Keratocan-deficient Mice Display Alterations in Corneal Structure*

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Keratocan (Kera) is a cornea-specific keratan sulfate proteoglycan (KSPG) in the adult vertebrate eye. It belongs to the small leucine-rich proteoglycan (SLRP) gene family and is one of the major components of extracellular KSPG in the vertebrate corneal stroma. The Kera gene is expressed in ocular surface tissues including cornea and eyelids during morphogenesis. Corneal KSPGs play a pivotal role in matrix assembly, which is accountable for corneal transparency. In humans, mutations of the KERA gene are associated with cornea plana (CNA2) that manifests decreases in vision acuity due to the flattened forward convex curvature of cornea. To investigate the biological role of the Kera gene and to establish an animal model for cornea plana, we generated Kera knockout mice via gene targeting. Northern and Western blotting and immunohistochemical analysis showed that no Kera mRNA or keratocan protein was detected in the Kera−/− cornea. The expression levels of other SLRP members including lumican, decorin, and fibromodulin were not altered in the Kera−/− cornea as compared with that of the wild-type littermates. Mice lacking keratocan have normal corneal transparency at the age of 12 months. However, they have a thinner corneal stroma and a narrower cornea-iris angle of the anterior segment in comparison to the wild-type littermates. As demonstrated by transmission electron microscopy, Kera−/− mice have larger stromal fibril diameters and less organized packing of collagen fibrils in stroma than those of wild type. Taken together, our results showed that ablation of the Kera gene resulted in subtle structural alterations of collagenous matrix and did not perturb the expression of other SLRPs in cornea. Keratocan thus plays a unique role in maintaining the appropriate corneal shape to ensure normal vision.

The vertebrate cornea is a tough and transparent tissue that provides greater than 60% of refractive power to the incoming light to cast a focused image on retina. To exact its proper functions, the cornea has to maintain transparent, appropriate curvature for refraction and toughness for protection. Genetic and epigenetic factors that cause the change of corneal curvature, reduction of transparency, and weakness in structure can lead to the impairment of visual acuity.

The corneal stroma consists of uniformly small collagen fibrils with an average diameter of 25 nm that are arranged in orthogonal lamellae. Stromal keratocyt es are responsible for the formation and maintenance of a unique collagenous matrix that is essential for proper corneal curvature and transparency (1, 2). The mechanisms that govern the assembly of different levels of stromal architecture are not well understood; however, proteoglycan-collagen and collagen-collagen interactions have been implicated. It has been suggested that the stoichiometry and interaction of different collagen types play an important role in modulating collagen fibril diameter (1, 3–5). The proteoglycans in the stroma are members of the small leucine-rich proteoglycan (SLRP) family and are thought to regulate collagenous matrix assembly in connective tissue because of their bifunctional character: the protein moiety that binds collagen fibrils and the highly charged hydrophilic glycosaminoglycans that regulate interfibrillar spacing (1, 2, 6–11). In addition to interactions with collagen fibrils, corneal stromal proteoglycans also play a role in corneal hydration due to the highly negative charge of their sulfated carbohydrate moieties and the glycosaminoglycan chains.

Keratocan, lumican, and mimecan/osteoglycin are the major keratan sulfate-containing proteoglycans in vertebrate corneal stroma. Their core proteins consist of 6–10 tandem repeats of 24 amino acids with hydrophobic residues in conserved positions. We and others have recently shown that mice lacking lumican revealed an age-dependent corneal opacity and a high proportion of abnormally thick collagen fibrils in the corneal stroma (12–14). In humans, the first SLRP gene that is directly linked to a disease is the keratocan gene (KERA). Mutations of the human KERA gene are associated with cornea plana (CNA2) in which the forward convex curvature is flattened, leading to a decrease in light refraction (15). The clinical synopsis of CNA2 includes reduced visual acuity, extreme hyperopia, hazy corneal limbus, corneal parenchymal opacities, thin corneal stroma, and indistinct sclerocorneal boundary. Moreover CNA2 with extremely flat cornea is often associated with angle closure glaucoma.

