Proteomics
www.proteomics-journal.com

A Proteomics Approach to Identify Candidate Proteins Secreted by Müller Glia that Protect Ganglion Cells in the Retina

*Noelia Ruzafa, Xandra Pereiro, Marlen F. Lepper, Stefanie M. Hauck, and Elena Vecino**

The retinal Müller glial cells, can enhance the survival and activity of neurons, especially of retinal ganglion cells (RGCs), which are the neurons affected in diseases such as glaucoma, diabetes, and retinal ischemia. It has been demonstrated that Müller glia release neurotrophic factors that support RGC survival, yet many of these factors remain to be elucidated. To define these neurotrophic factors, a quantitative proteomic approach was adopted aiming at identifying neuroprotective proteins. First, the conditioned medium from porcine Müller cells cultured in vitro under three different conditions were isolated and these conditioned media were tested for their capacity to promote survival of primary adult RGCs in culture. Mass spectrometry was used to identify and quantify proteins in the conditioned medium, and osteopontin (SPP1), clusterin (CLU), and basigin (BSG) were selected as candidate neuroprotective factors. SPP1 and BSG significantly enhance RGC survival in vitro, indicating that the survival-promoting activity of the Müller cell secretome is multifactorial, and that SPP1 and BSG contribute to this activity. Thus, the quantitative proteomics strategy identify proteins secreted by Müller glia that are potentially novel neuroprotectants, and it may also serve to identify other bioactive proteins or molecular markers.

1. Introduction

The vascularized mammalian retina contains three types of glial cells: microglia, astrocytes, and Müller cells (for a review see ref. 1). Müller cells are the main glial cells in the retina and they provide structural stability to the retina, constituting an anatomical link between all retinal neurons. In particular, Müller cell extensions surround the cell bodies and dendrites of retinal ganglion cells (RGCs).[2] Among the many functions of Müller cells, they are involved in maintaining extracellular homeostasis,[3] in the metabolism of glucose,[4] neurotransmitter recycling,[5] and in organizing the developing retina,[6] Müller cells adopt a gliotic phenotype in response to damage or disease[6] and, in addition, they provide neurotrophic factors that can directly or indirectly affect the survival of neurons in the retina.[1,6–9]

Müller cells can protect against the excitotoxic effects of glutamate, and they can enhance the survival and neuritogenesis of RGCs in culture under normal conditions, as well as during starvation.[9–12] This support is preferentially activated by anatomical interactions, although it has been demonstrated that Müller cell conditioned medium significantly enhances the survival of cultured adult porcine RGCs.[9,12] Indeed, the neuroprotection afforded by Müller cell condition media exceeds that observed in the retina itself.[13] Müller cell-derived trophic factors may regulate different aspects of neuronal circuits, such as synaptogenesis, differentiation, and the protection of neurons in the retina,[14] and previous studies have characterized the Müller cell secretome by combining high-resolution proteomics tools and in vitro models of Müller cells.[10,20] However, it must be noted that the proteome of Müller cells rapidly changes in culture due to their adaptation to the milieu.[7] Since the neurotrophic activity contained in the Müller cell secretome is multifactorial,[15] identifying molecules from this source that promote the survival of RGCs could be particularly beneficial to treat degenerative retinal conditions in which RGC death provokes irreversible blindness.

In addition to well-known growth factors, several factors have been identified in the retina as potential neuroprotective molecules that significantly augment photoreceptor survival,[15] including insulin-like growth factor-binding proteins (IGFBP5) and connective tissue growth factor (CTGF). Osteopontin (SPP1) was also identified and its neurotrophic activity was demonstrated on primary porcine photoreceptors, as well as on Pde6brd1 mouse mutant retinal explants.[16] Moreover, the C-X-C motif chemokine 10 (CXCL10) was also proposed to have neuroprotective properties[8] and it was validated using models of photoreceptor degeneration,[8] along with established neurotrophic factors like leukemia inhibitory factor (LIF)[17] and the iron-stress protective receptor transferrin.[18]

Dr. N. Ruzafa, Dr. X. Pereiro, Dr. E. Vecino
Department of Cell Biology and Histology
University of Basque Country UPV/EHU
Leioa, 48940, Vizcaya, Spain
E-mail: elena.vecino@ehu.es
Dr. M. F. Lepper, Dr. S. M. Hauck
Research Unit Protein Science
Helmholtz Zentrum München
German Research Center for Environmental Health GmbH
Neuherberg, D-80939, Germany

DOI: 10.1002/pmic.201700321

Significance Statement

A comprehensive study of the Müller cell secretome and its effect on the retinal ganglion cells has yet to be performed. Thus, we adopted a quantitative proteomic approach to analyze the secretome of primary cultures of adult Müller cells under three different conditions to help identify and select candidate neurotrophic proteins. The proteomic strategy used in this study allowed identifying proteins secreted by Müller glia, such as osteopontin or basigin, which are potentially novel neuroprotectants for retinal ganglion cells and can be candidate molecules to develop treatments in neurodegenerative disease. In addition, the functional assay-driven proteomics screening approach adopted can be also applied to the identification of other bioactive proteins or molecular markers as it allows single molecules derived from complex protein mixtures to be characterized.

As most studies have tested the effect of neurotrophic factors on photoreceptors, the objective of our study was to identify candidate proteins that enhance the survival of RGCs. While some Müller cell-derived neurotrophic factors have been identified, a comprehensive study of the Müller cell secretome has yet to be performed. Thus, we adopted a quantitative proteomic approach to analyze the Müller cell secretome, maintaining primary cultures of adult Müller cells under three different conditions to help identify and select candidate neurotrophic proteins.

2. Experimental Section

2.1. Animals

All animal experimentation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For Müller cell cultures, adult porcine eyes were obtained from a local slaughterhouse and transported to the laboratory in cold CO_2-independent medium (Life Technologies, Carlsbad, CA, USA) containing 0.1% gentamicin (Life Technologies, Carlsbad, CA, USA).

Eyes for RGC cultures were obtained from adult female Sprague Dawley rats (200–250 g). Animals were housed under a 12 hour light-dark cycle with ad libitum access to food and water, and they were sacrificed humanely by exposure to CO_2.

2.2. Müller Cell Culture

Adult porcine eyes were dissected within 1 to 2 h of enucleation and retinal Müller cell cultures were prepared according to a previously reported protocol[9] with the following minor modifications. Briefly, the major blood vessels were removed and the retina was washed in CO_2-independent medium. The retinas were dissected out and cut using an 8 mm diameter dissecting trephine (Biomedical Research Instruments, Silver Spring, MD, USA). The retinal tissue was dissociated for 30 min at 37 °C in 0.2% activated papain (Worthington, Lakewood, NJ, USA) with 10% DNAse I (Worthington, Lakewood, NJ, USA). Papain activity was stopped by the addition of further Müller medium and DNase I, and the tissue was disaggregated by gentle trituration using pipette tips of decreasing diameter.

Three types of Müller media were used: 1) DMEM (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS: Life Technologies, Carlsbad, CA, USA); 2) DMEM with 20% FBS; 3) Neurobasal A medium (NBA: Life Technologies, Carlsbad, CA, USA) with 10% FBS and supplemented with 2% B27 (Life Technologies, Carlsbad, CA, USA). In addition, 1% L-glutamine (2 mM: Life Technologies, Carlsbad, CA, USA) and 0.1% gentamicin (50 mg mL^{-1}: Life Technologies, Carlsbad, CA, USA) were added to all the media.

Dissociated cells were collected by centrifugation (1200 rpm, 5 min), resuspended in Müller medium and plated on poly-L-lysine (100 μg mL^{-1}: Sigma-Aldrich, St. Louis, MO, USA) and laminin (10 μg mL^{-1}: Sigma-Aldrich, St. Louis, MO, USA) coated 13 mm glass coverslips in 24-well plates. The cells were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2, and after 24 h, the unattached cells were removed by changing the entire medium. To maintain the cells half of the medium was replaced every 3 days.

