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“Selective Targeting of Tumor Cells and Protection of Mesenchymal Stem Cells”

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ZUSAMMENFASSUNG

Stromazellen sind Hauptbestandteil des Microenvironments und stehen im Verdacht, bei pathologischen Entwicklungen mitzuwirken, indem sie das Überleben, die Zellteilung und die Entwicklung der Tumorzellen unterstützen. Diese Zellen haben außerdem normale hämostatische, immunmodulierende und das Tumorwachstum supprimierende Fähigkeiten. Stromazellen stammen von mesenchymalen Stammzellen ab und unterliegen einer weiteren Differenzierung zu Osteoblasten, Chondrozyten, Adipozyten und Hämatopoese unterstützenden Stromazellen. Diese Zellen sind essenziell für Geweberegeneration, Wundheilung und Hämatopoese.

Die Anwendung einer Chemotherapie oder Radiotherapie sowie Zellalterung können zum Verlust der normalen physiologischen Funktionen oder gar zur Zerstörung dieser Zellgruppe führen. Daraus ergaben sich folgende Ziele, die wir in dieser Arbeit umsetzten:

1. Vorbeugung des Alterungsprozesses in aus dem Knochenmark stammenden mesenchymalen Stammzellen / Stromazellen durch Transfektion mit einer katalytischen Untereinheit des humanen Telomerase kodierendem Gen (hTERT).
2. Untersuchung des Effekts der hTERT Transfection auf die Differenzierungsfähigkeit der mesenchymalen Stammzellen / Stromazellen zu Osteoblasten und Adipozyten.
3. Untersuchung des Effekts der hTERT Transfection auf die Fähigkeit dieser Zellen, hämatopoetische Zellen zu unterstützen.

Die erhobenen Daten demonstrierten, dass Transfektion mit hTERT eine kurzzeitige (1-2 Wochen dauernde) Induktion der Zellteilung und dadurch einen Anstieg der Gesamtzellzahl bewirkt. Allerdings unterliegen die transfezierten Zellen einer normalen Alterung innerhalb der 4 Wochen Kultivierungszeit, die vergleichbar mit nicht transfezierten Zellen ist. Transfektion mit hTERT hat zudem keinen Effekt auf die Differenzierung der Knochenmark-Stromazellen zu Osteoblasten und Adipozyten sowie auf ihre Fähigkeit, das Überleben hämatopoetischer Zellen zu unterstützen.

Transfektion mit dominant negativen hTERT zeigte eine mäßige Hemmung der Osteogenese und eine starke Hemmung der Adipogenese. Wichtige Beobachtung war außerdem, dass hTERT Transfektion keinen Effekt auf den Zellphänotyp der Knochenmark-Stromazellen, sowie keinen Effekt auf die Expression der mit den mesenchymalen Stammzellen assoziierten Faktoren Oct4, Nanog, Lin28 und Sox4 hatte.

Zusammenfassend kann festgehalten werden, dass eine hTERT Transfektion der Knochenmark-Stromazellen zu einer Vorbeugung des Alterungsprozesses ohne Phänotypänderung und ohne Beeinträchtigung der normalen Funktionen führt und keine Umwandlung oder Immortalisation dieser Zellen hervorruft. Dies verdeutlicht die potentielle Möglichkeit, eine vorübergehende adenovirale Transfektion der mesenchymalen Stammzellen / Knochenmark-Stromazellen mit hTERT therapeutisch zu nutzen. Diese These bedarf jedoch weitergehender Untersuchungen und experimenteller Bestätigungen.

ABSTRACT

Stromal cells represent a major component of the tumor microenvironment. Although they may play a pathological role in supporting survival, proliferation and progression of tumors, these cells have normal homeostatic properties, immunomodulator and tumor suppressor functions. The stromal cells originate from mesynchymal stem cells (MSC) and undergo a process of differentiation and generate osteoblasts, chondrocytes, adipocytes and hematopoiesis supporting stromal cells. These cells are essential for tissue regeneration, wound healing and hematopoiesis. However, they might be damaged by chemotherapy, radiotherapy or senescence and loose their normal physiological functions. Therefore, the aim of this study was:

1. To investigate the value of transfection of bone marrow MSC/BMSC with the catalytic domain of human telomerase gene (hTERT) to prevent the process of aging in this cells.
2. To evaluate the effect of transfection with hTERT on the differentiation capacity of MSC/BMSC to generate osteoblasts and adipocytes
3. To study the effect of hTERT transfection on the hematopoietic supportive capacity of these cells

The obtained data demonstrated that hTERT transfection induces a short term (1-2 weeks) induction of cell proliferation and an increase in the number of bone marrow stromal cells. However, these cells undergo senescence process comparable to non-transfected cells within 4 weeks of continuous culture. The results also showed that transfection with hTERT does not affect the osteogenic and adipogenic differentiation of bone marrow stromal cells (BMSC) or their capacity to support survival of the hematopoietic cells. Transfection with dominant negative hTERT however, leads to moderate inhibition of osteogenic differentiation and a significant inhibition of adipogenic differentiation. Importantly, hTERT transfection does not effect the BMSC phenotype or the expression of MSC associated molecules including Oct4, Nanog, Lin28 and Sox4.

In conclusion, hTERT transfection may have the advantage of transiently preventing senescence of MSC/BMSC without affecting their phenotype and functions and does not lead to transformation or immortalization of these cells. This suggests that

transient adenovirus transfection of MSC/BMSC with hTERT may have a potential therapeutic application. This assumption will require further validation in extended studies beyond this diploma work.

INTRODUCTION

Malignant Diseases and Therapy

Cancer is the leading cause of death worldwide and accounted for 13% of all deaths in 2008 (WHO, 2011). Malignant diseases are very multifaceted and can affect any organ and any age group. Most common cancer types worldwide are lung, stomach, liver, colorectal and breast cancer (WHO, 2011).

Disease development is very variable in dependence of cancer type, patient age and personal disposition. Patients of the same age and with the same cancer type have sometimes very different therapy response and resulting lifespan. Commonly used types of treatment are chemotherapy, radiation therapy, surgical therapy, angiogenesis inhibitors therapy, bone marrow and peripheral blood stem cell transplantation. Nowadays new and different types of biological therapies and targeted cancer therapies have been implemented.

Chemotherapy is still the most common cancer therapy worldwide. Basis for this therapy type are cytostatic drugs which mostly affect cell growth, cell spreading or life span of the cells. This kind of drugs is not very selective and predominantly affects all kinds of high proliferative cells, therefore also healthy cells like mucosa cells, hair follicle, blood cells and mesenchymal stem cells are affected. In consequence patients face a variety of side effects such as defective hematopoiesis, anemia, bleeding problems, infections, as well as development of secondary diseases.

Depending on the cancer type and development stage a recurrence rate between 20 and 40% has to be expected. Secondary malignant diseases are more aggressive and highly resistant to common therapies and are very hard to handle. One of the possible solutions currently evaluated for these patients is the design of an individually adapted therapy for each patient with unique drug combinations and dosage.

Apart from this a supporting therapy will play a key role in the future cancer treatment.

Tumor Microenvironment

Cancer can develop from only one neoplastic cell. Surrounding tissues and soluble global factors such as hormones, growth factors and signaling molecules are essential for the progression of tumor growth. Today it is well known that cancer cells induce angiogenesis and therefore actively affect the surrounding tissues. Latest results in research show that mononuclear cells such as “T-” and “B-cells”, macrophages, “NK-cells” as well as stromal cells can infiltrate solid tumors and have pro malignance functions.

Besides some research groups demonstrated that gene expression of tumor cells is regulated by microenvironment factors (Witz et al 2009, Shehata et al 2010). Conclusively a tumor is a complex structure communicating with its surrounding environment and getting affected by it.

Tumor microenvironment can be defined as tumor supporting environment, which includes stromal components like stromal fibroblasts, mesenchymal stem cells, immune cells, blood cells, extracellular matrix and soluble molecules like cytokines and chemokines (American Association for Cancer Research, Matrisian et al. 2007).

Communication between cancer and its microenvironment involves three basic processes:

- Effect of soluble molecules (growth factors, immunoglobulins, hormones, etc.)
- Effect of microenvironment on cancer cells by cell – cell contact and cell - extracellular matrix contact
- Effect of cancer cells on microenvironment by cell – cell contact and cell extracellular matrix contact

This communication is responsible for the transformation from benign to malignant and from malignant to metastatic, as well as for the response to the chemotherapy and the development of a possible drug resistance (Shehata et al 2010).

The awareness of these processes makes it necessary to establish an in vitro drug testing model based on co-culture between cancer cells and the microenvironment according to the situation in vivo. Containing a variety of different components, microenvironment has a complex structure which needs to be simplified for in vitro experiments. Therefore we focused our research on stromal cells as a key player.

Stromal cells affected by cancer cells support their survival and proliferation, neglecting their normal functions such as supporting haematopoiesis and cell proliferation. A small part of these stromal cells (mesenchymal stem cells) still have the capacity to differentiate into specific tissue cells such as adipocytes, osteoblasts and chondrocytes (Prockop et al 1997), making them especially important for tissue repair functions in adults (Caplan et al 1991).

Destruction of these cells by chemotherapy means loss or at least strong inhibition of tissue repair capabilities, leading to visible symptoms of tissue repair failure and anemia after therapy (Paukovits et al 1990). In consequence a transplantation of own cell material might be a promising supporting therapy for cancer patients. Furthermore such tissue regeneration could be used for patients with fibrosis and wound healing defects and patients suffering from other degenerative diseases.

As stromal cells have only a short living period and a low proliferation rate in vitro non transformed stromal cell strains had to be developed by transient activation of hTERT catalytic domain. The hTERT activation should be temporary to preserve normal cell phenotype and avoid immortalization, transformation or the generation of uncontrolled growth or malignancy.

Mesenchymal Stem Cell (MSC)

Multipotent stem cells were proved to be present in almost every organ – skeletal muscle, fat, peripheral blood, bone marrow, etc. – as some kind of natural emergency service responsible for tissue repair.

In adults, the main source of stem cells is the bone marrow which contains two types of stem cells:

- HSCs – hematopoietic stem cells and
- MSCs - mesenchymal stem cells

Mesenchymal stem cells represent a very small population in the bone marrow, around 0,001% to 0,01% of isolated cells (Pittenger et al, 1999).

Mesenchymal stem cells may proliferate very slowly in vivo without differentiation. However, more information is needed to define their phenotype in vivo because all studies so far have been performed in vitro on isolated cells (Mesanobu Ohishi et al,

2010). For our research we focus on bone marrow mesenchymal stem cells (MSC) and bone marrow stromal cells (BMSC) which represent an early stage of differentiation of MSC isolated from bone marrow aspirates from CLL patients.

According to Friedenstein´s findings bone marrow MSC have fibroblast like morphology, adhere to plastic and are able to form confluent monolayers in vitro. They were identified to express a number of surface markers and to be

- positive for CD73, CD90, CD44, CD29, STRO-1, CD166, CD117 and CD105 and
- negative for hematopoietic and endothelial markers such as CD14, CD19, CD34, CD45, CD31, CD33, CD133 and HLA-DR (Bobis S. et al, 2006).

MSC are multipotent and have the ability to differentiate into adipocytes, chondrocytes, osteoblasts, myoblasts and ligament fibroblasts in vitro (Mesanobu Ohishi et al, 2010). MSCs have a high proliferation rate in vitro a few days after isolation. Bobis et al (2006) describe three phases, a lag phase 3 - 4 days after plating, a log phase and a stationary phase where the cells stop proliferation. This prevents further expansion of MSC and new approaches are needed to overcome this stationary phase without immortalization of MSC.

Telomeres and Replicative Senescence

Telomeres are complexes of G-rich, repetitive DNA sequences and special proteins, which protect DNA ends of chromosomal DNA from enzymatic attacks and shortening during replication (Ramiro et al, 2007). These DNA sequences were identified to be involved in life period limitation and cell senescence induction (Kong et al 2011). Continuous mitotic telomere shortening seems to be a natural hind of cancer prevention. Cancer, stem and germ line cells undergo a limitation of replication rounds by telomerase activity, an enzyme that synthesizes new telomeric repeats from a RNA template, or in some cases by alternative telomerase independent mechanism based on telomeric recombination.

Loss of telomeres means destabilization of the genome, such as chromosome fusion, increased level of recombination, growth arrest and cell death (Ramiro et al, 2007). Cells with critically short telomeres become growth arrested and undergo senescence losing their ability to respond to growth factors and to change their morphology and phenotype (Goldstein et al 1990). These changes are demonstrated in figure 1. Senescent cells stop proliferation and shut down their metabolic activity.

The reasons of senescence seem to diversify depending on species or maybe cell type. Human fibroblasts have telomere length dependent senescence, whereas mouse fibroblasts undergo senescence due to oxidative stress (Kong et al 2011).

