Let-7, Lin28 and Hmga2 Expression in Ciliary Epithelium and Retinal Progenitor Cells

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Purpose. Ciliary epithelium (CE) of adult mammalian eyes contains quiescent retinal progenitor/stem cells that generate neurospheres in vitro and differentiate into retinal neurons. This ability doesn't evolve efficiently probably because of regulatory mechanisms, such as microRNAs (miRNAs) that control pluripotent, progenitor, and differentiation genes. Here we investigate the presence of *Let-7* miRNAs and its regulator and target, Lin28 and Hmga2, in CE cells from neurospheres, newborns, and adult tissues.

Methods. Newborn and adult rats CE cells were dissected into pigmented and nonpigmented epithelium (PE and NPE). Newborn PE cells were cultured with growth factors to form neurospheres and we analyzed *Let-7*, Lin28a, and Hmga2 expression. During the neurospheres formation, we added chemically modified single-stranded oligonucleotides designed to bind and inhibit or mimic endogenous mature *Let-7*b and *Let-7*c. After seven days in culture, we analyzed neurospheres size, number and expression of *Let-7*, Lin28, and Hmga2.

Results. *Let-7* miRNAs were expressed at low rates in newborn CE cells with significant increase in adult tissues, with higher levels on NPE cells, that does not present the stem cells reprogramming ability. The Lin28a and Hmga2 protein and transcripts were more expressed in newborns than adults cells, opposed to *Let-7*. Neurospheres presented higher *Lin28* and *Hmga2* expression than newborn and adult, but similar *Let-7* than newborns. *Let-7*b inhibitor upregulated *Hmga2* expression, whereas *Let-7*c mimics upregulated *Lin28* and downregulated *Hmga2*.

Conclusions. This study shows the dynamic of Lin28-*Let-7*-Hmga regulatory axis in CE cells. These components may develop different roles during neurospheres formation and postnatal CE cells.

Keywords: ciliary epithelium, Let-7, Lin28, Hmga2, neurospheres

For the last 20 years[1,2] it has been known that a subset of cells located at the ciliary epithelium (CE) in mammalian eyes can be reprogramed to stem cells/retinal progenitors after stimuli.[3] Since its first identification, the progenitor potential of these cells has been tested by many experimental approaches such as injury models, transplantation, cell culture expansion, neurospheres formation, guided differentiation, cell sorting, electrophysiology analysis, and countless genetic and protein screening.[4–10] These experiments gathered strong evidence to prove the stem cell potential of the pigmented cells from CE.

The CE is a specialized secretory tissue that produces the aqueous fluid, responsible for the nourishment of the lens and cornea and also for the maintenance of eye pressure.[11] This tissue contains two cell layers, an inner nonpigmented epithelium (NPE) and an outer pigmented epithelium (PE). In the presence of growth factors, PE cells form neurospheres containing heterogenic retina progenitor cells, which expressed a variety of progenitor markers (*Pax6, Sox2, Vsx2, Rax, Lhx2, Nestin*).[5,8] When exposed to environmental conditions that mimic the early and late retinal development, CE cells differentiate in several retinal cell types, from photoreceptors to Müller glial cells.[12]

The progressive degeneration of retinal cells, especially photoreceptors, is one of the most frequent causes of severe visual impairment and blindness.[13] Treatments based on gene supplementation, optogenetics, prosthetic implants, and light-sensitive nanoparticles are very promising and generate immediate and robust responses from the remaining retinal cells.[14–18] Despite the promising results, the progressive degeneration of the retinal cells continued to the point it overcame the approach benefits, requiring a combined stem cells–based therapy to replace retinal dead cells, and CE cells could be a strong candidate for this therapy.[19]

Despite the CE's well-described regenerative potential, this is still limited. The differentiation of CE progenitor/stem cell population toward a therapeutically relevant cell type is still not effective and efficient for regenerative therapy.[19,20] We believe this limited potential is provided by the presence of differentiation-progenitor genes regulators within the CE cells, such as miRNAs, a small, noncoding RNA that links

to target mRNA and control gene/protein expression at the post-transcriptional level.[21]

Different analysis shows that members of the *Let-7* family of miRNAs are expressed in the retina and increase during development.[22–24] During retinal development, the expression of *Let-7* coincided with the shift from the early to late retinal histogenesis, which occurs between E16 and E18, with up-regulation of *Let-7* and generation of late-born neurons and Müller glial cells.[25]

The *Let-7* family of miRNAs target genes directly related to the retinal regeneration, including *c-Myc, Ascl1a, Lin-28, Pax6a, Pax6b, Mps1*e and *Hspd1 in zebrafish*.[26–28] The *Let-7* expression can be regulated by Lin28, an RNA binding protein expressed in undifferentiated and stem cells, responsible for maintaining the expression of specific pluripotency genes, and commonly associated with regenerative potential.[29,30] Lin28 directly regulates *Let-7* preventing its maturation.[31,32,33] Moreover, the influence of the heterochronic protein Lin28 on the neurogliogenic decision was observed to be independent of *Let-7*.[34]

On the other hand, *Let-7* targets Hmga2, a *H*igh *M*obility *G*roup protein that plays important roles in cell proliferation, differentiation, pluripotency induction and self-renewal.[35] The 3′ untranslated region (UTR) of Hmga2 contains complementary sequences to the *Let-7* miRNA family, and the binding of *Let-7* negatively regulates Hmga2 expression. Hmga2 expression is abundant during embryogenesis but very low in adult tissues, correlating inversely with *Let-7* expression. Overexpression of Hmga2 is often a consequence of chromosomal rearrangement that removes 3′ UTR of Hmga2, containing specific sites for *Let-7*.[36,37]

The regulatory axis formed by Lin28-*Let-7*-Hmga is present in several cell types, as retinal progenitor cells, cancer cells, and intestinal stem cells,[38–42] and regulates the progenitor cells progression to differentiation during specific histogenesis stages.

Here, we investigated the presence of Lin28-*Let-7*-Hmga regulatory axis in the CE cells from adult Wistar rats and compared the levels of expression to newborn animals. We also investigated the regulatory axis in progenitor neurosphere cells and its response after *Let-7*b and *Let-7*c overexpression and inhibition.

Methods

Animals

All experiments were conducted in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Biomedical Sciences Institute/University of São Paulo Ethical Committee for Animal Research (no. 75134-3). Newborn (1–3 days old) and adults (45–60 days) Wistar rats were kept in a 12-hour light/12-hour dark cycle, with food and water as desired.

