

Cell Survival Effects of Autophagy Regulation on Umbilical Cord-Derived Mesenchymal Stem Cells Following Exposure to Oxidative Stress

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What's Known

- Autophagy acts as a double sword, survival and cell death factor, in the cells.

What's New

- Inhibition of autophagy in umbilical cord-derived mesenchymal stem cells (UC-MSCs) enhanced cell survival following exposure to oxidative stress.

Abstract

Background: Due to oxidative stress, hypoxia, and serum deprivation, a large percentage of mesenchymal stem cells (MSCs) die in the early stages of transplantation. The present study aimed to address whether induction or inhibition of autophagy would affect the viability of MSCs after exposure to oxidative stress.

Methods: MSCs were isolated from umbilical cord tissue using the Ficoll gradient method. pCMV-GFP-LC-3 plasmid containing GFP-tagged LC3 was transfected into MSCs to assay autophagy level in these valuable cells. The four study groups were: MSC-LC3-Rapa, MSC-LC3-3MA, MSCs without any transfection, and MSC-GFP-LC3 (control groups). To induce autophagy, the MSC-GFP-LC3 was treated with different concentrations of Rapa for 24 hours and named MSC-LC3-Rapa. To inhibit autophagy in MSC-GFP-LC3, these cells were cultured in the presence of 3MA for 24 hours and named MSC-LC3-3MA. Non-treated MSC-GFP-LC3 and MSCs were considered as control groups. MSCs were exposed to lethal doses of H₂O₂ followed by cell viability evaluation with the water-soluble tetrazolium salt assay method. The data were analyzed with SPSS version 18.0 using one-way ANOVA test. P<0.05 was considered statistically significant.

Results: The results revealed that the enhancement of autophagy in MSC-LC3-Rapa sensitized them against oxidative stress (P=0.0006) and inhibition of autophagy in MSC-LC3-3MA led to resistance against oxidative stress (P=0.0003).

Conclusion: Inhibition of autophagy, as a non-genetic engineering method, in MSCs enhances cell viability following exposure to the oxidative stress. This may provide a novel strategy to promote the efficiency of MSC-based cell therapy for clinical applications.

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Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic stromal cells from different sources which can differentiate into many cell types such as osteocyte, adipose, cartilage, and muscle cells inside the human body and also in adequate cell culture media.¹⁻³ Nowadays MSCs are known as a promising cell source in the

field of cell-therapy and gene-therapy in order to cure most lethal and genetic diseases.^{4, 5} However, there are many obstacles in the clinical usage of MSCs which cause minimum survival of these cells shortly after transplantation, such as oxidative stress, hypoxia, and nutritional deficiencies.^{4, 6} A number of investigations in the field of MSCs-based therapy are in progress to address these obstacles. Overexpression of cytoprotective and cell survival genes by genetic engineering methods, preconditioning and pretreatment with stimulating agents and components are examples of strategies which are applied to withstand MSCs from the unfavorable micro-environments.⁷⁻¹²

Despite satisfactory results in each of these methods, the clinical utilization of MSCs is still challenging. For instance, when a survival gene like AKT (RAC-alpha serine/threonine-protein kinase) is up-regulated in MSCs by genetic engineering methods, the survival of the stem cells has increased permanently and apoptosis rate has decreased remarkably. Therefore, there had been a high risk of tumorigenesis in such cases.⁹ On the other hand, in the genetic engineering methods, the monitoring procedure takes a long time and is not financially feasible. It is noteworthy that safety treatment (a treatment with minimum complications) is the main principle of MSCs-based cell therapy. Therefore, to achieve this goal in the clinical usage of MSCs, we need a certain number of cells with adequate functional quality in phenotype and cell genotype.^{13, 14}

One of the safest and most effective strategies to increase the survival of MSCs could be a cellular garbage disposal system like autophagy. It is a catabolic path that indicates the formation of double-layer membrane vesicle called autophagosome. They deliver cytoplasmic components to the lysosomes. Autophagy has been considered as an adaptive response to stress such as starvation, hypoxia, and free oxygen radicals which promote cell survival by maintaining cellular energy levels.¹⁵⁻¹⁷ Cell death has increased by the elimination of autophagy-related genes. Also, in similar studies, an increase in apoptosis rate was reported through autophagy inhibition.¹⁸

