Retinal Progenitor Cell, Induced Pluripotent Stem Cell, and 3D Mini Retina Derived Extracellular Vesicles Contain Transcription Factors, MicroRNA and Protein Associated with Potency and Development

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Retinal progenitor cell, induced pluripotent stem cell, and 3D mini retina derived extracellular vesicles contain transcription factors, microRNA and protein associated with potency and development

Jing Zhou

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by

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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THE CITY UNIVERSITY OF NEW YORK
Abstract

Retinal progenitor cell, induced pluripotent stem cell, and 3D mini retina derived extracellular vesicles contain transcription factors, microRNA and protein associated with potency and development

By:

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Advisor: Stephen Redenti

Extracellular vesicles (EVs) have emerged as a novel cell-to-cell communication mechanism in recent years. EVs are membrane-covered cell fragments released into the extracellular environment by all cell types in the normal physiological and pathological conditions. These membranous extracellular organelles include exosomes (30-100 nm) and microparticles (100-1000 nm), which are believed to play a pivotal role in intercellular communication. EVs represent the way for intercellular transfer of proteins and RNAs. By transfer of genetic materials, EVs are involved in reprogramming and tissue repair. We predict that during retinal development, retinal progenitor cells release EVs containing temporally expressed mRNA, proteins and miRNA, which may influence functioning and fate of recipient cells. As the first step to validate this hypothesis, we isolated EVs from multi-passages of mouse retinal progenitor cells (RPCs). We describe the concentration, ultrastructure and contents of EVs released from mouse retinal progenitor cells (mRPCs). The data reveals that mRPC derived EVs contain mRNA, miRNA and proteins associated with multipotency and retinal development. EVs released from mRPCs contain mRNA for the transcription factors Nestin, Pax6, Hes1, Ki-67, and Sox2, each of which is involved in retinogenesis. Proteomic analysis revealed a wide number of transcription factors, growth factors and retinal morphogen proteins contained in mRPC EVs. Imaging revealed, EV binding to and uptake by mRPCs. Finally, mRPC-derived EVs were shown to transfer this material to non-GFP RPCs.
In summary, the data supports a novel 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.

In addition to RPC-derived EVs, we characterized the EVs from mouse induced pluripotent stem cell (iPSCs) content and fusion to retinal progenitor cells (RPCs) in vitro was analyzed. Electron microscopic analysis of iPSCs revealed cytoplasmic origin of EVs and release via lipid bilayer budding. The mRNA content of iPSC EVs was characterized and revealed the presence of the transcription factors Oct-3/4, Nanog, Klf4, and C-Myc. Isolated iPSC EVs were shown to fuse with RPCs in vitro at multiple points on the plasma membrane. These findings reveal that the mRNA and protein cargo in iPSC EVs have established roles in maintenance of pluripotency. Building on this work, iPSC derived EVs may be shown to be involved in maintaining cellular pluripotency and may have application in regenerative strategies for neural tissue.

To begin to characterize EVs present during human retinal development, we also analyzed the morphology and content of EVs released from hiPSC derived 3D retinas cups at three developmental time points, D42, D63 and D93, which are three important developmental stage for retinal lamination in vitro. Analysis of small RNAs contained in EVs using next generation sequencing revealed the presence of a range of non-coding RNAs such as micro, piwi- and transfer-RNA species with predicted regulatory functions. This work provides the first evidence of small RNAs contained within EVs released from a model of human retinal development.

Taken together, our work shows EVs from mouse retinal progenitor cells and hiPSC derived 3D retinas cups contain important genetic materials, which may involve in retinal development.

**Key words:** mouse retina progenitor cells; mouse induced pluripotent stem cell; human induced pluripotent stem cell; extracellular vesicles; exosomes; microparticles; retina cups;
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Chapter 1. Retina structure and cell type generation

The vertebrate retina is composed of six types of neurons and one glial cell type. All cell types in the neural retina are generated from multipotent progenitor cells during development. Mouse retinal development is initiated near embryonic day 11 (E11). Mouse retinal progenitor cells (mRPCs) proliferate, migrate and differentiate producing all major retinal cell types by postnatal day 10 (P10) [1]. During retinal development, mRPCs pass through a series of competence states during each of which different subsets of retinal cell types are produced with signature gene expression patterns. In a conserved temporal sequence, early mouse retinogenesis (embryonic day 8-14) generates ganglion, cone, horizontal, and amacrine cells. Similarly, 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 retinal progenitor cell fate is regulated by a combination of extrinsic and intrinsic cellular signaling, which are not yet fully characterized [1, 2]. To elucidate the dynamic process of retinogenesis, it is essential to further characterize positive and negative extrinsic signaling mechanisms.

In this chapter, we describe how retinal neural cells are generated and how extrinsic and intrinsic pathways regulates the differentiation of retinal progenitor cells.

1.1 Brief anatomy and physiology of the mammalian retina

1.1.1 Eye structure and generation of retina cells

The vertebrate retina is an accessible model for the study of neural system development. Neuron cells and Muller glia in retina organize into three cellular laminas and two synaptic or plexiform layers (Figure1a). The posterior most region contains the retinal pigmented epithelium (RPE), just anterior to, and interdigitated with are photoreceptor outer segments. The cell bodies of photoreceptors (rods, cones) localize in the outer nuclear layer (ONL). The inner nuclear layer (INL) contains the cell bodies of horizontal, bipolar, most amacrine cells and Muller glia. The ganglion cell layer (GCL) has cell bodies of ganglion and a small percentage of amacrine cells. Synapses formed between lamina include the outer plexiform layer(OPL), which has synapses between photoreceptors, horizontal and bipolar cells and the other is inner plexiform
layer (IPL), which has synapses between bipolar, ganglion and amacrine cells\[3\]. Ganglion cell (GC) axons form the nerve fiber (NF) layer (Figure 1a).

In the outer plexiform layer (OPL), the synaptic terminals of rods and cones connect with horizontal and bipolar cells. These two cell types modify the incoming signals from photoreceptors and then relay them to the dendrites of the amacrine and ganglion cells via synapses in the inner plexiform layer (IPL). The amacrine cells further process the incoming signals (e.g., motion detection), whereas the ganglion cells relay the visual information to the brain via their axons forming the optic nerve (Figure 1a).

![Figure 1. Mouse retina cellular structure and generation of cell types from progenitor cells](image)

Mature mouse retina contains six major cell types. Mature mouse retina contains six major cell types. (Trends in Neurosciences [3]).

Developmental time course and transcriptional factors involved in subtypes of retinal cells. According to birth-dating studies of across vertebrate species, seven types of retinal cell types are generated from an
initial pool of progenitor cells [4-6]. The birth order of different cell types is conserved, with some overlap between times of generation. Figure 1B displays birth order and cell types of mouse retina.

Retinal progenitor cells are multipotent, not limited generating only one type of cell, but capable of becoming any cell type in the retina. Differentiated cells generated from multipotent retinal progenitor cells are not fate restricted from the progenitor stage, but acquire terminal fate specification during development.[5-7]. During retinogenesis, ganglion cells are produced at the earliest time point, followed by horizontal cells, cone photoreceptors and amacrine cells. Postnatally, a small amount of amacrine cells, a large percentage of rod photoreceptors, bipolar cells and Muller glia are produced. The production time of each cell type overlaps. For example, at embryonic day 12, ganglion cells, horizontal cell, cone photoreceptors and amacrine cells are being produced; at embryonic day 14, in addition to cell types mentioned, a small percentage of rod photoreceptor starts to be produced [1, 3].

The fate of retinal progenitor cells is determined by intrinsic and extrinsic signaling during retinogenesis. Several studies suggest that, environmental signals only change the ratio of each cell type generated, but cell types produced at given time can’t be altered. The competence model of retinal development proposed that “retinal progenitor cells pass through a series of competence states during each of which the progenitors are competent to produce a subset of retinal cell types”[2]. Based on the competence model, cell fate is determined by intrinsic factors; within a given competence state, cell fate is influenced by extrinsic signals [4-7].

1.1.2 Extrinsic factors alter the production of retinal cell types

During retinal development, multipotent RPCs interact with diverse microenvironmental cues to determine the fate of progeny[1]. Evidence for extrinsic cue involvement in fate determination has been evaluated primarily by experiments of rod photoreceptor or ganglion cell development [8-14]. A smaller number of studies have studied amacrine and cone cell fate regulation by extrinsic cues [15]. The use of retinal-cell-conditioned media and extracts from postnatal retinal explants provided evidence that there were candidate signaling molecules, such as Taurine, as well as other factors, which influence the fate of early dividing
retinal progenitor cells toward rod development [16]. Several other secreted factors have been identified which are expressed in developing retina and either promote or inhibit development of rod cell type, including CNTF, Bfgf and Sonic Hedgehog [8-13]. The analysis of factors secreted by retinal tissue suggests that older retinal cell, early born, secrete factors to regulate the fate of younger, later born, cell populations; for example, differentiated retinal ganglion cells secrete factors that inhibit further production of new ganglion cells via a pathway separate from canonical Notch signaling [8]. Also, Shh secreted by newly born ganglion cells stimulates RPCs to both generate additional ganglion cells and secrete additional Shh [17]. Recently Nerve growth factor (NGF) was identified as released from differentiated ganglions and shown to regulate ganglion cell number by causing cell death in late migrating ganglion cells [18]. In addition to rod and ganglion cell fate determination, studies have shown that amacrine and cone cell fate are also regulated by extrinsic environmental cues. Using a re-aggregate culture system, in which progenitors from embryonic retina are cultured with postnatal retina, two signals from postnatal retina were found to influence embryonic cell choice of fate [15]. One from previously generated amacrine cells which, inhibits the production of new amacrine cells and the other affect the production of cone cells, suggesting amacrine cell number is controlled by feedback inhibition [15].

Taken together, these data support a role for soluble extrinsic factor involvement in regulation of retinal cell numbers, which may indirectly influence fate determination during retinogenesis.

1.1.3 Intrinsic properties of retinal progenitor cells guide fate determination

While environmental cues have been shown to regulate the proportions of retinal cell types, these factors have not been shown to induce RPC generation of temporally inappropriate cell types [15, 19, 20]. Interestingly, a key characteristic of the competence model is cellular heterogeneity during retinal development. Even at the same developmental stage, individual retinal progenitors exhibit diversity of transcription factor expression which has been correlated to generation of distinct retinal cell types [21]. Transcription factors well established intrinsic regulators of retinal cell fate, including basic helix-loop-helix (bHLH), homeodomain, forkhead families (Figure 1b) [22-24].
A canonical transcription factor that directs RPC fate is the basic motifleucine zipper (bZIP) protein (Nrl), which is necessary and sufficient to direct postmitotic photoreceptors precursors to the rod fate. Ganglion cell fate is guided by a combination of permissive transcription factors, such as the protein atonal homolog 7 (Atoh7/Math5), Neurod1, Isl1 and Pou41/2/3. Amacrine cell fate is influenced by transcription factors including Six3, Foxn4, Neurod1/2/4/6, Ptf1a, Bhlhb5, Barhl2, Isl1 and Stab2. Cone cell fate is guided by a combination of Neurod1, Ascl1, Otx2, Rorb, Prdm1, Sall3, Pias3, Thrb, Rxrg, Rora, Nr2f1/2 and horizontal cell determination guided by Foxn4, Six3, Neurod4, Ptf1a, Prox1 (Figure 1b) [25]. For each transcription factor network, dynamic expression is temporally and spatially restricted, leading to generation of specific retinal cell fates. For example, Neurod1, a bHLH transcription factor, regulates multiple functions in the developing neural retina, including neuron/glia cell fate determination, amacrine differentiation and a subset of photoreceptor survival and differentiation [26]. Expression of Neurod1 can first be detected at the period of peak amacrine cell genesis during rat retinal development, and then shows temporal and spatial expression with rod photoreceptor genesis [26]. The production of ganglion and bipolar cells, require peak Ath5 and Chx10 expression, respectively correlated to the peak time of exit from mitosis for each cell type, respectively [27, 28]. Additional work remains to be performed to fully map the level of transcription factor interactions guiding cell fate decisions in each major retinal cell type and subtypes.

To advance understanding of the molecular mechanisms involved in potency and retinogenesis, analysis of RPC, iPSC and 3D retina extracellular vesicles is essential. While a number of canonical genes have been associated with retinal cell fate determination during development, extracellular vesicle mediated gene regulation in the retinal microenvironment remains to be elucidated [29-31]. The objective of this work is to characterize extracellular vesicle genesis, content and cargo transfer between cells. It is proposed that EV signaling may prove to be involved in regulation of iPSC pluripotency, RPC multipotency and differentiation during retinal development.
1. Defined gene activity signaling during neural retina development

1.2 Gene expression during neural retina development

a. Genes of interest expressed in E14 retina

Analysis of gene expression at different stages of retinal developmental development reveals transcription factor regulatory networks that guide retinal progenitor cell multipotency and fate. Genes that regulate RPC fate specification have been well characterized using sequencing, microarray analysis and serial analysis of gene expression from early to late stage development [21, 32-35]. Gene expression identified in mouse embryonic day 14 (E14) retina reflect a complex regulatory network which includes transcription factors, signaling and cell cycle proteins and extrinsic factors [33]. Proliferating RPCs at this stage are guided toward differentiation into ganglion, cone and amacrine cells. A number of genes including Chx10, Pax6, Hes1, Hes5, Mash1, NeuroD, Neurogenin-2, OC-1, Six3, Math 5, Hes5, Sox9, Islet2 and mLHX2 involve in fate determination at E14 [33]. Cyclin D1 is also expressed in RPCs of the entire retina at E14.5, promoting cell-cycle progression through G1 [36]. Additional conserved retinogenic signaling pathways at this stage include Notch-Delta, Wnt-b-catenin, Ephrin-Eph, FGF, BMP-TGF-B and neurotrophin-P75 [33].

b. Genes of interest expressed in P1-4 retina

Postnatal day 1 (P1) mouse retina contain multipotent RPCs generating peak numbers of rod photoreceptors, bipolar and Muller cells. At this stage, genes involved in early retinogenesis including Sfrp2, Fgf15, Edr RNA, cyclin-D1 and cdk4 have rapidly decreased expression [34]. Genes associated with fate determination near P1 include Foxp1, Ety1 and Ety6 [21]. Sfrp2 was broadly expressed in the ONBL until E16 and then rapidly decreased; in contrast, Fgf15 and Edr expressed longer however after P0, neither of them was detected. Additional genes involved in differentiation and strongly expressed at this stage include Hes6, which suppresses the activity of Hes1 and supports activity of Mash1 to promote cell differentiation. Hes6 shows a significant overlap in expression with Mash1 [37]. Previous reports revealed that a low level of Hes1 expression is sufficient to block neuronal differentiation, which could be overcome by a positive regulator of Mash 1[38-40].
c. Genes of interest expressed in adult retina

After P4, near the end of retinogenesis, many genes, which were active in mitotic RPCs have decreased expression, including Rax, Sox13 and Six6 [32]. In contrast, expression of a few genes increase at or after P5, including genes Rbp3, Elovl4 and Abca4 [41, 42]. A number of transcription factors involved in rod photoreceptor differentiation are detected in newly postmitotic photoreceptors. Neurod1 exhibits robust expression near P5 and has a role in developing rod photoreceptor differentiation and survival [26]. The Rax homeodomain factor, strongly expressed in all progenitor cells, is transiently re-expressed in immature photoreceptors at P8 [34] and differentiated photoreceptors[43].

In contrast to these patterns, Nrl and NR2E3, two crucial transcription factors controlling rod photoreceptor commitment, have no detectable expression prenatally, and show peak expression near P6 [34]. Other functional classes of genes expressed in the first postnatal week include Pias and multifunctional protein Hrs [44, 45]; these two genes selectively inhibit STAT3, and might inhibit the action of ciliary-derived neurotrophic factor, a factor that has been shown to inhibit rod differentiation in rodents [11, 12, 14]. The expression of synaptic vesicle protein Cpx2 suggests that developing photoreceptors may be developing synaptic machinery, while the expression of Hrs also potentially suggests high levels of regulated endocytosis and remodeling of extracellular proteins [46].

1.2.2 MicroRNA activity during retinal development

MicroRNAs (miRNAs) are a class of small, noncoding RNAs, 18-24 nucleotides long, present in genomes of both invertebrates and vertebrates. It is believed that miRNAs are modulators of target mRNA translation and stability. Each miRNA is predicted to regulate the mRNA transcripts of approximately 200 genes, although most target mRNAs remain to be identified [47]. Expression of miRNAs has high developmental stage and tissue specificity, indicating their unique roles in differentiation of cell types at specific developmental stages [48]. Many studies show miRNAs are involved in the control of neural development by regulating cell-cycle and temporal gene expression [49-53]. A recent study demonstrated that miRNAs influence retinal cell fate by inhibiting activators for bipolar fate, Xvsx1 and Xotx2 [54]. Four miRNAs, miR-
miR-155, miR-214 and miR-222, were shown to shorten the cell-cycle length of the late retinal progenitors, thereby inhibiting production of bipolar cells. It is proposed that differentiation of RPCs is guided by temporally expressed sets of miRNAs [54]. To date, 78 miRNAs have been identified in adult mouse retina, 21 of which are retinal specific [55]. All identified 21 retina-specific miRNAs show little or no expression prior to E10 with increasing expression after E10 into adult retina; this pattern of miRNA expression is consistent with reports that miRNAs have a significant role in embryonic retinal differentiation and maturation, as well as maintenance in adult retina [56, 57].

a. MicroRNAs of interest in E14 retina

Four miRNAs, miR-335, miR-219, miR-194 and miR-185, show peak expression at E14 and E18, suggesting involvement in differentiation of retinal ganglion, cone, horizontal and amacrine cells [55]. While these miRNAs have strong expression in embryonic mouse retina, the mRNA targets have yet to be fully described. It remains to be seen if key retinal developmental regulatory genes are included in the targets of miRNA dependent regulation. Some miRNAs, which are not retina specific, also show high expression at E14, including miR-24, miR-125, miR-178 [58].

b. MicroRNAs of interest in P1-4 retina

MiRNAs, with known mRNA targets in postnatal mouse retina have recently been elucidated [55, 58]. Target mRNAs with putative binding sites for expressed miRNAs show significantly lower expression levels than non-target mRNAs in developing and adult retina [58]. At P4, miR-124, miR-125 and miR-9 have been shown to significantly effect target genes expression; the genes ACCN2, ETS1, KLF13, LIN28B and SH2B3 were identified as targets by miR-124, miR-125 and miR-9 and binding resulted in significant reduction of gene products [58]. At P4 miRNA mediated decrease in target mRNA levels is predicted to be involved in differentiation of cell in early postnatal mouse retina.

Twelve of the twenty-one miRNAs identified in mouse retina (miR-183, miR-182, miR-96, miR-9-AS, miR-181c, miR-210, miR-7, miR-320, miR-140_AS, miR-151_AS, miR-211 and miR-184) show increasing expression beginning near P1; increased expression indicate these miRNAs may involve events near P1,
such as late RPC differentiation of rod, bipolar and Muller cell types [55]. MiR-182, miR-183, and miR-96, members of a conserved sensory organ-enriched cluster, are highly expressed in photoreceptors and interneurons in the inner nuclear layer (INL). The members of this cluster have high sequence similarity and might have similar downstream targets. Targets of this cluster include genes known to have important roles in development including Pax6, Hes1, Nhlh2, Sox2 and Fgf2 in the retina [55]. MiR-7 is reported to regulate EGF receptor signaling and promote photoreceptor differentiation and is detectable in newly postmitotic photoreceptors [59]. miR-9 is expressed in glial cells with a wide range of predicted mRNA [60-62]. miR-184 has been shown to be involved in regulation of choroidal vascularization although the targets remain to be described [63].

c. MicroRNAs of interest in adult retina

In mouse retina, expression of miRNAs miR-106b, miR-25, miR-31, miR-92 and miR-9, peak at P10 [55]. miR-106b targets the transcriptional activator Clock and its regulated gene Cry2 involved in circadian functions of retina [64]. miR-92 and miR-9 are expressed significantly at later retinal developmental stages and predicted to be involved in the specification of photoreceptors, Muller and bipolar cells. miR-9 and miR-92 are highly expressed in the mitotically active ciliary marginal zone (CMZ) cells and are likely to be enriched in P10 to adult mouse progenitors [65]. Transfection of miR-9 induces a significant reduction of gene products including ACCN2, ETS1, KLF13, LIN28B, NFIB and SH2B3 [58]. Notably, Hes1, which maintains retinal progenitor pools during development, is a predicted target for miR-9 [50]. miR-31 has been shown to downregulate PDGF-B, HIF-1A and VEGF [64].

