Proteolytically activated, recombinant Anti-Müllerian Hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures.

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SUPPLEMENTAL DATA:

Supplemental Methods:

Preparation of protein extracts from zebrafish testes

Male zebrafish were anaesthetized in MESAB (0.25 g/l ethyl 3-aminobenzoate methanesulfonate) and decapitated. Testes (3-6) were dissected and immediately transferred to 0.3 ml of either cold Ringer solution (10 mM HEPES, pH 7.4, 3 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 5 mM CaCl2,) or cold lysis buffer (15 mM HEPES pH 7.9, 50 mM KCl, 6.25 mM MgCl2, 5 % (v/v) glycerol, 0.1 % (v/v) Nonidet P40, 1 mM EDTA containing Complete Protease Inhibitor Cocktail, Roche). The testes were homogenised using a pellet pestle motor. The Ringer homogenate was added to reducing SDS sample buffer, vortexed, boiled for 5 minutes and finally centrifuged at 12000 xg for 2 minutes and the supernatants were recovered. The lysis buffer-homogenate was centrifuged at 12000 xg, 15 minutes at 4 °C. The supernatant was recovered and added to reducing or non-reducing SDS sample buffer. Protein extracts were frozen at -20 °C until further analyses.

Recombinant zebrafish Amh production

Zebrafish amh cDNA (AY721604) was cloned into a pcDNA3.1/V5/His-vector (Invitrogen). Mutations were introduced using QuickChange II Site-directed mutagenesis kit, Stratagene. The presumed cleavage site, based on the human sequence, was optimized changing the RAQR-motif at amino acid position 439-442 to an RARR-motif (1) replacing CAG (Glu) at position 1321-1323 to CGG (Arg) with primers 5’-CAGAAAGAGCCCGGCGAGCAGCGAG and 5’-CTCGCTGCTCGCCGGGCTCTTTCTG. A six histidine-tag was incorporated behind Pro33 (based
on the histidine tag incorporation in human proline-30 by 1) using primers 5'-
30 GAGCAGGACAACACCACCATCACCACATACCCTCAACCAGGTTCAACCCG
30 and 5'- CGGGTTGACCTTCGGGTGATGGTGATGGTGATGGTTGTTGCTC.
31 The identities of these mutants were verified by DNA sequencing.
32
33 Human embryonic kidney (HEK293) were transfected using the calcium phosphate-method and
34 selected for positive cells with G418 (PAA Laboratories, 0.55 mg/ml) 48 hours post transfection.
35 Surviving colonies were picked and tested for recombinant Amh-production by immunocytochemistry
36 and grown at 37 °C in a 5% CO₂ in complete medium; Dulbecco’s Modified Eagle’s Medium
37 (DMEM, Gibco), supplemented with 10 % v/v fetal bovine serum (FBS), and penicillin/streptomycin
38 (BioWhittaker/Cambrex) containing 0.1 mg/ml G418. Cells were adapted to serum-free conditions,
39 first for 24 hours with reduced serum (5%) following serum-free medium for four days incubation. At
40 harvest, media were centrifuged for 2 minutes at 1000 xg to remove cell debris and then frozen at -20
41 °C until use. Medium was concentrated using Amicon Ultra Centrifugal Devices (Millipore), cut-off 5
42 kDa, following the manufacturer’s recommendations. Samples for SDS-PAGE were added reducing or
43 non-reducing SDS sample buffer while the remaining concentrated medium was frozen at -20 °C.
44 Recombinant zebrafish Amh was purified from medium (70-80 ml) on a 1 ml HisTrap HP Nickel
45 column, using Äkta Explorer system (GE Pharmacia) in 20 mM sodiumdihydrogen phosphate, pH 7.4,
46 0.2M NaCl, 20 mM imidazole and eluted by gradually increasing the imidazole concentration.
47 Imidazole was removed with a PD10 column (GE), in Leibovitz-15 medium (Gibco) and the protein
48 fraction was kept frozen at -80 °C until further use.
49
50 **Enzyme treatment of Amh**
51 Recombinant zebrafish Amh in concentrated cultured medium was treated with plasmin (0.015-0.24
52 mg/ml Sigma P1867) for 1 hour at room temperature. Testes protein extracts were homogenized in
53 Ringer solution based on Tris-HCl (pH 8.5) to achieve optimal conditions for the enzyme and treated
54 with plasmin (0.01-0.04 mg/ml) for 1 hour at room temperature. Reactions were stopped by adding
55 SDS sample buffer and frozen at -20 °C. Purified recombinant His-Amh-Q441R protein to be tested
for activity in organ cultures was cleaved using plasmin at concentrations 0.01 mg/ml and 0.2 mg/ml
for 2 hours at room temperature. Protein had been diluted in 1x reaction buffer (50 mM sodium
phosphate buffer pH 7). The reaction was stopped by adding cell culture tested Aprotinin (A3428,
Sigma) at equal concentrations as plasmin and frozen at -80 °C until further use.

