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Estrogen Receptor 1 (ESR1) Agonist Induces Ovarian Differentiation and Aberrant Müllerian Duct Development in Chinese Soft-shelled Turtle, *Pelodiscus sinensis*

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Estrogens play critical roles in ovarian and reproductive organ development, but the molecular signaling pathways in non-mammalian vertebrates are not well understood. Studies in reptiles have been indicated that an administration of exogenous estrogens during the embryonic development causes the ovarian differentiation and presumptive male to female sex-reversal. The Chinese soft-shelled turtle, *Pelodiscus sinensis*, belongs to the family Trionychidae and exhibits genotypic sex determination system with a ZZ/ZW sex chromosomes. In order to assess the role of estrogens and their signaling pathway on the process for sex determination and differentiation, the eggs were given a single administration of endogenous estrogen,17β-estradiol (E2) or a synthetic estrogen receptor 1 (ESR1) agonist, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) *in ovo* during gonadal differentiation, and the subsequent effects were examined at a final developmental stage prior to hatching. The administration of both E2 and PPT induced ovarian differentiation in genetic male embryos. Intriguingly, PPT but not E2 induced the Müllerian duct enlargement and aberrant glandular development. These data suggest ovarian differentiation and reproductive tract anomalies induced by the exogenous estrogen exposure act through ESR1 in the Chinese soft-shelled turtles.

Key words: Soft-shelled turtle, Estrogen receptor, Gonad, Müllerian duct, Ovarian differentiation.

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BACKGROUND

Steroid hormones are essential for development, reproduction and health throughout the life of vertebrates. Of those, estrogens play critical roles in sex determination and particularly in ovarian differentiation in non-mammalian vertebrates, regardless of the mode of sex determination. The medaka, *Oryzias latipes*, exhibits genotypic sex determination (GSD) with a distinct sex determination gene called DM-domain gene on the Y chromosome (dmy) (Matsuda et al. 2002). The administration of exogenous estrogens during the embryonic development causes complete male to female sex-reversal(Iwamatsu et al. 2005; Kobayashi and Iwamatsu 2005; Yamamoto 1962). Likewise, the induction of ovarian differentiation and sex reversal by exogenous estrogen has also been achieved in various fish species and amphibians (Hayes 1998; Nakamura 2010; Piferrer 2001). In the chicken, sex reversal phenotype can be experimentally induced by the injection of estrogens into the eggs or by the inhibition of estrogen production (Elbrecht and Smith 1992; Scheib 1983). Some reptiles exhibit temperature-dependent sex determination (TSD) that the ambient temperature determines the sex of the offspring. The administration of estrogens in alligator and turtle eggs incubated at a temperature that normally produces males results in female hatchlings (Bull et al. 1988; Crews et al. 1989). Thus, estrogens can override the effects of temperature on eggs at male-producing temperature. Although the estrogen function during ovarian development remains largely elusive, it has been reported that the exposure to estrogen during the early stages of gonadal development induces precocious downregulation of SRY (sex determining region Y)-box transcription factor 9 (SOX9) in the red-eared slider turtle (Barske and Capel 2010).

After ovarian development, differentiation of Müllerian duct subsequently takes place in the females. The Müllerian duct, a primordium of the female reproductive tracts in vertebrates, primarily develop in embryos of both sexes. Then, the Müllerian duct in females continues to develop and differentiate into oviduct, uterus and vagina, whereas in males, it regresses eventually under the influence of anti-Müllerian hormone (Amh; also called Müllerian inhibiting substance), which is secreted from testis. In birds, at least in part, differentiation of the Müllerian duct is

influenced by estrogens, which counter the effects of Amh, leading to left-side Müllerian duct (oviduct) development (Hermansson 2007). While no evidence has been reported for the role of endogenous estrogens on the Müllerian duct development during embryonic development in other animals, the administration of exogenous estrogens could disturb reproductive organ development. A synthetic estrogen, diethylstilbestrol (DES), was routinely prescribed to pregnant women for the prevention of miscarriages in the 1940s (Herbst 2000, 1981; Herbst et al. 1971; Robboy et al. 1984). To date, it is well-known that *in utero* exposure to DES induces vaginal clear-cell adenocarcinoma and various malformations in the reproductive tracts in young women. *In utero* exposure to DES also induces uterine atypical hyperplasia and adenocarcinoma in mouse as well (Newbold et al. 1990).

