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ORIGINAL PAPER

Lipocalin 2 decreases senescence of bone marrow-derived mesenchymal stem cells under sub-lethal doses of oxidative stress

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Abstract The regenerative potential of mesenchymal stem cells (MSCs) is impaired by cellular senescence, a multi factorial process that has various functions. However, pathways and molecules involved in senescence have not been fully identified. Lipocalin 2 (Lcn2) has been the subject of intensive research, due to its contribution to many physiological and pathophysiological conditions. The implication of Lcn2 has been reported in many conditions where senescence also occurs. In the present study, we evaluated the role of Lcn2 in the occurrence of senescence in human bone marrow-derived mesenchymal stem cells (hB-MSCs) under oxidative conditions. When hB-MSCs were genetically engineered to over-express Lcn2 (MSC-Lcn2) and exposed to H_2O_2, the proliferation rate of the cells increased. However, the number of colonies and the number of cells that made up each colony in both MSC-V and MSC-Lcn2 cells decreased compared to those cultivated under normal conditions. Our results revealed that over-expression of recombinant Lcn2 in hB-MSCs decreases senescence induced by H_2O_2 treatment. Senescent cells were observed in aged hB-MSCs; however, no alteration in the expression level of Lcn2 was detected compared to earlier passages. Finally, a higher amount of Lcn2 protein was detected in the plasma of the elderly than in young people. Our findings suggest that Lcn2 might restore the health and regeneration potential of MSCs by decreasing senescence.

Keywords NGAL/Lcn2 · Senescence · hB-MSCs · Oxidative stress

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Introduction

Senescence is defined as an irreversible blockage of cellular division. About 40 years ago, Hayflick and Moorhead observed that normal human fibroblasts had a limited proliferation capacity and ceased division after certain passages (Hayflick and Moorhead 1961). This was called cellular senescence. Today, it is known that senescence plays an important role in many physiological and pathological processes such as tumor suppression as well as aging and tissue repair (Rodier and Campisi 2011). The molecular mechanisms underlying senescence are not fully understood so far (Ben-Porath and Weinberg 2005). Hence, unveiling molecules implicated in senescence is of great interest for researchers in this field. Recently, the implication of certain genes/proteins in senescence has been identified including p21, p27, p16, etc. among which, p16/pRb or p53/p21 pathways play an important role especially in the onset of senescence. Stress-induced premature senescence (SIPS) is a kind of senescence that is induced when cells are exposed to sub-lethal doses of stress in vitro. Various factors can induce senescence in cells in vitro such as nutrient deficiency, the amount of reactive oxygen species in the environment and mild destructive agents. Among these factors, oxidative stress plays an important role in the induction of SIPS. It is noteworthy that SIPS occurs without affecting telomere length (Kuilman et al. 2011).

One of the key molecules induced under oxidative stress conditions is lipocalin 2 (Lcn2). Lcn2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is a relatively

small molecule whose expression is induced under various pathophysiological conditions such as infection (Berger et al. 2006), oxidative stress (Rodvold et al. 2012), cancer (Missiaglia et al. 2004; Santin et al. 2004), inflammation (Liu and Nilsen-Hamilton 1995; Nielsen et al. 1996), kidney injury (Mishra et al. 2004, 2005), cardiovascular disease (Mishra et al. 2005; Hemdahl et al. 2006), burn injury (Vemula et al. 2004), and intoxication (Tong et al. 2005). The precise role of Lcn2 remains unknown, but interestingly, stimulators of Lcn2 expression are similar to those implicated in cellular senescence.

The potential application of MSCs as autologous stem cell transplants for cell-based therapy and tissue engineering (Safford et al. 2002) is under intensive research. Thus, detailed information about the alterations imposed by age-related changes is necessary in order to develop cell-based therapies, especially in elderly patients. In addition, since MSCs appear to carry considerable regenerative potential, it seems that their insufficient number or deficient function may be associated with the aging process and with age-related diseases.

This study was conducted to investigate any relationship between the expression of Lcn2 and senescence of MSCs. First, we over-expressed Lcn2 in MSCs, and then evaluated them with regard to the occurrence of senescence following their exposure to sub-lethal doses of oxidative stress. Our findings might result in improving MSC-based therapy especially when aged autologous MSCs are used.

