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Mammalian retinal Müller cells have circadian clock function

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Purpose: To test whether Müller glia of the mammalian retina have circadian rhythms.
Methods: We used Müller glia cultures isolated from mouse lines or from humans and bioluminescent reporters of circadian clock genes to monitor molecular circadian rhythms. The clock gene dependence of the Müller cell rhythms was tested using clock gene knockout mouse lines or with siRNA for specific clock genes.
Results: We demonstrated that retinal Müller glia express canonical circadian clock genes, are capable of sustained circadian oscillations in isolation from other cell types, and exhibit unique features of their molecular circadian clock compared to the retina as a whole. Mouse and human Müller cells demonstrated circadian clock function; however, they exhibited species-specific differences in the gene dependence of their clocks.
Conclusions: Müller cells are the first mammalian retinal cell type in which sustained circadian rhythms have been demonstrated in isolation from other retinal cells.

Our vision is different at different times of day because our retina works differently at different times of day. Daily rhythms in visual function are not just simple responses to the daily light-dark cycle but are the overt expression of an endogenous, self-sustained circadian clock in the retina that drives many rhythms in retinal physiology and metabolism [1]. Numerous aspects of retinal function are under the control of an endogenous retinal circadian clock, including melatonin release [2,3], dopamine synthesis [4,5], gamma-aminobutyric acid (GABA) turnover rate and release [6], extracellular pH [7], electroretinogram (ERG) b-wave amplitude [8], rod disk shedding [9], and circadian clock gene expression [10-12]. In addition, the mammalian retinal clock and its outputs influence the cell survival and growth processes in the eye, including the susceptibility of photoreceptors to degeneration from light damage [13,14], photoreceptor survival in animal models of retinal degeneration [15], photoreceptor and retinal ganglion cell survival in aging [16], and the degree of refractive errors in primate models of myopia [17]. Circadian signals originating in the retina drive rhythms in the hypothalamic biologic clock, even in the absence of light-dark cycles [18].

Although the pervasive influence of the retinal circadian clock is well documented, the cell-specific organization of the mammalian retinal circadian clock is not completely understood. Mapping of the cell-specific expression of the core clock genes that generate circadian rhythms (*Bmal1, Clock, Per1, Per2, Cry1* and *Cry2*) suggests that multiple populations of retinal neurons may be capable of circadian rhythmicity [12,19,20]. Similarly, real-time gene expression imaging of molecular circadian rhythms has demonstrated circadian rhythmicity in the photoreceptor and inner nuclear layers of the retina [11,21,22].

Efforts to date at elucidating the cellular organization of the retinal clock have focused primarily on retinal neurons; however, retinal Müller cells are key glial cells that span the entire depth of the retina and integrate physiology and metabolism across the retinal cell layers [23]. In the central biologic clock of the hypothalamus, the suprachiasmatic nucleus, astrocyte glia express clock genes and exhibit endogenous molecular circadian rhythms [24]. Here, we report that retinal Müller cells express the full set of core clock genes and generate molecular circadian rhythms in isolation from other retinal cell types and that Müller cell circadian clocks have a clock gene dependence unique from the retina as a whole.

METHODS

Müller cell culture: Primary Müller cell cultures were derived from wild-type, *Per1−/−*, *Per2−/−*, and *Per1−/−/Per2−/−* transgenic mouse and human retinas. The *Period* gene knockout (KO) mouse lines were originally obtained as a gift from D. Weaver [25], backcrossed for more than 12 generations on the C57BL6 background, and then crossed to PER2::LUC bioluminescent reporter mice on the C57 background (a gift of J. Takahashi [26]) to generate *Period gene* knockout reporter mice [27]. Human Müller cells were isolated from human donor eyes using the method described in Hicks and Courtois (also described in the section on

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mouse Müller cells isolation below) [28]. Experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) and adhered to the ARVO Statement for Use of Animals.

To isolate mouse Müller cells, eyes were removed from 6- to 14-day-old pups and placed in soaking medium (low-glucose DMEM with 1% of antibiotic, 0.01% of trypsin [ThermoFisher Cat# 27250018] and 70 U/ml of collagenase [Sigma C9722, St. Louis, MO]) at room temperature in the dark overnight. The eyes were then incubated in digestion buffer (low-glucose DMEM with 1% of antibiotic, 0.01% of trypsin, and 70 U/ml of collagenase) for 40 min at 37 °C; the retinas were removed from the eyes, transferred into culture medium (low-glucose DMEM with 10% fetal bovine serum, FBS [Sigma F2442-500 ml]), and dissociated with repeated pipetting using 1 ml. Müller cells from both species were cultured in low-glucose DMEM containing 10% FBS and maintained at 37 °C in a 5% CO_2 incubator for 5–6 days to reach semiconfluence before being used in the experiments [28].

