Original Article

Importance of serum source for the *in vitro* replicative senescence of human bone marrow derived mesenchymal stem cells

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Abstract

Human mesenchymal stem cells (hMSCs) may be used for therapeutic applications. Culture conditions such as the serum source may impact on cell quality and the onset of replicative senescence. We have examined the effect of culturing hMSCs in autologous serum (AS) versus fetal bovine serum (FBS) on factors involved in *in vitro* replicative senescence. hMSCs from four donors were cultured in 10% FBS or 10% AS until they reached senescence. Cells were harvested at early passage and near senescence to study factors known to be involved in cellular senescence.The number of population doublings till senescence was similar for cells cultured in FBS, but varied greatly for hMSCs cultured in AS. FBS cells accumulated in S phase of cell cycle. This could not be explained by increased expression of cell cycle inhibitor proteins. Heat shock proteins were upregulated in AS compared to FBS cells. Reactive oxygen species and nitric oxide were upregulated in senescent FBS cells. Telomeres were shorter in senescent cells, more significantly in FBS cells. The source of serum was a determinant for the time till senescence in cultured hMSC. Serum source affected aspects of cell cycle regulation and the levels of heat shock proteins. Several mechanisms are likely to be responsible for replicative senescence in hMSC. Insight into the molecular details of how serum factors impacts on these mechanisms is important for the safe use of hMSCs in clinical applications.

Keywords: Bone marrow derived mesenchymal stem cells, fetal bovine serum, autologous serum, replicative senescence.

Introduction

Human mesenchymal stem cells (hMSCs) are defined as plastic adherent cells with the potential to differentiate into multiple mesenchymal tissues (Dominici, M. et al., 2006). hMSC's derived from bone marrow (hBM-MSCs) can proliferate *in vitro* and differentiate into lineages typical of bone, adipose tissue, cartilage and muscle (Caplan, A. I. & Bruder, S. P., 2001). Therefore, hBM-MSCs are interesting for tissue engineering strategies aiming to repair these tissues (Phinney, D. G. & Prockop, D. J., 2007). In addition, hMSCs are considered to be identical to the pericytes attached to endothelial cells in blood vessels *in vivo,* and thus may also be of importance for cell-based therapeutic neovascularization (Crisan, M. et al., 2009). However, hMSCs are rare within the BM aspirate, and need to be expanded *in vitro* for some passages in order to obtain clinical relevant numbers of cells. Although hMSCs are considered as stem cells based on self renewing capacity, differentiation capability and functional reconstitution following clonal transfer (Sacchetti, B. et al., 2007), they have a definitive life span and cease to proliferate after certain number of cell divisions. This state is referred as replicative senescence. It is characterized by cell enlargement and a certain metabolic activity, but the cells are completely refractory to mitogenic stimuli. This was first described in 1960s by Leonard Hayflick, and the number of population doublings (PD) inherent in a cell before the onset of replicative senescence is called the Hayflick limit (Hayflick, L., 1965).

Since adult stem cells are responsible for cell renewal and maintenance of tissue homeostasis, it has been argued that cellular senescence could be related to the aging process of the whole organism (Van, Zant G. & Liang, Y., 2003; Sharpless, N. E. & Schatten, G., 2009; Sharpless, N. E. & Depinho, R. A., 2004). *In vitro,* it has been shown that replicative senescence starts as soon as hBM-MSCs are placed in culture (Wagner, W. et al., 2008), and that their proliferation and differentiation capacity decrease with time in culture (Banfi, A. et al., 2000; Noer, A., Boquest, A. C., & Collas, P., 2007; Sethe, S., Scutt, A., & Stolzing, A., 2006). Therefore, analysis of the senescence status of hMSCs is an important part of the overall assessment of the suitability of the cells for therapeutic purposes.

Recently, the mechanisms underlying replicative senescence in stem cells have gained attention. Detailed investigations at the molecular level have revealed two major pathways for the induction of cellular senescence. One is dependent on telomere length, is mediated by the p53 and p21 cell signaling pathway and also encompasses the DNA-damage response mechanism. In contrast, a telomere-independent mechanism, typically activated by oxidative stress, is mediated by the Erk-p38^{MAPK} signaling pathway (Muller, M., 2009). There is evidence that this process also involves accumulation of cell cycle inhibitor proteins like p16^{INK4a} and heat shock proteins (Shibata, K. R. et al., 2007; Stolzing, A., Jones, E., McGonagle, D., & Scutt, A., 2008).

