The Phosphoinositide 3-Kinase Catalytic Subunit p110α is Required for Normal Lens Growth

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Purpose. Signal transduction pathways influence lens growth, but little is known about the role(s) of the class IA phosphoinositide 3-kinases (PI3Ks). To further investigate how signaling regulates lens growth, we generated and characterized mice in which the p110α and p110β catalytic subunits of PI3K were conditionally deleted in the mouse lens.

Methods. Floxed alleles of the catalytic subunits of PI3K were conditionally deleted in the lens by using MLR10-cre transgenic mice. Lenses of age-matched animals were dissected and photographed. Postnatal lenses were fixed, paraffin embedded, sectioned, and stained with hematoxylin-eosin. Cell proliferation was quantified by labeling S-phase cells in intact lenses with 5-ethyl-2'-deoxyuridine. Protein kinase B (AKT) activation was examined by Western blotting.

Results. Lens-specific deletion of p110α resulted in a significant reduction of eye and lens size, without compromising lens clarity. Conditional knockout of p110β had no effect on lens size or clarity, and deletion of both the p110α and p110β subunits resulted in a phenotype that resembled the p110α single-knockout phenotype. Levels of activated AKT were decreased more in p110α- than in p110β-deficient lenses. A significant reduction in proliferating cells in the germinative zone was observed on postnatal day 0 in p110α knockout mice, which was temporally correlated with decreased lens volume.

Conclusions. These data suggest that the class IA PI3K signaling pathway plays an important role in the regulation of lens size by influencing the extent and spatial location of cell proliferation in the perinatal period.

Keywords: cell proliferation, knockout animals, lens epithelium, phosphoinositide 3-kinase

Class I phosphoinositide 3-kinases (PI3Ks) are lipid kinases acting downstream of cell surface receptors to phosphorylate the 3'-hydroxyl group of phosphatidylinositol(4,5)P2. The phosphatidylinositol(3,4,5)P3 (PIP3) that is generated activates the 3-kinase catalytic subunit p110α is required for normal lens growth. Investigative Ophthalmol Vis Sci. 2016;57:3145-3151. DOI:10.1167/iovs.16-19607

The lens is composed of a monolayer of epithelial cells that cover the anterior surface and fiber cells that differentiate from epithelial precursors, filling the lens core.11 Cell proliferation drives lens growth and occurs predominantly in a ring of epithelial cells near the lens equator known as the germinative zone.12-14 It has been well documented that lens growth and development are strongly influenced by growth factor signaling. One of the best examples is that of the fibroblast growth factors and fibroblast growth factor receptors (FGFRs) that regulate lens induction, epithelial cell proliferation, and fiber differentiation.15-17 FGFRs are receptor tyrosine kinases that stimulate the mitogen-activated protein kinase (MAPK, or Ras-Raf-Mek-Erk) or the PI3K-AKT intracellular signaling pathways to regulate proliferation, differentiation, and cell survival.8,18,19 The roles of various components of the MAPK pathway have been extensively studied in the lens, often by genetic dissection, using transgenic mice.20-26 In contrast, exploration of the role(s) played by individual components of the PI3K-AKT branch of the intracellular signaling pathway has trailed behind. Two recent studies examined the lens-specific deletion of PTEN, the lipid phosphatase that negatively regulates AKT activity and antagonizes PI3K signaling by dephosphorylation of PIP3.27,28 In both cases, loss of PTEN resulted in significantly elevated levels of phosphorylated AKT, which had different consequences depending upon the developmental timing of tissue-specific deletion.29,30 When PTEN was deleted at the lens placode stage, elevated AKT rescued the cell death phenotype...
caused by knockout of the FGFR2 alone.\textsuperscript{30} In contrast, when PTEN was deleted at the lens vesicle stage, elevated AKT inhibited Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity, ultimately leading to lens cataract and rupture.\textsuperscript{29}

To date, the roles of different catalytic subunits of class IA PI3Ks have not been extensively studied in the lens. Complete knockout of the catalytic subunits p110\textsuperscript{a} and p110\textsuperscript{b} in mice resulted in embryonic lethality.\textsuperscript{31,32} To distinguish between different sources of stimulation for PI3K activity and elucidate nonredundant biological roles of these two PI3Ks in the lens, we generated lens-specific conditional knockouts of both of the enzymes alone or in combination. Loss of p110\textsuperscript{a} but not p110\textsuperscript{b} resulted in significantly reduced eye and lens growth that stemmed from an acute reduction in magnitude and altered spatial organization of epithelial cell proliferation on postnatal day 0 (P0). These findings show that the PI3K signaling pathway plays an important role in the regulation of lens size by influencing the extent and spatial location of cell proliferation in the perinatal period.

