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Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome

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The development of neuroprotective strategies to attenuate retinal ganglion cell death could lead to novel therapies for chronic optic neuropathies such as glaucoma. Intravitreal transplantation of mesenchymal stem cells slows retinal ganglion cell death in models of optic nerve injury, but the mechanism of action remains unclear. Here we characterized the neuroprotective effects of mesenchymal stem cells and mesenchymal stem cell-derived factors in organotypic retinal explant culture and an *in vivo* model of ocular hypertensive glaucoma. Co-culture of rat and human bone marrow-derived mesenchymal stem cells with retinal explants increased retinal ganglion cell survival, after 7 days *ex vivo*, by ~2-fold and was associated with reduced apoptosis and increased nerve fibre layer and inner plexiform layer thicknesses. These effects were not demonstrated by co-culture with human or mouse fibroblasts. Conditioned media from mesenchymal stem cells conferred neuroprotection, suggesting that the neuroprotection is mediated, at least partly, by secreted factors. We compared the concentrations of 29 factors in human mesenchymal stem cell and fibroblast conditioned media, and identified 11 enriched in the mesenchymal stem cell secretome.

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Treatment of retinal explants with a cocktail of these factors conferred retinal ganglion cell neuroprotection, with factors from the platelet-derived growth factor family being the most potent. Blockade of platelet-derived growth factor signalling with neutralizing antibody or with small molecule inhibitors of platelet-derived growth factor receptor kinase or downstream phosphatidylinositol 3 kinase eliminated retinal ganglion cell neuroprotection conferred by mesenchymal stem cell co-culture. Intravitreal injection of platelet-derived growth factor -AA or -AB led to profound optic nerve neuroprotection *in vivo* following experimental induction of elevated intraocular pressure. These data demonstrate that mesenchymal stem cells secrete a number of neuroprotective proteins and suggest that platelet-derived growth factor secretion in particular may play an important role in mesenchymal stem cell-mediated retinal ganglion cell neuroprotection. Furthermore, platelet-derived growth factor may represent an independent target for achieving retinal ganglion cell neuroprotection.

Keywords: mesenchymal stem cell; retinal ganglion cell; neuroprotection; glaucoma; platelet derived growth factor

Abbreviations: DMEM = Dulbecco's modified Eagle medium; IL = interleukin; MSC = mesenchymal stem cell; PDGF = platelet-derived growth factor; PEDF = pigment epithelium-derived factor; RGC = retinal ganglion cell; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling

Introduction

Progressive retinal ganglion cell (RGC) death and optic nerve damage characterize glaucoma, a neurodegenerative disease for which the only available treatment is pharmacological or surgical reduction of intraocular pressure (Quigley, 2011). There is growing interest in the development of neuroprotective therapies for glaucoma, which might be used adjunctively with ocular hypotensive approaches to reduce RGC death and attenuate loss of vision more effectively (Casson *et al.*, 2012; Chang and Goldberg, 2012). Previously investigated neuroprotective strategies include neurotrophic factor delivery, molecular suppression of pro-apoptotic signalling cascades, reduction of oxidative stress, and suppression of inflammation (reviewed in Danesh-Meyer, 2011; Vasudevan *et al.*, 2011; Almasieh *et al.*, 2012; Chang and Goldberg, 2012).

While early enthusiasm for the translational prospects of stem cell research in glaucoma focused on optic nerve regeneration, neuroprotective properties of various stem cell types are increasingly being appreciated (Bull *et al.*, 2008; Johnson and Martin, 2013). Mesenchymal stem cells (MSCs) are multipotent, with the ability to differentiate into adipocytes, osteoblasts, chondrocytes and myoblasts. *In vivo*, they are thought to play a role in maintaining the haematopoietic stem cell niche. MSCs possess immunomodulatory, antioxidant and neurotrophic characteristics that support neuronal survival when transplanted to ectopic sites of degenerative CNS stress (Kassis *et al.*, 2011; Uccelli *et al.*, 2011). These cells can be readily isolated from bone marrow aspirates from individual patients and expanded *in vitro* before autologous transplantation. For these reasons, MSC transplantation is presently being trialled as a therapy for multiple sclerosis, ischaemic stroke, spinal cord injury, Parkinson's disease, and other conditions (clinicaltrials.gov). In preclinical models of optic nerve neurodegeneration, MSC transplantation also appears to attenuate neuronal death. RGC neuroprotection has been noted with local MSC transplantation following ischaemia/reperfusion (Li *et al.*, 2009), optic nerve crush (Zhao *et al.*, 2011), optic tract transection (Zwart *et al.*, 2009), and ocular hypertension induced by episcleral vein ligation (Yu *et al.*, 2006) or photocoagulation of the trabecular meshwork (Johnson *et al.*, 2010). Although increases in retinal concentrations of a variety of trophic factors have been noted after MSC transplantation, the mechanism(s) by which MSCs confer neuroprotection to RGCs remains unclear. In this work, we employed *in vitro* organotypic retinal explant culture and *in vivo* experimental ocular hypertension as models to assess the effects of MSC co-culture and MSC-derived factors on RGC survival. In doing so, we demonstrated that both rat and human MSCs exhibited robust neuroprotective properties, and identified platelet-derived growth factor (PDGF) as a particularly potent neuroprotective MSC-derived protein, which may explain much of the neuroprotective effect of these cells.

Materials and methods

Animals

Adult (8–12 week old) Sprague Dawley rats were maintained in accordance with guidelines set forth by the National Eye Institute Committee on the Use and Care of Animals, the UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act (1986), and the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Visual Research.

Cell cultures

Rat MSCs were isolated from the femurs of 8-week-old transgenic Sprague Dawley rats engineered to express green fluorescent protein (GFP) under control of the chicken β-actin promoter (Okabe *et al.*, 1997; Hunt *et al.*, 2008). Femurs were obtained from euthanized rats and bone marrow was isolated by flushing the femurs with 1–2 ml of PBS. Mononuclear cells were plated at a density of 5×10^5 cells/cm^2 on plastic culture flasks in Dulbecco's modified Eagle medium (DMEM; 1 g/l glucose) containing 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml; all from Life Technologies). Non-adherent cells were removed by complete media exchange after 48 h, and then cells were cultured until 80% confluent before passage. Rat MSCs used in this study were from passages 12–20.

Human MSCs were purchased from Stem Cell Technologies and cultured in DMEM (1 g/l glucose) containing 10% foetal bovine serum, penicillin and streptomycin. The vendor verified >90% expression of CD29, CD44, CD105, and CD166 and <1% expression of CD14, CD34 and CD45 by flow cytometry. Human MSCs were passaged at 80% confluence. Cells used in this study were from passages 5–10. Human adult dermal fibroblasts were purchased from Life Technologies and cultured in Medium 106 with low serum growth supplement (both from Life Technologies). Fibroblasts were passaged at 80% confluence and used in this study at passages 5–10. The mouse embryonic fibroblast cell line NIH-3T3 (Jainchill *et al.*, 1969) was cultured in DMEM (4.5 g/l glucose) containing 10% foetal bovine serum, penicillin and streptomycin. Cells were passaged at 80% confluence.

The multipotencies of rat and human MSC cultures were verified experimentally by induction of differentiation into adipocytes and osteoblasts. This was accomplished by cell culture in StemPro osteogenesis or adipogenesis differentiation media according to vendor instructions (Life Technologies).

Flow cytometry

Live rat MSCs in single-cell suspension were blocked using 1% foetal bovine serum in PBS and then stained immunocytochemically for surface markers using the following antibodies: hamster anti-CD29 (1:100, Serotec), mouse anti-CD44 (1:50, Serotec), mouse anti-CD73 (BD Biosciences), PE-conjugated mouse anti-CD90 (1:50, BD Biosciences), mouse anti-CD34 (1:50, Santa Cruz Biotechnology Inc.), mouse anti-CD45 (1:100, Serotec), mouse anti-CD11b (1:50, Serotec), and mouse anti-CD68 (1:100, EMD Millipore). Secondary Alexa Fluor® 647-conjugated goat anti-hamster IgG or Alexa Fluor® 633-conjugated goat anti-mouse IgG (Life Technologies) were used where appropriate. Negative controls included cells with primary antibody omitted from the staining protocol. Analyses were carried out using a FACSCalibur flow cytometer (BD biosciences).

Organotypical retinal explant culture

Primary organotypical retinal explants were isolated and cultured as previously described (Johnson and Martin, 2008; Bull *et al.*, 2011). Briefly, 8–12 week old wild-type Sprague Dawley rats were euthanized and the globes were excised. The retina was dissected free from retinal pigmented epithelium and cut radially into quarters. Neural retina was then flat-mounted with the photoreceptor side down on polytetrafluoroethylene membranes (EMD Millipore) at an air-fluid interface overlying culture media composed of Neurobasal®-A media with 2% B27 supplement, 1% N2 supplement, L-glutamine (0.8 mM), penicillin, and streptomycin in humidified conditions at 35°C and 5% CO_2. Half of the culture media was exchanged on Day 1 of culture, and then every second day thereafter.

For co-culture experiments, dissociated cells were washed with PBS before being suspended at a concentration of 500–2500 cells/μl in retinal explant culture media. Using a standard 10 μl pipette, a 2 μl droplet of cell suspension was placed on the RGC surface of each retinal explant.

In some experiments, retinal explants were treated with cell culture conditioned media, addition of purified proteins (Table 2), and/or inhibitors of molecular signalling pathways. In all cases, these factors were diluted in the retinal explant culture media at the concentrations indicated. To ensure adequate exposure to the RGC layer, 2–5 μl of complete culture media containing experimental factors were pipetted directly onto the RGC surface of explants daily. Anti-PDGF antibody (EMD Millipore catalogue no. 06-127, goat polyclonal antibody recognizing PDGF-AA, -AB, and -BB) and normal goat IgG (Santa Cruz Biotechnologies) were each added to the culture media at 35 μg/ml. Small molecular inhibitors of PDGF receptor kinase (AG1296, EMD Millipore) or phosphatidylinositol 3 (PI3) kinase (LY294002, Cell Signaling Technologies) were added to culture media at 45 μM and 75 μM, respectively.

Conditioned media

To obtain cell-conditioned media, cell cultures were grown to 90% confluence in plastic culture flasks. Then, the growth media was removed, cells were rinsed three times with PBS, and serum-free DMEM was added. Cells were cultured for 72–96 h in this medium and then the conditioned media were collected. Conditioned media were concentrated using Amicon Ultra-15 centrifugal filter units (3 kDa normal molecular weight limit, EMD Millipore). Pre-concentrated and post-concentrated volumes were used to determine fold changes in protein concentration. To measure the concentration of specific cytokines, chemokines and other proteins (Table 1), conditioned media was analysed using commercially available multiplexed antibody-based assays (xMAP®, Luminex). All assays were conducted according to the manufacturer's recommendations. Twenty-five microlitres of concentrated conditioned media was analysed per sample in triplicate, using unconditioned, concentrated DMEM as a negative control. A minimum of three independent samples of concentrated conditioned media were analysed per cell type. Reported concentration measurements are corrected for fold-concentration of conditioned media that was analysed, and correlate to levels found in un-concentrated conditioned media. All xMAP® assays were analysed using a Luminex 200 platform and data were collected using Bio-Plex Manager™ software. To analyse the secreted concentration of thrombospondin 1, an ELISA kit (R&D Systems) was used according to the manufacturer's protocol using three independent samples of concentrated media per cell type, each run in triplicate.

