HHS Public Access

Author manuscript

Exp Eye Res. Author manuscript; available in PMC 2019 February 01.

Published in final edited form as:

Exp Eye Res. 2018 February ; 167: 18–24. doi:10.1016/j.exer.2017.11.002.

Expression of potassium-dependent sodium-calcium exchanger in the murine lens

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Abstract

Loss of intracellular calcium homeostasis may contribute to the opacification of lens tissue during cortical cataract formation. In healthy lenses, the concentration of intracellular calcium is maintained at levels far below electrochemical equilibrium but the identity of the calcium extrusion mechanism in lens fiber cells has remained elusive. Previous studies focused on the role of plasma membrane calcium ATPases and sodium-calcium exchangers. Here, we examined the expression of mRNA transcripts encoding potassium-dependent sodium-calcium exchangers (Nckx's, encoded by the *Slc24* gene family) in the mouse lens. The most abundant of the five *Slc24* family members was *Slc24a4* (Nckx4). Notably, *Slc24a4* was the only family member with increased expression in fiber cells. Using an antibody raised against recombinant mouse Nckx4, we showed that the protein is expressed strongly in the outer cortical fibers, consistent with results of in situ hybridization experiments and earlier mass spectrometry analysis. To test the role of Nckx4 directly, we generated mice in which *Slc24a4* was deleted conditionally in lens tissue. In conditional knockout animals, the level of Nckx4 protein was reduced to background levels without a discernible effect on lens growth or transparency. Thus, despite its relative abundance in the lens, Nckx4 does not appear to have an indispensable role in the maintenance of lens clarity.

Introduction

The concentration of calcium $[Ca^{2+}]_o$ in the humors surrounding the human lens is 1.34 mM (Ringvold et al., 1988) but the free calcium concentration $[Ca^{2+}]_i$ within the lens is orders of magnitude lower, varying from ≈ 100 nM in the epithelium (Riach et al., 1995) to 0.5–1 μM in the innermost fiber cells (Duncan et al., 1989; Gao et al., 2004). Lens cell membranes maintain a negative potential. In mice, this varies from ≈ −70 mV in the outer fiber layers to ≈ −60 mV in the innermost cells (Gong et al., 1998). The membrane potential of the young human lens is ≈ −50 mV, declining to −30 mV by 60 years of age (Duncan et al., 1989). Thus, in all cases $[Ca^{2+}]_i$, is maintained at levels far below electrochemical equilibrium.

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It has long been suspected that calcium accumulation contributes to lens pathology (Duncan and Jacob, 1984). An analysis of lenses with cortical opacities, for example, revealed that total calcium and free calcium were both elevated significantly, in comparison to clear, age-matched control lenses (Duncan and Bushell, 1975). Lens opacification can also be induced in vitro, by treatment with calcium ionophores (Sanderson et al., 1996, 2000) and blocked by co-incubation with EGTA, a calcium chelator (Sanderson et al., 2000). In animal models, calcium-activated proteases (calpains) have been implicated in the lens opacification process (Shearer et al., 1997; Tang et al., 2007), although their role in human cataractogenesis is less well established (Nakajima et al., 2006). Prompted by these and other observations, there has been a longstanding effort to identify the membrane-based mechanisms that contribute to calcium regulation in the lens.

In most cells, calcium is removed from the cytosol through the concerted activity of plasma membrane Ca^{2+} ATPases (PMCAs) and calcium exchangers. There is evidence that both mechanisms are present in the lens. PMCAs use energy derived from ATP hydrolysis to drive calcium from the cell (one Ca^{2+} is transported for every molecule of ATP hydrolyzed). Four isoforms (PMCA1-4) are known (Brini et al., 2013). All of them are expressed to some degree in lens epithelial cells (Marian et al., 2005; Marian et al., 2008), although expression is probably low compared to most other cell types (Lattin et al., 2008). The epithelium is the most metabolically active layer in the lens but constitutes only a small fraction of the total tissue volume. It is unclear, therefore, if the activity of PMCAs located in the epithelium is sufficient to regulate $[Ca^{2+}]_i$ throughout the lens or whether autonomous calcium extrusion mechanisms exist in the fiber cells. Significantly, PMCA expression is undetectable in fiber cells by PCR (Marian et al., 2008) and PMCAs were not among the transport proteins identified in the membrane proteome of mouse (Bassnett et al., 2009) or human (Wang et al., 2013) lens fiber cells.

