

Leptin receptor/CD295 is upregulated on primary human mesenchymal stem cells of advancing biological age and distinctly marks the subpopulation of dying cells

Gerhard Thomas Laschober, Regina Brunauer, Angelika Jamnig, Christine Fehrer, Brigitte Greiderer, Günter Lepperdinger *

Institute for Biomedical Aging Research, Austrian Academy of Sciences, Rennweg 10, 6020 Innsbruck, Austria

* Corresponding author: email: guenter.lepperdinger@oeaw.ac.at, phone: +43 512 58391940, fax: +43 512 5839198

Key words

mesenchymal stem cells, replicative aging, leptin receptor, self-renewal, cell death

Abbreviations

MSC: mesenchymal stem cells; LEPR: leptin receptor; qRT-PCR quantitative reverse-transcription polymerase chain reaction; 7-AAD: 7-amino-actinomycin D; DPI: diphenyliodonium

Abstract

During the lifetime of an adult organism, stem cells face extrinsic and intrinsic aging. Mesenchymal stem cells (MSC) can be expanded in culture, and the proliferation potential of individual cell isolates before growing senescent appear to be dependent on fitness and age of the donor, respectively. To date no molecular markers are available, which specifically reflect the degree of cellular aging in a population of MSC. Employing a genomic approach, we noticed that the gene encoding leptin receptor (also termed OB-R) is differentially regulated in MSC derived from aged donors as well as in MSC that had been stressed due to cultivation under hyperoxic conditions. We further observed that the leptin receptor transcript levels in primary MSC isolates are inversely correlated with the prospective number of generations that are ahead of these cells in culture, i.e., the number of population doublings that will occur in long term culture prior to cessation of growth due to replicative senescence. The MSC subpopulation, which exhibited distinctly elevated levels of leptin receptor or CD295 at the cell surface, is indistinguishable from dying cells. Considered together with the observation that primary MSC derived from healthy individuals showed proliferation capacities that declined at differentially increasing rates, we concluded that attenuation of MSC proliferation potential during aging greatly relies on the strictly increasing withdrawal of cells due to cell death.

Introduction

Medical application of adult stem cells is an emerging field. Stem cells hold tremendous promise for the treatment of many diseases including bone healing, cell death following nerve injury, inflammatory relief from arthritis, repair of suspensory ligament tears, and immune suppression in the context of graft rejection. Generally, a stem cell can be defined as a cell that is capable to self-renew, that is to divide and create additional stem cells, and of differentiating into at least into one lineage (Watt and Hogan, 2000).

Intrinsic stem cell factors and cell-cell and cell-matrix interactions provided by the stem cell niche control the delicate balance between self renewal and differentiation, concomitantly regulating the fate of the stem and daughter cell, and nurturing the stem cell in order to protect it throughout its life time. Presently few conclusive data are available, on how intrinsic and/or extrinsic aging processes conflict with stem cell properties. Hence, there is reason to believe that adult stem cells are deliberately defined as cells that are capable of undergoing continuous self-renewal, since many of the body's renewable cells show decreased regeneration and repair capacity. Such cells can divide to replace lost tissue, but in due course of these processes gradually loose their ability to proliferate further. Over the years, the cumulative effect may contribute to the aging of tissues. Understanding how stem cells are able to continuously self-renew may lead to a more complete understanding of how the body ages.

Mesenchymal stem cells (MSC) are known to exhibit stem cell properties with regard to non-hematopoietic tissues. MSC have been characterized by a stable undifferentiated phenotype, as well as by the ability to proliferate extensively while retaining the potential to differentiate along osteoblastic, adipocytic, and chondrocytic lineages *in vitro* (Sakaguchi et al., 2004). Besides bone marrow (Caplan, 1991), they can also be derived from other tissues (Barry and Murphy, 2004). MSC are part of a highly specialized microenvironment and thus generally believed to participate in the regulation of hematopoietic precursor cell differentiation into mature progeny (Muguruma et al., 2005; Prockop, 1997). They reside in a complex three-dimensional network, which is comprised of a plethora of other cell types such as, in the case of bone marrow, hematopoietic (stem) cells, adipocytes, endothelial cells and pericytes, supported by an

extracellular matrix consisting mainly of fibronectin, collagen I and IV, heparin sulphate, chondroitin sulphate, and hyaluronan (Dorshkind, 1990). Hence, there are multiple opportunities for MSC to interact at both the cellular and matrix level, and all of these elements may modify MSC behavior in particular with respect to organismic or cellular aging (Fehrer and Lepperdinger, 2005; Sethe et al., 2006).

