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MESENCHYMAL STEM CELLS SUPPORT DORSAL ROOT GANGLION NEURONS SURVIVAL BY INHIBITING THE METALLOPROTEINASE PATHWAY

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Abstract—The positive effect of adult undifferentiated mesenchymal stem cells (MSCs) on neuronal survival has already been reported, although the mechanisms by which MSCs exert their effect are still a matter of debate. Here we have demonstrated that MSCs are able to prolong the survival of dorsal root ganglion (DRG) neurons mainly by inhibiting some proteolytic enzymes, and in particular the pathway of metalloproteinases (MMPs), a family of proteins that are involved in many neuronal processes, including survival. The inhibition of MMPs was both direct, by acting on MT-MMP1, and indirect, by acting on those proteins that regulate MMPs' activation, such as Timp-1 and Sparc. The importance of the MMPs' down-regulation for neuronal survival was also demonstrated by using N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH), a wide range inhibitor of metalloproteinases, which was able to increase the survival of DRG neurons in a significant manner. The down-regulation of MMPs, obtained both by MSC contact and by chemical inhibition, led to the inactivation of caspase 3, the executor of apoptotic death in DRG neurons cultured alone, while caspase 7 was found to be irrelevant for the apoptotic process. The capacity of MSCs to prevent apoptosis mainly by inactivating the metalloproteinase pathway is an important finding that sheds light on MSCs' mechanism of action, making undifferentiated MSCs a promising tool for the treatment of many different neurodegenerative pathologies.

Key words: DRG neurons, mesenchymal stem cells, apoptosis, metalloproteinase inhibition.

The positive effect of adult undifferentiated mesenchymal stem cells (MSCs) on nerve regeneration, axonal growth and neuronal survival has already been reported (Akiyama et al., 2002; Zhao et al., 2002; Cuevas et al., 2004; Scuteri et al., 2006; Liu et al., 2010), although the mechanisms by which MSCs exert their effects are still unclear. Different explanations have been proposed: (1) undifferentiated MSCs are supposed to increase neuronal viability by transdifferentiation mechanisms (Sanchez-Ramos et al., 2000; Deng et al., 2006; Phinney and Prockop, 2007), or (2) by releasing neurotrophic factors (Crigler et al., 2006; Yoshida et al., 2007). It is, however, difficult to reproduce the transdifferentiation as it is strongly dependent on the experimental conditions and, therefore, this mechanism is still a matter of debate (Lu et al., 2004). On the other hand, the release of trophic factors, which is widely accepted, is a well-recognized mechanism, but not the only one, involved in MSCs' positive effect (Crigler et al., 2006). The release of other molecules, different from trophic factors, able to modify the expression of pro-survival genes should be also considered.

In a previous study (Scuteri et al., 2006) we demonstrated, by using both direct and indirect co-cultures and MSC conditioned medium, that undifferentiated MSCs were able to promote the differentiation and prolong the survival of dorsal root ganglia (DRG) dissociated neurons. DRG neurons survived up to 60 days or more when co-cultured with MSCs, while DRG neuronal primary cultures naturally underwent cellular death after a few weeks. This protective effect was due neither to transdifferentiation mechanisms (the number of neurons did not increase), nor to the release of soluble factors since direct contact between neurons and MSCs was essential for the long-lasting survival of DRG neurons. For this reason, to investigate the molecular mechanisms responsible for the effect of MSCs' direct contact on DRG neurons, we studied the modifications of the proteins that mediate cell contact, such as adhesion molecules and extracellular matrix proteins, by using a specific low-density microarray. Proteins important for the correct cellular contact include matrix metalloproteinases (MMPs), a family of proteolytic enzymes, some of which are membrane-bound and some others which are secreted, that also control many processes occurring in neurons, ranging from morphogenesis and aging to inflammation and apoptosis (Muroski et al., 2008). The death of neurons in cultures is mediated by MMP activation (Gu et al., 2005; Thirumangalakudi et al., 2007; Xue et al., 2009), which may occur both through the activation of the precursor zymogens and through inhibition, mainly by tissue inhibitors of metalloproteinases (TIMPs). The proteolitic activation of MMP precursor zymogens may be carried out by different MMPs or by other molecules such as Sparc that are able to activate MMP2 (Yan and Sage, 1999; McClung et al., 2007). However, changes in the control of their activity may result in neurological diseases.

In particular, it is known that MMPs are able to activate the apoptotic machinery in many cellular models (Lee et al., 2004) due both to their involvement in inflammation

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Abbreviations: DRG, dorsal root ganglia; ECM, extra-cellular matrix; MMPs, metalloproteinases; MSCs, mesenchymal stem cells; MT-MMP1, membrane-type matrix metalloproteinase-1; NNGH, N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid.

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pathways (Pagenstecher et al., 1998) and also to their ability to tamper with the correct cell-ECM (extra-cellular matrix) interaction which results in a form of apoptotic death termed anoikis (Valentijn et al., 2004). The importance of cellular adhesion for cell survival is widely recognized, and anoikis as a peculiar form of apoptosis has been studied and demonstrated in many adherent cells (Frish and Francis, 1994), especially in neurons (Bozzo et al., 2006). This process is mediated by the balance in the expression of pro/antiapototic proteins and by the activation of caspases, the final executors of the death pathway (Bozzo et al., 2006).

Here, we reported changes in the expression of MMPs in cultures of neurons alone (fated to early death), and in cultures of MSCs and neurons, destined to long-lasting survival, demonstrating, for the first time, that MSCs are able to prolong neuronal survival by inhibiting the MMP pathway and thus reducing apoptosis.

EXPERIMENTAL PROCEDURES

All the procedures on animals were carried out under anesthesia in accordance with the European Communities Council Directive 86/609/EEC.

All the experiments were repeated at least three times to validate the results.

