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Jacquelyn Gerhart

Philadelphia College of Osteopathic Medicine, jacquelynge@pcom.edu

Colleen Withers

Philadelphia College of Osteopathic Medicine, colleenwi@pcom.edu

Colby Gerhart

Liliana Werner

Nick Mamalis

See next page for additional authors

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Authors

Jacquelyn Gerhart, Colleen Withers, Colby Gerhart, Liliana Werner, Nick Mamalis, Arturo Bravo Nuevo, Victoria Scheinfeld, Paul FitzGerald, Robert Getts, and Mindy George-Weinstein

**Myo/Nog Cells are Present in the Ciliary Processes, on the
Zonule of Zinn and Posterior Capsule of the Lens Following
Cataract Surgery**

Jacquelyn Gerhart¹, Colleen Withers¹, Colby Gerhart¹, Liliana Werner², Nick
Mamalis², Arturo Bravo-Nuevo¹, Victoria Scheinfeld¹, Paul FitzGerald³, Robert
Getts⁴ and Mindy George-Weinstein^{1,†}

¹Philadelphia College of Osteopathic Medicine, Philadelphia, PA

²John A. Moran Eye Center, University of Utah, Salt Lake City, UT

³Department of Cell Biology and Human Anatomy, School of Medicine, University
of California Davis, Davis, CA

⁴Genisphere, LLC, Hatfield, PA

†Corresponding author:

307 Rowland Hall

Philadelphia College of Osteopathic Medicine

4190 City Avenue

Philadelphia, PA 19131

Email: mindygw@pcom.edu

Phone: 215-871-6654

Fax: 215-871-6359

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ABSTRACT

Myo/Nog cells, named for their expression of MyoD and noggin, enter the eye during early stages of embryonic development. Their release of noggin is critical for normal morphogenesis of the lens and retina. Myo/Nog cells are also present in adult eyes. Single nucleated skeletal muscle cells designated as myofibroblasts arise from Myo/Nog cells in cultures of lens tissue. In this report we document the presence of Myo/Nog cells in the lens, ciliary body and on the zonule of Zinn in mice, rabbits and humans. Myo/Nog cells were rare in all three structures. Their prevalence increased in the lens and ciliary body of rabbits 24 hours following cataract surgery. Rabbits developed posterior capsule opacification (PCO) within one month of surgery. The number of Myo/Nog cells continued to be elevated in the lens and ciliary body. Myo/Nog cells containing alpha smooth muscle actin and striated muscle myosin were present on the posterior capsule and overlaid deformations in the capsule. Myo/Nog cells also were present on the zonule fibers and external surface of the posterior capsule. These findings suggest that Myo/Nog contribute to PCO and may use the zonule fibers to migrate between the ciliary processes and lens.

Myo/Nog cells constitute a novel lineage with multiple functions in the embryo and adult. These cells were originally identified in the epiblast of blastocyst stage chick embryo by their expression of messenger RNA (mRNA) for the skeletal muscle specific transcription factor MyoD, the bone morphogenetic protein (BMP) inhibitor noggin and labeling with the G8 monoclonal antibody (mAb) (Gerhart et al., 2000; Gerhart et al., 2006; Gerhart et al., 2009; Strony et al., 2005). During early stages of morphogenesis, Myo/Nog cells are integrated from the epiblast into tissues and organs derived from all three germ layers, including the eyes (Gerhart et al., 2006; Gerhart et al., 2007; Gerhart et al., 2009). Myo/Nog cells continue to express MyoD and noggin regardless of their environment (Gerhart et al., 2001; Gerhart et al., 2007; Gerhart et al., 2009). Elimination of Myo/Nog cells in the epiblast results in hyperactive BMP signaling, malformations of the nervous system, body wall and face, an absence of skeletal muscle, and ectopic cardiac muscle (Gerhart et al., 2006; Gerhart et al., 2009; Gerhart et al., 2011). Additionally, embryos depleted of Myo/Nog cells develop eye defects ranging from anophthalmia to dysgenesis of the lens and retina (Gerhart et al., 2006; Gerhart et al., 2009).

