Functional distinctions associated with the diversity of sex steroid hormone receptors ESR and AR

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Highlights:

Sex steroid hormones play fundamental roles in reproductive activities.

Sexually dimorphic development depends on sex steroid hormones.

The functions of both ESR and AR have diverged during vertebrate evolution.

In this review we provide a comprehensive analysis of the diversification of ESR and AR, and their functional associations.

We first briefly describe the evolutionary background of steroid hormone receptors (SRs) and then illustrate the roles established for sex steroid hormones and their receptors in sexually dimorphic development, and how this relates to their diversity in vertebrates.

Abstract

Sex steroid hormones including estrogens and androgens play fundamental roles in regulating reproductive activities and they act through estrogen and androgen receptors (ESR and AR). These steroid receptors have evolved from a common ancestor in association with several gene duplications. In most vertebrates, this has resulted in two ESR subtypes (ESR1 and ESR2) and one AR, whereas in teleost fish there are at least three ESRs (ESR1, ESR2a and ESR2b) and two ARs (ARα and ARβ) due to a lineage-specific whole genome duplication. Functional distinctions have been suggested among these receptors, but to date their roles have only been characterized in a limited number of species. Sexual differentiation and the development of reproductive organs are indispensable for all animal species and in vertebrates these events depend on the action of sex steroid hormones. Here we review the recent progress in understanding of the functions of the ESRs and ARs in the development and expression of sexually dimorphic characteristics associated with steroid hormone signaling in vertebrates, with representative fish, amphibians, reptiles, birds and mammals.

1. Introduction

Steroid hormones serve important functions in regulating a wide range of physiological processes including cell growth, differentiation, development, reproduction, and in overall homeostasis and health, throughout the life of vertebrates. Among the sex steroid hormones, estrogens and androgens play important roles in sexual differentiation and reproduction, particularly in the development and expression of male and female sexual characteristics. These effects are principally mediated by specific receptors, the estrogen and androgen receptors (ESRs and ARs), which belong to the nuclear receptor superfamily. As the main regulators of sex hormone signaling, ESR and AR have key roles in the molecular processes mediating reproductive development and behavioral patterns of organisms, and their diversity and evolution.

Most vertebrates have two ESR subtypes (ESR1 and ESR2) and one AR. ESRs share a certain degree of sequence similarity and bind the endogenous estrogen 17β-estradiol (E_2) with a high affinity. However, the two receptors exhibit clear differences in the tissue distribution and their target genes [1-4] and hence, functional diversification has been suggested among the ESR subtypes. To date, distinct roles of ESRs have been characterized in only a limited number of mammalian species, including in mouse and human. In the teleost lineage, the *esr2* gene has been further duplicated through a teleost-specific whole genome duplication (WGD) event, but for *esr1* only one gene remains. As such, most teleosts possess three ESR subtypes encoded by separate genes: *esr1*, *esr2a* and *esr2b*. [The published nomenclature for classification has been confusing, particularly with regards to nomenclature for ESR2 (formerly ERβ) subtypes. For example, the medaka ERβ1 (NM_001104702) is orthologous to ERβ2 in other fish species, including carp (AB334724) and zebrafish (AJ414567), whereas medaka ERβ2 (NM_001128512) is orthologous to ERβ1 in carp (AB334723) and zebrafish (AJ414566). In human, the accepted nomenclature is "*ESR*" and this has subsequently also been adopted for other vertebrates in this review to avoid confusion]. The *ar* gene has also undergone duplication into $ar\alpha$ and $ar\beta$ in the teleost lineage, however, some fish species (*e.g.*, zebrafish and fathead minnow) have secondarily lost $ar\alpha$ [5]. The teleost-specific WGD event has led to the existence of more nuclear receptors in teleosts than in mammals (e.g., medaka has 69 nuclear receptors, whereas human and mouse have 48 and 49, respectively), with a difference also in functional diversity in fish compared with mammals. In this review, we provide a comprehensive analysis of the diversification of ESR and AR and their functional associations in a variety of vertebrate species, including fishes (teleosts such as medaka, stickleback, mosquitofish and zebrafish), amphibians (*Xenopus*), reptiles (alligator and turtle), birds (chicken, zebra finch and duck) and mammals (mouse and human). We first briefly describe the evolutionary background of steroid hormone receptors (SRs) and then illustrate the roles established for sex steroid hormones and their receptors in sexually dimorphic development, and how this relates to their diversity in vertebrates.

