Lipocalin-2-mediated upregulation of various antioxidants and growth factors protects bone marrow-derived mesenchymal stem cells against unfavorable microenvironments

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Abstract Despite many advantages of mesenchymal stem cells (MSCs) that make them suitable for cell therapy purposes, their therapeutic application has been limited due to their susceptibility to several stresses (e.g., nutrient-poor environment, oxidative stress, and hypoxic and masses of cytotoxic factors) to which they are exposed during their preparation and following transplantation. Hence, reinforcing MSCs against these stresses is a challenge for both basic and clinician scientists. Recently, much attention has been directed toward equipping MSCs with cytoprotective factors to strengthen them against unfavorable microenvironments. Here, we engineered MSCs with lipocalin 2 (Lcn2), a cytoprotective factor that is naturally induced following exposure of cells to stresses imposed by the microenvironment. Lcn2 overexpression not only did not interfere with the multidifferentiation capacity of the MSCs but also granted many protective properties to them. Lcn2 potentiated MSCs to withstand oxidative, hypoxia, and serum deprivation (SD) conditions via antagonizing their induced cytotoxicity and apoptosis. Adhesion rate of MSCs to coated culture plates

was also enhanced by Lcn2 overexpression. In addition, Lcn2 induced antioxidants and upregulated some growth factors in MSCs. Our findings suggested a new strategy for prevention of graft cell death in MSC-based cell therapy.

Keywords Mesenchymal stem cells · Lcn2 · Oxidative stress · Apoptosis · Antioxidants · Growth factors

Introduction

Over the past decade, among various types of stem cells, the mesenchymal stem cells (MSCs) represent a significant area of interest in the field of cell therapy. The ability of MSCs to home into injury sites and promote repair and regeneration of damaged tissues make them a significant cell type for transplantation into various disease models, such as renal failure (Hopkins et al. 2009), cardiac infarction (Bel et al. 2003; Berry et al. 2006; Chen et al. 2011; Hosoda et al. 2010; Price et al. 2006; Valina et al. 2007), injured skeletal muscle (de la Garza-Rodea et al. 2011; Natsu et al. 2004), and cerebral injuries (Chen et al. 2002; Hanabusa et al. 2005; Liu et al. 2011; Wei et al. 2005), and improves the outcome following traumatic brain injury (Kollar et al. 2009; Lindvall et al. 2004; McGinley et al. 2011; Park et al. 2012; Qian et al. 2008). Lack of MHC II, CD40, CD80, and CD86 is an additional benefit of MSCs, which permits allogeneic transplantation without immunosuppression (Berry et al. 2006). Recent studies on MSC therapy in animal models have confirmed an improvement in tissue repair (Higuchi et al. 2012; Quevedo et al. 2009), signifying safety and feasibility of this approach. Although several studies have proved that MSC-based therapeutic approaches for injury and diseases are promising (Rastegar et al. 2010), there are some shortcomings. Unfortunately, an extensive number of transplanted

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MSCs die within a few days after transplantation, mostly due to poor survival after transplantation (Kolossoff et al. 2006; Liu et al. 2008; Müller-Ehmsen et al. 2006; Zhu et al. 2006). Müller-Ehmsen et al. reported that 6 weeks after cell transplantation, only 2.0 % of MSCs were alive. In vitro and in vivo unfavorable conditions are the major reasons for the high death rates of the transplanted cells. During transplantation, cells are exposed to a nutrient-poor and hypoxic environment, and masses of cytotoxic factors involving a cytokine inflammatory storm surrounds the environment (Toma et al. 2002; Zhu et al. 2006). All of these might contribute to the MSCs' death. Improvement of stem cell survival during transplantation is a major concern in cell therapy. Therefore, equipping MSCs with cytoprotective strategies may improve their therapeutic potential by their strengthening toward the environment into which they will be transplanted (McGinley et al. 2011). Since in vivo manipulation is complex, most strategies for reducing in vivo cell death generally depend on in vitro physical, chemical, and pharmacological preconditioning and genetic manipulations of the cells before transplantation (Ramos et al. 2010). One of the strategies to reinforce the MSCs against toxic microenvironment is to equip them with cytoprotective genes (Xie et al. 2012). Exogenous manipulation of stem cells with chemical reagents has also been applied for improving cell survival (Niagara et al. 2007; Noiseux et al. 2012). Moreover, some studies suggested that combining apoptosis inhibitors, antiapoptotic genes, and some protective genes had beneficial effects on survival of MSCs and promoted differentiation and tissue recovery. Therefore, genetic modification could help to improve survival and differentiation rates (Chen et al. 2002; Gao et al. 2011; Hanabusa et al. 2005; Liu et al. 2011; Wei et al. 2005).

In this study, we employed one of the cytoprotective proteins called lipocalin 2 (Lcn2). The precise role of Lcn2 has not been fully understood yet. It is a magic protein with various functions that is involved in pathophysiological phenomena such as cancer (Missiaglia et al. 2004), infection (Berger et al. 2006), inflammation (Nielsen et al. 1996), kidney injury (Mishra et al. 2005), burn injury (Vemula et al. 2004), asthma, and arthritis (Mishra et al. 2004a, b). Lcn2 appears to be a protein that can protect against acute ischemic renal injury, inflammation, and infection (Berger et al. 2006). We previously showed that Lcn2 acts as a cytoprotective factor against oxidative stresses (Roudkenar et al. 2008a). Interestingly, in vitro and in vivo studies showed that Lcn2 has a protective effect against environmental physiological stresses that is a key point in medicine and transplantation.

These observations led us to hypothesize that overexpression of Lcn2 in MSCs could enhance cell viability following their exposure to a variety of stresses. Here, we present data showing that overexpression of Lcn2 in MSCs not only does not change their multipotent property but also confer beneficial abilities

including antioxidant, antiapoptotic, and anti-inflammatory properties to withstand against the lethal microenvironment.

