Introduction

Mammalian eye organogenesis is a multistep process of complex morphological events that involves interaction of the forebrain-derived optic vesicle (OV) with lens-competent head surface ectoderm (SE). If sufficient, this interaction leads to the coordinated invagination of both OV and SE resulting in formation of the optic cup (OC) [1]. The inner layer of OC is populated by retinal progenitor cells that further differentiate into seven retinal cell types: ganglion cells, amacrine cells, bipolar cells, horizontal cells, cone and rod photoreceptors and Muller glia cells. In previous three decades, several genes have been identified to play a crucial role in this process using the classical knockout strategies [2,3,4]. However, in some cases inactivation of such genes can lead to very early arrest of the eye development or even to the embryonic lethality, as can be demonstrated for the Pax6 gene [5,6]. This fact makes the dissection of cell autonomous function of a gene in early retinal progenitors of the OV and OC complicated. Nevertheless, the introduction of Cre-loxP-mediated tissue-specific gene inactivation can bypass embryonic lethality and allow functional study of the developmentally essential genes [7]. To perform conditional gene inactivation in retinal progenitors, utilization of a few Cre recombinase-expressing mouse lines has been reported. However, these lines display certain limitations and do not always offer sufficient strength at early stages [8,9,10,11] or the required specificity [12,13].

The homeodomain transcription factor Rx gene is one of the earliest genes expressed in the retinal lineage. It has been shown to be activated between embryonic day (E) 7.5 and E8.0 in the anterior neural plate and later strongly expressed in the optic vesicles and ventral forebrain [14,15]. Rx function is essential for vertebrate eye development. Loss of the Rx function results in loss of eyes in various vertebrate species [14,15,16,17,18,19,20], suggesting its conserved role in the eye development. The early onset of Rx expression in the retinal primordium suggests that the Rx locus could be utilized for driving the Cre expression at very early stages of retinal development.

One such Cre-expressing line, taking advantage of Rx expression, has been generated previously [12]. Based on the similarity in the Rx expression pattern in Japanese killifish medaka and mouse [20], a 4-kb DNA fragment upstream of the medaka Rx3 gene has been identified to contain an evolutionarily conserved region that can direct the Cre expression in the mouse retina [12]. However, when we reinvestigated this line we found that beside its activity in optic vesicles, it has a much broader scope of activity and this certain nonspecificity may complicate its use. Here, we have taken advantage of the Rx expression and have generated two transgenic mouse lines, Ab31-Cre and mRx-Cre, and compared their recombination potential with that of Rx-Cre. Our data demonstrate that among the three analyzed lines, mRx-Cre represents an ideal tool for gene manipulation in early retinal progenitors as judged by its specificity, strength and early onset.

Abstract

During mouse eye development, all retinal cell types are generated from the population of retina-committed progenitors originating from the neuroepithelium of the optic vesicle. Conditional gene inactivation provides an efficient tool for studying the genetic basis of the developing retina; however, the number of retina-specific Cre lines is limited. Here we report generation of the mRx-Cre BAC transgenic mouse line in which the expression of Cre recombinase is controlled by regulatory sequences of the mouse Rx gene, one of the earliest determinants of retinal development. When mRx-Cre transgenic mice were crossbred with the ROSA26R or ROSA26R-EYFP reporter lines, the Cre activity was observed in the optic sulcus from embryonic day 8.5 onwards and later in all progenitors residing in the neuroepithelium of the optic cup. Our results suggest that mRx-Cre provides a unique tool for functional genetic studies in very early stages of retinal development. Moreover, since eye organogenesis is dependent on the inductive signals between the optic vesicle and head surface ectoderm, the inductive ability of the optic vesicle can be analyzed using mRx-Cre transgenic mice.

