Increased Vascular Senescence and Impaired Endothelial Progenitor Cell Function Mediated by Mutation of Circadian Gene Per2

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Epidemiological studies have shown that shift or nighttime workers have a higher incidence of cardiovascular disease.1–3 Because blood pressure,4 heart rate,5 endothelial function,6 and the onset of myocardial infarction and stroke7,8 have distinct circadian patterns, it has been suggested that disruption of circadian rhythm may contribute to cardiovascular disease. The primary control of the circadian clock is located in the hypothalamic suprachiasmatic nuclei, which are used to synchronize peripheral clocks in organs and tissues. This synchronization is accomplished through a series of tightly regulated circadian genes such as CLOCK, BMAL1, NPAS2, Cry1/2, and Per1/2.9,10 Mutations in or knockout of these circadian genes lead to diverse pathophysiological disorders, including metabolic syndrome and obesity,11 premature aging,12 and abnormal sleep cycle.13 In particular, mice with a Per2 mutation (Per2m/m) suffer multiple abnormalities, including shortened lifespan, defect in thymocyte apoptosis,14 increased carcinogenesis,15 greater bone formation,16 increased alcohol consumption,17 and impaired endothelial function.6 The diverse phenotype observed in Per2m/m mice likely reflects the broad downstream targets of Per2, which include c-myc, Cyclin D1, Cyclin A, Mdm-2, and Gadd45α.14 In the cardiovascular system, the production of nitric oxide (NO) and vasoactive prostaglandins is altered in Per2m/m mice.6 However, the vascular consequences of Per2 mutation on NO-mediated angiogenesis and the underlying mechanism responsible for the phenotype have not been investigated or characterized in Per2m/m mice.

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Cardiovascular risks and disease occur at much higher rates in individuals with advanced age and vascular senescence. Vascular senescence as defined by a state of irreversible growth arrest, expression of negative regulators of cell cycle (such as p53 and p16), and increased senescence-related β-galactosidase staining is an important indicator of vascular dysfunction.18,19 Vascular senescence is associated with atherosclerosis,20 endothelial dysfunction,21 increased reactive...
oxygen species, and alteration of telomerase activity. However, the precise mechanisms contributing to vascular senescence are not known. We hypothesize that alteration of circadian rhythm leads to vascular senescence, decreased NO bioavailability, and impaired angiogenesis. Using Per2<sup>−/−</sup> mice, we investigated the effects of Per2 mutation on vascular senescence and the angiogenic response to distal limb ischemia.

**Methods**

**Animals**

Per2<sup>−/−</sup> mice with C57BL/6-<sup>Tyr<sup>−/−</sup></sup> background were obtained from The Jackson Laboratory (Bar Harbor, Me) and backcrossed to C57BL/6 background for 6 generations before experimental use. The wild-type (WT) and Per2<sup>−/−</sup> littermates were generated from heterozygote cross-breeding and kept in a regular light/dark cycle (ie, 12-hour cycle).

**Cumulative Population Doublings and Senescence Stain**

The lung and aortic endothelial cells were passaged every 3 to 4 days. Cumulative population doubling at each passage was calculated from the cell count with the following equation:

\[ N_d/N_1 = 2^X \] or \[ \log_{10}(N_d) - \log_{10}(N_1) = \log_{10}(2) = X \]

where \( N_1 \) is the inoculum number, \( N_d \) is the cell harvest number, and \( X \) is population doublings. The population doublings were added to yield cumulative population doubling. Replicative senescence is defined as \( X < 1 \) for 3 weeks. Senescence-associated β-galactosidase staining was performed as previous described at pH 5.5.