The signaling actions of estrogens are mediated by a specific receptor that belongs to the steroid nuclear receptor superfamily, estrogen receptors (ESRs). Two different genes encoding the two ESR subtypes, *ESR1*and *ESR2*, have been identified from many vertebrate species while, in the teleost lineage, the *esr2* gene has been further duplicated and consequently exhibit three esr subtypes, *esr1*, *esr2a* and *esr2b* (Tohyama et al. 2016). *Esr* knockout (KO) mouse studies have revealed the distinct functions of each receptor in mammals. In particular, ESR1 mediated estrogenic activity in mouse uterus in terms of cell proliferation and glandular development (Lubahn et al. 1993). Loss-of-function mutant analysis for ESR has not been applied in reptiles so far, but the functionalization of ESRs in reptiles has been analyzed using selective ESR1 and ESR2 agonists in the American alligator (*Alligator mississippiensis*). The exposure of alligator eggs to the ESR1-selective agonist 4,4’,4’’-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) induced ovarian differentiation at a male-producing temperature, whereas the ESR2-selective agonist 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070) had no effect (Kohno et al. 2015). Intriguingly, PPT-exposed embryos also showed the enlargement of the Müllerian duct, accompanied with advanced glandular development (Doheny et al. 2016; Kohno et al. 2015). These data suggest that ESR1 plays a central role in ovarian differentiation and also affects reproductive tract development in reptiles after exposure to exogenous estrogen.

Reptiles exhibit various modes of sex determination such that they employ TSD and GSD systems. For example, the sexual determination of the freshwater turtle, Malayemys macrocephala is under TSD system (Pewphone et al. 2020). The Chinese soft-shelled turtle, *Pelodiscus sinensis*, belongs to the family Trionychidae, and exhibits GSD with a ZZ/ZW sex chromosomes (Kawagoshi et al. 2009; Kawai et al. 2007; Mu et al. 2015). In this species, 18S-28S ribosomal RNA gene cluster is located in the micro-sex chromosomes (Kawai et al. 2007) and the genetic sex is hence determined by standard PCR method (Literman et al. 2004). The Chinese soft-shelled turtle is thus an excellent model for studying sex determination and gonadal differentiation as genetic and

intrinsic sexes can be distinguished. Ovarian differentiation by exogenous estrogens has been described in Trionychidae turtles, including the spiny soft-shelled turtle (*Apalone spinifera*) (Bull et al. 1988) and the Chinese soft-shelled turtle (Sun et al. 2017); however, the effects of ESR1-selective agonist *in ovo* has been elusive.

Here, we report the effects of pharmaceutical ESR1 agonist treatment on the gonadal and reproductive organ development in the Chinese soft-shelled turtles. We exogenously exposed E2 or ESR1-selective agonist PPT on the eggs and examine the effects on the ovarian differentiation and Müllerian duct development in ZZ embryos. The administration with both E2 and PPT could induce ovarian differentiation in genetic males. Intriguingly, PPT, but not E2, induces the Müllerian duct enlargement and aberrant glandular development. These data suggest that like alligator, ovarian differentiation and reproductive tract anomalies induced by exogenous estrogen exposure act through ESR1 during embryonic development in the Chinese soft-shelled turtles.

MATERIALS AND METHODS

Animals

Fertilized eggs of turtles were purchased from Tsujimura farm (Itoda, Fukuoka, Japan). The eggs were brought to the laboratory and were incubated in the moist vermiculite medium (Setogahara Kaen, Midori, Gunma, Japan) at temperatures ranging from 29 ± 1˚C. The developmental stages are estimated by candling technique and the criteria described by Tokita and Kuratani (Tokita and Kuratani 2001). In this study, stage 13 was determined by evaluating the pigmented eye development by the candling. All experiments were carried out under the guidelines specified by the Animal Care and Use Committee at the Tokyo University of Science.

