THE PATTERNS OF SEX DETERMINATION AND DIFFERENTIATION GENES IN GREEN SEA TURTLE (*Chelonia mydas*)

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ABSTRACT

Green sea turtle (C. mydas) is one of TSD (Temperature-dependent Sex Determination, TSD) animals which mean that their sex is determined by the egg's incubation temperature. Genotypic Sex Determination (GSD) homologous genes play a role in TSD process. Until now, research on the pattern of sex determination genes in C.mydas has not been conducted yet. The aim of this research is to reveal sex determination and differentiation genes expression in Mesonephros-Gonad (MG) complexes of C. mydas embryos which incubated in masculinizing temperature (MT) and feminizing temperature (FT). C. mydas eggs were incubated in 3 different stage of TSP (Thermosensitive Period) at masculinizing temperature (26±1^{0}C, MT) and feminizing temperature (31±1^{0}C FT). Mesonefros-gonad complexes were isolated at Pre-TSP stage (FT at 14th day, MT at 24th day), TSP stage (FT at 24th day, MT at 36th day) and differentiated stage (FT at 40th day, MT at 58th day). RNA from mesonephros-gonad (MG) complexes were converted into cDNA by RT-PCR process. Pattern of Sf1, Wt1, Aromatase, FoxL2, Sox9, Wnt4, Fgf9 and Rspo1 genes expression were analyzed by quantitative Real Time PCR (qPCR) method with β-actin gene as an internal control. Result of this study shown that expression pattern of Sf1, Wt1, Aromatase, FoxL2, Sox9, Wnt4, Fgf9, and Rspo1genes in gonadal embryo of C. mydas were increased during gonadal development stage. Four genes expression patterns (Wnt4, Fgf9, Rspo1, and FoxL2) have shown that these genes have role in sexual differentiation rather than in sexual determination.

Key words: C. mydas, temperature-dependent sex determination, masculinizing temperature, feminizing temperature.

INTRODUCTION

Green sea turtle (*C. mydas*) is one of TSD (Temperature-dependent Sex Determination) animal, which means that its sex determination and differentiation are affected by incubation temperature. Study about incubation temperature effect on sex ratio of *C. myda*s eggs was conducted by Hamid (1982). The results shown that eggs which incubated at 31° ± 10°C became 100% female, this temperature then called as a female producing temperature (FPT). Eggs which incubated at 26° ± 10°C became 100%, this temperature then called as a male producing temperature (MPT). In the development stage of TSD animal included *C. mydas*, there are crucial periods which assumed have important roles in sex determination and differentiation which called as thermo-sensitive period (TSP) (Chapeville et al., 1985). TSP stage in *C. mydas* embryos which incubated were on day 15-30 (FPT) and on day 28-43 (MPT) (Utanti, 2005).

On TSP period, homologous genes in GSD animal also take roles in sex determination and differentiation of TSD animal. That genes are Sf1, Aromatase, Wt1, Sox9, Mis, Dmrt1, Dax1, Wnt4, FoxL2, Fgf9 and Rspo1 (Ramsey et al., 2007; Shoemaker et al., 2007; Rhen et al., 2007; Smith, et al., 2008). Sox9 and Wt1 take role in testes development (Shoemaker et al., 2007). Aromatase and FoxL2 take role in ovary development (Ramsey et al., 2007; Valenzuela, 2008).Wnt4, Sf1, and Fgf9 take role in male and female gonadal development (Ramsey et al., 2007; Rhen et al., 2007; Crews et al., 2007).

Recently, study about sex determination and differentiation mechanisms in TSD animal were only focused on TSP stage. It can be assumed that gene expression patterns in TSP period possibly caused by genes expression patterns during pre-TSP period. The expression pattern of Sf1, Wt1, Aromatase, FoxL2, Sox9, Wnt4, Fgf9and Rspo1 in gonadal embryo development of *C. mydas* in pre-TSP period have not been revealed yet. Expression pattern of genes which take roles in sex determination and differentiation of *C. mydas* probably not the same as gene expression patterns of other TSD animals. This research aims to study the expression pattern of Sf1, Wt1, Aromatase, FoxL2, Sox9, Wnt4, Fgf9, and Rspo1 genes in pre-TSP period gonadal embryo of *C. mydas*

which incubated in male-producing temperature (MPT) and female-producing temperature (FPT) in order to reveal the mechanism of sex determination and differentiation in *C. mydas*.

