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Estrogen Receptor 1 (ESR1) Agonist Induces Ovarian Differentiation and Aberrant Müllerian Duct Development in the 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 of reptiles have indicated that administration of exogenous estrogens during embryonic development causes 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 ZZ/ZW sex chromosomes. In order to assess the role of estrogens and their signaling pathway on sex determination and differentiation, *P. sinensis* 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 during 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 that 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.

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 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

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2001). In the chicken, the sex reversal phenotype can be experimentally induced by injecting estrogens into the eggs or by inhibiting estrogen production (Elbrecht and Smith 1992; Scheib 1983). Some reptiles exhibit temperature-dependent sex determination (TSD) by which 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 temperatures. Although the function of estrogen in ovarian development remains largely elusive, it has been reported that 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, the Müllerian duct differentiates in females. The Müllerian duct, a primordium of the female reproductive tracts in vertebrates, primarily develops in embryos of both sexes. Then, the Müllerian duct in females continues to develop and differentiate into the oviduct, uterus and vagina, whereas in males, it regresses under the influence of anti-Müllerian hormone (Amh; also called Müllerian inhibiting substance), which is secreted from the testis. In birds, differentiation of the Müllerian duct is—at least in part—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 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 to prevent miscarriages in the 1940s (Herbst 1981 2000; Herbst et al. 1971; Robboy et al. 1984). It is now well-known that *in utero* exposure to DES induces vaginal clear-cell adenocarcinoma and various malformations in the reproductive tracts of young women. *In utero* exposure to DES also induces uterine atypical hyperplasia and adenocarcinoma in mouse (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 exhibits 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. One such study found that ESR1-mediated estrogenic activity in mouse uterus contributes to 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*). Exposing 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 an enlarged Müllerian duct and 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 through the TSD and GSD systems. For example, the sex determination of the freshwater turtle, *Malayemys macrocephala*, uses the TSD system (Pewphone et al. 2020). The Chinese soft-shelled turtle, *Pelodiscus sinensis*, belongs to the family Trionychidae, and exhibits GSD with ZZ/ZW sex chromosomes (Kawagoshi et al. 2009; Kawai et al. 2007; Mu et al. 2015). In this species, the 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 examined their effects on the ovarian differentiation and Müllerian duct development in ZZ embryos. The administration of 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, Chinese soft-

shelled turtles undergo ovarian differentiation and reproductive tract anomalies induced by exogenous estrogen exposure through ESR1 during embryonic development.

MATERIALS AND METHODS

Animals

Fertilized turtle eggs were purchased from Tsujimura farm (Itoda, Fukuoka, Japan). The eggs were brought to the laboratory and incubated in moist vermiculite medium (Setogahara Kaen, Midori, Gunma, Japan) at 29 ± 1°C. The developmental stages were estimated by the candling technique using 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 be approaching stage 16, a stage when 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) was 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 eggshells. 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 around stages 26 and 27 (37–41 days 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 the remaining tissue was stored at -80°C for genotyping.

Genetic sexing

The genetic sex of each embryo was determined by quantitative PCR as previously described (Literman et al. 2004), but instead of GAPDH, the 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. 18S ribosomal RNA (rRNA) gene copy number (forward: GAG TAT GGT TGC AAA GCT GAA A and reverse: CGA GAA AGA GCT ATC AAT CTG T) was normalized against the 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 more copies of the 18S rRNA gene than the Z chromosome (Literman et al. 2004). Hence, a higher value of ΔCt (β-actin - 18S rRNA) indicates a 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 a standard procedure. The histology of the Müllerian duct was analyzed at the level corresponding to the middle of the gonads. Phenotypic features of the gonads were determined by three independent blinded investigators.

RESULTS

Ovarian differentiation in genetic males by E2 and PPT treatments

In order to assess the role of estrogens on the sex determination and differentiation processes, 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 stages 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 that were administered 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).