Chemical treatments

When the embryos were estimated to reach near stage 16, a stage when the gonadal differentiation is about to begin (Sun et al. 2017) (5–7 days after stage 13), 5 µg of E2 (Sigma, St. Louis, MO, USA) or PPT (purity > 99.0%; Tocris Bioscience, Ellisville, MO, USA) per gram of egg weight (EW) were dissolved in 0.5 µl of 100% ethanol (EtOH) per gram of EW. The concentration is enough to induce male to female sex-reversal phenotypes in alligator embryos under incubation at male-producing temperature (Kohno et al. 2015). The dissolved chemicals

were, then, treated onto the surface of the eggshell. To facilitate chemical absorption into embryos, the outer shell membrane was partly exposed by scraping eggshell with a file before chemical treatment. Embryos were necropsied at stages of approximately 26 and 27 (37–41days after vehicle or chemical treatment). The gonad-adrenal-mesonephros (GAM) complex was dissected immediately and stored in 4% paraformaldehyde (PFA; Sigma). At the same time, a part of remaining tissue was stored in -80˚C for genotyping.

Genetic sexing

The genetic sex of each embryos was determined by quantitative PCR method as previously described (Literman et al. 2004), but instead of GAPDH, β-actin gene was used. Briefly, genomic DNA extracted by a conventional phenol-chloroform extraction was quantified and diluted to 1.25 ng/μl. Quantitative PCR was performed using StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with TB Green Premix Ex Taq II (Takara) according to the manufacturer's instructions. Copy number of the 18S ribosomal RNA (rRNA) gene (forward: GAG TAT GGT TGC AAA GCT GAA A and reverse: CGA GAA AGA GCT ATC AAT CTG T) was normalized against β-actin gene (forward: AAC TGG GAT GAC ATG GAG AAG A and reverse: AAC ATG ATC TGG GTC ATC TT) using the comparative Ct method of normalization. PCR conditions were as follows: 95˚C for 10 min and 45 cycles of 95˚C for 30 sec, 58˚C for 1 min and 72˚C for 1 min in 20 μl volumes. The W chromosome of *P. sinensis* has a larger number of copies of 18S rRNA gene than the Z chromosome (Literman et al. 2004). Hence, a higher value of ΔCt (β-actin - 18S rRNA) indicates female individual (female: > 8.0, male: < 7.0).

Histology

For histologic analyses, the tissues fixed in PFA were paraffin embedded, and 8 μm sections were stained with hematoxylin and eosin by standard procedure. Histology of Müllerian duct was analyzed at the level corresponding to the middle of gonads. Phenotypic features of the gonads were determined by three independent blinded investigators.

RESULTS

Ovarian differentiation in genetic males by E2 and PPT treatment

In order to assess the role of estrogens on the process for sex determination and differentiation, a single administration of E2 or PPT was given *in ovo* at the estimated stage 16 at 5 μg/g EW, and the effects of the chemical treatments were subsequently examined at stages 26–27 (the last stage prior to hatching). The gonads of the control ZZ-males showed well-developed seminiferous tubules with thin cortexes (Table 1 and Fig. 1A). By contrast, the gonads of the control ZW-female did not form such tubules but exhibited lacunae structures and thicker cortexes (Fig. 1B). Most of the ZZ-embryos administrated with E2 and PPT showed the ovarian differentiation with thicker cortexes, similar to the characteristics of the control ovary (Table 1). However, some of the ZZ-embryos administrated with E2 and PPT lacked lacunae structures (Table 1 and Fig. 1C, D). This phenotype was not observed in the ZW-embryos administrated with E2 and PPT, which developed lacunae structures (Table 1).

Table 1. Phenotypes of gonads in embryos exposed to E2 or PPT

Treatment	Genetic sex	Male phenotype; seminiferous tube development	Female phenotype; absence of seminiferous tube and thicker cortex	presence of lacunae
EtOH	ZZ (17)	17	0	0
	ZW (7)	0	7	7
E2	ZZ (7)	0	7	2
	ZW (4)	0	4	4
PPT	ZZ (7)	1	6	3
	ZW (13)	0	13	13