Autophagy is known as a vital process in biological evolution, the immune system, and cell death. It has an effective role in fatal disorders such as neurodegeneration, self-immune diseases, and cancer. Autophagy has a double function in cells, on the one hand, it increases the cell survival and on the other hand, it causes cell death in terminal stages.^{19, 20} Ubiquitous microtubule-associated protein 1A/1B-light chain

(LC3), the central protein in autophagy pathway, is expressed in two forms (I and II). This protein naturally exists in the cytoplasm which during the formation of autophagosome the type I modifies into type II and conjugates to the double-layer membrane of autophagosome. By identifying the LC3 on the membrane, autophagy can be indicated in cells.²¹

To date, there has been no agreement on the exact role of autophagy in the cell death process and divergent results from different research studies have fueled this controversy. Therefore, the present study aimed to evaluate the role of autophagy regulation, as a safe and novel strategy, to increase the survival of umbilical cord-derived MSCs in unfavorable microenvironments such as oxidative stress.

Materials and Methods

Plasmid and Bacterial Strain

pCMV-GFP-LC3 expression vector (Cell Biolabs Inc., USA), which human LC-3 gene is tagged in frame with green fluorescent protein (GFP) in the expression vector, was selected as the cloning and expression vector, and the bacterial strain *E. coli* (Invitrogen, USA) TOP10 was used as the prokaryote host. To analyze autophagy, plasmid pCMV-GFP-LC3 containing LC3 gene tagged with GFP was inserted into the intended cells and tracked through a fluorescence microscope.

MSCs Isolation and Characterization

Umbilical cord segments were taken from healthy women after cesarean section with written informed consent. MSCs were isolated from the umbilical cord mucous tissue as described previously.²² Briefly, small pieces of the umbilical cord were digested using a collagenase type IV and/or dispase II and 2% trypsin (Invitrogen, USA) cocktail. Following the separation of mononuclear cells of the umbilical cord using the Ficoll (Sigma, Germany) protocol, the cells were cultivated in specific cell culture media DMEM (Dulbecco's modified eagle's medium), low glucose (Gibco, Germany) with 10% FBS fetal bovine serum (Gibco, Germany), 1% Streptomycin (Cinnagen, Iran) in 37 °C, and 5% CO₂. After 48 hours, the non-adherent cells were eliminated by replacing fresh media and only the adherent ones remained. The fourth passage of MSCs was provided for the experiments. The MSCs were checked morphologically and the expression of MSCs CD-markers (cluster of differentiation) and no expression of hematopoietic CD-markers were evaluated using a flow cytometer instrument

(Part C PASIII, Germany) to confirm the cell identity. The MSCs ability for differentiation was also analyzed with specific differential osteogenesis and adipogenesis kits as described previously.²² Specific staining was performed to detect osteocyte and adipose cells following the 21st day of cultivation and the 7th day of MSCs cultivation in osteogenic and adipogenic media, respectively.

Transformation of pCMV-GFP-LC3 Expression Vector into the Prokaryotic Host

pCMV-GFP-LC3 expression vector (Cell Biolabs Inc., USA) was transformed into *E. coli* strain TOP10 using the cold CaCl₂ method. The transformed bacteria were cultivated in LB (Luria broth) agar media (Merck, Germany) that contained kanamycin (Cinnagen, Iran) for a night at 37 °C in a shaker incubator. The recombinant plasmid was extracted using the high pure plasmid isolation kit (Roche, Germany). Then, the quantity of the extracted plasmids was measured with a NanoDrop spectrophotometer (Thermo Scientific, USA) and their quality was checked with electrophoresis on 1% agarose gel.

Transfection pCMV-GFP-LC3 Expression Vector into MSCs

To analyze the rate of autophagy in MSCs and induce expression of LC3, 2 µg of pCMV-GFP-LC3 expression vector was mixed with 5 µl of FuGENE[®] HD transfection reagent (Roche, Germany) and added to 70% confluent MSCs. The transfected MSCs were named MSC-GFP-LC3. In order to have a stable expression of GFP-LC3 in MSC-GFP-LC3, optimized lethal Geneticin (Invivogen, USA) dose was added to the culture media.

