Controversial Issue: Is it safe to employ mesenchymal stem cells in cell-based therapies?

Günter Lepperdinger [1,*], Regina Brunauer [1], Angelika Jamnig [1], Gerhard Laschober [1], Moustapha Kassem [2]

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

2) Department of Endocrinology and Molecular Endocrinology Laboratory, Medical Biotechnology Center, University Hospital of Odense and University of Southern Denmark, J.B. Winsløws Vej 25, DK-5000 Odense C, Denmark

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

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Abstract:
The prospective clinical use of multipotent mesenchymal stromal stem cells (MSC) holds enormous promise for the treatment of a large number of degenerative and age-related diseases. However, the challenges and risks for cell-based therapies are multifaceted. The risks for patients receiving stem cells, which have been expanded in vitro in the presence of xenogenic compounds, can hardly be anticipated and methods for the culture and manipulation of "safe" MSC ex vivo are being investigated.

During in vitro expansion, stem cells experience a long replicative history and are thus subject to damage from intracellular and extracellular influences. While murine MSC are prone to cellular transformation in culture, human MSC do not transform. One reason for this striking difference is that during long-term culture, human MSC finally become replicatively senescent. In consequence, this greatly restricts their proliferation and differentiation efficiency. It however also limits the yield of sufficient numbers of cells needed for therapy.

Another issue is to eliminate contamination of expanding cells with serum-bound pathogenic agents in order to reduce the risks for infection. A recent technical advancement, which applies human serum platelet lysates as an alternative source for growth factors and essential supplements, allows the unimpaired proliferation of MSC in the absence of animal sera. Here, we present an update regarding cellular senescence of MSC and recent insights concerning potential risks associated with their clinical use.

Background

Stem cells are vitally involved in tissue regeneration and homeostasis in later life. Mesenchymal (skeletal) stem cells (MSC) (also known as multipotent stromal progenitor cells, or marrow stromal cells) are but one particular type of the so-called tissue-specific (or adult) stem cells. This term refers to stem cells derived from postnatal tissues, and this type of stem cell is generally believed to be multi- or oligopotent. MSC can differentiate into mesoderm-type cells, such as osteoblasts, chondrocytes, and adipocytes.

The clinical use of MSC is an emerging field, and part of the so-called regenerative medicine. MSC are envisaged to be employed for the treatment of a number of diseases including non-healing bone defects and fractures, inflammatory arthritis, and repair of suspensory ligament tears (Kassem and Abdallah, 2008). Furthermore, application of MSC is thought to enhance hematopoietic stem cell engraftment in the course of bone marrow transplantation (Giordano et al., 2007), to correct inherited disorders of bone and cartilage (Bolland et al., 2007), to ameliorate tissue damage after myocardial infarction (Abdel-Latif et al., 2007), and to treat graft-versus-host disease (Ringden et al., 2006). Also, attempts are being undertaken to employ MSC as a vehicle for gene therapy, e.g. in osteogenesis imperfecta (Chamberlain et al., 2008; Pochampally et al., 2005). For other clinical indications presently being tested, please see: www.ClinicalTrial.gov.

Since elderly patients are the main target population for this type of therapy, one important issue is to investigate the changes that occur in MSC with age. Studying this aspect of stem cell biology also offers the particular opportunity to define fundamental principles of how cells and tissues change during adulthood. Fundamental in this context is also research on the stem cells' niches, i.e. their microenvironment and the respective molecular composition, as well as the cellular response upon systemically acting endocrinological and immunological factors.

Aging, in vitro senescence and MSC

The relationship between aging and stem cells is important for a number of reasons. The effect of in vitro senescence on stem cell properties is an important topic for understanding the aging process per se, which is the basis of identifying those molecular targets that may delay or "rescue" the deleterious effects of senescence on stem cells. One option for cell-based therapies is autologous stem cell transplantation. In this respect, it is of prime interest to assess the impact of donor age on MSC biology. Often, there is a need for extensive cell amplification in vitro in order to obtain high enough numbers of cells to ensure the success of a cell-based therapy. However, in vitro expansion ultimately leads to cellular senescence and poses a risk of accumulating genetic and epigenetic changes to the cells, which in turn may also lead to cellular transformation and cancer (Figure 1). For this reason, it is a topic of major interest whether stem cells are bound to transform spontaneously in vitro.

MSC: biology and characteristics

Currently, it seems that there is no "universal" source for stem cells suitable for all conceivable clinical applications. It greatly believed that besides embryonic stem cells also different types of specialized, tissue-specific stem cells from adult donors will be employed for different therapies.

