Paternal H3K4 methylation is required for minor zygotic gene activation and early mouse embryonic development

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Abstract

Epigenetic modifications, such as DNA methylation and histone modifications, are dynamically altered predominantly in paternal pronuclei soon after fertilization. To identify which histone modifications are required for early embryonic development, we utilized histone K-M mutants, which prevent endogenous histone methylation at the mutated site. We prepared four single K-M mutants for histone H3.3, K4M, K9M, K27M, and K36M, and demonstrate that overexpression of H3.3 K4M in embryos before fertilization results in developmental arrest, whereas overexpression after fertilization does not affect the development. Furthermore, loss of H3K4 methylation decreases the level of minor zygotic gene activation (ZGA) predominantly in the paternal pronucleus, and we obtained similar results from knockdown of the H3K4 methyltransferase Mll3/4. We therefore conclude that H3K4 methylation, likely established by Mll3/4 at the early pronuclear stage, is essential for the onset of minor ZGA in the paternal pronucleus, which is necessary for subsequent preimplantation development in mice.

Keywords histone methylation; K-M mutant; minor ZGA; Mll3/4

Introduction

Epigenetic reprogramming in zygotes by erasing parental epigenetic marks such as DNA methylation and histone modifications is required for the establishment of totipotency. During mammalian embryonic development, this occurs at the pronuclear stage, during which zygotes have two distinct nuclei that are derived from the sperm [paternal pronucleus (PN)] and the oocyte [maternal pronucleus (PN)]. Genome-wide epigenetic alteration predominantly occurs in the paternal PN rather than the maternal PN, implying that paternal epigenetic reprogramming is more dynamic than maternal reprogramming [1–7]. For instance, many histone modifications in the paternal PN are unestablished or gradually become detectable as the pronuclear stage progresses, while there is no such dynamic alteration of histone modification in the maternal PN. In addition, localization of histone variants is also asymmetric between the paternal and maternal PNs. Particularly, histone H3.3, one of the histone variants that is incorporated into chromatin independently of DNA replication, is predominantly incorporated into the paternal PN soon after fertilization [5,8]. The impairment of K27 methylation and K36 methylation of H3.3 by using their lysine–arginine substituted mutants (i.e., H3.3-K27R and H3.3-K36R, respectively) has been reported to critically affect the development of preimplantation embryos; these effects were not observed when H3.1 was used [6,9]. Collectively, these findings demonstrate the critical role of histone methylation in embryonic development.

Immediately after fertilization, maternal RNAs and proteins are abundantly retained in zygotes. During pronuclear stages, these maternally derived components degrade rapidly, and transcription of the zygotic genome has to be initiated for subsequent embryonic development [10,11]. Gene transcription during the transition from maternal factors to those of the zygote is called zygotic gene activation (ZGA), which consists of two waves: the late pronuclear stage (minor ZGA) followed by the two-cell stage (major ZGA) [12,13]. Minor ZGA is the first activation of transcription from the zygotic genome and occurs in the middle pronuclear stages. Interestingly, it has been reported that the timing and global gene expression levels are different between the paternal and maternal PN: The paternal PN exhibits earlier and higher gene transcription compared with the maternal PN [14]. In addition, a recent study also reported that reprogramming factors are predominantly expressed from the paternal PN rather than the maternal PN [15]. The reason for these asymmetric properties is thought to be differences in their chromatin structures. However, exactly what kind of epigenetic factor(s) causes the asymmetric transcription in minor ZGA has not yet been elucidated.

Recently, a search for de novo mutations in pediatric glioma patients identified a genetic mutation of lysine 27 to methionine (K27M) in the histone H3.3 gene [16]. Interestingly, this mutation
not only abrogates K27 methylation in the H3.3 transcribed from the mutated genome, but also inhibits global endogenous K27 methylation in non-mutated H3, including H3.1 and H3.2, by preventing the methyltransferase activity of PRC2. In addition, the mechanism that prevents catalytic activity is often similar to other SET domain-containing methyltransferases; accordingly, K4M, K9M, and K36M mutants are able to inhibit endogenous K4, K9, and K36 methylation, respectively, when they are overexpressed. To take advantage of this system, we employed four K-M mutants (K4M, K9M, K27M, and K36M) to clarify the roles of histone methylation in ZGA.