Induction and Inhibition of Autophagy in MSC-GFP-LC3

To determine optimize autophagy inducing the dose of Rapamycin (Rapa) (Invitrogen, USA) as the autophagy inductor, the MSC-GFP-LC3 was treated with different concentrations of Rapa, including 100, 200, 500, 800 nM for 24 hours in a proper culture media.¹⁷ This group was named MSC- LC3-Rapa. In order to inhibit autophagy in MSC-GFP-LC3, these cells were cultured in the presence of 3-methyladenine (3MA) (Invitrogen, USA), as the autophagy inhibitor, in concentrations of 0.5, 1, 2, and 3 mM for 24 hours.²³ This group was named MSC-LC3-3MA. Non-treated MSC-GFP-LC3 and MSCs without any manipulation were considered as control groups. Then, these subjected cells were analyzed under a fluorescence microscope to assay autophagy induction or inhibition.

Oxidative Stress Induction using Different Concentrations of H₂O₂

About 10⁴ of MSC-LC3-Rapa and MSC-LC3-3MA cells were cultured in 96-well plate as well as MSCs without any transfection and MSC-GFP-LC3 separately as the control. To simulate an oxidative stress condition, 300 µM of H₂O₂ (as the lethal dose) was added to culture media for 24 hours.²⁴ Then, the viability of different groups was measured using the water-soluble tetrazolium salt (WST-1) assay method.

WST-1 Assay

The WST-1 reagent (Sigma, Germany) was used to assay the survival rate of different experimental cell groups. In accordance with manufacturer's recommendation, the reagent was mixed with culture media in the ratio of 1:10 for 4 hours and 450 nM was applied to read the absorbance of the samples (BioTek, Germany).

Statistical Analysis

The data were presented as mean±SEM and analyzed using the SPSS software (version 18.0). The mean was analyzed with the one-way ANOVA post hoc tests. P<0.05 was considered statistically significant. All experiments were performed in triplicates.

Results

The Isolated Cells from Umbilical Cord Expressed MSCs-Specific CD Markers and Differentiate to Osteocyte and Adipocyte.

The fibroblast-like morphology of the isolated cells expressed specific surface mesenchymal markers (CD73, CD90, and CD105) while it did not express hematopoietic markers (CD 45 and CD34). The cells also had the ability to differentiate to osteocyte and adipocyte lineages indicating their multipotent capacity.²²

Establishment of Stable Clone of MSC-GFP-LC3

As shown in figure 1, pCMV-GFP-LC3 expression vector in which human LC-3 gene is tagged in frame with GFP was selected as the cloning and expression vector. This vector had resistance gene to Kanamycin in order to screen the transformed clones in prokaryote host. It also had Geneticin resistance gene for screening the transfected stable clones in the eukaryotic cells (figure 1A). Evaluation of Geneticin treated MSC-GFP-LC3 under a fluorescence microscope and observation of green cells confirmed transfection of the cells (figure 1B).

500 nM Rapa Induce and 2 mM 3MA Inhibit Autophagy in MSC-GFP-LC3

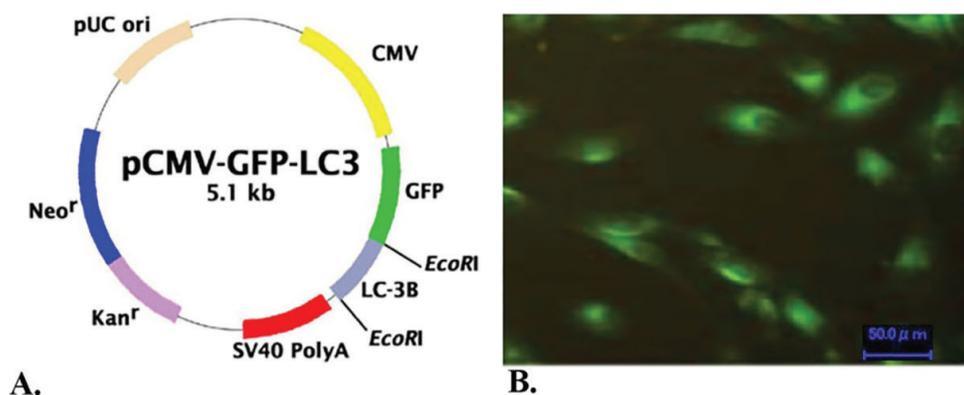


Figure 1: The transfection of pCMV-GFP-LC3 expression vector into umbilical cord MSCs. (A) pCMV-GFP-LC3 expression vector map containing GFP-fused LC3 gene, Kanamycin resistance gene as well as Geneticin resistance gene. (B) pCMV-GFP-LC3 was transfected into MSCs, known as MSC-GFP-LC3. Green fluorescent of MSC-GFP-LC3 under fluorescence microscope indicated their successful transfection.