Most MSC exhibit a spindle-shaped phenotype during initial stages with a gradual loss of these features over cultivation time (Baxter et al., 2004; Stenderup et al., 2004). In contrast to embryonic stem cells, which show no or little loss of proliferative potency, the number of population doublings MSC can achieve, greatly depends on the age of the donor (Stenderup et al., 2003). Conclusively, MSC face aging in vivo and those effects are retained by these stem cells when cultivated ex vivo. We have recently reported that the proliferative capacity of in vivo aged MSC can be increased when cultivating MSC at low oxygen tension (Fehrer et al., 2007). It is well known that many animal cells proliferate under reduced O₂ at an accelerated rate (Cooper et al., 1958; Zwartouw and Westwood, 1958). However, it has been recognized only recently that high O₂ tension may also promote and accelerate cellular aging, which in due course results in the loss of functional properties (Csete, 2005). These observations support the hypothesis that O₂, even though acting as the essential electron donor in the primary metabolic pathway of oxidative phosphorylation, affects cells as a potential stressor. To date however, the molecular and cellular consequences thereof are not well-defined.

In order to answer this question in more detail, we characterized the expression profile of MSC that had been derived from differently aged donors, and which had been grown under atmospheric conditions of normal and low O₂ concentration (3%). A variety of candidate marker genes have been elucidated in this manner. One particular candidate, which attracted our attention, was the gene encoding for the type I cytokine-like receptor CD295 or leptin receptor (LEPR). Leptin is a well-known adipostat, which is capable of regulating body weight by balancing food intake and energy expenditure. (Lavens et al., 2006). It is now generally accepted that leptin acts through LEPR in a pleiotropic fashion thereby regulating reproduction, glucose homeostasis, bone formation, wound healing

and the immune system (Blüher and Mantzoros, 2007; Cirmanova et al., 2008; Gao and Horvath, 2008; Hamrick et al., 2005; Malendowicz et al., 2007). However, whether leptin exhibits direct effects on adult MSC and what are the functional roles of its corresponding receptor with regard to stem cell aging has not been thoroughly investigated.

Material and Methods

Isolation and Culture of MSC

MSC were extracted from bone derived from the iliac crest of systemically healthy individuals, who were undergoing reconstructive bone surgery of defects. Isolation and culture of MSC was carried out as described previously. Briefly, MSC were cultivated (MEM supplemented with 20% fetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin,) at ambient atmosphere, 5% CO₂, 37°C (Heracell) as well as at 3% O₂, 5% CO₂, 37°C (Thermo Electron Corporation 3110). Before reaching confluence, cells were trypsinized and re-seeded at a density of 50 cells / cm² in order to select for MSC which exhibited clonogenic growth. In order to induce stress-induced premature senescence in culture, clonogenic MSC were left in culture after having reached confluency for 4 – 7 weeks with media change every 3-4 days. MSC were treated with recombinant human leptin (Biomol, Hamburg Germany; Cat. # 53290) at a concentration of 10⁻⁷ M for 3 days in culture medium at 3% O₂, 5% CO₂, 37°C.

RNA Isolation and quantitative PCR

RNA was isolated after homogenization in 4.2 M guanidinium thiocyanate, phenol extraction and ethanol precipitation. The resulting total RNA was further purified by LiCl precipitation (final concentration 4.5 M). For quantitative PCR, cDNA was synthesized from total RNA by reverse transcription using RevertAid H Minus -MMuLV-RT (Fermentas) and oligo(dT) primer (MWG, Germany). Quantitative reverse transcription PCR (qRT-PCR) assays were performed on a light cycler 480 instrument (Roche) with the LC-FastStart DNA Master SYBR Green I Kit (Roche). The 16 µL reactions contained 2 µM forward and reverse primer and 3 mM MgCl₂. After the activation of the enzyme at 95°C for 8 minutes, 50 cycles at 95°C for 15 seconds, 57°C for 8 seconds and 72°C for 15 seconds were performed. mRNA expression levels were calculated relative to eukaryotic translation elongation factor 1 alpha 1 (EEF1A1). Primers were as follows: elongation factor 1-alpha 1 (EF-1-alpha-1) (Ensembl transcript IDs ENST0000309268 / ENST0000316292 / ENST0000331523 / ENST0000358190), 5'-CAAGTGCTAACATGCCTTGGT-3' and 5'-GAACAGTACCAATACCACCAATTT-3'; leptin receptor precursor (LEPR)(Ensembl transcript ID ENST00000349533) 5'-