The conceptualization of the MSC as a prominent example of an adult stem cell was put forward by Owen and Friedenstein largely based on pioneering experimental studies performed in the 60s and 70s in Russia (Friedenstein et al., 1966; Luria et al., 1971; Owen and Friedenstein, 1988). The notion was popularized by Arnold Caplan in the late 80's and throughout the 90's (Caplan, 1991). Over the past decade, MSC have been the focus of a large number of experimental studies (overview of results see Figure 2). By now it is generally accepted that MSC can differentiate into a number of differently committed progenitor cell types, e.g. into osteoblasts, chondrocytes, and adipocytes as well as hematopoietic supportive stromal cells (Barry et al., 2001; Bruder et al., 1998; Cancedda et al., 2003; Caplan, 2007; Kassem et al., 1991; Le Blanc and Pittenger, 2005; Prockop, 1997). There have also been reports suggesting that these cells differentiate to cardiomyocytes (Wakitani et al., 1995), hepatocytes (Lee et al., 2004) or neuronal cells (Woodbury et al., 2000), but these incipient studies need further confirmation. Currently, bone marrow, subcutaneous

adipose tissue and umbilical cord blood are among the main sources for isolating MSC. Typically MSC are obtained from these tissue preparations by plastic adherence. Consequently, the resulting cell cultures contain not only MSC, but also lineage-committed progenitors (Kuznetsov et al., 1997; Post et al., 2008).

Due to the ease of obtaining these cells, MSC can be transplanted either directly as undifferentiated cells, allowing differentiation processes to take place through induction of locally-acting tissue factors, or alternatively, MSC can be expanded in culture first, before differentiating them appropriately in vitro, to obtain large numbers of cells with a distinctly defined phenotype.

MSC in vitro senescence

Both intrinsic and extrinsic aging processes affect stem cell properties (Kassem et al., 2003; Rando, 2006). One possible intrinsic cellular aging mechanism is replicative or cellular senescence. In 1961, Hayflick and Moorhead discovered that in vitro, human skin fibroblasts undergo only a limited number of population doublings (termed Hayflick limit), and that this number decreased with increasing donor age (Hayflick and Moorhead, 1961). Similar to other human diploid cells, MSC exhibit replicative senescence in vitro, as demonstrated by a number of investigators (Fehrer et al., 2007; Kern et al., 2006; Stenderup et al., 2003; Stenderup et al., 2004; Stolzing et al., 2008). The in vitro senescent phenotype includes the following characteristic features: (i) irreversible arrest of cell division (in contrast to quiescence, where this lock is reversible), (ii) resistance to apoptotic death, and (iii) the excretion of molecules normally secreted during wound repair and infection, such as inflammatory cytokines, proteases and growth factors, the latter having detrimental consequences for the surrounding tissue (Campisi, 2001). Some, but not all of these characteristics have also been described for MSC cultures (Fehrer et al., 2007; Kern et al., 2006; Stenderup et al., 2003; Stenderup et al., 2004; Stolzing et al., 2008)

The presence of cells in vivo, which closely resemble the phenotype of senescent cells replicatively aged in culture, has been observed in human skin

(Dimri et al., 1995; Ressler et al., 2006) as well as in the vascular system (Vasile et al., 2001). It is thus conceivable that also stem cells reach an equivalent state of senescence in vivo, but the proof of this hypothesis is still missing.

In vitro senescence and epigenetics:

Gene expression is controlled by transcription factors. Their respective activities are influenced by the chromatin structure. This in turn is modulated by chromatin remodeling factors, which modify the balance between euchromatin and heterochromatin. These particular factors are also known to be involved in determining self-renewal, proliferation and differentiation potential of stem cells (Boquest et al., 2006; Squillaro et al., 2008). Chromatin remodelers such as the ATP-dependent remodeling complexes which contain Brg1 are required for cell cycle control, apoptosis and cell differentiation. Moreover, they negatively affect stem cell self-renewal properties, because they appear to be involved in the induction of senescence and apoptosis (Napolitano et al., 2007). Other factors such as the polycomb group protein Bmi1, prevent senescence and thus maintain stem cell self-renewal at an uncommitted state (Park et al., 2004). In particular, BMI1 represses the transcription of the cell cycle regulator $p16^{INK4A}$ (Jacobs et al., 1999). In MSC cultivated in vitro, $p16^{INK4A}$ function is transcriptionally inactivated by DNA methylation in the core promoter region (Shibata et al., 2007). Down-regulation of $p16^{INK4A}$ expression results in the escape from senescence and the restoration of the cell's proliferating activity. These few examples clearly highlight the need for the careful balancing of site-specific chromatin remodeling to distinctly determine stem cell properties, while at the same time reducing enhanced cellular aging.