MATERIALS AND METHODS

Green sea turtle (*C. mydas*) eggs were taken from Pangumbahan Beach, Sukabumi, Jawa Barat, Indonesia. Eggs were incubated in two different temperatures, female-producing temperature (31° ± 10°C) and male producing temperature (26° ± 10°C). mRNA were isolated from Gonad-mesonephros complex in pre-TSP, TSP, and, differentiation stage. Isolated mRNA was then analyzed using Quantitative Real Time PCR.

RESULTS

Results of this study shown that expression pattern of Sf1, Wt1, Aromatase, FoxL2, Sox9, Wnt4, Fgf9, and Rspo1genes in gonadal embryo of *C. mydas* were increased during gonadal development stage. Study about protein level also supported the results in respective genes expression.

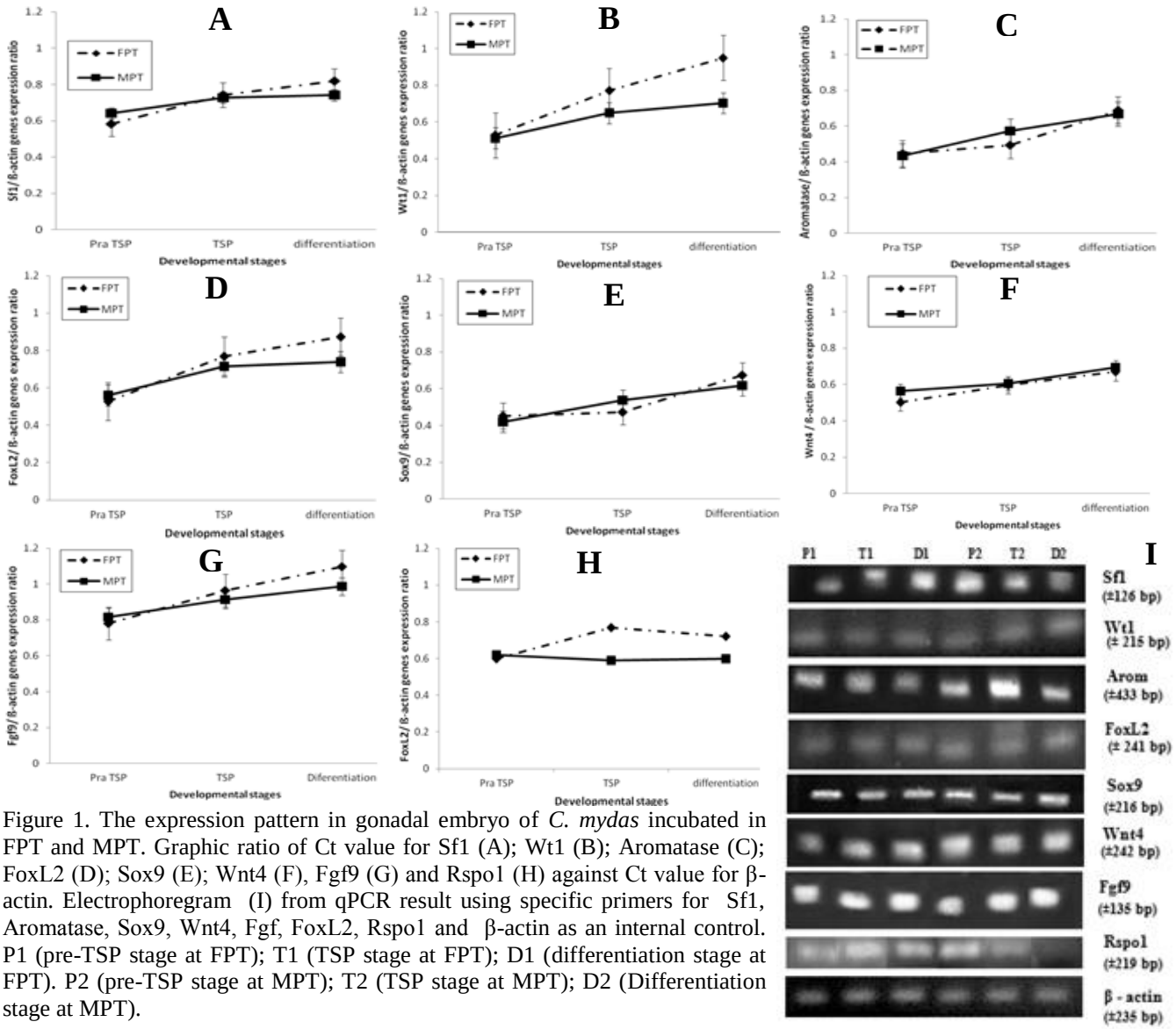