We have found previously that, unlike lumican or mimecan/osteoglycin, which is ubiquitously expressed by most interstitial connective tissues, keratocan expression is specific to the adult corneal stroma in the mouse. During embryonic development, Kera gene expression tracks ocular surface tissues mor-

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Western Blotting—To characterize keratocan in wild-type mice, two corneas were isolated from both eyes of normal FVB/N postnatal day 1, 7, and 14 mice. The corneas were minced and extracted in 1 m of 4 M guanidine-HCl containing 10 m sodium EDTA, 5 m p-aminobenzamidine, and 0.1 m e-amin-o-n-caproic acid at 4 °C overnight. The extracts were dialyzed exhaustively in distilled water at room temperature, and the water-insoluble fraction was dissolved in 0.1 M Tris acetate solution (pH 6.0) containing 6 m urea. The protein concentration was measured by spectrophotometer at A280 (mg), aliquots (100 m of protein) were incubated with or without endo-β-galactosidase (0.1 unit/ml) at 37 °C overnight. An equal volume of 2% SDS sample buffer was added into digested and undigested samples, boiled for 5 min, electrophoresed on an SDS-PAGE gradient (4–15%) or regular 10% gel, and transferred to polyvinylidene difluoride membrane. Affinity-purified antibodies against mouse keratocan peptide (VRQAYEQIADPDWYHDDFYC) were raised in rabbit and used as secondary antibody, and the immunocomplex was visualized with a Western Blue™ kit (Promega, Madison, WI). To detect decorin and fibromodulin of the mouse corneas, affinity-purified antibodies against mouse decorin peptide (IPYDPDNPPLSIC (17)) or mouse fibromodulin (CDR- VGRKVFSKLRHLER and CDPYDPYPYEPSEPYPYGVEE) (a generous gift from Dr. Larry Fisher, National Institutes of Health)2 were raised in rabbit and used as primary antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy and light) was used as secondary antibody, and the immunocomplex was visualized with ECL™ Western blotting detection substrate (Pierce).

Generation of Kera Knockout Mice by Gene Targeting—The keratocan genomic 6.6-kb BamHI-EcoRI fragment, which contains 3.1-kb promoter, exon 1, intron 1, exon 2, and 1.1-kb intron 2, was first cloned into pBluescript SK vector (Stratagene, La Jolla, CA), and the resulting plasmid was named pKera-BR6.6 (16). A 0.6-kb XhoI fragment was then deleted from the 5′ end of the pKera-BR6.6, and the plasmid was self-ligated. Next a 2.6-kb XhoI fragment containing 1.0-kb intron 1, entire exon 2, and 1.1-kb intron 2 was deleted from the 5′ end and replaced with a positive selection marker, neomycin resistance gene (pgk-pr-neoA) cassette in the antisense orientation with respect to the Kera gene. The resulting plasmid was designated as pKera5 pgk Neo. A 1.6-kb KpnI fragment, which contains 0.6-kb exon 2 and 1.0-kb intron 2, was excised from the pKera-BR6.6 and inserted into pkeras pgk KpnI. Finally, two positive selection markers, a diphtheria toxin A fragment (pgk-pr-Dta) cassette, was placed on the 3′ end of the targeting vector. Therefore, the Kera gene targeting vector, pKera-TV, contains a 3.1- and a 1.6-kb homologous sequence on 5′ and 3′ ends, respectively. The pKera-TV (80 μg) was linearized and transfected into an embryonic stem cell line (Duffy) via electroporation (250 microfarads and 800 Vcm). After 12 days of selection with Geneticin® (G418, 500 μg/ml), the G418-resistant colonies were individually picked and expanded. The homologous recombinant embryonic stem clones were identified by PCR-based analysis. PCR was carried out with the Expand Long Template PCR System (Roche Diagnostics). The primers used for 5′ PCR were as follows: kerans, 5′-gaggctgtgctgctgtcagc- caacgag-3′; kerac-2, 5′-cagcagtagttaccaagttggtg-3′; neo plus), 5′- egctctgctgctgctgcattagcctg-3′. The primers used for 3′ PCR were as follows: kera-3, 5′-gcgctgtgctgctgtcagc- caacgag-3′; kera-2, 5′-cagcagtagttaccaagttggtg-3′; neo-3′, 5′- egctctgctgctgctgcattagcctg-3′. Amplification of the 5′ PCR product was performed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 7 min. Amplification of 3′ PCR product was identical except the extension time at 72 °C was 3 min. The blastocyst microinjection and chimeric mice production were performed by the gene targeting core facility at the University of Cincinnati.

