. Our model provides new information on the mechanism of adipogenesis in BM MSCs.

We checked the influence of GSK3 inhibitors during the osteogenic differentiation of BM MSC progenitors. The inhibitors had stage-specific effects on the differentiation. The proliferation of progenitors during the first days of osteogenic induction was impaired by GSK3 inhibitors that reduced markedly the efficiency during the whole course of the differentiation. When applied on the later stages, GSK3 inhibitors increased the intensity of mineralization 3-9 fold (Fig. 29). Hence, GSK3 has a negative effect on the osteogenesis and/or Wnt/β-catenin might have a positive effect.

We have already discussed the controversy in the literature on the role of the canonical Wnt in BM MSC differentiation. Whereas it is commonly accepted that canonical Wnt negatively regulates the adipogenesis, the effect to the osteogenesis was considered as positive (Kang, Bennett et al. 2007), negative (Baksh, Boland et al. 2007), or neutral (Takada, Mihara et al. 2007). It is difficult to compile the results from studies that used different models and approaches. Nevertheless, we can presume the reasons, which might explain the discrepancies in those data. (1) The effect of the canonical Wnt on the osteogenesis might be stage-specific, therefore the observations might be opposing. Our data indirectly support this suggestion. (2) The effect of the canonical Wnt on the osteogenesis might be concentration-dependent, which is possible considering the gradients of signaling factors existing in the organism. Hence, the application of exogenous Wnt3a or Wnt10b might not reflect the physiological concentration of those factors. (3) The application of GSK3 inhibitors does not allow separating the effect of activation of Wnt/β-catenin pathway from the other functions of GSK3. (4) The cells *in vitro* do not receive the signals from the normal tissue context. In summary, the precise role of the canonical Wnt/β-catenin signaling in the osteogenesis is not known.

However, inhibition of GSK3 did not promote osteogenesis in non-osteogenic clones (“AC”, “A”) revealing that it is unlikely that canonical Wnt or GSK3 participate in the lineage commitment of BM MSC progenitors (Fig. 29). Additionally, Wnt/β-catenin activity was not detected in the osteogenic clones (“OC”, “O”) during the

attempt to induce adipogenesis. Hence, the Wnt/β-catenin pathway does not restrict non-adipogenic clones from adipogenic commitment. Finally, we didn't observe an increase in Wnt/β-catenin signaling in the cells during osteogenesis, at least the first days, indicating that the cells do not stimulate themselves by Wnt.

Taken together, we indeed observe the dual influence of GSK3/Wnt in terms of the inhibition of adipogenesis and the stimulation of osteogenesis. BM MSCs are responsive to the GSK3 inhibition during the differentiation *in vitro*, but we could not detect that they activate this pathway to control the differentiation endogenously. However, this is not a controversy. The progenitor cells are the parts of the complex tissues where they have to respond to the signals and maintain the integrity of the system. That is why those signals might be supplied from the other elements within the tissue in order to regulate or attenuate the differentiation, but not by themselves.

It should be noted that Wnt/β-catenin signaling is often considered as a candidate for the determinant of the lineage choice in BM MSCs, committing the multipotential cells into osteogenesis in expense of adipogenic potential (Kang, Bennett et al. 2007; Ling, Nurcombe et al. 2009). The models used in those studies represent multipotential cells capable for osteogenesis and adipogenesis, and Wnt favored to one differentiation lineage versus another. However, the opposite influence of Wnt to the two alternative pathways does not imply that it's a mechanism for the lineage choice. We apply different types of progenitors isolated from the same stromal population, which underwent the commitment step into osteo- or adipocyte direction. From our results, we don't observe the evidence that Wnt participates in the cell fate choice in BM MSCs.

4.7. The mechanism of adipogenic commitment in BM MSCs

The mechanism of adipogenic induction *in vitro* is well-described using the adipogenic fibroblasts 3T3-L1 and F442A, Fig. 2 (Tontonoz, Hu et al. 1994; Wu, Bucher et al. 1996). Nevertheless, it is often noted that L1 and F442 represent the cells arrested on the late stages of differentiation, pre-adipocytes. F442A are possibly more differentiated than L1, because Dex and insulin treatment is sufficient to induce adipogenesis in F442A, whereas additional stimulation of PPARγ or cAMP pathway is required for L1 (Wu, Bucher et al. 1996). Although these models provided important knowledge, they do not give information about the differentiation state of adipogenic progenitors from BM MSCs.