Figure 1. The expression pattern in gonadal embryo of *C. mydas* incubated in FPT and MPT. Graphic ratio of Ct value for Sf1 (A); Wt1 (B); Aromatase (C); FoxL2 (D); Sox9 (E); Wnt4 (F), Fgf9 (G) and Rspo1 (H) against Ct value for β-actin. Electrophoregram (I) from qPCR result using specific primers for Sf1, Aromatase, Sox9, Wnt4, Fgf, FoxL2, Rspo1 and β-actin as an internal control. P1 (pre-TSP stage at FPT); T1 (TSP stage at FPT); D1 (differentiation stage at FPT). P2 (pre-TSP stage at MPT); T2 (TSP stage at MPT); D2 (Differentiation stage at MPT).

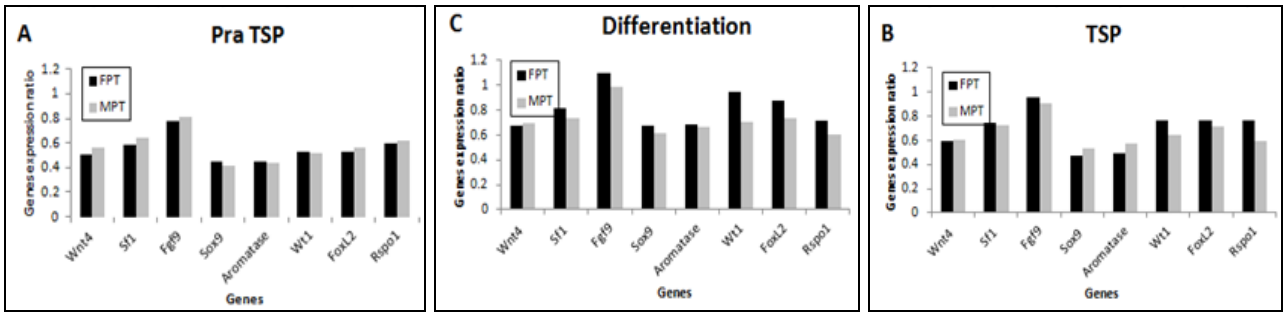

Figure 2. Hystogram of comparison between Sf1, Wt1, Aromatase, FoxL2, Sox9, Wnt4, Fgf9, and Rspo1 gene expression in gonadal complex of *C. mydas* incubated in FPT and MPT based on gonadal development stage. (A) Pre-TSP, (B) TSP, and (C) differentiation stage.