1.3 Extrinsic soluble factors influencing RPC fate during retinal development

Soluble factors showing high expression at E14 are FGF-15 and SFRP-2; both interact with cell cycle pathways and are expressed as extracellular diffusible ligands [34]. SFRP-2 expression decreases rapidly after E16, while FGF-15 decreases to undetectable levels after P0 [34]. FGF15 is expressed by cycling retinal progenitor cells and is a downstream target of Bmp signaling pathway, which is necessary for the patterning of the retinal dorsa-ventral axis [66]. SFRP-2 is a member of Frizzled-related class of secreted
proteins, which binds to Wnt receptors in mitotic progenitor cells [67]. In addition, during retinal development differentiated retinal ganglion cells secrete a range of factors, not fully defined, inhibiting further production of new ganglion cells [8]. In contrast, Shh is secreted by new ganglion cells and promote further production of ganglion cells by mitotic RPCs, which in turn secret Shh to promoter more ganglion cell production [17]. NGF is also released from differentiated ganglion cells, regulating ganglion cell number by killing later migrating ganglion cells [18].

Multipotent RPCs interact with diverse environmental cues that influence the fate of retinal progeny [1]. Evidence for the significance of extrinsic cues on RPC fate has been established in studies of rod cell fate regulation [8-15]. The analysis of medium conditioned from postnatal retinal explants provides evidence of candidate soluble signaling molecules capable of influencing the fate of multipotent RPCs [16]. Several secreted factors including taurine, CNTF, Bfgf and Sonic Hedgehog have been shown to influence rod cell fate determination[8-13]. In summary, retinal neural population numbers and fates have been shown to be guided by intrinsic and extrinsic signaling pathways which remain to be fully described.
Chapter 2. Extracellular vesicle genesis and forms

Previous studies have shown that cell communication is not limited to soluble factors, but various vesicles. In recent years, an increasing number of laboratories are studying a novel mechanism of cell-to-cell communication: extrinsic cell-to-cell communication, which involves in exchange of genetic material via secreted extracellular vesicles (EVs) [68, 69]. Materials enclosed in EVs include metabolites, lipids, carbohydrates and proteins present within the cell of origin. EV mediated exchange of material appears to facilitate stem and progenitor cell fate determination and trans-differentiation of injured cells during regeneration [70]. EV mediated communication has the potential to facilitate genetic signaling during retinal development. In this chapter, we will review EV genesis, content and signaling pathways.

2.1 A novel model of cell-to-cell genetic signaling in developing and adult retina

The temporally restricted patterns of gene, microRNA and soluble factor expression influencing retinal neural development described above serve as a model into which the mechanism of EV genetic signaling can be added. Several labs have detected functional miRNAs circulating in bodily fluids including serum, saliva, urine and CSF [71-74].

![Figure 2. Model predicting miRNAs packaging into extracellular vesicles.](image)
Interestingly, extracellular miRNAs exhibit resistance to extracellular ribonuclease (RNase) activity [71], increasing the possibility that they might be packaged in membrane enclosed EVs or in lipoprotein-miRNA complexes which provide miRNA stability. The mechanisms of miRNA targeting to EVs for release is summarized in Figure 2 [76]. EV mediated miRNA transfer has emerged as an important regulator in cell-to-cell communication [77, 78]. In addition to miRNA, a number of labs are investigating EV signaling involving mRNA, protein and DNA from originating cells [78-81].

### 2.2 Extracellular vesicle types and sizes

EVs are released by both eukaryotes and prokaryotes into extracellular space. It has long been known that both apoptotic cells and healthy cells release vesicles mediating intercellular communication. Subcategories of EVs are defined by, molecular markers, size and genesis pathways. There are three subtypes of EVs: microparticles, exosomes and blebbing bodies (Figure 3). During formation of all EVs, cell-derived materials are enclosed into vesicles that preserve membrane orientation and cytosolic proteins within lumen.

![Figure 3. Release of three types of extracellular vesicles.](image)

1. Ectosomes/microparticles are generated by direct outward budding.
2. Exosomes are released when multivesicular bodies fuse with plasma membrane

3. Apoptotic bodies are blebbing vesicles upon apoptosis with size about 500-2000 nm in diameter [82].

2.2.1. Ectosome/ microparticle formation

In general, the terms ectosomes and microparticles all describe EVs with 100nm-1000nm diameters. Microparticles were first described from blood platelets in 1967 [83]. These phospholipid-rich particles were initially considered as “platelet-dust”. Following separation by ultracentrifugation electron microscopy was used to characterize microparticles as defined today [83].

The morphology of microparticle is heterogeneous. The membrane of microparticles is composed of phospholipid bilayer. The shedding process of EVs starts from budding of small cytoplasmic protrusions, which then detach via fission from cells. Several enzymes are involved in the fission process including calpain, gelsolin, flippase and scramblase. During the releasing process, microparticle cargo includes a percentage of molecular material present in the originating cell [84]. Activation of cell surface receptors induces intracellular calcium increase followed by disruption and remodeling of phospholipid bilayered membrane, which finally leads to budding of microparticle from cell surface [85]. Release has been correlated to P2YR cell-surface receptor activation. P2YRs are expressed in many cell types, including immune cells, dendritic cells, neurons, muscle cells, epithelial and endothelial cells [86]. P2YR is a membrane-spanning, G-protein coupled receptor [87]; activation induces release of Ca2+ from intracellular stores and subsequent release of microparticles. In contrast, regeneration and release of exosomes is constitutive and regulated. Although the final step of microparticles and exosome release appears similar, the initial processes of generation are clearly different.

2.2.2 Apoptotic blebs

Cells undergoing apoptosis release membrane enclosed apoptotic blebs (Abs) into the extracellular environment via blebbing of the plasma membrane. Abs are released during the late stages of cell death and have average diameters of 50-500 nm. ABs have irregular morphology and represent a heterogeneous
population of vesicles. Abs also bud directly from the plasma membrane and are components of the final stages of the apoptotic cell death process.

2.2.3 Exosome formation and release

Exosomes were first discovered in the 1980s by members of the Johnstone lab. In mature reticulocytes, lab members observed plasma membrane associated elements in multivesicular body (MVB) derived vesicles. The novel vesicles were named exosomes [88, 89]. Exosomes were then defined by genesis pathway involving MVBs, size ranging from 40-100 nm and a number of transmembrane proteins including Tsg101 and CD63.

Exosomes are secretory products of endosomal origin. Endosomes are components of the endocytic membrane transport pathway originating from the Golgi membrane and categorized as either early, late or recycling. Molecules and ligands internalized by cells are delivered to early endosomes and face three possible fates: recycling, degradation or release [90, 91]. In the recycling pathway, early endosomes (EE) form recycling endosomes, which deliver materials back to membrane. Degradation and release pathways require maturation of EE into multivesicular bodies (MVBs). The destination of MVBs can involve fusion with lysomes to generate endolysosome (EL), or fusion with plasma membrane to release intraluminal vesicles into the extracellular space as exosomes [92].

During the maturation process of early endosomes to late endosomes, invagination of the limiting membrane and budding of vesicles into lumen of early endosome, MVBs are formed as intermediates [93]. The sizes of the MVB intermediates are much larger than average endosomes, with diameters near 400-500 nm. MVBs were identified using EM in the 1950s, as irregularly shaped membrane enclosed structures containing intraluminal vesicles (ILVs) [94, 95]. The ILVs within MVBs range in diameter from 30 nm to 100 nm in diameter and are the precursors of exosomes.

Protein sorting into MVBs and delivered to the lysosomal lumen is the main pathway for degrading post-Golgi integral membrane proteins. Some specific endocytic proteins such as Rabs, LAMPs have been used as MVB markers, which distinguish them from other internal membranized organelles. 

Compared to
proteasome, which can degrade integral membrane proteins in early secretory pathway through process of endoplasmic reticulum-associated degradation, MVBs involves in degrading proteins that travel past the Golgi [96].

There are two pathways which determine the MVBs. One is the intracellular degradation pathway, which degrades non-recycled integral membrane proteins and soluble luminal material via MVB targeting to late endosomes and lysosomes [92]. Upon fusion of MVBs with lysosomes, acidic hydrolytic enzymes degrade their cargo which can include transmembrane proteins and lipids [97, 98]. Endosome-lysosome events depend on activation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). In concert with SNARE and Rab 7 N-ethylmaleimide-sensitive factor (NSF) attachment proteins are required for targeted MVB-lysosome fusion [99-102]; additional required proteins for fusion include vesicle-associated membrane protein 7 (VAMP7), and syntaxin [103]. Changes in intracellular calcium levels are also required for MVB-lysosome fusion [104, 105]. Membrane proteins that are excluded from inner MVB vesicles remain within the limiting membrane of MVB and then transferred to limiting membrane of lysosome[106].

The other pathway of MVBs involves exocytosis, during which MVBs fuse with plasma membrane and then intraluminal vesicles are released to the extracellular space as exosomes. During this process, a subset of endosomal membrane proteins and lipids are sorted into the vesicles. How materials are sorted into ILVs for either extracellular release as exosomes or for degradation via lysosomes is still not completely understood.

Table 1. Summary of extracellular vesicles

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Exosomes</th>
<th>Ectosomes</th>
<th>ABs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane of assembly</td>
<td>Endosome derived MVB</td>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Diameter</td>
<td>50-100 nm</td>
<td>100-1000 nm</td>
<td>1000-5000 nm</td>
</tr>
<tr>
<td>ESCRT machinery</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>-----------------</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Markers</td>
<td>CD63, CD9, TSG101, HS, P70, Anexin V, Flotilin 2, Anexin V, DNA, Histone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formation</td>
<td>Endocytosis, exocytosis, Ourward budding, Ourward budding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>Cup-shaped, irregular, irregular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipids</td>
<td>Cholesterol, sphingomyelin, ceramide, Phosphatidylserine, Lipid rafts</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.4 ESCRT dependent pathway of Exosome formation

The sorting of many plasma membrane proteins destined for degradation into MVB and ILVs is guided by ubiquitination, which labels target proteins by adding ubiquitin protein. The ubiquitin protein is highly conserved in all eukaryotes such as yeast, mammals and higher plants [107-110]. After ubiquitination, proteins are internalized into clathrin-coated vesicles and transported to early endosomal compartments [111]. There, ubiquitin tags are recognized by the endosomal sorting complex required for transport (ESCRT) and packaged into MVB ILVs [112-115]. ESCRT machinery binds to ubiquitinated cargo which ensures proper sorting. Four ESCRT complexes have been are well studied and include ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III.
Figure 4. Exosome formation has two pathways, ESCRT dependent and ESCRT independent.

A, B) After ubiquitination, cargo proteins are sorted by ESCRT-0 into lipid domain. ESCRT-I and ESCRT-II then stabilizes neck part of forming vesicles and formation of ESCRT-III narrows neck. Final recruitment of the Vps4 complex to ESCRTIII initiates the scission of the vesicles neck and disassembly of the ESCRT-III complex. C) ESCRT-independent ILV formation depends on self-organizing lipid and cargo domains [116].

ESCRT-0

ESCRT-0 is a heterodimer consisting of two subunits: Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (Signal Transducing Adaptor Molecule) [117]. Structural analysis shows that ESCRT-0 consists of two GAT domains, each of which composed of two helices [110]. Two subunits Hrs and STAM both contain ubiquitin and clathrin binding domains [118], facilitating interactions with multiple partner proteins. Hrs can bind to endosomal lipid phosphatidylinositol 3-phosphate (PtdIns (3) P) through its FYVE zinc-finger domain, after which Hrs is recruited and then ESCRT-0 endosomal membranes [119].
ESCRT-0 interacts with ESCRT-1 through Hrs with the FYVE domain. In mammals, ESCRT-I is an 18-25 nm heterotetrameric complex composed of four subunits: Vps23 (Tsg101), Vps28, Vps37 and Mvb12 (Figure 5) [113]. In the ESCRT-I complex, three functional subunits have been identified: ESCRT-II binding domain, a 13nm-stalk and a tailpiece containing ubiquitin and the ESCRT-0-binding UEV domain. The UEV domains of Tsg101 and Vps23 bind to PSAP-like motifs in Hrs, and with additional interactions these motifs contribute to endosomal recruitment of ESCRT-I.
Figure 6. Organization of ESCRT-I and ESCRT-II.

ESCRT-I complex has a ESCRT-II domain and ESCRT-0 binding domain [120].

ESCRT-II

ESCRT-II is an additional heterotetramer made up of one VPS36, one VPS22 and two VPS25 subunits (Figure 6). Vps36 has a split pleckstrin homology domain and its 3-phosphorylated phosphoinositides is localized to the endosome membrane. This domain also binds ubiquitin [121]. ESCRT-II binds to the ESCRT-I Vps28 carboxy-terminal domain subunit through a helix immediately C-terminal to the GLUE domain. In yeast Vps36, the GLUE domain contains two inserted NZF zinc-finger domains, one of which binds ubiquitin while the other binds Vps28 [122]. In addition to the lipid-binding properties of the GLUE domain, the first helix of Vps22 also has (less specific) lipid-binding properties that are likely to participate in membrane recruitment of this complex [122].

ESCRT-III
ESCRT-III proteins consist of Vps2, Vps24, Snf7 and Vps 20 [112]. These small, highly charged subunits assemble into higher-order multimers on membranes. The inactive monomeric form of the metastable subunits is maintained by interactions between the auto-inhibitory C terminus and the N-terminal part of the subunit [120, 123]. All ESCRT-III related proteins have a similar organization with N-terminal basic and C-terminal acidic regions.

Figure 7. Organization of ESCRT-II and ESCRT-III.

a) Subunits of ESCRT-III b) Autoinhibited monomeric state of ESCRT-III subunits and c) ESCRT-III interaction network [120].

The assembly process involves conformational changes, relief of subunit autoinhibition and interactions of the ESCRT-III subunits. The core complex contains the subunits Vps20, Vps32, Vps24 and Vps2 and assembles in a highly ordered manner. Vps20 is the central component, which nucleates ESCRT-III
assembly on membrane. Vps20 interacts directly with the endosome membrane and binds to the Vps25 subunit, which functions in recruitment of Vps20. Vps20 interacts directly with Vps32, which is the most abundant of the ESCRT-III subunits and triggers assembly of Vps32 into filamentous oligomers capped by Vps24 (Figure 7). All ESCRT complex subunits in yeast and mammalian system are summarized in Table 2.

Table 2. The ESCRT complex subunits.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Yeast</th>
<th>Metazoan proteins</th>
<th>Mammalian synonym</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESCRT-0</td>
<td>Vps27</td>
<td>Hrs</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hse1</td>
<td>STAM1,2</td>
</tr>
<tr>
<td>ESCRT-I</td>
<td>Vps23</td>
<td>Tsg101</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Vps28</td>
<td>VPS28</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Vps37</td>
<td>VPS37A</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B, C, D</td>
<td>NA</td>
</tr>
<tr>
<td>Mvb12</td>
<td></td>
<td>MVB12A,B</td>
<td>NA</td>
</tr>
<tr>
<td>ESCRT-II</td>
<td>Vps22</td>
<td>Vps22</td>
<td>EAP30</td>
</tr>
<tr>
<td></td>
<td>Vps25</td>
<td>Vps25</td>
<td>EAP20</td>
</tr>
<tr>
<td></td>
<td>Vps36</td>
<td>Vps36</td>
<td>EAP45</td>
</tr>
<tr>
<td>ESCRT-III</td>
<td>Vps2</td>
<td>Vps2A,B</td>
<td>CHMP2A,B</td>
</tr>
<tr>
<td></td>
<td>Vps24</td>
<td>Vps20</td>
<td>CHMP3</td>
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<tr>
<td></td>
<td>Vps20</td>
<td>Vps24</td>
<td>CHMP6</td>
</tr>
</tbody>
</table>
In addition to ESCRT machinery, lipid related proteins such as phospholipid scramblase and phosphoinositide specific phospholipase C\(e^{124}\) play a role in exosome secretion, which is predicted to be independent of the ESCRT pathway (Figure 8). Under stress conditions, lysosomal A-SMase translocates from its intracellular location to outer leaflet of the plasma membrane, which is enriched in sphingomyelin [125]. Spingomyelin is hydrolyzed by lysosomal A-SMase at plasma membrane which results in formation of ceramide on microdomains of the membrane [125].

Accumulation of ceramide can induce coalescence of small microdomains into larger domains on plasma membrane, which triggers ILV formation and promotes domain-induced budding of vesicles from plasma membrane [126, 127]. These microdomains may contain high concentrations of sphingolipids from which ceramides are formed; ceramide might be used for generation of ILVs, which are destined for secretion as exosomes as opposed to lysosomal degradation [126].
2.3 Proteins that regulate extracellular vesicle formation and release

There are multiple intracellular transport pathways controlled by cytosolic proteins such as the Rab family of small GTPases and SNAREs. SNARE proteins have a central role in catalyzing the fusion of vesicles with target membranes [128]. The Rab family works upstream of the SNARE complex tethering of vesicles to target membranes (Figure 8).
Figure 9. Rab families involve in EV formation and release.

Rab 11 involves in secretory and endocytic pathways. Rab7 involves in EVS formation; Rab 4, 35 are related to recycling pathway [90].

SNARE complexes are localized to intracellular compartments with vesicle SNAREs (v-SNAREs) on the vesicles and target SNAREs (t-SNAREs) on target membranes [129-131]. Different SNARE complexes are associated with fusion events between different membrane compartment [129]. The function of SNARE complexes has been extensively studied. Stx3 is a plasma membrane t-SNARE, involved in targeted delivery of axonal cargo [132]; Stx1 functions via calcium-mediated fusion of synaptic vesicles leading to neurotransmitter release [133].

The Rab family are small guanosine triphosphatases (GTPases) which function in vesicular trafficking budding [134], ER-to-Golgi transport, endosomal recycling and fusion of transport vesicles with membrane acceptors [135, 136] (Figure 9). There are almost 70 Rab proteins and Rab-like proteins in human. Rab4 and Rab5 are primarily found on early endosomes.
Rab 11 is associated with secretory and endocytic pathways [135, 137]. Rab11 regulates transport from early endosomes to the trans-Golgi network [138], from the trans-Golgi network to the plasma membrane [139], suggesting that Rab11 may control the connection between the endocytic and secretory pathways. Overexpression of dominant-negative Rab11 mutant inhibits exosome release, supporting Rab11 modulation of exosome transport [140].

Rab proteins control vesicle traffic and timing of vesicle fusion through interactions with Actin and microtubules. A number of Rab proteins have been identified which control motor protein recruitment to specific target membranes [141]. For example, Rab11 recruit myosin and dynein, moving late endosomes along microtubules; Rab25 regulates docking on the plasma membrane via kinesin; during release of exosomes.

Rab 35 regulates recycling and transfer of York receptors. Rab35 also controls the recycling pathway form peripheral endocytic compartments to plasma membrane [142]; acting on late endosome/MVBs and regulating exosome release in oligodendroglial cells [143].

Rab 27a and Rab27b mediate secretion of granules [144-146] and lysosome-related organelles in mast cells [147]. Furthermore, they function in MVE docking on plasma membranes. Silencing Rab27a results in increased sized multivesicular endosomes. In contrast, Rab27b silencing induces redistribution of multivesicular endosomes towards the perinuclear region, the data suggests that Rab27a and Rab27b function differently regarding regulation of exosome formation and release [144].

Table 3. Rab GTPase and the control of motor driven transport

<table>
<thead>
<tr>
<th>Rab GTPase</th>
<th>Motor</th>
<th>Compartmen t</th>
<th>Interaction</th>
<th>Effector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab4</td>
<td>KIF3B</td>
<td>EE</td>
<td>IP</td>
<td>LIC</td>
<td>[148]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EE</td>
<td>Y2H</td>
</tr>
<tr>
<td>Rab5</td>
<td>DIC</td>
<td>EE</td>
<td>No</td>
<td>PI3K</td>
<td>[150]</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>----</td>
<td>----</td>
<td>------</td>
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</tr>
<tr>
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<td>EE</td>
<td>No</td>
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<td>[151]</td>
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</tr>
<tr>
<td>Rab6</td>
<td>P150 GLUED</td>
<td>Golgi</td>
<td>Y2H</td>
<td>BicD1/2</td>
<td>[152, 153]</td>
</tr>
<tr>
<td>P50 dynamitin</td>
<td>Golgi</td>
<td>Pull down</td>
<td>[153]</td>
<td></td>
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</tr>
<tr>
<td>RB6K/MKLP2</td>
<td>Golgi</td>
<td>Y2H, pull down</td>
<td>[154]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rab7</td>
<td>Dynein/dynactin</td>
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<td>RILP</td>
<td>[155]</td>
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<tr>
<td>Rab8</td>
<td>MyoVI</td>
<td>Golgi</td>
<td>Y2H, direct interaction</td>
<td>Optineurin</td>
<td>[156, 157]</td>
</tr>
<tr>
<td>Rab 11</td>
<td>NyoVb</td>
<td>Recycling compartment</td>
<td>Y2H</td>
<td>FIP2</td>
<td>[158]</td>
</tr>
<tr>
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<td>MyoVa</td>
<td>Melanosome</td>
<td>Y2H, IP</td>
<td>Melanophilin/Slac2</td>
<td>[160-162]</td>
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<tr>
<td>MyoVIIa</td>
<td>Melanosome</td>
<td>Pull down</td>
<td>MyRIP</td>
<td>[163, 164]</td>
<td></td>
</tr>
</tbody>
</table>

### 2.4 What is in the membrane of EVs?

EVs contain lipids, proteins, organelles and nucleic acids. EVs, mainly exosomes, are formed during fusion of multivesicular late endosomes with plasma membrane, therefore cholesterol is a common component of exosomal lipids. In addition, exosomes contain higher levels of cholesterol and glycosphingolipids than do cell membranes. Since exosomes originate from endosomes, they don’t contain protein originating from nucleus, mitochondria, Golgi apparatus or endoplasmic-reticulum [76]. Exosome membranes exhibit no asymmetrical distribution of phosphatidylethanolamines. The bilayers of exosomes are organized with a
random distribution of phosphatidylethanolamines. Exosome membranes display a tight packing of lipids at neutral PH, with a rapid flip-flop of lipids between the two leaflets. Exosomal membranes contain several proteins with inverse topology, presenting intracellular domains on their outer surfaces.

