Research Article

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Characterization and expression of dax1 during embryonic and gonad development in the carp (Cyprinus carpio)

Sazanlarda embriyo ve gonad gelişimi sırasında dax1 karakterizasyonu ve ekspresyonu (Cyprinus carpio)

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Abstract

Objective: The purpose of this study is to investigate the role of dax1, which causes adrenal hypoplasia congenita (AHC) with associated hypogonadotropic hypogonadism (HH) and dosage-sensitive sex reversal (DSS) during carp embryogenesis and gonadal development.

Methods: The dax1 gene was cloned from ovaries of adult carp using homologous cloning and RACE methods. Expression patterns during embryogenesis and various adult tissues were analyzed using RT-PCR (QRT-PCR) and in situ hybridization (ISH).

Results: Expression of dax1 was detected at beginning of the blastula stage and peaked at 80 days post hatching (dph), while expression in the ovary was stable. Expression of dax1 was detected in males and females, and showed sexually dimorphic pattern with high level expression in juvenile male liver and gonad. A dimorphic expression was observed in adult male gonad and heart, but was higher in liver of adult females. Whole-mount in situ hybridization revealed dax1 expression in the developing diencephalon at hatching. Later, at approximately 5 dph, a novel expression of dax1 was observed in the developing hindbrain of carp larvae.

Conclusion: These preliminary findings suggest that dax1 is conserved during vertebrate evolution and involved in a wide range of developmental processes including neurogenesis and sex differentiation in vertebrates.

Keywords: Carp; dax1; Embryo; Gonadal differentiation; Post hatching.

Özet

Amaç: Bu çalışmanın amacı, sazan embriyogenez ve gonadal gelişimi sırasında hipogonadotropik hipogonadizm (HH) ve dozaj duyarlı cinsiyet dönüşümü (DSS) ile Adrenal Hipoplazisi Congenita (AHC) neden olan dax1’in rolünün araştırılmasıdır.

Metot: Dax1 geni homolog klonlama ve RACE metodu ile yetişkin sazan yumurtalığından klonlanmıştır. Ekspresyon paternleri embriyogenez sırasında çok farklı dokularda RT-PCR (qRT-PCR) ve in-sitü hibridizasyonla(ISH) analizlenmiştir.

Sonuçlar: Dax1 geni homolog klonlama ve RACE metodu ile yetişkin sazan yumurtalığından klonlanmıştır. Ekspresyon paternleri embriyogenez sırasında çok farklı dokularda RT-PCR (qRT-PCR) ve in-sitü hibridizasyonla(ISH) analizlenmiştir.

Sonuçlar: Dax1 ekspresyonu blastula evresinin başlangıcında belirlenmiş ve 80. günde en yüksek seviyesine kulucka sonrasında ulaşmış, yumurtalıktaki ekspresyonu ise sabit kalmıştır. Dax1 ekspresyonu dişi ve erkeklerde tespit edilmiş, olarak genç erkek karaciğerleri ve gonadallarda dimorfik yaplar göstermiştir. Erişkinlerde dimorfik ekspresyon erkek gonad ve kalp dokusundada gözlenmiş, fakat dişi erişkinlerde karaciğerde daha fazladır. In-sitü hibridizasyona göre dax1 ekspresyonu yumurtadan çıkmadan aşamasında diensefalondan gelişimiinde görülmüştür. Yaklaşık 5 dphtan sonra, dax1 ekspresyonu sazan larvasının omurilik soğanının geliştiği aşamada gözlenmişdir.

Sonuç: Bu ön bulgular dax1 omurgalı evrim süresince korunmuş ve omurgalılarda nöron ve cinsiyet farklılaşması da dahil gelişimsel süreçlerin geniş bir aralığı yer aldığı göstermektedir.

Anahtar kelimeler: Sazan; Dax1; Embriyo; Gonad farklılaşması; Kuluka sonrası.
Introduction

The dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X chromosome, gene 1 (dax1 or Nr0b1) is a member of the nuclear receptor family [1–4], which, when mutated, causes adrenal hypoplasia congenita (AHC) with associated hypogonadotropic hypogonadism (HH), and when duplicated causes dosage sensitive sex reversal (DSS) [5]. Dax1 is thought to function as an anti-testicular or pro-ovarian factor during gonadal development [1]. However, dax1-deficient XY mice develop as phenotypic females, suggesting that dax1 is required for testis determination in mice. In contrast, humans (XY) with mutations in dax1 develop as males [6]. No differences in the pattern of expression of dax1 have been observed between the sexes in reptiles [7]. However, roles for dax1 in gonadal development have been suggested in other vertebrate classes. In the chicken, dax1 is down-regulated in the developing testis, but persists in the ovary [8]. Transcription of dax1 was stronger in the testis than in the ovary of frogs during metamorphosis, implying that it may be involved in testicular development in amphibians [3]. The expression of dax1 has also been associated with the development of the liver, adrenal cortex, kidney, teeth, and gut in fish [2, 4, 9–11]. Despite the potentially important roles of dax1 during fish development, it has yet to be cloned from carp. The common carp (Cyprinus carpio), which is cultured in over 100 countries world-wide for its delicious flesh and high nutritional value, account for up to 10% of global annual freshwater aquaculture production [12]. Due to its economic value in aquaculture, C. carpio has been intensively studied in terms of physiology, development, immunology, disease resistance, selective breeding, and transgenic manipulation. In addition, this species is also considered an alternative to zebrafish as a fish model [13]. More importantly, the mechanisms involved in primary sex determination of carp are complex, with genetic and environmental factors having effects on sex determination and differentiation. A role for dax1 in sex determination and differentiation has been established in many vertebrates, but whether this receptor is involved in gonadal development in carp is not clear.

The objective of this study was to obtain more detailed knowledge regarding the functions of carp dax1 in gonadal development. A dax1 cDNA was cloned from the ovary of carp, and its tissue distribution and pattern of expression throughout carp embryogenesis and gonadal development were examined in this study.

Materials and methods

Fish stocks

Carp (C. carpio) were obtained from the Henan Academy of Fishery Science (Zhengzhou, Henan Province, China) and maintained at the Genetic Laboratory (Henan Normal University, Xinxian Henan Province, China) in flow-through water tanks at 25±2°C under natural photoperiod for an initial acclimation period. Carp were fed Artemia nauplii twice a day for the duration of the experiment. No adult fish or larvae died during the experiment. Embryos were obtained by natural spawning and cultured in embryo medium following standard procedures. Staging of embryos was carried out according to Shaolian [14]. Carp embryos and larvae at 0, 5, 10, 15, 20, 25, 30, 40, 50, 65, and 80 days post hatching (dph) were sacrificed by briefly dipping them in ice-cold water as per animal ethics guidelines. As the gonad was impossible to isolate from larvae at 5 dph to 30 dph, the trunk region was sampled by removing most of the trunk muscle and head for total RNA isolation. As gonads are maintained in an undifferentiated state until 80 dph, they were isolated from 40 dph juvenile fish without separation of the sexes. After 80 dph, gonads and other tissues were selected from males and females separately. From 80 dph onwards, gonad was identified based upon gonadal histology. Such a strategy has been utilized previously for a similar ontogenic study in catfish [15]. Fifteen biological samples (n = 15) were taken at each time point. Various tissues (ovary, testis, brain, kidney, liver, and heart) were collected from 80 dph larvae and from adult fish (n = 5). The adult fish used for the tissue distribution analysis were sampled during the spawning phase in April of 2014. All tissue samples collected were snap frozen in liquid nitrogen and stored briefly at −80°C until use.

Preparation of total RNA

A total RNA extraction kit from RNAiso reagent (TaKaRa Bio, Shiga, Japan) was used to extract RNA. Approximately 20 mg of tissue were homogenized using the mortar containing liquid nitrogen. For the expression profile study, first-strand cDNA synthesis was carried out with Prime-Script reverse transcriptase (TaKaRa), using 1 μg of total RNA isolated from various stages of embryos, larvae, and different tissues.
Cloning the full-length cDNA of \textit{dax1}

A 586 bp \textit{dax1} fragment was amplified from ovary by RT-PCR using degenerate primers (Table 1). Then, the rapid-amplification of cDNA ends (RACE) technique was used to obtain the 5′- and 3′-cDNA ends of \textit{dax1} using the SMART RACE Kit (TaKaRa) according to the manufacturer’s instructions and using the primers (Table 1) designed according to the cloned cDNA fragment sequence. Amplification conditions were: 95°C for 3 min, followed by 30 cycles at 94°C (30 s), 56°C (30 s) and 72°C (60 s), ending with an extension at 72°C for 10 min. PCR products were separated by electrophoresis on 1% agarose gels and visualized by scanning with a UV imaging system (Bio-Rad, Hercules, CA, USA).

Protein alignment and phylogenetic analysis

The resulting sequences were confirmed by the BLASTx program on the NCBI Blast Server (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The deduced amino acid sequence of \textit{dax1} was aligned with those of other vertebrate members taken from GenBank, using ClustalW multiple alignment program software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A phylogenetic tree was constructed by the neighbor-joining algorithms in the MEGA6 program.