2. Evolutionary history of SR genes in vertebrates

Evolution of novel traits following genome duplication events has been considered to provide evolutionary innovations in the vertebrate lineage. Understanding the genetic mechanisms leading to functional diversity of SRs is one of the central challenges in comparative endocrinology and evolutionary biology. The SR family consists of ESR, AR, progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), and has been generated through a series of duplications of an ancestral

SR gene. Several gene duplication events, including two rounds of WGD occurring in the early vertebrate lineage, have lead to the current diversity of the SR family. The first duplication generated an *esr* and a 3-ketosteroid receptor from the ancestral SR [6]. The 3-ketosteroid receptor further duplicated into *a corticoid receptor* (*cr*) and a receptor for 3-ketosteroids (androgens, progestins). After the Cyclostome (jawless fish)-Gnathostome (jawed vertebrates) divergence, the *cr* and *3-ketosteroid receptor* each duplicated again, with the *cr* yielding the *gr* and the *mr*, and with the *3-ketosteroid receptor* leading to the creation of the *pr* and the *ar* [6]. As such, the four differently encoded genes, *mr*, *gr*, *pr* and *ar*, first appear in the common ancestor of gnathostome vertebrates [5, 7].

The evolution of *esr1* and *esr2* has been intensely studied (Fig. 1). Japanese lamprey (*Lethenteron japonicum*) (Cyclostomata; one of the earliest-branching lineages in vertebrates) has two distinct *esr* genes [8]. Some cartilaginous fish such as the elephant shark (*Callorhinchus milii*, Holocephali, a subclass of cartilaginous fish) also have two *esr* sequences similar to *esr1* and *esr2*. However, the catshark and whale shark (*Scyliorhinus torazame* and *Rhincodon typus*, Elasmobranchs, another subclass of cartilaginous fish) seem to have secondarily lost the *esr1* gene [9]. In Japanese lamprey, one Esr displays estrogen-dependent activation of gene transcription, whereas the other does not respond to E_2 [8], however, it remains controversial as to whether the two *esr* in lamprey are orthologs of vertebrate *esr1* and *esr2* or whether this duplication occurred after the split of cyclostomes from gnathostomes [8, 10]. Taken together, vertebrate *esrs* have emerged from an ancestral *esr* through a series of gene duplications. Duplication of the ancestral *esr* into *esr1* and *esr2* occurred early-diverging in the vertebrate lineage [6], however, additional ESR sequences from early diverging fish species are required for establishing definitive phylogenetic relationships.