Results and Discussion

H3K4 methylation of the paternal pronucleus is required for early preimplantation development

Global inhibitory effects of histone methylation by overexpressing H3.3 K4M, K9M, K27M, and K36M mutants were first confirmed in 293T cells (Supplementary Fig S1). For mouse embryos, overexpression was induced by injecting the mRNAs of these mutants into the cytoplasm of MI oocytes followed by intracytoplasmic sperm injection (ICSI), which ensured the translation of mutant proteins from the injected mRNAs by the time of fertilization and early zygotic stages (Fig 1A, red line). We also performed mRNA injection after in vitro fertilization (IVF), in which the mutant proteins were expressed from the late pronuclear stages (PN4–5) onwards (Fig 1A, blue line). In both experiments, embryonic development was monitored until the blastocyst stage. We found that injection of K4M mutants before fertilization, but not after fertilization, significantly decreased the embryo survival rate (Fig 1B and C), suggesting that although the retarded cell growth in K4M-injected embryos occurred after the 8-cell stage, the cause of the abnormality had already occurred in the zygotic stages. Other than the K4M mutant, the K36M mutant also exhibited significant growth retardation when it was injected before fertilization (Supplementary Fig S2A), consistent with previous work demonstrating the importance of H3.3 K36 methylation for early embryonic development [9]. In contrast, K9M and K27M mutants did not exhibit any significant abnormalities (Supplementary Fig S2B and C), which conflicts with a previous study demonstrating developmental alteration by H3.3 K27R-EGFP mRNA [6]. It is presumably due to the differences in terms of chromatin incorporation efficiency between H3.3 K27M and H3.3 K27R, as K27M is reported to associate with a SET domain of histone methyltransferases to block their catalytic activity, possibly causing less incorporation of chromatin attenuating its effectiveness, or the influence of an EGFP-tag, whose size is relatively large compared with the size of H3.3. Consistent with a previous report, we also confirmed that the H3.3 K4R mutant had no effect on embryonic development regardless of the order of mRNA injection and fertilization (Fig 1B and C) [6], implying that the inhibition of endogenous global H3K4 methylation is responsible for the phenotype.

H3K4 methylations in the paternal pronucleus are altered by the K4M mutant

To confirm the alteration of H3K4 methylation in K4M-injected embryos, we performed immunostaining in PN4–5 zygotes and 2-cell embryos. The results clearly demonstrated a significant reduction of both H3K4me1 and H3K4me3 predominantly occurred in paternal PNs of PN4–5 zygotes, whereas the level of H3K4 methylation in maternal PN was relatively stable (Fig 2A and C). Similarly, H3.3 K4M protein was preferentially incorporated into paternal PN compared with maternal PN (Fig 2A and C). These results suggest that the effect of the K4M mutant was dependent not only on the inhibition of SET domain-containing methyltransferases but also on chromatin incorporation and that the maternal PN exhibited less dynamic H3K4 methylation. Furthermore, overexpression of H3.1 K4M did not alter endogenous K4 methylation even in paternal PN despite its nuclear localization, whereas it worked properly when it was overexpressed in 293T cells (Supplementary Fig S3), supporting the idea that the effect of the K4M mutant in zygotes is incorporation dependent. At the 2-cell stage of K4M-expressing cells, both H3K4me1 and H3K4me3 exhibited reduced and uneven nuclear localization, likely because paternal PN-derived genomes are less methylated (Fig 2B and D). These uneven methylation patterns also suggested that the amount of overexpressed H3.3 K4M protein in 2-cell embryos is insufficient to replace endogenous H3.3 in maternal chromatin, or to inhibit the H3K4 methyltransferases. Recently, several studies demonstrated that depletion of H3.3 in zygotes by RNAi or morpholino resulted in impaired paternal pronuclear formation, as it was required for the nucleosome assembly followed by loss of DNA replication, transcription, and failure of zygotic cleavage, indicating the critical role of H3.3 itself for embryonic development [17,18]. As expected, the K4R mutant did not alter endogenous K4 methylation at either of the stages (Fig 2), supporting our hypothesis that K4M-induced growth retardation is likely due to the global reduction of H3K4 methylation.