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 the Müllerian duct is almost regressed in the control ZZ-embryos (Table 2). E2-treated embryos displayed an increase in the development of the prominent Müllerian duct in the genetic males (Table 2 and Fig. 2B). In embryos from PPT-treated eggs, the

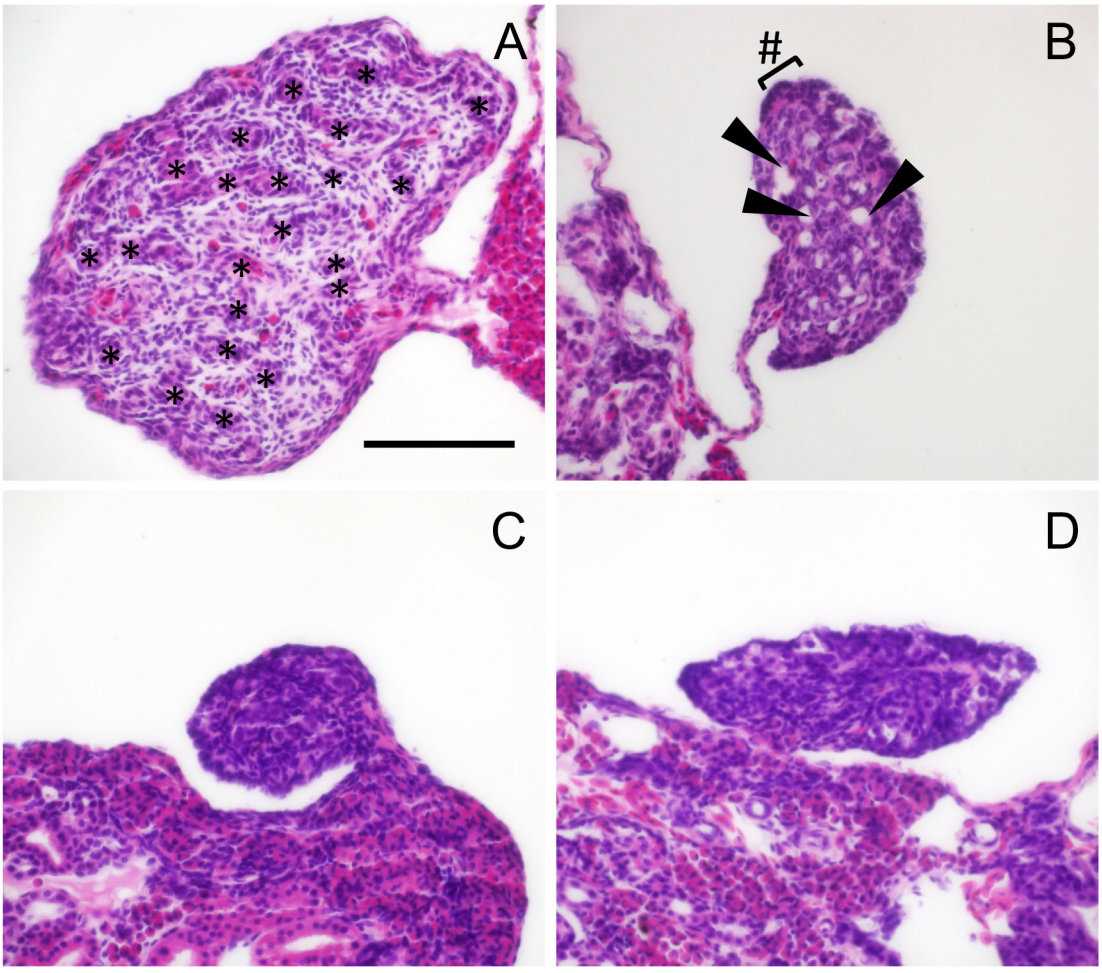

Fig. 1. Gonadal differentiation in embryos exposed to E2 or PPT. (A–D) Representative cross-sections of HE stained gonads at stage 26 for the control male (A), control female (B), E2-exposed male (C) and PPT-exposed male (D). Control testis showing well-developed seminiferous tubules (*); the control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

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

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.

DISCUSSION

The modes of sex determination among vertebrates exhibit remarkable diversity. In particular, reptiles comprise an intriguingly wide range of sex determination systems, ranging from GSD to TSD.