2.4.1 What is the cargo of EVs.

There is a large and growing body of data describing EV composition and cargo. The ExoCarta database (http://exocarta.org/, version 4) stores exosome content identification and lists 4563 proteins, 194 lipids, 1639 mRNAs and 764 miRNAs as export targets of exosomes collected from 146 published studies. Protein composition in Many ubiquitous proteins are found in EVs including MHC class-I and class-II proteins, cytoskeletal proteins (actin and tubulin), membrane transport and fusion proteins (annexins and Rabproteins), tetraspanins (CD63, CD8, CD9 and CD82, integrins including a4b1, aMb2 and b2), and heat-shock family proteins (Hsp70, Hsc70 and Hsp90) (Figure 10) [165].

A variety of proteins have been identified from EVs. A conserved set of proteins are found in EVs, even when the EVs originate from different cell types (Figure 10). Most conserved proteins are abundant in EVs and include ESCRTs, RABs, G proteins and 14-3-3 proteins. 14-3-3 proteins are one of the most abundant exosome protein, which interacts with LRRK2 leucine-rich repeat kinase 2 (LRRK2); LRRK-14-3-3 interaction are associated with process of secretion of LRRK into vesicles[166]. The 14-3-3 proteins are conserved proteins expressed in all eukaryotic cells and function of 14-3-3 are very diverse by interacting with numerous proteins[167]. Name 14-3-3 refers to elution and migration pattern, and 14-3-3 eluted on 14th fraction of bovine brain homogenate and on positions 3.3 of subsequent electrophoresis[168].

According to Mathivanan’s study, proteins involved in MVB biogenesis are abundant in EVs including Alix (68% of the studies), TSG101(37%) clathrin (37%); and HSP70 in 89% of studies [165]. In addition, a number of proteins reflecting origin cell/tissue identity can be found within EVs. As an example, Zhang et. Al. reported that exosomes from renal tissue contained aquaporin-1, -2, -7 and kidney-specific Na-K-Cl co-transporter.
Figure 10. Conserved proteins identified in EVs based on 19 proteomic studies.

Proteins in EVs are conserved and include Rabs, tetraspanins, transmembranes proteins, cytoskeleton protein, adhesion molecules, ATPase, Antigen presentation proteins, lipid raft proteins, ubiquitins[165].

A number of protein families appear across EV populations. Rabs and small GTPases are enriched in EVs. As discussed, Rabs together with other proteins regulate vesicle fusion with membranes. There are approximately 40 Rab proteins localized to EVs. Annexins and tetraspanins (CD63, CD81 and CD9) are other large protein families identified in EVs. EVs are also reported to transfer adhesion molecules and receptors; for example, CD41 integrin or CXCR4-6 and12 [79].

Heat shock proteins, HSP, are a family of functionally related proteins found in all eukaryotes and prokaryotes. HSP60, HSP70 and HSP90 have been shown to be enriched in EVs. HSPs are activated in response to high temperature and other stressful cellular conditions. The main functions of HSPs are targeting abnormal proteins for degradation, directing protein-folding, maintaining unfolded protein structure. HSPs are secreted via the exosome exocytosis pathway [169]. HSP70 protein, unlike other members of HSP proteins, lack a secretory signal sequence and are secreted within exosomes.
In addition to proteins used as general markers for EVs, each cell type incorporates cell-specific proteins into their secreted EVs. For example, urinary exosomes show the presence of carbonic anhydrase and aquaporin-2 [170]. Similarly, A33 is found in colon epithelial derived EVs; CD86 is found in antigen-presenting cells and pMFG-E8 lactadherin is found in immature dendritic cells.

2.4.2 RNA and DNA in EVs

It has been shown that EVs released from tumor cells carry both genomic and cDNA, retrotransposon elements, and amplified c-Myc oncogene sequences, all of which reflect the genetic status of the cells of origin [171]. More studies suggest that EVs transfer specific combinations of microRNA, mRNA that bi-directionally modulate gene expression, post-transcriptional modification, cell-cycle regulation, and differentiation [172-174].

The presence of mRNA and miRNA inside EVs was first reported in 2007 by Valandi et al [78]. According to their study, EVs contained mRNA from approximate 1300 genes, many of which were functional, leading to new protein formation in target cells following EVs mediated transfer. Interestingly, not all RNA species in EVs are expressed in the cells of origin. The mechanism of how EV-unique RNA formed remains unclear. The profile of mRNA characterized in EVs shows different expression patterns compared to the composition of the donor cells. Many mRNAs and miRNAs are highly enriched or exclusively present in EVs, suggesting that there is a selective mechanism for packaging mRNA and miRNA into EVs [175].

EVs have been shown to contain miRNA with predicted roles in differentiation. EVs released from human embryonic stem cells (hESCs) contain the ES-microRNAs, miR-290, miR-291-3p, miR-292-3p, miR-294 and miR-295 [79]. miRNAs are small, 21-23 nucleotide, single-stranded RNAs that regulate post-transcriptional modification by partial pairing to specific mRNAs [172, 176]. Interestingly, 121 miRNAs including let-7, miR-1, miR-15, miR-16 and miR-181 have been identified in EVs of mouse mast cell line (MC/9), which are predicted to interact with 24,000 mRNAs regulating expression of other mast cells [78]. Human ESC EVs are capable of reprogramming hematopoietic progenitors through transfer of genetic material, which suggests a larger yet to be defined role of EVs in proliferation and fate determination in
stem cell niches [79]. ESCs derived from totipotent cells of the early mammalian embryo, exist in a proliferative state receptive to extracellular fate influencing signals and EVs from neighboring cells are predicted to provide terminal differentiation cues [79, 173]. Traditionally, ES cells are expanded and partially fate directed in culture using combinations of exogenous factors (eg. transcription and growth factors) and then transplanted into host tissue where appropriate, often terminal differentiation occurs in response to cues from host tissue [177]. Some of these cues may involve EV signaling.

EVs encapsulate small RNA species and protect them from degradation in the extracellular environment. The membrane of EVs preserves cargo and protects genetic material from nuclease digestion. Mammalian mRNAs have average size from 400nt to 12,000nt. However, Most EV RNAs are smaller, with sizes between 25 and 700 nt. mRNA found in human plasma, saliva and breast milk have a smaller size which is < 700nt [178]. EVs derived from mesenchymal stem cells and tracheobronchial epithelial cells have mRNA species smaller than 500nt in length [179]. The functional significance of small RNAs transfer has been explored in a recent study that showed human CD34 cell derived EVs transferring Cy3 dye-labeled miRNA to target cells, regulating expression levels[180].

Next-generation sequencing has identification of large numbers of non-coding RNAs in EVs (data below). Newly emerging small RNAs in EVs, include snoRNA, vault RNA (vRNA) and transfer RNA (t-RNA) (dissertation data below). A study on from Donovani and Braziliens exosomal RNA using NGS technique reveals conserved RNA cargo mainly contains non-coding RNA species such as rRNA, tRNA and tRNA derived RNAs [181].

2. 5 Known and predicted function of EVs

EVs represent a heterogeneous population, differing in cellular origin, number, size (50nm to 1µm) and internal composition [77, 172]. EV membranes engulf cytoplasm through membrane blebbing leading to encapsulation of unique combinations of microRNA, mRNA and proteins similar to those present in the cells from which they originate [182]. Many mechanisms have been proposed to show the interaction between EVs and recipient cells. However, the functions of EVs remain to be more completely understood.
Characterized and predicted EV signaling include: EVs can bind to cells through ligand-receptor interactions, similar to cell-cell communication; EVs could fuse with target cells delivering EV surface proteins to both the plasma membrane and cytoplasm of target cells; EVs are internalized by target cells via endocytosis.

2.5.1 EVs involved in immune response

T cell activation upon immune response is through T cell receptor recognition of peptidic antigen fragments of MHCs displayed on antigen presenting cells. Interestingly, T cells can also be activated by EVs secreted by antigen presenting cells [183]. Higher amounts of EVs are released after infection of macrophages with mycobacteria, inducing pro-inflammatory responses [184]. A recent study shows that EVs are much more immunogenic when derived from mature rather than immature dendritic cells (DC), and are capable of activating T cells [185]. The analysis of the immunologic significance of EVs is still undergoing.

2.5.2 EV involvement in tissue repair

According to the study by Deregibus et al., EVs derived from injured tissues play a role in modifying the phenotype of endogenous adult stem cells involved in tissue homeostasis [186]. It has been established that bone marrow stem cells have the capacity to produce non-marrow cells in injured tissues after engraftment. It has been suggested that bone marrow niche EVs maintain the identity of endogenous bone marrow stem cells [177]. In support of this model, murine bone marrow cells exposed to EVs isolated from lung-epithelial and liver tissue were shown to internalize each type of EV and produce lung tissue specific surfactant B and C mRNA and albumin mRNA, respectively [173]. Importantly, lung-derived EVs also transferred microRNA, which negatively modulated EV introduced pulmonary epithelial specific mRNA in co-cultured bone marrow cells [173]. These findings support the hypothesis that tissue-specific EV signaling preserves adult stem and progenitor identity and that transplantation into a different tissue results in local EV-influenced reprogramming toward new tissue specificity.

Similarly, EVs released from stem cells at the site of tissue injury may induce de-differentiation of resident cells which survived injury, transiently inducing a stem cell-like phenotype, while activating cellular regenerative programs [172, 187]. The potential target cells are those, which survive injury and can be
influenced to re-enter the cell cycle, leading to repopulation and differentiation into mature tissue. In the kidney, transplanted stem cells or endogenous bone marrow-stem cells are recruited to the site of injury where they secrete EVs that influence tubular cells to dedifferentiate, proliferate and re-differentiate replacing functional epithelial cells [173]. These findings support a paradigm involving EV transfer of genetic material from stem cells to injured cells thereby directing cellular reprogramming and functional repair.

2.5.3 EV signaling in neural Tissue

Several studies have described processes involving the release of small (50-100nm) EVs from the cell walls of astrocytes and terminals of both cortical and retinal neurons [79, 188]. EVs released from mouse cortical neurons have been shown to contain neuronal membrane proteins including GluR2/3 subunits of the AMPA receptor and the cell adhesion molecule L1 [188]. Interestingly, EV content appears to be consistent with a positive role in cell communication and genetic material is selectively packaged for transfer to neighboring cells [173, 189]. Neuron-specific microRNAs have also been identified in cerebral spinal fluid, indicating that neural cells release EVs that may facilitate communication across the nervous system [189]. In addition, the transfer of neuronal EVs to co-cultured cells in vitro has recently been shown to involve transfer of genetic material [173]. Furthermore, bone marrow stem cells cultured in media containing EVs isolated from adult murine brain were shown to express neural specific beta-tubulin, glial fibrillary acidic protein, and neurofilament mRNA [80]. In addition to the delivery of transiently active molecules, EV transfer induces the long-term expression of delivered mRNA in recipient cells [80, 173]. This latter property may be involved in long-term fate determination facilitated through EV transfer of genetic materials [173].

2.6 Purification of extracellular vesicles

2.6.1 Differential centrifugation

EVs have a density in a linear sucrose gradient (2-0.25 M) ranging from 1.13 to 1.19 g /ml. The current gold standard and most precise method for purification of EVs is serial ultracentrifugation[190]. The initial step is centrifugation at 300–500 x g for 10 min, eliminating dead cells and large apoptotic debris. The next step of centrifugation varies from 1000x g to 10,000x g to remove more large vesicles, debris and cell fragments.
Before final ultracentrifugation, supernatant is passed through a 0.22 μm pore filter. Next ultracentrifugation at 100,000 x g isolates EV pellets. EV Pellets are resuspended in PBS and stored 80°C for further analysis (Figure 11). This protocol might change to meet the requirement of different users. Recovery of EVs varies due to different biofluid viscosity [191, 192]. A sucrose gradient centrifugation step might be added flowing last step of ultracentrifugation, which can provide a cleaner population of EVs.

![Ultracentrifugation protocol for isolation of EVs](image.png)

**Figure 11. Ultracentrifugation protocol for isolation of EVs.**

Exosomes is isolated by serial ultracentrifugation and final pellet after 100,000 x g is precipitating EVs [193].

Viscosity of fluid is significantly correlated with recovery of EVs. According to Momen-Heravi’s study, different chemical and molecular compositions of biofluids influences viscosity, consequently affecting movements of EVs. One additional consideration may be to dilute biofluids prior to ultracentrifugation; for instance, plasma and serum contain more protein and requires more energy for particles to move. Lower viscosity fluids yield more EVs, whereas higher viscous fluid show decreased EV yields [193, 194].

### 2.6. Alternative methods for EV purification.

Ultracentrifugation may not completely separate endosome-derived exosome and budding microvesicles. Ultracentrifuged EV pellets are usually a combination of exosomes, microvesicles, and occasionally protein
aggregations. In addition, vesicles might be damaged and yields reduced during repeated ultracentrifugation steps.

An improvement over ultracentrifugation is ultrafiltration [190], which purifies and concentrates samples. Compared to ultracentrifugation, ultrafiltration is more efficient taking 20 minutes, compared to nearly 2 hours of ultracentrifugation using same initial volume of medium.

An additional accurate EV isolation method is sucrose gradient centrifugation, which is based on differences in vesicle densities[195]. As described previously, exosomes have densities of 1.08-1.22 g/ml on sucrose gradients [196]. This density separates exosomes from endoplasmic reticulum at 1.18-1.25g/ml and Golgi compartments at 1.05-1.12 g/ml [197]. After sucrose gradient isolation the fraction containing exosomes is processed for ultracentrifugation.

New techniques using separation on an iodixanol (optiprepTM) gradient has recently been proposed to precisely separate exosomes from smaller virus particles. Using the exosome marker acetylcholinesterase (AChE), the fraction containing exosomes could be efficiently separated from HIV virions [195].

Another method to isolate exosomes are affinity capture onto magnetic beads [198]. This method is limited in that it will only capture populations of EVs expressing proteins that will bind with antibodies selected for use with magnetic beads. Another caveat is that exosomal proteins may lose function during magnetic bead capture and release.

A number of alternative methods have been recently introduced for rapid isolation of Evs, including microfluidic devices and commercial product such as: precipitation technologies ExoQuick, Exo-spin, and Izon qEV columns with OptiPrep density gradients. While OptiPrep density gradients generate the highest purification of exosomes, each product listed here provide similar EV size distributions profiles.

2.7 Summary of Background

Depending on the isolation and purification techniques, EVs populations can be analyzed multiple levels. In this work, we studied total EV populations to provide a comprehensive picture of the processes of release
rates, cargo characteristics and transfer. Our hypothesis for our first set of experiments was that retinal progenitor cells (RPCs) can release EVs that and will contain genetic content similar to that seen in parental cells. We also proposed that this cargo may be transferred to recipient cells, including other RPCs. Future studies may show that EV RPC signaling may play a role in retinogenesis as a whole.
Chapter 3. Retinal progenitor cells release extracellular vesicles containing developmental transcription factors, microRNA and membrane proteins

Here, we demonstrate that multipotent mouse retinal progenitor cells (mRPCs) release EVs with molecular cargo reflective of the expression states of the releasing cells. We show that mRPC EV release is modulated by calcium levels and that the content is transferred between RPC populations. The data reveals that EVs encapsulate miRNA and proteins involved in multipotency and differentiation. These data support a role for mRPC EVs in cell-cell communication potentially involved in retinal developmental, gene expression, post-transcriptional modification and fate specification.

3.1 Characterization of extracellular vesicles released from mRPCs

mRPCs were observed to release EVs consistently over the times studied. To confirm mRPC was healthy and viable during entire experiment, we firstly conducted WST-1 viability assay. By detection of the formazan level in the cells, we can quantify the cell number. Viability assay showed that mRPCs are viable and not dying (Figure 12).

![Graph showing WST-1 assay](image)

**Figure 12.** WST-1 assay revealed proliferation activity of mRPCs up to 48 hours in incubation.

The graph showed a positive linear slope indicating that the mRPCs were viable and not dying off prior to experimentation.
We then compared freshly prepared complete control medium and exo-depleted control medium to see if there are EVs in both medium using nanoparticle tracking analysis (NTA). Complete medium before removal of exosome has abundant EVs and average concentration of EVs in control medium is $1.79 \pm 0.23$ E8 as shown in Figure 13. After depletion of exosome form control medium by ultracentrifugation and filtration, the average concentrations of EVs/ml recorded were only $0.13 \pm 0.07$ E8, indicating very few EVs in control media (Figure 14). Exo-depleted complete medium was used for further use.

![Graph showing nanoparticles tracking analysis](image)

**Figure 13. Nanosight analysis of complete neurobasal medium.**

Data shows abundant of EVs in complete medium before depletion of exosome(n=3). Batch average results showed concentration of medium is $1.79+0.23$ E8 particles/ml and size distribution of EVs in complete medium is $122+6.8$ nm.
Figure 14. Nanosight analysis of exo-depleted complete neurobasal medium.

Data showed few EVs in complete medium after depletion of exosome (n=3). Batch average results showed concentration of medium is 0.13+/0.07 E8 particles/ml and size distribution of EVs in complete medium is 127+/− 6.6 nm.

The size range and concentrations of EVs released from mRPCs were characterized NTA (Figure 15). EV mean diameters and concentrations were analyzed from mRPC conditioned media at 12, 24 and 48 hours (Figure 15A-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 122 +/- 6.8 nm. We next analyzed mRPC released EV concentrations over time. The average concentrations of EVs/ml recorded were 0hr = 0.13 +/- 0.07 E8 (exo-depleted control medium), 12hr = 3.41 +/- 0.34 E8, 24hr = 4.91 +/- 0.35 E8 and 48hr = 5.93 +/- 0.22 E8. Data at time 0 h (control) was fresh media without cells. After normalization to control (concentration of EVs at different time point subtracted EVs concentration of control
media), the value was divided by cell number in culture; individual mRPCs were found to release approximately 4072 +/- 1003 EVs, 5871 +/- 929, 7104 +/- 976 EVs within 12h, 24h and 48 h, respectively (Figure 16).

Figure 15. Nanosight analysis of mRPC released EV diameters and concentrations.

A-C) mRPC released EVs were analyzed from conditioned media at 12, 24 and 48 hrs, respectively. Mean diameters for three samples at each of time point analyzed were: 12 hr = 133 +/- 3.2 nm, 24 hr = 130 +/- 0.6 nm and 48 hr = 132 +/- 1.8 nm. D) Sample 3D plot showing EV size/relative intensity at 24hrs. E) Arrows show light-scattering of individual EVs from a single frame of Nanosight tracking analysis at 24 hours. F) The average concentrations of EVs/ml recorded at 0hr = 0.13 +/- 0.07 E8, 12hr = 3.41 +/- 0.34 E8, 24hr = 4.91 +/- 0.35 E8 and 48hr = 5.93 +/- 0.22 E8.
After normalized to number of EVs in control medium, number of EVs at 12h, 24 and 48 h was calculated.

3.2 Characterization of mRPC extracellular vesicle ultrastructure

We next characterized mRPC released EV ultrastructure. The size and morphology of isolated mRPC and EV samples were analyzed using SEM and TEM. SEM analysis revealed the presence of EV structures on the soma and proximal processes of mRPCs (Figure 17 A, B). Isolated EVs appear in clusters during SEM analysis, with spheroid morphologies and selected sample diameters of 57.15 nm (Figure 17C, D). Immunogold TEM analysis identified EVs diameters ranging from 70 to 200 nm, positive for the EV marker CD63 (Figure 17 E, F). Immunogold in absence of antibody shows no staining (Figure 17G).
Figure 17. SEM and TEM characterization of mRPC surface and released EV ultrastructure.

A) SEM analysis of mRPC ultrastructure with EV structures apparent on the soma and left extended process, scale: 2μm, B) higher magnification of A), scale: 1μm. C) EVs isolated using ultracentrifugation appear as clusters under SEM analysis, scale: 300nm, 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. G) immunogold negative control of EVs in the absence of primary antibody, scale: 100 nm.