Experimental procedures

Isolation, culture, and characterization of rat bone marrow MSCs

All animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care with prior approval of the Institutional Animal Care and Use Committee. MSCs were prepared from rat bone marrow (4–6 weeks old) as described by Friedenstein et al. (1968). In brief, Sprague–Dawley (SD) rats weighing 90–110 g were selected, anesthetized with ketamin/xyline and then laid down on an operation station. The femurs and tibias were cut off from the back limbs, followed by removal of skin and muscles, and bone marrow was harvested. Bone marrow cells were introduced into 100-mm dishes and cultured in Dulbecco's modified eagle's medium (DMEM, Gibco-BRL, Eggenstein, Germany) containing 10 % fetal bovine serum, 100 U penicillin G per ml, and 100 mg streptomycin per ml. The culture dishes were kept in a 37 °C and 5 % CO₂ incubator for 1 week. Culture medium was replaced every 2 days and unattached cells were discarded. After 7 days, the cells were washed with PBS and trypsinized. Then, the cells were transferred into cell culture flasks and incubated until 90 % confluency was reached. Afterwards, the characteristics of MSCs were tested by immunophenotyping and their differentiation capacities. To confirm the identity of the cultured MSCs, they were harvested, washed with PBS, and labeled with the following fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Becton, Dickinson and Company) conjugated antibodies. After blocking for nonspecific binding with PBS buffer containing 1 % bovine serum albumin, the cells were incubated with 1:100, 1:50, 1:100, and 1:150 dilutions of anti-CD34/CD45 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-CD29, anti-CD105 (Novus Biologicals), and anti-CD44 (Cell Signaling Technology) antibodies, respectively, for 20 min at 4 °C. Matched isotype controls were purchased from AbD Serotec or BioLegend. In case of CD14, we used rabbit IgG (Santa Cruz Biotechnology, Inc.) as a negative isotype control in flow cytometry. At least 1×10^4 cells per sample were acquired and analyzed. The labeled cells were assayed by flow cytometry and analyzed with CellQuest Pro software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Isolation and cloning of Lcn2

For isolation of Lcn2 gene, HepG2 (human hepatoma) cell line was obtained from the National Cell Bank of Iran

(NCBI) and grown in RPMI-1640 medium (Gibco-BRL, Germany) containing 10 % fetal bovine serum (Gibco-BRL). Total RNA was extracted by TriPure reagent (Roache, Germany) according to the manufacturer's protocol. RNA quality was determined by electrophoresis. Following treatment of 500 ng of total RNA with DNase I (Invitrogen, Germany), cDNA was synthesized using a cDNA synthesis kit (Invitrogen). PCR was performed as described previously (Roudkenar et al. 2009). Taq platinum DNA polymerase was purchased from Qiagen and PCR was performed in GeneAmp PCR System 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA). Supplementary table 1 represents nucleotide sequences of the primers used for amplification of full-length human Lcn2 gene containing Kozak sequence, *EcoRI* (forward), *NotI* (reverse) restriction enzyme sites. The PCR annealing temperature was 59 °C for human Lcn2. PCR products were separated in 2 % agarose gel.

Stable Lcn2 expression by linearized pcDNA3.1/CT-GFP-Lcn2 in MSCs

To construct the Lcn2 expression plasmid, the amplified full-length human Lcn2 cDNA lacking termination codon was cloned into the *EcoRI* and *NotI* restriction sites of the mammalian expression vector pcDNA3.1/CT-GFP (Invitrogen). The fidelity of cloning was evaluated by DNA sequencing and the recombinant plasmid was designated as pcDNA3.1/CT-GFP-Lcn2. Next, the MSCs were transfected with 2 µg of linearized pcDNA3.1/CT-GFP-Lcn2 plasmid using FuGENE HD (Roche, Germany) transfection reagent according to the manufacturer's protocol. As a control, MSCs were transfected with pcDNA3.1/Ct-GFP (MSC-V). Then, the transfected cells were cultured in DMEM medium containing G418/ml (Sigma-Aldrich, USA) and cells stably expressing human Lcn2 (MSC-Lcn2) were selected after at least 20 days. The expression level of Lcn2 was evaluated by RT-PCR, real-time PCR, enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA) and Western blot analysis using 1:200 dilution of primary mouse anti-Lcn2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 1:2,000 dilution of HRP-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) as secondary antibody. For RT-PCR, RNA was isolated from MSC-Lcn2 and MSC-V using TriPure reagent (Roche, Germany) and RT-PCR was performed as described above.

Evaluation of antioxidant capacity

The antioxidant capacity of Lcn2 against oxidative stresses was measured with Antioxidant Assay Kit (Sigma-Aldrich). The antioxidant assay is based on formation of a ferryl myoglobin radical from myoglobin and hydrogen peroxide,

which oxidizes the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to produce a radical cation, ABTS⁺, a soluble chromogen that is green in color and can be determined at 405 nm. Antioxidants suppress the production of the radical cation in a concentration-dependant manner. The cell culture media and/or cell lysates were harvested and antioxidant capacity was measured in 96-well plates according to the manufacturer's protocol. Trolox (a water-soluble vitamin E analog) provided by the kit was used as a positive control. To determine the antioxidant capacity of NGAL, commercially available recombinant NGAL (iron free) (R&D Systems) was used. Absorbance was read at 405 nm using an ELx800 Absorbance Microplate Reader.

Induction of cell injury with different stresses and analysis of relative cell viability

The MSCs-Lcn2 and MSCs-V were seeded into 96-well culture plates at a density of 1×10^4 cells/well and kept at 37 °C with 5 % CO₂ for 1 day. Then, the MSCs were treated with various concentrations of 0.5–10 mM hydrogen peroxide (H₂O₂; Sigma-Aldrich). H₂O₂ was used to induce cytotoxicity and apoptotic cell death via release of reactive oxygen species (ROS). For the hypoxia stress, following 1 day of incubation in normoxia condition, the cells were incubated at 37 °C and in the presence of 5 % CO₂ and 1 % O₂ in N₂ (hypoxia) for various time lengths in a Galaxy 48 R Incubator (New Brunswick, Germany). For the serum deprivation (SD) stress, the MSCs were washed two times with PBS and serum-free medium and maintained at a serum-free medium for different time lengths. Following the mentioned treatments, the cytotoxic effects of H₂O₂, hypoxia, or SD were evaluated by trypan blue dye exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays at 570 nm with an ELx800 Absorbance Microplate Reader.