Teleosts experienced a teleost-specific WGD approximately 350 million years ago (MYA) [11, 12], which occurred after the split of the other ray-finned fish lineages (e.g. bichir, sturgeon, gar and bowfin) from the teleost lineage, and before the divergence of Osteoglossomorpha (e.g. arowana) and Elopomorpha (e.g. eel) [13, 14]. This teleost-specific WGD generated the additional copies of *gr* (*gr1* and *gr2*), *ar* (*ar*α and *ar*β) and *esr2* (*esr2a* and *esr2b*) compared with the gene repertoire in other jawed vertebrates [5, 15-17]. *Mr* and *pr* are also retained as single genes in teleosts [17]. To date, only a single *esr1* gene has been found from Silver arowana (*Osteoglossum bicirrhosum*) and Japanese eel (*Anguilla japonica*), suggesting that the *esr1* paralog has been lost in the early lineage of teleost fish species [10]. Two distinct paralogs of the *ar* gene, *ar*α and *ar*β, arose during the teleost-specific genome duplication and have been identified in a number of teleost fishes (Fig. 1) [5, 16]. In the history of the *ar* gene evolution, it is likely that the loss of the *ar*α gene occurred independently in Osteoglossiformes (e.g. arowana) [5], Cypriniformes (e.g. zebrafish and fathead minnow) [18, 19] and Siluriformes (e.g. catfish) [20]. Two *ar* genes have been identified in Salmoniformes [e.g. salmon and trout; and these diverged early in euteleost evolution [21], however, both are categorized into the *ar*β cluster [22]. Hence, the two *ar* genes in Salmoniformes arose by a lineage-specific gene duplication of *ar*β in the recent salmonid tetraploid event, estimated to have taken place 100-50 MYA [23], whereas, *ar*α gene might have been lost before this lineage-specific gene duplication.

3. Androgen-dependent secondary sex characteristics development in vertebrates

The development of vertebrate male reproductive organs and male secondary sexual traits is primarily regulated by androgens (Fig. 2). External genital organs have convergently evolved in vertebrates for efficient fertilization and reproduction. In mammals, the male external genitalia form a tubular urethra, as well as a well-developed prepuce and corporal body, and their development depends on androgens [24, 25]. Some fish species also have developed several types of copulatory organs for efficient sperm transport. In cartilaginous fishes, the midline pelvic fin is modified to form a tubular (glove-like) structure, termed the clasper in response to androgen [26]. In ovoviviparous fish such as Poecilidae (a group of Cyprinodontiformes), the development of a gonopodium (GP) through modification of the anal fin has generated a prominent male sexual characteristic [27-29]. The development of GP in ovoviviparous fish such as guppy, swordtail fish and mosquitofish enables internal fertilization. Oviparous fishes can also exhibit male-specific external structures associated with reproductive activities. For example, medaka (*Oryzias latipes*) exhibit a male-specific appendage structure, the elongation of fin rays and the formation of papillary processes in the anal fin [30, 31]. This enables mating males to embrace the posterior part of the female's body with the anal fin for efficient external fertilization [32].

Male secondary sexual characters also appear as an elongation of the fin ray, kidney hypertrophy, increase in skin thickness, and an appearance of breeding colors in some fishes [33]. Male stickleback (*Gasterosteus aculeatus*) produce spiggin in their kidneys in response to elevated circulating androgen levels and this glue protein is used during nest building. Sexually mature male stickleback also show a red coloration of their belly [34] and this prominent breeding color is attractive to females and simultaneously serves as warning for competing males [35]. A recent study indicates that androgen is a key factor in enhancing sensitivity to red light by regulating the expression of the opsin gene [36]. Such visual sensitivity might be important for territorial males to detect the presence of competitors [37, 38]. In mosquitofish, the transition from anal fin to GP is induced by androgen treatment in both juvenile fry and adult female [39, 40]. In medaka, castration causes regression of papillary processes, whereas transplantation of a testis to an adult female or the administration of androgens to females induces papillary processes formation [41, 42]. The androgen-dependent development of the anal fin with the papillary process in medaka, the GP outgrowth in mosquitofish, and the production of spiggin in stickleback have been used for the detection of chemicals having androgen action [43-48].

In amphibians, the development of a nuptial pad and vocal organ called the larynx are regulated by androgen [49, 50]. Adult male *Xenopus* form larger nuptial pads, which are used for grasping females during amplexus. Gonadectomized females implanted with a testosterone (T) pellet also form prominent nuptial pads [50]. The male larynx undergoes a profound transformation involving rapid growth, fiber addition, and conversion of fiber twitch type. Castration completely arrests fiber type conversion and retards muscle growth and fiber addition, indicating the androgen-dependency of these organs [49, 51].