DRG neuron primary cultures

DRG from 15-day-old embryonic Sprague–Dawley rats (Harlan Italy, Udine, Italy) were aseptically removed, pelleted and dissociated with trypsin. Neuronal cells, which are postmitotic neurons, were cultured onto a single layer of rat tail collagen surfaces in 35 mm dishes for 5 days in AN2 medium made up of MEM (Invitrogen, Carlsbad, CA, USA), plus 15% Calf Bovine Serum (Hyclone, Logan, UT, USA), 50 μg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA), 1.4 mM L-glutamine (Invitrogen), 0.6% glucose (Sigma Chemical Co.) supplemented with 5 ng/ml NGF (Invitrogen), and plated onto collagen-coated dishes. After 24 h, neurons were treated for 5 days with AN2 medium to which were added 5 ng/ml NGF and 2′-deoxy-5-fluorouridine (Fudr 10^{-5} M Sigma Chemical Co.) to remove satellite cells. Neurons were then incubated with AN2 medium with 5 ng/ml NGF.

The neuronal survival was evaluated by counting viable neurons, characterized by the presence of a refracting outline, at different time points in a blind manner (day: 0, 7, 14, 21, 30) (Scuteri et al., 2009).

MSC cultures

MSCs were obtained from the bone marrow of 10-week-old female Sprague–Dawley rats by flushing the femur and tibia diaphysis with 2 ml/bone of α-MEM to which was added 2 mM L-glutamine and antibiotics. MSCs were expanded in α-MEM medium (Lonza Group Ltd Switzerland) plus 20% ES cell screened Fetal Bovine Serum (FBS, Hyclone) (Donzelli et al., 2007).

For direct co-cultures, MSCs were added to neurons at a density of 100,000 cells/dish. Co-cultures were maintained in AN2 medium with 5 ng/ml NGF. The medium was changed once a week. Indirect co-cultures were established with neurons being seeded onto the upper surface of a collagen-coated cover glass resting on a single layer of MSCs plated at a density of 100,000 cells/dish. Indirect co-cultures were maintained in AN2 medium with 5 ng/ml NGF. The medium was changed once a week (Scuteri et al., 2006).

Microarray analysis

We used the Oligo GEArray System (SABiosciences Corporation, MD, USA) according to the manufacturer's instructions. The isolation of RNA was performed using the ArrayGrade TM Total RNA Isolation Kit. For the conversion of experimental RNA to labeled target cRNA, TrueLabeling-AMP TM 2.0 cRNA Synthesis and Labeling was used. The gene hybridization and detection were performed using Oligo GEArray (Hybtube protocol). The data analysis was carried out with the GEArray Expression Analysis Suite (SABiosciences Corporation).

The statistical analysis was performed using the ANOVA test and Tukey's Multi comparison Test with the GraphPad Prism (GraphPad Software, San Diego, CA, USA) statistical package.

In order to confirm the results obtained in the microarray analysis and to localize the proteins whose expression was significantly changed after co-cultures with respect to neurons and MSCs alone, we performed immunofluorescence studies (Scuteri et al., 2006). The primary antibodies used were anti-NeuN (Chemicon International, Temecula, CA, USA, 1:50), anti-Map2 (Chemicon, 1:200), anti-Sparc (Genetex Inc. San Antonio, TX, USA, 1:1000), anti-Timp-1 (Calbiochem, San Diego, CA, 1:50). Phalloidin (Invitrogen, 1:20) was used to visualize the actin filaments of MSCs.

Study with NNGH metalloproteinase inhibitor

N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH, metalloproteinase inhibitor, Biomol. Intern. LP, PA, USA) was dissolved in dimethilsulfoxide (DMSO, Sigma Chemicals Co., St. Louis, MO, USA) to make a stock solution of 2 mM concentration which was diluted with medium to obtain our working concentrations (1.3, 2 and 3 μM). The working solution concentration of DMSO was always very low and not cytotoxic for neurons (Scuteri et al., 2009). Neuronal cultures were exposed to NNGH for one month, the culture medium was changed twice a week and the NNGH effectiveness was confirmed by zymography.

Zymography

A common and simple method described in the literature to evaluate the activity of membrane-type matrix metalloproteinase-1 (MT-MMP1) is the study of the activity of some of its substrates, such as MMP2 and MMP9, by gelatin zymography (Yamamoto et al., 2008). Cells were washed twice with PBS, solubilized in lysis buffer (10 mM Tris–HCl, pH 7.5, 0.25% Triton X100) and sonicated. Protein quantification was performed using the Bradford method, then protein aliquots were solubilized in Laemmli buffer 5× without β-mercaptoethanol and run onto 10% SDS-Polyacrylamide gels (SDS-PAGE) with 2 mg/ml of gelatin (porcine skin) dissolved in bidistilled water. After the running onto the gel, the latter was washed twice with washing buffer (Triton X-100 2.5% w/v in bidistilled water) and then incubated in a buffer containing NaCl 150 mM, Tris–HCl pH 7.5 and $CaCl_2$ 5 mM for 48 h at 37 °C. Gels were then stained with Comassie Blue 0.25 g in 100 ml of a solution containing 45% methanol and 10% glacial acetic acid in bidistilled water. Reagents were purchased from Sigma Chemicals Co. After rinsing with demineralized water, gel images were acquired with a 100 Imaging System (Eastman Kodak Co, Rochester, NY, USA).

ELISA assays

The supernatants from DRG neurons, MSCs and co-cultures were analyzed at different time points (from 2 to 7 weeks) by ELISA for Timp-1 (Boster Biological Technology, Ltd, Malden, MA, USA) and Sparc (Cusabio Biotech Co., Newark, DE, USA) following the protocol provided by the manufacturers. Finally, the reaction was stopped and the optical density of each well was determined at 450 nm within 30 min.