For deglycosylation recombinant and endogenous zebrafish Amh were dialysed towards distilled
water (Slide-A-Lyzer dialysis cassette, cut-off 10 kD, Pierce) and treated with N-deglycosylating
PNGase F, and O-deglycosylating O-Glycosidase, Sialidase from the Enzymatic Carbo Release™ Kit
from QA-Bio (KE-DG01), following the manufacturer’s recommendations.

**Protein analysis**

Sodium dodecyl sulphate polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) and
immunoblotting with Immobilon-P™–PVDF membrane (0.2 µm) were performed following standard
procedures. Size standard was the Precision Plus Dual Colour and sizes are indicated in kD. Medium
had been concentrated 20.5 times (control) and 22 times the volume (recombinant). The SDS-PAGE
was performed with 12.5 % polyacrylamide gels, and the proteins were transferred to a 0.2 µm PVDF
membrane.

For N-terminal sequencing, fractions (300 µl) of untreated and plasmin-treated purified protein
(approximately 0.15 mg/ml) from conditioned medium were acetone-precipitated (80% acetone, 3
hours). SDS-PAGE and immunoblotting was performed and the membrane was washed twice in
distilled water and exposed to Coomassie stain for membranes (2). The N-terminal sequencing using
Edman degradation method was performed by the Proteomics Facility, University of Leeds. Maldi-
TOF analyses were performed by the staff at PROBE Proteomics Facility, University of Bergen.

Acetone precipitated purified protein was used to prepare peptide-in-gel samples of plasmin-treated
(0.01 µg/µl) and untreated Amh. Gels were fixed in 0.518 % (v/v) orto-phosphoric acid and stained
with Colloidal/Wita Coomassie (34% (v/v) methanol, 1.29 M Ammonium sulphate, 0.518 % (v/v)
Phosphoric acid, and 0.8 mM CBB G250) for 72 hours at room temperature with continuously
shaking. Proteins were cut out of the gel and dried using the vacuum centrifugation.
Amh directed antibodies

Two rabbit antisera were produced. Anti-N was raised against aa 53-67 –CVHRQQPTDQHATED- (AY721604) and anti-C, against aa 443-456 – AARADEDGPSASNQ located immediately downstream of the predicted proteolytic cleavage site. Antiserum and purified IgG were obtained from BioGenes, Germany. Western blot analysis were performed with zebrafish Amh anti-N and anti-C diluted primary antibodies 1:200 and mouse monoclonal anti-polyHis (H1029, Sigma) 1:3000. Secondary antibodies were horse peroxidase conjugated goat anti-rabbit IgG antibody (NA934V, Amersham) and sheep anti-mouse IgG (NA931V, Amersham) both diluted 1:2000. All blots were developed using Enhanced Chemiluminisence kit (ECL), Amersham. Silver staining of gels were performed as described in (3).

