Identification of a Novel Neurotrophic Factor from Primary Retinal Müller Cells Using Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)*

Christine von Toerne‡, Jacob Menzler‡, Alice Ly‡, Nicole Senninger‡, Marius Ueffing‡§, and Stefanie M. Hauck‡¶

Retinal Müller glial cells (RMGs) have a primary role in maintaining the homeostasis of the retina. In pathological situations, RMGs execute protective and regenerative effects, but they can also contribute to neurodegeneration. It has recently been recognized that cultured primary RMGs secrete pro-survival factors for retinal neurons for up to 2 weeks in culture, but this ability is lost when RMGs are cultivated for longer durations. In our study, we investigated RMG supernatants for novel neuroprotective factors using a quantitative proteomic approach. Stable isotope labeling by amino acids in cell culture (SILAC) was used on primary porcine RMGs. Supernatants of RMGs cultivated for 2 weeks were compared with supernatants from cells that had already lost their protective capacity. Using this approach, we detected established neurotrophic factors such as transferrin, osteopontin, and leukemia inhibitory factor and identified C-X-C motif chemokine 10 (CXCL10) as a novel candidate neuroprotective factor. All factors prolonged photoreceptor survival in vitro. Ex vivo treatment of retinal explants with leukemia inhibitory factor or CXCL10 demonstrated a neuroprotective effect on photoreceptors. Western blots on CXCL10- and leukemia inhibitory factor–stimulated explanted retina and photoreceptor lysates indicated activation of pro-survival signal transducer and activator of transcription signaling and B-cell lymphoma pathways. These findings suggest that CXCL10 contributes to the supportive potential of RMGs toward retinal neurons. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.033613, 2371–2381, 2014.

From the ‡Research Unit Protein Science, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany; §Institute for Ophthalmic Research, Centre for Ophthalmology, University of Tübingen, 72076 Tübingen, Germany.

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1 The abbreviations used are: RMG, retinal Müller glial cell; CXCL10, C-X-C motif chemokine 10; LIF, leukemia inhibitory factor; ONL, outer nuclear layer; PR, photoreceptor; SILAC, stable isotope labeling by amino acids in cell culture; SPP1, osteopontin; STAT, signal transducer and activator of transcription; pSTAT3, phosphorylated STAT3; BCL, B-cell lymphoma; GSH, glutathione; C-C motif, Chemokine; CCL5, ligand 5; IL8, interleukin, C-X-C motif, Chemokine; receptor 3, CXCR3.
We have previously established an in vitro model for studying primary RMG secretomes, demonstrating that these cells secrete a mixture of proteins that functionally prolong the survival of primary photoreceptors (PRs) (12). However, primary RMGs trans-differentiate during in vitro culture (13), resulting in the loss of PR-promoting survival properties (12). We performed an mRNA-based screening to compare neuroprotective and trans-differentiated RMG phenotypes, and we found that very few transcripts were altered between RMGs that had survival-promoting properties (day 14 cells) and those that had lost this activity (day 21 cells) (12). Unfortunately, the proteins related to the changed transcripts did not provide positive survival cues for PRs (data not shown). As our transcriptome screen did not reveal the identity of the RMG-derived molecules present in the secretomes, we established stable isotope labeling by amino acids in cell culture (SILAC) with primary RMGs. The cells were sufficiently labeled following 3 weeks in culture (day 21 cells), and we detected changes in the protein expression of cellular lysates that were indicative of trans-differentiation toward a fibroblast-like phenotype between day 14 and day 21 (13).

Using this model, we conducted a quantitative protein expression screen to examine differences in the RMG secretome, and we identified several potential neuroprotective molecules that correlated with the functional survival supporting phenotype. Along with previously established neurotrophic factors osteopontin (SPP1) (10), leukemia inhibitory factor (LIF) (14), and the iron-stress protective receptor transferrin (15, 16), we found the novel RMG-derived molecule C-X-C motif chemokine 10 (CXCL10) (previously known as IP-10). We examined the neurotrophic activity of transferrin, LIF, and CXCL10 on isolated primary PRs, and LIF and CXCL10 were further validated on retinal explants from a mouse model of retinal degeneration. Western blots of explanted porcine retina and porcine PR lysates indicated the activation of survival-prolonging signal transduction upon stimulation with CXCL10 or LIF. SILAC-based screening of a primary RMG model system therefore enabled the characterization of a mixture of neurotrophic factors secreted by RMGs.