Human MSC conditioned media was fractionated based on molecular weight by fast protein liquid chromatography using a Sephacryl® gel filtration column (HiPrep™ Sephacryl® S-200HR, GE Healthcare). A starting volume of 2 ml of concentrated human MSC conditioned media was applied to the column and run according to manufacturer recommendations at 0.5 ml/h in 50 mM NaPO4, 0.15 M NaCl running buffer. Eight samples of 40 ml each were collected during the fractionation and each was concentrated between 50- and 60-fold using Amicon® Ultra-15 centrifugal filter units (3 kDa normal molecular weight limit). Bicinchoninic acid (BCA) assays of the concentrated fractions showed that fractions 1–3 contained the majority of protein (0.266 mg/ml in fraction 1, 0.284 mg/ml in fraction 2, and 0.025 mg/ml in fraction 3), whereas little to no protein was detected in fractions 5–8. SDS-PAGE of fractions 1–3 shows separation of proteins by molecular weight (Fig. 3C). Because of their detectable protein levels, fractions 1–3 were further used to treat retinal explants. Retinal explants were treated with concentrated fraction 1, fraction 2, fraction 3 or concentrated column running buffer by applying a 2.5 μl bolus to the surface of the explants and 4 μl of fractionated media or running buffer to the base media once daily.

Animal glaucoma model

Ocular hypertension was induced using a modification of the method developed by Levkovitch-Verbin *et al.* (2002). Briefly, rats were anaesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) injected

Table 1 Analysis of human MSC and fibroblast secretomes

Secreted factor name	Concentration in human MSC conditioned media (pg/ml)	Concentration in human fibroblast conditioned media (pg/ml)	Secretion ratio human MSC : human fibroblast
Adiponectin	0.27 ± 0.39	0.02 ± 0.04	14.3
Basic fibroblast growth factor (FGF-2)	0.20 ± 0.04	0.23 ± 0.39	0.89
Brain derived neurotrophic factor (BDNF)	13.22 ± 2.63	0.02 ± 0.03	589***
Chemokine ligand 14 (CCL-14)	0.10 ± 0.04	0.09 ± 0.04	1.03
Ciliary neurotrophic factor (CNTF)	nd	0.02 ± 0.04	Human fibroblast only
Epidermal growth factor (EGF)	0.12 ± 0.13	0.75 ± 0.74	0.16
Epithelial neutrophil activating protein (ENA-78/CXCL-5)	0.44 ± 0.15	0.47 ± 0.18	0.92
Granulocyte colony stimulation factor (G-CSF)	nd	0.03 ± 0.06	hFibroblast only
Hepatocyte growth factor (HGF)	120.95 ± 59.70	178.84 ± 88.33	0.68
Insulin-like growth factor 1 (IGF-1)	nd	nd	N/A
Interferon gamma (IFN-g)	0.10 ± 0.02	nd	Human MSC only
Interleukin 10 (IL-10)	0.17 ± 0.14	0.01 ± 0.02	14.3
Interleukin 11 (IL-11)	2.22 ± 1.21	nd	Human MSC only
Interleukin 13 (IL-13)	0.007 ± 0.009	0.000 ± 0.000	47.5
Interleukin 17 (IL-17)	nd	nd	N/A
Interleukin 1a (IL-1a)	0.040 ± 0.034	0.006 ± 0.009	7.0
Interleukin 2 (IL-2)	0.011 ± 0.009	0.000 ± 0.001	25.5
Interleukin 4 (IL-4)	0.15 ± 0.10	0.02 ± 0.05	6.2
Interleukin 6 (IL-6)	116.23 ± 54.02	0.14 ± 0.20	811**
Leukaemia inhibitory factor (LIF)	1.80 ± 0.90	nd	Human MSC only
Melatonin	37.91 ± 5.94	17.48 ± 10.49	2.2*
Nerve grown factor (NGF)	5.96 ± 2.39	0.34 ± 0.12	17.5**
Platelet derived growth factor AA (PDGF-AA)	19.60 ± 12.30	0.04 ± 0.03	523*
Platelet derived growth factor AB/BB (PDGF-AB/BB)	0.44 ± 0.29	0.003 ± 0.005	126*
Stem cell factor (SCF)	0.87 ± 0.21	2.41 ± 1.10	0.36*
Thrombospondin 1 (TSP-1)	143.6 ± 76.7 ng/ml	2.7 ± 0.5 ng/ml	52.3*
Transforming growth factor beta (TGF-β)	2.67 ± 1.91	0.64 ± 0.76	4.17
Tumour necrosis factor alpha (TNF-α)	0.015 ± 0.005	0.010 ± 0.004	1.4
Vascular endothelial growth factor (VEGF)	24.77 ± 7.04	13.87 ± 3.79	1.79

Reported concentrations were corrected for centrifuge filtration and represent levels in non-concentrated conditioned media. Concentrations represent the mean of measurements from at least three independent samples obtained from at least three independent experiments. Data are presented as mean ± SD. Mean concentrations were compared between cell types using two-tailed unpaired Student's *t*-tests: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; nd = not detected.

intraperitoneally and were placed in front of a slit-lamp equipped with a 532 nm diode laser, which delivered 0.7 W pulses for 0.6 s. Fifty to 60 laser pulses (50 μM diameter) were directed to the trabecular meshwork 360° around the circumference of the aqueous outflow area of the left eye. Animals were treated twice, 1 week apart. Contralateral fellow eyes served as untreated controls. Immediately before each of the two laser treatments, PDGF or vehicle control was locally administered through 3 μl intravitreal injection through the superior nasal retina using a 30 G needle on a 5 μl glass Hamilton syringe. Solutions for injection were masked, and the researchers were blinded to the treatment or control status of each animal until final analysis of the entire experiment had been completed. Care was taken to ensure that the lens was not damaged and that the retinal blood supply was not affected. Three different treatment groups were analysed: PBS only ($n = 15$); PDGF-AA in PBS (1.5 μg delivered at 0.5 μg/μl; $n = 15$); and PDGF-AB in PBS (1.5 μg delivered at 0.5 μg/μl; $n = 15$). Intraocular pressure was measured bilaterally under anaesthesia before and 24 h after each laser treatment, and then weekly thereafter using the TonoLab rebound tonometer (Tiolat Oy). Tonometry was performed within 5 min of anaesthesia onset and always between the hours of 9:00 am and 11:00 am. Cumulative intraocular pressure exposure for each eye was calculated as the integral of the intraocular pressure over the 4-week period beginning with the first laser treatment.

Histology and histological analyses

Retinal tissue was processed to quantify survival of RGC layer cell populations, as described previously (Bull *et al.*, 2011). Retinal explant tissue was fixed by overnight immersion in 4% paraformaldehyde at 4°C, followed by cryoprotection in 30% sucrose for 24 h at 4°C. Tissue was frozen and cryosectioned at 14 μm. Transverse sections were simultaneously blocked and permeabilized by incubation in 0.1% Triton™ and 5% normal goat serum in PBS (blocking solution) for 1 h at room temperature. Sections were incubated in primary antibody diluted in blocking solution overnight at 4°C, before incubation with secondary antibody diluted in blocking solution for 3 h at room temperature. Nuclei were counterstained with DAPI. Primary antibodies used were: mouse anti-Islet-1 (clone 39.4D5, 1:500, hybridoma developed by T. Jessell and S. Brenner-Morton, concentrate from the Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-NeuN (1:100, EMB Millipore), mouse anti-β-III-tubulin (1:2000, Promega), mouse anti-human nuclear antigen (1:500, EMB Millipore), rabbit anti-glial fibrillary acid protein (GFAP, 1:500,

Dako), and rabbit anti-phospho (Y754)-PDGF receptor alpha (1:50, Abcam). Alexa Fluor®-conjugated secondary antibodies were obtained from Life Technologies. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was carried out, with appropriate modification, according to the manufacturer's instructions (GenScript Inc.). Tissue was processed on microscope slides. Samples were washed twice in PBS and then permeabilized with 0.1% Triton™ X-100 plus 0.1% sodium citrate (Sigma) in water for 5 min at room temperature. Samples were incubated in TUNEL reaction mixture, consisting of equilibration buffer, biotin-11-dUTP, and terminal deoxynucleotidyl transferase for 60 min at 37°C. dUTP binding was detected by incubation with Alexa Fluor® 594-conjugated streptavidin (Life Technologies) for 1 h at room temperature.

Islet-1$^+$, NeuN$^+$, β-III-tubulin$^+$ and DAPI$^+$ cell densities in the RGC layer were quantified by a masked investigator in at least five sections per explant, spaced equally across the entirety of the tissue, as assessed by epifluorescence in fields imaged with a ×20 objective. TUNEL$^+$ cells in the RGCL were quantified by a masked investigator in 1/10 of the sections obtained for each explant (typically at least 12 sections), spaced equally across the entirety of the tissue, and the total number of TUNEL$^+$ cells per explant was extrapolated by multiplying the total number counted by 10. Experimental groups contained at least four retinal explants. Data are presented as mean ± standard error of the mean unless otherwise indicated. Comparisons between two groups were made using two-tailed unpaired Student's *t*-tests. Comparisons between three or more groups were made with one-way ANOVA and (if $P < 0.05$) *post hoc* two-tailed Dunnett *t*-tests compared experimental groups to the control group.

To assess optic nerves of animals with laser-induced glaucoma 4 weeks after the onset of ocular hypertension, animals were perfused transcardially with 4% paraformaldehyde under terminal anaesthesia. Optic nerves were immersed in 4% paraformaldehyde/5% glutaraldehyde in phosphate buffer for 7 days at 4°C, post-fixed in 1% osmium tetroxide for 3 h, dehydrated and embedded in Araldite® resin for semi-thin sectioning. Semi-thin (1 μm) transverse sections were cut from the nerve 2–3 mm distal to the globe, dried onto slides and stained with 1% toluidine blue. Loss of RGC axons in the optic nerves of glaucomatous eyes was quantified using an established semi-quantitative optic nerve grading scheme, as we have described in detail previously (Marina *et al.*, 2010). Briefly, zones of apparently homogenous damage were identified under light microscopy (×100 magnification) and the percentage contribution of each zone to total optic nerve cross-sectional area was determined. All optic nerves were masked throughout the entire analysis process so that observers performing quantification were unaware of the treatment or control status of the animal. A representative photograph within each zone was captured at ×630 magnification (representing 0.004 mm^2 total optic nerve area) and the number of axons within each sample image was quantified using an automated image analysis program (ImageJ, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD http://rsb.info.nih.gov/ij/index.html). The number of axons within each damage zone was compared to the count obtained from a sample image of the uninjured companion eye to estimate the percentage of axonal survival. A weighted average calculation was then used to estimate the percentage of surviving axons in the total optic nerve for each animal based on the proportion of the total optic nerve cross-sectional area that each damage zone represented. Comparisons between the three groups were made with one-way ANOVA and (if $P < 0.05$) *post hoc* two-tailed Dunnett *t*-tests compared each PDGF group to the PBS control group.