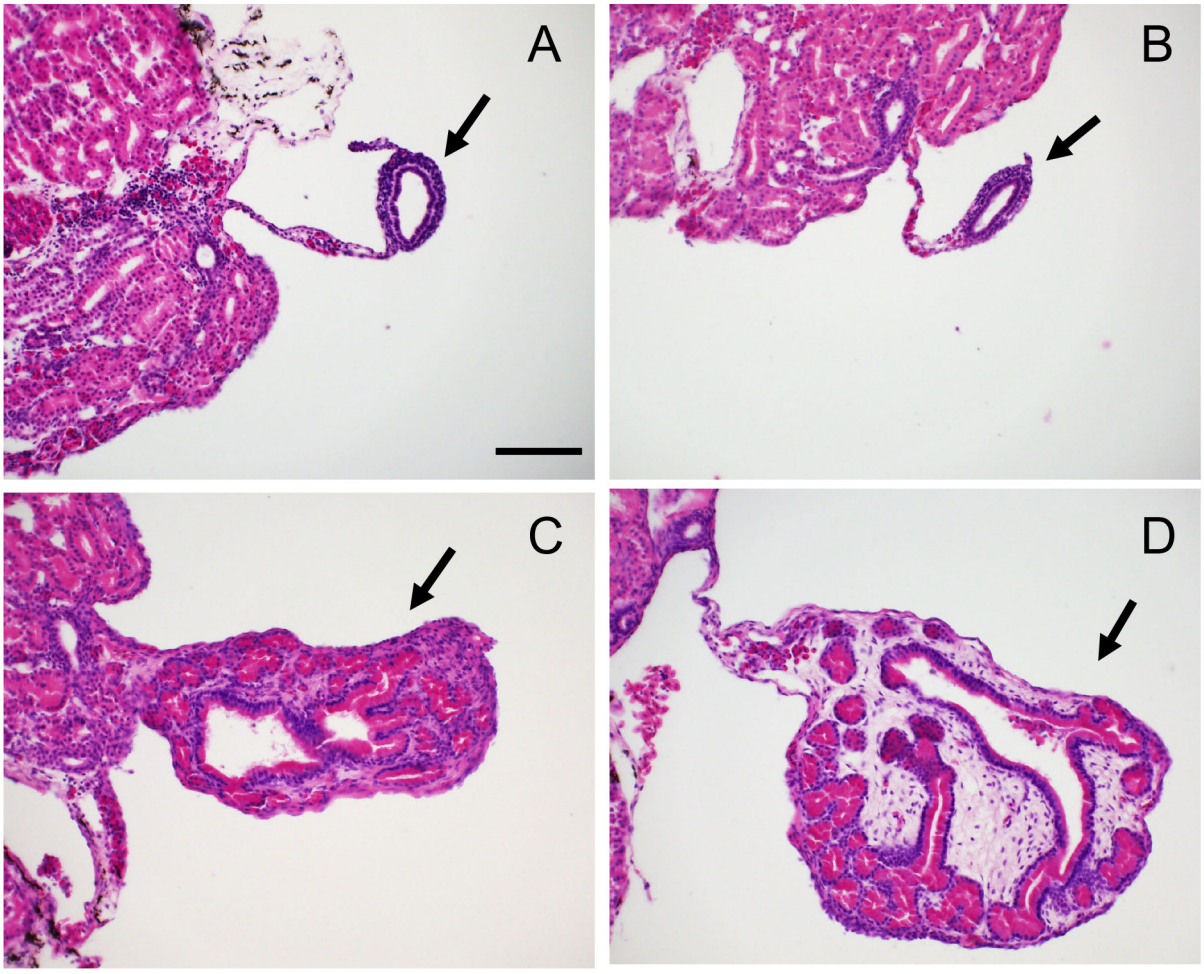

Fig. 2. Histology of the Müllerian duct in embryos exposed to E2 or PPT. (A–D) Representative cross-sections of HE stained Müllerian (arrows) ducts at stage 26 for control female (A), E2-exposed male (B), PPT-exposed males (C) and PPT-exposed females (D). Note the glandular development in the Müllerian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