3.3 Identification of EVs on and within cells

Next, expression of CD63 was used to characterize EVs isolated from mRPC conditioned media, present within and emerging from the mRPCs. Isolated EVs were fractioned on a sucrose-density gradient by ultracentrifugation (Figure 18). CD63 positive bands were detected in fractions at gradient-densities of 1.12-1.17g/cm³, which align well with other published studies characterizing EVs (Figure 18A) [199, 200]. To visualize CD63 positive EVs present in and emerging from mRPCs, anti-CD63 immunohistochemistry 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 (Figure 18 B-F).
3.4 Calcium induces release of extracellular vesicles from mRPCs

Increased intracellular calcium has been shown to induce release of EVs from a range of cell types [201, 202]. EV release from mRPCs was analyzed while modulating intracellular calcium [201, 203]. Treatment of mRPCs with the Ca\(^{2+}\) ionophore, ionomycin facilitated observed increases in EV formation and release into the extracellular space (Figure 19). Here, mRPCs were exposed to ionomycin and analyzed for the formation of PKH26 labeled vesicle structures. In non-treated mRPCs, baseline PKH26 labeling appeared uniformly distributed across the cell soma (Figure 19 C, D). Following exposure to ionomycin, vesicles were observed at higher numbers at the cell surface and emerging into the extracellular space (Figure. 19 G,H). The number of EV particles in each cell was counted. A comparison of mRPCs treated with ionomycin
to control mRPCs (non-treated) revealed a significant increase in CD-63 positive particles in treated cells (Figure. 19I).

![Images](image1.png)

**Figure 19. Calcium induced mRPC EVs release.**

A-D) GFP positive CD63+ mRPCs without ionomycin treatment, magnification: 60X. scale: 10 μm. E-H) Experimental GFP positive CD63+ mRPCs treated with ionomycin at 3 h, magnification, 60x; scale bar, 10 μm. Control and experimental cells were imaged using FITC (green), TRITC anti-CD63 (red) and DAPI (blue) in 20, 5 μm step z-stacks. I) At 60x, ionomycin treated mRPCs revealed significant increases in detected particles (34 +/- 12.6) compared to controls (19 +/- 4.3), mean number of EV particles per mRPC in control versus experimental group (mean± SD; * P<0.05). Average particle numbers for each RPCs were counted using n=8 cells /condition.

### 3.5 RNA and DNA content in mRPC EVs

EVs transfer specific combinations of microRNA and mRNA that may bi-directionally modulate post-transcriptional events, cell-cycle, differentiation and fate determination [173, 174]. Following the isolation of EVs from mRPCs, we characterized RNA and DNA content. Total RNA from EVs using Trizol reagent (Thermo Scientific) consists of RNA below 800nt and lacking 28S and 18S (Figure.20A). EVs from mRPCs are treated with RNaseA and no difference is detected between non-RNase-treated EVs, indicating the RNA of EVs are enclosed within the EV membrane. EVs contaminated by cell RNA show 28S and 18S (Figure 20A). Agarose gel electrophoresis didn’t detect DNA in EVs, indicating lower DNA amount in EVs.
EVs are enriched in small RNAs, which are below 500nt; there is no 28S and 18S rRNA detected by electrophoresis (Figure.20A).

**Figure 20. RNAs encapsulated in extracellular vesicles.**

A) 1.5% denaturing agarose gel loaded with total RNA from mRPCs and isolated EVs. Total RNA from EVs consisted primarily of species below 800 nucleotides (nt) lacking 28S and 18S rRNA. EVs were treated with RNase and no difference was detected when compared with non-treated EVs, indicating the RNA of EVs was enclosed within membrane. B) 1.5% agarose gel loaded with DNA from mRPCs and EVs. DNA from cells is digested by the restriction enzyme EcoRI and loaded into the gel. Cell DNA was present from mRPCs, yet DNA was not detectable in EVs using agarose electrophoresis.

### 3.6 Species of mRNA in mRPC released extracellular vesicles

To verify absence of retina specific transcription factors as well as mouse housekeeping genes in control medium, qPCR was performed using control medium derived “EVs”. 20 ml medium was used to isolate possible EVs from exo-depleted medium, which was same volume for mRPC derived EVs isolation. Then RNA was extracted from possible invisible “EVs” from control medium (n=3). qPCR result showed Ct value either > 37 or no signal at all, indicating absence of gene signal (Figure 21A). RT-PCR showed there was
no bands for each transcription factor (Figure 21 B). mRPCs cDNA was used as a control to show that qPCR / RT-PCR system was working properly.

### A

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### B

**Exo-depleted Medium** and **mRPCs**

![image](image.png)

**Figure 21.** PCR verified no mRPC–specific genes in exo-depleted complete medium.

A) Ct value of exo-depleted complete medium was beyond the normal range (either NA or > 37); B) there was no detectable band from Exo-depleted complete medium-derived EVs.

Results suggested that mRNA species in EVs from mRPCs reflected the expression state of the cells. mRNA was analyzed using realtime PCR. The results indicated that controls β-actin were present in higher levels in mRPCs when compared to EVs (Figure 22 A, B). In particular, important mRNA in both mRPCs and EVs included Nestin, Pax6, Hes1, Ki-67 and Sox2 (Figure 22B). These factors have been shown to be involved in different functions including multipotency, cell-cycle and fate specification in mRPCs throughout retinal development [204, 205]. To compare mRNA levels between EVs and mRPCs, the same amount of
initial RNA was used for qPCR. Transcription factors and GFP mRNA also appear in slightly higher levels in mRPCs when compared to EVs (Figure 22B). Interestingly, Nestin showed highest expression level in mRPCs but in EVs, Ki67 showed highest expression level, indicating gene expression pattern in cells and EVs was different.

**Figure 22.** qPCR analysis of factors in EVs.

Identical starting amounts of RNA, 800 ng, were used for both reverse transcription of mRNA from EVs and mRPCs. A) qPCR results indicate lower levels of β-actin in EVs when compared to mRPCs. B) Transcription factor mRNA identified in both mRPCs and EVs included Nestin, Pax6, Hes1, Ki67 and Sox2. GFP and GFAP mRNA were also detected in mRPCs and EVs. Cells were cultured in 4 replicates and RNAs were extracted for qPCR analysis. Data were combined from four independent biological replicates.
Figure 23. QPCR analysis of miRNA from EVs and mRPCs.

Identical starting amounts of RNA, 10ng, were used for both EVs and mRPCs. A) Results indicated lower levels of snU6 in EVs when compared to mRPCs. B) mRPCs specific miRNAs including let 7d, miR-9, miR-182, miR-204 were found in EVs but lower than in mRPCs. Data presented are from experiments performed in triplicates.

3.7 Extracellular vesicles of mRPCs contain miRNAs involved in retinogenesis

The results of agarose gel electrophoresis revealed that RNA species in EVs were below 2 kb; major RNA species in EVs were below 800 nt, with a percentage appearing below 100 nt (Figure 20A). To begin to characterize the small RNAs, qPCR was used to investigate the presence of miRNAs in both mRPCs and EVs. Four miRNAs, with established expression and studied function in retinal development, were chosen.
for analysis, including Let7d, miR-9, miR-182 and miR-2 [55, 206, 207]. Six miRNAs were chosen for analysis, including snU6, Let7d, miR-9, miR-182 and miR-2. snU6 was used as the endogenous control for PCR analysis of miRNA. miR-9 is highly expressed in neonatal retina and reaches maximum expression at P10. miR-182 is one of the photoreceptor-specific cluster genes, expression of which increases after birth and peaks at adult, indicating a significant role for photoreceptor function. miR-204 is expressed in the developing retina during rod photoreceptor differentiation and in mature retina and ciliary margin [206, 207]. Let-7d has important function in the nervous system of diverse species. In zebra fish retina, let-7b is specifically expressed in the peripheral region of the CMZ, and might be involved in the functions of early RPC development [65]. These five miRNAs are functionally important in mouse retina. The same amount of miRNA (10 ng) from EVs and RPCs were used for reverse transcription. Here, miRNA expression is not normalized to cell snU6, because even if the same amount of miRNA is used for reverse transcription, levels of miRNA in EVs and mRPCs are significantly different. Each small miRNA species tested appeared in higher levels in mRPCs, with lower detectable levels present in EVs (Figure 23A).

3.8 Proteins in mRPC EVs

3.8.1 EVs and mRPCs contain different protein profiles.

Silver staining of SDS-polyacrylamide gel result shows there are different proteins in EVs compared to conditional medium and parent mRPCs (Figure. 24A). Western blotting analysis revealed β-actin and GFP in EVs, albeit less than in mRPCs (Figure.24B). Important factors Nestin, Pax6, Hes1, Ki-67, GFAP and Sox2 were not detected in EVs, although were present in mRPCs (Figure. 24C).
Figure 24. Initial protein analysis in EVs and mRPCs.

A) Proteins were separated on a 4-12% SDS-polyacrylamide gel, stained using Biorad Silver Stain Plus kit. Equal amounts of protein were used from EVs and RPCs. Control media (CM) was used for negative control. B, C) Western blotting comparing β-actin, GFP, Nestin, Ki67, Hes1, GFAP and Pax6 in EVs and cells. Lane 1 = EVs and lane 2 = mRPCs. Protein concentrations of EVs and mRPCs were measured using Bradford protein assay and sample lysates containing 20 μg of protein were loaded for Silver staining. For western blotting, 30 μg protein was used for each condition.

3.8.2 Proteomic analysis of mRPC EVs

LC-MS/MS (Liquid chromatography–mass spectrometry) 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 fraction. 3,134 proteins were matched analyzing mRPC lysate. 223 of the proteins matched in the EV enriched sample where not matched in the cell lysate (Figure 25 A). The measured median iBAQ signal was ~4-fold lower in the EV enriched fraction Figure 25B. 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 Itgb1 (green circles) (Figure 25B), consistent with previous reports [208]. Analysis of mRPC derived EVs by LS-MS/MS revealed many common and previously described EV proteins such as cytoskeleton component (actins, myosins, actin interacting regulatory proteins cofilins andtubulins), heat shock proteins (HSP90-beta, HSP 90-alpha and HSPa4), G-proteins (Gprc5b), a great number of RabGTPase associated with membrane transport and fusion and the tetraspan family (Tspan4, Tspan7, Tspan14, Tspan9 and Tspan6).

Rab GTPase family proteins were also present in mRPC derived EVs. RabGTPases participate in multivesicular body (MVB) fusion and EVs genesis [209]. Two RabGTPases identified were Rab11 and Rab35. Rab11 plays a vital role in docking and fusion of MVBs [210]. Rab35 functions in MVB docking and subsequent release of EVs [211]. Our data shows that though Rab35 was measured in both samples the Rab35 signal was highly enriched in the EV sample (Table 4). Additional biogenesis-related proteins identified included vesicle assembly proteins and transportation (Annexins), many vacuolar proteins sorting-associated proteins (Snf8, Vps37c, Vps28, Vps4a, Vps36, Vps25 and Vps4b), and signal transduction proteins such as 14-3-3 and Sdcbp proteins. ESCRT proteins identified included ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, each with roles in biogenesis of MVBs and release of exosomes. In total, eighteen proteins, mostly vacuolar sorting-associated proteins, identified in this work, belong to the ESCRT sorting machinery (Table 5). Submitting proteins enriched 8-fold or more to a String analysis [212] (background proteome: mouse) revealed that indeed, ‘extracellular vesicles and exosomes’, ‘membrane-bound vesicles’ and ‘vesicles’ (GO: 1903561, 0070062, 00319888 and 0031982) were highly enriched (FDR better than 1e-70) among the enriched proteins. Figure 26 C shows a network of enriched EV proteins, including integrin, a protein family which were recently related to exosomes [213] and vacuolar protein sorting.
Figure 25. Qualitative proteomic evaluation of extracellular vesicle enriched fraction from mRPCs.

A) Venn diagram of unique and shared proteins measured in mRPCs (3,134 - blue) and the enriched EV fractions (1,829 - red). Proteins only measured in the EV enriched samples included Rab family proteins.

B) The distribution of iBAQ (intensity Based Absolut Quantitation) values of proteins matched in the two experiments. The measured median iBAQ signal is ~4-fold lower in the EV enriched fraction. Onto the Box Plot are mapped selected proteins belonging to 2 groups: Enriched/unique to EV (green and red) and not-enriched proteins (black/grey). Green indicates proteins often used as EV markers. Red is used for proteins with relevance to mRPC state and development. Squares are used for proteins not identified in the mRPC lysate, while diamonds indicate proteins matched in the mRPC lysate but that are highly enriched in the EV fraction. Black/grey bricks were used to map cellular proteins often observed because of their high abundance.

C) STRING-db (high confidence, 0.700) analysis presented using CytoScape 95. A. selected
network of proteins enriched 8-fold or more are shown. This network contains integrins, Rab GTP family, SLC transporters family and other vesicle related proteins.

In addition to EV proteins, many neural development associated proteins were identified from proteomic analysis. According to iBAQ signal, Lgsf8, Gprc5b, Dhx30, Klf14 and Mov10 were identified in EVs but not in the mRPC lysate; proteins including Ptgfmrn, Slc7a5, Adam10 and Prelp were matched in mRPC lysate but enriched in the EVs (Figure 25 C). Adam10 has been shown to be involved in maintaining progenitor cell pools and directing neural differentiation by regulating the Notch signaling [214, 215]. Additionally, Krueppel-like factor 14 (Klf14) is associated with positive regulation of cell cycle and regulation of transcription [216, 217] (Table 6).

GFP under the control of ubiquitous CAG was also found in both EVs and mRPCs. The signal of GFP is approximately 20 times lower in EVs than mRPCs (data not shown).

In Figure 25C, the selected network of proteins are enriched 8-fold or more in EVs than in cells. Based on this work, the network contains intergrin proteins such as itga4, itga6, itgb6, itgb1 which are enriched in exosomes and itga1, Itgav itga7 exclusively in exosomes. Integrins are a complex family of cell adhesion receptors mediating signaling of cell-cell and cell-extracellular matrix (ECM) protein interactions. Integrin α and β subunits form approximately 24 known pairs of heterodimer receptor complexes, which determine ECM cell binding sites [218]. In recent studies of integrins, Itgav was found to mediate multipotent adipose derived stem cells interaction with surrounding microenvironment and might be functional in cell cycle. In addition, Itgav is implicated in neural invasion, by malignant tumor cells, [219] and angiogenesis [220, 221]. Several solute carrier (SLC) transporters members were also found in EVs, including Slc1a2, Slc2a1, Slc6a1, Slc7a5, Slc12a7, Slc12a2 and Slc16a1 (Figure 25C and Table 6).

In addition to EV associated proteins, several proteins potentially associated with mRPC state and neural development were identified from proteomic analysis. According to the iBAQ signal, Ltsf8, Gprc5b, Dhx30, Klf14 and Mov10 were identified in EVs but not in the mRPC lysate; proteins including Ptgfmrn, Slc7a5, Adam10 and Prelp were matched in mRPC lysate but enriched in the EVs (Figure 25B). Adam10 has been
shown to be involved in maintaining progenitor cell pools and directing neural differentiation by regulating Notch signaling [214, 215]. Additionally, Krueppel-like factor 14 (Klf14) is associated with positive regulation of cell cycle and regulation of transcription [216, 217]. Additional EV proteins with predicted involvement in mRPC fate and neural differentiation are provided in Table 3. GFP under the control of ubiquitous CAG was also found in both EVs and mRPCs.

Table 4. RabGTPase proteins

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Table 6. Neural multipotency and developmental proteins.
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Table 7. Transport and channel proteins
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Table 8. Na+/K+-ATPase subunits

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3.9 Extracellular vesicles released from mRPCs bind to and are absorbed by target cells

Both EVs isolated from conditioned media and those released directly from living mRPCs were taken up by recipient cells. PKH26 was used to label isolated EVs and to label mRPCs, which then released EVs that could be tracked with TRITC imaging during target cell uptake. The PKH26 (exitation 551 nm/ emission 567 nm) stably incorporates a yelloworange fluorescent dye with long aliphatic tail to lipid regions of cell and vesicle membrane therefore label the lipid part.

For initial EV uptake analysis, isolated EVs stained with PKH26, were added and incubated with live mRPCs for 24 h. Then mRPCs were rinsed using PBS for three times and followed by microscopic imaging. To analyze 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 each panel of XY, XZ, YZ had red EVs, indicating stained EVs was internalized into cells (Figure 26). Assuming red EVs only localized to surface of cells, at least one panel of XY, XZ, YZ would not show red EVs.

Figure 26. Super-resolution microscopy reveals EV internalization by mRPCs.

A) 3D reconstruction of GFP+ mRPCs following 24hr incubation with PKH26 labeled extracellular vesicles. Red EVs are visible localized near the cell surface and within cytoplasm. The XZ axis shows the following channels: GFP (green), EVs (red) and nuclei (blue). B) The 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.
3.10 Transfer of EV encapsulated mRNA to target cells

Next, the transfer of stained EVs using PKH26 from GFP+ mRPCs to GFP+ mRPCs was analyzed using co-culture and qPCR. A co-culture system was used to analyze if EVs could transfer genetic materials to recipient cells (Figure 27). mRPCs were co-coltured in inserts and wells for four days, while separated by a transwell membrane with 400 nm pores. Images were taken at 48h, showing EVs released from PKH 26 stained mRPCs passed through filter and uptaken by mRPCs in wells(Figure 28 A-H).

**Figure 27. Diagram of co-culture system showing stained EVs transfer to target cells.**

PKH26 stained mRPCs (GFP+) and mRPCs (GFP+) were seeded in inserts with 400 nm sized pore and 6-well plates, respectively. Red EVs(GFP+) were released from passed filter and then incorporated into mRPCs (GFP+).
Furthermore, the transfer of a GFP mRNA from GFP+ mRPCs to non-GFP hRPCs was analyzed using coculture and qPCR. A co-culture system was used to analyze if EVs could transfer genetic materials to recipient cells (Figure 27). mRPCs and hRPCs were cocultured for four days, while divided by a transwell membrane with 400 nm pores. Total RNA from hRPCs alone was used as negative control. qRT-PCR and RT-PCR was performed to analyze if GFP was transferred to recipient cells by EVs. Following RT-PCR and sequencing, results showed that GFP mRNA was transferred to hRPCs within the 24hr period (Figure 28A, B). The data demonstrates the EV transfer GFP mRNA and support the possibility that transcription factors and miRNA identified in EV are also capable of being transferred between mRPCs.
**Figure 29.** Diagram of mRPC and hRPC co-culture system showed gene transfer through EVs.

mRPCs (GFP+) and hRPC(GFP-) was separated using 400 nm pore sized filter. EVs(GFP+) were released from mRPCs(GFP+), passed filter and then incorporated into human RPCs(GFP-).

**Figure 30.** GFP transfer between mRPC and hRPC.

(A) RT-PCR analysis of GFP mRNA transfer between from GFP+ mRPC EVs and non-GFP hRPCs. mRNA was treated using RNase-Free DNase Set to remove DNA contamination before cDNA synthesis. Non-GFP hRPCs served as negative control; GFP+ mRPCs was positive control. (B) GFP relative expression level.
level in hRPC, mRPC and hRPC with transfer(n=3). Intensities of RT-PCR images was measured with ImageJ software and normalized to GAPDH. Relative expression level of hRPC after transfer of EVs is significantly increased over negative control (hRPC only).

3.11 Discussion

Multipotent mRPCs are guided by genetic and epigenetic cues during differentiation and fate specification. In addition to differential gene expression and miRNA mediated post-transcriptional modifications within cells, a number of studies reveal that media conditioned from retinal cells provides soluble cues capable of influencing mRPC fate [8-13]. It is possible that factors secreted from developing retina, facilitating retinogenesis, are enclosed 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 decay [222]. 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 complex [223].

The data also reveals that mRPC EVs contain mRNA of transcription factors involved in retinal development including Nestin, Pax6, Hes1, Ki-67, and Sox2. Additionally, the miRNAs identified in EV 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 (data not shown). The differential distribution of specific mRNA species between mRPCs and EVs may indicate selective EV packaging of as a novel signaling mechanism guiding retinogenesis [224, 225].

A growing number of studies demonstrate the presence of functional miRNAs in extracellular spaces and in cerebral spinal fluid [71-74]. Additionally, extracellular miRNAs exhibit resistance to high extracellular ribonuclease (RNase) activity [71] increasing the probability of packaging in membrane bound EVs. EV
mediated miRNA transfer is emerging as a mechanism of cell-to-cell gene expression regulation across tissue types [77, 78].

The miRNA species present in mRPC EVs, miRNA9, miRNA182, miRNA204 and let7d, are present during retinal development. Analysis of mouse retinal miRNA transcriptome [55] 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 [206, 207]. Let-7d plays important roles in neural fate specification, with predicted function in RPC differentiation [65, 226]. Changes in expression levels of mRPC EV encapsulated miRNAs may provide a subtle level of cell-to-cell genetic regulation active during retinal development [55].