Table 1. Expression fold variation of adhesion-mediating genes after co-culture

	Neurons	Co-culture	*P*-value
MT-MMP1	2,445±0,392	0,901±0,047	$P<0.05$
Timp-1	0,647±0,074	2,625±0,265	$P<0.001$
Sparc	10,030±0,068	2,657±0,152	$P<0.001$
Basigin	13,257±0,693	3,069±0,050	$P<0.001$
Adamst1	0,194±0,009	0,041±0,010	$P<0.05$

Apoptosis detection

Flow Cytometry. DRG neurons, MSCs and co-cultures were detached used trypsin-EDTA, washed twice with PBS and then stained with FITC-Annexin V according to the manufacturer's protocol (Becton Dickinson, San Josè, CA, USA). The cells were acquired by FACSCanto (Becton Dickinson) and the analysis was performed on at least 10^5 events. For each sample the negative signal was set on unstained cells.

Electron microscopy. The cultures were fixed for 30 min in 4% paraformaldehyde and 2% glutaraldehyde in 0.12 M phosphate buffer and then post-fixed in 1% OsO_4 in cacodylate buffer for 30 min, dehydrated in ethanol and embedded in epoxy resin. Ultra-thin sections were stained with Uranyl Acetate and Lead Citrate and observed with a Philips CM10 transmission electron microscope (Philips Medical Systems S.p.A., Monza, Italy).

Immunofluorescence study of caspases. An immunofluorescence study of the activation state of the executor caspases 3 and 7 was performed as previously described (Scuteri et al., 2006) using antibodies directed against active-caspase 3 (Cell Signalling Technology, Danvers, MA, USA, 1:500) and active-caspase 7 (Calbiochem, 1:100).

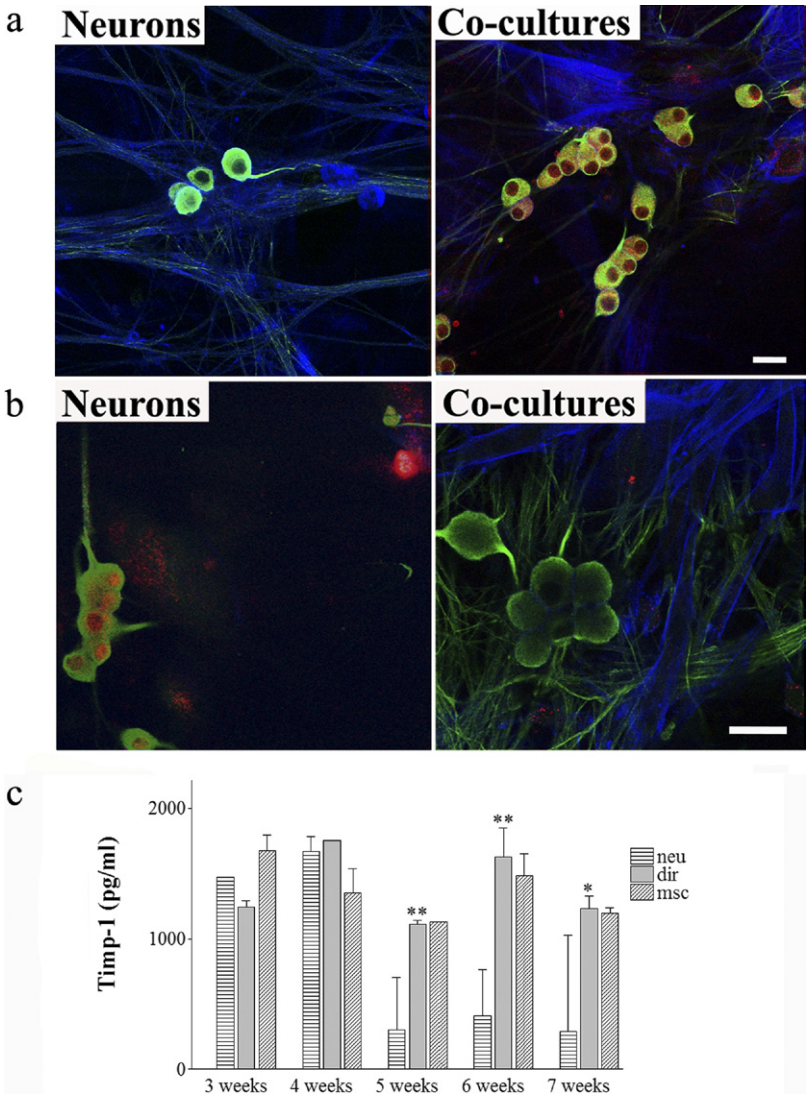

Fig. 1. Analysis of Timp-1 and Sparc. (a) Confocal microscopy detection of Timp-1: After 30 d of culture the protein was present only in co-cultured neurons and not in neurons alone (Timp-1 red, Map2 green, Phalloidin blue). Scale bar: 25 μm. (b) Confocal microscopy detection of Sparc: After 30 d of culture the protein was present only in neurons alone and not in co-cultured neurons (Sparc red, Map2 green, Phalloidin blue). Scale bar: 25 μm. (c) Elisa assay for Timp-1: The secreted form of Timp-1 was increased in co-culture medium with respect to the medium of neurons alone after 5 wk of culture. * $P<0.05$, ** $P<0.001$.

RESULTS

Analysis of adhesion and extracellular matrix molecule expression patterns

In the co-cultures of DRG neurons and MSCs, the microarray analysis (Table 1) evidenced a statistically significant down-regulation, with respect to the cultures of neurons and MSCs alone, of MT-MMP1 (also termed MMP14), a membrane-bound protein able to activate the metalloproteinase pathway by inducing MMP2 activation (Fillmore et al., 2001). Concomitantly, there was a statistically significant over-expression of the metalloproteinase inhibitor gene Timp-1 which is responsible for the activity inhibition of MMP9 (Rivera et al., 2002). The genes codifying for some anti-adhesive molecules, such as Sparc, Basigin and Adamst1, were also down-regulated in a significant manner.