Calcium exchangers can be classified into two main groups (Lytton, 2007): sodium-calcium exchangers (Ncx) and potassium-dependent sodium-calcium exchangers (Nckx). The best characterized are Ncx proteins (encoded by the *Slc8* gene family) which utilize the inwardly directed sodium gradient to drive calcium efflux ($3Na^+$: $1Ca^{2+}$ (Khananshvili, 2013)). There are three Ncx isoforms (Ncx1-3, encoded by *Slc8a1-3*) but only Ncx1 has been detected in lens. Western blot analysis identified Ncx1 in epithelial cell lysates from the rabbit lens (Tamiya and Delamere, 2006) and mass spectrometry has identified Ncx1 in fiber cell membranes prepared from the mouse lens (Bassnett et al., 2009). Nckx exchangers operate electrogenically, with a stoichiometry of $4Na^+$:($1Ca^{2+}$ + $1K^+$). Nckx exchangers use both the inward sodium gradient and the outward potassium gradient to drive calcium extrusion (although, if the sodium gradient collapses the mechanism can operate in reverse, leading to calcium accumulation). There are five members of this family (Nckx1-5; encoded by *Slc24a1-5*). Nckx1 was the first to be discovered and remains the best-studied family member, with an especially important role in the outer segments of vertebrate retinal rod photoreceptors (Schnetkamp, 2013). Unlike PMCA's, which are high affinity, low-capacity pumps, Ncx and Nckx represent low affinity, high-capacity calcium extrusion mechanisms (Clapham, 2007). They would appear well suited to regulating calcium in lens cells, where resting levels are higher than in many cell types. In neuronal cells, calcium exchangers are

known to play a role in the rapid removal of calcium from the cytoplasm during the generation of action potentials (Lee et al., 2002).

An Nckx family member, Nckx4 (encoded by *Slc24a4*), was detected in a mass spectrometric analysis of lens fiber cell membrane proteins (Bassnett et al., 2009). In that study, peptides derived from Nckx4 were more abundant than Ncx1 peptides, suggesting that Nckx4 could contribute significantly to lens fiber cell calcium extrusion. Visualizing the distribution of Nckx4 in the lens has been problematic, however, because commercially available antibodies against Nckx4 have generally proved to be neither sensitive nor specific (Bronckers et al., 2017).

In the current study, we quantified the expression of *Slc24a1-5* by quantitative PCR (Q-PCR). The expression of *Slc24a4*, the most abundant of the family members, was visualized using in situ hybridization. We also examined the distribution of the Nckx4 protein by immunofluorescence, employing a newly generated antibody, and analyzed the phenotype of mice in which the *Slc24a4* locus was conditionally inactivated in the lens.

2. Methods

2.1. Mice

Wild type mice (C57BL/6J) were obtained from the Jackson Lab (Bar Harbor, ME). Adult animals (1-month-old to 1-year-old) were killed by CO_2 inhalation, embryos were decapitated. The Animal Studies Committee at Washington University approved all procedures. To delete Nckx4 specifically in lens, we crossed *Slc24a4*$^{fl/fl}$ mice (Stephan et al., 2012) with *MLR10* mice; a transgenic line in which cre-recombinase is expressed specifically in lens epithelial and fiber cells (Zhao et al., 2004).

2.2. Quantitative PCR (Q-PCR)

For PCR analysis, lenses were dissected into epithelial and fiber cell samples. RNA was purified from pooled samples of 10 lenses from one-month-old wild type or *Slc24a4*$^{fl/fl}$;*MLR10*$^{-/Tg}$ mice using RNAeasy (Qiagen), and cDNA synthesis was performed with 0.5 – 1.0 μg of RNA as template (iScript reverse transcription kit; BioRad). Quantitative PCR (Q-PCR) experiments were designed and performed according to MIQE guidelines (Bustin et al., 2009) using SsoFast EvaGreen Supermix (Biorad). *Slc24a1-5* expression was compared initially to the expression of genes encoding two lens house-keeping enzymes (*Gapdh* and *Ppia*) and subsequently normalized to the expression of *Slc24a4* in the lens fiber cell sample.

The Q-PCR primers utilized in this study were:

Slc24a1 F 5′-CAGCCTATTAAACTGGCCGTC-3′, R 5′-TCCATTGCTATGCTAGGTGCT-3′;

Slc24a2 5′-GTCAATTCGAGTCATTGGCCT-3′, R 5′-TGTTGCTCTGAGAGTCTGTCT-3′;

Slc24a3 F 5′-CTGTGGTCGCACTCATTGTGT-3′, R 5′-TGGTGTATGCACGCATTGTATT-3′;

Slc24a4 (set 1) F 5′-GATTCTCAGCTCTAGCCCTCC-3′, R 5′-GCCATCCGTAGTCGAGTCCT-3′;

Slc24a4 (set 2) F 5′-TCGACACCAGAGCTGTTT-3′, R 5′-TGTCCAGCAAAGAGTCCA-3′

Slc24a5 F 5′-TTTGGGTCTCTGCATACCTGC-3′, R 5′-CGGGAAACTCCGATGCTGG-3′;

Note that two sets of primers were used to amplify *Slc24a4*. *Set 1* was used to determine relative expression levels of *Slc24a4* in epithelial and fiber cells of wild type mice. *Set 2* was used to compare *Slc24a4* expression in lens fiber cells from wild type and *Slc24a4* conditional knockout mice.