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

		Müllrian duct	
Treatment	Genetic sex (number of embryos)	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)

Primary factors of sex determination in reptiles, therefore, largely remain elusive. Recent studies have 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 the transforming growth factor β (TGFβ) signaling family, are strong candidates for a trigger that leads to 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 that other factors are involved in regulating the expressions of these genes and testicular differentiation. Although ovarian fate determination has not been cleared either, there is no doubt that estrogen signaling is the most critical mediator of ovarian differentiation, particularly in non-mammalian vertebrates. In general, the 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). For 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, the distinct roles of ESRs have only been characterized by a limited number of species, and thus ESR subtypes that mediate above estrogen-induced ovarian differentiation remain 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 the temperature-sensitive period), and the peak expression under the female-producing condition is higher than that under 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 the American alligator, exogenous exposure of PPT induces ovarian differentiation even under the male-producing condition, but such sex reversal is not observed under exposure to WAY 200070, an ESR2-specific agonist (Kohno et al. 2015), also suggesting a critical role of ESR1 in ovarian differentiation. These observations concur with the current results; however, further research is needed regarding the contribution of ESR2 in the soft-shelled turtle.

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

In addition, it has been shown that the exogenous exposure of PPT during embryonic development induces a 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 the American alligator (Doheny et al. 2016; Kohno et al. 2015) and red-eared turtle (Dodd and Wibbels 2008). We showed that this is 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 an over-dosage of estrogen influenced pituitary hormone levels and/or activity, and secondarily affected steroid hormone production in gonads, although it remains unclear whether feedback between gonads and the hypothalamo-pituitary system was established during embryos in reptiles. In the red-eared turtle, exogenous E2 administration *in ovo* inhibited Müllerian duct development, resulting in the loss of the oviduct in the hatchlings, regardless of ovarian development (Dodd and Wibbels 2008; Matsumoto et al. 2014), but all embryos that were administered chemicals in the current study 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 will help 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 turtle. 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 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.

REFERENCES

Barske LA, Capel B. 2010. Estrogen represses sox9 during sex determination in the red-eared slider turtle *Trachemys scripta*. Dev Biol **341:**305–314. doi:10.1016/j.ydbio.2010.02.010.

Bull JJ, Gutzke WH, Crews D. 1988. Sex reversal by estradiol in three reptilian orders. Gen Comp Endocrinol **70:**425–428. doi:10.1016/0016-6480(88)90117-7.

Crews D, Bull JJ, Wibbels T. 1991. Estrogen and sex reversal in turtles: A dose-dependent phenomenon. Gen Comp Endocrinol **81:**357–364. doi:10.1016/0016-6480(91)90162-y.

Crews D, Wibbels T, Gutzke WH. 1989. Action of sex steroid hormones on temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*). Gen Comp Endocrinol **76:**159–166. doi:10.1016/0016-6480(89)90042-7.

Diaz-Hernandez V, Marmolejo-Valencia A, Merchant-Larios H. 2015. Exogenous estradiol alters gonadal growth and timing of temperature sex determination in gonads of sea turtle. Dev Biol **408:**79–89. doi:10.1016/j.ydbio.2015.05.022.

Diaz-Hernandez V, Vazquez-Gomez A, Marmolejo-Valencia A, Montano LM, Merchant-Larios H. 2017. 17β-estradiol modulates cell proliferation of medullary cords during ovarian differentiation of the *Lepidochelys olivacea* sea turtle. Dev Biol **431:**263–271. doi:10.1016/j.ydbio.2017.09.008.

Dodd KL, Wibbels T. 2008. Estrogen inhibits caudal progression but stimulates proliferation of developing Mullerian ducts in a turtle with temperature-dependent sex determination. Comp Biochem Physiol A Mol Integr Physiol **150:**315–319. doi:10.1016/j.cbpa.2008.04.002.

Doheny BM, Kohno S, Parrott BB, Guillette LJ Jr. 2016. In ovo treatment with an estrogen receptor alpha selective agonist causes precocious development of the female reproductive tract of the American alligator (*Alligator mississippiensis*). Gen Comp Endocrinol **238:**96–104. doi:10.1016/j.ygcen.2016.02.026.