Immunohistochemistry—To examine keratocan and lumican expression, 5-μm paraffin sections were deparaffinized, blocked, and pro- cessed for indirect immunostaining using epitope-specific rabbit anti- body to synthetic mouse lumican 4 m guanidine-HCl, and extracts were subjected to gradient (4–15%) SDS-PAGE and “electrotransfer” with (D) or without (C) prior digestion with endo-β-galactosidase. Molecular mass markers are indicated on the left. Without endo-β-galactosidase treatment, keratocan appears as a smear. After enzyme digestion, keratocan appears as a ~50-kD single band (arrow).

Statistical Methods—In the initial analyses of fibril diameter, the fibril diameter measurements from two mutant animals were analyzed separately, and no significant differences were observed between the two. Therefore, in the final analyses data from different animals were

Keratocan Knockout Mice

FIG. 1. Western blotting analysis of the anti-mouse keratocan antibodies. Mouse corneas from postnatal day 1 (P1), day 7 (P7), and day 14 (P14) were extracted with 4 m guanidine-HCl, and extracts were subjected to gradient (4–15%) SDS-PAGE and “electrotransfer” with (D) or without (C) prior digestion with endo-β-galactosidase. Molecular mass markers are indicated on the left. Without endo-β-galactosidase treatment, keratocan appears as a smear. After enzyme digestion, keratocan appears as a ~50-kD single band (arrow).

Transcription Electron Microscopy—Corneas from keratocan-null and age-matched wild-type controls were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1% sodium cacodylate, pH 7.4, with 8.0 m CaCO3, pH 7.4 for 20 min. Finally the ribonucleoproteins were quantified by spectrophotometer, and 5 μg/ml was used for the hybridization. The mouse eyes and embryos were fixed with 4% parafomaldehyde at 4 °C and embedded in paraffin. The in situ hybridization protocol was performed on paraffin sections (5 μm) mounted on SuperFrost/Plus microscope slides (Fisher Scientific) as described previously (19). To remove nonspecifically bound probes, slides were sub- jected to a stringent wash with 0.5% SSC at 65 °C and treated with 20 μg/ml RNase (Sigma) at room temperature for 1 h followed by washing with 0.2× SSC at 65 °C. The hybridization signals were visualized with anti-digoxygenin antibody-alkaline phosphatase conjugates using pro- cedures recommended by Roche Diagnostics.

Transmission Electron Microscopy—Corneas from keratocan-null and age-matched wild-type controls were fixed in 4% paraformalde- hyde, 2.5% glutaraldehyde, 0.1% sodium cacodylate, pH 7.4, with 8.0 m CaCO3, pH 7.4 for 2 h on ice. The corneas were dissected and postfixed with 1% osmium tetroxide for 1 h. After dehydration in a graded ethanol series followed by propylene oxide, the corneas were infiltrated and imbedded in a mixture of Embed 812, nardy methyl anhydride, dodec- nylsuccinic anhydride, and DMP-30 (EM Sciences, Fort Washington, PA). Thick sections (1 μm) were cut and stained with methylene blue- azur blue for examination and selection of specific regions for further analysis. Thin sections were prepared using a Reichert UCT ultrami-icrotome and a diamond knife. Staining was with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid, pH 7.2 Sections were examined and photographed at 80 kV using a Hitachi 7000 transmission electron microscope. The microscope was calibrated using a line grating.

Fibril Diameter Measurements—The corneal stroma was divided into two regions for analysis: anterior and posterior stroma. The anterior stroma was defined as the 10 μm subjacent to the epithelium, and the posterior stroma was defined as the 10 μm adjacent to Descemet’s layer. Micrographs of appropriate regions were taken at 48,700× magnification. Calibrated micrographs from each region were randomly chosen in a masked manner. The micrographs were digitized, and diameters were measured using an R&M Biometrics-Bioquant (Memphis, TN) image analysis system.

