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Review - Biological and Applied Sciences

TGF-β Superfamily: an Overview of Amh Signaling into Sex Determination and Differentiation in Fish

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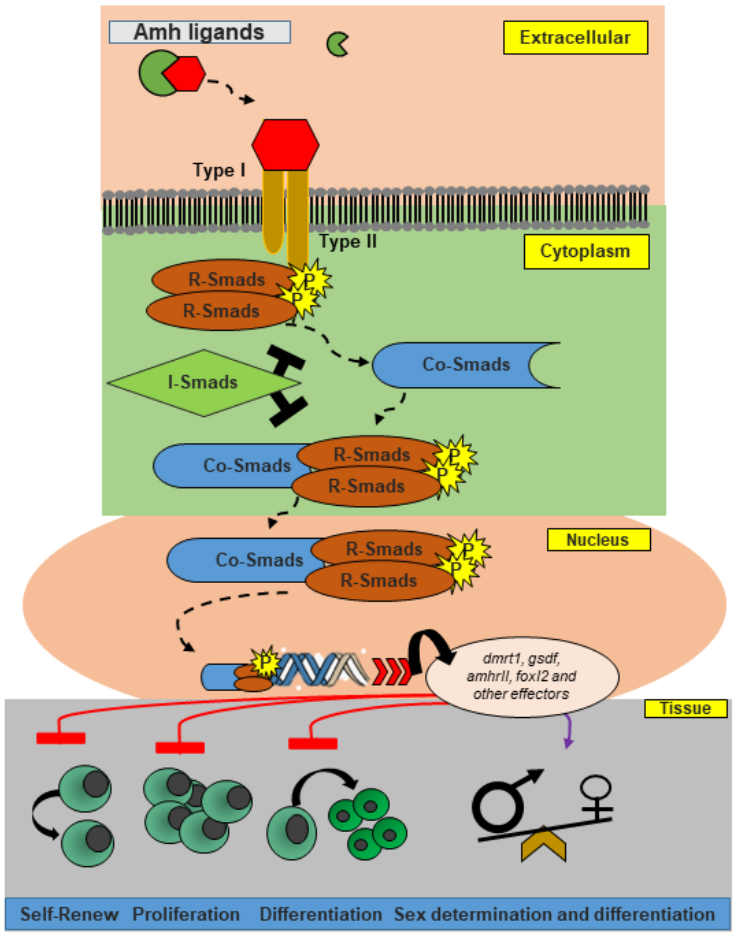
HIGHLIGHTS

- TGF-β superfamily members is crucial for cell differentiation and proliferation.
- TGF-β superfamily signaling are important to several biological system and the lack of these signaling can leads a several disorders and dysregulation of the tissue survive.
- Amh signaling have an important role during gonadal development in teleost fish.

Abstract: The decision whether the bipotential gonadal anlage will become a testis or ovary is a critical step during sex determination and differentiation in fish. This process involves a complex and coordinated genetic cascade, which result in the differentiation of the somatic cells into ovary or testis. In this context, important genes of TGF-β superfamily appears have a pivotal role in this biological process of fish development. In this review, we showed the breakthrough in the last decades that engage the Anti-Müllerian hormone (Amh) as an important effector in this decision. Here we exposed studies with different species of fishes around of world have paved the way for clarifying the role of Amh in the regulation of the germ cells proliferation, which may influence the spermatogenesis and, sex determination and differentiation decision on teleost fish.

Keywords: Amh; cell proliferation; fish; germ cell; sex differentiation and determination.

GRAPHICAL ABSTRACT



INTRODUCTION

Transforming growth factor-β superfamily (TGF-β) are active polypeptides related with growth factors that comprises several members in vertebrates [1].

