Retinal progenitor cells release extracellular vesicles containing developmental transcription factors, microRNA and membrane proteins

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A range of cell types, including embryonic stem cells, neurons and astrocytes have been shown to release extracellular vesicles (EVs) containing molecular cargo. Across cell types, EVs facilitate transfer of mRNA, microRNA and proteins between cells. Here we describe the release kinetics and content of EVs from mouse retinal progenitor cells (mRPCs). Interestingly, mRPC derived EVs contain mRNA, miRNA and proteins associated with multipotency and retinal development. Transcripts enclosed in mRPC EVs, include the transcription factors Pax6, Hes1, and Sox2, a mitotic chromosome stabilizer Ki67, and the neural intermediate filaments Nestin and GFAP. Proteomic analysis of EV content revealed retinogenic growth factors and morphogen proteins. mRPC EVs were shown to transfer GFP mRNA between cell populations. Finally, analysis of EV mediated functional cargo delivery, using the Cre-loxP recombination system, revealed transfer and uptake of Cre+ EVs, which were then internalized by target mRPCs activating responder loxP GFP expression. In summary, the data supports a paradigm of EV genetic material encapsulation and transfer within RPC populations. RPC EV transfer may influence recipient RPC transcriptional and post-transcriptional regulation, representing a novel mechanism of differentiation and fate determination during retinal development.

A growing number of studies are defining a novel form of cell-to-cell communication involving genetic material exchange via secreted extracellular vesicles (EVs)1–3. EVs include exosomes and microvesicles, which are lipid enclosed cell fragments with diameters ranging from approximately 30 nm to 1 μm, released from most cell
types studied including cancer cells, embryonic stem cells, hematopoietic stem cells, neurons and astrocytes\(^4\)–\(^8\). Exosomes have diameters of 30–150 nm and are formed through the endosomal-sorting complex required for transport (ESCRT) machinery\(^{9,10}\). Microvesicles range in diameter from 100–1000 nm and are formed by membrane budding mediated by interactions between cell wall cytoskeletal and phospholipid proteins\(^{11,12}\). The release of microvesicles is correlated to cytoplasmic calcium levels and signaling pathways involved in plasma membrane remodeling\(^13\). Comprehensive EV analysis has been performed on several bodily fluids, including blood, saliva, urine, cerebral spinal fluid\(^14\) and breast milk\(^{15,16}\). Across studies, EVs enclose cytoplasmic and lipid bilayer embedded molecules, leading to encapsulation of unique combinations of microRNA, mRNA and proteins similar to those present in the cells from which they originate\(^17\). DNA has been reported in EVs from tumor cells, which carry single- and double stranded DNA, retrotransposon elements, and amplified c-Myc oncogene sequences\(^18\). EVs derived from astrocytes have also been shown to contain mitochondrial DNA\(^19\).

Recently, oligodendrocyte derived exosomes have been shown to contain molecular cargo that can be functionally recovered in neurons, enhancing neuronal viability\(^20\). EVs from human embryonic stem cells (hESCs) are capable of reprogramming hematopoietic progenitors through transfer of oct-4, nanog and gata-4\(^{21,22}\), suggesting a larger yet to be defined role for EVs in pluripotency, progenitor proliferation and fate determination\(^23\). EVs derived from hESCs and iPSCs contain a range of microRNAs, suggesting a potential role of EVs in post-transcriptional regulation\(^15\). Similarly, by transfer of miRNAs and proteins, EVs released from adult progenitor cells in kidney, lung and liver, induce de-differentiation of differentiated resident cells into stem cell-like phenotypes, leading to activation of regenerative programs\(^{24,25}\).

Additional studies have described functional effects of adult neuron and neural progenitor EV signaling in differentiation and physiology\(^{26,27}\). Huttner et al.\(^{28}\) reported that during neurogenesis in developing mouse brain, neural progenitor derived EVs contain stem cell markers including prominin-1, with predicted involvement in proliferation regulation. Neural progenitor exosomes contain a high level of miRNA-128b and have been shown to direct neuronal differentiation in stem cell populations\(^25\). EVs released from cortical neurons and microglia contain cell-adhesion molecules and glutamate receptors with predicted function in synapse formation\(^8,28,27\). Emerging studies of EV content and function in the nervous system suggest potentially important roles in neurogenesis and physiology.

During retinal development, multipotent RPCs are enriched in early transcription factors and miRNAs, which are differentially expressed, influencing fate determination\(^28\)–\(^30\). During retinogenesis, multipotent mRPCs pass through a series of competence states, generating subsets of retinal cell types with signature expression profiles. In a conserved temporal sequence, early mouse retinogenesis (embryonic day 8–14) generates ganglion, cone, horizontal, and amacrine cells. In late retinogenesis (embryonic day 16 to postnatal day 12) bipolar, rod, and Muller cells are born. According to this competence model, the progeny of RPC fate is regulated by a combination of extrinsic and intrinsic cellular signaling, yet to be fully characterized\(^31,32\).

In this work, we demonstrate that mRPCs release EVs with molecular cargo reflective of the expression states of the releasing cells. Our results suggest that mRPC EVs encapsulate miRNA, mRNA and proteins involved in multipotency and differentiation. These data support that mRPC EVs are a novel mechanism of cell-cell communication that could potentially be involved in retinal development, regulating gene expression, post-transcriptional modification and fate specification.