Statistical Methods—In the initial analyses of fibril diameter, the fibril diameter measurements from two mutant animals were analyzed separately, and no significant differences were observed between the two. Therefore, in the final analyses data from different animals were

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2 See csbd.nidcr.nih.gov/csbd/frame_reagents.htm on the World Wide Web.
**Keratocan Knockout Mice**

A, the targeting vector, kera-TV, contains a 3.1- and a 1.5-kb homologous sequence on 5′ and 3′ ends, respectively. A 1.4-kb fragment from the XhoI site in intron 1 to a KpnI site in exon 2 was deleted and replaced with a positive selection marker, neomycin resistance gene (pgkpr-neopa) cassette in the antisense orientation with respect to the Kera gene. A negative selection marker gene, diphtheria toxin A fragment (pgkpr-Dta) cassette, was placed on the 3′ end of the targeting vector.

**Results**

Accumulation of Sulfated KS-keratocan in Cornea during the 1st and 2nd Week after Birth—To characterize keratocan protein expression in the wild-type mice, corneas were obtained from day 1, 7, and 14 mice. The keratocan core protein was detected by SDS-PAGE (10%). Western blotting analyses were performed with affinity-purified and epitope-specific antibody against keratocan.

### Fig. 2. Generation of Kera knockout mice via gene targeting.

A, the targeting vector, kera-TV, contains a 3.1- and a 1.5-kb homologous sequence on 5′ and 3′ ends, respectively. A 1.4-kb fragment from the XhoI site in intron 1 to a KpnI site in exon 2 was deleted and replaced with a positive selection marker, neomycin resistance gene (pgkpr-neopa) cassette in the antisense orientation with respect to the Kera gene. A negative selection marker gene, diphtheria toxin A fragment (pgkpr-Dta) cassette, was placed on the 3′ end of the targeting vector.

B, schematic diagram showing the organization and the restriction enzyme map of the mouse Kera gene. The three exons are represented as boxes and indicated by numbers. C, the predicted structure of a targeted mutant allele (KO) after homologous recombination. The primers used for identification of allele-specific recombination in PCR are also shown. D, the table shows the expected size of a 5′ PCR and a 3′ PCR product amplified by various primer pairs to distinguish wild-type and targeted mutant allele. E, PCR-based genotyping analysis using tail DNA. PCR was carried out with the Expand Long Template PCR system (Roche Diagnostics). The primers used for 5′ PCR were as follows: kera-1, 5′-gatggcctagtcggccatcactgcaaagag-3′; kera-2, 5′-ccacagagtattaaacagtttggggttgc-3′; neo (+), 5′-gctctctgtggtattagggggttcg-3′. The primers used for 3′ PCR were as follows: kera-3, 5′-cagacatggcgttaggtgtgtcgtgg-3′; kera-4, 5′-cgatggttagcaagaggggttgg-3′; pgkpr (+), 5′-gsgasagagagatgggtaggtg-3′. Amplification of the 5′ PCR product was performed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 7 min. Amplification of 3′ PCR product was identical except that the extension time at 72 °C was 3 min.

### Fig. 4. Keratocan but not lumican is missing in the Kera−/− cornea.

10 μg of 4 M guanidine-HCl protein extract was subjected to SDS-PAGE (10%). Western blotting analyses were performed with affinity-purified and epitope-specific antibody against keratocan. There was no keratocan detected (lanes 5 and 6), but lumican expression was not changed (lanes 7–12). U, undigested sample; D, endo-β-galactosidase-digested sample.

The analysis based on the full data (excluding outliers) is reported. The number of fibrils measured was 348 and 352 for the anterior regions and 783 and 897 for the posterior regions. All analyses were performed using SAS, version 8.2, software (SAS Institute Inc., Cary, NC).

**Fig. 3. In situ hybridization analysis of Kera+/+ and Kera−/− eyes.** Indigo color represents positive reaction of digoxigenin-labeled mouse antisense probes specifically hybridized to Kera mRNA (A–D) or Lum mRNA (E and F) on paraffin sections (5 μm). Kera mRNA was expressed specifically by the keratocytes in Kera+/+ (A and C) but was totally undetectable in the Kera−/− cornea (B and D cornea). Lum mRNA expression level, on the other hand, has no obvious difference between Kera+/+ and Kera−/− (E and F). It should be noted that Kera deficiency has a thinner cornea and a narrower cornea-iris angle. AC, anterior chamber; ep, epithelium; st, stroma. Scale bar represents 150 μm.