Differentiation of BM MSCs into adipocytes was accompanied by the increase in expression levels of regulators of adipogenesis, such as PPARγ, C/EBPα and C/EBPδ, whereas C/EBPβ stayed at the similar level (Fig. 30). We checked which of those factors were expressed in the undifferentiated progenitors of different types (Fig. 31). We found differential expression of PPARγ1 and C/EBPα between adipogenic and non-adipogenic clones, and the basal level of C/EBPβ and C/EBPδ was similar in the cells with different properties. We assumed that PPARγ1 and C/EBPα might determine the adipogenic potential in BM MSCs.

PPARγ2 isoform is specific for adipose tissue, whereas PPARγ1 is more abundant and widely expressed, that is why PPARγ2 is often considered as a regulator of adipocyte development. We haven't detected the PPARγ2 isoform in the undifferentiated cells and on the early stages of differentiation, since the transcript was found only on the late days (day 7-10). So, presumably PPARγ1 might act as a regulator of adipogenesis and PPARγ2 can serve as a marker for adipocytes. This conclusion is in agreement with data showing that both isoforms can fulfill adipogenic function, but PPARγ2 has higher activity (Mueller, Drori et al. 2002).

We found that activation of PPARγ with its agonist Troglitazone is a minimal sufficient condition for the differentiation of adipogenic cells (Fig. 32). Dexamethasone treatment led to the induction and activation of C/EBPβ;C/EBPδ complex resulting in the expression of C/EBPα at the low level (all conditions included Dex, "OC" clone, Fig. 33B). However, those conditions were not enough for the adipogenic differentiation in the cells. Insulin was rather acting through a different pathway in parallel with C/EBP's, because it did not influence the expression of the C/EBP's and PPARγ when it was applied alone. Indeed, the role of insulin in adipogenic differentiation *in vitro* is not exactly known, possibly, it acts through the IGFR1 (insulin-like growth factor receptor 1) pathway (MacDougald and Lane 1995). But the combination of Dexamethasone and insulin resulted in an increase of PPARγ1 without promoting adipocyte formation. The additional activation of PPARγ1 with Troglitazone to those conditions stimulated efficient adipogenesis. Activated PPARγ enhanced the expression of C/EBPα (all conditions included Tro, "AC" clone), but C/EBPα could not increase the PPARγ expression (all conditions included Dex, "OC" clone, Fig. 33B). Taken together, these data suggest that PPARγ is the main regulator of adipogenesis in BM MSCs.

We should note that we observed the effect of Dex on the expression of important regulators of differentiation in the cells, and at the same time Dex had to be applied constantly to the cells to induce conditional immortalization of BM MSCs from irtTA-GBD*-TAg mice. This is a disadvantage of the irtTA-GBD* system, and we have to confirm our data using irtTA-TAg cells (induction with Dox). However, we never observed the differences between the differentiation properties of BM MSCs induced with Dex/Dox and Dox only. We also noted that the expression level of C/EBPα, C/EBPβ, C/EBPδ is changing in the presence or absence of Dex, therefore we assume that the Dex influence is reversible. Additional evidence for that was obtained using mouse ES cells carrying irtTA-GBD*-TAg system. Comparison of the transcription profiles of the cells before induction, during induction and after deinduction of the T-antigen showed that the effects of Dex/Dox on the cells were reversible (Anastassiadis, Rostovskaya et al. 2010). The reason to use irtTA-GBD*-TAg cells in our study is connected to the excellent performance of this system in mice.

The role of PPARγ and C/EBPα as major regulators of adipogenesis was known before. Adipogenesis was stimulated in fibroblasts by the retroviral expression of PPARγ or C/EBPα, but it was much enhanced when PPARγ and C/EBPα were co-expressed (Freytag, Paielli et al. 1994; Tontonoz, Hu et al. 1994). It was also shown that PPARγ and C/EBPα cross-regulate each other, and both of the factors are critical for adipogenesis (Wu, Rosen et al. 1999). The knockout cells for PPARγ or C/EBPα failed to undergo adipogenesis. However, it was demonstrated that constitutive expression of PPARγ can rescue the adipogenic properties of C/EBPα$^{-/-}$ fibroblasts, but not the other way around (Rosen, Hsu et al. 2002). Hence, PPARγ is a primary and essential regulator of adipogenesis. The sequence of events upstream of PPARγ was also explored. Conditional ectopic expression of C/EBPβ induced PPARγ expression and stimulated adipogenesis in non-adipogenic cells, fibroblasts and myoblasts, suggesting a critical role in the induction of adipogenesis (Wu, Bucher et al. 1996). From all those data it is difficult to conclude, which factor is the primary determinant of adipogenic commitment in BM MSCs.

