MicroRNAs couple cell fate and developmental timing in retina

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Edited by Joseph G. Gall, Carnegie Institution of Washington, Baltimore, MD, and approved October 14, 2009 (received for review August 13, 2009)

Cell identity is acquired in different brain structures according to a stereotyped timing schedule, by accommodating the proliferation of multipotent progenitor cells and the generation of distinct types of mature nerve cells at precise times. However, the molecular mechanisms coupling the identity of a specific neuron and its birth date are poorly understood. In the neural retina, only late progenitor cells that divide slowly can become bipolar neurons, by the activation of *otx2* and *vsx1* genes. In *Xenopus*, we found that *Xotx2* and *Xvsx1* translation is inhibited in early progenitor cells that divide rapidly by a set of cell cycle-related microRNAs (miRNAs). Through expression and functional screenings, we selected 4 miRNAs—mir-129, mir-155, mir-214, and mir-222—that are highly expressed at early developmental stages in the embryonic retina and bind to the 3' UTR of *Xotx2* and *Xvsx1* mRNAs inhibiting their translation. The functional inactivation of these miRNAs in vivo releases the inhibition, supporting the generation of additional bipolar cells. We propose a model in which the proliferation rate and the age of a retinal progenitor are linked to each other and determine the progenitor fate through the activity of a set of miRNAs.

cell cycle | homeodomain | translational control | neurogenesis

During brain development, different types of nerve cells are generated according to a predictable schedule, through the coordination of cell cycle progression with the sequential expression of key genes of cell fate (1, 2). The time when a neural progenitor cell stops dividing and starts differentiating—the cell birth date—is related to its fate of differentiation (3), but the underlying molecular mechanisms are largely unknown. In vertebrates, retina development is an attractive model to investigate this issue (1).

Early retinal progenitors are multipotent (4, 5). However, if a progenitor exits the cell cycle early in development it is not able to become a bipolar neuron, which is the last type of retinal neuron to be generated. The homeobox genes *otx2* and *vsx1* act as positive key regulators of bipolar cell generation (6–10). Why do they act late in the retinal progenitor lineage? In *Xenopus*, *Xotx2* and *Xvsx1* are transcribed from very early retina developmental stages, but their translation is repressed in early progenitors through *cis*-acting signals contained in their 3' UTR (8). A crucial question concerns the mechanism able to release the translational inhibition of *Xotx2* and *Xvsx1* in late progenitor.

Cell cycle manipulation of retinal progenitors is sufficient to dissociate cell birth date and fate of differentiation (8, 11). The cell cycle length of vertebrate neural progenitor cells increases over time (12, 13), thus correlating with the age of a progenitor. Accordingly, in the *Xenopus* developing retina early progenitors have a shorter cell cycle compared with late progenitors (8). It has been shown that late retinal progenitors forced to divide as rapidly as early progenitors fail translating *Xotx2* (8). Conversely, here we show that lengthening the cell cycle of early progenitors supports and anticipates *Xotx2* and *Xvsx1* translation. Thus, these observations suggest that the cell cycle length of a progenitor might provide a mechanism of control for the translational inhibition of *Xotx2* and *Xvsx1*.

What is the molecular nature of the translational inhibition in early progenitors? Recent evidence implies microRNAs (miRNAs) in the control of neural development (14–16), cell cycle (17), and developmental timing (18), suggesting them as good candidates to mediate the translational inhibition of *Xotx2* and *Xvsx1*. Here we show that 4 miRNAs bind to the 3' UTR of *Xotx2* and *Xvsx1* mRNAs, inhibiting their translation and the generation of bipolar neurons.

Results

Sonic Hedgehog Inactivation Lengthens the Cell Cycle and Anticipates *Xvsx1* and *Xotx2* Translation in Early Retina Progenitor Cells. The pace of retinal progenitors proliferation is controlled by *sonic hedgehog* (*hh*) (19). Blocking *hh* signaling by cyclopamine lengthens G1 and G2 phases and delays cell cycle exit (20). By BrdU cumulative analysis (Fig. 1*A–F*), we confirmed that stage (st.) 33 retina progenitors treated with 50 μ M cyclopamine from st. 18 (after optic vesicle formation) undergo longer cell cycles compared with control progenitors, because the slope of BrdU increase over time is smaller (0.0270; Fig. 1*F*) compared with control (0.0538; Fig. 1*C*). We also confirmed that a higher proportion of progenitors remain cycling, because S index at 1 h is higher (0.18; Fig. 1*F*) compared with control (0.12; Fig. 1*C*).

