**Xrx1 controls proliferation and multipotency of retinal progenitors**

Simona Casarosa, Marcos A. Amato, Massimiliano Andreazzoli, Gaia Gestri, Giuseppina Barsacchi, and Federico Cremisi

*Sezione di Biologia Cellulare e dello Sviluppo, Dipartimento di Fisiologia e Biochimica, Università di Pisa, via Carducci, 13-56010 Ghezzano (Pisa), Italy

** Università degli Studi di Pisa, Centro di Eccellenza AmbiSEN, Pisa, Italy

** Scuola Normale Superiore, piazza dei Cavalieri, 7-56100, Pisa, Italy

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**Abstract**

We investigated the function of Xrx1 during Xenopus retinogenesis. Xrx1 overexpression lengthens mitotic activity and ectopically activates the expression of markers of undifferentiated progenitors in the developing retina. We assayed Xrx1 ability to support proliferation with a cell-autonomous mechanism by in vivo lipofection of single retinal progenitors. Xrx1 overexpression increases clonal proliferation while Xrx1 functional inactivation exerts the opposite effect. We also compared the effects of Xrx1 with those of the cyclin-dependent kinase cdk2, a strong mitotic promoter. Despite the similar increase in clonal proliferation displayed by both factors, Xrx1 and cdk2 act differently on retinal cell fate determination. cdk2/cyclinA2 lipofected retinas show a decrease in early-born cell types as ganglion cells and cones and an increase in late-born types such as bipolar neurons. On the contrary, Xrx1 lipofected retinas show no changes in the proportions of the different cell types, thus suggesting a role in supporting multipotency of retinal progenitors.

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**Introduction**

In the nervous system, the mechanisms coordinating cell proliferation and fate commitment of neuronal progenitors are fundamental for the construction of layered structures, such as the retina. In these structures, the generation of definite cell types in distinct layers is also influenced by the time when precursor cells exit the cell cycle (reviewed by Livesey and Cepko, 2001; Ohnuma et al., 2001). The development of the vertebrate retina recapitulates most of the general mechanisms governing neuronal development. Retinal cell types are generated through temporal waves that are distinct (even if partially overlapping) and conserved in the retinogenesis of different vertebrate species. One single multipotent retinal precursor can generate all retinal cell types, by means of multiple cell divisions, while undergoing a progressive restriction of its differentiation potential. Ganglion cells are generated first, followed by cones and horizontal cells, amacrine cells, rods, bipolar cells, and, finally, Müller glia (reviewed by Harris, 1997; Livesey and Cepko, 2001). Understanding how multipotent retinal precursors self-renew, or restrict their potential for generating the right number of cell types at the right time and in the appropriate retinal layer, is one of the main topics in the study of eye development.

Both extrinsic cues and intrinsic factors regulate proliferation versus differentiation during retinogenesis. Among extrinsic growth regulators, Notch and Delta (Artavanis-Tsakonas et al., 1999; Baker, 2000), Shh (Jensen and Wallace, 1997), and FGF family members (McFarlane et al., 1998) support mitotic activity. This effect is achieved either by downregulating genes that favor exit from the cell cycle and/or by sustaining the expression of genes driving the cell cycle. These same factors also contribute to cell fate decisions (Ezzeddine et al., 1997; Henrique et al., 1997; Furukawa et al., 2000; Lillien and Wancio, 1998; McFarlane et al., 1998). Intrinsic factors are part of the cell cycle machinery or belong to a numerous family of determination/differentiation factors that, in turn, regulate the cell cycle ma-
chirnery in concert with the above-mentioned extrinsic cues (Ohnuma et al., 2001). As intrinsic determination/differentiation factors, bHLH transcription factors are both activators and inhibitors of neuronal differentiation, acting to balance proliferation and cell differentiation (Kageyama and Nakanishi, 1997; Cepko, 1999). Most bHLH factors are expressed in all the nervous system and control neuronal differentiation with no evident cell-type specificity. Conversely, some of them are expressed in specific subsets of neuronal precursors and affect the differentiation of definite cell types of the retina, such as Math5 in mouse (Brown et al., 2001) and Xath5 and neurogenin in Xenopus (Kanekar et al., 1997; Perron et al., 1999).

