Dimorphic DNA methylation during temperature-dependent sex determination in the sea turtle *Lepidochelys olivacea*

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**Abstract**

Sex determination in vertebrates depends on the expression of a conserved network of genes. Sea turtles such as *Lepidochelys olivacea* have temperature-dependent sex determination. The present work analyses some of the epigenetic processes involved in this. We describe sexual dimorphism in global DNA methylation patterns between ovaries and testes of *L. olivacea* and show that the differences may arise from a combination of DNA methylation and demethylation events that occur during sex determination. Irrespective of incubation temperature, 5-hydroxymethylcytosine was abundant in the bipotential gonad; however, following sex determination, this modification was no longer found in pre-Sertoli cells in the testes. These changes correlate with the establishment of the sexually dimorphic DNA methylation patterns, down regulation of *Sox9* gene expression in ovaries and irreversible gonadal commitment towards a male or female differentiation pathway. Thus, DNA methylation changes may be necessary for the stabilization of the gene expression networks that drive the differentiation of the bipotential gonad to form either an ovary or a testis in *L. olivacea* and probably among other species that manifest temperature-dependent sex determination.

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**1. Introduction**

Development of the bipotential gonad to form either an ovary or a testis depends on the dimorphic expression of a conserved network of genes (Davidson and Erwin, 2006; Shoemaker and Crews, 2009). However, the mechanisms that trigger the establishment of male or female gene expression patterns differ among taxa. Most organisms have genetic sex determination, where sex is determined by the genetic background after fertilization (Ezaz et al., 2006). Therefore, differences in sex-specific alleles are responsible for activation of male or female differentiation pathways (Matsuda et al., 2007). Among mammals, genetic sex determination relies on the XX and XY sex chromosomes, in which the Sry gene encoded in the Y chromosome acts as the testis-determining factor (Koopman et al., 1991). Among birds, the *Dmt1* gene is encoded in sexual chromosome Z and dosage of this gene in paired ZZ and ZW chromosomes is important for sex determination (Smith et al., 2009).

Among vertebrates with environmental sex determination, sex depends on external cues, mostly in the absence of sex chromosomes (Rhen and Schroeder, 2010). Several species of turtles, lizards and all crocodiles are subject to temperature-dependent sex determination (TSD) (reviewed in Merchant-Larios and Díaz-Hernández (2013)). In these organisms, the molecular mechanism(s) involved in translation of temperature cues to dimorphic regulation of sex-determining genes in the gonad remain poorly understood. Epigenetic mechanisms play an important role in translating environmental and cellular stimuli into gene expression patterns and may also be involved in TSD (Navarro-Martin et al., 2011; Matsumoto et al., 2013; reviewed in Pifferer (2013)). Among epigenetic mechanisms, some directly affect DNA molecules, such as methylation of carbon 5 of cytosine in the CpG context (5mC), associated with transcriptional repression and stabilization of transposable elements. DNA methylation is important in inactivation of the X-chromosome among mammals and Z chromosome in birds (Simon et al., 2013; Teranishi et al., 2001; Yang et al., 2011). Furthermore, active DNA demethylation has recently been described in many embryonic cell types, proving

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DNA methylation and demethylation to be highly dynamic during development (Kinney et al., 2011; Szwagierczak et al., 2010). In particular, DNA demethylation involves hydroxylation of 5mC (5hmC) and its subsequent excision and replacement by a non-methylated cytosine (Sun et al., 2014; Ito et al., 2010; Koh et al., 2011). Another versatile epigenetic mechanism is that associated with a sophisticated set of histone post-translational modifications. Different histone residues can be targets for changes in acetylation, methylation, ubiquitination, or phosphorylation, among others that have direct effect on gene expression with biological significance (Gardner et al., 2011; Zentner and Henikoff, 2013).

