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Cultured Müller cells from mammals can synthesize and accumulate retinyl esters

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Research reports from our laboratory, as well as those from others, have shown that cone-dominant species such as chicken, ground squirrel and zebrafish possess a novel cone cycle which is dependent on the production of 11-*cis* retinal from Müller cells in the retina (Muniz A 2006; Muniz 2007; Wang and Kefalov 2011). This visual cycle operates differently from the classical (canonical) visual cycle, which is dependent on the synthesis of 11-*cis* retinal by an isomerohydrolase (RPE65 or Isomerase 1) located in the retinal pigment epithelium (RPE) (Fleisch VC 2010; Takahashi 2011). Using eyecup and isolated retina preparations, Kolesnikov et al. recently reported that the mouse retina promoted M/L-cone dark adaptation eightfold faster than the RPE alone (Kolesnikov 2011) suggesting that the initial rapid cone pigment regeneration is highly dependent on the synthesis of 11-*cis* retinal from Müller cells in the retina (Kolesnikov 2011). However, it is not clear if IRBP (Interphotoreceptor Retinoid Binding Protein) is essential for normal retinoid processing in the retinal cone cycle (Kolesnikov 2011; Parker 2011). Furthermore, it has recently been reported that different variants of isomerases are expressed in mouse cone photoreceptors (Tang, Wheless et al. 2011) as well as zebrafish Müller cells and ganglion cells (RPE65a and RPE65c) (Takahashi 2011).

Müller cells from the chicken retina have been shown to possess retinol isomerase (Das SR 1992; Mata NL 2005; Muniz 2009) and 11-*cis* retinyl ester synthase (Muniz A 2006) activities. Although limited biochemical data are now available on these visual cycle enzymes, they are collected from the retina or Müller cells in cone-dominant species. At present, it is not known if Müller cells from rod-dominant retinas also possess a cone visual cycle and specific visual proteins for the cone cycle.

Spontaneously immortalized human Müller cells were obtained from Dr. Astrid Limb (University College; London, UK). Cells were cultured in T25 flasks using Dulbecco's modified essential medium (DMEM) with 4.5g/L glucose and 10% fetal bovine serum (FBS) at 37°C + 5% CO₂ until confluent (approximately 5 days)(Lawrence 2007). SV-40 transformed rat Müller cells (rMC-1), was obtained from Dr. Vijay Sarthy (Northwestern

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University; Chicago, IL). Cells were cultured in T25 flasks using DMEM + 10% FBS at 37°C + 5% CO₂ until confluent (approximately 3–5 days)(Yego 2009).

In order to obtain retinas for primary culture of chicken, bovine and mouse Müller cells, ocular tissue was obtained and cell cultures were carried out as follows: freshly decapitated chicken heads were obtained from Tyson Foods Processing Plant in Seguin, Texas, and transported on ice to the laboratory. Eyes were enucleated and hemisected, vitreous and lens were removed from each eye, and retinas were dissected free from RPE using DMEM. Retinas were washed 3 times with 1X Hanks Buffered Saline Solution (HBSS) containing 1% Penicillin/Streptomycin. Retinas were incubated in 1X HBSS containing 0.1 mg/mL of Papain (Cat# P5306, Sigma Aldrich) for 60 minutes at 37°C + 5% CO₂. Retinas were then collected and centrifuged at 1,000 × G for 5 minutes. Supernatant was discarded and the pellet was washed 3 times with 1X HBSS and centrifuged once more at 1,000 × G for 5 minutes. Supernatant was discarded and the pellet re-suspended in DMEM containing 4.5 g/L glucose, 10% FBS, and 0.1% Penicillin/Streptomycin. DMEM + suspended cells were then introduced into T25 flasks (approximately 2–3 retinas per flask; approximately 1 × 10⁶ cells) and cultured at 37°C + 5% CO₂. Media was changed after the first 24 hrs in culture, then every 48 hrs afterward until confluent (approximately 10–14 days) (Muniz A 2006).