In all cases, measurements were made in triplicate and the statistical significance of the results was assessed using Student's *t*-test.

2.3. In situ hybridization

Eyes were enucleated and a small hole made in the back of the globe to allow the fixative to better penetrate the tissue. Samples were fixed for 24 h in 4% paraformaldehyde/PBS, dehydrated, embedded in paraffin, and sectioned in the mid-sagittal plane at 4 μm. Target probe sets were generated against a ≈1 kb region (nt 91-1093) of the *Slc24a4* gene (accession number 172152.2) and hybridized to the sections, as described (Shi et al., 2013; Wang et al., 2012). Following proprietary pre-amplification and amplification steps (RNAscope 2.0, Advanced Cell Diagnostics, Hayward, CA), correctly targeted probes were detected using an alkaline phosphatase-conjugated label probe with Fast Red as a substrate and Gill's Hemotaoxylin as a counter stain. As a negative control, some sections were hybridized with a target probe against *DapB*, a bacterial gene encoding dihydrodipicolinate reductase, an enzyme involved in lysine synthesis.

2.4. Immunofluorescence

An antibody against mouse Nckx4 was produced in rats using a recombinant His-tagged Nckx4 (from amino acid 229 to 366) as described (Vinberg et al., 2017). To visualize the expression of Nckx4 protein, eyes were lightly fixed with 4% paraformaldehyde/PBS for 15 minutes at room temperature, cryoprotected for 1 hour in 30% sucrose/PBS, and rapidly frozen in OCT (optimal cutting temperature compound; VWR). Blocking and antibody incubation were performed in 2% bovine serum albumin, 2% goat serum and 0.1% Triton X-100 in PBS. Anti-Nckx4 was used at 1:100 dilution. The secondary antibody was Alexa488-conjugated, goat anti-rat (Life Technologies, Carlsbad, Ca, 92008). Methyl Green was used as a nuclear counterstain. Samples were examined using an Olympus Fluoview 1000 confocal microscope.

3. Results

3.1 Q-PCR analysis of *Slc24* family members

The expression of *Slc24a1-5* in epithelial and fiber cells was assessed by Q-PCR and endpoint PCR (Figure 1). Expression of *Slc24a1* and *Slc24a5* was barely detectable in the wild type lens (Figure 1A). *Slc24a2* was expressed modestly in both epithelial and fiber cells. *Slc24a3* was relatively abundant in lens epithelium but its expression declined markedly in lens fiber cells. The most abundant of the potassium-dependent sodium-calcium exchangers in the lens (and the only family member with upregulated expression in the fiber cell compartment) was *Slc24a4* (encoding Nckx4). *Slc24a4* expression was 2–3 fold greater in fiber cells than in the epithelium. The expression of *Slc24a4* mRNA was also higher in lens fiber cells than in age-matched samples from brain (data not shown), a finding supported by published microarray analyses (Lattin et al., 2008). *Slc24a4* was cloned originally from brain where studies have identified an alternative transcript containing an additional 57-nucleotides (Li et al., 2002). Endpoint PCR resulted in the generation of two amplicons from lens cDNA but, interestingly, not from retinal DNA (Figure 1B). Sequencing confirmed that the larger of the two transcripts corresponded to the alternative transcript identified previously in brain (Li et al., 2002). Thus, lens cells express both the short and long *Slc24a4* mRNA transcripts. Q-PCR was also used to examine the expression of *Slc24* family members in lenses deficient in *Slc24a4* (Figure 1C). Those measurements confirmed that *Slc24a4* transcripts were largely eliminated from the knockout lenses (residual expression of Slc24a4 in the knockout lenses may have reflected low level mosaicism in the MLR10 cre-line, as reported by others (Kerr et al., 2014)). Significantly, there was no evidence for upregulation of other *Slc24* genes in the nominal absence of the *Slc24a4* transcript.

3.2 In situ hybridization analysis of *Slc24a4* expression in the lens

In situ hybridization analysis was used to localize *Slc24a4* expression within the developing lens. A strong hybridization signal was detected within the primary fiber cells (but not the epithelium) as early as embryonic day 12 (E12; data not shown). By E16.5, *Slc24a4* mRNA was detected in nucleated secondary lens fiber cells (Figure 2A). Expression was low in the central fiber cells which, at that stage of development, are undergoing nuclear degradation (Vrensen et al., 1991). *Slc24a4* mRNA expression in epithelial cells at E16.5 was almost undetectable, but upon fiber cell differentiation, transcripts rapidly accumulated, such that even the outermost fibers expressed *Slc24a4* strongly. At postnatal day 30 (P30; the oldest age examined) fiber cells continued to express *Slc24a4*. At that age, expression was restricted to fibers in the outer lens cortex (Figure 2B). Cells in the inner cortical region bordering the organelle-free zone (OFZ) of the lens did not express *Slc24a4* mRNA, nor did cells within the OFZ itself. Lens tissue did not hybridize with probes against *Dapb*, a bacterial gene, confirming the specificity of the in situ hybridization technique (Figure 2C).