MSC, telomere and telomerase

Telomeres are repetitive DNA sequences at the physical ends of chromosomes. They are responsible for the maintenance of chromosome stability, and therefore, dysfunctional telomeres affect genome stability, and the cell's proliferative capacity. The most terminal, single-stranded region of telomeres loop back and pair with an internal double-stranded location forming a stable loop structure - the telomeric cap (Griffith et al., 1999). Cells with telomeres in

their capped state exhibit enhanced proliferative capacity, whereas the uncapped form coincides with cell cycle arrest (Blasco, 2007b). In the absence of the enzyme telomerase, in many somatic cells including MSC, the telomeres gradually become shorter as the cell division proceeds The net impact is the successive telomere shortening during DNA replication with every cell division (Garcia et al., 2007; Harley, 2008; Shay and Wright, 2007). A direct consequence is the inactivation of genes closest to the telomere sequences, which is either directly, or indirectly by a positional effect, involving the formation of heterochromatin (Blasco, 2007a). Constitutive expression of the catalytic subunit of the telomerase gene, hTERT prevents replicative senescence and maintains "stemness" characteristics of MSC (Simonsen et al., 2002). Also regulated telomere maintenance by ectopic expression of hTERT sustains stem cell properties in MSC (Abdallah et al., 2005). It could be shown recently that treatment of MSC with trichostatin A resulted in transient expression of hTERT (Serakinci et al., 2006). In line with this, MSC that had been stalled in the cell cycle by serum deprivation, transiently expressed telomerase activity during the S phase (Zhao et al., 2008). Whether this periodic upregulation of telomerase activity is high enough to retain telomeric capping function, and thus inducing relevant biological effects, remains to be determined. Clearly, more research needs to be performed for the detailed understanding of how to counteract telomere shortening in MSC when expanding them in vitro.

MSC and cell-based therapies: concerns and potential hazards

MSC and serum-containing culture media

Most research laboratories culture MSC in media containing 10-20% fetal calf serum. The use of animal, in particular bovine products involves the risk of prion transmission, and/or yet unidentified zoonoses as well as the activation of the host immune system by biomolecules, which are foreign to the human species. Alternatives to that have been introduced lately: (i) freshly frozen plasma and platelets (Muller et al., 2006), or (ii) platelet lysate (Doucet et al., 2005). Careful side-by-side comparisons using media containing either calf serum or human platelet lysate (hPL) showed that MSC exhibit enhanced proliferation rates in hPL without changing morphology, immunophenotype or differentiation capacity. No apparent genetic abnormalities were detectable upon this treatment

(Bartmann et al., 2007; Lange et al., 2007). The cells also retained their immune-modulatory potential. More than that, they showed a more efficient suppression of allo-antigen-induced lymphocyte proliferation (Bernardo et al., 2007a). Furthermore, they were void of tumorgenicity when implanted into nude mice (Schallmoser et al., 2007). Another alternative to fetal calf serum is autologous patient serum. However, it appears to be less favorable, since serum derived from aged individuals interferes with MSC proliferation and/or differentiation capacity (Abdallah et al., 2006; Mannello and Tonti, 2007).

MSC and transformation risk

There are concerns that transplanted, culture expanded MSC may undergo spontaneous transformation. It is also thought that similar problems may arise, when MSC derived from aged donors are employed in cell therapy (Li et al., 2007). This conclusion however is based on extrapolations from studies performed on murine MSC. During long-term culture, murine MSC accumulate chromosomal aberrations and exhibit a malignant transformation phenotype (Zhou et al., 2006). The resulting MSC, when implanted into immune-compromised mice using hydroxyapatite/tricalcium phosphate as a carrier, as well as when injected into the tail vein led to sarcoma formation (Miura et al., 2006). Murine MSC also rapidly acquire chromosomal abnormalities in culture, and after infusion, these cells invaded lung parenchyma and formed tumor nodules with the characteristics of differentiated osteosarcomas (Aguilar et al., 2007). Compared to murine MSC, human MSC in long-term culture greatly differ in their behavior. Two recent reports demonstrated that human bone marrow-derived MSC are not bound to undergo transformation when propagated in long-term culture (Miura et al., 2006; Zhou et al., 2006), and under the same culture conditions, human MSC do not exhibit obvious chromosomal abnormalities at late passages (Bernardo et al., 2007b). Bernardo and colleagues performed extensive genetic characterizations using comparative genomic hybridization, karyotyping and subtelomeric fluorescent in situ hybridization analysis, telomerase activity as well as assessing the alternative lengthening of telomere at different stages of long-term culture. The authors found no evidence for transformation of human MSC during long term culture.

This issue is however still under debate, because two studies in the literature reported that MSC in vitro may exhibit spontaneous transformation. In one study, Rubino et al refer to adipose-tissue-derived MSC from young children do transform spontaneously in long-term culture (Rubio et al., 2005). The cells employed in these cultures were however not obtained from healthy individuals but isolated from subcutaneous fat during operation of complicated appendicitis and it is thus likely that they are not comparable to normal MSC (Rubio et al., 2005; Kassem et al., 2005). The second study broaches the issue of spontaneous transformation in the unphysiological case of immortalizing human MSC with hTERT, thereby gaining high levels of telomerase activity (Burns et al., 2005; Serakinci et al., 2004). Such MSC showed various genetic and epigenetic changes in spite of maintaining a normal karyotype (Burns et al., 2005; Serakinci et al., 2004). Taken together, there is to date good reason to believe that human bone marrow MSC that have been derived from healthy individuals in a standardized manner, do not readily transform in culture. This is in stark contrast to their instable murine counterpart and thus poses the question whether murine MSC are a suitable model system at any instance to understand their human relatives.