Freshly enucleated adult bovine eyes were obtained from Wiatrex Meat Market in Poth, Texas and transported to the laboratory on ice. Retinas were dissected free from the RPE and Müller cells cultured as described in 2.3 with the exception that each T25 flask contained about half of one bovine retina which yielded approximately 1 × 10⁶ cells. Freshly enucleated 8–12 day old C57BL/6 mice eyes were obtained from Dr. Joe Martinez (UT-San Antonio). Eyes were submerged in DMEM and kept at room temperature overnight under dark conditions. Approximately 12 hrs later, eyes were placed in a solution containing 0.1% Trypsin + collagenase (70U/ml) in DMEM and incubated at 37°C + 5% CO₂ for 60 minutes. Retinas were dissected and triturated in 2.5 mL of DMEM using a Pasteur pipette. Cell suspension was mixed with growth medium (DMEM + 10% FBS + 0.1% Penicillin/Streptomycin) and seeded into T25 flasks for culturing. Cells were then incubated at 37 °C + 5% CO₂ until confluent (approximately 10–14 days).

In order to verify Müller cells in culture, immunohistochemistry was performed. Müller cells were introduced onto 8 well tissue-tek slides (Cat # 154534; Thermo-Fisher) and grown for 3 days with culture medium and conditions described previously. Media was aspirated from each Müller cell type and cells were then washed with 1X Phosphate Buffered Saline + Tween 20 (PBST) and fixed in 4% paraformaldehyde (PFA) solution for 10 minutes. PFA was removed and cells were washed with 1X PBST 3 times for 5 minutes each wash. A blocking solution of 10% goat serum in 1X Phosphate Buffered Saline (PBS) was then added to each well and incubated for 30 minutes at room temperature. Blocker was then decanted without rinsing. Primary rabbit anti-GFAP (Glial fibrillary acidic protein) antibody was introduced to the cells at 1:1000 in goat serum and incubated for 1 hr at room temperature. Excess primary antibody was then removed and cells were washed with 1X PBST 3 times for 5 minutes each wash. Secondary antibody, goat-anti-rabbit with FITC conjugate (fluorescein isothiocyanate 1) was added at a 1:200 dilution in goat serum and then incubated for 5 minutes at room temperature. Cells were then lightly rinsed with 1X PBS 3 times for 5 minutes each wash. Chambers were then removed from the slides and cells were fixed with 4',6-diamidino-2-phenylindole (DAPI) fluorescence mounting media and cover-slipped. Controls from each Müller cell type were also DAPI fixed without antibodies. Cells were then viewed at a 10x magnification using a Zeiss Axioscope 2 Plus equipped with AxioVision Release 4.8.1 software.

Biochemical assays began with homogenates prepared from recovered cells and we then determined protein concentrations by Bradford method (BioRad protein assay dye Cat #500-0006, BioRad Laboratories) using a Helios spectrophotometer equipped with Vision 32 software (ThermoSpectronic). Retinoids were extracted with a 1:2 ratio of ethanol/hexane and analyzed using high performance liquid chromatography (HPLC with a 2996 photo-diode array on-line detector and Empower software, Waters Technologies) and identified by retention time and online UV spectra in comparison to authentic retinoid standards (n=3 for all experiments). Data were analyzed using students t-test and values 0.05 were considered to be statistically significant.

Human Müller cells (MIO-M1), rat Müller cells (MIO-M1), and primary Müller cells from bovine retinas were seeded individually into T25 flasks and then grown to confluence. Cells were serum starved for 6 hours before the addition of all-*trans* retinol (from Sigma-Aldrich; Cat #R7632) or 11-*cis* retinol (synthesized from 11-*cis* retinal, from Dr. Rosalie Crouch) and incubated at 37°C + 5% CO₂ (in dim red light). Retinoids were dissolved in ethanol, dispersed in 10% Bovine Serum Albumin and then added to the culture media; final retinol concentrations in culture media were 10 µM. MIO-M1, rMC-1, and primary bovine Müller cells were harvested at 0 hrs, 8 hrs, 16 hrs and 24 hrs. Retinoids were extracted and analyzed as described above.