Birds exhibit a diversified development of sex characteristics in appendicular and reproductive organs, including comb, wattle, syrinx, urogenital tract and gonads [52-57]. In birds, androgens play a role in the developmental program of these hormone sensitive tissues as well and therefore, AR expression in such tissues has been well analyzed [54, 58-61]. AR was exclusively detected in males in organs that display secondary sex characteristics, such as Wolffian duct and peripheral cloacal regions that develop into the prospective lymphobulbus [58]. By contrast, AR and ESR are both expressed in the developmental syrinx [58]. T treatment does not induce the male syrinx in female birds [62], while estrogen treatment feminizes the syrinx in zebra finch and duck [63, 64]. Thus, both hormones are involved in the sexual differentiation of vocal organ in birds, although a sole treatment of androgen or estrogen is not sufficient to induce sex reversed phenotypes [65].

Development of androgen-dependent secondary sexual characteristics in squamate reptiles is also well documented. Castration inhibits and T stimulates rapid growth in anole lizards, resulting in male-biased sexual size dimorphism [66]. T treatment increases AR mRNA and protein expression in the copulatory organ (hemipenis) in green anole [67].

The role of androgens in the development of sex characteristics has been studied by pharmacological and genetic analyses. In mice, administration of the anti-androgen flutamide, an AR antagonist [68-70] or the 5α-reductase inhibitors 4-methyl-4-aza-5-pregnan-3-one-20[s] carboxylate or finasteride [71, 72] interferes with the development of male external genitalia, resulting in a hypospadias-like phenotype. In human patients, hypospadias are a common malformation in which the urethral meatus is located at the ventral side of the penis [73]. Target mutation in *Ar* results in abnormalities in male sexual development including female-like external genitalia formation and cryptorchidism in mice [74-76].

It has been known that the ligand selectivity of AR is different among species. In mammals, T and 5α-dihydrotestosterone (5α-DHT) are considered to be effective ligands for AR [77]. 11-Ketotestosterone (11KT) is known as a potent androgen in teleost fishes [33]. Recent analyses, however, showed the presence of 11KT in early-branching actinopterygian fish (sturgeon) [78], urodele amphibian (*Necturus maculatus*) [79] and mammal (human) [80], suggesting a significant role of 11KT as an androgen in other vertebrates as well.

4. Molecular mechanisms of male sexual characteristics development; cross-talk between androgens and growth factors

Sexual differentiation is a remarkably complex process that depends on the orchestration of an intricate signaling network. Several effector genes that interact with androgen signaling have been identified [26, 39, 40, 52, 68, 81, 82]. Androgen-induced expression of *sonic hedgehog* (*shh*) is required for the formation of the GP in mosquitofish [40, 52], as well as the clasper function in cartilaginous fishes also [26]. During the androgen-induced transition from anal fin to GP, *shh* expression is closely associated with androgen-induced outgrowth of the anal fin, where *ar*s are expressed [40]. Flutamide treatment reduces cell proliferation in distal anal fin regions accompanied by reduced levels of the *shh* expression. These results suggest that androgen and hedgehog signaling are regulating cell proliferation and contributing to the development of new bone segments in the developing GP. It is clear that hedgehog signaling plays multiple roles on fin morphogenesis. The Shh is required for the anterior-posterior patterning of a developing fin [83], the growth and maintenance of the blastema, and patterning of the fin ray in adult fish, as illustrated following fin amputation [84, 85].

The androgen-dependent activation of hedgehog signaling is also necessary for male clasper development in cartilaginous fish [26]. By regulating *hand2*, androgens control the male-specific pattern of *shh* in pelvic fins [26]. In mouse, *Shh* is expressed in the embryonic external genitalia (genital tubercle, GT) throughout the embryonic development and is indispensable for protrusion of the GT precursor during early embryogenesis [86, 87]. Shh signal facilitates the masculinization processes by modifying androgen-responsive gene expression [88]. Conditional mutation of *Shh* during sexual differentiation has been shown to lead to abnormal development of male external genitalia. *Indian hedgehog* (*Ihh*), another member of the hedgehog gene family, is also responsible for the development of male external genitalia [89]. These results indicate the close association between androgen and hedgehog signaling during the development of sexual characteristics in vertebrates. In sexually dimorphic organs, androgen signaling may re-activate hedgehog gene expression, which is necessary for both early morphogenesis and sexual development. The latter is associated with the androgen-induced heterochronic event.