The conditioned medium was collected when the cultures had reached confluency (day 7), first washing the wells three times with NBA medium (NBA plus 1% L-glutamine and 0.1% gentamicin). Subsequently, NBA medium was added to each well and they were left for 3 h before the medium was changed to eliminate the rest of the FBS and B27. Fresh NBA medium was added and left for 2 days before it was collected and sterilized by passing through a 0.22 μm filter. The conditioned medium was frozen in aliquots at −20 °C. Finally, the Müller cells were fixed for 10 min with methanol at −20 °C. At least three replicates of each culture were made, and the procedure was performed in triplicate.

2.3. RGC Cultures

Retinal ganglion cell cultures were prepared as described previously.[19] Briefly, retinas were dissected and to obtain a mixed suspension of retinal cells, they were dissociated enzymatically using the Papain Dissociation Kit (Worthington Biochemical Lakewood, NJ, USA) according to the manufacturer's instructions. Briefly, the retinal tissue was digested for 90 min at 37 °C in 0.2% activated papain with 10% DNAse I and the tissue was disaggregated by gentle trituration using pipette tips of decreasing diameter. After purification, the dissociated retinal cells (250,000 cells per mL) were plated on 13 mm poly-L-lysine (100 μg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) and laminin (10 μg mL^{-1}: Sigma-Aldrich, St. Louis, MO, USA) coated glass coverslips in 24-well plates to test the activity of conditioned medium, or coated well in 96-well plates to test the activity of candidate proteins. The cultures were maintained in Neurobasal A medium (Life Technologies, Carlsbad, AC, USA) supplemented with 2% B27, and with 1% L-glutamine (2 mM: Life Technologies, Carlsbad, CA, USA) and 0.1% gentamicin (50 mg mL^{-1}: Life Technologies, Carlsbad, CA, USA). The RGCs were cultured for 6 days at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed every 3 days. The RGCs were fixed for 10 min with methanol −20 °C on day 6.

To test the activity of the conditioned medium and that of the candidate proteins, the RGCs were cultured in NBA/B27 medium:NBA medium (1:1, control); NBA/B27 medium:conditioned medium collected in NBA medium (1:1); or NBA/B27 medium:NBA medium plus protein (1:1). The proteins used were PDGF-CC as a positive control (50 ng mL^{-1} (3.73 nM): Peprotech, London, UK); osteopontin (SPP1, 200 ng mL^{-1} (6.08 nM): Life Technologies, Carlsbad, AC, USA); clusterin (CLU, 125 ng mL^{-1} (1.58 nM): R&D System, Minneapolis, MN, USA), basigin (BSG, 1000 ng mL^{-1} (21.36 nM): Life Technologies, Carlsbad, AC, USA). All the media contained 1% L-glutamine and 0.1% gentamicin. At least three replicates were performed for the analysis of the conditioned medium analysis and six replicates when analyzing the candidate proteins, repeating each independent experiment three times.

2.4. Immunochemistry

After fixing in methanol and washing with PBS (phosphate buffered saline, pH 7.0), the cells were immunostained as described previously.[11] After blocking the binding of non-specific antigens with blocking buffer (3% BSA and 0.1% Triton X-100 in PBS), the cells were incubated with antibodies against vimentin as a specific marker of Müller cells (mouse monoclonal antibody diluted 1:10,000: Dako, Glostrup, Denmark) and bIII-tubulin as a specific RGC marker (rabbit polyclonal antibody diluted 1:2000: Promega, Madison, WI, USA). After washing again, these antibodies were detected with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 goat secondary antibodies (Life Technologies, Carlsbad, CA, USA), both at a dilution of 1:1000, and the cells were counterstained with the nuclear marker DAPI (Life Technologies, Carlsbad, CA, USA), diluted 1:10 000.

2.5. Quantification of RGCs and Statistical Analysis

RGCs were analyzed on an epifluorescence microscope (Zeiss, Jena, Germany) coupled to a digital camera (Zeiss Axiocam MRM, Zeiss, Jena, Germany). At least three (for activity test of conditioned medium) or six (for activity test of candidate proteins) coverslips were analyzed for each experimental condition and from three independent experiments. The RGC density was quantified and the cells were classified as: 1) cells with no neurites; 2) cells with a longest neurite <50 μm; 3) cells with the longest neurite between 50 and 200 μm; and 4) cells with neurites longer than 200 μm. The total number of RGCs surviving in each condition was recorded.

The cell density was described as the mean (cells per mm^2) and standard error of mean, and this parameter was compared between the different conditioned media. The data were also normalized to the control to simplify the representation. Statistical analyses were carried out using IBM SPSS Statistics software v. 21.0 and the homogeneity of the variances was assayed with Levene´s test ($p < 0.05$). A Student's *t*-test and a Mann–Whitney *U* test were used to assess whether there were significant differences between the groups. The minimum value of significance for both tests was defined as $p < 0.05$.

2.6. Mass Spectrometry

LC-MS/MS analysis of the conditioned medium was performed on a QExactive HF mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Approximately, 1 μg of sample was automatically loaded onto a nano-trap column (300 μm inner diameter × 5 mm, packed with Acclaim PepMap100 C18, 5 μm, 100 Å: LC Packings, Sunnyvale, CA) and separated by reversed phase chromatography (PepMap, 25 cm, 75 μm ID, 2 μm per 100 Å pore size: LC Packings) on a nano-RSLC apparatus (Ultimate 3000, Dionex, Sunnyvale, CA). After 5 min, peptides were eluted from the trap column and separated using a gradient of increasing ACN concentrations in 0.1% formic acid over a period of 125 min: 90 min from 5 to 26%, followed by 5 min from 26 to 41% ACN, followed again by 5 min from 41 to 85% ACN. After 5 min at a fixed concentration of 85% ACN, the gradient was set back to 3% ACN over a period of 2 min and allowed to equilibrate for 18 min.

A high-resolution (60 000 full-width half maximum) MS spectrum was acquired in the Orbitrap with a mass range from 300 to 1500 *m/z*. The ten most abundant peptide ions were selected for fragmentation if they exceeded an intensity of at least 1xe4 counts and if they were at least doubly charged. MS/MS spectra were also recorded in the Orbitrap at a resolution of 15 000 with a maximum injection time of 50 ms. Dynamic exclusion was set to 30 s.

2.7. Label-free Analyses, Database Search, and Protein Identification

The acquired spectra of the different samples were loaded and analyzed using Progenesis QI software for label-free proteomics quantification (Version 2.0, Nonlinear Dynamics, Waters, Newcastle upon Tyne, UK), as described previously.[20] Briefly, the profile data of the MS scans were transformed into peak lists with respective *m/z* values, intensities, abundances, and *m/z* width. The MS/MS spectra were transformed similarly and then stored in peak lists comprising *m/z* and abundance. Using one sample as reference, the retention times of the other samples were aligned automatically to give a maximal overlay of the 2D features. After alignment and feature exclusion, the samples were allocated to their respective experimental groups and the raw abundances of all the features were normalized.

The MS/MS spectra were exported from the Progenesis QI software as Mascot generic files (mgf) and used for peptide identification with Mascot (version 2.5). Mascot was set up to search the Ensembl Pig protein database (Release 75—Sscrofa10.2, 11 660 809 residues; 25 859 sequences; http//www.ensembl.org), setting trypsin as the enzyme for digestion with one missed cleavage allowed, a fragment ion mass tolerance limited to 0.02 Da and a parent ion tolerance limited to 10 ppm. Carbamidomethylation of cysteines was a stable modification, as was methionine oxidation, and deamidation of asparagine or glutamine was specified as variable modifications for Mascot searches. A Mascot-integrated decoy database search calculated an average false discovery rate <1% when searches were performed with a Mascot percolator cut-off score of 13 and with an appropriate significance threshold (*p*).