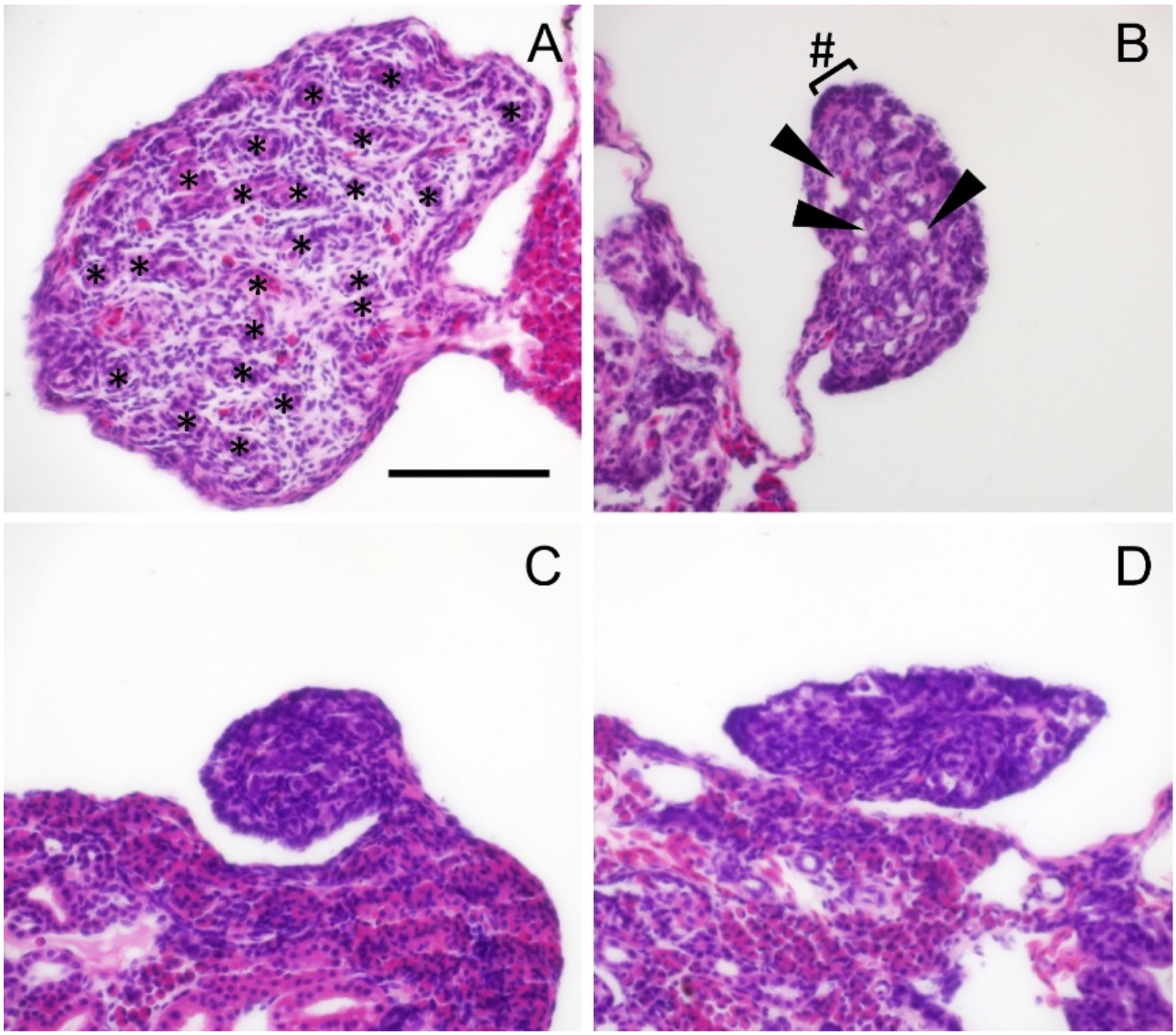

Fig. 1. Gonadal differentiation in embryos exposed to E2 or PPT. (A–D) Representative cross-sections of HE stained gonads at stage 26 for control male (A), control female (B), E2-exposed male (C) and PPT-exposed males (D). Control testis showed well-developed seminiferous tubules (*), whereas control ovary shows thicker cortex (#) and lacunae structure (arrowheads). Gonads from E2- and PPT-exposed males did not form seminiferous tubule and similar characteristics to control ovary. Scale bar = 100 μm.