CCTCTTCCATCTTATTGCTTGG-3' and 5'- GCTCAAACGTTTCTGGCTTCT-3';
cyclin-dependent kinase inhibitor 2A, isoform 4 (p16) (Ensembl transcript IDs
ENST00000304494 / ENST00000380151) 5'- CAACGCACCGAATAGTTACG-3' and
5'-AGCACCACCAGCGTGTC-3' (synthesized by MWG, Germany)

Flow cytometry

Cell viability was examined with the aid of argon laser-equipped flow cytometer (FACSCanto, Becton Dickinson) by monitoring 7-AAD fluorescence together with forward/side scatter analysis and the annexinV method. CD295 expression was detected using monoclonal anti-human leptin receptor/CD295-APC (R&D Systems, #FAB867A). Staining was performed as follows: After trypsination, 100,000 cells were stained with 20 μ L antibody and 20 μ g/mL 7-amino-actinomycin D (7-AAD) in 50 μ L PBS for 30 minutes at 4°C. Cells were washed with annexinV binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and stained with 5 μ L annexinV-FITC (Becton Dickinson) in 100 μ L annexinV binding buffer for 15 minutes at room temperature. Staining was stopped by addition of 400 μ L annexinV binding buffer. Data were analyzed with the aid of FACSDiva Software (Becton Dickinson).

Statistics

Values were expressed as means \pm standard deviation of the mean. Statistical differences of experimental scores were evaluated using Student's *t* tests. Differences were considered significant when the *p* value was less than 0.05.

Results

Age-dependent and stress-induced leptin receptor expression in MSC

Stromal cells, which we routinely isolate from the surface of trabecular bone and are subsequently selected for clonogenic growth, exhibit enhanced stem cell-like properties, such as high proliferation rates and multipotential differentiation capacity. Ex vivo, this particular cell type is referred to as an MSC.

In the course of a genomic study, which was undertaken in order to exploit the function of gene networks in the context of cellular and organismic aging (Fehrer et al., 2007; Lepperdinger et al., 2008), we observed that the expression level of the gene encoding LEPR was significantly enhanced when cultivating primary MSC at atmospheric conditions of 20% O₂ instead of 3%, the latter being within the physiologic range. Under these altered conditions the expression levels of bone morphogenetic protein receptor 1, epidermal growth factor receptor, leukemia inhibitory factor receptor as well as transforming growth factor beta receptor 1 remained unchanged. This initial observation was corroborated by means of qRT-PCR for MSC isolated from 12 systemically healthy individuals of different ages (Fig. 1). In order to assess the individual proliferation capacity, MSC were grown in long-term culture until reaching the state of replicative senescence. Recently we observed that MSC proliferation capacity significantly declines when cultivating MSC at 20% O₂ (Fehrer et al., 2007). In this context, we also noticed that LEPR transcript levels in primary MSC isolates inversely correlated with the prospective number of generations that are ahead of these cells in culture, i.e. the number of population doublings that will occur in long-term culture prior to ceasing growth due to replicative senescence (Figure 2). These results were further substantiated by the fact that MSC at late passages of cultivation exhibited enhanced LEPR expression (Figure 3A). In line with the latter results, MSC, which had faced extensive stress due to continuous cultivation after having reached confluency, exhibited distinctly elevated levels of LEPR expression (Figure 3B).

Effect of leptin on MSC stemness

MSC were treated with 10⁻⁷ M leptin for 3 days, a dose which induces apoptosis in bone

marrow stromal cells (Kim et al., 2003). In stark contrast, the death rate of leptin treated MSC slightly decreased. Moreover, the number of colonies formed by leptin-treated MSC remained unchanged (data not shown). In line with this, the transcript level of molecular stemness markers such as Nanog or Rex1 (Zfp-42) were also found to be unaffected.

Furthermore, MSC were treated with pro- and anti-inflammatory cytokines such as transforming growth factor beta I (20 pg/mL) , tumor necrosis factor alpha (1000 u/mL) or interferon gamma (100 u/mL) for 24 hours. No change in LEPR transcript level could be observed (data not shown). We furthermore noted that MSC, which were treated with various doses of diphenyliodonium (DPI), a drug that disturbs the intracellular electron transport process and thereby greatly enhances the rate of cell death, was accompanied by upregulated of LEPR gene expression.

LEPR / CD295 upregulation in dying MSC

Flow cytometric analyses of MSC employing anti LEPR / CD295 monoclonal antibodies revealed a distinct subpopulation of cells that displayed high level of CD295 immune reactivity at their surface (Figure 4). This particular population was indistinguishable from cells that are both annexinV positive and strongly staining for 7-AAD. These specific characteristics were by and large alike after treatment of MSC with 10^{-7} M human recombinant leptin for three days.