Historically**,** the medical and scientific importance of the TGF-β began in 1970**'**s, when a new revolutionary study identified and characterized the TGF-β signaling members. On 1975, Holley discovered that hormones or hormone-like are responsible for control of the growth of mammals cell [2]. Subsequently, in 1985, Derynck and colleagues [3] published the first molecular evidence of TGF-β members and your signaling pathway by cloning the complementary DNA (cDNA) from humans. In this work, the authors showed the expression of TGF-β mRNA was not restricted only in tumor cells (Wilms tumor, glioblastoma, bladder carcinoma and squamous cell carcinoma) but also in normal cells (placenta and peripheral blood lymphocytes). To identify the cDNA that encoding the protein, the author used the partial purified amino acid sequence from human [3].

Consequently, different research and methodologies to identified TGF-β superfamily member structure and component signaling, oncogenic function, developmental genetic, sex determination and differentiation, based on biochemical purification and cloning were employed over the years [4-12]. Interestingly, TGF-β superfamily members are highly conserved between the vertebrate [6-13].

Nowadays is known that the TGF-β superfamily members are responsible for mediate a wide range of embryonic and adult cell signaling that provide specific control of differentiation, proliferation, and cell-specific or tissue-specific signaling and control across many vertebrates species [1,2,6,8,11,13]. Interestingly, many members have been recognized in fish as initiator or crucial regulator in spermatogenesis and, sex determination and differentiation [1]. Among these members, the Anti-Müllerian Hormone (Amh) emerge as a fundamental play in testis formation, spermatogonial proliferation and differentiation, and sexual development in fish [1,13]. Thus, in this review, we will introduce the recent progress of the TGF-β superfamily

members, with focus on the Amh signaling in teleost fishes and its action during sex determination, differentiation, and spermatogenesis.

Transforming growth factor-β superfamily

Transforming growth factor-β superfamily (TGF-β) are characterized as a group of over 60 family members and all genes are expressed as precursors with an N_terminal signal peptide, a large prodomain, a protease cleavage site, and a C_terminal mature polypeptide that are secreted as homodimer protein which are activated and released by proteolytic cleavage [6,12,14,15].

The biological activities of TGF-β superfamily occurred across many species and are conserved between vertebrate [5-10,16]. TGF-β signaling controls the genes expression and growth factors that are important to several biological system and, the lack of these signaling can leads a several disorders and dysregulation of the cell homeostasis and tissue survive [8,10]. In teleost fishes for example, TGF-β signaling act in a wide range of cell types and play important roles mediating cell growth, cell proliferation, cell differentiation, cell apoptosis, stem cell self-renewal, stem cell differentiation and quiescence, differentiation and tissue morphogenesis providing tissue-specific regulation, testis development and, sex determination and differentiation [1,13,17-22].

TGF-β superfamily members include the TGF-β ligands whom inhibits proliferation and differentiation of many cell types and controls tumorigenesis-signaling [3,5,7,8,10,15,23-27]. Activins and inhibins, which are involved in embryogenesis, control of pituitary and gonadal hormone release [28-30]. Bone morphogenetic proteins (BMPs), which are involved in osteogenesis, cell growth, proliferation and differentiation [11] and, in the developing zebrafish ear and lateral line [31]. Nodal, which have a role as a regulator during the early cell fate decisions, organogenesis and adult tissue homeostasis in mammals [9] and in zebrafish, Nodal expression are required for dorsal mesoderm development [31] and mediate interactions between embryonic and extra-embryonic tissues [32]. Growth differentiation factors (GDFs), whom are involved in development of cartilage, joints and the growth of neuronal axons and dendrites in mammals [33,34] and, act as a regulator of appetite and energy homeostasis in mammal and in fish [35]. Anti-Müllerian hormone (AMH) is important for induce the regression of the Müllerian Ducts in males during sex differentiation in mammals [36-40] and, are involved in the spermatogonial proliferation and differentiation and, sexual developmentinteleost fish [1,13,41,42,43].