Elbrecht A, Smith RG. 1992. Aromatase enzyme activity and sex determination in chickens. Science **255:**467–470. doi:10.1126/science.1734525.

Gabriel WN, Blumberg B, Sutton S, Place AR, Lance VA. 2001. Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. J Exp Zool **290:**439–448. doi:10.1002/jez.1087.

Gustafsson JA. 2016. Historical overview of nuclear receptors. J Steroid Biochem Mol Biol **157:**3–6. doi:10.1016/j.jsbmb.2015.03.004.

Gutzke WH, Bull JJ. 1986. Steroid hormones reverse sex in turtles.

Gen Comp Endocrinol **64:**368–372. doi:10.1016/0016-6480(86)90070-5.

Hayes TB. 1998. Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. J Exp Zool **281:**373–399.

Herbst AL. 1981. Clear cell adenocarcinoma and the current status of DES-exposed females. Cancer **48:**484–488. doi:10.1002/1097-0142(19810715)48:1+<484::aid-cncr2820481308>3.0.co;2-x.

Herbst AL. 2000. Behavior of estrogen-associated female genital tract cancer and its relation to neoplasia following intrauterine exposure to diethylstilbestrol (DES). Gynecol Oncol **76:**147–156. doi:10.1006/gyno.1999.5471.

Herbst AL, Ulfelder H, Poskanzer DC. 1971. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med **284:**878–881. doi:10.1056/NEJM197104222841604.

Hermansson A. 2007. Effects on the reproductive system in domestic fowl (*Gallus domesticus*) after embryonic exposure to estrogenic substances. Dept. of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Iwamatsu T, Kobayashi H, Hamaguchi S, Sagegami R, Shuo T. 2005. Estradiol-17beta content in developing eggs and induced sex reversal of the medaka (*Oryzias latipes*). J Exp Zool A Comp Exp Biol **303:**161–167. doi:10.1002/jez.a.120.

Kawagoshi T, Uno Y, Matsubara K, Matsuda Y, Nishida C. 2009. The ZW micro-sex chromosomes of the Chinese soft-shelled turtle (*Pelodiscus sinensis*, Trionychidae, Testudines) have the same origin as chicken chromosome 15. Cytogenet Genome Res **125:**125–131. doi:10.1159/000227837.

Kawai A, Nishida-Umehara C, Ishijima J, Tsuda Y, Ota H, Matsuda Y. 2007. Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. Cytogenet Genome Res **117:**92–102. doi:10.1159/000103169.

Kobayashi H, Iwamatsu T. 2005. Sex reversal in the medaka *Oryzias latipes* by brief exposure of early embryos to estradiol-17β. Zoolog Sci **22:**1163–1167. doi:10.2108/zsj.22.1163.

Kohno S, Bernhard MC, Katsu Y, Zhu J, Bryan TA, Doheny BM, Iguchi T, Guillette LJ Jr. 2015. Estrogen receptor 1 (esr1; α), not esr2 (β), modulates estrogen-induced sex reversal in the American alligator, a species with temperature-dependent sex determination. Endocrinology **156:**1887–1899. doi:10.1210/en.2014-1852.

Literman R, Badenhorst D, Valenzuela N. 2004. QPCR-based molecular sexing by copy number variation in rRNA genes and its utility for sex identification in soft-shell turtles. Methods Ecol Evol **5:**872–880. doi:10.1111/2041-210X.12228.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA **90:**11162–11166. doi:10.1073/pnas.90.23.11162.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M. 2002. Dmy is a y-specific DM-domain gene required for male development in the medaka fish. Nature **417:**559–563. doi:10.1038/nature751.

Matsumoto Y, Hannigan B, Crews D. 2014. Embryonic PCB exposure alters phenotypic, genetic, and epigenetic profiles in turtle sex determination, a biomarker of environmental contamination. Endocrinology **155:**4168–4177. doi:10.1210/en.2014-1404.