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Generation of mRx-Cre Transgenic Mouse Line for Efficient Conditional Gene Deletion in Early Retinal Progenitors

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Results and Discussion

In order to perform gene inactivation specifically in retinal progenitors of the optic vesicle we first reinvestigated the previously generated Rx-Cre [12]. The Rx-Cre transgene is schematically depicted in Figure 1A. To define the kinetics and pattern of the Cre recombinase activity driven by Rx-Cre, transgenic mice were bred with the ROSA26R reporter mouse strain to generate Rx-Cre; ROSA26R double transgenic animals. These mice enabled detection of the Cre activity and lineage tracing of Cre-expressing cells using X-gal staining. Upon Cre recombination, the expression of LacZ under the ROSA promoter is activated by the removal of a stop cassette [21]. The Rx-Cre; ROSA26R embryos reproducibly showed a broad area of recombination (Figure 2A,D). Already at E9.0 X-gal staining was observed both in OV and in the overall head region, also targeting head surface ectoderm (Figure 2A,A’). By E10.5 expression was detected in the neuroepithelium of the optic cup and also in the invaginating structure of the lens pit (Figure 2D’). At the early stages of vertebrate eye development, some genes are expressed in both SE and OV that interact and induce their mutual development. To study such genes it is therefore essential to perform gene inactivation in both tissues separately in order to dissect their cell autonomous functions. For this reason, Cre activity in the ectodermal compartment can be undesirable. We thus analyzed recombination in ectoderm-derived structures in more detail using the ROSA26R-EYFP reporter line [22]. The Cre-mediated recombination in ROSA26R-EYFP results in the expression of fluorescent protein EYFP which enables a better signal resolution at the single cell level than ROSA26R. The analysis of Rx-Cre; ROSA26R-EYFP embryos confirmed Cre activity in SE and invaginating lens vesicle (Figure 3A,A’). Expression of EYFP also offered the opportunity to determine the degree of mosaic recombination based on the proportion of EYFP+ and DAPI+ retinal progenitor cells. The pattern of EYFP expression revealed that at E10.5 a considerable amount of cells residing in the retina escaped to Rx-Cre-mediated deletion (Figure 3A’).

To establish a genetic tool for directing gene inactivation selectively to the OV compartment, we decided to generate a new Cre-expressing line. We employed the same strategy as Swindell et al [12], having in mind that a nonspecific expression pattern of the short transgene Rx-Cre may depend on its integration site into the genome. The 4-kb DNA fragment upstream of the medaka Rx3 gene was used to drive the expression of Cre and generate MB31-Cre (Figure 1B). The coding sequence for fluorescent protein EGFP was linked to Cre via the IRES sequence for monitoring the recombinease expression. Pronuclear injection yielded nine transgenic founders with three animals exhibiting Cre activity specifically localized in the retina (not shown). One founder was chosen for further analysis using ROSA26R and ROSA26R-EYFP reporter lines. We were able to detect Cre activity in MB31-Cre; ROSA26R embryos as early as at E9.0, specifically in the OV compartment (Figure 2B,B’). At E10.5, as the optic vesicle had invaginated to form the optic cup, the X-gal+ progeny of Cre-expressing cells contributed to cells in the retina and retinal pigmented epithelium (Figure 2E,E’). In all analyzed embryos, no Cre activity was observed in ectodermal derivatives such as SE or lens pit or in other parts of the embryo (Figure 2B,E). This remarkable specificity was also demonstrated using MB31-Cre; ROSA26R-EYFP transgenic embryos, where EYFP was specifically localized in the retinal tissue (Figure 3B) and no EYFP+ cells were observed in the invaginating lens pit at E10.5 (Figure 3B’). These results indicate that MB31-Cre directed the recombinease activity specifically to the developing retina. However, it should be noted that at E10.5, in both MB31-Cre; ROSA26R and MB31-Cre; ROSA26R-EYFP embryos, Cre-mediated deletion did not target the whole progenitor cell population since not all retinal cells were X-gal+ or EYFP+, respectively (Figure 2E’ and Figure 3B’). Although the 4-kb DNA fragment upstream of the medaka Rx3 gene directed the MB31-Cre transgene expression specifically to the retina, it did not reconstitute the expression with regard to the onset and strength observed in the mouse Rx gene expression [14]. This may be attributed to the differences in the regulation of Rx expression in medaka and mice or to the insufficiency of the 4-kb fragment to cover all the regulation of Rx. It is possible that additional cis-regulatory elements are required for proper spatiotemporal expression of the Rx gene in mice. To circumvent these problems we decided to generate another Cre-expressing line, referred to as mRx-Cre, in which the Cre expression is driven by mouse Rx gene regulatory sequences.