Several growth factors also work as effectors in regulating reproductive organ formation in association with hormones. For example, the development of papillary processes is promoted by androgen-dependent increase of *bone morphogenic protein 7* (*bmp7*) and *lymphoid enhancer-binding factor-1* (*lef1*) expression. The Wnt/β-catenin signaling pathway has been identified as a masculine effector of androgen signaling in the development of both, papillary processes in medaka [81] and external genitalia in mouse [68]. The sexually dimorphic expression of several Wnt inhibitory genes, including *dickkopf 2* (*Dkk2*) and *secreted frizzled-related protein 1* (*Sfrp1*) have been identified in the developing external genitalia of mouse. These genes are more highly expressed in the female GT compared to males. In addition, loss-of-function and gain-of-function studies on *β-catenin* (*Ctnnb1*) mutants have shown impaired sexual differentiation of the GTs, indicating that AR-dependent inhibition of Wnt inhibitory genes is necessary for masculinization of external genitalia [68].

5. Contribution of sex steroid hormone receptors to gonadal differentiation

Although the relative importance of sex steroid hormones in sex determination apparently seems to diminish in mammals, estrogens play a critical role in sex determination and particularly in ovarian development in most non-mammalian vertebrates. Sex is genetically determined in the medaka and administration of exogenous estrogens shortly after fertilization causes male to female sex-reversal, with the formation of a functional ovary and reproductive capabilities [90-92]. Likewise, exposure to estrogens throughout the larval period results in the formation of ovaries in males [93, 94]. In the chicken, sex reversal can be also induced experimentally, at least in part, by injecting eggs with estrogens, or by inhibiting estrogen production [95, 96].

Sex determination in several species of reptiles involves temperature –a process called temperature-dependent sex determination (TSD) - where the incubation temperature of the egg, during a thermo-sensitive period (TSP) determines the sex of the offspring in, for example, all crocodilians studied, many turtles and some lizard species [97-99]. Gonadal differentiation in these species is also estrogen-sensitive. Administration of estrogens during the TSP induces male to female sex reversal even if eggs were incubated at a male-producing temperature. In general, expression of *cytochrome P450, family 19, subfamily a* (*cyp19a*; also named *aromatase*), which converts T to E_2, coincides with the later period of TSP in turtles and crocodilians [100-102] and thus, endogenous estrogen mediates terminal ovarian fate determination factor as a downstream signaling event in response to environmental temperature.

Expression pattern and distribution of *esrs* during the TSP have been studied extensively in the red-eared slider turtle (*Trachemys scripta*) and this has shown that *esr1* and *esr2* have distinct patterns of expression. *Esr1* mRNA expression peaks late during the TSP at both female- and male-producing temperatures (FPT and MPT), and at peak expression, gonadal *esr1* mRNA levels are 5-fold higher at FPT compared to MPT [103, 104]. By contrast, *esr2* expression increases after the TSP in the gonads that develop at FPT [103, 104]. It has been thus suggested that *esr1*, but not *esr2*, responds as an early target of estrogen-induced commitment to ovarian differentiation.

Functionalization of ESRs has been analyzed using selective ESR1 and ESR2 agonists in the American alligator (*Alligator mississippiensis*). 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 MPT, whereas the ESR2-selective agonist 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070), had no effect [105]. PPT-exposed embryos also show enlargement and advanced differentiation of the Müllerian duct, suggesting that ESR1 also plays a role in the development of the female reproductive tract [105]. In chicken, a sister group of crocodilians as Archosauria, PPT causes left-side ovotestis formation and retention of the Müllerian ducts in male embryos, whereas none of these effects are observed after exposure of embryos to the ESR2-selective agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) [106]. Taken together, these data suggest that ESR1 not only plays a central role in ovarian differentiation and the development of female reproductive organs, but also mediates induction of sex reversal in reptiles (and birds) after exposure to exogenous estrogen.

