Chapter 6
Revisiting the Role of Steroid Hormones in Gonadal Fate Determination

Kiyoshi Kikuchi and Takashi Koyama

Abstract Early in the twentieth century, it was posited that sexual differentiation in vertebrates is under the control of hormones. Referred to as the hormone theory of sex differentiation, it gave rise to the specific hypothesis that sex steroids control gonadal fate decisions. Although this hypothesis is no longer supported for placental mammals, there seems to be some uncertainty in the case of nonmammalian vertebrates, especially fish. Based on the success in generating complete sex reversals of medaka fish by exogenous steroid administration in the 1950s, Yamamoto hypothesized that sex steroids were endogenous sex inducers in fish embryos. While this model has provided a framework for research in fish developmental biology and physiology, the validity of the hypothesis has been brought into question, since experimental evidence has been lacking. However, the recent identification of master sex-determining (MSD) genes in some fish clearly supports part of this hypothesis, since estrogens play a role in determining gonadal fate. This commentary aims to give a brief overview of the developments related to the steroid theory and focuses on the role of sex steroids in gonadal sex determination and early sex differentiation in nonmammalian species, with a special emphasis on fish. The recent partial validation of Yamamoto's model should nevertheless lead to a better understanding of the evolution of sex-determining cascades in vertebrates and encourage the exploration of the target genes of estrogen signaling leading to gonadal fate specification.

Keywords Steroid hormones · Gonadal sex determination · Fish · Nonmammalian vertebrates

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K. Kikuchi (✉)
Fisheries Laboratory, University of Tokyo, Hamamatsu, Japan
e-mail: akikuchi@mail.ecc.u-tokyo.ac.jp

T. Koyama
Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki, Japan
e-mail: takashi.k1201@gmail.com

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6.1 Introduction

The idea that hormones can affect sexual dimorphism and sex determination can be traced back to the mid-nineteenth century (Berthold 1849; Josso 2008). However, more easily traceable history of this theory begins with Bouin and Ancel (1903), who suggested that the secretions of the fetal pig testes were responsible for the male characteristics in adults (termed the interstitial theory at that time) (Bouin and Ancel 1903; Scheib 1983; Josso 2008). However, there was scathing criticism of this theory (Klein and Bouin 1970), which was revived only in 1916 by Lillie, and independently by Keller and Tandler, all of whom studied freemartins, genetic female (XX) cattle that are infertile with partially masculinized phenotypes in gonads, reproductive tracts, etc. (Bouin and Ancel 1903; Lillie 1916; Scheib 1983). The hormonal theory inspired a long series of experiments such as grafting experiments in amphibians, initiated by Burns (1925) and pursued by Humphrey and Witschi (Burns 1961; Jost 1971; Josso 2008). In addition, the availability of purified sex hormones in the mid-1930s led to numerous administration experiments, first in birds and followed by amphibians and fish (for a review of experiments in fish, see Piferrer 2001, and for those in other vertebrates, see Burns 1961), which gave rise to the hypothesis attributed to the hormonal theory that sex steroids control gonadal fate determination. Although this idea is no longer supported for placental mammals, it appears to still have some traction in the case of nonmammalian vertebrates, especially fish.

After a series of experiments in the 1950s and 1960s with medaka fish (*Oryzias latipes*), in which exogenous sex steroids were administered and functional sex reversal was first obtained in fish (Yamamoto 1953, 1958, 1959a, b, 1961), Yamamoto hypothesized that the sex steroids were the endogenous sex inducers that determined gonadal fate in fish (Yamamoto 1962, 1969). Since then, numerous studies have experimented with steroid treatments in fish (e.g., Piferrer 2001; Guiguen et al. 2010). While Yamamoto's hypothesis provided an important framework for research that has continued until today, it has not been without controversy since many have questioned the validity of the hypothesis (Devlin and Nagahama 2002; Piferrer and Guiguen 2008; Guiguen et al. 2010; Li et al. 2019; Nagahama et al. 2021). As a result, there have been modifications to Yamamoto's framework (as detailed in Piferrer and Guiguen 2008; Guiguen et al. 2010), which can be summarized as follows.

1. Bogart (1987) proposed a theoretical model where the balance between androgens and estrogens, rather than their absolute amount, at a critical period during development, was the determinant of gonadal sex in vertebrates. This model has since been shown to not be the case in mammals (reviewed in Cutting et al. 2013).
2. Contrary to Bogart's modification of the Yamamoto hypothesis, it appears that changes in androgen levels (leading to changes in the balance between androgens and estrogens) may not play a critical role during early testicular differentiation after all, at least in the case of fish (Nagahama 2005). Rather, an estrogen-centric model was proposed, in which estrogens would be required for ovarian

differentiation, while their absence would be required for testicular differentiation (Kobayashi and Nagahama 2009; Guiguen et al. 2010).

3. In fish, 11-ketotestosterone (11-KT) rather than testosterone is one of the most potent steroids for male sex determination and/or differentiation (Piferrer et al. 1993; reviewed in Borg 1994; Tokarz et al. 2015).

In the following, I will provide a brief overview of the history of the hormonal theory of sex differentiation, while elaborating on some salient research selected from older studies in addition to the latest ones.

6.2 A Short History of the Hormonal Theory of Sex Differentiation

6.2.1 Hormones and Gonadal Sex Determination

The "hormone theory" has been thought to encompass hypotheses relating to hormonal control of several specific phenomena, such as gonadal sex determination, early gonadal differentiation, development of the sex cords and genital structure, and secondary sexual characteristics in adults (Burns 1961; Jost 1971). Among those, this commentary primarily focuses on models related to the gonadal sex determination by which the bipotential embryonic gonad commits to either the ovarian or testicular fate (Capel 2017). The subsequent process is often termed gonadal sex differentiation in which the molecular and cellular processes lead to the formation of morphologically and functionally distinct gonads, either the ovaries or testes (Devlin and Nagahama 2002; Adolfi et al. 2021). It should be noted that the conceptual distinction between the two processes, determination and differentiation, is not made in the old literature, and even today it is often difficult to clearly identify the transition between the two processes except for well-studied species such as mice.

