

# Clocking in on Diabetic Retinopathy

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**D**iabetic retinopathy (DR) is the leading cause of blindness in the working-age population in the U.S. and has created a massive health care burden. Chronic hyperglycemia leads to specific changes in the retina, including the loss of pericytes, basement membrane thickening, and impaired endothelial cell function. However, the biological explanation of these cellular events remains unclear. Eventually, these changes lead to the formation of microaneurysms, the breakdown of the tight junctions in the retinal vasculature, subsequent extravasation of fluid, and vision loss. DR often progresses to a more deleterious proliferative phenotype due to profound ischemia with neovascularization of the retina and vitreous hemorrhage. Given this substantial clinical problem, many researchers are focused on resolving molecular mechanisms in DR pathogenesis to identify potential new therapeutic targets. Although a significant cache of data has amassed in studies linking DR to proinflammatory mediators such as vascular endothelial growth factor-A, oxidative stress, and abnormal glycosylation, there is growing excitement over the recent discovery linking circadian dysfunction and diabetes and the implications of this biology in the eye.

The central mammalian clock located in the suprachiasmatic nucleus is sensitive to disturbances in the programmed 24-h circadian cycle controlled by the oscillatory expression of a clock gene cassette including *Period* (*Per*) 1–3, *Cryptochrome* (*Cry*) 1/2, *Clock*, and *Bmal1/2*. As the retina provides a critical signal entry point for both peripheral and central circadian clocks in mammals, this composite tissue provides an excellent platform to study potential interactions between circadian disruption and other cell types of vascular, neuronal, and immune origin. Specific nonvisual, light-sensing retinal ganglion cells harboring the photopigment melanopsin project axons to the suprachiasmatic nucleus, which then regulates the light cycle-dependent signal for circadian entrainment (1). Interestingly, the retina also runs its own internal clock to control photoreceptor turnover, dopaminergic responses, and visual sensitivity (2).

In this issue, Bhatwadekar et al. (3) present new physiological and molecular insights into the circadian control of vascular regulation in the retina by *Per2*. This work not only offers insight into the contribution of *Per2* to retinal vascular function and maintenance of the blood-retina

barrier (BRB) but traverses into mechanisms of circadian control of bone marrow-derived progenitor cell (BMPC) release (Fig. 1). The precise functions of *Per2* are minimally resolved, yet its expression is induced by the nuclear translocation of other master clock proteins, *Bmal1/Clock*, and it may contribute to a negative feedback loop to control the periodicity of circadian gene transcription (4). The *Per2* gene contains a glucocorticoid-responsive element that is important in glucose homeostasis (5), and hyperglycemia downregulates expression of *Per2* (6), giving the authors solid ground on which to base the current work. In a previous study, the authors clearly demonstrated the importance of diurnal rhythm and clock gene expression during BMPC release in a diabetic rat model (7). One of the most highly downregulated genes in BMPCs and retina in diabetic animals was *Per2*, suggesting that deficiency may be an important factor in DR pathogenesis. BMPC—specifically endothelial progenitor cell (EPC)—dysfunction correlates with diabetic disease severity and is hypothesized to decrease vascular repair capacity during disease progression (8). It is established that EPC release into the circulation in humans is under circadian control (9), and that disruption of *Per2* leads to murine EPC dysfunction and senescence (10). The authors report in this issue that mice deficient in *Per2* develop leaky retinal vasculature, acellular capillaries, and BMPC dysfunction, which recapitulate DR in both humans and other animal models.

The key strengths of this article are expert histologic analysis of retinal morphology and immunofluorescence to identify diabetic-like disease features and the identification of key mechanisms that may lead to vascular compromise. The authors first study glucose homeostasis in *Per2* mutant mice and observe normoglycemia and normal glucose tolerance tests. This is an important finding, as the authors were then able to dissect other potential diabetes-related mechanisms independent of hyperglycemia. The basal production of nitric oxide (NO) by endothelial NO synthase (eNOS) is critical for vascular homeostasis and maintenance of the BRB. Here, the authors observed decreased retinal eNOS protein and mRNA expression compared with wild-type animals and a later increase in inducible NOS (iNOS) at 12 months. Loss of basal eNOS expression and activation of iNOS results in overproduction of NO, leukostasis, and breakdown of the BRB in diabetic mice (11). Along with the high-quality trypsin digests demonstrating acellular capillaries in *Per2*-deficient retinas, these findings provide a glucose-independent mechanism through which the loss of circadian rhythm may induce significant retinal vascular permeability and derangements in highly organized retinal endothelial cell tight junctions.

