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# Myo/Nog cells expressing muscle proteins are present in preretinal membranes from patients with proliferative vitreoretinopathy.

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# Myo/Nog cells expressing muscle proteins are present in preretinal membranes from patients with proliferative vitreoretinopathy

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ABSTRACT

Proliferative vitreoretinopathy (PVR) is a complication of rhegmatogenous retinal detachment and ocular trauma. The disease is characterized by development of membranes that may apply traction to the retina and cause redetachment. Membrane contractions are attributed to myofibroblasts arising from retinal pigment epithelial cells, glia and fibroblasts. The progenitors of myofibrobasts in the lens are Myo/Nog cells that express the skeletal muscle transcription factor MyoD and bone morphogenetic protein inhibitor Noggin. The retina and choroid also contain Myo/Nog cells that respond to stress. We examined preretinal PVR membranes from three ocular trauma patients with retinal detachment for Myo/Nog cells and their expression of muscle proteins. Myo/Nog cells were identified by co-localization of antibodies to the G8 antigen and Noggin. Greater than 80% of all cells in sections from two of three patients expressed both G8 and Noggin. Myo/Nog cells lacked pigment. Alpha smooth muscle actin (α-SMA) and striated myosin II heavy chain were present in the majority of Myo/Nog cells in these two patients. Differentiation of Myo/Nog cells was paralleled by low levels of MyoD. Membrane sections from the third patient consisted mostly of connective tissue with very few cells. A small subpopulation in these sections expressed both G8 and Noggin, and muscle proteins were detected in only a minority of G8-positive (+) cells. In all three patients, greater than 99% of cells with MyoD, α-SMA and striated muscle myosin co-expressed G8. These findings suggest that contractile myofibroblasts in PVR membranes may be derived from differentiating Myo/Nog cells.

Proliferative vitreoretinopathy (PVR) is a vision threatening condition that occurs in 5–10% of patients following a rhegmatogenous retinal detachment (Pastor, 1998; Pastor et al., 2002; Morescalchi et al., 2013). The risk of developing PVR increases to up to 43% following ocular trauma with perforation (Cardillo et al., 1997). The disease is characterized by the formation of epi- and/or subretinal membranes that may distort tissue architecture and cause redetachment (Pastor, 1998). The process of retinal membrane formation resembles a wound healing response with proliferation of retinal pigment epithelial (RPE) cells that undergo an epithelial-mesenchymal transition and become migratory (Oberstein et al., 2011; Kroll et al., 2007; Heriot and Machemer, 1992; Asaria and Charteris, 2006; Anderson et al., 1981; Grisanti and Guidry, 1995; Casaroli-Marano et al., 1999; Baudouin et al., 1990). Other components of these membranes are glial cells, fibroblasts, myofibroblasts, leukocytes and extracellular matrix (Baudouin et al., 1990; Garweg et al., 2013; Tosi et al., 2014). The trigger for membrane formation is thought to be related to an injury-induced compromise of endothelial tight junctions of the dual-layered blood-retinal barrier and release of growth factors, mediators of chemotaxis and inflammatory cytokines (Sadaka and Giuliari, 2012). Retinal breaks and areas of detachment may result in leakage of fluid from the vitreous into the subretinal space and the creation of conduits for RPE migration onto the inner retinal surface (Morescalchi et al., 2013; Cowley et al., 1989).

The most significant pathological feature of PVR membranes is their ability to contract and produce traction on the retina (Glaser et al., 1987). The likely mediators of membrane contractions are myofibroblasts. The proposed sources of myofibroblasts in retinal membranes

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are transdifferentiated RPE cells and fibroblasts (Pastor et al., 2002; Oberstein et al., 2011; Kroll et al., 2007; Asaria and Charteris, 2006; Glaser et al., 1987; Bochaton-Piallat et al., 2000).

In this study, preretinal membranes were examined for the presence of an inherently myogenic cell type that we previously identified in the retina and choroid (Bravo-Nuevo et al., 2016; Brandli et al., 2017). These Myo/Nog cells were first discovered in the epiblast of the chick embryo by their expression of mRNA for the skeletal muscle specific transcription factor MyoD and bone morphogenetic protein inhibitor Noggin (Gerhart et al., 2000, 2004, 2006). They are integrated into the developing eye during early stages of development (Gerhart et al., 2006, 2009). Depletion of Myo/Nog cells in the epiblast results in severe malformations, including anophthalmia, microopathalmia, lens dysgenesis and overgrowth of the retina (Gerhart et al., 2006, 2009, 2011).

