Loss-of-function mutations with circadian rhythm regulator Per1/Per2 lead to premature ovarian insufficiency†

Yating Zheng 1, Chao Liu 1, Yan Li 1, Haijuan Jiang 1, Peixin Yang 2, Jing Tang 3, Ying Xu 4, Han Wang 5 and Yulong He 1,∗

1Cyrus Tang Hematology Center, Collaborative Innovation Center of Hematology, State Key Laboratory of Radiation Medicine and Protection, Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Soochow University, Suzhou, China; 2Department of Obstetrics, Gynecology and Reproductive Sciences, University of Maryland School of Medicine, Baltimore, USA; 3Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland; 4Cam-Su Genomic Resources Center, Soochow University, Suzhou, China and 5Center for Circadian Clocks, Soochow University, Suzhou, China

Correspondence: Cyrus Tang Hematology Center, Collaborative Innovation Center of Hematology, State Key Laboratory of Radiation Medicine and Protection, Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Soochow University, 199 Ren-Ai Road, Suzhou, China. Tel: +86-512-65880877; Fax: +86-512-65880929; E-mail: heyulong@suda.edu.cn

Grant Support: This work was supported by grants from the Ministry of Science and Technology of China (2012CB947600), the National Natural Science Foundation of China (91539101, 81770489, 91739304), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Received 15 March 2018; Revised 31 August 2018; Accepted 16 November 2018

Abstract

The mechanism underlying premature ovarian insufficiency remains incompletely understood. Here we report that mice with Per1m/m; Per2m/m double mutations display a decrease in female fertility starting approximately at 20 weeks old, with significantly less pups born from 32 weeks old onwards. Histological analysis revealed that a significant reduction of ovarian follicles was observed in the Per1/Per2 mutants compared with the littermate controls examined at 26 and 52 weeks old, while the difference was not statistically significant between the two groups at 3 and 8 weeks old. We further showed that vascular development including the ovarian follicle associated vascular growth appeared normal in the Per1/Per2 mutant mice, although clock genes were reported to regulate angiogenesis in zebrafish. The findings imply that loss-of-function mutations with Per1/Per2 result in a premature depletion of ovarian follicle reserve leading to the decline of reproductive capacity.

Summary Sentence

Disruption of circadian rhythm or its underlying regulatory network contributes to the premature depletion of ovarian follicle reserve.

Key words: PER1, PER2, ovarian follicle, ovarian insufficiency, knockout mouse.

Introduction

Premature ovarian insufficiency (POI) is characterized by a loss of ovarian function in women at reproductive ages, and is a heterogeneous condition with genetic, environmental, and other causes such as iatrogenic factors [1, 2]. The cyclic process of ovarian follicular development includes the follicle growth and maturation, ovulation,
as well as corpus luteum formation. The ovarian events are tightly controlled by the circadian system, as shown by the rhythmic hormone release in the hypothalamus–pituitary–ovary axis [3].

Circadian rhythms exist in diverse life forms and modulate the transcriptional activity of numerous genes in both suprachiasmatic nuclei (SCN) and peripheral tissues including the reproductive system [4–6]. The molecular machinery of the circadian clock is a feedback loop of core regulators including the transcriptional activators BMAL1 and CLOCK, as well as the repressors PER1–3 of the feedback loop of core regulators including the transcriptional activators BMAL1 and CLOCK, as well as the repressors PER1–3 [4–6]. The molecular machinery of the circadian clock is a feedback loop of core regulators including the transcriptional activators BMAL1 and CLOCK, as well as the repressors PER1–3 [4–6]. The molecular machinery of the circadian clock is a feedback loop of core regulators including the transcriptional activators BMAL1 and CLOCK, as well as the repressors PER1–3.

To assess reproductive performance, the doubly mutant (Per1/Per2) were generated by two rounds of mating. The genetic background of Per1/Per2 doubly mutant and control mice are C57/BL6. All animal experiments performed in accordance with the institutional guidelines of the Soochow University Animal Center. In all the phenotype analysis, littermates were used as control.

Materials and methods

Animal models

Mice with mutations targeting Per1 and Per2 genes were generated, as previously described [7, 8]. We obtained the breeding pairs of Per1 and Per2 single mutants, and the doubly mutant mice (Per1<sup>m/m</sup>; Per2<sup>m/m</sup>) were generated by two rounds of mating. The genetic background of Per1/Per2 doubly mutant and control mice are C57BL/6J. All animal experiments were performed in accordance with the institutional guidelines of the Soochow University Animal Center. In all the phenotype analysis, littermates were used as control.