Identifications were re-imported into Progenesis QI. For quantification, all unique peptides of an identified protein were included and the total cumulative normalized abundance was calculated by summing the abundances of all the unique peptides allocated to the respective protein. Statistical analysis with a Student's *t*-test was performed using normalized abundances and values of $p < 0.05$ were considered as significant for all further results. Two technical replicates were measured for each type of sample to verify the experimental reproducibility.

2.8. Heat Maps

A heat map of all quantified proteins was generated using the heat map function in the Excel (Microsoft) XLStat add-in included in the OMICS module (Addinsoft). Normalized protein abundances were used for the independent clustering of samples and proteins, applying hierarchical clustering based on Euclidian distances. The matrices are colored in green for highly abundant proteins and in red for low abundant proteins.

2.9. GProX Cluster Analysis

For visualization of dynamic protein expression throughout the different experimental conditions (10% FBS, 20% FBS, or NBA/27 10% FBS), proteins with $p < 0.05$ in at least one group comparison were clustered with GProX software (Version 1.1.16)[21] using mean normalized abundances per protein expressed as proportion of 100 for each condition. GProX settings for upper and lower regulation threshold were 2 and 0.5, respectively. Clustering was performed in standardized mode employing a minimum plot membership of 0.5.

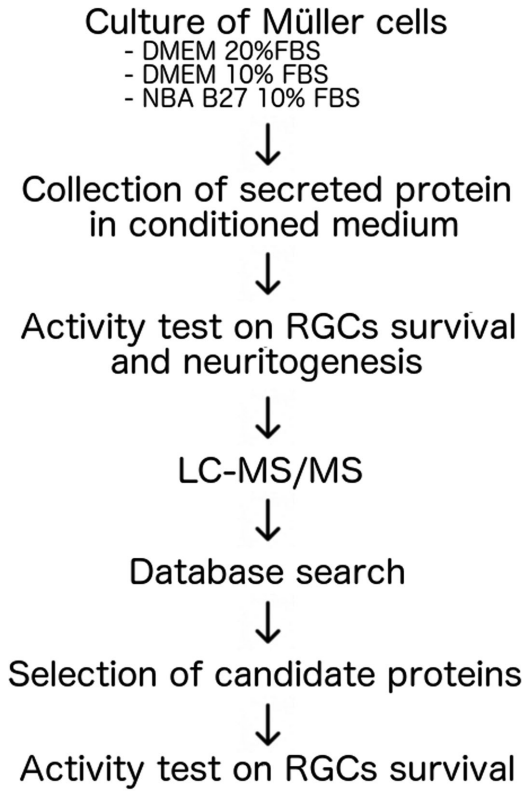

Figure 1. Proteomics work flow to identify proteins secreted by Müller cells that promote RGC survival. We start by culturing retinal Müller cells in different media and collecting the secretome of these cells. The effect of the conditioned medium on the survival and neuritogenesis of RGCs was tested in RGC cultures. The proteins in these conditioned media were analyzed by mass spectrometry (LC-MS/MS), allowing several candidate proteins to be identified. The proteins selected were analyzed thoroughly and their pro-survival activity tested in RGCs cultures.

3. Results

3.1. Proteomic Strategy to Identify Proteins Secreted by Müller Cells that Promote RGC Survival

In order to identify factors secreted by Müller cells that promote the survival of RGCs, a strategy was developed that integrates biological activity within a proteomics workflow (**Figure 1**). We started with the secretome of Müller cells cultured in three different media, each of which has a distinct effect on the survival and neuritogenesis of RGCs. As such, we established a possible means to select candidate neuroprotective proteins from the many proteins identified by mass spectrometry.

3.2. Effect of Müller Cell–Conditioned Medium on RGC Survival and Neuritogenesis In Vitro

Müller cells were cultured in three types of medium (DMEM + 10% FBS, DMEM + 20% FBS, and NBA/B27 + 10% FBS) and after collecting the conditioned medium from these cultures, the cells were labeled with an antibody against vimentin (**Figure 2**A–C). As a result, the Müller cells grown in DMEM were clearly more elongated than when maintained in NBA/B27. These differences were even more pronounced when the cells were maintained in DMEM + 20% FBS.

The RGCs were cultured in NBA/B27 medium:NBA medium (1:1, control) or in NBA/B27 medium:conditioned medium (1:1) from each of the three conditions of Müller cell culture. There were more RGCs and with longer neurites when the cells were maintained in the presence of medium conditioned by Müller cells grown in DMEM, whereas these parameters were similar or smaller to the control RGC cultures when RGCs were maintained in the presence of medium conditioned by Müller cells grown in NBA/B27 (Figure 2D–G).

Exposing the cultured RGCs to the conditioned media altered the number of the cells counted, increasing the total number of RGCs 3.29-fold when cultured with conditioned medium from Müller cells grown in DMEM + 20% FBS ($p < 0.01$) and 2.1-fold when cultured with medium conditioned by Müller cells grown in DMEM + 10% FBS ($p < 0.05$, Figure 2H). Conversely, the number of RGCs decreased by 69.4% when they were cultured with medium conditioned by Müller cells grown in NBA/B27 + 10% FBS ($p < 0.001$). Similarly, in the presence of medium conditioned by Müller cells grown in DMEM + 20% FBS, the number