To generate mRx-Cre transgenic mouse we selected a BAC clone (RP24-42D21) containing the entire mouse Rx gene as well as 95 kb upstream of the Rx translational start site and 100 kb downstream of the locus. We employed the method of BAC recombineering [23] to insert the Cre recombinase coding sequence into the first ATG of Rx gene (Figure 1C). As the 200-kb BAC clone is supposed to carry all the cis-regulatory sequences ensuring proper spatiotemporal expression, the expression pattern of Cre recombinase should imitate that of the endogenous Rx gene. The Cre-inserted BAC was used for pronuclear injection to generate mRx-Cre transgenic mice. We obtained three founders showing expression in the developing retina (not shown). One of them was chosen for further analysis using the ROSA26R reporter line. As already mentioned, endogenous Rx expression starts...
between E7.5 and E8.0 [14]. In agreement, we observed the mRx-Cre activity already from E8.5 in the optic sulcus, albeit with a frequent appearance of mosaic recombination (Figure 4A) of mRx-Cre; ROSA26R embryos. This slight delay can be explained by a short intermission between the Cre expression onset and recombination seen in ROSA26R. Consistent with the expression pattern of the endogenous Rx gene [14], strong X-gal staining was observed in the optic vesicles of E9.0 mRx-Cre; ROSA26R embryos (Figure 2C). Strikingly, at E10.5, the X-gal + progeny of Cre-expressing cells contributed to all cells of the retina and the majority of retinal pigmented epithelium cells (Figure 2F'). Therefore, mRx-Cre directed Cre activity in all optic vesicle derivates, mainly to the forming retina. This almost absolute recombination rate was further confirmed in mRx-Cre; ROSA26R-EYFP eyes at E10.5 showing that virtually all retinal progenitor cells were EYFP + (Figure 3C'). The observation that mRx-Cre targets all retinal progenitors in the early stages of eye development was further documented by uniform X-gal staining of all cellular layers of the mRx-Cre; ROSA26R adult retina (Figure 4E,E'). Importantly, no EYFP + cells were observed in the invaginating lens pit of mRx-Cre; ROSA26R-EYFP embryos (Figure 3C'). In addition, no cells showing recombination were observed in other parts of the embryo than eye, ventral forebrain and hypothalamus. Beside strong β-galactosidase activity in OV-derived structures, a strong although mosaic activity was found in the ventral part of forebrain and in prospective hypothalamus (Figure 4B–D'). Although strong X-gal staining was observed in E15.5 hypothalamus and forebrain after whole-mount staining.

**Figure 2. In vivo activity of Rx-Cre, MB31-Cre and mRx-Cre transgene products assessed using the ROSA26R line.** Whole-mounts (A–F) or coronal sections (A’–F’) were stained with X-gal at indicated stages to show the Cre activity in the eye primordium (white arrowheads). Surface ectoderm and developing lens are indicated with dashed lines. SE – surface ectoderm; OV – optic vesicle; RPE – retinal pigmented epithelium; RE – retina; vf – ventral forebrain.

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Sectioning of E15.5 and adult brains revealed that the mRx-Cre activity in the forebrain/cortex is strongly mosaic (Figure 4D',F). In contrast, the rate of recombination in the hypothalamus appeared very high and we propose that this driver line may be used for genetic studies in the hypothalamus.

In conclusion, we have generated two Cre-expressing transgenic mice based on Rx regulatory sequences and provided their comparison with previously reported and widely used Rx-Cre [12]. In our hands, mRx-Cre appears the optimal Cre-driver for retinal progenitors, exhibiting very early onset, strength, specificity, and very low degree of mosaicism. Furthermore, mRx-Cre provides a useful tool for the studies of molecular mechanisms facilitating the interaction between OV and SE since it shows selective activity in OV only.

Materials and Methods

Ethics Statement

Housing of animals and in vivo experiments were performed after approval by the Animal Care Committee of the Institute of Molecular Genetics (study ID #174/2010) and in compliance with national and institutional guidelines (ID #12135/2010-17210).