To determine retinyl ester synthase activity in Müller cells from different rod-dominant species, cultured Müller cells were incubated with 11-*cis* or all-*trans* retinol (added to the culture media; as described above) for 24 hrs. Retinoids were extracted from cell homogenates and analyzed by HPLC (n = 7 for human, rat, chicken and bovine Müller cells; n = 10 for mouse Müller cells; results from chicken Müller cells were obtained and included in these experiments to serve as a reference).

Immunohistochemistry results showed that all cell types (rMC-1, chicken, bovine, and mouse Müller cells in culture) were co-stained by antibodies against GFAP and by nuclear stain DAPI (to confirm identity of Müller cell and homogeneity of cells in culture; data not shown).

Results from our HPLC analyses show that cultured Müller cells did not contain any detectable amount of (endogenous) retinol or retinyl ester. However, incubation of human (MIO-M1) Müller cells with 10 µM of all-*trans* retinol yielded 17 ± 1.2 pmol all-*trans* retinyl ester/mg protein [mean $\pm$ SE(n=3)] at 8 hrs, 27 ± 1.0 pmol all-*trans* retinyl ester/mg (n=3) at 16 hrs, and 150 ± 1.0 pmol all-*trans* retinyl ester/mg (n=3) at 24 hrs, respectively (Fig. 1a). A similar rate of 11-*cis* retinyl ester synthesis was observed in these human Müller cells; 13 ± 0.5 pmol 11-*cis* retinyl ester/mg (n=3) at 8 hrs, 46 ± 1.2 pmol 11-*cis* retinyl ester/mg (n=3) at 16 hrs and 123 ± 1.0 pmol 11-*cis* retinyl ester/mg (n=3) at 24 hrs (Fig. 1a). Incubation of rat Müller cells (rMC-1) with 10 µM of all-*trans* retinol yielded 65 ± 3.0 pmol all-*trans* retinyl ester/mg (n=3) at 8 hrs, 120 ± 4.0 pmol all-*trans* retinyl ester/mg (n=3) at 16 hrs, and 192 ± 4.0 pmol all-*trans* retinyl ester/mg (n=3) at 24 hrs (Fig. 1b). Incubation of rMC-1 with 10 µM of 11-*cis* retinol yielded 22 ± 0.5 pmol 11-*cis* retinyl ester/mg (n=3) at 8 hrs, 190 ± 5.0 pmol 11-*cis* retinyl ester/mg (n=3) at 16 hrs, and 559 ± 18.5 pmol 11-*cis* retinyl ester/mg (n=3) at 24 hrs, respectively (Fig. 1b). Primary bovine Müller cells incubated with 10 µM all-*trans* retinol yielded 19 ± 0.5 pmol all-*trans* retinyl ester/mg (n=3) at 8 hrs, 75 ± 0.8 pmol all-*trans* retinyl ester/mg (n=3) at 16 hrs and 378 ± 0.9 pmol all-*trans* retinyl ester/mg (n=3) after 24 hrs, respectively (Fig. 1c). Primary bovine Müller cells incubated with 10 µM 11-*cis* retinol yielded 0.0 pmol 11-*cis* retinyl ester/mg (n=3) at 8 hrs, 3 ± 0.5 pmol 11-*cis* retinyl ester/mg (n=3) at 16 hrs, and 12 ± 0.5 pmol 11-*cis* retinyl ester/mg (n=3) after 24 hrs, respectively (Fig. 1c). In a separate set of experiments, retinyl ester synthase activities in different Müller cells of rat, human, chicken, bovine and mouse retinas