Several studies described the different sex determining and differentiation-related genes in the teleost fishes. One group of genes that is received great attention are belong to the TGF-β superfamily members. These included *gsdf*^{Y} (gonadal soma derived growth factor on the Y chromosome) in *Oryzias luzonensis* [16]; *gdf6* (growth differentiation factor 6) in the killifish, *Nothobranchius furzeri* [44]; *amhrII* (Anti-Müllerian Hormone receptor type 2) in tiger pufferfish, *Takifugu rubripes* [45]; *amhy* (Y-linked duplicates of the *amh)* in *Odontesthes hatcheri* [19], *amhby* (Y-chromosome-specific copy of *amh*) in *Esox lucius* [46] and Y-linked *amhr2* in ayu, *Plecoglossus altivelis* [47].

The TGF-β superfamily signaling pathway begins in the extracellular matrix where the dimeric ligands binds to a serine/threonine kinase type II and I membrane receptor that form a hetero-tetrameric complex. Thus, once formed the ligand/receptor II and I complex occurred a cascade of phosphorylations that initiated with the type II receptor phosphorylating type I receptor, which also by phosphorylation activates cytoplasmic mediator proteins called Smads [14,48-50].

In the cell, TGF-β signaling pathway initiate after the formation and activation of functional receptor complex with the phosphorylation of the C-terminal serine residues in R-Smads (Receptor-activated Smads). The R-Smads are Smad1, Smad2, Smad3, Smad5 and Smad8. After the R-Smad activation, an association of R-Smads with Co-Smad (common mediator Smad) occur and consequently form a Hetero-oligomerisation R-Smad-Co-Smad complex. To vertebrate cell, a common mediator Smad4.

In most cell types, TGF-β, Amh and Activin/Nodal receptors induce phosphorylation of Smad2 and Smad3, and BMP receptors induce phosphorylation of Smad1, Smad5 and Smad8 [14,15,22,27,51]. The R-Smad-Co-Smad complex moved to the nucleus where they bind to high-affinity DNA and associated with transcription factors to regulate the gene transcription [26,27,51]. Alternatively, beyond the positive Smad signaling, the inhibitory effect can be occur with action of the I-Smad (Inhibitory Smad), named Smad6 and Smad7. These Smads interact with the receptor complex inhibiting the R-Smad phosphorylation or the R-Smad-Co-Smad complex formation [22,51]. These evidences reveals that Smad proteins are important because transduce signals from TGF-β superfamily ligands to regulate various biological functions and gene transcription in various cell types.

Amh structure and gene activation

Anti-Müllerian Hormone (AMH) is a glycoprotein member of the TGF-β superfamily, which plays an important role in Müller's duct regression during male sexual differentiation in vertebrate tetrapods [36,37,39].

AMH is secreted as a 140-kDa homodimeric precursor, which consists of two 70-kDa monomers each. AMH is composed of a mature C-terminal region with 25-kDa. This region becomes bioactive after undergoing proteolytic cleavage and binding to the AMH receptor type 2 (AMHRII) inducing intracellular signals through Smads proteins [4,52,53]. The N-teminal region is called the pro-region. This part is important for the synthesis and transport of extracellular AMH. The precursor of AMH is cleaved between these two domains (pro-region and C-terminal). Then, a second cleavage occurs in the pro-region giving rise to three different regions: pro-semi-mature, semi-mature and mature [38,53,54]. The C-terminal region becomes biologically active when it is non-covalently associated with the pro-region. A new cleavage results in the dissociation of the pro-region with the mature C-terminal region. In this way, mature AMH is released into the extracellular matrix. The N-terminal portion is important for maintaining the biological activity of the C-terminal portion of AMH [38,53-55].

The sequences of the deduced protein from AMH show well-conserved characteristics among vertebrate species, such as the TGF-β domain in the C-terminal region and the Amh_N domain in the N-terminal region [40,48-50]. However, it is worth noting the differences in the cleavage site between vertebrates. In mammals and birds, the region where recognition by proteases for cleavage occurs is simple (R-X-X-R) [55,56], while in teleost fish the region is double (R-X-X-R-X-X-R). This cleavage is necessary for the processing of Amh [13,19,42,55-57].