Another concern is whether administered MSC promote the growth of a latent tumor. MSC can be recruited to the stroma of developing tumors when systemically infused in animal models for glioma, colon carcinoma, ovarian carcinoma, Karposi's sarcoma and melanoma (Lazennec and Jorgensen, 2008). There is also evidence that MSC, when injected subcutaneously together with tumor cells, can increase tumor growth by increasing the rates of tumor necrosis and angiogenesis (Zhu et al., 2006). In a breast cancer xenograft model, though having no effect on tumor growth per se, MSC greatly increased the metastasis rate through secretion of CCL5 (also called RANTES)(Karnoub et al., 2007). It has also been shown that highly malignant bone sarcomas contain self-renewing stem cells, which express stem cell markers such as Oct3/4 and Nanog, in parallel with the surface markers Stro-1, CD105 and CD44, a phenotype similar to MSC (Gibbs et al., 2005).

Transplanted bone marrow contains MSC, and hematologist have used these cells in clinical therapies for decades without recognising adverse effects comparable to the aforementioned scenarios. This let us to conclude that the relevance of these experimental studies for MSC-based cell therapies is perhaps limited. However, it should be also noted here that further investigations of how MSC and their stromal derivatives compromise cancer cells is a mandatory future research field in order to understand, and consequently influence tumor progression.

In conclusion, there is increasing evidence that with respect to the risk of MSC transformation and subsequent tumor formation initiated by MSC, human MSC appear to be safe. We however feel impelled to strongly recommend at this point careful quality control procedures for all cell preparations. These should be implemented for all kinds of cell-based therapies. Suffice it to say that before administering MSC to patients the cell preparations have to undergo carefull phenotypic, functional and genetic characterizations.

Concluding remarks:

The record of safety for MSC in general is excellent and we anticipate that after successful conclusion of ongoing preclinical and clinical tests, MSC will be gradually introduced into clinical practice for a number of disease conditions in the coming years. Careful pre-administration safety monitoring as well as close monitoring of the patients are important pre-requisites for the success of this novel form of therapy. Regulatory bodies such as the US Food and Drug administration (Halme and Kessler, 2006) and the European Union (http://eur-lex.europa.eu/LexUriServ/site/en/oj/2007/l_324/l_32420071210en01210137.pdf) have recently established a set of regulations for cell-based therapeutics. With continuous and open interactions between investigators, research institutions and regulatory bodies, successful, and most importantly, safe cell-based therapies will become routine for patients' treatment in the near future.

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Figure Legends

Figure 1: A model for stem cell propagation and aging in vitro - consequences for MSC progeny: the in vivo niche (magenta) is considered to control a stem cell's fate. In this niche, the cell rests in quiescence only being stimulated to self-renew at special occasions. Once being removed from its niche and put in culture, stem cells are capable of continuous proliferation that leads to cellular senescence, primarily caused by accumulation of damage to the genome, biomolecules and organelles. In due course, cells loose their proliferation potential and enter into a state of irreversible, senescence-associated growth arrest, or they exhibit alterations in their differentiation potential. Deviations from well-controlled regulation of lineage specific differentiation potential are thought to be the result of endogenous cellular alterations, but can be also caused by changes in the microenvironment.

Figure 2: A hypothetical model of the extrinsic feedback control of the MSC niche and regulation of MSC progeny: MSC reside in their niches. Tasks of MSC are to form tissues and organs during development and growth. They are also involved in regeneration and repair during adulthood. In addition, MSC nurture stromal compartments with cells, which themselves are important regulators of stem cell renewal, in particular in the case of hematopoietic stem and precursor cells. Moreover, MSC exert immune modulatory properties. These processes are intricately dependent on the supervision of the niche, which is but a part of the organ that constantly undergoes changes due to metabolic activity, pathologic insults and biological aging.

References

Abdallah, B. M., Haack-Sorensen, M., Burns, J. S., Elsnab, B., Jakob, F., Hokland, P., and Kassem, M. (2005). Maintenance of differentiation potential of human bone marrow mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene despite [corrected] extensive proliferation. Biochem Biophys Res Commun *326*, 527-538.

Abdallah, B. M., Haack-Sorensen, M., Fink, T., and Kassem, M. (2006). Inhibition of osteoblast differentiation but not adipocyte differentiation of mesenchymal stem cells by sera obtained from aged females. Bone *39*, 181-188.