To validate these findings at the protein level, we performed immunofluorescence studies which confirmed that the metalloproteinase inhibitor Timp-1 was present only in neurons co-cultured with MSCs while it was absent in neurons alone. The immunostaining for Timp-1 was present both in the nucleus and in the cytoplasm of neurons (Fig. 1a).

On the contrary, the anti-adhesive protein Sparc was present in the nucleus and in the cytoplasm of neurons alone but not in neurons co-cultured with MSCs (Fig. 1b).

The data obtained with microarrays concerning Basigin and Adamst1 failed to be validated at the protein level.

The Elisa assay performed to assess the release of Timp-1 in the different culture media evidenced a higher quantity of this protein in the medium of co-cultures with respect to the medium of neurons cultured alone, and the difference increased at later times (5–7 weeks) (Fig. 1c), thus confirming the Microarray results, while the Elisa assay performed to assess the quantity of Sparc released in the different culture media evidenced a very low amount of secreted-Sparc, close to the detectable threshold, in all the samples analyzed.

After 1 month of co-culture, MMP2 activity decreased in co-cultures with respect to neurons or MSCs alone, and the band corresponding to the active-MMP9, present in neurons at the beginning, disappeared in co-cultures (Fig. 2). MMP activity inhibition was not observed in indirect co-cultures of neurons and MSCs. In indirect co-cultures MSCs were unable to prolong the neuronal survival (Scuteri et al., 2006). We can, therefore, affirm that direct contact with MSCs induces the down-regulation of the MMP pathway and of Sparc in DRG neurons.

Effects of MSCs on neuronal apoptotic death

DRG neurons cultured alone underwent apoptotic death, as demonstrated by cytofluorimetric studies using Annexin V and by electron microscopy. The cytofluorimetric studies with Annexin V demonstrated that phosphatidylserine detection, a very early apoptotic sign, was strongly reduced in a significant manner in co-cultures with respect to neurons cultured alone (from more than 60% to 9%) even after only 15 days of culture (Fig. 3a). Electron microscopy evidenced the degeneration of the nucleus, that became denser than normal, and of the cytoplasm which was filled by electron-dense material (Fig. 3b). At later stages of degeneration there were a lot of electron-dense fragments which is consistent with an autophagic/apoptotic phenomenon (González-Polo et al., 2005). In the co-cultures, neurons had a normal appearance and MSCs formed multiple layers underneath (Fig. 3b).

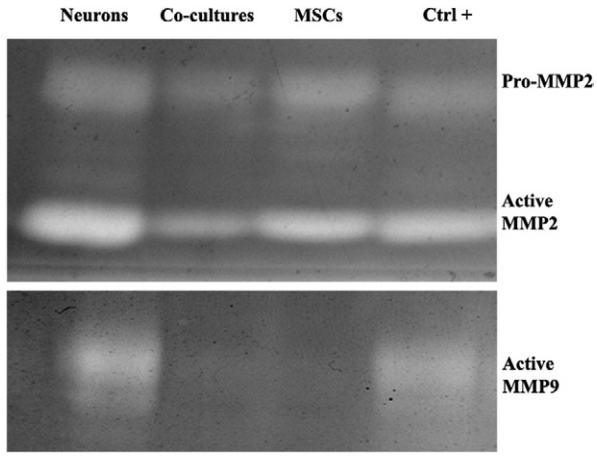

Fig. 2. Analysis of MMP activity by zymography. After 30 d of culture, both active-MMP2 and active-MMP9 forms decreased in co-cultures with respect to neurons and MSCs alone. Ctrl+represents the positive control of active MMP2 and active MMP9.

Moreover, the involvement of caspases, the executors of apoptotic death, was verified by immunofluorescence analysis of the active form of caspases 3 and 7. In the cultures of neurons alone, some nuclei were immunopositive for the active form of caspase 3, an executor of the apoptotic pathway, after 14 days of culture, while we did not observe the involvement of caspase 7. After 1 month of culture, the active form of caspase 7 was undetectable in all the cultures, while most of the neurons resulted immunopositive for caspase 3. Conversely, in the co-cultures of neurons and MSCs caspase 3 immunopositivity was absent (Fig. 3c).

Effect of metalloproteinase inhibition on neuronal survival

Having demonstrated that MSCs in contact with the DRG neurons inhibit the neuronal pathway of metalloproteinases directly, by acting on MT-MMP1, and indirectly, through the up-regulation of Timp-1 and the down-regulation of Sparc, we used MMPs' inhibition to study the action of these enzymes on neuronal survival. Since MT-MMP1 has a wide substrate specificity and given that the MMPs' pathway is crosslinked, it is plausible that many MMPs may be involved. For these reasons the NNGH metalloproteinase wide range inhibitor (Arendt et al., 2007) was used, at doses of 1.3 μM, 2 μM, and 3 μM. The lowest dose of NNGH (1.3 μM) was ineffective while NNGH 3 μM was toxic for neurons.

As shown in Fig. 4a, at the beginning of the experiment (T0), both untreated control neurons and neurons treated