Recent reports correlate epigenetic processes with sex differentiation: DNA methylation is necessary for Sertoli cell differentiation and formation of testicular cords in murine gonads (Mizukami et al., 2008). In birds, the DNA methylation over the promoter sequence of the aromatase gene CYP19A1 is more abundant in male than in female gonads (Ellis et al., 2012). In species with TSD, such as the European sea bass, it has also been found that the aromatase promoter shows higher levels of DNA methylation in males than in females (Navarro-Martin et al., 2011). This type of regulation has also been described in two reptile species: the red-eared turtle Trachemys scripta (Matsumoto et al., 2013) and the alligator Alligator mississippiensis (Parrott et al., 2014). Furthermore, in the alligator, a putative promoter sequence of the Sox9 gene showed higher DNA methylation levels in gonads from embryos incubated at female-promoting temperature (FPT) than when they were incubated at the male-promoting temperature (MPT) (Parrott et al., 2014). These results strongly corroborate the idea that epigenetic modifications are involved in the regulation of certain genes from the sex-determining networks in species with TSD. However, much has still to be clarified regarding global epigenetic changes to the DNA and epigenetic signals for specific gonadal cells involved in the establishment of TSD networks.

Sea turtles manifest TSD, where low and high temperatures produce male and female hatchlings, respectively. In the olive ridley turtle, L. olivacea, differentiation of the bipotential gonad into a testis or ovary takes several days, enabling the study of structural and molecular changes during sex determination and differentiation (Merchant-Larios and Díaz-Hernández, 2013). In the current study, we extended the investigation of this species using unbiased, global approaches and we provide evidence of dimorphism of DNA methylation patterns between ovaries and testes. We found a correlation between levels of 5hmC DNA modification in medullary gonadal cells involved in the establishment of TSD networks.

2. Methods

2.1. Embryos

Freshly laid eggs of the olive ridley turtle (L. olivacea) were collected at La Escobilla beach on the Pacific Coast of Oaxaca, Mexico (96.2701600W, 15.4003600N). The eggs were transported to the laboratory in Mexico City and incubated at MPT (26 °C ± 0.5) or FPT (33 °C ± 0.5). The total number of embryos used in the present study is shown in Supplementary Table 1. All protocols were approved by local animal rights committees from the Secretary for Environment and Natural Resources (SEMARNAT) and the National Autonomous University of Mexico. Embryos were sampled before and after sex determination: at Stages 24 and 27 for MPT and 23 and 27 for FPT; samples for immunofluorescence were taken also at stages 25 and 26 (Supplementary Table 1). Stages are according to Miller’s criteria (Miller, 1985).

2.2. Genomic DNA isolation

Urogenital complexes were removed from embryos incubated at MPT or FPT. Gonads were separated from the adjacent mesonephros and grouped into pools containing tissue from 10 embryos each. Gonads from embryos of at least 2 different nests were included in each pool. Samples were sliced into 5–8 pieces smaller than 2 mm³ and incubated in Lysis Buffer (10 mM NaCl, 10 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 2% SDS, 1 mg/ml Proteinase K and 150 µg/ml RNase) for 16 h at 65 °C. DNA was purified with phenol-chloroform-isooamyl alcohol and precipitated with ethanol. Biological replicates for other experiments were prepared from another 10 embryos originating from 2 different nests each time. Mouse DNA was obtained from a pool of 5 brains from newborn male CD-1 strain.