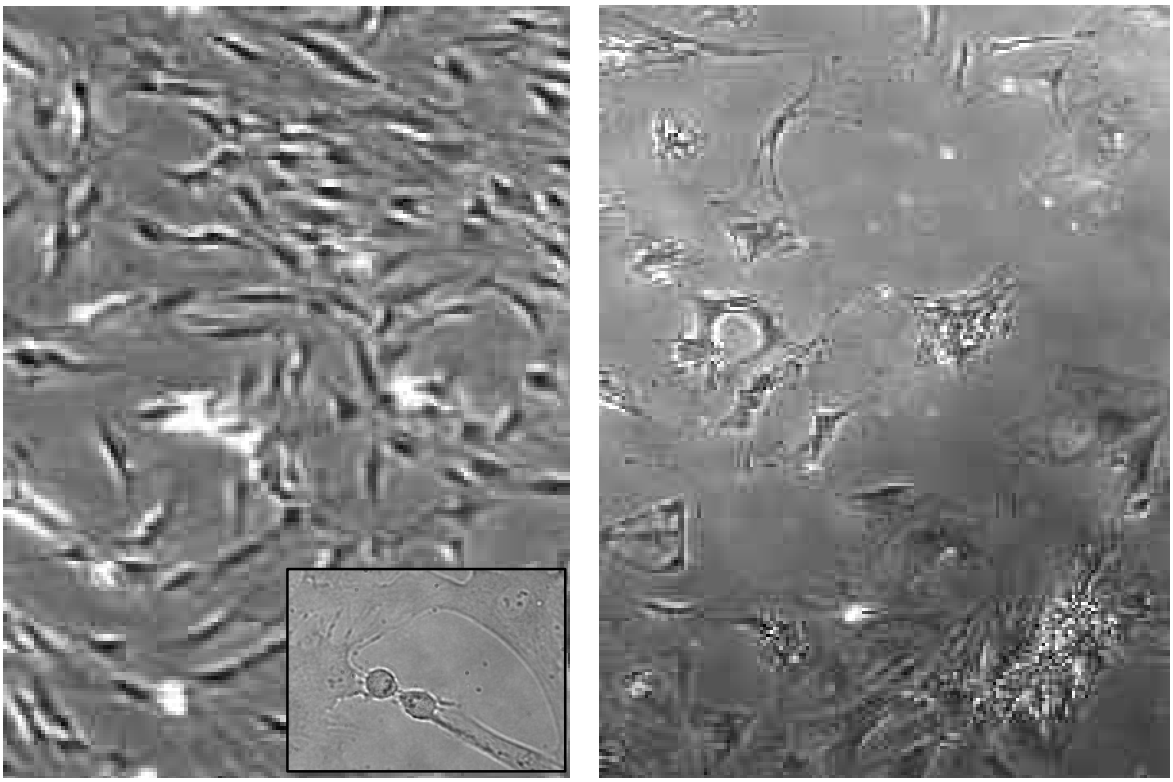


Figure 1: *Morphological difference between early and late passages bone marrow stromal cells (BMSC). Left image: BMSC in passage #2: and the inset demonstrates cells in mitosis. Right image demonstrates BMSC in passage #11. Primary fibroblasts in earlier passages have spindle form and undergo mitosis. Primary fibroblasts in higher passages have changed morphology, showing typical fried egg form and stop proliferation.*

Senescence Exit and Cell Immortalization

Spontaneous cell immortalization is the main step in cancer formation (Shay et al 1991). Immortalized cells escape senescence and apoptosis, as well as proliferation control and life period limitation. Immortalization is always associated with circumvention of the telomere replication problem. 90% of cancer cells are immortalized by the activation of telomerase genes normally inactive in somatic cells, 10% use a telomerase independent pathway of telomere elongation (Remiro et al, 2007).

The determination of cell immortalization in vitro is an important step for understanding cancer biology and development. Immortalized cell lines can be used as a model for drug testing and marker set establishment.

Common ways of in vitro cell immortalization are:

- Chemicals – carcinogenic agent
- Physical treatments – x-rays
- Oncogene activation
- DNA tumor viruses – HPV, SV 40, adenoviruses
- Retrovirus transfections
- Tumor suppressor genes inactivation (Shay et al 1991).

However, the major goal of this diploma work is to find methods for prolonging the life span of BMSC, prevent early senescence and maintain their normal physiological properties without inducing immortalization or transformation processes in these cells.

hTERT Activation

Human telomerase reverses transcriptase - enzyme responsible for telomere prolongation – consists of a protein domain (hTERT) and a RNA domain (hTR). The RNA domain contains a RNA template (TTAGGG) to be added to telomere ends. hTERT is the catalytic subunit of telomerase and limits its activity (Misiti et al, 2000).

Telomerase is known to be expressed in embryonic tissues and most cancer cell types. Somatic cells have no detectable telomerase activity except of germ line cells, stem cells and lymphocytes (Krikpatrick et al, 2004).

Tissue specific regulation of hTERT activity is probably linked to transcriptional regulation. Nevertheless a number of alternatively spliced hTERT mRNA as well as hTERT associated proteins, which have the ability to induce hTERT activity in vitro, were detected in different tissues and species (Ulaner et al, 1998).

Cell differentiation correlates with a loss of hTERT activity, which seems to be a natural prevention of malignancy in adult tissues. This hypothesis is backed up by the observation that induction of differentiation in immortalized cell lines also provokes loss of telomerase activity (Sharma et al, 1995).

Adenovirus Transfections

Human adenoviruses (hAdVs) of the *Adenoviridae* family, are non-enveloped, ssDNA containing, icosahedral viruses, transmitted via fecal-oral or respiratory routes and responsible for over 10% of the respiratory tract infection in children (Pabbaraju et al, 2011).

In molecular biology adenoviruses (Ad) are used for in vivo and in vitro gene transfer in different cell types and tissues. Adenoviruses can carry about 7,5 kb of foreign DNA, guarantee efficient uptake and allow reporter genes to be under the control of tissue specific elements (Kass-Eysler et al, 1993). These qualities allow the application of this vector for gene therapy. Adenovirus infection requires receptor mediated uptake of virus particles. The limitation of adenoviruses as vectors for gene therapy is inflammatory response based toxicity (Rogee et al, 2010) and a lower uptake rate in vivo.

Nevertheless, adenoviruses have a great advantage over other viral vectors as they do not integrate into the host genome eliminating the risk of tumor formation and reactivation of viral transgenes (Stadtfeld et al, 2008).

CLL (Chronic Lymphocytic Leukemia) microenvironment model

In order to understand the effect of transfection of BMSC with hTERT and the effect on the interaction between the hematopoietic cells and stromal cells, we applied our

CLL-microenvironment model. In this model we have demonstrated the dependence of CLL cells on bone marrow stromal cells for survival in vitro (Shehata et al 2010). CLL is a slowly developing malignant disease of blood and bone marrow. It is one of the most common leukemia's in Europe and occurs in higher frequencies in middle aged and aged patients. Symptoms and prognoses vary from patient to patient with some having no clinical signs of disease and not showing any life period limitation, unfortunately others need aggressive therapy and frequently die during the first five years after initial diagnosis (Byrd et al 2004).

CLL cells are malignant B-cells which typically co-express CD19, CD5 and CD23 antigenes. B-cells are immunoglobulin producing cells, developed from hematopoietic stem cells in the bone marrow and differentiate in the spleen. Two forms of CLL exist depending on the mutation status of the heavy chain of immunoglobulin (IgVH) genes.

CLL cases with IgVH mutations have a better prognosis and are referred to as mutated CLL (Stevenson et al 2004). CLL cells of unmutated CLL have no mutations in the IgVH region and have a poorer prognosis. Both CLL types have an imbalanced expression of pro- and anti-apoptotic genes and may show different mutations in tumor suppressor genes (p53), as well as mutated karyotype also correlating with the prognosis (Kalil et al 1999).

CLL cells can be easily isolated from peripheral blood and are applied in this work as a suitable experimental model for exploring the interaction between hematopoietic cells and BMSC.

Aims of the Study

This study was initiated to explore the value of transfecting bone marrow stromal cells/ bone marrow mesenchymal stem cells with hTERT using transient transfection with adenovirus which is not integrated in the DNA. The major aims of this study are as follows:

1. To test the effect of temporary hTERT activation or inactivation on life span and proliferation capacity, senescence and gene expression of BMSC.

2. To study the effect of hTERT transfection on the phenotype, gene expression and differentiation capacity of MSC/BMSC.

3. To explore the effect of hTERT transfection on the supporting capacity of BMSC to hematopoietic cells using CLL cells in co-culture as an experimental model.

4. To evaluate the therapeutic potential of hTERT transfected mesenchymal stem cells as supporting therapy in patients receiving chemotherapy or radiotherapy.

Work Plan

1. Transfection of BMSC with wild type or dominant negative hTERT

2. Long term and short term observation of the proliferation rate of BMSC by cell count and viability monitoring by MTT assay to demonstrate the effect of hTERT inhibition and activation on BMF lifespan.

3. Perform FACS analysis to study the effect of adenovirus transfection with hTERT on BMSC phenotype using antibodies against CD90, CD45, CD13, CD105, CD73 and STRO 1 and to compare their phenotype to retrovirus immortalized cell lines.

4. Using RT-PCR and western blotting to explore the effect of hTERT transfection on the expression of specific markers related to mesenchymal stem cells on RNA and protein levels.

5. The supportive capacity of hTERT transfected BMSC to hematopoietic cells will be evaluated using co-culture of CLL cells with hTERT transfected BMSC and cell viability will be monitored by Annexin V/PI staining and FACS analysis.

6. To study the effect of transfection on senescence of BMSC using ß-Gal assays and the effect on the differentiation of BMSC using osteogenic and adipogenic differentiation assays.

RESULTS

1. Effect of transfection with hTERT AV on the proliferation, viability, and phenotype of bone marrow stromal cells:

Bone marrow stromal cells (BMSC) were transfected with either eGFP vector, hTERT wild type, hTERT dominant negative vector as described in details in the materials and method section. As shown in figure 2, the transfection efficacy ranged between 60-80% as evaluated by fluorescence microscopy of eGFP transfected cells.

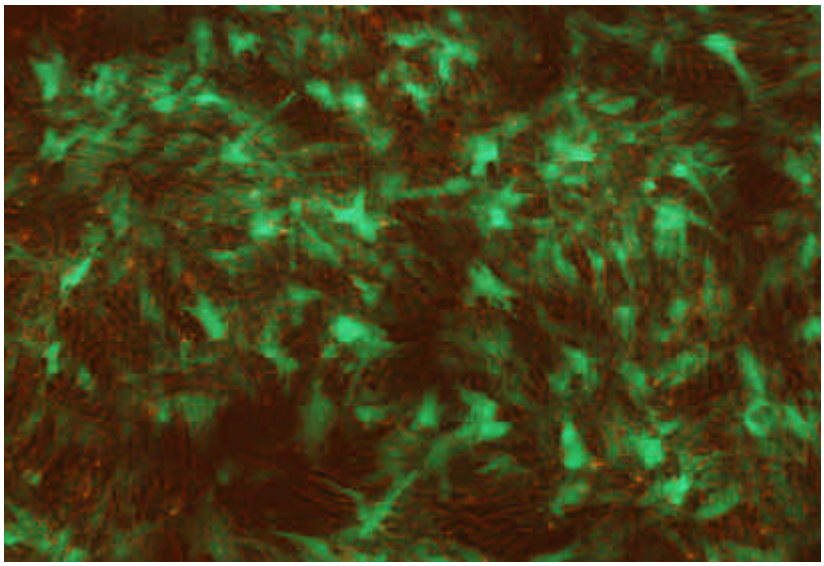


Figure 2: *Evaluation of the eGFP transfection rate via fluorescence microscope observation*. *Transfected cells fluoresce green, non transfected cells are seen in the background*.

A. hTERT transfection induce a short term increase in the proliferation of BMSC:

The influence of hTERT transfection on the proliferation rate was studied on 7 primary human BMSC strains in passage 1 - 4 isolated from bone marrow of CLL and ALL patients. One day after transfection, BMSC were trypsinized and cell count was performed using “Coulter” cell counter. Equal cell number of $1x10^6$ cells were plated in $75cm^2$ culture flasks and culture were continued. Estimation of cell number started

2 - 3 days after transfection and controlling of cell numbers continued every 3 – 4 days thereafter along the observation time of 40 days.

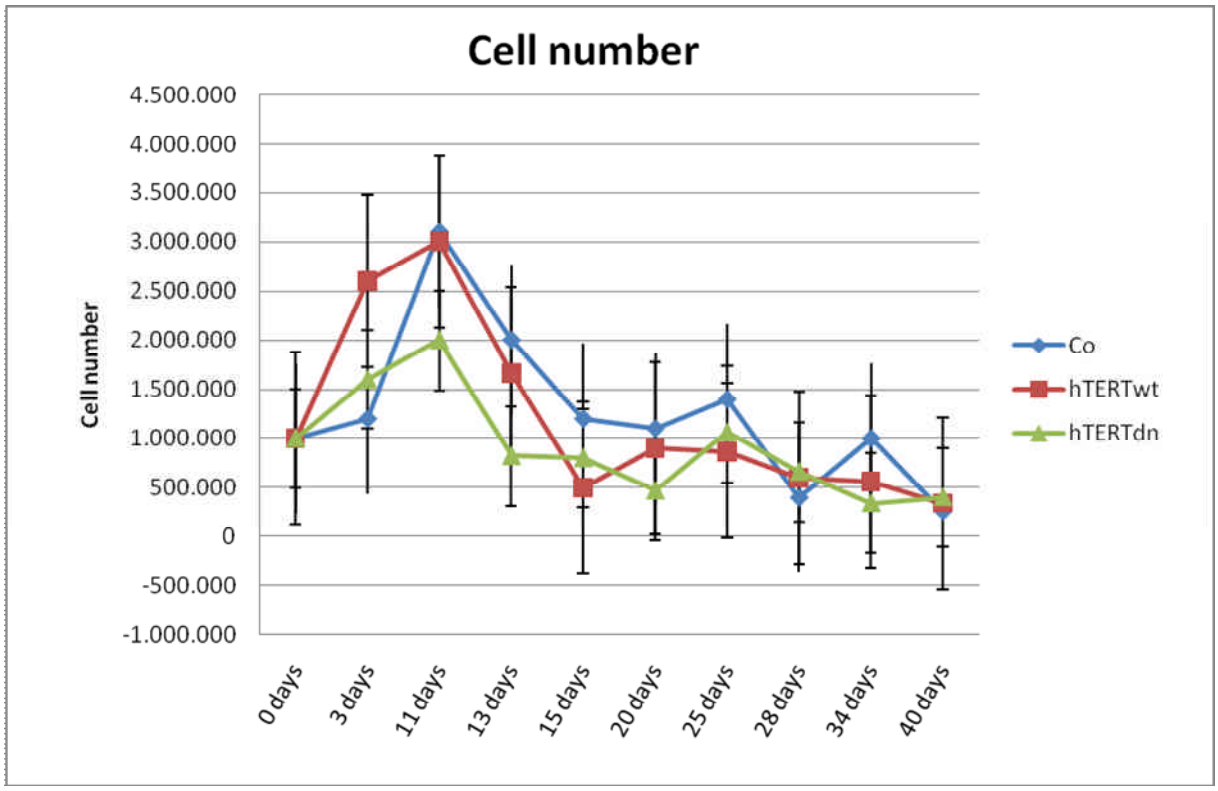


Figure 3A: *Long term effect of hTERT on cell number: Co: untransfected control cells, hTERTwt: hTERT wild type and hTERTdn: hTERT dominant negative) transfected cells.*