Combined, the field-to-field variability and correlation among measurements from the same field were accounted for by fitting a linear mixed effect model. For all but 2 of 24 fields, the assumption of normal distribution was appropriate after excluding 4.5% of the outliers. We fit the mixed effect model twice, with and without the two fields in question, and obtained similar estimates and consistent p values. Therefore,
are viable and fertile without any apparent abnormal phenotypes such as hazy cornea at the age of 4 weeks. *In situ* hybridization analyses showed that keratocan mRNA was completely undetectable in the *Kera* KO cornea, whereas the lumican mRNA expression level showed no difference between the wild-type and the *Kera* KO corneas (Fig. 3). Likewise the keratocan protein was completely missing as judged by an immunohistochemical staining (data not shown). As shown by the light microscopic picture (Fig. 3), the cornea-iris angle of the anterior segment was 30–40% narrower, and the corneal stroma was 40% thinner in the *Kera* KO as compared with the wild-type littersmates.

Western blotting analyses of the corneal extracts from 1-month-old animals also demonstrated that no keratocan protein could be detected in the *Kera* KO mice indicating *Kera* KO revealed a keratocan-null background (Fig. 4). The expression of lumican, fibromodulin, and decorin in the *Kera* KO cornea as determined by Western blotting analyses showed no significant alterations between the wild-type and the *Kera* KO corneas (Fig. 5).

**Ultrastructural Analysis**—The corneal stromata from keratocan-deficient and wild-type 7.5-month-old mice were analyzed using transmission electron microscopy. Since the lumican-deficient mouse had a spatially restricted phenotype in the corneal stroma (13), the anterior and posterior stromata were analyzed separately. Qualitatively there were consistent differences between the keratocan-deficient and wild-type stromata (Fig. 6). First, the collagen fibrils were larger in keratocan-deficient stromata. Second, there was less regular fibril packing in the deficient stromata. This was true throughout the stroma with no apparent differences between anterior and posterior stroma. The collagen fibrils in the keratocan-deficient stromata had regular, circular profiles characteristic of normal fibrils (Fig. 6). This was true for fibrils in both the anterior and posterior stroma. However, fibril diameters in both regions were larger in the mutant animals. Examination of the frequency distributions of collagen fibril diameters from wild-type and homozygous mutant mice indicates that mutant mice have significantly larger diameters in both the anterior and posterior regions of the corneal stroma (Fig. 7). Both the wild-type and mutant animals had a normal distribution of fibril diameters. There were no obvious populations of fibrils that were significantly different from the wild-type controls in either anterior or posterior regions of the mutant corneas. However, the distributions in both anterior and posterior regions of the mutant stromata showed a significant shift to larger diameters compared with the wild-type controls. The anterior fibrils had a mean diameter of 26.6 nm in the wild-type versus 30.7 nm in the keratocan-deficient stromata. This difference of 4.1 nm (95% confidence interval: 1.7, 6.7) was significant (*p* = 0.01). Likewise in the posterior stroma, fibrils had a mean diameter of 26.9 nm in the wild-type versus 32.0 nm in the mutant stromata. This difference of 5.1 nm (95% confidence interval: 3.8, 6.3) also was significant (*p* < 0.001). There was no statistical difference between anterior and posterior regions in the normal stroma; however, in the keratocan-deficient stromata the difference between anterior and posterior (1.2 nm 95% confidence interval: 0.3, 2.2) is significant (*p* = 0.02), suggesting that the posterior region may be affected to a greater degree by the keratocan deficiency.

**DISCUSSION**

In mice, eyelids are closed at birth and open in the 2nd week of postnatal age. We observed that the increase of protein level of keratocan during the 2nd postnatal week of the mouse coincided with the opening of the eyes at this stage (Fig. 1). However, the maximum accumulation of keratocan protein in the...
cornea does not correspond with the peak of keratocan mRNA (16). It is likely that the synthesis of keratocan may be regulated at translational and/or post-translational levels. This phenomenon has also been found in lumican (18) and collagen biosynthesis (20).