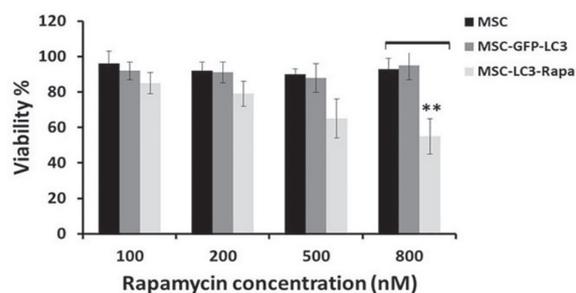
MSC-GFP-LC3 was treated with different concentrations of Rapa and 3MA separately, known as MSC-LC3-Rapa and MSC-LC3-3MA. The WST-1 assay results indicated that Rapa 100, 200, and 500 nM doses were not cytotoxic for MSC-GFP-LC3, but higher Rapa doses such as 800 nM led to cell death ($P=0.0044$) (figure 2A). We also determined that 500 nM Rapa not only had no extensive cytotoxicity for MSCs but also induced autophagy similar to our previous study.¹⁷ This condition allowed to have enough autophagy cells for further experiments. Then, 500 nM of Rapa was used for autophagy induction. As shown in figure 2B, among the different concentrations of 3MA, 2 mM and 3 mM led to severe cell death in comparison with the control group which was not primed with 3MA ($P=0.002$ and $P=0.0007$). A 1 mM of 3MA was selected as the optimized dose for other laboratory runs.

Generation of Dense Green Spots Confirmed Autophagy Induction after 500 nM Rapa Treatment

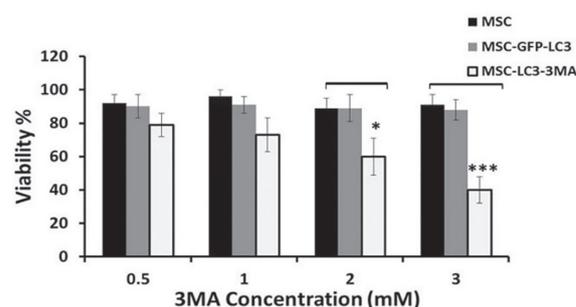
LC3 has a cytosolic form (LC3-I) that is converted to lipid-bound form (LC3-II) during formation of autophagosome.^{21, 25} It is worth mentioning that in the MSC-LC3-Rapa group, which autophagy was induced using Rapa, fusion of LC3 to GFP expressed itself in the form of dense shiny green spots under the fluorescence microscope (figure 3A). But in MSC-LC3-3MA, when the autophagy process was inhibited with 3MA, the GFP spread normally in the cytoplasm without any evidence of shiny spots (figure 3B).

Autophagy Induction Decreased Cell Survival Rate in MSCs while Autophagy Inhibition Increased this Process.

Rapamycin was used for the induction of autophagy and for the inhibition of 3-MA.



A.



B.

Figure 2: The determination of optimized Rapa and 3MA dose. MSC-GFP-LC3 was cultivated in the presence of Rapa (autophagy inducer) and 3MA (autophagy inhibitor) separately and was named MSC-LC3-Rapa and MSC-LC3-3MA, respectively. The WST-1 was performed to assay cell survival. (A) Rapa 800 nM dose has cytotoxic effects on MSC-LC3-Rapa cells, but Rapa 500 nM dose did not lead to severe cell death. (B) 2 mM and 3 mM of 3MA led to low MSC-LC3-3MA viability. 1 mM of 3MA was selected as the optimized non-toxic autophagy inducing dose ($P=0.002$, $P=0.0044$, $P=0.0007$, mean \pm SEM). The experimental groups were compared with control.