Abdel-Latif, A., Bolli, R., Tleyjeh, I. M., Montori, V. M., Perin, E. C., Hornung, C. A., Zuba-Surma, E. K., Al-Mallah, M., and Dawn, B. (2007). Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med *167*, 989-997.

Aguilar, S., Nye, E., Chan, J., Loebinger, M., Spencer-Dene, B., Fisk, N., Stamp, G., Bonnet, D., and Janes, S. M. (2007). Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. Stem Cells *25*, 1586-1594.

Barry, F., Boynton, R. E., Liu, B., and Murphy, J. M. (2001). Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res *268*, 189-200.

Bartmann, C., Rohde, E., Schallmoser, K., Purstner, P., Lanzer, G., Linkesch, W., and Strunk, D. (2007). Two steps to functional mesenchymal stromal cells for clinical application. Transfusion *47*, 1426-1435.

Bernardo, M. E., Avanzini, M. A., Perotti, C., Cometa, A. M., Moretta, A., Lenta, E., Del Fante, C., Novara, F., de Silvestri, A., Amendola, G.*, et al.* (2007a). Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. J Cell Physiol *211*, 121-130.

Bernardo, M. E., Zaffaroni, N., Novara, F., Cometa, A. M., Avanzini, M. A., Moretta, A., Montagna, D., Maccario, R., Villa, R., Daidone, M. G.*, et al.* (2007b). Human bone marrow derived mesenchymal stem cells do not

undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res *67*, 9142-9149.

Blasco, M. A. (2007a). The epigenetic regulation of mammalian telomeres. Nat Rev Genet *8*, 299-309.

Blasco, M. A. (2007b). Telomere length, stem cells and aging. Nat Chem Biol *3*, 640-649.

Bolland, B. J., Tilley, S., New, A. M., Dunlop, D. G., and Oreffo, R. O. (2007). Adult mesenchymal stem cells and impaction grafting: a new clinical paradigm shift. Expert Rev Med Devices *4*, 393-404.

Boquest, A. C., Noer, A., and Collas, P. (2006). Epigenetic programming of mesenchymal stem cells from human adipose tissue. Stem Cell Rev *2*, 319-329.

Bruder, S. P., Kraus, K. H., Goldberg, V. M., and Kadiyala, S. (1998). The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am *80*, 985-996.

Burns, J. S., Abdallah, B. M., Guldberg, P., Rygaard, J., Schroder, H. D., and Kassem, M. (2005). Tumorigenic heterogeneity in cancer stem cells evolved from long-term cultures of telomerase-immortalized human mesenchymal stem cells. Cancer Res *65*, 3126-3135.

Campisi, J. (2001). From cells to organisms: can we learn about aging from cells in culture? Exp Gerontol *36*, 607-618.

Cancedda, R., Bianchi, G., Derubeis, A., and Quarto, R. (2003). Cell therapy for bone disease: a review of current status. Stem Cells *21*, 610-619.

Caplan, A. I. (1991). Mesenchymal stem cells. J Orthop Res *9*, 641-650.

Caplan, A. I. (2007). Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol *213*, 341-347.

Chamberlain, J. R., Deyle, D. R., Schwarze, U., Wang, P., Hirata, R. K., Li, Y., Byers, P. H., and Russell, D. W. (2008). Gene targeting of mutant COL1A2 alleles in mesenchymal stem cells from individuals with osteogenesis imperfecta. Mol Ther *16*, 187-193.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995). A

biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.

Doucet, C., Ernou, I., Zhang, Y., Llense, J. R., Begot, L., Holy, X., and Lataillade, J. J. (2005). Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. J Cell Physiol *205*, 228-236.

Fehrer, C., Brunauer, R., Laschober, G., Unterluggauer, H., Reitinger, 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 lifespan. Aging Cell *6*, 745-757.

Friedenstein, A. J., Piatetzky, S., II, and Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol *16*, 381-390.

Garcia, C. K., Wright, W. E., and Shay, J. W. (2007). Human diseases of telomerase dysfunction: insights into tissue aging. Nucleic Acids Res *35*, 7406-7416.

Gibbs, C. P., Kukekov, V. G., Reith, J. D., Tchigrinova, O., Suslov, O. N., Scott, E. W., Ghivizzani, S. C., Ignatova, T. N., and Steindler, D. A. (2005). Stem-like cells in bone sarcomas: implications for tumorigenesis. Neoplasia *7*, 967-976.

Giordano, A., Galderisi, U., and Marino, I. R. (2007). From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. J Cell Physiol *211*, 27-35.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell *97*, 503-514.

Halme, D. G., and Kessler, D. A. (2006). FDA regulation of stem-cell-based therapies. N Engl J Med *355*, 1730-1735.

Harley, C. B. (2008). Telomerase and cancer therapeutics. Nat Rev Cancer *8*, 167-179.

