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Estrogen receptor subtype β2 is involved in neuromast development in zebrafish (*Danio rerio*) larvae

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ABSTRACT

Estrogens are known to play a role in both reproductive and non-reproductive functions in mammals. Estrogens and their receptors are involved in the development of the central nervous system (brain development, neuronal survival and differentiation) as well as in the development of the peripheral nervous system (sensory-motor behaviors). In order to decipher possible functions of estrogens in early development of the zebrafish sensory system, we investigated the role of estrogen receptor β_2 (ERβ_2) by using a morpholino (MO) approach blocking *er*β_2 RNA translation. We further investigated the development of lateral line organs by cell-specific labeling, which revealed a disrupted development of neuromasts in morphants. The supporting cells developed and migrated normally. Sensory hair cells, however, were absent in morphants' neuromasts. Microarray analysis and subsequent *in situ* hybridizations indicated an aberrant activation of the Notch signaling pathway in ERβ_2 morphants. We conclude that signaling via ERβ_2 is essential for hair cell development and may involve an interaction with the Notch signaling pathway during cell fate decision in the neuromast maturation process.

Introduction

A role of estrogens in the central nervous system (CNS) has been reported repeatedly (Duffy, 2006; Gruber et al., 2002; Hall et al., 2001). Besides sexual differentiation of the CNS, estrogens are involved in different aspects of brain development, as for example in neural differentiation and neuronal survival, or in the control of synaptic plasticity (Beyer, 1999; Meda et al., 2000; Nilsen et al., 2000; Wang et al., 2003). An increasing number of studies in mammals described estrogen functions that are not related to sexual activities, such as improvement of memory, blocking of apoptosis, maturation of motor behaviors and influence on the electrophysiological functions of neurons (Beatty and Holzer, 1978; McEwen and Alves, 1999). In zebrafish, a recent study showed that estrogens are essential for the proper developmental of sensory-motor behaviors, suggesting a novel role for these hormones in the peripheral nervous system (PNS) of fish (Nelson et al., 2008).

The molecular action of estrogens is mediated through two distinct types of signaling, often referred to as the non-genomic and the genomic pathway. In the latter pathway, estrogens form a complex with specific nuclear receptors, the so-called estrogen receptors (ERs), which then function as transcription factors by binding to specific DNA sequences in the promoters of estrogen target genes (Duffy, 2006; Gruber et al., 2002).

In mammals, there are two ER subtypes, ERα and ERβ. The role of each ER has been analyzed using knock-out mice deficient in ERα (ERαkos) and in ERβ (ERβkos) (Korach, 1994; Krege et al., 1998). Both knock-out animals exhibit distinct phenotypes, suggesting a distinct function for each ER (Gustafsson, 1999). ERα is mainly responsible for estrogen action in the uterus. ERβ may not be directly responsible for the regulation of reproductive physiology in rodents, but is suggested to mediate some of the many other effects of estrogens, such as neuronal survival and late embryonic development of the brain, as well as to have a function in memory and learning processes (Bodo and Rissman, 2006; Gustafsson, 1999; Kuiper et al., 1998; Lindner et al., 1998; Osterlund et al., 1998). Interestingly, ERβko mice suffered from a severe hearing defect caused by disruption of hair cells of the inner ear, where ERs are also expressed in mouse and rat (Hultcrantz et al., 2006; König, 2007; Stenberg et al., 1999).

Altogether, the non-reproductive roles of estrogens in the CNS and PNS, as cited previously, suggest that estrogen-dependent processes are broader than thought before. Therefore, research allowing increase in our understanding of estrogens physiological functions

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beyond the sexual system, is needed. In order to fulfill this gap, we investigated the role of the ER in embryonic development of the model organism zebrafish. Like in other teleosts, a second ERβ paralog has been identified in zebrafish (Filby and Tyler, 2005; Gruber et al., 2002; Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002; Nelson et al., 2007). These subtypes were previously named *erα*, *erβ$_1$* and *erβ$_2$* and then renamed by the Zebrafish Information Network (ZFIN) (http://zfin.org) to *esr1*, *esr2b* and *esr2a*, respectively. In order to facilitate comparison with other animals and previously published papers, we adopted the old nomenclature. In adult brain, *erβ$_2$* transcripts have been detected in the two neuroendocrine regions, the preoptic area and the mediobasal hypothalamus, and also in liver and gonads (Menuet et al., 2002). In early embryos, *erβ$_2$* was the most expressed subtype and its highest level was detected in freshly fertilized eggs, suggesting the presence of maternally loaded mRNA (Lassiter et al., 2002; Tingaud-Sequeira et al., 2004). The embryonic start of transcription for *erβ$_2$* is thought to be between 24 and 48 h post fertilization (hpf) (Bardet et al., 2002). *erβ$_2$* mRNA is expressed during early life stage in the epidermis, pectoral fin buds, hatching gland and developing brain. Surprisingly, *erβ$_2$* was also found to be expressed in the neuromasts, which are part of the lateral line organs, the superficial organs of the sensory system (Tingaud-Sequeira et al., 2004).

A rosette-like structure of neuromast is composed of two groups of cells, the supporting cells and the hair cells, the latter closely related to the inner ear sensory hair cells of mammals (Nicolson, 2005). This cluster of cells is innervated by sensory neurons projecting to the CNS (Lopez-Schier et al., 2004; Metcalfe et al., 1985). The function of lateral line organs has been reported to be mainly detection of directional water movements, facilitating prey capture and predator avoidance. The lateral line is divided into two major components: the anterior lateral line (ALL) which includes the neuromasts of head, jaw and opercle, and the posterior lateral line (PLL) which includes the neuromasts of the trunk and tail (Gompel et al., 2001).

Estrogen production enzyme, the aromatase, has been reported to be expressed in neuromasts and to play a role in hair cell development (Kalivretaki et al. unpublished). Although *erβ$_2$* mRNA was also detected in the supporting and hair cells of the mature neuromasts (Tingaud-Sequeira et al., 2004), its role has not been investigated so far. Therefore, the goal of this study was to examine whether *erβ$_2$* has a role in neuromast development. An elucidation of ERβ$_2$ function in the lateral line organs would give an indication of an estrogen action in the sensory system of zebrafish embryos and would further add to the understanding of multiple functions that ERs are involved in during various developmental processes.

ERβ$_2$ knock down with a morpholino (MO) indicated a crucial function of ERβ$_2$ in neuromast development. A more detailed analysis revealed that ERβ$_2$ plays an essential role in hair cell differentiation. Moreover, we suggest for the first time that ERβ$_2$ might interact with the Notch signaling pathway during hair cell differentiation. Based on these findings, we hypothesized that ERβ2 is involved in neuromast development by interacting with the Notch signaling pathway.

Methods

Zebrafish maintenance

The zebrafish strain was a wild-type mix obtained from a petshop. The zebrafish were reared in a recirculating flow-through system with tap water treated with active carbon and UV light, set at 29 °C. The adults were kept in 75 l glass aquaria in a density of around 30 fishes. Water was renewed weekly and the room was maintained on a 14-hour-light–10-hour-dark cycle according to Westerfield (1995). The adult diet contained mostly live food, *Artemia nauplia* but also vitamins and dry food flakes. The transgenic strain SqET4 was injected in Bettina Schmid's laboratory (Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for Alzheimer's and Parkinson's Disease Research, Ludwig-Maximilians-University, Munich, Germany).

Synthesis of DIG-labeled riboprobes

Erβ$_2$ cDNA, comprising the complete coding region, cloned into pcDNA3.1/V5-His-TOPO, was kindly provided by Menuet et al. (2004). For the synthesis of a riboprobe corresponding to the A/B domain of the zebrafish *erβ$_2$*, the relevant 429 bp fragment was amplified with PCR using primers fw: 5′-GTGTGGTATACCGCCTTGCT-3′ and rev: 5′-TGAGGAAACATGGCTGTGAG-3′, and cloned into pGemT-easy (Promega, Switzerland). For the sense riboprobe production, the plasmid was linearized with NcoI (Fermentas, Switzerland) and RNA was synthesized *in vitro* with Sp6 RNA polymerase (Promega, Switzerland). For the antisense riboprobe synthesis, the plasmid was linearized with SalI (Fermentas, Switzerland) and RNA was synthesized with T7 RNA polymerase (Promega, Switzerland).