Immunocytochemistry of transfected HEK293 cells

HEK cells were transferred to cover slips pre-incubated with collagen (Sigma), 25 µg/ml in 0.1 M acetic acid. Immunocytochemistry was performed as described in (4). Antibodies were diluted in ordinary complete medium. The anti-C Amh specific primary antibody was diluted 1:100. The Alexafluor 594 goat anti-rabbit IgG secondary antibody (A11012, Invitrogen Molecular probes) was diluted 1:500. In addition, primary antibody against Protein Disulphide Isomerase (ab2792, Abcam) was diluted 1:200 and secondary antibody Alexafluor 488-conjugated anti-mouse IgG (A11029, Invitrogen Molecular probes) was diluted 1:1000. Nuclei were stained with DAPI present in the mounting solution (Vectashield). All samples were analysed using a Leica DMI6000 B, inverse fluorescence microscope using the Leica Application suite software.

Immunocytochemical detection of endogenous Amh protein in adult zebrafish testes

Testes of adult males were dissected and fixed in 4% buffered paraformaldehyde for 4 h, dehydrated in ethanol, embedded in paraffin and sectioned as described previously (5). Sections were submitted to standard immunocytochemistry procedures as described before (6). Before over night incubation with the primary antibody (Amh anti C, purified 1 mg/ml used in dilution 1:200 in 1% bovine serum albumin), slides were subjected to antigen retrieval in 10mM citrate buffer (pH 6.0) for 10 min (slides
in 200 ml buffer, microwave set to 700W). The sections were counterstained with haematoxylin Gills #3 (Sigma). To evaluate the specificity of the immunocytochemistry reaction, the primary antibody (1mg/ml) was pre-incubated over night at 4 ºC with the peptide (5mg/ml) used to generate the antibody (Supplemental Fig. 4).

Biological activity of recombinant zebrafish Amh: effects on androgen release, and spermatogenesis in testis tissue culture

To study androgen release, both testes of 8 out bred males per condition were dissected and pre-incubated for 6 or 24 hrs with or without 10 µg/ml recombinant, plasmin-treated zebrafish His-Amh-Q441R. Then, to half of the testes (right or left of a given individual) incubations, recombinant zebrafish Fsh (100 ng/ml) was added to stimulate androgen release and the incubation was continued for another 18 hrs (as described in 7). This set-up resulted in the following conditions: 1. No Amh + no Fsh; 2. No Amh + Fsh; 3. Amh + no Fsh; 4. Amh + Fsh, where Amh had been present for either 6 or 24 hrs before Fsh was added. Eighteen hours after adding Fsh, incubation media were collected and analysed for 11-KT by radio-immuno assay as described before (7). Testis tissue was weighed to calculate 11-KT release as ng 11-KT/mg of testis tissue. The stimulatory effect of Fsh was calculated as fold-increase above basal androgen release (i.e. comparing androgen release from the two testes of one animal) and assessed statistically by a one sample paired t-test against the hypothetical value 1 (i.e. no stimulation), using Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, http://www.graphpad.com). The Fsh-induced fold-increases obtained in the presence or in the absence of Amh were compared by unpaired Student t-test. Next to plasmin-treated His-Amh-Q441R, we also tested His-Amh-Q441R before plasmin treatment with, however, only a single period (24 hrs) of His-Amh-Q441R pre-incubation before addition of Fsh.

To study spermatogenesis in tissue culture, testes were dissected from out bred adult zebrafish (n = 5-7) and cultured in parallel (i.e. one testis incubated under control, the contralateral one under experimental conditions), using a tissue culture system on agar blocks, as described previously (8). Two experiments were carried out. In the first experiment, one testis of each fish was incubated with
200 nM 11-KT (control condition), while the contralateral testis was incubated with 200 nM 11-KT and 10 µg/ml of plasmin-cleaved His-Amh-Q441R for 7 days. For the second experiment, adult males were exposed in vivo for three weeks to 10 nM of 17β-estradiol, as described previously (9) to reduce the spermatogonial proliferation and differentiation activity. The two testes of the oestrogen-treated males were then incubated in parallel in basal medium (control) as described previously, or in medium containing 10 µg/ml of plasmin-cleaved His-Amh-Q441R for 7 days. During the last 6 hours of incubation, 50 µg/ml of 5-bromo-2-deoxyuridine (BrdU) was added to the medium. Testis tissue was fixed in methacarn or 4% buffered paraformaldehyde, dehydrated, sectioned and embedded in Technovit 7100 as described before (8). Sections were either stained with toluidine blue, or subjected to antigen retrieval and peroxidase blocking, before BrdU immunodetection, as previously described (10). In the first experiment, we counted spermatogenic cysts containing single, undifferentiated type A spermatogonia (A_{und}), differentiated type A spermatogonia (A_{diff}), type B spermatogonia (B), spermatocytes (SPC) or spermatids (ST) (see 5). Normalization took place by expressing the results as number of cysts per mm² of counted testis section surface. Moreover, we determined the BrdU-labelling index of spermatogonia type A_{und}. In the second experiment, we also determined the BrdU-labelling index of spermatogonia type A_{und}, while testis morphology and BrdU incorporation in further advanced stages of germ cell development were examined qualitatively. Where appropriate, paired t-tests (GraphPad Prism 4.0) were carried out to calculate the statistical significance between the testes incubated with or without Amh, respectively.