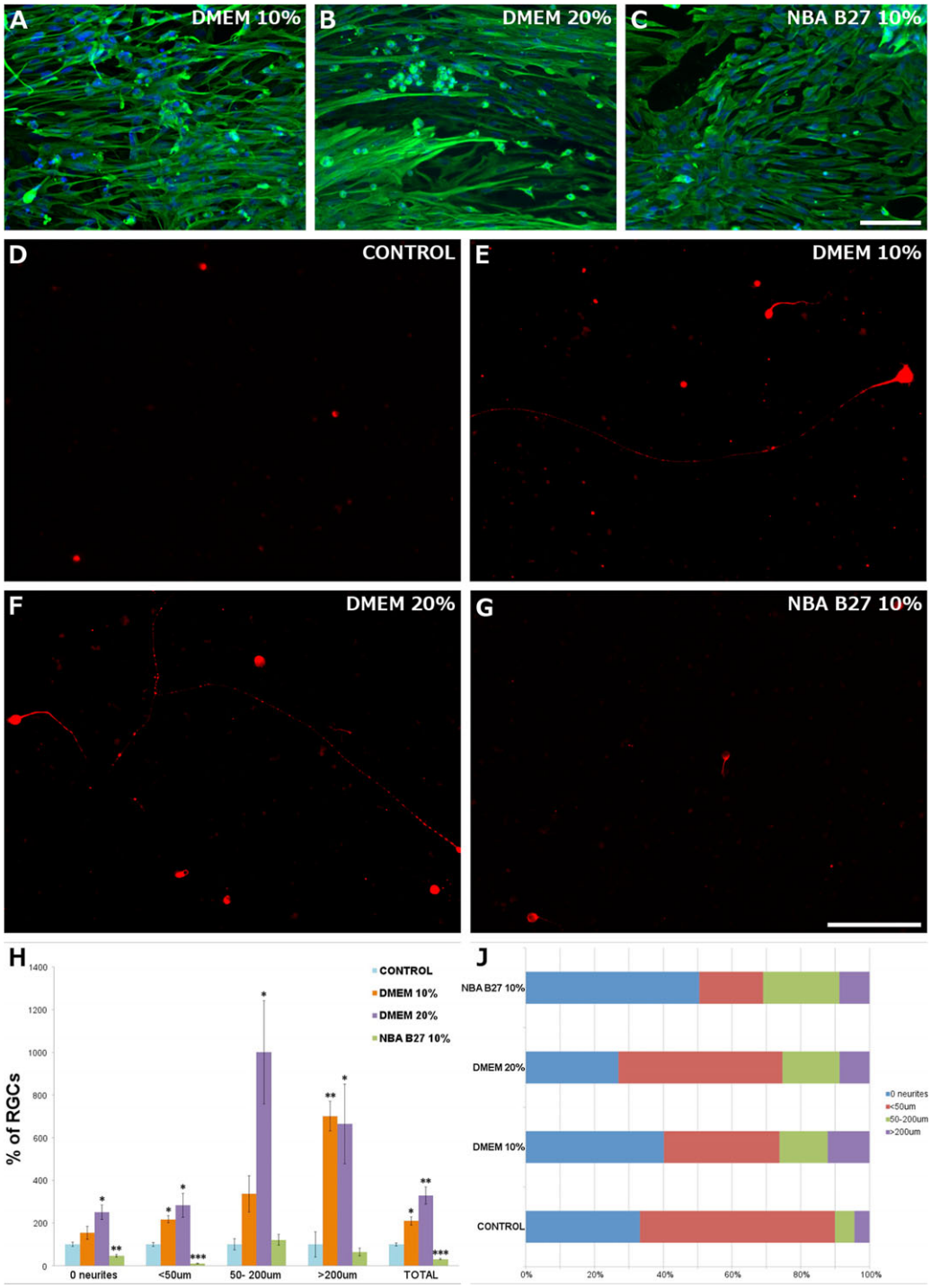

Figure 2. Effect of Müller cell conditioned medium on the survival and neuritogenesis of RGCs in vitro. Images of Müller cells cultured in A) DMEM + 10% FBS, B) DMEM + 20% FBS and C) NBA/B27 + 10% FBS. The cells were labeled with antibodies against vimentin (green) and the nuclei are marked with DAPI (blue). Images of RGCs in D) control conditions or E) cultured in conditioned media from Müller cells cultured in DMEM + 10% FBS, F) DMEM + 20% FBS, and G) NBA/B27 + 10% FBS. The cells were labeled with antibodies against β-III-tubulin (red). H) RGC survival in control conditions (blue) or in the presence of media conditioned by Müller cells cultured in DMEM + 10% FBS (orange), DMEM + 20% FBS (purple), and NBA/B27 + 10% FBS (green). To analyze neuritogenesis, the RGCs were classified as RGCs without neurites, with the longest neurite <50 μm, with the longest neurite between 50 and 200 μm, and with neurites longer than 200 μm. To analyze survival, the total number of RGCs was assessed and all the data were normalized considering the control as 100% for each condition. J) Percentage of RGCs without neurites (blue), with a longest neurite <50 μm (red), with the longest neurite between 50 and 200 μm (green), and with neurites longer than 200 μm (purple), for control cells and RGCs maintained in the presence of the three types of conditioned medium. Scale bar = 100 μm; *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.

of RGCs with no neurites increased 2.5-fold ($p < 0.05$) and it decreased by 53.7% in the presence of conditioned medium from Müller cells grown in NBA/B27 + 10% FBS ($p < 0.01$, Figure 2I). Similar effects were observed on the RGCs with neurites smaller than 50 μm, a 2.8-fold increase with medium conditioned by Müller cells grown in DMEM + 20% FBS ($p < 0.05$); a 2.2-fold increase in the presence of medium conditioned by Müller cells grown in DMEM + 10% FBS ($p < 0.05$); and a 90.1% decrease in conditioned medium from Müller cells grown in NBA/B27 + 10% FBS ($p < 0.001$). Moreover, the RGCs with a length of neurites between 50 and 200 μm increased 10-fold with medium conditioned by Müller cells grown in DMEM + 20% FBS ($p < 0.05$). Finally, the RGCs with neurites greater than 200 μm long increased 6.6-fold when RGCs were cultured with medium conditioned by Müller cells grown in DMEM + 20% FBS ($p < 0.05$) and 7-fold when the RGCs are cultured with medium conditioned by Müller cells grown in DMEM + 10% FBS ($p < 0.05$).

The proportion of each type of RGC was calculated for each culture condition to determine whether the conditioned medium affects RGC neurite length (Figure 2J). When RGCs were maintained in medium conditioned by Müller cells grown in DMEM + 10% FBS, the proportion of RGCs with the longest neurite greater than 200 μm increased from 4.4% (control) to 12.2% ($p < 0.001$). Similarly, the conditioned medium from Müller cells grown in DMEM + 20% FBS increase the proportion of RGCs with the longest neurite between 50 and 200 μm from 5.6% (control) to 16.6% ($p < 0.01$). By contrast, in the presence of the medium conditioned by Müller cells grown in NBA/B27 + 10% FBS, the percentage of RGCs with the longest neurite smaller than 50 μm decreased from 56.8% (control) to 18.6% ($p < 0.001$), in conjunction with an increase in the percentage of RGCs with the longest neurite between 50 and 200 μm from 5.6% (control) to 22.1% ($p < 0.01$). No significant differences were found in the proportion of RGCs without neurites.

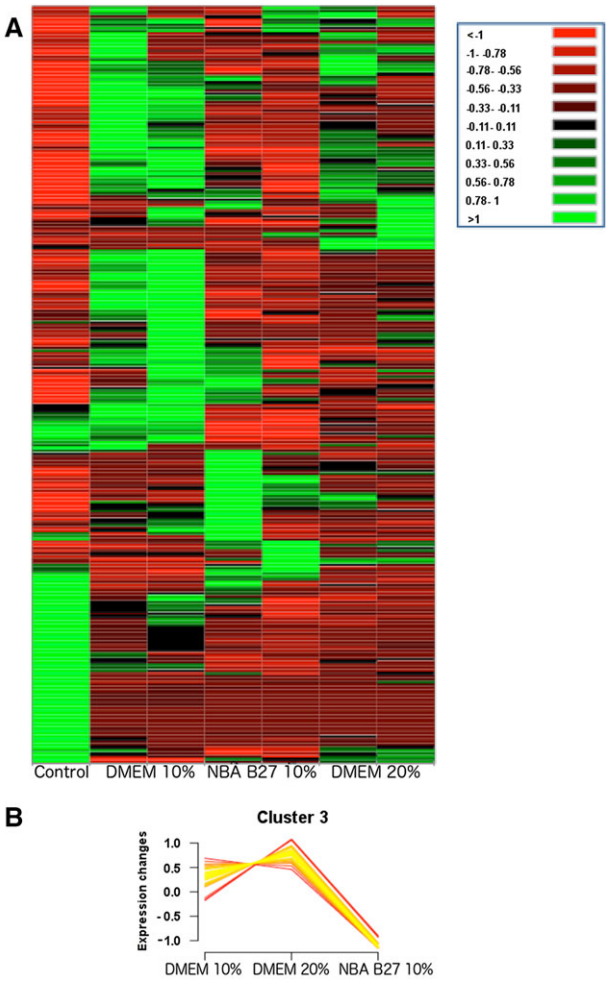

Figure 3. Protein identification, database search, and selection of candidate proteins. A) Heat maps of all the proteins identified in the three different samples of medium conditioned by Müller cells cultured in DMEM + 10% FBS, DMEM + 20% FBS, and NBA/B27 + 10% FBS. Two technical replicates were assessed for each sample type (two columns for each condition) to verify the experimental reproducibility and a column with NBA medium is shown as a negative control. The matrices are colored in green for highly abundant proteins and red for weakly abundant proteins. B) Clusters of the normalized protein abundance of the samples and proteins, applying hierarchical clustering based on Euclidian distances. Based on the Gprox analysis, all the proteins that were significantly altered ($p < 0.05$) in one of the comparisons and the proteins with similar behavior were combined into clusters. Although six clusters were generated, only cluster 3 is represented, as in this cluster the proteins in the different conditioned media behaved as expected.