In WT retina progenitors, *Xvsx1* and *Xotx2* are transcribed from st. 15 and st. 25, respectively (7, 10), whereas their translation is first detected at st. 37 (*Xvsx1*) or st. 39 (*Xotx2*) (8). Cyclopamine treatment from st. 18 induced a premature detection of *Xotx2* (Fig. 1*G* and *H*) and *Xvsx1* (Fig. 1*I* and *J*) proteins at st. 35, with a stronger effect on *Xotx2*. The cyclopamine treatment acted at the translational level, because it did not affect the mRNAs levels (Fig. 1*K*).

Selection of Developmentally and Cell Cycle Regulated Retina miRNAs Predicted to Bind the 3' UTR of *Xvsx1* and *Xotx2*.

We searched for developmentally regulated miRNAs that were predicted to bind the 3' UTR of *Xvsx1* and *Xotx2*. Because *Xotx2* and *Xvsx1* translation is de-repressed in late retina development, we reasoned that the amount of miRNAs targeting *Xotx2* and *Xvsx1* mRNAs ought to be high in early (st. 33) retinas and low in differentiated (st. 42) retinas. Furthermore, whereas cyclopamine removes the translational inhibition of *Xvsx1* and *Xotx2* (present data), we previously showed that hydroxyurea/aphidicolin (HUA) treatment maintains such inhibition (Fig. 1*L*) (8): therefore, the concentration of inhibitors

Author contributions: R.V., L.P., G.R., and F.C. designed research; S.D., D.B., S.M., X.W., and M.E. performed research; R.V., L.P., G.R., and F.C. analyzed data; and G.B. and F.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0909167106/DCSupplemental.

Fig. 2. Four developmentally regulated miRNAs inhibit the translation of *Xotx2* and *Xvsx1*. (A) Heat map shows log ratios (red to green) of miRNA expression from WT and treated retinas with respect to WT st. 42 retina (baseline), after normalization with U6 snRNA1–2. The 25 miRNAs giving the highest hybridization signal (log total intensity, white to blue) in the st. 33 array are shown. (B–E) ISH detection (red) of miR-129; nuclei are stained by Hoechst (blue). (F) Relative quantification of miRNAs at st. 33 (wt33, WT; cy33, cyclopamine-treated) and at st. 42 after HUA treatment (hua42) as compared with their expression levels in st. 42 WT retinas (baseline). Error bars show SEM. (G and H) *Xotx2* immunodetection (red) of lipofected cells (green, GFP detection). Arrows indicate double-positive cells. (I) Proportion of either *Xvsx1*- or *Xotx2*-positive lipofected cells. a-miR, antisense oligonucleotide to the indicated miRNA; a-miR-mix, equimolar mixture of antisense oligonucleotides to miR-129, miR-155, and miR-222 (*Xotx2* immunodetection) or to miR-129 and miR-222 (*Xvsx1* immunodetection). (n) number of cells analyzed. Error bars show SE. (L) lens; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

tiated neurons (outer nuclear layer and ganglion cell layer in Fig. 2E; see also Fig. S1A). Quantitative (q)RT-PCR results confirmed the pattern of expression observed by the microarray screening and by ISH (Fig. 2F).

In Vivo Inactivation of miR-129, miR-155, miR-214, and miR-222 Supports *Xvsx1* and *Xotx2* Expression. To decoy endogenous miRNAs, we lipofected antisense oligonucleotides into the optic vesicle. Compared with control (Fig. 2G and I and Fig. S2), the decoy of either miR-129 (Fig. 2H and I), miR-155, or miR-222, as well as the decoy of all of the 3 miRNAs together (Fig. 2I and Fig. S2) significantly increased the proportion of *Xotx2* translating cells, with a higher effect after the triple decoy. Conversely, only the decoy of miR-129 or miR-222, or their double decoy, increased, albeit to a lower extent, the proportion of *Xvsx1*-positive cells (Fig. 2I and Fig. S2). This result suggests that *Xotx2* mRNA is more sensitive than *Xvsx1* mRNA to translational repression and is consistent with the later translational onset of