**EXPERIMENTAL PROCEDURES**

**Preparation and Culture of RMGs—**Porcine RMGs were prepared as previously described (12). Major blood vessels were removed, and the retina was cut into smaller segments and then washed twice in Ringer’s solution. Retinal tissue was dissociated by treatment with activated papain (worthington) followed by a DNase (Sigma) digest and gentle trituration using a fire-polished Pasteur pipette. Dissociated cells were collected by centrifugation at 240 × g for 5 min and resuspended in SILAC-DMEM medium (PAA, GE Healthcare, Munich, Germany) supplemented with 0.23 mg/ml proline (Silantes, Munich, Germany), 4 mM L-glutamine (PAA), 10% dialyzed fetal bovine serum (FBS) (PAA), 50 units/ml penicillin (Introntecn, Heidelberg, Germany), and 0.05 mg/ml streptomycin (Invitrogen). 0.1 mg/ml [13C6]L-lysine and 0.1 mg/ml [15N4]L-arginine (Silantes) were added to the “heavy” medium, and normal amino acids (Silantes) to the “light” medium. Cells were plated onto 10-cm-diameter cell culture dishes (Nunc, Thermo Scientific, Dreieich, Germany) and incubated overnight at 37 °C. The following day, non-attached cells were removed by gentle panning, and the remaining RMG cells were cultured for 14 or 21 days to near confluence, with the media changed every 4 to 7 days. The day before harvesting, RMGs were washed three times with PBS and cultivated in FBS-free medium for 3 h. The medium was replaced once more, and cells were starved for another 18 h. A total of three SILAC experiments were performed with day 21 conditions heavy labeled.

**Preparation of RMG Supernatants—**After starvation, culture supernatants were collected, filtered (0.22 μm) to remove large cellular debris, and stored at −80 °C. The total protein content of RMG supernatants was measured using the Nanoquant protein assay (Roth, Karlsruhe, Germany) with concentrations ranging from 8 ng/μl to 14 ng/μl per sample. For every SILAC experiment, 40 μg of the respective day 14 and day 21 samples were mixed at a 1:1 protein ratio before precipitation with ice-cold acetone at a ratio of 1:5 at −20 °C overnight. Protein pellets were dissolved in urea buffer consisting of 4 M urea, 2 M thiourea, 4% CHAPS, and 4 mM DTT and prefractionated via one-dimensional SDS-PAGE.

**Prefractionation and Digestion of Samples—**A total of 80 μg per combined SILAC sample pair was loaded per lane on a 12% one-dimensional SDS-PAGE gel, and proteins were separated for 5 cm. The gel was fixed and stained using Coomassie dye (0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid). Each lane was excised, fractionated into six bands for the supernatants, and subjected to in-gel digestion as previously described (13).

**Mass Spectrometry—**Peptide samples were dissolved in 2% acetonitrile/0.5% trifluoroacetic acid by means of agitation and incubation for 30 min at room temperature. Before loading, the samples were centrifuged for 5 min at 4 °C. LC-MS/MS analysis was performed as previously described (17). Every sample was automatically injected and loaded onto the trap column at a flow rate of 30 μl/min in 5% buffer B (98% acetonitrile/0.1% formic acid in HPLC-grade water) and 95% buffer A (2% acetonitrile/0.1% formic acid in HPLC-grade water). Between each sample, the peptides were eluted from the trap column and separated on the analytical column by a 120-min gradient from 5% to 31% buffer B at 300 nl/min followed by a short gradient from 31% to 95% buffer B in 5 min. Between each sample, the gradient was set back to 5% buffer B and left to equilibrate for 20 min. From the MS prescan, the 10 most abundant peptide ions were selected for fragmentation in the linear ion trap if they exceeded an intensity of at least 200 counts and if they were at least doubly charged. During fragment analysis, a high-resolution (60,000 full width at half-maximum) MS spectrum was acquired in the Orbitrap with a mass range from 200 to 1500 Da.