The plasmids containing *claudinb* (*cldb*) and *keratin15* (*k15*) genes were linearized with NotI for *cldb* and with BamHI for *k15*. The antisense probe was synthesized using Sp6 RNA polymerase (Promega, Switzerland) for *cldb* and T7 RNA polymerase (Promega, Switzerland) for *k15*. The plasmid for *ngn1* was kindly provided by B. Schmid, (Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for Alzheimer's and Parkinson's disease Research, Ludwig-Maximilians-University, Munich, Germany). The plasmid was linearized with XhoI and T7 RNA polymerase was used for synthesis of the antisense probe. The plasmids for *notch3* (=*notch5*) and *notch1a* were received from the group of Jiang in Singapore (Ma and Jiang, 2007). The plasmid was linearized with BamHI and T3 RNA polymerase was used to synthesize the antisense probes.

The DIG dNTPs (Roche, Switzerland) were used for probe labeling. Template DNA was removed by RNase free DNase (Promega, Switzerland) treatment.

Whole mount in situ hybridization (ISH)

The embryos were depigmented in 0.003% PTU (1-phenyl-2-thiourea, Sigma-Aldrich, Buchs, Switzerland) in E_3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) after 24 hpf. The larvae were euthanized in MS222 (1 g/l 3-aminobenzoic acid ethylester (Sigma-Aldrich, Buchs, Switzerland), 600 mg/l $NaHCO_3$, pH 7) at 72 or 96 hpf and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS: NaCl 137 mM, KCL 2.7 mM, PO_4 0.02M) at 4 °C. After dehydration in 100% MeOH, larvae were stored at −80 °C. Prior to use, they were rehydrated with 75%, 50% and 25% MeOH/PBS and rinsed three times 5 min with PBT (PBS plus 0.1% Tween20 (polyoxyethylene-sorbitan monolaurate; Sigma-Aldrich, Buchs, Switzerland)). To permeabilize the larvae, proteinase K (5 μg/ml in PBT) digestion was performed for 30 min followed by a refixation in 4% PFA in PBS for 20 min. After washing several times in PBT, larvae were prehybridized for 4 h in HYBE (50% formamide (Fluka, Switzerland), 5× SSC, heparin 50 mg/ml, 0.1% Tween20, tRNA 10 mg/ml, citric acid 1 M) at 70 °C for *erβ$_2$*, 65 °C for *cldb*, *ngn1*, *notch3*, *notch1a* and 59 °C for *k15*. For hybridization, the labeled riboprobes were added to the pre-warmed HYBE (1:400) and larvae were incubated overnight at 70 °C. After washing at 70 °C two times for 30 min in 50% formamide/50% 2× SSC plus 0.1% Tween20, the larvae were rinsed in 2× SSC plus 0.1% Tween20 for 15 min, and then two times in 0.2× SSC plus 0.1% Tween20 for 30 min. The larvae were then transferred into blocking buffer (1× PBS, 0.1% Tween20, 100 mg/ml bovine serum albumin (BSA), 1% DMSO) to be blocked at room temperature for 3–4 h. The larvae were then incubated overnight at 4 °C in a solution with preabsorbed sheep anti-digoxigenin-AP Fab

fragments (Roche, Switzerland) at a 1:4000 dilution in blocking buffer. Before being transferred in staining buffer (0.1 M Tris–HCl pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl, 0.1% Tween20) in 6-well plates, the larvae were washed several times in PBT. Afterwards, the staining buffer was replaced with staining buffer plus 75 mg/ml NBT (Nitro blue tetrazolium chloride, Roche, Switzerland) and 50 mg/ml BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt, Roche, Switzerland) and the larvae were stained until a signal was detected. Finally, the larvae were washed several times in PBT and mounted in 60% glycerol (Fluka, Switzerland) on glass slides to be observed under bright field microscope or with differential interference contrast filter (Leica, Switzerland).

Microinjection

Prior to injections, the black ground egg collectors were sunk into the different aquaria. The first pool of eggs was collected after 15 min, 30 min later the second pool of eggs was collected. The eggs were washed with E_3 before and after injection. Injections were performed into the yolk of fertilized zebrafish eggs between the one- and four-cell stages. The injections were done using a PV820 Pneumatic Picopump (World Precision instruments, USA). The embryos were raised in E3 in petri dishes at 28 °C.

Morpholinos and capped RNA

The ERβ_2 morpholino (ERβ_2 MO) was designed against the flanking sequence to the ATG start codon of zebrafish *er*β_2 with the sequence 5′-ACATGGTGAAGGCGGATGAGTTCAG-3′. A standard control MO (coMO) 5′-CCTCTTACCTCAGTTACAATTTATA-3′ and a p53 MO 5′-GCGCCATTGCTTTGCAAGAATTG-3′ were used as controls for phenotype specificity. The MOs were obtained from Gene Tools, LLC (Philomath, Oregon, USA) and diluted for injection in 0.01% DEPC (Fluka, Switzerland) water with Phenol Red (0.005%) as injection indicator. ERβ_2 MO and coMO were injected at a concentration range between 15 μM and 100 μM. p53 MO was co-injected with ERβ_2 at a concentration of 15 μM for both. ERβ_2 capped RNA (cRNA) mutated in the MO binding region was co-injected with the ERβ_2 MO in a concentration of 300 ng and 15 μM, respectively. For the synthesis of ERβ_2 cRNA an insertion of 9 nucleotides into the complete *er*β_2 coding cDNA obtained from Menuet (Menuet et al. 2004) was performed with QuickChange® Site-Directed Mutagenesis kit (Stratagene, Switzerland). Primers were designed using Stratagene web page (http://www.stratagene.com\newline/qcprimerdesign). In a first step, 3 nucleotides (GAA) were introduced with primers fw: 5′-CTGAACTCATCCGCCGAATTCACCATGTCCGAG-3′ and rev: 5′-CTCGGACATGG TGAATTCGGCGGATGAGTTCAG-3′. In a second step, additional 6 nucleotides (GAATTC) were inserted with primers fw: 5′-CTGAACTCATCCGCCGAGAATTC ATTCACCATGTCCGAGT-3′ and rev: 5′-ACTCGGACATGGTGAATGAATTCTCGGCGGATGAGTTCAG-3′. For *in vitro* RNA synthesis, template DNA was linearized with NotI, the reaction was then purified with the MinElute Reaction Cleanup kit (Quiagen AG, Hombrechtikon, Switzerland). The cRNA was synthesized using mMessageMachine® kit (Ambion, Switzerland) and its concentration was measured with a Nanodrop spectrophotometer (Witeg, Littau, Switzerland).

Morphological and behavioral observations

Observation of injected embryos began at 24 hpf and was repeated every 24 h until 120 hpf. The embryos were observed under a stereomicroscope (Olympus Schweiz AG, Volketswil, Switzerland). Hatching rate and swimming behavior were recorded between 48 hpf and 72 hpf. Behavioral tests were conducted to observe swimming behavior and responses to a gentle touch of the tail with a needle at 72 hpf. The larvae that were not able to rapidly traverse the petri dish in a straight line and were swimming in circles were described as "larvae swimming in circles". Each larva was tested three times and, when it failed three times to swim straight, it was recorded as positive for swimming in circles. Finally, the number of larvae swimming in circles was expressed as percentage of the total number of alive larvae in the petri dish.

The staining of lateral line hair cells

The vital dye N-(3-triethylammoniumpropyl)-4-(dibutylamino) styryl) pyridinium dibromide (FM®1–43; Invitrogen, Switzerland) was used to label physiologically active hair cells within the neuromasts. The larvae were incubated for 30 s in 3 μM of FM®1–43, washed several times with E_3, and anaesthetized in MS222 (200 mg/l 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, Buchs, Switzerland), pH 7) at 72 hpf. They were then mounted in 3% methylcellulose (Fluka, Switzerland) and placed on their lateral side. The stained neuromasts were recorded. Imaging was processed with a green fluorescent protein (GFP) filter under a Fluorescent light microscope (Leica Microsystems, Heerbrugg, Switzerland).

Statistics

The statistical significance of the number of neuromasts of at least 35 individuals between uninjected embryos (blank) and ERβ_2 MO-injected was determined by one-way ANOVA with $p \leq 0.05$. A Post-Hoc Tukey test was performed to identify significant differences between the number of neuromasts in injected embryos and controls. All statistical analyses were computed using *Statistica 6.0* (Statsoft, Inc., USA). Values were reported in box plots performed with *SigmaPlot* (*Systat Software Inc.*, California, USA).