**Results**

**Extracellular vesicles are consistently released from mRPCs.** In these studies, mRPCs were observed to release EVs consistently over the time course analyzed. The robust viability of mRPCs during the time points of EV analysis was verified using a stable tetrazolium salt WST-based assay (Supplementary Figure S1). The size range and concentrations of EVs released from mRPCs were characterized using nanoparticle tracking analysis (NTA) (Fig. 1). EV mean diameters and concentrations were analyzed from mRPC conditioned media at 12, 24 and 48 h (Fig. 1A–D). Mean diameters for triplicate sample analysis at each time point were 133+/−3.2 nm, 130+/−0.6 nm and 132+/−1.8 nm, respectively. The average diameter of EVs in control media was 127+/−6.6 nm. We next analyzed mRPC released EV concentrations over time. The average concentrations of EVs/ml recorded were 0 h = 0.13+/−0.07 E8 (control), 12 h = 3.41+/−0.34 E8, 24 h = 4.91+/−0.35 E8 and 48 h = 5.93+/−0.22 E8. Data at time 0 h (control) was obtained from fresh media without cells that contained minimal amounts of EVs. After normalization to control, individual mRPCs were found to release approximately \(10^4\) EVs within 12 h. A sample of Brownian motion exhibited by mRPC EVs in solution at 24 h is provided in Supplementary Video 1.

**mRPC derived extracellular vesicles exhibit a spheroid morphology and CD63 expression.** The size and morphology of isolated mRPC and EV samples were analyzed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)\(^{22,23}\). SEM analysis revealed the presence of EV structures size and morphology of isolated mRPC and EV samples were analyzed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)33–35. SEM analysis revealed the presence of EV structures at gradient-densities of 1.12–1.17 g/cm\(^3\), which aligned well with other published studies characterizing
Figure 1. Nanosight analysis of mRPC released extracellular vesicle diameters and concentrations. (A–C) Nanometer diameter ranges and concentrations of mRPC released EVs were analyzed from conditioned media at 12, 24 and 48 h, respectively. Mean diameter for three samples at each time point analyzed were: 12 h = 133 +/− 3.2 nm, 24 h = 130 +/− 0.6 nm and 48 h = 132 +/− 1.8 nm. (D) Sample 3D plot showing EV size/relative intensity at 24 h. (E) Arrows showed light-scattering of individual EVs from a single frame of Nanosight tracking analysis at 24 h. (F) The average concentration of EVs/ml recorded at 0 h = 0.13 +/− 0.07 E8, 12 h = 3.41 +/− 0.34 E8, 24 h = 4.91 +/− 0.35 E8 and 48 h = 5.93 +/− 0.22 E8.

Figure 2. SEM and TEM characterization of mRPC surface and released extracellular vesicle ultrastructure. (A) SEM analysis of mRPC ultrastructure with extracellular vesicle structures apparent on the soma and left extended process, scale: 2 μm. (B) Higher magnification of (A), scale: 1 μm. (C) Released EVs isolated from conditioned media using ultracentrifugation appear as clusters under SEM analysis, scale: 300 nm. (D) Higher magnification of (C) allows for measurement of two vesicles at 57.15 nm, scale: 100 nm. (E) Immunogold TEM of isolated EV populations reveal the presence of the EV marker CD63, scale: 500 nm. (F) Higher magnification of (E), scale: 100 nm.
EVs (Fig. 3A)\textsuperscript{38,39}. An image of the entire sucrose gradient gel used to generate Fig. 3A appears in Supplementary Figure S4. To visualize CD63 positive EVs present in and emerging from mRPCs, anti-CD63 immunohistochemical analysis was performed. Confocal imaging and 3D reconstruction revealed CD63 labeling in the cytoplasmic spaces of mRPCs as well as on EVs emerging from lipid bilayer regions of both cell soma and processes (Fig. 3C–F).

Extracellular vesicles from a range of cell types have been shown to transfer combinations of mRNA, microRNA, and proteins\textsuperscript{40–42}. In this work, following the isolation of EVs from mRPC conditioned media, we analyzed mRNA, miRNA and protein content. Total RNA identified from EVs consisted primarily of species below 800 nucleotides (nt) lacking 28S and 18S rRNA (Fig. 4A). EVs were treated with RNase and no difference was detected when compared with non-treated EVs, indicating the RNA of EVs was enclosed within the vesicle membrane. Transcription factors, a cell-cycle regulator and intermediate filaments were identified in both mRPCs and EVs included Pax6, Hes1, Sox2, Ki67, GFAP and Nestin. The transcription factors identified are collectively involved in facilitating mRPC multipotency, cell-cycle and fate specification during retinogenesis. GFP, GAPDH and β-actin mRNAs were also detected in mRPCs and EVs. Next, the presence of miRNAs with established expression and function during retinogenesis were chosen for analysis. Selected miRNA species analyzed included Let7d, miR-9, miR-182 and miR-204. U6 snRNA was used as control. Data presented were combined from four independent replicates.