2.3. Amplification of Inter-methylated Sites (AIMS)

The AIMS method was adapted from the original (Frigola et al., 2002). Embryos came from the same 2 nests, sampled at different developmental stages. DNA samples from different incubation temperatures and developmental stages came from pools of gonads of 10 embryos, these being 5 embryos from each nest. A biological replicate used embryos from 2 other nests. (Supplementary Table 1). Each sample of 5 µg DNA was treated with 100 U methylation-sensitive endonuclease HpaII for 16 h at 37 °C and then purified with phenol-chloroform-isooamyl alcohol, as previously described. From each sample, 2 µg were treated with 10 U DNA polymerase I, Large (Klenow) fragment (New England Biolabs) following the manufacturer’s instructions, for 4 h at 30 °C until blunt ends remained. The reaction was arrested at 75 °C for 5 min and DNA was precipitated with 100% ethanol, 0.1 volumes of sodium acetate 3 M pH5 and 20 µg glycogen (Roche). This DNA was treated with 100 U methylation-insensitive MspI endonuclease for 16 h at 37 °C, and 20 U enzymeMsp1 was then added to the reaction and incubated for 4 h at 37 °C, prior to heat inactivation of the enzyme. DNA was once again precipitated, as previously described. To prepare the linkers, 2 nmoles of each primer Blue (5’-ATTGGAAAAACGTCTGATC-3’), and Blue-GC (Bios-5’-CGTCAAGCTTTGCGAAT-3’), were ligated to treated DNA with 1000 U T4 Ligase (New England Biolabs) for 16 h at 14 °C. Unincorporated linkers were removed with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and DNA was re-suspended in 250 µl DNA-free H2O. PCRs were prepared with 3 µl sample DNA and 1 µl [α-32P]dCTP with Hot Start Taq (Quagen), using 5 pmol of primer Blue1 (5’-ATTGCGAAAGCCTG CAGCGATTAG-3’), Blue2 (5’-ATTGCGAAAGCTCTGAGATC-3’), and Blue3 (5’-ATTGCGAAAGCTCTGAGGAT-3’). Reactions were performed in quadruplicate, using 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a last extended stage of 10 min at 72 °C. PCR products were precipitated as previously described, and re-suspended in 2 µl loading buffer. Products were run on 7.5% polyacrylamide denaturing gels, 0.4 mm thick with 7 M urea on TBE buffer. Gels were dried and autoradiographs were produced from X-ray films that had been exposed for 48 h. AIMS was performed twice for stage 24 and 3 times for embryos at stage 27, plus one extra time for a biological replicate to corroborate the results. Quantity One Analysis Software was used to generate histograms and evaluate relative intensity of the banding patterns. Only bands showing dimorphic intensity in every PCR and biological replicate were considered as indicating differences in DNA.
methylation. In order to elute DNA from acrylamide gels, bands were sliced and incubated in Elution Buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) overnight at 37 °C. DNA from the aqueous phase was then precipitated, as previously described.

### 2.4. Dot blot

DNA was obtained from *L. olivacea* gonads and brains as previously described. DNA from mouse brain was used as a positive control and pDrive vector was used as a negative control. Diverse amounts of DNA (30 ng, 50 ng, 500 ng, 1 μg and 2 μg) were diluted in 2 μl H₂O for each sample. This concentration gradient was used to detect 5hmC in tissues, which may present varied abundance. The greatest amount of DNA used from mouse brain was 500 ng, thus avoiding saturation. DNA was dotted on nitrocellulose membranes and dried at room temperature for 1 h. The membranes were blocked with blocking solution (0.1% Tween, 5% Svelty Light Powder Milk in PBS) for 1 h and then the primary antibody against 5hmC (1/10,000) was added and incubated overnight at 4 °C with gentle agitation. The membranes were then rinsed with 0.1% Tween PBS before incubation with secondary antibody diluted in blocking solution for 1 h at room temperature. The membranes were rinsed with PBS and revealed with X-ray films that had been exposed, using the kit as indicated by the manufacturer. In order to show that DNA was loaded for every sample, the membrane was stained with Ethidium Bromide (EtBr) to expose DNA under UV light.

### 2.5. Immunofluorescence

Urogenital complexes were dissected and fixed with freshly prepared 4% paraformaldehyde (Sigma) in PBS, for 16 h at 4 °C. The tissues were washed with PBS and sequentially incubated for 3 h in 10%, 20% and 30% sucrose, in PBS solutions. Samples were incubated overnight at 4 °C on a 1:1 mixture of 30% sucrose in PBS and Tissue-Tek CRYO-OCT Compound (Fisher Scientific). The complexes were then embedded in Tissue-Tek CRYO-OCT and frozen at −70 °C. Serial sections of 20 μm were obtained and vacuum dried prior to use. Tissue samples were permeabilized with 0.3% Triton X-100 on PBS for 15 min and blocked with 100 mM Ultra-pure Glycine (Sigma) on PBS for 1 h. Antigens were recovered by incubating slides in citrate buffer (10 mM sodium citrate pH = 6, 0.05% Tween) for 45 min at 92 °C. Slides were incubated overnight at 4 °C with primary antibody: anti-5hmC 1:500 (39769, Active Motif), anti-Pan Cytokeratin Plus AE1/AE3 + 8/18 1:100 (Biocare Medical). Incubation with secondary antibodies Alexa Fluor 1:200 (Invitrogen) was performed for 1 h. Finally, slides were incubated with 300 nm DAPI in PBS for 2 min and rinsed before being mounted with Fluorescence Mounting medium (DAKO). In order to quantify signal intensity, CK antibody was used to segregate stromal from epithelial cells. Afterwards, cells showing at least one nucleolus were marked and signal intensity of the mark was quantified with the Image J program. To group data from different images and biological samples, we calculated the average intensity value in every image and normalized the intensity of every quantified cell (both epithelial and stromal) against this value. Between 10 and 200 cells were quantified, from gonads of at least 3 embryos each from 2 different nests (Supplementary Table 1).

