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Loss of *Bmal1* decreases oocyte fertilization, early embryo development and implantation potential in female mice

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Summary

Biological clock genes expressed in reproductive tissues play important roles in maintaining the normal functions of reproductive system. However, disruption of female circadian rhythm on oocyte fertilization, preimplantation embryo development and blastocyst implantation potential is still unclear. In this study, ovulation, *in vivo* and *in vitro* oocyte fertilization, embryo development, implantation and intracellular reactive oxygen species (ROS) levels in ovary and oviduct were studied in female $Bmal1^{+/+}$ and $Bmal1^{-/-}$ mice. The number of naturally ovulated oocyte in $Bmal1^{-/-}$ mice decreased (5.2 ± 0.8 *vs* 7.8 ± 0.8, $P < 0.001$), with an increasing abnormal oocyte ratio (20.4 ± 3.5 *vs* $11.7 \pm 2.0\%$, $P = 0.001$) after superovulation. Significantly lower fertilization rate and obtained blastocyst number were observed in $Bmal1^{-/-}$ female mice either mated with wild-type *in vivo* or fertilized by sperm from wild-type male mice *in vitro* (all $P < 0.05$). Interestingly, *in vitro* fertilization rate of oocytes derived from $Bmal1^{-/-}$ increased significantly compared with *in vivo* study ($P < 0.01$). After transferring blastocysts derived from $Bmal1^{+/+}$ and $Bmal1^{-/-}$ female mice to pseudopregnant mice, the implantation sites of the latter decreased 5 days later (8.0 ± 0.8 *vs* 5.3 ± 1.0, $P = 0.005$). The intracellular ROS levels in the ovary on proestrus day and in the oviduct on metestrus day increased significantly in $Bmal1^{-/-}$ mice compared with that of $Bmal1^{+/+}$ mice. Deletion of the core biological clock gene *Bmal1* significantly decreases oocyte fertilization rate, early embryo development and implantation potential in female mice, and these may be possibly caused by excess ROS levels generated in ovary and oviduct.

Keywords: $Bmal1^{-/-}$ mice, Embryo development, Fertilization, Implantation, ROS

Introduction

Disruption of circadian rhythms due to rotating and night shift work or jet lag has been implicated in a variety of reproductive dysfunctions in women, including irregular, extended menstrual cycles, increased risk of pre-term birth, and overall reduced fecundity (Baker & Driver, 2007; Lawson *et al.*, 2011; Gamble *et al.*, 2013).

The circadian clock is cell autonomous and characterized by transcriptional–translational feedback loops (Reppert & Weaver, 2002). Previous work has shown that *BMAL1* (brain muscle arnt-like 1) protein (also known as MOP3 or ARNTL1), in a dimeric complex with the CLOCK protein, is an essential component that controls circadian driven gene expression (Lowrey & Takahashi, 2011). Loss of *Bmal1* expression disrupts behavioral rhythmicity and gene expression rhythms

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in the SCN and peripheral tissues (Bunger *et al.*, 2000). In human, polymorphisms in *BMAL1* and the CLOCK paralog NPAS2 also have been associated with altered pregnancy rates and risk of miscarriage (Kovanen *et al.*, 2010).

The effects of deletion of the core biological clock gene *Bmal1* in female mice on early embryo development are still unclear. The original report of the *Bmal1* null mice stated that they were viable, fertile, and with a normal distribution of genotype (Bunger *et al.*, 2000). Later studies demonstrated that *Bmal1* null mice are infertile, but reports on the early embryo development were not the same. Ratajczak *et al.* reported normal embryo development up to day 3.5 of gestation and suggested that the infertility of *Bmal1* null mice was due primarily to poor implantation (Ratajczak *et al.*, 2009). Boden *et al.* found *Bmal1* knockout mice ovulate, but with poor embryo development after mating in both natural and hormone stimulated cycles, and it was not clear from their study if the failure to produce mature blastocysts was because of poor oocyte quality or because the oviduct failed to support the developing embryo (Boden *et al.*, 2010).

Data obtained in different organisms have established a tight connection between the biological clock and cellular redox signalling (Patel *et al.*, 2014). Increased levels of reactive oxygen species (ROS) had been detected in some tissues of *Bmal1* null mice, and this was considered to be correlated with reduced lifespan and various symptoms of premature aging observed in this model animal (Kondratov *et al.*, 2006). In reproductive physiology, ROS is involved in normal ovulation, corpus luteum regression, while excess ROS is proven to be detrimental for reproductive function (Shkolnik *et al.*, 2011; Agarwal *et al.*, 2012; Al-Gubory *et al.*, 2012). We suppose the ROS levels in the reproductive organs of *Bmal1*$^{-/-}$ mice should also be changed and may be correlated with their reproductive function.

In this study, ovulation, *in vivo* and *in vitro* oocyte fertilization, embryo development, implantation and intracellular ROS levels in ovary and fallopian tubule were studied in female *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice.