6.2.2 Freemartins

Freemartin is a genetic female cattle with infertile ovaries and partially masculinized phenotype, typically as a result of being born as a twin to a male. At birth, freemartin ovaries are hypoplastic and depleted of germ cells, and they sometimes contain seminiferous tubules that would be formed in testes (Josso 2008; Harikae et al. 2012). In most cattle twins, the blood vessels between the two placentas become connected, thus creating a shared circulation (Fig. 6.1). In the early nineteenth century, it was hypothesized that masculinization was due to the transmission of a hormone produced by the gonads of the male twin to the female twin via the shared circulation (Keller and Tandler 1916; Lillie 1916; reviewed in Burns 1961; Padula 2005). Several hormones such as testosterone and anti-Müllerian hormone have been

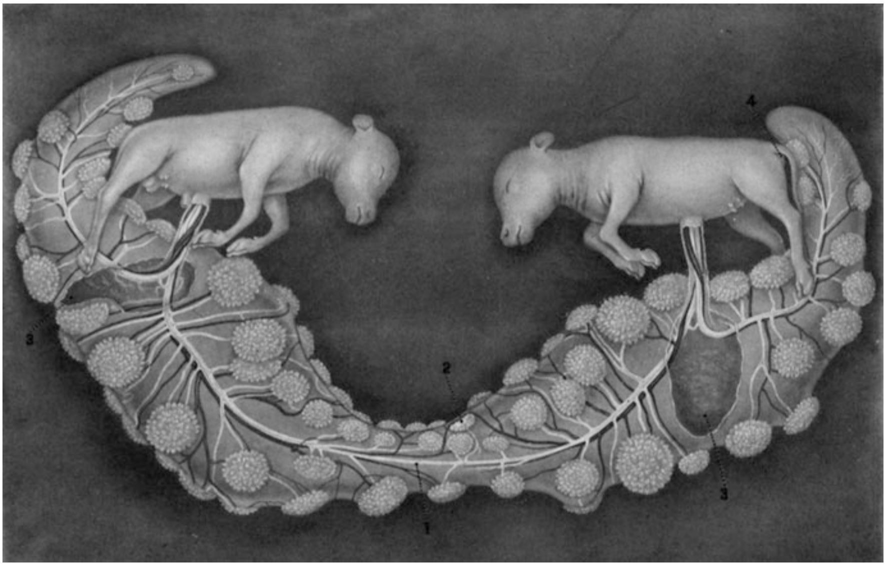

Fig. 6.1 Placentas connected between two cattle twins (Lillie 1916). The male is on the left and a freemartin on the right. (Reprinted with permission of Wiley-Liss, a subsidiary of John Wiley & Sons)

implicated, but the causative factor has been elusive (Padula 2005; Miura et al. 2019).

6.2.3 *Parabiosis and Gonadal Transplantation in Amphibians*

Parabiosis experiments conducted in the 1920s and 1930s on amphibian larvae (reviewed by Jost 1971) appeared to support the hormone theory (Fig. 6.2). In these experiments, the two embryos were conjoined in the manner of Siamese twins, providing an opportunity to test the possibility of hormonal action through a common circulation. Although the degree and direction (from the ovary to the testis or the reverse) varied greatly depending on the species under study and various experimental conditions, partially sex-reversed gonads were observed (reviewed in Burns 1961).

The parabiosis method was soon improved by the development of a technique by which the prospective gonad-forming tissue from a donor embryo was transplanted to a host (Humphrey 1928) (Fig. 6.3). This method has yielded results that have in general confirmed and extended those obtained by parabiosis. Remarkably, Humphrey (1945) obtained a fertile male by sex-reversing the prospective ovary (the left gonad) through orthotopic transplantation of the donor testis primordium to the right

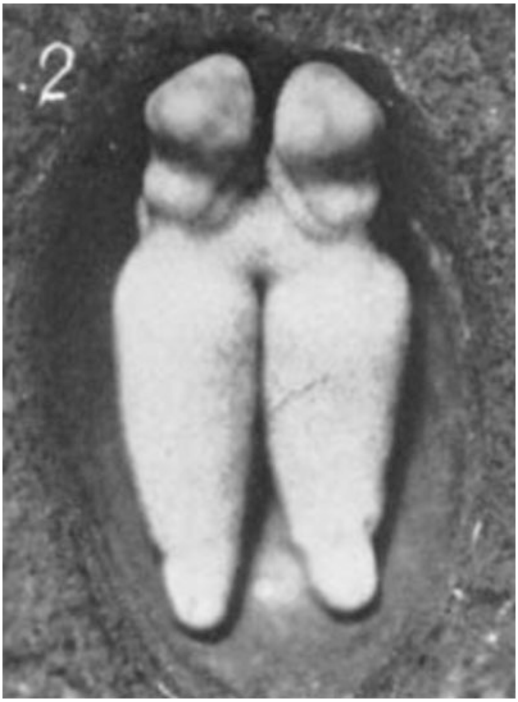

Fig. 6.2 A ventral view of two *Amblystoma* embryos joined in parabiosis (Burns 1925). (Reprinted with permission of Wiley-Liss, a subsidiary of John Wiley & Sons)

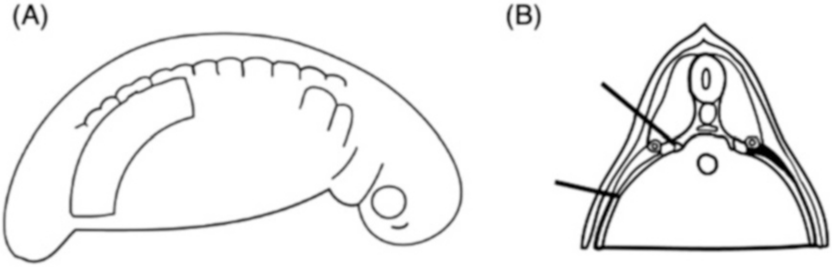

Fig. 6.3 Humphrey's orthotopic transplantation. (**a**) Position of the gonad- and mesonephros-forming area in the embryo (framed). This primordium was excised and replaced into the corresponding part of the donor embryo. (**b**) Cross-section of the host at a later stage shows the position of the implanted tissues (between thick lines) on the left. The Wolffian duct is lateral to the mass of primordial germ cells, and above the gonad- and mesonephros-forming regions. (Illustration modified from Humphrey (1928) and Burns (1961))

side of host embryos and confirmed the functionality of host-derived sperm and the sex chromosome constitution by means of crossing experiments.