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Extracellular vesicles encapsulate mRNA and miRNA expressed in mRPCs. QPCR results suggested that mRNA species contained in EVs from mRPCs reflect genetic expression patterns of the cells. In particular, identified transcription factor and neuron progenitor cell mRNAs in both mRPCs and EVs included Pax6,
pairs of heterodimer receptor complexes, which determine ECM cell binding sites. Recently, Itgav was found to be upregulated in conditioned media were taken up by recipient mRPCs. For initial extracellular vesicle uptake analysis, isolated EVs, containing Itgb1 (green circles) (Fig. 5B), consistent with previous reports. Analysis of mRPC derived EVs by LC-MS/MS revealed proteins, established as EV markers, were highly enriched in EVs, including CD81, CD9, TSG101, CD63 and ~4-fold lower in the EV enriched fraction (Fig. 5B). Using the protein iBAQ distribution as a reference, five of the 1,829 proteins were matched at 1% False Discovery Rates (protein and peptide) in a ‘bottom-up’ proteomics profiling of EV enriched sample. 3,134 proteins were matched against the EV lysate. 223 of the proteins matched in the EV enriched sample were not matched in the cell lysate (Fig. 5A). The measured median iBAQ signal was ~4-fold lower in the EV enriched fraction (Fig. 5B). Using the protein iBAQ distribution as a reference, five proteins, established as EV markers, were highly enriched in EVs, including CD81, CD9, TSG101, CD63 and Igfb1 (green circles) (Fig. 5B), consistent with previous reports. Analysis of mRPC derived EVs by LC-MS/MS revealed many common and previously described EV proteins such as cytoskeleton component (actins, myosins, actin interacting regulatory proteins cofilins and tubulins), heat shock proteins (HSP 90-beta, HSP 90-alpha and HSP a4), G-proteins (Gprc 5b), a great number of Rab GTPase associated with membrane transport and fusion and the tetraspanin families (Tspan4, Tspan7, Tspan14, Tspan9 and Tspan6).

Extracellular vesicles are enriched for protein species expressed in mRPCs. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to profile the proteome of mRPCs derived EVs. Overall, 1,829 proteins were matched at 1% False Discovery Rates (protein and peptide) in a ‘bottom-up’ proteomics profiling of EV enriched sample. 3,134 proteins were matched against the EV lysate. 223 of the proteins matched in the EV enriched sample were not matched in the cell lysate (Fig. 5A). The measured median iBAQ signal was ~4-fold lower in the EV enriched fraction (Fig. 5B). Using the protein iBAQ distribution as a reference, five proteins, established as EV markers, were highly enriched in EVs, including CD81, CD9, TSG101, CD63 and Igfb1 (green circles) (Fig. 5B), consistent with previous reports. Analysis of mRPC derived EVs by LC-MS/MS revealed many common and previously described EV proteins such as cytoskeleton component (actins, myosins, actin interacting regulatory proteins cofilins and tubulins), heat shock proteins (HSP 90-beta, HSP 90-alpha and HSP a4), G-proteins (Gprc 5b), a great number of Rab GTPase associated with membrane transport and fusion and the tetraspanin families (Tspan4, Tspan7, Tspan14, Tspan9 and Tspan6).

Extracellular vesicles released from mRPCs are taken up by target cells. EVs isolated from conditioned media were taken up by recipient mRPCs. For initial extracellular vesicle uptake analysis, isolated EVs, stained with PKH26, were incubated with live mRPCs for 24h, followed by fixation and microscopic imaging. A
low power image of mRPCs with PKH26 labeled EVs is provided as Supplementary Figure S2. For high power analysis of EV cell surface binding and internalization in recipient mRPCs, 3D super-resolution microscopy was utilized. 3D reconstruction with three channels (XY, XZ, YZ) overlayed, revealed that EVs were localized to the lipid bilayer, internalized to the cytoplasmic space and potentially to the nucleus of mRPCs (Fig. 6A,B). In addition, mRPC internalized PKH26 labelled EVs revealed co-localization with the EV marker CD63 (Supplementary Figure S3). Internalized EVs have been shown to be transported along microtubules, localized within the perinuclear region and nuclear envelope.

**Extracellular vesicles transfer encapsulated mRNA to target cells.** Next, the transfer of GFP mRNA from GFP+ mRPC EVs to non-GFP hRPCs was analyzed using co-culture and RT-PCR. GFP+ mRPCs and hRPCs were co-cultured for 96 hrs, while divided by a transwell membrane containing 0.4 µm pores. Total RNA from hRPCs without mRPC transfer was used as a negative control. RT-PCR was performed to analyze if GFP mRNA was transferred to recipient cells by EVs. Results showed no GFP signal in the negative control group (Fig. 6C). Following RT-PCR result showed that GFP mRNA was transferred to hRPCs within the 96 h period (Fig. 6C,D). The data reveals EV mediated transfer of GFP mRNA and supports the possibility that transcription factors and miRNA identified in EVs may also be transferred between mRPCs.

**Extracellular vesicles transfer functional Cre to reporter loxP mRPCs.** pCAG-Cre plasmid containing CMV enhancer and chicken β-actin promoter was used as Cre expression vector; pCALNL-GFP containing chicken β-actin promoter and EGFP sequence was used as reporter vector. P0 mouse retina electroporated with both pCAG-Cre and pCALNL-GFP plasmids, were dissociated into mRPCs and used as positive control. In the experimental group, retinas electroporated with only pCAG-Cre were dissociated and seeded into
transwell inserts, while pCALNL-GFP electroporated retinae were dissociated and seeded in the lower wells. Non-electroporated mRPCs in transwell inserts with reporter mRPCs seeded in the lower wells were used as negative control. GFP-positive cells were detected in experimental group starting from day 18 after electroporation (Fig. 7A–C); in contrast, negative control showed limited background without cell specificity (Fig. 7D–F). Analysis of electroporation efficiency in positive controls, indicated that approximately 9% of dissociated mRPCs expressed detectable levels of GFP (Fig. 7G–I). Cre genes in EV and mRPCs were verified by real time PCR (Fig. 7J). Ct values were 32 versus 26 in EVs and mRPCs, respectively; no free floating Cre was detected in supernatant after isolation of EVs (data not shown). Reporter mRPC GFP intensities were measured for experimental, negative control and positive control using ImageJ. The results demonstrated that, compared to negative controls, experimental mRPCs showed significant increases in GFP fluorescence consistent with EV mediated transfer and reporter activation (Fig. 7K).