With hMSCs becoming popular candidates in clinical settings, concerns have been raised regarding the culture of these cells in large scale for therapy. Most of the protocols use fetal bovine serum (FBS) as a supplement for cell attachment, growth factors and vital nutrients. However, FBS contain xenogenic proteins and potentially also zoonoses, with the risk of inducing immunological responses and transmitting pathogens. In addition, FBS is known to display considerable batch-to-batch variability (Heiskanen, A. et al., 2007; Honn, K. V., Singley, J. A., & Chavin, W., 1975; Sundin, M. et al., 2007). Still, in most of the clinical trials hMSCs have been cultured in FBS, and so far no reports have documented serious side effects (Berger, M. G. et al., 2006; Le, Blanc K., 2003; Mannello, F. & Tonti, G. A., 2007).. Other sources have been suggested as alternatives to FBS such as autologous or allogenic human serum (Shahdadfar, A., Fronsdal, K., Haug, T., Reinholt, F. P., & Brinchmann, J. E., 2005; Stute, N. et al., 2004), human platelet lysate (Bieback, K. et al., 2009; Schallmoser, K. et al., 2007) and thrombin-activated platelet rich plasma (Kocaoemer, A., Kern, S., Kluter, H., & Bieback, K., 2007). Currently much attention is focused on the use of autologous serum (AS) for MSC culture, because this would be an ideal solution to reduce the risk of immunoreactivity and transfer of pathogens (Takagi, M. et al., 2003). Our lab has previously reported that the source of serum can have profound effect on the proliferation and differentiation of hBM-MSCs (Shahdadfar, A., Fronsdal, K., Haug, T., Reinholt, F. P., & Brinchmann, J. E., 2005). In addition, AS has been shown to maintain long term genomic stability and preserve DNA methylation profile in hBM-MSCs (Dahl, J. A. et al., 2008). Systemic factors such as serum have also been suggested to play important roles in modulating key molecular pathways in tissue regeneration and repair during

aging (Conboy, I. M. et al., 2005; Mayack, S. R., Shadrach, J. L., Kim, F. S., & Wagers, A. J., 2010). However, to the best of our knowledge there is no study so far which has examined the differential effects of AS and FBS on the *in vitro* replicative senescence of hBM-MSCs. Therefore, in this study we have examined aspects of telomere dependent and independent mechanisms for *in vitro* replicative senescence in hBM-MSCs cultured to senescence in AS or FBS. We find that several mechanisms are likely to be involved in the senescence process, and that serum-intrinsic factors are important for determination of the Hayflick limit of these cells.

Materials and Methods

All reagents were purchased from Sigma-Aldrich (St Louis, MO). Unless otherwise stated

Isolation of hBM-MSC

BM was obtained from the iliac crest of healthy voluntary donors after written informed consent. The collection and storage of BM and hBM-MSC was approved by the regional committee for ethics in medical research. In this study, we have used specimens from healthy, young donors, 3 for AS and 4 for FBS. The aspirate was diluted 1:3 in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Paisley, UK). Mononuclear cells were obtained after Lymphoprep gradient separation (800 x g, 20 minutes), washed and resuspended in DMEM/F12 at 10^7 cells per ml. Cells were plated overnight in DMEM/F12 containing 10% AS or 10% FBS, and 1% PS. On day 3, nonadherent cells were discarded, cultures were washed with DPBS, and culture medium was replaced.

Expansion and sampling of hBM-MSC

hBM-MSCs were cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide with medium changes twice a week. After 7–20 days, initial colonies were trypsinized and re-plated in a new culture flask (passage 1, P1). Upon sub-confluent growth at a density of 50-60%, cells were harvested according to the standardized protocol and re-plated at a density of 200,000 cells per T175 flask (Nunc, Roskilde, Denmark). Photo documentation and cell counting was performed at every passage. Population doublings (PD) were calculated as described (Bieback, K.

et al., 2009). As cell numbers were first determined at P1, the population doubling number was first calculated for P2. For analysis, cells were harvested at early passage (P1/P2) and late passages (donor 1, D1: P8/AS, P9/FBS); (D2: P7/AS, P10/FBS); (D3: P7/AS, P8/FBS); (D4: P6/AS, P7/ FBS) (non proliferative, senescent) cells. Due to limitation of AS, long term culture experiments cultured in AS were performed only on three donors.