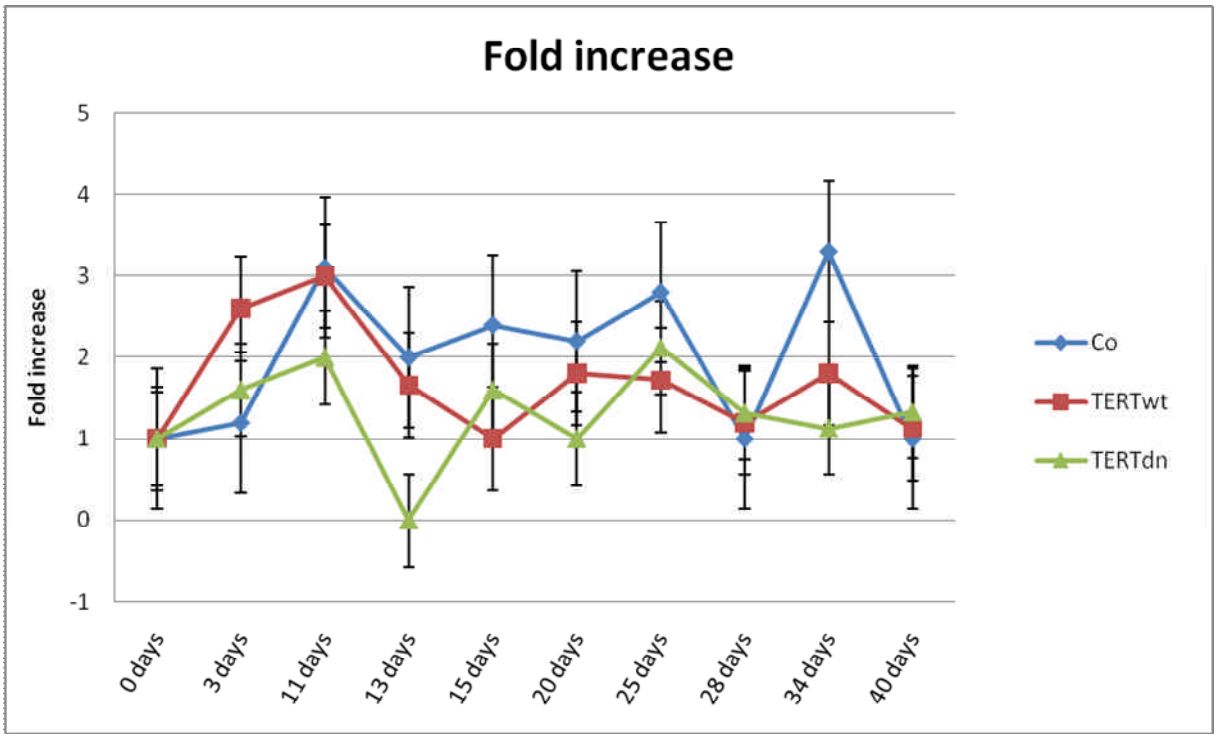


Figure 3B: *Long term effect of hTERT: Fold increase of cell number Co: untransfected control cells, hTERTwt: hTERT wild type and hTERTdn: hTERT dominant negative transfected cells.*

As shown in figure 3A, a remarkable increase in cell number was observed after 3 days of transfection with wild type hTERT compared to the non-transfected or hTERT DN transfected cells. The stimulatory effect of hTERTwt was also demonstrated by the fold increase in cell number particularly after 3 days of transfection (figure 3B). The proliferation rate of cells transfected with hTERTdn appeared to be lower in most of the cases. It is important to note that the proliferation rate of transfected cells returned to the base line as compared to the non-transfected cells during the second week of observation and remained comparable to the control BMSC thereafter.

In order to confirm this observation, another set of BMSC samples was transfected and closely observed for 9 days. As shown in figure 4A and 4B a transient increase in cell number is observed after 3 days of transfection with hTERTwt. In these experiments, the proliferation rates of control cells and cells transfected with hTERTdn were comparable.

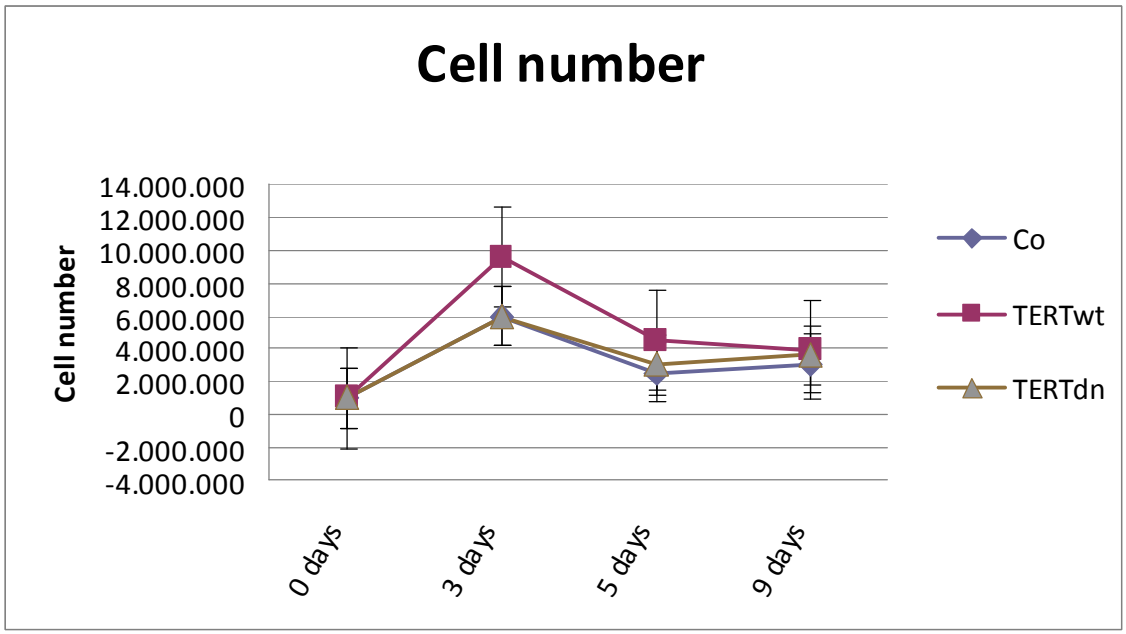


Figure 4A: *Short term effect of hTERT transfection on cell number: Co: untransfected control cells, hTERTwt: hTERT wild type and hTERTdn: hTERT dominant negative transfected cells.*

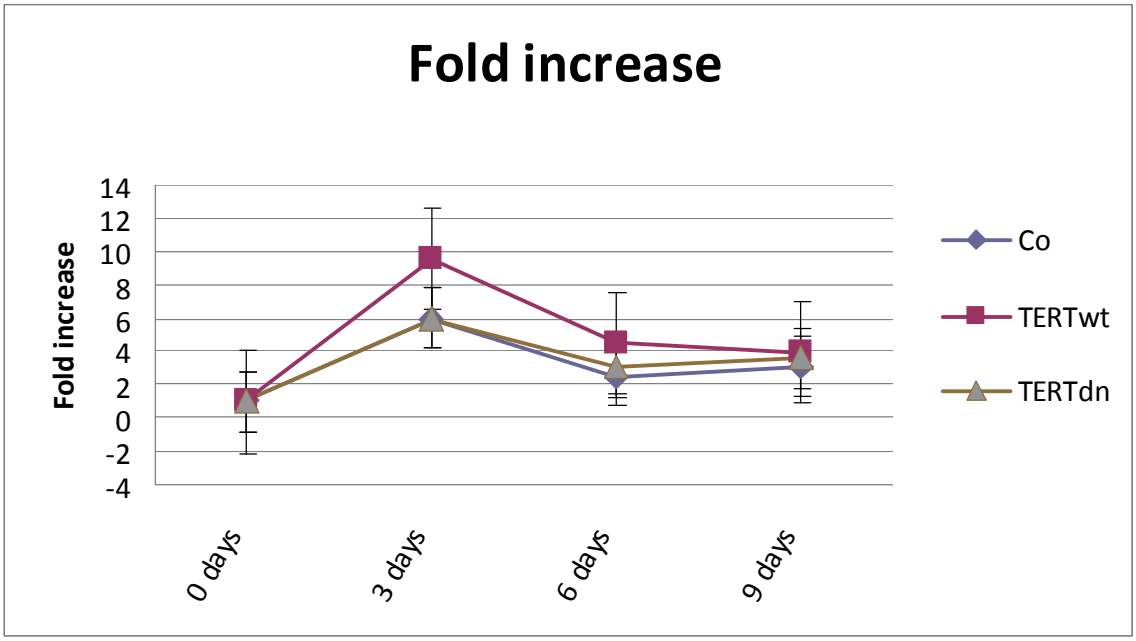


Figure 4B: *Short term effect of hTERT: Co: untransfected control cells, hTERTwt: hTERT wild type and hTERTdn: hTERT dominant negative transfected cells.*

Taken together, these experiments demonstrate that hTERTwt transfection provides BMSC with a short term proliferation advantage over the non-transfected cells. At the

same time the results also demonstrate that hTERTwt transfected cells do not undergo immortalization or transformation and retain a normal proliferation rate within 1-2 weeks after transfection.

B. Effect of hTERT transfection on senescence marker (ß-Gal):

The loss of the proliferative ability normally correlates with the differentiation stage and the cell senescence. The senescence stage was detected by using beta-galactosidase assay. ß-galactosidase activity is a marker for senescent cells and is not detectable in resting or immortalized cells. ß-Gal activity was detectable 3 - 5 weeks after transfection only in cells transfected with hTERTdn, but not in the control samples or after transfection with hTERTwt. In parallel to ß-Gal activity, microscopic observation showed morphological changes associated with cell senescence (see Figure 5).

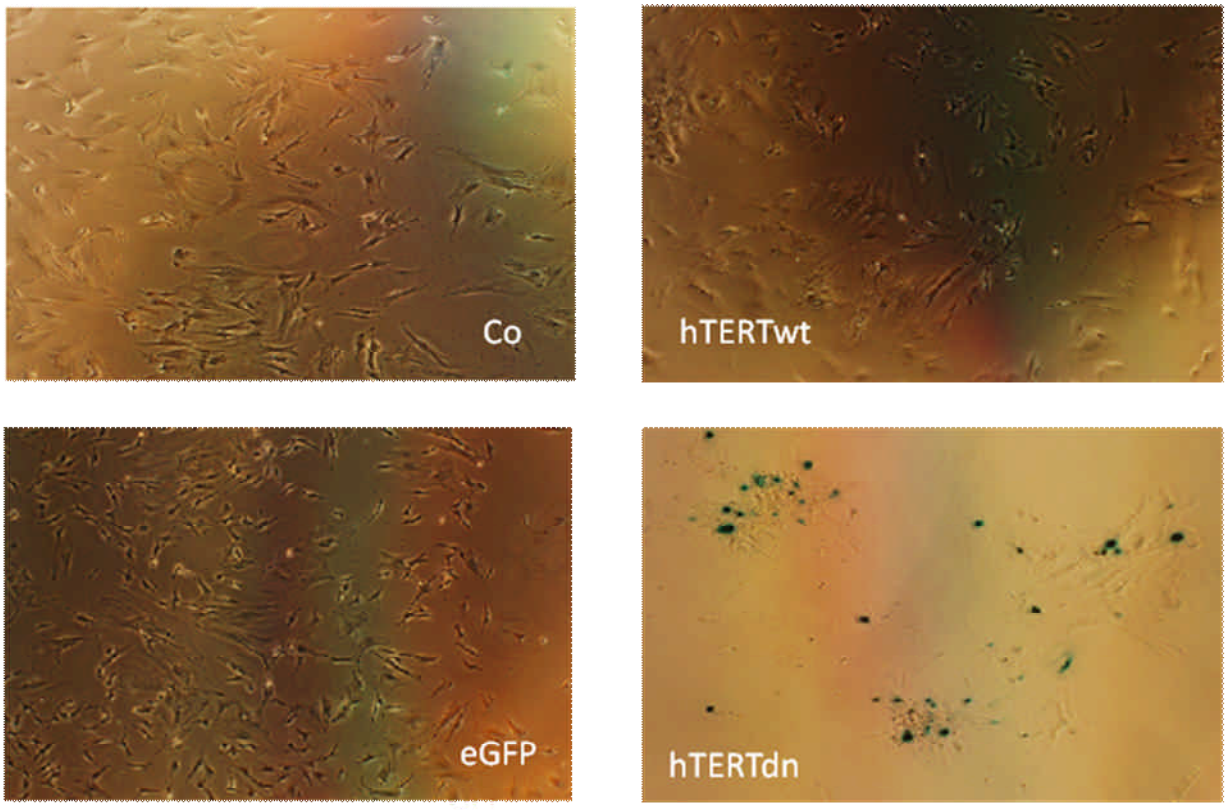


Figure 5: *Beta- galactosidase is an enzyme that is active only in senescent cells, and induces in a blue precipitate formation in a mixture of beta-gal substrate. Blue staining was detected only in hTERT deactivated cell strains, other cell types showed no precipitate formation.*

C. hTERT transfection dos not affect the BMSC Phenotype

In order to explore whether AV-hTERT transfection may have an effect of BMSC phenotype FACS analysis was performed using typical markers of bone marrow stromal cells which share similarities with mesynchymal stem cells being CD13+, CD73+, CD90+, and CD105+ while being CD45-. hTERT transfected and non transfected cells, primary BMSC strains, and hTERT retrovirus immortalized cell line (NK.tert) were compared to each other.