Materials and methods

Animals

Male and female heterozygous *Bmal1* knockout mice on C57BL/6J background (5–6 weeks of age, 18–20 g) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Homozygote animals were produced by breeding heterozygous pairs. The genotypes of the offspring were determined as previously described (Bunger *et al.*, 2000). Wild-type male and female C57BL/6J mice (5–6 weeks of age, 18–20 g) were purchased from the Guangdong Province Laboratory Animal Center (Guangzhou, China). All mice were synchronized with a 12 h light/dark cycle, with the lights on from 6 a.m. (Zeitgeber time 0, ZT 0) to 6 p.m. (ZT 12) for 2 weeks, and with free access to regular chow food and water. The indoor temperature was maintained at a 20–25°C and relative humidity of 40–70%. All experimental procedures were approved by the Ethical Committee of The First Affiliated Hospital of Sun Yat-Sen University, and all efforts were made to minimize suffering.

Natural ovulation

Estrous cycles were determined as described previously (Byers *et al.*, 2012). Bilateral oviducts of *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ female mice were dissected in the early hours of the estrus morning (ZT 2–3) to examine the number of naturally ovulated oocytes.

Superovulation

Wild-type and *Bmal1*$^{-/-}$ female mice were superovulated by an intraperitoneal (i.p.) injection of 10 IU pregnant mare serum gonadotrophin (PMSG; Ningbo Second Hormone Factory, Ningbo, China) at 16:00 (ZT 10), followed 48 h later by an i.p. injection of 10 IU of human Chorionic Gonadotropin (hCG, Ningbo Second Hormone Factory, Ningbo, China). For the observation of mice oocytes, cumulus-oocyte complexes were collected in 3-(*N*-morpholino)propanesulfonic acid (MOPS) medium (VitroLife, Sweden) 8 h after hCG injection, and then denudated with hyaluronidase (VitroLife, Sweden).

In vivo fertilization and embryo development

In the *in vivo* group, after injection with hCG, these females were placed with male C57BL/6J mice overnight. The following morning (ZT 2), female mice with the presence of vaginal plugs were considered pregnant as day 0.5. These mice were then humanely killed by cervical dislocation 1 or 3 days later for the determination of fertilization rate or blastocyst number, by dissecting oviducts or flushing dissected uteri, respectively. Fertilization was assessed by the formation of 2-cell embryos.

In vitro fertilization and embryo development

For the *in vitro* fertilization group, 15 h after injection with hCG, female mice were humanely sacrificed by cervical dislocation. Oviduct dissection was carried out at 37°C, and cumulus–oocyte complexes (COC) were collected in G-MOPS (VitroLife, Sweden)

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supplemented with 5 mg/ml human serum albumin (HSA). COC were then wash and placed in G-IVF (VitroLife, Sweden) supplemented with 5 mg/ml HSA under paraffin oil (VitroLife, Sweden) and incubated in a modular incubator chamber at 37°C in 6% CO_2, 5% O_2 for 4 h with sperm, collected from the cauda epididymis of male C57BL/6J mice, that had been previously incubated for 1 h in G-IVF media (VitroLife, Sweden) for capacitation. Putative zygotes were placed in G1 media (10 zygotes/20 μl drop; VitroLife, Sweden) and checked for fertilization/cleavage the next morning. Embryos (2-cell) were transferred to a new drop of G1 media for the initial 48 h of culture and then in G2 (VitroLife, Sweden) before hatching.

Blastocyst vitrification and thawing

Blastocysts derived from female wild-type and *Bmal1*$^{-/-}$ mice after *in vitro* fertilization by sperm from male wild-type mice were vitrified using a vitrification kit (Kitazato Biopharma Co. Ltd, Shizuoka, Japan) in combination with open pulled straws for vitrification. The vitrification and thawing procedure was carried out according to the protocols.

Blastocyst transfer

After thawing, blastocysts were transferred to G2 medium and cultured for 1–2 h before they were transferred to day 2.5 pseudopregnancy female wild-type mice. Pseudopregnant female mice were mated to vasectomized males 3.5 days prior to embryo transfer. The morning after mating, females were checked for the presence of a vaginal plug, and this was considered day 0.5 of pseudopregnancy. Embryo transfer with the NSET device (ParaTechs, Lexington, KY, USA) was performed as described previously (Green *et al.*, 2009). In brief, day 2.5 pseudopregnant mouse was placed on a wire-top cage and allowed to grip the bars. The small and large specula (ParaTechs) were placed sequentially into the vagina to open and expose the cervix. The NSET catheter was then inserted through the large speculum into the uterine horn. The device and specula were removed, and the mouse was returned to its home cage. Totally 12 blastocysts were transferred to uterine horn of pseudopregnant mouse. Pregnant females were killed 5 days later and implantation sites were evaluated.