Lentivirus vector transduction: Purified Müller cells were seeded into six-well plates and, when at 20%–30% confluence, were transduced with *Per2::luc* or *Bmal1::luc* lentiviral vectors [29] in DMEM containing 10 µg/ml of Polybrene (titer = 1×10^6, duration = 24 h) [EMD Millipore TR-1003-G, Billerica, MA]. Cell selection was performed with Blasticidin S (10 µg/ml; [InvivoGen ant-bl-1, San Diego, CA]) treatment for 3 days resulting in stable cell lines that expressed luciferase under the control of the *Per2* or *Bmal1* promoter.

siRNA transfection: For 1×10^5 cells, 1 µl of 10 µM of siRNA and 1 µl of Lipofectamine 2000 were added separately in two tubes containing 50 µl of Opti-MEM I Reduced Serum Medium (ThermoFisher 31-985-062, Grand Island, NY; prewarmed) for 15 min at room temperature; then, siRNA and Lipofectamine were combined, mixed gently, and incubated at room temperature for 25 min. Müller cells expressing *Per2::luc* or *Bmal1::luc* lentiviral reporters were serum starved overnight, trypsinized, and then incubated with siRNA (for mouse or human *Per1, Per2, Bmal1*, or a control siRNA, Santa Cruz) and Lipofectamine (ThermoFisher Cat#11668-027) for 25 min at room temperature. Cells were seeded on 35 mm dishes containing serum-free DMEM and incubated at 37 °C in 5% CO_2 and 95% for 6 h, switched to 10% FBS DMEM for 6 h, then changed into recording medium (medium 199 and 0.7 mM of L-glutamine [Sigma-Aldrich Cat#G7513], 2% of B27[ThermoFisher Cat# 17504-044], and 0.1 mM of luciferin [Promega Cat#E1601, Madison, WI]), and transferred to a Lumicycle multichannel luminometer (Actimetrics) for recording. The effectiveness and specificity of the siRNAs were confirmed with quantitative PCR (Appendix 1 and Appendix 2).

Data analysis: Experiments assessing the effects of gene knockouts or knockdowns were run with two to six independent replicates. Effects on rhythmicity were quantified by analysis using Lumicycle software with the threshold for considering a condition to be rhythmic being an average rhythmic power of ≥0.1 and goodness of fit ≥15% [30]. Values are reported as mean ± standard deviation.

RESULTS

To examine the circadian function of Müller cells, we first produced primary Müller cell cultures. The cultures were assessed with double-label immunofluorescence for 4',6-diamidino-2-phenylindole (DAPI), which stains all nuclei, and glutamine synthetase (GS), a enzymatic marker specific for Müller cells in the retina (Figure 1A,B). Mergers of the DAPI and GS staining patterns showed that the cell cultures were highly enriched for Müller cells (Figure 1C).

Expression of clock genes in Müller cells: To test for the expression of circadian clock genes in Müller cells, RNA was extracted from the Müller cells cultures, and reverse transcriptase PCR was performed for the core circadian clock genes: *Bmal1, Clock, Per1, Per2, Cry1*, and *Cry2*. Each gene was readily detected (Figure 1D), indicating that Müller cells as a population express key genetic components of the circadian clock.

Circadian rhythms of isolated Müller cells: To test whether Müller cells exhibit circadian clock function, cultures were isolated from the mice that harbored a bioluminescent circadian reporter gene, PER2::LUC [26]. Retinal whole mounts explanted from this mouse reporter line exhibit sustained circadian rhythms in PER2::LUC bioluminescence, allowing real-time readout of the cycling of the molecular circadian clock as the intensity of bioluminescence that varies with the abundance of the PER2::LUC fusion protein [11]. The PER2::LUC Müller cell cultures were switched from standard culture medium to bioluminescent recording medium containing luciferin, and then the bioluminescent activity recorded. These cultures exhibited robust circadian rhythms in PER2::LUC expression that persisted for several days following initiation of recording, and which could be partially restored in amplitude with subsequent media changes (Figure 2A).

To further test molecular circadian rhythms in isolated mouse Müller cells, Müller cell cultures from WT C57 mice were transduced with lentiviral constructs in which either the *Per2* gene promoter or the *Bmal1* gene promoter to drive the