Neural crest cells are important to the morphogenesis of various tissues during embryonic development. In ocular tissues, neural crest cells contribute to corneal stroma, iris, and eyelid mesenchyme. We have showed previously that keratocan expression is restricted to the keratocytes after embryonic day 16.5 (E16.5), but it is also transiently expressed by different sublineages of neural crest cells in several non-ocular tissues such as snout, ear, limb, and diaphragm during E13.5–E16.5. Keratocan knockout mice are viable and fertile, indicating that keratocan is dispensable in tissues that transiently express Kera during embryonic development. The expression levels of other SLRP members including lumican, decorin, and fibromodulin were not altered in the Kera−/− cornea as compared with that of the wild-type littermates, suggesting that the phenotypic changes observed in the Kera−/− mice resulted exclusively from the loss of function of Kera. Genetic evidence has shown that mice lacking a transcription factor, Lmx1b, decrease keratocan mRNA synthesis and exhibit abnormal corneal collagen fiber morphology at E15.5 (21). However, we did not find any significant alteration in collagenous matrix of the Kera−/− cornea at this stage (data not shown). The discrepancy of our data from the Lmx1b knockout mice is not known, but it is likely that ablation of Lmx1b actually may perturb the expression of genes other than Kera that are involved in collagen fibrillogenesis.

The corneas among keratocan, lumican, and mimecan/osteoglycin knockout mice show different but overlapping phenotypes. Lumican knockout mice have the most severe phenotypes with age-related corneal opacity, disorganized collagen fibrils, and thinner corneal stroma (12–14). Mimecan/osteoglycin knockout mice have slightly larger but uniform collagen fibrils with transparent cornea (22). Keratocan knockout mice also reveal larger diameters of corneal collagen fibers but less organized packing. However, like lumican-deficient mice, keratocan-null mice have a thinner corneal stroma and a narrower cornea-iris angle. Although the keratocan expression level is comparable to lumican in the corneal stroma, keratocan does not complement lumican functions and alleviate the corneal phenotype observed in the lumican-null mice. This result suggested that keratocan and lumican might bind to different sites of collagen fibril. The reduced thickness of corneal stroma seen in the keratocan-null mice was likely due to reduced hydration caused by a decrease of keratan sulfate proteoglycan content in Kera−/− cornea. Mimecan/osteoglycin expression level is the lowest in the cornea as compared with keratocan and lumican. Although lumican, keratocan, and mimecan/osteoglycin have been known to be corneal KS-LSLRPs in various species including human, bovine, and chick, it has been shown that, unlike lumican and keratocan, mimecan has no KS chain in mouse cornea. This may explain the subtle phenotypes observed in the cornea of mimecan knockout mice.

Human corneal plana (CNA2) is an autosomal recessive inherited disease that is directly linked to the mutation of KERA. So far at least three mutations have been identified. Forty-six Finnish patients are homozygous for a founder having AAC to AGC transition at codon 247 predicting an amino acid change from Asn to Ser. This amino acid substitution affects the Asn residue in the tandem leucine-rich repeat consensus motif (LXXLXLLXXNL) (15). Four affected Bangladesh patients are homozygous of sequence change from ACA to AAA at codon 215, which results in an amino acid change from Thr to Lys in the linker region between the sixth and seventh tandem leucine-rich repeat motif (23). In an American CNA2 patient of Chinese origin, a homozygous CAG to TAG transversion was found in exon 2 that causes a change of Gln to a stop codon, predicting a truncated protein of 173 amino acids (15). It is likely that all these mutations will yield a mutant keratocan gene product that may be quickly degraded in the cornea because they do not exhibit a dominant negative effect in a heterozygous mutant. To test this hypothesis, transgenic mice harboring recombinant human KERA cDNA (wild type or mutants) bred into a mouse with Kera-null background would become an ideal model to investigate the structure-function relationship of keratocan in vivo.

