Proteolytically Activated, Recombinant Anti-Müllerian Hormone Inhibits Androgen Secretion, Proliferation, and Differentiation of Spermatogonia in Adult Zebrafish Testis Organ Cultures


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Anti-Müllerian hormone (Amh) is in mammals known as a TGFβ type of glycoprotein processed to yield a bioactive C-terminal homodimer that directs regression of Müllerian ducts in the male fetus and regulates steroidogenesis and early stages of folliculogenesis. Here, we report on the zebrafish Amh homologue. Zebrafish, as all teleost fish, do not have Müllerian ducts. Antibodies raised against the N- and C-terminal part of Amh were used to study the processing of endogenous and recombinant Amh. The N-terminally directed antibody detected a 27-kDa protein, whereas the C-terminally directed one recognized a 32-kDa protein in testes extracts, both apparently not glycosylated. The C-terminal fragment was present as a monomeric protein, because reducing conditions did not change its apparent molecular mass. Recombinant zebrafish Amh was cleaved with plasmin to N- and C-terminal fragments that after deglycosylation were similar in size to endogenous Amh fragments. Mass spectrometry and N-terminal sequencing revealed a 21-residue N-terminal leader sequence and a plasmin cleavage site after Lys or Arg within Lys-Arg-His at position 263–265, which produce theoretical fragments in accordance with the experimental results. Experiments using adult zebrafish testes tissue cultures showed that plasmin-cleaved, but not uncleaved, Amh inhibited gonadotropin-stimulated androgen production. However, androgens did not modulate amh expression that was, on the other hand, down-regulated by Fsh. Moreover, plasmin-cleaved Amh inhibited androgen-stimulated proliferation as well as differentiation of type A spermatogonia. In conclusion, zebrafish Amh is processed to become bioactive and has independent functions in inhibiting both steroidogenesis and spermatogenesis. (Endocrinology 152: 3527–3540, 2011)
region (present as a homodimer after cleavage) enhances the activity of the C-terminal fragment (14). The C-terminal fragment contains the conserved TGFβ domain (2) with highly conserved cysteines involved in forming a cystine-knot structure of the active part (12, 15). A recent report has verified that cleavage is necessary for efficient receptor binding and that the N-terminal pro-region is present at receptor interaction but dissociates from the C-terminal homodimer as a consequence of receptor binding (16).

Amh signals through binding to a Amh type II transmembrane receptor (AmhRII) with serine/threonine kinase activity (17). This complex recruits and phosphorylates one of the activin-receptor kinases (ALK) mediating stimulatory (ALK2 and ALK3) or inhibitory (ALK6) effects (17, 18) that involve Smad proteins in the downstream signaling cascade (19). In the testis, AmhRII is expressed in Sertoli (20) and Leydig (21) cells. In fish, an AmhRII has been identified in medaka (*Oryzias latipes*) (22) and reported from black porgy, *Acanthopagrus schlegeli* (23).

In mammalian testes, Amh is highly expressed by immature Sertoli cells until puberty, when at the start of meiosis, increasing testosterone levels down-regulate Amh, mRNA and protein levels, that remain low during late puberty and adulthood (24). Amh expression in juvenile males is stimulated by Fsh when the androgen receptor is not activated in Sertoli cells (25). Moreover, Amh is a negative regulator of postnatal Leydig cell differentiation and steroidogenesis. Mice transgenic for human Amh show a decreased number of adult-type, differentiated Leydig cells, low plasma levels of testosterone, and reduced mRNA levels of steroidogenic enzymes, including Cyp17 (21). Also, Amh reduced LH-induced testosterone production by fetal and adult Leydig cells in rodents (26, 27). Amh-deficient mice, on the other hand, showed a remarkable hyperplasia of Leydig cells (28, 29). These observations suggest that in mammals, Amh has a negative regulatory role in the postnatal differentiation and function of Leydig cells.