Experimental procedures

Isolation, expansion, and identification of hB-MSCs

Bone marrow was obtained from healthy volunteers in the Hematopoietic Stem Cell Transplantation Facility of Shariati Hospital (Tehran, Iran) after obtaining written informed consent. Sample collection was authorized by the Hospital Ethics Committee. The human bone marrow-derived mesenchymal stem cells (hB-MSCs) were isolated and expanded according to our previous study (Halabian et al. 2010). Briefly, 1×10^6 cells/cm^2 of marrow were cultivated in Dulbecco's modified Eagle medium (DMEM) containing 1-g glucose/L (Gibco, USA), 10 % fetal bovine serum (FBS, Invitrogen, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, USA). Non-adherent cells were removed by medium replacement after 72 h. After two passages, the adherent cells were characterized by immunophenotyping as explained before (Halabian et al. 2010). For this purpose, phycoerythrin (Becton-Dickinson, USA) or fluorescein isothiocyanate-conjugated antibodies against CD34, CD45, CD90, CD105, and CD166 antigens were used. The samples were subjected to flow cytometry and data obtained from relevant gated populations were assayed using FloMax Software.

Isolation and cloning of Lcn2 cDNA

HepG2 cells were cultured in 25-cm^2 cell culture flasks, and grown in RPMI medium (Gibco-BRL, Germany) with 10 % FBS (Gibco-BRL, Germany). Then the cells were subjected to mRNA extraction using RNeasy Mini spin column kit (QIAGEN, Germany), and cDNA was synthesized using cDNA synthesis kit (Bioneer, Korea). Full-length human Lcn2 cDNA was amplified using a specific primer set (Roudkenar Habibi et al. 2009) and platinum *Taq* DNA polymerase (Invitrogen, USA). The amplified Lcn2 cDNA was digested and ligated to similarly digested ends of pcDNA3.1 (+) mammalian expression vector, which was then used to transform DH5α strain of *Escherichia coli*. Recombinant bacteria were selected on LB agar medium containing 100 μg/ml ampicillin. The presence of the cloned fragment was evaluated by PCR and finally, the fidelity of the cloned sequence was authenticated by DNA sequencing. The recombinant plasmid was designated as pcDNA3.1-Lcn2 (Roudkenar Habibi et al. 2009).

Plasmid transfection

The hB-MSCs were transfected with 2 μg of the pcDNA3.1 and pcDNA3.1-Lcn2 plasmids using FuGENE HD (Invitrogen, USA) according to the manufacturer's protocol. Then, the expression of Lcn2 mRNA and protein was evaluated by RT-PCR and ELISA.

RT-PCR to evaluate Lcn2 expression

RT-PCR was performed to evaluate the expression of Lcn2 mRNA following transfection. Total cellular mRNA was extracted as mentioned above and used at a concentration of 1 μg/μl to construct cDNA using a cDNA Synthesis kit (Bioneer, Korea) according to the manufacturer's protocol. Detection of the Lcn2 expression was evaluated with a primer pair (Table 1) that amplifies a 240-bp length fragment of human Lcn2 mRNA. Expression of β-actin was also evaluated as a housekeeping gene (Table 1). PCR conditions for amplification of both human Lcn2 and β-actin included a

Table 1 ELISA for Lcn2 immunoassay

Absorbance (450 nm)	Samples
1.75	10 ng/ml[$]
0.909	5 ng/ml[$]
0.523	2.5 ng/ml[$]
0.285	1.25 ng/ml[$]
0.045	MSC-V
1.095	MSC-Lcn2[a]

[$] Standard concentrations of human Lcn2 provided in the kit

[a] 20-fold dilution

primary denaturation step of 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 59 °C, and 20 s at 72 °C, and a final extension step of 5 min at 72 °C. Finally, PCR products were analyzed by electrophoresis on 2 % agarose gel.

H_2O_2 treatment

The hB-MSCs were cultured in DMEM low glucose (Gibco, USA) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5 % Co_2 in a humidified incubator. The hB-MSCs of earlier passages (between 4 and 6) were incubated with 150-μM H_2O_2 for 2 h (Brandl et al. 2011), then washed with PBS and cultivated with fresh medium.

Western blotting

Western blot analysis was performed to detect the expression of Lcn2 protein. After cultivation of MSCs in FBS-free DMEM medium, the cell culture medium was collected. Then, the samples were boiled for 5 min in a loading buffer containing 4 % sodium dodecyl sulfate (SDS), 20 % glycerol, and bromophenol blue. Then, proteins were resolved on 12 % SDS-PAGE and transblotted to polyvinylidene fluoride membrane (Roche, Germany), followed by blocking and subsequent overnight incubation at 4 °C with specific primary antibodies, i.e., β-actin (Sigma, USA) or anti-human NGAL rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Afterwards, the membranes were washed with tris-buffered saline containing 0.1 % Tween 20 and incubated with secondary horseradish peroxidase (HRP)-conjugated anti rabbit antibodies (Sigma). Finally, membranes were developed by DAB solution (Sigma).