Apoptosis assay via measurement of caspase 3 activity and annexin V/propidium iodide staining

Two methods were used for assessment of apoptosis induction in the MSCs-Lcn2 and MSCs-V following their treatment with various stresses. The relative caspase 3 activity was determined using a caspase 3 assay kit according to the manufacturer's instructions (Pharmingen, Germany). This assay is based on generation of free p-nitroanilide (pNA) chromophore following cleavage of the Asp-Glu-Val-Asp (DEVD)-pNA substrate by caspase 3 and subsequent absorbance read at 405 nm. Briefly, the MSCs-Lcn2 and MSCs-V were seeded in 24-well plates at a density of 2×10^5 cells/well and incubated at 37 °C in 5 % CO₂ atmosphere. Following treatment of the cells with various stresses (H₂O₂, hypoxia, and SD) for different time lengths, the cultured

MSCs were washed with PBS, trypsinized, harvested in lysis buffer, and centrifuged to eliminate cellular debris. Fifty-microliter aliquots of the cell extracts were subjected to caspase 3 measurement according to the manufacturer's protocol and the absorbance was read at 405 nm using an ELx800 Absorbance Microplate Reader. In addition, caspase 3 activity was confirmed by Western blot analysis. In this regard, following treatment of the cells with the various stresses, total cell protein was extracted by Complete Lysis-M reagent (Roche) according to the manufacturer's instruction. Next, the extracted proteins were boiled in loading buffer containing 4 % sodium dodecyl sulfate (SDS), 20 % glycerol, and bromophenol blue for 5 min. The samples were resolved by 12 % SDS-PAGE and *trans*-blotted onto PVDF membranes (Roche). The membranes were blocked and then incubated overnight at 4 °C with the following monoclonal antibodies: mouse anti-rat active caspase 3 antibody (Abcam, USA) and mouse anti- β -actin antibody (Sigma-Aldrich). After incubation with the primary antibodies, the membranes were washed with tris-buffered saline (TBS) containing 0.1 % Tween 20, followed by incubation with HRP-conjugated anti-mouse secondary antibody (Sigma-Aldrich). Finally, the membranes were developed by DAB solution (Sigma-Aldrich). Then, the expression level of caspase 3/ β -actin was analyzed using Image J software (version 1.42, National Institutes of Health, Bethesda, MD, USA). In addition, induction of apoptosis in MSC-Lcn2 and MSC-V by the various stresses was evaluated using annexin V-FLUOS staining kit (Roche Diagnostics) according to the manufacturer's instructions. In brief, following treatment of the cells with the stresses as mentioned above, approximately 2×10^5 cells were harvested, washed twice with PBS, and resuspended in incubation buffer containing annexin V-FITC and propidium iodide. The mixture was incubated at room temperature for 15 min and subjected to flow cytometry analysis using a Partec™ flow cytometer and FloMax software.

Assays for cell adhesion

Here, the effect of Lcn2 on cell adhesion was studied. In this regard, fibronectin (Sigma-Aldrich) was dissolved in PBS (pH 7.4) to yield a concentration of 10 μ g/ml and then added to the wells of plates at a final concentration of 2 μ g/cm². Afterwards, the plates were incubated at 4 °C for 24 h for being coated with fibronectin (Song et al. 2007). Then, wells of 96-well plates were blocked with a solution of 10 mg BSA (Sigma-Aldrich) per ml of PBS for 2 h at 37 °C. Following treatment of the MSC-Lcn2 and MSC-V with 100–400 μ M H₂O₂ (sublethal dose of H₂O₂) for various time lengths, they were washed with PBS and detached

by trypsinization. Then, they were washed once with DMEM medium containing 10 % FBS to deactivate trypsin and twice with serum-free DMEM medium to remove serum components. Viable MSCs (2×10^4) were then added to each well of the BSA-blocked plates and allowed to attach for 1 h at 37 °C and 5 % CO₂. This process was also performed for non-H₂O₂-treated MSC-Lcn2 and MSC-V. To verify the adhesion capability of the MSCs, the plates were carefully washed three times with PBS and then four separate fields were photographed by a phase contrast microscope. Some plates were washed with PBS, fixed with 3 % formaldehyde, stained with Coomassie blue, destained, and photographed.

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 % CO₂. Medium was refreshed every 2 days and cell proliferation was determined after a week using 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 MSC-Lcn2 and MSC-V with 100 μ M H₂O₂ (sublethal dose of H₂O₂), 3×10^3 cells/well were seeded in a 96-well plate and incubated at 37 °C with 5 % CO₂ and proliferation was assayed. In addition, 1×10^4 cells/well were seeded in a six-well plate with or without H₂O₂ treatment and incubated at 37 °C with 5 % CO₂. Afterwards, the cells were trypsinized and detached every 3 days and their viability was measured with 0.4 % trypan blue (Sigma-Aldrich, USA).

Analysis of antioxidant and growth factor gene expression by RT-PCR and quantitative real-time PCR

We previously showed that Lcn2 is a new antioxidant factor and it could be considered as a novel protective factor against oxidative stress. For evaluation of Lcn2 effects on expression of antioxidant and growth factors of the MSCs, the cells were washed with sterile PBS and their total RNA was isolated and reverse transcribed to complementary DNA in a 20- μ l reaction mixture using SuperScript III First-Strand Synthesis System (Invitrogen). Then, the cDNA was amplified using Taq DNA polymerase (Roche) and the specific primer pairs (Supplementary table 1) corresponding to heme oxygenase-1 (HO-1), metallothionein 1 (Mt1), and superoxide dismutase 1 (SOD1) as antioxidant genes; hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), transforming growth factor β 1 (TGF- β 1), and fibroblast growth factor 2 (FGF-2) as growth factors; and β -actin as housekeeping genes. Semiquantitative PCR was carried out in GeneAmp PCR System 9600. PCR condition included a primary denaturation step at 95 °C for 5 min followed by 35 cycles of 30 s