Neurospheres Assay

Isolation and culture of PE cells from newborns were performed as previously described.[2,12,43] Briefly, after newborn eye enucleation, the cornea, lens, iris, and retina were removed to eliminate any potentially dividing cells from these tissues as contaminants. The pigmented CE was incubated in HBSS, containing collagenase (78 U/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) and hyaluronidase (38 U/mL; Sigma-Adrich Corp.) for 35 minutes at 37°C, followed by dissociation in 0.25% trypsin, 1 mM EDTA, and 20 mg/mL DNase1 for another 35 minutes. PE cells were cultured in retinal culture medium[10] containing FGF2 (10 ng/mL; R&D Systems, Minneapolis, MN, USA) and EGF (20 ng/mL, R&D Systems) for seven days to generate the CE neurospheres.

mRNA and miRNA Expression Analysis

After newborn and adult eye enucleation, the cornea, lens, iris, and retina were removed with an Olympus SZ40 (Center Valley, PA, USA) stereo zoom microscope. CE cells (both PE and NPE) were manually dissected from each other in ice-cold PBS solution. Each sample contained a pool of PE or NPE from seven to 10 different animals. Samples proceeded to mRNA or miRNA extraction. A minimum of three samples (pools) were analyzed for each approach.

mRNA Expression. For *Lin28a* and *Hmga2* analysis, total RNA was isolated using MiniRNeasy Kit (Qiagen, Hilden, Germany). The cDNA (1 µg) was synthesized as previously described.[44] Briefly, specific transcripts were amplified with gene-specific forward and reverse primers, by using Quantifast SYBR Green PCR kit (Qiagen), on a 7300 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The gene-specific primers used are described in the Table. Amplification curves were normalized to the housekeeping gene Gapdh.

miRNA Expression. For *Let-7* analysis, the miRNAs were isolated using the specific miRNeasy Mini Kit (Qiagen). Ten nanogram of the miRNA were used for the reverse transcription with a Taqman MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), and the material was amplified with Thermo Fisher Scientific specific primers for mature *Let-7*a-5p (assay ID 000377), *Let-7*b-5p (assay ID 002619), *Let-7*c-5p (assay ID 000379), *Let-7*d-5p (assay ID 002283), *Let-7*e-5p (assay ID 002406), and *Let-7*f-5p (assay ID 000382). *SnoRNA* (assay ID 001718) was used as a housekeeping gene.

Immunofluorescence

Enucleated eyes from newborns and adults were fixed in 4% solution of paraformaldehyde in phosphate buffer (PB) 0.1 M (pH 7.4) for 30 minutes and cryoprotected in 30% sucrose in PB for at least 24 hours at 4°C. Later, fixed eyes were sectioned on cryostat (12 µm). Neurospheres were fixed in 4% paraformaldehyde for 30 minutes.

Eye sections and neurospheres were incubated overnight at room temperature with primary monoclonal rabbit antibodies against LIN28a (1:100, no. #8641; Cell Signaling Technology, Danvers, MA, USA), and HMGA2 (1:100 no. 8179S; Cell Signaling), diluted in PB 0.1 M containing 3% normal goat serum and 0.3% Triton X-100. After several washes in PB, sections were incubated for two hours with antisera against rabbit IgG (1:50), tagged to fluorescein isothiocyanate (Jackson Laboratories, West Grove, PA, USA). Slides were then coverslipped with VectaShield (Vector Laboratories, Burlingame, CA, USA), visualized under a Nikon PCM2000 (New York, NY, USA) or Zeiss LSM780 (Jena, Germany) confocal microscope. Figures were mounted with Adobe Photoshop (San Jose, CA, USA). Manipulation of the images was restricted to threshold and brightness adjustments to the whole image. Controls for the experiments consisted of the omission of primary antibodies; no staining was observed in these cases. Nuclei were counterstained

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Table. List of Specific Primers

Gene	Primer Sequence	Accession No	T°	Size
Hmga2	F: 5′-CTGGACGTCCGGTGTTGGT-3′ R: 5′-AACACCTTTCGGGAGACGGG-3′	NM_032070.1	60	131
Lin28A	F: 5′-CTTTTGCCAAAGCATCAGCCA-3′ R: 5′-GGTAGGGCTGTGGATCTCTT-3′	XM_006239062.1	60	121
Gapdh	F: 5′-ACAGTCCATGCCATCACTGCC-3′ R: 5′-GCCTGCTTCACCACCTTCTTG-3′	NM_017008	60	266

with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma-Aldrich)

Western Blotting

Dissected CE tissues were immersed in ice-cold 20 mM Tris/HCl (pH 8.0), in the presence of protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 20 µM leupeptin, 0.005 trypsin inhibiting U/mL aprotinin, 2 µg/mL soybean trypsin inhibitor) and homogenized. Cell debris was discarded by centrifugation (500*g* for 10 minutes at 4°C). Protein was determined by using Bradford assay (Bio-Rad Laboratories, Cambridge, MA, USA). Samples were subjected to SDS-PAGE (15% gel), and the proteins transferred to nitrocellulose membranes. Membranes were blocked with Superblock blocking solution (Pierce, Rockford, IL, USA) containing 3% BSA and 5% nonfat dried milk, incubated overnight at 4°C with antibodies against LIN28a and glyceraldehyde 3-phosphate dehydrogenase, and then with goat anti-rabbit IgG-peroxidase. Detection of labeled proteins was achieved with the enhanced chemiluminescent system (Amersham, Piscataway, NJ, USA).

Let-7 Mimic and Inhibitors

Customized *Let*-7b, *Let*-7c and *Let*-7e mimics and inhibitors (mirVana miRNA; Ambion, Austin, TX, USA) were used to promote and inhibit (respectively) miRNA activity of PE cells in culture during neurospheres assay. For transfection, Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) was mixed with the customized RNAs according to the manufacturer's instructions at different concentrations (mimics = 5, 10, and 30 nM; inhibitor = 10, 50, and 100 nM). After newborn PE dissection for neurospheres assay, mimic and inhibitor solutions were added to the cells and plated in 24-well culture plates at a density of 5.0×10^4 cells/well. After six hours, customized RNAs were removed by centrifugation, and retinal culture medium with growth factors was added to the cell culture. Cells were analyzed after seven days.

Gene Expression Datasets

EyeIntegration transcriptome database is available in the public domain at https://eyeIntegration.nei.nih.gov.[45,46] Human adult and fetal retina datasets were collected from SRP080002,[47] SRP080886,[48] SRP098761,[49] SRP015336,[50] SRP034875,[49] SRP105756,[51] SRP090040,[52] and SRP119766.[53] Adult retinal pigmented epithelium (RPE)– and embryonic stem cell (ESC)–derived RPE datasets were collected from the SRP064956,[54] SRP070938,[55] SRP062870,[56] SRP091675,[57] SRP094572,[58] SRP110135,[59] and SRP011895.[60] Downloaded data were given in transcripts per million and then normalized in $\log_2$. We compared *Let*-7 miRNAs, LIN28A, HMGA2, PAX6, NESTIN, and SOX2 expression. Data were analyzed by unpaired *t*-test. $P < 0.05$ was considered to be statistically significant.

Statistical Analysis

Statistical differences were calculated by unpaired Student's *t*-test (two-tailed), through comparative Ct ($2^{-\Delta\Delta CT}$) between treatments and respective controls, using GraphPad Prism Software (GraphPad, Inc., San Diego, CA, USA). Results were expressed as mean ± SEM (standard error of the mean), and a threshold of $P < 0.05$ was used for each test. The data were presented in arbitrary units.