Microarray analysis

Transcriptional profiling was performed using affymetrix microarrays at the Functional Genomic Center in Zürich, Switzerland (http://www.fgcz.ethz.ch). The spotted array contains 14,900 oligonucleotides (Affymetrix Inc., USA). Sequence information for this array was selected from the following public data sources: RefSeq (July 2003), GenBank (release 136.0, June 2003), dbEST (July 2003), and UniGene (Build 54, June 2003). Zebrafish embryos were injected with MOs before RNA isolation as described above. Total RNA of 72 hpf ERβ_2 MO- and coMO-injected embryos, as well as of uninjected embryos, was extracted using RNeasy mini kit (Qiagen, Switzerland). Concentration and purity of the isolated total RNA were assessed using Nanodrop spectrophotometer (Witeg, Littau, Switzerland) and gel electrophoresis.

The RNA was prepared using the biotin-labeling IVT Kit (Affymetrix Inc., USA) prior to hybridization done with fragmentation buffer (Affymetrix Inc., P/N 900720), hybridization controls and control oligonucleotide B2 (Affymetrix Inc., P/N 900454) according to the Affymetrix gene expression protocol. After hybridization of the labeled RNA with the oligos on the microarray surface, the array was washed using Affymetrix Fluidics Station 450 (FS450_0004 protocol) and subsequently scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Inc.) according to the protocol of the manufacturer. Raw data processing was performed using the Affymetrix AGCC software. After hybridization and scanning, probe cell intensities were calculated by means of the MAS5 algorithm. The data were then analyzed with Genespring (Agilent, USA). The data were normalized per chip (50th percentile) and per gene (to mean). Changes were identified by selecting transcripts with a fold change of >2 for increases and <0.5 for decreases.

As a second criteria, only the genes found to be differentially expressed in both arrays "ERβ_2 MO at 15 μM" and "ERβ_2 MO at 50 μM" were selected as being affected by the ERβ_2 MO.

Results

erβ2 mRNA presence in neuromasts

Erβ2 transcripts were localized in 96 hpf embryos using whole mount ISH with a sense and an antisense probe against *erβ2* mRNA. The presence of *erβ2* mRNA was detected in the neuromasts of the ALL and PLL, when using antisense probe (Figs. 1A–C). In the head, ALL expression is difficult to identify because of increased background staining, also detected using the sense *erβ2* probe (Figs. 1D–F). Neuromasts of the PLL were clearly visible in higher magnification (Fig. 1C). Beside *erβ2*, the two other subtypes' transcripts, *erα* and *erβ1*, were also localized with ISH, however, only weak expression in the head region and in the neuromasts has been detected (data not shown).

ERβ2 knock down phenotypes as a function of MO concentration

Expression of *erβ2* was blocked by injecting zebrafish eggs with a MO against *erβ2* mRNA (ERβ2 MO). The effects observed were MO concentration-dependent and were described as curved tail, lower hatching rate, disrupted neuromast development and circular swimming behavior. For schematic representation, these effects are plotted as a function of the MO concentration and the age of the morphants (Fig. 2). No effects were observed when a standard coMO was injected at the same concentration range.

In embryos injected with 100 μM MO the hatching rate decreased to 15% at 72 hpf compared to nearly 100% in controls. In addition to reduced hatching rate, 10% of the morphants receiving the high concentration of 100 μM MO developed a curved tail. The morphants did not survive longer than 96 hpf, independent of being hatched or not. Also at 50 μM MO the hatching rate was affected, as only 50% of the embryos were able to hatch at 72 hpf compared to nearly 100% in controls. Similarly to the 100 μM MO injected embryos, the embryos did not reach the age of 96 hpf. At 25 and 15 μM all embryos could hatch normally.

At all MO concentrations tested, we could observe an alteration of the swimming behavior and a reduction of neuromasts number, the latter being assessed with a specific hair cell staining, FM1–43.

The number of functional neuromasts is reduced in ERβ2 morphants

In order to assess the occurrence of neuromasts, functional hair cells were stained with a vital dye (FM1–43) in 72 hpf embryos (Fig. 3). After injection with 15 μM ERβ2 MO, we observed a significant reduction in number of stained hair cells. In the coMO-injected embryos and in the uninjected ones (blanks), the mean number of stained neuromasts was 13.1 ± 2.13 ($N = 48$) and 16.8 ± 2.76 ($N = 45$) respectively (Fig. 4A). The mean number of neuromasts stained in the ERβ2 morphants was reduced to 3.7 ± 2.08 ($N = 60$). When ERβ2 MO was co-injected with ERβ2 cRNA, the phenotype was rescued, although partially, to a mean number of neuromasts of 10.6 ± 5.05 ($N = 58$)

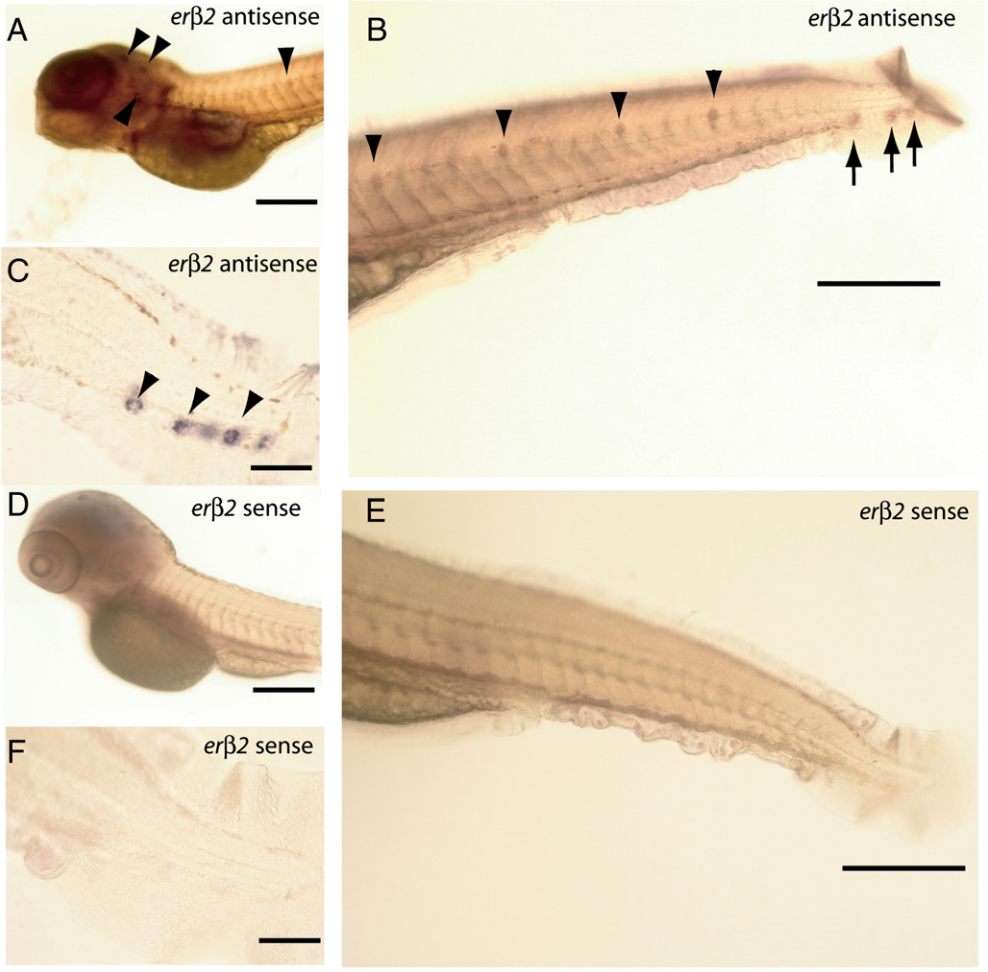

Fig. 1. Localization of *erβ2* mRNA with whole mount *in situ* hybridization in 96 hpf larvae. (A–C) Antisense probe was detected mainly in the brain and in the neuromasts (see black arrowheads). (D–F) With the sense probe, the signal was not detected in the neuromasts, but weakly in the brain. (A, B, D, E) Magnification 10×, scale bar = 200 μm. (C, F) Magnification 60×, scale bar = 50 μm.