As shown in the Figure 6A and 6B no obvious changes in the phenotype of BMSC could be detected after transfection with hTERT adenovirus based on the set of markers used in this study. The detected mean fluorescence level shown in the Figure 6C, as direct indicator for the level of surface marker expression, demonstrated that control cells, transfected cells and primary BMF strain are about 80% more positive for CD90 and CD73 than immortalized cell line NK.tert.

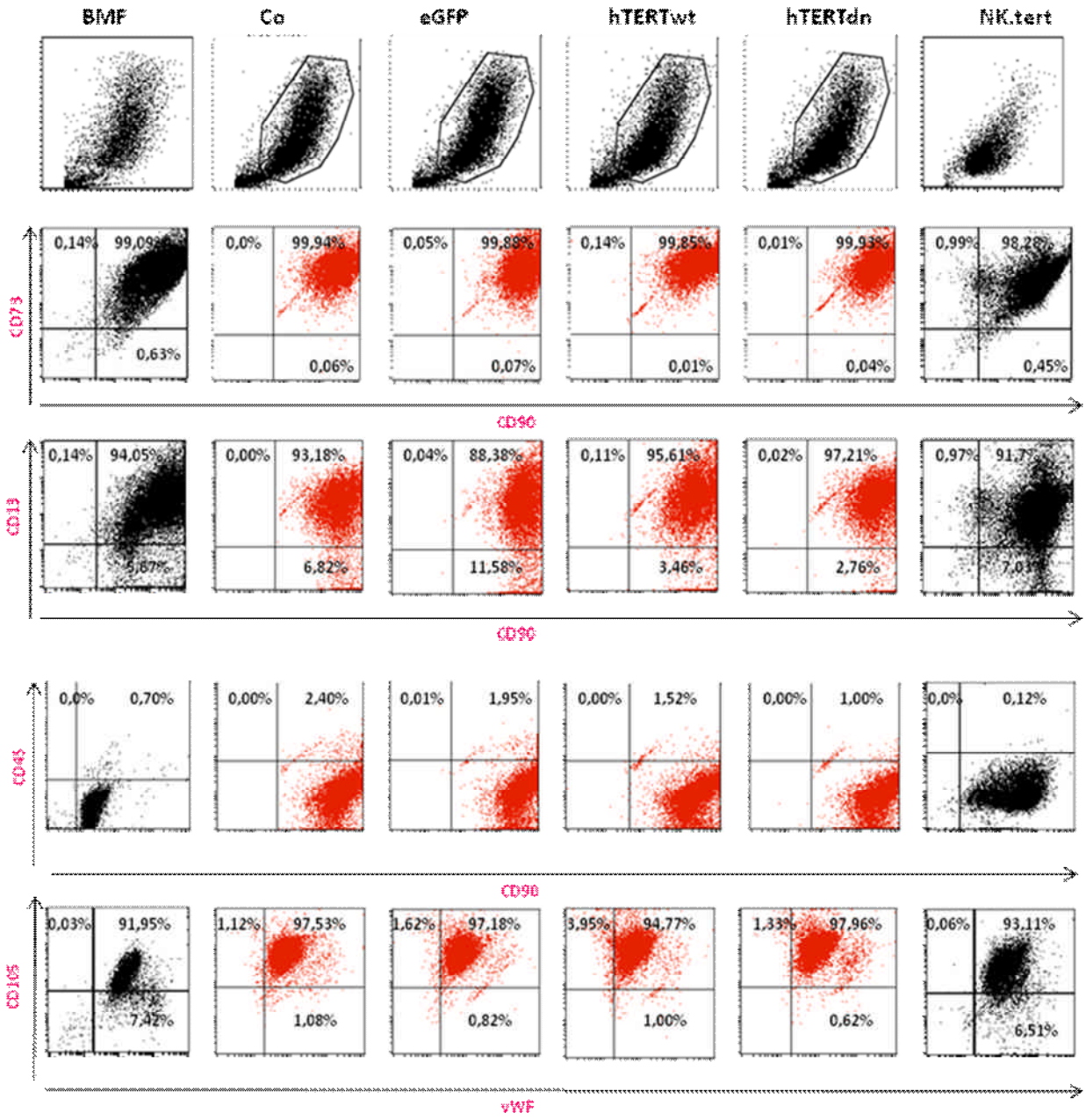


Figure 6A: *FACS analysis using the major markers of MSC (CD73+, CD90+, CD13+, CD105+) demonstrates that the transfection with hTERT may not significantly change the phenotype of BMSC. CD45 is used as marker for hematopietic cells which is not expressed on MSC.*

Markers	BMF	Co	eGFP	hTERTwt	hTERTdn	NK.tert
CD73+/CD90+	99,1	99,9	99,9	99,9	99,9	98,3
CD13+/CD90+	94,1	93,2	88,4	95,6	97,2	91,7
CD13-/CD90+	5,7	6,8	11,6	4,3	2,8	7,0
CD105+/vWF+	92,0	97,5	97,2	94,8	98,0	93,1
CD45+/90+	0,7	2,4	2,0	1,5	1,0	0,1
CD45-/90+	98,6	97,6	98,0	98,3	99,0	99,5

Figure 6B: *Summary of the FACS analysis data obtained from the dot plots shown above, confirming the phenotype similarity between untransfected and AV-hTERT or RV-hTERT transfected cells*

	BMF	Co	eGFP	hTERTwt	hTERTdn	NK.tert
CD73	1103,5	1016,8	1234,1	1277,4	1067	496,9
CD90 MFI	3399,7	3026,1	3923,7	3190,7	3370,8	1785,1
CD13 MFI	446,5	334,2	539,2	613,5	426,1	291,2
CD105 MFI	190	111,2	102,5	93,1	144,4	162,1

Figure 6C: *Mean fluorescence intensity obtained from FACS analysis confirming the similarities between untransfected primary BMSC and AV-hTERT or RV-hTERT transfected cells.*

In summary the AV-hTERT transfection had no effect on the phenotype of BMSC in terms of the surface expression of CD13, CD73, CD90 and CD105. However RV-hTERT transfection appeared to decrease the MFI of CD13, CD73 and CD90 surface expression.

D. Effect of hTERT transfection on the expression of MSC related genes:

To explore whether hTERT transfection may have effect on stem cell related genes or may induce reprogramming of BMSC, the following set of investigations was performed. Markers or transcription factors Oct4, Nanog, Sox2 and RNA binding protein Lin28 were identified to be the minimal set for the detection of pluripotent

stem cells. J. Yu from Thompson Laboratories (Yu J. et al 2007) used this factor set for reprogramming adult differentiated cells to pluripotent embryonic-like cells. Additionally a number of experiments and findings indicate the expression of these factors to be the key parameter for the identification of the cell differentiation stage. Furthermore they have been detected in different cancer types and may indicate malignancy (Ben-Porath I et al 2008).

Therefore, we investigated the effect of hTERT transfection on the expression of Oct4, Nanog, Sox2 and Lin28 as a marker for stages of differentiation or de-differentiation of BMSC/MSC. The optimal conditions and primers for RT-PCR analysis for each of the used markers were established.

As shown in figure 7A, RT-PCR analysis demonstrated that early passages of primary BMSC/MSC as well as NK.tert cells were positive for all four markers. However, the expression of these markers significantly decreases after 6 weeks in culture. As shown, the control cells expressed only Oct4 while hTERTwt or hTERTdn transfected cells showed positive, but relatively weaker expression of Oct4, Nanog and Sox2 mRNA compared to the primary BMSC and NK.tert.
The detection of an increased expression level for all four markers particularly Oct4 and Nanog in the primary BMF strain from passage 1 was unexpected. The detected expression level was higher than in immortalized cell line NK.tert.

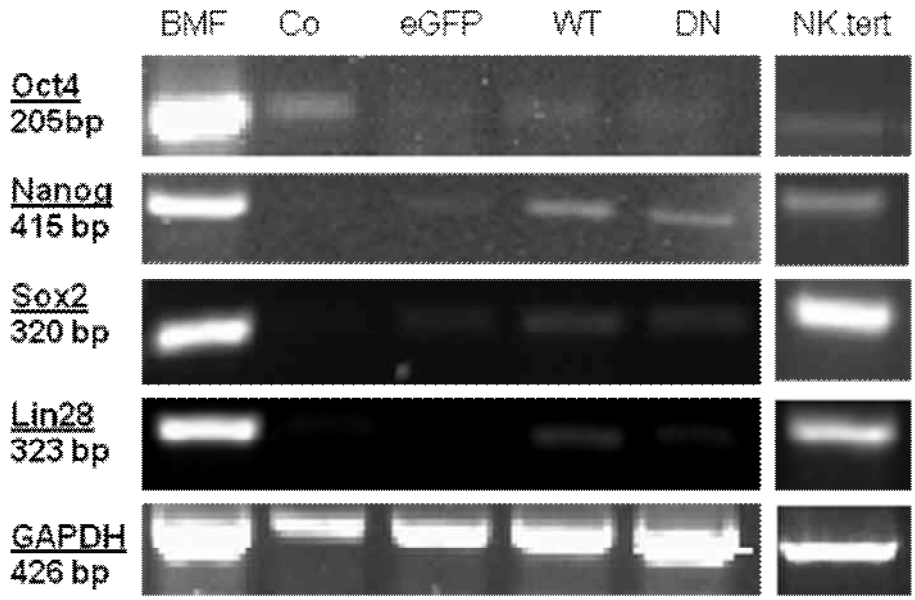


Figure 7A: *Stem cell markers expression on mRNA level. PCR under established conditions (for details see chapter "Methods").*

Western blot was established for the detection of Oct4, Nanog and Sox2 as described in details under "Method section". As shown in the figure 7B the detected protein level generally supported the data obtained by PCR (see Figure 7A). Oct4 was detectable in primary BMSC, and after transfection with hTERTwt or hTERTdn as well as in the embryonic kidney fibroblast cell line (HEK239). Sox2 was highly expressed in primary BMSC and in HEK239 cells but was undetectable or weakly expressed after 6 weeks in culture. Nanog was mainly detectable in early passage BMSC and remained detectable after 6 weeks in culture. NK.tert cell line was positive for all used markers.

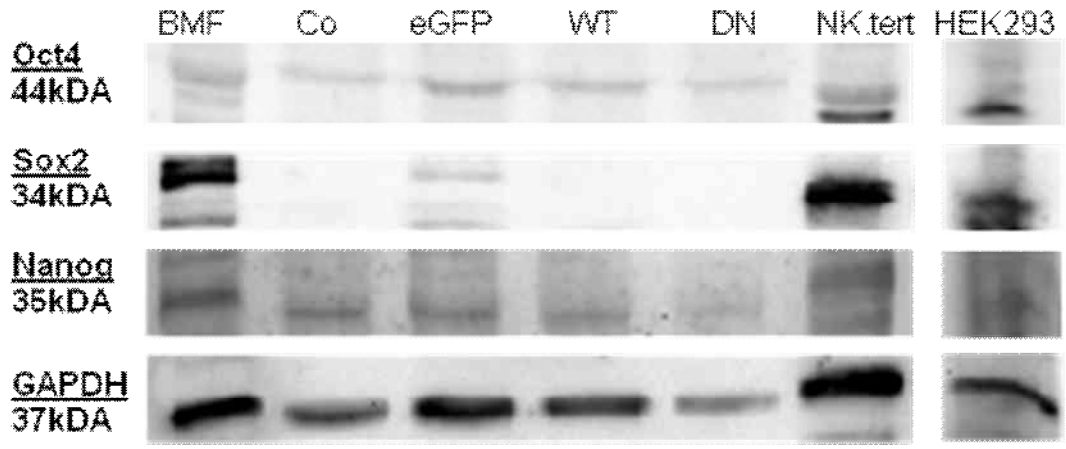


Figure 7B: *Detection of mesenchymal stem cell markers expression by Western Blot.*

Summary:

Figure 7A and 7B summarise evalueted data for stem cell marker expression. The observed signal intesity was graded as high (+++) or low (+) or undetectable (-). The data shows that the early passage of BMSC may retain the MSC-related genes and proteins (Oct4, Nanog, Sox2 and Lin28). Those MSC markers will be decreased or lost after several passaging of the cells or prolonged cell culture.

Cell type	Oct4	Nanog	Sox2	Lin28
BMF (passage 2)	+++	+++	+++	+++
Co	+	-	-	-
eGFP	+	+	+	+
hTERTwt	+	+	+	+
hTERTdn	+	+	+	-
NK.tert	+	++	+	+

Figure 8A: *Mesenchymal stem cell marker expression on the RNA level.*

Cell type	Oct4	Nanog	Sox2
BMF	++	+++	+++
Co	+	+	-
eGFP	+	++	++
hTERTwt	+	++	-
hTERTdn	+	+	-
NK.tert	+++	+++	+++
HEK293	+++	+++	+++

Figure 8B: *Mesencymal stem cell marker expression on the protein level.*

E. Effect of hTERT on the differentiation capacity of BMSC:

Differentiation ability is considered to be one of the main characteristic of pluripotent stem cells. We applied adipogenic and osteogenic differentiation to evaluate the influence of hTERT transfection on differentiation ability.

Adipogenic Differentiation: Figure 9 shows a representative experiment out of 3 carried out. Two of these experiments were performed with cells from a long-term (30 days) and one from a short-term (14 days) experiment. The presented images were taken 4 - 5 weeks after the start of the experiment. The evaluated data indicated that adenovirus hTERT activation had no influence on the adipogenic differentiation. Control cells, hTERT and eGFP transfected cells differentiated normally at a comparable rate of adipogenesis. However, transfection with hTERTdn showed a strong inhibition of adipogenesis and indicated a loss of adipogenic differentiation ability.