Results

Expression of *Let*-7 Family of miRNAs in Ciliary Epithelium Cells

To investigate the expression of mature *Let*-7 in CE cells, we compared *Let*-7a, *Let*-7b, *Let*-7c, *Let*-7d, *Let*-7e and *Let*-7f levels from both adult PE and NPE epitheliums (Fig. 1A). With the exception of *Let*-7f, all the members of the *Let*-7 family investigated were highly detected in adult PE and NPE cells, highlighting *Let*-7b ($P = 0.004$), *Let*-7c ($P = 0.0009$), and *Let*-7e ($P = 0.0006$), suggesting that these miRNAs have relevant roles in differentiated CE cells. In contrast, *Let*-7f presented the lowest expression. Interestingly, adult NPE cells demonstrated higher expression of all investigated *Let*-7 in comparison to PE cells.

Comparing adult *Let*-7 expression to newborn, we observed that both PE and NPE presented statistically lower expression of *Let*-7b, *Let*-7c, *Let*-7e, and *Let*-7f in newborn cells (Fig. 1B). *Let*-7a and *Let*-7d were highly expressed in adult NPE cells, but no statistical differences were found between PE newborn and adult tissues. In NPE cells, all *Let*-7 miRNAs investigated presented significantly lower levels in newborns in comparison to adults.

To understand the relationship between *Let*-7 expression and retinal progenitor genes profile, we performed bioinformatic analysis in human retinal and RPE cells during embryonic and adult stages. Our bioinformatic analysis from datasets available at EyeIntegration website indicated that RPE cells derived from ESC expressed lower levels of *Let*-7b in comparison to adult RPE immortalized cell line (Figs. 2A and 2B). The lower expression of *Let*-7b in ESC-RPE was followed by higher expression of retinal progenitor genes *PAX6*, *SOX2*, and *ASCL1*. Our bioinformatic analysis also demonstrated that human fetal retinas expressed lower levels of *Let*-7c and *Let*-7d, as well as higher levels of progenitor genes *PAX6* and *SOX2* (Figs. 2C and 2D) in comparison to adult retinal tissue. These results suggest the opposite correlation of *Let*-7 and retinal progenitor genes expression in retinal and pigmented epithelial cells.

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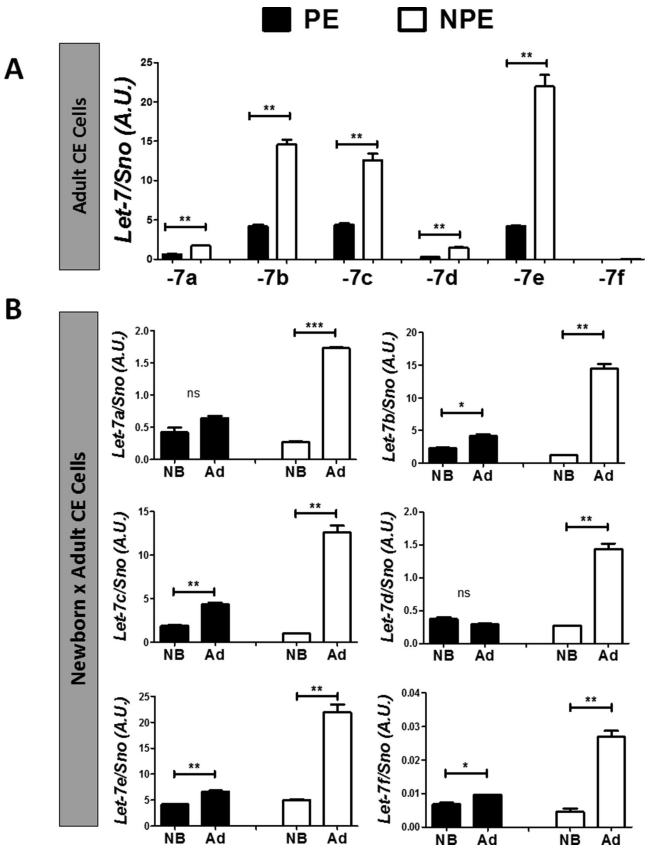

Figure 1. Expression of *Let*-7 miRNA in ciliary epithelium (CE) cells. (**A**) PCR analysis in adult CE cells showed high levels of *Let*-7a, *Let*-7b, *Let*-7c, *Let*-7d, and *Let*-7e in both PE and NPE cells, with statistically higher expression in NPE. (**B**) When compared to newborn CE cells, PCR results showed increased expression of *Let*-7b, *Let*-7c, and *Let*-7e in adult tissues. No differences were found between *Let*-7a and *Let*-7d adults and newborn PE cells. PE, pigmented epithelium; NPE, nonpigmented epithelium. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

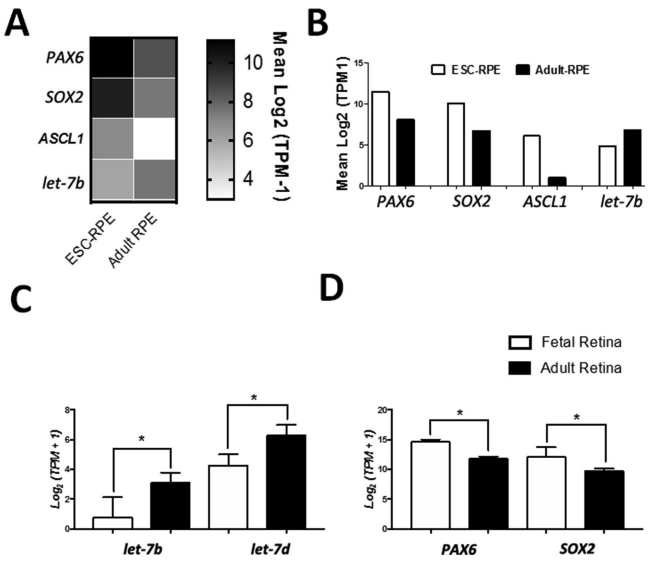

Figure 2. Bioinformatic analysis. Heatmap (**A**), and bar graphs (**B-D**), of the RNA-seq profile indicates the mean expression of *PAX6, SOX2, ASCL1,* and *Let*-7b (**A, B**) in embrionic stem cell (ESC)–derived RPE (n = 16), and adult RPE immortalized cell line (n = 32). Expression levels of *Let*-7b, *Let*-7d (**C**), *PAX6,* and *SOX2* (**D**) in human fetal retinas (n = 38), and adult retinas (n = 56). Results are given as mean after normalization by $\log^2$ of transcripts per million (TPM). The dark colors in the heatmaps indicate more reads obtained for each gene. Retrieved datasets from Eyeintegration database.[45,46]

Lin28 and Hmga2 Expression in CE Cells

Because Lin28 and Hmga2 are associated with *Let*-7 biosynthesis and transcriptional targeting, respectively, we investigated their expression in CE cells. We found very low transcriptional levels of *Lin28a* in both CE tissues from newborn and adult animals (Newborn PE = 0.0009 ± 4.9e-005; newborn NPE = 0.0003 ± 4.8e-005; adult PE = 0.0003 ± 3.4e-005; Adult NPE 0.0005 ± 4.6e-005) (Fig. 3B). However, newborn PE cells had significantly higher expression of *Lin28a* than adults (approximately threefold higher). Immunohistochemistry assay revealed few cells positive for LIN28a in newborn CE, with slight increase in PE in comparison to NPE cells (Fig. 3C). As expected, LIN28 localization was predominantly cytoplasmic.[34,61] In adult CE cells, LIN28a expression was undetectable in both PE and NPE.