Teleost fish lack Müllerian ducts, but Amh homologues have been identified in several species, such as Japanese eel (30), zebrafish (31, 32), tilapia (33), medaka (22), sea bass (34), Iberian chub (35), pejerrey (36), and rainbow trout (37, 38). In primary cultures of Japanese eel testis fragments, recombinant eel Amh (originally named spermatogenesis-preventing substance) inhibited 11-ketotestosterone (11-KT), the main androgen in zebrafish (39) and other fish species (40), and induced spermatogenesis by blocking the proliferation of type B spermatagonia (30). In medaka, there is an additional aspect of Amh bioactivity during early gonad differentiation in both sexes: gene knockdown experiments targeting Amh or AmhRII reduced germ-cell proliferation, and recombinant eel Amh counteracted this effect when added to medaka gonad tissue fragments from *amh* knockdown animals (41). In contrast, the medaka *amhrII/hotei* loss-of-function mutant displayed an increased number of germ cells in both sexes (42). Moreover, male mutants showed premature initiation of meiosis, and 50% underwent sex reversal.

Taken together, there is evidence for effects of Amh on germ-cell proliferation in fish, whereas the quality of the effect differs, being stimulatory during early stages of development (41) but inhibitory at the onset of puberty (30). However, it has remained unexplored whether Amh bioactivity described in mammals, such as the inhibitory effect on Leydig cell function (43), can be observed in fish and may thus be an evolutionary conserved feature. Moreover, it is not known whether Amh has an effect on spermatogenesis in adult animals. In this work, we first have investigated how zebrafish Amh is processed to become bioactive, before testing plasmin-cleaved, recombinant Amh in zebrafish testis cultures. To this end, we have investigated whether Amh modulates steroidogenesis or spermatogenesis in adult zebrafish testis. We also investigated the ontogenesis of *amh* expression and aspects of the endocrine regulation of *amh* expression, and we propose a model on the role of Amh on adult zebrafish testis functions.

**Materials and Methods**

Detailed material and methods are provided in the Supplemental data, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org. In brief, antisera directed to N-terminal and C-terminal regions of Amh were raised against peptides and provided as purified rabbit antibodies by BioGenes (Berlin, Germany). The C-terminal-directed antibody was used for immunocytochemical detection of endogenous Amh in adult zebrafish testis. Recombinant zebrafish Amh was produced in stably transfected human embryonic kidney 293 (HEK293) cells and purified from culture medium via a 6xHis tag introduced after Pro33 (*AY721604*). The presumed proteolytic cleavage site, based on sequence comparison, was optimized from RAQR motif (position 439–442) to RARR using QuickChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), and the product was named His-Amh-Q441R. Plasmin treatments were conducted as indicated in figure legends. The reaction was stopped with aprotinin (*A3428*; Sigma, St. Louis, MO) and the extract frozen at –80°C until further use.

Amh in distilled water was deglycosylated using the Enzymatic Carbo Release kit from QA-Bio (Palm Desert, CA) (KE-DG01). N-terminal sequencing using Edman degradation was performed (Proteomics Facility, University of Leeds, Leeds, UK) on SDS-PAGE separated Amh followed by electroblotting onto a sequencing-grade polyvinylidene fluoride membrane. Matrix-assisted laser desorption/ionization-time-
of-flight analyses (PROBE Proteomics Facility, University of Bergen) were performed on acetonitrile precipitated purified untreated and plasmin-cleaved peptide-in-gel samples. Untreated and plasmin-cleaved, recombinant zebrafish Amh (10 μg/ml or 140 nM, 70 nM if dimer) were added to primary zebrafish testis organ cultures (as described in Ref. 44) in the presence of recombinant zebrafish Fsh (100–500 ng/ml). Cultures were analyzed for 11-KT in media (44), and testis tissue for expression of selected genes (cytochrome P450, family 17, subfamily A, polypeptide 1 (cyp17a1), steroidogenic acute regulatory protein (star), insulin-like 3 (insl3), and androgen receptor (ar)) by real-time quantitative PCR (44). Relative mRNA levels of target genes (cyp17a1, star, insl3, and ar) were normalized to 18S rRNA (reference endogenous control gene) and expressed as fold induction compared with the control groups.

Gonadal amh mRNA was quantified as previously (44) in samples collected 4 wk postfertilization (wpf) (after completion of sex differentiation), 8 wpf (pubertal gonad growth ongoing), and 12 wpf (young adults), detailed in Supplemental Methods. To address the endocrine regulation of amh expression, adult testis tissue was incubated with androgens or with recombinant zebrafish Fsh in the absence or presence of a protein kinase A inhibitor H89 under different conditions (see Supplemental Methods), before amh mRNA levels were quantified as previously (44).