MSC-LC3-Rapa, MSC-LC3-3MA, MSCs without any transfection, and MSC-GFP-LC3 (control groups) were treated with a H_2O_2 lethal dose. Then, the cells were subjected to WST-1 assay. The viability of the above-mentioned cells was also determined in the normal condition without any stress induction. As shown in figure 4A, under normal conditions, there was

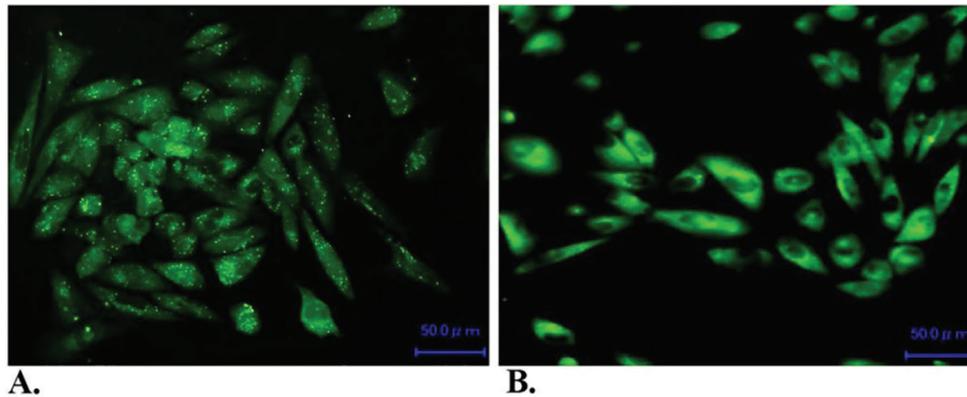


Figure 3: The confirmation of autophagy induction or inhibition with fluorescence microscope observation. (A) Conversion of LC3-I to LC3-II during autophagy process in MSC-LC3-Rapa led to the formation of dense shiny green spots in these cells. (B) The shiny green spots were not observed in MSC-LC3-3MA, which autophagy process was inhibited with 3MA.

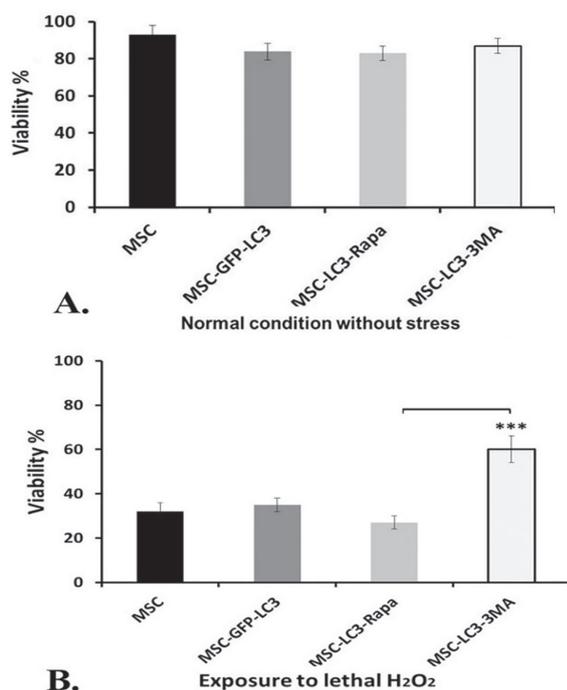


Figure 4: The evaluation of cell survival under normal condition and harsh lethal oxidative stress. (A) The viability of MSC-LC3-Rapa and MSC-LC3-3MA, non-transfected MSCs and MSC-GFP-LC3 (as controls) under normal conditions without any stress was estimated by WST-1 assay. There was no significant difference between the above-mentioned groups in term of cell viability. (B) MSC-LC3-Rapa and MSC-LC3-3MA were exposed to 300 $\mu\text{m}/\text{mL}$ of H_2O_2 as well as control groups. The results of WST-1 assay showed that the MSC-LC3-3MA had a higher survival rate than other groups and inhibition of autophagy strengthened them against severe oxidative stress ($P=0.0003$, mean \pm SEM). The MSC-LC3-3MA was compared with MSC-LC3-Rapa.

no significant difference between different cell groups in terms of viability. However, after exposure to the lethal dose of H_2O_2 , severe cell death occurred in the control groups as well as the MSC-LC3-Rapa group ($P=0.0006$) (figure 4B). Interestingly, in the MSC-LC3-3MA group, in the case of autophagy inhibition, the viability

of cells was significantly increased ($P=0.0003$) in comparison with the MSC-LC3-Rapa group (figure 4B).