**Amh expression during testicular and ovarian development**

Relative expression of amh levels during testicular and ovarian development were measured by qPCRs as described previously (41). To this end, total RNA from whole juveniles at 4 weeks post-fertilization (wpf) (n = 4 males/3 females), and gonads from animals at 8 (n = 7 males/6 females) and 12 wpf (n = 5 males/6 females) were extracted using the RNA Aqueous-Micro kit (Ambion). To distinguish males from females, vasa::egfp transgenic zebrafish were used (29), in which testes and ovaries were identified by the Egfp expression pattern and according the shape. After qPCR quantification, amh
mRNA levels were normalized to the levels of 18S rRNA, and expressed relative to the levels of amh. mRNA levels in males at the age of 4wpf.

Endocrine regulation of amh expression

Testes from sexually mature males (out bred) were dissected out and used for tissue culture as described previously (42). One testis was incubated with hormone, the contralateral one with medium only. First, we tested the effects of recombinant zebrafish follicle-stimulating hormone (Fsh) (500 ng/ml) on amh (and cyp17a1) expression in the presence or absence of 100 µM of the protein kinase A (PKA) inhibitor H89 (Sigma) for 24 hours incubation. To this end, one testis was incubated with 500 ng/ml Fsh, or with 500 ng/ml Fsh and 100 µM H89, the contralateral one in basal medium, or in medium containing 500 ng/ml Fsh, respectively. We had shown earlier that 100 µM of H89 strongly reduced the Fsh-induced androgen release in zebrafish testis (41), and the efficiency of the H89 treatment was confirmed by quantifying androgen release as described before (41). From the different treatments, testis total RNA (n= 6 treated/6 control) was extracted using the RNA Aqueous-Micro kit (Ambion), and qPCRs to determine the relative levels of amh and cyp17a1 mRNA were carried out as described above, except that target gene mRNA levels were normalized with the geometric mean of three housekeeping genes (18S, ef1α, and β-actin), and expressed as fold induction of the control (basal medium or Fsh only). To evaluate the effects of different concentrations of the androgen 11-ketotestosterone (11-KT) on amh expression, long-term tissue culture (7 days) was used. After dissection, one testis was incubated with 50, 100, 200 or 400 nM 11-KT, the contralateral one with basal medium without androgens. Testis total RNA (n= 4 for 50nM; n= 6 for 100nM; n= 4 for 200nM; n= 7 for 400nM) was extracted, and amh mRNA was quantified as described above, normalized to 18S rRNA levels.

Bioinformatic analyses

A leader sequence was predicted with SignalP version 3.0 (web-site: http://www.cbs.dtu.dk/services/SignalP/) using the hidden Markov model (11). Glycosylation sites
were predicted using NetNGlyc version 1.0 (web-site: http://www.cbs.dtu.dk/services/NetNGlyc/).