3.3. Protein Identification, Database Search, and Candidate Protein Selection

From the secretome of the cultured Müller glial cells, a total of 1325 proteins were identified and quantified by LC-MS/MS analysis (the complete list of all the proteins and peptides found in the conditioned media are presented in Tables S1 and S2, Supporting Information). Hierarchical clustering was performed on all quantified proteins for the samples obtained with the three different types of conditioned media, and fresh NBA medium that had not been in contact with Müller cells was used as a control. The three culture conditions clearly segregated and the heat map illustrated the fundamental differences between the distinct culture conditions (two technical replicates were used for each sample type to verify experimental reproducibility), with highly abundant proteins in the matrices colored green and low abundance proteins colored red (**Figure 3**A). The proteins were quantified and the ratio between the mean normalized abundance of each sample was compared with the other two samples. Secreted proteins of interest were identified in each of the Müller cell conditioned media, such as growth factors or adhesion molecules, including: LIF (leukemia inhibitory factor), SERPINF1 (or PEDF, pigment epithelium-derived factor), PDGF (platelet-derived growth factor), CTGF (connective tissue growth factor precursor), NCAM (neural cell adhesion molecule 1), BCAM (basal cell adhesion molecule) or ICAM (intercellular adhesion molecule 1 and 2). However, these proteins are present in all the samples and there were no significant differences in their abundance between them. As the three conditioned media do not have the same effect on the survival of RGCs, these proteins were therefore ruled out as candidate neuroprotective proteins.

Müller cell secreted molecules can enhance RGC survival in all the conditions in which they were cultured, yet we searched for candidate molecules with the strongest effect on RGC survival. Hence, we searched for proteins that were more abundant in the conditioned medium from Müller cells cultured in DMEM than in the conditioned medium from Müller cells cultured in NBA/B27. Due to its neuroprotective properties, we became particularly interested in osteopontin (SPP1), which is significantly more abundant in DMEM + 10% FBS than in NBA/B27 (16.0-fold, $p < 0.01$). However, although there appeared to be more osteopontin in DMEM + 20% FBS than in NBA/B27, this difference was not significant (9.1-fold, $p > 0.05$). As such, the effects of osteopontin may be concentration-dependent and they may be less effective when the concentration becomes too high.

All the proteins identified in each of the three conditions were represented in a heat map to illustrate the differences in their expression (Figure 3A). Normalized abundances were then used to independently cluster the protein abundances across samples, and all proteins that were significantly different in at least one of the comparisons were included in the analysis, resulting in clusters containing proteins with similar expression patterns. While six different clusters were generated (Figure S1, Supporting Information), cluster 3 was that which was of most interest to us (Figure 3B). The proteins in this cluster are those that were most abundant in the conditioned medium produced by Müller cells cultured in DMEM + 20% FBS, the medium that had the strongest effect on RGC survival. Moreover, the proportion of these proteins in the medium conditioned by Müller cells cultured in NBA/B27 + 10% FBS was the lowest, because this medium diminishes RGC survival (see **Table 1** for a list of the 39 proteins included in this cluster and their relative abundance). After analyzing the function of this list of proteins, CLU and BSG were selected as candidate proteins to evaluate their capacity to afford neuroprotection to RGCs.

3.4. Effect of Candidate Proteins on the Survival of RGCs In Vitro

To identify which proteins from the conditioned medium might be responsible for promoting the survival of RGCs, these cells were cultured separately in the presence of three of the candidate proteins, SPP1, CLU, and BSG (**Figure 4**). The effect of the isolated proteins was compared to that of conditioned medium from Müller cells cultured in DMEM + 20% FBS, and PDGF was used as a positive control promoting RGC survival. As indicated above, the conditioned medium increased the total number of RGCs 3.65-fold (from 1.106 ± 0.168 RGCs per mm^2 in the controls to 4.041 ± 0.691 RGCs per mm^2 in the presence of the conditioned medium: $p < 0.001$) and PDGF enhanced the survival of RGCs by 47.08% (to 1.627 ± 0.248 RGCs per mm^2: $p < 0.001$). SPP1 and BSG increased the survival of RGCs by 71.31% (1.895 ± 0.128 RGCs per mm^2: $p < 0.001$) and 55.81% (1.724 ± 0.285 RGCs per mm^2: $p < 0.001$), respectively, whereas CLU did not produce a significant change in RGC survival, although a tendency toward greater RGC survival was evident (36.02%, 1.505 ± 0.313 RGCs per mm^2). The combination of SPP1 and BSG did not exert a synergistic effect, these proteins together increasing the survival of RGCs by 86.79% (2.067 ± 0.065 RGCs per mm^2: $p < 0.001$). Thus, the action of the conditioned medium on the survival of RGCs appears to be dependent on the combination of more proteins than those identified, these two proteins failing to exert the full protective effect of the conditioned medium alone. As the analysis was carried out in 96-well plate to minimize the amount of recombinant proteins required, only a small proportion of RGCs developed neurites and thus, only RGC survival was assessed, although there appeared to be no significant difference in terms of neuritogenesis between each of the conditions tested.

4. Discussion

Endogenous neuroprotectants play crucial roles during development, as well as in the maintenance and repair of the central nervous system (CNS) under normal and pathological conditions. Therefore, protecting neurons when their survival is compromised is a good strategy to employ in the face of lesions to the nervous system and neurological disorders.[22] By interacting with specific targets, certain compounds can protect neurons from some of the deleterious effects of damage to the nervous system.[23] Consequently, identifying novel neuroprotective molecules could open the way to develop new neuroprotective strategies for CNS lesion and neurodegenerative diseases, including those that affect RGCs. Different strategies have been employed to identify novel neuroprotective molecules, such as a functional cloning to identify protective genes.[29] Proteomic strategies have also been shown to be suitable to identify neuroprotective proteins[11,20] and indeed, the proteomic analysis of the secretome of cultured adult Müller cells performed here identified proteins previously shown to enhance RGC survival, such as CNTF (ciliary neurotrophic factor),[30] FGF (fibroblast growth factor),[31] and BDNF (brain-derived neurotrophic factor).[32]

Primary cell cultures have been used to test the neuroprotective properties of the secretome or media conditioned by distinct cell types, identifying potential therapeutic proteins.[24] Hence, we analyzed the secretome of cultured Müller cells in an attempt to identify factors that might offer RGCs protection against compromising situations in the retina. It is known that some of the characteristic features of Müller cells may be altered or lost after 2 weeks in culture[25] and that the proteins expressed by Müller cells after 21 days in culture might reflect their transdifferentiation from a multifunctional, highly differentiated, glial cell to a dedifferentiated fibroblast-like phenotype.[7,26] In order to ensure that the features of differentiated Müller cells were preserved in vitro, the secretome of these cells was examined after 7 days in culture. Moreover, to take advantage of the adaptation of these cells to distinct in vitro conditions,[7] the secretome of these Müller cells was assessed in three different culture conditions. It is known that the secretome of Müller cells contains neurotrophic molecules, given that factors secreted by porcine Müller cells in culture promote RGC[9] and photoreceptors[27] survival. Müller cells synthesize known neurotrophic factors, such as BDNF[28], CTNF[29], basic fibroblast growth factor (bFGF[30]), pigment epithelium derived factor (PEDF[31]), or glial-derived neurotrophic factor (GDNF[32]). In addition, other neuroprotective factors have been seen to be secreted by Müller cells, such as

Table 1. List of the 39 proteins included in cluster 3.