Results

Isolation and identification of rat mesenchymal stem cells

Rat MSCs were isolated and purified from bone marrow aspirates obtained from the femurs of adult transgenic Sprague Dawley rats engineered to express GFP under control of the chicken β-actin promoter. Flow cytometric analysis demonstrated GFP expression in 96.2% of rat MSCs, as well as expression of CD29, CD44, and CD90 but not CD34, CD45, CD11b, CD68, or CD11b (Supplementary Fig. 1). In addition, rat and human MSCs underwent appropriate differentiation into adipocytes and osteoblasts after culture in induction media (data not shown).

Retinal ganglion cell neuroprotection by co-culture with rat mesenchymal stem cells

Previously, we showed that co-culture of retinal explants with rat MSCs could prolong RGC survival (Bull *et al.*, 2011). The current work expanded on those experiments to characterize further the ability of rat MSCs to protect RGCs from cell death. Rat MSCs were co-cultured on the inner retinal (vitreous) surface of organotypic retinal explants for 7 days. GFP expression allowed tracking of the rat MSCs within the co-cultures and revealed that rat MSCs did not migrate into the host neural retinal tissue, but instead remained as a multi-laminar structure outside of the retinal inner limiting membrane (Fig. 1), which is consistent with observations that were previously reported (Johnson and Martin, 2008; Bull *et al.*, 2011). Quantification of cell survival within the RGC layer of retinal explants demonstrated that rat MSC co-culture was associated with a significant attenuation of RGC death at 7 days, compared to without cell co-culture. The number of total surviving cells (labelled with DAPI) increased from 103 ± 4 to 213 ± 8 cells/mm ($P < 0.001$, Fig. 1I). To avoid quantification of astrocytes, microglia, and endothelial cells, which might be expected to survive more readily in explant culture given that they did not undergo direct cellular injury (axotomy) during the explantation process, neurons were immunofluorescently labelled with antibodies directed against Islet-1 and NeuN. Quantification confirmed a significant increase in the survival of both Islet-1$^+$ (13.9 ± 0.9 versus 41.5 ± 1.6 cells/mm, $P < 0.001$, Fig. 1A, B and I) and NeuN$^+$ (58.1 ± 2.0 versus 127.3 ± 8.3 cells/mm, $P < 0.001$, Fig. 1C, D and I) neurons in the RGC layer. Quantification of β-III-tubulin$^+$ neurons in the RGC layer also demonstrated a comparable level of neuroprotection (17.6 ± 1.1 versus 29.7 ± 3.7 cells/mm, $P < 0.05$, Fig. 1E–I). Although β-III-tubulin is a more specific marker for RGCs than either NeuN or Islet-1, immunoreactivity of neurite processes in addition to cell bodies made the precise quantification of these cells more difficult and potentially less accurate in transverse histological sections; for this reason, further experiments focused on quantification of nuclear/perinuclear antigens. In accordance with increased cell survival, rat MSC co-culture also reduced apoptosis in the RGC layer, as assessed by TUNEL, from 453 ± 70 to 127 ± 2 cells/

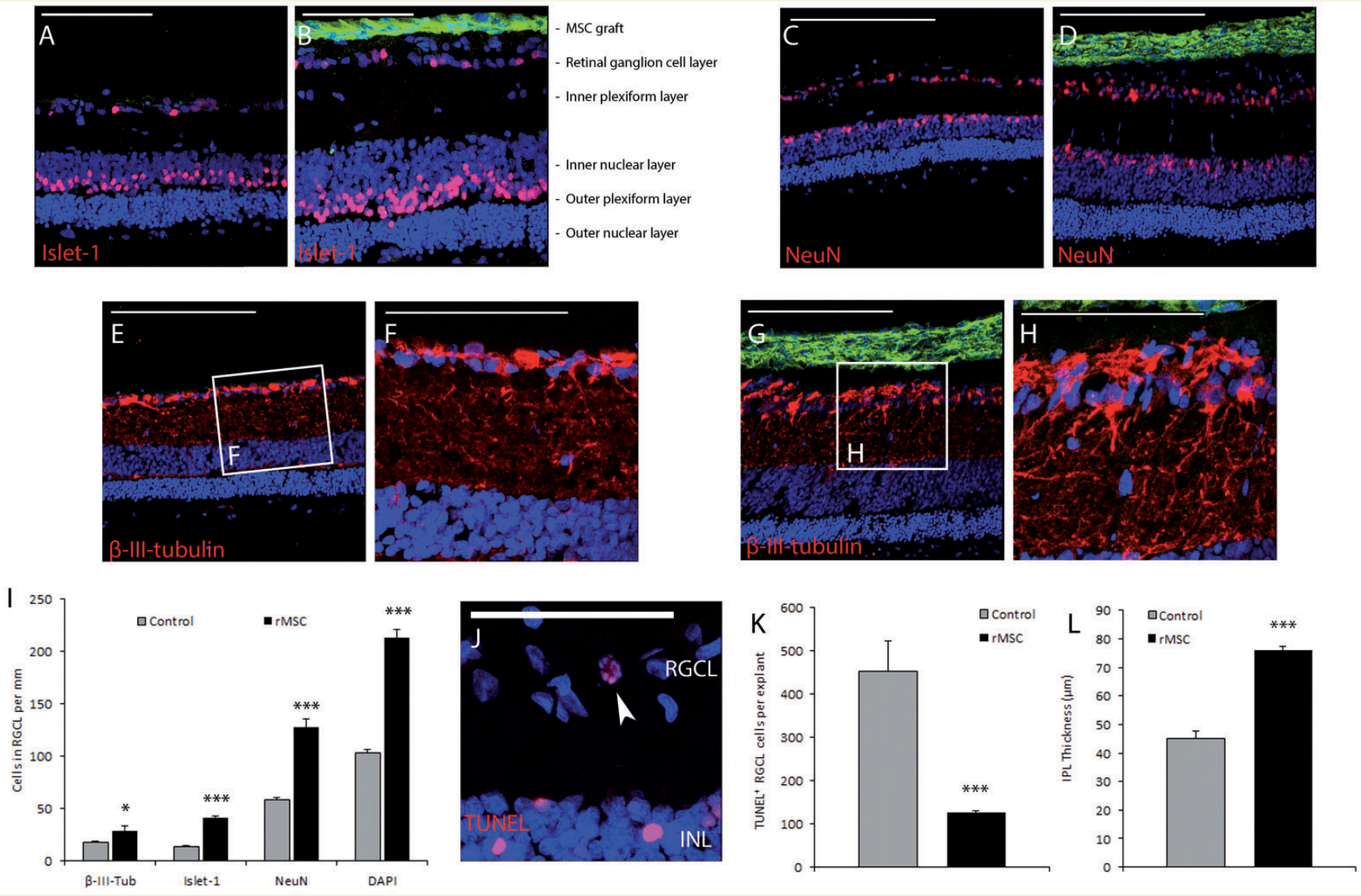

Figure 1 Retinal ganglion cell neuroprotection by rat mesenchymal stem cells. Green fluorescent protein-expressing rat MSCs (rMSC, green) were co-cultured on the RGC surface of retinal explants for 7 days (**B**, **D**, **G**, **H**). All nuclei in the RGC layer (RGCL) were visualized by DAPI staining (blue). RGC layer neurons were visualized by Islet-1 (**A** and **B**) and NeuN (**C** and **D**), or β-III-tubulin (**E–H**) immunofluorescence. RGC axons and dendrites were visualized with β-III-tubulin immunofluorescence (**E–H**). TUNEL was carried out and quantified as described (**J** and **K**). Arrowhead: TUNEL$^+$ karyorrhexic RGCL nucleus. Scale bars: **A**, **B**, **F**, **H** = 100 μm; **C**, **D**, **E**, **G** = 200 μm; **J** = 50 μm. White boxes in **E** and **G** shown in higher magnification in **F** and **H**, respectively. Quantification of neuronal survival in the RGC layer (**I**). Inner plexiform layer (IPL) thickness was measured in three areas per section, and in at least five sections per explant (**L**). $*P < 0.05$; $***P < 0.001$. Error bars represent standard error of the mean. Control indicates without cell co-culture.

explant ($P < 0.001$, Fig. 1J and K). Most TUNEL$^+$ nuclei were also pyknotic or karyorrhexic (Fig. 1J). In addition to increased cell soma survival, rat MSC co-culture was associated with enhanced preservation of RGC axons and dendrites. Qualitatively, retinal explants possessed a thicker nerve fibre layer and more discernible dendrites in the inner plexiform layer (Fig. 1E–H). Quantitatively, the inner plexiform layer thickness of rat MSC-treated explants increased from 45.2 ± 2.6 to $76.1 \pm 1.2\,\mu m$ ($P < 0.001$, Fig. 1L). Of note, other layers of the neural retina including the inner nuclear layer and outer nuclear layer appeared to be thicker in the context of rat MSC co-culture.

Retinal ganglion cell neuroprotection by co-culture with other cell types

Rat retinal explants were co-cultured with human MSC grafts to determine whether they could also alleviate RGC death. Similar to observations with rat MSCs, human MSCs remained as a bolus on the surface of the host neural retina and did not migrate into the explant (Fig. 2A and B). Quantification of cell density in the RGC layer demonstrated that human MSC co-culture was associated with increased survival of Islet-1$^+$ (17.4 ± 1.5 versus 36.6 ± 2.1 cells/mm, $P < 0.001$) and NeuN$^+$ (47.1 ± 2.3 versus 89.7 ± 4.4 cells/mm, $P < 0.001$) neurons compared to without cell co-culture (Fig. 2C). The specificity of this neuroprotective capacity was evaluated by co-culturing retinal explants with human fibroblasts or a cell line derived from mouse embryonic fibroblasts (NIH-3T3 cells). Co-culture with either of these two cell types resulted in only a modest increase in Islet-1$^+$ neuronal survival (21.3 ± 0.8 versus 24.3 ± 0.9 cells/mm for human fibroblasts, $P < 0.01$) and no other signs of neuroprotection compared to without cell co-culture (Fig. 2D and E). With only weak to no neuroprotective effects observed with fibroblast co-culture, it was concluded that the robust neuroprotective properties exhibited by MSCs from multiple species are not shared by all cell types.