Hayflick, L., and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. Exp Cell Res *25*, 585-621.

Jacobs, J. J., Kieboom, K., Marino, S., DePinho, R. A., and van Lohuizen, M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell

proliferation and senescence through the ink4a locus. Nature *397*, 164-168.

Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., Richardson, A. L., Polyak, K., Tubo, R., and Weinberg, R. A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature *449*, 557-563.

Kassem, M., and Abdallah, B. M. (2008). Human bone-marrow-derived mesenchymal stem cells: biological characteristics and potential role in therapy of degenerative diseases. Cell Tissue Res *331*, 157-163.

Kassem, M., Burns, J. S., Garcia Castro, J., and Rubio Munoz, D. (2005). Adult stem cells and cancer. Cancer Res *65*, 9601; author reply 9601.

Kassem, M., Risteli, L., Mosekilde, L., Melsen, F., and Eriksen, E. F. (1991). Formation of osteoblast-like cells from human mononuclear bone marrow cultures. Apmis *99*, 269-274.

Kassem, M., Stenderup, K., Justesen, J., and Kveiborg, M. (2003). In vitro senescence of human osteoblasts. In Aging of cells in and outside the body, S. C. Kul, and R. Wadhwa, eds. (UK, Kluwer Academic Publisher), pp. 67-84.

Kern, S., Eichler, H., Stoeve, J., Kluter, H., and Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells *24*, 1294-1301.

Kuznetsov, S. A., Krebsbach, P. H., Satomura, K., Kerr, J., Riminucci, M., Benayahu, D., and Robey, P. G. (1997). Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res *12*, 1335-1347.

Lange, C., Cakiroglu, F., Spiess, A. N., Cappallo-Obermann, H., Dierlamm, J., and Zander, A. R. (2007). Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. J Cell Physiol *213*, 18-26.

Lazennec, G., and Jorgensen, C. (2008). Adult Multipotent Stromal Cells and Cancer: Risk or Benefit? Stem Cells.

Le Blanc, K., and Pittenger, M. (2005). Mesenchymal stem cells: progress toward promise. Cytotherapy *7*, 36-45.

Lee, K. D., Kuo, T. K., Whang-Peng, J., Chung, Y. F., Lin, C. T., Chou, S. H., Chen, J. R., Chen, Y. P., and Lee, O. K. (2004). In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology *40*, 1275-1284.

Li, H., Fan, X., Kovi, R. C., Jo, Y., Moquin, B., Konz, R., Stoicov, C., Kurt-Jones, E., Grossman, S. R., Lyle, S.*, et al.* (2007). Spontaneous expression of embryonic factors and p53 point mutations in aged mesenchymal stem cells: a model of age-related tumorigenesis in mice. Cancer Res *67*, 10889-10898.

Luria, E. A., Panasyuk, A. F., and Friedenstein, A. Y. (1971). Fibroblast colony formation from monolayer cultures of blood cells. Transfusion *11*, 345-349.

Mannello, F., and Tonti, G. A. (2007). Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells *25*, 1603-1609.

Miura, M., Miura, Y., Padilla-Nash, H. M., Molinolo, A. A., Fu, B., Patel, V., Seo, B. M., Sonoyama, W., Zheng, J. J., Baker, C. C.*, et al.* (2006). Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells *24*, 1095-1103.

Muller, I., Kordowich, S., Holzwarth, C., Spano, C., Isensee, G., Staiber, A., Viebahn, S., Gieseke, F., Langer, H., Gawaz, M. P.*, et al.* (2006). Animal serum-free culture conditions for isolation and expansion of multipotent mesenchymal stromal cells from human BM. Cytotherapy *8*, 437-444.

Napolitano, M. A., Cipollaro, M., Cascino, A., Melone, M. A., Giordano, A., and Galderisi, U. (2007). Brg1 chromatin remodeling factor is involved in cell growth arrest, apoptosis and senescence of rat mesenchymal stem cells. J Cell Sci *120*, 2904-2911.

Owen, M., and Friedenstein, A. J. (1988). Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp *136*, 42-60.

Park, I. K., Morrison, S. J., and Clarke, M. F. (2004). Bmi1, stem cells, and senescence regulation. J Clin Invest *113*, 175-179.

Pochampally, R. R., Horwitz, E. M., DiGirolamo, C. M., Stokes, D. S., and Prockop, D. J. (2005). Correction of a mineralization defect by overexpression of a wild-type cDNA for COL1A1 in marrow stromal cells (MSCs) from a patient with osteogenesis imperfecta: a strategy for rescuing mutations that produce dominant-negative protein defects. Gene Ther *12*, 1119-1125.

Post, S., Abdallah, B. M., Bentzon, J. F., and Kassem, M. (2008). Demonstration of the presence of independent pre-osteoblastic and pre-adipocytic cell populations in bone marrow-derived mesenchymal stem cells. Bone.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science *276*, 71-74.