Gene	Protein name	DMEM 10%[a)]	Ratio DMEM 10%/DMEM 20%	*p*-Value DMEM 10%/DMEM 20%	DMEM 20%[a)]	Ratio DMEM 20%/NBA B27 10%	*p*-Value DMEM 20%/NBA B27 10%	NBA B27 10%[a)]	Ratio DMEM 10%/NBA B27 10%	*p*-Value DMEM 10%/ NBA B27 10%
ACO1	Aconitase 1	30	0.5	**0.015**	58	5.1	**0.033**	11	2.7	0.141
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif	29	0.6	**0.025**	49	2.3	**0.013**	22	1.4	**0.011**
SNRPG	Small nuclear ribonucleoprotein	37	0.8	**0.035**	45	2.6	0.172	18	2.1	0.272
COL23A1	Collagen, type XXIII, alpha 1	45	0.8	0.87	55	infinity	**0.002**	0	infinity	0.423
LMP2	Proteasome subunit, beta type, 9	44	0.9	0.64	47	4.8	**0.018**	10	4.5	**0.006**
ABCB11	ATP-binding cassette, sub-family B	45	0.9	0.066	50	9.3	**0.006**	5	8.4	**0.008**
SCAF11	SR-related CTD-associated factor 11	41	0.8	0.192	53	8.8	**0.017**	6	6.8	**0.010**
RAB11B	Member RAS oncogene family	41	0.9	0.341	47	3.8	**0.024**	12	3.3	**0.013**
ECM1	Extracellular matrix protein 1	40	0.8	0.067	51	5.7	**0.005**	9	4.4	**0.013**
CLU	Clusterin	36	0.7	0.06	51	4.1	**0.009**	12	2.9	**0.014**
TGM2	Transglutaminase 2	47	1.1	0.426	41	3.4	0.059	12	3.9	**0.017**
CLEC11A	C-type lectin domain family 11	31	0.7	0.09	47	2.1	**0.041**	22	1.4	**0.017**
CAPZA2	Capping protein muscle Z-line, alpha 2	38	0.8	0.186	47	3.1	**0.029**	15	2.5	**0.018**
SDCBP	Syndecan binding protein	43	0.8	0.527	51	9.1	**0.047**	6	7.6	**0.019**
KCTD12	Potassium channel tetramerization domain containing 12	42	1.0	0.974	42	2.7	**0.024**	16	2.7	**0.025**
ACP2	Uncharacterized protein	43	1.0	0.916	45	4.0	0.154	11	3.8	**0.026**
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	41	0.9	0.781	43	2.7	0.051	16	2.5	**0.033**
UFD1L	Ubiquitin fusion degradation 1 like	41	0.9	0.525	48	4.7	0.073	10	4.0	**0.034**
LGALS3	Galactoside-binding, soluble, 3	40	1.0	0.853	39	1.8	0.095	21	1.9	**0.036**
FBP1	Fructose-1,6-bisphosphatase 1	31	0.6	0.386	61	94.5	0.085	1	60.1	**0.034**
DF	Complement factor D	42	0.8	0.366	53	11.3	**0.019**	5	9.0	**0.038**
PFDN4	Prefoldin subunit 4	37	0.8	0.329	48	3.2	0.055	15	2.5	**0.040**
PTPN6	Tyrosine-protein phosphatase non-receptor type 6	42	0.8	0.301	56	24.4	**0.007**	2	18.5	**0.048**
PCSK1N	Proprotein convertase subtilisin/kexin type 1 inhibitor	29	0.5	0.075	59	5.0	**0.032**	12	2.5	**0.009**
MOB1A	Uncharacterized protein	37	0.6	0.206	63	108.2	**0.000**	1	63.8	0.121
RHO	Rhodopsin	48	1.0	0.982	49	15.7	**0.003**	3	15.4	0.427
IFITM1	Uncharacterized protein	45	0.9	0.908	50	10.4	**0.003**	5	9.2	0.458
ATP6V1H	ATPase, H+ transporting lysosomal, V1 subunit H	37	0.6	0.122	60	24.7	**0.006**	2	15.3	0.054

(*Continued*)

Table 1. Continued.

Gene	Protein name	DMEM 10%[a)]	Ratio DMEM 10%/DMEM 20%	*p*-Value DMEM 10%/DMEM 20%	DMEM 20%[a)]	Ratio DMEM 20%/NBA B27 10%	*p*-Value DMEM 20%/NBA B27 10%	NBA B27 10%[a)]	Ratio DMEM 10%/NBA B27 10%	*p*-Value DMEM 10%/ NBA B27 10%
GIMAP4	GTPase, IMAP family member 4	40	0.8	0.308	50	4.6	**0.009**	11	3.7	0.050
PNP	Purine nucleoside phosphorylase	49	1.1	0.738	44	5.7	**0.012**	8	6.3	0.088
IDE	Insulin-degrading enzyme	40	0.8	0.825	47	3.6	**0.017**	13	3.1	0.448
IDH1	Isocitrate dehydrogenase 1	40	0.8	0.319	49	4.7	**0.018**	10	3.9	0.059
BSG	Basigin	24	0.3	0.187	76	152.0	**0.020**	0	48.0	0.431
PEPD	Uncharacterized protein	39	0.9	0.871	41	2.0	**0.027**	20	1.9	0.332
VSIG4	V-set and immunoglobulin domain-containing protein 4	36	0.7	0.341	50	3.8	**0.030**	13	2.7	0.160
GBE1	Uncharacterized protein	37	0.7	0.639	55	6.3	**0.038**	9	4.2	0.490
PITPNB	Phosphatidylinositol transfer protein, beta	42	0.9	0.766	49	5.1	**0.041**	10	4.3	0.253
RAP1GDS1	GTP-GDP dissociation stimulator 1	40	0.7	0.499	57	22.9	**0.049**	3	16.1	0.152
CLIC4	Chloride intracellular channel 4	35	0.7	0.095	46	2.4	**0.050**	19	1.8	0.119

The test comparisons between the three conditions with fold changes and *p*-values are represented (*p*-values <0.05 are in bold); [a)] The genes encoding the proteins that are included in cluster 3, and the proportion (in % mean normalized abundance) of the proteins in the three different mediums are represented.

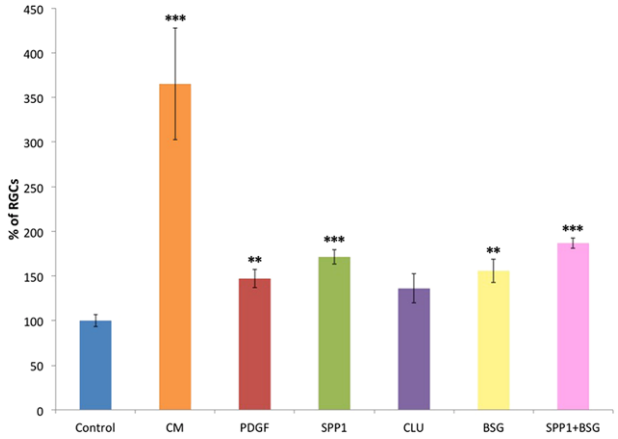

Figure 4. Effect of candidate proteins on the survival of RGCs in vitro. Histogram representing RGC survival after 6 days in culture in control conditions; in the presence of the medium conditioned by Müller cells maintained in DMEM + 20% FBS (CM); in the presence of PDGF, a protein with a demonstrated positive effect on RGC survival as a positive control; and in the presence of the candidate proteins osteopontin (SPP1), clusterin (CLU), and basigin (BSG), or the combination of SPP1 and BSG. The data are normalized considering the control as 100% for each condition: $^{**}p < 0.01$;$^{***}p < 0.001$.