6.2.4 Exogenous Sex Steroids

The hypothesized effects of diffusible substances on gonadal sex determination appeared to gain further traction by the sex reversals produced after 1935 through exogenous sex steroid administration in nonmammalian species (reviewed in Burns 1961; Bull et al. 1988). Male-to-female sex reversals occurred after treatment with estrogens in fish, amphibians, reptiles, and birds (reviewed in Piferrer 2001; Pieau and Dorizzi 2004; Lance 2009; Nakamura 2010; Flament 2016). While androgen treatments resulted in sex reversal from female to male in fish and some amphibians, it was far less effective and prone to paradoxical results (e.g., feminizing effect of androgens) in reptiles and birds (Burns 1961; Pieau et al. 1999; Ganesh and Raman 1995). Exposure to exogenous estrogens prior to gonadal sex differentiation can cause almost complete gonadal sex reversal in male marsupials as well (Burns 1955; Coveney et al. 2001; Pask et al. 2010). However, the embryonic gonads in placental mammals were found to be resistant to exogenous sex steroid (reviewed in Burns 1961), although estrogen appears to have a role in maintaining the postnatal ovary (Britt and Findlay 2002). As a result of these findings, it came to be considered that the hormonal theory on primary sex determination was only applicable to nonmammalian vertebrates (Burns 1961; Cutting et al. 2013).

Note that there appears to be a gradual evolutionary gain of resistance to exogenous estrogen in gonadal sex determination or differentiation, from fish to mammals (Cutting et al. 2013). It has been hypothesized that mammals discarded the use of steroid hormones in sex determination and/or early gonadal differentiation with the evolution of placenta and intrauterine development to resist the influence of maternal estrogen (Wolf 1999; Cutting et al. 2013; Capel 2017).

6.2.5 Fish

The earliest experiments that administered sex steroid hormones to fish to control their gonadal sex can be traced to 1937 (Berkowitz 1937; Castelnuovo 1937; Padoa 1937). However, intersex fish were produced in the early experiments (reviewed in Piferrer 2001). Yamamoto (1953) appears to have been the first to achieve complete (functional) sex reversal in which male-to-female reversal of medaka fish was induced by oral administration of estrogen. In this work, the genetic sex of the fish and the fertility were experimentally confirmed. Based on a series of experiments that followed (e.g., Yamamoto 1958, 1959a, b), Yamamoto (1962, 1969) hypothesized that endogenous estrogens are female-inducing substances (female inducers) that drive ovarian differentiation during the sex determination period in fish, and androgens are male-inducing ones (male inducers) that trigger testicular differentiation (Yamamoto 1962, 1969) (Fig. 6.4).

The term, “inducers,” appears to have been adapted by Yamamoto from that of Witschi (1914) who had postulated that, in amphibians, the inducing substances, named medullarin and corticin, behaved like the hypothetical-inducing substances in

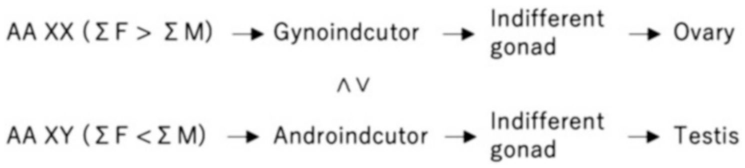

Fig. 6.4 Yamamoto's scheme for the steroid theory of sex inducers. Yamamoto assumed a polygenetic system in which sex is determined mainly by sex-determining genes on the sex chromosomes (X and Y) with influences of many modifiers on autosomes (A), based on (Winge 1934). The genetic information is realized through the action of sex steroids (gynoinductor and androinductor) on indifferent gonads. (Illustration modified from Yamamoto (1969))

the Spemann organizer (Spemann and Hilde 1924), being transmitted by diffusion and having localized effects to induce the formation of either female or male gonads (Witschi 1914; Burns 1961; Gorbman 1979). Interestingly, Witschi consistently assumed the sex inducers to be proteins, rather than sex steroids.

6.3 Are Steroids Natural Inducers of Gonadal Sex Differentiation?

6.3.1 Controversies

Since Yamamoto's experiments, a large number of studies in many fish species have been conducted on the influence of steroid hormones on sex determination and/or differentiation (for review Hunter and Donaldson 1983; Pandian and Sheela 1995). In most cases, the results have shown that when fish are treated with the appropriate dose of sex steroids during the critical time window of sex determination, exogenous estrogen and androgen are able to induce female and male sex reversals, respectively (Piferrer 2001).

While these studies appear to support Yamamoto's model, the effects of exogenous steroid treatments do not necessarily reflect the physiological role of endogenous steroids (e.g., Stewart et al. 2020). Thus, the actual role of endogenous steroids in sex determination, in other words, whether sex steroids are endogenous sex inducers ("direct sex determiners" or "natural sex inducers") has continued to be debated.

6.3.2 Androgen in Model Fish

The controversy regarding the role of androgens during sex determination has been well covered elsewhere (Piferrer and Guiguen 2008; Guiguen et al. 2010; Devlin and Nagahama 2002; Ijiri et al. 2008; Fernandino et al. 2012; Yamaguchi et al. 2010), and therefore, we only briefly mention it here.