EVs derived from mRPCs may also provide protein cargo to guide morphogenic processes. Morphogenesis proteins identified in mRPC EVs include, Latrophilin-3 (Lphn3) [227], which facilitates cell adhesion and synapse formation, Integrin alpha-6 (Itga6) [228], active in retinal lamination and neurite outgrowth as well as neural cell adhesion molecule 1 (Ncam1) [229], associated with retinogenesis (Table 3). Potential mediators of mRPC physiology, contained in EVs include, the Ca\(^{2+}\)-activated chloride channel, twenty homolog 2 (Ttyh2) [230], the voltage-dependent calcium channel gamma-7 subunit (Cacng7) [231], which regulates the trafficking and gating properties of AMPA-selective glutamate receptors and the Monocarboxylate transporter 1 (Slc16a1) [232], 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 Tables 4 and 5, respectively.

Solute carrier (SLC) transporters are a family of membrane proteins, involved in cellular uptake of small molecules. mRPC EVS are enriched in SLC transporter proteins including. Slc1a2, Slc2a1, Slc6a1, Slc7a5, Slc12a7, Slc12a2 and Slc16a1 (Figure 25 and Table 6). Slc1a2 is a glutamate transporter, mainly expressed in neurons, involved in removing synaptic glutamate and terminating glutamatergic transmission [233]. Slc2a1 is also involved in uptake of glucose [234, 235]. An additional mRPC EV amino acid
transporter is ASCT1 (Slc1a4) which facilitates electroneutral exchange of amino acids [236]. Lactate transporter Slc16a1 is involved in regulation of lactate transport, which increases during glycolysis [237, 238]. In addition, Slc3a2 regulates the function of integrins 5-8 and modulates amino acid transport [239-241].

Future work may help explain some predicted functions of mRPC EV cargo transfer. While progenitors pass through stages of retinal development, a number of time specific gene products, transcription factors and miRNA are present in cells and potentially released in EVs. mRPC EVs may modulate genotypic and phenotypic dynamics of neighboring cells. We assume EVs from retina, at different developmental stages including E14 (embryonic day 14), P1-4 (postnatal day 4) and adult will contain functional miRNA, mRNA and proteins which can be delivered to recipient cells. We predict that the characterization of EVs collected from E14, P4 and adult retina will show mRNA and microRNA similar to the expression patterns of the originating mRPC populations at each developmental time point. Enclosing genetic materials in EVs and deliver them to neighbor local cells is a native process during pathology and physiologic state. Alongside these native effects, EV-based therapy holds great hope for possible therapy, in which EVs has been used as vectors to deliver genetics or drugs to recipient cells.

EVs released from mRPCs E14 might contain mitotic progenitor cell-enriched gene products including Chx10, Pax6 and Hes1 which could influence RPC multipotentiality and other transcription factor such as Hes5, Mash1, NeuroD, Neurogenin-2, OC-1, Six3, Math 5 (Atoh7), Hes5, Sox9, Islet2 and mLHX2 which function in early ganglion cell fate determination.

The genetic composition in EVs isolated from P1-4 RPCs may show decreased E14 products. And that the transcription factors Hes6 and Mash1 involved in promoting cell differentiation might be detected. Also, EVs from P4 may contain a number of rod specific genes such as Rbp3, Elovl4 and Abca4, Rax homeodomain factor, Nrl and NR2E3. EVs isolated from adult retina contain Ddx5, Hmgb3, Kdd3 which are highly expressed in late stage and adult retinal tissue.
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, 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 in mRPC derived EVs supports the possibility that additional 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.

3.12. Materials and Methods

3.12.1 mRPC isolation and culture

Actin promoter-GFP mRPCs were isolated from post-natal day one mice and cultured as previously described [242]. Briefly, mRPCs were grown at 37°C and 5% CO₂ in neural basal complete media containing 2 mM glutamate, 1x EV-depleted B27 supplement, 1x N2 supplement, 20 ng/ml recombinant epidermal growth factor (rEGF), 50μg/ml nystatin, penicillin-streptomycin (10 IU ml⁻¹ and 20μg ml⁻¹), respectively. mRPCs were plated onto T-75 flasks and medium replaced every two days. EVs were depleted in B27 by first centrifuging at 100,000g for 70 min and then filtering using a 0.2 μm pore size filter.

3.12.2 Isolation of mRPC EVs

EVs were isolated from media conditioned by 1x10⁷ mRPCs cultured in T75 flasks for 48 h. Media was centrifuged at 300g for 10 minutes at 4°C to pellet cell debris. Supernatant was transferred to an ultracentrifuge tube (Beckman Coulter, USA) and spun at 10,000g for 20 minutes using 60Ti rotor (Beckman Coulter ultracentrifuge) at 4°C; supernatant was filtered through a 0.22 μm filter and centrifuged at 100,000g for 70 minutes 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.
3.12. 3 Cell Viability Assay

Mouse retinal progenitor cell suspension was prepared from one T-75 flask at 80% confluency. Cell suspension was then centrifuged at 300 x g for five minutes and old media was removed. Five milliliter of Neurobasal Media was added and cell pellet was re-suspended. The number of viable cells was then counted using an EVE Cell Counting Chamber before plating. mRPCs were plated at the concentration of $1.0 \times 10^5$ cells/cm$^3$ with 2ml of media in 24 well plates. There are four samples (n=4) for the following incubation period: 0, 24, and 48 h. After incubation at 0, 24, and 48 h, a viability assay was performed using WST-1 reagent (Cell Proliferation Reagent WST-1, CELLPRO- RO ROCHE). 200ul 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 450nm. WST-1 is a colorimetric assay that uses a soluble tetrazolium salt, which are then cleaved to a colored product known as formazan by the reductase system of metabolically live cells.

3.12.4 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 hour. Samples were then rinsed in 0.1 M phosphate buffer, pH 7.2 three times, 5 minutes 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). Sample edges were blotted during each solution change, taking care to not let samples dry out. Samples were then dried on a glass surface, coated with gold sputter coating and examined using a Zeiss Supra 55VP microscope.

3.12.5 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 hours. Cells were post-fixed in osmium tetraoxide for 30 min, washed with distilled water and subsequently dehydrated using increasing ethanol concentrations (70%, 85%, 95% and 100%), each for 10 minutes. Sample dehydration was followed by immersion in propylene for 20 minutes twice. Samples were infiltrated with a 1:1 mixture of propylene oxide and Spurr’s Resin for
1 hour, 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 minutes, rinsed in water and stained for 90 seconds 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.

3.12.6 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 24 h at 4 °C. After 4 washes in PBS, a 1:50 dilution of 2 nM 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 Forvar 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. After rinsing with water, grids were stained in 4% uranyl acetate solution, rinsed and dried. Samples were imaged using a FeiTechnai Spirit Transmission Electron Microscope operated at 80 kV and AMT digital imaging software.

3.12.7 NanoSight analysis

EV size and concentration were assessed using the NanoSight NS500 system. Collection of supernatant was identical to isolation of EVs described above, with the exception that supernatant just prior to 100, 000g
ultracentrifugation was used for NanoSight analysis. Control media, non-conditioned, was processed under identical conditions. Final supernatant was diluted at 1:20 in PBS and triplicates of 1 ml samples were used for NanoSight 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 graph, 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 are denoted with asterisks: * (p<0.05), ** (p<0.01), *** (p<0.001), **** (p<0.0001); “ns” indicates no significant difference.

### 3.12.8 Sucrose gradient analysis

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, USA). An EV pellet isolated from 27 ml of conditional medium was resuspended in 0.5 ml of PBS, loaded on top of the layered sucrose gradient and centrifuged at 18,000g at 4°C for 15 h. Fractions containing EVs were harvested and the densities determined by weighing each fixed volume. Each 1ml fraction was then diluted in 26 ml of PBS, and ultracentrifuged for 1h at 100,000g. Pellets were solubilized in sample buffer, electrophoresed, and immunoblotted as described above. The protein concentration each sample was quantified using the NanoOrange Protein Quantification kit (Life Technologies Corporation).

### 3.12.9 Western blot analysis

RPC and isolated EV protein was measured using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, USA). RPCs and 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), 1x Halt Protease inhibitor Cocktail (Thermo Scientific), 100mM NaCl, 1mM EDTA and 2mM MgCl$_2$ at pH7.4. Aliquots of sample lysates containing 20 μg protein, as determined by the BCA Protein Assay, were used for 4% to 12% Sodium Dodecyl Sulfate Polyacrylamide gel
electrophoresis (SDS-PAGE) and further processed for silver staining (Bio-Rad, USA) or electroblotted onto nitrocellulose, respectively. The following primary antibodies were used: paired box gene 6 (Pax6) (Rabbit Polyclonal, Abcam) 1 μg/ml, and glial fibrillary acidic protein (GFAP) (Rabbit Polyclonal, Abcam) 1:5000, enhancer of split 1 (Hes1) (Rabbit monoclonal, Abcam) 1:1000, (sex determining region Y)-box2 (Sox2) (Rabbit Polyclonal, Abcam) 0.5 μg/ml, Nestin (DSHB) 1:5000, Ki67 (Life Technologies) 1:5000, β-actin (Sigma), CD63 (Rabbit Polyclonal, Santa Cruz) 1:500, GFP (Sigma) 1:2000.

3.12.10 Mass spectrometry

Whole cell lysate and exosome-enriched samples from mRPCs were denatured in 8M 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 6h at room temperature. The digestion was quenched with 2% formic acid and resulting peptide mixtures were [243]. Samples were dried and resolubilized in 2% acetonitrile and 2%formic acid. Approximately 1 μg of each sample was injected for analysis by reversed phasenano-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 x 2 cm, Thermo Scientific) peptides were separated using a 12 cm x 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% BufferB (0.1% formic acid in acetonitrile) / 95% Buffer A (0.1% formic acid) to 40% Buffer B / 60%Buffer A, over 140 minutes. All LC-MS/MS experiments were performed in data dependent mode with lock mass of m/z 445.12003[244]. Precursor mass spectra were recorded in a 300-1400m/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.

3.12.11 Protein profiling analysis

Mass spectrometry data were searched against a Uniprot mouse database (July 2014) using MaxQuant (version 1.5.0.30 [244]). 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) [245]. 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.

3.12.12 DNA extraction, RNA extraction and Quantitative real-time PCR

EVs isolated from 20 ml of conditioned media was used for DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega USA). Concentration was measured using a Nanodrop 2000. Half of the DNA extractions from EVs was used for agarose gel analysis and the other half was used for EcoRI digestion (NEB). For the RPC sample, 0.5 μg of extracted DNA was used for each agarose lane.

3.12.13 RNA extraction and Quantitative real-time PCR

EVs isolated from 20 ml of conditioned media were used for total RNA extraction using RNeasy Mini Kit (Qiagen). Prior to RNA and miRNA extraction, EVs were treated with 100 ug/ml RNAse (Thermoscientific) for 30min 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[246, 247]. 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. Primers were listed in Table 9.

Table 9. Primers used for realtime PCR.
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<td>R: TCAAGGGTATTAGGCAAGGGG</td>
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<td>Sox2</td>
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miRNA was extracted using a MirVana RNA isolation kit (Thermo Fisher Scientific). The same amount 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 10.

### Table 10. miRNAs assay IDs

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<td>001973</td>
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3.12.14 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 instructions. Briefly, EVs were collected after the 100,000g ultracentrifugation, and incubated in PKH26 for 5 min at room temperature. Excess dye was removed by rinsing with serum-free medium, followed by three rinses in PBS. EVs were re-suspended in serum-free medium and incubated with mRPCs for 4 hours at 37°C. mPRCs were cultured on coverslips coated with mouse laminin (Life Technologies, USA) 24 hours before culturing with PKH26 labeled EVs. Images of EV binding and internalization were captured with a Nikon confocal spinning disk microscope using a 60X objective.
3.12.15 Ionomycin EV release and immunocytochemistry

To measure calcium induced EV release, mRPCs were cultured on 6-well plates and treated with 1 μM ionomycin (Sigma) based on manufacturer’s instructions. mRPCs were then fixed using 1% paraformaldehyde in PBS for 30 min, blocked and permeabilized for 30 min by incubation in PBS with 0.1% Triton X-100 and 3% bovine serum albumin (BSA). Anti-CD63 (Rabbit Polyclonal, Santa Cruz) was added and incubated for 1h at 37°C. Next, mRPCs were incubated with a fluorescein conjugated secondary antibody (DyLight 594 AffiniPure Goat Anti-Mouse IgG, Jackson Immuno) and ProLong Gold mounting media with DAPI (Thermofisher). Images were taken from time 0-10 min and analyzed for the increased presence of labeled (541/572) EVs using a Leica SP2 AOBS confocal microscope with a 40X objective. Each image was acquired with identical laser intensities and amplifier gains. Imaged mRPC fluorophores included GFP (490/520), CD63 protein (541/572) and DAPI (358/461), excited independently and detected sequentially.

3.12.16 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. 5x10^5 GFP+ mRPCs were plated on transwell inserts (0.4 μm pore diameter) above non-GFPhRPCs in each 6-well plate. Cells were grown at 37°C and 5% CO₂ in neural basal complete media containing 2 mM glutamate, 1x B27, 1x 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 3 days, rinsed using PBS and processed for mRNA extraction.
Chapter 4. Characterization of Induced Pluripotent Stem Cell Extracellular Vesicle Genesis, Morphology and Pluripotent Content

In this section of the work we analyze EVs released from induced pluripotent stem cells (iPSCs). We characterized iPSC EV genesis, content and fusion to retinal progenitor cells (RPCs) in vitro. Nanoparticle tracking revealed that iPSCs released approximately 2200 EVs cell/hour in the first 12 h with an average diameter of 122 nm. Electron microscopic analysis of iPSCs revealed cytoplasmic origin of EVs and release via lipid bilayer budding. The mRNA content of iPSC EVs was characterized and revealed the presence of the transcription factors Oct-3/4, Nanog, Klf4, and C-Myc. The protein content of iPSCs EVs, detected by immunogold electron microscopy, revealed the presence of Oct-3/4, Nanog and TSG101, a EV marker. Isolated iPSC EVs were shown to fuse with RPCs in vitro at multiple points on the plasma membrane. These findings reveal that the mRNA and protein cargo in iPSC EVs have established roles in maintenance of pluripotency. Building on this work, iPSC derived EVs may be shown to be involved in maintaining cellular pluripotency and may have application in regenerative strategies for neural tissue.

4. 1 Introduction

EVs have unique molecular compositions derived from the type and activation state of the cell of origin and have been shown to contain cell specific subsets of proteins, mRNA, microRNA and organelles[248]. Embryonic stem cell (ES) derived EVs have been shown to contain ES specific mRNA with the potential to influence target hematopoietic progenitor cell gene expression. Hematopoietic progenitor cells co-cultured with ES derived EVs exhibited enhanced survival and upregulated expression of pluripotent genes[249]. Also, EVs from adult human bone marrow and mesenchymal stem cells were shown to horizontally transfer mRNAs to recipient kidney tubular cells, contributing resistance to apoptosis and repair of acute kidney injury [248]. Similarly, EVs from human liver stem cells were shown to accelerate the morphological and functional recovery of liver tissue in a rat hepatectomy model[250].

In this study, mouse iPSC derived EVs were characterized for intracellular origin, morphology and molecular composition. We demonstrate that iPSC derived EVs contain a group of iPSC-specific pluripotent
transcription factors. We also evaluated iPSC-derived EVs release rate and fusion to target cells in vitro. Results from this study suggest that iPSC-derived EVs are vehicles for transfer of pluripotent genetic material to target cells with potential application toward regenerative strategies in neural tissue. Analysis of iPSC-derived EVs may enhance understanding of disease pathogenesis and be useful in developing personalized medicine.

4.2 Materials and Methods

4.2.1 Cell culture

All cells were cultured under sterile conditions and maintained in a 95% O₂/5% CO₂ humidified incubator at 37 °C. Primary mouse Nanog-GFP iPSCs were purchased from Stemgent (Cambridge, MA, USA) and cultured in Knockout DMEM (GIBCO, Invitrogen), supplemented with 15% fetal bovine serum (FBS; Sigma), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 5 mg/ml penicillin/100 mg/ml streptomycin, and leukemia inhibitory factor (1 x 10⁷ U/ml). The iPSCs used for EV analysis were plated and cultured without MEFs on 0.1% gelatin-coated T75 culture flasks. Actin promoter-GFP mouse RPCs were isolated from post-natal day one mice and cultured as previously described [251].

4.2.2 Cell membrane labeling

To visualize lipid membranes, iPSCs were labeled with the TRITC fluorescent lipophilic dye PKH26 (Sigma). iPSCs (2 x 10⁷ cells) were suspended in PKH26 diluent-C and mixed with 4x10⁻⁶ M PKH26 dye and incubated at 25°C for 5 min. The staining reaction was quenched by addition of an equal volume of DMEM supplemented with 1% BSA. Labeled iPSCs were then centrifuged, and re-suspended in pre-warmed media for further analysis.

4.2.3 Isolation of EVs

EVs were isolated using a modified protocol based on previous work by Yuan et al.[252]. Exosomes in FBS were depleted by first centrifuging FBS at 110,000 x g for 70 min and then filtering using a 0.2 μm pore size filter. To collect iPSC EVs, cells were cultured from 24-48 h, and conditioned media was collected and
transferred to centrifuge tubes (polypropylene conical bottom) and centrifuged at 500x g for 10 min to pellet cells at room temperature; supernatant was collected and centrifuged at 10,000x g for 20 min at 4°C (Beckman Ultracentrifuge, Rotor 60ti) to remove cell fragments and debris; final supernatant was spun at 100,000 x g for 70 min at 4°C to pellet the EVs. EVs were resuspended in phosphate-buffered saline (PBS) and stored at −80°C for further analysis.

4.2.4 NanoSight analysis of EV size and concentration

EV size and number were assessed with NanoSight NS500 system. From 12-48 h after culture in T-75 flasks, conditional media was collected and transferred to centrifuge tubes. Collection of supernatant was identical to previously described isolation of EVs, except supernatant before 100,000x g ultracentrifugation was used for NanoSight analysis. Control media, non-conditioned, was processed at the same time. Final supernatant was diluted at 1:20 in PBS and 1ml was used for NanoSight analysis. The NanoSight system uses a laser light source to illuminate nano-scale particles, detected individually as light-scattered points moving via Brownian motion. Polydispersity was quantified, and we used Nanoparticle Tracking Analysis (NTA) software 2.3 to track and size nanoparticles on an individual basis. Results are displayed as a frequency sized distribution graph describing the number of particles per ml.

4.2.5 Preparation of PKH26-labelled iPSC samples for confocal microscopy

After labeling iPSCs with fluorescent PKH26, cells were incubated at 37°C for 3 h on glass cover slips. iPSCs were then fixed on coverslips with 4% PFA for 5 min and washed with PBS 3 times at 10 min intervals. The samples were imaged at 40x using a Leica SP2 AOB5 confocal microscope. Excitation was achieved using a HeNe laser. The excitation wavelength was 543 nm and the emission range was 553-650nm. Z-stacks were obtained and a 3-dimensional reconstruction was done using Nikon Elements software.

4.2.6 Transmission electron microscopy (TEM)

Cultured iPSCs (4 x 10^7 cells) were fixed in 2.5% glutaraldehyde with 4% paraformaldehyde for 2.5 h and washed in PBS for 2-4 h. Cells were post-fixed in osmium tetraoxide for 30 min, and washed with distilled water and subsequently dehydrated using increasing ethanol concentrations (70%, 85%, 95% and 100%),
for 10 min each, followed by immersion in propylene for 20 min, two times. Next, cells were infiltrated with a 1:1 mixture of propylene oxide and Spurr’s Resin for 1 hand then left in 100% Spurr’s Resin overnight. They were then embedded in Beem Capsules using fresh Spurr’s Resin and left in an oven at 70 °C to polymerize. Excess resin was trimmed and 90 nm sections of cells were made on a Leica Ultracut Ultra microtome. Sections were placed on 200 mesh copper grids, stained with saturated uranylacetate in 50% ethanol for 6 min, followed by rinsing in water and staining for 90 s in lead citrate. Grids were then washed in water, dried on filter paper and viewed under a FeiTecnai transmission electron microscope rated at 80 kV. Images were obtained using an AMT camera with AMT digital software.

4.2.7 Preparation of EVs for TEM

EVs released from iPSCs were isolated from 50 ml of culture supernatant using differential centrifugation. Then 5 µl of suspension containing isolated EVs was dropped on a Zoo-mesh Carbon Formuar grid; EVs were allowed to absorb for 20 min at room temperature. Excess suspension was wicked off and grids were submerged in 25% gluturadelyde/4% PFA with 25% tannic acid in PBS for 10 min. Grids were then washed in distilled water and viewed on a FeiTecnai transmission electron microscope, operated at 60kv. Digital images were obtained using an AMT digital camera and software.