Determination of intracellular ROS

Intracellular ROS levels were determined using the fluorescent marker 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; S0033; Beyotime, China), according to the manufacturer's instructions. Briefly, mice in specific estrus stage were killed before bilateral oviducts and ovaries were quickly and gently cut using fine forceps under a dissecting microscope, homogenized in phosphate-buffered saline (PBS) on ice and filtered. Cells were collected and incubated with DCFH-DA at a final concentration of 10 μM for 20 min at 37°C in the dark. After washing with serum free cell culture medium twice, cells were resuspended in PBS in the presence of 2 mM freshly prepared H_2O_2 or not. Samples with or without H_2O_2 stimulation were analyzed by fluorescence activated cell sorting (FACS) Canto instrument flow cytometer system (Becton Dickson, Inc.), with an excitation at 488 nm and emission at 529 nm. Ten thousand events were analyzed in each sample. The fluorescence data were further analyzed with WinMDI 2.9 software (Scripps Research Institute, Jupiter, FL, USA). Intracellular ROS levels were expressed as the average dichlorodihydrofluorescein (DCF) fluorescence intensity of the cells.

Statistical analysis

Statistical analyses were performed using the *t*-test as appropriate. All *P*-values quoted are two-sided, and values <0.05 indicate statistical significance. Analyses were performed using the SPSS statistical package.

Results

Female *Bmal1*$^{-/-}$ mice are infertile, even though we detected vaginal plugs after mating to wild-type males.

The naturally ovulated oocyte number of *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice was 7.8 ± 0.8 and 5.2 ± 0.8 ($n = 6$), respectively. Compared with *Bmal1*$^{+/+}$mice, the obtained oocyte number of *Bmal1*$^{-/-}$ mice decreased ($P < 0.001$). No major difference was found in the obtained oocyte number between *Bmal1*$^{-/-}$ and *Bmal1*$^{+/+}$ mice after superovulation (26.9 ± 3 versus 30.1 ± 5.2, $n = 6$, $P = 0.283$) (Fig. 1*A*). However, the total abnormal oocyte (degeneration, fragmentation, etc.) ratio increased in *Bmal1*$^{-/-}$ mice (20.4 ± 3.5% versus 11.7 ± 2.0%, $n = 6$, $P = 0.001$).

In *Bmal1*$^{+/+}$ wild-type mice, no significant difference was found in the fertilization rate (70.3 ± 5.7% versus 66.1 ± 5.9%, $n = 6$, $P > 0.05$) (Fig. 1*B*), blastocyst number (15.2 ± 1.9 versus 13.9 ± 2.1, $n = 6$, $P > 0.05$) (Fig. 1C) between *in vivo* and *in vitro* conditions.

However, significantly lower levels of fertilization rate (33.2 ± 3.3% versus 70.3 ± 5.7%, $n = 6$, $P < 0.001$) (Fig. 1*B*) and obtained blastocyst number (3.0 ± 0.8 versus 15.2 ± 1.9, $n = 6$, $P < 0.001$) (Fig. 1C) were detected in *Bmal1*$^{-/-}$ mice compared with that of *Bmal1*$^{+/+}$ mice after superovulation and mated with wild-type male mice *in vivo.*

Similarly, significantly lower levels of fertilization rate (57.4 ± 2.0% versus 66.1 ± 5.9%, $n = 6$, $P <$

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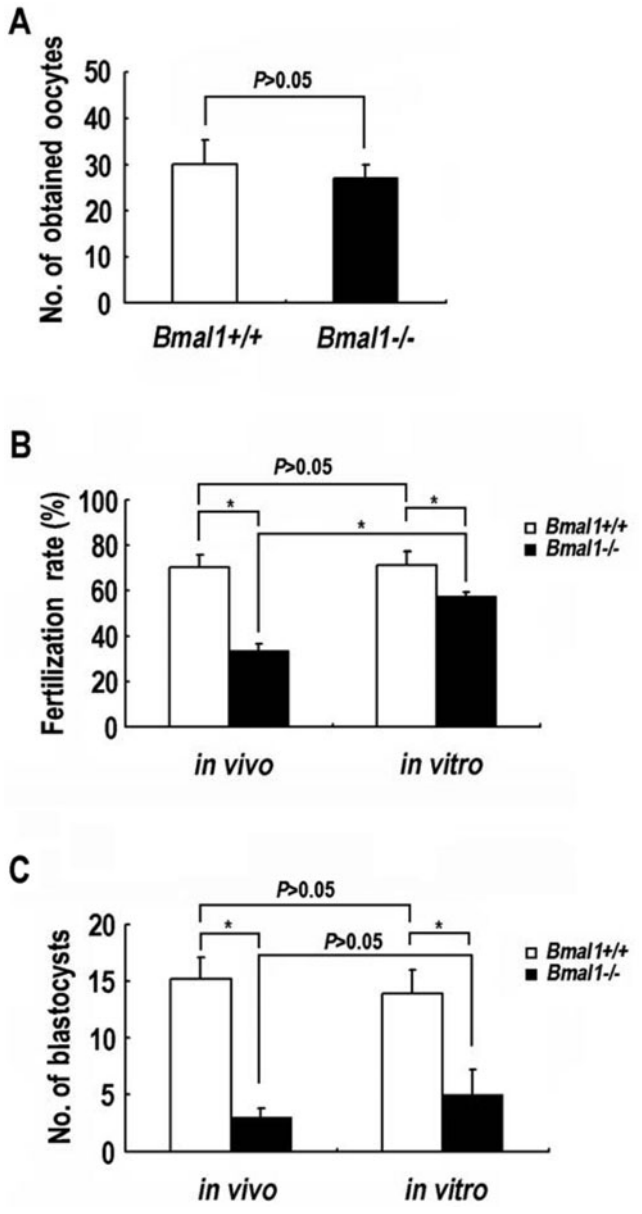