Myo/Nog cells are also present in adult mammalian tissues (Bravo-Nuevo et al., 2016; Brandli et al., 2017; Gerhart et al., 2012, 2014, 2018, 2019a, 2019b). In the lens, they are progenitors of contractile myofibroblasts that deform the capsule (Gerhart et al., 2014, 2019a; Walker et al., 2010). Targeted depletion of Myo/Nog cells during cataract surgery in rabbits significantly reduces the emergence of myofibroblasts, capsular wrinkles and posterior capsule opacification (Gerhart et al., 2019a).

The retina contains a low number of Myo/Nog cells under normal conditions (Bravo-Nuevo et al., 2016; Brandli et al., 2017). Hypoxia and light damage stimulate the proliferation and migration of Myo/Nog cells to areas of stress and cell death (Bravo-Nuevo et al., 2016; Brandli et al., 2017). Elimination of Myo/Nog cells in the mouse model of retinopathy of prematurity results in increased neuronal cell death, whereas their addition to the vitreous following light damage reduces photoreceptor loss and improves visual function in rats (Bravo-Nuevo et al., 2016; Brandli et al., 2017). While these studies indicate that Myo/Nog cells are neuroprotective in the retina, their presence in the ganglion cell layer, the interface between the inner nuclear and outer plexiform layer, and choroid in the normal retina, along with their potential to differentiate into myofibroblasts in the eye, led us to test the hypothesis that Myo/Nog cells contribute to retinal membranes.

Preretinal membranes from three PVR patients that had suffered retinal detachment from ocular trauma were screened for Myo/Nog cell and muscle markers. Membranes were procured from patients in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of California, Davis. Informed consent was obtained before vitrectomy. Patients 1, 2 and 3 were males ages 34, 40 and 15, respectively. Six and eight weeks after vitrectomy for retinal detachment, patients 1 and 2 again underwent surgery for recurrent detachment with PVR. Patient 3 had a limited retinal detachment from ocular trauma that was not repaired. His PVR preretinal membrane was removed four years after injury to improve vision. Surgical specimens were fixed immediately in 4% formalin, embedded in paraffin and sectioned at 5 μM.

Tissue sections were stained with hematoxylin and eosin or double labeled with the G8 mouse monoclonal antibody (mAb), a specific marker for Myo/Nog cells (Gerhart et al., 2001, 2006, 2007, 2009, 2012, 2018, 2019a, 2019b), and an anti-Noggin goat polyclonal antiserum (AF719; R&D Systems, Minneapolis, MN), as described previously (Gerhart et al., 2001, 2006). Double labeling also was carried out with G8 and either the anti-MyoD IgG1 mAb (MA5-12902, ThermoFisher Scientific, Rockford, IL) or anti-MyoD rabbit polyclonal antiserum (ab203383, Abcam, Cambridge, MA), anti-α-SMA mAb (Sigma-Aldrich, St. Louis, MO) and anti-striated muscle myosin II heavy chain IgG 2b mAb (MF20 mAb; Developmental Studies Hybridoma Bank, Iowa City, IO). The α-SMA mAb was directly conjugated with fluorescein. Other primary antibodies were tagged with AffiniPure Fab fragment subclass and species-specific secondary antibodies conjugated with Rhodamine Red or Alexa 488 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The level of background fluorescence was assessed by labeling tissue sections with secondary antibodies only.

**Table 1**
**Quantitation of cells expressing Myo/Nog cell markers and muscle proteins in PVR membranes.** Tissue sections were double labeled with antibodies to G8 and Noggin, α-SMA or striated muscle myosin. Percent positive = number of fluorescent cells ÷ total number of cells X 100. Percentage of G8+ cells labeled with another primary antibody = number of double labeled cells ÷ total number of G8+ cells X 100. Percentage of Noggin+, MyoD+, α-SMA + or myosin + cells labeled with G8 = number of double labeled cells ÷ total number of fluorescent cells X 100. α-SMA stained cells surrounding blood vessels were not counted. Results are the mean ± standard deviation. The number of sections scored is indicated in parenthesis.