Cell proliferation assay

In order to verify the influence of Lcn2 on cell proliferation, 3×10^3 cells/well were seeded in a 96-well plate, and incubated at 37 °C with 5 % CO_2. The medium was refreshed every 2 days and cell proliferation was determined after 1 week using a WST-1 assay kit (Roche, Germany) as instructed by the manufacturer, and the absorbance was read at 450 nm using an ELx800 Absorbance Microplate Reader. On the other hand, following treatment of the hB-MSCs transfected with recombinant pCDNA3.1-Lcn2 vector (MSC-Lcn2) and hB-MSCs transfected with empty pCDNA3.1 vector (MSC-V) with 150-μM H_2O_2 (sub-lethal dose of H_2O_2), 3×10^3 cells/well were seeded in a 96-well plate, incubated at 37 °C with 5 % CO_2, and their proliferation was assessed. In addition, 1×10^4 cells/well were seeded in a 6-well plate with or without H_2O_2 treatment, and incubated at 37 °C with 5 % CO_2. Afterwards, the cells were trypsinized and detached every 3 days and their viability was measured with 0.4 % trypan blue solution (Sigma, USA).

Senescence-associated β-galactosidase staining

Senescence β-galactosidase staining was performed using a commercial kit according to the manufacturer's instructions (Cell Signaling, Beverly, MA, USA). Briefly, the culture medium was removed and the cells were washed with PBS followed by fixing with a solution containing (2 % formaldehyde and 0.2 % glutaraldehyde). Then, the cells were washed twice with PBS, incubated with a staining solution containing 5-bromo-4-chloro-3-indolyl-D-galactopyranoside, citric acid/sodium phosphate (pH 6.0), 1.5-M NaCl, 20-mM $MgCl_2$, potassium ferrocyanide, and potassium ferricyanide. After 11 to 14 h, the cells were observed under simple light microscopy, and the number of blue cells and the total number of cells were counted. Finally, the percentage of blue cells (which represent senescent cells) over the total cell count was measured in 30 random fields.

Real-time PCR analysis

SYBR green PCR master mix (Takara, Japan) was used to amplify the desired fragments using specific primers including $p16^{INK4a}$ forward:5′-GACATCCCCGATTGAAAGAA3′and reverse: 5′TTTACGGTAGTGGGGGAAGG3′; $p21^{WAF1/CIP1}$ forward: 5′GACACCACTGGAGGGTGACT-3′and reverse: 5′-CAGGTCCACATGGTCTTCCT-3′; $P27^{KIP1}$ forward: 5′-TACAGACCCCCGCGGCCC-3′ and reverse: 5′-TCCGCT AACCCCGTCTGGCT-3′; p53 forward: 5′-GGCCCACTTC ACCGTACTAA-3′ and reverse: 5′-GTGGTTTCAAGGCC AGATGT-3′; and Lcn2 forward: 5′-TCACCTCCGTCCTG TTTAGG-3′ and reverse: 5′-CGAAGTCAGCTCCTTGGT TC-3′. Two-microliter cDNA was used per reaction in a mixture including 25-μl SYBR green PCR master mix (Takara, Japan) and 10 pmol of each gene-specific primer. The reaction was performed in a Rotor gene RG-3000 (Corbett, Germany) and the PCR conditions included 1 min of pre-incubation at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at a suitable annealing temperature of each primer pair, and 30 s at 72 °C, then a step of 10 s at 82 °C followed by melting curve analysis. Data analysis was performed using the Rotor Gene software. Relative expression of the target genes was determined after normalization against β-*actin* expression as housekeeping gene and reported as fold changes compared to the non-transfected cells.

Gimsa staining

For the evaluation of cell sizes, cells were cultivated on slides and incubated overnight and then the culture medium was removed and the cells were washed with PBS. Then, the slides

were soaked in absolute methanol for 10 min, followed by washing with water and soaking in Giemsa stain for 15 min. Finally, the slides were washed with water and the cells were identified under light microscopy as violet-stained cells.

Evaluation of lipocalin plasma levels by ELISA

For this experiment, 30 volunteers were selected from nonsmokers with no pulmonary or renal disorders and the samples were classified into three groups. The first group included individuals between 18 and 22 years of age, the second group included those between 38 and 42 years of age, and the third group included those from 58 to 62 years of age (elderly cases were not included in this study because of their medical condition). The Lcn2 protein level was determined with a Human Lipocalin-2/NGAL Immunoassay kit (R&D Systems, USA) according to the manufacturer's protocol. Briefly, 100-μL RD1-52 was added to each well, and then a 50-μL sample was added to the wells and incubated for 2 h at 4 °C. Next, the wells were washed four times with wash buffer, followed by the addition of 200-μL HRP conjugated anti Lcn2 antibody to each well. Again, the plates were incubated for 2 h at 4 °C and washed with wash buffer. Afterwards, 200-μL substrate solution was added to each well and kept in the dark for 30 min. Finally, 50 μl of stop solution was added to each well and the plates were subjected to an absorbance reading at 450 nm using a microplate reader.

Statistical analysis

Analysis of variance and Student's *t* test were used to evaluate the statistical difference of the results. In all tests, $p<0.05$ was considered significant.