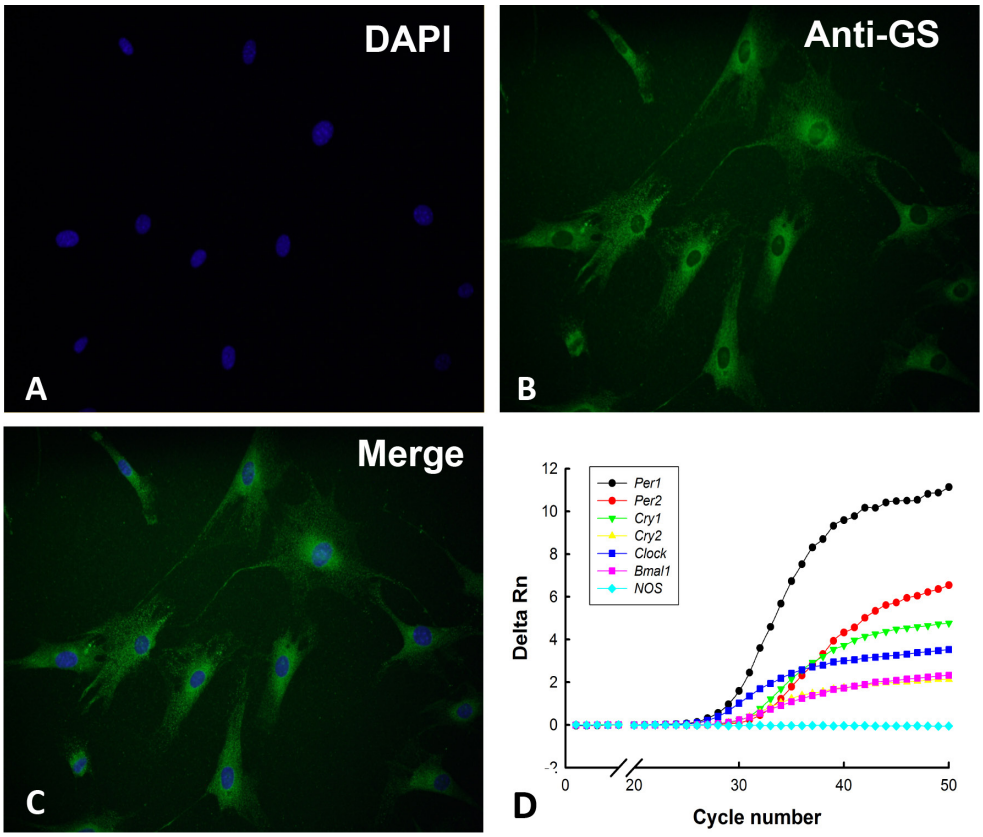

Figure 1. Mouse retinal Müller cells express the canonical circadian clock genes. **A**: 4',6-diamidino-2-phe-nylindole (DAPI) staining showing all cell nuclei (blue) in a fluorescence photo-micrograph of a purified Müller cell culture. **B**: Glutamine synthase (GS) antibody staining showing Müller cells (green). **C**: Merged image of A and B, showing the high degree of purity of the Müller cell culture. **D**: Clock gene expression from a purified Müller cells culture with quantitative PCR. Nitric oxide synthase (NOS), which is present in many retinal neurons but not in Müller cells, was used as a negative control for the purity of the cultures.

expression of firefly luciferase [29]. Similar to the cultures from the PER2::LUC reporter mice, Müller cells transduced with lentiviral *Per2::luc* and *Bmal1::luc* exhibited robust circadian rhythms in bioluminescence, which were sustained for days, and then could be partially restored in amplitude with a change to a fresh culture medium (Figure 2B,C).

Clock gene dependence of Müller cell rhythms: In the whole retina, expression of *Per1* and *Bmal1* is necessary for molecular circadian rhythms, whereas expression of *Per2* is not [8,27]. Here, we tested for the necessity of these three clock genes in maintaining molecular circadian rhythms in mouse Müller cell cultures. The dependence on *Period* genes was tested in the mice by isolating Müller cells from *Period*

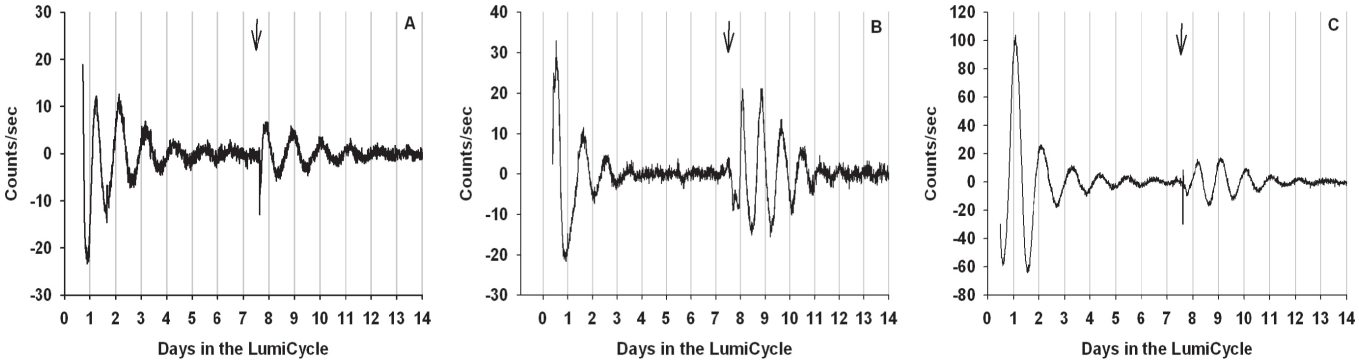

Figure 2. Mouse Müller cells exhibit molecular circadian rhythms. **A**: Bioluminescent rhythms recorded from Müller cells cultured from PER2::LUC mice. **B**: Bioluminescence rhythms from WT C57 mouse Müller cells transduced with *Per2::luc* lentivirus. **C**: Bioluminescence rhythms from WT C57 mouse Müller cells transduced with *Bmal1::luc* lentivirus. Arrows indicate the introduction of the new culture medium.