Discussion
Multipotent mRPCs are guided by genetic and epigenetic cues during retinal differentiation and fate specification. In addition to cell-autonomous differential gene expression and miRNA mediated post-transcriptional modifications, a number of studies revealed that media conditioned from retinal cells provides soluble cues capable of influencing mRPC fate69–74. It is possible that factors secreted from the developing retina, that influence retinogenesis, may be contained in EVs. In this work, mRPC EVs were shown to contain mRNA species associated with transport and transcription including poly(A)-binding protein cytoplasmic 4 (Pabpc4) with demonstrated function in supporting mRNA expression via increased stability and protection from decay75. Another identified protein in EVs with potential roles in mRNA transport and transcription is the mediator of RNA polymerase II transcription subunit 23 (Med23), required for transcriptional activation and assembly of the pre-initiation complex76.

Figure 6. Extracellular vesicle internalization and transfer of GFP mRNA (A) Super resolution 3D reconstruction of GFP+ mRPC following 24 h incubation with PKH26 labeled extracellular vesicles. Red vesicles are visibly localized near the cell surface and within cytoplasm. In the XZ axis, GFP (green), EVs (red) and nuclei (blue, DAPI). (B) Same as (A) with GFP (FITC) channel removed to increase visibility of PKH26 (TRITC) labeled EVs. Each panel contains three cross-sectional views (xy, xz, and yz). Scale: 5 µm. (C) RT-PCR analysis of GFP mRNA transfer between GFP+ mRPCs and non-GFP hRPCs. Non-GFP hRPCs served as negative control; GFP+ mRPCs served as positive control. GAPDH served as the internal control gene. EVs were treated using an RNase-Free DNase Set to remove DNA contamination before cDNA synthesis. (D) Intensities of RT-PCR images were measured with ImageJ software and normalized to GAPDH. Relative levels of hRPC GFP after transfer of EVs is significantly higher than negative control.
This work also demonstrates that mRPC EVs contain mRNA of transcripts involved in retinal development. mRPC EVs were observed to contain transcription factors including Pax6, Hes1 and Sox2. Additionally, the miRNAs identified in EVs included Let7d, miR-9, miR-182 and miR-204. Interestingly, the ratio of RNA species in mRPCs and EVs differed. For example, Ki67 showed higher expression compared to other mRNAs in mRPCs, while in EVs, Nestin mRNA was present in higher levels than in mRPCs. The differential distribution of RNA species between mRPCs and EVs may indicate selective packaging of transferable RNA as a mechanism involved in modulating retinogenesis. While, a number of molecular mechanisms are suggested to play roles in the differential distribution of RNA species in EVs, including, 3′-adenylation, cell RNA levels and RNA affinities to the MVBs outer surface, the exact process remains to be fully defined.

A number of studies demonstrate the presence of functional miRNAs in extracellular spaces and in cerebral spinal fluid. Additionally, extracellular miRNAs exhibit resistance to high extracellular ribonuclease (RNase) activity, increasing the probability of packaging in membrane bound EVs. EV mediated miRNA transfer is emerging as an important regulator of cell-to-cell regulation of gene expression in a number of tissue types.

Figure 7. Extracellular vesicle functional transfer of Cre in dissociated mRPCs. P0 retina were electroporated with Cre+ plasmid, dissociated into mRPCs and plated in 0.4µm pore transwell inserts. Dissociated mRPCs electroporated with reporter constructs were placed in lower wells beneath Cre+ mRPCs. (A–C) EV mediated functional transfer of Cre leading to activation of GFP expression in reporter RPCs as can be observed in experimental wells following 14 days of culture. (D–F) Negative controls, containing reporter constructs electroporated mRPCs, did not show GFP expression. (G–I) Positive controls, electroporated with both Cre+ and reporter constructs, showed robust GFP expression. Scale: 100µm. (J) Real time PCR verified Cre+ signal in EV derived from Cre+ electroporated cells. (K) Analysis of GFP intensity in mRPCs revealed a significant difference between experimental and neg. control (experimental: mean 341.41, sd115.92, neg.control mean 70.39 sd 31.65) P value < 0.0001 experimental vs. negative control and between experimental and pos.control (751.42 sd 271.1293979) P value = 0.05 experimental vs positive control.
miRNA204 and Let7d. Analysis of mouse retinal miRNA transcriptome reveals that miR-9 is highly expressed in neonatal retina, with peak expression near P10; miR-182 interacts with a photoreceptor-specific cluster of genes and increases expression after P1. MiR-204 is expressed in the developing retina during rod photoreceptor differentiation and later in the ciliary margin. Let7d plays important roles in neural fate specification, with predicted function in RPC differentiation. Changes in expression levels of mRPC EV encapsulated miRNAs may provide a level of cell-to-cell genetic regulation active during retinal development.