4.2.8 Preparation of EVs for scanning electron microscopy (SEM)

EVs released from iPSCs were isolated from 50 ml of culture supernatant using differential centrifugation. The isolated EV sample was added to membrane filter discs and incubated in 3% glutaraldehyde (dissolved in PO₄ buffer) for 1 h at room temperature. Glutaraldehyde was then aspirated, and the EV sample was washed with PO₄ buffer 4 times, at 10 min. intervals. To dry the sample, PO₄ buffer was exchanged with ethanol. To avoid osmotic shock, ethanol concentration was gradually increased (10, 30, 50, 70, and 90%). The final step was performed in 100% ethanol for 1 h with three changes of ethanol. Samples were dried with liquid CO₂ and sputtered with gold. Samples were imaged using a ZeissSupra 55VP scanning electron microscope.
4.2.9 Total RNA purification

Total RNA from iPSCs and EVs were isolated using a Max-96 total RNA kit (Life Technologies). Briefly, 20 μL of bead mix was added to iPSCs or EV pellets, and shaken for 5 min. Then, RNA was magnetically captured by the RNA binding beads and washed with 150 μL wash solutions 1 and 2, followed by shaking for 1 min per wash. 50 μL of Diluted TURBO DNase was added to the sample followed with shaking for 10–15 min at room temperature. After adding 100 μL of RNA rebinding solution, the sample was shaken for 3 min. 150 μL of wash solution 2 was added twice to the sample, followed by shaking for 2 min. Next, 50 μL of elution buffer was added to the sample followed by vigorous shaking for 3 min. The supernatant was transferred to a nuclease-free container. Isolated RNA was measured for quality using a Nanodrop ND/1000 spectrophotometer and analyzed by 2% gel electrophoresis.

4.2.10 Real-time PCR analysis

Total RNA (400~600 ng) from iPSCs and EVs was reverse-transcribed to generate cDNA using the AMV first strand cDNA synthesis kit (New England Biolabs). 10 ng of reverse-transcribed single strand cDNA was used as a template for real-time PCR in 50μL of RT-PCR mix. Forty cycles of PCR were performed on cDNA samples using SYBR Green ER qPCR ER Supermix (Invitrogen) and six primers: c-Myc, Klf4, Nanog, β-actin, GAPDH and Oct-3/4. The PCR reaction consisted of an initial enzyme activation step, at 95°C for 10min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s, then followed by melting curve analysis. RT-PCR was carried out in triplicates for each pair of primer. Student’s t-test was used to identify significant differences in mRNA between two groups and p values of less than 0.05 were considered statistically significant.

4.2.11 Immunoelectron microscopy

Resuspended 2% paraformaldehyde fixed EVs were put on glow discharged formvar-carbon coated nickel grids. After washing with PBS, the grids were incubated with 50 mM glycine/PBS for 3 min. The grids were blocked for 10 min with either 1% coldwater fish skin gelatin (Sigma-Aldrich) for the surface immunolabeling (Tsg101, Abcam), or 5% BSA, 5% goat serum, 0.1% cold-water fish skin gelatin and 0.1% saponin in PBS
for inner membrane protein labeling (Nanog and Oct4, Abcam). Primary antibodies in blocking solution for Tsg101 or in antibody incubation buffer (0.1% BSA and 0.1% saponin) for Nanog and Oct4 were applied for 2 hours at room temperature. After washing with PBS, Nanogold-labeled Fab’ anti-rabbit or anti-mouse (Nanoprobes, NY), or 5nm, 10nm gold conjugated goat anti-mouse antibodies (Ted Pella Inc. Redding, CA) were applied in the correlated antibody incubation buffer for 1 hour. The grids were then washing with PBS, fixed in 1% glutaraldehyde for 5 min. After thoroughly washed with distilled water, the grids were either directly go to methylcellulose embedding for 5nm or 10nm gold, or continue with silver enhancement for nanogold. For the silver enhancement, the grids were washed with 0.02 M sodium citrate (pH 7.0), and performed silver enhancement in the dark using HQ Silver enhancement kit (Nanoprobes, NY) at room temperature for 8 min. After washing with distilled water, the grids were contrasted and embedded in a mixture of 3% uranyl acetate and 2% methylcellulose in a ratio of 1 to 9. Stained grids were examined under Philips CM-12 electron microscope and photographed with a Gatan (1k x1k) digital camera. All antibodies were purchased from Abcam, USA. Anti-Tsg101 (1:200), Anti- Nanog (1:400) and Anti-Oct4 (1:200) were diluted according to manufacturer’s instruction.

4.2.12 Transfer and fusion of PKH26-labelled iPSC EVs to mRPCs

EVs from PKH26-labelled iPSCs, were isolated from 50 ml of culture supernatant using differential centrifugation. PKH26-labelled EVs were incubated with retinal progenitor cells (RPCs) in culture at 37°C for 3 h. mRPCs were used to demonstrate iPSC-EV binding within a robust neural progenitor population. RPCs with fused PKH26-labelled EVs were rinsed 3 times with PBS and then were fixed in 4% PFA for 20 min and washed with PBS 3 times,10 min each. Samples were imaged using a Leica SP2 AOBS confocal microscope with 40x oil immersion objective and digital zoom. A HeNe laser was used for PKH26-labelled EVs. The excitation wavelength was 543 nm and the emission range was 553-650nm. A 488 Argon Laser was used to image for GFP RPCs.
4.3 Results

4.3.1 Size and Concentration of EVs derived from iPSCs

EVs isolated from iPSCs were analyzed using nanoparticle tracking analysis technology (NTA). NTA tracking allowed robust analysis of secreted vesicle size and release rate (Figure. 31). Data shows that iPSC released EVs have diameters that fall within the range reported for secreted microparticles (100 nm-1 um)[253, 254]. EVs derived from iPSC conditioned media at three time points 12, 24 and 48h had consistent mean diameters of 122 ± 2.3 nm, 124 ± 6.0 nm and 122 ± 2.2 nm, respectively. The concentration of released EVs was calculated and the average number of EVs in conditioned medium at 12 h = (1.69 ±0.66)* 10^8, 24 h = (3.80 ±0.77)* 10^8 and 48 h = (4.20 ±0.40)* 10^8 (Figure.1F). Number of EVs released per cell/hr was also calculated after data was normalized to control media. At 12 h post-plating of 1x10^6 cells, individual iPSCs had released an estimated 2200± 884 EVs; at 24 h the number increased to 5000± 1023 EVs and at 48 h 5300± 220 EVs were released (data not shown).

![Figure 31](image.png)

**Figure 31.** iPSC EV diameter and release rate.
A, B, C) EV nanometer diameter range and concentration were derived from iPSC conditioned media 12 h, 24 h and 48 h, respectively. EV diameters and concentrations for three samples at each time point are indicated in colored traces. Mean diameters and standard deviation for each time point remained consistent; 12h 122 ± 2.3nm, 24h 124 ±6.0nm, 48h 122 ± 2.2nm. D) Arrows show light-scattering of individual EVs from a single frame of Nanosight tracking analysis at 24 h. E) 3D plot showing EV size/relative intensity. F) Average number of EVs in conditional medium at 12 h=(1.69 ±0.66)* 10^8, 24h =(3.80 ±0.77)* 10^8 and 48 h=(4.20 ±0.40)* 10^8. Error bars represent standard deviation. Student’s t-test was performed to assay the difference between iPSCs and EVs. p < 0.05 was considered statistically significant. “ns” represents not significantly different.

TEM analysis revealed release of EVs from iPSCs (Figure. 32A). TEM shows a microparticle (>100 nm) budding from the plasma membrane into the extracellular space (Figure.32A, arrowhead). To further confirm lipid involvement in release of EVs from iPSCs, we utilized confocal analysis of PKH26 labeled cell membrane and imaged the release of EVs emerging from iPSCs (Figure. 32B,C,D). Our analysis suggests that EVs, both exosomes and microparticles, were released from PKH26-lablelled iPSCs. Exosome budding appeared across the plasma membrane with release occurring periodically in cluster sites.

![Image A](image1.png) ![Image B](image2.png) ![Image C](image3.png) ![Image D](image4.png)

Figure 32. Analysis of iPSC EV formation.
4.3.2 Morphology of EVs isolated from iPSCs

The morphology and size of isolated iPSC EVs were analyzed using SEM and TEM. Visualization of iPSC EVs isolated via ultracentrifugation and imaged using SEM revealed heterogeneous spheroid morphologies with sizes ranging from 30 to 300 nm. SEM shows microparticles (Figure 33A) to be approximately 100-300 nm, while exosomes ranged from 30-95 nm (Figure 33B). The smooth shape of the EV surface, observed on the SEM image, can be attributed to the physical properties of the phospholipid bilayer. Using TEM analysis Figure 33C and D showed a sample of an isolated EV. TEM images revealed that iPSC exosomes have a cup-shaped appearance, which uniquely differentiates them from microparticles. Microparticles appeared consistently spheroid in shape. This level of TEM analysis aligns with previous studies describing microparticles as heterogeneous in shape and larger than exosomes with diameters reaching up to 1 µm [211].

Figure 33. SEM and TEM analysis of iPSC derived EVs.
SEM analysis of EV populations isolated from iPSCs revealed a heterogenous population of spheroid vesicles ranging in size from 30-300 nM. A) SEM of an EV population with a sample microparticle measured with a diameter of 103 nM, scale bar: 200 nm. B) A higher magnification SEM image showing measured exosomes with diameters of 73.60 and 93.42 nm. C) TEM analysis of EVs isolated from iPSC supernatant showed characteristic cup-shaped morphology and size (arrowhead) and D) spheroid exosome (arrowhead) with 100 nm diameter, scale bar: 100 nm.

4.3.3 RNA and protein identified in EVs derived from iPSCs.

The total RNA profile of iPSC EVs was determined by native agarose gel electrophoresis. As displayed in Figure. 34A, the 18S and 28S ribosomal RNA bands are clearly visible in the total RNA profile of iPSCs (lane2); in contrast, the total RNA from iPSC derived EVs does not contain 28S and 18S ribosomal RNA (lane1). The largest fraction of total RNA isolated from the EV population appeared as a lower molecular weight band suggesting the presence of mRNA and small RNA species. We then confirmed the presence of selected mRNA species in iPSC EVs. Isolated total RNA from iPSC EVs (1 µg) was reverse-transcribed and used as a template for amplification using the primers shown in Table 11. The house keeping genes β-actin and GAPDH were tested as positive controls and were present in both iPSCs and EVs (Figure. 34B). Both β-actin and GAPDH displayed much lower expression level in EVs than in iPSCs (p<0.05), so β-actin and GAPDH were not used as normalizers in our experiments. To compare expression level of transcription factors, same amounts of RNA was used in reverse-transcription for further analysis. Four transcription factors including Oct-3/4, Nanog, Klf4, and C-Myc were found in both cells and isolated EVs, but expression levels were lower in EVs than in iPSCs (Figure. 34B). The RNA composition of the EVs was also compared to that of iPSCs of origin, showing EVs lack 18 S and 28 S rRNAs. (Figure. 34A). With the exception of Klf, each RNA species analyzed was present higher levels in cells than EVs.
Figure 34. iPSC EVs contain pluripotent transcription factor mRNA.

A) Total RNA isolated from iPSC derived EVs lacks 28S and 18S rRNA and consists primarily of RNA below 200 bp as revealed through 2% denaturing agarose gel loaded with total RNA from iPSC EVs and iPSCs.

B) To verify the presence of selected iPSC derived mRNA, EV mRNA was analyzed using real-time PCR and contained factors involved in the maintenance of iPSC pluripotency including Oct-3/4, Nanog, Klf4, and C-Myc, Positive control β-actin and GAPDH was present in iPSCs and EVs. Significant differences between cells and EVs are denoted with asterisks: * (p<0.05), ** (p<0.01), *** (p<0.001), **** (p<0.0001); “ns” represents not significantly different.

Table 11. Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Myc</td>
<td>CAGAGGAGGAACGAGCTGAAGCGC TTATGCACCAGAGTTTCGAAGCTGTTCG</td>
<td>228 bp</td>
</tr>
<tr>
<td>Nanog</td>
<td>AGGTCTGACTGAGTGCTGCTCTG CAACCACCGTTTCTGACCAG</td>
<td>228 bp</td>
</tr>
<tr>
<td>Oct-3/4</td>
<td>CTGAGGGCCAGGCAGGAGCACGAG CTGTAGGGCTTCCGGGCACTT</td>
<td>485 bp</td>
</tr>
</tbody>
</table>
The protein content of iPSC EVs was visualized using immunogold TEM. In Figure 35, black punctate regions indicate immunogold labeling of the homeodomain transcription factor Nanog, the homeodomain transcription factor Oct-3/4 and the tetraspanprotein TSG101 protein within EVs. Each pluripotent protein identified within EVs has established roles in maintaining undifferentiated iPSC self-renewal.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klf4</td>
<td>CACCATGGACCCGGGCGTGCTGCCAGAAA</td>
<td>TTAGGCTGTCTTTTTCCGGGCCACGA</td>
<td>739 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGTTACCAACTGGGACGACA</td>
<td>ACCTGGGTCATCTTTTCACG</td>
<td>150 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGGCAAAATGGGAGATTGTGTC</td>
<td>AAGATGGGTGATGGGCTTCCCG</td>
<td>150 bp</td>
</tr>
</tbody>
</table>

**Figure 35. Immunogold EM analysis of EVs Protein Content.**

Immunogold EM images reveal the presence of A) the homeodomain transcription factor Nanog, B) the homeodomain transcription factor Oct-3/4, C) the tetraspanprotein Tsg101, a canonical EV marker, and D) control. Each pluripotent protein identified within EVs is involved in undifferentiated iPSC self-renewal. Samples were viewed under a FeiTecnai Transmission Electron Microscope. Scale: 50 nm.
4.3.4 Fusion of EVs derived from iPSCs with RPCs in vitro

The red fluorescent lipophilic membrane dye PKH26 was used to label isolated iPSC derived EVs, which were then incubated with target RPCs to evaluate EV docking and fusion (Figure 36). Following three hours of co-incubation, iPSC derived EVs attached to RPCs and a percentage remained adherent following multiple rinses with media. Imaging revealed the binding of single and clustered EVs across the surface of GFP+ RPCs with varying morphologies, as single cells and within larger colonies. EV binding appeared to be facilitated via lipid fusion as EVs were visible only on cells and not on cell-free culture surfaces following rinsing.

**Figure 36. iPSC EVs fuse to target cells in vitro.**

A) iPSC EV co-cultured GFP+ RPCs remain viable expressing GFP ubiquitously throughout the cytoplasm and showing robust branching morphology. B) Dendritic processes and cell-to-cell contacts are visible using phase contrast. C) EVs isolated from iPSCs labeled with the red fluorescent lipophilic dye PKH26 fuse to GFP+ RPCs. D) Overlay A-C. scale 10µm.
4.4 Discussion

In this study we observed that iPSC EV generation and release are consistent with previous studies describing this process [255[S, 2013 #531]. We discovered that the cargo of iPSC derived EVs incudes mRNA and protein species involved in self-renewal and pluripotency[256[Takahashi, 2013 #534, 257]. In addition, SEM and TEM analysis of iPSC derived EVs was consistent with previous findings, describing microparticles as heterogeneous in shape, and exosomes with cup-shaped morphologies[165, 248].

In the process of electron microscopic analysis, we observed nano-sized, grain-like structures (>20 nm) in the background of SEM images (Figure.33A). These structures are gold grains formed as a result of gold sputtering during sample preparation for SEM. Additional studies may advance EV analysis by use of flow cytometry. An advantage of flow cytometry is that no sputtering procedures are needed. A disadvantage would be that the structural features of the EVs would not be visible[258] and most systems cannot reliably acquire data on the smaller size EVs (>500 nm). To address these current technical challenges in the field of EV analysis, the Scientific Standardization Committee of the International Society on Thrombosis and Hemostasis are evaluating standardized protocols for EV isolation and analysis [259].

Our findings support existing data describing the process of microparticle and exosome genesis. However, the signaling mechanisms of initiation and termination of EV genesis remain to be fully elucidated[254]. Using TEM, we showed how microparticles were formed by the process of lipid bilayer budding[255]. We also observed exosome secretion from multivesicular bodies. Among membranous vesicles, exosomes are the only type that originate from intracellular MVB compartments [165]. According to a previous study, MVBs either fuse with lysosomes to degrade their bioactive load or fuse with the plasma membrane to release their content as exosomes into the extracellular environment [260].

In agreement with a number of studies, our findings support that EVs contain a subset of mRNA and protein derived from the cell type of origin [77, 248]. In particular, we have shown that iPSC EVs contain mRNA species, which encode for transcription factors critical to maintaining iPSC pluripotency. In addition, iPSC EVs are enriched in the transcription factor Nanog and Oct-3/4, important for iPSC self-renewal. Current
understandings of cell targeting of subsets of RNA and proteins to EVs remains limited [73]. A recent study suggest that ES EVs transfer a specific subset of miRNAs and mRNA in a highly selective manner possibly regulated by proteins found in EV membranes[77]. Cargo selection for EVs may also be understood by comparing the expression profile of mRNA and miRNA in the cells of origin and the presence of ribonucleoproteins that function in the intracellular transport [261]. For example, EVs released from MSCs(mesenchymal stem cells) have been shown to contain both cell specific mRNA and ribonucleoproteins that function in RNA storage, transport and stability. Current studies suggest that EV cargo uptake is facilitated by clathrin-mediated processes with ribonuleoproteins targeting mi[262]RNA and mRNA and N-linked glycosylation directing protein cargo [261-263]

In this work, our confocal analysis confirms earlier studies showing that an essential mechanism through which EVs communicate with target cells by fusing directly with the plasma membrane (Figure.36). Here FITC labeled EVs remained bound to, and potentially became internalized in, RPCs following a brief incubation. Our 3D confocal reconstruction of EV fusion in S2 shows robust fusion at several points on target RPCs. EVs have been reported to release their contents into the cytoplasm of target cells with the potential of influencing gene expression states, viability and plasticity [264, 265]. Recent findings indicate that fusion is the initial step required for horizontal transfer of EV genetic cargo as well as proteins, phospholipids and organelles [174, 266]

Further analysis of the process of iPSC EV transfer of pluripotent transcripts and protein will build on data presented here. EVs derived from ES and iPSCs may influence expression patterns toward pluripotency in target cells[267]. Recently it has been shown that ES EVs can alter the gene expression of retinal Muller cells contributing to trans differentiation, an initial step toward regeneration [253]. The finding that ES EVs may switch on early programs of pluripotency suggests that EVs may serve as therapeutic agents to restore the regenerative potential of the retina. In line with this research, iPSCs may be developed as a patient specific vehicle for delivery of genetic cargo toward nervous system repair. In support of the in vivo neural protective potential of EVs in retinal disease, a recent study has examined the role of exosome transfer of crystallin (a biomarker in age related macular degeneration) between retinal pigment epithelial cells and
photoreceptors. The authors demonstrated that EV-secreted crystalline was neuro protective for photoreceptors [268]. Additional studies suggest that miRNAs may be potential therapeutic targets for age related macular degeneration[269]. Since it may be harmful to inject naked siRNAs into the eye, EVs may also be useful to transfer miRNA to treat retinal disease [253].
Chapter 5. Extracellular Vesicles of Developing Human iPSC Derived 3D retinas as a model of human retinal development

Noncoding small RNAs in retina regulate gene expression by targeting and repressing mRNA. Recent studies demonstrated that small RNAs are secreted by extracellular vesicles (EVs).

Extracellular vesicles (EVs) are newly identified mechanism of cell-to-cell communication, with potential information transfer comparable to paracrine signaling. EVs provide snapshots of the originating cell’s genotype and phenotype, encapsulating and transferring cell-specific combinations of DNA, RNA and protein. To begin to characterize EVs present during human retinal development, we analyzed the morphology and content of EVs released from iPSC derived 3D retinas at three developmental time points, D42, D63 and D93. Analysis of small RNAs contained in EVs using next generation sequencing revealed the presence of a range of non-coding RNAs such as micro, piwi- and transfer-RNA species with predicted regulatory functions. This work provides the first evidence of small RNA species contained within EVs released from a model of human retinal development.

5.1 Introduction

As previously described, EVs have the potential to facilitate horizontal transfer of DNA, mRNA, miRNA, and protein between cells without direct cell-to-cell contact [270, 271]. During release of EVs, mRNA, small RNAs and proteins are specifically sorted and accumulated in EVs. Recent studies suggest that small RNAs including microRNA (miRNA) are particularly abundant in exosomes and EVs can transfer a select subset of microRNA that might influence cellular functions.

A novel group of studies are investigating genetic material in EVs released from neural tissue [272, 273]. Extracellular vesicle encapsulation, release and transfer of genetic material has been shown to facilitate stem and progenitor cell fate determination[70] . Studies also show that EVs are released by cortical neurons, microglia, oligodendrocytes, and astrocytes in the central nervous system and Schwann cells in the peripheral nervous system[270, 274, 275] . According to recent findings, mRNA and miRNA are
selectively packed into Schwann cell EVs, which influence broad changes in the transcriptional profile of recipient neurons [171].

During the development of the neural retina, retinal progenitor cells pass through a series of competence states, during each of which different subsets of retinal cell types are produced with signature gene expression patterns [276]. In a conserved temporal sequence, early retinogenesis generates ganglion, cone, horizontal, and amacrine cells. In late retinogenesis bipolar, rod, and Muller cells are born. According to this model, the progeny of retinal progenitor cell fate are regulated by a combination of extrinsic and intrinsic cellular signaling, which are not yet fully characterized [171][Livesey, 2001 #67]. To analyze EV release and content during human retinal development, human induced pluripotent stem cells (hiPSCs) were directed to differentiate into laminated 3D retinas in vitro [277][Mellough, 2012 #666]. Differentiation of hiPSCs into 3D retina occurs within a dynamic microenvironment with expression patterns and cell–cell signaling aligned with the competence model of in vivo mammalian retinal development[171].