Western blotting analysis indicated very low levels of LIN28 protein in newborn cells from both PE and NPE (Fig. 3D). Similarly to immunohistochemistry results, no expression was detected in adult tissues. We compared the LIN28 expression from CE newborn cells to the expression found in nine-day embryos (E9), known to highly express LIN28 (Fig. 3E). E9 embryos presented significantly higher levels of LIN28 protein expression in comparison to CE cells. These results indicated that CE cells decreased LIN28 expression during development and despite the low transcriptional and protein levels, newborn CE cells still retain a vestige of LIN28 expression.

Higher levels of *Hmga2* transcripts were found in newborn tissues from both PE and NPE cells in comparison to adults (Fig. 4B), with significantly higher expression of *Hmga2* in newborn NPE (newborn PE = 0.067 ± 0.003; newborn NPE = 0.19 ± 0.025). Immunofluorescence analysis revealed an evident nuclear expression of HMGA2 restricted to the lens, peripheral retina, and CE cells from newborn animals (Fig. 4C). In contrast, HMGA2 was undetected in adult CE cells. Together, these results indicated lower Lin28/Hmga2 expression in adult CE in comparison to newborn cells. The bioinformatic analysis from *LIN28* and *HMGA* expression in RPE (Figs. 5A, 5B) and retina (Figs. 5C, 5D) cells indicated similar higher transcriptional expression in embryonic tissues than adults or differentiated cells.

CE Progenitor/Stem Cells Present the Lin28/*Let*-7/Hmga2 Axis

Next, we investigated the axis Lin28-*Let*7-Hmga2 in neurospheres assay. For this analysis, we chose to evaluate *Let*-7b, *Let*-7c, and *Let*-7e in particular, once they were the highest expressed *Let*-7 in the adult CE cells. To enrich retinal progenitor properties, newborn PE cells were cultured in the presence of EGF and FGF for seven days to generate neurospheres (Fig. 6A). NPE cells were not able to form neurospheres (data not shown), and we did not proceed the studies with these cells.

After seven days in culture with growth factors, retinal progenitor cells in the neurospheres expressed low

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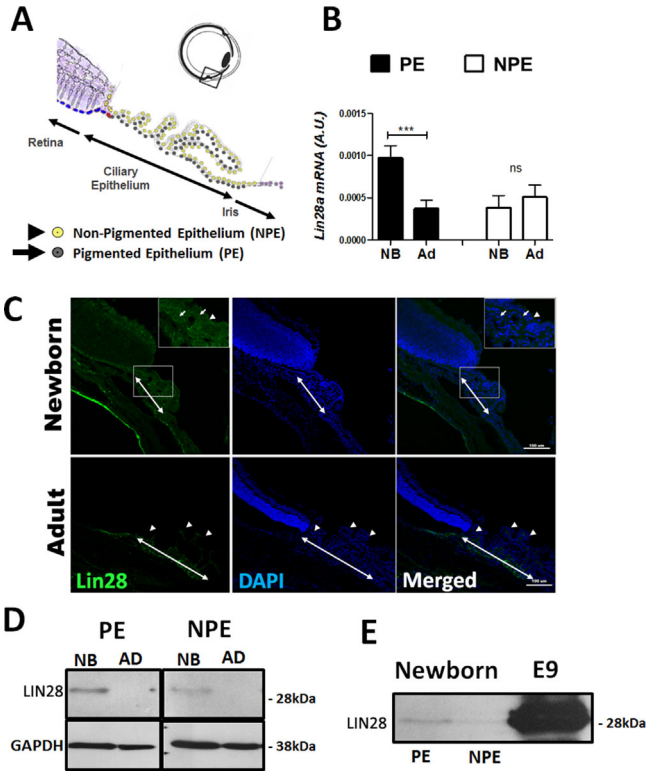

Figure 3. Lin28 expression in ciliary epithelium (CE) cells. (**A**) Schematic diagram of rat CE located between the peripheral retina and the iris (indicated by the *double-head arrow* below the tissue). CE consists of two epithelial cell layers, an inner nonpigmented epithelium (*yellow circles*) and an outer pigmented epithelium (*gray circles*). (**B**) Quantitative PCR analysis showed that the transcriptional level of *Lin28* decreased in adult PE cells, but showed no differences between newborn and adult NPE cells. (**C**) Immunofluorescence analysis in newborn CE sections (*top row*), revealed LIN28 positive cells (*green*) in both PE (*arrow*) and NPE (*arrowhead*). Detail in higher magnification of the image (*inset*). No LIN28-positive cells were detected in adult CE (*bottom row*). (**D**) Western blotting analysis for LIN28 protein between newborn and adults tissues from pigmented epithelium and nonpigmented epithelium indicating higher leves in newborn tissues. Cells from embrionic day 9 were used as positive control (**E**). PE, pigmented epithelium; NPE, nonpigmented epithelium; NB, newborn; Ad, adult; Ns, nonsignificant; A.U., arbitrary unit. *Doubledhead arrows* are located below the CE, for orientation. *Arrowheads* indicate NPE cells. *Single arrows* indicate PE cells. Nuclei were stained with DAPI (*blue*). *Scale bar:* 100 µm. ***$P < 0.0005$.