Glandular development in the Müllerian duct by PPT treatment

At stages 26–27, the ZW-control female embryos exhibited Müllerian duct development (Fig. 2A), whereas Müllerian duct is almost regressed in the control ZZ-embryos (Table 2). E2-treated embryos displayed an increase in the prominent Müllerian duct development in the genetic males (Table 2 and Fig. 2B). In embryos from PPT-treated eggs, Müllerian duct was greatly enlarged in width compared to the controls (Fig. 2C, D). In addition, 4 of 7 (ZZ) and 11 of 13 (ZW)

PPT-administrated embryos showed multiple glandular structures in the Müllerian duct (Fig. 2D). None of the other treatment groups exhibited any of these structures.

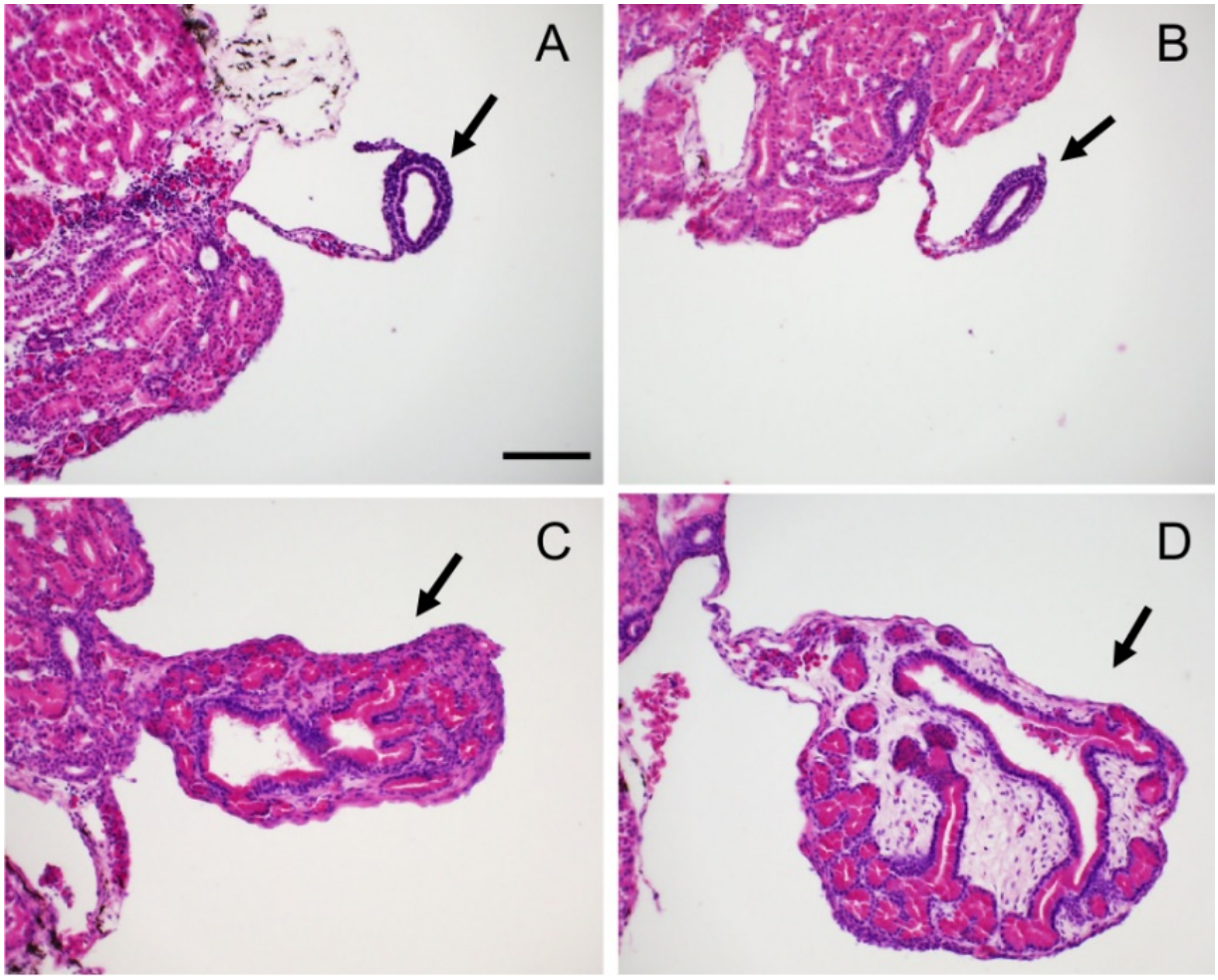

Fig. 2. Histology of Müllerian duct in embryos exposed to E2 or PPT. (A–D) Representative cross-sections of HE stained Müllerian (arrows) ducts at stage 26 for control female (A), E2-exposed male (B), PPT-exposed males (C) and PPT-exposed females (D). Note the glandular development in the Müllerian duct of embryos exposed to PPT. Scale bar = 100 μm.

Table 2. Phenotypes of Müllrian duct in embryos exposed to E2 or PPT

Treatment	Genetic sex (number of embryos)	Müllrian duct	
		Absence	Presence (hyperplasia with granduar development)
EtOH	ZZ (17)	17	0
	ZW (7)	0	7 (0)
E2	ZZ (7)	0	7 (0)
	ZW (4)	0	4 (0)
PPT	ZZ (7)	0	7 (4)
	ZW (13)	0	13 (11)

DISCUSSION

The modes of sex determination among vertebrates exhibit remarkable diversity. In particular, reptiles intriguingly comprise wide range of sex determination system, ranging from GSD to TSD. Primary factors of sex determination in reptiles, therefore, largely remain elusive. Recent studies have been suggested that *Doublesex and mab-3 related transcription factor 1* (*DMRT1*), which encodes a putative transcription factor with a conserved (dsx and mab-3) DM domain, and *Anti-Müllerian hormone* (*Amh*), a member of transforming growth factor β (TGFβ) signaling family, are strong candidates for a trigger of male gonadal differentiation in the Chinese soft-shelled turtle (Sun et al. 2017; Zhou et al. 2019). However, both genes are mapped to autosomes in this species (Kawai et al. 2007) suggesting the involvement of other factors in the regulation of these gene expression and testicular differentiation. Although ovarian fate determination has not been cleared either, there is no doubt that estrogen signaling is the most critical mediator for ovarian differentiation particularly