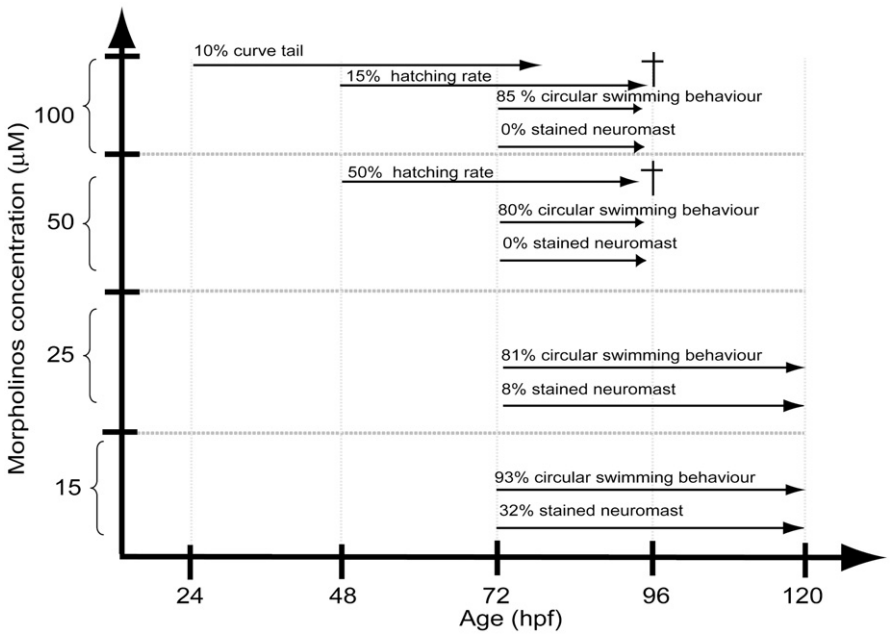

Fig. 2. Concentration-dependent endpoints. The morpholino against ERβ$_2$ (ERβ$_2$ MO) was used at concentrations ranging from 15 µM to 100 µM, as plotted on the *Y* axes. The *X* axes represent the age of observed larvae in hours post fertilization (hpf). The black arrows point to the time where the specific phenotype was observed. Four biological endpoints were observed: a curved tail at 100 µM, a lower hatching rate at 100 and 50 µM, a disturbed swimming behavior at all concentrations and a disruption in the neuromast development at all concentrations.

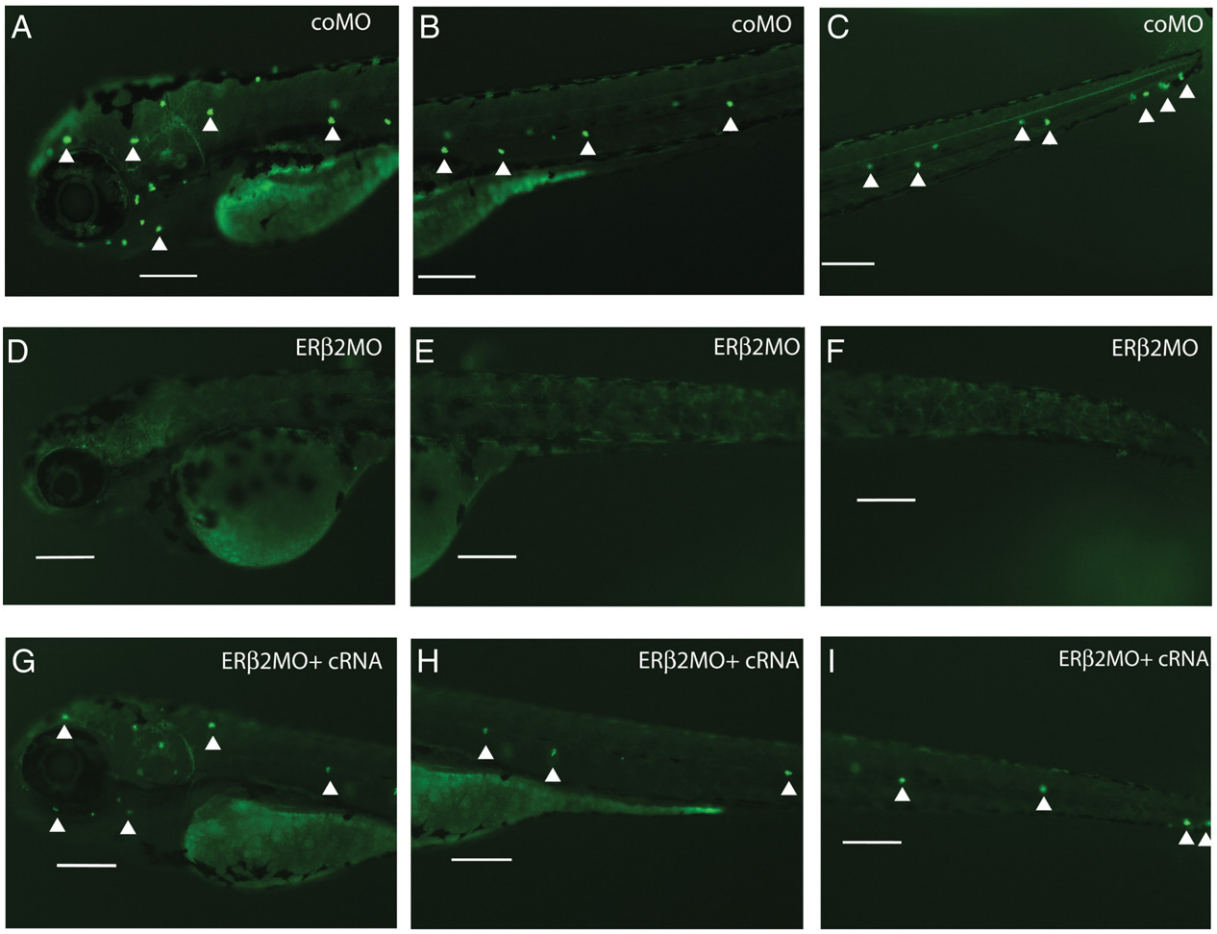

Fig. 3. Neuromast detection with live staining (FM1–43) in 72 hpf larvae. (A–C) Larvae injected with coMO at 15 µM. (D–F) Larvae injected with ERβ$_2$ MO at 15 µM. (G–I) Larvae injected with ERβ$_2$ MO at 15 µM and cRNA at 300 ng. Arrowheads point to neuromasts with functional hair cells. Scale bar = 200 µM.