Retinal ganglion cell neuroprotection by mesenchymal stem cell-conditioned media

Based on observations that MSCs secrete relatively high levels of signalling factors that could play a role in neuroprotection, the effects of media conditioned by 3 days of rat or human MSC culture were evaluated. Initial experiments demonstrated no detectable effects on RGC survival when rat MSC conditioned media was added to retinal explant culture media without further refinement

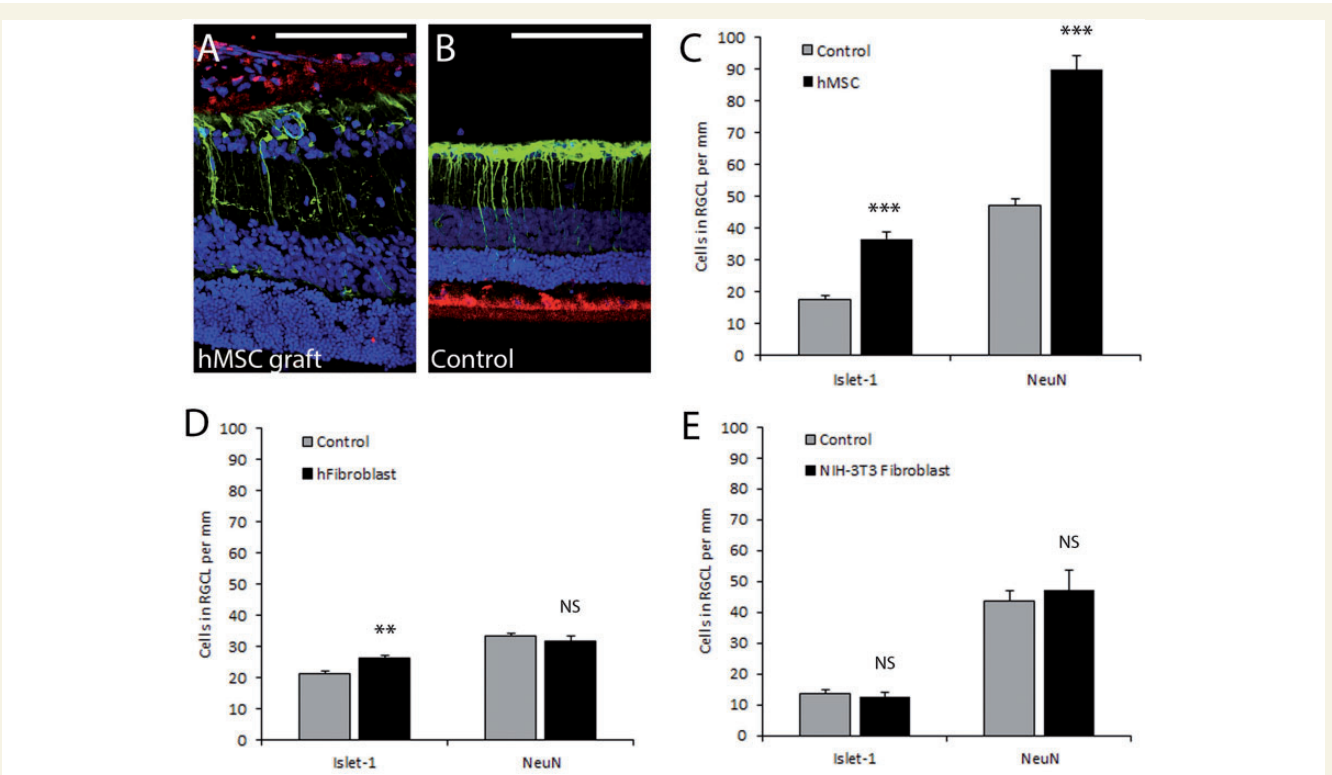

Figure 2 Retinal ganglion cell neuroprotection by human mesenchymal stem cells. Human mesenchymal stem cells (hMSCs), human fibroblasts (hfibroblast), or mouse embryonic fibroblasts (NIH-3T3) were co-cultured on the retinal ganglion cell (RGC) surface of retinal explants for 7 days. Human MSCs were visualized by anti-human nuclear antigen immunofluorescence (**A** and **B**, red). Glial cells were visualized with glial fibrillary acid protein (GFAP) immunofluorescence (**A** and **B**, green). Müller glial endplate processes at the inner limiting membrane delineated the boundary of the neural retina. Red fluorescence at the outer retina (**B**) represents autofluorescence. All nuclei were stained with DAPI. Scale bars: **A** and **B** = 100 μm. Quantification of neuronal survival in the RGC layer (**C–E**). $^{**}P < 0.01$; $^{***}P < 0.001$; NS = not significant. Error bars represent standard error of the mean. Control indicates without cell co-culture.

(data not shown). We hypothesized that secreted factors may have been diluted to subtherapeutic concentrations in conditioned media whereas paracrine signals from MSCs situated in near proximity to RGCs reach higher concentrations in experiments with cell co-culture. Therefore, filter centrifugation was used to concentrate rat MSC conditioned media by 25–35-fold. Addition of concentrated rat MSC conditioned media to the explant culture media (1:133) and daily placement of a 2.5 µl bolus of concentrated rat MSC conditioned media directly onto the inner retinal surface of the explants resulted in significant neuroprotection of Islet-1$^+$ (13.7 ± 0.6 versus 21.9 ± 0.3 cells/mm, $P < 0.001$) and NeuN$^+$ (66.9 ± 3.0 versus 79.6 ± 1.9 cells/mm, $P < 0.01$) neurons compared to similar treatment with concentrated, unconditioned DMEM (Fig. 3A). Thus, the neuroprotective effect of MSC-derived factors appeared to be concentration-dependent.

The neuroprotective potentials of concentrated (25–55-fold) human MSC and human fibroblast conditioned media obtained were also investigated. Whereas human fibroblast conditioned media had no effect on RGC survival in retinal explants, factors secreted from human MSCs demonstrated a modest but statistically significant increase in Islet-1$^+$ neuron density in the RGC layer (24.5 ± 1.6 versus 30.6 ± 1.4 cells/mm, $P < 0.05$, Fig. 3B) compared to treatment with concentrated, unconditioned DMEM.

To determine whether a particular fraction of the MSC secretome could be identified as accounting for the RGC neuroprotection observed, human MSC conditioned media was fractionated using fast protein liquid chromatography. Three fractions each contained protein (Fig. 3C) and were used to treat retinal explants. Only fraction 1 demonstrated neuroprotective activity, with an increase in Islet-1$^+$ neurons from 24.6 ± 1.3 to 36.4 ± 2.6 cells/mm ($P < 0.05$) and an increase in NeuN$^+$ neurons that did not reach statistical significance (56.1 ± 8.8 versus 65.5 ± 2.6 cells/mm, $P = 0.3$, Fig. 3D) compared to treatment with concentrated, unconditioned DMEM. It was notable that RGC cell survival was generally greater when rat MSCs or human MSCs were co-cultured directly on the explant surface as compared to treatment with rat MSC or human MSC conditioned media, or fractions thereof.

Analysis of the human mesenchymal stem cell secretome and identification of neuroprotective components

A clear effect on RGC survival of factors secreted by MSCs into conditioned media prompted a careful analysis of the human MSC secretome, focusing specifically on proteins with

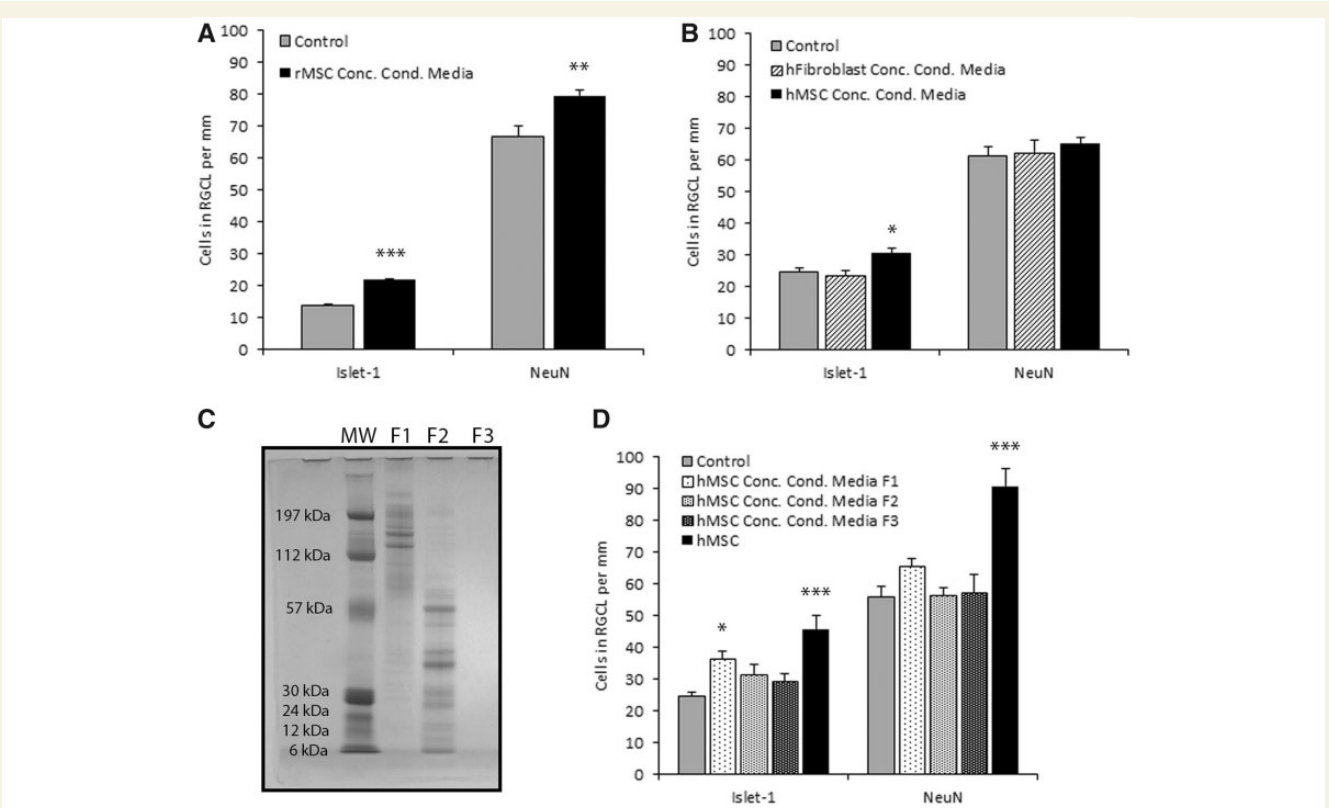

Figure 3 Retinal ganglion cell neuroprotection by mesenchymal stem cell conditioned media. DMEM was conditioned by 72–96 h incubation with rat or human mesenchymal stem cells (hMSCs) or human fibroblasts. Control DMEM was unconditioned. Conditioned media was then concentrated 25 to 55-fold by centrifuge filtration. Concentrated rat MSC (**A**) or human MSC (**B**) conditioned media was added to retinal explant media and to the retinal ganglion cell (RGC) surface of retinal explants daily for 7 days. Neuronal survival in the RGC layer was quantified. Human MSC conditioned media was fractionated by fast protein liquid chromatography (**C**). Retinal explants were treated with individual human MSC conditioned media fractions (F1, F2, F3) or with human MSC co-culture as a positive control, and RGC survival was quantified (**D**). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Error bars represent standard error of the mean.

known or purported effects on neuronal survival. Also analysed were factors involved in regulating inflammation, as MSCs possess well-documented immunomodulatory properties that have been proposed to play a role in neuroprotection in other models. Since human fibroblast conditioned media demonstrated no effect on RGC survival, it was used as a negative control. Conditioned media from these two cell cultures were analysed using multiplexed xMAP® factor arrays and ELISAs. The results are presented in Table 1. Eleven proteins were identified that were secreted at significantly higher levels by human MSCs as compared to human fibroblasts including brain-derived neurotrophic factor (BDNF), interferon gamma, interleukin (IL)-11, IL-6, leukaemia inhibitory factor (LIF), melatonin, nerve growth factor (NGF), platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB, stem cell factor, and thrombospondin 1.