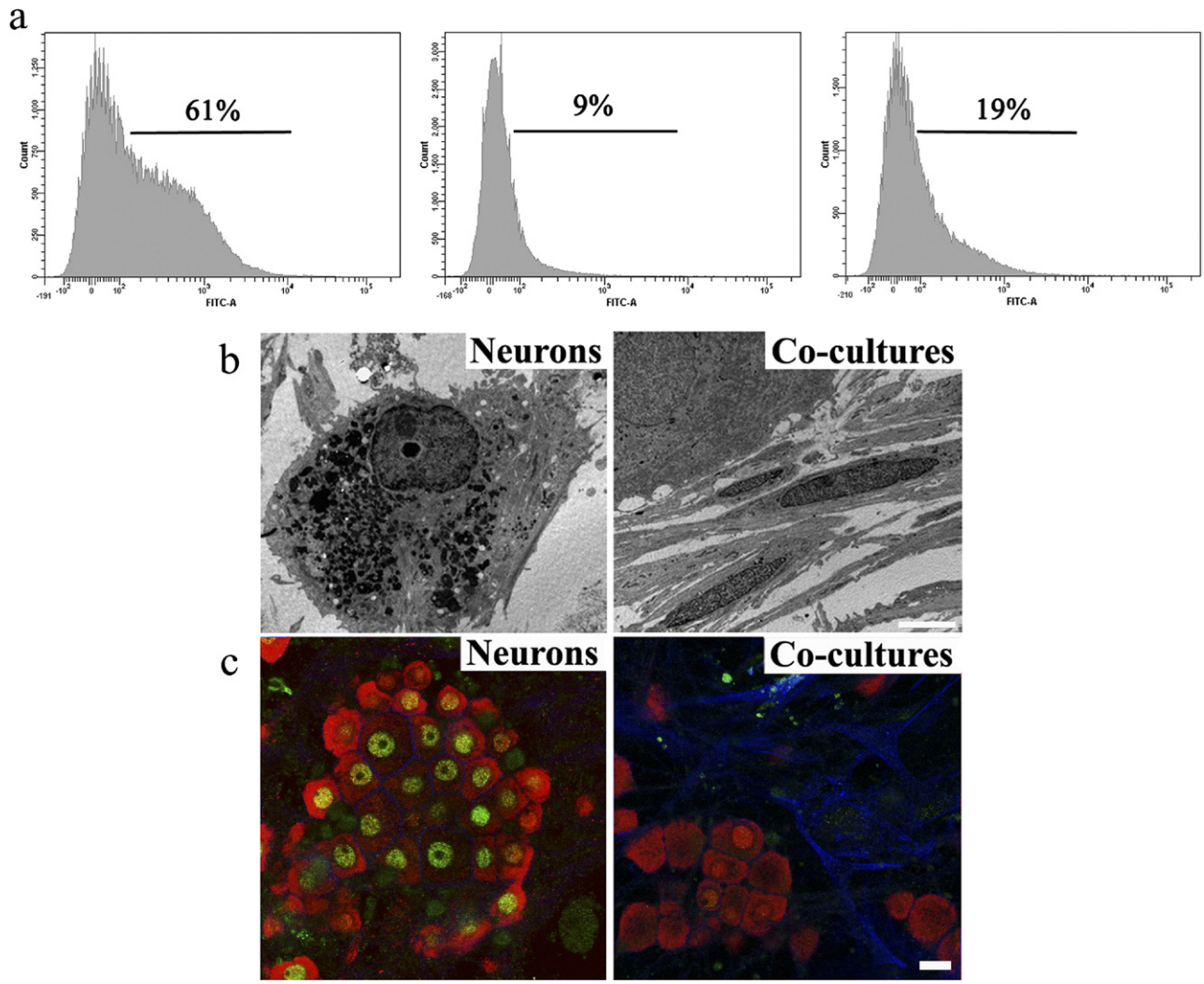

Fig. 3. Apoptosis detection. (a) Annexin V test: In neurons cultured alone for 14 d there are more cells positive for annexin V (61%) with respect to co-cultures (9%) and MSCs alone (19%) at the cytofluorimetric analysis. (b) Electron microscopy: After 14 d neurons showed nuclear degeneration and a cytoplasm with electron-dense material. In the co-cultures, both neurons (top left) and the underlying MSCs had a normal appearance. Scale bar: 5 μm. (c) Confocal microscopy: Active-caspase 3 was present in neurons alone but not in neurons co-cultured with MSCs after 14 d of culture. (Active-caspase 3 green, NeuN red, Phalloidin blue). Scale bar: 10 μm.

with NNGH were vital and had numerous processes. After 14 days of exposure to NNGH, untreated control cultures were suffering, with some signs of cellular degeneration, as demonstrated by the presence of cellular debris. On the contrary, NNGH-treated cultures maintained a healthy appearance. After 30 days, untreated control neurons showed evident signs of cellular degeneration while neurons treated with NNGH were only marginally affected.

The count of viable neurons demonstrated that the survival of neurons alone was greatly decreased after 14 days of culture, and it got worse until 30 days. On the contrary, neurons cultured with NNGH survived longer than neurons alone in a statistically significant manner (Fig. 4b). Finally, neurons co-cultured with MSCs did not show any signs of cellular degeneration and were able to survive up to 30 days of culture (Fig. 4b).

NNGH was also able to prevent the activation of caspase 3, since the percentage of neurons positive for active-caspase 3 decreased in NNGH-treated cultures (about 20%) with respect to cultures of neurons alone (more than 90%) (Fig. 5). Since the inhibition of caspase 3 activation occurred also in the co-culture of neurons with MSCs, this observation demonstrates that the MSC-dependent inhibition of MMPs is important for the protection of neurons from apoptosis.

DISCUSSION

In the present study, we have demonstrated that the positive role of MSCs on neuronal survival is due to the down-regulation of the MMP pathway. Microarray analysis shows that co-cultures present a down-regulation of some MMPs that are known to activate other MMPs involved in cellular apoptotic death. Furthermore, in co-cultures there is also a down-regulation of Sparc, which in turn activates MMPs. All these findings, together with the observed up-regulation