Myo/Nog cells are also present in adult mammalian tissues (Brandli et al., 2017; Bravo-Nuevo et al., 2016; Gerhart et al., 2017; Gerhart et al., 2014; Gerhart et al., 2012). A neuroprotective function of Myo/Nog cells was revealed in the retina of neonatal mice exposed to hyperoxia and adult rats with light induced retinopathy (Brandli, 2017; Bravo-Nuevo, 2016). Anterior lens tissue removed from patients during cataract surgery contains Myo/Nog cells that

surround wounds in the epithelium and wrinkles in the capsule (Gerhart et al., 2017; Gerhart et al., 2014). Depletion of Myo/Nog cells in human lens explant cultures prevents the emergence of single nucleated skeletal muscle cells designated as myofibroblasts (Gerhart et al., 2017; Gerhart et al., 2014).

In this report, we document the presence of Myo/Nog cells in the mouse, rabbit and human lens, ciliary processes and on the zonule of Zinn, a circumferential system of fibers that connect the ciliary body and lens (Zinn, 1755). The zonule of Zinn, also known as the ciliary zonule, is responsible for lens centration and transmitting the force of contraction from the ciliary body muscle to the lens for accommodation. Zonule fibers, consisting of bundles of microfibrils composed of fibrillins 1 and 2, microfibrillar-associated protein 2, collagens, proteoglycans and other proteins, insert into the lens in the zone of proliferating epithelial cells (Cain et al., 2006; Collin et al., 2015; De Maria et al., 2017; Hubmacher et al., 2014; Inoue et al., 2014; Shi et al., 2013).

The following methods were employed in this study. The globes of two C57 black 6 mice were enucleated, frozen in dry ice-cooled propane, fixed in 97% methanol and 3% acetic acid, and stored at -80° for 48 hours (Sun et al., 2015). Eyes were warmed slowly, transferred to 100% ethanol and then xylene, and embedded in paraffin. Tissue was sectioned at 4 µm.

Three human eyes from three donors were procured 3-13 hours postmortem through the National Disease Research Interchange (Philadelphia, PA). The eyes were placed in a modified Davidson's fixative containing 14% ethyl alcohol, 14% formalin and 6.25% glacial acetic acid (Excalibur Pathology,

Inc., Oklahoma City, OK). Anterior segments were embedded in paraffin and sectioned at 10 μm .

Anterior segments also were obtained from two female New Zealand white rabbits weighing between 2.8 and 3.2 kg. Additional rabbits underwent cataract surgery as described previously (Bozukova et al., 2015; Kramer et al., 2015; Li et al., 2016). Briefly, the pupil was dilated, a 3.0-mm corneal-scleral incision was made and a 5.0-mm capsulorhexis was performed after injection of viscoelastic material. The residual cortex was removed with the irrigation/aspiration handpiece following hydrodissection and phacoemulsification (INFINITI® Vision System, Alcon, Fort Worth, TX). Viscoelastic material was used to expand the capsular bag and the intraocular lens (IOL) (SA60AT; single-piece hydrophobic acrylic; AcrySof lens manufactured by Alcon, Fort Worth, TX) was then inserted into the capsular bag using the recommended injection system (Monarch III injector with “C” cartridges, Alcon). The wound was closed with 10.0 monofilament nylon suture after removal of viscoelastic material and injection of balanced salt solution.

Four and 13 globes were enucleated at 24 hours and four weeks following surgery, respectively. Posterior capsule opacification (PCO) was scored on day-30 by slit lamp examination as described previously (Nishi and Nishi, 1999; Werner et al., 2001). Eyes were placed in 10% neutral buffered formalin for a minimum of twenty-four hours and bisected coronally just anterior to the equator. Tissue was embedded in paraffin and sectioned at 10 μm .