The in situ hybridization results were generally in accordance with the Q-PCR measurements. However, we noted that while the Q-PCR data indicated modest expression of *Slc24a4* in the lens epithelium (Fig. 1A), the in situ hybridization data suggested that epithelial expression was very low (Fig. 2B). A plausible explanation for this discrepancy is

that the epithelial sample used for Q-PCR analysis may have contained a small contingent of newly-formed fiber cells. Young fiber cells express *Slc24a4* strongly and if even a few such cells were co-isolated with the epithelium during dissection, it would skew the Q-PCR epithelial expression data.

3.3. Immunofluorescence analysis

In situ hybridization data suggested that *Slc24a4* transcription was strongly upregulated during the early phases of fiber differentiation but that transcripts were absent from the nucleated cells of the inner cortex and the anucleated cells of the OFZ. We used an antibody raised against recombinant Nckx4 protein to localize Nckx4 in the lens. Preliminary studies suggested that the epitopes recognized by the antibody do not survive processing of paraffin embedded tissue or lengthy exposure to aldehyde fixative (data not shown). Consequently, to preserve antigenicity, we used a brief fixation method to stabilize the tissue prior to preparing cryosections of the lens. Because Nckx4 has been notoriously difficult to detect using immunologic techniques (Bronckers et al., 2017), it was important to include appropriate negative controls (in our case, tissue from conditional *Slc24a4* knockout mice) in the immunofluorescence experiments. Nckx4 protein was detected in fiber cells in the lens cortex (Figure 3). Positive immunofluorescence was also observed in fiber cells located within the OFZ. High magnification images of the surface regions of the lens confirmed the near absence of Nckx4 from the lens epithelium. In sections from $Slc24a4^{fl/fl}; MLR10^{tg/-}$ mice, the immunofluorescence was reduced to low, background levels, confirming the specificity of the antibody. Although Nckx4 was expected to reside in the plasma membrane, the immunofluorescence observed in our samples was diffuse in nature, encompassing both the cytoplasm and the fiber cell membranes. The significance of this observation is uncertain. It is possible that some of the cytoplasmic immunofluorescence emanated from protein associated with vesicular structures within the cell, as has been proposed for other lens membrane proteins (Petrova et al., 2015). Alternatively, the brief fixation/cryopreservation technique necessary to maintain the antigenicity of the Nckx4 epitope(s) might not have adequately preserved the subcellular structures.

3.4. Lens phenotype in Nckx4 deficient mice

To determine whether Nckx4 plays an essential role in lens homeostasis, we generated mice in which the *Slc24a4* locus was disrupted specifically in lens tissue and examined the resulting lens phenotype (Figure 4). To delete *Slc24a4* in the lens, we crossed $Slc24a4^{fl/fl}$ mice with MLR10 mice. The MLR10 line expresses cre-recombinase in both epithelial and fiber cells and has been used widely in conditional knockout studies. We examined lenses from one-month-old knockout mice and compared them with wild type or heterozygous littermates. In all cases, the size and transparency of the lenses were indistinguishable between genotypes, suggesting that Nckx4 does not have an indispensable role in the maintenance of lens clarity. We also examined lenses from older animals (9 and 12 months) to determine whether age-related opacities might develop in the absence of Slc24a4. There was no difference in lens size or transparency in age-matched wild type and knockout lenses.

4. Discussion

Previous studies of calcium extrusion mechanisms in the lens focused on the role of PMCA's and sodium/calcium exchangers. In the current work, we examined the expression of another class of calcium transport proteins, the family of potassium-dependent sodium/calcium exchangers (Nckx1-5 encoded by *Slc24a1-5*).

Slc24a4 was the most strongly expressed member of the *Slc24* family in the mouse lens. Outside the lens, the *Slc24a4* gene is also expressed in murine retina (Vinberg et al., 2017) and other regions of the central nervous system, as well as in lung tissue and vascular wall (Li et al., 2002). The findings in the current paper are consistent with results of an earlier proteomic study of mouse lens fiber cell membrane proteins, which showed that Nckx4 was a relatively abundant component of the fiber cell membrane proteome, and the only member of the Nckx family detected (Bassnett et al., 2009). Microarray analysis of murine tissues suggests that *Slc24a4* transcripts are expressed at higher levels in the lens than in any of the ninety one cell types examined (Lattin et al., 2008). Interestingly, in a recent study of human lens fiber cells, Nckx2 (rather than Nckx4) was the most abundant *Slc24* family member of the membrane proteome (Wang et al., 2013), implying that different mammalian species may express different members of the *Slc24* family in the lens. *Slc24* family members have distinct tissue distribution patterns (Schnetkamp, 2013) but, functionally, Nckx1-4 are almost indistinguishable (Jalloul et al., 2016). It is unclear, therefore, why certain family members are expressed in one tissue rather than another.