In the extracellular matrix, mature AMH binds to a complex of type I and type II serine/tyrosine kinase membrane receptors. Type II receptors have an extracellular domain for specific association with ligands [48,50]. From the formation of the ligand / receptor complex II and I, a phosphorylation cascade, initiated with the type II receptor phosphorylating the type I receptor, which also by phosphorylation, activates cytoplasmic mediating proteins called Smads [48,50,58].

Smads are divided into 3 groups according to their function. As R-Smads are associated with type I receptors and mediate the membrane signal towards the nucleus; they are Smads 1, 2, 3, 5 and 8. Co-Smad (Smad 4) is associated with R-Smads in the cytoplasm and how they translocate to the cell nucleus. Finally, as I-Smads (Smads 6 and 7), which are antagonists of the R-Smads that activate inhibitory activity under stimulation [50,51,58]. The R-Smads are activated by the type I and II ligand / receptor complex according to a distribution that allows us to group the TGF-β pathway into two large subfamilies [48,50,51].

AMH has R-Smads 1, 5 and 8 as mediators. Smad4 transports the phosphorylated R-Smads (1, 5 and 8) from the cytoplasm to the cell nucleus. This complex of the Smads proteins is translocate to nuclei and associate to DNA and act in the regulation of gene expression, in association with Co-activators, Co-repressors and other transcriptional regulators [14,49,58]. Thereon, several downstream mechanism beginning and the Amh signaling was triggered [14,51]

Regarding its structure, Amh presents itself as a single copy gene in vertebrates. The AMH gene has 5 exons in mammals and birds [52,59,60]. In humans and mice, the protein formed contains 554 and 560 amino acid residues, respectively [52,59]. In chickens, AMH has 644 amino acid residues [60,61]. In teleost fishes, the *amh* gene consists of 7 exons that encode a protein of 500 amino acid residues [13,41,62,64]. In the other hand, in the *Oreochromis niloticus* [65], *O. hatcheri* [19] and in *E. lucius* [46] *amh* are duplicated, and this second copy, called *amhY* and *amhb-Y* (Y-chromosome-specific copy of *amh*), respectively, acts as a sex determining gene. Interestingly, a study by Nakamoto and colleagues [47] identified that *amhr2bY* is critical for gonadal sex determination in *P. altivelis.*

Sex determination and differentiation in fish: The role of *amh* signaling

The undifferentiated gonad is composed of the somatic cells and germ cells, the later give rise to the gametes. At the sex determination stage, the somatic cells begin to differentiate into Sertoli and Leydig cells in males, or granulosa and theca cells in females [66,67]. The germline components are derived from the primordial germ cells, which migrate into the developing gonad, where the gametogenesis takes place [68,69]. Once the sex was determined, the germ cells follows a unique path of development, the cells differentiate as spermatogonia or oogonia [21,70].

In mammals, during embryonic formation, the undifferentiated gonad is formed by paramesonephric ducts (Müller) and mesonephric ducts (Wolf). Müller's ducts will be responsible for the formation of the uterus, fallopian tubes and the formation of upper parts of the vagina. Wolf's ducts form the epididymis, the vas deferens, and the seminal vesicles. The undifferentiated gonad differentiate into a testis or ovary by activating a gene cascade [39,71,72]. In humans, this cascade is well described and primarily occurs by activating *SF-*

1 (Steroidogenic factor 1) e *WT1* (Wilms' tumor *1*) which in turn activate the *SRY* (Sex determining Region Y) expression. In the presence of *SRY*, as Sertoli cells initiate the production of *AMH*. The *AMH* promote the regression of Müller's ducts, leading to male differentiation with the development of the tests. In the absence of *SRY*, *WNT4* (Wingless-type MMTV integration 4) and *DAX-1* (Dosage-sensitive sex reversal - Adrenal hypoplasia congenital critical region on the X chromosome 1) gene expression occurs activating the cascade of female sex differentiation [56,71,73-76].