Quantification of reproductive performance

To assess reproductive performance, the doubly mutant (Per1<sup>m/m</sup>; Per2<sup>m/m</sup>, 8 weeks old) and littermate control female mice (Per1<sup>m/m</sup>; Per2<sup>m/m</sup>) were mated with age-matched male mice (B6 background). The total number of litters and pups for each female mouse were recorded up to 52 weeks old.

Histological analysis of ovarian follicles

Ovaries of Per1<sup>m/m</sup>; Per2<sup>m/m</sup> mutant and littermate control female mice (3, 8, 26, or 52 weeks old) were fixed in 4% PFA (paraformalde-
Premature ovarian follicle insufficiency in Per1/Per2 mutants

Although a number of genes and nongenetic factors have been identified contributing to the occurrence of POI, the involvement of circadian clock and its regulators in this pathogenesis is unclear. To analyze the effect of double Per1/Per2 mutations on ovarian follicle development, ovaries from different ages of mice (3, 8, 26, and 52 weeks old) were collected for histological analysis of follicle number at primordial, primary, secondary, and antral stages (Figure 2A). Quantification of follicle number revealed that Per1m/m; Per2m/m doubly mutant mice had similar number of primordial, primary, secondary, or antral ovarian follicles at the stages of 3 weeks old (Figure 2B) in comparison with those of control littermates. There was also no significant difference in the total number of ovarian follicles observed in the Per1/Per2 mutant ovaries of 8-week-old mice (Figure 2C). However, a significant decrease in the number of ovarian follicles was detected at ages of 26 weeks old (Figure 2D) and 52 weeks old (Figure 2E) between the two groups (Table 2). Analysis by immunostaining with cleaved caspase-3 showed that there was no statistically significant difference in apoptotic cell number between the Per1m/m; Per2m/m doubly mutant and control mice (Supplementary Figure S1; Per1m/m; Per2m/m: 53.35 ± 2.03, n = 3; Control: 51.69 ± 2.68, n = 3; P = 0.4406).

Angiogenesis in ovaries of Per1/Per2 doubly mutant mice

It has been shown that morpholino-mediated knockdown of per2 or bmal1 altered the developmental angiogenesis in zebrafish [22]. To find out whether Per1/Per2 mutations would affect vascular develop-

Table 1. Litter size and pup number of Per1m/m; Per2m/m doubly mutant and control mice.

| Age (weeks) | Number of accumulated litters | n (Per1m/m; Per2m/m) | n (Control) | P value |
|-------------|-----------------------------|---------------------|-------------|---------|
|             | Per1m/m; Per2m/m | Control |              |         |         |
| 8           | 0                      | 0       | 12           | 11      |         |
| 12          | 0.50 ± 0.52            | 0.64 ± 0.50 | 12           | 11      | 0.53182939 |
| 16          | 1.25 ± 0.75            | 1.36 ± 0.67 | 12           | 11      | 0.70798791 |
| 20          | 2.00 ± 0.74            | 2.36 ± 0.67 | 12           | 11      | 0.23254607 |
| 24          | 2.75 ± 0.62            | 3.09 ± 0.70 | 12           | 11      | 0.22987128 |
| 28          | 3.42 ± 0.90            | 3.73 ± 0.79 | 12           | 11      | 0.39011313 |
| 32          | 3.92 ± 1.08            | 4.64 ± 0.67 | 12           | 11      | 0.07253613 |
| 36          | 4.17 ± 1.19            | 5.36 ± 0.92 | 12           | 11      | 0.01431332 |
| 40          | 4.58 ± 1.00            | 6.27 ± 1.19 | 12           | 11      | 0.00132172 |
| 44          | 4.92 ± 1.16            | 7.18 ± 1.25 | 12           | 11      | 0.00001971 |
| 48          | 5.08 ± 1.31            | 7.82 ± 1.25 | 12           | 11      | 4.6302E-03 |
| 52          | 5.25 ± 1.29            | 8.45 ± 1.29 | 12           | 11      | 6.6428E-06 |