| Percent Positive | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| **G8+** | 87 ± 7 (12) | 88 ± 6 (12) | 11 ± 3 (12) |
| **Noggin+** | 79 ± 8 (3) | 88 ± 5 (3) | 12 ± 4 (3) |
| **G8+ with Noggin** | 100 (3) | 100 (3) | 100 (3) |
| **Noggin+ with G8** | 100 (3) | 100 (3) | 99.9 ± 0.05 (3) |
| **MyoD+** | 4 ± 1 (3) | 0.3 ± 0.1 (3) | 6 ± 2 (3) |
| **G8+ with MyoD** | 5 ± 1 (3) | 0.3 ± 0.1 (3) | 47 ± 23 (3) |
| **MyoD+ with G8** | 99.9 ± 0.05 (3) | 100 (3) | 100 (3) |
| **α-SMA+** | 66 ± 10 (3) | 72 ± 6 (3) | 3 ± 3 (3) |
| **G8+ with α-SMA** | 74 ± 7 (3) | 80 ± 3 (3) | 42 ± 41 (3) |
| **α-SMA with G8** | 100 (3) | 99 ± 1 (3) | 100 (3) |
| **Myosin+** | 58 ± 3 (3) | 64 ± 15 (3) | 1 ± 1 (3) |
| **G8+ with Myosin** | 63 ± 4 (3) | 75 ± 11 (3) | 16 ± 14 (3) |
| **Myosin + with G8** | 100 (3) | 100 (3) | 100 (3) |

Tissues were analyzed with the Nikon Eclipse E800 epifluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with 10x, 60x and 100x lenses, the Evolution QE Optronics video camera and Image Pro Plus image analysis software program (Media Cybernetics, Rockville MD). All cells were scored for the presence or absence of one or both fluorescent antibodies in each section. The number of α-SMA stained cells reported in Table 1 did not include smooth muscle surrounding blood vessels. Photographs were adjusted for brightness and contrast, and figures were annotated with Adobe Photoshop CC 2014 (Adobe Inc., San Jose, CA).

Tissue sections of preretinal membranes from the three patients varied in size and appearance (Fig. 1A–C). Sections from patient 2 contained the largest amount of tissue. Membranes from patients 1 and 2 consisted of an abundance of cells and some collagenous extracellular matrix. Sections from patient 3 contained few cells overall and was mostly composed of fibrous connective tissue. The differences in the cellularity and extent of fibrotic tissue in these sections reflects the age of the membrane itself which had been present for four years in patient 3 versus six to eight weeks in patients 1 and 2. Pigment was visible in very few cells in the membrane of patient 1 (Fig. 1 A). More pigmented cells were present in patient 2's membrane but they were only a minor population within the tissue (Fig. 1B). In patient 3's membrane, some pigment appeared in fibrotic tissue lacking nuclei (Fig. 1C).

Sections of membranes from PVR patients 1 and 2 contained an abundance of G8-positive (+) cells, whereas only a small subpopulation bound the G8 mAb in patient 3 (Table 1). G8 and Noggin were localized to the same cells in all three patients (Table 1; Fig. 1D–F). Noggin + cells lacking detectable levels of G8 were extremely rare (less than 0.1%) in patient 3 (Table 1). Low numbers of G8+ cells in patients 1–3 contained detectable levels of MyoD protein (Table 1). All cells with MyoD cells were co-labeled with the G8 mAb except for tissue from patient 1 in which only a few MyoD+/G8-cells were observed (Table 1; Fig. 1G–I).

PVR membrane sections also were screened for muscle filament proteins. Levels of α-SMA were high in patients 1 and 2, and very low in patient 3 (Table 1; Fig. 1J-L). The majority of G8+ Myo/Nog cells had