**SILAC Analysis—**Sample compositions of the six supernatant fractions of each of the three independent SILAC experiments were analyzed using MaxQuant software (version 1.2.0.25, Max Planck Institute of Biochemistry, Martinsried, Germany) (18) with its internal search engine Andromeda (19). Default search parameters were used, allowing two missed cleavages, a fragment ion mass tolerance of 0.5 Da, and a parent ion tolerance of 20 ppm. Carbamidomethylation was set as a fixed modification; methionine oxidation and N-terminal acetylation were allowed as variable modifications. Labeling was set to doublets (0/0 und 8/10). The Andromeda search engine was configured for the Ensembl Pig protein database (version 62, 8,747,138 residues, 19,083 sequences) including a decoy database as well as a common-contaminants database. The MaxQuant output was filtered for reverse identifications, contaminants, findings “only identified by site,” and findings identified with more than one peptide. For analysis of the supernatants, only candidates identified in a least two out of three
experiments were pursued. Neurotrophic candidates were selected based on the fold change with the following criteria: a minimum of 10 SILAC pairs per quantification, a maximum SILAC-pair variability of 100%, and a maximum standard deviation between the fold changes of 50% per protein across experiments. 30 candidates with a 2-fold greater abundance at day 14 were loaded into Ingenuity Pathway Analysis (INGENIUTY System) (20), and only candidates with an extracellular localization were pursued further.

Photoreceptor Preparation and Survival Assay—Porcine PRs were prepared and cultured as described elsewhere (12). Human recombinant CXCL10 (100 ng/ml; Prospective, Recchovot, Israel), human Hologenin (1000 ng/ml; Prospective), human recombinant LIF (1 and 100 ng/ml; Prospective), human recombinant CCL5 (2 and 10 ng/ml; Peprotec, Hamburg, Germany), and human recombinant IL8 (10 and 100 ng/ml; Peprotec) in DMEM/F-12 medium (Invitrogen) or medium alone (negative control) were applied to the PR cultures 20 h after preparation. PR survival was monitored via an esterase calcein-fluorophore assay (Molecular Probes, Darmstadt, Germany) in a 96-well assay format. Total fluorescence per well is linearly correlated to the number of living cells per well (12) and was measured daily for 6 days using a fluorescence reader (Synergy HT, BioTek, Bad Friedrichshall, Germany) and compared with the initial fluorescence. All PR survival assays experiments, with the exception of 100 ng/ml LIF treatment, were performed at least three times.

Retinal Explants—Retinal explant experiments were performed using retinas from C57/HeH mice carrying the Pde6β<sup>−/−</sup> mutation. Animals were bred and maintained in ventilated cages at 22 °C ± 1 °C with a 12-h light/dark cycle and free access to water and food. All animals in this study were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Long-term Mouse Organotypic Explants—Retinas were isolated from 5-day-old mice, with the day of birth defined as postnatal day 0, with the retinal pigment epithelium attached as described previously (10, 21). Encuclated eyes were incubated at 37 °C for 15 min in R16 serum-free medium (Invitrogen) containing 0.12% proteinase K (M Biomedicals, Santa Ana, CA). The enzymatic digestion was stopped by the addition of 10% FBS (Invitrogen); the anterior segment, lens, vitreous, sclera, and choroid were carefully removed, and the retina was dissected free. The retina was cut perpendicular to its edges, resulting in a coverleaf-like shape, and transferred to a 0.4-μm polycarbonate membrane insert (Costar, Fisher Scientiffic, Schwerte, Germany) with the retinal pigment epithelium directly facing the membrane. The insert was placed into a six-well culture plate and incubated in R16 nutrient medium at 37 °C. For stimulation experiments, retinas were incubated in media containing LIF (50 ng/ml) or CXCL10 (100 ng/ml) for 24 h. The factors were absent from the media in control experiments. After 24 h, the retinal tissue was directly lysed and used for Western blot analyses.