EVs derived from mRPCs may also provide protein cargo to facilitate morphogenetic processes. Morphogenesis proteins identified in mRPC EVs include, Latrophilin-3 (Lphn3), which facilitates cell adhesion and synapse formation, Integrin alpha-6 (Itga6), active in retinal lamination and neurite outgrowth, as well as neuronal cell adhesion molecule 1 (Ncam1) associated with retinogenesis. Potential mediators of mRPC physiology contained in EVs include the Ca2+-activated chloride channel, twenty homolog 2 (Ttyh2), the voltage-dependent calcium channel gamma-7 subunit (Cacng7), which regulates the trafficking and gating properties of AMPA-selective glutamate receptors and the Monocarboxylate transporter 1 (Slc16a1) known to regulate lactate exchange between neurons and glia, localized in late development in Müller processes and plexiform layers. Additional channel proteins and transport proteins are listed in Supplementary Tables 4 and 5, respectively.

In conclusion, data presented in this work provides the first analysis of release rate, morphology, content and transfer of EVs derived from mRPCs. As predicted, the mRNA, miRNA and protein contents of EVs reflect the general expression states of mRPCs, while the ratio difference in levels of mRNA species in EVs compared to mRPCs suggests a possible mechanism of selectivity involved in determining cargo. The transfer of GFP mRNA and Cre+ in mRPC derived EVs supports the possibility that a range of functional mRNA, miRNA and protein species may be transferred in EVs in vitro and in vivo. Taken together, the data suggest that mRPC EV cell-to-cell transfer of molecular cargo may present a novel form of genetic regulation and soluble signaling involved in RPC differentiation and retinal development.

Materials and Methods

mRPC isolation and culture. All animal procedures were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research, and were approved by the City University of New York, Lehman College Animal Care and Use Committee (IACUC). Actin promoter-GFP multipotent mRPCs (mRPCs) were isolated from post-natal day one mice and culture expanded as previously described. Briefly, mRPCs were grown at 37 °C and 5% CO2 in DMEM containing 10% FBS, 1 mM sodium pyruvate, 20 ng/ml recombinant epidermal growth factor (rEGF), 50 ug/ml nystatin, penicillin-streptomycin (10 IU ml-1, 20 ug ml-1), respectively. For EV isolation, multipotent mRPCs, at passage 15 were grown in T-75 flasks while supplemented with 2 M glutamate, 1× Neurobasal complete media containing 2 M glutamate, 1× EV-depleted B27 supplement, 1× N2 supplement, 20 ng/ml recombinant epidermal growth factor (rEGF), 50 ug/ml nystatin, penicillin-streptomycin (10 IU ml-1) and 20 ug ml-1, respectively. For EV isolation, multipotent mRPCs, at passage 15 were grown in T-75 flasks while proliferating and growing as both single cells and neurospheres. EVs were depleted from culture media by first centrifuging at 100,000 × g for 70 min and then filtering using a 0.22 µm pore sized filter.

Cell viability assay. mRPC suspension was prepared from one T-75 flask at 80% confluency. Cell suspension was then centrifuged at 300 × g for 5 min. Cell pellet was re-suspended in 5 ml complete neurobasal media as above. The number of viable cells was counted and plated at the concentration of 1.0 × 10⁶ cells/cm² with 2 ml of media in 24 well plates. Cells were incubated for 0, 24 and 48 h (n = 4 each). After incubation at 0, 24, and 48 h, a viability assay was performed using WST-1 reagent (WST-1, CELLPRO ROCHE). 200 ul of WST-1 reagent was added per well and incubated for 2 h before its quantification using a microplate reader (Benchmark Microplate Reader; BIO-RAD) at 450cy5 nm. WST-1 is a colorimetric assay that uses a soluble tetrazolium salt, which is then cleaved to a colored product known as formazan by the reductase system of metabolically live cells.

Isolation of mRPC EVs. EVs were isolated from media conditioned by 1 × 10⁷ mRPCs cultured in T75 flasks for 48h. Media was centrifuged at 300 × g for 10 min at 4 °C to pellet cell debris. Briefly, supernatant was transferred to an ultracentrifuge tube (Beckman Coulter), spun at 10,000 × g for 20 min using 60Ti rotor (Beckman Coulter ultracentrifuge) at 4 °C; supernatant was filtered through 0.22 µm filter and centrifuged at 100,000 × g for 70 min to pellet the EVs. All centrifugation was performed at 4 °C to minimize degradation of EVs. EVs were suspended in phosphate-buffered saline (PBS) and stored at ~80 °C for analysis. While ultracentrifugation was used in this work, the authors acknowledge that different EV isolation techniques can affect protein and RNA profiles of isolated EVs.

Scanning electron microscopy (SEM). EVs were fixed with 2% glutaraldehyde (EM grade) in 0.1 M phosphate buffer, pH 7.2, at room temperature for 1 h. Samples were then rinsed in 0.1 M phosphate buffer, pH 7.2 three times, 5 min each. Samples were then dehydrated in the following solutions for 5 min each: 10% ethanol, 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol (1), 100% ethanol (2), 100% ethanol (3). Samples were then dried on a glass surface, coated with gold sputter coating and examined using a Zeiss Supra 55VP microscope.
Transmission electron microscopy (TEM). EVs isolated from conditioned media were fixed in 2.5% glutaraldehyde with 4% paraformaldehyde (EM grade) for 2.5 h and washed in PBS for 24 h. Cells were post-fixed in osmium tetroxide for 30 min, washed with distilled water and subsequently dehydrated using increasing ethanol concentrations (70%, 85%, 95% and 100%), each for 10 min. Sample dehydration was followed by immersion in propylene for 20 min twice. Samples were infiltrated with a 1:1 mixture of propylene oxide and Spurr’s Resin for 1 h, left in 100% Spurr’s Resin overnight. Samples were then embedded in beem capsules using fresh Spurr’s Resin at 70 °C for polymerization. Excess resin was trimmed and 90 nm sections of samples were made using a Leica Ultramicrotome. Sections were placed on 200 mesh copper grids, stained with saturated uranyl acetate in 50% ethanol for 6 min, rinsed in water and stained for 90 s in lead citrate. Grids were then rinsed in water, dried on filter paper and viewed under a Fei Tecnai transmission electron microscope operated at 80 kV. Images were obtained using an AMT camera with AMT digital software.