Rando, T. A. (2006). Stem cells, ageing and the quest for immortality. Nature *441*, 1080-1086.

Ressler, S., Bartkova, J., Niederegger, H., Bartek, J., Scharffetter-Kochanek, K., Jansen-Durr, P., and Wlaschek, M. (2006). p16INK4A is a robust in vivo biomarker of cellular aging in human skin. Aging Cell *5*, 379-389.

Ringden, O., Uzunel, M., Rasmusson, I., Remberger, M., Sundberg, B., Lonnies, H., Marschall, H. U., Dlugosz, A., Szakos, A., Hassan, Z.*, et al.* (2006). Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation *81*, 1390-1397.

Rubio, D., Garcia-Castro, J., Martin, M. C., de la Fuente, R., Cigudosa, J. C., Lloyd, A. C., and Bernad, A. (2005). Spontaneous human adult stem cell transformation. Cancer Res *65*, 3035-3039.

Schallmoser, K., Bartmann, C., Rohde, E., Reinisch, A., Kashofer, K., Stadelmeyer, E., Drexler, C., Lanzer, G., Linkesch, W., and Strunk, D. (2007). Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion *47*, 1436-1446.

Serakinci, N., Guldberg, P., Burns, J. S., Abdallah, B., Schrodder, H., Jensen, T., and Kassem, M. (2004). Adult human mesenchymal stem cell as a target for neoplastic transformation. Oncogene *23*, 5095-5098.

Serakinci, N., Hoare, S. F., Kassem, M., Atkinson, S. P., and Keith, W. N. (2006). Telomerase promoter reprogramming and interaction with general transcription factors in the human mesenchymal stem cell. Regen Med *1*, 125-131.

Shay, J. W., and Wright, W. E. (2007). Hallmarks of telomeres in ageing research. J Pathol *211*, 114-123.

Shibata, K. R., Aoyama, T., Shima, Y., Fukiage, K., Otsuka, S., Furu, M., Kohno, Y., Ito, K., Fujibayashi, S., Neo, M.*, et al.* (2007). Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. Stem Cells *25*, 2371-2382.

Simonsen, J. L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S. I., Jensen, T. G., and Kassem, M. (2002). Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat Biotechnol *20*, 592-596.

Squillaro, T., Hayek, G., Farina, E., Cipollaro, M., Renieri, A., and Galderisi, U. (2008). A case report: bone marrow mesenchymal stem cells from a rett syndrome patient are prone to senescence and show a lower degree of apoptosis. J Cell Biochem *103*, 1877-1885.

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.

Stolzing, A., Jones, E., McGonagle, D., and Scutt, A. (2008). Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev *129*, 163-173.

Vasile, E., Tomita, Y., Brown, L. F., Kocher, O., and Dvorak, H. F. (2001). Differential expression of thymosin beta-10 by early passage and senescent vascular endothelium is modulated by VPF/VEGF: evidence for senescent endothelial cells in vivo at sites of atherosclerosis. Faseb J *15*, 458-466.

Wakitani, S., Saito, T., and Caplan, A. I. (1995). Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve *18*, 1417-1426.

Woodbury, D., Schwarz, E. J., Prockop, D. J., and Black, I. B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res *61*, 364-370.

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.

Zhou, Y. F., Bosch-Marce, M., Okuyama, H., Krishnamachary, B., Kimura, H., Zhang, L., Huso, D. L., and Semenza, G. L. (2006). Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. Cancer Res *66*, 10849-10854.

Zhu, W., Xu, W., Jiang, R., Qian, H., Chen, M., Hu, J., Cao, W., Han, C., and Chen, Y. (2006). Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. Exp Mol Pathol *80*, 267-274.