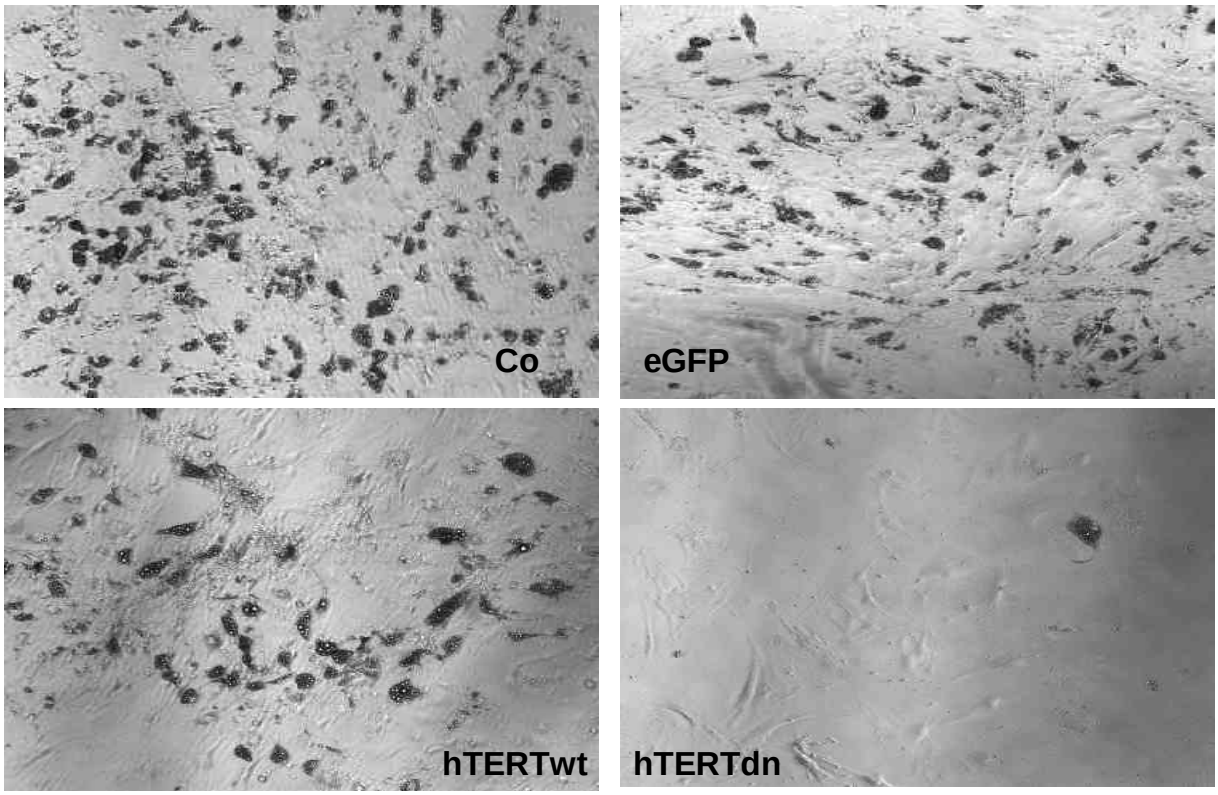


Figure 9: *Adipogenic differentiation. Control cells, eGFP and hTERT transfected cells had a comparable adipogenic capacity, hTERT deactivated cells exhibited a strong inhibition of adipogenic differentiation ability.*

Osteogenic Differentiation: Results of the osteogenic differentiation are summarized in the Figure 10 showing a representative experiment of three performed experiments and indicated no influence of hTERT transfection on the differentiation ability. All cell types differentiated normally with a comparative rate of osteogenesis. However, a relatively weaker staining was observed in hTERTdn transfected cells suggesting that active hTERT might be essential for full osteogenic differentiation capacity.

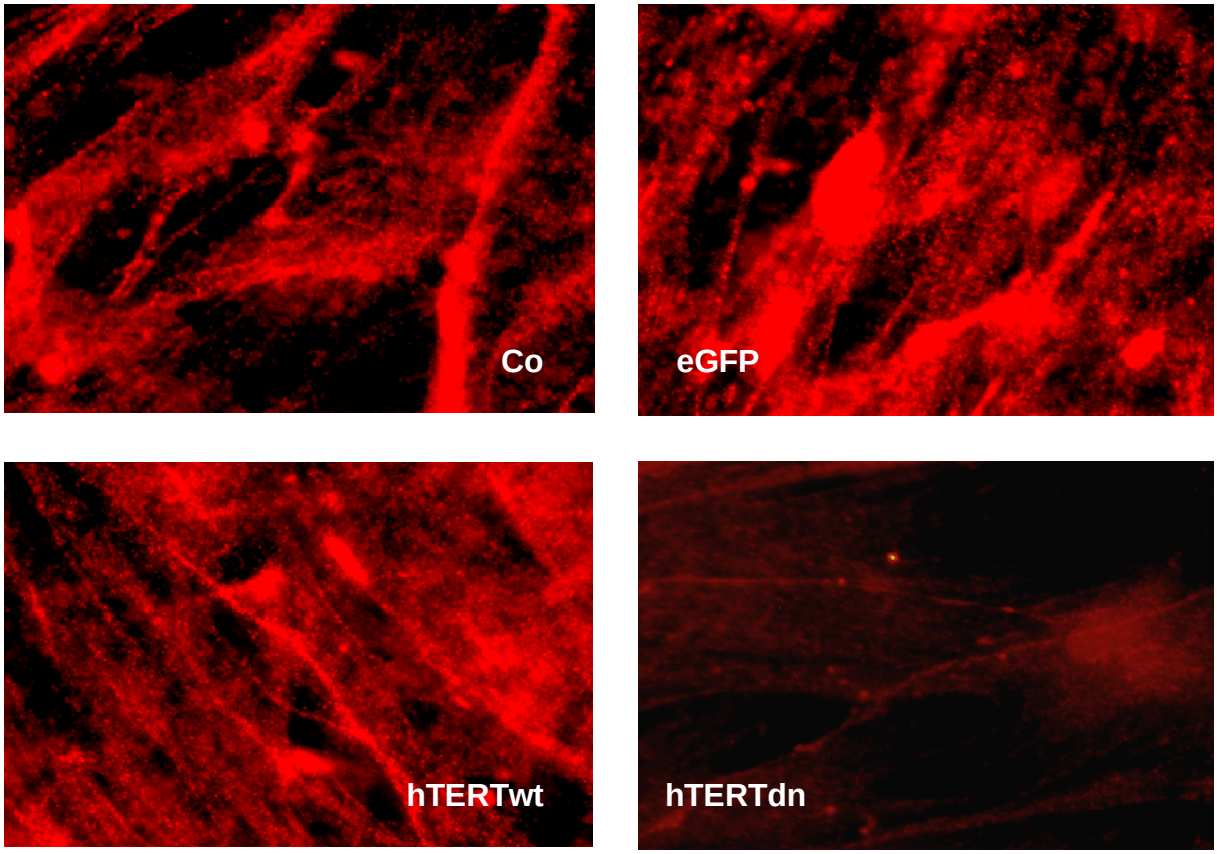


Figure 10: *Osteogenic differentiation. All cell types differentiated with a comparative rate of osteogenesis with exception of hTERTdn transfected cells.*

2. Effect of hTERT transfection of hTERT gene and protein expression:

To explore the effect of hTERT transfection we examined hTERT expression on the RNA and protein levels.

A. RT-PCR:

As shown in figure 11A, after 4 weeks of transfection, control BMSC, eGFP transfected cells as well as early passage BMF expressed low levels of hTERT mRNA while hTERT tarnsfected cells and NK.tert (retrovirally transfected cells) expressed high levels of hTERT mRNA.

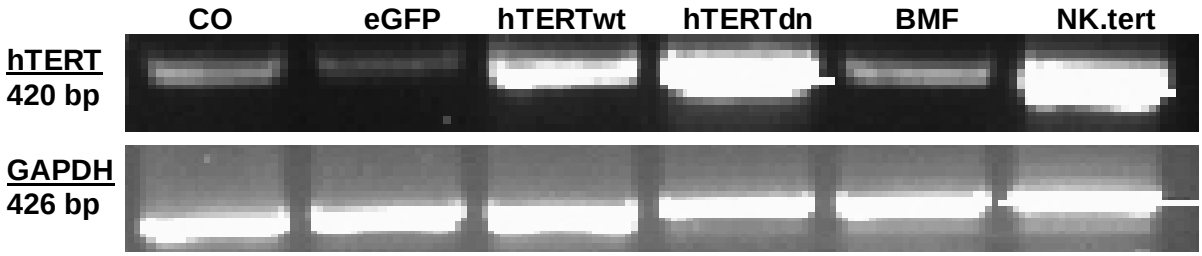


Figure 11A: *High expression of hTERT mRNA in hTERT-transfected cells compared to low expression in controls and eGFP transfected cells.*

B. Western Blot:

We analysed hTERT expression on the protein level four weeks and four days after trasnfection.

As shown in Figure 11B, after 4 weeks of transfection, hTERTwt and eGFP transfected cells showed detectable levels of hTERT protein. However, hTERTdn transfected cells as well as the control cells and an early passage BMF (3rd passage) showed a very weak expression of hTERT protein.

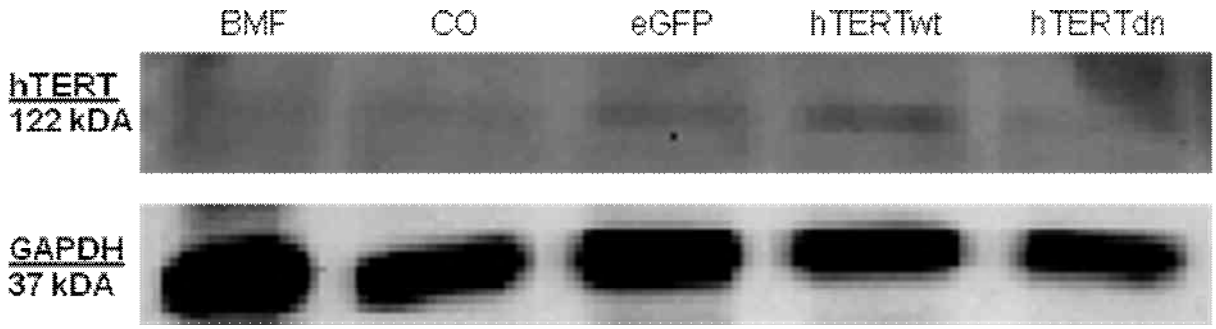


Figure 11B*: After 4 weeks of transfection***,** *hTERT was found to be expressed mainly in the transfected cells but at relatively low levels.*

After 4 days of transfection the expression of hTERT protein was comparable between control and hTERT transfected cells but was rather lower than in hTERT immortalized NK.tert BMSC.

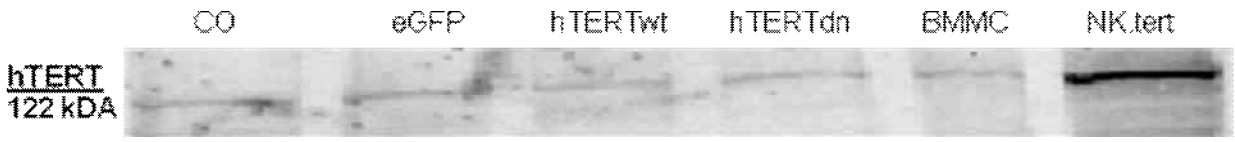


Figure 11C: *hTERT expression on the protein level four days after transfection.*

3. Effect of hTERT transfection on the supportive capacity of BMSC of hematopoietic cells

BMSC play a significant role in supporting survival and differentiation of hematopoietic cells and normal and leukemic B cells (CLL) (Shehata et al 2010). Therefore, we evaluated the effect of transfection with hTERTwt and hTERTdn on the supportive effect of BMSC. PBMC of 14 CLL patients and 1 healthy person were either co-cultured with non-transfected BMSC or with eGFP, hTERTwt, hTERTdn transfected cells or cultured in suspension cultures without BMSC. The CLL cell viability was evaluated every 3 days using Annexin V/ Propidium Iodide (PI) staining and FACS analysis. Cells which are positive of Annexin V represent early apoptotic process, while cells which are double positive for Annexin V and PI represent late apoptotic or necrotic cells. The viability was evaluated at the 3rd and 5th day in 14 patients. 11 patients were followed for 7 days, 6 patients were followed for 10 days and 3 patients were followed for 12 days.

