Pax2 Expression and Retinal Morphogenesis in the Normal and Krd Mouse

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The Kidney and retinal defects (Krd) mouse carries a 7-cM transgene-induced deletion on chromosome 19 that includes the Pax2 locus. Adult mice heterozygous for the Krd deletion (Krd/+) are haploid for Pax2 and have a variable, semidominant phenotype characterized by structural defects of the kidney, retina, and optic disc. Renal and ocular anomalies present in heterozygous Pax2 mutants in both mice and humans support the hypothesis that haploinsufficiency of Pax2 underlies the Krd phenotype. To understand the embryonic basis of ocular defects observed in adult Krd/−/− mice, we used immunohistochemistry, digital three-dimensional reconstructions, and quantitative morphometry to examine Pax2 protein distribution and ocular development in normal and Krd/−/− embryos from E10.5 to P2. In Krd/−/− embryos, Pax2 immunopositive (Pax2+) cells demarcate the embryonic fissure as it forms in the ventral optic cup and optic stalk. After closure of the embryonic fissure, Pax2 immunostaining disappears from the ventral retina, but persists in a cuff of cells encircling the developing optic disc, the site where ganglion cell axons exit the retina. In Krd/−/− embryos, Pax2+ cells in the posterior optic cup and the optic stalk undergo abnormal morphogenetic movements and the embryonic fissure fails to form normally. This results in an abnormal organization of the Pax2+ cells and ganglion cell axons at the nascent optic disc. The abnormal morphogenetic movements of the Pax2+ cells in the embryonic retina and optic stalk and the initial misrouting of the ganglion cell axons give rise to retinal and optic disc defects observed in the adult Krd/−/− mice. Key Words: development; embryo; retina; optic cup; optic disc; optic stalk; embryonic fissure; gene expression; immunohistochemistry; reconstruction.

INTRODUCTION

Morphogenetic movements of embryonic epithelial layers establish the basic architecture of many tissues. This is true for the eye, where the neuroepithelium undergoes a series of complex movements as it transforms from a simple balloon-like optic vesicle into a bilayered optic cup. In mammals, the ventral epithelium of the optic vesicle and optic stalk invaginates to form the embryonic fissure (Lopashov and Stroeva, 1964; Mann, 1964). The embryonic fissure can be subdivided into two contiguous parts: the retinal fissure, which forms a transient cleft within the ventral optic cup, and the optic groove, which extends proximally toward the diencephalon as an invagination of the ventral optic stalk epithelium (Lopashov and Stroeva, 1964; Silver and Robb, 1979; Silver and Sapiro, 1981). As development progresses, the two sides of the retinal fissure make contact and fuse, leaving no trace in the adult retina (Mann, 1964; Silver and Robb, 1979; Hero, 1990). At the back of the optic cup, there is a transition between the retinal fissure and optic groove. Here, cells of the ventral neuroepithelium invaginate and encircle the nascent optic disc, the site where ganglion cell axons will exit the optic cup to form the optic nerve (Silver and Robb, 1979; Silver and Sapiro, 1981; Brown and Tasman, 1983). Abnormal formation and/or closure of the embryonic fissure is believed to result in an array of congenital retinal and optic disc anomalies collectively referred to as colobomas (Mann, 1957, 1964; Lopashov and Stroeva, 1964; Kindler, 1970; Brown and Tasman, 1983; Apple, 1984; Silver et al., 1984; Schimmenti et al., 1995).

The growing array of murine mutations affecting ocular development has begun to provide new insight into the genetic and molecular basis of congenital defects of the eye and the retina (reviewed by Beebe, 1994; Reh and Cagan, 1994; Graw, 1996; MacDonald and Wilson, 1996). One example is Kidney and retinal defects (Krd) mice which carry a 7-cM transgene-induced deletion on chromosome 19 (Kel-
Pax2 is a member of the Pax gene family of transcription factors that are characterized by the presence of a highly conserved DNA binding domain, the paired box (Stuart et al., 1993; Strachan and Read, 1994; Stuart and Gruss, 1995, 1996). Several Pax genes share an uncommon genetic trait, haploinsufficiency, wherein the presence of one wild-type allele cannot compensate for a second mutated or deleted allele (Gruss and Walther, 1992; Stuart et al., 1993; Stuart and Gruss, 1995). Krd/+ mice, which are heterozygous for the Krd deletion, are haploid for Pax2.

Based on concordance of the known expression of Pax2 in the developing kidney, retina, and optic stalk (Nornes et al., 1990; Puschel et al., 1991; Stoykova and Gruss, 1994; Torres et al., 1995, 1996) with the sites of congenital anomalies in Krd/+ mice, haploinsufficiency of Pax2 was proposed as the molecular genetic basis for the Krd phenotype (Keller et al., 1994). This proposal is consistent with reports of severe renal and retinal defects resulting from both heterozygosity and homozygosity for induced and spontaneous Pax2 mutations in mice (Torres et al., 1995, 1996; Favor et al., 1996). Likewise, congenital renal-cataract syndrome in several human families has been shown to result from haploinsufficiency for Pax2 mutations (Sanyanusin et al., 1995a,b; Schimenti et al., 1995, 1997).