at 94 °C, 35 s at corresponding annealing temperature of each primer pair, and 40 s at 72 °C. To perform the quantitative real-time PCR, total RNA was isolated by TriPure reagent (Roache), and cDNA was synthesized using 1 µg total RNA as described above. Two microliters of the cDNA was used per reaction and the quantitative real-time PCR analyses were carried out using primers corresponding to *Rattus norvegicus* gene sequences represented by Supplementary table 1. Twenty-five microliters of real-time PCR mixture including SYBR Green PCR Master Mix (Takara, Japan), 10 pmol of each gene-specific primer, and 1 µl of diluted cDNA were reacted in Rotor-Gene RG-3000 (Corbett, Germany). The PCR condition was as follows: 1 min of preincubation at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at suitable annealing temperature of each primer pair, and 30 s at 72 °C and then a step of 10 s at 82 °C followed by melting curve analysis. With the Rotor-Gene software, the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene. Data analysis was performed using the Rotor-Gene software. Relative expression of the target genes was determined after normalization against β -actin expression as a house-keeping gene and reported as fold changes compared to the nontransfected MSCs.

Measurement of SOD and HO-1 activities

HO-1 activity was quantified by evaluation of bilirubin concentration in the culture media as reported by Tsai et al. (2006) and Turcanu et al. (1998). The method is based on formation of picomoles of bilirubin per milligram of cell protein and it was performed as described previously. The SOD activity was determined by measuring sample-mediated inhibition of xanthine oxidase-dependent O_2^- (superoxide radical) production using Superoxide Dismutase Assay II kit as instructed by the manufacturer (Calbiochem, USA). One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical.

Quantification of growth factors by enzyme-linked immunosorbent assay

ELISA analysis of the HGF (R&D Systems), IGF-1 (Immunodiagnostic Systems, Germany), FGF-2 (Biorbyt, USA), TGF- β 1 (Abcam), and Mtl (ABIN416217, kangtizaixian.cn) was performed as instructed by the manufacturers. In a few words, cell lysates or conditioned supernatants from MSC-Lcn2 and controls were collected, centrifuged at 1,800 rpm for 10 min to remove cells and debris, and were used for measurement of the proteins' concentration. The concentrations were calculated according to the standard curves created using standard samples of the kits.

MSCs' differentiation assay

In vitro differentiation capability of the MSC-Lcn2 to adipogenic, chondrogenic, and osteogenic lineages was evaluated. Briefly, for induction of differentiation to adipocytes, MSC-Lcn2 and MSC-V were plated in six-well plates at a density of 1×10^4 cells/well and cultivated in adipogenic medium for 2 weeks (Gibco-BRL, Invitrogen). The medium was refreshed every 4 days. For induction of differentiation to osteoblasts, MSC-Lcn2 and MSC-V were cultivated in StemPro osteogenic medium for 4 weeks (Gibco-BRL, Invitrogen). LipidTOX dye and Alizarin Red S staining were performed to identify the adipocytes and osteoblasts, respectively. Also, for induction of chondrogenesis differentiation, 10μ l of a 1×10^6 cell/ml solution was seeded in center of wells. After 2 h, warmed chondrogenesis medium (Gibco-BRL, Invitrogen) was added to the wells and then incubated at 37 °C and in the presence of 5 % CO_2 . The culture medium was replaced every 2–3 days. At certain intervals during cultivation, chondrogenic pellets were subjected to Alcian Blue staining. The levels of osteocalcin, the accumulation of triglycerides and glycosaminoglycans (GAG), and the determinants of osteogenic, adipogenic, and chondrogenic differentiation, respectively, were also evaluated to determine whether Lcn2 expression affects the differentiation capability of the cells. The osteocalcin level was determined using a specific ELISA test according to the manufacturer's protocol (BioSource International, USA). Accumulation of triglycerides was measured using BioVision's adipogenesis assay kit as instructed by the manufacturer (BioVision Research Products, USA). The GAG was also measured by a GAG assay kit (Kamiya Biomedical Company, USA) according to the manufacturer's protocol.

Statistics

Cell culture experiments were performed in three independent tests of triplicates. All values were expressed as mean $\pm$ SD and considered significantly different when p value was <0.05 . ANOVA was performed for all statistical analyses using the Tukey–Kramer post hoc test for multiple comparisons between all groups.

Results

MSC isolation and characterization

MSCs were first isolated from mixed cultures with hematopoietic cells based on their attachment to the culture plate. The successful isolation and expansion of the MSCs were confirmed by two methods. First, the expanded cells showed spindle-shaped morphology, and second, they were trypsinized and evaluated for expression of CD

markers. FACS analysis demonstrated that the obtained cells were entirely negative for CD34 and CD45, which are two specific markers of hematopoietic cells. In addition, they were positive for CD44, CD105, and CD29, with 99 % purity (Supplementary fig. 1). Also, the MSCs retained a fibroblastic morphology through repeated passages.

Expression of human Lcn2 in MSCs

Similar to our previous studies (Roudkenar et al. 2009), the 624-bp-length Lcn2 cDNA was isolated from HepG2 cells (data not shown), and the PCR product lacking a stop codon was directionally cloned into pCDNA3.1/CT-GFP vector producing recombinant pCDNA3.1/CT-GFP-Lcn2 plasmid. Four days post-transfection of the MSCs with the recombinant pCDNA3.1/CT-GFP-Lcn2 plasmid, GFP was detected under a fluorescent microscope. Subsequently, stable cells containing either Lcn2 (MSC-Lcn2) or empty vector (MSC-V) (Fig. 1a) was obtained. The expression of Lcn2 was confirmed by semiquantitative PCR with both total and

internal primer sets (Fig. 1b), Western blotting (Fig. 1c), and ELISA (Supplementary table 2) after 3 weeks. No detectable amount of Lcn2 protein was found in MSC-V. MSC-Lcn2 (1) and MSC-Lcn2 (2) denote two different stable clones expressing Lcn2.