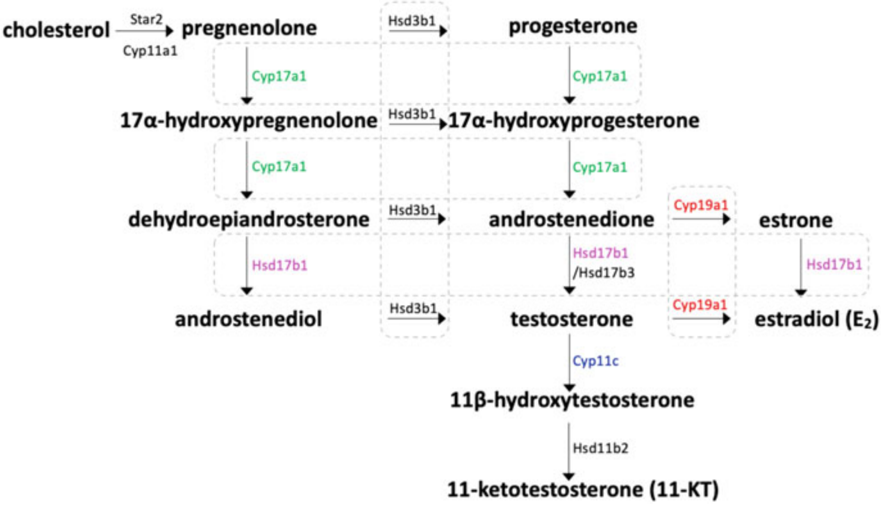

Fig. 6.5 Postulated major pathway of steroidogenesis in teleost fishes. Key enzymes are shown next to arrows at each step of biosynthesis according to (Tokarz et al. 2015; Koyama et al. 2019; Nagahama et al. 2021). Note that all pathways and enzymes are not shown. *Cyp* cytochrome P450, *Hsd* hydroxysteroid dehydrogenase. Cyp17a1 (P450c17-I), shown in green, is required for producing both estrogens and androgens. Cyp11c, shown in blue, is involved in synthesis of 11-ketotestosterone (11-KT) that is one of the most potent androgens in fish (Piferrer et al. 1993). Cyp19a1 (aromatase), shown in red, is required for the synthesis of estradiol (E_2), a major estrogen in ovarian differentiation. Hsd17b1, shown in magenta, is also required for estradiol production. It is speculated that male Seriola homozygous for the hypomorphic allele of *Hsd17b* (the Z-type Hsd17b) can synthesize testosterone/11-KT via postlarval expression of *Hsd17b3* gene (Koyama et al. 2019)

Yamamoto's hypothesis predicts that the level of androgens is higher in the prospective testes during sex determination, as is that of estrogens in the prospective ovaries. However, there have not been many studies on their levels in fish embryonic gonads, likely due to the technical difficulty in measuring steroid levels in the very small fish gonads during the early developmental stages (Guiguen et al. 2010). In contrast to the paucity of studies of the early stages, it has been shown in many fish species that the gonads exhibit contrasting properties in the synthesis of sex steroids during the differentiating stage or later, with marked androgen (specifically 11-KT, a potent androgen in fish) synthesis in the differentiating testes and significant estrogen production in the differentiating ovaries (Guiguen et al. 2018).

While these observations are consistent, if not in direct agreement, with the steroid hypothesis, some studies on gene expression related to androgen production appear to question the role of androgens during sex determination. For example, expression analysis of the *Cyp11c/Cyp11b* gene that encodes a steroid enzyme involved in 11-KT production in the rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) (shown in green in Fig. 6.5) suggested that their expression in the male gonad could not be detected before the first sign of morphological gonadal differentiation (Vizziano et al. 2007; Ijiri et al. 2008; Kobayashi and Nagahama 2009). The implication of these results was that androgens are not yet actively produced at this stage in these species.

More decisive evidence came from the experiments that blocked estrogen synthesis with aromatase (Cyp19a) inhibitors, originally conducted with the aim of clarifying the role of estrogen (Piferrer et al. 1994; Guiguen et al. 2010). Cyp19a1 is a key enzyme in the biosynthesis of estrogens (Nagahama and Yamashita 2008; Lubzens et al. 2010) (shown in red in Fig. 6.5). In these experiments, complete female-to-male sex reversals were obtained in many fish species including the rainbow trout and Nile tilapia (Guiguen et al. 1999; Kitano et al. 2000; Rashid et al. 2007; Kobayashi and Nagahama 2009) (but see an exception below). Given that the presumed levels of androgens are very low at the sex determination period, the results suggested that the mere absence of estrogens is sufficient to drive testicular differentiation, and androgens are not inducers for the development of testes (Kobayashi and Nagahama 2009). Summarizing these results, Guiguen et al. (2010) proposed an estrogen-centric model in which estrogens are required for ovarian differentiation, while their absence would be sufficient to trigger testicular differentiation. The dispensability of androgens in the early testicular differentiation was further confirmed in medaka, tilapia, and zebrafish (*Danio rerio*) by mutant analysis in which the genes required for the production of testosterone and/or 11-KT (*Cyp17a1* or *Cyp11c*) were disrupted (Fig. 6.5) (Sato et al. 2008; Zhang et al. 2020; Zheng et al. 2020).

6.3.3 Androgen in Fish Undergoing Temperature-Induced Masculinization

Apart from their role in sex determination in the absence of stress mentioned above, androgens have recently been suspected to be endogenous drivers for testicular differentiation in temperature-induced masculinization (Fernandino et al. 2012). For example, in the pejerrey (*Odontesthes bonariensis*) and Japanese flatfish (*Paralichthys olivaceus*), larvae exposed to masculinizing temperature during a critical period of sex determination tend to develop testes rather than ovaries, with increased levels of whole-body cortisol (Hattori et al. 2009; Yamaguchi et al. 2010). It was hypothesized that the masculinizing effect could be due to either the concomitant synthesis of 11-KT with cortisol inactivation by Hsd11b (shown in blue in Fig. 6.6) (Fernandino et al. 2012) or the suppressing role of cortisol on the aromatase (Cyp19a1a) gene expression (Yamaguchi et al. 2010) (shown in red in Fig. 6.6).

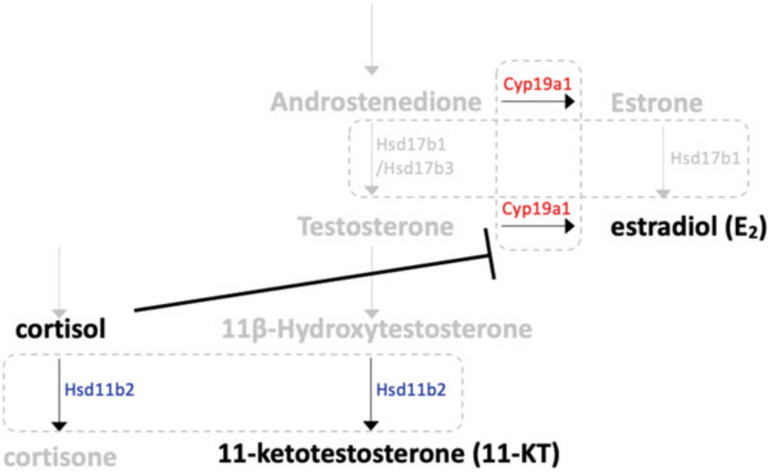

Fig. 6.6 Simplified pathways of biosynthesis of glucocorticoids and sex steroids in fish and two proposed models for temperature-induced masculinization. Yamaguchi et al. (2010) proposed that masculinization is due to the direct suppression of cortisol induced by the temperature stress on the aromatase/Cyp19a1 (red) gene expression (shown as "-|"). On the other hand, Fernandino et al. (2012) postulated that masculinization is due to the concomitant synthesis of 11-KT by Hsd11b2 (blue) whose expression is increased via the inactivation process of cortisol induced by the temperature stress