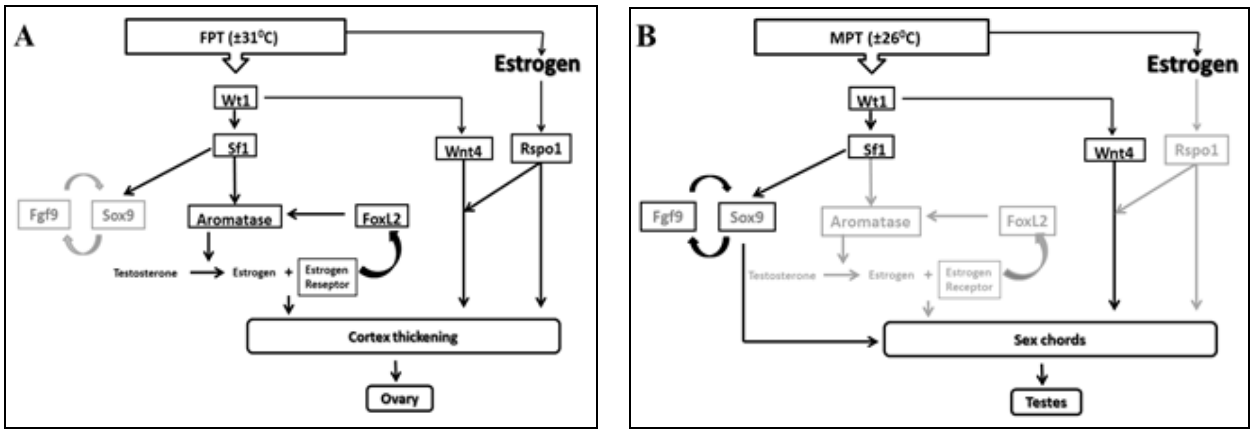

Figure 3. Hypotetical diagram of sex determination and differentiation genes correlation in gonadal embryo *C. mydas* differentiation mechanism while incubated in FPT and MPT. → shows the sex differentiation pathway, and ▭ shows target genes.

DISCUSSION

Sf1 gene expression in MPT was higher than in FPT (Figure 1A). Expression of Sf1 then increased both in MPT and FPT of TSP stage. It was higher in FPT than MPT of differentiation stage. Pattern of Sf1 gene expression in all stages of *C. mydas* gonadal development was similar to the expression pattern of other TSD animal, such as *Trachemys scripta*, *Chrysemys picta*, and *Chelydra serpentina* (Ramsey, et al., 2007; Valenzuela, et al., 2006; Rhen, et al., 2007). The expression of Sf1 gene on pre-TSP, TSP, and differentiation stages during gonadal embryo development which incubated in FPT and MPT was related to Sf1 protein level (Vebiyanti, 2007).

Wt1 expression in gonadal embryo of *C. mydas* can be seen in Figure 1B. The expression of Wt1 was increase in TSP and differentiation stages. The expression was higher in FPT compared than in MPT. The expression pattern of Wt1 in pre-TSP stage of *C. mydas* was also similar to other TSD animal expression pattern, such as *T. scripta* and *C. picta* (Shoemaker & Crews, 2009). However, in differentiation stage, its expression was different with the other species which indicated that Wt1 expression is specific. Study about Wt1 protein expression level has shown different pattern of mRNA level for pre-TSP and TSP stage. This phenomenon was assumed could occur because of transcriptional regulation. Alternative splicing mechanism generally happened in Wt1 either in TSD or GSD animal. This hypothesis was supported by the research of Spotila & Hall (2003) in *T. scripta* and Wagner et al. (2003) in mammals.

Aromatase gene expression of *C. mydas* gonadal which incubated in both of MPT or FPT was almost similar. In TSP stage, expression pattern of aromatase in MPT incubation was higher than FPT incubation. Surprisingly,

aromatase expression pattern in FPT incubation was significantly increased than in MPT incubation. As it is shown in the result of Aromatase expression in protein level by Equilibrina (2007), it was expressed in every stage of gonadal development both in MPT and FPT incubation with highest expression on differentiation stage.

Another gene which suspected take the role in female-producing pathway is FoxL2. Expression pattern of FoxL2 in pre-TSP was similar to its expression pattern in MPT and FPT incubation. The expression pattern of FoxL2 was significantly increased in TSP and differentiation stages after FPT incubation. This finding has shown that FoxL2 was specific in female sex determination mechanism. It is supported by result study of Shoemaker, et al. (2010) and Loffler, et al (2003) in *T. scripta* which shown that FoxL2 has been found higher in female-producing pathway. Correlation between FoxL2 and the other genes in sex determination and differentiation mechanism is still open to be discussed because of limited study of it.