Lcn2 exhibits antioxidant properties

For evaluation of antioxidant effect of Lcn2 on MSCs, MSCs-Lcn2 and controls (MSCs and MSC-V) were exposed to H₂O₂. The cell culture medium of the MSCs expressing Lcn2 (MSC-Lcn2) showed increased antioxidant activity (Fig. 2). However, the corresponding cell lysate revealed low antioxidant activity due to the secretion of Lcn2 into cell culture medium (data not shown). The antioxidant activity in cell culture medium of the MSCs expressing Lcn2 was increased, but the antioxidant assay of MSC-Lcn2 cell lysates showed a low level of antioxidant activity in accordance with the secretion property of Lcn2 (Fig. 2). However, it must be noted that although the level of free radicals was significantly reduced, it could not be

Fig. 1 Expression of Lcn2 by the MSCs transfected with the recombinant pCDNA3.1/CT-GFP-Lcn2 plasmid. **a** Transfected MSCs were cultivated in the presence of G418 to produce stable cells overexpressing Lcn2. **b** *Upper image* RT-PCR analysis showed high levels of Lcn2 expression by the stable MSCs. No detectable amount of expression was observed in MSC-V and nontransfected MSCs. The MSC-Lcn2 showed a 242-bp fragment (lanes 3 and 4) and also the full-length Lcn2 (640 bp) (lanes 7 and 8), whereas no expression was detected in nontransfected MSCs and MSCs transfected with the nonrecombinant pCDNA3.1/CT-

GFP plasmid (lanes 1, 2, 5, and 6). *M* 100-bp DNA marker. *Lower image* Expression of β-actin was used for normalization. *M* 100-bp DNA marker. **c** Western blot analysis of Lcn2 expression. Control MSCs revealed no detectable Lcn2 protein expression compared to the MSC-Lcn2. *Lanes 1 and 2* nontransfected MSCs and MSCs transfected with the nonrecombinant pCDNA3.1/CT-GFP plasmid. *Lanes 3 and 4* MSCs transfected with the recombinant pCDNA/CT-GFP-Lcn2. *Lower figure* represents the assessment of β-actin expression as a control for both groups. *MSC-Lcn2 (1)* and *MSC-Lcn2 (2)* denote two different stable clones expressing Lcn2

Fig. 2 Determination of antioxidant capacity of MSCs overexpressing Lcn2. This assay was used to quantify ROS in the culture medium. The antioxidant capacity of the MSCs expressing Lcn2 was higher than both MSC-V and MSC. There was no significant difference between MSC-Lcn2 (1) and (2) stable clones. Clearly, the cells expressing Lcn2 were also able to scavenge free radicals. Torlox was used as a positive control in kit. It should be noted that when a molecule has antioxidant property, the number of free radicals is lower so less dye is produced and absorbance is lower. (Mean $\pm$ SD; *** p <0.001; number of replicates, 3)

obviously inferred as a result of free radical scavenging. In other words, it is possible that Lcn2 prevents the formation of

free radicals rather than reacting with them after they are formed.

Lcn2 improved the adhesion capability of MSCs

Usually, cell adhesion property changes following exposure to stresses that could also affect cell viability (Song et al. 2010). In addition, during our work, we found that following trypsinization, the Lcn2-expressing cells were detached harder than the control cells. This observation led us to determine the effect of Lcn2 expression on adhesion capability of MSCs. As shown in Fig. 3, adhesion of MSCs to the BSA-coated surface of cell culture plates was approximately enhanced by 45 % due to Lcn2 expression. To further evaluate the effect of Lcn2 on improving cell adhesion, the MSCs were exposed to H_2O_2 before cell adhesion assay. As shown in Fig. 3, H_2O_2 treatment resulted in significant decreased adhesion; however, Lcn2 interestingly maintained the adhesion property of the cells. In other words, following H_2O_2 treatment, a higher number of suspended cells was detected in the MSCs and MSC-V cultures than in the MSC-Lcn2. Next, the positive effect of Lcn2 on cell attachment was further investigated by cell proliferation.

Fig. 3 Assessment of the Lcn2

effects on adhesion of MSCs. **a** The adhesion of MSCs, MSC-V, and MSC-Lcn2 to coated plates were measured quantitatively for 2 h. Adhesion of MSCs to the coated culture plastic surfaces was enhanced by approximately 45 % in case of Lcn2 expression. When the MSCs were exposed to H_2O_2 , their adhesion markedly decreased, but interestingly, Lcn2 maintained their adhesion property. 1×10^4 cells were seeds per well. The mean attachment values $\pm$ SD were 35 ± 1.2 in MSCs and 65 ± 1.96 in MSC-Lcn2. After treatment with H_2O_2 , the mean attachment values $\pm$ SD on fibronectin (Fn) were 25 ± 0.65 in MSCs and 48 ± 0.99 in MSC-Lcn2. **b** The number of attached cells was estimated by a phase contrast microscope. Following trypsinization and seeding of cells in the coated wells, the MSCs-Lcn2 was able to attach to the wells better than the MSCs or MSC-V. Arrows indicate the number of cells that attached on coated well (mean $\pm$ SD; *** p <0.001; number of replicates, 3)

Fig. 4 Effects of Lcn2 on proliferation of MSCs. Proliferation rate of the MSC-Lcn2 and MSC-V were determined by WST-1 assay. **a** Lcn2 expression resulted in increased cell proliferation for 2 weeks. **b** Proliferation of MSCs after treatment with 100 μ M H_2O_2 for 6 h followed by incubation under normal condition; although treatment with H_2O_2 decreased proliferation rate of the MSCs, it was maintained

by Lcn2 expression. Moreover, proliferation rate of the MSC-Lcn2 and MSC-V was also determined by cell counting over a number of passages (every 3 days). **c** Cell count under normal condition and **d** cell count following treatment of the MSC-Lcn2 and MSC-V with 100 μ M H_2O_2 (Mean $\pm$ SD; * p < 0.5, ** p < 0.01, and *** p < 0.001; three independent experiments were carried out)

Effect of Lcn2 on MSC proliferation

To determine whether Lcn2 affects the proliferation of MSCs, cell proliferation was measured with WST-1. Lcn2 increased cell proliferation rate during 2 weeks, as is shown by Fig. 4a. Interestingly, treatment of the cells with 100 μ M H_2O_2 for 6 h followed by their incubation under normal condition decreased the proliferation rate of the control MSCs, but Lcn2 maintained it (Fig. 4b). Cell proliferation was also evaluated by cell count over a number of passages and, again, similar findings were obtained (Fig. 4c and d). Taken together, these results indicate that Lcn2 enhances cell proliferation even in the presence of oxidative stress.