| Age (weeks) | Number of accumulated pups | n (Per1m/m; Per2m/m) | n (Control) | P value |
|-------------|-----------------------------|---------------------|-------------|---------|
| 8           | 0                      | 0       | 12           | 11      |         |
| 12          | 3.58 ± 3.78            | 3.82 ± 3.79 | 12           | 11      | 0.88319599 |
| 16          | 8.58 ± 5.55            | 10.82 ± 5.13 | 12           | 11      | 0.32893069 |
| 20          | 14.08 ± 6.42           | 19.00 ± 6.45 | 12           | 11      | 0.0813109 |
| 24          | 19.73 ± 4.99           | 24.09 ± 7.12 | 12           | 11      | 0.10293094 |
| 28          | 24.08 ± 6.56           | 28.91 ± 7.42 | 12           | 11      | 0.11268427 |
| 32          | 27.17 ± 7.71           | 36.18 ± 8.75 | 12           | 11      | 0.01573296 |
| 36          | 28.42 ± 9.22           | 41.73 ± 10.04 | 12           | 11      | 0.00329245 |
| 40          | 30.83 ± 8.62           | 47.55 ± 11.80 | 12           | 11      | 0.00082026 |
| 44          | 33.00 ± 8.44           | 51.64 ± 11.58 | 12           | 11      | 0.00022759 |
| 48          | 33.42 ± 8.50           | 54.45 ± 12.13 | 12           | 11      | 8.5033E-05 |
| 52          | 33.92 ± 7.86           | 57.55 ± 12.23 | 12           | 11      | 1.604E-05 |
Figure 2. Analysis of ovarian follicle development in \( \text{Per1}^{\text{m/m}}; \text{Per2}^{\text{m/m}} \) mutant and control mice. (A) Ovarian follicular development in wild-type mice at different stages including primordial, primary, secondary, and antral stages. (B–E) Histological analysis and quantification of ovarian follicle number at different ages, including 3, 8, 26, and 52 weeks old. Arrows indicate the follicles with oocyte nuclei for quantification. Scale bar: 20 \( \mu \)m in A, 50 \( \mu \)m in B, 200 \( \mu \)m in C–E.

Discussion

We show in this study that mice with \( \text{Per1}/\text{Per2} \) double mutations displayed an early onset of decrease in the female fertility. However, there was no obvious change with vascular growth associated with the ovarian follicles and in other tissues examined in the mutant mice. Interestingly, we found that there was a significant reduction of the ovarian follicle number in the \( \text{Per1}/\text{Per2} \) mutants from the middle-aged stage onwards compared with the littermate controls. The process of ovarian follicle development is tightly controlled by many factors. It is possible that \( \text{Per1}/\text{Per2} \) loss-of-function mutations may alter gene expression involved in the follicular development, leading to the depletion of the ovarian follicle reserve and the decline of reproductive capacity.

It was previously demonstrated that deletion of \( \text{Per1} \) or \( \text{Per2} \) affected female fertility at the age of 9–12 months old displaying abnormal oestrous cycle and reduced implantation success, but there was no obvious difference observed with young adult mutants (2–6 months old) [15]. In this study, we found that the doubly \( \text{Per1}/\text{Per2} \)
**Table 2.** Quantification of ovarian follicle number at different development stages of Per1<sup>m/m</sup>; Per2<sup>m/m</sup> doubly mutant and control mice (3, 8, 26, 52 weeks old).

| Ovarian follicle stages | Ovarian follicle number (3 weeks old) n (Per1<sup>m/m</sup>;Per2<sup>m/m</sup>) | n (Control) | P value |
|-------------------------|---------------------------------|-------------|---------|
|                         | Per1<sup>m/m</sup>;Per2<sup>m/m</sup> |                        |         |
| Primordial              | 1356.00 ± 307.05                 | 1642.00 ± 634.34 | 5       |
| Primary                 | 162.00 ± 23.61                   | 165.00 ± 53.85  | 5       |
| Secondary               | 343.00 ± 102.02                  | 337.00 ± 45.36  | 5       |
| Antral                  | 55.00 ± 12.75                    | 53.00 ± 54.15   | 5       |
| Total                   | 1916.00 ± 370.18                 | 2197.00 ± 692.08| 5       |
| Ovarian follicle number (8 weeks old) |                               |                       |         |
| Primordial              | 151.67 ± 80.04                   | 222.00 ± 95.43    | 9       |
| Primary                 | 177.78 ± 57.29                   | 177.00 ± 61.85   | 9       |
| Secondary               | 211.11 ± 70.70                   | 174.00 ± 86.16   | 9       |
| Antral                  | 91.11 ± 24.21                    | 61.00 ± 25.38    | 9       |
| Total                   | 631.67 ± 172.92                  | 634.00 ± 248.26  | 9       |
| Ovarian follicle number (26 weeks old) |                               |                       |         |
| Primordial              | 60.00 ± 43.09                    | 95.91 ± 41.76    | 11      |
| Primary                 | 94.38 ± 51.79                    | 148.18 ± 40.64   | 11      |
| Secondary               | 72.50 ± 35.36                    | 138.18 ± 24.73   | 11      |
| Antral                  | 41.88 ± 13.61                    | 53.64 ± 32.41    | 11      |
| Total                   | 268.75 ± 103.71                  | 435.91 ± 85.81   | 11      |
| Ovarian follicle number (52-week-old) |                               |                       |         |
| Primordial              | 27.14 ± 19.19                    | 51.25 ± 35.87    | 14      |
| Primary                 | 25.00 ± 19.12                    | 52.50 ± 12.88    | 14      |
| Secondary               | 23.93 ± 11.12                    | 41.67 ± 25.35    | 14      |
| Antral                  | 12.14 ± 7.26                     | 22.50 ± 13.23    | 14      |
| Total                   | 88.21 ± 48.70                    | 167.92 ± 59.29   | 14      |
An important role in the amplitude and timing of ovarian steroid expression of FSH and LH with a diurnal rhythm [14]. Furthermore, rhythmic clock genes as well as clock-controlled genes, including the release in the SCN provides a central control for the expression of core mal hormonal signaling including the follicle-stimulating hormone and folliculogenesis [1, 2]. Among the causative candidates, abnor-