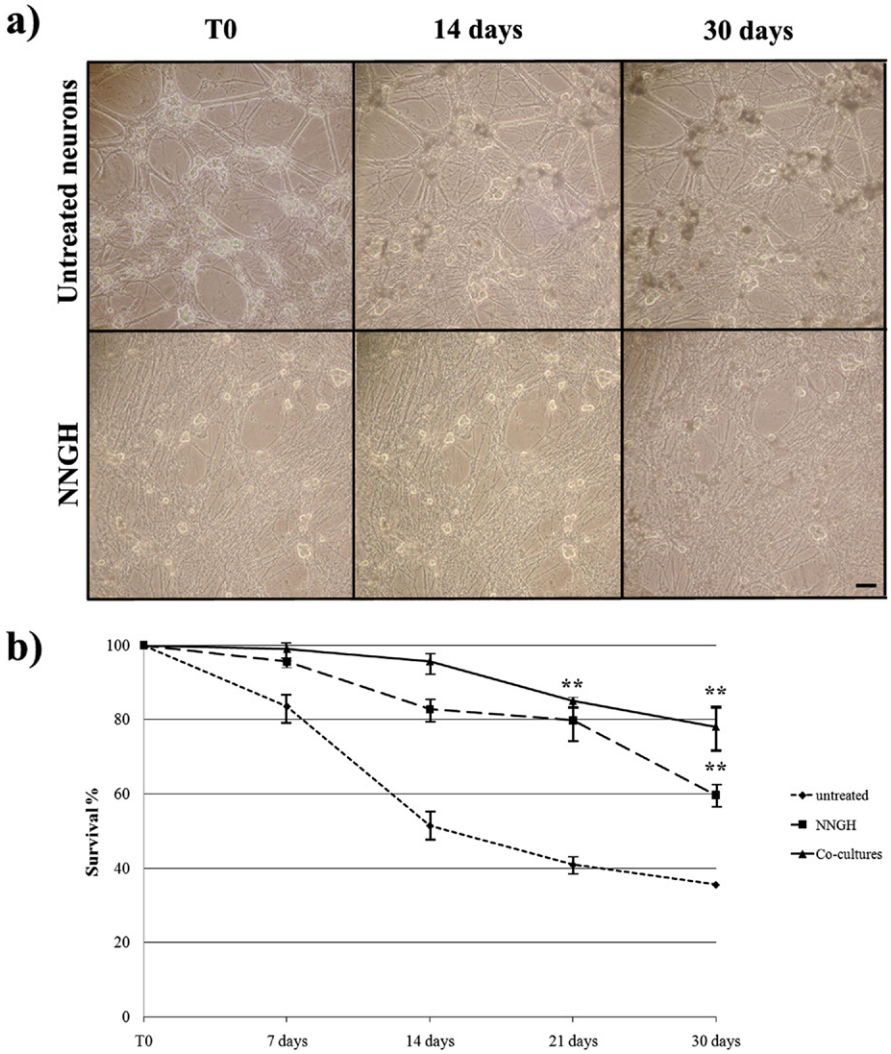

Fig. 4. Evaluation of NNGH effect on neuronal survival. (a) Untreated neurons showed degeneration signs after days 14 and 30 of culture. NNGH-treated neurons were still vital after 30 d of culture. Scale bar: 50 μm. (b) Cell viability was expressed as a percentage±SD. $P<0.001$.

of Timp-1 and its increased release in the culture medium, and the pro-survival effect of an inhibitor of MMPs, suggest that MMPs have a specific role in the survival of neurons co-cultured with undifferentiated MSCs.

The involvement of MMPs in neuronal death has already been demonstrated both *in vivo* (Romanic et al., 1998; Lee et al., 2004; Gu et al., 2005; Kiaei et al., 2007) and *in vitro* (Guedez et al., 1998) by using chemical inhibitors or knockout animals. These studies have evidenced that the lack or deficiency of some MMPs protects various neuronal populations from apoptosis. In the same way, the increase in MMPs' activity, obtained by recombinant MMP administration, was able to increase the cell death of various neurons exposed to toxic stimuli (Jourquin et al., 2003; Manabe et al., 2005; Muroski et al., 2008). In some of these models MMP9 and MMP2 are directly involved in neuronal apoptosis and neuronal damage (Gu et al., 2005; Dang et al., 2008) as also occurred in our study. In our model, the chemical inhibition of the MMP neuronal pathway was able to determine an increase in neuronal viability even if the pro-survival action was less effective than in the MSC co-culture, suggesting that the effect of MSCs does not occur only through MMP pathway inhibition. In fact, the MMP inhibitor does not block the activity of Sparc, a protein that acts not only on MMP pathways, but which is also able to directly activate the caspase 3-dependent apoptotic process (Tang and Tai, 2007).

A high level of MMP activity, which may depend on a down-regulation of the TIMP family, has been reported in many degenerative disorders of the CNS (Gu et al., 2005; Kiaei et al., 2007; Kim et al., 2007; Thirumangalakudi et al., 2007). In our model, where neuronal survival was increased by MSC co-culture, Timp-1 expression was up-regulated, thus protecting neurons from death. This observation is in accordance with a study in which the over-expression of Timp-1 in transgenic mice, as well as the treatment with recombinant Timp-1, was neuroprotective for cortical neurons (Tejima et al., 2009).

In our model the expression of the anti-adhesive protein Sparc was down-regulated in co-cultured neurons with

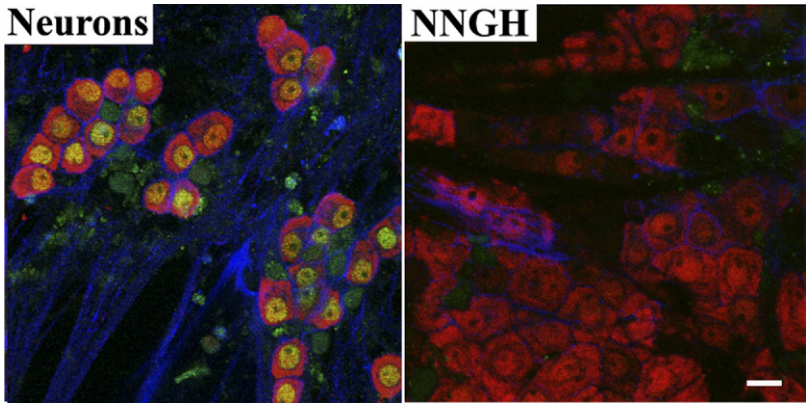

Fig. 5. Apoptosis detection by immunofluorescence. After 30 d of culture active-caspase 3 was present in the most part of neurons alone while in NNGH-treated cultures the number of active-caspase 3 positive neurons was greatly reduced. (Active-caspase 3 green, NeuN red, Phalloidin blue). Scale bar: 10 μm.

respect to neurons cultured alone, but the total amount of secreted Sparc was very low in all the culture media analyzed. However, besides being a secreted glycoprotein, Sparc also has many biological functions carried out inside the cells (Yan and Sage, 1999) ranging from cell cycle regulation, to apoptosis, to activation of MMPs' pathway (Yan and Sage, 1999; McClung et al., 2007). For this reason it is plausible that in our model Sparc acts as a metalloproteinases and/or caspases activator rather than by exerting its counteradhesive properties.