Effects of recombinant zebrafish Amh on spermatogenesis [bromodeoxyuridine (BrdU) incorporation into type A undifferentiated (Aunu) spermatogonia and frequency of germ-cell types] were analyzed using a previously described tissue culture system (45). Testes were collected from untreated, adult males, and tissue was incubated in the presence of 200 nM 11-KT to test the effect of recombinant zebrafish Amh (10 μg/ml), or testes were collected from adult males exposed for 3 wk to 10 nM estradiol-17β to induce androgen insufficiency and inhibit spermatogenesis (46) before the tissue was incubated in the absence or presence of Amh (10 μg/ml). Incubation conditions, fixation, and morphological and morphometrical analysis of the samples are detailed in the Supplemental Methods.

Significant differences between two groups were identified using Student’s t test (paired and unpaired) (P < 0.05). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test (P < 0.05). GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA) was used for all statistical analysis. Sequence analyses were performed as described in Supplemental Methods.

Results

Amh is processed by proteolytic cleavage

The zebrafish Amh cDNA sequence (AY721604) predicts a 549-aa protein with a molecular mass of 61.1 kDa, including a 21-residue leader sequence, that after cleavage gives a 58.7-kDa protein (Fig. 1A and Supplemental Fig. 1). Two N-glycosylation sites were predicted at N334 (NSST) and N510 (NRSL), where the first site is conserved among fish species and is close to the human Amh glycosylation site (NLSD position 329–332, BC049194) (Supplemental Fig. 1). The predicted C-terminal TGFβ domain (aa 457–549) has seven conserved cysteine residues where C514 is expected to be involved in dimer formation. Human AMH is known to be cleaved after RAQR at position 448–451, which align to a putative cleavage site in zebrafish Amh at position 439–445 (Supplemental Fig. 1B).

Immunoblots of testes protein extracts under reducing and nonreducing conditions revealed two fragments probably generated by proteolytic cleavage of the full-length protein: N-27 and C-32 (Fig. 1, B and C). An alternative lysine buffer produced two candidate full-length Amh proteins of 66 and 71 kDa detected with the anti-C antibody (Fig. 1C). A 140-kDa protein detected under nonreducing conditions may represent a dimerized form. We observed no molecular mass shift between reducing and nonreducing conditions of the presumptive processed C-32 form of the protein. This finding implies that the 32-kDa C-fragment is present as a monomer in zebrafish testes and not as the expected cysteine-bridged dimer typically seen in the TGFβ class of protein (IPR021203; http://www.ebi.ac.uk/interpro).

Recombinant zebrafish Amh

Recombinant Amh (wild-type sequence) produced in HEK293 cells was recognized as a 70-kDa precursor in cells lysates and in concentrated medium by the two specific antibodies anti-N and anti-C (see Fig. 1, E and F). Treatment with plasmin, a serine protease that cleaves after arginine or lysine (9), gave three cleavage products from the N terminus (N-24.5-26.5-28.5), whereas the C-terminal antibody detected three matching products (C-34-36-39) (Fig. 1, E and F). Increased plasmin concentrations produced several less abundant C-fragments (C-16-19-21.5), all larger than the predicted C-product of 11.7 kDa assuming TGFβ class of maturation (Fig. 1A and Supplemental Fig. 1C).

Amh in testis extracts from zebrafish was apparently cleaved by an endogenous enzyme to give the C-terminal 32-kDa protein. This fragment was susceptible to plasmin treatment, because it was degraded even at low protease concentration and no degradation products were observed (see Fig. 1D).