**Statistical Analysis**

Results are expressed as mean ± SEM. Differences between 2 groups were examined by the Student \( t \) test. Laser Doppler perfusion images were analyzed with repeated-measures ANOVA with posthoc analysis. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Impaired Proliferation and Vascular Network Formation in Endothelial Cells From Per2 Mice**

To determine whether mutation of Per2 affects endothelial function and angiogenesis, we isolated endothelial cells from WT and Per2<sup>−/−</sup> mice. Cells from WT and Per2<sup>−/−</sup> mice have similar appearances and sizes (cell diameters: WT, 16.1 ± 0.4 μm; Per2<sup>−/−</sup>, 15.9 ± 0.3 μm; \( n = 120; P = NS \)). However, the ability of endothelial cells from Per2<sup>−/−</sup> mice to form vascular networks (ie, total length of cords) was substantially reduced compared with endothelial cells from WT mice (WT, 13 246 ± 1420 μm; Per2<sup>−/−</sup>, 8181 ± 1192 μm; \( n = 9; P < 0.05 \)) (Figure 1A and 1B). Interestingly, endothelial cell migration and adhesion were not altered by Per2 mutation (Figure 1C and 1D). In agreement with decreased vascular network formation, cell proliferation was impaired in endothelial cells from Per2<sup>−/−</sup> mice (WT, 20 520 ± 1319 cells per well; Per2<sup>−/−</sup>, 13 473 ± 1291 cells per well; \( n = 11; P < 0.05 \)) (Figure 1E).

These results indicate that mutation of Per2 impairs the ability of endothelial cells to proliferate and form vascular networks.

**Per2 Mutation Leads to Vascular Senescence**

To determine whether mutation of Per2 leads to senescence, endothelial cells from WT and Per2<sup>−/−</sup> mice were subjected to serial passages to test for replicative capacity. WT endothelial cell cultures undergo growth arrest after 10 to 12 passages (Figure 2A). However, Per2<sup>−/−</sup> endothelial cells exhibited earlier growth arrest at passages 8 to 10. Similar results were obtained from 4 independent Per2<sup>−/−</sup> and 2 independent WT endothelial cell cultures. To determine whether endothelial cells from Per2<sup>−/−</sup> mice show increased senescence compared with WT mice, the aortas from 12-month-old mice were stained for senescence-related β-galactosidase activity. The aortas from Per2<sup>−/−</sup> mice showed increased β-galactosidase staining compared with WT aortas (Figure 2B). When endothelial cells from 12-month-old Per2<sup>−/−</sup> mice were isolated, they also showed an increased percentage of β-galactosidase–stained cells compared with WT mice (Per2<sup>−/−</sup>, 74 ± 22%; WT, 12 ± 13%; \( n = 6; P < 0.05 \)) (Figure 2C). Furthermore, the
expression of endothelin-1, plasminogen activator inhibitor-1, thromboxane A2, hypoxia inducible factor-1α, and vascular endothelial growth factor (VEGF) in endothelial cells from WT and Per2\textsuperscript{m/m} mice was not different (data not shown). These findings indicate that Per2 mutation is associated with increased endothelial cell senescence.

Increased Akt Signaling in Per2\textsuperscript{m/m} Mice

The Per2\textsuperscript{m/m} mice have a shortened lifespan and are prone to developing teratomas and lymphomas.\textsuperscript{14} Furthermore, their thymocytes are relatively resistant to apoptosis in response to γ-radiation. Because oncogenesis and senescence have been shown to be associated,\textsuperscript{26} they are sometimes regulated by similar signaling pathways. We investigated whether Per2 could regulate an oncogene that could be responsible for cellular senescence. We postulate that protein kinase B/Akt, which was initially found to be an oncogene in murine thymic cell lines, mediates vascular senescence. We postulate that protein kinase B/Akt, which initially found to be an oncogene in mouse thymic cell lines, mediates vascular senescence. Because Per2\textsuperscript{m/m} mice have a shortened lifespan and are prone to developing teratomas and lymphomas,\textsuperscript{14} we investigated whether these 2 pathways are altered in Per2\textsuperscript{m/m} mice.

Compared with endothelial cells from WT mice, endothelial cells from Per2\textsuperscript{m/m} mice exhibited high constitutive levels of Akt Ser\textsuperscript{473} phosphorylation (pAkt; Figure 3A and 3B). Treatment with VEGF increased pAkt levels in WT endothelial cells but not in Per2\textsuperscript{m/m} endothelial cells (Figure 3A and 3B). Similarly, aortas from Per2\textsuperscript{m/m} mice have 2-times-higher pAkt levels and >50% lower p27 levels (Figure 3C and 3D).