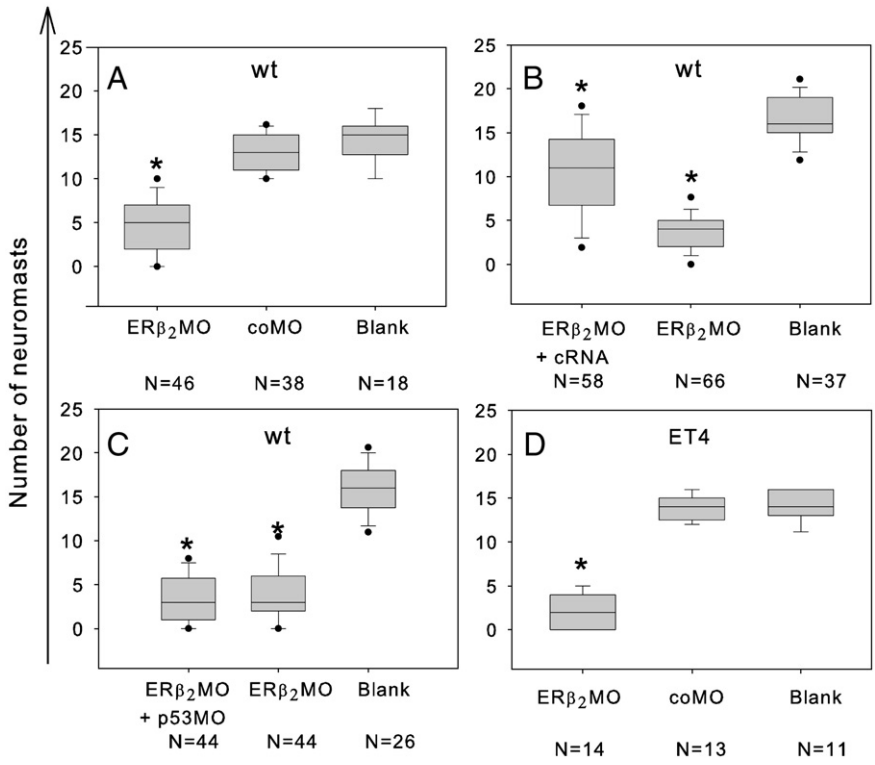

Fig. 4. Number of neuromasts assessed using live staining (FM1–43) in wild-type (A–C) at 72 hpf and with GFP visualisation in transgenic line (ET4) (D). The boxes symbolize the interquartile space of each distribution; the median is indicated with a horizontal line within the box, and dots indicate extreme values (5th/95th percentile). The number of individuals recorded for elaboration of the box plots is indicated with N = number of individuals. Numbers of neuromasts are shown in (A) ERβ$_2$ MO- or coMO-injected wild-type larvae (both MOs at 15 μM); (B) ERβ$_2$ MO-injected wild-type larvae (15 μM) co-injected with cRNA (300 ng); (C) ERβ$_2$ MO-injected wild-type larvae co-injected with p53MO (both at 15 μM). (D) ET4 larvae injected with ERβ$_2$ MO or coMO (both at 15 μM). One-way ANOVA following the Tukey test were performed to test the differences between blank (uninjected) and injected embryos and significances were indicated with * when $p \leq 0.05$.

instead of 3.71 ± 2.08 (N = 60) in the ERβ$_2$ MO injected embryos (Fig. 4B). Injection of higher cRNA concentrations caused embryos to die, and hence the numbers of rescued embryos could not be increased. Contrary to the morphants, the rescued larvae developed functional neuromasts in the ALL and the PLL, reinforcing the specificity of knockdown phenotype (Fig. 3).

The p53 gene is known to be activated upon injection of several MOs resulting in off-targeting effects (Langheinrich et al., 2002), and its knock down is therefore an additional control for phenotype specificity. To exclude p53-provoked off-targeting effects as a reason for the ERβ$_2$ MO morphant phenotype, we co-injected embryos with ERβ$_2$ MO and p53 MO. The morphant phenotype did not change, the mean number of neuromasts in the co-injected embryos was at 3.32 ± 2.5 (N = 58), compared to 3.8 ± 2.9 (N = 48) in the treated embryos at 72 hpf (Fig. 4C). Thus, the results of co-injection of p53 MO and ERβ$_2$ MO eliminated the eventual off-targeting effects and reinforced the specificity of the ERβ$_2$ morphant phenotype.

Our results showed that ERβ$_2$ is essential for the normal development of functional neuromasts. As a next step we investigated the presence and development of hair cells and supporting cells, which compose the neuromast, in the ERβ2 morphants.

Fewer hair cells are developed in ERβ$_2$ morphants

In order to investigate if ERβ$_2$ plays a role in the development of the hair cells of the neuromast, the ERβ$_2$ MO was injected into a transgenic line (ET4) expressing GFP in the hair cells (Parinov et al., 2004). GFP expression was reduced in the neuromasts of ERβ$_2$ MO injected ET4 larvae (Fig. 4D). There are 2.2 (± 1.8) neuromasts in ERβ$_2$ morphants, in comparison with 13.7 (± 1.4) in coMO and 13.9 (± 1.7) in blanks. These results confirmed that the reduced FM1–43 staining observed in morphants corresponded to an absence of hair cells in neuromasts and showed that normal hair cell development is disturbed in ERβ$_2$ MO treated embryos.

The alteration of swimming behavior gave further indications of the hair cell disruption in morphants. Normally, zebrafish embryos swim straight away when mechanically stimulated (needle). The embryos injected with all concentrations of ERβ$_2$ MO showed an abnormal response when stimulated; the morphants were swimming in circles. The average of three biological replicates is plotted in Fig. 5. Of the larvae missing ERβ$_2$ (ERβ$_2$ morphants), 93% (± 4.7) were swimming in circles and showed an abnormal response to tail stimulus when injected with 15 μM of ERβ$_2$ MO. This phenotype could be rescued after co-injection of ERβ$_2$ MO with the ERβ$_2$ cRNA and was not rescued after co-injection with the p53 MO.

Presence of supporting cells in ERβ$_2$ morphants

Besides the hair cells, the neuromasts are composed of two types of supporting cells, the sustentacular cells and the mantle cells. The ability of the morphants to develop supporting cells was investigated. To assess the presence of supporting cells in normal larvae and in ERβ$_2$ morphants, we used cellular markers specific for supporting cells. In an *in situ* hybridization screen *keratin15* (*k15*) was found to be a marker of peripheral (mantle) cells (data not shown). *Claudinb* (*cldb*) detects all supporting cells (Lopez-Schier et al., 2004). After ISH with the probe against *k15*, mantle cells were visible in morphants injected with 15 μM ERβ$_2$ MO until the tip of the tail, while the expression of *cldb* was observed in the ALL as well as in the PLL of larvae injected with 15 μM ERβ$_2$ MO (Fig. 6).

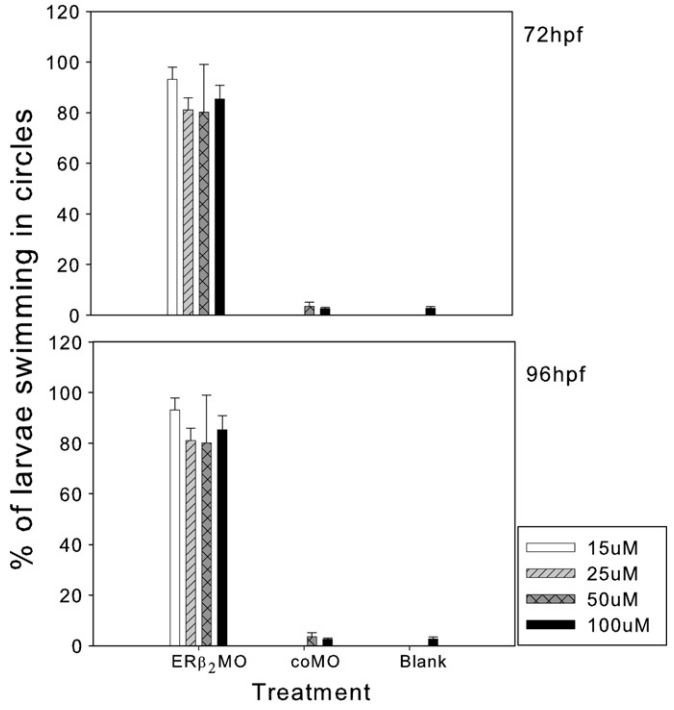

Fig. 5. Percentage of larvae that were swimming in circles after injection with the ERβ$_2$ MO or with a coMO at different concentrations (15, 25, 50, 100 μM) or uninjected (blank) at 72 hpf and 96 hpf. Values are reported as arithmetic means ± standard deviation of biological triplicates.

As a next step in unraveling the molecular mechanisms underlying the disturbed neuromast development in ERβ$_2$ MO injected embryos, gene expression levels were examined using affymetrix gene arrays.

Up-regulation of proneural genes in ERβ$_2$ morphants

We aimed at investigating the genes affected by ERβ$_2$ suppression in early development by analyzing modified gene expression levels in morphants using microarrays. Two independent affymetrix microarray sets were performed. One set comprised samples of 15 μM ERβ$_2$ MO injected embryos, 15 μM coMO injected embryos and uninjected embryos. The second set comprised analogue samples at 50 μM.

In the first set, using the total RNA from 50 μM ERβ$_2$ MO injected larvae, 2666 genes were differentially expressed when comparing with larvae injected with 50 μM coMO. Twenty-one genes were differently expressed between blank (uninjected) RNA and RNA from 50 μM coMO injected larvae. These genes were mostly hypothetical proteins with unknown function.

In the second array, using total RNA from 15 μM MO injected larvae, 1046 genes were differentially expressed when compared with larvae injected with 15 μM coMO.

After comparing the list of genes differentially expressed in morphants injected with 15 μM and 50 μM ERβ$_2$ MO, 631 genes were found to be affected by both MO concentrations in larvae.