In contrast, teleost fishes are perhaps the most complex group of animals in the mechanism of sex determination and differentiation. In the fishes, the sex development begging with a trigger a complex transformation process of bipotential gonad into a differentiated gonad, either testis or ovary [70,77-80] (Figure 1). The gonadal sex are specifically determined by a complex developmental process includes fate determination and cell differentiation, and both programs are regulated and tuned by cascades or networks of genes [81] (Figure 1).

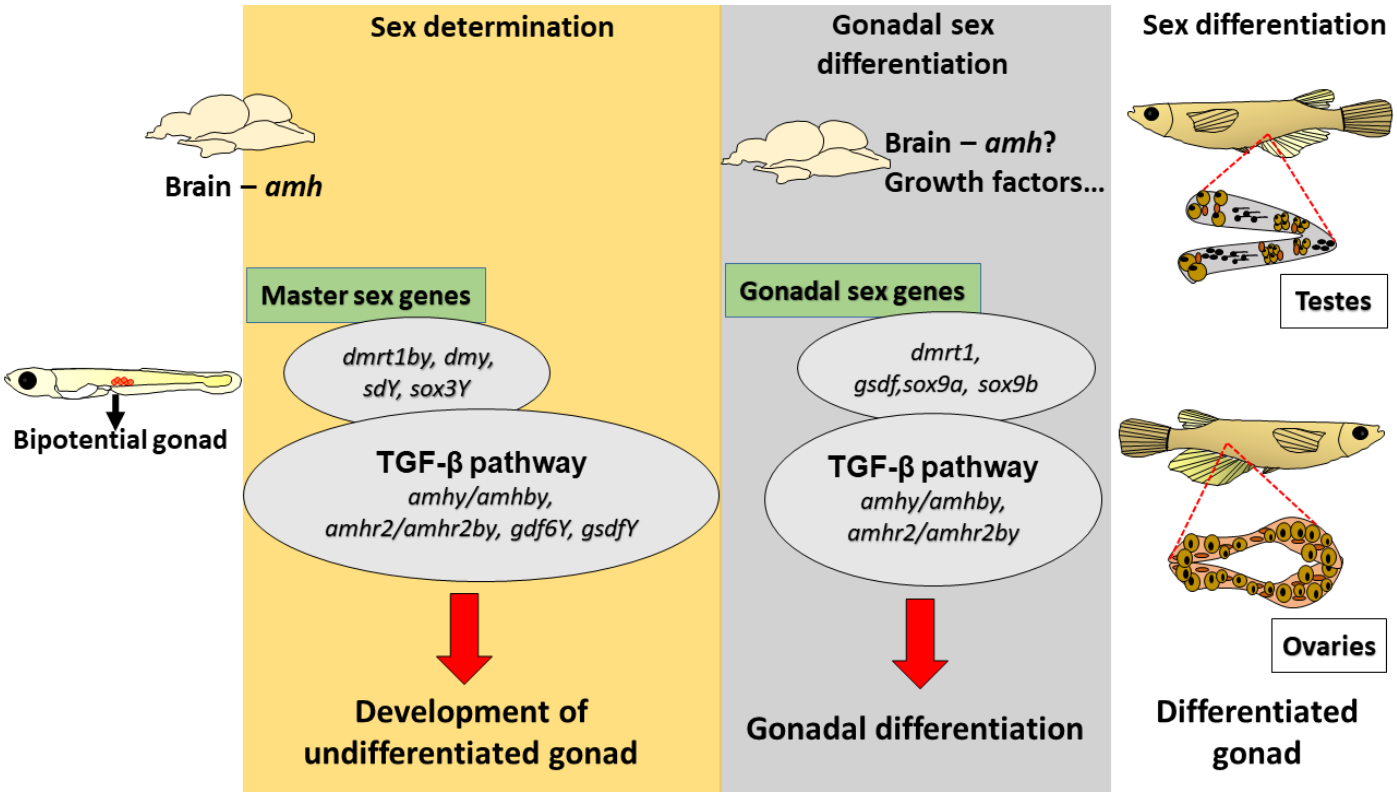


Figure 1. First, the bipotential gonad that was formed by primordial germ cells and somatic cells is genetically determined. During this moment, the expression of the master genes of sex determination occurs. In some species, amh expression occurs in the bipotential gonad phase, which favors the differentiation pathway for males. After this stage, the gonad begins its differentiation process. At this moment, the expression of genes related to the formation of testes in males and of ovaries in females beginning (genes linked to the formation of ovaries not shown in the figure). At this time, amh expression is high in males and low in females. After gonadal differentiation, phenotypic sex differentiation occurs in animals with the formation of testis in males and ovaries in females.

The genetic machinery controlling gonad development is broadly conserved, where downstream components tend to converge upon the regulation of common effectors. However, comparisons of the sex determination cascades in different organisms show an impressive diversity of 'master sex-determining genes' at the top of the genetic hierarchies [77,80,82].

In the last years, different master genes to sexual determination and differentiation have been identified between teleost fishes (Table 1), showing a great diversity and plasticity in this vertebrates group [83].

Table 1. Male sex-determining genes identified in fishes.

Gene	Gene-full name	Species	References
