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Short Communication

Molecular mechanism of temperature-dependent sex determination and differentiation in Chinese alligator revealed by developmental transcriptome profiling

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Sex determination (SD) is a critical embryonic development process that has profound impacts on the morphology of individuals and the survival of populations [1]. In vertebrates, sex can be determined by either genetic or environmental factors [2], with the underlying molecular mechanism unknown for most species except for a few model organisms [3,4]. Unlike mammals and birds, crocodilians lack sex chromosomes and their sexes are determined by incubation temperature of eggs during a specific developmental period called the temperature-sensitive period (TSP) [5]. Many genes that participate in the crocodilian temperature-dependent sex determination (TSD) process have been identified (e.g., *SOX9*, *AMH*, *FOXL2*, etc.) [6], but their exact regulatory pathways still remain elusive. Chinese alligator is a critically endangered freshwater crocodilian that is endemic to China and its genome has been analyzed by our group [7]. It has a female-male (FM) pattern of TSD, with eggs incubated at 34 °C producing all males, and those incubated at 29 °C producing all females [8]. Here, we collected embryonic genital ridges or gonadal tissues from Chinese alligator eggs incubated at 34 °C (male-producing temperature, MPT) and 29 °C (female-producing temperature, FPT) at the pre-, mid- and post-TSP stages (Fig. S1), and used strand specific RNA sequencing and small RNA sequencing to explore the molecular and regulatory mechanisms underlying the TSD of Chinese alligator (Data S1 and S2).

The differential expression analysis revealed that as gonadogenesis progressed, the numbers of sex-biased differentially expressed mRNA-encoding genes (DEGs) or miRNA genes (DEmiRs) increased with development. At the pre-TSP stage, we identified 21 sex-biased DEGs (0.127% of active genes) and 4 DEmiRs (0.763% of active miRNAs) (Fig. S2, Table S1). None of these DEGs or putative DEmiR target genes had an ortholog that had been reported to participate in the SD of another vertebrate (Table S2). This provides molecular support of the hypothesis that the sexual fate is not affected by temperature prior to TSP [6]. The corresponding numbers increased to 2177 (13.121% of active genes) and 21 (4.008% of active miRNAs) at the mid-TSP stage and 5280 (31.824% of active genes) and 44 (8.397% of active miRNAs) at the post-TSP stage (Fig. S2, Table S1). These transcriptome patterns are also consistent with the morphological pattern, as the gonads of Chinese alligator do not differentiate until the end of TSP [9].

Our principal component analysis (PCA) of genes/miRNAs suggested that the SD process of this species seems to be driven mainly by changes in the male gonads. The MPT and FPT samples clustered together at the pre-TSP stage (Fig. S3), whereas those of the other MPT stages (mid- and post- stages) clustered separately from one another and from the FPT samples. This indicates that the gonads exist a bipotential state before TSP, and that the expression pattern changes much less under FPT than under MPT. Consistent with this notion, we found that much fewer genes exhibited altered expression levels over the developmental course under FPT than under MPT: 30.9% of the active genes showed up- or down-regulation as gonads developed from the pre- to mid-TSP stages under MPT (Fig. 1a), compared to 1.8% of the active genes under FPT (Fig. 1b). The results show that the gene expression changes under MPT contributed much more to the sex-biased DEGs than those under FPT (Fig. S4). A similar pattern for miRNA

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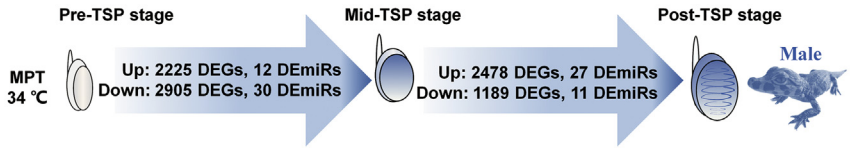
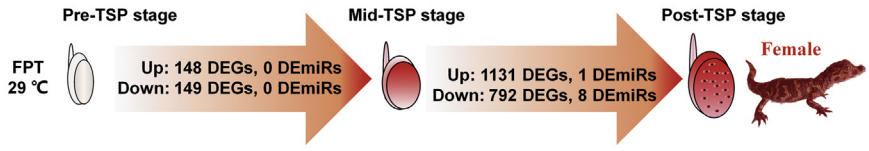
(a)



(b)



(c)