Lens expression of *Slc24a4* was detected as early as E12.5 (i.e., shortly after the completion of primary fiber cell elongation). At later stages, transcripts were restricted to the differentiating fiber cells located near the lens surface. The intracellular calcium concentration in the lens has been measured using microinjected Fura2 and varies from 300 nM in the outer layers to 700 nM in the core of the lens (Gao et al., 2004). Such a gradient is consistent with a model in which the calcium extrusion mechanism is concentrated near the lens surface.

The lack of a phenotype in *Slc24a4* deficient lenses suggests that either the protein is normally nonfunctional in the lens or that lens cells can effectively compensate for its absence. We favor the latter explanation and note that compensatory mechanisms have been reported in cone photoreceptors, where loss of either Nckx2 or Nckx4 has surprisingly mild effects on the cone photo-response (Sakurai et al., 2016; Vinberg et al., 2017), whereas absence of both proteins triggers cone degeneration. In our study, we did not find evidence that the transcription of other Slc24 family members was increased in the absence of Slc24a4. However, it is possible that the *activity* of other Slc24 family members was increased, compensating for the absence of Slc24a4. It is also possible that increased flux through the sodium/calcium exchanger Ncx1, a known component of the fiber cell membrane proteome, could help compensate for the absence of Nckx4.

We did not measure directly whether fiber $[Ca^{2+}]_i$ was elevated in *Slc24a4* conditional knockout lenses. Such measurements are technically challenging because the lens tissue is optically active, interfering with the fluorescent signals produced by microinjected calcium

indicators. However, large increases in cytosolic calcium in the knockout lenses appear unlikely. Work in human (Sanderson et al., 2000), bovine (Marcantonio et al., 1986), rat (Sanderson et al., 1996) and mouse lenses, suggests that lens transparency is very sensitive to increases in intracellular calcium concentration. For example, in mice deficient in the gap junction protein, Cx46, the concentration of calcium in the center of the lens increases from 700 nM to >1.5 μM (Gao et al., 2004) and there is a concomitant increase in light scatter in the central region of the lens. Opacification is likely due to calcium-induced activation of calpain 3, which results in proteolysis of spectrin, crystallins, and other critical substrates (Tang et al., 2007). Because the *Slc24a4* knockout lenses remained perfectly transparent (even in aged mice), it is reasonable to conclude that the absence of Nckx4 did not trigger a significant rise in intracellular calcium. Direct measurements of $[Ca^{2+}]_i$ would be necessary to confirm this point, however.

In summary, our data indicate that *Slc24a4* mRNA is expressed strongly in the mouse lens, in accord with immunofluorescence data and the findings of an earlier proteomic study (Bassnett et al., 2009). Significantly, Nckx4 appears to be the only *Slc24* family member with increased expression in the fiber cell population. The known physiological properties of the Nckx4 exchanger and the distribution of both the mRNA transcript and protein suggest that that Nckx4 may contribute to calcium homeostasis in lens fiber cells. However, conditional knockout of *Slc24a4* in the lens did not result is tissue opacification, suggesting that the absence of this potassium-dependent sodium/calcium exchanger is effectively compensated for by another calcium extrusion mechanisms.

Acknowledgments

Supported by National Institutes of Health Grants EY09852 and EY024607 (SB), Core Grant for Vision Research P30 EY02687; and an unrestricted grant to the Department of Ophthalmology and Visual Sciences from Research to Prevent Blindness. The authors are indebted to Dr. Michael Robinson (Miami University) for kindly providing the MLR10 mice and Dr. Vladimir Kefalov (Washington University School of Medicine) for the Nckx4 antibody.

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Highlights

- At least four potassium-dependent sodium-calcium exchangers are expressed in the mouse lens.
- Transcription of the most abundant family member, Nckx4, is upregulated in the outer cortical layers.
- Nckx4 protein expression persists in the organelle-free zone.
- Conditional knockout of *Slc24a4*, the gene encoding Nckx4, does not compromise lens transparency.