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References
1. Linsenmayer, T. F., Fitch, J. M., and Birk, D. E. (1990) Ann. N. Y. Acad. Sci. 580, 114–160
2. Hay, E. D. (1980) Int. Rev. Cytol. 63, 263–322
3. Doane, K. J., Babiarz, J. P., Fitch, J. M., Linsenmayer, T. F., and Birk, D. E. (1992) Exp. Cell Res. 202, 82–95
4. Hahs, R. A., and Birk, D. E. (1992) Development 114, 383-393
5. Haida, J. A., Cornwell, P. K., and Hassell, J. R. (1993) Exp. Eye Res. 56, 615–648
6. Weber, I. T., Harrison, R. W., and Izoz, R. V. (1996) J. Biol. Chem. 271, 31767–31770
7. Iozzo, R. V. (1997) Crit. Rev. Biochem. Mol. Biol. 32, 112–174
8. Scott, J. E. (1996) Biochemistry 35, 8795–8799
9. Bettelheim, F. A., and Plessy, B. (1975) Biochim. Biophys. Acta 381, 203–214
10. Rawe, I. M., Tuft, S. J., and Meek, K. M. (1992) Histochem. J. 24, 311–318
11. Hassell, J. R., Cintrun, C., Kublin, C., and Newsome, D. A. (1983) Arch. Biochem. Biophys. 222, 362–369
12. Chakravarti, S., Magnuson, T., Lass, J. H., Jepsen, K. J., LaMantia, C., and Carrell, H. (1998) J. Cell Biol. 112, 987–996
13. Chakravarti, S., Petroll, W. M., Hassell, J. R., Jester, J. V., Lass, J. H., Paul, J., and Birk, D. E. (2000) Investig. Ophthalmol. Vis. Sci. 11, 3365–3373
14. Suika, S., Shiraishi, A., Liu, C.-Y., Funderburgh, J. L., Kao, C. W., Converse, R. L., and Kao, W. W.-Y. (2000) J. Biol. Chem. 275, 2607–2612
15. Pellegata, N. S., Dieguez-Lucena, J. L., Joensuu, T., Lau, S., Montgomery, K. T., Krahe, R., Kivela, T., Kucherlapati, R., Forsius, H., and de la Chapelle, A. (2000) Nat. Genet. 25, 91–95
16. Liu, C.-Y., Shiraishi, A, Kao, C. W., Converse, R. L., Funderburgh, J. L., Corpuz, L. M., Conrad, G. W., and Kao, W. W.-Y. (1998) J. Biol. Chem. 273, 22584–22588
17. Fisher, L. W., Stabbs, J. T., III, and Young, M. F. (1995) Acta Orthop. Scand. Suppl. 266, 61–65
18. Ying, S., Shiraishi, A., Kao, C. W., Converse, R. L., Funderburgh, J. L., Swiergiel, J., Roth, M. R., Conrad, G. W., and Kao, W. W.-Y. (1997) J. Biol. Chem. 272, 30306–30313
19. Smith, R. S., Zabeleta, A., John, S. W. M., Bechtold, L. S., Ikeda, S., Relyea, M. J., Sundberg, J. P., Kao, W. W.-Y., and Lu, C.-Y. (2002) in Systematic Evaluation of the Mouse Eye: Anatomy, Pathology and Biomethods (Smith, R. S., ed) pp. 285–297, CRC Press, Boca Raton, FL
20. Kao, W.-W.-Y., Mai, S. H., Chou, K. L., and Ebert, J. (1983) J. Biol. Chem. 258, 7779–7787
21. Pressman, C. L., Chen, H., and Johnson, R. L. (2000) Genesis 26, 15–25
22. Tasheva, E. S., Koester, A., Paulsen, A. Q., Garrett, A. S., Boyle, D. L., Davidson, H. J., Song, M., Fox, N., and Conrad, G. W. (2002) Mol. Vis. 8, 407–415
23. Lehmann, O. J., El-ashry, M. F., Ebenezer, N. D., Osakam, L., Francis, P. J., Wilkin, S. E., Patel, R. J., Ficker, L., Jordan, T., Khaw, P. T., and Bhattacharya, S. S. (2000) Invest. Ophthalmol. Vis. Sci. 42, 3118–3122
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