6.3.4 Androgen in Fish Undergoing in Sequential Sex-Change

In sequential hermaphroditic fishes (sex-changing fishes), it is now generally considered that a rapid decrease of estrogens is important for the initiation of gonadal sex change from females to males, while androgens play roles in the maintenance of the testicular function (Ortega-Recalde et al. 2020; Li et al. 2019; Nagahama et al. 2021). However, the endogenous androgen (11-KT) is suspected to be the potential trigger of the gonadal sex change in some protogynous fishes that exhibit female-to-male sex change. For example, an increase in 11-KT levels without a rapid decrease in estradiol levels was observed in an early transition phase from female to male in the Honeycomb grouper (*Epinephelus merra*) (Bhandari et al. 2003; Alam et al. 2006). Moreover, a recent study revealed that many grouper species commonly contain androgen-producing cells in the tunica of the ovaries, which are likely involved in the increase in 11-KT in the early transition phase, and hence in the onset of the sex change (Murata et al. 2021).

6.3.5 Androgen in a Frog Species

Outside of teleost fish, the endogenous androgen (testosterone) is suspected to play a key role in the early steps of gonadal sex differentiation in a species of amphibians, the Japanese wrinkled frog (*Glandirana* (formerly *Rana*) *rugosa*) (Miura 2007; Oike et al. 2017). In this species, the androgen receptor (*Ar*) gene resides on both sex chromosomes (W and Z) along with *Sox3* and *Sf-1* (Miura 2017), but the expression of the W-type *Ar* allele is barely observed (Oike et al. 2017). Overexpression and knockdown of *Ar* in this frog resulted in the formation of ovotestis, suggesting that *Ar* expression is required fore normal testis development (Oike et al. 2017).

6.3.6 An Inconvenient Truth: Estrogen in Medaka Fish

In contrast to the case of androgens, the importance of estrogens in the gonadal fate determination or the early ovarian differentiation in fish has been well accepted (Piferrer and Guiguen 2008; Guiguen et al. 2010; Devlin and Nagahama 2002). However, Yamamoto's model was criticized soon after it was proposed, ironically based on studies using medaka. Specifically, it was reported that the steroid-producing cells were clearly observed in medaka only after the initiation of sexual dimorphic differentiation in the ovary as well as in the testis (Satoh 1974; Kanamori et al. 1985), implying that sex steroids are not involved in determining the gonadal fate in this species.

6.3.7 Medaka Depleted in Both Androgens and Estrogens

Experimental evidence against the hypothesis was obtained from genetic studies using medaka. As already touched on in the section on androgen, Cyp17a1 (P450c17-I) is involved in the steroidogenic pathway that produces both estrogens and androgens (shown in green in Fig. 6.5) (Devlin and Nagahama 2002; Zhou et al. 2007; Sato et al. 2008). Therefore, the synthesis of estrogens as well as androgens is expected to be severely impaired when the function of Cyp17a1 is lost. However, in the mutant medaka with a female genotype (XX), early oogenesis and folliculogenesis appeared not to be affected (Sato et al. 2008). Later, the mutant developed a gonad with both oocytes and spermatozoa, suggesting that estrogens have a role in maintaining the ovarian fate in later stages.

6.3.8 Estrogen in Medaka

Evidence more directly related to estrogen was obtained when a pharmacological inhibitor of aromatase/Cyp19a1 was administrated to medaka to block estrogen synthesis (shown in red in Fig. 6.5). Repeated oral administration of the aromatase inhibitor to hatching larva revealed that the inhibitor treatment did not affect early oogenesis in genetically female medaka, and spermatogenesis was occasionally observed later in the ovaries (Suzuki et al. 2004). A similar phenotype in oogenesis was also obtained in a mutant medaka deficient in ovarian aromatase (Cyp19a1): the gonads first differentiated into ovaries with follicles, and the ovaries subsequently degenerated, followed by the appearance of testicular tissues (Nakamoto et al. 2018). These results suggest that endogenous estrogens are likely not essential for either sex determination or early oogenesis/folliculogenesis in female medaka but are required for the maintenance of ovarian fate at a later stage. Of note, the master sex-determining (MSD) gene in medaka was identified as *Dmy* (a duplicated copy of *Dmrt1*) (Matsuda et al. 2002; Nanda et al. 2002). However, the pathway linking this gene to steroid synthesis remains elusive.

6.3.9 Estrogen in Nile Tilapia and the Rainbow Trout

Contrary to the medaka studies, however, evidence supporting at least the requirement of estrogen for early stages of gonadal development, if not gonadal sex determination itself, has been accumulating in other fish such as the Nile tilapia (Nakamura et al. 2003) and rainbow trout (Vizziano et al. 2007). For example, *Cyp19a1*-positive cells became detectable before morphological sex differentiation in the prospective ovary of the Nile tilapia and rainbow trout (Nakamura et al. 1998; Vizziano et al. 2007). Gene expression profiling during the critical period of sex differentiation in both species confirmed that key genes for estrogen synthesis including *Cyp19a1* have been expressed before ovarian morphological differentiation (Vizziano et al. 2007; Ijiri et al. 2008). Oral administration of an aromatase inhibitor resulted in female-to-male sex reversal in 100% of the rainbow trout and 75.3% of the Nile tilapia (Guiguen et al. 1999). In *Cyp19a1*-deficient tilapia with a female genotype (XX), the gonads differentiated into testes rather than ovaries (Zhang et al. 2017). Remarkably, a sign of sex reversal in this mutant was observed at a very early stage of gonadal development when a sexually dimorphic difference in the germ cell number appeared. This histological differentiation precedes other morphological differentiation such as the formation of a testicular efferent duct or ovarian cavity in the Nile tilapia (Kobayashi and Nagahama 2009).