gene knockout mice: *Per1−/−, Per2−/−* and *Per1−/−;Per2−/−* double-knockout mice [25]. The circadian clock gene activity of the *Per1−/−* Müller cells was monitored using the PER2::LUC knockin transgene reporter, while the *Per2−/−* and *Per1−/−;Per2−/−* double-knockout Müller cells were monitored using lentiviral *Per2::luc* or *Bmal1::luc* reporters. Dependence on *Bmal1* was tested by isolating retinas and Müller cells from *Bmal1* KO mice crossed with PER2::LUC reporter mice. Individual example traces are shown in Figure 3, Figure 4, and Figure 5 while summary analysis of rhythmicity is shown in Table 1.

Knockout of *Per1* led to the loss of PER2::LUC rhythms in the whole retina as well as in isolated Müller cells (Figure 3A–C), similar to the strong effect of *Per1* knockout on retinal rhythmicity previously reported [27]. *Bmal1* knockout retinas and Müller cells also showed severely blunted circadian rhythms (Figure 3D,E), again mimicking the results from the whole retina [27]. Of note, Müller cells derived from mice harboring a *Per1::luc* transgene reporter [31] did not exhibit circadian rhythms in *Per1* activity (Figure 3F). Knockout of *Per2* left Müller cell rhythmicity intact, while the double knockout of *Per1* and *Per2* resulted in arrhythmicity as would be expected from *Per1KO* alone (Appendix 3).

To test whether acute suppression of the *Per1* or *Bmal1* clock gene, as opposed to genetic knockout, may also disrupt Müller cell molecular rhythms, experiments were performed using gene knockdowns with siRNA. In control experiments, neither the Lipofectamine transfection reagent itself nor transfection of a control siRNA sequence that had no known target in the mouse genome altered Müller cell circadian rhythms as measured with PER2::LUC bioluminescence (Figure 4A–C). Application of siRNA targeted to *Per1*, however, produced arrhythmic Müller cell cultures after an initial set of transients, and rhythmicity was restored after the culture medium was changed (Figure 4D). Application of *Per2* siRNA also led to the loss of rhythm (Figure 4E), likely due to direct