Lcn2 inhibits the cytotoxic effects of H_2O_2 , hypoxia, and serum deprivation stresses on MSCs

To determine whether Lcn2 can protect MSCs against stresses that they will inevitably face, the Lcn2-expressing cells were exposed to hydrogen peroxide, hypoxia, and SD stresses for different time lengths and then subjected to cytotoxicity and proliferation assays. The data represented by Fig. 5 show that after 2 h, those MSCs producing measurable amounts of Lcn2 clearly resisted 3–5 mM H_2O_2 , but the non-Lcn2-expressing MSCs were dead at this concentration. These results confirmed

the cytoprotective effect of Lcn2 against hydrogen peroxide cytotoxicity. Interestingly, exposure of the cells to 1 and 2 mM hydrogen peroxide for 2 h did not considerably change the viability of both MSC-Lcn2 and MSCs. It showed that MSCs could tolerate low concentration of H_2O_2 . However, MSCs, MSC-V, and MSC-Lcn2 died at 7 mM and higher concentrations (Fig. 5a). In addition to the H_2O_2 stress, the cells were also exposed to SD or hypoxia conditions for different time lengths followed by assessment of their viabilities (Fig. 5b and c). Results indicated that both hypoxia and SD had a cytotoxic effect on MSCs and MSC-V, but sufficient concentration of Lcn2 had protective effects on MSCs against these stresses. All together, the results confirmed protective effects of Lcn2 on MSCs against H_2O_2 -, hypoxia-, and SD-induced toxicities.

Lcn2 reduces stress-induced apoptosis in the MSCs

To analyze the effect of Lcn2 on apoptosis, the MSCs, MSC-V, and MSC-Lcn2 were exposed to various stresses and subjected to evaluation of caspase 3 activation and annexin V staining. Our results indicated the regulatory effect of Lcn2 on apoptosis. Whereas the controls (MSCs and MSC-V) showed increased apoptosis after exposure to different stresses, the apoptotic MSC-Lcn2 was significantly reduced by about 2.5-fold (Fig. 6a). In other words, when MSCs were treated with

Fig. 5 Evaluation of cytotoxic effects of H₂O₂, hypoxia, and serum deprivation on MSCs by MTT assay. **a** H₂O₂: the cytotoxic effect of various concentrations of H₂O₂ on the MSCs, MSC-V, and MSC-Lcn2 were determined by MTT assay after 2 h of exposure. At H₂O₂ concentrations of 3, 4, and 5 mM, the viability of the MSC-Lcn2 was higher than the MSCs or MSC-V. So, Lcn2 had a significant cytoprotective effect against H₂O₂ stress. **b** Serum deprivation (SD): there was not a significant difference between MSCs, MSC-V, and MSC-Lcn2 following their incubation under SD condition for 5 h. However, after 12 h of incubation of the cells under SD, the number of viable cells of MSCs and MSC-V was significantly lower than those of MSC-Lcn2. **c** Hypoxia: following 6 and 12 h of incubation of the cells under hypoxia conditions, Lcn2 protected MSC-Lcn2 against hypoxia compared to the MSCs and MSC-V. In fact, Lcn2 decreased cell death. (Mean±SD; **p*<0.05, ***p*<0.01, and ****p*<0.001; three independent experiments were carried out)

350 μM hydrogen peroxide for 24 h, the number of apoptotic MSCs and MSC-V was higher than apoptotic MSC-Lcn2

(Fig. 6a). Similarly, the number of apoptotic MSC-Lcn2 was significantly lower than the apoptotic MSCs and MSC-V following their exposure to hypoxia and SD stresses (Fig. 6a). Furthermore, the level of activated caspase 3 was also assessed by a caspase 3 assay kit and Western blotting that revealed higher levels of the activated caspase 3 in MSCs and MSC-V compared to the MSC-Lcn2 (Fig. 6b, c, and d). Taken together, these results confirmed the antiapoptotic effect of the overexpressed Lcn2 on the MSCs against the H₂O₂, SD-, and hypoxia-induced apoptosis.

Direct relation between induction of antioxidants and growth factors in the MSCs overexpressing Lcn2

Expression level of antioxidant genes was determined by semi-quantitative (Supplementary fig. 2a) and real-time PCR (Fig. 7a(I)). As shown in Supplementary fig. 2a, the basic expression level of the HO-1, SOD-1, and Mt1 antioxidant genes was higher in MSC-Lcn2 compared to MSCs. Following exposure of the cells to the H₂O₂, hypoxia, and SD conditions, the expression level of the HO-1 and Mt-1 antioxidant genes was upregulated in all MSCs compared to the nontreated cells. However, the overexpression of Lcn2 resulted in higher upregulation of the genes. These findings were further confirmed by real-time PCR, showing that the expression level of these genes was upregulated in MSC-Lcn2 and following exposure to the stresses (Fig. 7a(I)). On the other hand, the activity of SODs and HO-1 was also measured by commercially available kits. Lcn2 also enhanced the activity of SODs and HO-1 (Fig. 7b (I–III)). In consistence with the RT-PCR and real-time PCR results, following exposure of the cells to H₂O₂ and hypoxia, the SOD activity was not increased significantly. The concentration of H₂O₂, percent of hypoxia, and exposure time could account for these findings.

This result confirmed our finding that antioxidant upregulation in MSC-Lcn2 in stress condition is higher than MSCs and MSC-V. These findings suggest that the cytoprotective effects of Lcn2 may be attributed to the induction of these enzymes.