Taken together, these results suggest that estrogens are needed in the very early stages of ovarian differentiation in these species (Fig. 6.7). Furthermore, a recent study on the MSD gene, *sdY*, in the rainbow trout sheds light on a link between its MSD gene and estrogen synthesis (Bertho et al. 2018). The rainbow trout *sdY*

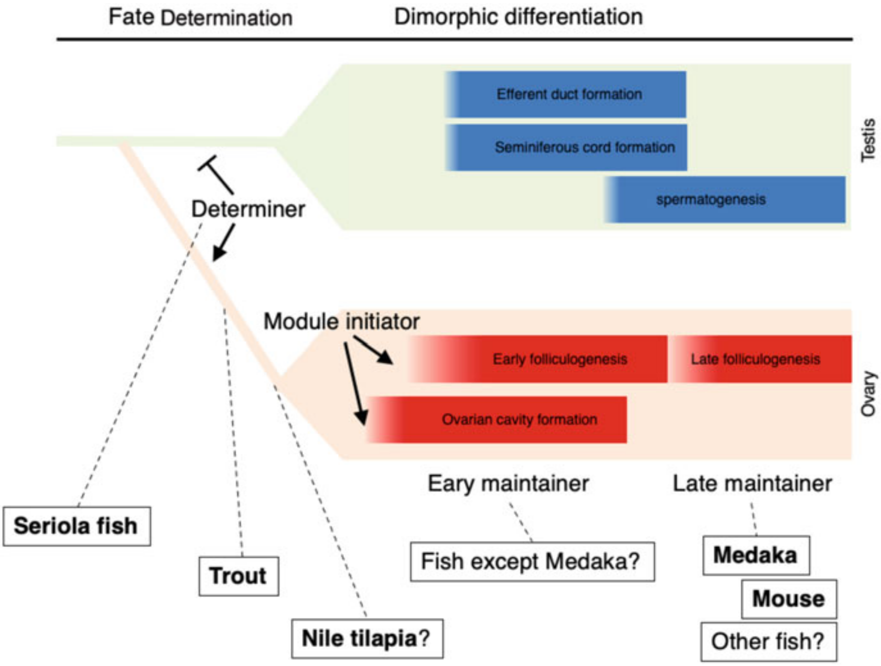

Fig. 6.7 The role of estrogens in the sex determination and differentiation period varies among fish species

encodes a truncated form of an immune-related protein and directly modulates *Cyp19a1* expression in cooperation with Foxl2 and Nr5a1, resulting in depletion of estrogens in a male genotype (XY) (Bertho et al. 2018) (Fig. 6.7).

6.3.10 Estrogen in Birds

Outside of teleost fish, it is well recognized that estrogen plays a major role in the early stages of ovarian differentiation in birds (Lambeth et al. 2013), while a very likely candidate MSD gene has been *DMRT1* that resides on the Z chromosome but not on the W chromosome (Smith et al. 2009). Estrogen treatment of genetic male (ZZ genotype) embryos results in testis-to-ovary sex reversal, and inhibition of estrogen synthesis in genetic female (ZW) embryos promotes ovary-to-testis sex reversal (Elbrecht and Smith 1992; Burke and Henry 1999). A recent genome editing experiment in chicken (*Gallus domesticus*) showed that loss of one copy of *DMRT1* in genetic males ($ZZ^{DMRT1+/DMRT1-}$) caused testis-to-ovary sex reversal, confirming that *DMRT1* is the key switch gene necessary for testis development (Ioannidis et al. 2021). In addition, the experiment with an aromatase inhibitor revealed that the production of estrogen is also a key factor in primary sex determination in chicken by suppressing the testis pathway activated by DMRT1 (Ioannidis et al. 2021).

6.3.11 Estrogen in Reptiles

Sex determination in reptiles is very complicated, ranging from temperature-dependent sex determination, genetic sex determination, and their coexistence, in which temperature-dependent sex determination prevails in crocodiles, most turtles, and some lizards (Barske and Capel 2008; Holleley et al. 2016; Nagahama et al. 2021). Among the reptiles, the red-eared slider turtle (*Trachemys scripta elegans*) has been relatively well studied concerning the sex determination process (Nagahama et al. 2021; Garcia-Moreno et al. 2018; Yao and Capel 2005). In this species, eggs incubated at a male-producing temperature with exogenous estrogen developed into females, whereas administration of an aromatase inhibitor to an egg incubating at female-producing temperature resulted in male development (Crews and Bergeron 1994; Wibbels and Crews 1994; Lance 2009; Pieau and Dorizzi 2004). Moreover, *Cyp19a1* expression is higher in embryonic gonads at the female-producing temperature than at the male-producing temperature (Czerwinski et al. 2016), suggesting that estrogens play a role in sex determination or subsequent differentiation. While recent studies in this species suggest that DNA methylation of the *Dmrt1* promoter might be key to determining gonadal fate by temperature (Ge et al. 2017), the direct link between Dmrt1 and estrogen production or the precise role of estrogens in sex determination is still unknown.

6.3.12 Estrogen in Amphibians

In many amphibians, gonadal differentiation is responsive to steroid manipulation, but the extent of the response varies among species (Nagahama et al. 2021; Flament 2016). Among amphibians, two species, *Xenopus laevis* and *G. rugosa*, have been well studied concerning the mechanism of sex determination and sex differentiation. Female-biased expression of the *Cyp19a1* gene in the gonads is observed just after the sex determination step or during early gonadal differentiation in both species (Maruo et al. 2008; Mawaribuchi et al. 2014). While their MSD genes or candidates have been known: *Dmw* in *X. laevis* and *Ar*, *Sox3* or *Sf-1* in *G. rugosa* (Yoshimoto et al. 2008; Miura 2017), the link between estrogen production and their MSD genes or the precise role of estrogens in sex determination is still unknown.