dates, which are involved in various processes such as oogenesis genetic origin, more than 50 genes have been identified as POI can-

may involve a wide spectrum of causes. Of the causative factors of causes as previously reported [15]. Consistently, the reduced female fertility caused by Per1/Per2 double mutations is not due to the alteration of vascular components associated with ovarian follicle development. It is possible that the distinct vascular phenotype may result from the species difference between zebrafish and mouse in the regulation of vascular formation by circadian clock genes. However, due to the potential for off-target effects by the use of antisense morpholinos [28], it is necessary to validate the vascular phenotypes using knockout models targeting zebrafish per2 or bmd1.

In summary, we found in this study that Per1/Per2 double mu-
tations caused a significant decrease of ovarian follicles in mutant mice from the middle-aged stage onwards. Although the detailed mechanism remains a topic for further investigation, findings of this study showed that the alteration of circadian rhythm and its underlying regulatory network could be an important factor contributing to the pathogenesis of POI. Future studies in this direction may yield more mechanistic insights into POI and provide potential therapeutic targets for the disease.

Supplemental Data

Supplemental data are available at BIOLRE online.

Supplemental Figure S1. Analysis of apoptosis in ovaries of Per1m/m; Per2m/m doubly mutant and control mice. (A) Immunostaining analysis of apoptotic cells in ovaries (3 weeks old) of Per1m/m; Per2m/m mutant and control mice (cleaved caspase 3, red; PECAM-1, green; LYVE-1, red; DAPI, blue). (B) Quantification of apoptotic cells (per grid, ×40 magnification). Scale bar: 50 μm in A.

Supplemental Figure S2. Analysis of vascular growth in retina and skin of Per1m/m; Per2m/m doubly mutant and control neonatal mice (P5). (A, B) Immunostaining analysis of blood vascular and lymphatic vessels in retina (A) and skin (B) of Per1m/m; Per2m/m mutant and control mice (P5, postnatal day 5; PECAM-1, green; LYVE-1, red). Quantification of retina vascularization area showed that there was no significant difference detected among Per1/Per2 mutant and control mice (A; WT: 46.25 ± 2.93%, n = 6; Per1+/m; Per2+/m: 46.97 ± 5.16%, n = 6; Per1m/m; Per2m/m: 48.52 ± 5.39, n = 9). Scale bar: 100 μm in A and B.

Supplemental Figure S3. Analysis of vascular growth in adult tissues of Per1m/m; Per2m/m doubly mutant and control mice. (A–C) Immunostaining analysis of blood vascular and lymphatic vessels in ear skin (A, 7 weeks old), trachea (B, 7 weeks old), and uterus (C, 8 and 52 weeks old) of Per1m/m; Per2m/m mutant and control mice (PECAM-1, green; LYVE-1, red). Scale bar: 100 μm in A, B, and C.
Acknowledgment

We thank the staff in Animal facility of Soochow University for technical assistance.