(incubated with all-*trans* retinol or 11-*cis* retinol for 24 hrs) were obtained based on the amount of retinyl ester synthesized within a 24 hr incubation period (Fig 2). Rat (rMC-1) Müller cells synthesized 192 ± 18.0 pmol all-*trans* retinyl esters/mg ($n = 7$) and 560 ± 42.7 pmol 11-*cis* retinyl ester/mg ($n = 7$). Human (MIO-M1) Müller cells synthesized 150 ± 14.3 pmol all-*trans* retinyl ester per mg (total cell proteins; mean $\pm$ SE; $n = 7$) and 123 ± 7.9 pmol 11-*cis* retinyl ester/mg ($n = 7$). Primary chicken Müller cells synthesized 30 ± 0.6 pmol all-*trans* retinyl ester/mg ($n = 7$) and 145 ± 0.0 pmol 11-*cis* retinyl ester/mg ($n = 7$). Primary bovine Müller cells produced 378 ± 91.7 pmol all-*trans* retinyl ester/mg ($n = 7$) and 12 ± 1.5 pmol 11-*cis* retinyl ester/mg ($n = 7$). Primary Mouse Müller cells produced 31 ± 2.3 pmol all-*trans* retinyl ester/mg and $8,280 \pm 14.3$ pmol 11-*cis* retinyl esters/mg ($n = 10$).

Although Müller cells from the cone-dominant chicken retina have been shown to synthesize retinyl esters from exogenously added 11-*cis* and all-*trans* retinol (Das SR 1992; Muniz A 2006), this is the first study to report that Müller cells from human retina, as well as from other rod-dominant retinas (rat, bovine and mouse) also possess a significant retinyl ester synthase enzyme activity. The increase in retinyl ester accumulation observed in human, rat and bovine Müller cells appears to be time-dependent (Fig. 1). These data strongly support the suggestion that an retinyl ester synthase enzyme exists in cultured Müller cells to synthesize retinyl esters from exogenously added retinols.

In a separate set of experiments, we obtained additional experimental results to show that retinyl ester synthesis in Müller cells are dependent on the isomeric configuration of the retinol substrate and the species from which these Müller cells were derived. For example, there is a high selectivity for the synthesis of 11-*cis* retinyl esters (vs all-*trans* retinyl esters) by rat and mouse Müller cells (3:1 and 200:1). However, bovine Müller cells synthesized five times more all-*trans* than 11-*cis* retinyl esters (1:0.2) and human Müller cells were not selective in their synthesis of *cis* vs. *trans* retinyl esters (1:1). In terms of the rate of retinyl ester synthesis (amount of retinyl ester accumulation over a 24 hr period), mouse (primary) Müller cells exhibited a significantly higher rate of 11-*cis* retinyl ester synthesis (8280 pmol/mg in 24 hrs or 5.8 pmol/min/mg) than all other Müller cells examined in the present study. At present, it is not known if such species differences are due to protein expressions in different types of cultured cells. However, comparison of synthase activity among cells from primary cultures (derived from mouse, chicken and bovine retinas) clearly shows a large species difference.

In the present study, we have obtained evidence that cultured Müller cells from rod-dominant (human, rat/mouse, bovine) retinas accumulate 11-*cis* and all-*trans* retinyl ester when corresponding isomers of retinols were added to the culture media. The rate of retinyl ester synthesis is isomer-specific and species-dependent. We believe that retinyl esters in Müller cells provide (via the hydrolysis of retinyl esters) all-*trans* retinol to serve as the substrate for retinol isomerase (or Isomerase II, Takahashi 2011) in the Müller cells and to provide 11-*cis* retinol for transfer to cones (it is known that the addition of 11-*cis* retinol to cones led to the recovery of cone sensitivity (Travis GH 2010). In some cone-dominant retinas, retinyl esters are found in significant quantity (Bustamante 1995) which is consistent with our observation on retinyl ester accumulation in culture Müller cells from the cone-dominant chicken retina. However, most rod-dominated mammalian retinas do not contain any significant amount of retinyl esters. Nevertheless, cultured Müller cells from these retinas also accumulate retinyl esters. It is possible that Müller cells from both rod- and cone- dominant retinas have an inherent ability to synthesize and accumulate retinyl esters. However, the level of retinyl esters existing in a particular type of retina (rod-dominant) may be based on the balance between ester synthesis vs hydrolysis (in support of pigment regeneration).