Minor ZGA in the paternal pronucleus is altered by the K4M mutant

As H3K4 methylation is well known to be highly associated with active transcription, we next examined global transcription levels in H3.3 K4M-expressing embryos using a 5-ethynyl uridine (EU) incorporation assay at the PN4–5 stages and the 2-cell stage, corresponding to the minor and major ZGA, respectively. Consistent with the levels of H3K4 methylation shown in Fig 2, transcription was significantly altered in the paternal PN of H3.3 K4M-expressing zygotes, whereas H3.3 K4R had no such effect on transcription (Fig 3A). On the other hand, although there was a modest decrease in transcription in H3.3 K4M-expressing cells at the 2-cell stage, no significant change in transcription was observed between samples (Fig 3B). Collectively, these data indicate that the growth retardation by H3.3 K4M mutants is caused by the alteration of minor, rather than major, ZGA in the paternal PN, and its onset is dependent on H3K4 methylation.

To further confirm the inhibitory effect of the H3.3 K4M mutant on paternal transcription during minor ZGA, the mRNA level of a paternally transcribed gene was compared between H3.3 wild-type (WT) versus K4M injection conditions. For this particular experiment, we utilized a transgenic (Tg) mouse line which carries a histone H4-Venus fusion transgene under the regulation of an endogenous histone H4 promoter (Fig 3C) [19]. We found weak but substantial expression of H4-Venus in PN4–5 zygotes obtained from mating pairs of H4-Venus homozygous Tg sperm × WT oocytes.
Comparison of the Venus mRNA levels between H3.3 WT- and H3.3 K4M-injected 1-cell embryos found a reduced level of Venus in H3.3 K4M-injected embryos (Fig 3E). This result provided another piece of evidence that suppression of H3K4 methylation by H3.3 K4M attenuates paternal allele-derived minor ZGA.

Mll3/4 expression in the PN stage is necessary for early preimplantation development

There are six major histone methyltransferases that catalyze H3K4 methylation—Setd1a, Setd1b, Mll1, Mll2, Mll3, and Mll4—which are divided into three distinct groups according to their sequence similarity and complex formation: Set1a/b, Mll1/2, and Mll3/4 [20–22]. Previously, Mll2 was reported to be required for early embryogenesis, based on the observations of decreased H3K4me2/3 in maternal PNs concomitant with ~30% reduction of major ZGA and developmental arrest mainly at 2-cell stages, when it was knocked out [23]. To determine the enzyme responsible for H3K4 methylation during minor ZGA, we first investigated their expression levels from MII oocytes to 2-cell stage embryos using the Database of Transcriptome in Mouse Early Embryos (DBTMEE, http://dbtmee.hgc.jp/index.php) [24,25]. The database indicated that among

![Diagram](image-url)
Figure 2. H3.3 K4M overexpression causes a significant reduction of global H3K4me1 and H3K4me3 in paternal PN.

A–D Immunostaining against H3K4me1 and H3K4me3 was performed for PN zygotes (A, C) and 2-cell embryos (B, D). Left top pictures are representative images, and left bottom schemes indicate which type of H3K4 mRNA was injected into embryos corresponding to the representative images. Left middle pictures in (B) and (D) are magnified images of the nucleus in each 2-cell embryo. Right graphs are boxplots for relative intensities of H3K4me1 or H3K4me3 from three independent experiments. Statistical analyses performed were the Games–Howell test (A), Tukey–Kramer test (B, D), and Steel–Dwass test (C). Scale bars, 20 μm. *P < 0.01; **P < 0.05.
these H3K4 methyltransferases, Mll3 showed transient expression highest at the 1-cell stage with a FPKM score of 81.5485, which was equivalent to that of Actb (90.2577) and higher than that of Gapdh (23.5705) (Supplementary Table S1A). Mll2 also displayed a high FPKM score (31.451), whereas Mll4, Setd1a, and Setd1b were expressed to much lower degrees, and Mll1 was barely detectable (Supplementary Table S1A). Interestingly, among these six enzymes, only Mll3 and Mll4, both of which are orthologs of Drosophila Trr mediating H3K4me1 at enhancer regions rather than promoter regions [26–28], are categorized as “minor ZGA” depending on their expression patterns during the preimplantation stages (Supplementary Table S1A). Because these six methyltransferases are composed of three distinct protein complexes (Supplementary Table S1A) [29], we further examined the expression of molecules unique to each complex. The results showed that Paxip1, Ncoa6, and Kdm6a, unique components of the Mll3/4 complex, as well as factors that commonly exist in all three complexes (Dpy30, Rbbp5, and Wdr5), were relatively abundantly expressed in preimplantation

Figure 3. H3.3 K4M overexpression causes paternal PN-specific reduction of global transcription.