### 2.6. Analysis software

The following software was used: Image J open-access software to analyse and quantify signal intensity in IF images and Image Quant 5.2 Analysis for gel analysis.

### 2.7. Statistical analyses

To analyse 5hmC distribution, individual nuclei (n > 10 per micrograph) from both stromal or epithelial cells were identified and their average fluorescent intensity quantified using Image J Software. To normalize intensity values, all quantified nuclei from both epithelial and stromal cells were identified, and median intensity was calculated for each of these. Values obtained were used to calculate an average intensity for the entire micrograph. Intensity values relating to every nucleus were normalized against the mean intensity value of each micrograph. Several organisms were quantified for each developmental stage and incubation temperature (St. 27 n = 8; St. 26 and 25 n = 3 for each temperature). Independent graphs were generated for each organism (mean ± S. D. n > 10) (Fig. 3B–C; Supplementary Figs. 4 and 5).

Average values were calculated for each cell type (epithelial medullary cords or stromal), developmental stage and incubation temperature using the normalized values from each organism. Comparison between groups was performed using ANOVA. Verifying ANOVA assumptions: (a) Cumulative frequency distributions showed symmetrical distributions of the values for each organism; since the dependent variable used is the average for each organism, averages calculated from big numbers (n > 20 for symmetrical distributions) have a normal distribution according with the Central Limit Theorem; for those cases where averages were calculated with n < 21 normality was proved using Anderson-Darling test. (b) A Levene’s test showed homogeneity of variance between groups (P = 0.226). Significant difference for multiple comparisons was evaluated using a Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ) P < 0.05 (Fig. 3D). (SAS Version 9.0 2002; SAS Institute Inc., Cary, NC, USA).

### 3. Results

#### 3.1. Differential DNA methylation patterns between ovaries and testes

To explore epigenetic mechanisms involved in TSD, we looked for epigenetic differences between differentiated ovaries and testes from *L. olivacea* embryos incubated at MPT (26 °C) or FPT (33 °C), respectively. With the aim of identifying whether particular differences in DNA methylation patterns correlate with incubation temperature, we performed amplification of inter-methylated sites (Aims) (Frigiola et al., 2002). AIMS is an unbiased technique used to expose differences in DNA methylation patterns, based on the cleavage of genomic DNA with methyl-sensitive endonucleases (Supplementary Fig. 1).

The results revealed differences in DNA methylation distribution between ovaries and testes, evidenced by the presence of differentially enriched bands in acrylamide sequencing gels (Fig. 1A). These differences were maintained in PCR reactions and biological replicates. Histograms were generated to analyse banding patterns using Image Quant 5.2 Analysis software. Bands showing dimorphic intensity in every replicate PCR reaction were identified as differentially methylated. These bands were cut from either ovary or testis samples, depending on where they showed greater intensity (hence higher DNA methylation), and were eluted from the acrylamide gels. Eluted bands were used as templates for PCR re-amplification with the same primers used for AIMS. re-amplified bands were cloned and sequenced for identification (Fig. 1B); however, several sequences were found in each band. We performed a Genome browser BLAT analysis of the sequences obtained from the AIMS technique against the reported genome from Chrysemys picta, (Supplementary Tables 2 and 3). Unfortunately, it was impossible to determine which sequence was responsible for the dimorphic band intensity shown in the gels.
3.2. Dimorphic DNA methylation patterns may be stabilized during sex determination

During the thermo-sensitive period, embryos have bipotential gonads responsive to temperature (Merchant-Larios et al., 1997). In order to discover whether the dimorphic DNA methylation patterns were established before or after the thermo-sensitive period, we performed AIMS in morphologically undifferentiated gonads from embryos incubated at MPT or FPT (Fig. 2, Supplementary Fig. 2).