Based on the hypothesis that ocular abnormalities observed in adult Krd/+ mice arise from haploinsufficiency of Pax2 during early embryogenesis, we characterized Pax2 expression and ocular morphogenesis in normal and Krd/+ embryos beginning with the period of embryonic fissure formation. We confirm and extend the reported expression of Pax2 based on in situ hybridization (Nornes et al., 1990; Torres et al., 1996) and immunohistochemistry (Puschel et al., 1992). Within the optic cup, Pax2+ cells form the lips of the retinal fissure as they come together and fuse in the ventral optic cup. Pax2 cells of the ventral optic stalk invaginate to form the optic groove. After closure of the retinal fissure, Pax2 immunoreactivity is lost from the ventral retina; however, cells that encircle the nascent optic disc continue to express Pax2+ into late embryogenesis. In Krd/+ embryos, although there is apparently normal formation and closure of the retinal fissure, abnormal morphogenetic movements of Pax2+ cells within the posterior retina and ventral optic stalk lead to a failure of optic groove formation and malformation of the optic disc and optic stalk. This is seen as a spatial disorganization of Pax2+ cells that normally encircle the developing optic disc and is associated with anomalies in the organization and trajectory of ganglion cell axons at the disc and subsequent laminar defects within the adjacent retina. These results indicate a requirement for full diploid expression of Pax2 for normal morphogenesis of a portion of the embryonic fissure, the optic groove, and provide insight into the origins of the retinal and optic disc defects observed in Krd/+ mice and other Pax2 heterozygotes.

**MATERIALS AND METHODS**

**Animals**

Wild-type and Krd/+ embryos were collected from timed matings of C57BL/6J females with Krd/+ males. Twenty-two litters of embryos (7–10 embryos per litter) were collected at ages between E9.5 and P2. Three litters were analyzed at E11.5 and E16.5, and 4 were analyzed at E10.5 and E12.5; one litter was examined for each other time point. Embryos were dissected from the uterus in phosphate-buffered saline (PBS), and their predicted developmental age was verified using crown to rump length, heart development, limb bud morphology, and extent of ocular melanization (Kaufman, 1992). Heads were removed and placed directly into fixative (see below). For embryos older than E15, the head ectoderm and skull overlying the brain were removed prior to fixation, and heads were hemisected along the midline prior to further processing (see below). The posterior portion of each embryo was frozen at −80°C for subsequent DNA isolation and genotyping.

**DNA Isolation and Genotyping**

Krd/+ embryos were identified by PCR amplification of genomic DNA using primers complementary to the transgene, as previously described (Keller et al., 1990). DNA was isolated from embryonic tissue by proteinase K (Boehringer Mannheim, Indianapolis, IN) digestion, phenol/chloroform extraction, and ethanol precipitation. The transgene was detected in 47% of the embryos and pups, consistent with the expected Mendelian inheritance of 50%.

**Tissue Preparation**

Tissues were fixed and embedded using routine procedures. Heads for immunostaining were fixed in 4% paraformaldehyde in 0.08 M phosphate buffer, pH 7.2, for 90 min and washed in phosphate buffer. Following overnight cryoprotection at 4°C in 20% sucrose, heads were equilibrated and frozen in a 2:1 mixture of 20% sucrose/OCT (TissueTek, Miles, Inc., Elkhart, IN). Cryosections were cut at 10 μm in the coronal, horizontal, or sagittal plane and air-dried onto microscope slides coated with 3-aminopropyltriethoxysilane (TESPA, Sigma, St. Louis, MO). All tissues and sections were stored at −80°C prior to use.

Heads processed for embedding in glycomethacrylate were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.08 M phosphate buffer, pH 7.2, overnight at 4°C. Following multiple washes in 0.08 M phosphate buffer, heads were dehydrated in ethanol, infiltrated with catalyzed glycomethacrylate (JB4, Polysciences Inc., War- rington, PA), and cast in plastic molds. Sections were cut at 5 μm in either the coronal or parasagittal plane with a Leitz rotary microtome, collected at variable intervals onto microscope slides coated with poly-L-lysine or gelatin, and stained with toluidine blue. For eyes used for digital reconstructions and morphometric analysis, the interval between each section was tabulated during sectioning.
Pax2 and Retinal Morphogenesis

Immunostaining

Tissue sections were warmed to room temperature and air-dried for 15 min prior to immunostaining. All reagents were applied directly to the sections on slides in a humidified chamber. Initial PBS washes were followed by blocking of nonspecific binding of secondary antibodies by incubation in PBS/0.2% Triton X-100 with 20% normal goat serum (NGS) for 30-90 min at room temperature. Polyclonal Pax2 antibodies (a gift of G. Dressler, HHMI, University of Michigan), diluted 1:50 in PBS/0.2% Triton X-100/2% NGS, were applied to sections and incubated for 2 h at room temperature. Prior to the application of secondary antibodies, endogenous peroxidase activity was eliminated, in most cases, by application of 3% hydrogen peroxide in 70% methanol. Antibodies against Pax2 were detected using peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG; Sigma), with a diaminobenzidine (SigmaFast-DAB, Sigma) color substrate. For fluorescence immunostaining, goat anti-rabbit (IgG)-TRITC (Sigma) (diluted 1:64 in PBS/0.2% Triton X-100/2% NGS) was substituted for the peroxidase-conjugated secondary antibodies and DAB. Slides were coverslipped using Gelmount (Biomedica, Foster City, CA) and photographed using differential interference contrast or epifluorescence illumination.

Imaging

Digital images were captured with an Optronics CCD camera (Optronics Engineering, Goleta, CA) attached to an Olympus Van-nox compound microscope and Capture+ software (TrueVision, Indianapolis, IN) running on a Macintosh PowerPC. Adobe Photoshop 3.0 software was used to prepare all photomontages from either the original digital images or from digitized 35-mm slides.