We assumed the role of C/EBPα and PPARγ in the adipogenic cell fate determination in BM MSCs due to the differential expression in adipogenic versus non-adipogenic BM MSCs progenitors. From the experiment above, non-adipogenic cells could express C/EBPα upon Dex stimulation, but they failed to express PPARγ

in all the conditions. Hence, we grant a primary role in the adipogenic commitment of BM MSCs to PPARγ.

4.8. Conversion of non-adipogenic BM MSCs progenitors to the adipogenic lineage

We overexpressed transiently the main transcription factors regulating adipogenesis in the non-adipogenic cells ("OC" clone) and treated with adipogenic inducers Dexamethasone, insulin and Troglitazone (Fig. 35). We observed adipocyte formation in the cells overexpressing one of C/EBPα, C/EBPβ or C/EBPδ factors. Unexpectedly, PPARγ1 and PPARγ2 overexpression did not result in adipogenesis.

We conclude that factors of C/EBP family C/EBPα, C/EBPβ, C/EBPδ can start adipogenic programm in non-adipogenic bone marrow stromal progenitors in the overexpression conditions. The inability of PPARγ1 and PPARγ2 to induce adipogenesis can be explained by the insufficient level of the expression (as it was observed in the validation of the expression constructs in 293 cells, Fig. 34). However, these results do not oppose our previous conclusion that PPARγ is a major determinant of adipogenesis in the cells, and C/EBP's are unlikely. The overexpression often leads to very high, non-physiological levels of the proteins, and this can be seen from the validation of C/EBP expression constructs. In this situation, the factors might cause the effects, which do not happen in the usual conditions.

Our experiment confirms the adipogenic potential of C/EBPα, C/EBPβ, C/EBPδ and their role in the adipogenesis in BM MSCs. In the past, the role of PPARγ and C/EBP's in adipogenesis was elucidated in the experiments using ectopic expression of those factors (Freytag, Paielli et al. 1994; Tontonoz, Hu et al. 1994; Wu, Xie et al. 1995). However, there were some discrepancies in the results of different groups in the overexpression tests. For example, it was considered as difficult to obtain cells overexpressing C/EBPα due to its anti-mitogenic activity. All the stable clones obtained from the retroviral transfections of C/EBPα expression constructs into 3T3-L1 cells were negative for C/EBPα (Umek, Friedman et al. 1991). However, the other groups of authors reported the generation of clones from different lines of fibroblasts (i.e. 3T3-L1, BALB/C, NIH-3T3, Swiss 3T3, totally 10 lines) by retroviral expression of C/EBPα, which proliferated and could be induced for adipogenesis (Freytag, Paielli et al. 1994; Tontonoz, Hu et al. 1994). In the same

report, C/EBPβ was also transduced into the fibroblasts lines, but the cells expressing C/EBPβ failed in the adipogenic differentiation. It was concluded that C/EBPβ is not able to promote adipogenesis. However, the other studies demonstrated that the conditional ectopic expression of C/EBPβ in NIH-3T3 fibroblasts converted them to preadipocytes revealing the role of C/EBPβ in the adipogenesis (Wu, Xie et al. 1995). In the study above only one half of the stably transfected clones was efficient for C/EBPβ expression and adipogenesis (4 out of 8 shown clones), whereas the other half failed.

In fact, overexpression of factors can provide important information about the mechanisms and even cause some interesting unnatural processes (for example, cell reprogramming, (Takahashi and Yamanaka 2006)). The chain of the reactions leading to the adipogenesis stimulated by C/EBPα, C/EBPβ and C/EBPδ seems to be important to investigate further. But overall, the conclusion about the precise role of the transcription factors in the cellular processes should be made avoiding the overexpression tests.

Taken together, non-adipogenic BM MSC progenitors can be converted to the adipogenic lineage, and non-adipogenic clones are not blocked from the adipogenesis by mutations, loss of the chromosomes and other stochastic reasons, because the differentiation can be stimulated potentially. The factors of C/EBP family C/EBPα, C/EBPβ, C/EBPδ are powerful regulators of adipogenesis.