Discussion

Ideally, stem cell self-renewal brings forth two daughter cells, one of which is identical to the mother cell. Consequently, self-renewal and replicative aging of stem cells are mutually exclusive processes. Events, which cause cellular aging over the course of the lifespan of an adult organism, and the extent, by which these events are compromising basic stem cell properties *in vivo* are currently being studied extensively.

It is generally accepted that human MSC exhibit a limited proliferative potential during long-term *in vitro* culture (Banfi et al., 2000; Bonab et al., 2006; Bruder et al., 1997; Digirolamo et al., 1999). Replicative capacity of MSC has been shown by several research groups to be inversely correlated with increasing donor age (Baxter et al., 2004; Pittenger et al., 1999; Stenderup et al., 2003). *In vitro*, the gradual loss of proliferation potential, and finally, the acquisition of replicative senescence are paralleled by progressive telomere shortening. The prime reason for this telomere shortening is that cultured human MSC lack telomerase reverse transcriptase activity (Baxter et al., 2004; Zimmermann et al., 2003). In contrast however, freshly isolated primary human MSC have chromosomes, which display only slightly shortened telomeric ends, most likely because MSC appear to transiently express telomerase in the S phase of the cell cycle, therefore of sufficient expression to keep their telomeres extended (Zhao et al., 2008). In parallel, other factors such as the accumulation of damage by various cellular molecules consequently leads to structural and biochemical alteration of intracellular compartments and processes. Taken together, MSC are thus pushed to enter the vicious cycle of cellular aging, which eventually ends in MSC senescence.

Previously we were able to show that human MSC protected from accelerated aging *in vitro* showed enhanced proliferation capacity when cultivated at conditions of low atmospheric oxygen (Fehrer et al., 2007). We furthermore assessed whole-genome expression profiles of primary MSC, which had been isolated from an infant as well as from an aged individual, both being systemically healthy, after cultivating the cells for a short period at atmospheric conditions of high and low oxygen. The expression level of many genes changed significantly. Amongst these, LEPR transcript numbers were

found increased in MSC from the old donor as well as in cells cultivated at elevated O₂. In vitro replicatively aged MSC exhibited markedly enhanced LEPR mRNA levels. This suggested that aged or stressed MSC may become more susceptible to leptin. Seeding MSC at low density leads to clonal proliferation and the characteristic growth of colonies in vitro. Colony formation is also commonly used to assay MSC proliferation potential and is a generally accepted indicator of MSC stemness (Abdallah and Kassem, 2008). Treatment of MSC with leptin however did not interfere with colony formation. Leptin resistance in MSC can, at least in part, be explained by high levels of SOCS3 (Fruhbeck, 2006). This particular intracellular leptin signaling inhibitor is indeed present in clonogenic MSC (unpublished results).

In an independent set of experiments, in which the cellular electron transport was impaired by treatment of MSC with DPI, we observed greatly enhanced rates of cell death (unpublished data). In parallel to that, LEPR mRNA levels were boosted. In light of these results, MSC were stained with cell-death specific reagents, 7-AAD and annexinV as well as with anti-LEPR/CD295. Cells, which are double positive for the necrosis marker 7-AAD as well as for the apoptosis marker annexinV are generally considered to be dying cells. Consequently, this particular MSC subpopulation, which is cell-death-prone, can be discriminated by an elevated level of LEPR exposed at the cell surface. We therefore concluded that enhanced brightness in CD295 representatively stands for dying MSCs. In the context of proliferating MSC, the appearance of CD295 bright cells emphasizes that the rate of cell death corresponds with the number of cells that fail to self-renew.

A senescent stem cell is a contradiction in terms. Stem cells, which neither give rise to progeny nor self-renew anymore, are consequently eliminated from the stem cell pool. It is virtually impossible to detect senescent MSC in primary cell isolates derived from healthy individuals, even if donors were of advanced age. Most likely, this is due to the fact that the culture conditions applied greatly favor rapidly growing cells. Only when MSC are being propagated in culture for many generations, does accumulation of senescent cells become apparent to the experienced observer. Unfortunately, there are no

MSC-specific molecular markers available, which allow us to label and/or separate presenescent or senescent stem cells.

Taken together, these considerations support the notion that the attenuated proliferation potential of MSC, which are derived from elderly individuals, is determined yet by another process. We hereby propose that the reduction of MSC potency greatly relies on the withdrawal of cells by cell death, be it necrosis or apoptosis (Figure 5). This assumption is supported by the experimental observations reported here, which demonstrate that dying MSC express enhanced levels of LEPR. Furthermore, MSC pools from aged donors, which display slowing growth kinetics, also exhibit increasing levels of LEPR mRNA. In the long run, such MSC cultures cease growth before their youthful equivalents show signs of cellular senescence. We therefore conclude that within a given MSC pool, the number of dying cells is a criterion and a previously undefined measure for stem cell aging.

