Laser Capture Microdissection-Directed Profiling of Glycolytic and mTOR Pathways in Areas of Selectively Ablated Müller Cells in the Murine Retina

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PURPOSE. We have reported previously down-regulation of key metabolic pathways, the glycolytic and mTOR pathways, from a global retinal microarray analysis after selective Müller cell ablation in a novel transgenic model. The purpose of the present study was to examine changes in expression of key molecules of glycolytic and mTOR pathways specifically in patches of Müller cell loss.

METHODS. Eyes were enucleated 1 and 3 months after induced Müller cell ablation, directly embedded in optimal cutting temperature medium, and snap frozen in liquid nitrogen. Laser capture microdissection (LCM) was conducted to dissect patches of Müller cell loss for quantitative RT-PCR (qRT-PCR) analysis of key genes of the glycolytic (glyceraldehyde-3-phosphate dehydrogenase, enolase 1 and 2, lactate dehydrogenase A and B) and mTOR pathways (insulin-like growth factor receptor 1, phosphatidylinositide-3-kinase, Akt1, and regulatory-associated protein of mTOR). Protein validations were performed by immunohistochemistry.

RESULTS. The LCM-directed qRT-PCR analysis of Müller cell ablated specimens demonstrated reduced transcription of genes involved in the glycolytic and mTOR metabolic pathways. Of the proteins we chose to study, only enolase 1 was expressed by Müller cells. Other glycolytic and mTOR pathway proteins were expressed by photoreceptor inner and outer segments, which were lost in patches of Müller cell ablation.

CONCLUSIONS. We found suppression of genes encoding various glycolytic and mTOR pathway-associated enzymes in areas of Müller cell loss. This appeared mainly to be due to loss of photoreceptor inner and outer segments. The consequences of metabolic derangement caused by Müller cell ablation warrant further investigation.

Keywords: Müller cells, glycolysis, mTOR pathway, photoreceptors, laser capture microdissection

Müller cells are the principal glial cells of the mammalian retina. They provide an anatomic and functional link between retinal neurons and their surrounding compartments, since they span the entire thickness of the retina from the microvilli projecting from the outer limiting membrane, formed by the apical processes of Müller cells, to the Müller cell end feet, which form the inner limiting membrane. They have critical roles in maintaining retinal homeostasis, such as producing neuroactive substances through a neurotransmitter recycling system, maintaining the blood–retinal barrier (BRB), and regulating ion, water content, and pH. Müller cells also provide metabolic support and nutrients to neurones via metabolic pathways, such as the glycolytic pathway. There is strong evidence that the Müller cell is one of the major cells responsible for metabolizing glucose into pyruvate and lactate to fuel neurons in the retina.

The retina is a metabolically active tissue whose energy demands normally are met through the uptake of glucose and oxygen from the inner retinal and choroidal circulations. Glycolysis, a form of energy metabolism that converts glucose to lactate, is one of the sources of cellular ATP in the retina. The photoreceptor inner segment, where most of the mitochondria are located for oxidative phosphorylation in the retina, is highly metabolically active to produce energy and nucleotides for phototransduction in the outer segment. There is evidence that the photoreceptor outer segment also is involved in anaerobic glycolysis and contributes to retinal energy production. It has been reported that impairment of photoreceptor energy metabolism contributes to photoreceptor degeneration in several retinal diseases.

The mTOR pathway, one of the key regulators of cellular metabolism that is coupled with energy and nutrient abundance, regulates cellular growth and division. It consists of two distinct complexes: mTOR complexes (mTORC) 1 and 2. Of these, mTORC 1 is involved largely in mitochondrial metabolism and biogenesis. Schieke et al. demonstrated that inhibition of mTOR and one of its catalytic subunits, regulatory-associated protein of mTOR (RAPTOR), with rapamycin lowered mitochondrial membrane potential, oxygen consumption, and ATP synthetic capacity, and also caused alterations in the mitochondrial phosphoproteome. Cunningham et al. described a target transcription factor of mTOR, called yin-yang
Tissues were cut at 10°C without fixation, and snap frozen in liquid nitrogen. 