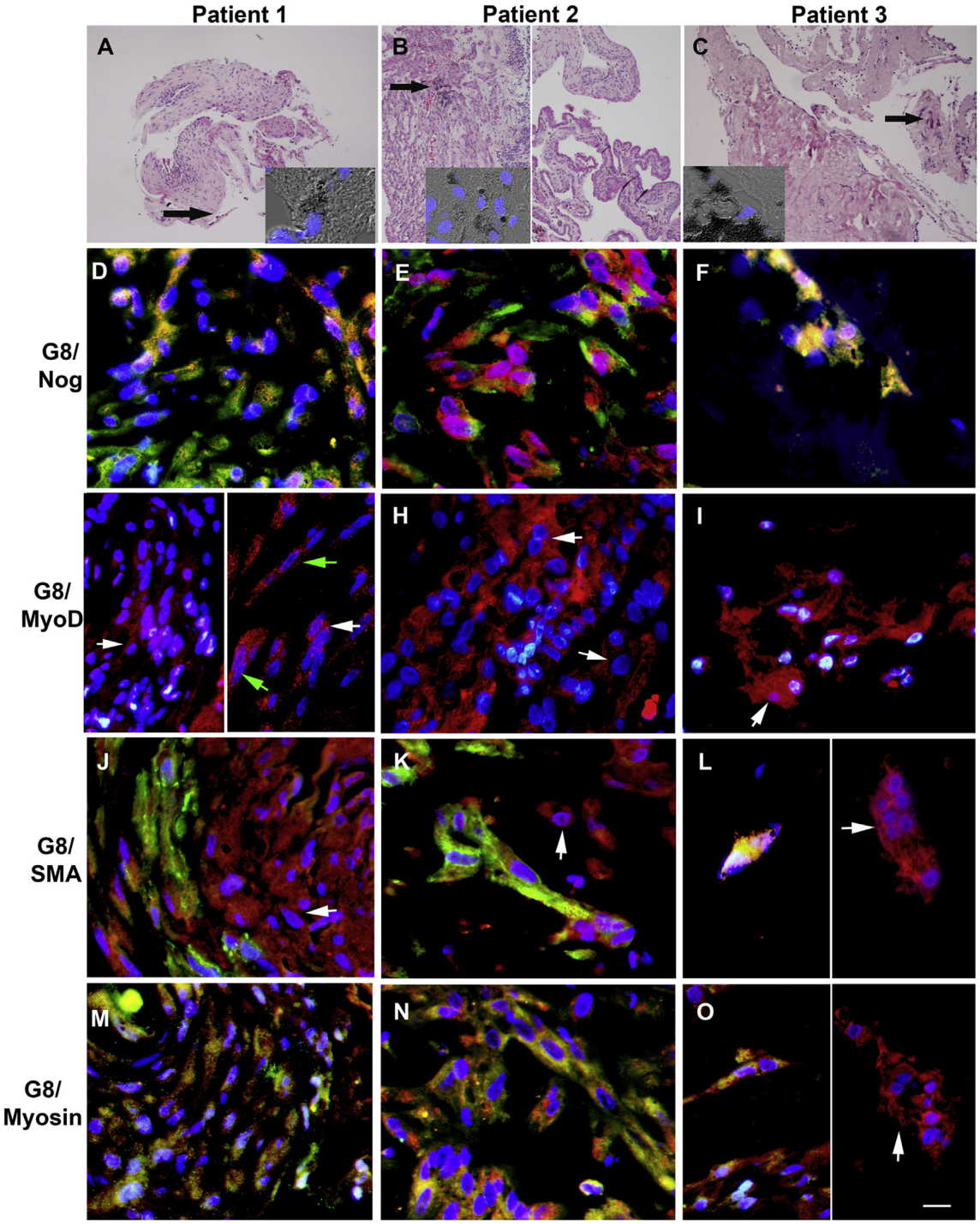


**Fig. 1. Co-expression of Myo/Nog and muscle proteins in PVR membranes.** Tissue sections of preretinal membranes from patients 1–3 were stained with H&E or double labeled with the G8 mAb (red) and antibodies to Noggin (Nog), MyoD, α-SMA or striated muscle myosin (green). The overlap of red and green appears yellow in merged images. Single labeled cells are indicated with white arrows. Green arrows in G indicate cells that appear multinucleated. Areas with pigment are shown in the insets in A-C. Bar = 54 μM in A-C and 9 μMin D-O. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

synthesized α-SMA in the first two patients. Less than 20% of G8+ cells were stained for α-SMA in two out of three sections from Patient 3 (Fig. 1L). A single cluster of double labeled cells was present in the third section. All α-SMA + cells contained G8 with the exception of patient 2 in which α-SMA+/G8-negative (−) cells were rare (< 1%) (Table 1; Fig. 1J-L).

Similar results were obtained for the expression of striated muscle myosin. The majority of G8+ cells were labeled with the myosin mAb in patients 1 and 2, whereas only a small number of Myo/Nog cells in patient 3 were myosin+ (Table 1). All cells with myosin were labeled with the G8 mAb in all three patients (Table 1; Fig. 1M–O). Some G8+ cells appeared to be multinucleated in membrane sections from patient 1 (Fig. 1G).

The results of this study demonstrate that Myo/Nog cells are present in trauma-induced preretinal membranes from PVR patients and the cells are in different states of maturation. The majority of G8+ cells in

the two patients with recently formed membranes had undergone differentiation, as evidenced by their expression of α-SMA and striated muscle myosin, and low levels of MyoD that is downregulated with skeletal muscle maturation (Hinterberger et al., 1991). The number of Myo/Nog cells was low in the third patient whose membrane contained few cells overall. In this patient's tissue sections, the majority of G8+ cells were not labeled with antibodies to MyoD, α-SMA and striated muscle myosin. This suggests that most G8+/Noggin + cells in patient 3 were quiescent progenitors because MyoD mRNA translation is not detected until Myo/Nog cells are activated (Gerhart et al., 2001, 2012, 2014, 2019a; George-Weinstein et al., 1996; Strony et al., 2005).