Paraffin embedded tissue sections from all three species were stained

with hematoxylin and eosin (H&E) or labeled with fluorescent antibodies as described previously (Gerhart et al., 2001; Gerhart et al., 2000; Gerhart et al., 2006). Sections were permeabilized with 0.1% Triton X-100, treated with 0.1% sodium citrate and incubated with the G8 IgM mouse mAb diluted 1:40 (Gerhart et al., 2001), G8 and a goat polyclonal antiserum to noggin diluted 1:100 (AF719; R&D Systems, Minneapolis, MN), or combinations of G8 or noggin antibodies and mAbs to alpha smooth muscle actin (α -SMA) directly conjugated with fluorescein diluted 1:250 (Sigma-Aldrich, St. Louis, MO) or vimentin diluted 1:400 (AMD-17b mAb, Developmental Studies Hybridoma Bank, Iowa City, IA). Sections also were double labeled with the MF20 antibody to striated muscle myosin heavy chain (Bader et al., 1982) diluted 1:40 (Developmental Studies Hybridoma Bank) and an antibody to MyoD1 diluted 1:100 (Vector Laboratories, Inc., Burlingame, CA). The 11C1.3 antibody to fibrillin 1 (Ashworth, J, Br J Ophthalmology 2000, 84, 1312) diluted 1:250 (Fisher/Invitrogen, Philadelphia, PA) was used to stain the zonule fibers in rabbit and human tissue sections. The polyclonal antibody to fibrillin 2 (ThermoFisher Scientific, Waltham, MA) was applied to tissue sections from the mouse.

Primary antibodies were visualized with affinity purified, F(ab')₂ goat anti-mouse IgM μ chain conjugated with rhodamine, donkey anti-goat IgG conjugated with Dylight 488, goat anti-mouse IgG2b conjugated with rhodamine, goat anti-mouse IgG1 and goat-anti-mouse IgG conjugated with fluorescein (Jackson ImmunoResearch, West Grove, PA). Secondary antibodies were diluted 1:400. Nuclei were labeled with Hoechst dye. Background fluorescence was assessed

by incubating tissue sections with secondary antibodies alone. Lens fiber cells had a stronger, homogeneous, fluorescent hue than the rest of the tissue (not shown). Occasionally, a low level of punctate fluorescence was observed within the lens capsule.

Antibody labeling was analyzed with the Nikon Eclipse E800 epifluorescence microscope equipped with the Evolution QE Optronics video camera and Image Pro Plus image analysis software program (Media Cybernetics, Rockville, MD), and the Nikon Eclipse 90i with Roper camera and Nikon Elements Advanced Research software. Figures were annotated and adjusted for brightness and contrast with Adobe Photoshop CC 2014.

Means and standard deviations were calculated for cells labeled with antibodies to G8 and noggin, G8 or noggin and α -SMA, and sarcomeric myosin heavy chain and MyoD1 in the lens and ciliary body. The numbers of cells were compared between normal eyes and rabbit eyes 24 hours and four weeks after cataract surgery by the t-test.

The length and width of the ciliary processes of normal rabbit eyes and those that had undergone cataract surgery were measured in H&E stained sections that were photographed with a Nikon Eclipse Ti-inverted microscope with a Nikon Ds-Qi2 camera. The mean \pm standard deviation was calculated for the length and width of 11 ciliary processes of two normal rabbit eyes, and 21 and 24 ciliary processes from three eyes 24 hours and 30 days after cataract surgery, respectively. The t- test was used to compare the ciliary processes of normal and post-operative eyes.

Rabbits and mice were treated in accordance with guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the IACUC committees of the University of California, Davis and University of Utah. Human tissue procurement by the NDRI followed The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained for all patients.

We previously reported that Myo/Nog cells expressing MyoD mRNA, noggin and the G8 antigen were present in the ciliary body and lens of the human eye (Gerhart et al., 2014). In this report, we demonstrate G8-positive (+)/noggin+ Myo/Nog cells in the anterior segment of mouse and rabbit eyes (Fig. 1). Myo/Nog cells were found in the equatorial and bow regions of the lens in mice (3.4 ± 1.1 / section, $n = 5$ sections from 2 eyes), rabbits (2.6 ± 0.8 cells/section, $n = 21$ sections/2 eyes) and humans (4.9 ± 3.6 cells/section, $n = 36$ sections/3 eyes) (Fig. 1B, C, G, H, L and M). The ciliary processes also contained low numbers of Myo/Nog cells within the stroma in all three species (mice: 5.4 ± 0.9 cells/section, $n = 5$ sections/2 eyes; rabbits: 2.5 ± 1.4 cells/section, $n = 11$ sections/2 eyes; humans: 2.6 ± 1.9 cells/section, $n = 36$ sections/3 eyes) (Fig. 1D, I and N). In the rabbit, these cells were occasionally found among the ciliary epithelial cells (Fig. 1I). A few Myo/Nog cells (1-4) were associated with the zonule in mice, rabbits and humans (Fig. 1E, J and O); however, cells were not observed on most zonule fibers. In all three structures, some cells appeared to contain cytoplasmic G8 mAb staining (Fig. 1). This finding is consistent with our previous study demonstrating that Myo/Nog cells in

human lens explant cultures internalize G8 antibody/antigen complexes into acidic compartments of the cytoplasm (Gerhart et al., 2017).