Mu Y, Zhao B, Tang WQ, Sun BJ, Zeng ZG, Valenzuela N, Du WG. 2015. Temperature-dependent sex determination ruled out in the Chinese soft-shelled turtle (*Pelodiscus sinensis*) via molecular cytogenetics and incubation experiments across populations. Sex Dev **9:**111–117. doi:10.1159/000373903.

Murdock C, Wibbels T. 2003. Cloning and expression of aromatase in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol **130:**109–119. doi:10.1016/s0016-6480(02)00573-7.

Nakamura M. 2010. The mechanism of sex determination in vertebrates-are sex steroids the key-factor? J Exp Zool A Ecol Genet Physiol **313:**381–398. doi:10.1002/jez.616.

Newbold RR, Bullock BC, McLachlan JA. 1990. Uterine adenocarcinoma in mice following developmental treatment with estrogens: A model for hormonal carcinogenesis. Cancer Res **50:**7677–7681.

Pewphong R, Kitana J, Kitana N. 2020. Chronology of gonadal development in the Malayan snail-eating turtle *Malayemys macrocephala*. Zool Stud **59:**20. doi:10.6620/ZS.2020.56-20.

Piferrer F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture **197:**229–281. doi:10.1016/S0044-8486(01)00589-0.

Ramsey M, Crews D. 2007. Steroid signaling system responds differently to temperature and hormone manipulation in the red-eared slider turtle (*Trachemys scripta elegans*), a reptile with temperature-dependent sex determination. Sex Dev **1:**181–196. doi:10.1159/000102107.

Robboy SJ, Noller KL, O'Brien P, Kaufman RH, Townsend D, Barnes AB, Gundersen J, Lawrence WD, Bergstrahl E, McGorray S, et al. 1984. Increased incidence of cervical and vaginal dysplasia in 3,980 diethylstilbestrol-exposed young women. Experience of the national collaborative diethylstilbestrol adenosis project. JAMA **252:**2979–2983. doi:10.1001/jama.1984.03350210027024.

Scheib D. 1983. Effects and role of estrogens in avian gonadal differentiation. Differentiation **23 Suppl:**S87–92. doi:10.1007/978-3-642-69150-8_15.

Sun W, Cai H, Zhang G, Zhang H, Bao H, Wang L, Ye J, Qian G, Ge C. 2017. *Dmrt1* is required for primary male sexual differentiation in Chinese soft-shelled turtle *Pelodiscus sinensis*. Sci Rep **7:**4433. doi:10.1038/s41598-017-04938-5.

Tohyama S, Miyagawa S, Lange A, Ogino Y, Mizutani T, Ihara M, Tanaka H, Tatarazako N, Kobayashi T, Tyler CR, Iguchi T. 2016. Evolution of estrogen receptors in ray-finned fish and their comparative responses to estrogenic substances. J Steroid Biochem Mol Biol **158:**189–197. doi:10.1016/j.jsbmb.2015.12.009.

Tokita M, Kuratani S. 2001. Normal embryonic stages of the Chinese softshelled turtle *Pelodiscus sinensis* (Trionychidae). Zoolog Sci **18:**705–715. doi:10.2108/zsj.18.705.

Yamamoto T. 1962. Hormonic factors affecting gonadal sex differentiation in fish. Gen Comp Endocrinol **Suppl 1:**341–345. doi:10.1016/0016-6480(62)90107-7.

Yatsu R, Miyagawa S, Kohno S, Parrott BB, Yamaguchi K, Ogino Y, Miyakawa H, Lowers RH, Shigenobu S, Guillette LJ Jr, Iguchi T. 2016. RNA-seq analysis of the gonadal transcriptome during *Alligator mississippiensis* temperature-dependent sex determination and differentiation. BMC Genomics **17:**77. doi:10.1186/s12864-016-2396-9.

Zhou Y, Sun W, Cai H, Bao H, Zhang Y, Qian G, Ge C. 2019. The role of anti-Müllerian hormone in testis differentiation reveals the significance of the TGF-β pathway in reptilian sex determination. Genetics **213:**1317–1327. doi:10.1534/genetics.119.302527.