Because Akt phosphorylation is regulated by phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin–rictor pathway,\textsuperscript{27} we examined whether these 2 pathways are altered in Per2\textsuperscript{m/m} mice. Compared with WT mice, the protein levels of mammalian target of rapamycin components, rictor, raptor, and p70 S6 kinase, were not different in Per2\textsuperscript{m/m} mouse aorta (data not shown). Treatment with the PI3K inhibitors LY294002 (LY) and wortmannin decreased pAkt levels in both WT and Per2\textsuperscript{m/m} endothelial cells (Figure 3E), suggesting that constitutive pAkt in Per2\textsuperscript{m/m} mice is due in part to chronic PI3K activation. However, both phosphatase and tensin homolog (P=0.04) and protein phosphatase 2A (P=0.014), which negatively regulate Akt phosphorylation, showed compensatory increases in Per2\textsuperscript{m/m} mice. In contrast, the levels of phosphatidylinositol-dependent kinase-1 (PDK1) in Per2\textsuperscript{m/m} mice are ∼2.3 times higher than those of WT mice (Figure 3F). These findings suggest that increased PDK1/Akt signaling in Per2\textsuperscript{m/m} mice may contribute to the observed increase in vascular senescence.

Decreased Angiogenesis in Per2\textsuperscript{m/m} Mice

Because Per2\textsuperscript{m/m} mice exhibit endothelial dysfunction\textsuperscript{8} and senescence, we investigated whether angiogenic function in Per2\textsuperscript{m/m} mice is altered. To study the angiogenic response in vivo, we performed subcutaneous implantation of matrigel with or without VEGF. The matrigel was removed 5 days after implantation, and the incorporated hemoglobin, which correlates with vessel formation, was then quantified. Compared with WT mice, the hemoglobin content of the matrigel was reduced in the Per2\textsuperscript{m/m} mice in the absence of VEGF (Per2\textsuperscript{m/m}, 33±7%; WT, 100±17%; n=6; P<0.05) (Figure 4A and 4B). Although treatment with VEGF produced a similar induction of hemoglobin content in matrigel implanted into WT and Per2\textsuperscript{m/m} mice, the absolute amount of hemoglobin content was substantially less in Per2\textsuperscript{m/m} mice compared with that of WT mice (Per2\textsuperscript{m/m}, 145±11%; WT, 313.6±33%; n=6; P<0.05). These findings indicate that Per2 mutation leads to impaired angiogenesis in vivo.

To determine the in vivo relevance of Per2 mutation in postnatal ischemia-induced neovascularization, we performed hind-limb ischemia in WT and Per2\textsuperscript{m/m} mice. Surprisingly, all Per2\textsuperscript{m/m} mice developed autoamputation following hind-limb ischemia after 2 weeks, whereas none of the WT mice developed autoamputation (Figure 5A). This correlated with impaired blood flow recovery after ischemia in Per2\textsuperscript{m/m} mice compared with WT mice after 1 week (Figure 5B). These findings indicate that the circadian gene, Per2, is essential for postischemic blood flow recovery in adult mice.

Because bone marrow (BM) cells may contribute to ischemia-induced revascularization, we investigated the role of Per2 mutation in BM-derived endothelial progenitor cells (EPCs). We first investigated EPC levels in WT and Per2\textsuperscript{m/m} mice. EPCs were characterized by positive staining for acetylated low-density lipoprotein, lectin, and endothelial NO synthase.\textsuperscript{28} Interestingly, there were no significant differences in the baseline EPC levels between WT and Per2\textsuperscript{m/m} mice (Per2\textsuperscript{m/m}, 21±11 per hpf; WT, 20±13 per hpf; n=9; P>0.05). Furthermore, the ability of EPCs from Per2\textsuperscript{m/m} mice to proliferate in vitro was not different compared with that of EPCs from WT mice (data not shown). However, 5 days after hind-limb ischemia, the increase in EPC was substantially less in Per2\textsuperscript{m/m} mice compared with WT mice (Per2\textsuperscript{m/m}, 31±6 per hpf; WT, 51±13 per hpf; n=9; P<0.05) (Figure 5C). To further characterize the EPC re-
response, we administered VEGF_{165} (500 \mu g \cdot kg^{-1} \cdot d^{-1} SC for 3 days) to WT and Per2^{-m/m} mice. The increase in EPC numbers in Per2^{-m/m} mice was much less compared with EPC numbers in WT mice (Per2^{-m/m}, 43±16 per hpf; WT, 65±12 per hpf; n=9; P<0.05) (Figure 5C).