5. Summary

Although the existence of mesenchymal stem cells in the bone marrow was confirmed years ago (Friedenstein, Petrakova et al. 1968), the identity of those cells still remains elusive, and the complex population of their descendants is the only available system to study the principles of differentiation and commitment into mesodermal lineages. At present, the conditions for the reliable expansion and maintenance of bone marrow mesenchymal stromal cells (BM MSCs) are not known, hence, the conditional immortalization provides an invaluable tool to study these cells. The transgenic mouse with improved tetracycline-regulated system of conditional immortalization using SV40 Large T-antigen was generated in our group previously (Anastassiadis, Rostovskaya et al. 2010). We confirmed the excellent properties of the Large T-antigen induction of those mice, and suggested as a tool for isolation of rare and low-proliferative cell types. We isolated BM MSCs from the transgenic mice and proved the maintenance of their identity. We showed the advantages of the system of conditional immortalization in (1) indefinite expansion of cell populations, (2) possibility to perform cellular cloning and (3) prevention from spontaneous differentiation *in vitro*.

We focused our attention on the heterogeneity of the BM MSCs population. We demonstrated that colony forming units-fibroblasts (CFU-F's) considered as close descendants of mesenchymal stem cells represent a heterogeneous population of progenitors with various differentiation potential. To our knowledge, this is a first report about the differentiation potential of the expanded mouse CFU-F's.

We identified 6 types of progenitors within BM MSCs population based on their differentiation potential ("OAC", "OA", "OC", "AC", "O", "A"). The diversity of the differentiation properties within a progenitor pool suggested a complex model of BM MSC hierarchy. We hypothesized that the imprint for each of the three mesenchymal lineages (osteogenic, adipogenic, chondrogenic) might exist in the progenitors independently.

The differentiation efficiency can be attenuated by the Wnt/β-catenin signaling pathway and GSK3 activity. We observed positive regulation of the osteogenesis and negative regulation of the adipogenesis in BM MSCs by the canonical Wnt. However, it is unlikely that Wnt is utilized endogenously by BM MSCs to trigger the cells to the differentiation.

We dissected the mechanism of adipogenic stimulation of BM MSCs *in vitro* and identified PPARγ1 as a key regulator of the adipogenesis in BM MSCs. By comparison of the adipogenic and non-adipogenic progenitors, we revealed that PPARγ1 expression determines the adipogenic potential of BM MSCs progenitors in the undifferentiated state. The demonstration of PPARγ1 role in the BM MSC cell fate determination adds more insight to the known functions of PPARγ1 in adipogenesis.

Finally, we could convert the non-adipogenic BM MSCs progenitors to the adipogenic lineage using ectopical expression of the transcription factors C/EBPα, C/EBPβ and C/EBPδ. The mechanisms of the adipogenic induction by C/EBP's have to be investigated further.

Taken together, we enclose two major values in our study. First, we validated conditionally immortalized progenitor cells from the transgenic mice as a model to study differentiation and lineage commitment. Second, we applied an approach to characterize a large spectrum of mesenchymal stem cell descendants in order to elucidate the mechanism of differentiation and determination. Our findings provide a novel insight into the molecular mechanisms of BM MSC lineage commitment.

6. Abbreviations

BM MSCs – bone marrow mesenchymal stromal cells

CFU-F – colony forming unit-fibroblasts

SV40 – simian virus 40

TAg – Large T-antigen

tet - tetracycline

(i)rtTA – (improved) reverse tetracycline transactivator

GBD* - mutated glucocorticoid bunding domain

Dex - Dexamethasone

Dox – Doxycycline

Tro - Troglitazone

Ins - Insulin

O – osteogenic potential or lineage

A – adipogenic potential or lineage

C – chondrogenic potential or lineage

CAFC – cobblestone area-forming cells

Wnt – wingless-type MMTV integration site

GSK3 – glycogen synthase kinase 3

TCF/LEF – T-cell factor/lymphocyte enhancer factor

PPAR – peroxisome proliferator-activated receptor

C/EBP – CCAAT/enhancer-binding proteins

LAP – liver activated protein

LIP – liver inhibitory protein

Klf5 – krueppel-like factor 5

Egr2 – early growth response 2

RUNX2 (Cbfa) – runt-related transcription factor 2 (core-binding factor a)

Osx - osterix

pRb – retinoblastoma protein

tsA58 – temperature-sensitive Large T-antigen

CD – cluster of differentiation

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Declaration according to §5.5 of the doctorate regulations

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from August, 2006, to July, 2010, under the supervision of Prof.Dr. Francis Stewart at Biotec, TU Dresden, Department of Genomics.

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