Gene Name | Forward Primer Sequence, 5’→3’ | Reverse Primer Sequence, 5’→3’ | Amplicon Size 
--- | --- | --- | ---
GAPDH | AAGATGTTGATGGGCTTCCGG | TGGCAAAGTGAGATTGTGGCC | 150
LDHa | TGTCCTACGAAGACTTCTGT | GACGTGATCTGGCAATGTGGGAA | 155
LDHb | AGATGACGTAGTTGGGGTGG | TTTTGAGGCTCTGGAGGACAAA | 164
ENO1 | GCTGCCTCAGGCTGTTGCAG | GCAGGAGTTCGGTCACAGAC | 325
ENO2 | ATCAAGCTCAAGGTTATCCCGTG | TGGCGATAGGGGCGAGAATG | 167
IGF1r | TCTACACCCGACGACTTGGC | TGGGCGTTGCGCCGAGATATG | 103
PI3K | ACAACACGGGTTGAGACGTTCG | GCTTGACAGCAATGGGCCAT | 140
AKT1 | ATCAAGCCAGATCGTGGCCA | TTTTGACCAAAATGGGCCCAT | 116
RAPTOR | TTGGTCTCAGCTGCTCCTGCAATG | GCTACCTCAGCTCCTGCTCCTG | 86
18SrRNA | TGGACGGAAGGGCCACCAAG | GCACACACCACCAACAGAAT | 130

Slides were thawed, and tissues were fixed with 50% ethanol for 15 seconds and 75% ethanol for 30 seconds, and washed with diethyl pyrocarbonate water for 15 seconds. Morphology was exposed with HistoGene staining solution (KIT0415; Arcturus, Sunnyvale, CA). Laser capture microdissection (Carl Zeiss Meditec) was applied directly after HistoGene staining. In brief, a microbeam was used to cut tissues and a laser catapult was applied to collect the tissues into collecting tubes. RNA was extracted with the Qiagen RNeasy Microkit (74004; Qiagen, Venlo, The Netherlands), and the quality and quantity were assessed with bioanalyzer RNA pico chips. RNA amplification and cDNA synthesis were performed with Ovation PicoSL WTA System V2 (NUG-3302-12; Nugene, Chicago, IL) according to the manufacturer's instructions and the Qiagen MinElute Reaction Cleanup Kit was applied for cDNA purification (28204; Qiagen).

## Materials and Methods

### Conditional Müller Cell Ablation in Transgenic Mice

Rlbp-CreER-DAT176 transgenic mice and Rlbp-CreER-LacZ control mice have been described previously. Müller cell ablation was induced by daily intraperitoneal injection of tamoxifen (TMX, 3 mg in 0.2 mL sunflower oil) for 4 consecutive days at 6 to 8 weeks of age.

### Tissue Preparation

All animal experiments adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The project was approved by the Animal Ethics Committee of the University of Sydney. All animals were kept in a 12-hour light and dark cycle, and fed with standard chow and water. The 36 mice were divided into 2 groups, with 18 Rlbp-CreER-DAT176 mice for conditional Müller cell ablation and 18 Rlbp-CreER-LacZ mice as controls. Mice were euthanized in a carbon dioxide chamber followed by cervical dislocation at 1 and 3 months after TMX injection (n = 9/group/time point). Eyes were enucleated, then embedded directly in optimal cutting temperature media (OCT) without fixation, and snap frozen in liquid nitrogen. Tissues were cut at 10 μm onto membrane slides (415190-9041-000; Carl Zeiss Meditec, Jena, Germany) and stored in a −80°C freezer until use.

### Immunofluorescence

Immunofluorescence studies were performed as described previously. In brief, slides were thawed at room temperature, and washed with PBS 3 times, 5 minutes each time. The sections were incubated with blocking buffer (5% normal goat serum in PBS), washed with PBS 3 times for 5 minutes, and then incubated with primary antibody at 4°C overnight. Secondary antibody incubation was conducted on the next day at room temperature for 3 hours. Slides were washed with PBS 3 times for 5 minutes each and Hoechst staining was applied for visualization of cell nuclei. Primary antibodies included anti-glutamine synthetase synthetase (MAB302; Chemicon, Temecula, CA), anti-GAPDH (2118; Cell Signaling Technology, Beverly, MA), anti-LDHa (2012; Cell Signaling Technology), anti-18SrRNA (130).
Lectin peanut agglutinin (PNA)-488 (21409; Invitrogen, Carlsbad, CA) and lectin wheat germ agglutinin (WGA)-594 (W11262; Invitrogen) were used to label cone and rod photoreceptor segments, respectively. Stained sections were examined by confocal microscopy.