The anterior segments of rabbits that had undergone cataract surgery were examined for Myo/Nog cells. A day after surgery, G8+ cells were visible in the equatorial region of the lens (Fig. 2B). The number of Myo/Nog cells was elevated (5 ± 0.8 cells/section, $n = 8$ sections from 4 eyes) compared to normal eyes ($p = 0.0001$). An increase in Myo/Nog cells following surgery also was observed in the ciliary processes (7 ± 5 cells/section, $n = 21$ sections/6 eyes, $p = 0.06$) in which they were present in the stroma (Fig. 2C) and associated with the epithelium (Fig. 2C-E). The width of the ciliary processes appeared enlarged (2.4 ± 1.1 cm, $n = 21$ processes/3 eyes) compared to eyes that had not undergone surgery (0.95 ± 0.42 , $n = 11$ processes/2 eyes, $p = 0.0002$). Myo/Nog cells were associated with some but not all zonules fibers (Fig. 2F).

One month after cataract surgery, rabbit lenses had PCO scores of 2.1 ± 0.8 ($n = 13$ eyes) reflecting mild to moderate opacification with areas of capsular folding. The number of Myo/Nog cells in the lens remained elevated (23 ± 26 , cells/section, $n = 30$ sections from 8 eyes) compared to normal eyes ($p = 0.01$). The interior surface of the posterior capsule contained G8+/ α -SMA+ (Fig. 2H and I) and noggin+/ α -SMA+ cells (22 ± 29 cells/section, $n = 22$ sections/10 eyes). Eighty eight percent of the G8+ and noggin+ cells were stained for α -SMA (Fig. 2 H and I). Cells labeled for α -SMA alone were more numerous than double labeled cells but could not be accurately quantified due to their presence in large aggregates (Fig. 2I). Contractility of the α -SMA+ cells is indicated by their

association with deformations in the lens capsule (Fig. 2H and I). Single G8+/ α -SMA+ overlaid less pronounced wrinkles in the capsule than aggregates of these cells (Fig. 2H and I).

Striated muscle myosin heavy chain also was detected in cells associated with capsular wrinkles (7 ± 4.6 cells/section, $n = 6$ sections from 6 eyes) (Fig. 2J and K). Approximately 43% of the myosin+ cells were labeled with an antibody to MyoD1 (2.2 ± 1.9 cells/section, $n = 6$) (Fig. 2J and K). MyoD was localized mostly in the cytoplasm (Figure 2J). The significance of cytoplasmic MyoD in rabbit lenses with PCO is unknown. Although cultured myofibroblast cell lines established from the liver and kidney also express myosin heavy chain, MyoD was located within the nucleus (Mayer and Leinwand, 1997). Cytoplasmic MyoD in the lens could be attributable to the presence of an inhibitor of nuclear localization (Chen et al., 1996), differential rates of nuclear versus cytoplasmic MyoD degradation (Lingbeck et al., 2003) or position of the cells within the cell cycle (Hecker et al., 2011). In addition to their presence on the internal surface of the posterior capsule, Myo/Nog cells expressing the intermediate filament protein vimentin were occasionally found on the capsule's external surface (Fig. 2L).

The number of Myo/Nog cells in the ciliary processes four weeks after cataract surgery (7 ± 5 cells/section, $n = 27$ sections from 4 eyes) was higher than in normal eyes ($p = 0.06$), but fewer than 24 hours post-surgery ($p = 0.003$) (Fig. 2C-E and N). The processes continued to be wider (2.2 ± 1.1 , $n = 24$ processes from 8 eyes) than those in unoperated eyes ($p = 0.001$). Differences

between the length of the ciliary processes 24 hours and 30 days after surgery and normal eyes were not significant. As was the case in normal eyes and those 24 hours after surgery, Myo/Nog cells were present on some of the zonule fibers a month after surgery (Fig. 2O). Extravascular α -SMA was not detected in the ciliary processes (Fig. 2N) or in cells on the zonules (not shown). Neither of these structures contained visible staining for striated muscle myosin or MyoD protein (not shown), suggesting that the Myo/Nog cells were not translating the MyoD mRNA expressed in G8+/noggin+ cells (George-Weinstein et al., 1996; Gerhart et al., 2001; Gerhart et al., 2000; Gerhart et al., 2014; Gerhart et al., 2012) or differentiating into myofibroblasts.