Category	Pattern	Gene numbers	Putative sex determination/differentiation genes	miRNA numbers	Putative sex determination/differentiation genes targeted by miRNAs
Up2		1559	*FGFR2, MAP3K4, PDGFB, PDGFRB, p38α-1, p38α-2; FST-1; TRPC6, TRPV2, CACNA1D, CACNA2G1, CACNA2G2, HSP47, HSP40B4, DACY4, DACY8, PKA, PKC*	11	*LHX9, HSD17B2, HSD17B6, CYP11A, StAR, ERβ, MAP3K1*
Up3		1140	*IkB-2, SOX8, DAX1, CBLN4*	13	
Up4		97	*IP3R; PTCH1*	0	
Up5		438	*p38γ, p38β, FOG2, PDGFRA, TGFβ2-1*	0	
Down1		88	*CIRBP*	0	
Down2		1599	*LHX9, SF1, StAR, CYP11A, HSD17B1; DMRT1; HSPB1*	17	*FGFR2, VNN1, p38α-2*
Down3		555	*P2RX5,HSP40A1,PI3K; β-Catenin*	11	*p38α-1, p38α-2, VNN1, IkB-2, NFkB, SOX9, HES1*
Down5		635	*WNT4, GATA2; CYP17, EMX2*	13	*DACY4, FGFR2*
Down6		439	*TRPM6, WT1, HSD17B2, HSD17B6, HSD3B*	0	
Steady		17922	*FGF9, FGFR1, GATA4, MAP3K1, SOX3, SOX9, SOX10, AMH, VNN1, PGDS, HHAT, SIX4, WNT5A, PDGFA, HES1, SMAD4-1, SMAD4-2, SMAD2, TGFβ2-2, TGFBR3, AR, IkB-1, NFkB, P38δ, CBX2; RSPO1, FOXL2, FST-2, FST-3, BMP2-1, BMP2-2, CYP19A, ERα-1, ERα-2, ERβ; TRPM2,CACNB2, CACNA1H, HSPA12A, HSP90AA1, HSPH1, CREB, AKT*	1106	*PDGFA, MAP3K1, p38α-1,p38α-2; WT1; WNT4, ERβ*

(d)

Category	Pattern	Gene numbers	Putative sex determination/differentiation genes	miRNA numbers	Putative sex determination/differentiation genes targeted by miRNAs
Up3		1040	*CYP19A; P38δ; HSD17B1, HSD17B6*	1	
Up5		108	*FDGFRB*	0	
Down3		678	*DACY8, HSP47; TRPM2,CACNA1H; LHX9; GATA4, PDGFRA, TGFβ2-1, TGFβ2-2, p38α-1*	8	
Down5		84	*HSPB6*	0	
Steady		22732	*DMRT1, FGF9, FGFR1, FGFR2, FOG2, MAP3K1, MAP3K4, SOX3, SOX8, SOX9, SOX10, PTCH1, AMH, VNN1, CBLN4, PGDS, DAX1, KDM3A, HHAT, SIX4, WNT5A, PDGF-A, PDGF-B, HES1, SMAD4-1, SMAD4-2, SMAD2, TGFBR3,AR, IkB-1, IkB-2, NFkB, p38γ, p38α-2; RSPO1, β-Catenin, FOXL2, FST-1, FST-2, FST-3, WNT4, BMP2-1, BMP2-2, ERα, ERβ, GATA2; EMX2, M33, WT1, SF1, StAR, CYP11A, CYP17, HSD3B, HSD17B2; TRPC6, TRPV2, TRPM6, CACNA1D, CACNA2G-2, CACNB2, IP3R1, P2RX5, HSPH1, HSP90AA1, HSP40B4, HSP40A1, HSPA12A, DACY4, PKA, CREB, PKC, PI3K, AKT*	1163	*IkB-2, NFkB, SOX9, HES1, MAP3K1, p38α-1, p38α-2, FGFR2, VNN1, PDGFA; WNT4， ERβ; LHX9, HSD17B2, HSD17B6, CYP11A, StAR, WT1; DACY4*

Male determination/differentiation genes
Female determination/differentiation genes
Putative thermosensitive genes and their downstream genes
Steroidogenic genes and their regulators

Fig. 1. The developmental transcriptome profiling of Chinese alligator embryonic gonads. Numbers of DEGs and DEmiRs under MPT (a) and FPT (b) between sequential stages across gonadogenesis. The numbers inside the arrowheads represent the numbers of genes/miRNAs that showed significantly up- or down-regulated expression (FDR < 0.05) when we compared stage 23 (mid-TSP) with stage 17 (pre-TSP) or stage 27 (post-TSP) with stage 23 (mid-TSP). (c, d): Categories of expressional trends for genes and miRNAs during gonadogenesis under MPT (c) and FPT (d).

expression was also observed between the pre- and mid-TSP stages (Fig. 1a, b). These results suggest that the SD process, which is triggered by the yet-unknown temperature sensor (s), mostly occurs between the pre- and mid-TSP stages, and that the MPT transcriptome shows much more dramatic changes compared to the FPT transcriptome, which is relatively stable over the developmental course. This pattern supports the hypothesis proposed by Deeming and Ferguson [5,10] that alligator embryos develop as female through the preset program unless they are exposed to the high MPT during TSP.