In order to assign the affected genes into functional classes based on the gene ontology, information was taken from public databases using NCBI website (http://www.ncbi.nlm.nih.gov/sites/entrez). We classified the genes in 10 different functional groups, namely: egg and embryo development, energy metabolism, intra- and intercellular signaling, transcription and translation, cell growth and development, cell structure and cytoskeletal organization, detoxification and defense, intra- and intercellular transport and unknowns (Table 1). Of the differentially expressed genes, 45% had an unknown function, 18% were involved in energy metabolism, 10% were involved in transcription and translation and another 10% in intra- and intercellular transport, 2.6% of the genes were involved in

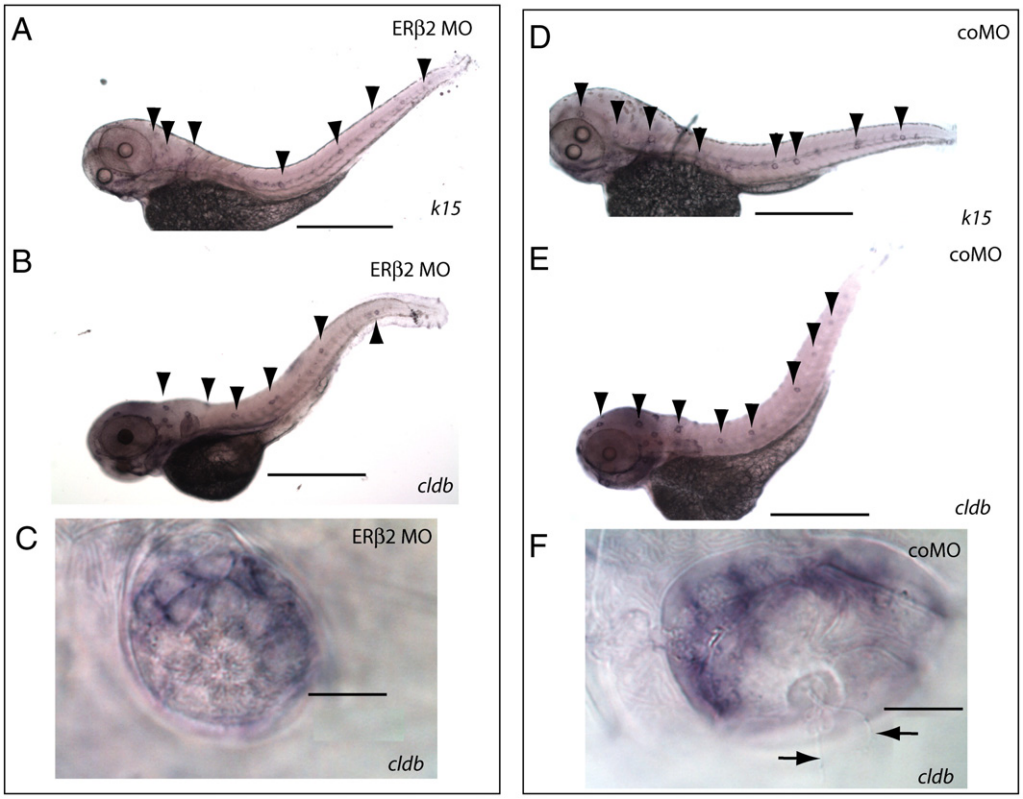

Fig. 6. Detection of sustentacular cells and mantle cells with whole mount *in situ* hybridization against *keratin15* (*k15*) (A, D) and *claudinb* (*cldb*) (B, C, E, F) in 72 hpf larvae injected with 15 μM ERβ$_2$ MO (A–C), and with 15 μM coMO (D–F). (A, B, D, E) Magnification 10×, scale bar = 500 μM, Arrowheads point to neuromasts. (C, F) Magnification 100×, scale bar = 10 μM. Arrowheads point to visible hair cells.

Table 1
Differentially expressed genes at 72 hpf in 15 μM ERβ$_2$ MO and in 50 μM ERβ$_2$ MO injected embryos, divided in functional classes.

Gene description		Number of genes	
		Up-regulated (>2)	Down-regulated (<0.5)
Egg and embryo development	17	11	6
Energy metabolism	118	25	93
Intra- and intercellular signaling	54	17	37
Transcription and translation	67	56	11
Cell growth and development	3	2	1
Cell structure and cytoskeletal organization	11	3	9
Detoxification and defense	10	2	8
Intra- and intercellular transport	66	14	52
Unknown	285	128	157
Total	631		

embryo development. The raw data were submitted to GEO (Gene Expression Omnibur) website and the accession number is GSE13771, http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE13771.

Based on the combination of microarray and literature data, interesting genes involved in neurogenesis were selected. The *ngn1* gene was 2.6× and 7.8× up-regulated in 15 μM and 50 μM ERβ$_2$ MO injected embryos, and the *nrd* gene was 2.2× and 3.3× up-regulated in 15 μM and 50 μM ERβ$_2$ MO injected embryos, respectively. The *ngn1* gene was selected for more detailed analysis. ISH in ERβ$_2$ morphants confirmed a differential expression of the *ngn1* gene in the morphants, where the *ngn1* expression pattern was disrupted (Fig. 7). In morphants *ngn1* was expressed in the motor neurons, contrary to coMO-injected larvae, where *ngn1* expression was detected in neuromasts, corresponding to the sensory neurons. Expression of *ngn1* was higher in morphants' heads than in heads of control larvae.

Neuromast differentiation follows lateral inhibition mechanisms (Itoh et al., 2003). In this cell fate decision process in neuromasts, the *notch* gene has been described to play a role. We therefore searched for differential expression of *notch* family members in morphants. Microarray data showed that *notch1a* was 3.6× and 4.3× up-regulated in 15 μM and 50 μM ERβ$_2$ MO injected embryos respectively. The *notch3* gene was 2.2× up-regulated in 50 μM ERβ$_2$ MO injected embryos. Even if the induction of *notch3* has not been found in the 15 μM ERβ$_2$ MO, ISH analysis showed differential expression of the *notch3* gene in morphants injected with 15 μM ERβ$_2$ MO (Figs. 8A–D). More *notch3* staining was detected in supporting cells of the morphants compared to controls. ISH with a probe against *notch1a* gene detected no clear up-regulation of mRNA staining in supporting cells of morphants injected with 15 μM ERβ$_2$ MO (Figs. 8E, F).

Discussion

ERs are known to play a major role in reproduction in vertebrates. They are also involved in diverse non-reproductive processes. In zebrafish larvae, ERs were found to be expressed in neuromasts (Tingaud-Sequeira et al., 2004, present study), however, their function in these lateral line organs remained unclear. Current work used a MO knock down technique to investigate the role of ERβ$_2$ in the early development of zebrafish larvae, and particularly in neuromasts. Of the three ER subtypes present in zebrafish, ERβ$_2$ exhibits the highest expression during early development (Bardet et al., 2002; Lassiter et al., 2002; Tingaud-Sequeira et al., 2004); Froehlicher et al., unpublished). The knock down of two other subtypes, ERα and ERβ$_1$, was not performed in this work. We cannot exclude that ERβ$_2$ action may be taken over by the two other receptors in ERβ$_2$ morphants, however, severity, dose- and time-dependency, as well as specificity of the observed morphant phenotype suggest that this is not the case. Thus, although we cannot draw any conclusion regarding the possible involvement of ERα and ERβ$_1$ in neuromast development, the results