Three-Dimensional Reconstructions

Three-dimensional reconstructions were generated from digital images of a continuous series of 5-μm glycolmethacrylate sections cut in the sagittal plane. The intervals between sections varied within the eye, with smaller intervals in regions of rapid change, such as the transition from optic cup to optic stalk. One eye was reconstructed from a normal and a mutant embryo from litters at E10.5, E11.5, and E12.5 and, in the final reconstructions, each eye included multiple ocular components as separate objects: E10.5, the optic cup (retina + pigmented epithelium), the lens, and the subretinal space; E11.5, the optic cup and lens; and E12.5, the optic cup, lens, and optic nerve fibers. A more detailed description of the methodology for the three-dimensional reconstructions will be published separately. Briefly, captured images were exported into Adobe Photoshop 3.0, aligned manually as layers of a single file, and exported to NIH Image 1.60 (available on the Internet by accessing http://rsb.info.nih.gov/nih-image) to create a multi-image stack. Ocular components were isolated by removing unwanted portions of the original images from duplicate copies of the stack file. Using custom programs (written by E. S. using Metrowerks Code Warrior), X, Y, and Z coordinates, defining the perimeter of each image and the intervals between sections, were used to generate contours which were subsequently transformed into 3D-DXF meshes using Nuages, a freeware program running on a Sun Sparc 2 workstation. Strata Studio Pro software was used to align and smooth the 3D-DXF mesh images, to assign color and opacity, and to generate final surface renderings.

Quantitative Morphometric Analysis

For quantitative morphometric analysis, a series of 5-μm glycolmethacrylate sections were traced using a drawing tube attached to an Olympus compound microscope. In each eye, the posterior pole of the lens was used as a landmark, allowing the identification of comparable regions through the posterior optic cup and the optic stalk for analysis. For each eye at E10.5, 16 sections were traced at 10-μm intervals spanning 1/2 of the optic cup and the distal 1/2 of the optic stalk (+/+, n = 4 eyes; Krd/+, n = 6 eyes). For each eye at E11.5, 13 sections were traced at 20-μm intervals spanning 1/2 of the optic cup and the distal 1/2 of the optic stalk (+/+, n = 5 eyes; Krd/+, n = 6 eyes). All tracings were digitized using a drawing tablet (Kurta Corp., Phoenix, AZ) and NIH Image 1.60 software. Individual components of the eye (lens, retinal neuroepithelium, pigmented epithelium, subretinal space) and optic stalk (optic stalk epithelium, optic stalk lumen) were identified by the morphology of individual cellular elements and/or position. The area (μm²) of each digitized image was measured using NIH Image 1.60. Measurements of individual components (i.e., lens, subretinal space) were summed for each eye and mean values for total areas were compared by independent sample t tests, using SPSS for Windows 6.1. This method tests the null hypothesis that the mean of the individual ocular component measurement from the Krd/+ embryos is equal to the mean of the corresponding measurement from age-matched +/- embryos. Values for P = 0.05 were considered statistically significant.

RESULTS

We use the convention of describing anatomical relationships within the eye in terms of the longitudinal axis of the eye itself, rather than that of the embryo. Thus, structures that lie closest to the overlying head ectoderm, including the cornea, lens, and iris, form the anterior structures of the eye, and those near the optic stalk and nerve, including the retina and optic disc form the posterior structures. The terms proximal and distal are also used to describe relative position of histological sections within the optic cup and optic stalk. In this context, a proximal section lies closer to the embryonic midline and diencephalon, whereas a distal section lies closer to the anterior segments of the eye and the overlying head ectoderm (see diagram, Fig. 2G).

Pax2 Protein Expression in the Normal Optic Cup and Stalk

We characterized Pax2 protein expression in sections through the optic cup and optic stalk from embryos at E10.5, 11.5, 12.5, 14.5, 16.5, and adults, confirming and extending the previous reports (Nornes et al., 1992; Puschel et al., 1992; Torres et al., 1996). At E9.0, Pax2 expression presages the sites within the optic vesicle where the neuroepithelium will invaginate to form the double-layered optic cup and ventral embryonic fissure (Nornes et al., 1990; Puschel et al., 1992). At E10.5 (data not shown) and E11.5, Pax2+ cells in the ventral optic cup form the lips of the retinal fissure, with the most darkly stained cells, presumably those expressing the highest levels of Pax2, aligned at
the ventral midline of the optic cup where the two sides of the retinal fissure meet (Figs. 1A and 1B), Pax2 immunoreactivity continues uninterrupted into the optic stalk where, by E11.5, all cells express Pax2 and cells of the ventral optic stalk epithelium have invaginated to form the optic groove (Fig. 1C; see also Fig. 3E). In the posterior optic cup, at the transition between the retinal fissure and optic groove, Pax2+ cells invaginate and encircle the site within the retina where the optic disc will form. In cross section, these cells form a wedge within the retinal neuroepithelium flanking the fissure (Figs. 1C and 1D). From E11.5 to 12.5, as the retinal fissure closes, the Pax2 immunostaining in the ventral optic cup decreases, and by E16.5, Pax2+ cells are no longer found in the ventral retina (data not shown). In contrast, Pax2 immunostaining persists in cells at the posterior pole of the retina, so that by E12.5, Pax2+ cells encircle the ganglion cell axons and hyaloid artery at the nascent optic disc (Figs. 1E and 1F). Similarly, at E14.5 (data not shown) and at E16.5 (Fig. 1G), Pax2+ cells remain as a thin cuff of cells that surround the optic disc, now filled with the axons of ganglion cells.

During these periods of retinal development, Pax2+ cells are also present within the optic stalk (Figs. 1C, 1H, and 1I) and the lateral wall of the diencephalon (Figs. 1A and 1C; see also Nornes et al., 1990; Puschel et al., 1992). By E16.5, after the stalk is transformed into the optic nerve, Pax2+ cells are present in the optic nerve and at the vitreal boundary of the optic disc (Fig. 1G). A similar pattern of Pax2 mRNA expression has been observed at E18.0 (Nornes et al., 1990). We did not determine the stage at which Pax2 protein expression ceases, but there were no Pax2+ cells in the retina, optic disc, or nerve of adult mice (data not shown).