Preparation of EVs for immunogold electron microscopy. EVs were isolated from 25 ml of culture supernatant using differential centrifugation, permeabilized and blocked in filtered 1% bovine serum albumin (BSA) with 0.05% Triton-X in PBS, pH 7.4 for 1 h at RT. Anti-CD63 (1:100 rabbit anti-mouse antibody; Santa Cruz USA) was applied for 24h at 4 °C. After 4 washes in PBS, a 1:50 dilution of 2nm goat anti-rabbit antibody was applied and left for 1 h at room temperature. After 4 rinses, EVs were fixed in 4% glutaraldehyde for 2.5 h and rinsed four times over 6 h in PBS. EVs were placed in Oso4 for 1 h, rinsed 3 times over 1 h in water and then placed in an increasing ethanol dehydration series. EVs were then placed in propylene oxide for 20 min, two times, and then infiltrated for 3 h in a 1:1 propylene oxide/spurr resin mixture. EVs were left to infiltrate in pure Spurr overnight and were then embedded in Spurr overnight at 70 °C. Samples were sectioned into 90 nm slices and placed on Nickel Formvar covered 200 mesh grids. Aurion silver enhancement was brought to room temperature, 20 drops of enhancement solution was mixed with one drop of developer, and grids were left to incubate for 10 min, which enlarged the 0.5 nm diameter gold nanoparticles to 20 nm. After rinsing with water, grids were stained in 4% uranyl acetate solution, rinsed and dried. Samples were imaged using a Fei Technai Spirit Transmission Electron Microscope operated at 80 kV and AMT digital imaging software.

NanoSight analysis. EV size and concentration were assessed using the NanoSight NS500 system. Collection of supernatant at three time points following plating, 12 h, 24 h and 48 h, was identical to isolation of EVs described above, with the exception that the supernatant was used just prior to 100, 000 × g ultracentrifugation for NanoSight analysis. Control media, non-conditioned, was processed under identical conditions. Based on the NanoSight protocol, to ensure accurate readings, final supernatant was diluted at 1:20 in PBS and triplicates of 1 ml samples were used for analysis. The NanoSight system uses laser to illuminate nano-scale particles, detected individually as light-scattered points moving via Brownian motion. Polydispersity was quantified, and Nanoparticle Tracking Analysis (NTA) software 2.3 used to track size and diffusion of nanoparticles. Results are displayed as a frequency size distribution graphs, describing the number of particles per ml. Significance was calculated using Student’s t-test with three independent experiments. The error bars represent standard deviation of the mean. Significant differences were denoted with asterisks: *(p < 0.05), ***(p < 0.01), ****(p < 0.0001); “ns” indicates no significant difference.

Sucrose gradient analysis and Western blot. EVs were analyzed using 10%-40% sucrose (w/v) density gradient solution. A linear sucrose gradient was prepared with 12.6 ml of 10% (w/v) and 12.6 ml of 40% (w/v) sucrose solutions, mixed in a sucrose gradient device (Life technologies). An EV pellet isolated from 27 ml of conditioned medium was resuspended in 0.5 ml of PBS, loaded on top of the layered sucrose gradient and centrifuged at 18,000 × g at 4 °C for 15 h. Fractions containing EVs were harvested and the densities were determined by weighing each fixed volume. Each 1 ml fraction was then diluted in 26 ml of PBS, and ultracentrifuged for 1 h at 100,000 × g. EVs were lysed at 4 °C for 1 h in a lysis buffer containing 50 mM Tris-HCl, 1% Triton X-100, 2 mM PMSF (Sigma Aldrich), 1 × Halt Protease inhibitor Cocktail (Thermo Scientific), 100 mM NaCl, 1 mM EDTA and 2 mM MgCl2 at pH 7.4. Aliquots of sample lysate from each 1 ml fraction were all used for 4% to 12% Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. Protein bands were visualized with NBT/BCIP (Sigma) after membrane incubation with primary antibody anti-CD63 antibody (1:500; Rabbit Polyclonal, Santa Cruz) and secondary antibody conjugated to alkaline phosphatase (1:10, 000; Abcam) (Figure s4).