A, B Left pictures are representative fluorescent images of zygotes overexpressing H3.3 WT, H3.3 K4M, and H3.3 K4R after EU treatment for PN zygotes (A) and 2-cell embryos (B). Left bottom schemes indicate which type of H3.3 mRNA was injected into embryos corresponding to the representative images. Right graphs are boxplots for relative intensities of EU from four independent experiments.

C Scheme of the experimental procedure for (D) and (E).

D Representative image of PN4–5 zygotes expressing sperm-derived H4-Venus. Dotted circles indicate pronuclei according to the DAPI staining (red pseudo-color). PB: polar bodies.

E Relative expression levels of H4-Venus by single-cell RT–qPCR. Sample numbers are as indicated; the normalization method is described in the Supplementary Materials and Methods.

Data information: Statistical analyses performed were the Tukey–Kramer test (A), Steel–Dwass test (B), and Student’s t-test (E). Scale bars, 20 μm. *P < 0.01; **P < 0.05.
embryos and had a ‘minor ZGA’ pattern of expression, supporting our hypothesis that \( \text{Mll3/4} \) is involved in H3K4 methylation in minor ZGA (Supplementary Table S1B). We further verified the gene expression dynamics of the six methyltransferases according to pronuclear stage by RT–qPCR, because we supposed that the enzymes that function in minor ZGA (= the late pronuclear stages) could be transcribed in the early pronuclear stages so that there is enough time for them to be translated. Indeed, we found a transient increase in \( \text{Mll3} \) expression in the early pronuclear stages, suggesting that Mll3 is likely translated by the time of minor ZGA (Supplementary Fig S4A and B). To further consolidate this idea, embryos were treated with cycloheximide (CHX), an inhibitor of protein synthesis, at the time of insemination. Unlike the effect of CHX on H3K9me2 that impairs the asymmetric methylation pattern in zygotes [30], CHX did not cause global changes of H3K4me1 or H3K4me3 (Supplementary Fig S4C and D). Interestingly, however,
it induced alteration of the subnuclear localization of H3K4me1 in PN4–5 (Supplementary Fig S4E). Notably, retained H3K4me1 in CHX-treated embryos was accumulated in heterochromatic areas such as perinuclear and pericentromeric regions, where transcription was generally silenced (Supplementary Fig S4E). Although this experiment did not specify MLL3/4 as responsible for zygotic H3K4 methylation, it supported the idea that de novo protein synthesis is required for full H3K4 methylation in minor ZGA.

Based on the gene expression pattern, we next tested the contribution of Mll3 and its paralog Mll4 in early preimplantation development. We knocked down Mll3 and Mll4 by siRNA injection followed by ICSI using the same procedure as we performed.

Figure 5. Double knockdown of Mll3 and Mll4 causes paternal PN-specific reduction of global H3K4me1, H3K27ac, and global transcription.
A, B Immunostaining against H3K4me1 and H3K27ac (A) and EU treatment (B) were performed for PN zygotes. In each figure, top pictures are representative images, and bottom schemes indicate which type of siRNA was injected into embryos corresponding to the representative images.
C–E Boxplots for relative intensities of H3K4me1 and H3K27ac or EU from three independent experiments for each expression as in (A) and (B). Statistical analyses performed were the Steel–Dwass test (H3K4me1) and Tukey–Kramer test (H3K27ac and EU). Scale bars, 20 μm. *P < 0.01.
for K4M mutants. To ensure that the siRNA was fully functional, we microinjected siRNA into MII oocytes 8 h before ICSI, in accordance with the protocol of a previous report [31], and confirmed that both MII3 and MII4 mRNA levels were decreased by ~85% and ~60%, respectively, at the 2-cell stage (Fig 4A). Similar to the effects of overexpression of H3.3 K4M, single knockdown of MII3 caused developmental retardation at the 8-cell stage and arrest at the morula stage, whereas such alterations were not observed when siMII3 was microinjected after fertilization (Fig 4B and C). On the contrary, single knockdown of MII4 did not significantly affect embryonic development, regardless of the order of mRNA injection and fertilization (Fig 4B and C). However, simultaneous knockdown of MII3 and MII4 (siMII3/4) synergistically affected early embryonic development only when the knockdown was performed before fertilization (Fig 4B and C), suggesting that the MII3/4 is essential in early embryonic development.