Fluorescent real-time quantitative reverse transcription (QRT)-PCR

Expression profiles of \textit{dax1} were investigated using a QRT-PCR assay on an ABI 7500 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with a SYBR green fluorescent label. Data were normalized to β-actin expression to account for differences among samples. The primers used are shown in Table 2. PCR amplification was performed as follows: 95°C for 10 s, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using the 7500 system SDS Software v 1.4 (Applied Biosystems) and the 2−ΔΔ\textit{C}\textit{t} method was used to analyze the expression level of \textit{dax1}. Negative controls were included in all runs to confirm the absence of DNA contamination.

In situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and stored in methanol at −20°C until use. Gonad specimens were dissected and pre-fixed for 20 h in 4% PFA at 4°C, placed in 30% sucrose in PBS overnight at 4°C, and finally embedded in Tek OCT (Leica, Wetzlar, Germany). Sections (20 μm thick) were obtained using a cryostat and collected on Superfrost plus slides. In situ hybridization (ISH) for \textit{dax1} was performed on carp embryos and gonad specimens using a DIG-labeled \textit{dax1} riboprobe. A \textit{dax1} cDNA fragment was obtained by RT-PCR (primers shown in Table 2) and was cloned into the Pspt18 vector (Roche, Basel, Switzerland). Digoxigenin-labeled RNA probes were synthesized from \textit{XbaI} linearized \textit{dax1} cDNA using T7 RNA polymerase from an RNA labeling kit (Roche). The probes were detected with an alkaline phosphatase (AP)-conjugated anti-DIG antibody and visualized with BM Purple AP substrate (Roche, Basel, Switzerland). Specimens were photographed under Normaski optics on a Zeiss Axio microscope system (Carl Zeiss, Oberkochen, Germany).

### Table 1: Primers for RT-PCR and RACE-PCR amplification.

| Names         | Oligonucleotide sequence (5′→3′) | Length (bp) |
|---------------|----------------------------------|-------------|
| DAX1-1 (Fw)  | C(CG)CTG(AG)(CG)TT(AG)T(AG)AA(AG)AACGT | 22          |
| DAX1-2 (Rv)  | TA(CT)TT(TT)CC(AG)TA(AG)AACCTC     | 20          |
| DAX1-3 (3′RACE-F1) | AGCGAGGCGAACCGAGCCTT | 21          |
| DAX1-4 (3′RACE-F2) | GGTCGCCGGTCTCCTCTCAAAC | 23          |
| DAX1-5 (5′RACE-R1) | CACCTGCGGGTTATTACTCTG | 21          |
| DAX1-6 (5′RACE-R2) | GCCATGCCAAGCACCAGCAG | 20          |

### Table 2: Primers for fluorescent quantitative RT-PCR and ISH.

| Names         | Oligonucleotide sequence (5′→3′) | Length (bp) |
|---------------|----------------------------------|-------------|
| dax1-1 (Fw)  | GGTGGCCGGTCTCTTCTTCAAAC         | 23          |
| dax1-2 (Rv)  | CCATTCACTGCAAACTGCC             | 19          |
| β-actin (Fw) | GATGATGAAATTGCCGCACTG           | 21          |
| β-actin (Rv) | ACCAACCATGACACCCTGATGT          | 22          |
| ISH-F        | TGCTCTAGAAACCGAGCGAGCAGCAGAAG  | 28          |
| ISH-R        | CCGGAATTCTGCGGATTAGGTCAGTCTCAAC | 31          |
Statistical analysis

Data were analyzed using SPSS version 13 and presented as mean ± standard error. All parameters were tested for normality using the Shapiro-Wilk test. Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA). Differences were considered statistically significant when p < 0.05.

Results

Molecular cloning of carp dax1

The full length dax1 cDNA obtained by RACE was 1228 bp long, with a 52 bp 5'-untranslated region (UTR), a 384 bp 3'-UTR, and a 792 bp open reading frame (ORF), which encoded a protein of 264 amino acid residues (Figure 1).

Protein alignment and phylogenetic analysis

The amino acid sequence of the putative carp dax1 was compared with dax1 orthologues in zebrafish, tilapia, frog, alligator, chick, mouse, and human (Figure 2). Multiple sequence alignment revealed that the deduced carp dax1 protein sequence shared the highest identity (94.5%) with that of Carassius auratus ssp. High sequence identities with dax1 in other teleosts, alligator, and chicken (> 60%) were also found, but identities were lower when compared to mammals (< 60%) (Table 3). In addition, the predicted carp dax1 contained only the second and fourth of the four LXXLL motifs found in mammals, although the core of the AF-2 carboxy-terminal ligand binding-like motif was conserved. Based on the alignment results, a phylogenetic tree of dax1 proteins in vertebrates was constructed, and this demonstrated that carp dax1 clusters with dax1s from other teleost fish. The teleost grouping was also separate from the other vertebrate classes, including mammals, birds, reptiles, and amphibians (Figure 3).