A growing body of data supports the presence of a range of RNA sizes and species within EVs released from neural tissue. A percentage of the small RNA species contained within EVs falls within the range of 20 to 200nt. Small non-coding RNAs (snRNAs) have shown regulatory functions in a number of organisms. There are diverse types of non-protein coding RNAs, including micro RNA (miRNA), piwi-interacting RNA (piRNA), tRNAs and long-non-coding RNA(lncRNA). miRNAs play an essential role in regulation of neuronal differentiation throughout development [278]. During the temporal progression of retinal development, RPC competency changes occur, activating cell fate transcription factors and responses to extrinsic factors, leading to a stereotyped schedule of generation of different types of neurons [276, 279]. miRNAs, in particular let-7, microRNA-125, and microRNA-9, are involved in temporal regulation of RPC differentiation and fate specification during development [280, 281]. A number of studies reveal that miRNA species involved in retinal development and function circulate in the extracellular space and can be isolated from CSF, aqueous and vitreous humor [272, 282].
piRNA (Piwi-associated RNA) or piRNA-like molecules, are longer than miRNA, ranging in size from 26 to 31 nt. [283, 284]. Several studies have reported that piRNAs are a complex population of small RNAs protecting the animal germline by silencing transposons [285, 286]. More recently, roles for piRNAs in regulation of gene expression have been described [285]. In addition, piRNAs are expressed abundantly in the CNS and regulate retrotransposons [287]. Finally, a number of piRNA have been identified in ocular tissue, retina and retinal pigment epithelium (RPE) and lens [288, 289].

In addition to miRNA and piRNA, there is abundant tRNA present in developing tissue and required for translation. One way tRNA differs from miRNA is that it is restricted to the cytoplasm. tRNAs have been found to act as regulatory mechanisms during retinal development [290, 291]. tRNAs are also known to be active as regulators of cellular housekeeping, essential for neural signaling and basic cellular physiology [292].

Inducing hiPSC to 3D retinal cup is a simple and in vitro strategy to mimic the in vivo microenviroments [293]. In this system, hiPSC recapitulate each of the main steps during native retinal development and form 3D retinas. Formed photoreceptors begin to form outer-segment and photosensitivity. Differentiation of ES/iPS cells into retinal cells brings great potential for future possible therapies for retina disease. According to Zhong’s report, culturing hiPSCs in vitro in particular chemically defined neural differentiation media, form aggregates and eye field during D10 to D 17 (Figure37); by D21-D28, neural retina domains formed, which are enriched in progenitors. RPE at this point was removed and neural retina was cultured in suspension. As differentiation progressed, 3D mini retina cup formed. From time of NR domain was collected to D35, NR retina showed similar molecule features resembling human embryonic retina at the same age. from D42 to D 93 is time for retina lamination. Starting from D42 (W7), neural retinal cells spontaneously began to differentiate and migrate to corresponding layers. By D93 (W13–W14), RCs (retina cups) showed distinguishable layers containing the precursors of most of the major neuronal cell types. We are interested in retinal lamination from D42 to D93 (Figure 37). We predict that there are EVs released from 3D retina cup from D42 to D93 which might contain important genetic materials [293].
To advance understanding of molecular signaling during retinogenesis, analysis of EV content is an essential and timely step. While a range of canonical genes have been associated with retinal cell fate determination during development, EV mediated gene regulation in the retinal microenvironment remains to be elucidated [30, 31, 294]. In this part of the dissertation research we set out to determine the microRNA, tRNA, and piRNA composition of EVs secreted from hiPSC derived 3D retinas at three developmental time-points: D42, D63, and D93. Nanosight and TEM analysis were used to determine 3D retinas derived EV diameters, release concentrations and ultrastructure. NGS was used to identify small RNA composition (microRNA, tRNA, and piRNA) in both hiPSC derived 3D retinas and released EVs. These findings will be helpful in characterizing developing retinal EVs and to help structure functional of EV signaling during retinal development.
5.2 Results

5.2.1. hiPSC derived 3D retinas as a human retinal model

hiPSC derived 3D retinas is an in vivo model of human retinal development recapitulating established stages of retinogenesis including proliferation, interkinetic nuclear migration, lamination and temporally ordered fate specification (Figure 38). hiPSC derived 3D retinas was used in this study. We did histological sections of hiPSC derived 3D retinas immunolabeled for the detection of ganglion, amacrine and horizontal cells and photoreceptors showing the progression of cell differentiation and lamination at different developmental state (b) 42, (c) 63, and (d) 93. Hu C/D is used as postmitotic precursor for amacrine, ganglion, horizontal cells[295, 296]

![Figure 38. hiPSC-derived 3D retinas: Retinal progenitors differentiated following the typical central-to-peripheral pattern.](image)
(a) Macroscopic image representative of 3D hiPSC derived 3D retinas used in this study. (b-d) Histological sections of hiPSC derived 3D retinas immunolabeled for the detection of ganglion, amacrine and horizontal cells (HU C/D in green) and photoreceptors (OTX2 in red) showing the progression of cell differentiation and lamination at (b) 42, (c) 63, and (d) 93 days of differentiation. Scale bar: 50 µm (done by Dr. Miguel Flores Bellver at Johns Hopkins medicine school)
5.2.2 Size and concentration of EVs released from hiPSC derived 3D retinas

EVs isolated from hiPSC derived 3D retinas were analyzed using nanoparticle tracking analysis technology (NTA). Size and release rate of EVs were analyzed (Figure 39). EV nanometer diameter and concentration distributions derived from hiPSC 3D retinas media conditioned at D42, D63, D93. The diameter ranges at 96hrs for D43 = 30nm-550nm, D62 = 30nm-530nm and D93 = 30nm-570nm (Figure 39 A-C). Next, the concentration of hiPSC derived 3D retinas released EVs was calculated at D42 24hr = -4.1± 1.76, 96hr = 4.4 ± 2.21; D63 24hr = -1.7 ± 2.00, 96h r= 2.7±1.60E8 and D93 24hr = 9.9±2.91E8, 96hr = 14.8±4.43E8. Data was normalized to control media (non-conditioned) at each time point (Figure 39E). A trend of increased EV concentrations above controls was visible at 96 h for each developmental time point. The average diameters of EVs released from 3D retinas were analyzed and showed D42 24hr = 148 ± 38nm, 96hr = 134 ± 28nm; D63 24hr = 142 ± 31nm, 96hr = 159 ± 32nm and D93 24hr = 156 ± 33nm, 96hr = 135 ±28nm (Figure 39F). Diameter analysis suggested that EV analyzed from hiPSC derived 3D retinas contained both exosomes and microvesicles.

Figure 39. 3D retinas released EV diameter and concentration.

A, B, C,) EV nanometer diameter and concentration distributions derived from 3D retinas media conditioned at D42, D63, D93. The diameter ranges at 96hrs for D43 = 30nm-550nm, D62 = 30nm-530nm and D93 =
30nm-570nm. D) A sample 3D plot showing EV size/relative density at from D93 at 96 hr. E) Concentration of released EVs from n=10 3d retinas each at D42 24hr = -4.1 ± 1.76, 96hr = 4.4 ± 2.21; D63 24hr = -1.7 ± 2.00, 96h r= 2.7±1.60E8 and D93 24hr = 9.9±2.91E8, 96hr = 14.8±4.43E8. Concentration data are normalized to control media for each time point. F) Average diameters of EVs released from 3D retinas D42 24hr = 148 ± 38nm, 96hr = 134 ± 28nm; D63 24hr = 142 ± 31nm, 96hr = 159 ± 32nm and D93 24hr = 156 ± 33nm, 96hr = 135 ±28nm. error bar: standard error of mean.

5.2.3 Ultrastructure of EVs isolated from 3D retinas.

Using immunogold and standard transmission electron microscopy (TEM) protein localization and hiPSC EV ultrastructure were determined. Immunogold TEM images showed the localization of canonical exosome (CD63) and microparticle (Tsg101) transmembrane proteins at the surface of hiPSC EVs (Figure 40 A, B)[165, 208]. Control TEM was processed in the absence of primary antibodies. Standard TEM images revealed a heterogeneous population of spheroid and cup-shaped EV morphologies, with an average diameter near 110nM, consistent with the known EV size and morphology (Figure 40 D-F) [211].

Figure 40. TEM analysis of hiPSC derived EVs.

TEM analysis of EV populations isolated from hiPSC derived 3D retinas. A) Immunogold TEM of an EV sample isolated from D63 iPSC retina revealed localization of CD63, scale 100nm. B) Labeling of an EV sample derived from D96 iPSC retina showed labeling for TSG101, scale 100nm. C) Immunogold TEM
control, scale 100nm. D-F) TEM sample EVs isolated from D63 iPSC retina exhibit spheroid and cup-shape morphology with diameters ranging from approximately 50-200nm, scale 250nm.

5.2.4 Deep sequencing analysis of small RNA from EVs

Sequencing was quality controlled using a Nanodrop ND/1000 spectrophotometer(Table 12). To validate the RNA sequencing data, RT-qPCR analysis was performed for small RNA and miRNA purified from all samples. Small RNA and miRNA isolated from matched experimental tissue, EVs and controls for D42, D63 and D92 exhibited good sample quality and quantity. Good RT-qPCR amplification was observed for both the 5S rRNA and miR-26a with good Ct. values from 3D retinas and EVs, respectively. Table 12 shows the quality and quantity of both small RNA and miRNA. All sample quality was identified as good.

Table 12. hiPSC derived 3D retinas extracellular vesicle QC for Next Generation Sequencing.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sample Type</th>
<th>RNA Conc. (ng/ul)</th>
<th>RNA yield (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Media D42</td>
<td>EVs</td>
<td>0.233</td>
<td>11.65</td>
</tr>
<tr>
<td>Experimental Media D42</td>
<td>EVs</td>
<td>2.6</td>
<td>130</td>
</tr>
<tr>
<td>Experimental Tissue D42</td>
<td>3D retinas</td>
<td>103.1</td>
<td>5.155</td>
</tr>
<tr>
<td>Control Media D63/D92</td>
<td>EVs</td>
<td>0.109</td>
<td>5.45</td>
</tr>
<tr>
<td>Experimental Media D63</td>
<td>EVs</td>
<td>0.762</td>
<td>38.1</td>
</tr>
<tr>
<td>Experimental Tissue D63</td>
<td>3D retinas</td>
<td>108.3</td>
<td>5.155</td>
</tr>
<tr>
<td>Experimental Media D93</td>
<td>EVs</td>
<td>0.049</td>
<td>2.45</td>
</tr>
<tr>
<td>Experimental Tissue D93</td>
<td>3D retinas</td>
<td>46.1</td>
<td>2.305</td>
</tr>
</tbody>
</table>
Figure 41. Comparative NGS Heatmaps of miRNA expression between hiPSC retina and released EVs at D42, D63, D93.

Each heat map shows a clear separation of miRNA expression between hiPSC derived 3D retinas and released EVs at three time points, D42, D63 and D93. Many miRNAs showed similar expression patterns between tissue and EVs at all three time points.
Following NGS data were analyzed to determine expression patterns of small RNAs present in hiPSC derived 3D retinas EVs and control (Figure 41). To illustrate unique and common miRNAs in 3D retinas and EVs at three time points, Venn Diagrams were generated. Unique and shared miRNAs in samples at three time points are described.

**Figure 42. NGS Venn Diagrams, tables and heat maps for miRNA in retinal cups and EVs**

A) The Venn diagrams show the number of miRNA expressed >5 RPM in the 3D retinas, EVs, or both samples. D42, D63, and D93 represent the time (in days) when the samples were collected. B) miRNA that are expressed >5 RPM in EVs at any time points. C: expressed >5 RPM in 3D retinas; e: expressed >5 RPM in EVs. Those without annotation are either expressed below the threshold or expressed in controls. C) Comparison of miRNA expression between samples. miRNA that are expressed >5 RPM in EVs at any time points are shown. The RPM values are log-transformed and standardized across samples for
visualization. For each RNA, red-colored samples represent higher expression values than blue colored samples. The color bar shows the range of standardized expression values.

In addition, EV samples at D42, D63 and D93, each had 10, 2, and 1 shared miRNA with 3D retinas (Figure 42). The majority miRNA expression in 3D retinas samples occurred at D63 time point, and decreased from 199 miRNAs at D63, to 53 miRNAs at D93. However, more species of miRNA expression in EV samples was apparent at D42 time point, and decreased from 12 miRNAs at D42, to 3 miRNAs at D63, and 2 miRNAs at D93.

**Figure 43. NGS Venn Diagrams, tables and heat maps for piRNA in 3D retinas and EVs.**

A) The Venn diagrams show the number of piRNA expressed >5 RPM in the 3D retinas, EVs, or both samples. D42, D63, and D93 represent the time (in days) when the samples were collected. B) piRNA that are expressed >5 RPM in EVs at any time points. C: expressed >5 RPM in retinal cups; e: expressed >5 RPM in EVs. Those without annotation are either expressed below the threshold or expressed in controls.
C) Comparison of piRNA expression between samples. piRNA that are expressed >5 RPM in EVs at any time points are shown. The RPM values are log-transformed and standardized across samples for visualization. For each RNA, red-colored samples represent higher expression values than blue colored samples. The color bar shows the range of standardized expression values.

Figure 44. NGS Venn Diagrams, tables and heat maps for selective tRNA in 3D retinas and EVs.

A) The Venn diagrams show the number of tRNA expressed >5 RPM in the retinal cups, EVs, or both samples. D42, D63, and D93 represent the time (in days) when the samples were collected. B) selective tRNA (23 out of 54) that are expressed >5 RPM in EVs at any time points. C: expressed >5 RPM in retinal cups; e: expressed >5 RPM in EVs. Those without annotation are either expressed below the threshold or
expressed in controls. C) Comparison of tRNA expression between samples. tRNA that are expressed >5 RPM in EVs at any time points are shown. The RPM values are log-transformed and standardized across samples for visualization. For each RNA, red-colored samples represent higher expression values than blue colored samples. The color bar shows the range of standardized expression values.

Compared to other small RNA species, tRNA was identified with the highest numbers of species contained within EVs released from developing hiPSC retinal cups. According to Figure 44A, numbers of tRNA species present were changed at all three time points. The common tRNAs between cells and EVs at three time points were shown in Figure 44A. EV samples at D42, D63 and D93 each had 39, 28 and 9 tRNAs with >5 reads per million, respectively. In addition, EV samples at D42, D63 and D93, each had 36, 27, and 6 shared tRNA with retinal cups.

5.2.5 Prediction of targets of miRNAs derived from EV of hiPSCs

To explore potential roles of miRNAs in EVs during retinal development, miRNA targets were identified using miRTarBase, the experimentally validated microRNA-target interactions database [297]. The reference retina transcriptome includes 12, 792 genes (ArrayExpress id: E-MTAB-4377) [298]. We identified total of 1,566 MicroRNA-Target pairs for 12 miRNAs. Gene ontology analysis of the target mRNAs revealed that these mRNA encode proteins primarily involved in cellular metabolic processes, anatomical structure development and neuronal development (Figure 45). We then focused on miRNA targets involved in neural development.

In terms of retina transcriptome, has-miR-1298-5p has 34 targets, hisa-miR-181b-5p has 295 targets, has-miR-184 has 17 targets, has-miR-204-3p has 81 targets, has-miR-204-5p has 266 targets, has-miR-4301 has 61 targets, has-miR-4488 has 50 targets and has-miR-92b-3p has 532 etc (Figure 45A). We then determined if each target was targeted by 1, 2 or 3 miRNAs. The data revealed a total of 1252 targets, each of which is targeted by one of 12 miRNAs; 139 genes are targets by 2 miRNA and there are 12 genes that are targeted by 3 of 12 miRNAs (Figure 45B).
Figure 45 C is a further analysis of the genes targeted by 2 or more miRNA of the 12 miRNAs identified. This was done to evaluate if genes targeted by 2 miRNAs were always targeted by the same 2 miRNAs or rather by different combinations of miRNAs (same for 3 or more miRNAs). The data suggests it is combinatorial: different sets of 2 miRNAs target different genes.

A.

B.

C.
Figure 45. Bioinformatics analysis of targets of miRNA in the top 20 categories for GO stats biological processes and heatmap.

A) Number of genes are targeted by each of the 12 miRNAs; B) Number of genes that are target only by 1 miRNA; 2 miRNAs, 3 or more miRNAs; C) A second level analysis for the genes targeted by 2 or more miRNAs represented in the pie diagram. Size of slice corresponds to number of targets per miRNA in slice plus one or two additional miRNA from another slice.
Figure 46. Gene Ontology and Pathway Enrichment Analysis.

The heat map shows the P value significance of enriched GO categories at D42, D63 and D93. GOs here are from unbiased analysis and shows the most significant functional categories represented among predicted target genes. The color scale shows in white (low, p-value ≥ 10⁻⁰), light orange (medium, 10⁻¹ < p-value < 10⁻⁰), and red (high, p-value ≤ 10⁻¹). P value significance is determined using Fisher's exact test.
To predict the functional distribution of miRNA targets involved, we performed Gene Ontology (GO) enrichment analysis (Figure 46). Gene ontology (GO) has three basic domains: cellular component, biological process and molecular function. As we see from Figure 46, the groups include protein stabilization (molecular function), negative regulation of translation (molecular function), translation (molecular function), transcription (molecular function), negative/positive regulation of translation (molecular function), positive regulation of cell proliferation (molecular function), cytoskeleton (cellular component), nucleus (cellular component), plasma membrane (cellular component), Ran GTP binding (molecular function), core promotor binding (molecular function), Poly(A) RNA binding (molecular function), signaling pathway (biological process) and signaling pathways regulating pluripotency of stem cells (biological process). A number of GO categories exhibited significantly higher enrichment in the gene data set at D42; including GO terms for transcription, RNA binding, protein binding, positive regulation of cell proliferation and nuclear transcription factor binding. Specifically, hsa-miRNA-181b-50 targets show significant enrichment in transcription related to transcription (DNA templated), transcription from RNA polyperase II promoter, regulation of transcription (DNA templated). hsa-miRNA-926-3p targets show significant enrichment in translation related to protein stabilization and negative regulation of translation (Figure 46). While the data from GO and pathway enrichment analysis shows that targets of miRNA at specific developmental stages may involve specific functions, it also indicates that certain miRNAs target may function in cellular processes, which do not overlap with others miRNA targets or functions.
Mechanisms with potential relevance to retinal development are selected such as cell proliferation, cell differentiation, neuron projection and neuron projection development.

Figure 47 provides a more complete picture of mechanisms that EV-derived miRNAs target. Mechanisms predicted for identified miRNAs are very diverse, including extracellular exosomes, negative regulation of cell proliferation, cell proliferation, cell division, cell differentiation, neuron projection, neurogenesis, regulation of cell proliferation, regulation of cell shape, positive regulation of cell migration, regulation of cell cycle, neuron projection development, negative regulation of cell differentiation, etc. (For more details of each GO category in terms of function, : http://amigo.geneontology.org/amigo/term/GO:0042127)
The miRNA targets at D42, D63, D93 that appear relevant to retinal development include negative regulation of cell proliferation (values at D42, D63, D93 are 1.5/2.3/2.3, respectively), cell proliferation (values at D42, D63, D93 are 1.5/2.5/2.5, respectively), and cell differentiation (values at D42, D63, D93 are 1.3/1.7/1.9, respectively), in which enrichment value is > 1. In terms of neural related mechanism, including neuron projection (value at D42, D63, D93 are 1.3, 1.4, 1.2, respectively), neurogenesis (values at D42, D63, D93 are 1.2, 0.97, 1.1, respectively), neuron projection development (values at D42, D63, D93 are 1.2, 1.3, 1.5, respectively), negative regulation of neuron projection development (value at D42, D63, D93 are 1.8, 1.6, 1.7, respectively), regulation of cell migration (values at D42, D63, D93 are 1.4, 1.4, 1.5, respectively), positive regulation of neuron differentiation (values at D42, D63, D93 are 0.86, 1.2, 1, respectively) (Figure 47).

Interestingly, enrichment heatmap shows miRNA targets at D42 are more relevant to neuron stem cell population maintenance (values at D42, D63, D93 are 2.4, 0, 0, respectively), neurodegenaration (values at D42, D63, D93 are 0.95, 0.24, 0.27, respectively), positive regulation of neuron projection development (values at D42, D63, D93 are 1.1, 0.78 0.85, respectively), central nervous system development (values at D42, D63, D93 are 0.92, 0.71, 0, respectively), neuron migration (values at D42, D63, D93 are 0.95, 0, 0, respectively), regulation of neuron apoptotic process (values at D42, D63, D93 are 3.2, 0, 0, respectively), cell morphogenesis (values at D42, D63, D93 are 1.1, 0, 0, respectively), neuron differentiation (values at
D42, D63, D93 are 0.77, 0, 0, respectively), retina development in camera-type eye (values at D42, D63, D93 are 1.1, 0, 0, respectively), eye development (values at D42, D63, D93 are 1.1, 0, 0, respectively).