Results

Characterization of hB-MSC

hB-MSCs were isolated and identified as described previously (Halabian et al. 2010). Flow cytometry analysis of the isolated and expanded cells showed that they were positive for CD166, CD105, and CD90 markers, and negative for hematopoetic surface markers, i.e., CD45 and CD34 (data not shown).

Oxidative stress-induced Lcn2 expression in hB-MSCs

Expression of Lcn2 in hB-MSCs was low, but when they were exposed to sub-lethal doses of H_2O_2, Lcn2 expression was slightly induced. This was revealed by RT-PCR (data not shown) and further confirmed by real-time PCR and Western blot analysis (Fig. 1a, b).

Ectopic expression of Lcn2 in hB-MSCs

In order to over-express Lcn2, hB-MSCs were transfected with pcDNA3.1-Lcn2 plasmid. Expression of Lcn2 was evaluated by real-time PCR and ELISA. As shown in Fig. 2, up-regulation of Lcn2 was observed in MSC-Lcn2 cells compared to MSC-V cells (which were transfected with empty pcDNA3.1 vector). These findings were also confirmed by ELISA indicating ectopic over-expression of Lcn2 in the cells (Table 1).

Lcn2 over-expression increased proliferation of the hB-MSCs in normal and oxidative stress conditions

One of the well-known functions of Lcn2 is modulation of cell proliferation; 3×10^3 cells/well were seeded in a 96-well plate, and incubated at 37 °C with 5 % CO_2. The medium was

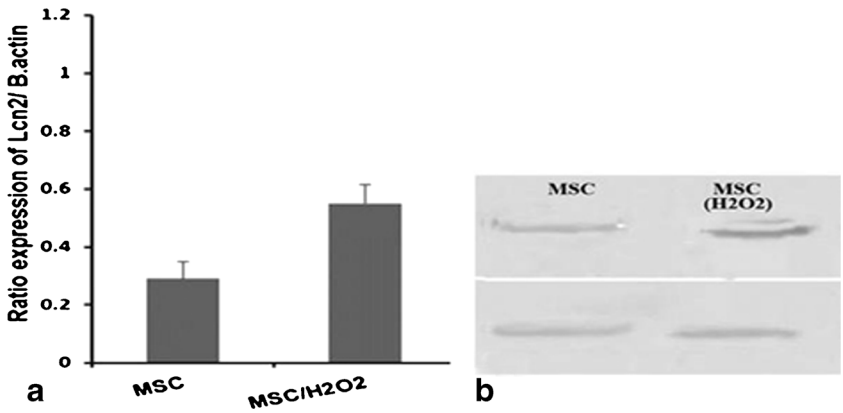

Fig. 1 **a** Assessment of Lcn2 expression in MSCs following H_2O_2 treatment with real-time PCR. The ratio of Lcn2 to β-actin expression was evaluated before and after treatment with H_2O_2. The expression of Lcn2 was slightly up-regulated following H_2O_2 treatment in MSCs, but it was not significant. **b** Western blot analysis of Lcn2 expression in H_2O_2 treated or non-treated MSCs. Lcn2 expression under normoxic conditions was low in normal MSCs and up-regulated after treatment with H_2O_2

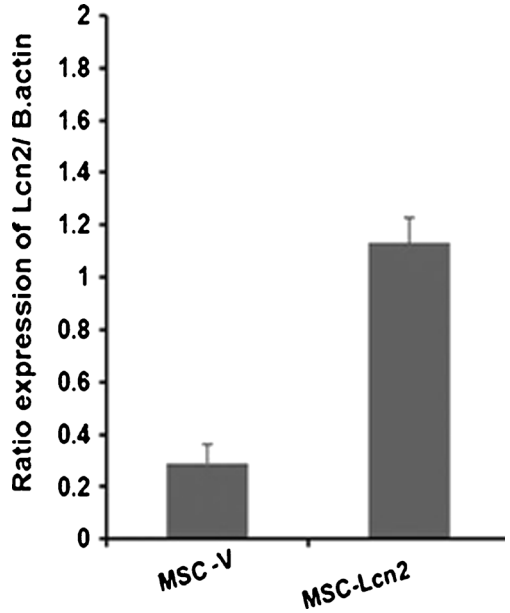

Fig. 2 Evaluation of Lcn2 mRNA expression in MSCs by real-time PCR. The ratio of Lcn2 to β-actin expression was evaluated in MSC-V and MSC-Lcn2. After transfection of the pcDNA3.1-Lcn2 plasmid, the expression of Lcn2 mRNA in MSCs was increased (data represents mean ± SD; number of replicates=3, $^{**}P<0.01$, $^{***}P<0.001$)