Figure 1 Oocyte number, fertilization rate and blastocyst number in female *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice under *in vivo* and *in vitro* conditions after superovulation. (*A*) Obtained oocyte number in female *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice after superovulation. (*B*) Fertilization rate in female *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice under *in vivo* and *in vitro* conditions after superovulation. (*C*) Obtained blastocyst number in female *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice under *in vivo* and *in vitro* conditions after superovulation. All values represent the mean ± standard error of the mean (SEM) ($n = 6$). $^{*}P < 0.05$.

0.001) (Fig. 1*B*) and blastocyst number (5.0 ± 2.2 versus 13.9 ± 2.1, $n = 6$, $P < 0.05$) (Fig. 1*C*) were observed in *Bmal1*$^{-/-}$ mice after superovulation and *in vitro* fertilized with sperm from wild-type male mice.

Interestingly, *in vitro* fertilization rate of oocytes derived from *Bmal1*$^{-/-}$ increased significantly compared with *in vivo* study (57.4% ± 2.0 versus 33.2 ± 3.3%, $n = 6$, $P < 0.01$) (Fig. 1*B*). There was also a trend for blastocyst number to increase *in vitro*, but no significant difference was found (5.0 ± 2.2 versus 3.0 ± 0.8, $n = 6$, $P > 0.05$) (Fig. 1*C*).

Obtained blastocysts derived from female *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice under *in vitro* environment were transferred to pseudopregnant wild-type mice, the implantation sites 5 days later was 8.0 ± 0.8 and 5.3 ± 1.0, respectively ($n = 4$, $P = 0.005$), as shown in Fig. 2.

At ZT 10 and 14 on the day of proestrous, the ROS level in the ovary cells of *Bmal1*$^{-/-}$ mice significantly increased compared with that of *Bmal1*$^{+/+}$ mice ($n = 4$, both $P < 0.01$) (Fig. 3); Similarly, the ROS level of oviduct cells in *Bmal1*$^{-/-}$ mice significantly increased compared with that in *Bmal1*$^{+/+}$ mice at ZT4 on the day of metestrus, ($n = 4$, $P < 0.001$) (Fig. 4).

Discussion

Previous reports on the early embryo development of *Bmal1* null mice were not consistent. In this study, oocyte morphology, *in vivo* and *in vitro* oocyte fertilization, early embryo development and their implantation potential of female *Bmal1*$^{-/-}$ mice were investigated. Our study, consistent with recent studies (Ratajczak *et al.*, 2009; Boden *et al.*, 2010), showed that female *Bmal1*$^{-/-}$ mice were infertile. Chu *et al.* found that *Bmal1*$^{-/-}$ females tends to yield fewer oocytes than wild-type, but the difference did not reach significance; Furthermore, they observed that cumulus–oocyte complexes in distended ampullae of female *Bmal1*$^{-/-}$ mice were morphologically indistinguishable from wild-type controls (Chu *et al.*, 2013). Our result also showed that female *Bmal1*$^{-/-}$ mice were able to ovulate spontaneously, but relatively fewer oocytes were retrieved, indicating the process of follicle development and/or ovulation was at least partly affected. Due to the fewer number of naturally ovulated oocytes, experiments on *in vivo* and *in vitro* oocyte fertilization and embryo development were carried out after superovulation. No significant difference was observed in the obtained oocyte number, this is consistent with Borden's result (Boden *et al.*, 2010). However, after removing cumulus cells, we first found that the ratio of abnormal oocytes, including degeneration, big polar body and fragmentation increased significantly in female *Bmal1*$^{-/-}$ mice, indicating adverse environment existed before ovulation.

Ratajczak *et al.* found normal embryo development up to day 3.5 of gestation (Ratajczak *et al.*, 2009). Later, Boden firstly reported lower insemination rate and embryo development in *Bmal1*$^{-/-}$ mice *in vivo*.