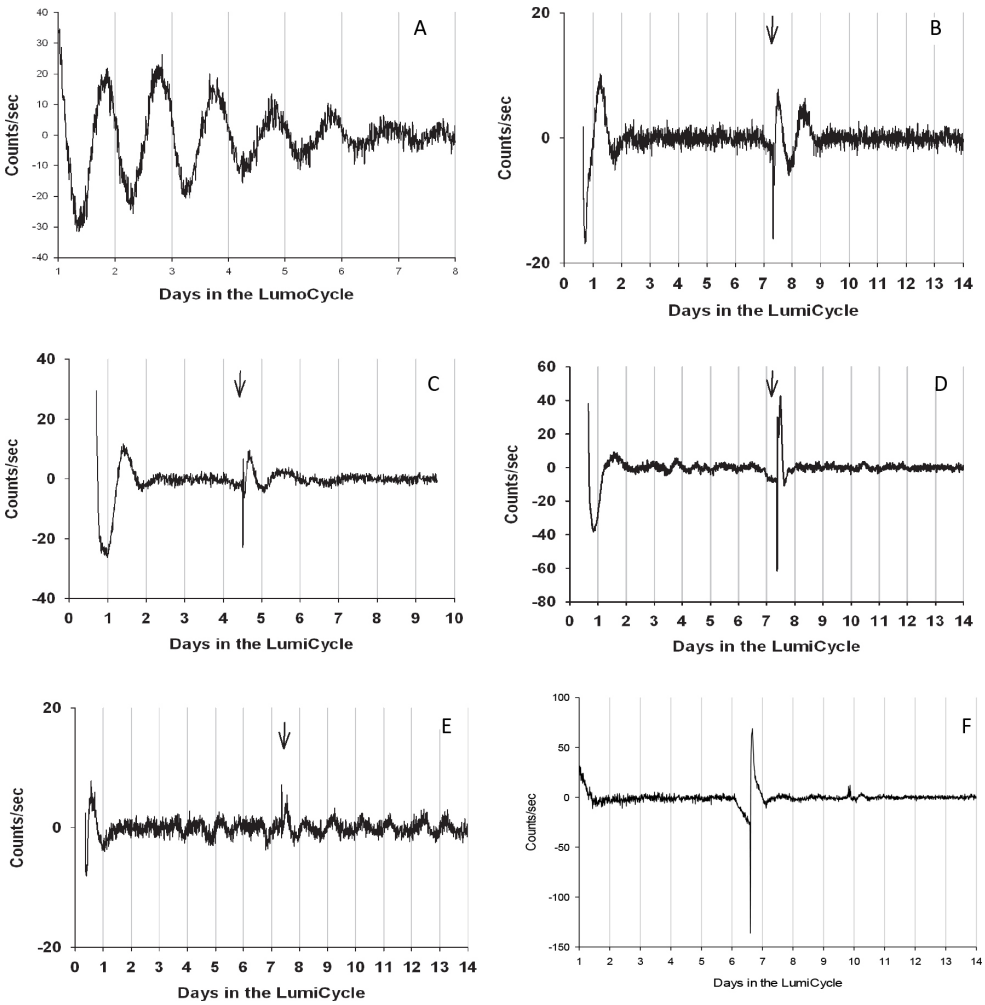

Figure 3. Mouse Müller cell culture rhythms are dependent on *Per1* and *Bmal1* genes. **A**: *Per1::luc* retinal rhythms. **B**: Absence of PER2::LUC rhythms from the *Per1* KO retina. **C**: Absence of rhythms in the Müller cell cultures derived from the *Per1−/−* PER2::LUC mice. **D**: Absence of rhythms from the whole retina derived from the *Bmal1−/−* PER2::LUC mice. **E**: Absence of rhythms from the Müller cells derived from the *Bmal1−/−* PER2::LUC mice. **F**: Absence of *Per1::luc* rhythms in the Müller cell cultures derived from the *Per1::luc* mice.

suppression of the PER2::LUC reporter, as *Per2*−/− tested with a *Bmal1* reporter did not disrupt rhythmicity (Appendix 3). Application of siRNA for *Bmal1* also negated PER2::LUC rhythms in cultured Müller cells, which were restored after the culture medium was changed (Figure 4F). Thus, ongoing expression of *Per1* and *Bmal1* is necessary for intact robust mouse Müller cell molecular circadian rhythms, and the loss of rhythmicity in gene knockouts is unlikely to be due to purely developmental effects.

To test whether human Müller cells also exhibit molecular circadian rhythms, we transduced purified human Müller cell cultures with the *Per2::luc* or *Bmal1::luc* constructs delivered by lentivirus as above. Similar to the mouse Müller cells, the human Müller cell cultures exhibited robust circadian rhythms in *Per2* and *Bmal1* expression as measured by the bioluminescence output (Figure 5A–C). We also examined the gene dependence of these rhythms by siRNA knockdown as above but used the human *Per1*, *Per2*, and *Bmal1* sequences for targeting. In contrast to the mouse, knockdown of *Per1* left the human Müller cell rhythms intact, as measured by *Per2::luc* (Figure 5D), as did knockdown of *PER2*, measured with *Bmal1::Luc* (Figure 5E). Knockdown of *Bmal1*, however, resulted in the loss of rhythms measured with *Per2::Luc* (Figure 5F).

DISCUSSION

Our findings indicate that mammalian retinal Müller cells express the canonical circadian clock genes, are capable of sustained circadian oscillations in isolation from other cell types, and exhibit unique features of their molecular circadian clock compared to the retina as a whole. These are the first mammalian retinal cell type to be shown to exhibit sustained circadian rhythms in isolation from other retinal cell types in purified cell cultures.

A fundamental question regarding the circadian organization of the mammalian retina is which cell types are the loci of endogenous rhythms generation. Previous studies have mapped the coordinate expression of the complete set of