Fig. 3. In vivo inactivation of miR-129, miR-155, miR-214, and miR-222 supports the bipolar cell fate. (A–G) Sections of retinas, lipofected with control (A) or antisense to miRNAs (a-miRNAs, B–G) oligonucleotides, show morphology of lipofected cells (GFP positive, white detection) in the differentiated cell layers: outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Bipolar cells are located in the INL. a-miR-mix is an equimolar mix of antisense oligonucleotides to miR-129, miR-155, miR-214, and miR-222. (H) Heat map shows the percentage ratio (red to blue) of neuronal (ganglion, horizontal, amacrine, photoreceptor, and bipolar) and glial (Müller) cells in retinas lipofected with antisense oligonucleotides to miRNAs (a-miR) as reported in Table S3.

Xotx2 compared with *Xvsx1* in retinal development (8). Notably, the high increase of *Xotx2*-positive cells after the knockdown of miR-129, miR-155, and miR-222 is comparable to the increase observed after cyclopamine treatment (Fig. 2I and Fig. S2), which decreases the amount of these miRNAs (Fig. 2A and F).

In Vivo Inactivation of miR-129, miR-155, miR-214, and miR-222 Supports the Bipolar Cell Fate. We investigated the effects of miRNAs decoy on cell fate (Fig. 3A–H). Compared with control, the decoy of either miR-129, miR-155, miR-214, or miR-222 significantly increased the proportion of the bipolar cells in the inner nuclear layer from 32% to 44.9%, 40.9%, 39.8%, and 38.2%, respectively. Consistently, lipofecting a mixture of the 4 antisense oligonucleotides increased the proportion to 49.2%. Decoy of other miRNAs decreased the proportion of bipolar cells or had no effect (Fig. 3 and Table S3). The lipofection of antisense oligonucleotides to the 4 miRNAs did not increase the ratio of TUNEL-positive apoptotic cells compared with the lipofection of a control antisense (Fig. S3).

miR-129, miR-155, miR-214, and miR-222 Target the 3' UTR of *Xvsx1* and *Xotx2* in HEK 293 Cells and in Vivo. miR-129, miR-155, and miR-222 were predicted to target the 3' UTR of *Xotx2*, whereas all of the 4 miRNAs were expected to target the 3' UTR of *Xvsx1* (Fig. 4A and Fig. S4). We confirmed the activity of the identified miRNAs binding sites by measuring the translational rate of reporter constructs (sensors), carrying GFP upstream of WT or mutated 3' UTR of *Xvsx1* and *Xotx2*, after cotransfection with either mature miRNAs or antisense oligonucleotides to miRNA, in HEK293 cells (SI Text and Figs. S5 and S6).

The 4 miRNAs target the 3' UTR of *Xvsx1* and *Xotx2* also in

Fig. 4. miR-129, miR-155, miR-214, and miR-222 target the 3' UTR of *Xotx2* and *Xvss1*. (A) miRNA target sites as evaluated by functional assay in HEK 293 cells (Figs. S5 and S6). (B) In vivo sensor assay. (C) In vivo translational efficiency of GFP sensors as in B. Bars indicate GFP/RFP relative intensity ratio after lipofection. Cyc, cyclopamine. Mix, an equimolar mixture of antisense oligonucleotides to miR-129, miR-155, and miR-222 (for *Xotx2* 3' UTR), or to miR-129, miR-155, miR-214, and miR-222 (for *Xvss1* 3' UTR). (n) number of retinal sections. Error bars show SE. Asterisks as in Fig. 2. (D–G) Examples of cells colipofected with sensors and control antisense oligonucleotide. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Layers did not form properly in cyclopamine-treated retinas (E and G), as previously described (20).