IGFBP5, CTGF,[15] SPP1,[16] CXCL10,[8] LIF,[17] and transferrin.[18] However, it seems unlikely that all the proteins responsible for the neuronal survival-promoting effects of the Müller cells have been identified. The strategy employed here intended to demonstrate the importance of endogenous neurotrophic activities in the retina. As such, we monitored the secreted protein pool for its RGCs survival-promoting activity, classifying the secretomes from each of the three conditions in terms of RGC protection and the neuritogenesis promoted. The secretome of Müller cells cultured in the presence of a higher proportion in FBS (20%) better promoted RGC survival and neuritogenesis, whereas the secretome of Müller cells cultured in NBA, a medium more specific to neurons than glial cells, had the worst effect. These differences were also evident when the conditioned media were analyzed by mass spectrometry, and the distinct effects of the media on RGC survival facilitated the comparison between the proteins analyzed, enabling them to be classified into clusters according their relative abundance in medium with a strong, medium, or weak positive effect on RGC survival. This quantitative or comparative proteomics strategy is essential to characterize a complex proteome and compare it with others.[33] Using LC-MS/MS, the relative abundance of each labeled peptide can be compared in two or more samples by analyzing peptides identical in sequence but differing in mass.[34] As this method is semi-quantitative, it can detect changes in protein abundance with high confidence[35] and it is suitable for high-throughput, multisample proteome profiling.[34] Moreover, the proteomic clustering analysis provides a means of identifying and visualizing relationships in complex mixtures with many interacting elements.[36]

We used this tool to select a cluster of proteins whose relative abundance was correlated with the neuroprotective effect of the three conditioned media. This cluster contained the proteins that are more abundant in the medium and which have a positive

effect on RGC survival, and those that are less abundant in the media that negatively affect RGCs. After analyzing the functions of the 39 proteins in the chosen cluster, two candidate proteins were selected, clusterin and basigin. Clusterin was chosen because it is implicated in DNA repair, cell cycle regulation, and apoptotic cell death, and it also exerts a pro-survival role during cell death and confers resistance against cytotoxic agents.[37] Basigin was selected because it is required for normal retinal development and function, and for normal neuron-glia interactions in the visual system.[38] In addition, SPP1 was selected as a candidate molecule after confirming its presence in the conditioned medium because it is a neuroprotective factor for photoreceptors,[16] and its protective effect on RGCs has not yet been fully analyzed. The concentrations of these recombinant proteins used in the RGCs culture were concentrations of the proteins found in plasma[39] or those used previously in culture.[40,41]

The role of the selected candidate proteins in RGC survival was assessed in primary cell cultures of adult RGCs, using PDGF as a positive control as it is a potent neuroprotective factor known to enhance RGC survival.[42] Indeed, we have confirmed that PDGF is present in the secretome of Müller cells and as expected, it produced a significant increase in RGC survival in culture. Although CLU appeared to enhance RGC survival, these changes were not significant with respect to the controls. However, we cannot rule out any neuroprotective effects when CLU is available at other concentrations. Conversely, the concentration of BSG used significantly enhanced RGC survival, a novel neuroprotective property of this protein. The presence of SPP1 in the culture medium further improved RGC survival in culture at the concentration used. These data are consistent with data from ex vivo cultured glaucomatous retinas treated with SPP1 in which cell degeneration within the ganglion cell layer was inhibited.[40] Moreover, the essential role of SPP1 in the retina has been demonstrated in the SPP1 knock-out mouse, where the lack of SPP1 induces a decrease in RGC number.[43]

Although the SPP1 and BSG exert a positive effect on RGC survival, the use of one protein or the sum of both does not fully replicate the neuroprotective effect exerted by the conditioned medium. This indicates that the protective activity in the Müller cell conditioned medium is multifactorial, SPP1 and BSG forming only part of this activity, or that these proteins act in a synergistic manner. SPP1 and BSG are both proteins that have neuroprotective properties and thus, they merit further study in this system, particularly as they may potentially serve as therapeutic agents in neurodegenerative diseases in which RGCs are affected.

In conclusion, primary adult porcine Müller cells cultures represent a suitable model to study the neuroprotection of RGCs provided by Müller cells. The functional assay–driven proteomics screening approach adopted, based on the survival of RGCs, not only served to validate known neurotrophic factors but also identified novel proteins secreted by Müller cell as potential neuroprotectants. This strategy can be also applied to the identification of other bioactive proteins or molecular markers as it allows single molecules derived from complex protein mixtures to be characterized.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors acknowledge the support of Retos-MINECO Fondos Fender (RTC-2016-48231) and Grupos Consolidados del Gobierno Vasco (IT437-10) to E.V.

Conflict of Interest

The authors declare no conflict of interests.

Keywords

glia, Müller cells, neuroprotection, retinal ganglion cells, secretome

Received: August 28, 2017
Revised: November 20, 2017
Published online:

[1] E. Vecino, F. D. Rodriguez, N. Ruzafa, X. Pereiro, S. C. Sharma, *Prog. Retin. Eye Res.* **2016**, *51*, 1.
[2] E. Newman, A. Reichenbach, *Trends Neurosci.* **1996**, *19*, 307.
[3] a) A. Reichenbach, J. U. Stolzenburg, W. Eberhardt, T. I. Chao, D. Dettmer, L. Hertz, *J. Chem. Neuroanat.* **1993**, *6*, 201; b) A. Bringmann, T. Pannicke, J. Grosche, M. Francke, P. Wiedemann, S. N. Skatchkov, N. N. Osborne, A. Reichenbach, *Prog. Retin. Eye Res.* **2006**, *25*, 397; c) E. A. Newman, D. A. Frambach, L. L. Odette, *Science* **1984**, *225*, 1174.
[4] C. L. Poitry-Yamate, S. Poitry, M. Tsacopoulos, *J. Neurosci.* **1995**, *15*, 5179.
[5] a) N. L. Barnett, D. V. Pow, *Invest. Ophthalmol. Vis. Sci.* **2000**, *41*, 585; b) A. Derouiche, T. Rauen, *J. Neurosci. Res.* **1995**, *42*, 131; c) E. Willbold, J. Berger, M. Reinicke, H. Wolburg, *J. Hirnforsch.* **1997**, *38*, 383.
[6] a) A. Reichenbach, A. Bringmann, *Glia* **2013**, *61*, 651.
[7] S. M. Hauck, S. Suppmann, M. Ueffing, *Glia* **2003**, *44*, 251.
[8] C. von Toerne, J. Menzler, A. Ly, N. Senninger, M. Ueffing, S. M. Hauck, *Mol. Cell. Proteomics* **2014**, *13*, 2371.
[9] M. Garcia, V. Forster, D. Hicks, E. Vecino, *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 3735.
[10] a) A. Kawasaki, Y. Otori, C. J. Barnstable, *Invest. Ophthalmol. Vis. Sci.* **2000**, *41*, 3444; b) V. Heidinger, D. Hicks, J. Sahel, H. Dreyfus, *Glia* **1999**, *25*, 229; c) Y. Izumi, C. O. Kirby, A. M. Benz, J. W. Olney, C. F. Zorumski, *Glia* **1999**, *25*, 379.
[11] N. Ruzafa, E. Vecino, *Archivos de la Sociedad Espanola de Oftalmologia* **2015**, *90*, 522.
[12] D. M. Skytt, A. K. Toft-Kehler, C. T. Braendstrup, S. Cejvanovic, I. S. Gurubaran, L. H. Bergersen, M. Kolko, *BioMed Res. Int.* **2016**, *2016*, 1087647.
[13] R. A. Reis, M. C. Cabral da Silva, N. E. Loureiro dos Santos, E. Bampton, J. S. Taylor, F. G. de Mello, R. Linden, *J. Neurobiol.* **2002**, *50*, 13.
[14] R. A. de Melo Reis, A. L. Ventura, C. S. Schitine, M. C. de Mello, F. G. de Mello, *Neurochem. Res.* **2008**, *33*, 1466.