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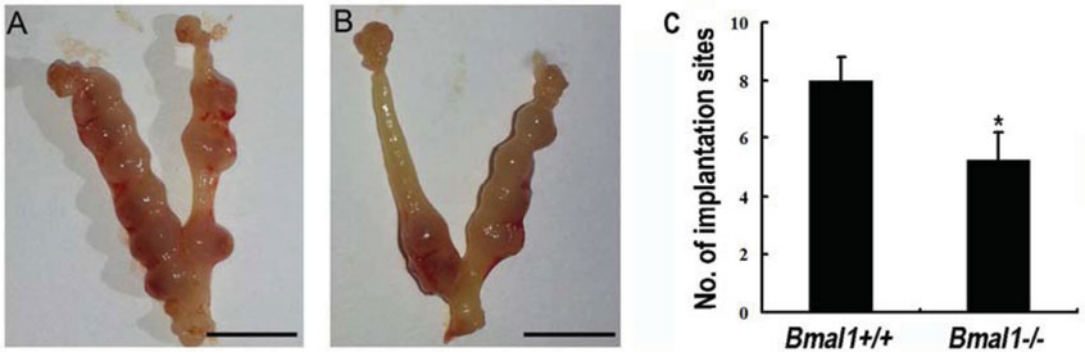

Figure 2 Number of implantation sites in pseudopregnant $Bmal1^{+/+}$ mice 5 days after transfer of blastocysts derived from female $Bmal1^{+/+}$ or $Bmal1^{-/-}$ mice. (*A*) The transferred blastocysts were derived from female $Bmal1^{+/+}$ mice. (*B*) The transferred blastocysts were derived from female $Bmal1^{-/-}$ mice. (*C*) Blastocysts derived from female $Bmal1^{-/-}$ formed fewer implantation sites compared with that derived from female $Bmal1^{+/+}$ mice ($^*P < 0.05$). Scale bar = 1 cm. Values represent the mean ± standard error of the mean (SEM) ($n = 6$).

Under both *in vivo* and *in vitro* conditions, we found that oocyte fertilization rate and early embryo development decreased in female $Bmal1^{-/-}$ mice. It was not possible to remove abnormal oocytes before fertilization, so it is reasonable that the decreased oocyte fertilization rate in female $Bmal1^{-/-}$ mice was at least partly due to the increased abnormal oocytes rate. To our surprise, we found that the *in vitro* fertilization rate of oocytes derived from $Bmal1^{-/-}$ increased significantly compared with *in vivo* study. Moreover, the blastocyst number also had a trend to increase under *in vitro* condition. In our experiment, oocytes were collected for *in vitro* fertilization 15 h after hCG injection, and fertilization was assessed by the formation of 2-cell embryos in the next moring (ZT 2) for both *in vivo* and *in vitro* fertilization. *In vivo*, the oviduct is the site of fertilization and early embryo development. This result indicated that detrimental factors existed in oviduct of $Bmal1^{-/-}$ female, except for the decreased oocyte quality.

In recent years, the existence of circadian rhythm in the ovary has been demonstrated extensively (Sellix & Menaker, 2010). It is also known that the oviduct rhythmically expresses the core clock genes, several transcription factors and enzyme regulators important for the protection of the embryo (Kennaway *et al.*, 2003). Tight connections between biological clock and cellular redox signaling have been established in different organisms. As we speculated, the ROS level in the ovary cells on the day of proestrus at ZT 10 and 14, and in the oviduct cells on the day of metaestrus at ZT 4 were significantly higher in $Bmal1^{-/-}$ mice compared with that of $Bmal1^{+/+}$ mice, respectively. In wild-type mice, luteinizing hormone (LH) surge generally occurs just prior to the active period on proestrus day (approximately ZT 11) (Chu *et al.*, 2013). Our results indicated that around the time of ovulation and fertilization, oocytes/early embryos are exposed to the environment of excess ROS in $Bmal1^{-/-}$ females. Previous studies demonstrated that oxidative stress impairs oocyte quality, fertilization and embryo development (Matsuzuka *et al.*, 2005; Tamura *et al.*, 2008). So the decreased oocyte quality, fertilization and embryo development *in vivo* in $Bmal1^{-/-}$ females may be caused by excess ROS in these organs.

In our embryo transfer experiment, blastocysts derived from $Bmal1^{+/+}$ and $Bmal1^{-/-}$ female mice were transferred to wild-type pseudopregnant mice, whose ovary and uterus were normal. Furthermore, the blastocysts derived from female $Bmal1^{-/-}$ mice were all heterozygous and it has been indicated the developmental potential of heterozygous *Bma11* embryos were not influenced (Boden *et al.*, 2010). Thus, the decreased implantation potential of blastocysts derived from female $Bmal1^{-/-}$ mice observed in our experiment indicated their quality was negatively affected. Ratajczak *et al.* suggested that implantation failure due to impaired steroidogenesis cause infertility of $Bmal1^{-/-}$ females. After receiving daily injections of progesterone, 38% (five of 13) $Bmal1^{-/-}$ females displayed implantation sites at day 10.5 (Ratajczak *et al.*, 2009). Using a cell-specific knockout mice model, in which *Bmal1* was deleted in the steroidogenic cells, Liu *et al.* observed embryonic implantation failure. Through supplementation of progesterone or transplantation of wild-type ovaries to these conditional knockout female mice, they showed that clock gene *Bmal1* in ovary steroidogenic cells is crucial for embryonic implantation (Liu *et al.*, 2014). Although blastocyst quality was not determined in these studies, after progesterone supplementation, lower implantation rate observed in global $Bmal1^{-/-}$ female mice (38%) compared with that of the conditional