**METHODS**

**Generation of Knockout Mice**

To generate lens-specific knockouts of the PI3K catalytic subunits p110\textsuperscript{a}floxflox and p110\textsuperscript{b}floxflox animals (in a mixed C57BL/6j129Sv genetic background)\textsuperscript{33} were interbred with MLR10-Cre mice (in a FVB/N genetic background).\textsuperscript{34} Genotypes were verified by PCR of tail DNA, and littermate controls were used for all experiments. All animal experiments used male and female mice and conformed to the ARRIVE statement for the use of animals in ophthalmic and vision research and were approved by the Stony Brook University IACUC.

**Growth Analysis and Lens Photography**

Age-matched littermate animals between 1 and 24 weeks old were weighed. After they were euthanized by CO\textsubscript{2} inhalation, their eyes were dissected, weighed, and transferred to a Petri dish containing 37\degree C Tyrode solution on a warm stage. Lenses were dissected and photographed using a model SZX16 dissecting microscope equipped with a digital camera (Olympus, Waltham, MA, USA). Lens diameters were measured and used to calculate lens volume, assuming a spherical shape.\textsuperscript{35}

**Western Blotting**

Lenses were dissected from eyes and transferred to Tyrode solution. The lens capsule was then peeled away from the fiber cell mass by using fine forceps. For Western blotting, capsules were transferred to 3X sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Blots were probed using rabbit monoclonal antibodies against PI3K p110\textsuperscript{a} or p110\textsuperscript{b}; mouse monoclonal antibodies against serine 473 phospho-Akt (Cell Signaling Technology, Danvers, MA, USA); or rabbit polyclonal antibodies against total AKT1/2/3 (Santa Cruz Biotechnology, Dallas, TX, USA). Peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA), or sheep anti-mouse (GE Healthcare, Pittsburgh, PA, USA) secondary antibodies were used prior to enhanced chemiluminescence detection. Band densities from independent blots were quantified using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).\textsuperscript{36}

**Histology**

Mouse eyes were dissected and fixed in a 4\% formaldehyde solution in PBS for 16 to 24 hours at room temperature. Fixed eyes were rinsed with PBS, dehydrated using an ethanol series, and embedded in paraffin. Sections of 2 to 3 \textmu m were cut on a diamond knife, deparaffinized, and stained with hematoxylin-cosin. Histological sections were viewed using a model BX51 microscope and photographed with a DP72 digital camera (Olympus).

**5-Ethynyl-2\textsuperscript{-}Deoxyuridine Staining**

Pregnant females or neonatal pups were injected with 50 \mu g/g 5-ethyl-2\textsuperscript{-}deoxyuridine (EdU; Click-IT; Thermo Fisher Scientific, Waltham, MA, USA). Embryonic lenses were dissected 4 hours afterward, and postnatal lenses were dissected 2 hours after EdU injection, and were then photographed and fixed in a 4\% formaldehyde in PBS for 1 hour. Lens permeabilization and EdU staining were performed according to the manufacturer’s instructions and published protocols.\textsuperscript{37} Fluorescing z-stack images were obtained using an Axiocam 200M microscope (Zeiss, Thornwood, NY, USA), and flattened. Image postprocessing and statistical analysis were performed using ImageJ or MATLAB software (MathWorks, Portola Valley, CA, USA). Images were converted to grayscale by using the rgb2gray function of MATLAB, and background noise hue was removed. For whole-lens analysis, a computational mask was created by thresholding the fluorescing region, and the total amount of fluorescence signal was quantified for each lens. For line-scan analysis, six lenses of each genotype at each age were measured across the lens diameter with the plot profile function in ImageJ software. Diameters were normalized within genotypes at each age, and mean ± standard error (SE) values were plotted.