[15] S. M. Hauck, C. J. Gloeckner, M. E. Harley, S. Schoeffmann, K. Boldt, P. A. Ekstrom, M. Ueffing, *Mol. Cell. Proteomics* **2008**, *7*, 1349.
[16] P. Del Rio, M. Irmler, B. Arango-Gonzalez, J. Favor, C. Bobe, U. Bartsch, E. Vecino, J. Beckers, S. M. Hauck, M. Ueffing, *Glia* **2011**, *59*, 821.
[17] S. Joly, C. Lange, M. Thiersch, M. Samardzija, C. Grimm, *J. Neurosci.* **2008**, *28*, 13765.
[18] E. Picard, L. Jonet, C. Sergeant, M. H. Vesvres, F. Behar-Cohen, Y. Courtois, J. C. Jeanny, *Mol. Vis.* **2010**, *16*, 2612.
[19] E. Vecino, J. P. Heller, P. Veiga-Crespo, K. R. Martin, J. Fawcett, *PLoS One* **2015**, *10*, e0125250.
[20] S. M. Hauck, J. Dietter, R. L. Kramer, F. Hofmaier, J. K. Zipplies, B. Amann, A. Feuchtinger, C. A. Deeg, M. Ueffing, *Mol. Cell. Proteomics* **2010**, *9*, 2292.
[21] K. T. Rigbolt, J. T. Vanselow, B. Blagoev, *Mol. Cell. Proteomics* **2011**, *10*, O110 007450.
[22] H. Hayashi, N. Takagi, *Biol. Pharm. Bull.* **2015**, *38*, 1104.
[23] a) F. M. Longo, S. M. Massa, *NeuroRx* **2004**, *1*, 117; b) F. J. Vajda, J. Clin. *Neurosci*. **2002**, *9*, 4.
[24] a) F. Mbeunkui, B. J. Metge, L. A. Shevde, L. K. Pannell, *J. Proteome Res.* **2007**, *6*, 2993; b) S. K. Sze, D. P. de Kleijn, R. C. Lai, E. Khia Way Tan, H. Zhao, K. S. Yeo, T. Y. Low, Q. Lian, C. N. Lee, W. Mitchell, R. M. El Oakley, S. K. Lim, *Mol. Cell Proteomics* **2007**, *6*, 1680; c) J. C. Dreixler, J. N. Poston, I. Balyasnikova, A. R. Shaikh, K. Y. Tupper, S. Conway, V. Boddapati, M. M. Marcet, M. S. Lesniak, S. Roth, *Invest. Ophthalmol. Vis. Sci.* **2014**, *55*, 3785; d) T. V. Johnson, N. W. DeKorver, V. A. Levasseur, A. Osborne, A. Tassoni, B. Lorber, J. P. Heller, R. Villasmil, N. D. Bull, K. R. Martin, S. I. Tomarev, *Brain* **2014**, *137*, 503; e) C. H. Lin, C. H. Wang, S. L. Hsu, L. Y. Liao, T. A. Lin, C. M. Hsueh, *PLoS One* **2016**, *11*, e0146692.
[25] C. Guidry, *Invest. Ophthalmol. Vis. Sci.* **1996**, *37*, 740.
[26] J. Merl, M. Ueffing, S. M. Hauck, C. von Toerne, *Proteomics* **2012**, *12*, 1902.
[27] E. Balse, L. H. Tessier, C. Fuchs, V. Forster, J. A. Sahel, S. Picaud, *Invest. Ophthalmol. Vis. Sci.* **2005**, *46*, 367.
[28] E. Vecino, M. Ugarte, M. S. Nash, N. N. Osborne, *Neuroreport* **1999**, *10*, 1103.
[29] a) K. D. Rhee, X. J. Yang, *Adv. Exp. Med. Biol*. **2010**, *664*, 647; b) D. J. Zack, *Neuron* **2000**, *26*, 285.
[30] a) A. Bringmann, I. Iandiev, T. Pannicke, A. Wurm, M. Hollborn, P. Wiedemann, N. N. Osborne, A. Reichenbach, *Prog. Retin. Eye Res.* **2009**, *28*, 423; b) M. Hollborn, K. Jahn, G. A. Limb, L. Kohen, P. Wiedemann, A. Bringmann, Graefes Arch. *Clin. Exp. Ophthalmol.* **2004**, *242*, 414.
[31] X. Zhou, F. Li, L. Kong, J. Chodosh, W. Cao, *Mol. Vis.* **2009**, *15*, 438.
[32] a) S. M. Hauck, N. Kinkl, C. A. Deeg, M. Swiatek-de Lange, S. Schoffmann, M. Ueffing, *Mol. Cell Biol.* **2006**, *26*, 2746; b) C. Harada, T. Harada, H. M. Quah, F. Maekawa, K. Yoshida, S. Ohno, K. Wada, L. F. Parada, K. Tanaka, *Neuroscience* **2003**, *122*, 229.
[33] a) S. E. Ong, M. Mann, *Nat. Chem. Biol.* **2005**, *1*, 252; b) R. D. Unwin, C. A. Evans, A. D. Whetton, *Trends Biochem. Sci.* **2006**, *31*, 473.
[34] Y. Kinoshita, T. Uo, S. Jayadev, G. A. Garden, T. P. Conrads, T. D. Veenstra, R. S. Morrison, *Arch. Neurol.* **2006**, *63*, 1692.
[35] J. Colinge, D. Chiappe, S. Lagache, M. Moniatte, L. Bougueleret, *Anal. Chem.* **2005**, *77*, 596.
[36] K. Jablonowski, *Methods Mol. Biol.* **2017**, *1555*, 99.
[37] a) B. Shannan, M. Seifert, K. Leskov, J. Willis, D. Boothman, W. Tilgen, J. Reichrath, Cell Death Differ. **2006**, *13*, 12; b) I. P. Trougakos, E. S. Gonos, *Free Radic. Res.* **2006**, *40*, 1324; c) I. P. Trougakos, M. Lourda, M. H. Antonelou, D. Kletsas, V. G. Gorgoulis, I. S. Papassideri, Y. Zou, L. H. Margaritis, D. A. Boothman, E. S. Gonos, *Clin. Cancer Res.* **2009**, *15*, 48.
[38] a) J. M. Fadool, P. J. Linser, *Dev. Dyn.* **1993**, *196*, 252; b) J. D. Ochrietor, P. J. Linser, *Dev. Neurosci.* **2004**, *26*, 380; c) K. D. Curtin, R. J. Wyman, I. A. Meinertzhagen, *Glia* **2007**, *55*, 1542; d) M. Munro, Y. Akkam, K. D. Curtin, *Gene* **2010**, *449*, 50.
[39] M. Thambisetty, Y. An, A. Kinsey, D. Koka, M. Saleem, A. Guntert, M. Kraut, L. Ferrucci, C. Davatzikos, S. Lovestone, S. M. Resnick, *Neuroimage* **2012**, *59*, 212.
[40] M. T. Birke, C. Neumann, K. Birke, J. Kremers, M. Scholz, *Invest. Ophthalmol. Vis. Sci.* **2010**, *51*, 5759.
[41] R. J. Belton, Jr., L. Chen, F. S. Mesquita, R. A. Nowak, *J. Biol. Chem.* **2008**, *283*, 17805.
[42] C. He, C. Zhao, A. Kumar, C. Lee, M. Chen, L. Huang, J. Wang, X. Ren, Y. Jiang, W. Chen, B. Wang, Z. Gao, Z. Zhong, Z. Huang, F. Zhang, B. Huang, H. Ding, R. Ju, Z. Tang, Y. Liu, Y. Cao, X. Li, X. Liu, *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 14806.
[43] a) Z. Tang, P. Arjunan, C. Lee, Y. Li, A. Kumar, X. Hou, B. Wang, P. Wardega, F. Zhang, L. Dong, Y. Zhang, S. Z. Zhang, H. Ding, R. N. Fariss, K. G. Becker, J. Lennartsson, N. Nagai, Y. Cao, X. Li, *J. Exp. Med.* **2010**, *207*, 867; b) N. Ruzafa, X. Pereiro, P. Aspichueta, J. Araiz, E. Vecino, *Mol. Neurobiol.* **2017**, *55*, 213.