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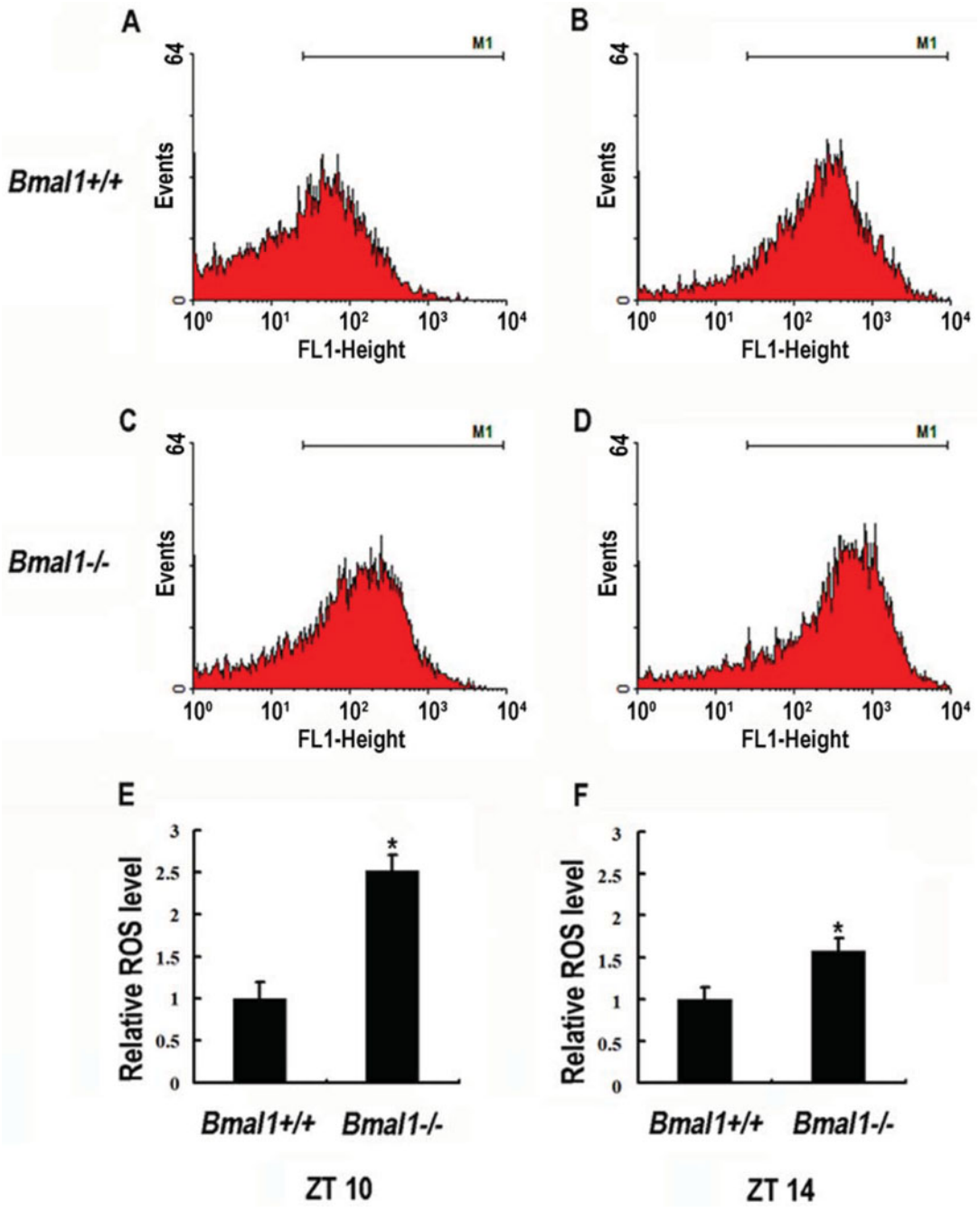

Figure 3 ROS levels in the ovary cells of *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice at ZT10 and ZT 14 on the day of proestrous. Representative histogram of flow cytometric analysis of intracellular ROS staining with DCFH-DA at ZT 10 and 14 in the ovary cells of *Bmal1*$^{+/+}$ (*A*, *B*) and *Bmal1*$^{-/-}$ (*C*, *D*) mice, respectively. Comparative ROS levels (expressed as fluorescence intensity) in the ovary cells of *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice at ZT 10 (*E*) and 14 (*F*) on proestrous day. Values represent the mean ± standard error of the mean (SEM) ($n = 4$, in each time point and mice type). $^*P < 0.05$, compared with respective *Bmal1*$^{+/+}$ mice.

knockout mice (62.5%), in which oviduct biological rhythm was not disrupted, also suggested blastocyst quality was impaired in female *Bmal1*$^{-/-}$ mice. Taking together, we consider the decreased blastocyst quality also contributes to the implantation failure in *Bmal1*$^{-/-}$ female mice.