in non-mammalian vertebrates. In general, expression of *cytochrome P450, family 19, subfamily a* (*cyp19a*; also named aromatase), which converts testosterone to E2, coincides with the later period of gonadal differentiation, and thus, estrogens seem to determine the gonadal fate in turtles and crocodilians (Gabriel et al. 2001; Murdock and Wibbels 2003; Yatsu et al. 2016). In case of turtles, the exogenous estrogen has been shown to be associated with ovarian differentiation in both TSD and GSD species; *e.g.*, red-eared slider turtle (*Trachemys scripta*) (Crews et al. 1991), snapping turtle (*Chelydra serpentina*) (Crews et al. 1989), painted turtle (*Chrysemys picta*) (Gutzke and Bull 1986), spiny soft-shelled turtle (Bull et al. 1988) and Chinese soft-shelled turtle (Sun et al. 2017). Most vertebrates possess two ESR subtypes, ESR1 and ESR2; however, to date, distinct roles of ESRs have been characterized in only a limited number of species, and thus, ESR subtypes which mediate above estrogen-induced ovarian differentiation remains elusive. The current study employed pharmacological manipulation using PPT, which is available for ESR-specific agonist in reptiles (Kohno et al. 2015) and showed that PPT induced ovarian differentiation in ZZ-embryos of the Chinese soft-shelled turtle.

Previous reports also supported the idea that ESR1 is essential for ovarian differentiation. In the red-eared turtle, a TSD animal, ESR1 expression levels spike at stage 19 (during temperature-sensitive period), and the peak of the expression at female-producing condition is higher than that of the expression at the male-producing condition. While ESR1 expression falls during ovarian differentiation, ESR2 mRNA increases at stage 23 (after the sex is determined) at female-producing

condition (Ramsey and Crews 2007). These data suggest that both ESR1 and ESR2 are involved in ovarian differentiation sequentially, but ESR1 could play more important roles for determining the ovarian fate in the red-eared turtle. In American alligator, exogenous exposure of PPT induces ovarian differentiation even at male-producing condition, but such sex reversal is not observed by the exposure of WAY 200070, an ESR2-specific agonist (Kohno et al. 2015), also suggesting a critical role of ESR1 for ovarian differentiation. These observations coincide with the current results; however, further study is needed regarding the contribution of ESR2 in the soft-shelled turtle.