Previous studies have linked the over-expression of MMPs and Sparc to alterations in the interactions between cells and the extracellular matrix causing a particular kind of apoptotic death termed anoikis (Rivera et al., 2002; Lee et al., 2004; Valentijn et al., 2004; Tang and Tai, 2007; Yang et al., 2007), a model of apoptosis in which the "toxic stimulus" is represented by the loss of cellular adhesion to the ECM, or at least to an incorrect cell-ECM interaction (Valentijn et al., 2004). In anoikis, caspase 3, rather than caspase 7, is the executor of the apoptotic death (Feng et al., 2007). Similarly, in our paradigm caspase 3 was the executor of apoptosis in DRG neurons.

Neuronal apoptosis may be modulated by MMPs' pathway through the cell surface death receptor (Wetzel et al., 2004), and our hypothesis is that MSCs, by the direct cellular contact, inhibit some of the surface death receptors linked to MMPs' pathway thus preventing the neuronal apoptosis.

CONCLUSION

The capacity of MSCs to prevent apoptosis through the inactivation of the metalloproteinase pathway represents a very important feature of MSCs as an alternative to drug treatment of neurodegenerative diseases. A high level of MMP activity is indeed a feature of many different CNS disorders (Gu et al., 2005; Kiaei et al., 2007; Kim et al., 2007; Thirumangalakudi et al., 2007). From this point of view MSCs are, therefore, a promising tool for the treatment of different neurological pathologies.

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REFERENCES

Akiyama Y, Radtke C, Kocsis J (2002) Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. J Neurosci 15:6623–6630.

Arendt Y, Banci L, Bertini I, Cantini F, Cozzi R, Del Conte R, Gonnelli L (2007) Catalytic domain of MMP20 (Enamelysin)—the NMR structure of a new matrix metalloproteinase. FEBS Lett 581:4723–4726.

Bozzo C, Sabbatini M, Tiberio R, Piffanelli V, Santoro C, Cannas M (2006) Activation of caspase-8 triggers anoikis in human neuroblastoma cells. Neurosci Res 56:145–153.

Crigler L, Robey R-C, Asawachaicharn A, Gaupp D, Phinney D-G (2006) Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. Exp Neurol 198:54–64.

Cuevas P, Carceller F, Dujovny M, Garcia-Gomez I, Cuevas B, Gonzalez-Corrochano R, Diaz-Gonzales D, Reimers D (2004) Peripheral nerve regeneration by bone marrow stromal cells. Neurol Res 24:634–638.

Dang A-B, Tay B-K, Kim H-T, Nauth A, Alfonso-Jaume M-A, Lovett D-H (2008) Inhibition of MMP2/MMP9 after spinal cord trauma reduces apoptosis. Spine 33:576–579.

Deng J, Petersen B-E, Steindler D-A, Jorgensen M-L, Laywell E-D (2006) Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. Stem Cells 24:1054–1064.

Donzelli E, Salvadè A, Mimo P, Viganò M, Morrone M, Papagna R, Carini F, Zaopo A, Miloso M, Baldoni M, Tredici G (2007) Mesenchymal stem cells cultured on a collagen scaffold: *in vitro* osteogenic differentiation. Arch Oral Biol 52:64–73.

Feng J, Yang S, Xu L, Tian H, Sun L, Tang X (2007) Role of caspase-3 inhibitor in induced anoikis of mesenchymal stem cells *in vitro*. J Huazhong Univ Sci Technol Med Sci 27:183–185.

Fillmore H-L, VanMeter T-E, Broaddus W-C (2001) Membrane-type matrix matalloproteinases (MT-MMPs): expression and function during glioma invasion. J Neuroonc 53:187–202.

Frish S-M, Francis H (1994) Disruption of epitelial cell-matrix interaction induces apoptosis. J Cell Biol 124:619–626.

González-Polo R-A, Boya P, Pauleau A-L, Jalil A, Larochette N, Souquère S, Eskelinen E-L, Pierron G, Saftig P, Kroemer G (2005) The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. J Cell Sci 118:3091–3102.

Gu Z, Cui J, Brown S, Fridman R, Mobashery S, Strongin A-Y, Lipton S-A (2005) A highly specific inhibitor of matrix metalloproteinase- 9 rescues laminin from proteoysis and neurons from apoptosis in transient focal cerebral ischemia. J Neurosci 25:6401–6408.

Guedez L, Stetler-Stevenson W-G, Wolff L, Wang J, Fukushima P, Mansoor A, Stetler-Stevenson M (1998) *In vitro* suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. J Clin Invest 102:2002–2010.

Jourquin J, Tremblay E, Décanis N, Charton G, Hanessian S, Chollet A-M, Le Diguardher T, Khrestchatisky M, Rivera S (2003) Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate. Eur J Neurosci 18:1507–1517.

Kiaei M, Kipiani K, Calingasan N-Y, Wille E, Chen J, Heissig B, Rafii S, Lorenzl S, Beal M-F (2007) Matrix metalloproteinase-9 regulates TNF-alpha and FasL expression in neuronal, glial cells and its absence extends life in a transgenic mouse model of amyotrophic lateral sclerosis. Exp Neurol 205:74–81.