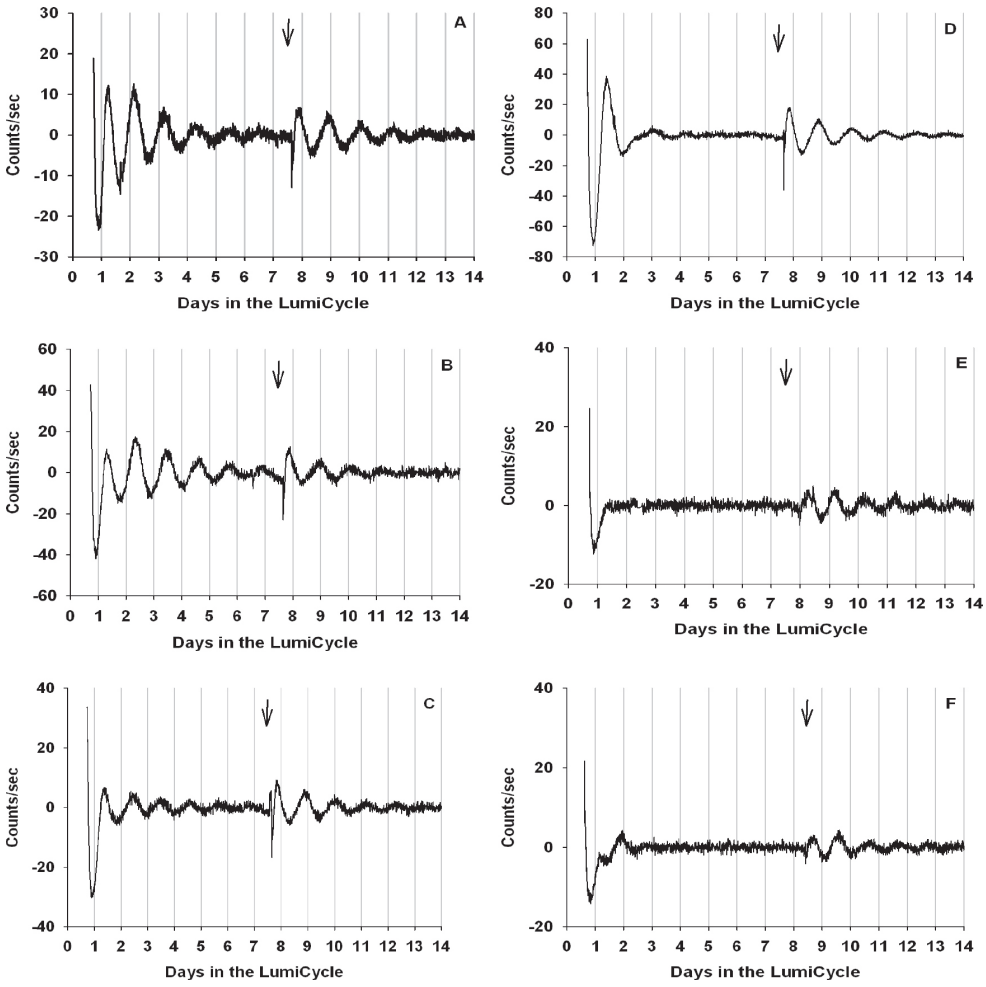

Figure 4. Mouse Müller cell PER2::LUC rhythms depend on ongoing *Per1* and *Bmal1* expression. **A**: Bioluminescent rhythms recorded from the Müller cells cultured from PER2::LUC mice. **B**: PER2::LUC rhythms from the Müller cell cultures treated with control siRNA. **C**: PER2::LUC rhythms from the Müller cell cultures treated with the Lipofectamine transduction reagent. **D**: Loss of PER2::LUC rhythms from the Müller cell cultures treated with *Per1* siRNA. **E**: Loss of PER2::LUC rhythms from the Müller cell cultures treated with *Per2* siRNA. **F**: Loss of PER2::LUC rhythms from the Müller cell cultures treated with *Bmal1* siRNA. Arrows indicate the introduction of the new culture medium.

canonical clock genes to all major retinal neuronal subtypes, with the possible exception of rods [12,19,20], and shown that the photoreceptor layer (rods and cones) and the inner retina (horizontal cells, bipolar cells, amacrine cells, ganglion cells, Müller cells) can each express molecular circadian rhythms in isolation [11,21]. In the intact in vivo mouse retina, dopaminergic amacrine cells, nitric oxide synthase (NOS)–positive amacrine cells, and cones have also been shown to express *Per1* clock gene rhythms [20,32,33]. These studies suggest that multiple cell types within the retina could be a source of the circadian rhythm, but do not distinguish between endogenously rhythmic versus passively driven cell types.

We have shown that Müller cells produce endogenous circadian rhythms in the cycling of canonical clock genes in isolated purified cell cultures, demonstrating that Müller cells have endogenous circadian clocks that likely contribute to retinal circadian rhythms. Müller cell nuclei reside in the inner nuclear layer (INL) and likely contribute to the high amplitude molecular rhythms observed in that region of the retina with the *PER2::LUC* reporter transgene [11]. In addition, as glial cells that span the entire depth of the neural retina, Müller cells have the potential to influence many retinal cell types and processes, through their regulation of ion fluxes and neurotransmitter uptake [23]. One of the principal functional circadian rhythms in the retina is in the amplitude of photopic light responses, which can be quantified by the ERG b-wave [34,35]. Through regulation of ionic and neurotransmitter transport, Müller cell circadian function may play a role in the circadian rhythm in retinal light responsiveness.

The overall molecular basis of the mouse Müller cell circadian clock is similar to that of the mouse retina as a whole. Gene knockout and knockdown experiments showed that mouse Müller cell rhythms require expression of the clock gene *Per1* or *Bmal1*, but not *Per2*, as does the intact retina [27]. However, although Müller cells require *Per1* to express circadian oscillations in PER2::LUC bioluminescence, *Per1* promoter activation, as read out by *Per1*::Luc,