Acknowledgements

We would like thank Drs. Robert Gassner and Frank Kloss of the University Clinics Innsbruck for continuous support and fruitful discussions regarding questions related to clinical research. This study was supported by the Austrian Science Fund (FWF), NRN project S9305. RB is a DOC-fFORTE fellow of the Austrian Academy of Sciences.

Figure Legends

Figure 1: Differential LEPR expression. By means of qRT-PCR, mRNA levels of LEPR were assessed in MSC derived from 12 systemically healthy donors of both sexes within age range of 7-78 years. Cells were cultivated under atmospheric conditions of 3 or 20% oxygen.

Figure 2: LEPR expression and MSC proliferation capacity. MSCs were isolated from 18 systemically healthy donors of both sexes within age range of 7-78 years. Cells were cultivated at atmospheric conditions of 3% oxygen. The number of population doublings until reaching replicative senescence was assessed. LEPR expression levels of individual primary cultures were assessed by means of qRT-PCR.

Figure 3: LEPR expression in senescent MSC. (A) mRNA levels of the senescence marker p16 and LEPR were examined in primary cultures (p1) and late passages (p4/5) of pre-senescent MSC cultures, which had been clonally expanded in long-term cultivation from three independent donors (D1-3). (B) MSCs were stressed by prolonged cultivation after having reached confluency (c) and p16 and LEPR mRNA levels were compared to growing cells (g).

Figure 4: Flow cytometric analysis: Viability of MSC treated with 10^{-7} M leptin for 3 days was examined using 7-AAD and annexinV in order to label dying cells together with LEPR/CD295 expression. 7-AAD / annexinV – double positive events were labeled in magenta. Left and middle columns: dot plot pictogram, right column: histogrammatic illustration of results presented in the middle column.

Figure 5: Model of long-term MSC proliferation. Self-renewal capacity during aging is compromised by the gradually increasing rate of cell death. This reduces the net proliferation potential of the MSC population. Emergence and accumulation of senescent MSC is characteristic at later stages.

References

- Abdallah, B. M., and Kassem, M. (2008). Human mesenchymal stem cells: from basic biology to clinical applications. *Gene Ther* 15, 109-116.
- Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quarto, R. (2000). Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. *Exp Hematol* 28, 707-715.
- Barry, F. P., and Murphy, J. M. (2004). Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 36, 568-584.
- Baxter, M. A., Wynn, R. F., Jowitt, S. N., Wraith, J. E., Fairbairn, L. J., and Bellantuono, I. (2004). Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. *Stem Cells* 22, 675-682.
- Bluher, S., and Mantzoros, C. S. (2007). Leptin in reproduction. *Curr Opin Endocrinol Diabetes Obes* 14, 458-464.
- Bonab, M. M., Alimoghaddam, K., Talebian, F., Ghaffari, S. H., Ghavamzadeh, A., and Nikbin, B. (2006). Aging of mesenchymal stem cell in vitro. *BMC Cell Biol* 7, 14.
- Bruder, S. P., Jaiswal, N., and Haynesworth, S. E. (1997). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* 64, 278-294.
- Caplan, A. I. (1991). Mesenchymal stem cells. *J Orthop Res* 9, 641-650.
- Cirmanova, V., Bayer, M., Starka, L., and Zajickova, K. (2008). The effect of leptin on bone - an evolving concept of action. *Physiol Res*.
- Cooper, P. D., Burt, A. M., and Wilson, J. N. (1958). Critical effect of oxygen tension on rate of growth of animal cells in continuous suspended culture. *Nature* 182, 1508-1509.
- Csete, M. (2005). Oxygen in the cultivation of stem cells. *Ann N Y Acad Sci* 1049, 1-8.
- Digirolamo, C. M., Stokes, D., Colter, D., Phinney, D. G., Class, R., and Prockop, D. J. (1999). Propagation and senescence of human marrow stromal cells in culture: a

- simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 107, 275-281.
- Dorshkind, K. (1990). Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu Rev Immunol* 8, 111-137.
- Fehrer, C., Brunauer, R., Laschober, G., Unterluggauer, H., Reitingger, S., Kloss, F., Gully, C., Gassner, R., and Lepperdinger, G. (2007). Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their life span. *Aging Cell* 6, 745-757.
- Fehrer, C., and Lepperdinger, G. (2005). Mesenchymal stem cell aging. *Exp Gerontol* 40, 926-930.
- Fruhbeck, G. (2006). Intracellular signalling pathways activated by leptin. *Biochem J* 393, 7-20.
- Gao, Q., and Horvath, T. (2008). Crosstalk between estrogen and leptin signaling in the hypothalamus. *Am J Physiol Endocrinol Metab*.
- Hamrick, M. W., Della-Fera, M. A., Choi, Y. H., Pennington, C., Hartzell, D., and Baile, C. A. (2005). Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. *J Bone Miner Res* 20, 994-1001.
- Kim, G. S., Hong, J. S., Kim, S. W., Koh, J. M., An, C. S., Choi, J. Y., and Cheng, S. L. (2003). Leptin induces apoptosis via ERK/cPLA2/cytochrome c pathway in human bone marrow stromal cells. *J Biol Chem* 278, 21920-21929.
- Lavens, D., Piessevaux, J., and Tavernier, J. (2006). Review: Negative regulation of leptin receptor signalling. *Eur Cytokine Netw* 17, 211-219.
- Lepperdinger, G., Grillari, J., Madeo, F., Breitenbach, M., Minois, N., Berger, P., Grubeck-Loebenstien, B., Fröhlich, K. U., Zwerschke, W., and Jansen-Dürr, P. (2008). Differentiation and cell death during cellular aging. *Frontiers in Bioscience in press*.
- Malendowicz, L. K., Rucinski, M., Belloni, A. S., Ziolkowska, A., and Nussdorfer, G. G. (2007). Leptin and the regulation of the hypothalamic-pituitary-adrenal axis. *Int Rev Cytol* 263, 63-102.

- Muguruma, Y., Yahata, T., Miyatake, H., Sato, T., Uno, T., Itoh, J., Kato, S., Ito, M., Hotta, T., and Ando, K. (2005). Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood* *107*, 1878-1887.
- Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* *284*, 143-147.
- Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* *276*, 71-74.
- Sakaguchi, Y., Sekiya, I., Yagishita, K., Ichinose, S., Shinomiya, K., and Muneta, T. (2004). Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow aspirates. *Blood* *104*, 2728-2735.
- Sethe, S., Scutt, A., and Stolzing, A. (2006). Aging of mesenchymal stem cells. *Ageing Res Rev* *5*, 91-116.
- Stenderup, K., Justesen, J., Clausen, C., and Kassem, M. (2003). Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* *33*, 919-926.
- Stenderup, K., Rosada, C., Justesen, J., Al-Soubky, T., Dagnaes-Hansen, F., and Kassem, M. (2004). Aged human bone marrow stromal cells maintaining bone forming capacity in vivo evaluated using an improved method of visualization. *Biogerontology* *5*, 107-118.
- Watt, F. M., and Hogan, B. L. (2000). Out of Eden: stem cells and their niches. *Science* *287*, 1427-1430.
- Zhao, Y. M., Li, J. Y., Lan, J. P., Lai, X. Y., Luo, Y., Sun, J., Yu, J., Zhu, Y. Y., Zeng, F. F., Zhou, Q., and Huang, H. (2008). Cell cycle dependent telomere regulation by telomerase in human bone marrow mesenchymal stem cells. *Biochem Biophys Res Commun* *369*, 1114-1119.

- Zimmermann, S., Voss, M., Kaiser, S., Kapp, U., Waller, C. F., and Martens, U. M. (2003). Lack of telomerase activity in human mesenchymal stem cells. *Leukemia* 17, 1146-1149.
- Zwartouw, H. T., and Westwood, J. C. (1958). Factors affecting growth and glycolysis in tissue culture. *Br J Exp Pathol* 39, 529-539.