Further experiments explored the effect on RGC survival of a subset of these proteins (BNDF, IL-6, LIF, melatonin, NGF, PDGF-AA, PDGF-AB, and thrombospondin 1) as well as pigment epithelium-derived factor (PEDF), IL-2, IL-13, secreted protein acidic and rich in cysteine (SPARC, also known as osteonectin), and galectin-1, which are secreted by MSCs and may play a role in neuronal survival or regeneration (Sholl-Franco *et al.*, 2001; Bampton *et al.*, 2005; Okada *et al.*, 2005; Yabe *et al.*, 2010). First, retinal explants were cultured in standard media with the addition of purified proteins as a cocktail at previously reported bioactive concentrations (Table 2). Treatment with this secreted factor cocktail significantly increased survival of Islet-1$^+$ (28.7 ± 2.1 versus 37.2 ± 1.8 cells/mm, $P < 0.05$, Fig. 4A) and NeuN$^+$ (79.5 ± 6.6 versus 103.5 ± 4.5 cells/mm, $P < 0.05$, Fig. 4B) neurons compared to vehicle treatment. The cocktail was then split into subgroups based on signalling mechanism/molecular family, along with two residual 'miscellaneous' subgroups composed of molecules that did not fit clearly into the other subgroups (Table 3). Treatment of retinal explants with subgroups of purified protein revealed that several groups conferred protection to RGCs (Fig. 4C and D). The only subgroup that was not associated with increased RGC survival was that composed of IL-6, IL-11, and LIF. In contrast, the subgroup consisting of PDGF-AA, PDGF-AB, PEDF, and IL-2 was associated with the greatest level of RGC survival compared to vehicle treatment (Islet-1: 23.1 ± 0.6 versus 37.3 ± 2.0 cells/mm, $P < 0.001$; NeuN: 34.9 ± 3.5 versus 59.3 ± 2.3 cells/mm, $P < 0.001$), similar to that achieved with human MSC co-culture (Fig. 4C and D). Finally, retinal explants were treated with each factor in the PDGF subgroup individually. This revealed that PDGF-AA and PDGF-AB alone protected RGCs to roughly the same extent as the complete subgroup of four factors, whereas IL-2 and PEDF alone were not associated with significant changes in RGC survival (Fig. 4E and F). Treatment of retinal explants with a combination of PDGF-AA and PDGF-AB revealed dose-dependent RGC neuroprotection (Fig. 4G).

Given that PDGF seemed to confer neuroprotection to RGCs in retinal explant culture and was highly secreted by human MSCs, immunohistochemistry was used to identify PDGF receptor activation in RGCs under various conditions. In untreated explants, few RGCs were immunopositive for phosphorylated (activated) PDGF receptor (Fig. 5A). After co-culture with human MSCs (Fig. 5B and E) or treatment with purified PDGF protein (Fig. 5C), marked PDGF receptor activation was observed in retinal neurons co-labelled with antibodies against NeuN. Activation of the PDGF receptor could also be found co-localized with RGCs expressing β-III-tubulin (Fig. 5F). In addition, PDGF receptor activation was observed outside of the RGC layer, likely in retinal astrocytes or Müller cell processes (Fig. 5F). Interestingly, strong PDGF receptor activation also took place within the human MSC graft, suggesting that PDGF signalling may play a role in human MSCs in co-culture. Addition of a soluble anti-PDGF antibody blocked the PDGF receptor activation that occurred in RGCs in response to treatment with purified PDGF protein (Fig. 5D). The relationship between PDGF receptor activation and RGC survival was assessed by blocking PDGF signalling. Incubation with anti-PDGF neutralizing antibody accelerated RGC death in untreated retinal explant cultures, and also completely blocked the neuroprotective effects of both human MSC co-culture and treatment with purified PDGF protein (Fig. 5G–I). In contrast, non-specific species-matched IgG had no

Table 2 Composition of secreted factor cocktail

Factor name	Final concentration (ng/ml)	Vendor source
Brain-derived neurotrophic factor (BDNF)	50	Sigma #B3795
Galectin 1	10	PeproTech #450-39
Interleukin 13 (IL-13)	50	PeproTech #200-13
Interleukin 2 (IL-2)	25	PeproTech #200-02
Interleukin 6 (IL-6)	50	PeproTech #200-06
Leukaemia inhibitor factor (LIF)	50	Sigma #L5283
Melatonin	100	Sigma #M5250
Nerve growth factor (NGF)	50	PeproTech #450-01
Pigment epithelium derived factor (PEDF)	50	PeproTech #130-13
Platelet derived growth factor (PDGF)-AA	50	PeproTech #100-13A
Platelet derived growth factor (PDGF)-AB	50	PeproTech #100-00AB
Secreted protein acidic and rich in cysteine (SPARC)	25	PeproTech #120-36
Thrombospondin 1 (TSP-1)	100	Sigma #SRP4805

Representative data from experiments comparing treatment of retinal explants with secreted factor cocktail versus vehicle are presented in Fig 4A and B. Indicated factors were obtained in purified protein form and added at the indicated concentrations to retinal explant culture media.

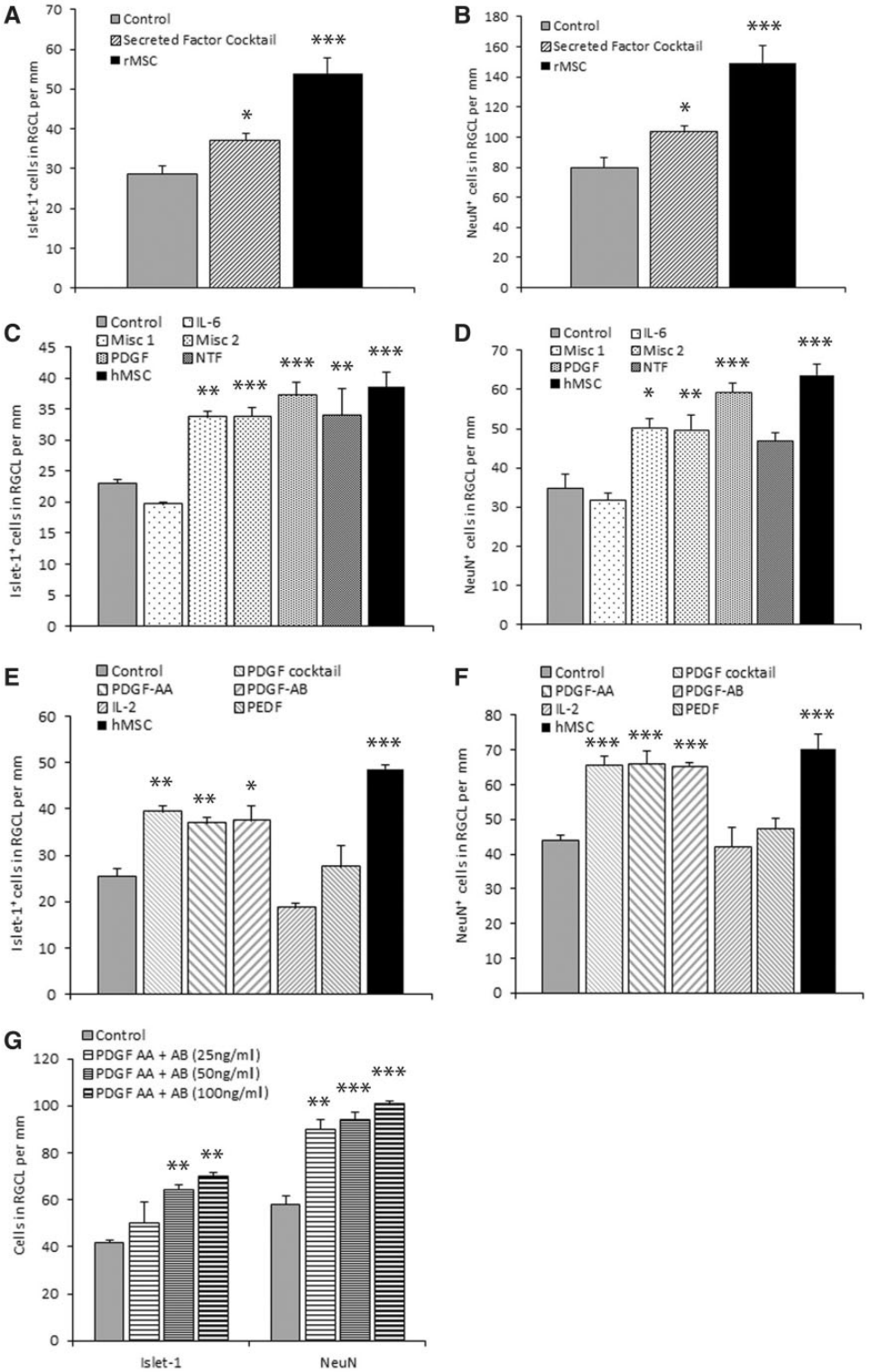

Figure 4 Retinal ganglion cell neuroprotection by factors enriched in the MSC secretome. Retinal explants were cultured using standard protocols with or without the addition of various purified proteins or vehicle (control) to the culture media. A cocktail of 13 factors detailed in Table 2 was added to the culture media (**A**, **B**). Factors were sub-divided as indicated in Table 3 and added to the culture media (**C**, **D**). Factors in the PDGF group were further sub-divided and added to the culture media at the same final concentrations as indicated in Table 3 (**E**, **F**). The dose-dependence of PDGF neuroprotection was evaluated by adding each of these factors at the indicated concentration to the culture media (**G**). Treatment with rat MSC (rMSC) or human MSC (hMSC) co-culture was used as a positive control for comparison. Neuronal survival in the RGC layer was quantified. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Error bars represent standard error of the mean.