In both the optic cup and stalk, there is a reciprocal pattern of melanin pigment and Pax2 expression (see also Torres et al., 1996). Within the optic cup at E11.5, Pax2 is expressed in cells of the prospective pigmented epithelium that do not yet contain melanin granules (Fig. 1D). Melanization of the pigmented epithelium follows a characteristic pattern beginning in the dorsal optic cup and progressing ventrally toward the ventral midline and proximally toward and into the optic stalk (Silver and Sapiro, 1981; Colello and Jeffery, 1991). In the optic cup, there is a distinct junction between melanin-containing cells and those lacking melanin, but expressing Pax2 (Fig. 1D). Likewise, during the transient melanization of the dorsal optic stalk (Silver and Sapiro, 1981; Colello and Jeffery, 1991), melanin-containing cells do not express Pax2 and vice versa (Fig. 1H), although, in unpigmented regions of the optic stalk, all cells are Pax2+ (Fig. 1I).

Ocular Development and Pax2 Immunostaining in Krd/+ Embryos

Sectioned optic cups and optic stalks/nerves from 55 Krd/+ animals at E10.5 to P2 were examined and compared to 59 normal littermates. A single litter of animals at E9.5, the only litter not genotyped, was also examined, and none of these embryos exhibited malformations in either the optic vesicles or optic stalks. In contrast, in embryos E10.5 and older, the ocular phenotype invariably matched the genotype that was determined using PCR.

In normal embryos at E10.5, parasagittal sections through the head, which cut the optic cup en face (see Fig. 2G), reveal the retinal fissure as a cleft in ventral retina filled with migrating mesenchymal cells that are destined to form the hyaloid vasculature (Figs. 2A and 2C). The ventral optic groove confers a characteristic horseshoe shape to the optic stalk (Fig. 2E), leading to the eventual occlusion of the optic stalk lumen. In Krd/+ embryos at E10.5, the retinal fissure forms in the distal optic cup and mesenchymal cells are visible migrating into the optic cup (Fig. 2B). However, the retinal fissure has not progressed into the posterior optic cup, resulting in an alteration in the distribution of cells in the ventral optic cup and a somewhat misshapen profile (Fig. 2D). The optic groove fails to form in the ventral optic stalk resulting in the retention of a round or oval shape and a patent lumen (Fig. 2F).

FIG. 1. Photomicrographs of Pax2 immunostaining in the normal embryonic eye. Horizontal sections through the ventral (A, B) and mid optic cup (C, D) at E11.5; B and D are higher magnification views of A and C, respectively. (A, B) Pax2+ cells are present in the ventral retina, where they are aligned at the embryonic fissure (arrowheads in B). (C) Pax2+ cells extend from the neuroretina in the posterior eyecup (large arrows) through the optic stalk (arrowheads) and into the diencephalon (see arrowheads in A). (D) Unpigmented cells within the outer layer of the optic cup (solid arrowhead) are Pax2+, whereas adjacent pigmented cells (open arrowhead) are not. Intense staining of erythrocytes (RBC in C; see also A, D) reflects endogenous peroxidase activity which was not quenched prior to application of the diaminobenzidine substrate. In parasagittal sections through the posterior optic cup (E, F) and optic stalk (H, I) of E12.5 embryos, Pax2+ cells extend from the ventral margin of the optic cup, along the site of the erstwhile embryonic fissure (arrowheads in E), and encircle the optic disc (F). Ganglion cell axons within the optic disc appear lightly stained because, in our hands, the Pax2 antibody shows weak cross-reactivity to all axons. In regions of the optic stalk just behind the optic cup (H), Pax2+ cells (solid arrowhead) are present in the ventral unpigmented half, whereas the pigmented cells (open arrowhead) in the dorsal optic stalk show no immunostaining. In sections through the optic stalk closer to the diencephalon (I), there are no pigmented cells and all cells are Pax2+ (arrowheads). (G) A frontal section through the posterior optic cup and optic disc at E16.5 reveals that Pax2+ cells form a boundary between the neuroretina and optic nerve (large arrowhead) and Pax2+ cells, presumptive astrocyte precursors, are also present within the optic nerve (small arrowhead) and at the optic nerve head (small arrow). (J) Cross section through the optic stalk at E12.5 (similar to H), where the primary antibody was omitted (Note: see Fig. 2G for orientation of sections in E, F, H--J). NE, neuroepithelium; L, lens; HA, hyaloid artery. Scale bar: 140 μm in A and C; 70 μm in B and D; 150 μm in E; 50 μm in F, H, I, and J; 100 μm in G.
FIG. 2. Photomicrographs of representative parasagittal sections through the optic cup and optic stalk of normal (A, C, E) and Krd+/-(B, D, F) embryos, E10.5. In the distal optic cup of both normal (A) and Krd+/-(B) embryos, the embryonic fissure (arrowheads) is present, whereas in more proximal portions of the optic cup (D) and in the optic stalk (F) of Krd+/+ embryos, the embryonic fissure is absent. Note the visible subretinal space (arrow) present in the dorsal optic cup of Krd+/+ embryos (B, D). Because of the lateral orientation of the optic cup, parasagittal sections through the head cut the optic cup en face and the optic stalk in cross section, as illustrated by a cartoon (G), indicating the plane of section (gray) through the optic cup and posterior lens shown in C and D. The lens (L) lies in the anterior portion of the optic cup with the optic stalk at the posterior pole. The terms distal and proximal refer to the relative position of structures and sections, relative to the diencephalon (Di) and the embryonic midline, with distal structures farthest from and proximal structures nearest to the diencephalon. Scale bar, 100 μm in A–F.