We further tracked the trends of mRNA and miRNA gene expression throughout the three stages, in an effort to identify candidate thermosensitive genes as the initial sex-determining cue, as well as male or female differentiation pathway genes that act under the different incubation temperatures. Based on previous studies in other vertebrate species [3,4], we compiled lists of genes that are likely to participate in male determination/differentiation pathways (MDGs), female determination/differentiation pathways (FDGs), and steroid hormone biosynthesis, as well as putative thermosensitive genes and their downstream targets (Table S3). We then classified all 24,747 mRNA genes and all 1172 mature miRNAs by 13 transient modes of expression throughout alligator gonadogenesis (Fig. 1c, d, Fig. S5). As expected, we observed a significant (Fisher's exact test, $P = 2.54 \times 10^{-4}$) enrichment of the alligator orthologs of MDGs in the up-regulated categories ('Up2', 'U3', 'Up4' and 'Up5') throughout TSP. In addition, the thermosensitive genes and their downstream targets (e.g., Ca^{2+} channel genes) were significantly (Fisher's exact test, $P = 1.17 \times 10^{-7}$) enriched in the 'Up2' category (Fig. 1c). There was no significant enrichment of FDGs in the 'Up' categories or MDGs in the 'Down' categories under FPT, which is consistent with the notion that these animals develop into females by default [5,10].

Among the thermosensitive genes known in mouse, the transient receptor potential (TRP) cation channels and heat shock proteins (HSPs) are the two best-studied gene families whose orthologs are likely to act as the initial cue for TSD in Chinese alligator [11,12]. In particular, *TRPV4* was recently implicated as contributing to the TSD of American alligator through Ca^{2+} signaling [11]. Interestingly, we have not found *TRPV4* show a sex-biased expression during TSP *in ovo* in Chinese alligator (Fig. S6d, Table S4). Instead, three other TRP family genes, *TRPV2*, *TRPC6* and *TRPM6*, are specifically activated in male or female gonads at mid-TSP (Fig. 1c, d; Fig. S6d, e; Table S4). We also found that the term 'calcium signaling pathway' was significantly enriched among the genes that were up-regulated from the pre-TSP to mid-TSP stages under MPT (FDR = 0.025) and the male-biased genes of the post-TSP stage (FDR = 0.003), as assessed by both KEGG (Table S5) and GO (Table S6) analyses. These results suggest that TRP family genes may act as the initial temperature sensor and then modulate downstream SD genes through Ca^{2+} signaling (Fig. S7) [11]. We also found that seven of the 72 examined HSP genes showed sex-biased expression during TSP (Fig. S8a, d, f; Table S7). Interestingly, six HSP genes were activated at mid-TSP under MPT, while all but only one were activated at post-TSP under FPT (Fig. S8d). This suggests that HSPs may not play an important role in initiating female determination in Chinese alligator.

As mentioned, the genes up-regulated under MPT were enriched for MDGs (Fig. 1c), mutations in many of which (e.g., *MAP3K4*, *FOG2*, *DAX1*, *FGFR2*, and *PDGFRA*) have been associated with sex reversal or defective testis development in mouse [3,4]. In particular, from pre- to mid-TSP, genes encoding components of the PDGF, p38-MAPK, and FGF signaling pathways were up-regulated (Fig. 1c, 'Up2' and 'Up5' categories; Fig. S9; Table S3). Many other MDGs showed relatively steady expression levels (Fig. 1c, 'Steady') or even down-regulation ('Down2') throughout MPT, possibly because they were specifically activated in males before TSP, or because their degrees of up-regulation did not reach the threshold for any of the 'Up' categories (e.g., *SOX9* and *FGF9*; Fig. S9c, Table S3). Most of the 'Steady' MDGs maintained significantly higher expression levels than FDGs from pre-TSP onward under MPT (Mann-Whitney test, $P < 0.05$) (Fig. S10a), and maintained higher expression levels than the corresponding levels seen under FPT beginning at mid-TSP (Fig. S10b). This supports the notion that MDGs play a conserved role in male determination or differentiation in Chinese alligator. With reference to the SD pathway of other vertebrate species [3,4] and the gonadal gene expression patterns found in this work, we summarize the putative temperature-dependent male determination pathway of Chinese alligator in upper panel of Fig. S7.