Immunofluorescence—CXCL10 expression was examined with immunofluorescence in porcine retina. Porcine eyes were dissected with the lens, cornea, and vitreous removed. The posterior eye cup was separated from the vitreous and transferred to a Petri dish with 0.4-% trypsin (Sigma, Munich, Germany) or a combination of 100 ng/ml CXCL10 and 50 ng/ml LIF (or kept as an untreated control) and coverslipped. All sections were photographed with constant variables on an AxioImager Z1 with an ApoTome attachment (Zeiss).

Western Blot Analysis—Western blot analysis was used to determine the protein levels of phosphorylated STAT3 (pSTAT3) and BCL-2 from 20 μg of total cell lysates with α-tubulin as the loading control. For Western blot analysis, PRs were prepared as described in Refs. 10 and 12 using a pool of 20 eyes per experiment to ensure sufficient material. After cultivation overnight, PRs were starved for three hours and stimulated using human recombinant CXCL10 (1000 ng/ml, Prospective) or human recombinant LIF (100 ng/ml, Prospective) or left in starving medium for four hours. Blots were incubated overnight with the following primary antibodies: rabbit anti-pSTAT3 (1:7000; #9145, Cell Signaling, Leiden, The Netherlands) BCL-2 (1:1000; B3170, Sigma, Munich, Germany) or α-tubulin (1:10,000; ab6160, Abcam, Cambridge, United Kingdom). Appropriate secondary horseradish peroxidase-coupled antibodies were used in a dilution of 1:10,000. Protein signals were visualized using the ECL Plus enhanced chemiluminescence kit (GE Healthcare, Munich, Germany). Semi-quantitative analysis of intensities was performed using ImageJ software.

Data Analysis—Data were evaluated using Prism software (version 6, GraphPad) and Excel 2010 (Microsoft). For Table I, fold changes between 0 and 1 were transformed according to the formula Neg $\log_{10}$ Fc = −1 × Fc<sup>−1</sup> to achieve negative values. The photoreceptor survival assays were analyzed using Student’s t test. For explants, a nonparametric analysis of variance was performed comparing the experimental groups with the Kruskal–Wallis test followed by Dunn’s post-test, correcting for multiple testing. p values less than 0.05 were considered to indicate statistically significant differences.

RESULTS

RMG culture supernatants are known to contain neurotrophic factors (24–26), and a previous study demonstrated the survival-prolonging effect of porcine RMG supernatants on photoreceptors in vitro (12). In this study, we
used a quantitative mass spectrometric approach on primary RMGs to identify currently unknown neurotrophic factors (Fig. 1).

**Labeling Efficiency of Primary Porcine RMGs Using the SILAC Approach**—Sufficient labeling is a prerequisite for reliable quantifications using SILAC and requires the use of dialyzed FBS as a supplement to cell culture medium to avoid all sources of nonlabeled amino acids. The ability of primary RMGs cultivated under SILAC conditions to produce neurotrophic factors was examined and confirmed using PR survival assays (Fig. 2). In order to control for labeling efficiency, our primary cells grown under SILAC heavy condition were analyzed as described (13). Cells cultured for 21 days reached a median labeling efficiency of more than 90% (supplemental Fig. S1), which is in accord with other studies using primary cells (27, 28). As primary RMGs cultured for 14 days reached a median labeling of only 50% (supplemental Fig. S1), no reverse SILAC experiment (heavy labeling of day 14 primary cells) was performed. All three SILAC experiments were therefore performed with day 21 cells grown under SILAC heavy conditions compared with day 14 cells grown in light (unlabeled) medium.

**Differential Quantification of RMG Supernatants Identifies Neurotrophic Candidate Proteins**—A total of 376 protein groups were reliably identified in the RMG supernatants. Protein abundances are displayed as log transformed heavy-to-light ratios between cells cultured for 21 days and cells cultured for 14 days, independent of their intensity. Negative ratio values represent a greater abundance of proteins on day 14 than on day 21 (black dots). For the identification of neurotrophic candidates, stringent filtering criteria were applied to proteins with differential abundances with respect to quality of quantification, secretion, and fold change.

**Fig. 1.** Identification of neurotrophic candidate proteins using SILAC quantification. A total of 376 protein groups were reliably identified in the RMG supernatants. A, protein abundances are displayed as log transformed heavy-to-light ratios between cells cultured for 21 days and cells cultured for 14 days, independent of their intensity. Negative ratio values represent a greater abundance of proteins on day 14 than on day 21 (black dots). B, for the identification of neurotrophic candidates, stringent filtering criteria were applied to proteins with differential abundances with respect to quality of quantification, secretion, and fold change.