Preparation of autologous serum

From each BM donor, 300–400 ml of whole blood was drained into blood bags (Baxter, Deerfield, IL, http://www.baxter.com), quickly transferred to 10-ml vacutainer tubes without anticoagulants (BD, Plymouth, U.K.), and allowed to clot for 2 hours at room temperature. Subsequently, the blood was centrifuged at 1800 x *g* for 15 minutes. Serum was collected and filtered through a 0.2-μm membrane (Sarstedt, Numbrecht, Germany, http://www.sarstedt.com). Aliquots of the sterile autologous serum were stored at −20°C.

Immunophenotyping of cultured hBM-MSC

Early-passage (P1/P2) hBM-MSC were harvested by trypsinization and cells were stained with directly conjugated monoclonal antibodies against CD90, CD73, CD34, (BD Biosciences, San Diego,CA) CD45, CD105, HLA DR (Diatec, Oslo, Norway), CD44 (Serotec, Oxford, U.K.) CD14. Irrelevant control antibodies were included for all fluorochromes. *In vitro* expanded cells were incubated with monoclonal antibodies for 15 min in the dark, washed and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

In vitro differentiation

In vitro differentiation of hBM-MSCs to adipogenic or osteogenic lineages was performed by using cells at both early and late passages. The culture medium was replaced with specific differentiation inductive medium that was changed every third day. For adipogenic differentiation confluent cultures were incubated in DMEM/F12 containing 10% FBS or 10% AS , 0.5 mM 1-methyl-3 isobutylxanthine, 1 μM dexametasone, 10 μg/ml insulin (Novo Nordisk, Copenhagen, Denmark), and 100 μM indomethacin (Dumex-Alpharma, Copenhagen, Denmark). For osteogenic differentiation, cells were incubated at 3000 cells per cm^2 in DMEM/F12 containing

10% FBS or 10% AS, 100 nM dexametasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2- phosphate. After three weeks of differentiation, cells were harvested and used for RNA isolation.

RNA isolation, cDNA synthesis and real-time RT PCR

Total RNA was extracted using Ambion RNaqueous (Austin, TX) according to the manufacturer's instructions and quantified with a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA) according to protocols from the manufacturer using 200 ng of RNA. The genes examined and their respective TaqMan assay numbers are given in Supplementary Table 1. Quantification of gene expression was performed with a 7300 Real-time RT-PCR system from Applied Biosystems using reagents and following protocols from the TaqMan Gene Expression Assay Protocol (Applied Biosystems). Each sample was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for adipogenic and mitochondrially encoded ATP syntase (MT-ATP6) for osteogenic differentiation, as these were the most stable internal controls.

Senescence associated β-galactosidase staining

Expression of pH-dependent senescence associated β-galactosidase (SA-β-gal) activity was analyzed simultaneously in different passages of hBM-MSC using the SA-β-gal staining kit (Biovision Research Products, CA) according to manufacturer's instructions.

Flow cytometry assays

Cell cycle related distribution of DNA in early and late passage cells was done by using Click-iT™ EdU (Molecular Probes) as per the manufacturer's instructions. Briefly, cells were pulsed with 10μM EdU for 2 hours in normal culture conditions. Cells were then washed, fixed and permeabilized as per the kit instructions. For the other intracellular staining assays the hBM-MSCs were fixed and permeabilized using BD Cytofix/Cytoperm buffer (BD Biosciences), and then incubated for 30 minutes in the dark with the followin reagents:, directly conjugated antibodies specific for heat shock proteins Hsp27, Hsp60 (1:100) and Hsp70 (1:10) (Stressgen, Ann Arbor, MI) or specific for cell cycle proteins p53, p16 (1:5) (BD Biosciences) and p21

(1:50) (Calbiochem). Finally cells were washed and analyzed by flow cytometry using an excitation beam of 488 nm.

Determination of intracellular reactive oxygen species and nitric oxide

For the measurement of reactive oxygen species (ROS) and nitric oxide (NO), cells were incubated with H2DFFDA (5μM) and DAF-FM (5μM) (Molecular Probes, Eugene, OR), respectively, in the dark for 30 minutes. Cells were then washed with DMEM/F12 without phenol red medium and analyzed by flow cytometry.

Telomere length assay

Genomic DNA was isolated from 10^6 cells in early and late passages using DNA Isolation Kit (Roche, Mannheim, Germany). Analysis of telomere length was performed using the Telo TTAGGG Telomere Length Assay (Roche) as described previously (Lund, T. C., Glass, T. J., Tolar, J., & Blazar, B. R., 2009). In brief, 2μg DNA was digested with the restriction enzymes HinfI and RsaI, separated by electrophoresis for 4 h at 50 V on a 0.8% agarose gel and transferred on positively charged nylon membranes (Roche). Terminal restriction fragments were detected by hybridization to a digoxigenin-labeled telomeric oligonucleotide according to the manufacturer's protocol. Mean telomere length was determined as per protocol using Kodak image station 2000R (Woodbridge, CT).