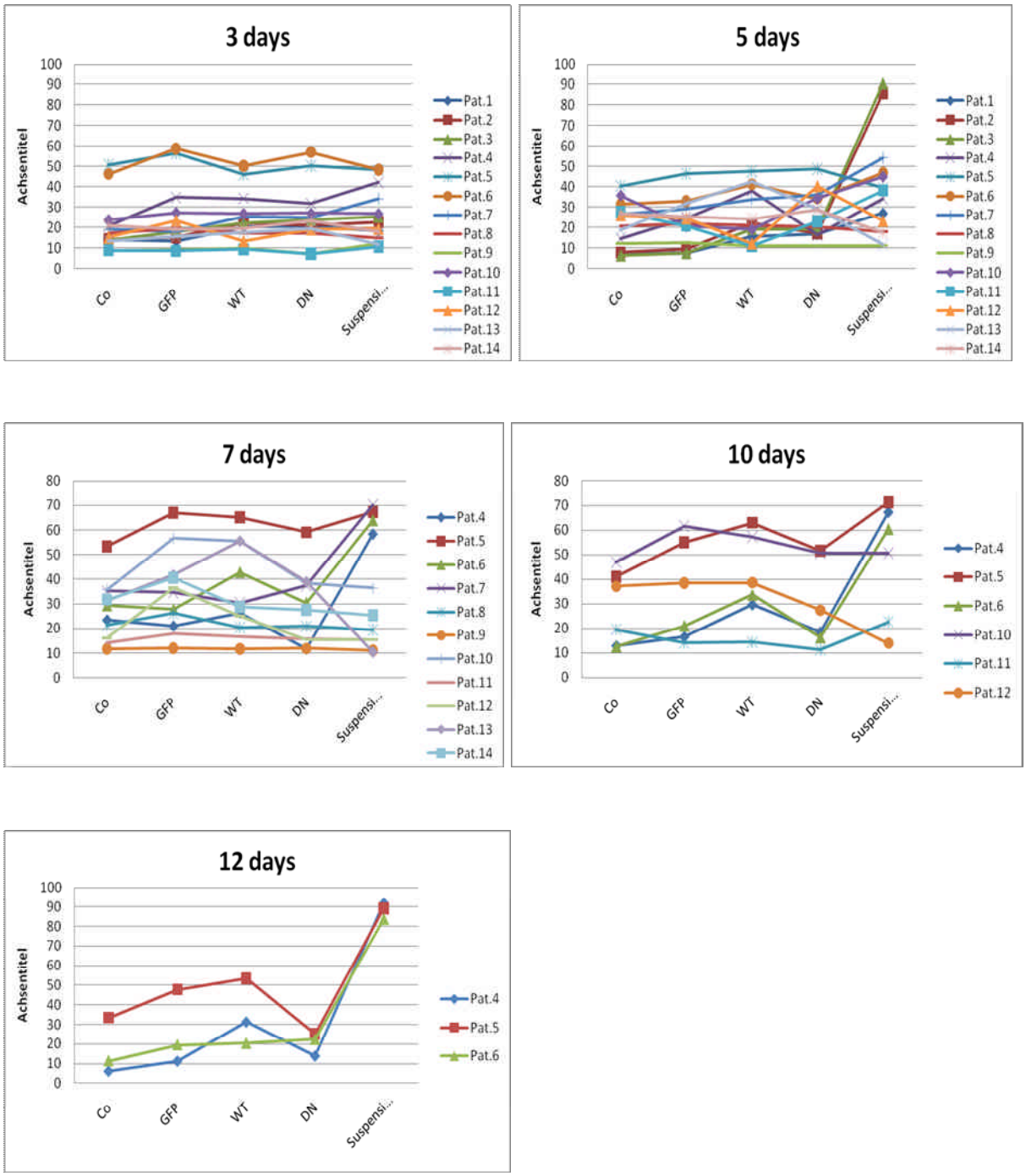
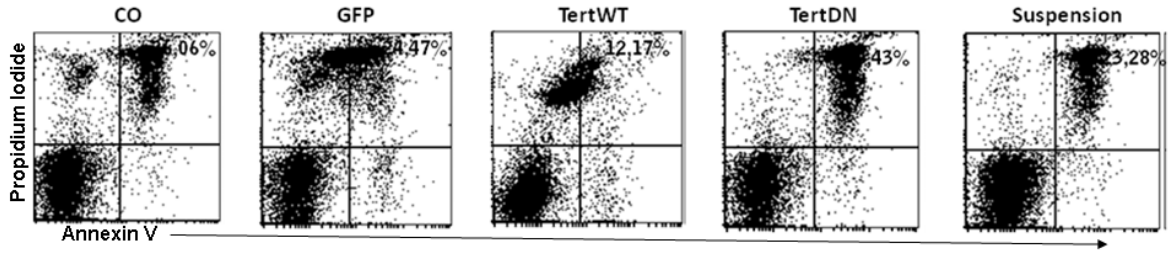


Figure 12: *Time dependent CLL viability in co- culture with different cell types.*

As shown in the figure 12, cell viability was variable between patients in the control co-culture and in suspension. However, the highest percentage of apoptotic cells was detected in the suspension where the apoptotic rate increased 5 days after the experiment start and continued to increase during the next 7 days confirming our recently published data (Shehata et al 2010). Cell viability was generally comparable

in co-culture with control BMSC or with eGFP, hTERTwt and hTERTdn transfected cells. This observations indicate that the transfection did not have a major influence on the supportive capacity of hTERT transfected BMSC on their capacity to support survival of the hematopoietic cells and that the viability in co-culture with hTERT transfected cells were significantly higher than in suspension culture without BMSC.

To analyze the detected variation between patients we divided the patients into 3 groups according to sample viability after 5 days in culture as shown in the Figures 13A, 13B and 13C.



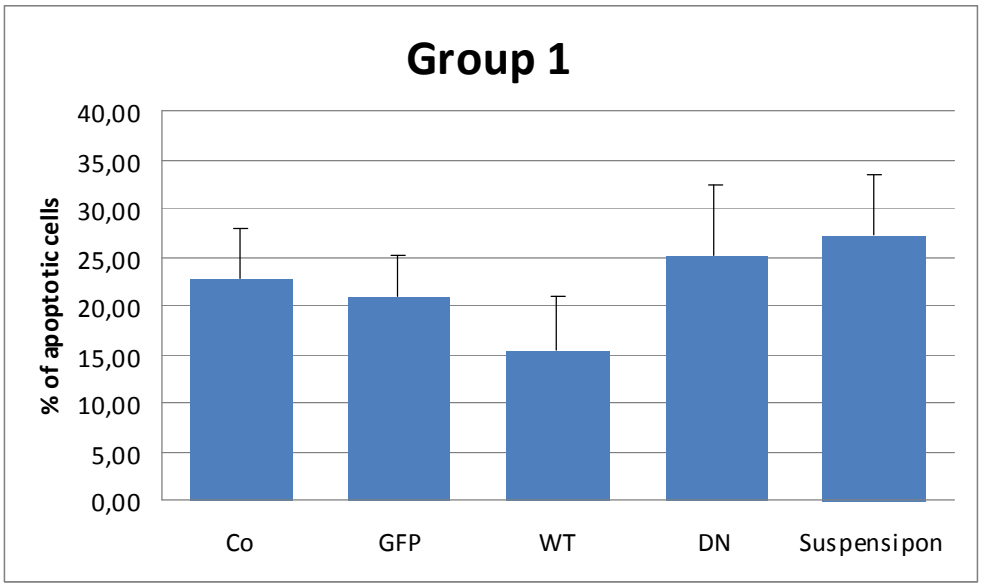
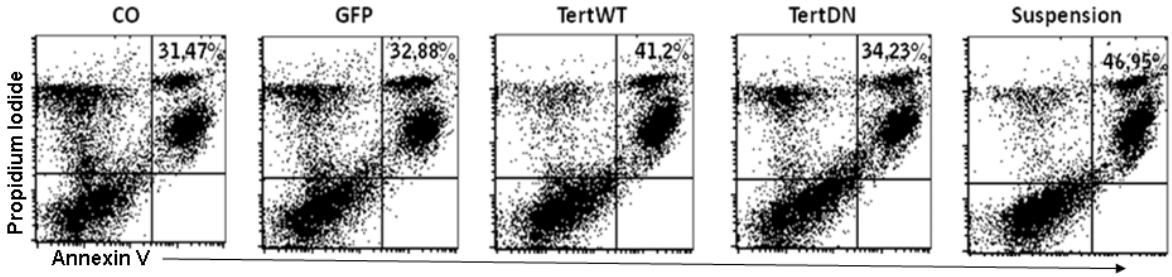


Figure 13A: *Apoptotic rate is demonstrated in dot plots of a representative case of group 1 in the upper panel and the mean value + SD of 3 patients is presented in the lower panel.*

Group 1: As demonstrated in figure 13A, **Group 1** of patients (3 patients) showed enhanced cell viability in co-culture with hTERTwt transfected

BMSC and the viability was remarkably higher than in co-culture with hTERTdn transfected cells and in suspension cultures.



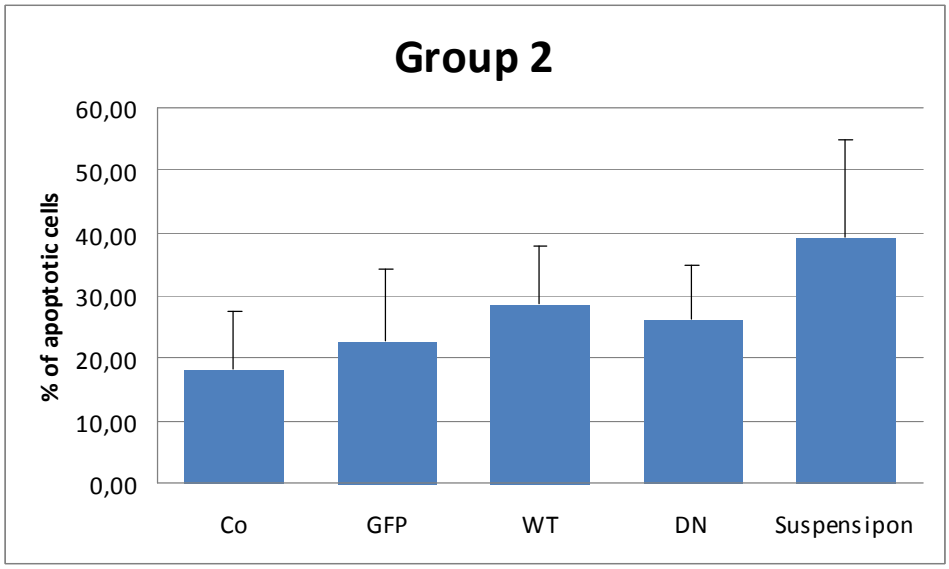


Figure13B: *Apoptotic rate is demonstrated in dot plots of a representative case of group 2 in the upper panel and the mean value + SD of 7 patients is presented in the lower panel.*

Group 2: As shown in figure 13B, samples of 7of 13 patients showed the highest cell viability in co-culture with non-transfected BMSC. While transfection appeared to partially reduce the supportive effect of BMSC. However, the viability of CLL cells in co-culture with hTERT transfected BMSC was remarkably higher than the viability in suspension cultures.

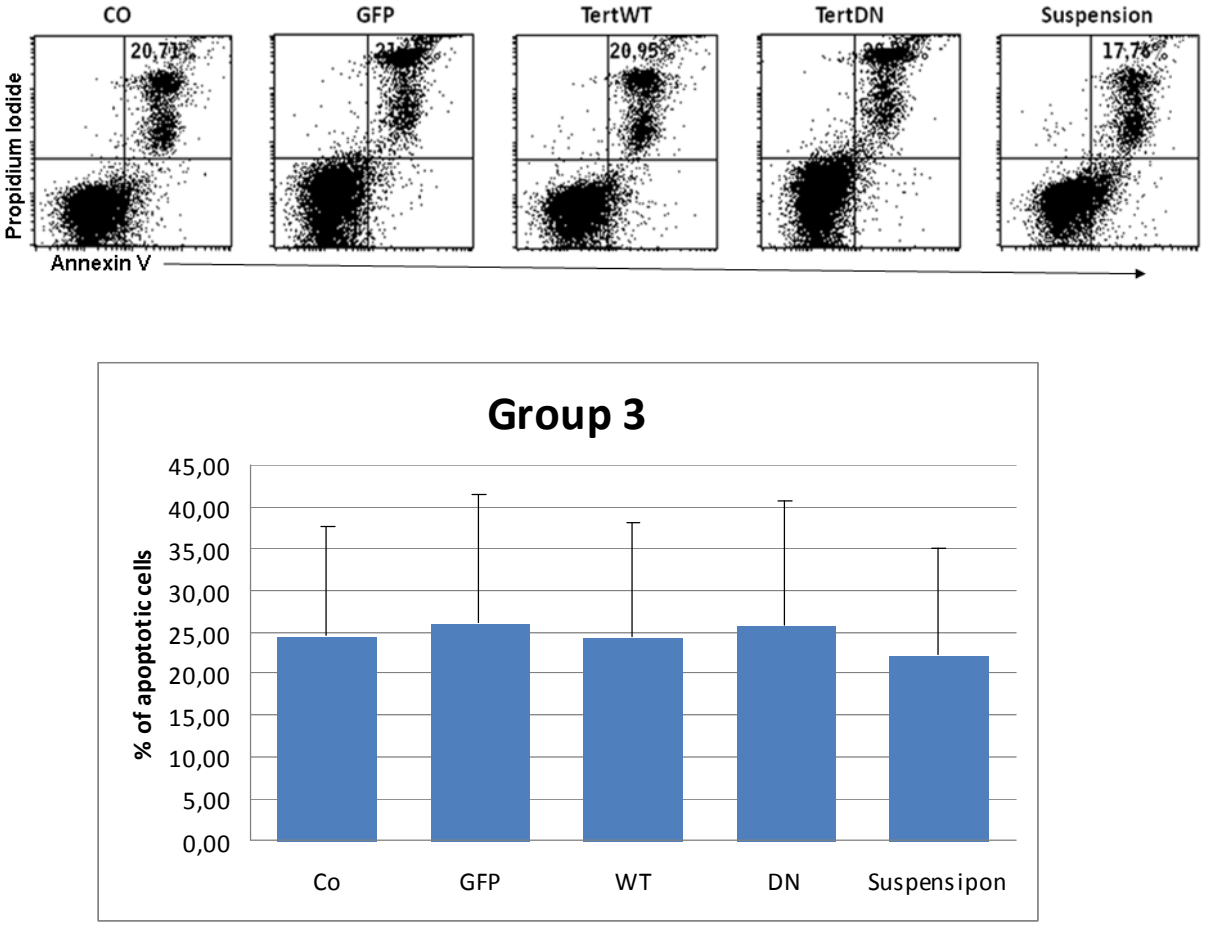


Figure 13C: *Apoptotic rate is demonstrated in dot plots of a representative case of group 3 in the upper panel and the mean value + SD of 3 patients is presented in the lower panel.*

Group 3: In this group of patients (3 patients), no differences in the apoptosis rate between CLL cells co-cultured with control and hTERT transfected BMSC as well as in suspension culture were detected. These results suggest that these cells appear to exhibit a higher endogenous survival capacity independent of the presence or absence of BMSC.