Table 3 Composition of secreted factor subgroups

Group name	Factors in group	Final concentration (ng/ml)	Vendor source
IL-6	Interleukin 6 (IL-6)	50	PeproTech #200-06
	Interleukin 11 (IL-11)	50	PeproTech #200-11
	Leukaemia inhibitory factor (LIF)	50	Sigma #L5283
Misc 1	Melatonin	100	Sigma #M5250
	Thrombospondin 1 (TSP-1)	100	Sigma #SRP4805
	Secreted protein acidic and rich in cysteine (SPARC)	25	PeproTech #120-36
	Galectin-1	10	PeproTech #450-39
Misc 2	Interleukin 13 (IL-13)	50	PeproTech #200-13
	Interferon gamma	50	PeproTech #300-02
PDGF	Platelet derived growth factor (PDGF)-AA	50	PeproTech #100-13A
	Platelet derived growth factor (PDGF)-AB	50	PeproTech #100-00AB
	Pigment epithelium derived factor (PEDF)	50	PeproTech #130-13
	Interleukin 2 (IL-2)	25	PeproTech #200-02
NTF	Brain derived neurotrophic factor (BDNF)	50	Sigma #B3795
	Nerve growth factor (NGF)	50	PeproTech #450-01

Representative data from experiments comparing treatment of retinal explants with secreted factor subgroups versus vehicle are presented in Fig. 4C and D. Indicated factors were obtained in purified protein form and added at the indicated concentrations to retinal explant culture media. Proteins in the IL-6 group signal through the gp130 receptor complex. Proteins in the PDGF group signal through, among many pathways, PI3 kinase. Proteins in the neurotrophic factor (NTF) group signal through Trk receptors.

effect on human MSC-mediated neuroprotection (Fig. 5G). Furthermore, suppression of PDGF signalling using a small molecule inhibitor of PDGF receptor kinase, AG-1296, had little effect on RGC survival in untreated explant cultures but attenuated the neuroprotective effects of both human MSC co-culture and treatment with purified PDGF protein (Fig. 5J and K). Similar results were obtained using a small molecule inhibitor of PI3 kinase (Fig. 5L and M), which lies downstream in the PDGF receptor signalling cascade (Fantl *et al.*, 1992).

Platelet-derived growth factor-mediated retinal ganglion cell neuroprotection in an *in vivo* glaucoma model

Having demonstrated that PDGF-AA and PDGF-AB confer robust neuroprotection to RGCs in a retinal explant culture model, it was hypothesized that these proteins would be similarly protective to the optic nerve in an *in vivo* model of RGC neurodegeneration. To test this, ocular hypertension was induced in one eye of adult rats by laser photocoagulation of the trabecular meshwork while the fellow eye served as a control. Laser treatments were administered twice, spaced 1 week apart, and in both cases immediately preceded by intravitreal injections of PDGF-AA (1.5 μg, $n = 15$), PDGF-AB (1.5 μg, $n = 15$), or PBS as a vehicle control (3 μl, $n = 15$). Animals were sacrificed 4 weeks following the initial induction of ocular hypertension and optic nerves were processed to quantify RGC axonal survival. Tonometry demonstrated a strong, consistent, and transient rise in intraocular pressure induced by laser treatment compared with fellow untreated eyes (Fig. 6A-C). Peak intraocular pressure, mean intraocular pressure, and cumulative integral intraocular pressure exposure (expressed as mmHg × days) were significantly greater in treated eyes compared with fellow controls in all three treatment groups ($P < 0.001$ for all comparisons, Fig. 6D–F). The peak intraocular pressures (Fig. 6D), mean intraocular pressures (Fig. 6E) and cumulative integral intraocular pressure exposures (Fig. 6F) of each of the two PDGF groups were statistically similar to those of the PBS control group for ocular hypertension eyes ($P > 0.05$). PBS treatment was associated with a survival of $68.2 \pm 4.4\%$ of RGC axons at 4 weeks after the initial laser treatments, whereas treatment with PDGF-AA was associated with increased RGC axonal survival to $93.6 \pm 2.9\%$ ($P < 0.001$) and PDGF-AB with increased RGC axonal survival to $96.7 \pm 2.0\%$ ($P < 0.001$, Fig. 6G). To account for variability in intraocular pressure elevation between animals and the fact that the PDGF-AB group experienced overall greater intraocular pressure exposure than the PBS control group, RGC axonal loss was normalized to cumulative excess intraocular pressure exposure (RGC axonal loss divided by integral intraocular pressure exposure in the ocular hypertension eye minus integral intraocular pressure exposure in the control eye). RGC axons were lost at a rate of 2.9×10^{-3} per cent/mmHg × day with PBS treatment, whereas the rate of RGC axonal loss was reduced to 9.3×10^{-4} per cent/mmHg × day by PDGF-AA treatment ($P < 0.05$) and to 2.9×10^{-4} per cent/mmHg × day by PDGF-AB treatment ($P < 0.001$, Fig. 6H). Representative photomicrographs from optic nerves of animals treated with PBS (Fig. 6I), PDGF-AA (Fig. 6J), and PDGF-AB (Fig. 6K) are shown.

Discussion

The present study demonstrates that MSCs derived from both human and rat bone marrow possess a potent neuroprotective capacity capable of enhancing RGC survival in retinal explant culture. Moreover, our comparison of more than 29 factors in the secretomes of human MSCs and human fibroblasts identified PDGF as a particularly potent neuroprotectant molecule that was highly secreted by MSCs. In fact, the neuroprotective effect observed in the present study by intravitreal administration of

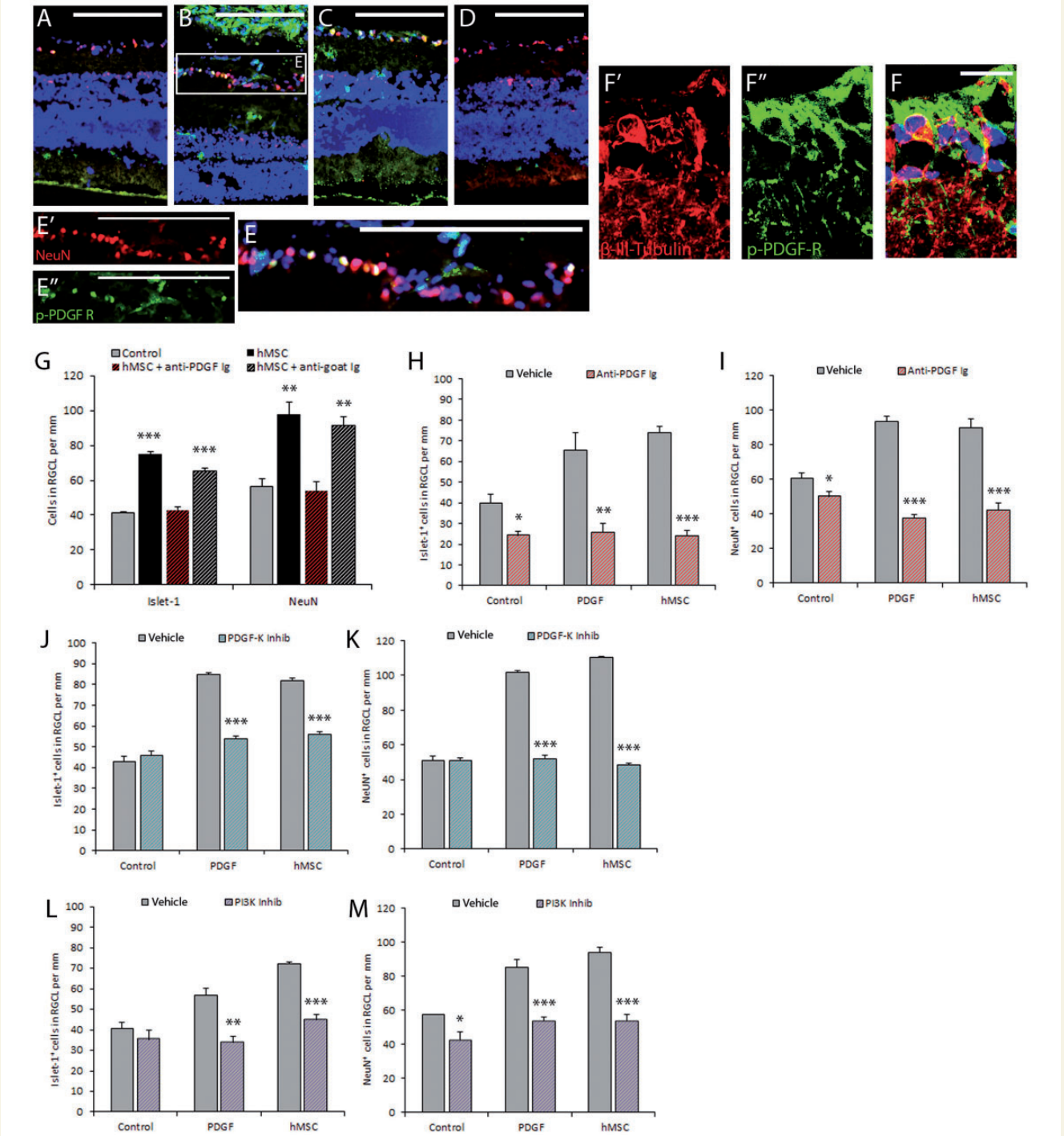

Figure 5 Role of PDGF signalling in retinal ganglion cell neuroprotection by MSCs. Retinal explants were cultured alone (**A**); with human MSCs (**B, E** and **F**); with PDGF-AA + PDGF-AB (50 ng/ml) added to the culture media (**C**); or with PDGF-AA + PDGF-AB (50 ng/ml) plus anti-PDGF IgG (35 μg/ml) added to the culture media (**D**). Retinal ganglion cell (RGC) layer neurons were visualized with NeuN (**A–E**) or β-III-tubulin (**F**) immunofluorescence (red) and PDGF signalling was detected with phospho-PDGF receptor immunofluorescence (green). Nuclei were visualized with DAPI (blue). Higher magnification image of the RGC layer in **B** is depicted as individual channels in **E′** and **E″** and merged in **E**. Scale bars: **A–E** = 200 μm; **F** = 25 μm. Retinal explants were cultured alone, or with the addition of PDGF-AA + PDGF-AB (50 ng/ml) to the culture media, or with human MSC co-culture for 7 days. Half of the explants from each group were additionally treated with non-specific anti-goat IgG (35 μg/ml, **G**); anti-PDGF IgG (35 μg/ml, **G–I**); small molecular inhibitor of PDGF receptor kinase, AG1296 (45 μM, **J–K**); or small molecule inhibitor of PI3 kinase, LY294002 (75 μM, **L–M**). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Error bars represent standard error of the mean. Control indicates without cell co-culture or addition of PDGF to growth media.