**Mutation of Per2 in BM-Derived Cells Impairs Angiogenesis**

To determine the role of Per2 in ischemia-induced neovascularization, we performed BM transplantation (BMT) from WT or Per2^{-m/m} mice into irradiated recipient WT mice. Four weeks after BMT, the number of BM-derived EPCs was similar in the 2 groups of recipient mice (Per2^{-m/m}, 17±9 per hpf; WT, 16±12 per hpf; n=6; P=0.25), and hind-limb ischemia was then induced. Compared with mice receiving WT BM (WT→WT BMT mice), Per2^{-m/m}→WT BMT mice exhibited 27±0.9% less blood flow recovery at 28 days after surgery (Figure 6A and 6B). The microcapillary density in the ischemic adductor skeletal muscle tissue also was substantially less in Per2^{-m/m}→WT BMT mice compared with WT→WT BMT mice (Per2^{-m/m}→WT, 1543±652/mm²; WT→WT, 2542±523/mm²; n=6; P<0.05) (Figure 6C). These findings suggest that mutation of Per2 in BM-derived cells impairs ischemia-induced neovascularization.

To determine whether infusion of WT EPCs rather than BMT can restore the angiogenic response and prevent the autoamputation observed in Per2^{-m/m} mice after hind-limb ischemia, we infused EPCs (2×10^5 cells) from either WT or Per2^{-m/m} mice into recipient Per2^{-m/m} mice and then monitored these mice (WT→Per2^{-m/m} and Per2^{-m/m}→Per2^{-m/m} mice) for blood flow recovery and autoamputation. Surprisingly, the autoamputation phenotype was completely rescued in both groups of mice (Figure 6D and 6E). However, consistent with our findings, the Per2^{-m/m} mice receiving EPCs from Per2^{-m/m} mice showed less blood flow recovery compared with mice receiving EPCs from WT mice (Per2^{-m/m}→Per2^{-m/m}, 34±6%; WT→Per2^{-m/m}, 61±15%; n=6; P<0.05). Quantitative histological analysis of neovascularization revealed that capillary density was lower in the ischemic adductor muscle of Per2^{-m/m} mice receiving EPCs from Per2^{-m/m} mice compared with similar mice receiving EPCs from WT mice (Per2^{-m/m}→Per2^{-m/m}, 1298±118/mm²; WT→Per2^{-m/m}, 1762±661/mm²; n=6; P<0.05) (Figure 6F). Taken together, our findings indicate that the autoamputation observed in Per2^{-m/m} mice could be rescued by intravenous infusion of EPCs from either WT or Per2^{-m/m} mice but that, in contrast to EPCs from Per2^{-m/m} mice, only EPCs from WT mice have the ability to restore ischemia-induced neovascularization and blood flow recovery in Per2^{-m/m}.
mice. These findings strongly suggest that Per2 in BM-derived cells, particularly in EPCs, mediates ischemia-induced revascularization.

Inhibition of Akt Signaling Rescues the Impairment of Cellular Function in Per2m/m Mice

Next, we examined whether inhibition or knockdown of excessive Akt signaling could restore and improve endothelial function such as proliferation or vascular network formation in Per2m/m mice. Akt1 is the predominant isoform in endothelium, and Akt1 haploinsufficiency leads to less Akt activation.29 Because Akt1 also mediates tumor and ischemic angiogenesis,30 we investigated the role of Akt1 in Per2m/m mice by crossing Per2m/m mice with Akt1+/− mice to generate Per2m/m/Akt1+/− mice. Consistent with previous reports, we found that Akt1+/− mice have less pAkt (Figure 7A). Despite less Akt activity, there were no significant differences in endothelial cell proliferation or vascular network formation between Akt1+/− and Akt1+/+ mice (Figure 7B and 7C). Haploinsufficiency of Akt1, however, restored the impaired vascular network formation and endothelial cell proliferation in Per2m/m mice (Figure 7B and 7C). These findings suggest that excessive Akt activation in Per2m/m mice is probably responsible for the impaired vascular function in these mice.