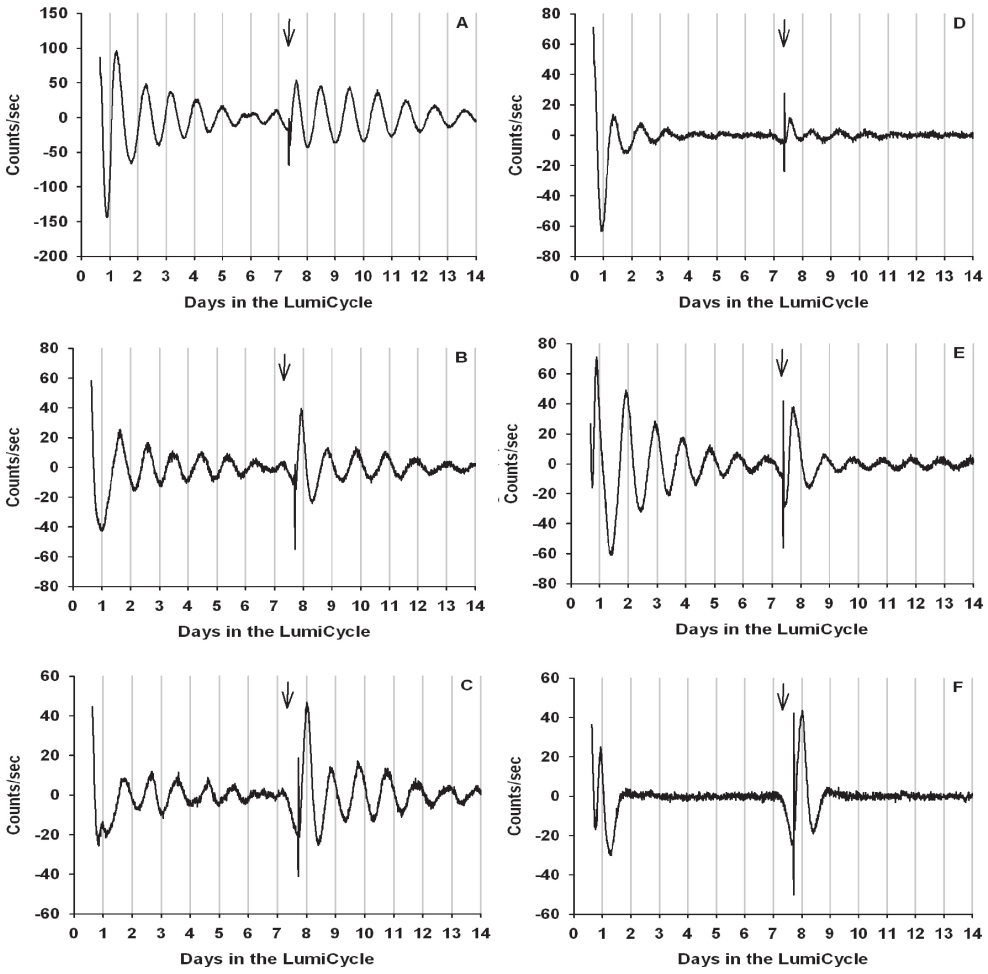

Figure 5. Human Müller cells exhibit molecular circadian rhythms that depend on *Bmal1*. **A**: Bioluminescence rhythms from the human Müller cells transduced with *Per2::luc* lentivirus. **B**: Bioluminescence rhythms from the human Müller cells transduced with *Per2::luc* lentivirus and control siRNA. **C**: Bioluminescence rhythms from the human Müller cells transduced with *Per2::luc* lentivirus and Lipofectamine. **D**: Bioluminescence rhythms from the human Müller cells transduced with *Per2::luc* lentivirus and *Per1* siRNA. **E**: Bioluminescence rhythms from the human Müller cells transduced with *Bmal1::luc* lentivirus and *Per2* siRNA. Absence of bioluminescence rhythms from the human Müller cells transduced with *Per2::luc* lentivirus and *Bmal1* siRNA.

Table 1. Summary analysis of rhythmicity for retinal and Müller cell preparations.

Preparation	Period (h)	Power	Goodness (%)	N
Per2::Luc **MMC**	24.05±0.11	0.170±0.121	27.616±12.00	6
MMC with ***Per2::Luc*** **lentivirus**	22.67±2.11	0.163±0.114	15.07±4.53	4
MMC with ***Bmal1::Luc*** **lentivirus**	23.98±0.12	0.147±0.105	23.86±9.22	7
Per1::Luc **Retina**	24.1±3.89	0.197±0.20	20.42±13.64	6
Per1−/−/Per2::Luc **Retina**	ns	0.010±0.008	8.98±5.60	3
Per1−/−/Per2::Luc **MMC**	ns	0.032±0.018	20.57±4.47	3
Bmal1−/− Per2::Luc **Retina**	ns	0.0087±0.0089	17.87±6.27	3
Bmal1−/− Per2::Luc **MMC**	ns	0.051±0.039	8.31±1.96	3
Per1::Luc **MMC**	ns	0.00034±0.000016	15.45±0.99	4
Per2::Luc **MMC with control siRNA**	23.99±0.12	0.193±0.171	25.85±22.78	4
Per2::Luc **MMC with lipofectamine**	23.96	0.26	29.8	1
Per2::Luc **MMC with Per1 siRNA**	ns	0.032±0.007	9.19±1.06	2
Per2::Luc **MMC with Per2 siRNA**	ns	0.0172±0.018	4.055±3.31	2
Per2::Luc **MMC with Bmal1 siRNA**	ns	0.006±0.004	0.02±0.66	4
HMC with ***Per2::Luc*** **lentivirus**	23.32±1.51	0.474±0.076	54.2±9.37	5
HMC with ***Per2::Luc*** **lentivirus control siRNA**	22.87±1.95	0.45±0.072	55.8±9.47	3
HMC with ***Per2::Luc*** **lentivirus lipofectamine**	22.37±2.48	0.43±0.014	52.65±13.93	2
HMC with ***Per2::Luc*** **lentivirus *Per1* siRNA**	22.87±1.96	0.16±0.107	20.99±10.97	3
HMC with ***Per2::Luc*** **lentivirus *Per2* siRNA**	24.03±0.099	0.396±0.104	42.68±9.53	5
HMC with ***Per2::Luc*** **lentivirus *Bmal1* siRNA**	ns	0.035±0.050	12.5±2.36	3