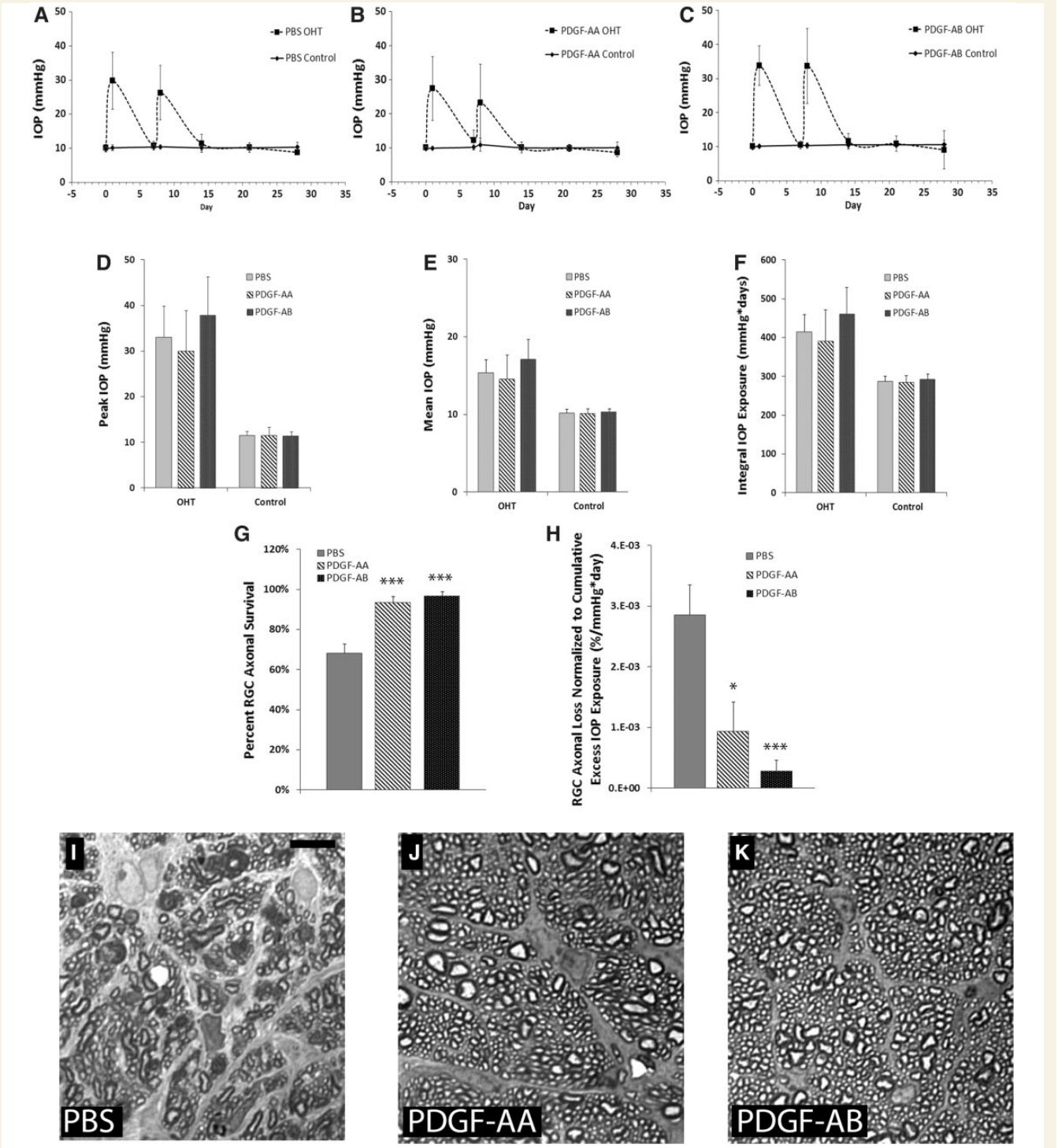

Figure 6 Optic nerve protection by PDGF treatment in an *in vivo* glaucoma model. Unilateral ocular hypertension (OHT) was induced by laser trabecular meshwork photocoagulation on Days 0 and 7 of the experiment, immediately preceded by intravitreal injection of PBS (3 µl, $n = 15$), PDGF-AA (1.5 µg, $n = 15$), or PDGF-AB (1.5 µg, $n = 15$). Intraocular pressure (IOP) measurements over time are shown in **A** (PBS), **B** (PDGF-AA), and **C** (PDGF-AB). Peak intraocular pressure (IOP; **D**), mean intraocular pressure (**E**), and cumulative integral intraocular pressure exposure (**F**) were calculated for ocular hypertension and control eyes in each group. Optic nerve survival was quantified as per cent surviving RGC axons in the ocular hypertension optic nerve as compared to the contralateral control eye (**G**). The rate of RGC axon loss normalized to cumulative excess intraocular pressure exposure is shown in **H**. Representative photomicrographs of optic nerve cross sections are shown for treatment with PBS (**I**), PDGF-AA (**J**), and PDGF-AB (**K**). $*P < 0.05$, $***P < 0.001$, compared to the PBS control group. Error bars for intraocular pressure data represent standard deviation. Error bars for RGC survival data represent standard error of the mean. Scale bar = 10 µm.

PDGF protein in rats with laser-induced ocular hypertension was extremely robust, and even stronger than that seen following intravitreal grafting of rat MSCs derived from two different strains of rat, where RGC axonal survival in the context of a comparable level of injury increased from 74.7% to 92.1% and from 61.7% to 89.3% (Johnson *et al.*, 2010). Interestingly, the current study also identified a necessity for PDGF signalling in the mechanism of action of MSC-mediated neuroprotection, such that blockade of PDGF signalling or downstream pathways eliminated the beneficial effect of the MSCs.

The present experiments extend the findings published in previous studies. Yu *et al.* (2006) noted a 16% increase in RGC survival at 12 weeks following induction of intraocular pressure elevation by episcleral vein ligation when MSCs were injected into the vitreous at Week 8. Li *et al.* (2009) reported a 33% increase in RGC survival at 4 weeks after ischaemia/reperfusion injury to the retina with concurrent MSC transplantation. We previously showed that intravitreal transplantation of rat MSCs at the time of ocular hypertension induction by laser photocoagulation of the trabecular meshwork leads to a >60% reduction in the loss of RGC axons in the optic nerve (Johnson *et al.*, 2010). MSCs derived from umbilical cord blood also appear to confer neuroprotection to RGCs following optic nerve crush injury (Zhao *et al.*, 2011) and optic tract transection (Zwart *et al.*, 2009). Moreover, we previously reported on the effects of rat MSC co-culture with organotypic retinal explants and, in agreement with the current study, observed significant increases in RGC survival at both 4 and 7 days *ex vivo* (Bull *et al.*, 2011). Some studies have attempted to delineate the mechanism by which MSC-mediated RGC neuroprotection occurs, and in most cases have focused on the elaboration of various neurotrophic factors. Factors found to be expressed by MSCs *in vivo* or upregulated in the eye after MSC transplantation, at the messenger RNA or protein levels, include BDNF, NGF, CNTF, GDNF, NT-3, HGF, b-FGF, and TGF-β (Yu *et al.*, 2006; Li *et al.*, 2009; Zwart *et al.*, 2009; Zhao *et al.*, 2011). However, in most cases the number of factors evaluated was relatively narrow, the direct effects of many of these factors on RGC survival were not evaluated, and the necessity of these factors in conferring RGC protection by MSCs was not tested. Each of those limitations was addressed by the present study.

The MSC secretome is being increasingly recognized as a potential source of factors capable of protecting patient tissues throughout the body in the context of insult, injury or disease. The relevance of MSC-derived factors to ischaemic heart disease was recently reviewed (Ranganath *et al.*, 2012). It has also been shown that intravenous administration of MSC conditioned media to rats with experimental kidney disease improves renal function (van Koppen *et al.*, 2012). Although MSCs secrete an extremely large number of factors at varying concentrations that likely exert wide-ranging bioactive effects, attempts at characterizing the MSC secretome have led to the identification of specific molecules that might be relevant to treating various pathological processes. Liquid chromatography tandem mass spectrometry identified the protein Cyr61 as being highly secreted by MSCs, and elaboration of this factor accounted for the angiogenic effects of MSC conditioned media *in vivo*, which could prove useful in promoting wound healing (Estrada *et al.*, 2009). The same method identified prosaposin as being highly secreted by a subset of beta-III-tubulin-expressing mouse MSCs cultured under specific conditions; prosaposin was subsequently found to reduce apoptosis in neuronal cultures stressed with 6-hydroxydopamine (Li *et al.*, 2010). Antibody array screening of MSC conditioned media, combined with fractionation based on heparin binding affinity, was used to generate an MSC-derived chemokine cocktail effective in dampening the inflammatory process in hepatic failure (Parekkadan *et al.*, 2007). Thus, continued research aimed at fully characterizing the MSC secretome and evaluating the bioactive effects of its constituents could prove useful to the clinical translation of therapies for many different sorts of pathology, either by identifying the mechanisms by which MSC transplantation confers benefit or by identifying molecules that could be delivered independently of MSC transplantation to treat disease.

The PDGF family contains four members, PDGF-A, -B, -C, and -D that homodimerize, or in the case of PDGF-AB, heterodimerize. Secreted PDGF dimers signal through receptor tyrosine kinases consisting of homo- or heterodimers of PDGF receptor α and β. PDGFs are known to play a significant role in development and also in the maintenance and pathology of various mesenchymal tissues (Andrae *et al.*, 2008). In addition, PDGFs appear to play a role in the susceptibility of CNS neurons to cell death after insult. For example, knock out of PDGF receptor β in neurons makes them more susceptible to excitotoxic death (Ishii *et al.*, 2006). Administration of PDGF-B confers neuroprotection after NMDA administration whereas knockdown of PDGF-B exacerbates neuronal death (Egawa-Tsuzuki *et al.*, 2004). More specifically, PDGFs may play a role in the survival and degeneration of RGCs. PDGF-CC has been identified as a potent neuroprotective molecule for RGCs, which reduces RGC death after axotomy by modulating GSK3β signalling (Tang *et al.*, 2010). PDGF receptors α and β are expressed in the RGC layer and their activation leads to downstream signalling through the PI3 kinase/Akt pathway (Biswas *et al.*, 2008). PDGF-B is expressed by RGCs and its level declines after optic nerve transection, which has been hypothesized to play a role in RGC death after injury (Mekada *et al.*, 1998). Finally, treatment with PDGF-AA has been shown to attenuate oxidative stress-induced cell death in RGC-5 cells via PI3 kinase signalling (Kanamoto *et al.*, 2011). The results of the present study extend these results by demonstrating that PDGF-AA and PDGF-AB confer neuroprotection to RGCs in the context of axotomy and *ex vivo* culture in a PI3 kinase-dependent manner. Moreover, we have now also demonstrated that intraocular injection of PDGF-AA or PDGF-AB protects the optic nerve in an *in vivo* model of experimental glaucoma. Thus, while the neuroprotective effects of PDGF family members have been demonstrated in other contexts previously, the present results certainly suggest that PDGF signalling may represent a target for RGC neuroprotection in glaucoma that should be explored further. In addition, to our knowledge, these data are the first to identify PDGF signalling as a key player in the mechanism of MSC-mediated neuroprotection.