refreshed every 2 days and cell proliferation was determined after 1 week using a WST-1 assay kit. Results showed that the number of hB-MSCs-Lcn2 was higher than hB-MSCs-V under normal conditions (data not shown). We also performed a colony assay. Equal numbers of the MSC-Lcn2, MSC-V (about 5×10^2cells) were seeded in 1-well plates and allowed to grow separately and form a colony. The number of colonies and the number of cells in each colony were counted 7 days post cultivation. The results revealed that colony numbers and the number of cells that made up each colony were higher when lipocalin-2 was over-expressed in the cells (Fig. 3). Next, we performed the same experiments under oxidative stress conditions, i.e., treatment of the cells with a sub-lethal concentration of H_2O_2. As in the above findings, colony numbers and the number of cells that made up each colony were higher in MSC-Lcn2 compared with MSC-V. However, the numbers were lower compared with the results obtained from non-treated cells. In other words, colony numbers and the number of cells that made up each colony of both MSC-V and MSC-Lcn2 cells decreased following H_2O_2 treatment.

Furthermore, under oxidative stress conditions, the morphology of the MSC-Lcn2 cells was changed and they became enlarged. These observations prompted us to ask whether under sub-lethal doses of oxidative stress, Lcn2 exerts any protective effect on the cells and if so, by which biological mechanism.

Lcn2 reduced senescent cells in hB-MSCs exposed to oxidative stress conditions

We hypothesized that Lcn2 exerts its cytoprotective effects under sub-lethal doses of H_2O_2 through senescence. First, we observed that the number of enlarged MSCs treated with H_2O_2 was considerable by Giemsa staining (Fig. 4a (II)). Next, we studied the senescence with a B-gal staining kit. Blue cells indicated the senescent cells (Fig. 4a (III)). Figure 4b shows the number of senescent cells in hB-MSCs-V and hB-MSCs-Lcn2, in normal and oxidative conditions. Lcn2 slightly reduced the number of blue cells under normal conditions.

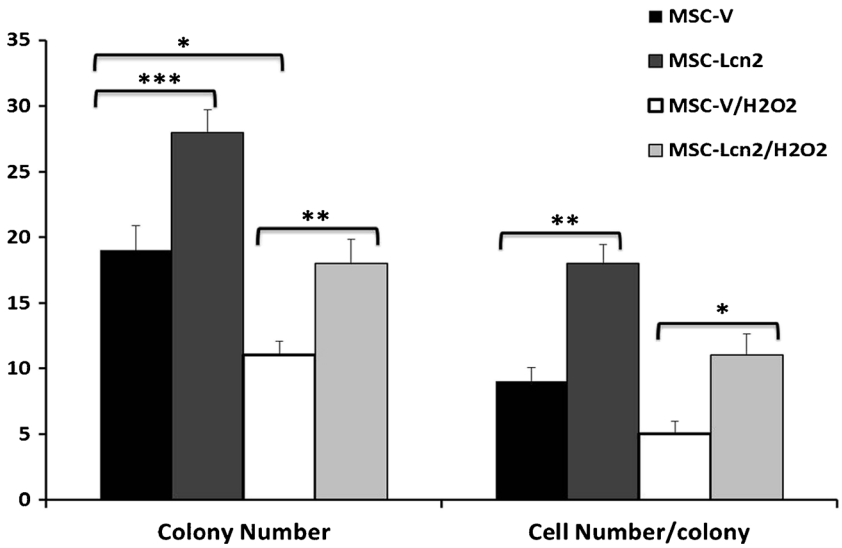

Fig. 3 Number of colonies and the number of cells per colony of the MSC-V and MSC-Lcn2 with or without H_2O_2 treatment. After Gimsa staining, in non-H_2O_2-treated samples, not only the number of colonies of the MSCs-Lcn2 cells, but also the number of cells per colony was higher than MSC-V. After H_2O_2 treatment, similar results were observed. However, the number of colonies and the number of cells that made up each colony in both MSC-V and MSC-Lcn2 decreased when compared to cells cultivated under normoxia conditions (data represents mean ± SD; number of replicates=3, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$)