The increase in Myo/Nog cells in the ciliary processes following cataract surgery may reflect a response to inflammation 24 hours post-operatively, mechanical stress from the surgery itself or implantation of the IOL. Although the source of additional Myo/Nog cells in the ciliary body following cataract surgery is unknown, their increase may result from proliferation of resident Myo/Nog cells or an influx from neighboring tissues or the vasculature. The presence of Myo/Nog cells in the ciliary body warrants further study given the function of this structure in accommodation and producing and regulating the flow of aqueous humor (Delamere, 2005; Tamm, 2015).

Myo/Nog cells have been implicated in the development of PCO that occurs in some adults and most children following cataract surgery (Gerhart et al., 2017; Gerhart et al., 2014; Walker et al., 2010). The decline in visual acuity in PCO results, in part, from myofibroblast contractions that produce wrinkles in

the lens capsule (Wormstone et al., 2009). Killing Myo/Nog cells with either the G8 mAb and complement, or G8 conjugated to 3DNA nanocarriers intercalated with the cytotoxin doxorubicin, eliminated myofibroblasts in explant cultures of human lens tissue (Gerhart et al., 2017; Gerhart et al., 2014). Targeting Myo/Nog cells at the time of cataract surgery is predicted to reduce contractile cells, deformations of the capsule and PCO. The appearance of Myo/Nog cells on the zonule raises the possibility that they may use the zonule fibers to traffic between the ciliary processes and lens in response to stress.