**Fig. 2.** Transferrin, LIF, and CXCL10 directly promote PR survival in vitro. Isolated and cultured porcine PRs were used to assess the neurotrophic capacities of either RMG-conditioned medium or the individual candidate neurotrophic factors identified via the quantitative SILAC approach. PRs were treated with supernatant from day 14 or day 21 cultured RMGs or medium containing neurotrophic candidates transferrin (1000 ng/ml transferrin), LIF (1 ng/ml), or CXCL10 (100 ng/ml). After 6 days in vitro, the day 14 supernatants and all three candidates were found to significantly enhance PR survival relative to controls and day 21 supernatant (t test p < 0.05). FC, fold change.

**Table I.** Summary of the 11 potential neurotrophic candidate proteins that were assigned to the extracellular space.
Among the most differentially expressed proteins between the experimental groups were the known neurotrophic factors SPP1 (10) and LIF (29). The extracellular receptor transferrin demonstrated an almost 10-fold higher expression on day 14 and has been reported to play a role in photoreceptor survival (16). Our quantitative approach identified not only known neurotrophic factors, but also a novel candidate that has yet to be discussed in a neuroprotective context. The chemokine CXCL10 exhibited the greatest fold change between days 14 and 21 of all our identified proteins (Fig. 1B). Based on these results, the following analyses focused on the cytokine candidates, SPP1, LIF, CXCL10, and the iron receptor transferrin.

Survival Assays on Primary Porcine Photoreceptors Confirm Neurotrophic Activity of Candidates—Photoreceptor survival assays were performed to validate the neuroprotective potential of transferrin, LIF, and CXCL10. To test the validity of our filtering approach, chemokine ligand 5 (CCL5) and interleukin 8 (IL8) were also tested, as they were more abundant on day 14 but did not meet all filtering criteria. Survival of PRs was monitored by a calcein-esterase assay (12). After 6 days in vitro, transferrin, LIF, and CXCL10 significantly enhanced PR survival (Fig. 2). Neither CCL5 nor IL8 enhanced PR survival relative to controls (supplemental Fig. S2).

CXCL10 Is Expressed in Porcine Retina in Vivo—We addressed the question of whether CXCL10 is also present in healthy retina or induced by culture conditions. First, expression of CXCL10 was confirmed on total retinal tissue by means of RT-PCR (data not shown). To gain more insight on the CXCL10 distribution and the cells of origin, an immunofluorescence assay was conducted on porcine retina. We found intense and specific CXCL10 labeling within the ganglion cell layer and some amacrine cells of the inner nuclear layer, in addition to weak labeling of other cells in the inner nuclear layer and diffuse fluorescent labeling throughout the retina, which is expected for a secreted protein (Fig. 3A). This confirmed CXCL10 in vivo expression in the retina and suggests that CXCL10 is produced in vivo by several cell types.

LIF and CXCL10 Decrease Cell Death in Pde6brd1 Retina Explants—To assess whether the neurotrophic effects of LIF and CXCL10 counteracted PR degeneration, we conducted ex vivo retina explant cultures to monitor cell survival in intact tissue over extended periods of time (10, 30). Retinal explant cultures were prepared from C3H/HeJ mice, which carry the...
**FIG. 4.** LIF and CXCL10 showed protective effects in ex vivo retina explants. Retinas from mice with the \textit{Pde6brd1} mutation (rd1-positive) were isolated 5 days after birth and cultivated for 14 days. All micrographs are of the central retina. \textit{A}, rd1-positive mice lost 90% of photoreceptors, as reflected by the small size of the ONL, which was reduced to a single cell layer. Rd1-positive retinas treated for 12 days with (\textit{B}) 50 ng/ml of CXCL10 or (\textit{C}) 100 ng/ml LIF demonstrated rescue of ONL size. \textit{D}, a combination treatment of 50 ng/ml CXCL10 and 100 ng/ml LIF resulted in slightly increased ONL size relative to the singly treated explants. \textit{E}, a retinal section of a 20-day-old C57/Bl6 mouse showing the normal size of the ONL in a healthy, non-explanted retina. \textit{F}, mean ONL thickness for treated and untreated explants. All treatments resulted in significantly increased ONL thicknesses relative to untreated samples (untreated, 9 \(\mu\)m \(\pm\) 3.9; CXCL10, 21 \(\mu\)m \(\pm\) 5.5, \(p < 0.05\); LIF, 35 \(\mu\)m \(\pm\) 7.3, \(p < 0.05\)), as well as to the combined treatment (CXCL10/LIF, 38 \(\mu\)m \(\pm\) 7.9, \(p < 0.05\)). None of the treatments restored ONL thickness to the level of C57/Bl6 (wild-type) mice (62 \(\mu\)m \(\pm\) 11.7). All data presented as mean \(\pm\) S.D. Scale bar: 50 \(\mu\)m. ONL, outer nuclear layer; INL, inner nuclear layer; wt, wild type.