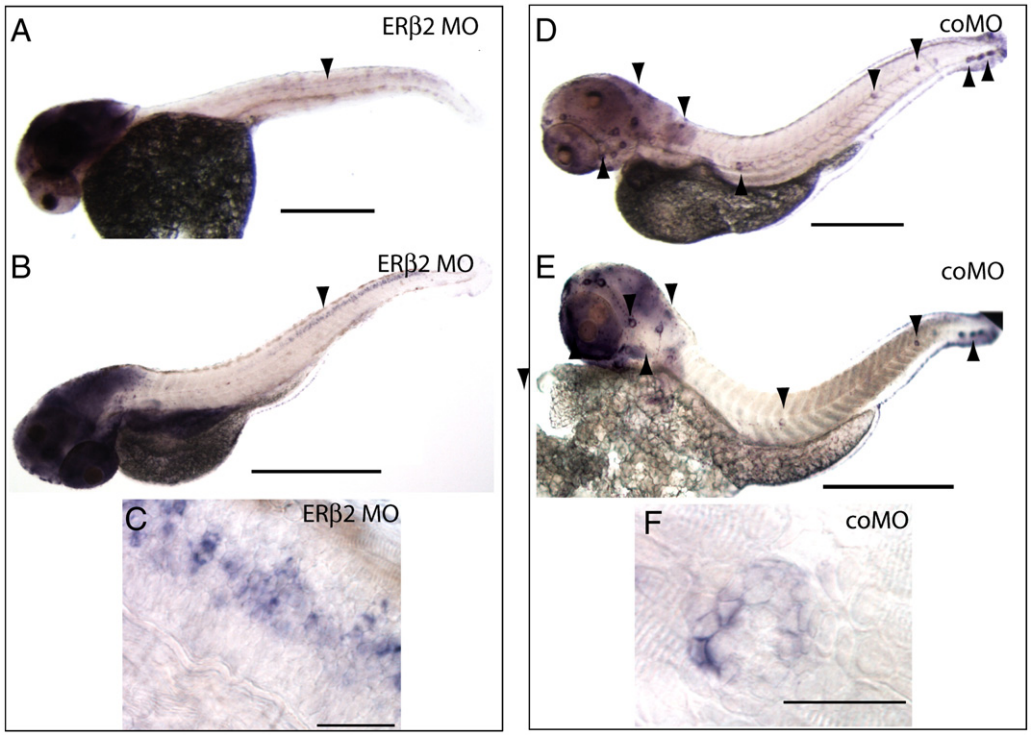

Fig. 7. Localization of *ngn1* mRNA with whole mount *in situ* hybridization in 72 hpf larvae. (A) In 50 μM ERβ$_2$ MO-injected embryos; (B, C) in 15 μM ERβ$_2$ MO injected embryos; (D) in 50 μM coMO injected embryos; (E, F) in 15 μM coMO injected embryos. (A, B, D, E) Magnification 10×, scale bar = 500 μm. (C) Magnification 60×, scale bar = 20 μm. (F) Magnification 100×. Scale bar = 20 μm. Arrowheads point to *ngn1* expression in the tail.

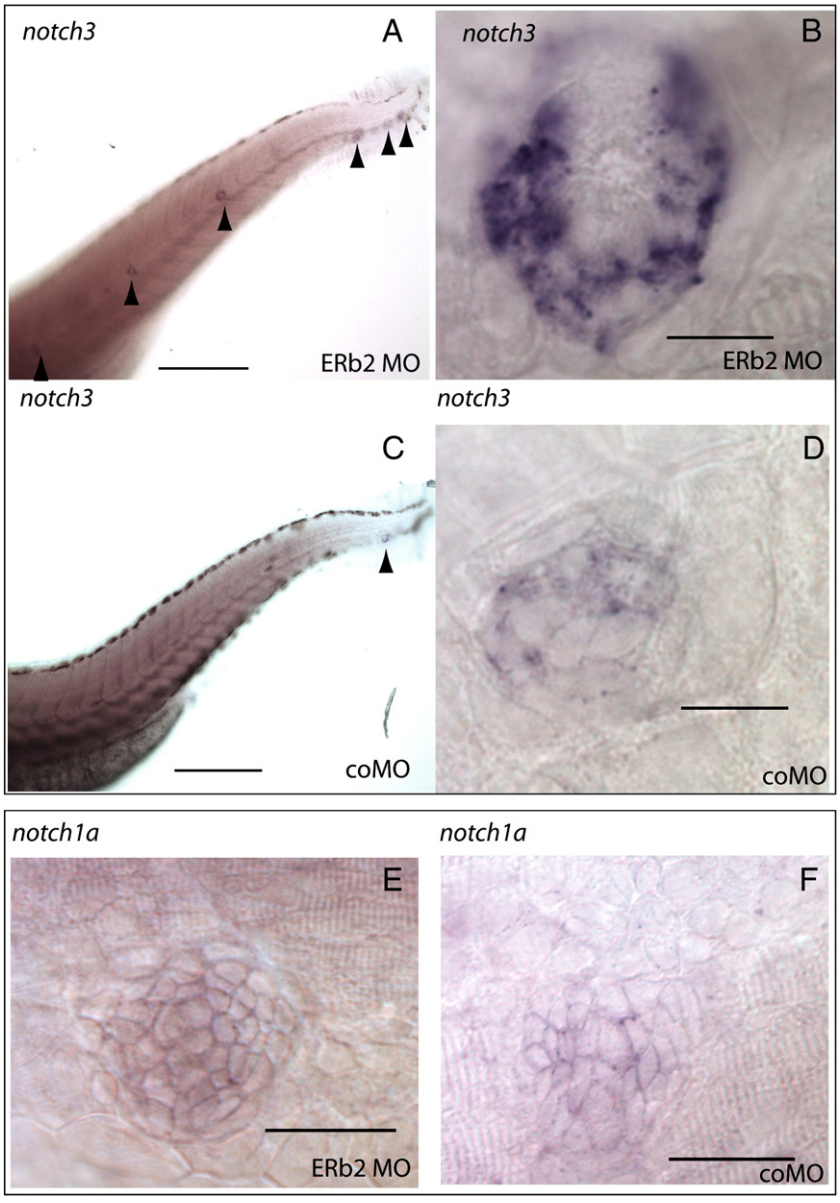

Fig. 8. Localization of *notch3* (A–D) and *notch1a* (E, F) mRNA with whole mount *in situ* hybridization in 72 hpf larvae. (A, B) *Notch3* expression in 15 μM ERβ$_2$ MO injected embryos. (C, D) *Notch3* expression in 15 μM coMO injected embryos. (E) *Notch1a* expression in 15 μM ERβ$_2$ MO injected embryos. (F) *Notch1a* expression in 15 μM coMO injected embryos. (A, C) Scale bar = 200 μm. (B, D) Scale bar = 10 μm. (E, F) Scale bar = 20 μm. Arrowheads point to *notch3* expression in neuromasts.

of our work provide a strong evidence for an important role of ERβ$_2$ in this process.

ERβ$_2$ is involved in hair cell development

The neuromasts are superficial sense organs of the mechanosensory lateral line, a sensory system closely related to that of the inner ear of mammals (Nicolson et al., 1998). Neuromasts develop from pro-neuromasts deposited by primordium migrating from head to tail. They comprise a cluster of cells which are innervated by sensory neurons projecting to the CNS (Lopez-Schier et al., 2004; Metcalfe et al., 1985). Hair cells form the core of the neuromast, surrounded towards periphery by the supporting cells of two types, sustentacular and mantle. Supporting cells constantly proliferate and are able to differentiate into hair cells, creating a continuous turnover of hair cells (Williams and Holder, 2000). This potential for the hair cells to regenerate may explain the phenomenon of hair cells recovery in the neuromasts of ERβ$_2$ morphants after MO dilution with time.

A significant reduction in the number of neuromasts stained with vital dye FM1–43, marking functional hair cells in these organs, was observed in the ERβ2 morphants. This endpoint was dose-dependent (Fig. 2) and ERβ$_2$-specific, as it was not observed after injection of coMO and could be partially rescued by co-injection of ERβ$_2$ cRNA (Figs. 3, 4). It was also not caused by off-targeting effects brought about by possible p53 induction, as co-injection of p53 and ERβ$_2$ MOs did not protect the morphants from reduction of number of stained neuromasts. Our further experiments were designed to characterize the cell type affected in the neuromasts of ERβ$_2$ morphants more specifically and to elucidate the possible reasons leading to this disruption.

Injection of ERβ$_2$ MO into transgenic line ET4 expressing GFP in the hair cells of neuromasts led to significant reduction of GFP fluorescence (Fig. 4), providing further evidence that hair cells were not only non-functional, but also not differentiated in neuromasts of morphants, as ET4 GFP labeling is a marker of differentiated hair cells in zebrafish neuromasts (Lopez-Schier and Hudspeth, 2006; Parinov et al., 2004; Sarrazin et al., 2006). At the same time, with use of

supporting cell markers we could show that the primordium migration was not disrupted in ERβ$_2$ morphants, and supporting cells of both types were present in neuromasts deposited until the tip of the tail (Fig. 6). Taken together, our data show that the neuromast disruption in ERβ$_2$ morphants concerned exclusively the hair cells of these organs, strongly arguing for the role of ERβ$_2$ in hair cell development. Another recent investigation has shown the presence of estrogen production in neuromasts, using ISH to detect the expression of aromatase *cyp19a1* in these organs and MO knock down to show its indispensability for the differentiation of hair cells (Kallivretaki et al., unpublished). Thus, locally produced estrogens may play a role in the development of the zebrafish sensory hair cells in the neuromasts, most probably through the classical genomic pathway mediated by ERβ$_2$.