PDGF is also known to play an important role in MSC proliferation (Qiu *et al.*, 2013) and differentiation, particularly into osteoblasts and chondrocytes (Ng *et al.*, 2008). Furthermore,

PDGF signalling is important for maintaining the commitment of MSCs to mesenchymal lineage and blockade of PDGF signalling increases the multipotentiality of these cells (Ball *et al.*, 2012). In the present study, we observed strong activation of PDGF receptors in MSCs co-cultured with retinal explants, suggestive that PDGF signalling in MSCs could play an important role in the retinal neuroprotective effects observed after co-culture. It should be noted that although loss of RGC neuroprotection was seen following blockade of PDGF signalling in retinal explant/MSC co-cultures, it is unclear whether this effect was mediated specifically by reduced PDGF signalling in RGCs, in MSCs, or a combination of the two. It is possible that blockade of PDGF signalling in the MSC graft altered its phenotype such that additional neuroprotective MSC properties (beyond PDGF delivery to RGCs) were modified. Indeed, identification of high secretion levels of numerous factors by MSCs, which independently conferred RGC protection, suggests that PDGF delivery by MSCs is likely not the sole mechanism underlying the observed neuroprotection.

We have also demonstrated that MSCs secrete high levels of several neurotrophic factors, including BDNF and NGF, which are known to support RGC survival, and were shown here to protect RGCs in retinal explant culture. Furthermore, we identified a high molecular weight fraction of MSC conditioned media that possessed enhanced neuroprotective activity. One high molecular weight protein in our secreted factor cocktail, thrombospondin 1, is a trimer of three glycoprotein chains having molecular weights of ~140 kDa each. Thrombospondin 1 was included in the Misc1 factor subgroup cocktail, which demonstrated RGC neuroprotective activity in the current study (Fig. 4C and D), and thrombospondin 1 has been shown previously to be an MSC-derived factor capable of protecting RGCs in dissociated cell culture (Yu *et al.*, 2008). It is likely that thrombospondin 1, and possibly other yet undefined factors from the MSC secretome, contribute to RGC neuroprotection following transplantation or co-culture. The nearly complete elimination of MSC-mediated RGC neuroprotection by PDGF signalling blockade that was observed in the current study might indicate that PDGF not only promotes RGC survival by direct action, but also supports MSC behaviours that confer additional RGC neuroprotection. The role that PDGF signalling may play in controlling MSC neuroprotective actions warrants further investigation. Nonetheless, a protective role for PDGF signalling within RGCs was clearly defined by the present study.

The current study failed to demonstrate a neuroprotective effect on RGCs after treatment with soluble IL-2 or PEDF, which is in contrast to previous studies that noted increases in RGC survival conferred by these factors. The IL-2 receptor is expressed in the inner retina at postnatal day (PND)-2 and treatment of retinal explants derived from PND-2 rats increased RGC survival at 2 and 5 days of culture in a JAK and ERK1/2-dependent manner (Marra *et al.*, 2011). PEDF increases RGC survival in dissociated cells cultured under both normoxic and hypoxic conditions (Unterlauft *et al.*, 2012), as well as following neurotrophic factor withdrawal and glutamate-induced excitotoxicity (Pang *et al.*, 2007). Moreover, intraocular viral gene transfer of PEDF improved RGC survival and electrophysiological function in rats following ischaemic-reperfusion injury and NMDA-induced excitotoxicity (Miyazaki *et al.*, 2011). It is unclear why the current experiments demonstrated a lack of neuroprotection conferred by these two factors in retinal explants, but differences in experimental systems including the age of host tissue, type of host tissue (e.g. retinal explant culture versus dissociated cell culture), type of RGC injury, and dosing regimens could account for the disparity between results. A possible neuroprotective role of IL-2 and PEDF should be investigated further using *in vivo* models.

The current study extends our previous findings by showing that human MSCs are capable of protecting RGC cell bodies and neurite processes following injury. Furthermore, several secreted factors that could account for this bioactivity, the most potent of which was PDGF, were identified. These observations, combined with a growing body of literature that has demonstrated RGC neuroprotection using *in vivo* models of RGC/optic nerve degeneration, support our hypothesis that intravitreal MSC transplantation might be applied to the long-term management of chronic optic neuropathies such as glaucoma. MSCs inherently possess neuroprotective properties that could attenuate the loss of RGCs over time. In addition, studies have demonstrated that the neuroprotective phenotype of MSCs can be augmented to greater-than-baseline levels, for instance by *in vitro* treatment with defined signalling factor cocktails (Levkovitch-Verbin *et al.*, 2010) or viral transduction to overexpress growth factors (Harper *et al.*, 2011). This approach has been shown to improve both structural and functional outcomes in models of optic nerve neurodegeneration. The theoretical benefits of local stem cell transplantation for the treatment of chronic conditions such as glaucoma include potentially sustained long-term delivery of neuroprotective substances independent of patient adherence to therapy. Moreover, MSCs appear to target multiple neuroprotective pathways through the simultaneous secretion of a multitude of factors, a characteristic that could be especially beneficial in a process like glaucoma where many pathogenic pathways appear to contribute to disease progression. The safety of local MSC transplantation could potentially be increased by encapsulating the graft in a device that is permeable to soluble factors, but which sequesters the transplanted cells beyond the visual axis and offers the possibility of complete surgical removal should an adverse event occur. In fact, such a device loaded with cells that secrete CNTF is the focus of a registered trial for patients with glaucoma (NCT01408472). Alternatively, continued research aimed at elucidating the mechanism(s) of MSC-mediated neuroprotection may lead to the discovery of as yet unappreciated neuroprotection targets. The identification of an unexpectedly large effect size for PDGF-mediated neuroprotection in the present study suggests that it may represent such a target for the treatment of glaucoma. In fact, direct injection of PDGF protein at regular intervals could represent a viable treatment option for late-stage glaucoma when conventional treatments have failed to arrest progression towards blindness, given that monthly intravitreal injections of anti-VEGF agents have become routine clinical care in the treatment of neovascular age-related macular degeneration.

In summary, we have demonstrated that human MSCs are able to enhance the survival of RGCs under the harsh conditions of *in vitro* culture following complete axotomy, as observed

previously for rat MSCs (Bull *et al.*, 2011). Among many factors secreted by MSCs that appear to promote RGC survival, PDGF family members conferred a particularly strong neuroprotective effect both *in vitro* and *in vivo*, which appears to be dependent on PI3 kinase signalling. Future work will evaluate the translational potential of local MSC transplantation for the treatment of glaucoma, alongside targeting PDGF signalling pathways through stem cell-independent methods, such as through drug development.

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Supplementary material

Supplementary material is available at *Brain* online.

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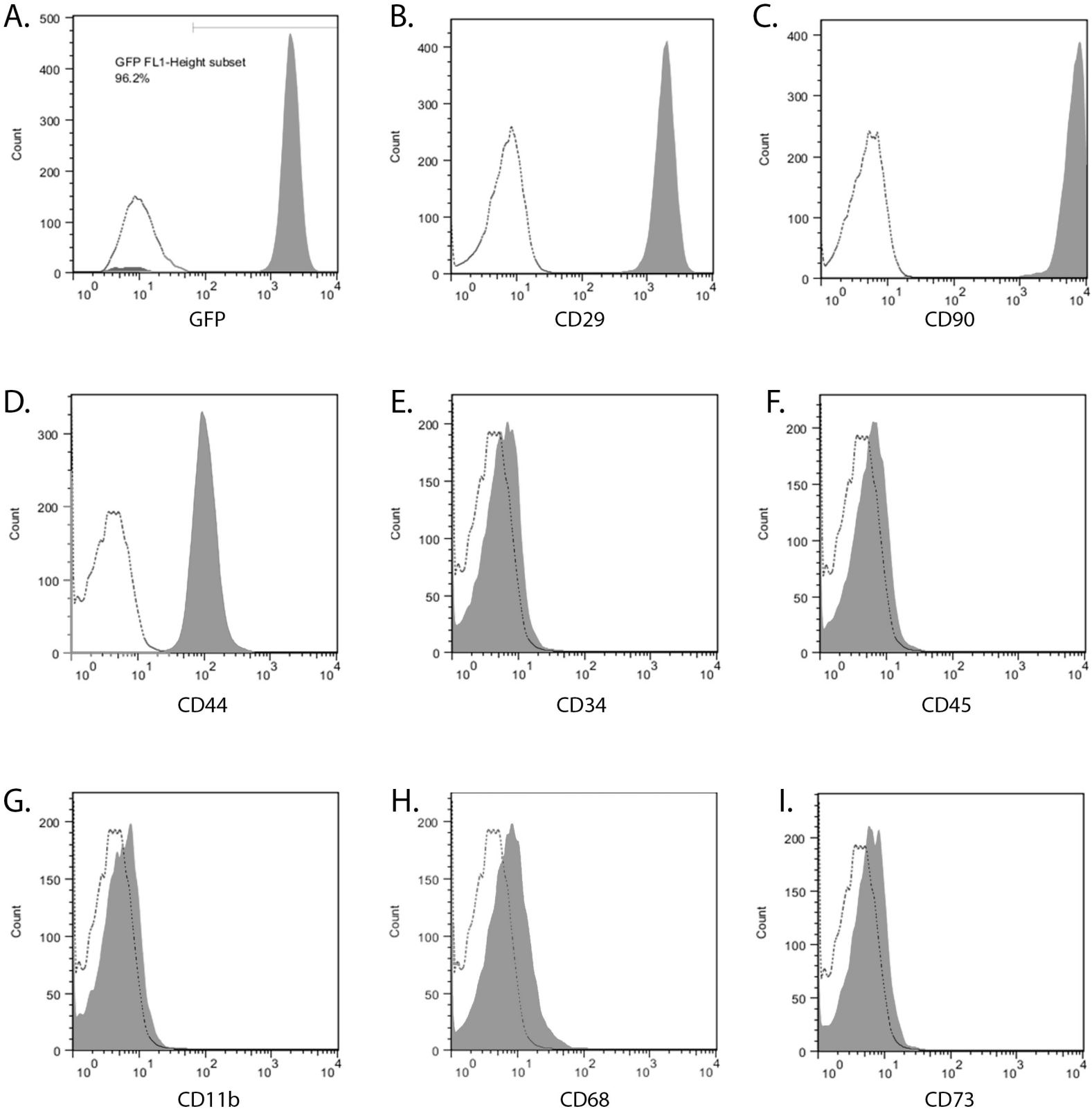
Figure S1. Flow cytometric analyses of rat mesenchymal stem cells. Green fluorescent protein (GFP)-expression rat mesenchymal stem cells (rMSCs) were dissociated and analysed for GFP expression and expression of cell surface receptors associated with mesenchymal or hematopoietic lineage as indicated in Methods. Cells were gated by GFP fluorescence (A), Alexa Fluor-647 fluorescence (B), PE fluorescence (C), or Alexa Fluor-633 fluorescence (all others). Negative controls (dotted lines) included rMSCs from wild type animals (A) or rMSCs in which the primary antibody was omitted from the immunofluorescence protocol (B-I).


A.
GFP FL1-Height subset
96.2%
Count
GFP
B.
CD29
C.
CD90
D.
CD44
E.
CD34
F.
CD45
G.
CD11b
H.
CD68
I.
CD73