Bedaiwy *et al.* found high day 1 ROS levels in culture media were associated with lower pregnancy rates in both *in vitro* fertilization and intracytoplasmic sperm injection cycles in human (Bedaiwy *et al.*, 2004). In murine, melatonin treatment reduced ROS production and increased the efficiency of blastocyst

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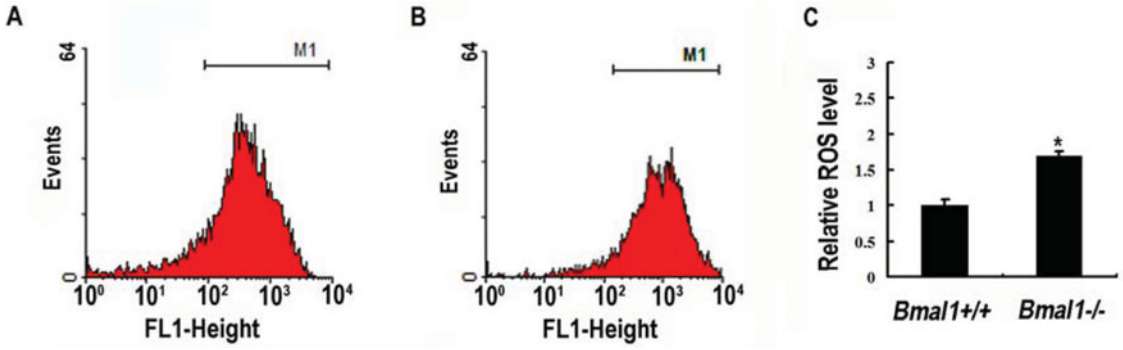

Figure 4 ROS levels in the oviduct cells of *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice at ZT4 on the day of metestrus. Representative histogram of flow cytometric analysis of intracellular ROS staining with DCFH-DA at ZT 4 in the oviduct cells of *Bmal1*$^{+/+}$ (*A*) and *Bmal1*$^{-/-}$ (*B*) mice. Comparative ROS levels (expressed as fluorescence intensity) in the oviduct cells of *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ mice at ZT4 on metestrus day (*C*). Values represent the mean ± standard error of the mean (SEM) ($n = 4$, in each mice type). $^{*}P < 0.05$ compared with respective *Bmal1*$^{+/+}$ mice.

implantation (Wang *et al.*, 2013). The increased intracellular ROS levels detected in our experiment in oviduct of female *Bmal1*$^{-/-}$ mice may explain the lower implantation rate of blastocyst derived from these mice.

Generally, *in vitro* fertilization is also affected by excessive ROS in embryo culture media. However in our experiment, in wild-type female mice, no significant difference was found in the fertilization rate and blastocyst number between *in vivo* and *in vitro* conditions, although blastocyst morphology seemed better under *in vivo* conditions. It is possible that, in *Bmal1*$^{-/-}$ females, the relatively higher fertilization rate and blastocyst number *in vitro* was due to the effect of potent antioxidant contained in G-series culture mediums we used in our experiment; while *in vivo* the oocytes/early embryos were exposed to excess ROS in the oviduct.

Widely held view is that the proestrus LH surge is an obligatory prerequisite for ovulation. The phenomenon that natural ovulation was unaffected in *Bmal1* null mice has been considered surprising, since the loss of central rhythmicity should have compromised the LH surge mechanisms (Boden *et al.*, 2010). After detecting blood samples taken at consecutive time points on proestrus and estrus day, Chu *et al.* surprisingly found that *Bmal1*$^{-/-}$ females lack the proestrus LH surge, with only a trend towards slightly higher LH abundance at one hour before lights off (ZT 11) (Chu *et al.*, 2013). These authors challenged the traditional view and considered that LH surge is not required for spontaneous ovulation. H_2O_2 could fully mimic the effect of LH, bringing about an extensive mucification/expansion of the follicle-enclosed cumulus–oocyte complexes, which is essential for normal ovulation (Shkolnik *et al.*, 2011). Our result that excess ROS existed in the ovary of *Bmal1*$^{-/-}$ females around the time of ovulation may explain why natural ovulation was not affect in this animal model.

To sum up, we have determined that disruption of circadian rhythms in ovary decreases oocyte quality and subsequent fertilization; loss of circadian rhythms in oviduct also decreases fertilization rate and embryo developmental potential. However, we could not exclude the influence from central and other periphery system, which were also affected by the disruption of biological clock. In conclusion, deletion of the core biological clock gene *Bmal1* significantly decreases oocyte quality, fertilization, embryo development and implantation potential in female mice, and these may be caused by excess ROS levels generated in ovary and oviduct. However, due to the relatively small sample size, more research will be needed to confirm this.

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References

Agarwal, A., Aponte-Mellado, A., Premkumar, B.J., Shaman, A. & Gupta, S. (2012). The effects of oxidative stress on female reproduction: a review. *Reprod. Biol. Endocrinol.* **10**, 49.

Al-Gubory, K.H., Garrel, C., Faure, P. & Sugino, N. (2012). Roles of antioxidant enzymes in corpus luteum rescue from reactive oxygen species-induced oxidative stress. *Reprod. Biomed. Online* **25**, 551–60.