RESULTS

qRT-PCR on Laser Capture Microdissection (LCM)–Directed Tissues

We have demonstrated previously that photoreceptor degeneration occurred in areas of Müller cell ablation with protrusion of outer nuclear layer into the subretinal space.\(^ {26} \)

This feature was seen clearly on histogene staining, which showed minimal disruption of tissue structure without sacrificing RNA quality (Fig. 1).

The quality and quantity of RNA from the captured tissue were assessed with a Bioanalyzer. Samples with the highest RNA integrity number (higher than 6) and quantity of more than 200 pg/\( \mu l \) were selected for amplification. Six samples were selected from each group for qRT-PCR analysis. Quantification of the relative expression ratio was generated by REST2009\(^ {28} \) (Fig. 2). After normalization to the reference gene (\( 18S \)rRNA), a ratio (Müller cell ablated area from Rlbp1-CreER-DTA176 mice versus Rlbp1-CreER-LacZ control mice) of >1 was considered as upregulation, whereas a ratio of <1 was considered as down-regulation of gene expression.

All 5 genes involved in the glycolytic pathway that we examined were significantly down-regulated in patches of Müller cell loss 1 month after it had been induced by administration of tamoxifen (Fig. 2A). After 3 months, all 5 genes were down-regulated again, though the reduction in the relative expression ratio of \( LDHb \) was not statistically significant (Fig. 2C). \( ENO1 \) was the most down-regulated gene 1 and 3 months after induced Müller cell ablation.

All mTOR pathway–related genes that we studied by qRT-PCR also were down-regulated in patches of Müller cell loss after 1 and 3 months (Figs. 2B, 2D). However, only \( PI3K \) and \( RAPTOR \) were significantly down-regulated 1 month after tamoxifen-induced Müller cell ablation (Fig. 2B). \( PI3K \) also was statistically significantly down-regulated after 3 months (Fig. 2D). The reductions of these metabolic pathway-associated genes in patches of Müller cell ablation generally were consistent with our microarray analysis of whole retinal samples.\(^ {27} \)

![Diagram showing LCM after histogene staining on cryosections. (A) A section was fixed with alcohol and morphology was exposed with histogene staining solution. (B) Identification of an area with protrusion of photoreceptor cell bodies into the subretinal space (asterisk) before microdissection. (C) Tissue was cut with a microbeam and the laser catapult captured the tissue into a collecting tube (inset, arrow). PS, photoreceptor segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 150 \( \mu m \).

![The qRTPCR analysis of glycolytic (A, C) and mTOR (B, D) pathways 1 month (A, B) and 3 months (C, D) after Müller cell ablation. Expression ratio (Rlbp1-CreER-DTA176 mice versus Rlbp1-CreER-LacZ control mice) >1 represents upregulation, whereas expression ratio <1 represents down-regulation. Error bars indicate maximum and minimum value of the expression, and the dot lines in the box plots show the median. *P < 0.05; †P < 0.01; n = 6 in each group.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933598/ on 09/23/2017)
We performed immunohistochemistry to investigate the posttranslational effects of the genes of interest in patches of Müller cell loss. Staining for glutamine synthetase (GS), which is expressed exclusively by Müller cells, revealed a significant loss of Müller cell bodies in the inner nuclear layer of Rlbp1-CreER-DTA176 mice (Fig. 3, white arrows), compared to Rlbp1-CreER-LacZ controls. Overall, we found reduced immunostaining for ENO1 and 3 months after Müller cell ablation in Müller cell bodies (Fig. 4) and absence of protein expression of GAPDH, ENO1, and ENO2 in the photoreceptor inner and outer segments (Fig. 5) compared with control group (A–C). Asterisks indicate loss of lectin PNA and GAPDH expression (D, E), and protrusions of photoreceptor nuclei into the subretinal space (F) in areas Müller cell loss. Scale bars: 50 μm.

**Immunohistochemistry**

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outer segments in areas of Müller cell loss (Figs. 3–8). Similar changes in the chosen proteins involved in the mTOR pathways were found in the photoreceptor inner and outer segments in regions of Müller cell loss (Figs. 9, 10).