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At E11.5, the anterior regions of the optic cup of both normal and Krd animals are morphologically similar, with a well-formed retinal fissure (Figs. 3A and 3B). However, malformations within the posterior optic cup and optic stalk at E10.5 (Figs. 2D and 2F) persist and are relatively unchanged at E11.5 (Figs. 3D and 3F). Three-dimensional
FIG. 4. Three-dimensional reconstructions of representative normal (A) and Krd/+(B) optic cups at E11.5, viewed from a perspective that shows the ventral surface of the optic cup and optic stalk. Although the retinal fissure is present in the optic cup of Krd/+ embryos (B), it fails to extend through the posterior pole of the retina into the optic stalk. Arrows indicate the approximate location of sections illustrated in Fig. 3.

reconstructions of optic cups from normal and Krd/+ embryos at E10.5 (data not shown) and at E11.5 (Figs. 4A and 4B) confirm that the apparent difference in optic cup morphogenesis between normal and Krd/+ embryos is a failure of the embryonic fissure to progress proximally beyond the optic cup and into the optic stalk.

Differences between normal and Krd/+ embryos are not a consequence of an overall developmental delay in Krd/+ embryos. This is based on two sets of observations. First, differences in the size and apparent developmental stage of the embryos (e.g., crown to rump length; extent of melanization of the optic cup) did not correlate with the genotype of the animals. Second, quantitative comparisons of optic cups at E10.5 (Fig. 5A) and E11.5 (Fig. 5B) indicate there are no differences in the average size of the optic cup or lens in normal and Krd/+ embryos. At E10.5, however, there is a statistically significant difference in the relative proportions of inner retinal neuroepithelial and outer pigmented epithelial layers. In the optic cups of Krd/+ mice, there is less tissue within the inner layer and more in the outer layer of the optic cup (Fig. 5A) when compared to their normal littermates. Because in our analysis, epithelial layers were identified by position only, we believe that these differences result from the abnormal formation of the embryonic fissure in Krd/+ mice; neuroepithelial cells destined for the inner retinal layer are present in the outer pigmented epithelial layer. In addition, at E10.5, the decreased size of the retinal neuroepithelium and concomitant increase in the size of the pigmented epithelium creates a spatial mismatch between the two layers of the optic cup resulting in an increase in the amount of subretinal space (Fig. 5A; see also Figs. 2A–2D).

Quantitative differences observed at E10.5 are transient and differences in the amount of retina, pigmented epithelium, and subretinal space are no longer present by E11.5 (Fig. 5B), although the posterior optic cup and optic stalk continue to be visibly malformed (see Figs. 3D and 3F). During early embryogenesis, there is significant daily growth of the optic cup, and by E11.5, the area of the malformation within the posterior optic cup of Krd/+ embryos constitutes a much smaller portion of the total optic cup than it did at E10.5. Thus, in this analysis, the overall similarity of the optic cups from normal and mutants embryos at E11.5 outweighs the differences associated with the malformation at the forming optic disc.

There is apparently normal closure and fusion of the retinal fissure in Krd/+ embryos (data not shown), and in adults, defects are not observed at the former site of the retinal fissure within the ventral retina (Keller et al., 1994). In contrast, within the posterior optic cup, the failure of the optic groove to form has consequences for the growth of ganglion cell axons out of the retina. In the retinas of normal embryos at E12.5, Pax2+ cells encircle the fascicles of ganglion cell axons which form a crescent dorsal to the hyaloid artery (Figs. 6A–6C; see also Figs. 1E and 1F). As development proceeds, the number of axons exiting the eye increases and the dorsal crescent of axon fascicles expands ventrally to surround the artery (Silver and Robb, 1979), all the while remaining within the cuff of Pax2+ cells (see Fig. 1G). In the Krd/+ embryos, Pax2+ cells are present in the approximate location of the nascent optic disc, but do not form a coherent annulus surrounding the ganglion cell axons (Figs. 6D–6F). With the failure of optic groove formation, Pax2+ cells that do not invaginate to surround the
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Despite the failure of the embryonic fissure to invaginate in the posterior optic cup and optic stalk of Krd/+ embryos, ganglion cell axons are able to exit the optic cup, as evidenced by the presence of axon fascicles in sections through the optic stalk of embryos at E12.5 (Fig. 6L) and later (Figs. 7B–7D). In the distal optic stalk of normal embryos at this stage, Pax2+ cells are confined to the ventral aspect of the optic stalk and, as in the retina, are associated with fascicles of ganglion cell axons (Figs. 6G, 6H, and 6K). Similarly, in Krd/+ embryos, Pax2+ cells are also present in the ventral optic stalk in close association with ganglion cell axons, although the failure of the embryonic fissure to extend proximally results in a stalk with an abnormal morphology (Figs. 6I, 6J, and 6L).

In embryos older than E12.5, although there are no apparent defects in other regions of the optic cup, malformations of the optic nerve, optic disc, and the adjacent retina persist as features diagnostic of the Krd/+ genotype. In the Krd/+ animals, the ganglion cell axons appear to penetrate the eye, not as a coherent bundle, but as isolated fascicles. At the optic disc, ganglion cell axons traverse the retinal neuroepithelium, creating "islands" of neuroepithelial cells among the axons (Fig. 7B). In areas of the retinal neuroepithelium adjacent to the optic disc, there are clefts and folds within the neuroepithelium (Fig. 7D). These neuroepithelial "islands" and folds are likely the origins of the rosettes of photoreceptor cells and laminar defects seen in the adult retina (Keller et al., 1994). In addition, although not quantified, there appear to be fewer cell bodies present among the ganglion cell axons of the optic disc and nerve (Figs. 7B and 7C). In the eyes of normal mice at E16.5 and later, there is a distinct boundary between the pigmented epithelium and the unpigmented optic nerve (Fig. 7A). In the eyes of Krd/+ littermates, pigmented cells extend along the surface of the forming optic nerve (Figs. 7B–7D) and the retinal neuroepithelium often extends into the optic nerve just beneath these pigmented cells (Fig. 7B). In homozygous Pax2 mutants, similar but more extensive pigmentation defects have been reported (Torres et al., 1996).