DNA methylation patterns in the bipotential gonads (stages 23 and 24) were similar at both temperatures (Fig. 2); however, they differed in differentiated testis and ovaries. Thus, the establishment of a sex-specific pattern of DNA methylation may gradually evolve between the stages 24 and 26. Importantly, events of DNA methylation as well as demethylation took place during the thermo-sensitive period, setting the sexually dimorphic DNA methylation patterns found in differentiated gonads (Fig. 2, Supplementary Fig. 2). These results suggest that methylation and demethylation processes are both involved in the establishment of the DNA methylation patterns of ovaries and testes during the thermo-sensitive period.

3.3. 5-Hydroxymethylcytosine DNA modification in nuclei of stromal and epithelial cells shows dimorphic distribution in ovary and testis

Even though both methylation and demethylation processes are involved in the establishment of a sex-specific DNA methylation pattern, due to the difficulties involved in acquiring samples, we decided to investigate whether an active demethylation process may be involved. For this purpose, the presence of 5hmC was analyzed, as DNA demethylation involves hydroxylation of 5mC and its subsequent excision and replacement by a non-methylated cytosine (Sun et al., 2014; Ito et al., 2010; Koh et al., 2011).

We first evaluated the presence of 5hmC in L. olivacea gonads and brain by Dot-Blot assay, using a 5hmC antibody (Supplementary Fig. 3). As 5hmC modification is reported to be abundant in mouse brain (Kinney et al., 2011), we used genomic DNA from that tissue as a positive control. We compared brain and gonad genomic DNA from embryos at stages 23–24 (bipotential gonads) and at stage 27 (differentiated ovaries and testes) of L. olivacea. 5hmC mark was found in both bipotential and differentiated gonads, whether the embryos were incubated at FPT or at MPT. Thus 5hmC modification is present in gonads as well as in the brain of L. olivacea (Supplementary Fig. 3).
To study the distribution and relative abundance of 5hmC modification in distinct cell types within developing ovaries and testes, we performed confocal immunofluorescence on frozen sections. Gonads were isolated from 8 embryos of each sex, sampling four nests (Fig. 3). Interestingly, in all embryos the intensity of 5hmC signal in differentiated gonads at stage 27 showed an opposed distribution depending on the gonadal sex (Fig. 3): in ovaries, the nuclei of cells in fragmented medullary cords showed higher signal intensity than did the surrounding stromal cells; the reverse was found in the testes, where levels were lower in seminiferous cords than in stromal cells.

To discover whether this sexually dimorphic distribution originated from enrichment or losses of 5hmC modification, early developmental stages were analyzed (Supplementary Figs. 4 and 5). The paucity of stromal tissue precluded distinction between the staining of nuclei of epithelial and stromal cells in bipotential gonads at stage 24 (data not shown). The sexually dimorphic distribution of 5hmC becomes apparent in gonads at stage 25 (Supplementary Fig. 4A–D). The differences in 5hmC intensity between cell types increase at stage 26 (Supplementary Fig. 5A–D) and become clear in differentiated gonads from stage 27 (Fig. 3). The 5hmC signal of medullary cords was considerably diminished in embryos incubated at MPT through gonad differentiation. Some clusters of cells at stage 25 and even stage 26 still maintained a high intensity of 5hmC (Supplementary Figs. 4C and 5C). These observations suggest abundant 5hmC modification in epithelial cells of bipotential gonads. In ovaries, however, a difference in signal intensity between epithelial and stromal cells first became evident at stage 26 (Supplementary Fig. 5E–H). These results suggest a gradual dimorphic loss of 5hmC modification among cells that depends upon the temperature.