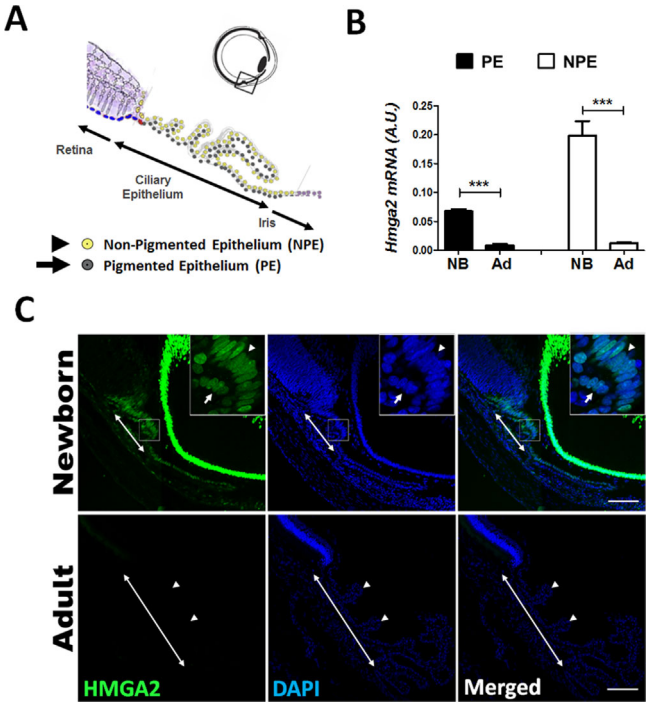

Figure 4. Hmga2 expression in ciliary epithelium (CE) cells. (**A**) Schematic diagram of rat CE located between the peripheral retina and the iris (indicated by the *double-head arrow* below the tissue). CE consists of two epithelial cell layers, an inner nonpigmented epithelium (*yellow circles*), and an outer pigmented epithelium (*gray circles*). (**B**) Quantitative PCR analysis showed higher expression of *Hmga2* in PE and NPE of newborns in comparison to adults. (**C**) Immunofluorescence analysis in newborn CE sections (*top row*), revealed HMGA2-positive cells (*green*) in both PE (*arrow*) and NPE (*arrowhead*). Detail in higher magnification of the image (*inset*). No HMGA 2–positive cells were detected in adult CE (*bottom row*). PE, pigmented epithelium; NPE, nonpigmented epithelium; NB, newborn; Ad, Adult; A.U., arbitrary unit. *Doubledhead arrows* are located below the CE, for orientation. *Arrowheads* indicate NPE cells. *Single arrows* indicate PE cells. Nuclei were stained with DAPI (*blue*). *Scale bar:* 100 µm. ***$P < 0.0005$.

levels of *Let-7*b and *Let-7*c, similarly to newborn tissue (Fig. 6B). *Let-7*e expression was higher in neurospheres than in the newborn cells (2.5 folds). As expected, adult PE cells expressed higher levels of all *Let-7* investigated than neurospheres and newborn cells. Progenitor cells in the neurospheres indicated significant higher expression of *Lin28a* and *Hmga2* transcripts than newborn cells (6.5- and 11-fold higher, respectively) despite the low expression in both tissues (Figs. 6C, 6D). Adult PE cells presented very low transcriptional levels of *Lin28* and *Hmga2*, significantly less than neurospheres and newborn cells.

The low expression of *Lin28*a transcripts reflected in the low protein expression found in neurospheres cells (Fig. 6E). Despite faint and diffuse, the presence of LIN28 in the neurospheres progenitor cells was confirmed by confocal microscopy. The orthogonal axis analysis indicated diffuse cytoplasmatic staining of LIN28 within the cells of the neurospheres (Fig. 7A), and the fluorescence profile analysis indicated low levels of LIN28 within the cells that co-localized with the DAPI fluorescence, showing different intensity from the background noise detected outside the cells (Figs. 7B, 7C).

On the other hand, despite the low levels of *Hmga2* transcripts detected in neurospheres, they were able to induce significant levels of protein expression in progenitor cells (Fig. 6F). HMGA2 was detected in the nucleus of several cells within the neurospheres, with different fluorescence intensity and different from the background noise detected outside the cells (Figs. 7D–7F).

Effect of *Let-7* in Lin28 and Hmga2 Expression in the Neurospheres

To understand the role of *Let-7*b, *Let-7*c and *Let-7*e miRNAs in Lin28 and Hmga2 expression at the beginning of the progenitor cells activation, we exposed the cells to synthetic RNAs oligonucleotides to mimic or inhibit the miRNA activity (Fig. 8A). First, we optimized the concentration efficiency of each miRNA mimics/inhibitors (Supplementary

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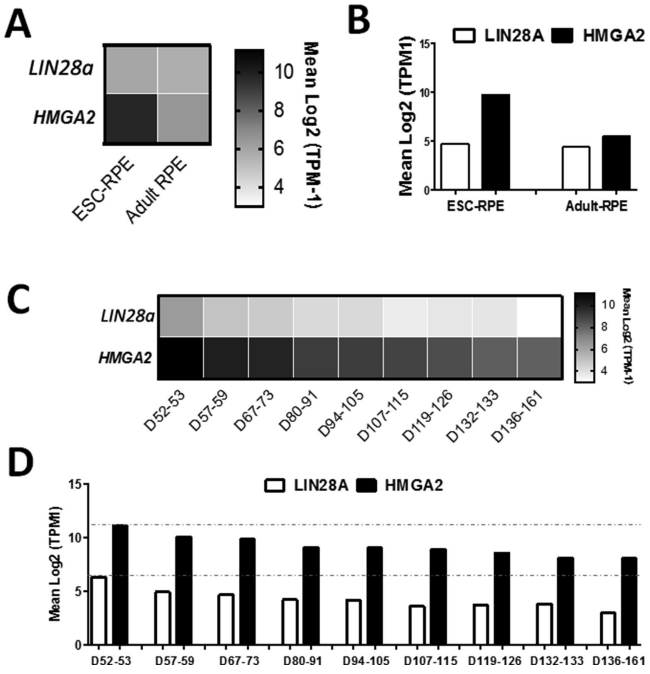

Figure 5. Bioinformatic analysis. Heatmap (**A, C**), and bar graphs (**B, D**), of the RNA-seq profile indicates the mean expression of *LIN28A* and *HMGA2* in embrionic stem cell (ESC)–derived RPE and adult RPE immortalized cell line (**A, B**) and in fetal human retinal development (**C, D**). Results are given as mean after normalization by $\log^2$ of transcripts per million (TPM). The dark colors in the heatmaps indicate more reads obtained for each gene. Retrieved datasets from Eyeintegration database.[45,46]

Fig. S1). The selected concentrations of the mimic agents were highly efficient to upregulate *Let*-7b, *Let*-7c, and *Let*-7e levels (7500-, 4000-, and 18,000-fold, respectively), whereas specific inhibitors' best efficient concentrations reduced the expression of *Let*-7b in 53%, *Let*-7c in 48%, and *Let*-7e in 60%, in comparison to control conditions. Then we confirmed the specific mimic/inhibitors efficiency after seven days in culture, which is the time needed for neurosphere formation (Supplementary Fig. S1). All specific mimic oligonucleotides kept the *Let*-7 investigated levels significantly upregulated after seven days in culture. The specific inhibitors for *Let*-7b and *Let*-7c maintained the levels downregulated at 55.4% and 62%, respectively. However, the specific inhibitor for *Let*-7e failed to keep the downregulation after seven days in culture, and, for that reason, we excluded the *Let*-7e from the next analysis.