The precise relationships among control of the cell cycle machinery, extrinsic and intrinsic cues, and generation of specific retinal cell types are not yet well understood in the retina. Moreover, little is known about patterning genes that affect cell proliferation of specific vertebrate CNS structures cell-autonomously, such as genes coding for transcription factors that have an effect on those same cells in which they are expressed. The Xenopus homeobox-containing gene Xrx1 is a possible candidate for this function in the retina (Casarosa et al., 1997; Furukawa et al., 1997). Xrx1 is one of the master regulators of vertebrate eye development, together with Pax6 and Six3 (Mathers et al., 1997; Chow et al., 1999; Zhou et al., 2000). Among them, Xrx1 shows the most eye-specific pattern of expression, covering first most of the presumptive forebrain at early neurula stage and later the eye vesicle, the pineal gland, and a small ventral diencephalic region (Casarosa et al., 1997). Xrx1 expression progressively disappears from differentiating retinal cells, while it is retained in the ciliary marginal zone (CMZ), which is a source of retinal stem cells in the adulthood of fish and amphibians (Harris and Perron, 1998). Gain- and loss-of-function studies of rx in different species have shown this gene to be necessary for the formation of anterior brain structures and eye, and sufficient to induce retina and neural tube hypertrophy (Mathers et al., 1997; Andreazzoli et al., 1999; Winkler et al., 2000; Chuang and Raymond, 2001; Loosli et al., 2001). However, the mechanisms of rx action are still poorly understood. Moreover, since these studies were performed on early embryos, they do not analyze the role of rx1 in the proliferation and differentiation of retinal progenitors.

Here we report that Xrx1 overexpression is able to induce retina and neural tube hypertrophy through a territory-restricted increase in cell proliferation, which is due to a longer persistence of dividing precursor cells during development. Moreover, we show that Xrx1 promotes clonal proliferation and maintains multipotency of single retinal progenitors throughout retinogenesis, without affecting their differentiation. We thus suggest a role for Xrx1 in supporting retinal stem cells.

Results

Xrx1 promotes proliferation of retinal progenitors and delays neuronal differentiation

During retinogenesis, Xrx1 is expressed in undifferentiated progenitors and in CMZ cells, where its expression persists as retinogenesis is terminated (Mathers et al., 1997; Perron et al., 1998). Double labeling with a Xrx1 probe and an antibody to phosphohistone-H3 (which recognizes cells undergoing mitotic prophase) (Hendzel et al., 1997) shows that all M-phase cells of the CMZ express Xrx1 (Fig. 2A, arrows). Thus, Xrx1 seems to be expressed in undifferentiated, proliferating retinal precursors. To understand whether Xrx1 plays a role in the generation and/or in the support of undifferentiated retinal precursors, we performed an over-expression study making use of markers of either cell proliferation or differentiation. Previous studies showed that Xrx1 overexpression at early stages generates an expanded neural retina (Mathers et al., 1997; Andreazzoli et al., 1999). We analyzed the effect of the injection of 100 pg of Xrx1 mRNA in one dorsal animal blastomere of four-cell-stage embryos (Andreazzoli et al., 1999) on the expression of cyclin D1, a proliferation marker, and N-tubulin, a marker of neuronal differentiation, at different stages of retinogenesis. At stage 37 we can see an increase of cyclin D1 and a decrease in N-tubulin expression, in both the diencephalon and the retina of the injected side compared with the control side (Figs. 1A, 1B, 1E, 1F, 1Q, 1R). In the control side, N-tubulin marks mainly the external, differentiated region of the neural tube (Fig. 1R) and, in the eye, the ganglion cell layer (GCL) and the most central region of the inner nuclear layer (INL), where differentiation has already occurred (Fig. 1B). Conversely, in the injected side at this stage N-tubulin is down-regulated in the neural tube as well as in the retina (Figs. 1F, 1R). In the control retina, cyclinD1 expression is somewhat complementary to that of N-tubulin, marking the dividing precursors of the CMZ (Fig. 1A). In the injected retina the domain of expression of cyclinD1 is largely expanded with respect to the control, comprising not only the CMZ but also a good portion of the central retina (Fig. 1E). A similar situation is found in the anterior neural tube (Fig. 1Q). On the whole, the expression pattern of these two markers at stage 36/37 can be interpreted as an enlargement of the proliferating retina, after Xrx1 injection. In accordance with the induction of cyclin D1 expression, the domain of Xotch expression is also enlarged in the injected retina compared with the control (Figs. 1D, 1H). At this stage, Xotch is downregulated in the GCL of the control retina (Fig. 1D), which contains differentiated ganglion cells, while it is still expressed in the GCL of Xrx1-overexpressing retinas (Fig. 1H).

To better evaluate the state of commitment of cells, we then analyzed the expression of markers of either general neuronal commitment (Xdelu, not shown) or more specific retinal commitment (Xath5). We observed a small but con-
sistent increase in the expression level of both markers in the injected retina. \textit{Xath5} is expressed in a larger number of cells compared with the control (compare Fig. 1C with 1G). We also show that \textit{Xath5} is not expressed in the hypertrophic neural tube (Fig. 1S). This observation indicates that the hypertrophic tissue generated by \textit{Xrx1} overexpression in the anterior region, outside of the eye, has a neural, but not a retinal identity. Interestingly, when we analyze these markers at the end of retinogenesis, we observe that the domains of expression found in the injected side are comparable to those found in the uninjected side (Figs. 1I–1P). So, \textit{Xrx1} is able to extend the proliferation of neuronal precursors and to delay their differentiation, but does not cause a total block of differentiation.