Baker, F.C. & Driver, H.S. (2007). Circadian rhythms, sleep, and the menstrual cycle. *Sleep Med.* **8**, 613–22.

https://doi.org/10.1017/S0967199416000083 Published online by Cambridge University Press

Bedaiwy, M.A., Falcone, T., Mohamed, M.S., Aleem, A.A., Sharma, R.K., Worley, S.E., Thornton, J. & Agarwal, A. (2004). Differential growth of human embryos *in vitro*: role of reactive oxygen species. *Fertil. Steril.* **82**, 593–600.

Boden, M.J., Varcoe, T.J., Voultsios, A. & Kennaway, D.J. (2010). Reproductive biology of female *Bmal1* null mice. *Reproduction* **139**, 1077–90.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S. & Bradfield, C.A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103**, 1009–17.

Byers, S.L., Wiles, M.V., Dunn, S.L. & Taft, R.A. (2012). Mouse estrous cycle identification tool and images. *PLoS One* **7**, e35538.

Chu, A., Zhu, L., Blum, I.D., Mai, O., Leliavski, A., Fahrenkrug, J., Oster, H., Boehm, U. & Storch, K.F. (2013). Global but not gonadotrope-specific disruption of *Bmal1* abolishes the luteinizing hormone surge without affecting ovulation. *Endocrinology* **154**, 2924–35.

Gamble, K.L., Resuehr, D. & Johnson, C.H. (2013). Shift work and circadian dysregulation of reproduction. *Front. Endocrinol. (Lausanne)* **4**, 92.

Green, M., Bass, S. & Spear, B. (2009). A device for the simple and rapid transcervical transfer of mouse embryos eliminates the need for surgery and potential post-operative complications. *Biotechniques* **47**, 919–24.

Kennaway, D.J., Varcoe, T.J. & Mau, V.J. (2003). Rhythmic expression of clock and clock-controlled genes in the rat oviduct. *Mol. Hum. Reprod.* **9**, 503–7.

Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V. & Antoch, M.P. (2006). Early aging and age-related pathologies in mice deficient in *BMAL1*, the core component of the circadian clock. *Genes Dev.* **20**, 1868–73.

Kovanen, L., Saarikoski, S.T., Aromaa, A., Lonnqvist, J. & Partonen, T. (2010). ARNTL (*BMAL1*) and NPAS2 gene variants contribute to fertility and seasonality. *PLoS One* **5**, e10007.

Lawson, C.C., Whelan, E.A., Lividoti, H.E., Spiegelman, D., Schernhammer, E.S. & Rich-Edwards, J.W. (2011). Rotating shift work and menstrual cycle characteristics. *Epidemiology* **22**, 305–12.

Liu, Y., Johnson, B.P., Shen, A.L., Wallisser, J.A., Krentz, K.J., Moran, S.M., Sullivan, R., Glover, E., Parlow, A.F., Drinkwater, N.R., Schuler, L.A. & Bradfield, C.A. (2014). Loss of *BMAL1* in ovarian steroidogenic cells results in implantation failure in female mice. *Proc. Natl. Acad. Sci. USA* **111**, 14295–300.

Lowrey, P.L. & Takahashi, J.S. (2011). Genetics of circadian rhythms in mammalian model organisms. *Adv. Genet.* **74**, 175–230.

Matsuzuka, T., Ozawa, M., Nakamura, A., Ushitani, A., Hirabayashi, M. & Kanai, Y. (2005). Effects of heat stress on the redox status in the oviduct and early embryonic development in mice. *J. Reprod. Dev.* **51**, 281–7.

Patel, S.A., Velingkaar, N.S. & Kondratov, R.V. (2014). Transcriptional control of antioxidant defense by the circadian clock. *Antioxid. Redox Signal* **20**, 2997–3006.

Ratajczak, C.K., Boehle, K.L. & Muglia, L.J. (2009). Impaired steroidogenesis and implantation failure in *Bmal1*$^{-/-}$ mice. *Endocrinology* **150**, 1879–85.

Reppert, S.M. & Weaver, D.R. (2002). Coordination of circadian timing in mammals. *Nature* **418**, 935–41.

Sellix, M.T. & Menaker, M. (2010). Circadian clocks in the ovary. *Trends Endocrinol. Metab.* **21**, 628–36.

Shkolnik, K., Tadmor, A., Ben-Dor, S., Nevo, N., Galiani, D. & Dekel, N. (2011). Reactive oxygen species are indispensable in ovulation. *Proc. Natl. Acad. Sci. USA* **108**, 1462–7.

Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., Taketani, T., Matsuoka, A., Yamagata, Y., Shimamura, K., Morioka, H., Ishikawa, H., Reiter, R.J. & Sugino, N. (2008). Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J. Pineal Res.* **44**, 280–7.

Wang, F., Tian, X., Zhang, L., Tan, D., Reiter, R.J. & Liu, G. (2013). Melatonin promotes the *in vitro* development of pronuclear embryos and increases the efficiency of blastocyst implantation in murine. *J. Pineal Res.* **55**, 267–74.