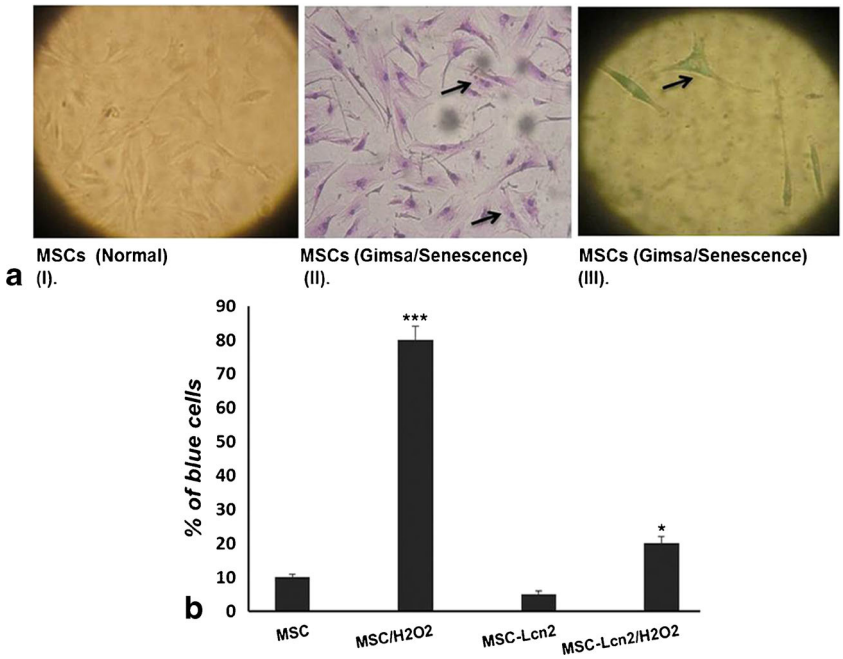

Fig. 4 Effect of a sub-lethal dose of H_2O_2 on induction of senescence in MSCs. **a** *I* Normal cells. *II* Gimsa staining, and *III* Beta-Gal staining. H_2O_2 induces cell enlargement. Typical presentation of forward scatter, reflecting the average cell size. **b** Quantitative assay of Beta-gal-positive cells. The sub-lethal dose of H_2O_2 induces senescent phenotype in MSCs but it showed no significant effect on MSCs-Lcn2 senescence. H_2O_2 treatment increased senescence in MSCs, but it did not significantly affect MSC-Lcn2 senescence

However, when hB-MSCs were treated with H_2O_2, the senescent cells increased significantly (MSC-H_2O_2 in Fig. 4b). Interestingly, in the case of H_2O_2 treatment of the MSC-Lcn2, Lcn2 decreased the number of blue cells considerably (MSC-Lcn2-H_2O_2 in Fig. 4b). Taken together, these results indicated that Lcn2 reduces senescent cells in hB-MSCs particularly under oxidative stress conditions.

Over-expression of Lcn2 down-regulates senescence-related genes in hB-MSCs following their exposure to oxidative stress conditions

Next, we studied the expression of certain genes whose expression has been attributed to senescence (Lafferty-Whyte et al. 2010). To do this, the hB-MSCs were treated with 150-μM H_2O_2 for 2 h, a condition in which cells survive but senescence is induced (as previously described by Brandl et al. (2011)) followed by RNA extraction, cDNA synthesis, and real-time PCR analysis of p16, p27, and p53 gene expression. Relative expression of the genes was determined after normalization against expression of the β-*actin* housekeeping gene and reported as fold changes.

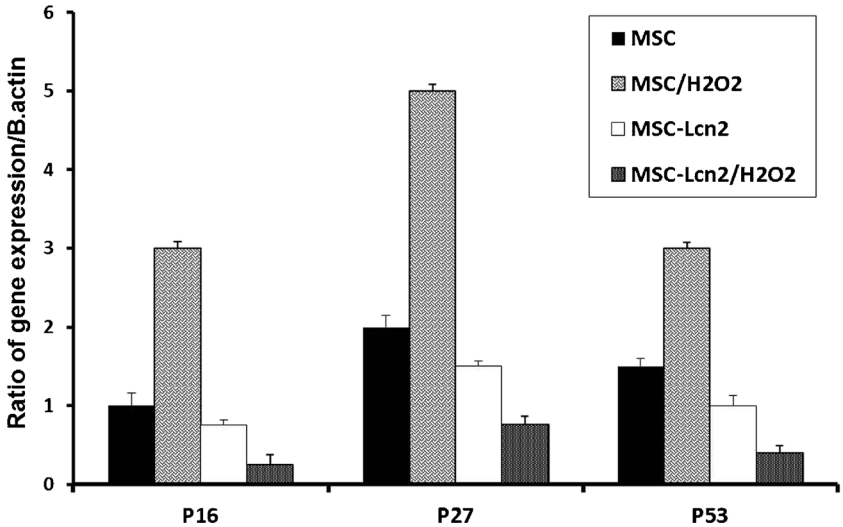

Fig. 5 Expression of senescence-related genes in MSCs/MSC-Lcn2 with or without exposure to oxidative stress. Quantitative assessment of expression of senescence-related genes in MSCs and MSC-Lcn2 with or without H_2O_2 treatment by real-time PCR. Up-regulation of P16, P27, and P53 mRNA was observed in normoxia conditions in MSCs compared to the MSC-Lcn2. In addition, following H_2O_2 treatment, expression of the senescence-related genes was increased in MSCs. The highest over-expression of senescence-related genes was observed in p27 of the H_2O_2-treated MSCs

It was found that oxidative stress up-regulated the expression of p16, p27, and p53 genes in hB-MSCs. Interestingly, expression of these genes was down-regulated with Lcn2 (Fig. 5). Consistent with our previous findings, Lcn2 is shown to decrease the rate of senescence in hB-MSCs following exposure to H_2O_2.