\textbf{Regional competence of \textit{Xrx1} proliferative activity}

We wanted to further understand the relationship between increased cell proliferation and the hypertrophic neural tissue produced by \textit{Xrx1} overexpression. To this aim, we analyzed the expression of phosphohistone-H3 in the eye and in the neural tube, after unilateral injection of \textit{Xrx1} mRNA in four-cell-stage embryos. We focused our analysis on stage 42 embryos, since at this stage proliferation of neuronal precursors is almost completed in the retina, except for the CMZ (Perron et al., 1998). Our findings show that, in the injected side, the number of phosphohistone-H3 labeled cells is higher in both the eye and anterior neural tube, with respect to the corresponding structures in the control side (Figs. 2B, 2E, 2F). In addition, phosphohistone-H3-positive cells are widespread through all retinal regions (Fig. 2E), and not confined to the CMZ (Fig. 2F). On the contrary, analysis of more posterior sections of the neural tube shows no evident difference between the injected and control sides (Fig. 2C).

We carried out a quantitative analysis on serial sections of 20 injected embryos. We counted phosphohistone-H3-positive cells of both retina and neural tube and compared their numbers in the injected side with respect to the control side (Fig. 2G). An anteroposterior (A/P) subdivision of the
neural tube was taken into account for the comparison: we analyzed forebrain and hindbrain separately (Figs. 2B, 2C, 2G). The resulting figures show that Xrx1 induces a significant increase in mitotic activity both in the retina (6.1 mitoses/section in injected retina vs 4 mitoses/section in control) and in the forebrain (5 mitoses/section in injected neural tube vs 3.2 mitoses/section in control), but not in the hindbrain (1.9 vs 1.8 mitoses/section, not significant). This latter result does not depend on a differential distribution of the injected mRNA along the A/P axis, since the co-injected tracer (GFP mRNA) diffused up to the very caudal regions of the neural tube in all embryos analyzed (not shown).

As a further control of our experiments, we tested the ability of activators of the cell cycle machinery, namely cdk2 and cyclinA2, to increase the mitotic rate in the hindbrain. Overexpression of these molecules by means of mRNA injection is known to cause an enlargement of the neural tube in Xenopus embryos (Zuber et al., 1999), as they inhibit exit from the cell cycle of neural precursors. In fact, when analyzed at stage 42, sections of both forebrain and hindbrain highlighted a significant increase in mitoses in the cdk2/cyclinA2-injected side (Figs. 2D, 2G). The average number of mitoses per section increased significantly from 2.63 to 3.91 in the forebrain and from 2.40 to 3.54 in the hindbrain.

Finally, we performed control experiments to assay the ability of Xrx1 overexpression to inhibit naturally occurring programmed cell death. After Xrx1 mRNA injection in one blastomere at two cell stage, we compared cell death between injected and control side by TUNEL assay. Analysis carried out at stage 31–35 showed no decrease of apoptosis in the injected side (not shown), thus excluding a direct involvement of Xrx1 in the control of the apoptotic program during retinogenesis.

Xrx1 promotes clonal proliferation of retinal progenitors

One way to further investigate the proliferative activity of Xrx1 in retinal precursors is the analysis of cell number in clones overexpressing it. We thus compared the clone size of control retinal progenitors with that of progenitors misexpressing Xrx1 or other genes controlling cell proliferation. To this aim we cotransfected appropriate expression constructs, with a GFP plasmid as a clonal tracer, in the optic vesicle of stage 17/18 embryos, by means of lipofection (Holt et al., 1990). Previous studies described that isolated clusters of transfected cells may actually correspond to the clonal progeny originated by single retinal

cyclinA2/cdk2 injection in FB and HB, respectively, cause significant increase of mitotic activity with respect to control, as evaluated by Student’s t-test analysis (P < 0.02). Error bars indicate SEM. Cell count: inj eye, n = 609; con eye, n = 400; inj FB, n = 1011 (Xrx1), n = 180 (cyclinA2/cdk2); con FB, n = 655 (Xrx1), n = 121 (cyclinA2/cdk2); inj HB, n = 245 (Xrx1), n = 170 (cyclinA2/cdk2); con HB, n = 227 (Xrx1) n = 115 (cyclinA2/cdk2).
progenitors, under certain experimental conditions (Zuber et al., 1999) (see Experimental methods). We analyzed the transfected cells as detected by fluorescence on cryostat sections of stage 42 retinas. Figs. 3 and 4 show examples of clusters cotransfected with GFP plus the control pCS2 plasmid (Figs. 3A, 4A), pCS2Xrx1 (Figs. 3B, 4B) or the dominant-negative construct pCS2Xrx1–EnR (Figs. 3C, 4C). Each of the three pictures of Fig. 3 has been obtained by superimposing four layers corresponding to four serial sections of 12 μm each, covering an entire cluster of transfected cells. The four different pseudocolors—white, blue, green and red—correspond to the GFP fluorescence detected in the four different sections. The figure illustrates the effects of Xrx1 on clone size: its overexpression makes more than twice as many cells per cluster than the control transfection (compare B with A), while the clonal size is reduced to 30% of the control after Xrx1–EnR transfection (compare C with A). Fig. 5A reports the statistical analysis on clone size after transfection. The average clone size is 10.4 cells per clone in the control, 21.38 in Xrx1 transfected retinas, and 3.62 after Xrx1–EnR transfection. Transfection of a control plasmid containing the EnR domain alone affects neither clone size nor cell type differentiation (results not shown).