sdY	Sexually dimorphic on the Y-chromosome	*Oncorhynchus mykiss* *O. masou* *Salmon salar* *S. trutta* *Salvelinus alpinus* *S. fontinalis* *S. malma malma* *Hucho hucho* *Parahucho perryi*	[84]
sdf/gsdfY	Gonadal soma derived Factor/Gonadal soma derived factor on the Y-chromosome	*Anoplopoma fimbria* *O. luzonensis*	[16] [85]
gdf6Y	Growth differentiation factor 6 on the Y-chromosome	*Turquoise killifish* *Nothobranchius furzeri*	[44]
dmy	DM-domain on the Y-chromosome	*O. latipes*	[86]
dmrt1	Doublesex and mab-3 related transcription fator 1	*Scatophagus argus* *Cynoglossus semilaevis*	[87] [88]
dmrt1bY	Doublesex and mab-3 related transcription fator 1b on the Y chromosome	*O. latipes,* *O. curvinotus*	[89] [90]
sox3Y	Sry-related high mobility group-box gene 3 on the Y chromosome	*O. dancena*	[91]
amhr2	Anti-Müllerian hormone receptor type 2	*T. rubripes*	[45]
amhr2by	Anti-Müllerian hormone receptor type 2 b on the Y chromosome	*Perca flavescens* *P. altivelis*	[47]
amhy	Y-linked anti-Müllerian hormone	*O. Hatcheri* *O. Niloticus* *Sebastes schlegelii* *Hypoatherina tsurugae*	[19] [70] [92] [93]
amhby	Y-linked anti-Müllerian Hormone b	*E. lucius*	[46]

In this context, *amh* emerge as important effector during the sex determination and differentiation process. Although there is information in several studies pointing to the importance of *amh* in both processes, there is a lack of data on its importance in the event, especially the expression timing and your role during gonadal differentiation. For a better understanding of which biological processes can underlie the molecular mechanisms of interaction between *amh-amhr2*, an associative gene network was constructed (Figure 2). *amh-amhr2* are involved in gonadal development, sex determination and differentiation in fishes and are relatively well connected between them and with other gonadal development and sex differentiation genes inside the associative gene network. To create a gene interaction, was used two species that have full genomic data available (https://www.ncbi.nlm.nih.gov/; https://www.ensembl.org/index.html).