H3K4me1, H3K27ac, and minor ZGA in the paternal pronucleus are altered by siMII3/4

Recent studies have uncovered distinct functions for each Mll complex. Particularly, the MII3/4 complex is reported to be preferentially involved in enhancer activation through H3K4me1, in cooperation with Utx and p300, to remove H3K27me3 and ensure H3K27ac, respectively, rather than in promoter activation through H3K4me3 [32–34]. To examine whether siMII3/4 affected H3K4 methylation, immunostaining was performed in siMII3/4 zygotes followed by ICSI. However, no remarkable change in global H3K4me1 or H3K4me3 was observed in siMII3 or siMII3/4 embryos, respectively (Supplementary Fig S5A and B). Nuclear accumulation of RNA polymerase II (RNAP II) CTD, including the non-phosphorylated form, the phosphorylated form at serine 5 representing transcriptional initiation, and the phosphorylated form at serine 2 representing transcriptional elongation, was also maintained in the knockdown embryos (Supplementary Fig S5A–C). Similarly, BRG-1, a catalytic subunit of the SWI-SNF chromatin remodeling complex, and previously reported to be involved in H3K4me2 and major ZGA at the 2-cell stage [35], was unaffected by siMII3/4 (Supplementary Fig S5C).

These results suggested at least three possibilities. The first is that since minor ZGA is a very weak transcriptional wave compared with major ZGA at the 2-cell stage, global changes in H3K4 methylation may not be especially correlated with transcriptional levels. The second is that MII3/4 may simply not be responsible for global H3K4 methylation in zygotes. The third is that injection of siMII3/4 at 8 h prior to ICSI is too late for the siRNA to be fully functional and induce global changes of H3K4 methylation. To verify the second and third possibilities, siMII3/4 was injected into MII oocytes, followed by in vitro maturation for 30 h and ICSI. As a result, a significant decrease of H3K4me1 in paternal PNs rather than in maternal PNs was observed in PN4–5 zygotes, confirming that MII3/4 is responsible for paternal H3K4me1 in zygotes (Fig 5A, left panel and C). Furthermore, reduction of H3K27ac was also induced by siMII3/4, implying a role of MII3/4 in enhancer regions (Fig 5A, middle panel and D). Consistently, depletion of p300, the mRNA expression of which was significantly increased in PN1–3 stages, via siRNA injection also induced developmental arrest at around the 4-cell stage, also indicating the importance of enhancers in minor ZGA (Supplementary Fig S5D and E). Finally, we tested the effect of siMII3/4 on transcription via an EU incorporation assay and observed a significant reduction in transcription in the paternal PN of siMII3/4 double-knockdown zygotes (Fig 5B and E).

Collectively, we conclude that paternal H3K4me1 catalyzed by the MII3/4 complex is required for minor ZGA in the paternal genome, and its alteration causes developmental arrest in 4- to 8-cell stage embryos, which is distinct from major ZGA phenotype (i.e., arrest in 2- to 4-cell stages) induced by α-amanitin treatment, or depletion of Mi2 or Brg-1 [23,35]. Interestingly, this delayed growth retardation may be explained by a “stepwise” gene activation model; that is, one transcriptional wave triggers the following transcriptional waves during preimplantation development, and thus, ZGA transcripts and their protein products would be required for the progression of embryos beyond the 4-cell stage [36].

In conclusion, our study not only demonstrates the importance of MII3/4-mediated H3K4 methylation in the onset of minor ZGA in the paternal genome by utilizing a forward genetic and siRNA approaches, but also suggests the importance of enhancer activation for minor ZGA. These findings provide new insights into how the genome adapts during the maternal–zygotic transition, that is, the transformation from a transcriptionally quiescent state at fertilization to the robust but not promiscuous gene activation thereafter. Further investigations into this topic may rely on innovative technical improvements such as a small-scale (~ a few hundred cells) ChIP sequence.