Our observations indicate that Xrx1 overexpression promotes clonal proliferation of retinal precursors, while its functional impairment produces the opposite effect. Notably, the increase in clone size due to Xrx1 lipofection is very similar to that obtained after cdk2/cyclinA2 lipofection (Figs. 4D–4F, 5A).

The decreased clone size after Xrx1–EnR lipofection could be due to an impairment of mitotic activity, to increased cell death, or to both. We previously showed Xrx1–EnR expression to induce apoptosis (Andreazzoli et al., 1999). Here we show that cdk2/cyclinA2 cotransfection cannot rescue Xrx1 functional inactivation by Xrx1–EnR (3.92 cells/clone) (Figs. 4G, 5A). Such observations are in accordance with a possible apoptotic effect of Xrx1 functional inactivation. Nonetheless, a main goal remains to investigate the proliferative potency of retinal progenitors after Xrx1 functional inactivation. To this purpose, we aimed to rescue apoptosis induced by Xrx1–EnR transfection by
means of the antiapoptotic Bcl2 gene, which is an inhibitor of specific cell death pathways in the nervous system (Chen et al., 1997). Bcl2 structural and functional conservation has been demonstrated, and the human gene was found to rescue apoptosis induced by inhibitors of transcription or translation in Xenopus embryos (Hensey and Gautier, 1997). Transfection of Bcl2 alone increases the average clone size (17.47 cells/clone, Figs. 4H, 5A) compared with control (10.4 cells/clone). Nevertheless, Bcl2/Xrx1–EnR cotransfected clones show lower size than Bcl2 transfected clones (10.91 cells/clone) (Figs. 4I, 5A), thus suggesting that Xrx1 functional inactivation decreases the mitotic activity of retinal progenitors.

**Xrx1 supports multipotency of retinal progenitors**

We addressed the question of whether Xrx1 could be involved in cell fate commitment of neural precursors. We analyzed the cell types generated after lipofection of Xrx1 or cdk2/cyclinA2. Fig. 4 shows examples of the results obtained after transfection of the different constructs assayed in the present work. The relative percentages of the different retinal cell types in control transfected clones are similar to those already reported (Fig. 6B) (Kanekar et al., 1997; Zuber et al., 1999). Transfection of Xrx1 had no effect on the relative proportions of the various cell types, compared with the control (Figs. 4A, 4B, 5B). In some experiments we have further confirmed the identity of the cells transfected with Xrx1 using specific antibodies. Fig. 6C shows that Xrx1 lipofected cells in the GCL effectively express Islet-1, a marker of ganglion cells (Dorsky et al., 1997). Analysis of R5 immunoreactivity (a marker of Müller glial cells) (Drager et al., 1984) confirms that Xrx1 does not increase Müller glia (Fig. 6D), conversely to what was observed in mouse (Furukawa et al., 2000) (see Discussion). Transfection of cdk2/cyclinA2 generated a significant decrease in ganglion cells, and cones, as shown by calbindin immunoreactivity (Fig. 6B), and an increase in bipolar cells (Figs. 4A, 4D–4F, 5B). The different phenotypes exhibited by Xrx1 and cdk2/cyclinA2 may point to different roles for these two kinds of molecules, even though both are involved in stimulating cell proliferation (see Discussion).

The ability of Xrx1 transfected cells to generate all retinal cell types in the same proportions as the control cells favors the possibility that Xrx1 activity is compatible with the maintenance of precursors in a multipotent state. To investigate this point, we analyzed the pattern of BrdU incorporation of the different cell types generated by Xrx1 transfected progenitors. BrdU was administered at stage 35–36, when all early-born cells, namely, ganglion cells, have already left the cell cycle and have started differentiation. Fig. 7A shows a section of a nontransfected retina at stage 42 after BrdU immunostaining (green), over Hoechst counterstaining (blue). In all retinas analyzed (n = 23) BrdU is consistently distributed in a gradient decreasing from the CMZ toward the central part of the retina, where labeled cells are mostly in the INL. Notably, labeled cells were never detected in the GCL. Conversely, BrdU-positive cells, mostly bipolar cells of the INL and photoreceptors, are detectable in the central retina, thus suggesting that the BrdU was incorporated by later precursors.