Data analysis

Delta mean fluorescence intensity (ΔMFI) was calculated as mean fluorescence of isotype staining subtracted from staining with specific antibody. Results were expressed as the mean ± SEM unless otherwise stated. Statistical analysis was performed using the Student t-test by using Graphpad prism software, (La Jolla, CA). Data was considered significant different when *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Characterization of long term growth kinetics and morphology of hBM-MSCs

There was a tendency for hMSCs grown in AS to reach P1 earlier than hMSCs grown in FBS, but as soon as log phase of growth was established, the serum supplement did not seem to greatly

affect PD time (Figure 1A). The most obvious difference between the serum supplements was the number of PD required to reach senescence. For hBM-MSCs cultured in their own serum this was 20, 12, 16 and 7 PD for Donors 1-4, respectively, while for hMSCs grown in FBS this was 20, 24, 20 and 18, respectively. During log phase of growth the hMSCs showed typical fibroblastoid morphology (Figure 1B). As the cells reached their Hayflick limit they became large and irregular, with increased expression of SA-beta-gal (60-80%), an established biomarker for senescence (Figure 1C). hBM-MSCs from all donors cultured in both AS and FBS showed phenotypical characteristics of MSC: expression of CD90, CD105, CD73, CD44 and absence of expression of CD34, CD45, CD14 and HLA DR (data not shown) (Dominici, M. et al., 2006).

Effect of serum supplement and long term culture on the differentiation potential of hBM-MSCs

Another defining parameter for hBM-MSCs is that they should differentiate into various mesenchymal lineages. Here, we analyzed the effect of serum supplement on the differentiation potential of hBM-MSCs at early and late passage. The potential for adipogenic differentiation was evaluated by using three different genes which are known marker for adipogenesis; lipoprotein lipase (*LPL*), peroxisome proliferator activated receptor (*PPARγ*) and CCAAT-enhancer binding protein α (*C/EBPα*), while osteogenesis was quantified by measurement of osteomodulin (*OMD*) and alkaline phosphatase (*ALPL*) mRNA. There was a certain constitutive expression for all these genes. For all the adipogenesis genes the differentiation-induced expression was higher in early than in late passage, and higher in cells cultured in FBS than in AS (Figure 2: A,B,C). There was a tendency towards greater difference between the serum sources than between the individual donor cells. For the induced expression of *OMD* we found a trend towards reduced differentiation in late passage AS cells, while no evidence was found of osteogenic differentiation in late passage cells using the marker *ALPL* (Figure 2: D,E).

Serum source impacts on the proportion of cells in S phase of cell cycle

To correlate the proportion of cells in different stages of cell cycle with passage and serum source, we performed EdU-based cell cycle analysis on cells in early and late passage. The results are presented in Figure 3A and B. As expected, we observed a significant drop in the proportion of cells in S phase between early and late passage cells. However, to our great surprise we found

a much higher proportion of early passage hMSCs cultured in FBS in S phase compared with early passage hMSCs cultured in AS. This result was consistent for all the donors. There was a difference between cells cultured in the different serum supplements also in late passage, but at a much lower level of cells in S phase.

Analysis of proteins involved in the cell cycle

In order to further evaluate proteins involved in the regulation of cell cycle, and also known to impact on the induction of replicative senescence, we studied the tumor suppressor protein p53 and cell cycle inhibitors, $p21^{CIP1}$, $p16^{INK4a}$ (Figure 4A). Of these, only p53 showed expression ≥ 2 in all donors under all conditions. For p53 all the expression levels in late passage were higher than the levels for the same serum supplement and donor in early passage. Also, all the results for p53 in early passage AS cells were higher than those observed in early passage FBS cells. Expression of $p21^{CIP1}$ was expressed at very low levels, slightly higher in cells cultured in AS than in FBS in both early and late passages. Finally, all the levels for $p16^{INK4a}$ in late passage AS cells were higher than the corresponding levels in early passage AS cells, but none of these results reached significance for the groups.

Quantification of NO and ROS

In order to show the role of oxidative stress we quantified the level of NO and ROS in the hMSCs in both serum supplements in early and late passage (Figures 4B and D). A trend was seen for increase in NO from early to late passage cells in both serum conditions. For ROS, a very high level was observed in hMSCs cultured in FBS in late passage. This level was significantly higher than the level observed for these cells in early passage, and also significantly higher than the level observed in hMSCs cultured in AS at late passage. For hMSCs cultured in AS we also observed an increase in ROS levels from early to late passage for all three donors analyzed, but this did not reach significance for the group as a whole.