In summary, the data obtained from 10 of 13 CLL patients (70%) showed an increased cell viability in co-culture in comparison to suspension culture, suggesting dependence of CLL cells from the BMSC. In 3 cases however, it appeared that CLL cells may also have an endogenous capitity to survive in vitro independent from BMSC.

To get further insight into the influence of hTERT transfection on the supportive effect of BMSC for CLL cells, we also used the hTERT-retrovirus immortalised bone marrow cell line NK.tert, BMF from earlier passage and IMR90 – foetal lung fibroblasts to compare them with adenovirus hTERT transfected cell strains (see Figure 14). PBMC from CLL patients and from a healthy person (HD) were used for co-culture.

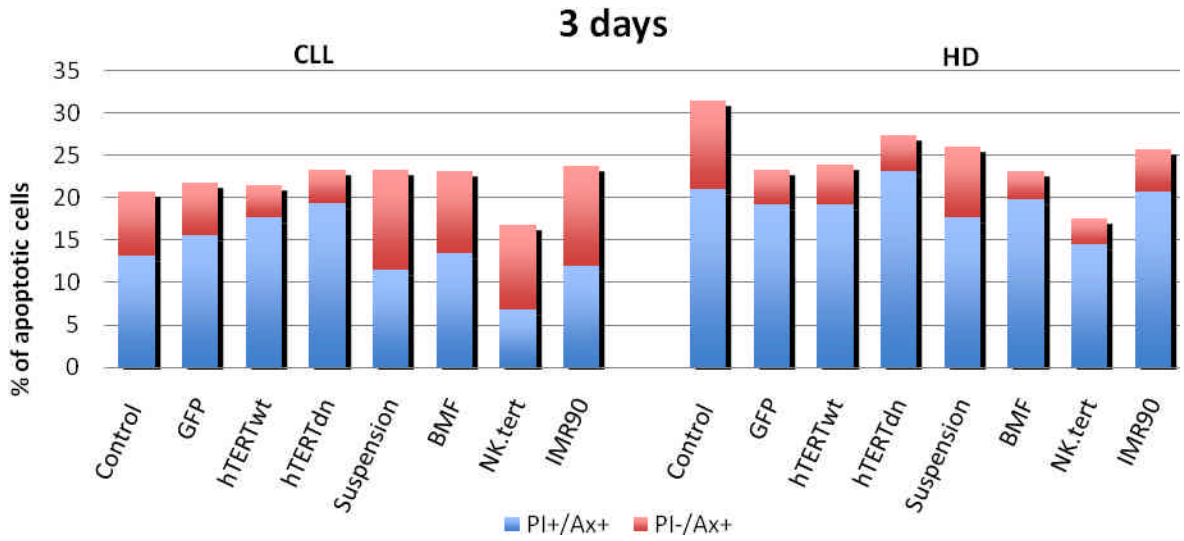


Figure 14: *Time dependent comparison of apoptotic rates between CLL cells and mononuclear cells from healthy person (HD) in co-culture with different cell lines and cell strains. In co-culture with hTERT immortalised cell line the apoptosis rate in both, CLL cells and cells from healthy persons was lower than in co-culture with AV-hTERT transfected and control BMSC.*

As shown in figure 14, the viability of CLL cells appeared to be comparable in co-culture condition with primary BMSC, adenovirus hTERT transfected cells, suspension culture and in co-culture with lung fibroblasts. However the cell viability was further enhanced in co-culture with hTERT retrovirus immortalized BMF (NK.tert) cells. On the other hand, the viability of PBMC of healthy person was enhanced in co-culture. In this set of experiment, cell viability was relatively high in co-culture with immortalized NK.tert cell line.

4. Effect of pharmacological inhibition of hTERT

To further explore the effect of hTERT on cell viability, we compared the influence of pharmacological hTERT inhibitor BIBR 1532: 2-[[(2E)-3-(2-Naphthalenyl)-1-oxo-2-butenyl1-yl]amino]benzoic acid on primary non-transfected BMSC and hTERT transfected BMSC. BIBR 1532 is a selective hTERT inhibitor that induces telomere shortening by hTERT inhibition and has a direct toxic effect in higher dosage (up to 80µM) (El-Daly et al 2005). It also selectively inhibits the proliferation of cancer cells and has been tested on different cancer types.

To test direct toxicity and short-term effects we applied only short exposure times of maximum 48 hours. As shown in figure 15A, only of the highest used concentration of a maximum 20µM a detectable influence on viability was observed. Control cells were more sensitive to the inhibitor than hTERT transfected cells. This indicated that transfection with WT-hTERT provides a decreased sensitivity to pharmacological inhibition of hTERT.

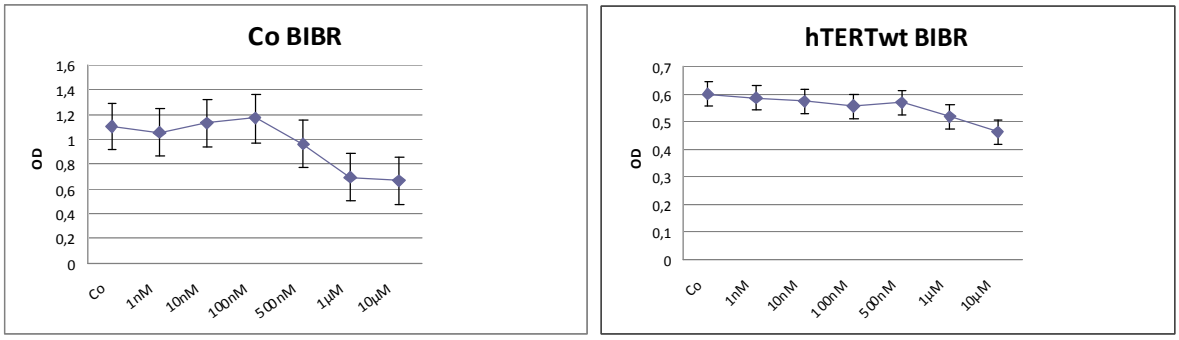


Figure 15A: *BIBR effect on hTERT transfected and control cells (passage 8) after 24 hours exposure time. Control cells (left panel) showed more growth inhibition than hTERT transfected cells (right panel).*

Further experiments with primary BMF strains indicated that earlier passages (passage 2) are less sensitive for BIBR, even if it is used in higher concentration and for a longer incubation time.

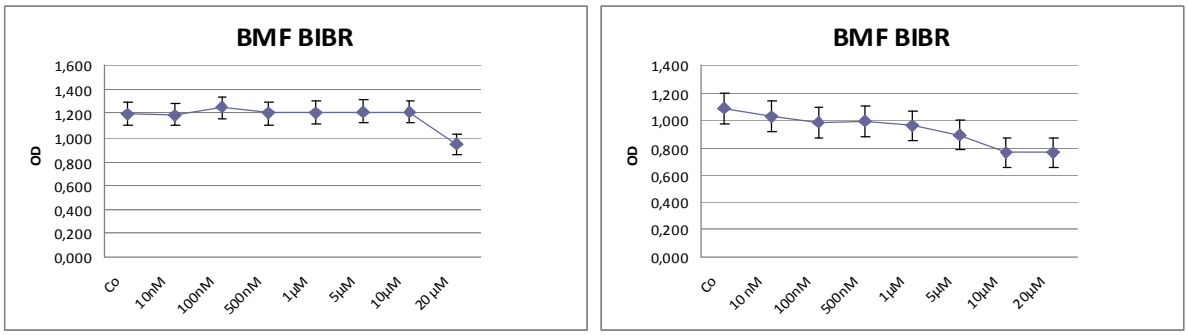


Figure 15B: *BIBR effect on primary BMF in earlier passages (passage 2) 24 hours (left) and 48 hours (right) exposure time.*

These data indicate that transient transfection of BMSC with WT-hTERT provide the cells with a remarkable protection against pharmacological inhibitors of hTERT.

Discussion

The main goals of this work are to explore the effect of hTERT adenovirus transfection on the viability, lifespan, proliferation, differentiation and functions of the bone marrow mesenchymal stem cells (MSC) and bone marrow stromal cells (BMSC).

The obtained data from this work indicates that activation of hTERT in BMF strains provided a short term proliferative advantage in comparison to the control cells and had no major influence on total cell number at the end of observation period. hTERT transfected cells showed an increased proliferation rate in the first week after transfection and seemed to lose this proliferation advantage in parallel to the loss of the virus DNA.

The data also indicated that adenoviral transfections have no effect on the native cell phenotype and do not induce malignant changes. This could be demonstrated through monitoring surface expression of CD13, CD45, CD73, CD90 and CD105, and the detection of gene expression changes for stem cell marker set on the mRNA and protein levels, as well as by the monitoring of differentiation capacity of transfected BMSC.

Another important observation in this work is the expression of Oct4, Nanog, Sox2 and Lin28 on the early passages of BMSC. These markers have been described, as characteristic markers for pluripotent stem cell, cancer and germ line cells. Therefore, this observation suggests the presence of MSC with BMSC population.

In terms of the capacity of hTERT transfected BMSC to support hematopoietic cells, this work applied B-CLL co-culture model which is based on the recently published data on the dependence of B cells on the stroma for survival and inhibition of spontaneous apoptosis in suspension culture (Shehata et al 2010). FACS analysis using Annexin V/PI staining confirmed that co-culture of CLL cells with hTERT transfected BMSC was associated with enhanced cell viability which was comparable to their viability in co-culture with primary BMSC. Under both conditions the viability of

B-CLL cells was much higher than in suspension cultures. Thus, the data suggest that hTERT transfection may not influence the endogenous capacity of transfected BMSC to support hematopoietic cells.

In addition, differentiation assays demonstrated that hTERTwt transfected BMSC undergo adipogenic and osteogenic differentiation process in a comparable pattern to primary BMSC. However, hTERTdn transfection resulted in a decrease of the adipogenic and to some extent of the osteogenic differentiation capacity of BMSC.
Furthermore, this work demonstrated that hTERT transfection had a detectable effect on hTERT expression on the mRNA and protein level. hTERTwt and hTERTdn transfected cells were positive for hTERT expression on the mRNA level. Although hTERTdn transfected cells express hTERT mRNA they showed no detectable hTERT expression on the protein level. hTERT gene used for creation of hTERT inactivated cells carried a mutation that circumvented production of the normal protein. That means hTERT inactivation in this case had no influence on the transcriptional expression level, but prevented normal protein folding and might provide protein disassembly explaining the absence of the signal by Western Blot detection.

Another significant observation in this work was that hTERT inhibitor had a lower proapoptotic effect on BMF in early passages, hTERT activated BMF strain and immortalized cell line. This data indicate that early passaged BMSC are less sensitive to pharmacological inhibition of hTERT compared to those cells which are in later passages. Possibility of the use of hTERT inhibitor BIBR 1532 as cancer therapy should be reconsidered. It seemed to have no toxic effect but also provided no inhibition of cell viability.

Taken together, the data generated from this diploma work demonstrates that transfection of BMSC with hTERT using adenovirus system may have a major advantage in providing a transient and limited phase of increased cell proliferation while keeping the original phenotype, differentiation capacity and support of hematopoietic cells.

Conclusion

This diploma thesis indicates a possibility of advantage of transient transfection of BMSC with hTERT using adenovirus system. By FACS analysis, using defined surface marker panel, we proved that temporary hTERT transfection does increase hTERT expression rate on the mRNA and protein levels, but does not influence native cell phenotype and does not induce malignant transformation or immortalization. The work also demonstrated that Oct4, Nanog, Sox2 and Lin28 expression is detectable in BMF strains in the early passages and that expression level decreases in higher passages.

This diploma work thus provides preliminary evidence on the advantage of hTERT transfection using adenovirus as a self controlled approach for temporary expansion of human mesenchymal stem cells / stromal cells. This information might have valuable clinical relevance and potential applications in patients with cancer who are receiving chemotherapy and / or radiotherapy which cause damage of MSC. However, confirmation experiments and consolidation studies in vitro and in vivo in animal models are absolutely essential for the verification of this hypothesis. Substantial sets of experiments and extension studies are planned as a follow up for this diploma work.

Material and Methods

All used materials for cell isolation and cell cultivation should be sterile. It is also advisable to disinfect working surface of the lamina and hands.

Isolation of Monucleare Cells from Peripheral Blood

The simplest way of blood components separation is density gradient centrifugation. Through Ficoll-Hypaque density gradient mononuclear cells (lymphocytes and monocytes) collect near the top of Ficoll gradient, granulocytes and erythrocytes pellet at the tube bottom.
Blood sample was transferred into the sterile 50 ml falcon and PBS was added till total sample volume of 35 ml. The mixture was carefully underlain with 15 ml of Ficoll. Sample was centrifuged 30 min at 1850 X g at room temperature with no brake.
Than mononuclear cells were transferred at a new sterile 50 ml falcon and washed twice with PBS.

Cell Count

Now building pellet cells were resuspended at 10 ml sterile PBS. 10 µl was added to 10 ml of Coulter Isoton II Diluent. To prevent cell number falsification by erythrocytes, still present at the sample, 3 drops of Zap-oglobin II Lytic Reagent were added. This reagent initiates erythrocytes lyses.
Coulter Z1 particle counter was used for cell count.

Isolation of Primary BMFs from Bone Marrow

Analog to cell isolation from peripheral blood, bone marrow samples were transferred onto a Ficoll layer and centrifuged 30 min at 1850 X g at room temperature with no brake. The BMMC layer was collected. Cells were at least washed twice with PBS and than cultivated in αMEM/20%FSC.

Cultivation of Adherent Cells

Cells for cell count experiments as well as cell lines and BMF cell strains were cultivated in αMEM medium containing 20% FCS in CO2-Incubator Heraeus® cytoperm® 2. Incubation conditions: 37°C, 5% CO2, and 95% humidity. We normally used 75cm² flasks TPP 90075. For cell harvest we used Trypsin-EDTA 0.05%/0,02% in PBS.