It is not clear whether natural testicular development in reptiles requires androgen signaling. *In ovo* exposure of alligator or turtle embryos to the non-aromatizable androgen 5α-DHT or the anti-androgen flutamide have no effects on gonadal differentiation at both FPT and MPT, respectively [107, 108]. In contrast, at the pivotal temperature, that produces an approximately 1:1 sex ratio, exposure to androgens resulted in the male-biased hatchling production in turtles [109]. Androgens thus appear to play a more subtle role in gonadal fate determination. In the red-eared slider turtle, gonadal *ar* expression pattern is similar to *esr1*, which shows a spike late in the TSP [104]. By contrast, *ar* expression in the American alligator increases significantly over developmental time, but does not vary between MPT and FPT [110]. This implies different AR-mediating signaling pathways during gonadal differentiation between these two TSD species. Intriguingly, a spliced form of the AR, which lacks 7 amino acids within the ligand-binding domain, is expressed in the gonads of American alligator. This variant shows no response to androgens and perturbs intact AR transactivity as a dominant negative form [110].

6. Roles of ESR and AR in mammals as assessed via knockout studies

Since the establishment of the *Esr1* knockout (KO) mouse [111], the distinct roles of ESR subtypes have been extensively investigated. In mice, ESR1 plays an indispensable role in maintaining reproductive function. Although offspring were born without any gross effects on the gonad with normal reproductive organ morphogenesis, both female and male were infertile because of conditions including anovulation, hypoplastic reproductive organs, lack of any normal sexual behaviors, failure of response to estrogen in females, and abnormal water absorption in the epididymis in males [111-114]. The possible role of ESR2 in reproductive functions and fertility, on the other hand, remains controversial. Several *Esr2* KO mouse lines have been established with phenotypic variation in terms of fertility, probably due to variation of residual ESR2 function [113, 115-118]. Taken together, *Esr* KO mice studies revealed that receptor subtypes exhibit distinct functions, which cannot be compensated by each other. One exception is maintenance of ovarian differentiation in mature animals where *Esr1* and *Esr2* double KO mice show transdifferentiation of ovarian somatic cells into testicular Sertoli cells, whereas this is not the case in single *Esr* KO mice [119].

In mammals, AR functional abnormalities cause a spectrum of disorders of androgen insensitivity syndrome (AIS) or testicular feminization mutation (Tfm) [77, 120, 121], showing that ARs are indispensable for male development. *Ar* KO male mice exhibit female-type external appearance and absence of seminal vesicles, vas deferens, epididymis and prostate, but retain a small inguinal testes with severely arrested spermatogenesis [75, 122], suggesting that although AR was not required for the formation of testis, it was essential for the development of male reproductive organs and spermatogenesis. AR-mediated androgen signaling also plays an important role in the female reproductive system. Female *Ar* KO mice show normal growth but are subfertile resulting in significantly fewer pups per litter compared to control mice. In the ovary of *Ar* KO mice, folliculogenesis is impaired with an increase in the number of atretic follicles [123].