Analysis of heat shock proteins

The heat shock proteins Hsp60, 70 and 27 were expressed at moderate to high levels in cells from all donors in both supplements at all stages of culture, with Hsp27 expressed at much higher levels than the two other heat shock proteins (Figure 4C). Significantly increased expression was

observed both for Hsp60 and Hsp27 between early and late passage for cells cultured in FBS, and also a significantly higher expression for Hsp27 in early passage for cells cultured in AS versus FBS. In addition, increased expression was observed for all donors also for Hsp60 and Hsp70 in late passage AS cells compared with early passage, for Hsp70 in late passage FBS cells compared with early passage and for all donors for Hsp27 in late passage cells cultured in FBS compared with AS. However, these latter changes did not reach significance.

Telomere length

In accordance with the fact that adult stem cells express very low levels of telomerase, the telomeres slowly shorten (Sethe, S., Scutt, A., & Stolzing, A., 2006). Also, telomere length attrition is the hallmark for somatic cells undergoing replicative senescence. Therefore we studied the effect of serum on the telomere length in hBM-MSCs in early and late passages. We found that there was a significant decrease in mean telomere restriction fragment length in FBS from early (9918±325.5 bp) to late (7636±418.2bp) passage. There was a similar decrease in AS from early (10189± 731.6bp) to late (8878±150.8bp) passage, but this change was not found to be significant (Figure 5A and B). Also, no significant difference was seen between late passage FBS and late passage AS cells. This result shows that the source of serum did not seem to impact on telomere length at neither early nor late passage in these cells.

Discussion

In the last decade, hMSCs have received attention for their potential role in therapeutic applications, and this is reflected by the increasing number of ongoing clinical trials (www.clinicaltrial.gov). Recently, there have been several reports showing the importance of serum for the stable, clinical scale propagation of human MSCs (Bieback, K. et al., 2009; Kocaoemer, A., Kern, S., Kluter, H., & Bieback, K., 2007). Like other somatic cells, hMSCs have a definitive replicative life span consistent with a Hayflick model of cellular aging. Therefore, in the present study we have examined the relationship between serum from two commonly employed sources (AS and FBS) and replicative senescence in hMSCs. We show that serum-intrinsic factors are important for determination of the Hayflick limit of these cells. We also show that several pathways are likely to be involved in the establishment of replicative

senescence in hMSCs. Although senescence is induced at different numbers of PD depending on the serum source, the mechanisms responsible for senescence appear to be similar.

As hMSCs from all the donors were expanded in the same FBS and under identical cell culture conditions, differences observed in this part of our study should provide information about donor-specific, cell-intrinsic factors involved in replicative senescence. As the same cells were also cultured, under identical conditions, in serum from the individual MSC donors, results observed in the MSC/AS cultures that differ from the MSC/FBS results should be the consequence of donor-specific, serum-intrinsic factors. For the PD curves, only minor differences were observed for the cells from different donors cultured in the same batch of FBS, while the hMSCs cultured in AS mostly exhibited different PD curves from the cells cultured in FBS, and different PD curves also from the other MSC/AS combinations. Similar conclusions could be drawn from other assays performed here, particularly the cell cycle assay, where great differences were observed between cells cultured in FBS and cells cultured in AS, but also for some of the cell cycle and heat shock proteins. Taken together, these results show that there are factors in the serum added to the culture medium which strongly impacts on the longevity of hMSCs *in vitro*. The presence of these factors are different between FBS and AS, and also different between AS from different individuals. From the early days of MSC research, where the importance of checking out a large number of FBS batches in order to find a batch which would support prolonged MSC growth *in vitro* was always strongly emphasized, we know that these factors are differentially present also in different FBS batches. Finally, these results are fully consistent with conclusions derived from experiments performed both *in vitro* and *in vivo*, which show that old cells placed in young serum acquire a young phenotype, and vice versa (Conboy, I. M. et al., 2005; Mayack, S. R., Shadrach, J. L., Kim, F. S., & Wagers, A. J., 2010). In the present paper we show some of the differences within aging pathways that may be observed in cells cultured in different serum sources. However, the full picture of the interactions between the pathways involved, and particularly the identity of the serum factor(s) responsible for the differences in the Hayflick limit must await further research.