We then analyzed retinal cells transfected at stage 17 either with pCS2 or with pCS2Xrx1, together with a myc-tag vector suitable for immunodetection. Figs. 7B–7E show examples of results. Again, none of the control transfected retinas analyzed (n = 11) show BrdU incorporation in the GCL. Arrows in Figs. 7B and 7C point to one control-transfected cell of the GCL that did not incorporate BrdU. Some control-transfected cells belonging to the same clone are BrdU-positive (Figs. 7B, 7C) in the INL and in the photoreceptor layer, thus demonstrating that BrdU incorporation was effective. Typical examples of Xrx1 transfected cells are shown in Figs. 7D and 7E. As in the control-transfected clones, in Xrx1 transfected clones we find BrdU-positive cells in both the INL and photoreceptor layer. Nonetheless, unlike the control, most of the Xrx1 transfected retinas analyzed (n = 12) show BrdU-positive transfected cells in the GCL as well (arrows in Figs. 7D, 7E). Thus, Xrx1 over expression is likely to maintain early retinal precursors, i.e., those fated to generate also ganglion cells, in a proliferating condition beyond the due time (Fig. 8) (see Discussion).

**Discussion**

In this article we show evidence that Xrx1 supports proliferation and multipotency of retinal progenitors by cell-autonomous mechanisms and with a region-specific competence. The way Xrx1 acts on retinal progenitors is different from that of a general signal promoting cell proliferation, such as the cdk2 cyclin-dependent kinase. Thus, Xrx1 represents a new, intrinsic cue acting on the control of retinal proliferation.

**Xrx1 controls cell proliferation in retina and forebrain**

The net effect of Xrx1 overexpression in the early embryo is an overgrowth of neural tissue restricted to the territories of Xrx1 expression, retina and forebrain (Mathers et al., 1997; Andreazzoli et al., 1999; this article). Similarly, expansion of neural retina has been shown when overexpressing rx1 and rx2 in zebrafish and rx3 in medaka, although the authors propose different mechanisms for these effects (Chuang and Raymond, 2000; Loosli et al., 2001). In agreement with the results obtained in medaka, we show direct evidence that the retinal overgrowth is achieved in Xenopus by increased mitotic activity. Moreover, the fact that Xrx1 overexpression is not sufficient to induce expression of Xath5, a marker of retinal progenitors, in the ectopic neural tube confirms that the hypertrophic neural tissue induced by Xrx1 is not ectopic retina. This supports the idea
that the Xrx1 phenotype is due to a region-restricted increase in proliferation rather than to a transformation toward retinal fate of other embryonic territories. Interestingly, Xrx1 overexpression does not affect neuronal commitment since neuronal cell differentiation is recovered by stage 45, when the expression of the markers mentioned above becomes comparable to that of control retina.

Another key regulator of retinal development, the transcriptional repressor Xopt2, induces retinal overgrowth by supporting clonal proliferation of retinal progenitors. Notably, Xopt2 is turned on after Xrx1 onset of expression (Zuber et al., 1999) and Xrx1 overexpression is able to induce Xopt2 expression (M.A. et al., submitted), thus suggesting the view that Xopt2 might be downstream of Xrx1 in a regulatory cascade. We also found that Xrx1 can positively regulate both the Delta/Notch pathway and the cell cycle machinery, by delaying the arrest of expression of Xotch and cyclinD1 in the developing retina. These observations are examples of how tissue-specific genes such as Xrx1 can regionally regulate cell proliferation by controlling general extrinsic and intrinsic cues.

Two effects can account for the increased mitotic activity of neural progenitors: a shortening of the cell cycle or a lengthening of the proliferative state. Xrx1 overexpression increases phosphohistone-H3 immunoreactivity in retina and forebrain at stage 42, when normally most of the progenitors of these regions have already left the cell cycle. This observation suggests that Xrx1-overexpressing cells lengthen their proliferative condition. The same conclusion is also supported by the analysis of markers of undifferentiated cells, such as cyclinD1 and Xotch (see Results and Fig. 1).