Kim Y-S, Choi D-H, Block M-L, Lorenzl S, Yang L, Kim Y-J, Sugama S, Cho B-P, Hwang O, Browne S-E, Kim S-Y, Hong J-S, Beal M-F, Joh T-H (2007) A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. FASEB J 21:179–187.

Lee S-R, Tsuji K, Lee S-R, Lo E-H (2004) Role of matrix metalloproteinases in delayed neuronal damage after transient global cerebral ischemia. J Neurosci 24:671–678.

Liu L, Cao J-X, Sun B, Li H-L, Xia Y, Wu Z, Tang C-L, Hu J (2010) Mesenchymal stem cells inhibition of chronic ethanol-induced oxidative damage via upregulation of phosphatidylinositol-3-kinase/Akt and modulation of extracellular signal-regulated kinase 1/2 activation in PC12 cells and neurons. Neuroscience 167:1115–1124.

Lu P, Blesch A, Tuszynski M-H (2004) Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J Neurosci Res 77:174–191.

Manabe S, Gu Z, Lipton S-A (2005) Activation of matrix metalloproteinase-9 via neuronal nitric oxide synthase contributes to NMDA-induced retinal ganglion cell death. Invest Ophthalmol Vis Sci 46:4747–4753.

McClung H-M, Thomas S-L, Osenkowski P, Toth M, Menon P, Raz A, Fridman R, Rempel S-A (2007) SPARC upregulates MT1MMP expression, MMP2 activation, and the secretion and cleavage of galectin 3 in U87MG glioma cells. Neurosci Lett 419:172–177.

Muroski M-E, Roycik M-D, Newcomer R-G, Van den Steen P-E, Opdenakker G, Monroe H-R, Sahab Z-J, Sang Q-X (2008) Matrix metalloproteinase-9/gelatinase B is a putative therapeutic target of chronic obstructive pulmonary disease and multiple sclerosis. Curr Pharm Biotechnol 9:34–46.

Pagenstecher A, Stalder A-K, Kincaid C-L, Shapiro S-D, Campbell I-L (1998) Differential expression of matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase genes in the mouse central nervous system in normal and inflammatory states. Am J Pathol 152:729–741.

Phinney D-G, Prockop D-J (2007) Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair-current views. Stem Cells 25:2896–2902.

Rivera S, Ogier C, Jourquin J, Timsit S, Szklarczyk A, Miller K, Gearing A- J-H, Kaczmarek L, Khrestchatiski M (2002) Gelatinase B and TIMP-1 are regulated in a cell- and time dependent manner in association with neuronal death and glial reactivity after global forebrain ischemia. Eur J Neurosci 15:19–32.

Romanic A-M, White R-F, Arleth A-J, Ohlstein E-H, Barone F-C (1998) Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. Stroke 29:1020–1030.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman T-B, Saporta S, Janssen W, Patel N, Cooper D-R, Sanberg P-R (2000) Adult bone marrow stromal cells differentiate into neuronal cells *in vitro*. Exp Neurol 164:247–256.

Scuteri A, Cassetti A, Tredici G (2006) Adult mesenchymal stem cells rescue dorsal root ganglia neurons from dying. Brain Res 1116: 75–81.

Scuteri A, Galimberti A, Maggioni D, Ravasi M, Pasini S, Nicolini G, Bossi M, Miloso M, Cavaletti G, Tredici G (2009) Role of MAPKs in platinum-induced neuronal apoptosis. Neurotoxicology 30:312–319.

Tang M-J, Tai I-T (2007) A novel interaction between procaspase 8 and sparc enhances apoptosis and potentiates chemoterapy sensitivity in colonrectal cancers. JBC 282:34457–34467.

Tejima E, Guo S, Murata Y, Arai K, Lok J, Van Leyen K, Rosell A, Wang X, Lo E-H (2009) Neuroprotective effects of over-expressing tissue inhibitor of metalloproteinase TIMP-1. J Neurotrauma 26:1935–1941.

Thirumangalakudi L, Yin L, Rao H-V, Grammas P (2007) IL-8 induces expression of matrix metalloproteinases, cell cycle and pro-apoptotic proteins, and cell death in cultured neurons. J Alzheimers Dis 11:305–311.

Valentijn A-J, Zouq N, Gilmore A-P (2004) Anoikis. Biochem Soc Transac 32:421–424.

Wetzel M, Tibbitts J, Rosenberg G-A, Cunningham L-A (2004) Vulnerability of mouse cortical neurons to doxorubicin-induced apoptosis is strain-dependent and is correlated with mRNAs encoding Fas, Fas-Ligand, and metalloproteinases. Apoptosis 9:649–656.

Xue M, Fan Y, Liu S, Zygun D-A, Demchuk A, Yong V-W (2009) Contributions of multiple proteases to neurotoxicity in a mouse model of intracerebral haemorrhage. Brain 132:26–36.

Yamamoto H, Noura S, Okami J, Uemura M, Takemasa I, Ikeda M, Ishii H, Sekimoto M, Matsuura N, Monden M, Mori M (2008) Overexpression of MT1-MMP is insufficient to increase experimental liver metastasis of human colon cancer cells. Int J Mol Med 22:757–761.

Yan Q, Sage E-H (1999) SPARC, a matricellular glycoprotein with important biological functions. J Histochem Cytochem 47:1495–1505.

Yang R, Liu H, Williams I, Chaqour B (2007) Matrix metalloproteinase-2 expression and apoptogenic activity in retinal pericytes: implications in diabetic retinopathy. Ann N Y Acad Sci 1103:196–201.

Yoshida T, Hakuba N, Morizane I, Fujita K, Cao F, Zhu P, Uchida N, Kameda K, Sakanaka M, Gyo K, Hata R (2007) Hematopoietic stem cells prevent hair cell death after transient cochlear ischemia through paracrine effects. Neuroscience 145:923–930.

Zhao L-R, Duan W-M, Reyes M, Keene C-D, Verfaillie C-M, Low W-C (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174:11–20.

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