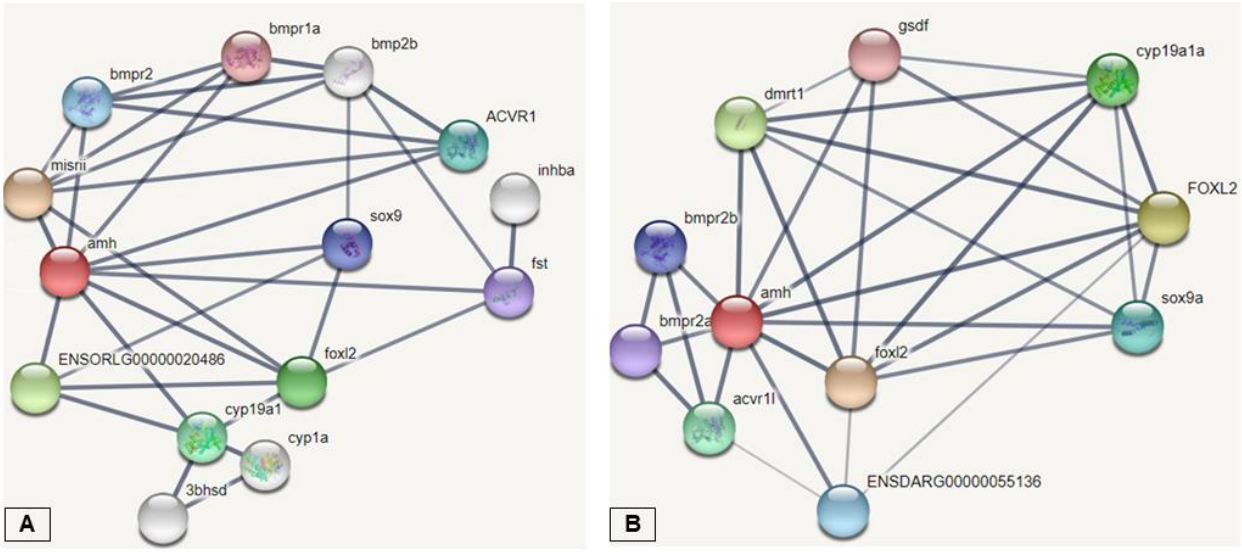


Figure 2. An associative gene network illustrating the interactions of the *amh-amhr2* with related-genes for gonadal development, sex determination and differentiation in two teleost fishes. Observing the branches is possible to identified that *amh-amhr2* have an important role controlling the molecular cascade of both biological process. To create the interaction was used the gene sequences from (A) medaka (*O.latipes*) and (B) zebrafish (*D. rerio*) and the network was reconstructed with STRING software [94].

The Amh was first described in the Japanese eel (*Anguilla japonica*) and in this first moment was called eel spermatogenesis related substances 21 (eSRS21) or spermatogenesis-preventing substance [43]. The authors described that eSRS21 prevents the beginning of spermatogenesis and, consequently, your suppression is necessary to spermatogenesis beginning. On the other hand, Oliveira and colleagues [13], showed that the common carp (*Cyprinus carpio*) *amh* transcripts were down-regulated during the reproductive developing phase, which is characterized by an increased proliferation of type A undifferentiated spermatogonia and Sertoli cells in the spermatogenesis process. The results reveals that Amh is crucial to improve or inhibiting the male sexual maturity in this teleost fish.

Nowadays, the role of *amh* as a master sex-determining gene are demonstrated in different species of teleost fishes, Patagonian pejerrey (*O. hatcheri*) [19], Nile tilapia (*O. niloticus*) [70,95] and Northern pike (*E. lucius*) [66]. Interestingly, in these species, the authors identified a Y-chromosome-specific duplicated copy of *amh.* In Northern pike*,* Pan and colleagues [66], named the duplicated copy of the *amhb-Y.* In the other hand, in pejerrey [19] and Nile tilapia [70,95], the authors classified as *amhY.* In all species, a duplicate copy of *amh* are necessary to trigger testicular development, playing key role in the sex determination being high expressed in the male gonadal primordium, supporting the evidence that *amhy* is a master sex-determining gene.