**RESULTS**

Loss of p110\textsuperscript{a} but not p110\textsuperscript{b} reduced lens and eye size. Male and female mice with lens-specific knockout of either the p110\textsuperscript{a} or the p110\textsuperscript{b} catalytic subunit of PI3K exhibited normal overall growth (Fig. 1A). However, p110\textsuperscript{a} knockout animals had noticeably smaller eyes throughout life than their control littermates. Dissecting and weighing the eyes showed that at all postnatal ages tested, p110\textsuperscript{a} knockout eyes were \textapprox 20\% smaller by wet weight than wild-type eyes (P < 0.05, Student’s \textit{t}-test) (Fig. 1B). This difference was accompanied by a \textapprox 25\% reduction in lens volume in p110\textsuperscript{a} knockout animals (P < 0.05). Deletion of p110\textsuperscript{b} did not affect lens (or eye) size, and double deletion of p110\textsuperscript{a} and p110\textsuperscript{b} did not cause reductions greater than that of knockout of p110\textsuperscript{a} alone (Fig. 1C). These data suggested that the activity of p110\textsuperscript{a} but not p110\textsuperscript{b} is required by the lens to achieve normal ocular growth.

Loss of PI3K did not affect lens clarity or structure. Although loss of p110\textsuperscript{a} reduced lens and eye size, conditional deletion of p110\textsuperscript{a} or p110\textsuperscript{b} alone or in combination did not disturb lens clarity. All lenses from PI3K single- and double-knockout animals between 1 and 24 weeks of age were transparent and free of cataract (Fig. 2). This was corroborated by histological analysis of PI3K single- and double-knockout lenses. Eyes from 1-week-old mice were dissected, fixed, sectioned, and stained with hematoxylin-cosin. Sagittal sections through the central region of wild-type lenses appeared normal (Fig. 3A), as did sections from p110\textsuperscript{a} knockout (Fig. 3B) and p110\textsuperscript{b} and p110\textsuperscript{a} double-knockout lenses (Fig. 3C). Higher-magnification views of sagittal sections from mice of all genotypes showed the normal differentiation of lens fibers.
from equatorial epithelial cells (Fig. 3D–F). Thus, deletion of p110\(\alpha\) and/or p110\(\beta\) from the lens failed to disrupt lens morphology or transparency.

Phosphorylated AKT levels were differentially reduced in p110\(\alpha\) and p110\(\beta\) knockout lenses. To determine how PI3K signaling was affected in p110\(\alpha\) and p110\(\beta\) knockout lenses, epithelial cell extracts were probed using Western blot analysis. Wild-type epithelial cells expressed both the p110\(\alpha\) (Fig. 4A) and the p110\(\beta\) (Fig. 4B) catalytic subunits of PI3K, which were absent in cells from the respective conditional knockout mice. Levels of phosphorylated AKT, the principal downstream effector of PI3K signaling, were reduced in both p110\(\alpha\) and p110\(\beta\) knockout epithelial cells compared to those of wild-type cells, and total AKT levels were not affected by p110\(\alpha\) or p110\(\beta\) deletion. Quantitation of band densities (Fig. 4C).

**FIGURE 1.** Deletion of p110\(\alpha\) significantly reduced sizes of eyes and lenses. (A). There were no significant differences in overall body sizes among wild-type, p110\(\alpha\) KO, p110\(\beta\) KO, and p110\(\alpha/p110\beta\) double-KO animals up to 24 weeks of age (\(P > 0.05\)). (B) In contrast, eye mass was reduced 18% at 1 week and 22% at 12 weeks in p110\(\alpha\) KO animals compared to that in wild-type littermates (\(P < 0.05\)). (C) Plotting lens volume versus age for all knockouts showed that p110\(\alpha\) KO lenses were 27% smaller at 1 week and 26% smaller at 12 weeks than those of wild-type animals (\(P < 0.05\)). p110\(\beta\) KO lenses were similar to those of wild-type mice at all ages examined, and the size of p110\(\alpha/p110\beta\) double-KO lenses resembled those of the p110\(\alpha\) single-KOs. Data are mean ± SD; \(n = 11–46\) animals of each genotype at each time point.