the developing retina. We colipofected either *Xotx2* or *Xvss1* sensor with a red fluorescent protein (RFP) reporter construct into st. 18 optic vesicles and analyzed the GFP/RFP intensity ratio in lipofected retinal cells at st. 39 (Fig. 4 B and C). *Xotx2* (Fig. 4 D and E) and *Xvss1* (Fig. 4 F and G) sensors translated GFP more efficiently in cyclopamine-treated retinas (Fig. 4 E and G) than in control retinas (Fig. 4 D and F; 155% and 119%, respectively), with a higher effect on the *Xotx2* sensor (Fig. 4C). Thus, the sensors reproduce the translational regulation of *Xotx2* and *Xvss1* (Fig. 1 G–L) in vivo. Compared with control miRNA, a mixture of the mature ds miRNAs that affected sensor translation in HEK293 cells (Fig. S5) was able to repress the translation of *Xotx2* sensor by 30% (Fig. 4C). A much less significant repression of *Xvss1* sensor was also detected. The triple mutation of miR-129, miR155, and miR-222 target sites completely abolished the responsiveness of *Xotx2* sensor to miRNAs. In addition, the miRNAs mixture was able to counteract the effects of cyclopamine on *Xotx2* sensor, indicating that

cyclopamine de-repression of *Xotx2* sensor translation may work through depletion of these miRNAs.

miR-129, miR-155, miR-214, and miR-222 Act Downstream of the Cell Cycle Setting a Bipolar Cell Fate. To investigate whether miRNAs affect cell fate through effects on cell cycle, we measured the proportion of lipofected cells that were postmitotic at st. 34 by 5-ethynyl-2'-deoxyuridine (EdU) birthdating (Fig. 5A and Fig. S7). The depletion of miR-129, miR-155, miR-214, and miR-222, which increases the proportion of bipolar cells by approximately 65% compared with control (Fig. 5B), did not affect the ratio of dividing progenitors significantly (26% vs. 28% of control; Fig. 5A); conversely, this proportion was decreased by lipofecting progenitors with the cell cycle inhibitor *Xgadd-45- α* (23) (18%; Fig. 5A), according to previous data (8). Progenitor cells forced to withdraw early from the cell cycle by *Xgadd-45- α* lipofection produced, compared with control, more of the early retinal neurons—namely ganglion cells—and fewer bipolar neurons (Fig. 5B) (8). The

Fig. 5. miR-129, miR-155, miR-214, and miR-222 act downstream of the cell cycle setting a bipolar cell fate. (A) Fraction of EdU-positive lipofected cells. Error bars indicate SE. (n) number of cells. (B) Proportion of different lipofected retinal cell types. Error bars indicate SEM. Asterisks as in Fig. 2. (C) Model representing regulation of the timing of the translational inhibition of *Xotx2* and *Xvss1* by miR-129, miR-155, miR-214, and miR-222 (miRNAs). S is the synthesis phase of the progenitor cell cycle. Early, mid, and late refer to cell birthdates in B and to retinal developmental stages in C.

depletion of miR-129, miR-155, miR-214, and miR-222 counteracted both the increase of ganglion cells and the decrease of bipolar cells due to *Xgadd-45- α* lipofection (Fig. 5B), with no effect on the ratio of dividing progenitors (18%; Fig. 5A). Therefore, the depletion of these miRNA in *Xgadd-45- α* lipofected cells switches the fate of early progenitors from an early to a late type of retinal neuron.

Discussion

Mechanisms accounting for the generation of distinct types of neurons with a precise timing schedule have been described. *ikaros*, the mouse ortholog of *hunchback* setting early neuroblast fate in *Drosophila* (24), is sufficient to generate early-born neurons when misexpressed in late progenitors of the retina (25). In mouse developing cortex, the double knockdown of the 2 transcription factors COUP-TFI and -II causes sustained neurogenesis and prolonged generation of early-born neurons (26). However, how *ikaros* and COUP-TFI and -II are down-regulated in late progenitors remains unclear.

In the vertebrate retina, *vax1* and *otx2* are both necessary and sufficient to specify the identity of the last-born neurons, the bipolar cells (6–10). In *Xenopus*, progenitors of st. 25–37 embryos transcribe *Xvax1* and *Xotx2*; upon cell cycle exit these early progenitors generate all of the retinal neurons but the late-born bipolar cells (8, 11, 27). Only after st. 37–39, when *Xvax1* and *Xotx2* are translated, does differentiation of bipolar cells occur (8). Thus, translational control in frogs plays a crucial role in establishing a specific cell type at a precise developmental time.