As one of specific male-producing genes, Sox9 has shown higher on pre-TSP stage than on MPT incubation. On the contrary, expression pattern of Sox9 has significantly increased in TSP stage and become much higher in MPT than FPT. However, Sox9 expression pattern has decreased in differentiation stage at similar level as in pre-TSP stage. Its expression in FPT was higher than in MPT. Observation result of Sox9 protein expression level has shown that it was only expressed in pre-TSP and TSP stage in FPT. Sox9 protein expression level then decreased in differentiation stage (Utami, 2010). The expression level of Sox9 protein and mRNA were similar in pre-TSP and TSP stage, but these expression levels were not similar in differentiation stage. Post transcriptional regulation could be occurred in differentiation stage of FPT which caused Sox9 mRNA untranslated. These results proved that Sox9 had specific role to downstream the male-producing pathway of differentiation stage.

Figure 1F shows that in pre-TSP stage the expression level of gonadal embryo in MPT was higher than in FPT. The expression was increased in TSP and differentiation stage, it was shown the same pattern in both of MPT and FPT. The enhancement of Wnt4 gene expression in FPT incubation was also confirmed in research using gonadal embryo of *T. scripta* (Ramsey et al., 2007). However, the expression pattern of Wnt4 gene in gonadal embryo of *C. mydas* in MPT incubation showed different pattern with *T. scripta*. Wnt4 gene expression in gonadal embryo of *T. scripta* which incubated in MPT was decreased during gonad development, whereas it was increased in *C. mydas*. mRNA expression of Wnt4 was similar with its protein expression level (Vanawati, 2009).

During pre-TSP stage, the expression level of Fgf9 was almost similar in gonadal embryo which incubated at both of MPT and FPT (figure 1G.). Fgf9 expression level was increased in TSP and differentiation stage. At FPT, the expression of Fgf9 was higher at MPT. Research about Fgf9 gene expression in gonadal embryo of *C. mydas* has never been done before in both of mRNA and protein level. However, in other TSD species (*Chelydra serpentine)* level of FgF9 mRNA expression observation has been done by Rhen et al (2007). Fgf9 expression during gonad development in *C. mydas* and *C. serpentine* were similar. In GSD animal, Fgf9 was expressed and escalated in male gonad, but it was decreased in female gonad (Kim et al, 2006). gf9 gene expression in TSD animal still need to be explored for further, especially *C. mydas*.

Rspo1 expression patterns were almost similar in pre-TSP stage at both of FPT and MPT incubations. It was increased in TSP of FPT incubation. However, the expression level of Rspo1 was almost at the same level in pre-TSP stage until differentiation stage at MPT incubation. This results have shown that Rspo1 specifically work in female-producing pathway as mentioned in Smith et al., (2008) study using another TSD animal. Rspo1 considered as new discovered gene in sex determination mechanism

of TSD or GSD. Therefore, more information about Rspo1 role is needed to be explored.

Gene expression pattern results of Sf1, Wt1, Aromatase, Fox12, Sox9, Wnt4, Fgf9, and Rspo1 have shown that they were expressed during developmental stage of gonadal embryo in both of MPT and FPT incubations (Figure 2). None of these gene expressed specifically only at MPT or FPT incubations. Therefore, it was difficult to determine which one of genes that has significant role in gonadal sex determination of *C. mydas*. Among those 8 genes, Wnt4 and Sf1 were not significantly different expressed at pre-TSP stage in both of FPT and MPT incubations.

Candidate genes which have important role at TSP should be more considered in *C. mydas* regarding to that TSP was determined at pre-TSP. Expression patterns of four genes Wnt4, Fgf9, Rspo1, and FoxL2 have shown that these genes were possibly have role in sexual differentiation rather than sexual determination.Further studies are needed to be done in order to determine the role of those eight genes in gonadal sex determination and differentiation of *C. mydas* quantitatively. Figure 3 shown has resumed the gonadal sex determination and differentiation mechanism based on the patterns of genes expression results in *C. mydas* at FPT and MPT incubations.

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