Discussion

An interesting and important finding of this study is that Per2 is a critical mediator of vascular senescence, angiogenesis, and EPC function. This finding is in agreement with previous studies showing that Per2 is an important mediator of endothelial function because mutation of Per2 impairs NO release and vascular relaxation.6 However, the mechanism and pathophysiological consequences of endothelial dysfunction in Per2m/m mice were not known. In this study, we provide a potential underlying mechanism by showing that Per2 mutation leads to premature vascular senescence, which correlates with impaired EPC function and angiogenesis. The association of disruption or mutation of the circadian rhythm with senescence or aging remains controversial, despite evidence suggesting that the robustness and integrity of the circadian rhythm decrease with age.31 For example, BMAL1 knockout mice exhibit decreased lifespan, sarcopenia, cataracts, and other aging symptoms.12 It is not known if the circadian rhythm itself is important in the aging process or if a specific circadian gene is responsible for the aging phenotype. In support for gene specificity rather than general
circadian rhythm disturbance is the observation that Cry1/Cry2 double-knockout mice, which have a disturbance in the circadian rhythm, do not exhibit the vascular senescence phenotype that Per2m/m mice do (C.-Y.W. and J.K.L., unpublished observation).

Decreased replicative capacity refers to a condition when cells lose their ability to replicate after a finite number of cell divisions. Reduced cellular replicative capacity is a marker of senescence and correlates with aging. Although a change in cellular replicative capacity often is associated with aging or senescence, it also is observed sometimes in tumor suppression.26,32,33 In this study, we have shown that endothelial cell senescence as defined by decreased replicative capacity is associated with impaired vascular function and angiogenesis.

Our findings indicate that vascular senescence and dysfunction are associated with chronic upregulation of Akt signaling. Overactivity of Akt has been linked to increased vascular senescence through increased reactive oxygen species generation and decreased NO bioavailability.34 Indeed, excessive Akt activation and impaired angiogenesis are observed in VEGF receptor-1 (VEGFR-1 or flt-1) haploinsufficient mice as a result of unmitigated VEGFR-2 (flk-1/KDR) signaling.35 We find that mutation of Per2 leads to greater Akt phosphorylation via the PI3K-dependent pathway and upregulation of PDK1. PDK1 is an important regulator of insulin and growth factor–stimulated protein kinases such as Akt,36 p70 ribosomal S6 kinase,37 and p90 ribosomal S6 kinase.38 PDK1 also is important in regulating murine developmental physiology.39

Figure 6. Per2 in BM-derived cells mediates ischemia-induced neovascularization and restoration of blood flow recovery in Per2m/m mice. A, Decreased neovascularization after ischemia in WT mice transplanted with Per2m/m BM. Quantitative analysis of the ischemic-to-nonischemic laser Doppler blood flow ratio in WT→WT BMT or Per2m/m→WT BMT mice (n=6 in each group). *P<0.05 vs WT→WT BMT mice. B, Representative laser Doppler blood flow images of WT→WT and Per2m/m→WT BMT mice at 28 days after induction of hind-limb ischemia (n=6 in each group). C, Microcapillary density analysis in ischemic adductor muscle tissue of WT→WT and Per2m/m→WT BMT mice at 28 days (n=6 in each group). Capillary density was expressed as the number of capillaries stained with Isolectin IB4 per 1 mm². *P<0.05 vs WT→WT BMT mice. D, Decreased neovascularization after ischemia in Per2m/m mice infused with Per2m/m EPCs. Quantitative analysis of the ischemic-to-nonischemic laser Doppler blood flow ratio in WT→Per2m/m or Per2m/m→Per2m/m (n=6 in each group). *P<0.05 vs WT→Per2m/m EPC mice. E, Representative LDBF images of WT→Per2m/m and Per2m/m→Per2m/m EPC mice at 28 days after induction of hind-limb ischemia. F, Microcapillary density analysis in ischemic adductor muscle tissue of WT→Per2m/m and Per2m/m→Per2m/m EPC mice at 28 days. *P<0.05 vs WT→Per2m/m BMT mice; n=6.