\textit{Pde6brd1} mutation resulting in fast-degenerating PRs (31). Retinas were explanted at postnatal day 5 and cultured for 14 days with or without LIF or CXCL10, and the ONL, where PR cell bodies are located, was measured. Fig. 4 shows photomicrographs of explanted \textit{Pde6brd1} retinas and a freshly dissected wild-type C57/Bl6 retina. The ONLs of the \textit{Pde6brd1} untreated retinas displayed only single layers of nuclei in the ONL (Fig. 4\textit{A}), whereas the LIF-treated (Fig. 4\textit{B}), the CXCL10-treated (Fig. 4\textit{C}), and the combined CXCL10/LIF-treated (Fig. 4\textit{D}) retinas showed comparatively increased ONL size. None of the treatments, however, led to wild-type ONL thickness (Figs. 4\textit{E} and 4\textit{F}). Statistical analysis indicated that retinas treated with LIF (35 \(\mu\)m \(\pm\) 7.3), CXCL10 (20 \(\mu\)m \(\pm\) 5.5), or the combined treatment (38 \(\mu\)m \(\pm\) 7.9) exhibited significantly thicker ONLs than untreated \textit{Pde6brd1} retinas (9 \(\mu\)m \(\pm\) 3.9).
Activation of Pro-survival Signaling through CXCL10 and LIF—Western blot analysis was used to investigate effects of CXCL10 or LIF treatment on intact retinal tissue and isolated PR signal transduction in vitro. We focused on two known survival-associated pathways: signal transducer and activator of transcription (STAT) and B-cell lymphoma (BCL) signaling. Active STAT signaling has been associated with survival in photoreceptors (32) and other cells of the retina (33–35), and BCL-2 is an important anti-apoptotic protein in the retina (36). pSTAT3 levels were markedly increased in explanted porcine retina stimulated with LIF for 24 h, but they were only slightly increased by CXCL10 stimulation (Fig. 5A). Both factors slightly increased BCL-2 levels in explanted retina (Fig. 5A). Stimulation of isolated PRs with LIF also strongly induced pSTAT3 protein levels and only slightly increased BCL-2 levels (Fig. 5B), whereas stimulation with CXCL10 resulted in a stronger induction of BCL-2 levels (Fig. 5B) as compared with pSTAT3 levels.

DISCUSSION

RMGs in Health and Disease—The loss of sight is an increasing problem in our progressively aging society, and the search for neurotrophic factors that might alleviate vision loss has been ongoing for the past 40 years. RMGs have been the subject of many investigations, as they have many functions essential to the maintenance of the normal retinal environment, such as metabolite transport (6), control of K⁺ and water homeostasis (3, 8), and uptake and recycling of neurotransmitters (37). In addition, RMGs release factors that protect neurons, particularly PRs, against cell death (4, 9, 10). Under pathological conditions, RMGs are known to up-regulate glial fibrillary acidic protein (38, 39), exhibit abnormal neurochemical metabolism (40), and have altered expression and function of inwardly rectifying K⁺ channels (41). Given the importance of RMGs in retinal function, it is important to understand the roles these cells play under normal conditions and how they are altered in disease.