Many studies have reported that a number of proliferative growth factors and cytokines such as FGF-2, FGF-4, FGF-9, HGF, IGF-1, and TGF-β1 are synthesized by MSCs. These proteins could improve growth, proliferation, and adhesion properties of the MSCs and have protective effects on these cells (Kollar et al. 2009). Furthermore, recent studies have shown that secretion of these proteins by MSCs could be regulated under immunosuppression and proinflammation conditions. On the other hand, we have reported that Lcn2 possesses antioxidant, cytoprotective, and proliferative effects that can be mediated by enzymatic and nonenzymatic pathways including SOD, HO-1, catalase, and GSH peroxidase. Hence, here, we assessed the expression level of the HGF, IGF-1, FGF-2, and TGF-β1 growth factors in the MSC-V and

Fig. 6 Apoptotic effects of the H₂O₂, serum deprivation (SD), and hypoxia treatment on the MSCs were evaluated with annexin V staining, caspase 3 assay kit, and Western blotting. **a** Annexin V staining and flow cytometry. After treatment of the cells with 350 μ M H₂O₂ or hypoxia for 24 h, or 2 days of serum deprivation, the number of apoptotic cells was higher in the MSCs and MSC-V compared to the MSC-Lcn2. **b** Detection of apoptotic activity using caspase 3 assay kit: following the conditions described above, the

lowest level of the activated caspase 3 was observed in MSC-Lcn2, while the MSCs and MSC-V showed the highest levels of the activated caspase 3. **c** Western blot analysis of activated caspase 3: it was showed that the lowest level of the activated caspase 3 was observed in MSC-Lcn2. **d** Quantification of the expression levels of caspase 3 protein normalized against β -actin in the MSCs, MSC-V, and MSC-Lcn2 under the stress conditions (mean $\pm$ SD of three independent experiments; ** p <0.01 and *** p <0.001)

MSC-Lcn2 by real-time PCR and ELISA. The basic expression level of the growth factors, especially FGF-2 and HGF, was increased in MSC-Lcn2 in comparison with the controls (MSCs and MSC-V). Following exposure to the H₂O₂, hypoxia, and SD stresses, the expression of HGF, TGF β 1, IGF-1, and FGF-2 was upregulated in comparison with the controls (normoxia). However, the upregulation was considerable in MSC-Lcn2 (Supplementary fig. 2b and Fig. 7a(II)). These results were also confirmed at protein level by ELISA (Fig. 7b(IV–VII)). Altogether, our findings indicated that there is a direct relation between upregulation of antioxidants and growth factors by Lcn2 in the MSCs.

Multidifferentiation capacity maintained in MSC-Lcn2

Differentiation of MSCs into multiple lineages (such as bone, adipose tissues, and chondrocyte) is one of the famous properties of MSCs. This ability is considered a functional criterion for MSC precursor cells. Therefore, to determine whether the MSCs transfected with Lcn2 were able to differentiate same as native MSCs, and in other words, to verify whether the Lcn2 overexpression interferes with the functional criterion of the MSCs, control MSCs and the stable MSCs-Lcn2 were treated with adipogenic, chondrogenic, and osteogenic differentiation media. After 28 days, there was a positive reaction for Alizarin Red S staining (Fig. 8a(II) and A(II*)) confirming the ability of the cells to differentiate into osteocytes. After 15 days

of induction of MSCs and stable MSCs-Lcn2 toward chondrogenic lineage, blue staining reaction indicated the synthesis of proteoglycans by chondrocytes (Fig. 8a(III and II)) and after 21 days, a characteristic morphological change with accumulation of lipid vacuoles was observed following induction toward an adipogenic lineage supplementary (Fig. 8a(IV) and a(IV*)). Next, the amount of osteocalcin and accumulation of triglycerides and GAG were measured. The ability of the MSCs to differentiate into adipogenesis was induced in presence of Lcn2, but it was not significant (Fig. 8b). Differentiation level to chondrocyte and osteogenesis was the same in both untransfected MSCs and MSC-Lcn2. Taken together, these results showed that the MSC-Lcn2 retained their multidifferentiation potential into bone, adipogenic, and chondrogenic lineages.

Discussion

Despite promising therapeutic application of cell transplantation in some diseases, a majority of transplanted stem cells die because of the hostile environment they encounter (Xie et al. 2012). Recently, new approaches have gained much interest to enhance the viability of MSCs, and some studies focused on increasing the viability of MSCs via application of various survival, antioxidant, and growth factor genes (Gao et al. 2011; Hanabusa et al. 2005; Song et al. 2009;

Fig. 7 Assessment of expression of some antioxidants and growth factors by the MSCs under normoxia, H₂O₂, hypoxia, and serum deprivation conditions. **a** Real-time PCR. **a(I)** Quantitative assessment of the expression levels of antioxidants was performed with real-time PCR. In normoxia condition, the MSC-Lcn2 showed higher levels of HO-1 and Mt1 mRNA compared to the MSCs and MSC-V. Interestingly, in H₂O₂, hypoxia, and SD stresses, expression level of the antioxidant genes by MSC-Lcn2 was obviously higher than MSCs and MSC-V. **a(II)** Quantitative assessment of the growth factor expression by real-time PCR. Results showed upregulation of the growth factors in MSC-Lcn2 compared to the MSCs and MSC-V. In addition,

expression of the growth factors was increased in stress conditions; however, the rate of upregulation was higher in the MSC-Lcn2 compared to the MSCs and MSC-V. **b** proteins assay. **b(I–III)** Total SOD and HO-1 activities and the concentration of Mt-1 protein were compared between MSC-Lcn2 and the controls (MSCs and MSC-V). Results showed significant enhancement of activities or concentration of these proteins in the MSC-Lcn2. **b(IV–VII)** The expression level of the growth factors was compared between MSC-Lcn2, MSCs, and MSC-V. Considerably, the expression of the growth factors was upregulated in the presence of Lcn2. Normoxia: without stress (mean±SD; * p <0.5, ** p <0.01, and *** p <0.001; number of replicates, 3)

Fig. 7 (continued)