Recombinant zebrafish Amh optimized for downstream processing

To produce a biologically active recombinant zebrafish Amh designed for simple purification and use in functional experiments, a strategy used with human recombinant AMH (47) was adapted. The putative protease cleavage motif RAQR at aa position 439–442 was changed to a RARR motif, and a histidine tag was inserted just before proline at position 33 (similar to 47). Medium from stably transfected HEK293 cells producing His-Amh-Q441R re-
revealed both the apparent 70-kDa precursor form and different processed forms (Fig. 2A). Nonreducing conditions gave a 140-kDa protein, suggesting a dimerized form of the 70-kDa protein. A 50-kDa protein (N-50) was detected with anti-N corresponding to the predicted 48-kDa protein (K263 or R264 in the native Amh). Theoretically, this cleavage should give an N-fragment of 27.8 kDa (27 kDa without histidine tag) and a C-fragment of 31.8 kDa (Fig. 1 and Supplemental Fig. 1).

Identification of signal peptide and possible site of proteolytic cleavage

The predicted 21-aa leader sequence (Fig. 1 and Supplemental Fig. 1) was verified by N-terminal sequencing (Edman degradation) of purified 70-kDa zebrafish His-Amh. Identification of the six first aa showed that the signal peptide had been cleaved off between aa C21 and A22 (see Supplemental Figs. 1 and 2A).

Two products from plasmin-treated recombinant Amh, N-28.5 and C-36, were analyzed by mass spectrometry to reveal the cleavage site (Supplemental Fig. 2B). Aligning the fragments from the mass spectrometry analysis indicated cleavage after K269 or R270 in the sequence of the modified protein (K263 or R264 in the native Amh). Theoretically, this cleavage should give an N-fragment of 27.8 kDa (27 kDa without histidine tag) and a C-fragment of 31.8 kDa (Fig. 1 and Supplemental Fig 1).

Zebrafish Amh is glycosylated in human cultured cells but not in zebrafish testes

Mammalian Amh is glycosylated, and two N-glycosylation sites were predicted in the zebrafish protein (Sup-
from 8 to 12 wpf (see figure 5A). In females, expression levels increased approximately 10-fold from 4 to 8 wpf but then remained constant (see figure 5A). In adult zebrafish testes, Amh was detected in Sertoli cells surrounding early germ-cell generations, such as type $A_{\text{und}}$ (Fig. 4, A–E) and type $A_{\text{diff}}$ spermatogonia (Fig. 4, F and G), the former often located near to the interstitial compartment (Fig. 4, B and C). The Amh-specific staining was much weaker or absent from Sertoli cells surrounding later stages of germ-cell development, such as type B spermatogonia, spermatocytes, and spermatids (Fig. 4, A, D, and F). Preabsorption of the antibody with the peptide fragment used to generate the antisera abolishes the staining (Supplemental Fig. 4), demonstrating the specificity of the immunocytochemical reaction.

Testes from adult zebrafish were studied in a primary, short-term tissue culture system to investigate the effect of Amh on gonadotropin-stimulated androgen release. In zebrafish, the pituitary gonadotropin Fsh has a strong steroidogenic potency that exceeds the one of LH, and Leydig cells express the receptor for both gonadotropins, Lh and Fsh (44). Thus, the release of 11-KT, the major androgen in fish, was stimulated using recombinant zebrafish Fsh. Preincubation of zebrafish testes with purified plasmin-treated recombinant zebrafish Amh for 6 or 24 h significantly reduced, or abolished, respectively, Fsh-stimulated androgen release (Fig. 5B). When testing uncleaved recombinant Amh, Fsh-stimulated 11-KT production was not compromised (Fig. 5B, to the right of dashed line). Expression analysis revealed that the Fsh-induced up-regulation of cyp17a1, star, and insl3 transcript levels was significantly reduced after 24 h of preincubation with Amh (Fig. 5C, Supplemental Fig. 5); expression of ar remained unaltered under all conditions.

Amh mRNA in testis tissue cultures was down-regulated by Fsh treatment, independent of the steroidogenic activity of Fsh (Fig. 5, D and E). Increasing doses of 11-KT did not change amh mRNA levels significantly. Moreover, the down-regulatory effect of Fsh is possibly also independent of the cAMP/PKA pathway (Fig. 5, D and E).