Still in relation the role of *amh* as sex-determining gene, Kamiya and colleagues [45] suggest that a missense SNP in the anti-Müllerian hormone receptor type II (*amhr2*) is a master sex-determining gene in fugu (*T. rubripes*). Similar to fugu, a duplicate copy of *amhr2* on the Y-chromossome (*amhr2bY*) is critical for sex determination in ayu (*P. altivelis*) [47]. Taken together, all these results shown that the *amh-amhr2* pathway is critical for gonadal differentiation in male teleost fishes.

Amh are produced and released by Sertoli cells and have play an important function in fish gonadal development, with a higher *amh* expression in males than females, suggesting therefore that Amh might be important for testicular differentiation [13,96-98]. In Japanese eel, zebrafish, medaka, sea bass, Iberian chub, common carpand rainbow trout the *amh* expression can be found both male than female with high expression in testis and, have a role during the male sex determination and differentiation and, act inhibiting both steroidogenesis and spermatogenesis [13,17,41,43,63,99-101].

The fact that Amh signaling to be linked to sexual differentiation in fish was observed in studies carried out on teleost fish medaka. Studies on the medaka mutant *hotei* showed an over-proliferation of germ cells and 50% of male-to-female sex reversal in the *hotei* homozygous [102]. The *hotei* phenotypes is caused by a mutation of the *amhr2*. Characterization of this mutant showed that the Amh signaling acts in supporting cells to regulate the proliferation of the mitotically active germ cells but does not trigger quiescent germ cells to proliferate in the developing gonad.

In this way, Lin and colleagues [80], using a CRISPR/Cas9 technology carried out the knockout of *amh* in zebrafish to understood the *amh* role during sex differentiation. These authors showed that the loss of *amh*

function led to gonadal hypertrophy due to the accumulation of undifferentiated spermatogonia and dysregulation of rate sexual development The authors also reported an increase in the rate of females in homozygous mutants (71%) while in heterozygous the rate was 46% of the females.

In protogynous orange-spotted grouper (*Epinephelus coioides*) the Amh to play roles in regulating male differentiation during the female-to-male sex change and, in the inhibiting type-A spermatogonia-like cell proliferation and differentiation during male-to-female sex change [87]. On the other hand, in the protandrous black porgy (*Acanthopagrus Schlegeli*), a hermaphrodite fish, elevate levels of *amh* expression are associates with beginning of testicular differentiation and the levels are maintained during natural female-to-male sex change. Still, during natural male-to-female sex change, *amh* expression decreased drastically to development and growth ovarian [103-105].

Interestingly, it is noteworthy that *amh* have expression extragonadal, specifically in the fish brain. For example, in the brain of larval Nile tilapia [106] and Atlantic salmon [107] *amh* had expression before the beginning of gonadal *amh* expression when the gonads are still bipotential. This result showed that sexual differentiation can occurred earlier in brain than in the gonads. In this specific case, early male-specific *amh* expression in the brain suggests an auto - or paracrine regulation in the larval brain [106,107].

Take together, these data suggest that the *amh-amhr2* pathway is key to both testicular development and male sex determination and differentiation in fish and,in this latter case, is noteworthy the requirement to duplicated and had undergone functional diversification, supporting the network between gonadal plasticity and genetic factor in teleost fish sex.

CONCLUSIONS

The discovered of *amh-amhy-amhr2* as a “master sex determining-gene” in teleost fish has shed light on the molecular mechanism of the sex determination and differentiation in fish and open the genetic toolbox to better understanding this important mechanism in all fishes.

In summary, this review showed the pivotal role of *amh-amhy-amhr2* signaling. Overall, the information herein provide the genetic evidence and gonadal plasticity for the important role of the *amh* to sex determination and testicular differentiation and shown that the Amh signaling is an important effector in the decision whether the undifferentiated gonad anlage will become a male or female.

Conflicts of Interest: The authors declare no conflict of interest.

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