Mass spectrometry. Whole cell lysate and exosome enriched samples from mRPCs were denatured in 8 M urea, reduced with 10 mM DTT, and alkylated with 50 mM iodoacetamide. This was followed by proteolytic digestion with endoproteinase LysC (Wako Chemicals) overnight, and with trypsin (Promega) for 6 h at room temperature. The digestion was quenched with 2% formic acid and resulting peptide mixtures were desalted using in-house made C18 Empore (3M) StAGE tips. Samples were dried and resolubilized in 2% acetonitrile and 2% formic acid. Approximately 1 μg of each sample was injected for analysis by reversed phase nano-LC-MS/MS (Ultimate 3000 coupled to a QExactive Plus, Thermo Scientific). After loading on a C18 trap column (PepMap, 5 μm particles, 100 μm × 2 cm, Thermo Scientific) peptides were separated using a 12 cm × 75μm C18 column (3 μm particles, Nikkyo Technos Co., Ltd. Japan) at a flow rate of 200 nL/min, with a gradient increasing from 5% Buffer B (0.1% formic acid in acetonitrile) / 95% Buffer A (0.1% formic acid) to 40% Buffer B / 60%Buffer A, over 140 min. All LC-MS/MS experiments were performed in data dependent mode with lock mass of m/z 445.12003110. Precursor mass spectra were recorded in a 300–1400 m/z range at 70,000 resolution, and fragment ions at 17,500 resolution (lowest mass: m/z 100). Up to twenty precursors per cycle were selected for fragmentation and dynamic exclusion was set to 60 s. Normalized collision energy was set to 27.
Protein profiling analysis. Mass spectrometry data were searched against a Uniprot mouse database (July 2014) using MaxQuant (version 1.5.0.30110). Oxidation of methionine and N-terminal protein acetylation were allowed as variable modifications, while all cysteines were treated as being carbamidomethylated. Precursor mass tolerance was set at 4.5 ppm while a 20 ppm tolerance was allowed for fragment ions. Two missed cleavages were allowed for specific tryptic search. The “match between runs” option was enabled. False discovery rates at the protein and peptide level were set to 1%. Protein abundances were represented by LFQ (Label Free Quantitation) and iBAQ (intensity-Based Absolute Quantitation). iBAQ values were log2(x) transformed and further used to create box plots to depict the distribution and changes in protein expression between two samples.

RNA extraction and quantitative real-time PCR. EVs were isolated from 20 ml of media conditioned by 1 × 10⁷ mRPCs cultured in T75 flasks for 48 h and all EVs were used for RNA extraction. The same amount of EV-depleted complete control media was used as a control to verify absence of retina specific transcription factors and mouse house keeping genes in control medium. Total RNA extraction was performed using an RNeasy Mini Kit (Qiagen). Isolated RNA was measured for quality using a Nano drop ND/2000 spectrophotometer and analyzed by 2% gel electrophoresis. Prior to RNA and miRNA extraction, EVs were treated with 100 ug/ml RNAse (Thermoscientific) for 30 min at 37 °C according to manufacturer’s instruction. DNA contamination was removed using an RNase-Free DNase Set (Qiagen). cDNA was synthesized using equal amounts of RNA samples (800 ng), according to the AMV First Strand cDNA Synthesis Kit instructions (NEB Biolabs). β-actin and GAPDH were used as a housekeeping gene controls for mRNA analysis. Quantitative real time PCR (qPCR) was performed using SYBR GreenER™ qPCR SuperMix (Life Technologies) on a Biorad system. Significance was calculated using Student’s t-test with three independent experiments. For miRNA analysis, the same amount of starting material (10 ng) from extracellular vesicles and mRPCs was used for reverse transcription. The primers used for qPCR are described in Table 1. miRNA was extracted using a MirVana RNA isolation kit (Thermo Fisher Scientific). Equal amounts of miRNA from EVs and RPCs was converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit. QPCR was performed using the Taqman Universal Master Mix II and Taqman assays (Life technologies). Analyzed miRNA assay IDs are provided in Table 2.

| Gene   | Primer sequence (5′-3′)            | (bp) |
|--------|-----------------------------------|------|
| Nestin | F: AACTGGCAACACCTCAAGATGT         | 235  |
|        | R: TCAGGGGTATTAGGCAAAGGG          |      |
| Sox2   | F: CACAACCTGGGGATACGCACAA         | 190  |
|        | R: CTCCCGGAACGGCGTTAAGCTTA        |      |
| Pax6   | F: AGTGAAATGGGCGGAATGTGATG        | 132  |
|        | R: ACTTGAGGAGAAGTGACAC            |      |
| Hes1   | F: CCCCACCTCTCTCTCTCTGAGC         | 185  |
|        | R: AGGGGCAACCCCTCAATAGGAAC        |      |
| Ki-67  | F: CCGTACTGGGGAATGCAGCAAA         | 170  |
|        | R: CAGTCTTACGGGCGCTCTGTCA         |      |
| GFAP   | F: AGAAAACCGCATCCACATTCA          | 184  |
|        | R: TCCATGACAGCTCTCTTGTGA          |      |
| β-actin| F: ACCTGGGATGCGTTCAAGG           | 100  |
|        | R: GCAGAATTCCTCTCTCTGTCA          |      |
| GAPDH  | F: ACCACAGTCCATGGCATCAC           | 452  |
|        | R: TCCACCACCTGTTGTCGTGA          |      |
| EGFP   | F: AAGTTCATCTGTACCCCGG          | 533  |
|        | R: TCCAGCAGACACATGCTGCGC          |      |
| CRE    | F: CTGACGCTGGGGAATGGTAAT         | 271  |
|        | R: TCATCCTTAGCGCCGTAAATC          |      |

Table 1. Primers and product sizes for qRT-PCR.

| miRNAs | Assay IDs |
|--------|-----------|
| miR-182| 002599    |
| miR-96 | 000186    |
| miR-204| 005008    |
| miR-9  | 000583    |
| Let-7d | 002283    |
| U6 sn RNA | 001973 |

Table 2. miRNAs assay IDs.