In the present study, the single observation that most clearly separated cells cultured in FBS from cells cultured in AS was the percentage of cells in S phase in early passage. These studies were

performed in passage 1 or 2, at a time when the cells were in log phase of growth, and there was no systematic difference between the PD time for the two culture conditions. For cells cultured in FBS, more than 20% of the cells were in S phase for every donor, with >30% being in S phase for one of the donors. These are exceptional accumulations of cells in S phase, and may suggest that these cells experience some hinderence in the course of DNA replication. The fact that PD time was not prolonged suggest that these cells traverse other phases more rapidly. A rapid transition through G1, for instance, may result in the cells not being quite ready for DNA replication, which may be why they accumulate in the S phase. Cells cultured in AS, on the other hand, transit S phase very rapidly, with only around 4% of the cells being in this phase. The cell cycle proteins examined in this study can all inhibit cell cycle progression, but none of them are known to induce cell accumulation in S phase. Also, all of them tended to be expressed slightly higher in AS cells, which again would not explain S phase accumulation for the FBS cells. Thus, we have no explanations at this time for these surprising differences, but are planning experiments to unravel the mechanisms involved.

To account for the differences observed for the time to senescence we believe that we should look both in early and late passage cells. Differences observed in senescent cells cultured in serum from different sources would suggest that serum factors induce fundamentally different aging pathways. This could in itself explain the differences in the time to the Hayflick limit observed here. Differences observed in early passage cells would suggest that the impact of serum factors was in the differential rate of induction of senescence pathways, and not necessarily in the induction of different pathways. The only assay that showed significant differences in early passage was Hsp27, which was higher in AS cells than in FBS cells. However, the participation of heat shock proteins in the aging mechanisms of hMSCs was further substantiated by the increased expression in late passage versus early passage FBS cells for both Hsp27 and Hsp60, and a trend for increased expression in late passage versus early passage also for Hsp60 in AS cells and for Hsp70 in both serum supplements. This leads to the theory that heat shock proteins are importantly involved in aging mechanisms in hMSCs, and that AS induces upregulation of heat shock proteins compared with FBS, with Hsp27 being upregulated at the earliest time point. Our data are contradictory to a recent report which showed that the level of heat shock proteins in hMSCs decreases with age (Stolzing, A., Jones, E., McGonagle, D., &

Scutt, A., 2008). However, it has also been shown that heat shock proteins have an inducing effect on cellular senescence. Increased expression of Hsp27 was seen in H_2O_2 treated fibroblast (Chen, J. H. et al., 2004), and its overexpression lead to senescence in bovine arterial endothelial cells (Piotrowicz, R. S., Weber, L. A., Hickey, E., & Levin, E. G., 1995). Increased expression of heat shock proteins could be an adaptive response of the MSCs to due to stress or misfolding of proteins. At some stage the increased levels of heat shock proteins may fail to satisfy the demand, which would result in accumulation of misfolded proteins (Soti, C. & Csermely, P., 2002) . This might contribute to the induction of senescence, earlier in cells cultured in certain autologous sera. Interestingly, small heat shock proteins such as Hsp27 have been known to act as sensors of cellular redox change. Specifically, Hsp27 has been described to keep glutathione in its reduced form, thereby reducing intracellular ROS and maintaining the redox state of the cell (Arrigo, A. P., 2007; Arrigo, A. P. et al., 2005) . Therefore, it is conceivable that the low level of ROS in late passage cells in AS could be the effect of high expression of Hsp27.

As for the other mechanisms of aging investigated here, they may well contribute to the induction of senescence, but they do not seem to explain how serum supplements may affect the time to senescence. The levels of ROS and NO were always higher in FBS cells, with ROS being greatly upregulated in aging hMSCs cultured in FBS. The accumulation of oxygen breakdown products may be an important senescence mechanism in cells cultured in FBS, but does not explain the early senescence observed in AS cells. For the cell cycle pathway dominated by p53 and p16 we observed significant differences between early and late passage cells, consistent with their known participation in the mechanism of aging (Sharpless, N. E. & Depinho, R. A., 2007). However, except for p53 the level of expression of these proteins was very low, which may suggest that their role in senescence for hMSCs is not dominant. Similarly we observed a reduction in the telomere length, but not to very low levels, and not in a way which may explain the difference between the serum supplements.

In conclusion, our results suggest that systemic factors like serum not only play role in repair and regeneration process of stem cells but also impacts on the longevity of hMSC's culture *in vitro.* Also, our data indicates that replicative senescence involves intertwined multiple complex mechanisms involving telomere dependent and independent pathways and at some stage serum intrinsic factor shows a profound effect to govern these processes. While data presented in this

paper reveal some of the differences observed in cells cultured in AS and FBS serum sources, the full picture, and particularly the identity of the factor(s) responsible for the early/delayed Hayflick limit observed for cells cultured in certain sera must await further research.