In the current experiment, some of the ZZ-embryos administrated with E2 and PPT exhibited thicker cortexes but with the absence of lacunae structure. This is probably due to delayed gonadal growth and development, which is induced by E2 or PPT administration. In sea turtle (*Lepidochelys olivacea*), E2 administration can induce male to female sex reversal, but their ovaries exhibited a remarkably small size (Diaz-Hernandez et al. 2015; Diaz-Hernandez et al. 2017).

In addition, it has been shown that the exogenous exposure of PPT during embryonic development induces significant enlargement of the Müllerian duct with the epithelial hypertrophy and precocious gland development, whereas E2 only induces hypertrophic phenotypes in the luminal epithelium in a previous study of American alligator (Doheny et al. 2016; Kohno et al. 2015) and red-eared turtle (Dodd and Wibbels 2008). We showed that this was also the case in the soft-shelled turtles. ESR1 basically mediates estrogen effects in female reproductive tracts in mouse while ESR2 antagonized ESR1-mediated estrogenic action. Thus, the two ESRs often have a yin/yang relationship (Gustafsson 2016). E2 can activate both ESR1 and ESR2, but PPT only stimulates ESR1 pathway. It is possible that an excessive ESR1-mediated action promotes glandular development in the Müllerian duct. It may also be possible that over-dosage of estrogen treatment influenced pituitary hormone levels and/or activity, and secondarily affected steroid hormone production in gonads, although it remains elusive whether feedback between gonads and hypothalamo-pituitary system was established during embryos in reptiles. In the red-eared turtles, exogenous E2 administration *in ovo* inhibits the Müllerian duct development, resulting into the loss of oviduct in the hatchlings regardless of ovarian development (Dodd and Wibbels 2008; Matsumoto et al. 2014), but all embryos administered with chemicals in the current study showed developed the Müllerian ducts.

To date, several lines of analyses implicate estrogenic endocrine disrupting chemicals in reproductive abnormalities in wildlife. However, knowledge of the reptilian endocrine system has been limited. The current results would be helpful to understand the mechanisms of endocrine disruption issues in reptiles.

CONCLUSIONS

The current study has revealed that ESR1 plays an important role in ovarian differentiation by the exogenous estrogen administration in the Chinese soft-shelled turtles. The activation of ESR1 also induced the Müllerian duct enlargement and aberrant glandular development. Although the relative importance of ESR2 warrants further study, the balance between ESR1 and ESR2 may be important for the normal Müllerian duct development.

List of abbreviations

Amh, Anti-Müllerian hormone
Cyp19a, cytochrome P450, family 19, subfamily a.
DES, diethylstilbestrol.
Dmrt1, DM (Doublesex and mab-3) related transcription factor 1.
Dmy, DM (Doublesex and mab-3) -domain gene on the Y chromosome.
E2, 17β-estradiol.
Esr, estrogen receptor.
EW, egg weight.
GAM, gonad-adrenal-mesonephros.
GSD, genotypic sex determination.
KO, knockout.
PFA, paraformaldehyde.
PPT, 4,4’,4’’-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol.
rRNA, ribosomal RNA.
Sox9, SRY (sex determining region Y)-box transcription factor 9.
TGFβ, transforming growth factor β
TSD, temperature-dependent sex determination.

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Authors’ contributions: SMi and KT conceived and designed the study. SMa and SS carried out

sampling and histology with KT, KM, GY, HA and SMi. KT and SMa performed genotyping. SMa, HA and SMi performed data analysis, and SMi produced majority of the figures and wrote the manuscript. KT, SMa, SS, KM, GY and HA handled eggs and administered the chemicals. All authors discussed the results and contributed to the manuscript.

Competing interests: All authors declare that they have no conflict of interest.

Availability of data and materials: The datasets during the current study available from the corresponding author on request.

Consent for publication: Not applicable

Ethics approval consent to participate: Ethics approval consent to participate: All animal experiments were carried out under the guidelines specified by the Animal Care and Use Committee at the Tokyo University of Science.

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