Figure 1

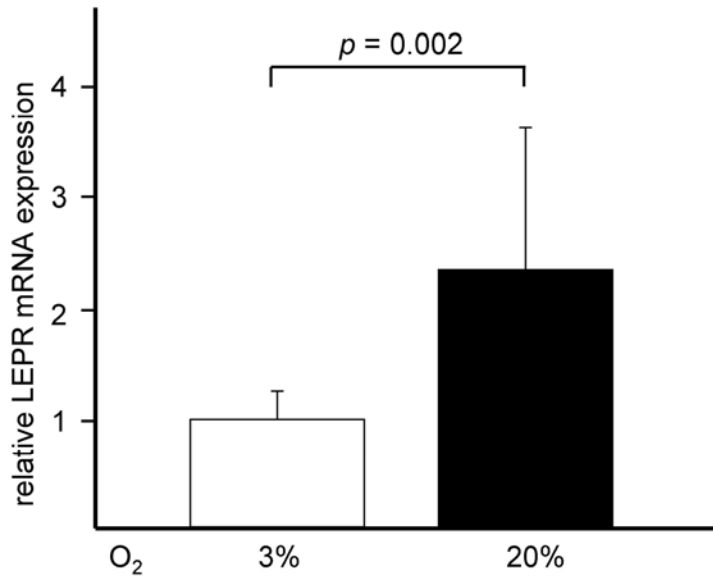


Figure 2

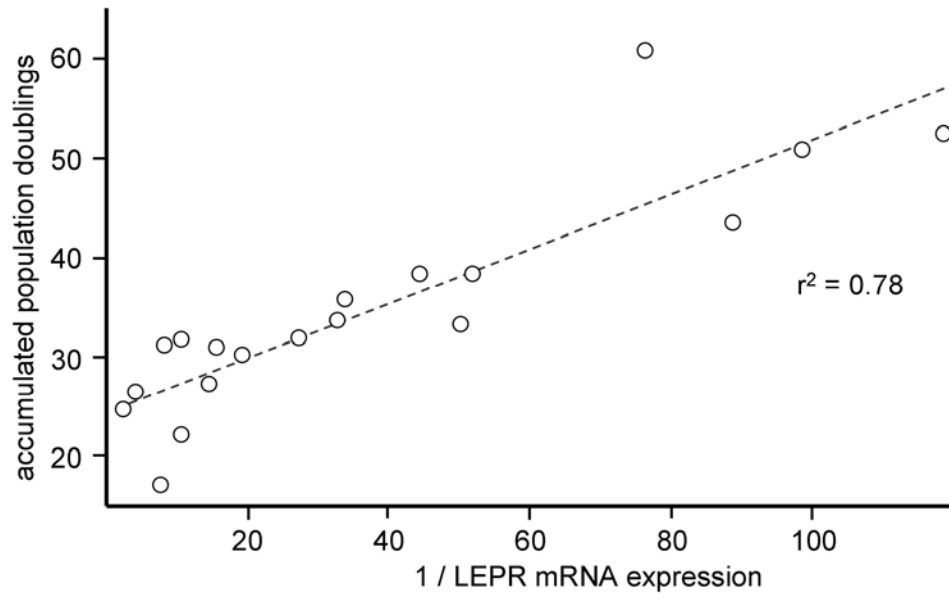