This study introduces a previously undescribed cell type into the PVR disease process. While the origin of Myo/Nog cells in preretinal membranes is unknown, the retinal ganglion cell layer, interface of the inner nuclear and plexiform layer, and choroid normally contain Myo/Nog cells (Bravo-Nuevo et al., 2016; Brandli et al., 2017). An additional potential source of Myo/Nog cells is the ciliary body (Gerhart et al., 2018, 2019a). In the retina, ciliary body and lens, Myo/Nog cells increase in number and migrate in response to stress and injury (Bravo-Nuevo et al., 2016; Brandli et al., 2017; Gerhart et al., 2018, 2019a). These properties, along with their stable commitment to the skeletal muscle lineage regardless of their environment (Gerhart et al., 2007) and tendency to differentiate into myofibroblasts that express striated muscle proteins after injury to the lens (Gerhart et al., 2014, 2017, 2019a; Walker et al., 2010), suggest that Myo/Nog cells contribute to retinal membrane formation and contractions.

Myo/Nog cells do not express markers of the monocyte/macrophage lineage in the retina and elsewhere (Bravo-Nuevo et al., 2016; Brandli et al., 2017; Gerhart et al., 2012), and therefore, it is unlikely that microglia transdifferentiated into myofibroblasts expressing G8 and Noggin. Fibroblasts and fibroblast-like cells also have been implicated as a source of myofibroblasts in retinal membranes (Grisanti and Guidry, 1995; Baudouin et al., 1990); however, they are morphologically similar to Myo/Nog cells. Our study cannot rule out the possibility that RPE cells transdifferentiated into Myo/Nog cells and myofibroblasts. While RPE cells are capable of expressing α-SMA *in vitro* (Kurosaka et al., 1996; Stocks et al., 2001; Ando et al., 2000; Si et al., 2013), to our knowledge they have not been screened for expression of striated muscle proteins. Furthermore, Myo/Nog cell markers were not detected in RPE cells *in vivo* even under conditions of stress (Bravo-Nuevo et al., 2016; Brandli et al., 2017). Pigment was not observed in G8+ cells within the retina (Bravo-Nuevo et al., 2016; Brandli et al., 2017) or PVR membranes, although loss of pigment accompanies the epithelial to mesenchymal transition of RPE cells (Campochiaro et al., 1991; Lee et al., 2001). Screening of RPE cells for markers of Myo/Nog cells and striated muscle proteins *in vitro* and models for inducing PVR *in vivo* may reveal whether there is a lineage relationship between the two cell types. In this context, it is important to note that once Myo/Nog cells are depleted in the chick embryo and human lens tissue, they are not replaced by stem or transdifferentiating cells (Gerhart et al., 2006, 2009, 2011, 2014, 2017, 2019a; Sadaka and Giuliari, 2012; Telander et al., 2011).

Regardless of the origin of Myo/Nog cells in retinal membranes, targeting them for elimination during surgical repair of a detached retina is worthy of consideration.

While the anatomic success rate of surgery is estimated at 60–80% for redetachments caused by PVR, only 40–80% of patients experience functional success with an ambulatory vision of 5/200 or greater (Sadaka and Giuliari, 2012). Current adjunct pharmacological approaches to prevent PVR include administration of drugs and biologics that inhibit inflammation, proliferation, oxidation, epithelial to mesenchymal transition, adhesion, remodeling of the extracellular matrix and cell migration (Morescalchi et al., 2013; Sadaka and Giuliari, 2012). These approaches are effective in reducing PVR in animal models (Sadaka and Giuliari, 2012; Telander et al., 2011), but thus far, limited success has been achieved with those drugs that have been tested in clinical trials (Morescalchi et al., 2013; Sadaka and Giuliari, 2012).

Myo/Nog cells have been killed in explants of human lens tissue and in the rabbit lens during cataract surgery using a drug consisting of the targeting G8 mAb conjugated to 3DNA intercalated with the cytotoxin doxorubicin (Gerhart et al., 2017, 2019a). In both studies, off-target effects of the drug were not observed and treatment prevented the emergence of myofibroblasts and contractions. Injection of a drug that specifically targets Myo/Nog cells in animal models of PVR will help resolve questions surrounding the origin of contractile cells in retinal membranes and simultaneously test this approach for disease modifying effects.

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