Here we report that miR-129, miR-155, and miR-222 target the 3' UTR of *Xvax1* and *Xotx2*, inhibiting their translation both in HEK 293 cells and in vivo. The in vivo decoy of each of the 4 miRNAs—miR-129, miR-155, miR-214, and miR-222—suppresses the generation of extra bipolar cells. However, miR-214 inactivates the translation of a reporter carrying *Xvax1* 3' UTR in HEK293 cells, but its in vivo decoy is not effective on *Xvax1* translation. We speculate that the ability of miR-214 to affect the generation of bipolar cells may be due to its action on genes other than *Xvax1* and *Xotx2*. Conversely, miR-129, miR-155, and miR-222 could affect the bipolar cell proportion by directly inhibiting *Xvax1* and *Xotx2* translation.

The proliferation rate of a progenitor decreases over time during neural development as the progenitor cell cycle length increases (8, 12, 13). By microarray analysis, ISH, and qRT-PCR, we showed that miR-129, miR-155, miR-214, and miR-222 are highly expressed in fast cycling, early retinal progenitors of st. 30–33 embryos that do not translate *Xvax1* and *Xotx2* and are down-regulated in slowly cycling, late progenitors of st. 34–37 and in st. 42 postmitotic cells. Emerging evidence indicates that the expression of these miRNAs is under the control of the cell cycle machinery. The 4 miRNAs are up-regulated in tumor cells (28–31). Moreover, the expression of miR-155 and miR-214 is directly related to the proliferation rate of primary fibroblasts (32). A functional relationship

between cell cycle speed and expression of the 4 miRNAs is also supported by their decrease upon the cell cycle lengthening exerted by cyclopamine.

Interestingly, miR-222 is up-regulated as glioblastoma cells progress beyond the G1–S phase transition (33). If miR-129, miR-155, miR-214, and miR-222 were produced in a constant window of the cell cycle of retinal progenitors such as the G1–S phase transition, their amount in these cells would depend on cell cycle length because rapidly dividing cells spend a higher proportion of time in the G1–S phase transition compared with slowly dividing cells. This is consistent with the up-regulation of miR-222 (and of the other 3 miRNAs) after treatment with HUA (Fig. 2A), which blocks cells in G1–S.

We propose that miR-129, miR-155, miR-214, and miR-222 could be part of a mechanism coupling the determination of the bipolar cell identity with a low proliferation rate of retinal progenitors: they would act by inhibiting translation of the key homeobox genes *Xotx2* and *Xvax1* early in development (Fig. 5E). As a consequence, these miRNAs would play a major role in matching the bipolar cell identity with a late cell birth date. A crucial question is how the expression of the 4 miRNAs may be regulated during development and, in particular, what might be the nature of the signal supporting their high level of expression in highly proliferating cells. *hh* signaling, which supports proliferation of embryonic retinal cells (19, 20), may be a candidate worth scrutiny.

The first isolated miRNAs were originally identified as regulators of the developmental timing in *Caenorhabditis elegans* (18). The *Drosophila* counterpart of a heterochronic miRNA gene from *C. elegans*, *let-7*, was recently shown to regulate the timing of neuromuscular tissue development, thus suggesting a widespread use of miRNAs in temporal regulation of animal development (34). Here we show that miR-129, miR-155, miR-214, and miR-222 play a similar role in the vertebrate neural development, controlling the timing of the generation of retinal bipolar cells.

Materials and Methods

BrdU cumulative analysis was performed as described previously (8). MiRNA microarrays (Exiqon miRCURY LNA Array version 8.1) were hybridized according to the manufacturer's protocol; data were analyzed using Axon Genepix and Axon Aquity software. The miRanda algorithm (version 1.0b) was used to predict binding of the selected miRNAs. MiRNAs qRT-PCR was performed according to the miScript-System kit (Qiagen). Lipofection experiments were carried out as described previously (8, 10, 11). The Click-it kit (Invitrogen) was used to detect EdU. ISH and immunohistochemistry were performed as described previously (7, 8, 10) with minor modifications. More details are described in *SI Text*.

ACKNOWLEDGMENTS. We thank Paolo Capelli, Viviana Guadagni, and Paola Iacopetti for assistance; and Marcella Simili, Laura Mariani, Massimiliano Andreazzoli, Simona Casarosa, Paolo Malatesta, Giuseppe Macino, Rongqiao He, and Magdalena Goetz for discussion. This work was supported by Telethon, Ministero Università e Ricerca Scientifica, Ministero Affari Esteri, and Scuola Normale Superiore.

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