7. Roles of ESR and AR in fish, as assessed via knockout studies

Above we illustrate the established fundamental roles of Esr and Ar in reproduction in mammals, as established through gene KOs. Such detailed information relating to the distinct roles of each subtype of Esr and Ar in non-mammalian vertebrates is still limited. Recently the generation of *esr* KO zebrafish (*Danio rerio*) and medaka by TALEN and CRISPR/Cas9 methods has been reported [124, 125]. Unexpectedly, KO of a single *esr* subtype alone showed normal reproductive development and function in both female and male zebrafish [125]. By contrast, double and triple KO (*esr2a*$^{-/-}$;*esr2b*$^{-/-}$ and *esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) develop all male phenotypes and thus, Esr2a and Esr2b are, despite of the presence of functional redundancy among Esr subtypes, essential for female development [125]. Zebrafish are juvenile hermaphrodites, where all fish develop a so-called juvenile ovary and it followed by sexual differentiation into testis or true ovary [126]. Some double and triple KO fish appear to exhibit sex reversal and loss of Esr2s leads an arrest of folliculogenesis resulting in female to male sex reversal, as intersexual gonadal phenotypes were often observed after the window of natural sex differentiation stage [125]. In the zebrafish, all *esr* subtypes are expressed in the mature ovary, and *esr2a* is most highly expressed during folliculogenesis. *Esr2a* is also expressed in the oocytes and *esr2a* KO eggs showed the unique phenotype of weakened chorion and early hatching [125]. It is thus suggested that Esr2a is the most predominant Esr subtype contributing to ovarian development in zebrafish.

The medaka exhibits XX-XY heterogamety with a distinct sex determination gene called *DM-domain gene on the Y chromosome* (*dmy*) [127]. Hence, medaka is an excellent model for studying sex determination and differentiation during early gonadal development as genetic and intrinsic sexes can be identified. In our own studies we have established *esr1* KO medaka and these did not show any significant defects in gonadal development, sexual characteristics and reproductive activity [124] as in the case of zebrafish. Intriguingly, *esr2a* KO female medaka show abnormal abdominal swelling with ovarian expansion and are infertile (Fig. 3). Hence, even within the teleost lineage, roles and functions of Esr are diverged. The development of *esr* KO zebrafish and medaka provides important insights into receptor subfunctionalization between mammals and fish and offers a powerful prospect for better understanding the distinct roles of the different Esrs in vertebrates.

The hepatic *vitellogenin* (*vtg*) is a representative estrogen-responsive gene in oviparous animals [128] and it has been shown that all three Esr subtypes are functionally involved in E_2-induced *vtg* expression. Esr2a-mediated upregulation of *esr1* induces enhanced *vtg* expression in primary hepatocytes of goldfish (*Carassius auratus*) [129]. The need of both Esr1 and Esr2a for the induction of *vtg* has furthermore been shown through morpholino (MO)-knockdown of each *esr* mRNA in zebrafish embryos [130]. Estrogen stimulation significantly up-regulates *esr1* expression in *in vivo* medaka study, while *esr2a* and *esr2b* expressions are unchanged, indicating that *esr1* is the most highly expressed hepatic Esr subtype [124]. These results suggest that estrogen stimulation primes and upregulates *Esr1* expression by either Esr2 subtype and resulting in a continued *vtg* expression through augmented Esr1 in the liver. In fact, *vtg* expression is significantly lower in the liver of *esr1* KO medaka than that of controls. However, the finding that *esr1* KO medaka show no significant effects on reproductive activities suggests that Esr1 function could be partly compensated for by one or both Esr2 subtypes.

Intriguingly, *Ar* is not primarily required for male sexual differentiation in the zebrafish, as it is in mice, it is required for the development of secondary sexual characteristics, and for proper organization of the testis in males and for oocyte maturation in females [131]. The *ar* mutant male zebrafish fails to release sperm and courtship behavior is significantly less [131]. To further understand functions of AR in fish, we are currently establishing *ar*α and *ar*β KO medaka.