We show by in vivo lipofection of single retinal progenitors that Xrx1 misexpression affects cell proliferation with a cell-autonomous mechanism. However, while the role of Xrx1 in supporting mitotic activity in gain-of-function experiments has been elucidated (see above), the same cannot be easily predicted in functional inactivation experiments. In fact, we previously showed that Xrx1 loss of function by means of Xrx1–EnR mRNA injection induces apoptosis at early neural plate stage (Andreazzoli et al., 1999), which does not allow analysis of the proliferative potency of Xrx1 targeted cells. To overcome this technical restriction, we aimed to keep cells alive in the absence of Xrx1 function by taking advantage of the antiapoptotic Bcl2 gene. Bcl2 lipofection increases retinal clone size. This indicates that a Bcl2-sensitive pathway of cell death contributes to regulate the clone size of progenitors, as a proportion of retinal cells normally undergoes cell death during development (Bahr, 2000; Gonzalez-Hoyuela et al., 2001). Nonetheless, clone size after Bcl2/Xrx1–EnR cotransfection is lower than clone size after transfection of Bcl2 alone. These data could be interpreted by considering that Xrx1 loss of function inhibits mitotic activity, since apoptosis by itself cannot entirely account for the smaller clone size of Xrx1–EnR clones.

Xrx1 supports proliferation without affecting cell fate commitment

Although transfection of cdk2/cyclinA2 produced an increase in clone size that is comparable to that induced by Xrx1, the role of Xrx1 does not seem to be restricted to control of the cell cycle. Indeed, cotransfection of cdk2/cyclinA2 cannot rescue the reduced clone size due to Xrx1–EnR. This would suggest that, in the absence of normal Xrx1 function, cdk2/cyclinA2 are not limiting factors in retinal cell proliferation or survival. Moreover, it also suggests that Xrx1 can influence molecular pathways different than those controlled by cdk2/cyclinA2. This hypothesis is also supported by the different effects exerted by Xrx1 and cdk2/cyclinA2 on the differentiation of retinal cell types. Unlike Xrx1 lipofection, the lipofection of cdk2/cyclinA2 generates an alteration of the normal proportions of cell types, namely, an increase in bipolar cells and a decrease in cones and ganglion cells. In principle, cdk2/cyclinA2 could either interact with other factors affecting neuronal cell fates or influence neuronal fate commitment by altering the normal timing of neuronal generation. Genes involved in the control of cell proliferation and differentiation, such as Xotch, Delta, p27Xic1, and Xath5, were reported both to affect cell differentiation and to modify the relative proportion of the cell types of the Xenopus retina (Dorsky et al., 1995, 1997; Kanekar et al., 1997; Ohnuma et al., 1999). In some instances, models have been proposed to explain the effect of these genes on cell fate as a consequence of changes in the “timing” of cell type generation (Kanekar et al., 1997; Ohnuma et al., 1999; Moore et al., 2002). Then, a possible interpretation of the results obtained after cdk2/cyclinA2 lipofection is that a prolonged proliferation of progenitors during retinogenesis depletes multipotent precursors and increases the number of neuronal precursors committed to later fates. As a result, at the time when precursors eventually exit the cell cycle they would produce more late-type neurons, such as bipolar neurons, and fewer early-type neurons, such as ganglion cells and cones.

We show that Xrx1 supports cell proliferation without affecting cell fate commitment, since its overexpression generates no alteration of the proportions of the different retinal cell types. Different results were reported after overexpression of the Xrx1 mouse homologue, rax, which increases the number of Müller glial cells in transduced clones (Furukawa et al., 2000). However, different types of retinal progenitors were targeted in the two approaches, as retinal progenitors in newborn mouse retina have a restricted cell fate commitment and generate late-born cell types, mostly rods, bipolar cells, and Müller glia (Furukawa et al., 2000). Moreover, gliogenesis was recently shown to be differentially regulated in mouse and Xenopus retinas (reviewed by Vetter and Moore, 2001), with key molecules like Notch...
and p27 playing different roles in Müller cell specification in these two species (Dorsky et al., 1995; Onhuma et al., 1999; Furukawa et al., 2000; Dyer and Cepko, 2001; Moore et al., 2002). Our results can be explained considering that Xrx1 supports multipotent progenitors. In fact, the results of BrdU incorporation after lipofection indicate that Xrx1 overexpression delays the time of exit from the cell cycle of cells fated to the GCL, namely, the multipotent retinal precursors (see Results and Fig. 8). This supports the idea that Xrx1-overexpressing progenitors generate early cell types at a late time of retinogenesis. Interestingly, Xrx1 overexpression supports transcription of both Xotch and Xath5, which are markers of proliferating and retina-committed progenitors, respectively. In particular, Xath5 expression marks an early step of neuronal differentiation common to multipotent retinal progenitors (Kanekar et al., 1997). In the Xenopus retina, Xotch lipofection maintains transfected cells as undifferentiated proliferating progenitors, while Xath5 increases the number of differentiated ganglion cells. Thus, the capability of Xrx1 to induce the expression of both genes would suggest Xrx1 sustains proliferating progenitors like those that generate ganglion cells, that is, multipotent progenitors that are competent to differentiate into all retinal neurons. In summary, we propose that Xrx1 acts as an intrinsic cue in supporting multipotency and mitotic activity of embryonic retinal progenitors.