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Figure 1.

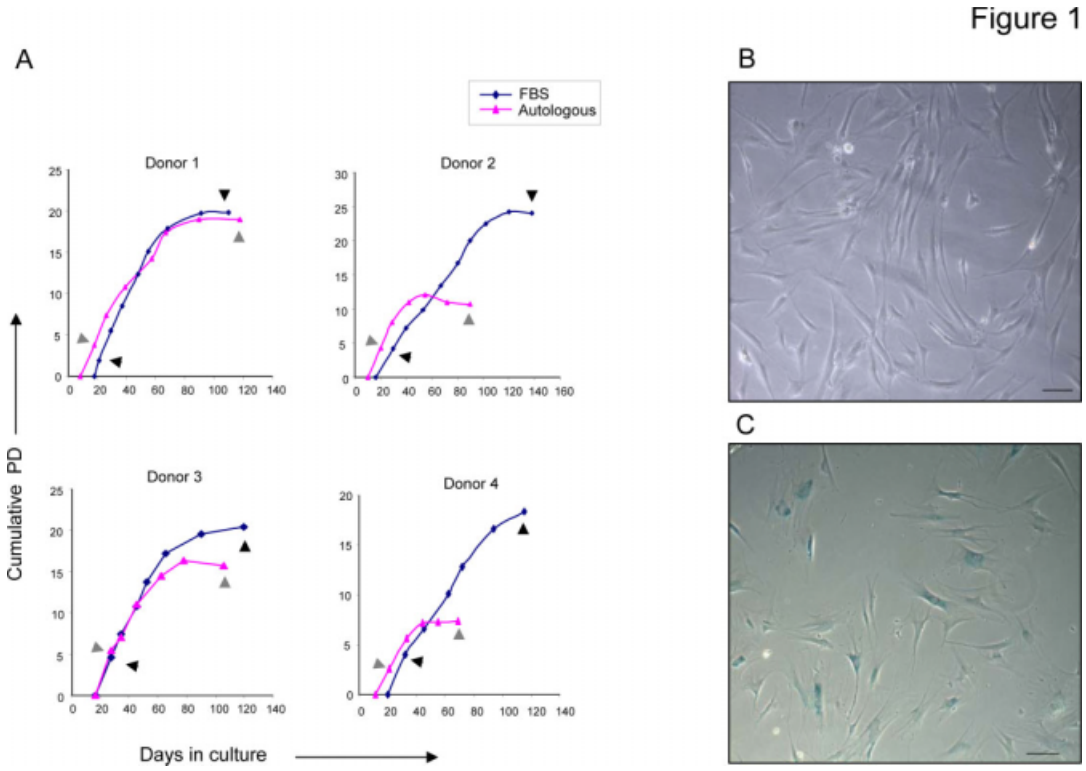

Figure 2.