7. Conclusion

Sex steroid hormone receptors are associated with the regulation of reproductive actions in vertebrates, and are most likely subject to directional selection. Cross-species comparative analyses from various vertebrates has revealed species differences in ESR sensitivity in response to endogenous estrogens, notably via the use of luciferase reporter gene assays [132]. For example, teleost Esr1s do not show much difference in responsiveness to E_2, whereas species differences are more pronounced in tetrapods [133, 134]. Amphibian Esrs appear to be less sensitive to E_2 generally [135, 136]. From vertebrates studies to date, the ESR1 in snakes - the Okinawa habu (*Protobothrops flavoviridis, Viperidae*) and Japanese four-striped rat snake (*Elaphe quadrivirgata, Colubridae*), have the highest estrogen sensitivity, followed by other reptilian and avian species [133, 137]. ESRs from high sensitive animals may respond more quickly and have a lower demand for the amount of hormone required to trigger hormone activity compared with low sensitive animals. However, the biological implications of such species differences in estrogen sensitivity have yet to be determined.

The presence of multiple SR subtypes, in particular in teleosts, may have significant bearing on the responsiveness and effects of steroid hormones. There are clearly different responses between receptor subtypes for the Esr in fish. As in the case for Esr1, inter-species differences in response to E_2 for both Esr2a and Esr2b are small. However, across the Esr subtypes Esr2a is generally the most sensitive to E_2 (i.e., Esr2a can be activated by the lowest concentration of E_2). An exception here is in the zebrafish, where Esr2b is the most sensitive Esr subtype [138]. The transactivation property of teleost Arβ is similar with tetrapod and cartilaginous fish Ars, indicating that Arβ retains the original and common function throughout vertebrates. By contrast, teleost Arα shows a unique intracellular localization and significantly higher transactivating properties [5, 52, 139]. This has been observed for Arαs from spiny-rayed fishes (Acanthomorpha), but not for Japanese eel (Elopomorpha, an earlier branching lineage among teleosts), suggesting that *arα* has evolved after the divergence of the Elopomorpha lineage. The amino acids that are responsible for Arα specific hyper-transactivation and constitutive nuclear localization have been identified and are highly conserved in spiny-rayed fish Arα, but differ in Japanese eel [139]. Insertion of spiny-rayed fish type amino acids into Japanese eel Arα recapitulates the evolutionary novelty of euteleost Arα, indicating these substitutions generate a new functionality of Arα in the teleost genome after the divergence of the Elopomorpha lineage [139]. Such evolutionary novelty of protein function in *ar* genes might facilitate the emergence of divergent sex characteristics in teleost lineage.

Taken together, this review serves to illustrate that divergence of the sex steroid receptors, most notably for the estrogen receptor associates with functional complexity. Recent progress in genome editing approaches now allow for more practical capability to effectively target specific gene manipulations. Although adoption of these approaches has been reported in a few species only, application in future studies to genetically modify the estrogen and androgen receptors in animals throughout the vertebrate lineage is likely to enable the rapid advancement in our understanding of the evolution and functionalization of steroid hormone signaling.

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Figure legends

Fig. 1

Composite phylogeny of vertebrates with the hypothesized scenario of ESR and AR evolution. The evolutionary tree illustrates that Chondrichthyes (shark), the earliest branching group of living jawed vertebrates, possess ESR1, ESR2 and AR. The teleost-specific whole genome duplication (WGD) gave rise to two different teleost ARs (ARα and ARβ) and ESRs (ESR2a and ESR2b). Figure modified from Ogino *et al*., 2016, Tohyama *et al*., 2016.

Fig. 2

Androgen-dependent development of sex characteristics. (A) Male mosquitofish and bone staining of gonopodium (GP). The distal portion of the GP is composed of the 3rd, 4th, and 5th fin rays and the distal tip is equipped with spines, serrae, an elbow, and hooks. (B) Male medaka and bone staining of papillary processes that develop as an outgrowing bone nodule from the anal fin rays. (C) Mouse external genitalia in male. The development of copulatory organs is one of the representative models to investigate androgen-dependent organogenesis. (D) A schematic diagram of the possible signaling cross-talk between androgen and growth factor signaling for development of secondary sex characteristics. Figure modified from Ogino et al. 2004.

Fig. 3

Esr2a KO female medaka exhibit abnormal abdominal swelling and are infertile. (A) Wild-type female, (B) *esr2a* KO female.