Potential role of Xrx1 in retinal stem cells

We show that Xrx1 is also expressed in the dividing cells of the CMZ, as other genes involved in the control of cell proliferation (Perron et al., 1998). This observation suggests that Xrx1 could also support proliferation of multipotent stem cells of CMZ. The precise contribution of Xrx1 to the capability of the stem cells of the CMZ to self-renew and maintain their potential to generate all retinal cell types remains to be established.

We speculate that Xrx1 could exert in the stem cells of the adult CMZ the same effects observed in embryonic progenitors. In a possible model, the effect of the intrinsic

![Fig. 5. Statistical analysis of retinal progenitors proliferation and differentiation after lipofection. Statistics on the results shown in Fig. 4 and 5. In (A), the Y-axis reports the average clone size after lipofection with different combinations of vectors (X-axis). Error bars represent SE. Clone count: pCS2, n = 62; Xrx1, n = 24; Xrx1-EnR, n = 84; Bcl2, n = 15; Bcl2 + Xrx1-EnR, n = 44; cyclinA2/cdk2, n = 9; cyclinA2/cdk2 + Xrx1-EnR, n = 33. Student’s t test was performed to evaluate clone size increase or decrease compared with control (pCS2). Clone size was significantly increased by Xrx1, Bcl2, and cyclinA2/cdk2 (P < 0.001, P < 0.02, and P < 0.02, respectively) and decreased by Xrx1-EnR and cyclinA2/cdk2 + Xrx1-EnR (P < 0.001). (B) Percentage of specific retinal cell types, after lipofection of control (n = 1245 cells), Xrx1 (n = 1101 cells), and cyclinA2/cdk2 (n = 1743 cells) vectors. Error bars represent SEM. The number of cells lipofected with Xrx1-EnR was not sufficient for a statistical analysis of the cell types. cyclinA2/cdk2 lipofection significantly increased the number of bipolar cells (P < 0.02) and decreased the number of ganglion cells and photoreceptors (P < 0.02) with respect to control, as evaluated by Student’s t test.

![Fig. 6. Analysis of cell type-specific markers in lipofected retinas. (A, B) calbindin staining (red) on control (A) and cyclinA2/cdk2 (B) lipofected cones (green, GFP reporter). Arrows point at calbindin-negative rods; arrowheads indicate calbindin-positive cones where double staining with GFP results in yellow color. cyclinA2/cdk2 lipofection reduces the percentage of cones compared with control. Cones account for 57% of control (190, SE = 4.9), not shown), and 27% of Xrx1 lipofected photoreceptors (n = 102, SE = 8). The decrease in cones in cyclinA2/cdk2 lipofected retinas is reflected in a decrease in the total number of photoreceptors (see B). (C) Islet-1 staining (red) in the GCL of Xrx1 lipofected retinas (green, GFP reporter). All Xrx1 transfected cells (arrowheads) show yellow double staining. (D) R5 staining (red) of Xrx1 lipofected cells (green, GFP reporter). R5 labels Müller glia processes; note that none of the GFP-positive Xrx1 transfected cells show yellow double staining. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.]
Xrx1 signal would integrate with the external cues (see Introduction), which would push cells to exit cell cycle and choose the proper differentiation type in the mature central retina, or keep them proliferating in the CMZ.

**Experimental methods**

**Embryos and histology**

Induction of ovulation of females, in vitro fertilization, and embryo culture were carried out as described by Newport and Kirschner (1982). Staging was according to Nieuwkoop and Faber (1994). For both in situ hybridization and immunohistochemistry, embryos were fixed in MEMFA at room temperature (Harland, 1991), cryoprotected with 30% sucrose in PBS O/N at 4°C, and stored at −70°C until cryosectioning.

*In situ hybridization, immunohistochemistry, and BrdU staining*

Digoxigenin (DIG)-labeled antisense RNA probes were generated for Xrx1 (Casarosa et al., 1997), Cyclin D1 (provided by Matt Cockerill, Xenopus Molecular Marker Resource), N-tubulin (Richter et al., 1988), Xotch (Dorsky et al., 1995), X-Delta-1 (Chitnis et al., 1995), and Xath5 (Kanekar et al., 1997) as described in Harland (1991). In situ hybridization on 12-μm cryosections was carried out as described in Kanekar et al. (1997). Signal detection with either NBT/BCIP or Fast Red substrate (Roche) was performed according to the manufacturer’s instructions.

Immunostaining was performed on 12-μm cryosections using the following primary and secondary antibodies. Primary antibodies: anti-phosphohistone-H3 (1:200, Upstate Biotechnology), anti-myc (9E10, 1:500, Sigma), anti-Islet-1 (1:100, Developmental Studies Hybridoma Bank), anti-calbindin (1:500, Oncogene), and anti-R5 (1:1) (Drager et al., 1984). Secondary antibodies: TRITC-conjugated donkey anti-rabbit IgG (1:400, Sigma), TRITC-conjugated goat anti-mouse IgG (1:100, Sigma).