Materials and Methods

Ethics statement

All experimental procedures involving animals were approved by the Animal Experiment Ethics Committees at the Institute of Molecular and Cellular Biosciences, The University of Tokyo (#23015, #2509).

Oocyte preparation

Four- to eight-week-old BDF1 mice (C57BL/6 × DBA/2 strain; CLEA Japan, Tokyo, Japan) were used for all experiments. H4-Venus transgenic mice were prepared as previously reported [19]. MII oocytes collected from superovulated female mice were cultured in HTF medium with 3 mg/ml bovine serum albumin. Forty-six hours after PMSG injection, GV oocytes were collected from ovaries by puncturing antral follicles and then cultured with TAM medium supplemented with 5% FBS [37]. After about 2 h of GVBD, MI oocytes were subjected to siRNA injection. The siRNA-injected MI oocytes were cultured with TAM medium for 28–30 h, and the cells that successfully matured to MI oocytes were subjected to ICSI.

In vitro fertilization

In vitro fertilization was performed in HTF medium. After 2 h, fertilized oocytes were washed by pipetting with mouth pipettes in HTF medium and cultured at 37°C with 5% CO2. The following day, 2-cell embryos were transferred to KSOM medium (ARK Resource,
Kumamoto, Japan) and cultured at 37°C with 5% CO₂ until they became blastocysts.

Plasmid construction and in vitro transcription

The cDNA of Flag-HA-tagged H3.1 and H3.3 were obtained from pOZ-e-H3.1 and e-H3.3, respectively [38]. These cDNAs were subcloned into a pcDNA3.1-poly(A)83 vector [39] and used for transcription or in vitro transcription (IVT). IVT was performed using the RibomAX Large Scale RNA Production System T7 (Promega, Madison, USA) according to the manufacturer's instructions.

Microinjection of mRNAs and siRNAs and intracytoplasmic sperm injection (ICSI)

Approximately 3–5 pl (= polar body size) of 40 ng/μl mRNAs or 90 μM siRNAs (Thermo Fisher Scientific, Massachusetts, USA) was microinjected into the cytoplasm of MII oocytes or zygotes in M2 medium (ARK Resource). ICSI was performed 4 h after mRNA microinjection or 8 h after siRNA microinjection in M2 medium.

RT–qPCR

cDNA was extracted from MII oocytes, zygotes, and 2-cell embryos using SuperScript III CellsDirect cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Results were normalized based on the geometric mean of the expression levels of three reference genes (Ppia, H2afz, and Hprt1) [40,41]. Primer sequences for qPCR are listed in Supplementary Table S2.

Immunostaining

Zona pellucida-removed embryos were fixed with 4% paraformaldehyde (PFA) at room temperature (RT) for 15 min or at 4°C overnight. They were then permeabilized with 0.5% Triton X-100 at RT for 15 min. After washing with PBS containing 0.05% Triton X-100 (PBST) and blocking with 5% goat serum at RT for 1 h, cells were incubated with primary antibodies in 5% goat serum at RT for 1 h followed by detection with secondary antibodies. Antibodies used in the experiments are described in Supplementary Materials and Methods.

Global transcription assay

Global transcription assays were performed using a Click-it RNA Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, zygotes were incubated with 2 mM EU at 37°C for 2 h, treated with Tyrode's solution, fixed with 4% PFA, and permeabilized with methanol at −30°C for 15 min or 0.5% Triton X-100 as described above for immunostaining. Zygotes were then incubated in the Click-it reaction cocktail at RT for 30 min and then washed with the Click-it reaction rinse buffer.

Data quantification and statistical analysis

Briefly, image data quantification was performed using ImageJ. Obtained (pro)nuclear intensities were used to calculate relative intensities (paternal PN/maternal PN for zygote, and nucleus/cytosol for 2-cell embryo [42]). Statistical analysis for embryonic survival rates was performed using the chi-square test [43,44]. For other experiments, detailed information about quantification and statistical analyses is described in the Supplementary Materials and Methods.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

KA, HS, and YO conceived and designed the experiments. KA analyzed the data. KA and YO wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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