Reference

1. Tucker EJ, Grover SR, Bachelot A, Touraine P, Sinclair AH. Premature ovarian insufficiency: new perspectives on genetic cause and phenotypic spectrum. Endocr Rev 2016; 37:609–635.
2. Jiao X, Ke H, Qin Y, Chen ZJ. Molecular genetics of premature ovarian insufficiency. Trends Endocrinol Metab 2018; 29:795–807.
3. de la Iglesia HO, Schwartz WJ. Minireview: timely ovulation: circadian regulation of the female hypothalamo-pituitary-gonadal axis. Endocrinology 2006; 147:1148–1153.
4. Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci 2012; 35:445–462.
5. Albrecht U. Timing to perfection: the biology of central and peripheral circadian clocks in mammals. Nature 2019; 400:169–173.
6. Xu Y, Toh KL, Jones CR, Chin YJ, Fu YH, Pracek LJ. Modeling of a human circadian mutation yields insights into clock regulation by PER2. Cell 2007; 128:59–70.
7. Fahrenkrug J, Georg B, Hannibal J, Hindersson P, Gras S. Diurnal rhythmicity of the clock genes Per1 and Per2 in the rat ovary. Endocrinology 2006; 147:3769–3776.
8. Karman BN, Tischkau SA. Circadian clock gene expression in the ovary: effects of luteinizing hormone. J Neuroendocrinol 2005; 17:119–130.
9. Zitmann BD, Lemus DR, Ortinger MA, Urbanski HF. Effects of age on clock gene expression in the rhesus macaque pituitary gland. Neurobiol Aging 2010; 31:696–705.
10. Sellix MT. Circadian clock function in the mammalian ovary. J Biol Rhythms 2015; 30:7–19.
11. Piazzoli V, Steinlechner S. Low reproductive success in Per1 and Per2 mutant mice females due to accelerated aging? Reproduction 2008; 135:559–568.
12. Chappell PE. Clocks and the black box: circadian influences on gonadotropin-releasing hormone secretion. J Neuroendocrinol 2005; 17:119–130.
13. Sitzmann BD, Lemus DR, Ortinger MA, Urbanski HF. Effects of age on clock gene expression in the rhesus macaque pituitary gland. Neurobiol Aging 2010; 31:696–705.
14. Sellix MT. Circadian clock function in the mammalian ovary. J Biol Rhythms 2015; 30:7–19.
15. Piazzoli V, Steinlechner S. Low reproductive success in Per1 and Per2 mutant mice females due to accelerated aging? Reproduction 2008; 135:559–568.
16. Miller BH, Olson SL, Turek FW, Levine JE, Horton TH, Takahashi JS. Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Cell Biol 2004; 14:1367–1373.
17. Ratajczak CK, Boehler KL, Muglia LJ. Impaired steroidogenesis and implantation failure in Bmal1−/− mice. Endocrinology 2009; 150:1879–1885.
18. Tsujii T, Kiyoumi K, Akiyama K, Kunieda T. CNP/NPR2 signaling maintains oocyte meiotic arrest in early antral follicles and is suppressed by EGFR-mediated signaling in preovulatory follicles. Mol Reprod Dev 2012; 79:795–802.
19. Liu Y, Johnson BP, Shen AL, Wallisser JA, Krentz KJ, Moran SM, Sullivan R, Glover E, Parlow AF, Drinkwater NR, Schuler LA, Bradfield CA. Loss of BMAL1 in ovarian steroidogenic cells results in implantation failure in female mice. Proc Natl Acad Sci USA 2014; 111:14295–14300.
20. Merenesis AL, Murphy ZC, Forrestell AC, Butler S, Ko C, Richards JS, Sellix MT. Conditional deletion of Bmal1 in ovarian theca cells disrupts ovulation in female mice. Endocrinology 2016; 157:913–927.
21. Viswanbhbaran H, Carvas JM, Antic V, Marcic A, Jud C, Zaug CE, Ming XF, Montani JP, Albrecht U, Yang Z. Mutation of the circadian clock gene Per2 alters vascular endothelial function. Circulation 2007; 115:2188–2195.
22. Jensen LD, Cao Z, Nakamura M, Yang Y, Brautigam I, Andersson P, Zhang Y, Wahlberg E, Lanne T, Hosaka K, Cao Y. Opposing effects of circadian clock genes Bmal1 and Period2 in regulation of VEGF-dependent angiogenesis in developing zebrafish. Cell Rep 2012; 2:231–241.
23. Cerra F. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25:581–611.
24. Chu M, Li T, Shen B, Cao X, Zhang X, Zhou F, Ma W, Jiang H, Xie P, Liu Z, Dong N et al. Angiopoietin receptor Tie2 is required for vein specification and maintenance via regulating COUP-TFII. eLife 2016; 5:e21032.
25. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 2001; 30:525–536.
26. Zangen D, Kaufman Y, Zeligson S, Perlberg S, Fridman H, Kanaan M, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 2001; 30:525–536.