Discussion

The umbilical cord-derived MSCs were transfected by a vector expressing both LC3 (marker of autophagy) and GFP to investigate autophagy effects on cell survival. Among different concentrations of Rapa (a well-known inducer of autophagy) and 3MA (inhibitor of autophagy), 500 nM dose of Rapa and 1 mM dose of 3MA were selected as the optimized dose for further experiments. After exposure to harsh oxidative stress, the results of the WST-1 assay indicated that the modulation of autophagy in MSCs led to a higher survival rate in comparison with other groups.

The most important issue in treatment by using these cells has been their death occurring shortly after transplantation, which makes the procedure much less efficient than expected.^{26,27} In this regard, Toma and colleagues reported that less than 0.44% of MSCs had survived within 4 days after transplantation into the heart of SCID mice.⁶ Different factors play a role in the early death of MSCs in a transplantation micro-environment. Hodgetts and colleagues showed that oxidative stress and ischemic damages (nutrient deficiency along with hypoxia) were the main causes of the early death of MSCs during the first days after transplantation.²⁸

In the present study, the effect of autophagy on MSCs survival in an inappropriate transplantation micro-environment was investigated. Autophagy played an important role in biological development, immune system, and cell death.^{19, 20, 29} Oxidative stress, serum deficiency, and hypoxia which had been the main causes of low survival of MSCs after

transplantation could induce autophagy. Matsui and Scherz-Shouval showed that oxidative stress, nutrient deficiencies, malfunctioning mitochondria, and endoplasmic reticulum stress can cause autophagy induction.^{30, 31} Yu and colleagues proved that cell death under oxidative stress had occurred through apoptosis and due to caspases action. By caspase inhibition, still, catalase degradation and an increase in ROS amounts had continued and caused cell death, which was due to the autophagy process.³²

Autophagy was induced by using Rapamycin and 3MA and utilized for autophagy inhibition. According to our previous study, Western blot analysis confirmed that 500 nM Rapa induced autophagy.¹⁷ In that experiment, we applied suppressing shRNA vector to down-regulate ATG7, a key autophagy gene. MSCs viability in the case of exposure to different stress conditions was improved by this strategy.¹⁷ The results implied that autophagy inhibition in MSCs could improve cell resistance in reaction to oxidative stress (H₂O₂) and improve the cell survival. In the of autophagy inhibition and its effect, Chen and colleagues showed that when HEK-293, U87, and HeLa cell lines had been exposed to different concentrations of H₂O₂, cell death occurred. By autophagy inhibition, they could decrease the lethal effect of H₂O₂ without apoptosis inhibition. However, this lethal rate had no significant change by apoptosis inhibition without the inhibition of autophagy.³³ Kuwahara showed that Rapamycin-induced-autophagy in radiation-resistant cancer cells conferred them sensitive to irradiation. They also showed that autophagy inhibition by 3MA could make the turmeric cells much more resistible to radiotherapy.³⁴

In mammary cancer cells and glioma, hypoxia can cause cell death through autophagy. Autophagy also plays an important role in prostatic cancer development.³⁵ Kanzawa and colleagues showed that chemical medicines like arsenic trioxide or tumor suppressors could cause cancer cell death through autophagy induction path and could cure the disease.³⁶ Lin and colleagues reported that autophagy inhibition could make thyroid papillary carcinoma cells more resistible to radiotherapy. They concluded that autophagy induction could possibly be effective in the treatment of this type of cancer and killing the resistant tumor cells.³⁷ It is noteworthy that the focus of the present study was in vitro; however, preclinical and in vivo studies should be undertaken.

Conclusion

The results showed that the induction of

autophagy not only did not protect the MSCs against oxidative stress-induced cell death but also exacerbated them in terms of cell death. However, inhibition of autophagy had the protective effects. Therefore, inhibition of autophagy in MSCs could possibly provide a strong defense mechanism against unfavorable conditions in vitro.

Conflict of Interest: None declared.

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