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| miR-9  | 000583    |
| Let-7d | 002283    |
| U6 sn RNA | 001973 |

Table 2. miRNAs assay IDs.
PKH26 labeling of EVs and uptake by mRPCs. Isolated EVs were labeled with the red fluorescent lipophilic dye PKH26 (Sigma) according to the manufacturer’s instruction. The use of fluorescence microscopy to image PKH26 labeled EVs has been demonstrated in a number of studies97–116,118. Briefly, EVs were collected after the 100,000 g ultracentrifugation, and incubated in PKH26 for 5 min at room temperature. Excess dye was removed by rinsing with EV-depleted complete media followed by three rinses in PBS. EVs were re-suspended in serum-free medium and incubated with mRPCs for 24 h at 37 °C. mRPCs were cultured on coverslips coated with mouse laminin (Life Technologies, USA) 24 h before culturing with PKH26 labeled EVs. mRPCs were fixed in 4% PFA for 10 min and washed with PBS 3 times, 10 min each. Low and high power images of EV binding and internalization were captured with a Nikon confocal spinning disk microscope using a 20× or 60× objective20,117,118. A HeNe laser was used for PKH26-labelled EVs (551/567). A 488 Argon Laser was used to image mRPC GFP. As a control, co-localization of CD63 with PKH26 labelled EVs was analyzed by adding PKH26 labelled EVs to mRPCs for 24 h, as above. mRPCs were then rinsed three times with PBS, fixed in 4% PFA for 30 min, blocked and permeabilized for 30 min in 0.1% Triton X-100 and 3% bovine serum albumin (BSA) in PBS. Anti-CD63 (1:50; Rabbit Polyclonal, Santa Cruz) was added to mRPCs for overnight incubation at 4 °C. After rinsing three times with PBS, mRPCs were incubated with a fluorescein conjugated secondary antibody, Cyamine 5 (1:500; goat anti-rabbit IgG, ThermoFisher) for 1 h at room temperature. After rinsing once for 15 min and thrice for 5 min with wash buffer, mRPCs were mounted using ProLong Gold media with DAPI (ThermoFisher). Images were taken using a Nikon Ti microscope with a 40× objective and the fluorophores Cy5 (649/666), PKH26 (551/567) and DAPI (358/461).

RT-PCR analysis of GFP mRNA EV content transfer between mRPCs and hRPCs. The transwell system was used for EV content transfer between GFP + mRPCs and non-GFP hRPCs. The use of a transwell culture system to evaluate EV mediated transfer of miRNA and mRNA between cell populations has been successful in a number of published studies19–121. Briefly, 5 × 10^5 GFP + mRPCs were plated on transwell inserts (0.4 μm pore size) above non-GFP hRPCs in each 6-well plate; the same number of hRPCs in plate, without mRPCs, were used as negative control. Cells were grown at 37 °C and 5% CO2 in neural basal complete media as above, containing 2 mM glutamate, 1 × EV-depleted B27, 1 × N2 supplement, 20 ng/ml recombinant epidermal growth factor (rEGF), 10 ng/ml rhbFGF, 50 μg/ml nystatin, penicillin-streptomycin (10 IU ml^-1 and 20 μg ml^-1, respectively. hRPCs were collected after 96 h, rinsed using PBS and processed for mRNA extraction.

Fluorescence analysis of Cre EV content transfer between mRPCs. P0 mouse retina were isolated and electroporated with pCAG-Cre plasmid and pCALNL-GFP plasmid as described previously122. The pCAG-Cre and pCALNL-GFP plasmids were gifts from Connie Cepko (Addgene plasmids #13775 and 13770)68. For electroporation efficiency, P0 retina were electroporated and then dissociated into mRPCs for analysis. Briefly, retina were placed into an electroporation chamber filled with plasmids/1 × PBS and 5 pulses of 25 volts were applied to drive plasmids into retinas. Electroporated retina were placed on a polycarbonate filter (0.2 μm pore size) for 24 h in media containing 45% DMEM, 45% Ham’s F12 Nutrient Mixture (Life Technologies), 10% fetal calf serum, L-glutamine (2 mM) penicillin (100 U/ml), and streptomycin (0.1 mg/ml) and then manually dissociated into mRPCs. Electroporation efficiency was determined by counting the number of GFP positive mRPCs dissociated from retina that were electroporated with both plasmids. Dissociated mRPCs with or without pCAG-Cre were cultured in transwells with 0.4 μm pores and dissociated retina cells containing the LoxP plasmid were seeded in 6 well plates for 3 weeks. Images were taken in phase and FITC every other day at 20× using an EVOS FLoid Cell Imaging Station (ThermoFisher). GFP expressing reporter+ cells were counted from n = 3 experimental and n = 3 control wells and intensity quantified using ImageJ. Significance was calculated using Student’s t-test with three independent experiments. The error bars represent standard deviation of the mean. Significant differences were denoted with asterisks: *(p < 0.05), **(p < 0.01), ****(p < 0.001), *****(p < 0.0001); “ns” indicates no significant difference.

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Author Contributions

J.Z., H.M., M. V.C-S. and S.R. designed the experiments. J.Z., A.BM, J.M, L.C, S. Ghoroghi, M.W, H.W, S.G, P.B, M.E., M.TM, H.M and SR conducted experiments and analyzed the data. M.Y, R.G and M. V.C-S. edited the manuscript. J.Z, A. BM, H.M, H.PS, and S.R wrote the manuscript. S.R supervised the project. All authors reviewed the manuscript.

Additional Information

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