At all time points examined, there is considerable variation in the extent of ocular defects between littermates and often between eyes of the same animal. However, we find that all Krd/+ embryos at E10.5 and older show at least a subset of these defects. The defects which most consistently predicted the mutant genotype were the failure of optic groove formation and patent optic stalk lumen during morphogenesis and the abnormal pigmentation of the optic nerve during later development.

DISCUSSION

Pax2 Haploinsufficiency and Ocular Defects in Krd/+ Mice

Krd mice carry a 7-cM deletion that includes the Pax2 gene and may encompass as many as 400 other loci (Keller et al., 1994). Although we cannot dismiss the possibility that hemizygosity of other genes within this large deletion

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FIG. 6. Pax2 immunostaining and cellular morphology in photomicrographs of parasagittal sections through the optic disc and optic stalk of normal (A–C, G, H, K) and Krd+/− (D–F, I, J, L) embryos at E12.5. (A, B) Pax2+ cells surrounding the optic disc in a normal embryo. (C) Comparable section from a glycomethacrylate-embedded eye illustrating the elongated morphology of Pax2+ cells (arrowheads) arrayed about the hyaloid artery and ganglion cell axons (arrow). (D, E) Pax2+ cells in the retina of a Krd−− embryo. (F) Comparable section from a glycomethacrylate-embedded eye. Note the relative spatial disorganization of the Pax2+ cells in the area of the optic disc (D, E; arrowheads in F), and the paucity of axon fascicles (arrow in F). (G, H) Pax2+ cells in the optic stalk of a normal embryo. (I, J) Pax2+ cells in the optic stalk of a Krd+/− embryo. Note that, as in the normal embryo, Pax2+ cells are present in the ventral optic stalk, although the lumen remains patent. (L) Comparable glycomethacrylate section illustrating the presence of axon fascicles (arrow) at the ventral surface of the optic stalk. (A, D, G, I) Double exposures using differential interference contrast and epifluorescence illumination. Pax2 immunostaining was consistently less intense on sections from Krd+/− embryos, and exposure time for Pax2 immunofluorescence of sections from Krd+/− embryos was twice that of the normal controls. HA, hyaloid artery. Scale bar: 100 µm in A and D; 50 µm in B, C, E, and F; 80 µm in G, H, I, and J; 60 µm in K and L.
FIG. 7. Photomicrographs of frontal sections through the optic discs and nerves from normal (A) and Krd/− (B−D) mice at E16.5. B−D illustrate representative optic disc malformations observed in Krd/− mice: Arrowheads indicate islands of neuroepithelial cells within the optic nerve (B) and laminar defects in the retinal neuroepithelium (D). Pigmented cells, continuous with those of pigmented epithelium (arrowhead in C, see also B and D), and retinal neuroepithelial cells (arrow in B) extend along the optic nerve, beyond their normal boundary at the junction of the optic cup and optic stalk (arrowhead in A). Scale bar, 150 μm.

 contributes to the Krd phenotype, we believe haploinsufficiency of Pax2 is primary in the etiology of the congenital anomalies found in Krd/− mice. First, we find no evidence for generalized developmental delay in Krd/− embryos that might indicate nonspecific deletion effects. Second, Pax2 is expressed during embryogenesis in precisely those tissues affected in the mutants. Indeed, we find that the initial defects observed in the retina are specifically associated with abnormal morphogenetic movements of Pax2-expressing cells. Third, the congenital anomalies present in the Krd/− mice, including aplastic, hypoplastic, and cystic kidneys, pan-retinal hypocellularity, reduced electroretinogram, and optic disc malformations (Keller et al., 1994; Hitchcock et al., 1995; Otteson et al., 1996), are remarkably
similar to those found in other Pax2 heterozygotes, both murine (Torres et al., 1995, 1996; Favor et al., 1996) and humans (Sanyanusin et al., 1995a,b; Schimenti et al., 1995, 1997). Although ocular malformations of the optic disc and/or optic stalk were present in all Krd+/+ animals examined, defects were reported to be variable in their occurrence in mice heterozygous for induced Pax2 mutation (Torres et al., 1996). This may reflect differences in genetic background or a failure to identify the full spectrum of retinal defects present. Alternatively, hemizygosity for other genes within the Krd deletion may compound or enhance the retinal defects associated with hemizygosity of Pax2.

**Pax2+ Cells and Optic Groove Morphogenesis**

The analysis of adult phenotypes provides only indirect insight into the origins of congenital defects; therefore, we undertook a detailed comparative analysis of early ocular morphogenesis and Pax2 protein expression in normal and Krd+/+ mice. It has previously been shown that Pax2 is expressed in cells of the ventral retina and optic stalk at the site where the embryonic fissure forms (Nornes et al., 1990; Puschel et al., 1992; Torres et al., 1996). Our results confirm and extend these results to show that within the posterior retina, Pax2+ cells of the ventral neuroepithelium invaginate to demarcate the retinal site of the optic disc. Here, the Pax2+ cells persist into late embryogenesis as a cuff of cells that encircles the forming optic disc, creating a boundary between the ganglion cell axons and the retinal neuro-epithelium. Pax2-expressing cells that encircle the disc are continuous with those of the optic stalk and may represent an extension of the optic stalk into the optic cup. Both the Pax2+ cells that encircle the optic disc and those present in the developing optic nerve have been shown to be astrocyte precursors that derive from cells of the optic stalk (Edwards et al., 1990; Huxlin et al., 1992; see also MacDonald et al., 1997). In the Krd+/+ embryos, although the retinal fissure forms within the ventral optic cup, the ventral optic stalk epithelium does not invaginate to form the optic groove. At the junction between these two portions of the embryonic fissure, the lack of invagination of the ventral epithelium is associated with malformation of the optic disc. Specifically, it is the Pax2+ cells that fail to invaginate and, as a consequence, fail to form a coherent annulus surrounding the nascent optic disc.