Theoretical molecular weights were estimated with ProtParam tool (web-site: http://au.expasy.org/tools/protparam.html). Alignment was conducted with the Cobalt Multiple Alignment tool, NCBI and T-COFFEE at Swiss Institute of Bioinformatics(http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee cgi/index.cgi)
2. Dunn MJ 1999 Detection of Total Proteins on Western Blots of 2-D Polyacrylamide gels In: Link AJ ed. 2-D Proteome Analysis Protocols. Totowa, New Jersey: Humana Press; 319-329
10. van de Kant HJ, de Rooij DG 1992 Periodic acid incubation can replace hydrochloric acid hydrolysis and trypsin digestion in immunogold--silver staining of bromodeoxyuridine incorporation in plastic sections and allows the PAS reaction. Histochem J 24:170-175
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Legends to supplemental figures.

Supplemental Figure 1: Signal peptide, N-glycosylation and cleavage site predictions in zebrafish Amh with comparison to the human homologue.

Alignment of the aa sequences of zebrafish (Dr) and human (Hs) AMH, A. and schematic of protease cleavage pattern of recombinant Amh, B. Indicated are position of the N-terminal signal peptides (green), peptides used to generate zebrafish Amh-specific antibodies (red), N-glycosylation sites (coloured blue), primary proteolytic cleavage sites in human AMH (RAQR position 448-451, bold and underlined) and predicted protease cleavage site in zebrafish Amh (RAQRAAR position 439-445, underlined), primary experimentally verified cleavage site in zebrafish Amh (KR 263-4, bold and underlined) and second cleavage site in human AMH (R254, bold and underlined), other possible plasmin cleavage sites in zebrafish Amh close to the verified cleavage site (indicated in dark grey), 6xhistidine tag insertion points at proline, positions 33 in zebrafish and 30 in human AMH (purple). The conserved TGFβ-domain predicted over amino acids 457-549 in zebrafish Amh contain seven conserved cysteines (light grey) that form covalent bridges (red lines). The expected N- and C-fragments after cleavage are shown as black lines with theoretical molecular weights.

Supplemental Figure 2: Proteolytic processing of recombinant zebrafish Amh

Purified recombinant His-Amh-Q441R precursor protein subjected to N-terminal sequencing of the six most N-terminal amino acids showed that signal peptide had been cleaved off before secretion. The alanine is corresponding to amino acid number 22 of the primary amino acid sequence (A). The 70 kD, N-28,5 and C-36 fragments of plasmin-treated (0,01 mg/ml) His-Amh-Q441R-protein were cut out from a Wita Coomassie- stained polyacrylamide gel and subjected to trypsin digestion and mass spectrometry (B). Molecular weight Precision plus protein standard was used and the molecular weights are given in kD. Mass spectrometry peptide fragment distribution revealed plasmin cleavage at KRH263-265 behind Lysine or Arginine. Expected molecular weights after cleavage behind R264
without signal peptide and including histidine tag would have been 27.8 and 31.8 kD for the N- and C-
fragments after cleavage, respectively (Fig. 1B and 1C).

**Supplemental Figure 3: Recombinant Amh is located to ER.** Immunocytochemistry of HEK cells
producing His-Amh-Q441R co-localised Amh to ER with protein disulphide isomerase (PDI). Primary
antibodies were rabbit anti-C and mouse anti-PDI (C). Secondary antibodies were Alexafluor 594-
conjugated anti-rabbit IgG (red) and Alexafluor 488-conjugated anti-mouse IgG (green). DAPI stained
nuclei blue. Yellow colouring indicates co-localisation in the merged image.

**Supplemental Figure 4.** Zebrafish testis section used as a negative control for the
immunocytochemical detection of Amh. The primary antibody was first incubated over night at a
concentration of 1 mg/ml with the 5mg/ml peptide used to generate the antibody.

**Supplemental Figure 5.** Scatter plot to check for the stability of $\beta$-actin1 (○), 18S rRNA (●), and ef1-
α (▲) mRNAs as housekeeping genes for the normalization of the expression of the selected testicular
genes (cyp17a1, star, insl3, and ar) in the different experimental conditions. Each dot in the scatter
plot represents the average Ct-value of duplicate measurements for each testis per condition. No
significant differences (P>0.05) were found for the Ct-values between the different experimental
groups.
A  Sequence:  Ala-Thr-Val-Arg-His-Glu

Initial yield 15 pmole

B

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Wita Coomassie

70
C-36
N-28.5