Mouse Lines

mRx-Cre. A 200-kb Bacterial Artificial Chromosome (BAC) (RP24-82D21) harboring all coding exons, 5' and 3' region of the mouse Rx gene was purchased from Children's Hospital Oakland Research Institute. To generate mRx-Cre BAC, the open reading frame of Cre recombinase was inserted into the exon 1 containing the translation initiation codon of Rx using a method of BAC recombineering [23]; [http://web.ncifcrf.gov/research/brb/protocol.aspx]. The recombineering construct pCS-Cre-FRT-neo-FRT was generated by replacing the DsRed coding sequence in pCS2+DsRed+FRTKanFRT (provided by James D. Lauderdale [24]) by the Cre coding sequence. The Cre-FRT-kan-FRT targeting cassette was PCR-amplified from pCS-Cre-FRT-neo-FRT using Rx forward and reverse targeting primers: mRxCreF: 5'-AGGGAACCGGGCATCGAGCTCCAGTTGCAAAAGTGCACTCCCTCCTCACCATGTCCAATTTACTGACCGTACA-3'; mRxCreR: 5'-CTTGGTAAAGCCCAGGATGGCTTCGATGCTGTGCAAACGCGACGTCTCTATTCCAGAAGTAGTGAGGAG-3'. The PCR product was purified using Qiagen Gel extraction Kit and treated with DpnI to dispose of the template plasmid backbone. The PCR product was then electroporated into RP24-82D21 BAC-carrying bacterial strain EL250, and double resistant colonies (Cmr, Kanr) were tested for homologous recombination by PCR (primers: F: 5'-AGCACCAAAGCTCCAGTTACC-3'; R: 5'-CGTTGCATCGAGCAGCTTCGAGCCAAAGGATGGTCATGCTGTGCAAACGCGACGTCTCTATTCCAGAAGTAGTGAGGAG-3'). The kanamycin resistance cassette was further removed by induction of flipase activity in EL250 cells and the colonies were tested for kanamycin sensitivity. Modified mRx-Cre BAC was isolated, treated with NotI and applied to a Sepharose 4B-CL column to remove the BAC backbone according to the protocol [http://www.med.umich.edu/tamc/BACcol.html]. Fractions were collected and insert integrity was analyzed using pulsed field gel electrophoresis. DNA was used for pronuclear injection. Injection gave us three founders one of which was chosen for further analysis. Mice were genotyped using

Figure 3. Activity of Rx-Cre, MB31-Cre and mRx-Cre in the eye primordium analyzed using the ROSA26R-EYFP reporter line. (A–C) Whole-mounts showing EYFP expression (green) in the overall embryo at E10.5. (A’–C’) Coronal sections through the eye region co-stained with DAPI (blue) showing Cre activity in the retina, retinal pigmented epithelium and invaginating lens pit (dashed line) at E10.5. doi:10.1371/journal.pone.0063029.g003
primers that recognize the recombination junction, with forward primer located upstream of the \(Rx\) translation start site (F: 5\'-AGCACCAAAGCTCCAGTTACC-3\') and reverse primer located in \(Cre\) recombinase (R: 5\'-CGTTGCATCGACCGGTAATGCA-3\'). For analysis of \(Cre\) activity, the eighth generation from the original founder was used for the generation of presented embryos. For stages E8.5–E10.5, twenty embryos from three to five independent litters were used. For stages E12–E15.5, eight embryos from three litters were used. For adult stages (6 weeks), tissues from three independent animals were used. All embryos reproducibly exhibited the same expression pattern with highly comparable strength. Presented expression pattern was stable from F1 generation.

\textbf{MB31-Cre.} The coding region of \(Cre\) recombinase was cloned into pIRES2-EGFP (Clontech) and the \(Cre\)-IRE-EGFP cassette was fused to the medaka \(Rx\) gene promoter (provided by Jochen Wittbrodt). DNA was used for pronuclear injection and nine founders were analyzed for activity using \(ROSA26R\) mice. The least mosaic line, designated \(MB31\), was characterized further. For analysis of \(Cre\) activity, the fifteenth generation from the original founder was used for the generation of presented embryos. For stages E9.5 and E10.5, twenty embryos from three to five independent litters were used. All embryos reproducibly exhibited the same expression pattern with highly comparable strength.