The early defects associated with reduction or loss of Pax2 expression in mice result from altered movements of epithelial cells (see Keller et al., 1994; Torres et al., 1995, 1996; Favor et al., 1996). It is well known that precise regulation of the expression of cell adhesion molecules is required for the morphogenetic movements of simple epithelia as they form the complex shapes of mature organs (reviewed by Hynes, 1994; Fagotto and Gumbiner, 1996; Marrs and Nelson, 1996; Redies and Takeichi, 1996; Roussant and Obrink, 1996; Steinberg, 1996). Although it is unknown which genes are regulated by Pax2 expression in the eye, there is evidence that other Pax genes regulate the expression of cell adhesion molecules. Pax6 binds to a sequence in the promoter region of the cell adhesion molecule, L1 (Chalepakis et al., 1994b), and Pax3 (Chalepakis et al., 1994a), Pax6 (Holst et al., 1997), and Pax8 (Holst et al., 1994) can regulate in vitro transcription of the neural cell adhesion molecule, N-CAM. Several cell adhesion molecules are known to be expressed in the developing eye, including R-cadherin and N-cadherin (Redies and Takeichi, 1993; Riehl et al., 1996). Interestingly, a novel cadherin, K-cadherin, has been shown to be expressed in many tissues that express Pax2, including the developing kidney and brain (Xiang et al., 1994) and, within the embryonic eye, the developing optic disc and nerve (P. Hitchcock, unpublished data).

**Pax2+ Cells and Axon Guidance at the Forming Optic Disc**

Based on morphology and position, we believe that the Pax2+ cells at the optic disc play a role in guiding ganglion cell axons out of the retina. The Pax2+ cells that encircle the optic disc are a subset of the channel-forming cells first described by Silver and Robb (1979). These cells create extracellular spaces or channels among their endfeet at the inner surface of the pia that are believed to play a role in guiding ganglion cell axons out of the retina into the optic nerve (Silver and Robb, 1979; Silver and Sidman, 1980; Horsburgh and Sefton, 1986; Colello and Guillery, 1992; Britts and Silver, 1995). In addition, N-CAM, a secreted protein that is required for axon guidance of commissural neurons within the developing brain (Serafini et al., 1996; see also Serafini et al., 1994), is expressed by the Pax2+ cells at the optic disc and optic nerve, and Netrin-1 mutants show significant axon pathfinding defects at the optic disc (Deiner et al., 1997). In the Krd+/+ embryos, the disorganization of the Pax2-expressing cells at the optic disc would be predicted to result in both an abnormal organization of the network of extracellular channels and a dispersion of the associated chemoattractant guidance cues to ectopic locations. Either or both of these events could give rise to the intraretinal defects in axon pathfinding observed in Krd+/+ embryos.

We observe an initial reduction in the number of identifiable axon fascicles at E12.5 in Krd+/+ mice (Fig. 6L). However, at E16.5 (Fig. 7D) and E18.5 (data not shown), size of the optic nerve as it exits the cup does not appear qualitatively different than in normal littermates. This is somewhat surprising since adult Krd+/+ mice have reduced numbers of ganglion cells within the retina and correspondingly reduced numbers of axons within the optic nerve (Green et al., 1997). However, this is a highly variable phenotype and the cause and time course of ganglion cell loss is not yet known (see also below).

**Ocular Anomalies in Later Development**

Defects observed during ocular morphogenesis in Krd+/+ embryos provide insight into the origin of the ocular defects observed adult Krd+/+ mice and, by extension, in other Pax2
FIG. 8. Proposed model of optic disc formation in Krd/+ and Pax2 heterozygous mice. Diagrams based on three-dimensional reconstructions of the optic cup and forming optic nerve at E12.5 and on histological preparations of later stages of development in +/+ (A, B) and Krd/+ (C, D) mice. Ganglion cell axons (yellow) follow the surface of the retina (gray) toward the posterior pole of the optic cup where they exit the eye among Pax2+ cells (red). (A) In normal mice, prior invagination of the optic groove has created a cuff of Pax2+ cells that encircle the axons and guide them to the optic stalk. (The optic stalk (OS) is shown as a cutaway to illustrate the formation of the ventral optic groove.) (B) Later in development, continued addition of axons fills the optic disc and Pax2+ cells are no longer detected in the retina. The former optic stalk, now filled with axons, has become the optic nerve. (C) In Krd/+ and Pax2 heterozygous mice at E12.5, the failure of optic groove formation alters the position of the posterior retina and the Pax2+ cells. Axons exit the eye along the retinal surface, traveling obliquely to reach the ventral optic stalk. The transected optic stalk shows the lack of optic groove formation and the patent optic stalk lumen (OSL) which creates a space behind the retina. Subsequent addition of ganglion cell axons at the disc pushes the adjacent retina into this space (arrow in C). (D) Displacement of retina into the optic stalk may give rise to laminar defects and the concave morphology of the optic disc observed in Krd/+ mice and Pax2 heterozygotes, both murine and human. Note that diagrams depicting different stages of eye development are not drawn to scale. The pigmented epithelium is indicated by the thick black line. In both normal (A) and mutant (C) eyes, pigmentation extends along the dorsal optic stalk at E12.5. Only mutant mice (D) show pigmentation in the optic nerve at later stages of development. ON, optic nerve; L, lens.
forming optic disc. The first ganglion cells are born in the
dorsal retina immediately adjacent to the forming optic disc
(Mann, 1964; Sidman, 1961; Kahn, 1973; Silver and Robb,
1979) and their axons are thought to be constrained from
projecting away from the forming disc by the presence of
inhibitory cues within the peripheral retina (Snow et al.,
1991; Brittis et al., 1992). They must therefore project to-
ward posterior pole of the optic cup and the site of the
forming optic disc where they encounter the Pax2+ cells
and their associated guidance cues (Fig. 8A; see discussion
above). In Krdd/+ embryos, ganglion cell axons are appar-
ently still guided toward the posterior pole of the optic cup,
but at the optic disc, they encounter a disorganized array
of Pax2+ cells. Despite the failure of the optic groove to
form, the retina maintains its continuity with the ventral
optic stalk epithelium allowing the ganglion cell axons to
follow the surface of the retina to reach the Pax2+ cells
and the ventral optic stalk (Fig. 8C). During subsequent
development, ganglion cell axons fill the disc and may force
the adjacent retinal neuroepithelium into the occult space
created by the persistent optic stalk lumen. Such epithelial
displacement could cause the retinal folding observed near
the concave optic disc (Fig. 8D; see also Fig. 7D).