4. Discussion

Genes involved in sex determination and differentiation are highly conserved in vertebrate Amniota; however, their spatial and temporal expression varies among species, owing to the evolution of the multiple layers of regulatory mechanisms (Rhen et al., 2015). Due to difficulties for obtaining samples, in...
study only a few developmental time points were tested. Thus, the overall representation of all molecular events underlying gonadal development remains unclear. However, the sampled gonads analyzed correspond to two critical developmental stages: bipotential and sexually differentiated (ovary or testis). A dimorphic DNA methylation pattern was found between ovaries and testes that depended upon the incubation temperature. The DNA methylation profile in the bipotential gonad is modified by methylation or demethylation events so that the profile differs between differentiated ovaries and testes.

Cell-cell and cell-matrix interactions are crucial prior to and after remodeling of the bipotential gonad under the influence of temperature (Mork et al., 2014; Pieau and Dorizzi, 2004). Similar to the red-eared turtle (Barske and Capel, 2010), the bipotential gonads of the olive ridley turtle have two kinds of epithelial cells: Sox9-negative cells at the cortex and Sox9-positive cells in the medullary cords. Medullary cords are delimited by stromal cells formed by blood vessels, mesenchymal cells and fibroblasts. In the current study, the nuclear staining signal of 5mC modification was more intense in the surface epithelium than in medullary cells.

Fig. 3. 5hmC distribution between nuclei of epithelial and stromal cells of testes and ovaries at stage 27. (A) 5hmC (red) distribution differed between ovary and testis at Stage 27. In testis, stroma cells show a higher intensity than cords, whereas the opposite is observed in the ovary. Cytokeratin CK (green) marks epithelial cells of gonadal medullary cords: they become seminiferous cords in testes or fragmented medullary cords in ovaries. Dotted lines: the border between epithelial and stromal cells. DAPI (blue) marks cell nuclei. Scale bar, 20 μm. (B and C) 5hmC signal intensity was quantified in individual stromal or epithelial cells (n > 10) from ovaries or testes of each organism sampled *(mean ± S.D.). (C) Summary graph shows average 5hmC intensity values of stromal and epithelial cells in gonads from embryos of different incubation temperatures: MPT or FPT and developmental stages: St. 25 (n = 3), St. 26 (n = 3) or St. 27 (n = 8) (mean ± S.D.). RGEOWQ grouping (a–d) shows significant difference among groups (P < 0.05). Groups with different letters are statistically different. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
in both the ovary and the testis. This suggests that DNA methylation may be involved in the dimorphic specification of the surface epithelium, prior to segregation of cells negative or positive for Sox9 (Díaz-Hernández et al., 2012).

Ten-eleven translocation (TET) proteins, responsible for 5mC oxidation into 5hmC, have been associated with poised promoters that present bivalent histone marks H3K4me3/H3K27me3 and are prepared to be activated or silenced during development (Sun et al., 2014; Ito et al., 2010; Koh et al., 2011). It has also been suggested that DNA methylation patterns in pluripotent cells are not maintained by Dnmt1 maintenance methyl-transferase, but rather by the equilibrium that exists between de novo methyl-transferases and TET proteins (Ficz et al., 2011). Concurring with those observations, we found high levels of 5mC modification in bipotential gonads, whereas this modification appears to cease in pre-Sertoli cells. Thus, the decrease in this DNA modification in the testes may be associated with the loss of bipotentiality after the thermo-sensitive period. It is interesting to note that differences in 5hmC signal became evident at stage 25 in testes and stage 26 in ovaries, correlating with the ending of the thermo-sensitive period reported for MPT and FPT respectively and the loss of Sox9 expression in ovaries (Merchant-Larios and Díaz-Hernández, 2013).

Changes in DNA methylation may be necessary for the stabilization of gene expression networks that drive the differentiation of the gonadal tissue to form either an ovary or a testis. The dimorphic 5mC DNA modifications are spatially and temporally correlated with major structural and molecular events that occur during gonadal development in L. olivacea. Considering that the current study is the first to analyse the global DNA methylation and the dimorphic 5mC DNA modifications in a species with TSD, comparison with other species needs to be carried out.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygeno.2016.06.026.

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