To examine whether zebrafish Amh prevents androgen-stimulated adult spermatogenesis, akin to the inhibi-
tion of the onset of spermatogenesis described in juvenile Japanese eel (30), we incubated adult zebrafish testis tissue (45) for 7 d with 11-KT only (control), or with 11-KT and Amh. All stages of spermatogenesis were present (i.e., undifferentiated and differentiated type A spermatogonia, type B spermatogonia, spermatocytes, spermatids, and spermatozoa) (see Fig. 6, A, C, and E) in testes incubated with 11-KT only. Qualitatively, all these stages were also present after incubation with 11-KT and Amh. However, morphometric analysis revealed significant differences. The number of cysts with type A und spermatogonia was higher in testis tissue exposed to Amh, whereas the number of cysts containing type B spermatogonia, spermatocytes, spermatids was significantly reduced (Fig. 6, A, D, and F). The increased number of type A und spermatogonia can reflect a block of their differentiation, leading to their accumulation, or an increase in their proliferation. Therefore, we studied BrdU incorporation, which revealed a reduction of the BrdU-labeling index of A und spermatogonia in the presence of Amh (Fig. 6B), excluding the possibility that Amh stimulated the proliferation of A und spermatogonia. The inhibitory effect of Amh on 11-KT-induced proliferation of further advanced germ cells was reflected in the reduced number of BrdU-positive cysts in testis exposed to 11-KT and Amh (Fig. 6, D and F).

Quantitatively clearer results as regards spermatogonia type A und were obtained when using testis tissue for primary cultures from males pretreated with estrogen in vivo. Previous studies showed that estrogen treatment blocked differentiation and reduced proliferation of A und but also of A diff spermatogonia (46). Spermatogonial proliferation and differentiation recovered from the estrogen-induced inhibition when testis tissue was cultured ex vivo under basal conditions, as indicated by the presence of clones of type B spermatogonia (Fig. 7B), a BrdU-labeling index of spermatogonia type A und of 40% (Fig. 7C), and a high number of BrdU-positive germ cells (Fig. 7E), both single cells and differentiating clones of spermatogonia. This spontaneous recovery was suppressed in the presence of Amh: differentiating germ cells were rare and spermatogonia type A und were frequently present (Fig. 7A), whereas their BrdU-labeling index was reduced more than 3-fold (Fig. 7B). In general, the number of BrdU-positive cells was lower (Fig. 7D). We conclude that Amh reduced proliferation and prevented differentiation of spermatogonia type A und.

Discussion

This communication focuses on how Amh is processed to a biologically active protein and elucidating its functions in fish. Using zebrafish as model organism, Amh was analyzed in testes extracts and as recombinant protein. We found that zebrafish Amh, proteolytically cleaved to become fully active, near abolished Fsh-stimulated androgen production, inhibited spermatogenesis, and was down-regulated by Fsh but not by androgens.

Endogenous Amh

The endogenous zebrafish protein was detected as a N-27 and a C-32 fragment in contrast to processed endogenous rat Amh, reported to be 48 and 12 kDa, respectively, at reducing conditions (11). The eel homologue was detected as a 30-kDa protein in immature testes using an
antiserum against the N-terminal 243 aa of the protein (30). This suggests that Amh is proteolytically processed both in mammals and fish but with different cleavage sites. In human Amh, the C-terminal fragment includes 109 aa and forms a homodimer (2), whereas the zebrafish protein C terminus extends over 280 residues and is a monomer. This is unusual for members of the TGFβ family, because dimerization of a C-terminal fragment is considered a general characteristic of this class of proteins (48). However, the recombinant variant of zebrafish Amh appeared as a disulfide linked dimer in the range of 65–76 kDa and monomer size of 34, 36, and 39 kDa detected with C-terminal-directed antibodies.

Two endogenous precursor candidate proteins of 66 and 71 kDa were recognized with the anti-C antibody (Figs. 1C and 3B) and a possible dimer of 140 kDa at nonreducing conditions. The eel Amh is 60 kDa at reducing conditions and 120 kDa at nonreducing conditions due to disulfide bonding (37). Full-length zebrafish Amh consists of 549 aa, whereas full-length eel protein consists of 614 aa, coinciding with theoretical molecular masses of 58.7 and 62.6 kDa without signal peptides, respectively.