In contrast, enrichment heatmap shows miRNA targets at D63 and D93 are more specific for Negative regulation of cell proliferation (values at D42, D63, D93 are 1.5, 2.3, 2.3, respectively), cell proliferation (values at D42, D63, D93 are 1.5, 2.5, 2.5, respectively), cell cycle (values at D42, D63, D93 are 1.5, 2.5, 2.8, respectively), nervous system development (values at D42, D63, D93 are 1.4, 2.6, 2.2, respectively), regulation of cell shape (values at D42, D63, D93 are 1.4, 2.8, 2.4, respectively), cell migration (values at D42, D63, D93 are 1.2, 2, 2.2, respectively), neural tube closure (values at D42, D63, D93 are 1.6, 2, 2.2, respectively), negative regulation of neuron apoptotic process (values at D42, D63, D93 are 0.92, 1.3, 1.4, respectively), negative regulation of neuron projection (values at D42, D63, D93 are 1.3, 3.1, 3.4, respectively), neural tube development (values at D42, D63, D93 are 1.7, 3, 3.3, respectively), neural crest cell migration (values at D42, D63, D93 are 1.3, 2, 2.2, respectively), cellular response to retinoic acid (values at D42, D63, D93 are 1.2, 1.7, 1.9, respectively), retinoic acid receptor signaling pathway (values at D42, D63, D93 are 2.2, 3.8, 4.2, respectively), lens fiber cell differentiation (values at D42, D63, D93 are 3.7, 13, 7 respectively), glial cell differentiation (values at D42, D63, D93 are 3, 5.2, 5.7, respectively) (Figure 47).

A.
Figure 48. GO term-Target gene Heatmap.

A) The heatmap presents significant targets of miRNA with putative category correspondences. The gene heatmap here represents the genes that fall into each of the categories. Colored spots indicate the targets
of miRNA that are significantly enriched in corresponding category at right side. B) The Venn diagram represents degrees of overlap there is between related GO categories. A number of miRNA target mRNA in categories independent of other categories.

To visualize overlap between related GO categories, we further analyzed GO categories, for example “regulation of cell differentiation” vs “positive regulation of cell differentiation” vs “negative regulation of cell differentiation” (Figure 48). Most overlapping areas in Figure 48 B show “0”; indicating that most of the miRNA targeted genes do not show overlap with other miRNA which target separate genes.

Table 13. EV miRNA target genes involved in relevant terms.

<table>
<thead>
<tr>
<th>GO</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0046548~retinal rod cell development</td>
<td>NTRK2, ALMS1, RORB</td>
</tr>
<tr>
<td>GO:0001754~eye photoreceptor cell differentiation</td>
<td>AGTPBP1, STAT3</td>
</tr>
<tr>
<td>GO:0042462~eye photoreceptor cell development</td>
<td>VEGFA, RORB</td>
</tr>
<tr>
<td>GO:0001750~photoreceptor outer segment</td>
<td>MYO5A, GNAQ, MAP1B</td>
</tr>
<tr>
<td>GO:1902336~positive regulation of retinal ganglion cell axon guidance</td>
<td>VEGFA</td>
</tr>
<tr>
<td>GO:0090259~regulation of retinal ganglion cell axon guidance</td>
<td>VEGFA</td>
</tr>
<tr>
<td>GO:0046549~retinal cone cell development</td>
<td>RORB</td>
</tr>
<tr>
<td>GO:0061549~sympathetic ganglion development</td>
<td>SEMA3F</td>
</tr>
<tr>
<td>GO:0003406~retinal pigment epithelium development</td>
<td>PAX2</td>
</tr>
<tr>
<td>GO:0042574~retinal metabolic process</td>
<td>ALDH1A2</td>
</tr>
</tbody>
</table>
We also analyzed predicted targets according to their function. Table 13 showed GO categories related to ganglion cells and photoreceptors. Genes were also identified within the pool of targeted genes that are associated to mechanisms relating to GC and photoreceptors differentiation/maturation. Possible targets were grouped by various function, including neural development, retinal targets, retinoic acid, regulation of cell fate. For example, hsa-miR-181b-5p had predicted retinal targets such as RBBP7, OPN5, IMPG1, CALB1; predicted targets such as MSI2, LRRN1, NCALD, NEGR1, NRXN1, NPTN might involve in neural development and NOTCH2 and WIF1 might involve in regulation of cell fate. hsa-miR-4516 had predicted retinal targets including BSN, RHO, GNAT1, OPN1MW2, OPN1LW, OPALIN, OPN1MW, GABBR2 NGFR, GFRA1; NRARP, WNT4 involved in regulation of cell fate. hsa-miR-6748-3p had predicted retinal targets including GDNF, RS1, GFRA1, GABBR2, ABCF2, NFASC, THRA, GR8IK5.

5.3 Discussion

The discovery that exosomes are an important carrier of miRNAs holds great interest as it may serve potential vehicles for miRNA based intercellular communication as well as source of diagnostic extracellular miRNA biomarkers. Although this small size (~100 nm) limits its capacity to sample the RNA repertoire, there have been several studies showing certain miRNA sequences are enriched in exosomes relative to
their cells of origin [78] Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells [299].

Differentiation of ES and iPS cells into retinal cells brings great potential for future posible therapies for retina disease. This is a simple and in vitro strategy for inducing hiPSC to autonomously into 3D retinal tissue and this protocol mimics the vivo microenvironment where normal retinal cell specification occurs. In this system, hiPSC recapitulate each of the main steps during retinal development and form three-dimentional 3D retinas that contains all major retinal cell types; formed photoreceptors are able to reach an advanced stage of maturation and begin to form outer-segment and photosensitivity. To better understand development of hiPSC induced 3D retinas development in vitro, we characterized miRNA in hiPSC derived EVs in microenvironment and compared the miRNA in EVs to retina cups [293].

NGS is a powerful tool to discover novel and large numbers of small RNA species. By using small amounts of starting RNA input, NGS can detect all small RNA populations without prior knowledge of them. These small RNAs may act as regulatory elements to modulate gene activity. In addition, small RNAs offer great hope as biomarkers of disease and regular development [300]. We report a large variety of small RNA in EVs from hiPSC derived 3D retinas and including miRNAs, tRNA and piRNAs. This work represents the first analysis of small RNA species contained within EVs released from developing hiPSC derived 3D retinas at three time points, D42, D63 and D92, which provide snapshots of changing expression profiles. We found a number of differentially expressed miRNAs, piRNA, and tRNA in hiPSC derived 3D retinas compared with EVs.

miRNAs targets are involved in diverse cellular processes including proliferation, apoptosis, neuronal development and fate specification by targeting and controlling networks of hundreds of mRNA species. Several studies have analyzed miRNA expression in developing retina and identified a number of miRNAs enriched in retinal tissue[277, 301] . We identified 12 miRNAs in EVs derived from 3D retinas at different developmental stages.
Extracellular miRNAs exhibit resistance to high extracellular ribonuclease (RNase) activity[302], increasing the possibility of packaging in membrane enclosed vesicles or in conjunction with a lipoprotein-miRNA complexes providing stability. Therefore, compared to cellular RNAs, EVs have higher proportion of RNAs that are more stable, and resistant to physical degradation[291, 303, 304].

Some profiling studies suggest that the loading of miRNAs into EVs is not a random event and a common mechanism for selective miRNA export exists [305, 306]. In this study, hsa-miR-4488, hsa-miR-1246, hsa-miR-4516 were identified as the three most abundant miRNA species in EVs derived from 3D retinas. This finding is consistent with those of Stevanato, et al, who identified hsa-miR-1246, hsa-miR-4488, hsa-miR-4508, hsa-miR-4492 and hsa-miR-4516 as the 5 most abundant miRNA types in EVs derived from human neural stem cells [272]. Hsa-miR-1246, was one of the most abundant miRNAs in EVs derived from our hiPSC derived 3D retinas [307] and has been demonstrated to play an important role in regulating cell growth and apoptosis [272]. Also, in our data, miR-24 was present in both hiPSC derived 3D retinas and EVs. MiR-24 has recently been shown to repress apoptosis and plays a crucial role in proper retinal development [308].

A number of miRNA species including miR- 125, miR-124 and miR-9 have been independently reported to be highly expressed in developing and adult retina [308]. Our analysis showed that while these miRNAs were present in hiPSC derived 3D retinas at the three time points analyzed, they were not detected in EVs. In another retinal development study, miR-129, miR-155, miR-214, and miR-222 were down regulated as retinal development proceeded [280]. In this analysis, these miRNAs were not detected in hiPSC derived 3D retinas or EVs, at three time points studied.

Although a few studies have reported the presence and function of piRNA in the nervous system, exact roles for piRNAs in nervous system tissues are not fully understood[278]. piRNA in the mammalian brain is involved in silencing of retro transposons; although piRNA levels are low brains. Putative piRNAs may facilitate changes in genome-wide DNA methylation and animal behavior. Predicted roles for piRNA beyond maintaining genome integrity, includes modulating stability and translation of mRNA encoding proteins
required for spermatogenesis and targeting genes that controls spine shape. A recent study showed that piRNAs are present in mouse brain throughout postnatal development are only present in adult stage of brain development[309]. To the best of our knowledge, this is the first study detecting piRNAs in hiPSC derived 3D retinas and EVs throughout development. This work indicates that EV enclosed piRNAs are present and may play a role in retinal development.

tRNA in hiPSC derived 3D retinas EVsOur NGS data of hiPSC derived 3D retinas and EVs revealed a highly enriched number of tRNA species enclosed in EVs. This result is consistent with those of Bellingham et al., who indicated that neuronal cell- derived EVs are enriched in tRNA species[310]. tRNA in cell is not most abundant small RNAs in cells [311], however it accumulates to higher level than other small RNAs, indicating a selective mechanism for EVs transfer. The role of varying species of tRNA in EVs remains to be defined further.

Target analysis of mirNA from EVs.

We are interested in some important target gens which relate to eyes and retina. Selected GO category include retinal rod cell development (GO:0046548), eye photoreceptor cell differentiation(GO:0001754), eye photoreceptor cell development (GO:0042462) photoreceptor outer segment(GO:0001750), positive regulation of retinal ganglion cell axon guidance(GO:1902336), regulation of retinal ganglion cell axon guidance(GO:0090259), retinal cone cell development (GO:0046549), sympathetic ganglion development(GO:0061549), retinal pigment epithelium development(GO:0003406),retinal metabolic process(GO:0042574), photoreceptor cell maintenance(GO:0045494), negative regulation of photoreceptor cell differentiation(GO:0046533), photoreceptor outer segment membrane(GO:0042622), photoreceptor connecting cilium(GO:0032391), retinal dehydrogenase activity(GO:0001758), retinal binding(GO:0016918). Targets gene of miRNA involves in but not limited to these signaling pathways.

ALMS1 gene has been implicated in function, formation, and maintenance of primary cilia; in photoreceptors, mutation of ALMS1 results in dysfunction of primary cilia and further leads to human
monogenic disorders, such as ciliophthies [312, 313], including plural systemic disease. ALMS1 mutations lead to dysfunction of the connecting cilium and affect retina function a{314-316}.

Rorb gene, which encode retinoid-related orphan nuclear receptor RORβ expresses in brain, pineal gland, and retina. Rorb is expressed in all neural retina layers from early stages with a peak at neonatal stages and might play important roles in rods and cones. According to Jia Li's report, Robs is critical for rod differentiation and lies upstream of Nrl in the rod transcriptional pathway. Rorbeta lacks a known physiological ligand and activates the Opn1sw promoter alone but strongly in synergy with retinal cone-rod homeobox factor (CRX) and play an enhancing role; Rorb deficient mice is not able to induce S opsin during postnatal cone development and mice with deficient Rorb lack outer segments; Rorb with Crx synergistically induce the S opsin promoter[317] and regulates opsin expression in color visual system.

STAT (signal transducer and activator of transcription) proteins is initial isolated form interferon-stimulated transcriptional complexes. STAT proteins involves in modulating transcription and mediating signaling in the determination of rod photoreceptor cell fate in mouse retina. Activation of STAT3 by cytokines and growth factors plays a critical role during rod photoreceptor determination[318].

VEGF-A was first identified as an endothelial cell mitogen and vascular permeability factor. By binding to various receptor including VEGFR2/Flik1/KDR and VEGFR1/Flt-1s. VEGF-1 has been stated to be neuroprotective in models of ischemic/hypoxic injury in CNS and now more roles are demonstrated in retina. Use of VEGF-A neutralizing therapies in the treatment of macular edema and choroidal neovascularization associated with the wet from of age related macular degeneration (AMD) indicate it might play critical role in developmental vascularization of the retina [319]. Now it has been proved that VEGF-A directs angiogenic in growth and acts as a survival factor, has a nonvascular role in the retina[320, 321]; adding VEGF-A induces differentiation of photoreceptor cell[322]. However, few data is known about the function of VEGF-A in the adult, either vascular or nonvascular cells.

The MYO5A encodes a protein called myosin Va, which is part of a group of proteins called unconventional myosins. These proteins, which have similar structures, each play a role in transporting molecules within
cells. In human, dysfunction of MYO5A leads to both pigment and neurological abnormalities. Myosin Va involves in synaptic terminals in the retina and brain. Myosin Va mutant mice shows physiological abnormal photoreceptor synapses thus myosin Va is required for normal photoreceptor signaling, suggesting that it might function in central nervous system synapses in general, with aberrant synaptic activity potentially underlying the neurological defects observed in dilute lethal mice and patients with Griscelli syndrome type 1 and Elejalde syndrome[323].

GNAP, together with GNA11, GAN14, GAN 15 are members of GANQ/11 family. GANP express within retina and more specifically, within melanopsin expression retinal ganglion cells. Full function analysis is not known and studies yet, localization of GNAP indicates it might involve in photoreceptor function.

Semaphorins are known modulators of axonal sprouting and angiogenesis. Sema3A is identified from retina outer layer where are avascular. It shows that Sema 3F potential role in anti-angiogenic on both retina and choroidal vessels. Sema 3F acts as vasorepulsive cue to maintain physiologic avascularity[324]. A most recent study shows Sema3f levels can be modulated to protect the physiologically avascular outer retina from invasion by retinal as well as choroidal pathological neovascularity[325].

Pax 2 is co-expressed with Pax in the entire optic vesicle and concomitant with the establishment of distinct neuroretinal, retinal, pigmented epithelial and optic stalk progenitor domains. Pax6 is expressed highly throughout the early optic vesicle (OV). Pax 6 is a master regulator of eye development [326]. Lacking of Pax6 results in no functional eye structures forming in frog and fly[327]. It has been shown that during optic nerve formation at ~E12.5, Pax2 expression becomes restricted to the ventral NR that surrounds the closing optic fissure. According to Nicole’s study[328], redundant activities of Pax2 and Pax6 are required and sufficient to direct the determination of RPE.

Notch signaling pathway regulates both cell cycle exit and cell fate specification during retinal development[329, 330]. Removal of Notch1, either in early or late stages of retinal development, results in precocious cell cycle exit and overproduction of cone and rod photoreceptors photoreceptors[329-331]. Notch signaling involves in maintaining RPCs in a cycling state and determine cell type identify by inhibiting
the photoreceptor fate. In developing retina, progenitor cells generate restricted types of postmitotic progeny which respond differentially to Notch signaling.

We have characterized miRNA transcriptome from EVs released from hiPCS derived retina cup. We selected different time points during retina development. For comparative analysis, we have profiled miRNA transcriptome from retina cup at D42, D63 and D93, too. Expressed miRNAs in retina cup and EVs showed overlapping, although there are only a few of them.

5.4. Conclusion

This work demonstrates for the first time the release of EVs from developing hiPSC derived 3D retinas in vitro. NGS data revealed the presence of a wide range of small RNA species contained within EVs and released from developing hiPSC retina. Future studies may build on these findings to determine what biological function these molecules may perform in guiding retinal development and fate specification.

5.5 Methods

5.5.1 hiPSC derived 3D retinas culture and conditioned media sample collection

hiPSC retina were cultured as previously described [293]. Briefly, hiPSC were cultured on Matrigel (growth factor reduced)-coated plates with mTeSR1 medium. Cells were passaged every 5-7 days at 80% confluence. hiPSC derived 3D retinas were generated from hiPSC around day 30 after differentiation and then maintained with different media. D42 Medium basically contains DMEM/F12 and B27; D63/D92 medium had FBS, taurine and RA added to D42 medium.

Media was collected at D42, D63 and D92 and frozen at -80 until further analysis. This work was kindly done by our collaborating lab Dr. Maria Canto Soler and postdoc fellows Dr. Miguel Flores Bellver and Dr. Xiufeng Zhong.

5.5.2 NanoSight analysis of EV size and concentration[293]

EV size and number were assessed with Nano-Sight NS500 system. From D42, D63 qand D93 hiPSC derived 3D retinas culture. After retina culture conditioned media was collected and transferred to centrifuge
tubes. Collection of supernatant was identical to previously described isolation of EVs, except supernatant before 100,000x g ultracentrifugation was used for Nano-Sight analysis. Control media D42 and D63/92, non-conditioned, was processed at the same time. Supernatant was then diluted at 1:20 in PBS and 1ml was used for Nano-Sight analysis. The NanoSight system uses a laser light source to illuminate nano-scale particles, detected individually as light-scattered points moving via Brownian motion. Polydispersity was quantified, and we used Nanoparticle Tracking Analysis (NTA) software 2.3 to track and size nanoparticles on an individual basis. Results were displayed as a frequency sized distribution graph describing the number of particles per ml.

5.5.3 Extracellular Vesicle Isolation

EVs were isolated using a modified protocol based on previous work by Yuan et al [77]. To collect EVs, hiPSC derived 3D retinas were cultured as previously [293]. Briefly, conditioned media was collected, transferred to centrifuge tubes (polypropylene conical bottom) and centrifuged at 500x g for 10 min to pellet cells at room temperature; supernatant was collected and centrifuged at 10,000x g for 20 min at 4°C (Beckman Ultracentrifuge, Rotor 60ti) to remove cell fragments and debris; final supernatant was spun at 100,000 x g for 70 min at 4°C to pellet the EVs. EVs were re-suspended in phosphate-buffered saline (PBS) and stored at −80°C for further analysis.

5.5.4 TEM EV Analysis

EVs released from hiPSC retina were isolated from 25 ml of culture supernatant using differential centrifugation. Then 5 µl of suspension containing isolated EVs was dropped on a Zoo-mesh Carbon Formuar grid; EVs were allowed to absorb for 20 min at room temperature. Excess suspension was wicked off and grids were submerged in 25% glutaradeliyde/4% PFA with 25% tannic acid in PBS for 10 min. Grids were then washed in distilled water and viewed on a FeiTecnai transmission electron microscope, operated at 60kv. Digital images were obtained using an AMT digital camera and software.
5.5. 5 EV RNA Isolation from hiPSC derived 3D retinas

Exosomal RNA was isolated from samples Control D42, Experimental D92 and D63 using the Cell Culture Media Exosome Purification and RNA Isolation Midi Kit (10 mL-20 mL) (Cat# 60800) and from samples Experimental D42 and Control D63/92 using the Cell Culture Media Exosome Purification and RNA Isolation Maxi Kit (20 mL-35 mL) (Cat#60900) according to the manufacturer's instructions (Norgen Biotek, Thorold, ON, Canada). The purified RNA sample was stored at –80°C until further analysis.

5.5.6 RNA Isolation from iPSC Retina

RNA was isolated from samples TN-1, TN-2 and TN-3 using the Cell Animal Tissue RNA Purification Kit (Cat# 25700) according to the manufacturer's instructions (Norgen Biotek, Thorold, ON, Canada). The purified RNA sample was stored at –80°C until further analysis.

5.5. 7 NGS of hiPSC derived 3D retinas and EVs small RNA library preparation and sequencing

Small RNA Libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina (NEB, Ipswich, MA). For Exosomal RNA isolated from Cell-Culture Media, about 1 ng was used as input for library generation. For RNA isolated from Tissue Cultured Retina, about 500 ng was used as input for library generation. The libraries were prepared according manufacturer's instruction. The amplified libraries were resolved on a Novex® 6% TBE Gels (Thermo Fisher, Waltham, MA). Library fragments were excised and further purified using RNA Clean-up and Concentration Micro-Elute Kit (Norgen Biotek, Thorold, Ontario). The indexed libraries were quantified using the High Sensitivity DNA Kit (Agilent, Santa Clara, CA). All libraries were pooled together for a final pooled library at a final concentration of 4 nM. The pooled library was then sequenced on an Illumina MiSeq using version 3 reagents by Norgen Biotek Corp (Figure 49). The Small RNA expression profile was generated from the resulting FASTQ files using the exceRpt small RNA-seq pipeline [332].
Figure 49. Flow chart showing small RNA-seq Data Analysis Pipeline for EV-RNA Profiling.
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