Let-7b mimic or inhibitor treatment didn't induce changes in the *Lin28* transcriptional levels after 7 days in culture (Fig. 8B). Similarly, *Let*-7b mimic did not regulate *Hmga2* levels in comparison to the control (Fig. 8C). In contrast, the *Let*-7b downregulation was able to significantly increase the expression of *Hmga2* in 2.4-fold (Figs. 8C and 8E). *Let*-7c mimics significantly increased the expression of *Lin28a* in approximately fivefold (Fig. 8F) and decreased the *Hmga2* transcriptional levels in half (Figs. 8G, 8I). However, *Let*-7c inhibitors were not able to induce significant changes in *Lin28a* or *Hmga2* transcripts.

Interestingly, we found that mimic treatment induced an endogenous indirect expression of the other miRNA investigated. After the cells were exposed to *Let*-7b mimic oligonucleotides, the levels of *Let*-7c were 33% higher than the control (Fig. 8D). Similarly, the treatment with *Let*-7c mimic doubled the expression of endogenous *Let*-7b (Fig. 8H). These results suggest a possible positive feedback between *Let*-7 members.

Finally, we observed that *Let*-7b and *Let*-7c mimic and inhibitor treatment induced no significant differences in neurospheres sizes or quantity (Supplementary Fig. S2). Most of the PE cells in culture formed groups of cells smaller than 20 µm and fewer neurospheres from 20 to 100 µm were found in all treatment groups. Taken together, these results suggest that *Let*-7b and *Let*-7c regulated *Lin28* and *Hmga2* transcripts expression in progenitor cells from PE; however, they were not able to interfere directly in neurosphere growth.

Discussion

In this study, we investigated the presence of Lin28-*Let*-7-Hmga2 axis in two different states of CE cells: postnatal development and progenitor cells activated by growth factors. We suggest that Lin28 and *Let*-7 may act differently on CE cells depending on the cell state, through the regulatory axis in postnatal development and axis-independent in neurospheres cells.

Here, we showed that *Let*-7 miRNAs are highly expressed in adult CE cells, with low expression of Lin28 and Hmga2. As expected, newborn cells demonstrated inverse Lin28-*Let*-7-Hmga2 expression in comparison to adults, with lower expression of *Let*-7, and higher expression of Lin28 and Hmga2. These results suggested that Lin28-*Let*-7-Hmga2 regulatory axis might be involved in CE cells postnatal development or differentiation. Numerous evidence indicated that *Let*-7 family of miRNAs are important regulators of the process related to cellular aging and senescence.[62] In retinal tissue, the *Let*-7 family was previously associated with cell differentiation, being responsible for maintaining the Müller glial cells differentiated and controlling the cell cycle entry.[63] It is known that *Let*-7 levels are strictly controlled in stem cells because of their ability to suppress neural stem cells self-renewal,[64] repress progenitor/stem cell genes such as *c-Myc, Ascl1a, Lin-28, Pax6a, Pax6b, Mps1*e, and *Hspd1*,[26–28] and to act as a tumor suppressor.[65] In mouse embryonic stem cell lines, for example, *Let*-7 negatively regulated the targets genes *Pou5f1/Oct4, Sox2, Nanog, Tcf3*, and *Myc*, leading to cell differentiation.[66]

As expected, we found the *Let*-7 miRNA family poorly expressed in newborn CE cells, with increased expression in adult CE tissues, suggesting a role of *Let*-7 miRNAs in differentiated CE cells. The PE from ciliary body shares the same embryonic precursors with RPE, and *Let*-7 has a documented role in RPE cell differentiation and increased pigmentation. The *Let*-7 mimics (particularly *Let*-7a-5p) promoted RPE differentiation at the expense of neural differentiation and downregulated VEGF at the mRNA level.[67] Besides the embryonic origin, both PE and RPE cells participate in retinal regeneration in lower vertebrates.[68,69] Both PE and RPE contain a subpopulation of quiescent progenitor/stem cells that can be stimulated in vitro to generate multipotent self-renewing cells able to clonally proliferate under both adherent and neurosphere-forming conditions.[1,2,6–8,70,71]

The stem cell properties found in PE were never observed in NPE cells, and we associated this lack of progenitor property to the higher levels of *Let*-7 found in NPE in comparison to PE from adult tissues.

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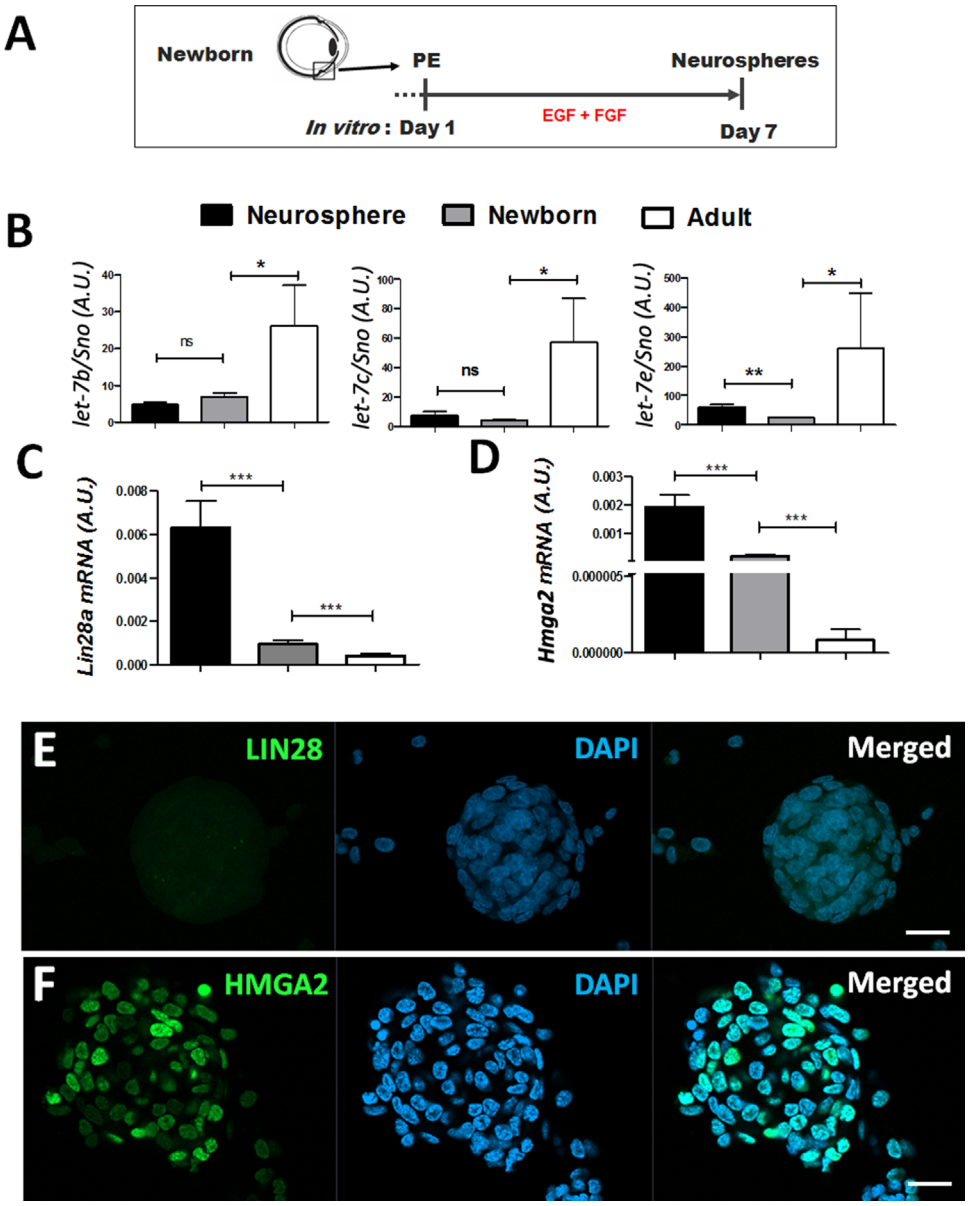