FIGURES

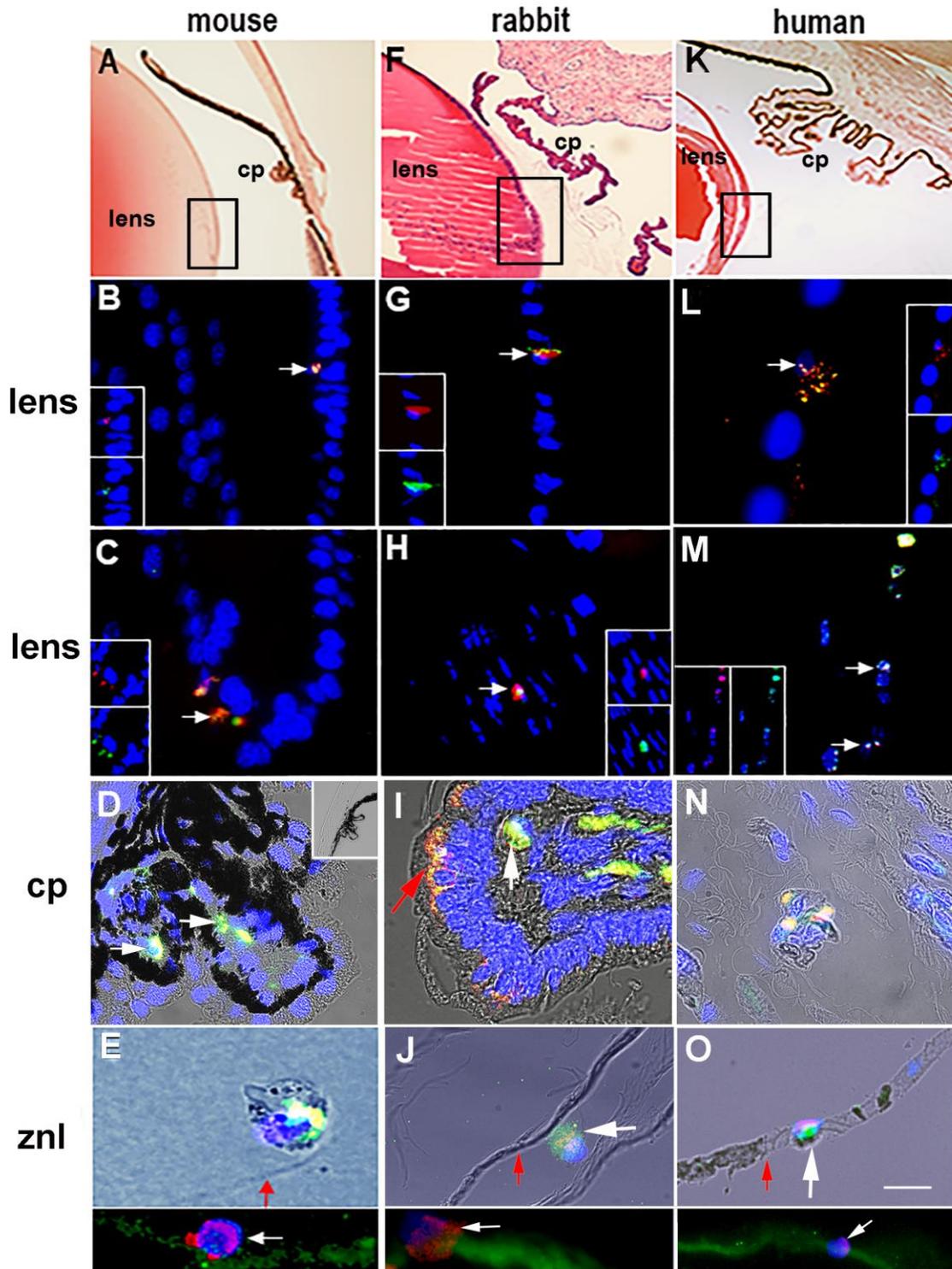


Figure 1. Distribution of Myo/Nog cells in the anterior segments of mice, rabbits and humans. Tissue sections from mice, rabbits and humans were stained with H&E (A, F and K) or double labeled with antibodies to G8 (red) and noggin (green) (B-O), G8 (red) and fibrillin 2 (green) (inset in E) or G8 (red) and fibrillin 1 (green) (insets in J and O). Nuclei were stained with Hoechst dye (blue). Unmerged images are shown in the insets in B, C, G, H, L and M. Overlap of red and green appears yellow in merged images. Fluorescence and DIC images are merged in D, E, I, J, N and O. Low magnification images of the ciliary process are included as insets in D, I and N. The areas within the black boxes in the H&E photographs are shown at high magnification in the fluorescence photomicrographs of the lens. Myo/Nog cells (white arrows) were present in the equatorial (B, G and L) and bow regions (C, H and M) of the lens in mice, rabbits and humans. G8+/noggin+ cells also were present in the stroma of the ciliary processes (cp) in all three species (white arrows in D, I and N) and among the epithelial cells of the ciliary processes in the rabbit (red arrow in I). Myo/Nog cells (white arrows) were found on the zonules (znl) (red arrows) (E, J and O) in all three species. Bar = 135 μ M in F and L, 54 μ M in A and 9 μ M in the fluorescence photomicrographs.