Senescence occurs in aged hB-MSCs, but no alteration in Lcn2 expression level is observed

Since our results showed that ectopic over-expression of Lcn2 prevents hB-MSCs from senescence, we wanted to determine whether Lcn2 expression increases throughout aging or not. The hB-MSCs were cultivated for 18 passages. Initial signs of senescence were detected in the 10th passage as determined by SA-B-gal staining. However, in the 18th passage, a majority of the cells stained blue (data not shown). Expression of some senescence genes including p16, p27, and p53 and also Lcn2, was evaluated by real-time PCR in different passages. Consistent with the SA-B-gal staining results, up-regulation of the genes began after 10 passages and reached the highest level in the 18th passage (Fig. 6). Interestingly, the Lcn2 expression level was approximately constant throughout the 18 passages (Fig. 6). Together, these results indicated that the basal level of Lcn2 expression in hB-MSCs is low, and also that the Lcn2 expression level does not change in aged hB-MSCs.

Plasma level of Lcn2 in the elderly is higher than in the young

It has been shown that there is a positive correlation between aging and senescence. Therefore, we evaluated the plasma level of Lcn2 protein in various cases. In this regard, 30 healthy volunteers were selected and classified into three groups of age ranges including 18–22 (n=10), 38–42 (n=11), and 58–62 years old (n=9), and their Lcn2 plasma level was determined by ELISA. As shown in Fig. 7, the plasma level of Lcn2 was the lowest in the youngest group (18–22 years old) ($P<0.001$), and the highest level was found in the oldest group (58–62 years old), $P\leq0.001$).

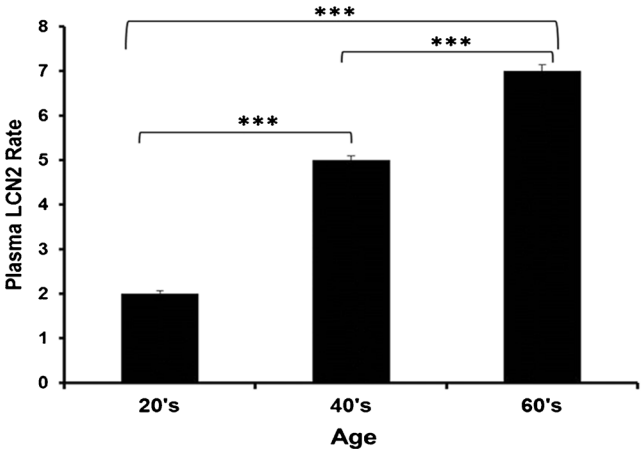

Fig. 7 Fold changes of Lcn2 concentration obtained by ELISA in plasma samples. ELISA was performed to evaluate the Lcn2 expression in protein levels. Plasma level of Lcn2 increased consistent with increase in age (Data represents mean ± SD; ***$P<0.001$)

Discussion

Cellular senescence is a complex phenotype that entails changes in both function and replicative capacity. In this study, we investigated the responses of hB-MSCs to ectopic up-regulation of Lcn2. Under normal conditions, over-expression of Lcn2 enhanced cell proliferation. Our results strongly suggested that Lcn2 plays an important role in a cellular phenomenon called senescence. In naïve cells such as hB-MSCs, Lcn2 was slightly up-regulated when exposed to sub-lethal doses of H_2O_2. This could be due to the presence of a huge antioxidant depository in MSCs (Kim et al 2011;

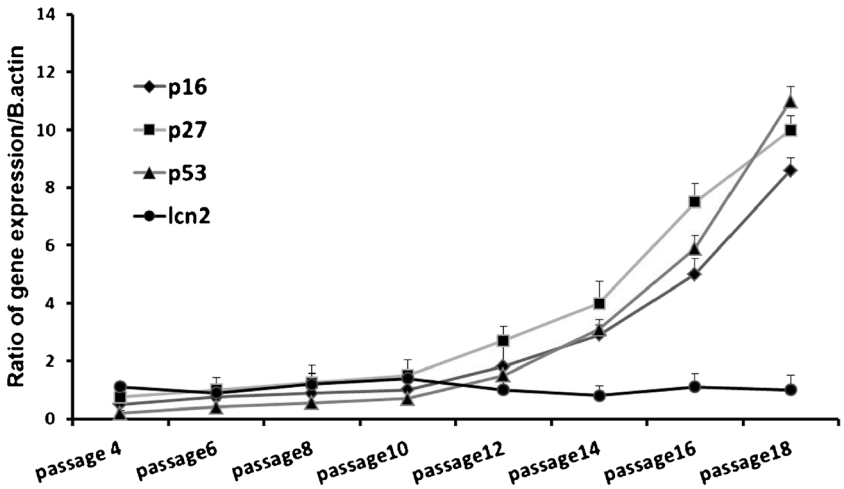

Fig. 6 Assessment of expression of senescence-related genes in different passages of MSCs. Quantitative evaluation of the expression of the senescence-related genes in different passages of MSCs by real-time PCR. Results showed that after 10 passages, P16, P27, and P53 mRNAs were up-regulated in MSCs. There was no up-regulation of Lcn2 in different passages