**FIGURE 2.** Loss of PI3K did not affect lens clarity. (A–D) Dissected wild-type lenses remained transparent for 1 to 24 weeks. (E–H) p110\(\alpha\) KO. (I–L) p110\(\beta\) KO, and (M–P) p110\(\alpha/p110\beta\) double-KO lenses also remained clear and free of cataracts throughout the time period examined, although p110\(\alpha\) KO and p110\(\alpha/p110\beta\) double-KO lenses were noticeably smaller than those of wild-type or p110\(\beta\) KO mice. Scale bar: (A): 1 mm.

**FIGURE 3.** PI3K knockout lenses lacked histological abnormalities. (A) On P7, sagittal sections through the central region of wild-type lenses appeared normal (200×). (B) p110\(\alpha\) KO and (C) p110\(\alpha/p110\beta\) double-KO lenses also lacked any structural anomalies, except they were smaller than those of wild-type mice. (D–F) Higher magnification (400×) views showed normal differentiation of lens fibers from equatorial epithelial cells in all three genotypes of lenses.
mice. (type lens epithelial cells expressed p110
both p110
similar in both wild-type and PI3K KO animals. (were reduced 63% in p110
(0.05). Data are mean
in the embryonic or postnatal period, as has been observed
mice could have resulted from abnormal epithelial cell mitosis
altered on P0. The smaller lenses observed in p110
knockouts and 30% in the p110
4A) showed that, compared to wild-type lenses, the phospho-
ATK-to-total AKT ratios were reduced 63% in the p110α
knockouts and 30% in the p110β KO epithelial cells (P < 0.05). These
data implied that the loss of PI3K catalytic activity resulted in
differentially reduced levels of phospho-AKT, which in the case
of the p110α subunit, produced a reduction of ~25% in lens
volume.

The pattern of mitosis in p110α knockout lenses was
altered on P0. The smaller lenses observed in p110α knockout
mice could have resulted from abnormal epithelial cell mitosis
in the embryonic or postnatal period, as has been observed
for other mouse knockout models with microphthalmia,36,39 or was due to an increased level of apoptosis. TUNEL staining,
or immunostaining for cleaved caspase 3, showed that any
apoptosis was very difficult to detect in both the wild-type
and the p110α-deficient lenses, with no increase observed in
knockout animals (data not shown). To examine whether loss of
p110α influenced the magnitude or pattern of mitosis, lenses were labeled with EdU between embryonic day 14 (E14) and P2. From E14 through E17, both the wild-type (Fig
5A–D) and the knockout (Fig 5G–J) lenses displayed similar
patterns of robust EdU labeling, with the highest level of
fluorescence in the germinative zone near the lens equator as
previously reported.40,41 On P0, wild-type lenses continued
to show the greatest level of proliferation at the equator (Fig.
5E), whereas the p110α knockout lenses lacked the ring of
increased labeling at the equator and showed a more
homogeneous pattern of EdU incorporation (Fig 5K). By
P2, both the wild-type (Fig 5F) and the knockout (Fig 5L)
 lenses once again displayed the greatest levels of proliferation
in the equatorial germinative zone. To quantify this transient
change in mitotic pattern, line scans were performed using
EdU-labeled images from wild-type and p110α knockout
lenses on P0 (Fig. 6A) and P2 (Fig. 6B), and the mean (±SE)
values were plotted against the position along the lens
diameter. On P0, wild-type lenses had clear peaks of
fluorescence intensity near the equator. In contrast, p110α
knockout lenses lacked the equatorial peaks and had
maximum fluorescence values 41% lower than those in
wild-type lenses (P < 0.05) lenses. On P2, both the wild-
type and the knockout lenses displayed clear peaks of EdU
signal in the equatorial germinative zone, and the mean value
of maximum fluorescence in p110α-deficient lenses was only
12% lower than those in wild-type lenses (P > 0.05). These
data suggested that the significant reduction in lens size
following p110α deletion could be caused by a transient loss
of proliferating epithelial cells in the germinative zone on P0.