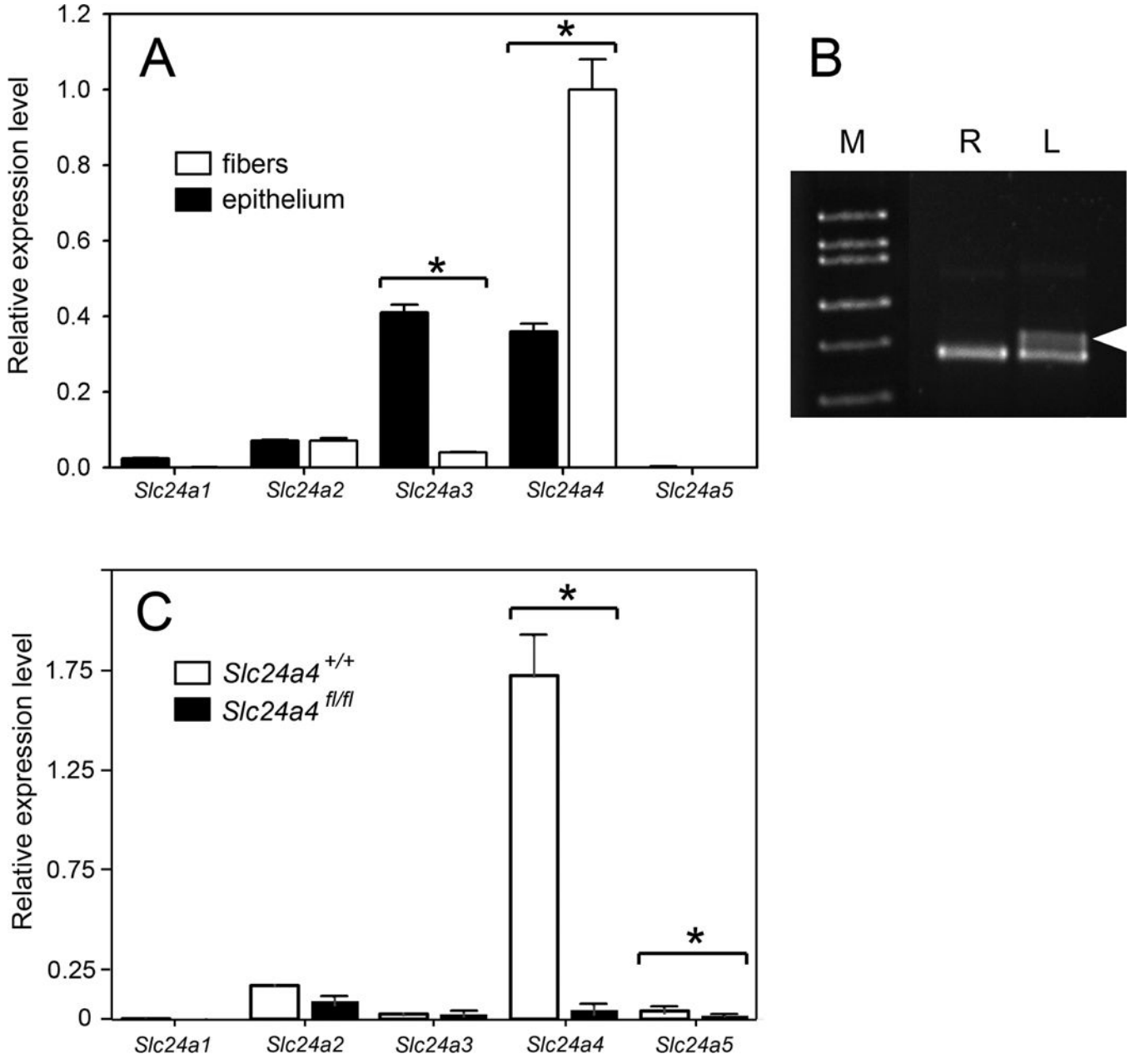

Figure 1.

Expression of potassium-dependent sodium-calcium exchanger mRNA in lens. (A) Q-PCR analysis of *Slc24a1-5* expression in wild type lens epithelial cells (filled bars) and fibers (open bars) normalized to *Slc24a4* expression in fiber cells. *Slc24a4* (encoding Nckx4) is the most abundantly expressed of the five *Slc24* genes and the only transcript to show increased expression in fiber cells. (B) Endpoint PCR analysis identifies an alternative *Slc24a4* transcript (arrowed) in lens (L) cDNA but not retinal (R) cDNA. (C) Q-PCR analysis of lenses from *Slc24a4* conditional knockout mice (filled bars) confirms the near-absence of the *Slc24a4* transcript (data are normalized to the expression in retina from mice of the

corresponding genotype). There is no evidence of compensatory upregulation of other family members in the absence of *Slc24a4*. (*) = $p < 0.01$