Figure 7. Inhibition of Akt signaling rescues the impairment of cellular function in Per2m/m mice. A, Decreased pAkt level in Akt1−/− mice (n=4). B, Quantitative assessment of vascular network formation (tube length) (n=9). C, The decrease in cell proliferation in Per2m/m mice is rescued when Per2m/m mice are crossed onto Akt1−/− background. (n=10). *P<0.05 compared to WT.
ompent and multiple cellular functions. A potential downstream target of PDK1/Akt that may mediate senescence is FOXO. For example, the insulin/PI3K/Akt/FOXO pathway has been shown to alter the lifespans of organisms from worms to mice, and insulin signaling has been shown to regulate circadian rhythm through FOXO. Thus, insulin signaling, aging, and circadian rhythm may be linked through chronic, excessive PDK1/Akt activation. However, further studies are required to determine how Per2 regulates Akt activation through PI3K/PDK1.

We found that mutation of Per2 leads to decreased EPC levels in response to ischemia or VEGF. However, the ability of EPCs from Per2<sup>m/m</sup> mice to proliferate in vitro was not different compared with that of EPCs from WT mice. These findings suggest that the defect in EPC function in Per2<sup>m/m</sup> mice is due to EPC mobilization rather than proliferation. This is in agreement with previous studies showing that NO is required for EPC mobilization and that NO bioavailability is impaired in Per2<sup>m/m</sup> mice. Furthermore, several studies suggest that the circadian rhythm affects stem cell function. For example, hematopoiesis and phagocytosis by BM-derived cells have circadian variations. Disturbances or disruption in circadian genes is likely to affect the function of BM-derived cells, including EPCs. Because Per2 mutation leads to both endothelial senescence and decreased EPC mobilization, both phenotypes may contribute to the autoimmunization observed in Per2<sup>m/m</sup> mice after hind-limb ischemia. Indeed, blood flow recovery after hind-limb ischemia in WT→Per2<sup>m/m</sup> BMT mice was still substantially lower than that of WT→WT BMT mice (ischemic-to-nonischemic limb ratio, 0.35 versus 0.62), suggesting that impaired intrinsic vascular function, in addition to impaired EPC function, contributes importantly to the altered angiogenic response and autoimmunization in Per2<sup>m/m</sup> mice.

We find that alteration of the circadian rhythm leads to vascular senescence. Although Per2 mutation leads to increased Akt signaling and excessive Akt activation is linked to increased vascular senescence by oxidative stress, Akt may not be the only factor that mediates the senescence phenotype. Because Per2<sup>m/m</sup> mice exhibit a wide array of phenotypic abnormalities, other factors that are downstream of Per2 may cooperate with Akt in causing replicative senescence. These factors may include genes that regulate the cell cycle such as c-myc, p27<sub>Kip1</sub>, Cyclin D1, and Cyclin A. In particular, p27<sub>Kip1</sub> is important in cell cycle control, and disruption of the cyclin-dependent kinase-inhibitory domain of p27 enhances growth of mice. Disruption of p27 increases cell proliferation, cell cycle progression, and senescence, which is compatible to our findings. As mentioned, the decrease in NO bioavailability in Per2<sup>m/m</sup> mice also may contribute to senescence, although senescence itself may ultimately lead to decreased NO bioavailability and endothelial dysfunction. Regardless of the precise mechanism by which mutation of Per2 causes vascular senescence, Per2<sup>m/m</sup> mice have circadian rhythm disturbances and have impaired EPC function and angiogenic response to ischemia. These findings suggest that polymorphisms or alteration of circadian genes may be linked to cardiovascular disease. It remains to be determined, however, whether restoration of circadian gene function could ameliorate age-related vascular diseases.

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**Disclosures**

None.

**References**

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