Used Cell Lines

Cell line	Description
NK.tert	derived from human bone marrow cells immortalized with human telomerase reverse transcriptase (hTERT)
IMR90	fibroblast strain derived from the lungs of a 16-week female fetus
HEK293	generated by transformation of human embryonic kidney cell cultures with sheared adenovirus 5 DNA

BMF Transfection

In replication-deficient recombinant adenovector E1 was replaced by the cytomegalovirus-immediate early promoter (CMV) region gene promoter/enhancer. Virus vectors were charged with hTERT, hTERT DN or eGFP coding gen cassettes. Virus particles were amplified in HEK293 cell line and than collected by CsCl-gradient centrifugation. Used virus particles were compounded by working group of Klaus Holzmann according to the protocol of G. Zaccagnini.

Ratio of virus particles: 3,00E+0,8 for 3,00E+0,6 cells.

Virus particles MOI 100 were added to 80% confluent BMF (1,00 E + 0,6) and incubated for 24 hours under normal cultivation conditions. After the incubation cells were washed 3x with αMEM/20%FSC.

Senescence-Associated beta-Galactosidase Assay

Materials

All solutions were prepared fresh before use.

Cells

1 X PBS

Fixing solution

1xPBS, 2% (v/v) formaldehyde, 0,2% (v/v) glutaraldehyde

Glutaraldehyde

200 µl 25% (v/v) glutaraldehyde, 9,8 ml 1 x PBS

Staining solution

1 ml 1 mg/ml Xgal (5-bromo-4-chloro-3-idolyl-beta-galactopyranosode) in DMF

4 ml citric acid/sodium phosphate solution

1 ml 100mM potassium ferrocyanide

1 ml 100mM potassium ferricyanide

0,6 ml 5M NaCl

40 µl 1M $MgCl_2$

12 ml dH_2O

Senescence-associated beta-galactosidase stain

40 mM citric acid/sodium phosphate pH 6,0

1 mg/ml Xgal

5 mM potassium ferricyanide

5 mM potassium ferrocyanide

150 mM NaCl

2 mM $MgCl_2$

Cells were seeded 24 hr before staining in 6-well plates and incubate under standard conditions. 50% confluence is a supposition for this assay.
Cells were washed twice with 1xPBS for 2 min at the room temperature. To make wash steps more efficient we used orbital shaker.
After removing of 1xPBS, 1,5 ml per well fixing solution was added. Fixing solution should cover the cell monolayer completely.
Fixation time - 5 min at room temperature.
After aspiration of fixing solution, 3 ml 1xPBS per well was added.
After removal of PBS 1,5 ml staining solution per well was added.
Plates should be incubated in a 37°C incubator, till blue coloration is detected via microscope observation.
Than plates should be washed with 1xPBS or distillated water and retained at 4°C.

Differentiation Kit – Human Mesenchymal Stem Cell Functional Identification KIT (R&B Systems)

Materials

Alpha Minimum Essential Medium (Alpha MEM) Invitrogen ™
D-MEM/F-12 (1x) Invitrogen
Fetal Bovine Serum Invitrogen
PBS (Phosphate Buffered Saline)
Penicilin-Streptomycin-Glutamin 100x Invitrogen
1% BSA in PBS
0,3% Triton 100x, 1%BSA, 10% normal donkey serum in PBS
Mounting medium
Secondary developing reagents
Distilled water
Adipogenic or osteogenic supplement
Cell type specific antibodies goat anti-mause FABP-4, mouse anti-human Osteocalcin

Adipogenic Differentiation

Adipogenic Differentiation Medium

500 µl of 37°C warm Adipogenic Supplement were added to 50 ml of Alpha MEM with 20% fetal bovine serum and mixed gently.
For this assay should be used 100% confluent cells in 6 well plates (or 16 well plates). After removal of normal 20% Alpha MEM medium 1 ml of differentiation inducing medium per well were added. Medium change was made every 3 days.
After three medium changes first lipid vacuoles were detected via microscope.
Staining of adipocytes was normally made between four and five weeks after experiment start.

Immunocytochemistry of Adipocytes

Cells was washed twice with 1x PBS and fixed with 0,5 ml of 4% paraformaldehyde in PBS by incubating for 20 min at room temperature.
After fixation cells were washed three times with 1 ml of 1% BSA in PBS for 5 min. Aspiration of wash and fixation solutions was made gently by pipetboy without jet.
Than cells was permeabilized and block with 0,5 ml of 0,3% Triton X-100, 1% BSA, 10% normal donkey serum in PBS at room temperature for 45 minutes.
After blocking cells were incubated with 300µl/well of goat anti-mouse FABP-4 antibody diluted in 1% BSA and 10% normal donkey serum containing PBS over night at 2-8°C. And concentration of diluted antibody should be 5 µg/ml.
Past over night incubation cells were three times washed with 1ml PBS containing 1% BSA for 5 minutes at room temperature by gently shaking.
As second antibody was used anti-goat Alexa 594 diluted 1:400. Incubation with second antibody took place at the dark at room temperature and required 60 minutes.
Then, cells were washed three times for 5 minutes with 1 ml/well PBS containing 1% BSA.
After aspiration of wash solution 1 ml/well PBS was added and visualization with fluorescence microscope followed.
In this stage cells can be kept by 2-8°C at the dark.

Osteogenic differentiation

Osteogenic Differentiation Medium

Medium was prepared according to the description (see Adipogenic Differentiation Medium Preparation) using Osteogenic Supplement.
Cells were cultivated in 6-well plates under normal conditions for 3-5 weeks. Differentiation progression was monitored by specific staining.

Immunocytochemistry of Osteocytes

Cell were fixated according to the protocol (see Immunocytochemistry of Adipocytes) and subsequently incubated over night with 300µl/well of mouse anti-human Osteocalcin dilution 5 µg/ml. Rabbit anti-mause Alexa 647 was used as second antibody in dilution 1:400.

RNA Isolation

Cell pellets were well resuspendet at approx. 1mL Trizol (Invitrogen). 200µL CHCl3 were added and sempels were vortext. After incubation time of 2 minutes by room temperature samples were centrifuged at 12000rpm/4°C for 15min. Aqueous phase was collected and mixed with 500 ml cold isopropanol. After an incubation time of 30 minutes at -20°C samples were centrifuged at 12000rpm/4°C for 10min.
Pellets were washed twice with cold 75% ethanol by centrifugation at 7500rpm/4°C for 5min and dried than at the room temperature. Dry pellets were resuspendet in 50µl deionized, steril water. RNA concentration was determined by photometer measuring.

cDNA Synthesis

0,5µL oligo(dT)15 primer (Promega) were added to 2 µg isolated RNA (see below) and incubated at 72°C for 5min. Afterwards samples were keeped on ice and 5µL 5xBuffer (Promega), 0,625µL RNasin (Promega), 1,25µL dNTP mixture (10mM), and 1µL M-MLV reverse transcriptase (Promega) were edded.

Mixture was incubated for 60 minutes at 37°C. cDNA concentration was determined photometrically.

RT-PCR

For PCR equal rate of cDNA from each sample was mixed with GoTaq® PCR Mix (1,5mM MgCl2 (GoTaq® Reaction Buffer), 0,2mM dATP, 0,2mM dCTP, 0,2mM dGTP, 0,2mM dTTP, 1,25U GoTaq® DNA Polymerase), 0,5nM forward-primer and 0,5nM reverse-primer. Pre-PCR conditions 2min/94°C. Optimal PCR conditions were established for each primer (see below).

Primers

All used primes were obtained from VBC Genomics.

1. TERT
   PCR conditions 60°C, 30 cycles.
   TERT-S: 5' – GAC GGG CTG CTC CTG CGT TT– 3'
   TERT-AS: 5' – GGT GCA CAC CGT CTG GAG GC – 3'

2. Oct-4
   PCR conditions 61°C, 38 cycles.
   Oct-4-S: 5' – ACA GGC CGA TGT GGG GCT CA – 3'
   Oct-4–AS: 5' – CTT TCG GGC CTG CAC GAG GG – 3'

3. Nanog
   PCR conditions 54°C, 35 cycles.
   Nanog-S: 5' – GTT GGA GCC TAA TCA GCG AGG – 3'
   Nanog-AS: 5' – ATC TAT AGC CAG AGA CGG CAG – 3'

4. Sox2
   PCR conditions 58°C, 30 cycles.
   Sox2-S: 5' – AGG ACC AGC TGG GCT ACC CG – 3'
   Sox2-AS: 5' – GGC GCC GGG GAG ATA CAT GC – 3'

5. Lin28
   PCR conditions 58°C, 30 cycles.
   Lin28-S: 5’ – CTG GGC CCG GTG AAA AGG CC – 3’
   Lin28-AS: 5’ – AGG GCA GGA CAC AGG CTG CA – 3’

6. GPDH
   PCR conditions 54°C, 28 cycles.
   GAPDH-S: 5’-GTC AGT GGT GGA CCT GAC CT-3’
   GAPDH-AS: 5’-TGT GAG GAG GGG AGA TTC AG – 3’

Cell Phenotyping

All used cell lines and cell strains were phenotyped by FACS analysis using BMF and MSC surface markers: CD13, CD45, CD73, CD90, CD105, vWF. All used antibodies were obtained from BDBiosciences.

FACS Surface Staining

Cell pellets of at least $1x10^6$ cells washed with PBS were resuspended in 20% BSA and incubated for 30 minutes by room temperature. Blocked cells were than incubated with fluorochrome-conjugated antibody for 15 minutes on ice at the dark. Hereafter cells were washed with PBS/0,3%BSA/0,1%NaN3 and resuspended in 250 µL of the wash solution.

Viability Staining

For Annexin V –FITC/PI staining we used Annexin V kits (BenderMed Systems). Cell pellets were resuspended in 200µL Annexin-binding buffer containing 5µL AnnexinV-FITC. Cell solutions were incubated in the dark at RT for 10 min. After that samples were washed with 1 ml PBS and resuspended at 200µL Annexin-binding buffer containing 10µL PI.

MTT Assay

For comparison of cell viability from transfected and control cells, 2,5-5x10^3 cells per well were incubated in 96 well plates for at least 24 hours using RPMI medium containing 10% FCS. For inhibitor testing - inhibitor was added and plates were incubated further 24 or 48 hours. Than cell viability was monitored by measurement of formazan derivate formation with microplate reader (Dynatech) at 450nm wavelengths. We used EZ4U proliferation assay (Biomedica). .

Inhibitors

BIBR 1532 (Tocris) selective telomerase inhibitor was used as DMSO solution with stock concentration of 10mM.

Protein Isolation

Cell pellets were resuspendet in RIPA –buffer, contening: 50mM Tris-HCL (pH7,4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% deoxycholic acid sodium salt, 0,1% SDS, 1mM PMSF, 1mM Na2OV4 and Complete Protease Inhibitor CocktailTM (Roche) .
Sampels were vortext every 5 minets during the incubation on ice. After the incubation period of 30 minutes lysates were centrifugated for 10 minutes at 14000rpm and 4°C. Supernatant was transferred in a new tube and protein concentration was determined by Bradford assay via BSA standard curve. Than equal volume of 2xSDS-sample buffer (100mM Tris-HCL (pH6,8), 25% gycerol, 2% SDS, 0,01% bromphenol blue, 10% 2-mercaptoethanol) was added to each sample and incubate at 99 °C for 5 minutes. Protein SDS-sampel buffer mixture can be stored at -20°C.

SDS-PAGE

Separating gel: 4,05ml H2O, 2,5 ml 1.5M Tris HCl pH8,8, 100µl 10% SDS, 3,3 ml 30% Acrylamid, 80µl 10% APS, 15µl TEMED
Stacking gel: 6,1ml H2O, 2,5ml 0,5M Tris HCl pH6,8,100µl 10% SDS, 1,33 ml 30% Acrylamid, 100µl 10% APS, 20µl TEMED

Gels were run in electrophoresis buffer (25mM Tris base, 192mM glycine, 0,1% SDS) at 200V using Mini-Protean II® system (Bio-Rad). 20µg of total protein were loaded.

Western Blot

Subsequently proteins were transferred in blotting buffer (25mM Tris base, 192mM glycine, 20% methanol) to Hybond-C Nitrocellulose membrane (Amersham) using MiniTrans-Blot Electrophoretic Transfer Cells (Bio-Rad) by 100V. Poinceau S staining was used as indicator of transfection quality. Membrane was blocked for an hour in 3%BSA TBS/T (1M Tris pH7,5, 5M NaCl, Tween20) and than washed 3x5 min. with TBS/T. First antibody was diluted in TBS/T according to the data shit or experimental established data and membrane was incubated over night at 4°C or 1hour RT. Than membrane was washed 3x 10 min. with TBS/T and second antibody was added. Second antibody dilution: goat-anti-rabbit HRP antibody (Amersham) 1:50000 in TBS/T; goat-anti-mouse HRP antibody (Amersham) 1:25000 in TBS/T. Incubations conditions 1 hour by gentle shaking RT. Subsequently membrane was washed 3x 10 min. with TBS/T. For detection we used Immun-Star™Western C™ (BioRad).

Antibodies

1. 1hTERT C-term (RabMAb) was used 1:300 in combination with goat-anti-rabbit HRP antibody (Amersham).
2. Oct-4 (Chemicon) was used 1:500 in combination with goat-anti-mouse HRP antibody (Amersham)
3. Nanog (Millipore) was used 1:1500 in combination with goat-anti-rabbit HRP antibody (Amersham).
4. Sox 2 (Santa Cruz) was used 1:500 in combination with goat-anti-rabbit HRP antibody (Amersham).
5. GPDH (Santa Cruz) was used 1:5000 in combination with goat-anti-rabbit HRP antibody (Amersham).

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List of Figures and Tables:

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