The period of time required to accumulate retinyl esters in cultured cells is attributable to time required for the diffusion of exogenous retinol from culture media into cells and also time required for the isomerization/esterification reactions to take place. In comparison to *in-vitro* studies using cell homogenates or microsomal membranes in buffer, the transferring of retinol to an intracellular location may explain why retinyl ester accumulation required 24 hrs to complete.

Because rod-dominant retinas (human, rat, mouse and cow) do not store any significant amount of retinyl esters under both light and dark adapted states (Rodriguez 1989; Mata 2002), what is the role of retinyl ester accumulation in Müller cells? It is possible that the synthesis of retinyl ester is required for the function of a related visual protein such as retinol isomerase. This suggestion is consistent with our previously published report where we provided data to show that ARAT (a retinyl ester synthase enzyme) activity is found in Muller cells and is required for retinol isomerase activity (Muniz 2009). Results from the present study clearly reveal some novel and distinct properties of Muller cells in culture. Further studies are needed to provide additional information to show how such properties support visual pigment regeneration.

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Abbreviations

IRBP	Inter-photoreceptor Retinoid Binding Protein
RPE	retinal pigment epithelium
rMC-1	rat Müller Cells
MIO-M1	human Müller Cells
PalmCoA	Palmitoyl Co-Enzyme A
IRBP	Inter-photoreceptor Retinoid Binding Protein
FBS	Fetal Bovine Serum
HBSS	Hanks Buffered Saline Solution
DMEM	Dulbecco's Modified Eagle Medium
BSA	Bovine Serum Albumin
11-<i>cis</i>RP	11- <i>cis</i> retinyl ester
all-<i>trans</i>RP	all- <i>trans</i> retinyl ester
HPLC	High Performance Liquid Chromatography
DAPI	4',6-diamidino-2-phenylindole
FITC	fluorescein isothiocyanate 1

GFAP	Glial fibrillary acidic protein
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween 20
PFA	paraformaldehyde
ATOL	all- <i>trans</i> retinol
11cisOL	11- <i>cis</i> retinol

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Figure 1. Accumulation of all-*trans* and 11-*cis* retinyl esters in cultured Müller cells incubated with all-*trans* retinol or 11-*cis* retinol

Retinyl esters extracted from a) Human Müller cells (MIO-M1) incubated with all-*trans* retinol or 11-*cis* retinol for 8 hrs, 16 hrs, 24 hrs. b) Rat Müller cells (rMC-1) incubated with all-*trans* retinol or 11-*cis* retinol for 8 hrs, 16 hrs, and 24 hrs. c) Bovine Müller cells from primary culture incubated with all-*trans* retinol or 11-*cis* retinol for 8 hrs, 16 hrs, 24 hrs [mean $\pm$ SE (n = 3) for all time points].

Figure 2. Retinyl ester synthase activities in cultured Müller Cells incubated with all-*trans* retinol or 11-*cis* retinol for 24 hrs

Synthase activity in human (MIO-M1) Müller cell line, rat (rMC-1) Müller cell line, chicken Müller cells (primary culture), bovine Müller cells (primary culture) and mouse Müller cells (primary culture) when incubated with all-*trans* or 11-*cis* retinol for 24 hrs at 37°C + 5% CO₂ [mean ±SE(n = 7) for all data points except mouse where n = 10].