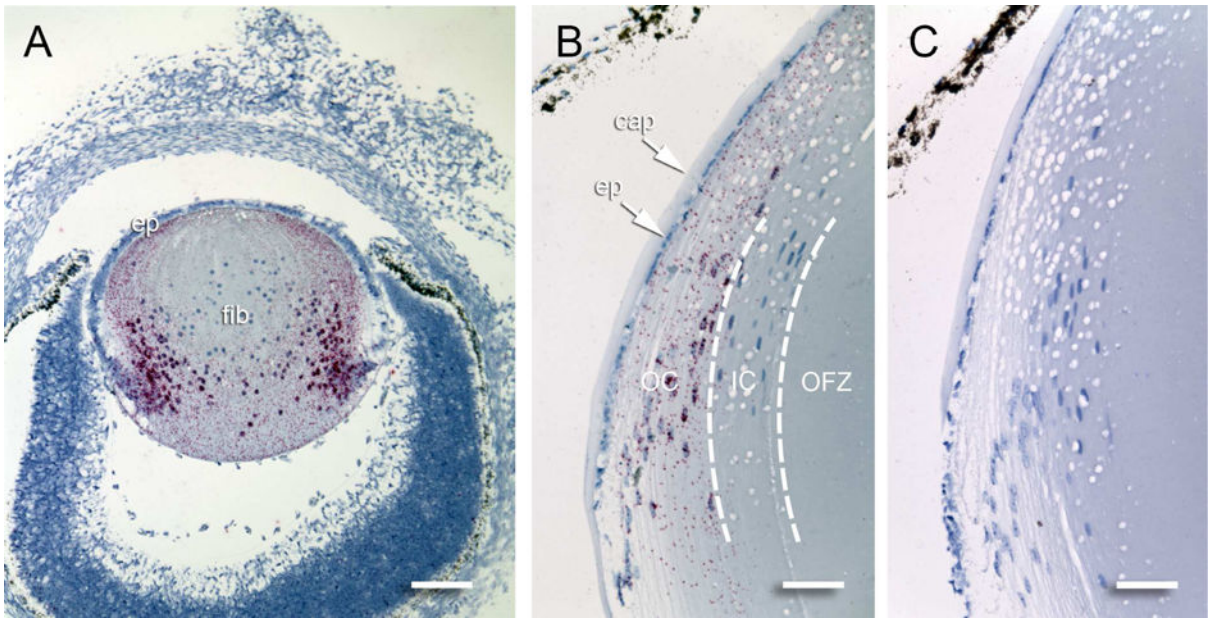

Figure 2.

In situ hybridization analysis of *Slc24a4* mRNA in the developing lens. *Slc24a4* transcripts (red dots) are abundant in lens fiber cells (but not epithelial cells) at E16.5 (A) and P30 (B). At both ages, expression is strongest in differentiating fiber cells. At P30, *Slc24a4* transcripts are restricted to the outer cortical layer. Transcripts are not detected in nucleated inner cortical fiber cells or in the organelle-free zone of the lens. Sections hybridized with probe against *DapB*, a bacterial gene and negative control, show no signal (C). ep, lens epithelium; fib, lens fibers; cap, capsule; OC, outer cortex; IC, inner cortex; OFZ, organelle-free zone. Scale bars: A=100 μm; B,C = 25 μm.