Wang et al. 2012; Wei et al. 2005; Yuan et al. 2011). In the present study, we equipped MSCs with NGAL/Lcn2 that is a multifunctional protein. Previous works of our group revealed the ability of Lcn2 to protect cells under oxidative stresses (Roudkenar et al. 2008b, 2009). Hence, overexpression of Lcn2 (an antioxidant and cytoprotective factor) in MSCs could be useful for increasing their survival. Within our expectation, here, we report that overexpression of Lcn2 in MSCs protected them against the encountered stressed condition. However, it seems that full protection is achieved through recruitment of other growth factors and genes. Interestingly, our results revealed that Lcn2 induces the expression of some growth factors, which are not only essential for survival and proliferation of MSCs but also augments them against harmful microenvironments. It has been shown that Lcn2 enhances adhesion and proliferation of cells (Tong et al. 2010). However, its precise mechanism in this regard has not been clarified yet. In this study, we showed that Lcn2 enhances the adhesion capability of MSCs even following their exposure to oxidative stresses. This finding suggests that one of the probable mechanisms of the Lcn2 cytoprotective effects is the enhancement of MSCs adhesion.

The absence of adhesion is probably the main cause of poor cell survival after cell transplantation (Kolossoy et al. 2006; Liu et al. 2008; Müller-Ehmsen et al. 2006). Supporting this notion, it has been shown that Lcn2 protein, which interacts with the

integrin and E-cadherin, plays a crucial role in integrin-mediated cell adhesion and signaling (Tong et al. 2010). More recently, Song et al. (2009) reported that overexpression of an adhesion molecule, called integrin-linked kinase (ILK), improved efficacies of MSCs after transplantation. They also reported that ILK increased the Bcl-2/Bax ratio and inhibited caspase 3 activation. Similarly, here, we reported that expression of Lcn2 in MSCs resulted in inhibition of caspase 3 activation. Furthermore, Lcn2 itself can also mediate proliferation and antiapoptotic actions following exposure of cells to oxidative and physical stresses (Roudkenar et al. 2009). It is noteworthy that the survival potency of MSCs is reduced following their exposure to various stresses. Hence, the expression of Lcn2 in MSCs to withstand the arduous microenvironments seemed reasonable. On the other hand, with presence of Lcn2, stem cells could be more stable in unpleasant microenvironments. In addition, stronger MSCs clearly could perform their physiological functions after transplantation. Lcn2 has been shown to act as an antioxidant, antiapoptotic, and anti-inflammatory agent following exposure to stressed conditions (Roudkenar et al. 2011). In the present study, we also exploited these cytoprotective properties of Lcn2. The differentiation assessments in the present study showed that both MSC-V and MSC-Lcn2 were able to differentiate to osteoblasts, chondrocytes, and adipocytes, suggesting that gene transfer did not alter the characteristics of the MSCs.

Fig. 8 Differentiation capacity of the MSC-Lcn2. **a** Using specific induction media and conditions for differentiation, MSCs and MSC-Lcn2 were successfully differentiated into osteoblasts as demonstrated by positive staining with alizarin red (**a(II)** and **a(II*)**), chondrocytic lineages as visualized using Alcian Blue (**a(III)** and **a(III*)**), and adipocytes that stained with LipidTOX staining (**a(IV)** and **a(IV*)**). The arrow indicates lipid droplets. **a(I)** and **a(I*)**) represents control MSCs and MSC-Lcn2 cultivated in normal medium (DMEM low glucose). **b** Accumulation of triglycerides, and the amount of glycosaminoglycan (GAG) and osteocalcin showed no significant difference between the MSC-Lcn2 and the MSC-V differentiation capacities

In this study, we also reported that Lcn2 upregulates the expression of some beneficial growth factors and antioxidants in MSCs. Interestingly, the rate of upregulation is augmented following exposure of the MSCs to harmful conditions, i.e., H_2O_2 , hypoxia, and SD, where the expression of growth factors seems to be essential to maintain the viability of the MSCs. On the other hand, the paracrine/endocrine effects of these growth factors might protect MSCs in harmful conditions and enhance their viability. Supporting our results, in an in vivo study, Abdel-Mageed et al. (2009) showed that intravenous administration of

MSCs following their genetic modification with extracellular SOD improves their survival in irradiated mice. Additionally, Tang et al. (2005) demonstrated that modification of MSCs by a hypoxia-regulatable HO-1-expressing vector increases the tolerance of engrafted MSCs to hypoxia-reoxygenation injury in vitro and improves their viability in ischemic hearts. More recently, in an in vitro study, we showed that adenovirus-mediated expression of the HO-1 protein within MSCs decreased cytotoxicity of oxidative stresses and inhibited the apoptosis induced by them (Hamedi-Asl et al. 2011). We also

showed that Nrf-2 overexpression in MSCs reduces oxidative stress-induced apoptosis and cytotoxicity (Mohammadzadeh et al. 2012). Wang et al. (2009) also expressed HSP20 in MSCs using adenoviral expression system, and an improved heart function was observed in an in vivo study. It should be noted that there is a major safety concern in clinical application of genetically manipulated MSCs. Therefore, further studies to address this concern are also essential. These studies may include the assessment of beneficial effects of MSC cultivation in the presence of secretome of the MCS-Lcn2 or improvement of endogenous expression of Lcn2 in nonrecombinant MSCs via synergistic effects mediated by the surrounding cells or microenvironments that might alleviate the need for exogenous overexpression of Lcn2 in recombinant MSCs.

In conclusion, for the first time, we reported the beneficial properties of Lcn2 to strengthen MSCs for cell therapy purposes. These advantageous effects are as follows:

1. Lcn2 has protective effects on MSCs against the stressed conditions in vitro,
2. Lcn2 overexpression in MSCs can strengthen the proliferation rate of stem cells due to antiapoptotic, proliferative, and protective effects of Lcn2,
3. Overexpression of Lcn2 in MSCs upregulates expression of some growth factors and antioxidants, which in turn, can protect the cells against various stresses and enhance their viability, and
4. Lcn2 improved the adhesion capability of MSCs.

Overall, our findings could be used to design a strategy for prevention of graft cell death in MSC-based cell therapy. It also highlights that management of cellular stress responses can be used for practical applications.

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