Figure 6. Expression of Lin28, *Let-7*, and Hmga2 in CE progenitor/stem cells neurospheres. (**A**) Schematic representation of neurospheres formation from PE newborn cells in culture. (**B**) After seven days in culture with FGF and EGF, PCR analysis of *Let-7*b and *Let-7*c indicated neurospheres expression levels similarly to newborns, but *Let-7*e expression was statistically higher. Real-time PCR analysis indicated (**C**) *Lin28a* and (**D**) *Hmga2* higher expression in neurospheres in comparison to newborns and adults. Neurospheres immunofluorescence analysis revealed the presence of (**E**) LIN28a- and (**F**) HMGA2-positive cells (*green*). A.U., arbitrary unit; Ns, nonsignificant. Nuclei were stained with DAPI (*blue*). *Scale bar:* 20 µm. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

Similar to newborn PE cells, the neurosphere progenitor cells presented lower levels of miRNAs *Let-7*b and *Let-7*c in comparison to adult tissues. It is known that stem cells and tumors negatively regulate the expression of *Let-7* family members to reactivate the pro-proliferative genes.[72] For instance, lung cancer cells indicated lower levels of *Let-7* in comparison to normal tissues, and *Let-7* overexpression leads to inhibition of the cancer growth.[73] In addition, *Let-7* has also been observed downregulated in colorectal cancer tissue, hepatocellular carcinoma, gastric adenocarcinoma, pancreatic cancer, ovarian cancer, prostate cancer, Burkitt's lymphoma, renal carcinoma, and melanoma.[74,75]

The fine control of *Let-7* expression can be regulated by Lin28.[76] Lin28 is highly expressed in stem cells, responsible for maintaining the expression of specific pluripotency/progenitor genes,[61,77] and is associated with the regenerative capacity of a cell or tissue.[29] Lin28 regulates *Let-7* through its binds to pre-*Let-7* in a region called the precursor element, located in the hairpin loop,[31,78,79] an important area for the maturation of pre-*Let-7*.[32]

Here, we observed an overexpression of *Lin28*a in neurospheres in comparison to newborn cells. Despite the higher levels of *Lin28a* in neurospheres, the levels of *Let-7* were similar to newborns and not lower as expected, suggesting that *Lin28* had little influence on *Let-7* regulation in neurosphere cells. This Lin28-*Let-7* independent activity was observed by Romer-Seibert and collaborators. The group showed that biological role of Lin28 in mouse subventricular

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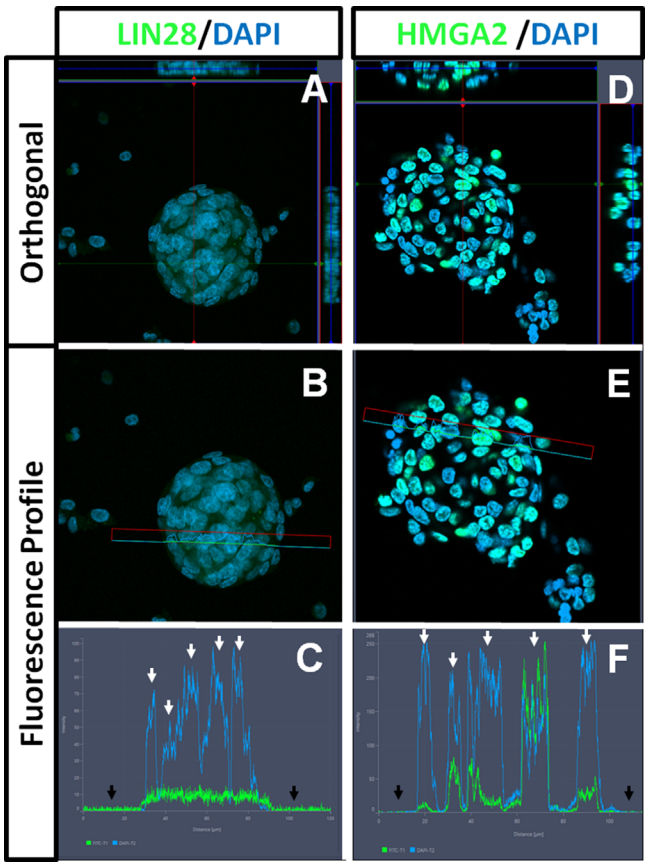

Figure 7. Microscopy characterization of Lin28a and Hmga2 expression in neurospheres. (**A**) Confocal orthogonal analysis showed LIN28a expression mainly in the cytosol of progenitor cells, and HMGA2 was restricted to the nuclei of several progenitor cells within the neurospheres (**D**). A small group of cells was selected for fluorescence intensity analysis for LIN28 (**B**), and HMGA2 (**E**). The nuclei of the cells were stained with DAPI (*blue*), and the fluorescence profile of each single cell was indicated by the *white arrows* in **C** and **F**. (**C**) The green fluorescence of LIN28 presented low intensity inside the cells (*white arrows*), but higher than the background fluorescence found outside the cells (*black arrows*). (**F**) The green fluorescence of HMGA2 presented different levels of intensity inside the cells (*white arrows*), significantly higher than the background fluorescence found outside the cells (*black arrows*).