The \(Rx-Cre\) mice were described previously and were provided by Milan Jamrich [12]. For analysis of \(Cre\) activity, fifteen embryos from three litters were used. All embryos exhibited comparable expression pattern. Both \(Rx-Cre\) and \(MB31-Cre\) were genotyped using primers: F: 5\'-CATTTGTGAAGTGCTTGAAGGAAT-3\'; R: 5\'-AGAGGAAGGCAGCAGCTGATGAAA-3\'.

Generation of \(ROSA26R\) (stock no. 003309) and \(ROSA26R-EYFP\), both purchased from Jackson laboratory, was described previously [21,22]. The genotype was determined by PCR analysis of genomic DNA obtained from tail biopsies.

|   | E8.5 | E9.0 | E12 | E15.5 | adult (6 weeks) |
|---|------|------|-----|-------|----------------|
| A | ![image](image1.png) | ![image](image2.png) | ![image](image3.png) |   |   |
| B | ![image](image4.png) | ![image](image5.png) | ![image](image6.png) |   |   |
| C | ![image](image7.png) | ![image](image8.png) | ![image](image9.png) |   |   |
| D | ![image](image10.png) | ![image](image11.png) | ![image](image12.png) |   |   |
| E | ![image](image13.png) | ![image](image14.png) | ![image](image15.png) |   |   |
| F | ![image](image16.png) | ![image](image17.png) | ![image](image18.png) |   |   |

**Figure 4.** The \(mRx-Cre\) activity in the eye, forebrain and hypothalamus analyzed using the \(ROSA26R\) reporter line. Whole-mounts or sections were stained with X-gal at indicated stages to show the \(mRx-Cre\)-mediated \(Cre\) activity. (A) The X-gal\(^*\) cells were first observed in the optic sulcus of E8.5 embryo. (B–D) The \(Cre\) activity in developing brain. Whole-mounts (C, D, D'), coronal sections (C', D') and transversal section (C) showing \(Cre\) activity in embryonic brain. (F) Coronal section of adult brain showing \(Cre\) activity in the hypothalamus and cortex. (E–E') Sections through the adult eye showing strong uniform \(Cre\) activity in all layers of the retina. OS-optic sulcus; F-forebrain; GE-ganglionic eminences; H-hypothalamus; OB-olfactory bulbs, C-cortex; RPE-retinal pigmented epithelium; ONL-outer nuclear layer; INL-inner nuclear layer; GCL-ganglion cell layer.

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Tissue Collections and Histology

Mouse embryos were harvested at several developmental stages from timed pregnant females. The morning of vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos were fixed in 4% paraformaldehyde (w/v) on ice for time depending on embryonic stage (from 20 minutes up to 2 hours). Embryos were washed several times with cold PBS, cryopreserved by overnight incubation in 30% sucrose (w/w), frozen in OCT (Tissue Tek, Sakura Finetek) and sectioned.

X-Gal Staining

For β-galactosidase assay, embryos were fixed on ice in 0.4% formaldehyde (w/v) in PBS, washed 3×20 minutes with the rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM MgCl₂, 20 mM Tris pH 7.3, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) and incubated in X-Gal staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg/ml X-gal) overnight at room temperature. For sectioning, embryos were fixed in 4% paraformaldehyde (w/v), washed with PBS, cryopreserved by overnight incubation in 30% sucrose (w/w), frozen in OCT (Tissue Tek, Sakura Finetek) and sectioned.

Immunohistochemistry

For observation of EYFP expression, the cryosections were permeabilized with PBT (PBS with 0.1% Tween-20) for 15 minutes, washed 3×10 min with PBT, stained 10 min with DAPI (1 μg/ml) in PBT and mounted into Mowiol (Sigma).

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Author Contributions

Conceived and designed the experiments: ZK LK. Performed the experiments: OM JL LK. Analyzed the data: LK OM ZK. Contributed reagents/materials/analysis tools: ZK RS. Wrote the paper: LK OM ZK.

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