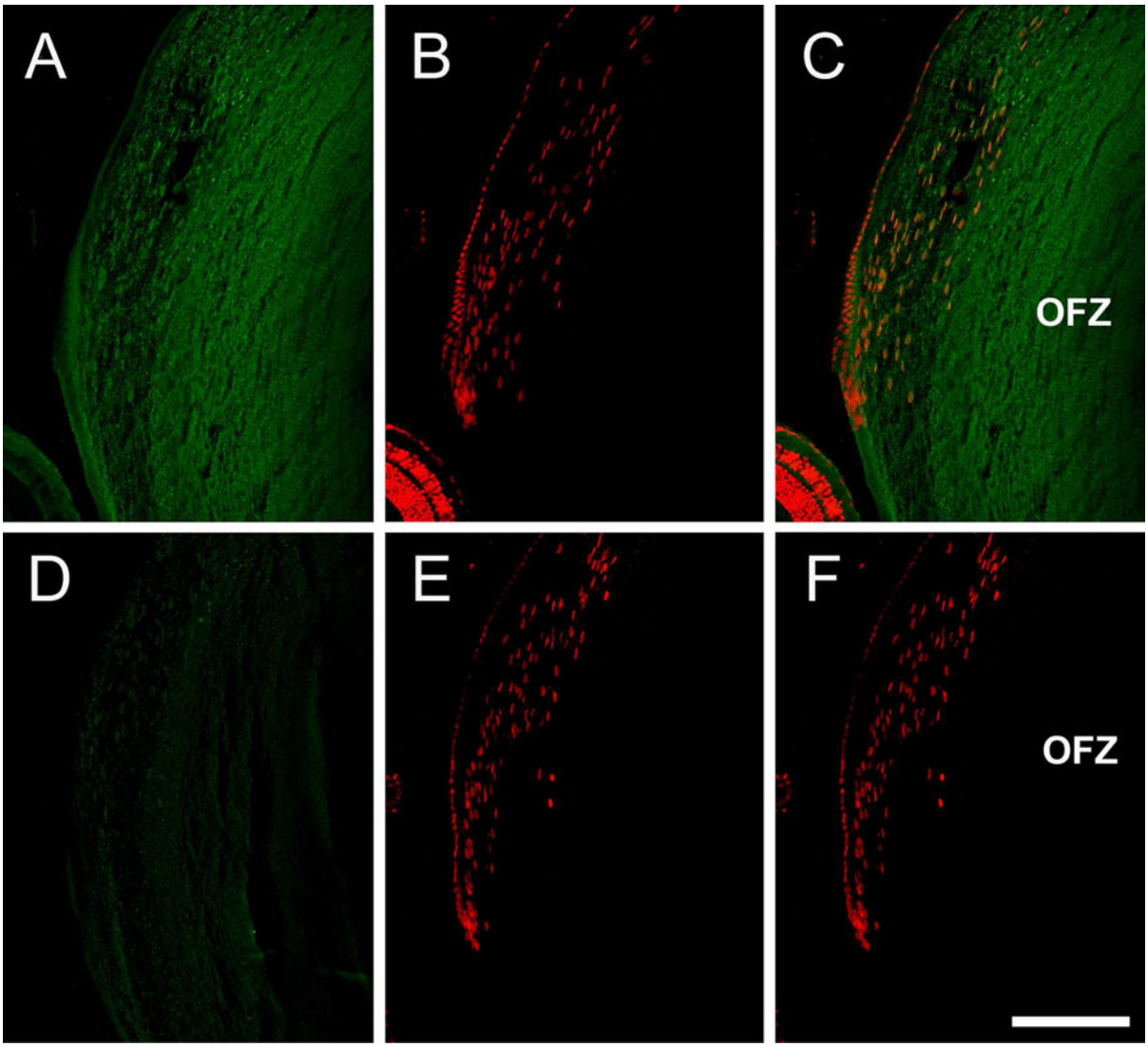

Figure 3.

Nckx4 expression in lenses from one-month-old mice. Lens cryosections were prepared from wild type mice (A–C) or animals in which *Slc24a4* was deleted conditionally in lens tissue (D–F). Sections were immunolabeled with anti-Nckx4 (green). Methylgreen (red) was used as a nuclear counterstain. In wild type lenses, immunofluorescence was detected in both nucleated and non-nucleated regions of the lens cortex. Immunofluorescence was reduced to low, background levels in lenses from conditional *Slc24a4* knockout mice. Scale bar = 250 μm.

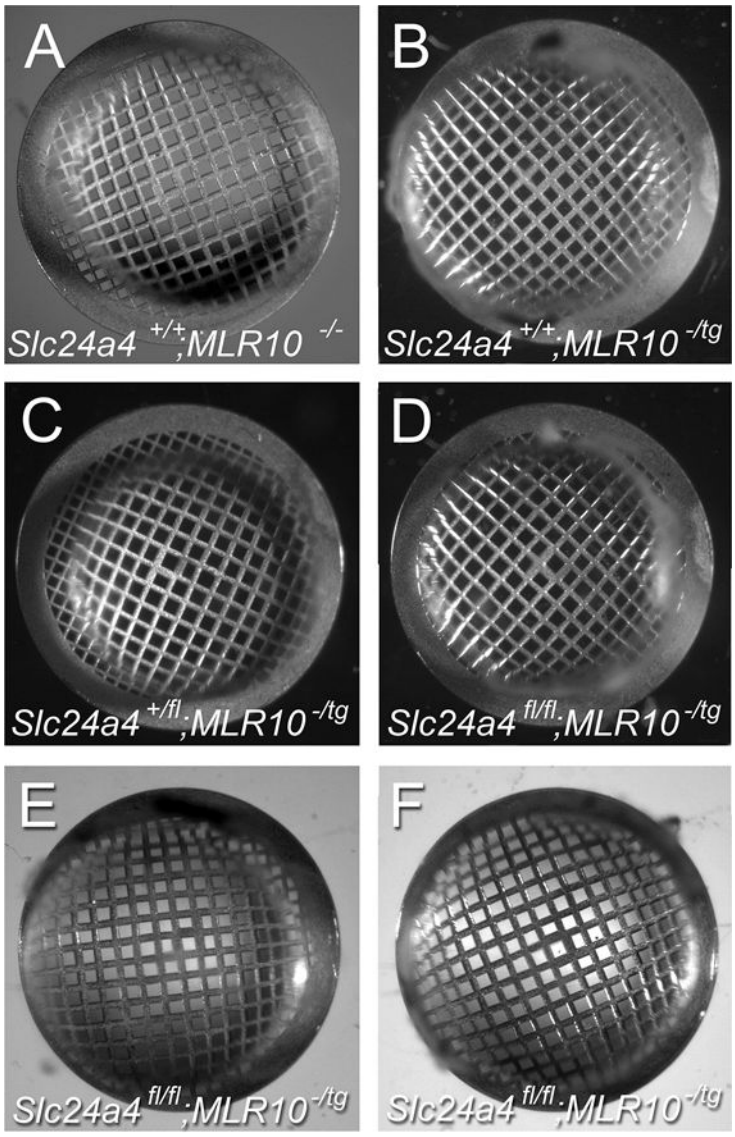

Figure 4.

Lens phenotypes in conditional *Slc24a4* knockout animals, wild type or heterozygous littermates. Lenses are photographed above a copper electron microscopy grid to better judge their transparency and refractive properties. Note that lens clarity is maintained in all cases, and that the lenses from the conditional knockout animals (D–F) are indistinguishable from the other genotypes. Lenses are from one-month-old (A–D), 9-month old (E) or 1-year-old (F) mice.