Effects on rhythmicity were quantified using Lumicycle software with the threshold for rhythmicity set at an average rhythmic power of ≥0.1 and goodness of fit ≥15% [30]. Values are reported as mean ± standard deviation. Period in hours is shown for those preparations that met the criteria for rhythmicity. MMC=mouse Müller cells; HMC=human Müller cells.

does not appear to be expressed with a circadian rhythm in mouse Müller cells (Figure 4F). The role of *Per1* in mouse Müller cell clocks may be analogous to *Period* in *Drosophila* photoreceptors in which the presence of the *Per* gene is required, but not rhythmic cycling of the promoter, for the generation of molecular circadian rhythms in the fly eye [36]. *Per1* cycles in abundance in the mouse retina as a whole but with a somewhat damped amplitude [12], possibly due to non-circadian expression in Müller cells but circadian expression in retinal neurons, including dopaminergic and NOS amacrine cells, and cone photoreceptors [20,32,33].

Human Müller cells also exhibit in vitro circadian rhythms in clock gene expression, as read out by the lentiviral *Per2* and *Bmal1* bioluminescent constructs. Surprisingly, the rhythm in these cells may not depend on *Per1* expression, as molecular rhythms are maintained following the knockdown of *Per1* with siRNA in human Müller cells with siRNA. This is distinct from what was observed in mouse Müller cells in which either knockout or knockdown of *Per1* led to arrythmicity. Although no germline knockout can be tested in human Müller cells, *Per1* siRNA was approximately twice as effective in humans versus mice, reducing *Per1* mRNA levels to ca. 25% of the control in humans versus 50% in mice (Appendix 1, Appendix 2, Appendix3). Thus, human Müller cells may have a decreased dependence in *Per1* expression for rhythmicity.

Although our results show clearly that Müller cells as a cell class generate circadian rhythms, our data do not address directly whether individual Müller cells may be autonomous clock cells, or whether they require communication with each other to be rhythmic. Additional experiments, using isolated individual Müller cells, or low-density cultures, instead of the high-density cultures we have used, will be necessary to resolve this question. Carbenoxolone, a blocker of gap junctions, a principal form of Müller cell communication in culture, does not disrupt retinal molecular rhythms [11], suggesting gap junctional communication is not necessary for the generation of retinal circadian rhythms.

Another interpretational limitation of our study is that we cannot rigorously exclude the possibility that the gene knockouts and knockdowns we performed led to the loss of rhythmicity in cell populations but not at the individual cell

level. We consider this possibility unlikely, however, as loss of *Per1* or *Bmal1* has been shown to result in the loss of single cell as well as population rhythms in cellular clocks from several tissues (e.g., [37]).

In summary, mouse and human retinal Müller cells generate endogenous molecular circadian rhythms in purified cell culture, establishing Müller glia as a candidate clock cell population in the mammalian retina. Further studies may shed light on the role that Müller cells play in the cellular organization of the mammalian retinal circadian clock.

APPENDIX 1. EFFECT OF *PER1, PER2* OR *BMAL1* SIRNA ON RNA EXPRESSION IN MOUSE MÜLLER CELLS.

To access these data, click or select the words "Appendix 1".

APPENDIX 2. EFFECT OF *PER1*, *PER2* OR *BMAL1* SIRNA ON RNA EXPRESSION IN HUMAN MÜLLER CELLS.

To access these data, click or select the words "Appendix 2".

APPENDIX 3. A. BIOLUMINESCENCE RHYTHMS FROM PER2KO MOUSE MÜLLER CELLS TRANSDUCED WITH *BMAL1* REPORTER B.

To access these data, click or select the words "Appendix 3". Lack of bioluminescence rhythms from Per1/Per2KO double knockout mouse Müller cells transduced with *Bmal1* reporter. Arrows indicate introduction of new culture medium.

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