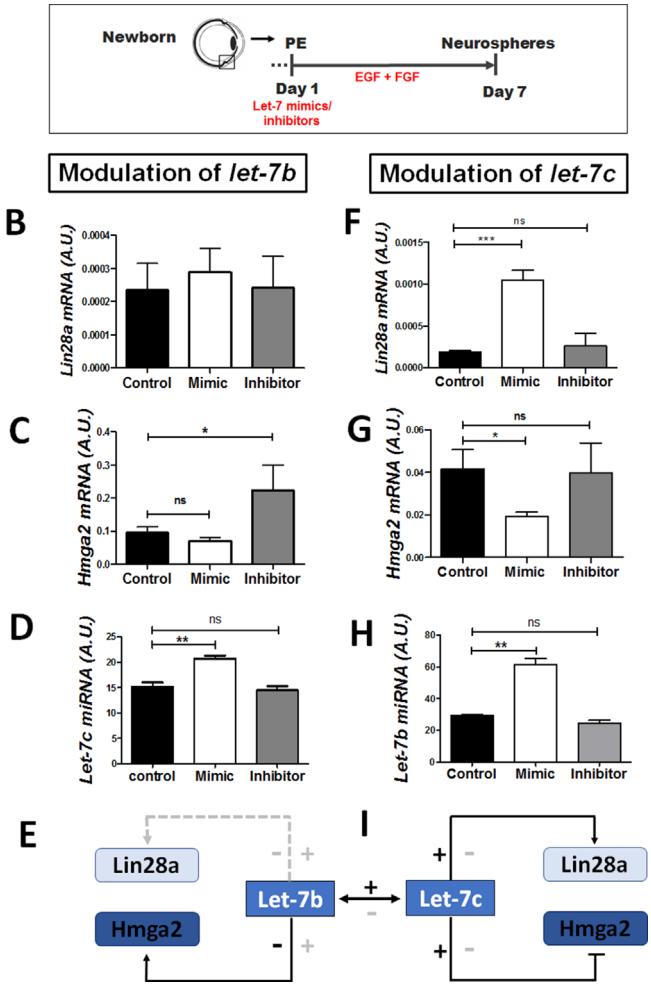

Figure 8. Effect of *Let*-7b and *Let*-7c modulation in Lin28 and Hmga2 expression. (**A**) Schematic representation of PE newborn cells in culture in the presence of *Let*-7 mimic/inhibitor treatments. Real-time PCR revealed that *Let*-7b mimic or inhibitor induced no transcriptional changes in *Lin28a* expression in comparison to control (**B**). *Hmga2* was increased after *Let*-7b inhibitor but presented levels similar to control after mimic treatment (**C**). *Let*-7b mimics also induced increased expression in *Let*-7c (**D**). *Let*-7c mimic increased the transcriptional levels of *lin28*, whereas the inhibitors induced no effect (**F**). On the other hand, *Let*-7c inhibitor increased the transcriptional levels of *Hmga2*, whereas the inhibitors induced no effect (**G**). *Let*-7c mimics also induced increased expression in *Let*-7b (**H**). (**E, I**) Schematic representation of the *Let*-7 modulation results. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

zone was independent from its known activity of blocking *Let*-7.[80] The authors correlated Lin28 expression to neural fate determination of mouse subventricular zone at a post-stem cell/pre-differentiation step, increasing the proportion of neuroblasts and reducing the number of astrocytes. Moreover, Balzer and collaborators also demonstrated that constitutive Lin28 favored neural differentiation after blocking completely the accumulation of glial cells in embryonic carcinoma cell line.[34] *Lin28*a and *Lin28*b deletion during retinal histogenesis can lead to premature maturation of Müller cells, whereas its ectopic expression converted Müller cells to a neural phenotype, indicating its important role in the regulation of retinal progenitor cell fate.[81]

Once the PE cells in the neurospheres express neural progenitor genes that favor neuronal differentiation, it is possible that Lin28 could control neural fate determination in PE-derived neurospheres, independent of *Let*-7 downregulation.

We demonstrated that neurosphere treatment with *Let*-7b and *Let*-7c inhibitors had no influence on PE-derived neurosphere formation and *Lin28* expression. However, *Let*-7c mimic induced an unexpected increase in *Lin28* expression that should be analyzed with caution. First, it is known that Lin28 regulates *Let*-7 expression while *Let*-7 itself binds to the 3′ UTR of Lin28 mRNA to regulate negatively Lin28 expression, thereby establishing a well-known double-negative feedback loop.[82–84] This negative feedback is common but not observed in all of the cases. Trophoblast transfected with *Let*-7e or *Let*-7f mimics, for example, did not significantly alter LIN28B expression.[85] Here, we observed an opposite response of *Lin28* to *Let*-7c increase, suggesting that this negative feedback is suppressed in PE neurospheres, and the upregulation of Lin28 could be a

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compensatory mechanism to maintain the progenitor cells undifferentiated.

Second, Triboulet and colleagues[86] reported that Lin28-*Let*-7c regulatory mechanism is slightly different from the other Let-7 miRNAs, and can bypass LIN28-mediated regulation. They found that the 5-nt-long sequence forming the short apical stem-loop of Let-7c-2 precursor element precludes LIN28A binding in vitro and LIN28A-mediated repression in cells.

These findings suggest that Lin28-Let-7 mutual interaction should be analyzed with care. Lin28 may not repress Let-7 in all cell types or developmental stages. Thence, further investigation on the mechanisms involved in Lin28-*Let*-7 interaction in progenitor cells from CE neurospheres are necessary to clarify the role of these actors in progenitor and differentiation activities.

Differently from the unexpected results found between the Lin28-*Let*-7 interaction in the neurospheres, the Hmga2 results were consistent in postnatal and progenitor cells. Hmga2 was highly expressed in neurospheres and newborn cells with inversely proportional *Let*-7 expression. Interestingly, inhibition of *Let*-7b increased *Hmga2* expression in neurospheres, whereas *Let*-7c mimics decreased its expression, confirming the *Let*-7 regulation of *Hmga2* in PE neurospheres. In many cell types, the expression of *Let*-7 is inversely proportional to the expression of Hmga2, and the ectopic expression of this protein promotes cell proliferation, even in the presence of *Let*-7.[36] Hmga2 knockout animals show reduced muscle development and proliferation of myoblasts, while their overexpression promotes the growth of myoblasts and prevents their differentiation.[87] In neural progenitor cells, Hmga1 and Hmga2 knockout animals show chromatin condensation and early astrocytic differentiation.[88]

Last, we observed that *Let*-7 perturbation in neurospheres did not lead to significant changes in the neurosphere size or proliferation. Hmga2 is commonly upregulated in neurosphere culture and regulates self-renewal of retinal progenitor cells[89] through decreased expression of CDK inhibitors p16(Ink4a) and p19(ARF).[64,90] Overexpression of Hmga2 increased the number of retinal progenitor cells neurospheres and negatively influences cell differentiation through overexpression of *Ki67* and *Pax6* and decrease in levels of *Junb/p19^{arf}* transcripts.[25] We observed an increased *Hmga2* expression after *Let*-7b inhibitor treatment with no influence on neurosphere proliferation. To understand this, further analysis on cell cycle regulators is required.

In summary, we have shown that postnatal CE cells express Lin28- *Let*-7-Hmga2 regulatory axis, suggesting that it might be involved in CE cells postnatal development or differentiation. On the other hand, neurosphere progenitor cells expressed higher levels of *Lin28a* than newborn CE cells, but the levels of *Let*-7 were similar in neurospheres and newborns, suggesting that Lin28 could be involved in neurosphere maintenance, independently from the *Let*-7 regulation. These results reinforce the importance to investigate the Lin28-*Let*-7-Hmga2 contribution in different cell contexts.

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