Kasper et al. 2009) that eliminates the need for high induction of Lcn2 and its protective effects.

Similar to our study, Brandl et al. reported that oxidative stress induces senescence in MSCs (Brandl et al. 2011). More recently, we also reported that engineering of hB-MSCs with lipocalin 2 granted them many protective properties including strengthening them to withstand oxidative, hypoxia, and serum-deprivation conditions, besides the induction of antioxidants and the up-regulation of some growth factors (Halabian et al. 2013). According to the present study, another property might be added to the previous properties of Lcn2, which is its capability to decrease senescence in cells following their exposure to oxidative stress. It is strongly recommended that for MSC-based cell therapy, lower passages (passage 4 or 5) must be used. It is also known that hB-MSCs finally become senescent, a feature they share with normal somatic cells (Brandl et al. 2011). It should be noted that the regenerative potential of hB-MSCs is impaired by cellular senescence (Brandl et al. 2011). Therefore, overexpression of Lcn2 in hB-MSCs not only reduces their senescence rate, but also might restore their regenerative potential. In other words, Lcn2 protects hB-MSCs against early aging. In support of this notion, Stolzing et al. (2008) evaluated the "fitness" of human MSCs obtained from various donors of different ages. In this regard, they assessed various markers routinely used to characterize the aging of somatic cells, related to their differentiation capacity and functionality. In their study, they found an age-related reduction in CFU-f and CD45 low/D7fib+ve/LNGF+ve cell numbers, in addition to reduced proliferation and differentiation capacity of the cells.

Interestingly, our results showed that in higher passages of hB-MSCs, in which the number of senescent cells is high, there is no alteration in the expression level of Lcn2, when compared with lower passages, suggesting that Lcn2 does not play any role in the natural aging process of hB-MSCs.

It has been known for more than 40 years that cellular senescence is a process limiting the growth (proliferation) of normal human cells in culture (Hayflick and Moorhead 1961). Recently, however, it seems that this process entails more than the simple stopping of cell growth. Cellular senescence has been implicated in four complex biological processes including tumor promotion, tumor suppression, aging, and tissue repair, some of which have opposing effects.

Finally, we also found that the expression level of Lcn2 was higher in elderly people than in young people. This could be due to higher levels of oxidative stress in the elderly as an inducer of Lcn2/NGAL (Roudkenar Habibi et al. 2007). A higher level of Lcn2 in the aged also suggests that Lcn2 might be considered as a new aging marker. In accordance with this notion, it has been shown recently that lipocalin-2 deficiency protects mice from aging (Law et al. 2010).

Organ failure and death are proposed to result from detained tissue regeneration following induction of senescence (Knapowski et al. 2002). In addition, it has been suggested that secretion of various factors by senescent cells, such as degradative enzymes, inflammatory cytokines, and growth factors, stimulates tissue aging (Krtolica and Campisi 2003). However, the relevance of these factors to in vivo aging is still controversial (Hornsby 2002).

There are two important issues regarding the relation between aging and MSCs, including how aging affects MSCs and how MSCs contribute to the aging of an organism. In this case, the primary concern is to find whether internal factors result in the aging of MSCs (intrinsic theory), or if the proliferative silence of the MSCs are imposed through alterations within surrounding tissue (extrinsic theory).

Taken together, in the present study, we found that the plasma level of Lcn2 is elevated by increasing age, which could be due to a compulsory response. Of course, this up-regulation doesn't necessarily indicate any role for Lcn2 in senescence, and necessitates further investigation.

In conclusion, in this study, we reported for the first time the implication of Lcn2/NGAL in cellular senescence especially when oxidative stress is a challenge. In other words, our results suggest that Lcn2 regulates cell survival through senescence under harmful conditions. Our findings might also provide new insight into how cells react to stress and how this cellular response can affect complex processes such as aging in an organism. Our results also suggest that application of Lcn2 would be beneficial to retain the fitness of MSCs for cell therapy purposes in elderly people, who are susceptible to many diseases in which autologus MSC-based therapy is convenient. However, further and complementary studies are required to clarify the mechanisms underlying Lcn2/NGAL modulatory effects on cell senescence.

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