A number of studies from our laboratory have identified neuroprotective factors secreted by RMGs (10, 12). This study continued the search for novel neuroprotective factors by using the quantitative SILAC approach to examine differences in the secretomes of primary porcine RMGs cultivated for either 14 or 21 days. Here, we report the detection SPP1, LIF, transferrin, and CXCL10 in the secretomes of RMGs cultured for 14 days and demonstrate their neuroprotective abilities (Figs. 2 and 4).

Neurotrophic Candidate Proteins—Primary RMGs start losing their neuroprotective capacities when cultivated for over 2 weeks. For this reason, we were interested in proteins that were more abundant on day 14 and assigned to the extracellular compartment. We shortlisted 11 proteins (Table I), of which one candidate, SPP1, had been previously identified and confirmed as a neuroprotective factor via transcriptome analysis (10). In that study, Del Rio et al. induced SPP1 se-
Iron is crucial for cell survival, and stable transferrin expression has been demonstrated to play a major role in iron homeostasis within the retina (43). Iron storage is regulated by ferritin and iron export by proteins such as ceruloplasmin, ferroportin, and hephaestin, and transferrin is involved in iron import into the cell (44). Extracellular transferrin binds iron in circulation to its receptor, which is then internalized through clathrin-coated pits into endosomes (44, 45). Although iron is a co-factor for numerous enzymes and necessary for cell survival, free iron can produce the highly reactive hydroxyl radical, which can damage proteins, lipids, and nucleic acids (16, 43). Iron-induced oxidative stress has been implicated in age-related neurodegenerative diseases affecting the brain and the retina (46). Elevated iron levels have been observed in PRs, the retinal pigment epithelium, and the Bruch’s membrane in studies of age-related macular degeneration (16, 45, 46). While examining treatments for increased iron levels in disease, Hadziahmetovic et al. observed beneficial effects when studying the effect of iron chelation on retinal integrity in ferroxidase-deficient mice (47). Picard et al. (16) demonstrated that overexpressed or intraperitoneally injected human transferrin in rd10 mice prevented PR degeneration in vivo, thereby highlighting the important role of transferrin in iron homeostasis in the retina and its neuroprotective capacity. Our observation of greater transferrin abundance in the secretomes of RMGs cultivated for 14 days and demonstration that transferrin promoted PR survival are in accord with these previous studies and might represent a change in RMG metabolism accompanied by the phenotypic de-differentiation toward a fibroblast-like cell. An inability of these de-differentiated cells to bind free iron sufficiently might partially explain their lack of survival-prolonging capacities.

In addition to SPP1 and transferrin, we identified higher levels of LIF in RMG secretomes at day 14 than at day 21. LIF belongs to the IL6 family, also termed glycoprotein 130 cytokines (48). Other members of this cytokine family include ciliary neurotrophic factor, IL11, and oncostatin M, all of which signal through a common glycoprotein 130 receptor (48). LIF exhibits multiple biological effects in the developing vertebrate retina (49) and has been widely investigated as a neurotrophic factor in the retina (14, 50–52). The identification of three previously described neuroprotective molecules validates our quantitative proteomic approach. CXCL10, which has not previously been identified as having neurotrophic properties, was found to have the greatest differential abundance between day 14 and day 21 RMG secretomes, and it was the only cytokine/chemokine other than SPP1 and LIF to demonstrate a differential abundance. CXCL10 is a highly inducible, primary-response protein from the C-X-C chemokine superfamily (53). Pleiotropic biological effects include stimulation of monocytes, natural killer and T-cell migration, regulation of T-cell and bone marrow progenitor maturation, modulation of adhesion molecule expression, and inhibition of angiogenesis (53). CXCL10 and its receptor the chemokine (C-X-C motif) receptor 3 (CXCR3) are expressed throughout the nervous system and have been reported to play a role in acute inflammation (54). Using immunofluorescence, we confirmed that CXCL10 was expressed in healthy porcine retina in vivo (Fig. 3).

Secreted by neurons in response to viral infections, CXCL10 has been discussed as a protective agent that acts by increasing effector T-cell traffic, thereby alleviating viral burden in the brain (55). Experiments on mixed rat hippocampal neuronal and glial cell cultures showed chronically expressed CXCL10 to be protective in certain neuroinflammatory conditions (56). During chronic ocular toxoplasmosis, CXCL10 is required to maintain T-cell populations and to control parasite replication (57). In the retina, CXCL10 has recently been discussed as playing a role in diabetic retinopathy (58).