Both FDGs and steroid hormone synthesis-related genes were found to be repressed during MPT of Chinese alligator, some of them probably by miRNAs, whereas they were activated under FPT (Fig. 1c, d, Fig. S11, Table S3). Under MPT, we observed a significant (Fisher's exact test, $P = 4.12 \times 10^{-9}$) enrichment of steroid hormone biosynthesis pathway genes (eg. *CYP11A* and *HSD17B2*; shown in yellow in Fig. 1c) and their regulators (*LHX9*, *WT1* and *SF1*), in the down-regulated categories of 'Down2', 'Down5', and 'Down6', all of which showed decreased expression following entry into TSP under MPT (Fig. 1c, Fig. S11). This contributes to the excess of female-biased genes seen at the mid-TSP stage (Fig. S4). KEGG pathway analysis revealed that the 'steroid hormone biosynthesis' pathway was significantly (FDR = 0.004) enriched in female-biased DEGs at mid-TSP stage (Table S5). Under FPT, in contrast, these genes (e.g., *CYP19A*) or their regulators were up-regulated after TSP, when the ovary starts to form, or maintained a significantly (Mann-Whitney test, $P < 0.05$) higher expression level than MDGs as 'Steady' genes (Fig. S10b). These expression patterns suggest that steroid hormone genes play a more important role in female development than in male development, and the functions and regulatory structures of the key involved genes are conserved between Chinese alligator and other vertebrates (lower panel of Fig. S7).

In addition, we identified a total of 21 sex-biased DEmiRs putatively targeting 410 genes at the mid-TSP stage, and 44 sex-biased DEmiRs putatively targeting 4207 genes at the post-TSP stage. Similar to the patterns of mRNA gene expression, the expression patterns of miRNAs were more dynamic under MPT than under FPT, and many more miRNA genes distributed to the 'Up' or 'Down' expression modes under MPT than under FPT (Fig. 1c and d). The expression changes of miRNAs tended to show trends opposite to those of their putative target mRNAs. This makes sense given that miRNAs usually bind to the 3′UTRs of mRNAs to down-regulate their target genes [13]. Under MPT, miRNAs putatively targeting FDGs or steroid hormone genes were significantly (Fisher's exact test, $P = 1.4 \times 10^{-9}$) enriched in the 'Up2' category (Fig. 1c, Fig. S12). This suggests that the female determination and/or differentiation pathways are actively repressed at least partially via miRNAs under MPT. In addition, miRNAs targeting MDGs were significantly (Fisher's exact test, $P = 1.47 \times 10^{-11}$) enriched in the 'Down2', 'Down3', and 'Down5' categories under MPT (Fig. 1c, Fig. S12), likely enabling the sustained/up-regulated expressions of MDGs during MPT. We have not observed any up-regulation of MDG-targeting miRNAs under FPT (Fig. 1d, Fig. S12), suggesting that the male development pathway might not be actively suppressed by miRNA under FPT. In particular, from pre- to mid-TSP, upstream genes of the steroidogenic pathway, such as *StAR*, *CYP11A*, and *ERβ*, are probably repressed by the up-regulation of their corresponding miRNAs (Fig. S7), perhaps blocking the entire steroidogenic pathway early during MPT. Indeed, most of the steroidogenic genes showed constantly low expression levels or underwent down-regulation from pre- to mid-TSP (Fig. S11b, c). Interestingly, different miRNAs targeting the same gene were often

activated at different developmental time points, appearing to enable the constant suppression of FDGs under MPT. For example, both miR-189 and miR-1662 were predicted to target *ERβ* (Fig. S12). The former was down-regulated from the pre-TSP to mid-TSP stages under MPT, while the latter was up-regulated beginning at mid-TSP (Fig. S13); together, these expression changes would appear to result in the constant suppression of *ERβ* expression throughout MPT. Similar patterns were observed for miRNAs targeting *StAR*, *CYP11A*, *HSD17B1*, and *HSD17B2* (Figs. S12, S13). Conversely, we also observed cases in which the down-regulation of miRNAs was associated with the up-regulation of their corresponding target genes. For example, miR-novel182, which putatively targets *SOX9*, was down-regulated from mid- to post-TSP, corresponding to the up-regulation of *SOX9* (Figs. S12, S13). These results provide the first clue to suggest that miRNAs participate in TSD by suppressing the SD genes of the opposite sex.

Overall, the transcriptome profiling suggests that the male-determination process of Chinese alligator involves both commitment to the male developmental fate and suppression of the preset female development fate. This is likely to be realized by miRNAs, whose expression changes tended to oppose those of their putatively targeted sex-determining genes. These results reveal the molecular and regulatory mechanisms underlying the TSD of Chinese alligator (Fig. S7), and may facilitate the conservation of other TSD reptile species whose sex ratios are being skewed by climate change.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.scib.2018.01.004.

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