An interesting characteristic observed in ERβ$_2$ morphants was an alteration of swimming behavior: the larvae were unable to swim straight and performed circling movements instead. This biological endpoint was affected even at the lowest concentration of MO injected, when all other endpoints (survival, hatching rate, presence of curved tail phenotype) did not differ from those of controls. Similar phenotypes have been described in animals carrying mutations that affect the balance systems and/or the swimming behavior, so-called motility mutants (Ashmore, 1998). The inner ear was found to be affected in the mutants that exhibit the circling behavior (Nicolson et al., 1998; Whitfield et al., 1996). Thus, the circling swimming behavior observed in our morphants indicated a disruption of inner ear functions. As the gross morphology of the inner ear in morphants developed normally (data not shown), the circling is most probably due to the inability of ERβ$_2$ morphants to differentiate functional hair cells not only in the neuromasts but also in the inner ear.

This study showed for the first time the role of ERβ$_2$ in the development of the hair cells in the lateral line of zebrafish. Our findings also support different reports documenting the role of estrogens in the inner ear development. ER expression was detected in the inner ear of mouse and rat (Stenberg et al., 1999). Hearing was disrupted in ERβko mice (Hultcrantz et al., 2006). In humans, a study looking at menopause women revealed that estrogen therapy may slow down hearing loss (Kilicdag et al., 2004). In fish, ERα has been detected in the inner ear of midshipman (Forlano et al., 2005). Reduced levels of estrogens prevented midshipman females from hearing the reproductive songs of the male (Sisneros et al., 2004). The overall morphology of the hair cells of the inner ear and lateral line is conserved among vertebrates. Moreover, a number of genes required for hair cell function in the zebrafish have been associated with auditory defects in mice and humans, thus revealing the conservation of function of these genes (Nicolson, 2005). These facts along with the reports documenting the role of estrogens in the inner ear development provide further support for the role of ERβ2 in the development of hair cells in zebrafish, discovered in the present study.

Notch family members are up-regulation in ERβ$_2$ morphants

We have performed DNA microarray analyses to investigate further the genetic mechanisms leading to disruption of hair cell differentiation in the neuromasts of ERβ2 morphants. The acquired data showed an up-regulation of two homologous *notch* genes, *notch1a* and *notch3*. The latter was found to be up-regulated only in the morphants injected with higher concentration of ERβ$_2$ MO. Interestingly, two of the Notch ligands (*deltaA* and *deltaB*) were found to be up-regulated in morphants injected with both MO concentrations, which supports the activation of the Notch signaling pathway.

The involvement of Delta/Notch signaling in hair cell differentiation has been shown before in both neuromasts and inner ear of zebrafish. Increased Notch expression prevents supporting cells from differentiating into hair cells, while a disruption in this signaling leads to abnormal increase in hair cell numbers. This mechanism has been investigated in the *mind bumb* (*mib*) mutants, which are characterized by presence of supernumerary hair cells in the PLL neuromasts and a disruption in the Notch signaling pathway (Itoh et al., 2003). In the inner ear it has been shown that the nascent hair cells, expressing Delta protein, the ligand of Notch, inhibit their neighbors from differentiating into hair cells, forcing them to be supporting cells instead (Haddon et al., 1998). Studies in mice also revealed this mechanism of lateral inhibition that plays a major role in determining the fate of the neuroepithelial cells of the inner ear (Bryant et al., 2002).

The *notch3* gene was found to be up-regulated in the high MO concentration array and was clearly induced in the neuromasts of the low concentration MO-injected embryos', as shown with ISH (Figs. 8A–D). The fact that *notch3* up-regulation has not been found in one of the screens may be related to the consideration that arrays have been performed on RNA extracted from the whole organism, thus masking the occurrence of local induction. Opposite to *notch3*, *notch1a* was found to be up-regulated in both screens but locally its expression was weak and no clear induction could be detected with ISH (Figs. 8E, F). These findings may suggest that the main Notch receptor playing a role in cell fate decisions in neuromasts is the Notch3 or/and that these two are collaborating, as has been already shown in other systems in zebrafish, for example in the epidermal ionocytes (Hsiao et al., 2007) or in the pronephric duct (Ma and Jiang, 2007).

A recent study showed that after ablation of hair cells by neomycin treatment, the supporting cells started to proliferate and later differentiated into new hair cells (Ma et al., 2008). Different members of the Notch signaling pathway (*notch3*, *deltaA*, *atoh1a*) were found to be up-regulated during the time of maximum proliferation of supporting cells and formation of hair cell progenitors, suggesting a regulation via Notch signaling. Although in the present study we did not look directly into proliferation of supporting cells and hair cells renewal after ERβ$_2$ knock down, the discovery of up-regulated *notch* expression in the supporting cells of neuromasts in ERβ$_2$ morphants may suggest that Notch signaling is involved in the suppression of hair cell differentiation.

Our findings indicate a direct or indirect interaction between transcription factor ERβ$_2$ and members of Notch signaling pathway, supporting a few other studies reporting on the interaction between Notch pathway players and steroid hormones. Notch activation has been observed in the cells treated with tamoxifen, an ER antagonist (Rizzo et al., 2008). Another study revealed a cross-talk between Notch and androgen pathways in human prostate (Belandia and Parker, 2006; Belandia et al., 2005). In order to get more information about a cross-talk between estrogens and Notch signaling, it would be interesting to see if hair cells would differentiate in ERβ$_2$ morphants after additional knock down of *notch* or after injection of ERβ$_2$ MO in the *mib* mutant which has a disrupted Delta/Notch signaling.

ERβ$_2$ role in neurogenesis

The Delta/Notch signaling pathway is also known to be involved in neurogenesis, where the neurogenic genes *delta* and *notch* together with the proneural genes, *neurogenin* (*ngn1*) and *neuroD* (*nrd*), the two most important transcription factors involved in neurogenesis, establish the nervous system, including the sensory system of the embryos (Bertrand et al., 2002; Sarrazin et al., 2006). Gain- and loss-of-function studies revealed that *ngn1* activates a generic neuronal program and determines with local signals the particular subtype of neuron that is formed (Bertrand et al., 2002). It is also essential for the development of the sensory system of most of the cranial ganglia, including those innervating the lateral line (Blader et al., 1997). Besides the loss of sensory neurons, *ngn1* knock down revealed also a strong down-regulation of *nrd* (Andermann et al., 2002).

Our microarray data provided indications that the overall process of neurogenesis is altered in ERβ$_2$ morphants. The genes *ngn1* and *nrd*

were found to be strongly up-regulated in the microarrays, also confirmed with ISH for *ngn1* (Fig. 7). Interestingly, *ngn1* was not only up-regulated in morphants, but also showed an altered expression pattern. It was found to be exclusively expressed in motor neurons along the neural tube instead of localizing in sensory neurons along the lateral line. Thus, the disruption in the development of sensory neurons may explain why the morphants were not reacting to tactile stimuli. It would be interesting to investigate if the pattern of *nrd* expression is also altered in morphants, as it is expressed in the lateral line neuromasts (Sarrazin et al., 2006) and plays a role in neuronal differentiation together with *ngn1* (Bertrand et al., 2002). Further studies should investigate if the neurons that innervate the hair cells of the neuromasts are affected by the lack of ERβ$_2$ or by the up-regulation of the *ngn1* and *nrd* genes itself.

We show that ERβ$_2$ function is required for the normal development of the sensory system, which arises from the CNS. In mammals, ERβ plays a predominant role in the CNS, as was shown with the ERβko mice model (Bodo and Rissman, 2006; Gustafsson, 1999). Elucidation of the role of ERβ$_2$ in the development of the CNS was not the goal of this study, however, our data suggest that ERβ$_2$ might play a role in neuronal development in zebrafish.

Conclusions

The present study added to the knowledge on the multiple functions of estrogens in vertebrates. With loss-of-function analysis we have shown that ERβ$_2$ is needed for the hair cell differentiation in the neuromasts, supported the possible interaction between estrogens and Notch signaling pathways and provided indications for the involvement of ERβ$_2$ in the process of neurogenesis during early development of zebrafish. Further research should concentrate on the identification of the exact mechanisms by which ERβ$_2$ mediates these multiple functions and of the specific roles that different receptors play during development.

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