BrdU in vivo administration, labeling, and detection were carried out as described in Zuber et al. (1999) with minor modifications. Detection of the BrdU antibody was performed as described in Harland (1991).
performed by using FITC-conjugated sheep anti-mouse IgG F(ab\(^{-}\))\(_{2}\) (1:100, Sigma) followed by blocking with goat anti-mouse IgG F(ab\(^{-}\))\(_{2}\) fragment (1:100, Sigma). Myc tag detection was then carried out as described above. In all immunohistochemistry experiments, Hoechst 33258 (Sigma, final concentration 0.12 \(\mu\)g/ml) was added to secondary antibody for counterstaining of cell nuclei.

**Plasmids and embryo mRNA microinjection**

All cDNAs used for either RNA in vitro transcription or in vivo DNA lipofection were subcloned in the pCS2 vector. The full-length cDNA sequence of human Bcl2 was provided by J.C. Martinou. The pCS2MT vector, carrying a myc tag, is a kind gift from M. Zuber. Capped synthetic RNAs were generated in vitro by SP6 transcription from pCS2Xcdk2 and pCS2mtXcdk2 (provided by T. Hunt; Paris et al., 1991; Howe et al., 1995) and from pCS2GFP (provided by M. Zuber). The capability of the dominant-negative \(\text{X}_{\text{rx1}}–\text{EnR}\) construct to antagonize endogenous \(\text{X}_{\text{rx1}}\) function has been described (Andreazzoli et al., 1999). The following amounts of mRNAs were injected unilaterally at the four-cell stage: pCS2Xrl, 100 pg; pCS2mtXcyclinA2 and pCS2mtXcdk2, 1 ng. Five hundred picograms of pCS2GFP was always coinjected as a tracer. The injected embryos were collected under an epifluorescence microscope following detection of the GFP reporter activity.

**In vivo DNA lipofection**

DNA was lipofected into the anterior neural region of stage 17 embryos as previously described (Holt et al., 1990; Dorsky et al., 1995). DNA was lipofected at a final concentration of 0.25 mg/ml with a 1:3 (w/w) DNA:DOTAP (Roche) ratio. pCS2GFP or pCS2MT reporter vectors were cotransfected with a 1:3 (w/w) ratio with respect to the construct(s) of interest, to mark the transfected cells. Either GFP-positive or myc-tagged cells were counted and the cell types were identified based on their laminar position and morphology, as described in Dorsky et al. (1995, 1997).

For the clone size analysis, the amount of lipofected DNA was reduced to 0.05 mg/ml to obtain isolated clones of transfected cells. At limiting DNA dilution, most retinas contained no transfected cells or one single cluster, with an average number of cells that did not vary when the percentage of transfected retinas became very low, thus suggesting targeting of clonal progeny. When DNA was lipofected at such low doses, each transfected cluster appeared compact and oriented in a vertical column, as it is observed in clonal analysis performed by using retrovirial vectors, or direct injection of enzymatic/fluorescent tracers (Price et al., 1987; Turner and Cepko, 1987; Wets and Fraser, 1988). When analyzing clone size, we considered batches of injected embryos with one or less than one cluster of transfected cells per retina. When more than a cluster was detected in the same retina, we scored them as different clones only when they were well separated. As an additional criterion to identify single clones, we considered the spatial distribution of cells through serial sections. A clonal progeny usually covered few serial sections of 12 \(\mu\)m. Serial collections lacking even a single section were not considered in this analysis.

**Cell counts**

For the analysis of mitosis in mRNA-injected embryos, we counted phosphohistone-H3 positive cells in either the control or injected side, as detected by the analysis of GFP reporter activity. The analysis was carried out on 12-\(\mu\)m serial serial cross sections, by scoring the number of cells per section in either retina or neural tube. Three or more sets of experiments were carried out for each type of mRNA injected. Weighted averages of the number of cells per section for both injected and control sides were then compared as shown in Fig. 2.

Lipofected cells were considered to have a clonal origin as described here and in Zuber et al. (1999). According to previous studies (Dorsky et al., 1995, 1997; Kanekar et al., 1997; Ohnuma et al., 1999; Zuber et al., 1999), we scored the different cell types on the basis of their morphology and laminar position. We sometimes confirmed cell identity with specific antibodies such as: Islet-1 for ganglion cells (Dorsky et al., 1997), R5 for Müller glia (Drager et al., 1984), calbindin for cones (Chang and Harris, 1998). Cell type percentages were calculated as weighted averages, after analysis of three or more independent sets of experiments (Kanekar et al., 1997; Zuber et al., 1999).

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