6.4 Insights from Master Sex-Determining Genes

6.4.1 Limitations of Loss-of-Function Experiments

As discussed above, pharmacological and genetic inhibition of the endogenous enzymes that catalyze sex steroids resulted in sex-reversal phenotypes in several species, providing strong evidence to support the requirement of estrogens to trigger ovarian differentiation, promote early differentiation, or maintain ovarian identity. However, loss-of-function experiments alone are not sufficient to distinguish the role of estrogens among them because estrogens are often essential for maintaining gonadal identity in nonmammalian vertebrates (e.g., Paul-Prasanth et al. 2013; Takatsu et al. 2013). Thus, late developmental phenotypes in loss of function experiments, i.e., gonadal sex reversal, can obscure early developmental phenotypes. Furthermore, in the case of teleost fish, the existence of two paralogs of the steroidogenic enzyme gene in the genome due to an ancient genome duplication does not permit a simple interpretation of the genetic inhibition experiments (Tenugu et al. 2021; Zhou et al. 2021; Force et al. 1999; Jaillon et al. 2004).

6.4.2 Master Sex-Determining Genes

Alternative evidence demonstrating the steroid hypothesis could be obtained if there is a species whose gonadal sex is determined by the master control gene that affects sex steroid levels. Although the search for such species has been previously considered to be impractical due to the difficulty for the identification of MSD genes in nonmodel organisms, recent advances in DNA sequencing technology and its commoditization enabled us to identify MSD genes (or strong candidates) in many vertebrate species, in particular, in fish, by using genome-wide association studies (e.g., Table 1 in Nagahama et al. 2021; Pan et al. 2021).

As mentioned in the previous section for some examples, more than a dozen different MSD genes have been identified in nonmammalian vertebrates to date. However, the majority of them encode Dmrt1, the Sox-family proteins, or proteins involved in the TGF-β pathway (e.g., Matsuda et al. 2002; Smith et al. 2009; Hattori et al. 2012; Kamiya et al. 2012; Takehana et al. 2014; reviewed in Kikuchi and Hamaguchi 2013; Bachtrog et al. 2014; Nagahama et al. 2021; Pan et al. 2021). Thus, the direct link between MSD genes and sex steroid levels has been unclear until very recently, although it can reasonably be surmised that steroidogenesis acts downstream of these MSD genes.

In 2018, a link between the MSD gene and sex steroid levels was reported in rainbow trout, as mentioned above (Bertho et al. 2018). An even more direct link was reported in four *Seriola* fish (the California yellowtail *S. dorsalis*, the greater amberjack *S. dumerili*, the Japanese amberjack *S. quinqueradiata*, and the yellowtail kingfish *S. lalandi*) in 2018 and 2019 (Purcell et al. 2018; Koyama et al. 2019).

In three Seriola fish including the greater amberjack, the Japanese amberjack, and the yellowtail kingfish, a Z-specific single nucleotide missense substitution in the coding region of *Hsd17b1* has been identified as the sole polymorphism associated with phenotypic sex (Koyama et al. 2019). Biochemical and molecular dynamics analyses revealed that Z-type Hsd17b1 protein is a hypomorphic form attenuating production of estradiol relative to the allelic product from the W chromosome (shown in magenta in Fig. 6.5), by disrupting the hydrogen bond network between the steroid and the enzyme's catalytic residues. Therefore, fish homozygous for the hypomorphic allele ($\mathrm{ZZ}^{\mathrm{Hsd17bz/Hsd17bz}}$) develop into males due to the depletion of estrogens (especially estradiol) during the sex determination period (Koyama et al. 2019). In the California yellowtail, a W-specific 61-base deletion upstream of the *Hsd17b1* gene has been identified. It was hypothesized that the deletion disrupts a putative silencer motif of *Hsd17b1* and thereby enhances estrogen production, leading to ovarian development in a female genotype ($\mathrm{ZW}^{+/\mathrm{del}}$) (Purcell et al. 2018).

Besides, in two species of *Trachinotus* fish (*T. ovatus* and *T. anak*) as well, allelic variation at the *Hsd17b1* locus, an intronic polymorphism in the splicing site this time, has been shown again to be associated with the phenotypic sex (Fan et al. 2021; Guo et al. 2021). Furthermore, in two species of tuna fish (*Thunnus orientalis* and *T. maccoyii*), the male-specific *Sult1st6Y* gene encoding an estrogen sulfotransferase appears to trigger testicular differentiation through inactivation of estrogens by sulfation in genetic males ($\mathrm{XY}^{-/\mathrm{Sult1st6Y}}$) (Nakamura et al. 2021).

In addition, an allelic variation of an estrogen signaling-related gene, *Bcar1*, has been proposed as the candidate MSD allele in the channel catfish (*Ictalurus punctatus*) (Bao et al. 2019). Since human BCAR1 interacts with estrogen receptor α (ERα) in human breast carcinoma cells (Cabodi et al. 2004) and the channel catfish *Bcar1* resides on the Y chromosome, it was assumed that the male-biased expression of this gene during the sex determination period resulted in an inhibition of estrogen receptor signaling and drives testicular development in catfish with a male genotype ($\mathrm{XY}^{\mathrm{Bcar1x/Bcar1y}}$).

6.5 Conclusions and Future Perspective

Together, these genetic studies clearly indicate that estrogens can be endogenous sex inducers by triggering ovarian differentiation in some species of fish, but not in others. In other words, the role of estrogens in cell fate specification and maintenance at the early gonadal differentiation likely vary among species even within teleost fish (Fig. 6.7). The situation in many species may fall between two extremes: (1) Seriola fish in which estrogen production is the initial trigger for sex determination, and (2) medaka fish in which estrogens are required neither for gonadal sex determination nor early ovarian differentiation (Fig. 6.7).