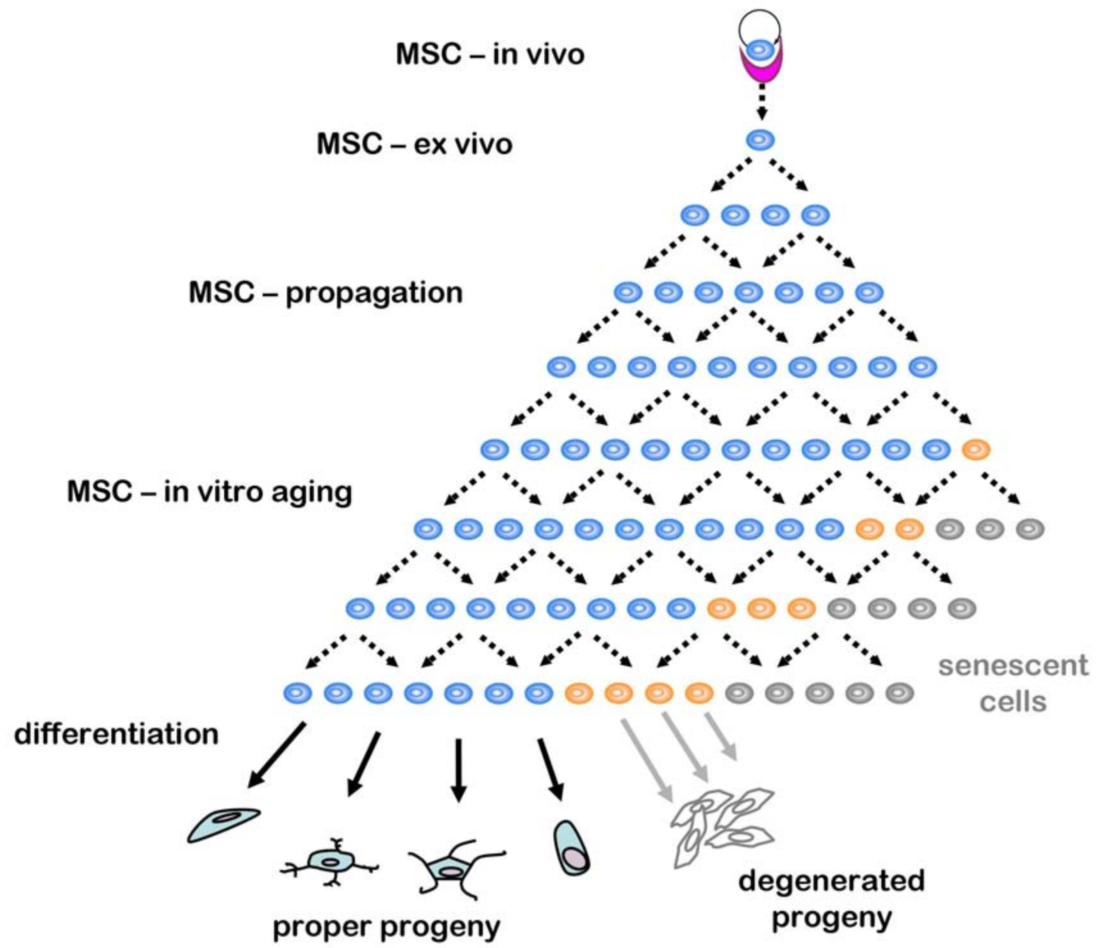

Figure 1

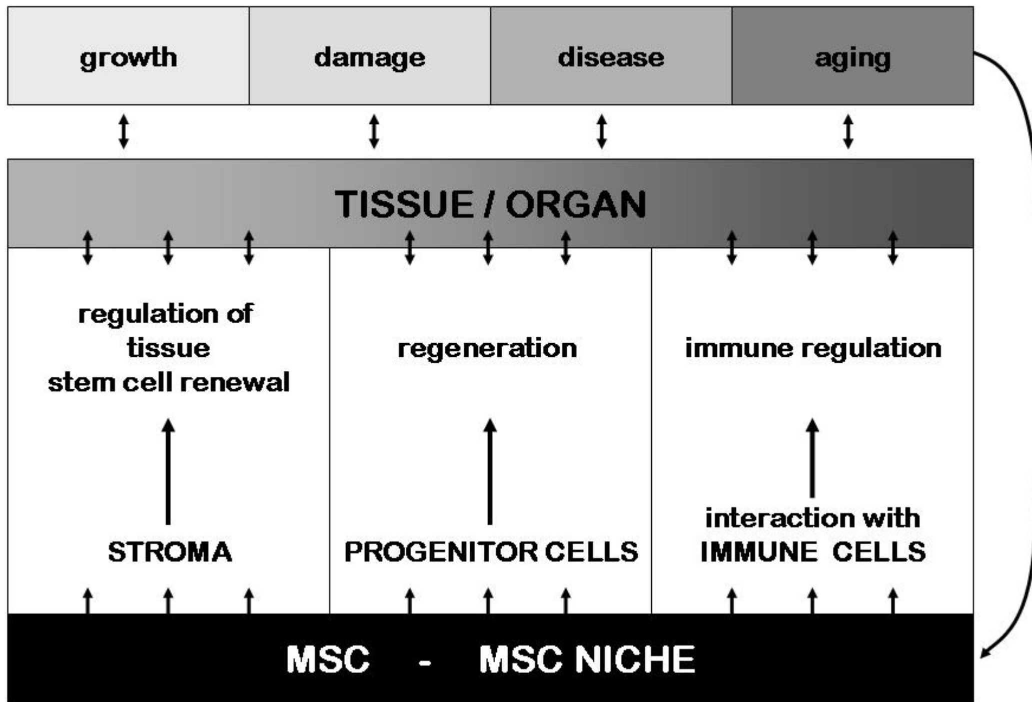

Figure 2