Proteolytic activation of zebrafish Amh differs from human Amh

Signal peptides of secreted proteins are cleaved off by signal peptidase in the endoplasmatic reticulum (ER) before secretion (49). N-terminal sequencing of the purified recombinant Amh verified cleavage of a 21-residue long signal peptide. The recombinant Amh was further located to ER in the HEK293 cells and colocalized with the ER-protein disulfide isomerase (Supplemental Fig. 3).

Zebrafish His-Amh-Q441R was designed to be in vivo processed in the HEK293 cells similar to modified mammalian Amh (47). Although mammalian Amh was cleaved at the optimized site only (47), we never detected the predicted C-terminal 11.7-kDa zebrafish fragment (Supplemental Fig. 1). The N-terminal-directed antibody detected a major 50-kDa protein and several weaker fragments (N-32-34-36). In addition, C-fragments (with C-19 as major product) were found. The pattern of fragments agrees with the occurrence of at least one extra cleavage of zebrafish Amh in HEK293 cells compared with modified human protein. Proteolytic activation of human AMH takes place by cleavage after residue 451 at RAQR/S, although a potential alternative cleavage may occur after R254 at PR/S (50). Human AMH with the optimized RARR451/S cleavage site is spontaneously cleaved in HEK293 cells to give a biologically active protein (51). The sequence variant RAQR451/R needed plasmin cleavage to be activated. Treating zebrafish Amh with plasmin resulted in a different cleavage pattern compared with human AMH, with preference for larger C-fragments than the reported bioactive 25-kDa human homodimer protein. The predicted plasmin cleavage site (KR264/H) in
zebrafish Amh (Supplemental Fig. 1) seems to be in a position similar to the secondary mammalian cleavage site (R254) (50).

Modification by glycosylation

Endogenous and recombinant Amh appeared as 70-kDa proteins, whereas sequence predictions suggested proteins of 58.7 and 59.6 kDa (excluding leader sequences), respectively. Glycosylation of the recombinant protein was evident in HEK293 cells and could explain much of the observed differences (Fig. 3A). The discrepancy between observed and theoretical molecular mass of endogenous Amh remains unclear, because glycosylation was not detected (Fig. 3C). The in vivo function of glycosylation remains obscure, but studies of gonadotropins point at a general impact on improved secretion and stability (52, 53).

Deglycosylation of recombinant Amh C-terminal cleavage product C-34-36-39 yielded a 32-kDa product similar to the theoretical size and endogenous nonglyco-
sylated Amh. This suggests that the verified cleavage site KR/H could be a natural cleavage site of zebrafish Amh yielding a 27-kDa N-fragment and a 32-kDa C-fragment. Endogenous protease(s), which have Amh as natural substrate, is/are not known. However, proprotein convertase 5 has been suggested as the natural protease in rat (11).

FIG. 6. Biological activity of recombinant zebrafish Amh (rzfAmh) on zebrafish spermatogenesis in testis tissue culture from untreated adult males. A, Ex vivo spermatogenesis supported by 11-KT (200 nM; white bars) was inhibited by Amh (10 μg/ml; black bars). Bars indicate the number (mean ± ssm) of cysts/mm² of testis containing type A.Rem spermatogonia, type A.Rem spermatogonia, type B spermatogonia, spermatocytes (Sc), and spermatids (ST). B, BrdU-labeling index of type A.Rem spermatogonia from zebrafish testes cultured for 7 d with 11-KT or 11-KT + Amh. *, Values are significantly different (P < 0.05) between 11-KT and 11-KT + Amh in A and B. C and D, Low magnifications of zebrafish testis sections immunostained for BrdU (brown) and counterstained with hematoxylin. Amh decreased the number of BrdU-positive spermatogenic cysts containing germ cells advanced beyond the stage of type A spermatogonia (arrows) and led to an accumulation of type A.Rem spermatogonia. E and F, High magnifications illustrate that type A.Rem spermatogonia are often BrdU-positive (black arrowheads) in tissue incubated with 11-KT, whereas type A.Rem spermatogonia accumulated in the presence of Amh and were then often BrdU-negative (white arrowheads).
The proprotein convertases 1 (enzyme commission number 3.4.21.93) and 2 (enzyme commission number 3.4.21.94) cleave proteins at KR-X sites. Further investigations will have to reveal if these proteases are present in zebrafish testes and if they can cleave zebrafish Amh. Zebrafish Amh, monomer, or dimer?