Absence of GAPDH expression was found in the photoreceptor inner and outer segments in patches of Müller cell ablation (Fig. 3, asterisks). High magnification of confocal microscopy (×40) revealed that there was loss of photoreceptor inner and outer segments in the area of outer nuclear layer protrusion, and, consequently, the absence of GAPDH protein expression (Fig. 5).

Expression of ENO2 by photoreceptor inner and outer segments was similarly absent (Fig. 6, asterisks) in patches of Müller cell loss, with occasional expression of ENO2 in the areas of outer nuclear layer protrusion (Fig. 6, pink arrowheads). We occasionally found migration of cells from the inner nuclear layer into the outer nuclear layer 3 months after Müller cell ablation (Fig. 6, yellow arrows). We also observed a unique staining pattern of ENO2 in the outer region of the outer nuclear layer and inner segment layer (Fig. 6, white arrows). To

Along with strong expression in inner and outer segments, colocalization studies in the control retinae revealed that ENO1 was expressed in Müller cell bodies in the inner nuclear layer, and their processes in the inner plexiform layer and ganglion cell layer (Fig. 4). In contrast, selective Müller cell ablation resulted in reduced expression of ENO1 in the inner nuclear layer, with a few Müller cells expressing ENO1 in Rlbp1-CreER-DTA176 mice (Fig. 4, yellow arrows). Sporadic expression of ENO1 also was found in the areas of outer nuclear layer protrusion, where the photoreceptor inner and outer segments were absent after Müller cell ablation (Fig. 4, pink arrowheads).

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study this further, we performed double-immunofluorescence labeling using ENO2 in combination with lectin PNA and lectin WGA, which label cone photoreceptor inner segments and rod photoreceptor outer segments, respectively. Colocalization studies revealed these structures in ENO2 stain were cone photoreceptors (Figs. 7, white arrows). Moreover, no cone photoreceptor inner and outer segments were seen in patches of Müller cell loss where the outer nuclear layer protruded into subretinal space (Fig. 7, asterisks), and occasional loss of photoreceptor cell bodies near the boundaries of outer nuclear layer protrusion also was found (Figs. 7, yellow arrows). Lectin WGA strongly labeled the rod photoreceptor outer segments in the control group; however, absence of ENO2 expression also was observed in areas where Müller cell ablation induced loss of rod photoreceptor outer segments (Fig. 8, asterisks).

Absence of P3K and RAPTOR expression was seen in areas where patchy Müller cell ablation induced loss of photoreceptor inner and outer segments (Figs. 9, 10). We presented photomicrographs of P3K and RAPTOR staining (Figs. 9, 10), since the expression of genes encoding these proteins was down-regulated most significantly in the LCM-directed qRT-PCR analysis.

**DISCUSSION**

We have studied alterations in the glycolytic and mTOR pathways in patches of Müller cell loss using LCM-directed qRT-PCR and immunofluorescence analysis in a transgenic model, in which Müller cells can be selectively and inducibly ablated. We found that transcription of the genes involved in the glycolytic (GAPDH, LDHαa and -b, and ENO1 and -2) and mTOR (IGF-1, P3K, Akt1, and RAPTOR) pathways was suppressed. Immunofluorescence studies indicated that loss of these proteins was mainly from photoreceptor inner and outer segments, as well as from the inner nuclear layer where ENO1 had been shown to colocalize with Müller cell bodies in control mice. These results complemented those of our previous study, in which we found suppression of the glycolytic and mTOR pathways after selective Müller cell ablation in whole retinal samples, but without indicating precisely where it was occurring. Together with the previous report, the present study indicated that suppression of the glycolytic and mTOR pathways occurred mainly in photoreceptor inner and outer segments in regions of Müller cell loss.