Another major strength in this article is the authors' analyses of bone marrow dysfunction and BMPC release in *Per2* mutant mice. They have previously shown bone marrow neuropathy and significantly decreased circulating BMPCs in a diabetic rat model (7), providing necessary evidence to support a system by which circadian control

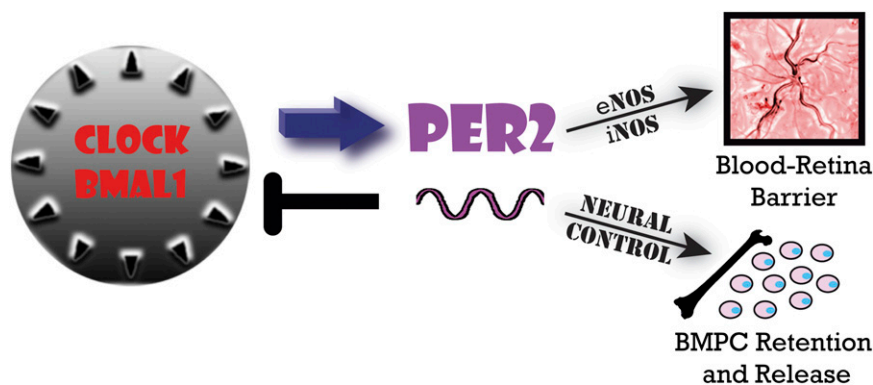
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**FIG. 1. Loss of *Per2* expression results in diabetic-like retinal vascular and bone marrow phenotype. The illustration demonstrates *Clock* gene control and the feedback loop of *Per2*, with deficiency leading to multiple downstream effects, including increased retinal vascular permeability through an imbalance of eNOS and iNOS production and retention of BMPCs by neural dysregulation.**

of adrenergic signaling within the bone marrow regulates BMPC reserve and release. Recent data from an independent group demonstrated that a similar mechanism is active during leukocyte recruitment (12). In *Per2*-deficient mice, increased retention of BMPCs in bone marrow and decreased circulating BMPCs in peripheral blood were observed. With rigorous immunohistochemical analyses, a significant loss of bone marrow innervation and vasa nervorum were revealed, offering a clear mechanistic explanation for their findings.

This work offers novel insight and further excitement for the field of circadian biology and metabolic disease, yet efforts in transcript profiling in this animal model were more limited. Imbalanced NOS expression and acellular capillaries could explain increased vascular permeability, but elevated expression of matrix metalloproteinases (MMPs) may also play a role (13,14). Studies of peripheral vascular disease identify both MMP-2 and -9 as critical factors in vascular stiffness in mice with mutations in *Per1-3* knockout mice (15). *Clock* gene function is complex, and other *Per* genes require study, as their function especially in regulating retinal versus central circadian clocks may be selective (16). Still, this study provides a robust dataset from which to confidently proceed into further investigations of retinal vascular function and BMPC biology in circadian mutants.

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#### REFERENCES

1. Tu DC, Zhang D, Demas J, et al. Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells. *Neuron* 2005;48:987–999
2. Ruan GX, Zhang DQ, Zhou T, Yamazaki S, McMahon DG. Circadian organization of the mammalian retina. *Proc Natl Acad Sci USA* 2006;103:9703–9708
3. Bhatwadekar AD, Yan Y, Qi X, et al. *Per2* mutation recapitulates the vascular phenotype of diabetes in the retina and bone marrow. *Diabetes* 2013;62:273–282
4. Sasaki M, Yoshitane H, Du NH, Okano T, Fukada Y. Preferential inhibition of BMAL2-CLOCK activity by PER2 reemphasizes its negative role and a positive role of BMAL2 in the circadian transcription. *J Biol Chem* 2009;284:25149–25159
5. So AY, Bernal TU, Pillsbury ML, Yamamoto KR, Feldman BJ. Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. *Proc Natl Acad Sci USA* 2009;106:17582–17587
6. Hirota T, Okano T, Kokame K, Shirohata-Ikejima H, Miyata T, Fukada Y. Glucose down-regulates *Per1* and *Per2* mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J Biol Chem* 2002;277:44244–44251
7. Busik JV, Tikhonenko M, Bhatwadekar A, et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. *J Exp Med* 2009;206:2897–2906
8. Fadini GP, Sartore S, Albiero M, et al. Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. *Arterioscler Thromb Vasc Biol* 2006;26:2140–2146
9. Thomas HE, Redgrave R, Cunningham MS, Avery P, Keavney BD, Arthur HM. Circulating endothelial progenitor cells exhibit diurnal variation. *Arterioscler Thromb Vasc Biol* 2008;28:e21–e22
10. Wang CY, Wen MS, Wang HW, et al. Increased vascular senescence and impaired endothelial progenitor cell function mediated by mutation of circadian gene *Per2*. *Circulation* 2008;118:2166–2173
11. Leal EC, Manivannan A, Hosoya K, et al. Inducible nitric oxide synthase isoform is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2007;48:5257–5265
12. Scheiermann C, Kunisaki Y, Lucas D, et al. Adrenergic nerves govern circadian leukocyte recruitment to tissues. *Immunity* 2012;37:290–301
13. Das A, McGuire PG, Eriqat C, et al. Human diabetic neovascular membranes contain high levels of urokinase and metalloproteinase enzymes. *Invest Ophthalmol Vis Sci* 1999;40:809–813
14. Kowluru RA, Mohammad G, dos Santos JM, Zhong Q. Abrogation of MMP-9 gene protects against the development of retinopathy in diabetic mice by preventing mitochondrial damage. *Diabetes* 2011;60:3023–3033
15. Anea CB, Ali MI, Osmond JM, et al. Matrix metalloproteinase 2 and 9 dysfunction underlie vascular stiffness in circadian clock mutant mice. *Arterioscler Thromb Vasc Biol* 2010;30:2535–2543
16. Ruan GX, Gamble KL, Risner ML, Young LA, McMahon DG. Divergent roles of clock genes in retinal and suprachiasmatic nucleus circadian oscillators. *PLoS ONE* 2012;7:e38985