Based on the gel patterns of recombinant zebrafish Amh under reducing and nonreducing conditions (Fig. 2C), it seems that a cysteine close to the C terminus is involved in dimerization of the protein. The N-terminal fragments failed to form homodimers, in contrast to human AMH (Fig. 4C) (9). Homodimerization of the recombinant Amh C-peptide correlates well to data on mammalian AMH (9) but disagrees with our observation that the endogenous C-32 is present as a monomer in zebrafish testes. Japanese eel Amh was detected as a 120-kDa cysteine-bridged dimer in its full-length form in testes extracts but the cysteine-bridge where absent in a 30-kDa N-terminal part in the recombinant eel Amh variant, suggesting a similar organization as for the recombinant zebrafish Amh (30). The human Amh required cleavage for biological activity to generate the active C-terminal 25-kDa homodimer, the N-terminal part remains in the complex and enhances receptor binding but is lost during receptor activation (16).

Cleaved Amh inhibits Fsh-stimulated androgen production and inhibits spermatogenesis

Teleost fish lack Müllerian ducts but nevertheless express Amh, suggesting that other than the namesake functions can be investigated in fish. In mammals, AMH inhibited Leydig cell differentiation and LH-stimulated androgen release (27, 43, 54). Our studies revealed for the first time in a lower vertebrate a suppressive role of Amh on gonadotropin-stimulated Leydig cell gene expression (star, insl3, and cyp17a1) and androgen release (Fig. 5). In vivo (55) and in vitro (27) experiments demonstrated that Amh inhibition of testosterone production also in rodents involved reduced Cyp17 expression, and low levels of testosterone in Amh-overexpressing mice coincided with decreased expression of steroidogenic genes (21). We conclude that Amh-mediated down-regulation of star and cyp17a1 in Leydig cells is dominant over stimulatory effects of steroidogenic gonadotropin on the expression of these genes and is an evolutionary conserved function of Amh.

Amh reduced Fsh-induced increases in insl3 mRNA levels. In mammals, INSL3 is required for the testicular descent in embryonic life and acts as a male germ-cell survival factor in adults (56, 57). Transcription factors such
Amh, androgens stimulate germ-cell differentiation from type A und spermatogonia into type Adiff spermatogonia, and type B spermatogonia (B). The suppressive role of Amh on insl3 expression, though there is no mechanistic information regarding its promoter in mouse, rat, and humans (58). Hence, allogenic enzyme-encoding genes and regulate the subfamily 4 group A member 1) regulate certain steroidogenesis genes such as star and cyp17a1, also in the presence of Fsh. In the absence of Amh, androgens stimulate germ-cell differentiation from type A und spermatogonia into type AAdiff spermatogonia, and type B spermatogonia (B). The suppressive role of Amh on androgen biosynthesis might have secondary effects on the expression of other Leydig cell genes, such as insl3 (partially down-regulated). Similar to the mammalian testis, Amh receptors are expressed by Sertoli cells but not by germ cells in fish (22), so that an autocrine Amh loop might trigger a downstream signaling mechanism in Sertoli cells, which would prevent early spermatogonial differentiation. On the other hand, when Sertoli cells expressing high levels of Amh respond to Fsh, Amh-mediated inhibition of germ-cell development and androgen production would wane, whereas Fsh-stimulated androgen and possibly growth factor release would stimulate the progression of germ cells toward meiosis.

Our observations verify this in a third and unrelated order of teleost fish, suggesting that Amh-mediated inhibition of spermatogenesis may be a typical aspect of Amh action in fish. In addition, we report for the first time on effects of Amh on adult spermatogenesis. BrdU incorporation analysis showed that although Amh reduced the mitotic index of type A und spermatogonia, that are the germ-cell population containing the spermatogonial stem cells (59), still the number of type A und spermatogonia increased. This apparent contradiction can be explained by the Amh-mediated block of the further differentiation of the slowly (viz. low mitotic index) accumulating type A und spermatogonia. Inhibiting differentiation of type A und spermatogonia would also explain the decreased number of more differentiated germ cells observed after Amh exposure. The present data, however, do not allow excluding an independent effect of Amh on later germ-cell stages. Taken together, our studies provide direct evidence for an inhibitory effect of Amh on the proliferation of type A und spermatogonia and moreover for a block of their differentiation. Hence, type A und spermatogonia accumulate while rapidly proliferating type B spermatogonia (and later stages of spermatogenesis) become depleted. In rat testis, the highest levels of expression of AMH and its receptor are in Sertoli cells in epithelial stage VII (60), postulating a relation between AMH signaling and the low mitotic activity of spermatogonia in stage VII. It might therefore be interesting to examine experimentally the possibility that AMH modulates proliferation/differentiation of spermatogonia also in adult mammals.