Reduced cell division in p110α knockout lenses on P0 was
temporarily correlated with decreased lens volume. To quantify
global changes in proliferation between E14 and P2, total EdU
fluorescence was plotted for wild-type and p110α knockout
mice (Fig. 7A). Between E14 and E16, levels of EdU
fluorescence were slightly lower in the p110α-deficient lenses;
however, on P0, total EdU fluorescence in knockout lenses was
maximally reduced by 38% compared to that in wild-type. On
P2, EdU fluorescence levels had partially recovered in
knockout lenses to a level that was 22% less than that in
wild-type lenses. To relate cell proliferation to organ growth,
the mean (±SE and are derived from 2-week-old mice.

DISCUSSION

We generated and characterized mice with lens-specific
deletions of the p110α and p110β catalytic subunits of PI3K. Loss of p110β partially reduced levels of phosphorylated AKT
in mouse lenses but failed to noticeably alter lens growth,
clarity, or development. Knockout of p110α more substantially
reduced phosphorylated AKT levels, in addition to producing a
discrete growth defect on P0 that resulted in reduced lens size
throughout life. This same phenotype was observed whether
p110α alone was deleted or was deleted in combination with
p110β, and was caused by a transient reduction in the number
of proliferating lens epithelial cells present in the germinative
zone during the first day of life.
The process of rodent lens growth undergoes a transition at approximately P0, the time where we observed a significant decrease in dividing epithelial cells in p110α knockout lenses. During the preceding embryonic period, the lens diameter increases at a constant linear rate, and the lens volume grows as a smooth exponential.42,43 In the early postnatal period, the growth of the lens becomes oscillatory in a manner that corresponds to the timing of the epithelial cell cycle, and that can be mimicked in organ culture by the pulsatile administration of growth factors.44,45 Our data showed that, on E17 and P2, the germinative zone was clearly present in p110α knockout and wild-type lenses but transiently vanished in p110α-deficient mice at approximately P0. This suggests that both the embryonic constant growth and the postnatal pulsatile growth mechanisms produce maximum cell division in the germinative zone and that this does not require the activity of p110α. In contrast, the transitional period between these two growth mechanisms was sensitive to the loss of p110α activity but not p110β. The precise mechanism whereby PI3K activity is required for the preservation of normal germinative zone proliferation around P0 is currently not known.

Significant reductions in lens size have been linked to discrete changes in epithelial cell proliferation in other mouse lens knockout models. Deletion of Cx50 or its functional replacement with Cx46 resulted in mouse lenses that were significantly smaller than those of wild-type mice and specifically failed to achieve a postnatal pulse of epithelial cell proliferation between P2 and P3.35,38,39,46 Interestingly, Cx50 has the greatest level of functional activity in epithelial cells in the early postnatal period when lens mitosis switches to the pulsatile growth pattern, and Cx50 conductance can be upregulated by p110α activity.39,47 Thus, one possibility is the absence of p110α results in a delay in the upregulation of Cx50 gap junction channels in epithelial cells at P0, producing a transient decrease in germinative zone proliferation. The availability of a lens-specific p110α knockout mouse model will

![Figure 5](image-url) Distribution of proliferating cells is altered in p110α KO lenses. (A–F) Wild-type lenses displayed a characteristic pattern of EdU labeling between E14 and P2, with the greatest proliferation observed in the circumferential germinative zone near the lens equator. (G–J) Between E14 and E17, p110α KO lenses had a pattern of proliferation similar to that of wild-type littermates. (K) On P0, EdU staining in the circumferential germinative zone was greatly reduced in p110α KO mice. (L) On P2, the characteristic pattern of EdU labeling was restored in p110α KO lenses.

![Figure 6](image-url) Quantification of germinative zone staining on P0 and P2. (A) On P0, p110α knockout lenses (red line) had peak fluorescent intensity values near the equator that were 41% lower than those in wild-type (black line \( P < 0.05 \)). (B) By P2, there were clear peaks of EdU labeling near the equator of p110α-deficient lenses, and the maximum fluorescence was only 12% lower than that in wild-type \( P > 0.05 \). Data are mean ± SE (gray lines) and aligned at the center of the lens. The diameters of p110α KO lenses were 11% to 12% smaller on P0 and P2. \( n = 6 \) lenses of each genotype at each time point.
allow future testing of this hypothesis through interbreeding with Cx50-deficient mice to test for functional epistasis.

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