**Roles of CXCL10 and LIF in Neuroprotection**—We investigated signal transduction in PRs to elucidate how LIF and CXCL10 mediate neuroprotection. Together with other glycoprotein-130-related cytokines, LIF activates the Janus kinase and STAT pathways (48). The capacity of LIF to counteract oxidative stress (29, 51) fits well with the described role of transferrin in the retina, and as our study found both proteins at reduced levels in de-differentiated cells concomitant with a loss of survival-prolonging capacities, this suggests an important role of RMGs in buffering oxidative stress in the healthy retina. It has been proposed that RMG-derived LIF controls an intrinsic protective mechanism that supports PR cell survival (14). Experiments on RMGs from ciliary neurotrophic factor and LIF knockout mice demonstrated that the LIF-induced glycoprotein 130/Janus kinase/STAT3 pathway is required for the initiation of the astrogliosis-like reaction of RMGs following optic nerve injury (59). In the context of embryonic stem cells, LIF has recently been shown to induce STAT3 signaling and thereby suppress differentiation. When LIF is withdrawn from the culture medium, the signaling mode of these cells is switched, leading to differentiation (60). If the same signaling mechanism applies to our primary RMGs, reduced LIF production might not only be involved in the loss of neuroprotection but also trigger the de-differentiation process we observed after 21 days in culture.

Western blot analysis demonstrated an increase of pSTAT3 in LIF- and CXCL10-stimulated PRs (Fig. 5). This finding agrees...
with the literature on LIF and, together with our finding that LIF/CXCL10 combined treatment had only a slight additive effect on PR survival in explanted retina, might indicate that CXCL10 and LIF partially act through the same cellular signaling pathway, possibly STAT3 signaling.

So far, little is known regarding the beneficial effect of inflammation in the context of RMG-mediated neuroprotection. While CXCL10 knock-out mice (B6.129S4-Cxcl10tm1Ad/J, The Jackson Laboratory) are viable, albeit with impaired immune functions, no problems in visual function are reported in the literature (61–63). As we were able to demonstrate the neurotrophic potential of CXCL10 in vitro and ex vivo, it can be assumed that CXCL10 acts as an ancillary factor in vivo in concert with other neurotrophic factors. In the context of regeneration, CXCL10 was shown to be hepatopo-regenerative in a murine model of acute liver injury (64). In retinal ganglion cells, a role for inflammation in the context of regeneration has been discussed, yet the factors involved and the mediating cells remain controversial (65–67).

In addition to STAT signaling, we found BCL-2 to be up-regulated upon CXCL10 stimulation in primary PRs (Fig. 5). Ectopic expression of BCL-2 in PRs of mice with retinal degenerative disease even slowed the progression of disease (68). A recent study by Park et al. reports that changes in retinal glutathione (GSH) levels affect BCL-2 expression in mouse retina (36). GSH plays a critical role in cellular defense against oxidative stress in neurons, and maintenance of GSH levels in the retina is controlled by RMGs (36). The finding that CXCL10-mediated neuroprotection might be BCL-2 associated links an inflammatory molecule to PR survival. One common denominator of neuroprotective factors seems to be the protection against oxidative stress, as demonstrated for transferrin, LIF, and GSH, and future studies will elucidate whether this is also the mechanism for CXCL10. Furthermore, the connection between BCL-2 and GSH expression indicates the loss of neuroprotective capacity of RMGs in cultivation as a lack of detoxification due to the differentiation of RMGs.

CONCLUSION

Primary porcine RMGs are a suitable model for studying RMG-associated neuroprotection. Our quantitative proteomic approach not only validated known neurotrophic factors but also identified a novel candidate in the RMG secretome. Insights into the signaling processes of photoreceptors could help to unravel RMG-mediated neuroprotective signal transduction.

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To whom correspondence should be addressed: Stefanie M. Hauck, Research Unit Protein Science, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. E-mail: hauck@helmholtz-muenchen.de.

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