We have reported previously that patches of Müller cell loss were accompanied by photoreceptor degeneration, which we hypothesized was due to the failure of Müller cells to produce lactate to support photoreceptors. Of the 5 glycolytic enzymes we studied, however, only ENO1 was expressed clearly by Müller cells and was subsequently lost after Müller cell ablation. ENO1 also was expressed sporadically in some cells in areas of outer nuclear layer protrusion into the subretinal space, which we have shown previously is related topographically to zones of Müller cell ablation. This might reflect a compensatory response from the surviving photoreceptor cell bodies. Glycocalyx occurs in Müller cells and photoreceptors. Müller cells constantly are converting glucose to lactate to provide energy to photoreceptors, and photoreceptors can obtain glucose from the choroidal circulation, which they convert to pyruvate for their own energy production. Glycolysis involves a sequence of 10 enzymatic reactions. Of the enzymes involved in the glycolytic pathway, we chose 5 to analyze in this study: GAPDH catalyzes the phosphorylation of glyceraldehyde-3-phosphate; LDH catalyzes the interconversion of pyruvate and NADH to lactate and NAD+; and ENO is involved in the later stages of glycolysis by converting 2-phosphoglycerate to phosphoenolpyruvate. We are unable to say whether other glycolytic pathway enzymes apart from the 5 we chose to study are expressed normally by Müller cells.

While all the other 4 enzymes were expressed by photoreceptor inner and outer segments in the control group, they subsequently were lost after Müller cell ablation. We believe, however, that the loss of photoreceptor inner and outer segments, consequently the loss of glycolytic enzymes in the area, is secondary to Müller cell ablation. We occasionally found increased expression of ENO2 in some cells in the outer nuclear layer 3 months after Müller cell ablation (Fig. 6). These appeared to be INL cells that were displaced by the structural derangement of the deep retina in areas corresponding to patches of Müller cell loss (yellow arrows in Figs. 6H, 6I). We also saw some cone photoreceptor nuclei protruding into the subretinal space that were expressing ENO2 (pink arrowheads in Figs. 6H, 6I).

Together with disruptions of Müller cell, and photoreceptor inner and outer segments, cone photoreceptor cell bodies occasionally were seen to be absent in regions bordering patches of Müller cell loss, which appeared otherwise normal. Considering that Müller cell end feet processes are located in outer limiting membrane, occasional absence of cone photoreceptor cell bodies near patches of Müller cell loss showed that Müller cell ablation strongly affected the photoreceptors with which the Müller cells are in direct contact, but also other photoreceptors with which they have no direct contact.

Another finding from the study is suppression of genes and proteins involved in the mTOR signaling pathway in photoreceptor inner and outer segments in areas of Müller cell loss. The mTOR pathway has an important role in photoreceptor survival, since it positively regulates glycolysis through growth factor signaling, such as insulin receptor activation. Reduced phosphorylation of mTOR in cone photoreceptors has been found in a mouse model of photoreceptor degeneration, in which cone photoreceptors survival was improved by stimulation of the mTOR pathway with insulin treatment. It has been reported that deletion of the p85 subunit of P3K specifically in cone photoreceptors resulted in their degeneration and functional impairment in a transgenic model. Hsu et al. demonstrated that photoreceptor outer segments obtain energy not only from their own glycolysis, but also from the photoreceptor inner segments through a phosphocreatine shuttle. However, as we showed with the glycolytic pathway enzymes, we believe that loss of the mTOR pathway enzymes is a secondary consequence of photoreceptor degeneration in our model. However, their loss may have significance in retinal diseases that affect photoreceptor health.

In summary, we have used successfully LCM-directed qRT-PCR and immunohistochemistry to examine changes in glycolytic and mTOR pathways to localize their changes more precisely after induced Müller cell loss. While we confirmed that reduced transcription and expression of the proteins we selected did occur in areas of Müller cell loss, our immunofluorescence studies revealed that this occurred mainly because of degeneration of the inner and outer segments, which we believe was a secondary effect of photoreceptor degeneration due to loss of trophic support from Müller cells, the nature of which remains obscure. We also found evidence that photoreceptor degeneration in this model appeared to occur also at the boundaries of patches of Müller cell loss, suggesting that loss of the trophic effect from Müller cells on photoreceptors may be achieved by diffusible factors as well as direct contact. Further biochemical studies of glycolytic pathway activity in this model are warranted to identify...
metabolic mechanisms of photoreceptor degeneration after Müller cell ablation.

Acknowledgments
The authors thank Louise Cole for LCM training. Supported by grants from Lowy Medical Research Institute, National Health and Medical Research Council (APP1028395; NHMRC, Cannaberra, Australia), Ophthalmic Research Institute of Australia, Rebecca L. Cooper Medical Research Foundation, and the University of Sydney Bridging Grants scheme, and by an NHMRC Practitioner Fellowship (MCG).

Disclosure: S.H. Chung, None; W. Shen, None; M.C. Gillies, None.

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