Figure 3

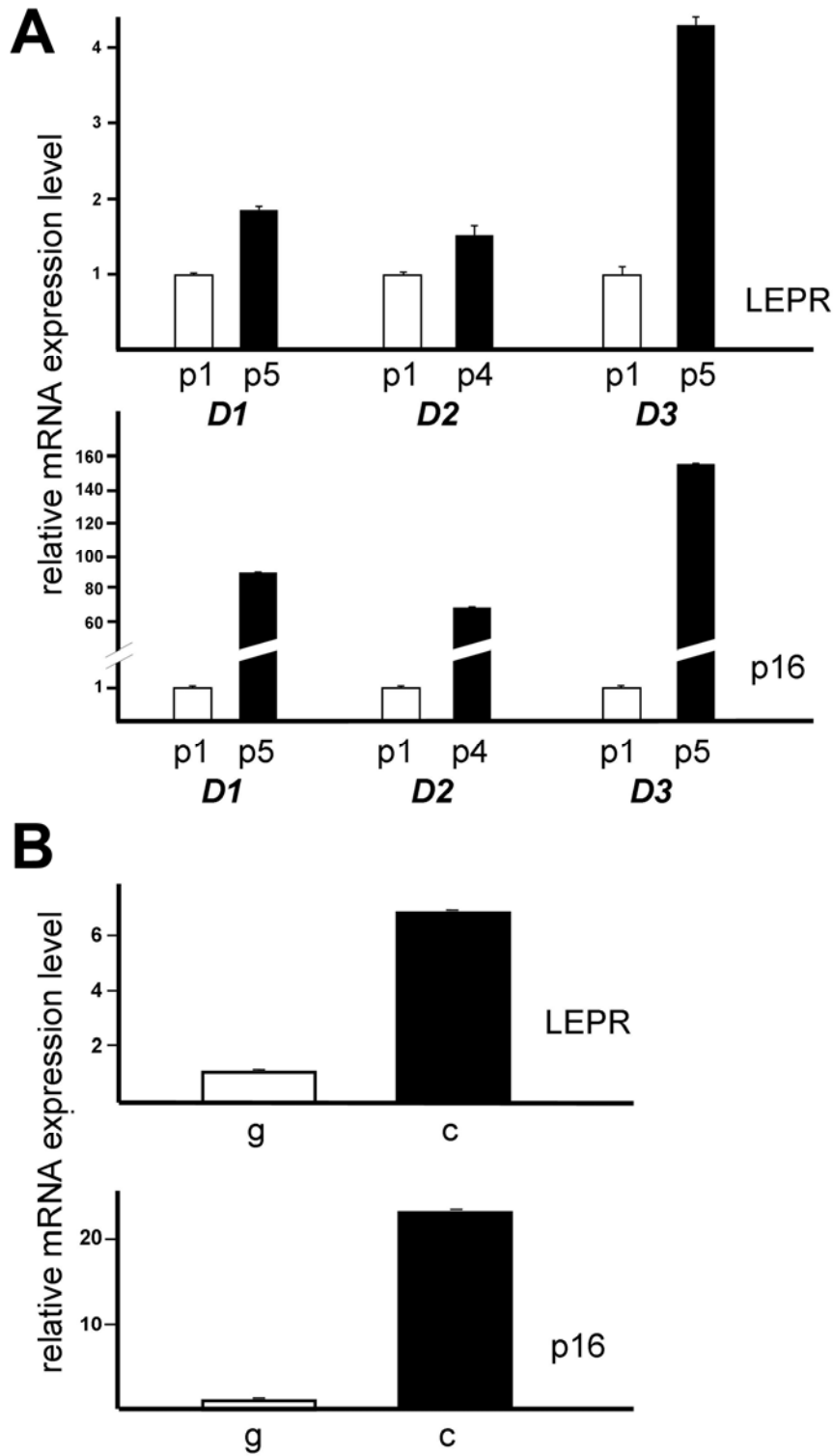


Figure 4

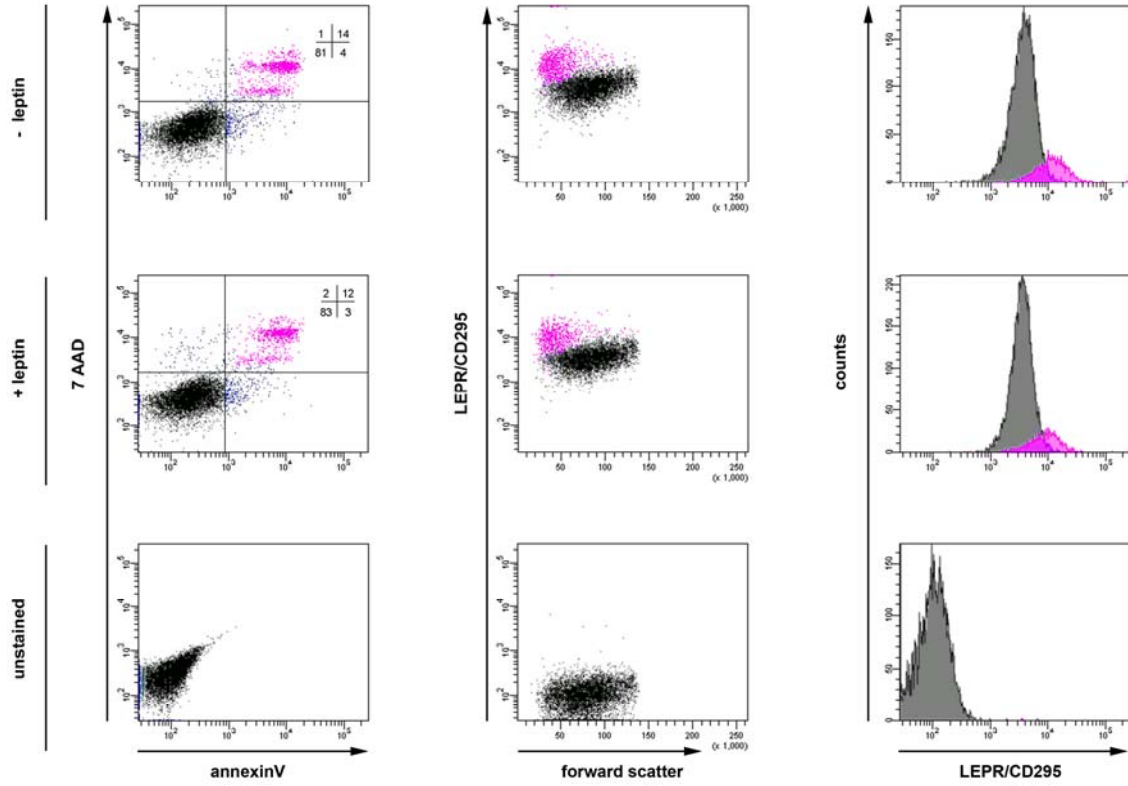


Figure 5