Apart from the optic disc anomalies and the retinal hypo-
plasia observed in the retinas of Krdd+/ and Pax2mice
(Keller et al., 1994; Favor et al., 1996; Green et al., 1997),
the remainder of the optic cup is similar to that of age-
matched controls (data not shown). This is consistent with
the apparently normal growth of the optic cup and mor-
phogenesis of the retinal fissure in these mutants, and with
the limited domain of Pax2 expression within the develop-
ing optic cup. With the failure of optic groove formation,
it is possible that the optic disc may be displaced toward mal-
formed fissure. However, we were unable to discern if this
was true or not in Krdd+/ mice, suggesting that any malposi-
tioning of the disc is likely to be subtle.

**Pax2 Gene Expression and Coloboma**

Pax2 is important in the morphogenesis of the embryonic
fissure, and reduction or loss of Pax2 expression results in
coloboma of the optic disc and/or retina (Hitchcock et al.,
1995; Sanyanusin et al., 1995a,b; Schimenti et al., 1995,
1997; Favor et al., 1996; Otteson et al., 1996; Torres et al.,
1996; see also MacDonald et al., 1997). A comparison of
phenotypes observed in heterozygous and homozygous Pax2
mutant mice shows different requirements for Pax2 during
morphogenesis of the embryonic fissure. Full diploid ex-
pression of Pax2 is required for formation of proximal por-
tions of the embryonic fissure and the lack of one copy of
Pax2 results in a failure of optic groove formation in Krdd+/mice. The presence of optic disc coloboma in other Pax2
heterozygotes (Favor et al., 1996; Torres et al., 1996) and
the lack of optic groove formation in Pax2 homozygous
mice (Favor et al., 1996) indicate that this is likely to be
true for all Pax2 mutants. In contrast, the retinal fissure is
less sensitive to Pax2 gene dosage. Pax2 is required for clo-
sure, but not formation of the retinal fissure. In contrast
to optic groove formation, haploid expression of Pax2 is
sufficient to mediate closure of the retinal fissure. Bilateral
retinal colobomas, resulting from failure of retinal fissure
closure, are present in homozygous, but not heterozygous
Pax2 mutant mice (Favor et al., 1996; Torres et al., 1996).

In seven independent human pedigrees, patients hetero-
zygous for mutations in Pax2 have been identified, all with
autosomal dominant renal anomalies and bilateral optic
disc colobomas. This is a relatively uncommon type of colo-
oboma, similar to that present in Pax2 heterozygous mice,
that is characterized primarily by an enlarged, funnel-
shaped optic disc extending into the retina (Sanyanusin et
al., 1995a,b; Schimenti et al., 1995, 1997). The develop-
mental mechanisms underlying this type of coloboma have
not been directly examined in humans, although it has gen-
erally been thought that both retinal and optic disc colobo-
mas arises from a failure of closure of the embryonic fissure
(Mann, 1957, 1964; Lopashov and Stroeva, 1964; Brown and
Tasman, 1983; Apple, 1984; Silver et al., 1984; Schimenti
et al., 1995). The early development of the optic vesicle and
eye is highly conserved among mammalian species (Mann,
1964; Lopashov and Stroeva, 1964) and phenotypes present
in humans and mice heterozygous for Pax2 mutations are
remarkably similar. This suggests that the murine Pax2
mutations provide a model for understanding the develop-
ment of these malformations in humans. Based on our anal-
ysis of the development of optic disc malformations in
Krdd+/ mice, we propose that optic disc anomalies observed
in Pax2 heterozygotes, both murine and human, represent
a unique subclass of coloboma that does not result from
abnormal closure of the retinal fissure, but rather from the
abnormal formation of the optic groove.

**ACKNOWLEDGMENTS**

The authors thank the following individuals who contributed
to this study: Dr. Gregory Dressler for his kind gift of the Pax2 anti-
body; Dr. Miriam Meisler for generously providing the Krdd mice
used in this study; Dr. David Sretavan for communicating results
from the largest study; Dr. Gregory Dressler for his kind gift of the Pax2 anti-
todd antibody; and the following individuals who contributed to
this study: Dr. Gregory Dressler for his kind gift of the Pax2 anti-
body; Dr. Miriam Meisler for generously providing the Krdd mice
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