While it is now evident that the whole process of gonadal sex determination can lie downstream of estrogen activity in some fishes, there are still many unanswered questions about the detailed molecular processes that lead from estrogens to the

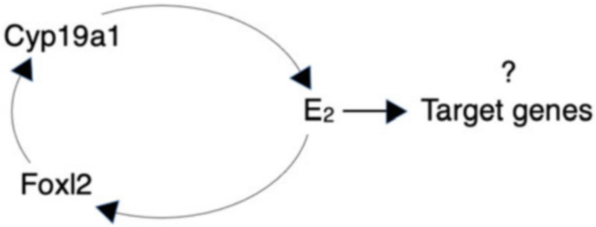

Fig. 6.8 Target genes of estrogens responsible for the gonadal fate determination and its maintenance. The action of estrogens is likely maintained by a positive feedback loop of Cyp19a, estrogens (represented by E_2), and Foxl2 in fish (Wang et al. 2007; Bertho et al. 2018). However, target genes of estrogens during sex determination/differentiation have been yet to be determined

gonadal fate determination. For example, although the action of estrogens should be mediated by estrogen receptors (ERs) (Nagahama et al. 2021) and likely maintained by a positive feedback loop of Cyp19a, estrogens, and Foxl2 in fish (Wang et al. 2007; Bertho et al. 2018) (Fig. 6.8), the mode of mediation of estrogen's effects and their target genes during sex determination/differentiation have been elusive. Indeed, despite a large number of studies reporting transcriptome analysis of differentiating ovaries and testes in fish (e.g., Sreenivasan et al. 2008; Tao et al. 2013; Koyama et al. 2019), direct target genes of ERs leading to ovarian development have yet to be identified (Fig. 6.8).

Given that an enormous number of ER target genes have been reported in human breast cancer cells and mouse mammary gland (Carroll et al. 2006; Palaniappan et al. 2019), it is reasonable to speculate that estrogens control many ER target genes during the period of sex determination and early steps of ovarian differentiation in nonmammalian vertebrates. Consistent with this expectation, of 337 female-biased genes in the differentiating gonads of the American alligator *Alligator mississippiensis*, 116 genes are in predicted estrogen-responsive (ER1-targeted) genomic regions (Rice et al. 2017). However, recent studies in zebrafish raised the intriguing possibility that the number of target genes may be limited. By generating double mutants of *cyp19a1* and *dmrt1*, Wu et al. (2020) showed that disruption of *dmrt1* rescues an initiation defect of folliculogenesis observed in the *cyp19a1a* mutant. In this double mutant, ovarian follicles developed normally up to the previtellogenic stage. This result suggests that estrogens are dispensable for early folliculogenesis in the absence of dmrt1 function (Wu et al. 2020), and thus, the ER target genes may be restricted to dmrt1-activated genes or *dmrt1* itself (Fig. 6.9). A similar dispensability of estrogen in early ovarian differentiation in the absence of DMRT1 function was recently reported in chicken (Ioannidis et al. 2021), though this study focused on earlier stages than those of folliculogenesis.

From the above discussion, the conclusions that could be drawn are as follows.

1. Although Yamamoto proposed that androgens can act as endogenous testes inducers in fish, it is not likely the case in many fishes except for fish that undergo temperature-induced masculinization or sequential sex change. Testes are likely

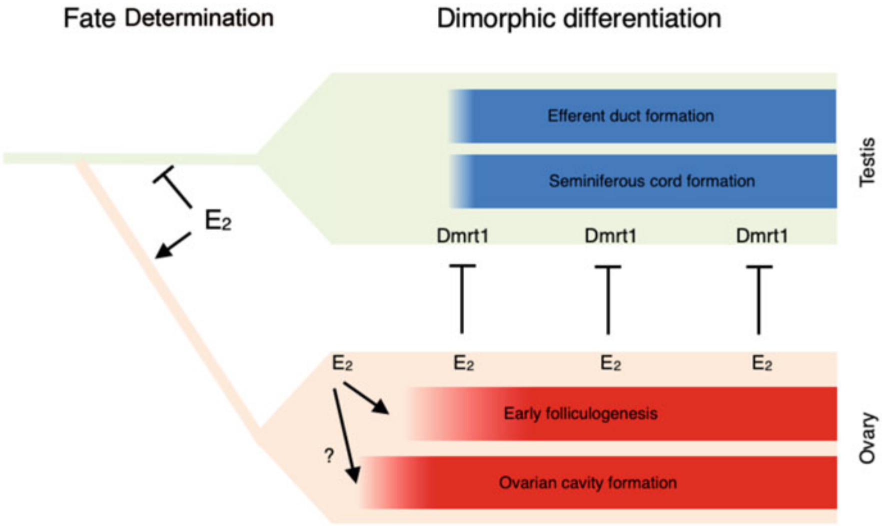

Fig. 6.9 A model for the role of estrogens in the sex determination and early sex differentiation. The role of estrogens (represented by E_2) as a gonadal sex determiner would be to activate the ovarian, while repressing the testicular pathway, or both during the sex determination period. To maintain the ovarian fate, continuous repression of the Dmrt1-activated pathway by E_2 is required. E_2 may play no active role in the developmental module of early folliculogenesis except for the initiation, contrary to a traditional view in nonmammalian vertebrates. The role of E_2 at the sex determination period can be performed by other MSD genes. Furthermore, the role of E_2 in the subsequent differentiation can be performed by other unknown factors

the default gonads in many fishes as far as sex steroid signaling is concerned: the undifferentiated gonads autonomously develop into testes in the absence of sex steroids. With some caution, the default state of gonads with respect to MSD gene in a species can be changeable with the replacement of their MSD gene. The role of androgens in sex determination remains to be elucidated in amphibians and reptiles.

2. As Yamamoto predicted, estrogens (namely estradiol) are most likely natural inducers that trigger ovarian differentiation in some fishes such as *Seriola* (Fig. 6.7). However, this role of estrogen to turn on or off the ovarian pathway can be performed by other MSD genes such as *Dmrt1*, *Sox*, and *Tgfb*-related genes. Currently, no direct link between these MSD genes and estrogens has been shown except for *sdY* in rainbow trout. It would be interesting to see whether such divergent MSD signaling converged into estrogen signaling or if estrogens only play a part in their downstream pathway in other species. From this view point, the sex determination mechanism of sharks would be also interesting. The precise role of estrogens in sex determination remains to be elucidated in amphibians and reptiles.
3. In a simplified model (Fig. 6.9), the role of estrogens as the gonadal sex determiner would be achieved by either activating the ovarian pathway or repressing the

testicular pathway during the sex determination period, and continuous repression of the pro-testis factors such as Dmrt1 during the early differentiation period. Estrogens may play no active role in the developmental module of the early folliculogenesis, and thus, the number of genes directed by estrogens in sex determination and early ovarian development could be far fewer than previously thought. It should be emphasized that direct target genes of the estrogen signaling leading to ovarian development have yet to be identified (Fig. 6.8).

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