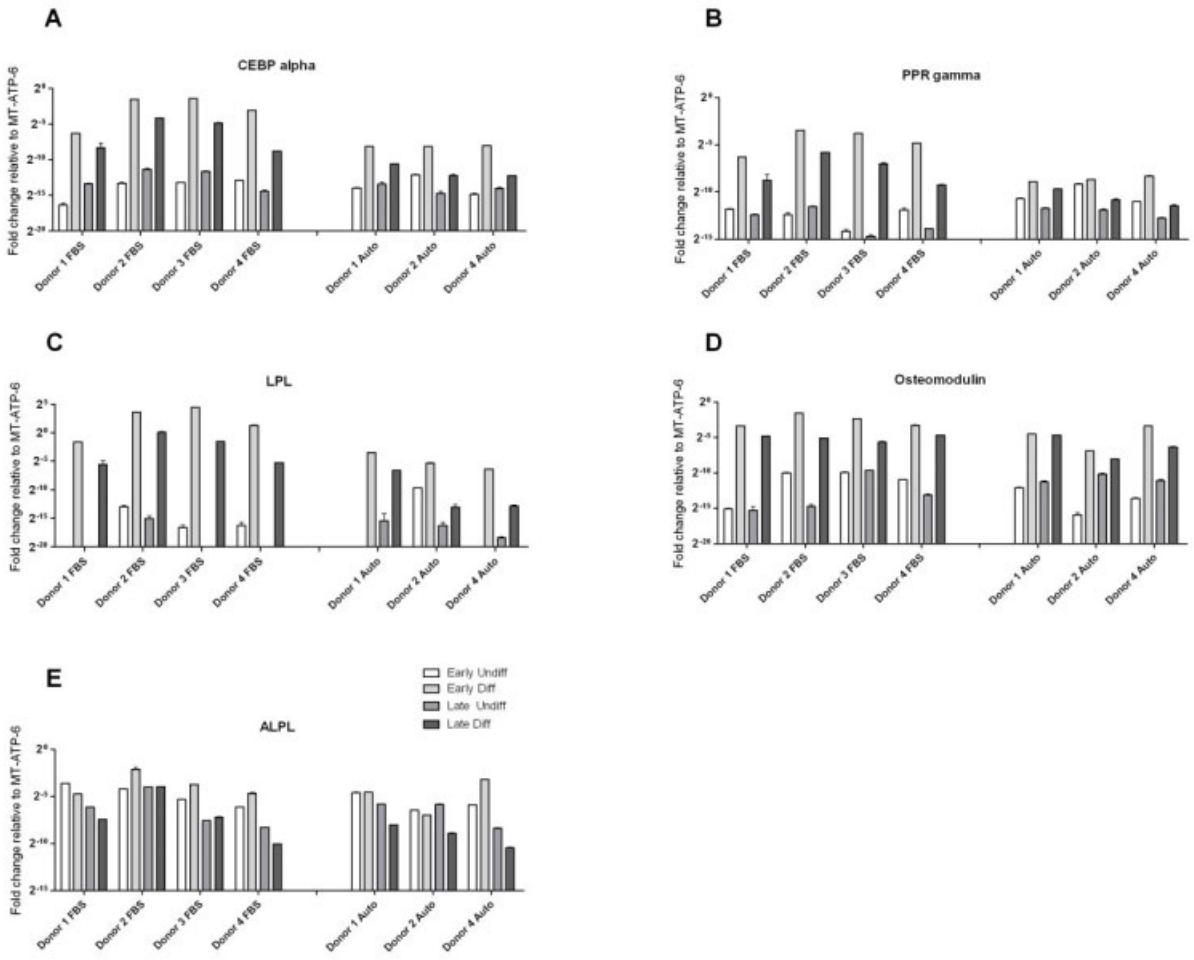

Figure 3

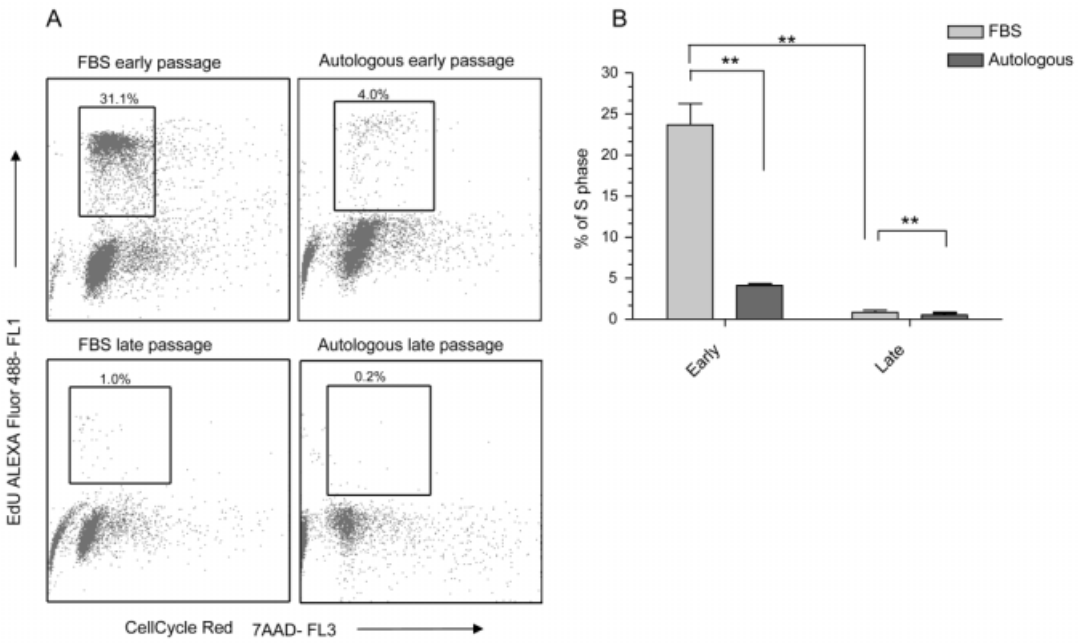

Figure 5