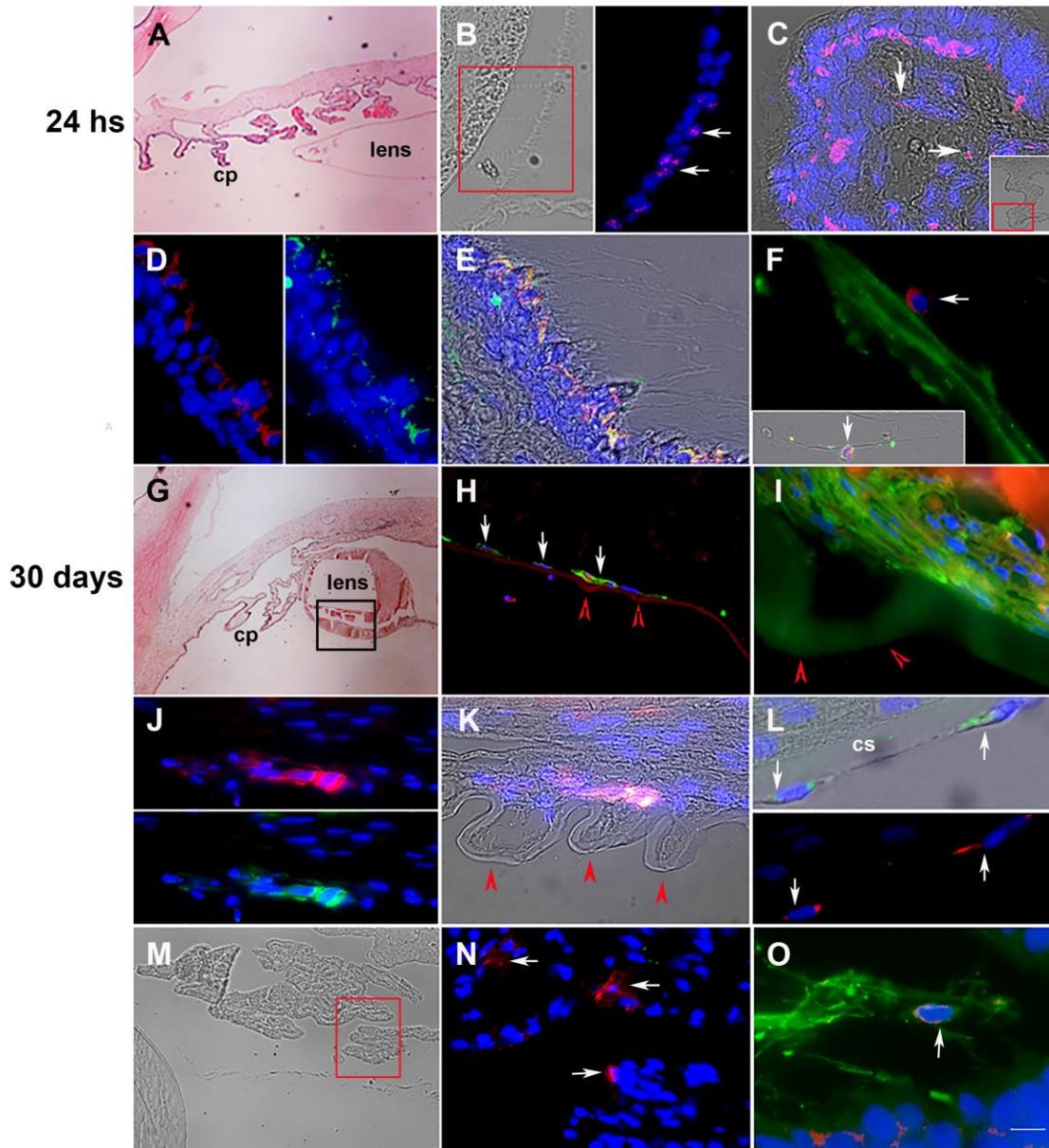


Figure 2. Distribution of Myo/Nog cells in the anterior segments of rabbits following cataract surgery. Tissue sections from anterior segments of rabbits 24 hours (A-F) and 30 days after cataract surgery (G-O) were stained with H&E (A and G). Other sections were labeled with the G8 mAb (red in B and C) or double labeled with antibodies to G8 (red) and noggin (green) (D, E and inset in F), G8 (red) and α -SMA (green) (H, I and N), G8 (green) and vimentin (red) (L),

or G8 (red) and fibrillin 1 (green) (F and O). Double labeling also was performed with antibodies to striated muscle myosin (red) and MyoD (green) (J and merged in K). Nuclei were stained with Hoechst dye (blue). Unmerged images are shown in D, J and L. Overlap of red and green appears yellow in merged images (E, F, H, I, K, N and O). Fluorescence and DIC images are merged in C, E, inset in F, K and L. Areas within the boxes in B, inset in C, G and M indicate similar areas shown at high magnification in the fluorescence photomicrographs in B, C, H-L and N, respectively. Myo/Nog cells were present in the equatorial region of the lens 24 hours after cataract surgery (B). G8+/ α -SMA+ and myosin+/MyoD+ cells overlaid wrinkles in the posterior capsule 30 days after surgery (red arrowheads in H, I and K). G8+/vimentin+ cells were found on the external surface of the capsule (cs) (L). The ciliary processes (cp) contained Myo/Nog cells within the stroma (arrows in C) and among the epithelial cells (C-E and N) at both time points. G8+ cells lacked α -SMA in the ciliary processes one month after surgery (N). G8+ cells were present on the zonules that were stained for fibrillin 1 (F and O). Bar = 135 μ M in A and G, 54 μ M in the inset in C and M, 27 μ M in H, and 9 μ M in B-F, I-L, N and O.

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