Sertoli cell AMH expression is high before puberty in mammals (2), is up-regulated by FSH (25), and greatly reduced at the pubertal increase in androgen levels and Sertoli cell androgen receptor expression (2). In zebrafish, however, amh expression progressively increased with puberty and adulthood. Two related observations seem important in this regard. First the Amh protein level is high in Sertoli cells contacting type A spermatogonia. And second, these Sertoli cells are not terminally differentiated in fish. Therefore, increasing amh expression during ontogeny can be explained with the increase in the number of spermatogenic cysts containing type A spermatogonia that accompanies pubertal testis growth and the further development toward adulthood (61).
Amh expression patterns during ontogenesis change differently in mammals vs. zebrafish and may reflect differences in the regulation of expression. In mammals, Fsh-stimulated androgen hormone excretion and inhibits Amh expression, whereas in zebrafish Fsh, down-regulated amh expression and androgens had no significant effect. For androgens, we speculate that the cystic mode of spermatogenesis in fish, characterized by undifferentiated Sertoli cells in contact with type A spermatogonia (i.e. showing high levels of Amh) also in the adult testis, is a situation incompatible with a mechanism where the pubertal increase in androgen production would induce a testis-wide down-regulation of amh expression. The down-regulation triggered by zebrafish Fsh contrasts with the up-regulation observed in mammals (25). We have not examined if cAMP is required in zebrafish in this context, but PKA signaling appears to be of minor relevance. Fsh-mediated down-regulation of amh expression fits well into the spectrum of biological activities of Amh. Two aspects are relevant in this context, namely that Fsh is a potent steroidogenic hormone in fish (58), including zebrafish (44), and that unpublished work in our laboratory shows that the proliferation/differentiation of spermatogonia is stimulated by Fsh in testis tissue culture in an androgen-independent manner (Nobrega, R. H., and R. W. Schulz, unpublished data). Both these effects of Fsh would be counteracted by Amh, so that Fsh-mediated down-regulation of amh expression seems an integral component of Fsh-mediated stimulation of zebrafish testis functions.

Despite the different regulatory mechanisms, reduced Amh signaling may permit germ-cell development both in fish (continuously occurring after puberty on the level of individual spermatogenic cysts) and mammals (occurring as a testis-wide event during puberty) and could be the second aspect of evolutionary conserved Amh bioactivity across vertebrates.

The biological activities of Amh in zebrafish are summarized graphically in Fig. 8. Amh effects in zebrafish are consistent with keeping germ cells in an immature state of development. In view of the Fsh-mediated suppression of Amh, we predict that Sertoli cells expressing high levels of Amh may have a limited responsiveness to Fsh. In Leydig cells, Amh inhibits androgen production, so that in the vicinity of Sertoli cells expressing high levels of Amh (i.e. contacting type A spermatogonia), androgen levels may be locally lower, resulting in an area were germ-cell differentiation is less likely to occur. In Sertoli cells, yet elusive signaling mechanisms would be activated in response to Amh to prevent differentiation of early spermatogonial generations (Fig. 8). When Sertoli cells expressing high levels of Amh become responsive to Fsh, Amh would be down-regulated, thereby permitting Fsh to stimulate germ-cell proliferation/differentiation and androgen production. For future work, several aspects are of interest, such as the biological activity of fish Amh as monomeric protein; information on the signaling systems used by Amh to modulate steroidogenesis via Leydig cells, and spermatogenesis via Sertoli cells; or finally, the integration of the mainly inhibitory Amh signaling with presumably existing, stimulatory signaling to achieve a coordinated regulation of testis functions.

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