Tissue-specific expression of dax1 in juvenile (80 dph) and adult carp

The mRNA expression patterns of dax1 in tissues including the brain, kidney, liver, heart, and gonad of both juvenile (80 dph) and adult carp were determined using QRT-PCR analysis. In adult female carp, dax1 was expressed predominantly in ovary, liver, and brain. In adult males, dax1 mRNA levels were higher in testis and heart, and a sexually dimorphic pattern was also observed in these tissues, with expression levels being 11.5-fold and 6.32-fold higher than that in the ovary and heart of females, respectively. However, dax1 expression was higher in the liver of adult females when compared to adult males (Figure 4A).

Expression of dax1 during developmental stages

To investigate dax1 gene expression during embryogenesis and gonadal differentiation, samples were collected from the blastocyst stage until 80 dph. Expression of dax1 was detected from the beginning of the blastula stage and there were no significant changes observed from the gastrula stage to 50 dph. Thereafter, expression of dax1 increased marginally over that at the gastrula stage, beginning at 65 dph, and it was significantly higher in the testis at 80 dph compared to the gastrula stage (p < 0.05). However, the expression of dax1 in the ovary was stable at 80 dph, and was 9.8 times higher in the testis than that in the ovary at this time. Moreover, dax1 expression at the gastrula stage and at 65 dph were higher than at other stages, but these differences were not significant (Figure 5).

In situ hybridization

To determine the cell types that expressed dax1 mRNA in gonads, expression of dax1 in tissue sections was analyzed by in situ hybridization. In oocytes, dax1 expression was detected in the cytoplasm. In the testis, dax1 mRNA was detected in the cytoplasm of germline and somatic cells, especially within spermatocytes (Figure 6). Whole-mount in situ hybridization revealed that dax1 was widely expressed in many embryonic stages (Figure 7), and strongly expressed in the rostral diencephalon between
the eye fields at hatching. There were also bilateral dax1-expressing structures located in the dorsal part of the trunk, emerging as two clusters of cells on either side of the body midline. Later, at about 5 dph, de novo expression of dax1 in the hindbrain was observed. Another intriguing ISH result was that dax1 expression was detected in the liver at 5 dph (Figure 7).

**Discussion**

A carp dax1 cDNA containing two LXXLL-related motifs was cloned and characterized in the present study. It has been well documented that the LXXLL-related motifs are involved in nuclear receptor binding [16, 17] and nuclear localization [18]. Human dax1 contains four
LXXLL-related motifs; three in the DNA/RNA nuclear receptor-binding domain and one in the ligand-binding domain [17]. However, many non-mammalian dax1 proteins contain only two (typically the second and fourth) LXXLL-related motifs (Figure 2). The carp dax1 also contains two conserved LXXLL-related motifs, and this is consistent with dax1 in zebrafish, tilapia, frog, alligator, and chicken [3, 4, 7, 8]. The functions of the first three LXXLL-related motifs appear to be redundant as they act as negative regulators of the farnesoid X receptor (FXR) through a physical interaction with FXR. The transcriptional activity of FXR was repressed by the LXXLL-related motif of dax1, a function which could be accomplished by any of the individual LXXLL-related motifs [19]. Verrijn Stuart also described the functional redundancy among these motifs [20]. However, several studies have demonstrated that the first motif plays a more important role in the nuclear localization of dax1 than the third or the second motifs [18]. As the first three of the LXXLL-related motifs generally compensate for each other, and the second LXXLL-related motif of dax1 is conserved among most vertebrates, it is likely that the second motif also performs various primary functions in transcriptional regulation in vertebrates. However, more evidence is needed to support this assumption.

Previous studies have demonstrated that the tissue distribution of dax1 mRNA varies with species among vertebrates. In human, mouse, chicken, and medaka, dax1 expression was down-regulated in the developing testis, but persisted in the ovary [8, 17, 21–23]. Curiously, there are no sex-related differences in dax1 expression patterns in the alligator and some teleosts [2–4]. In frogs, rainbow trout, and black porgy, dax1 is important for testicular development [3, 24, 25]. The role of dax1 in gonadal development in carp is currently not clear. Differences between the sexes in the expression of dax1 were observed in the present study, with expression in the testis being higher than that in the ovary. The expression of dax1 was also observed to be very low from the blastocyst stage up to 50 dph. However, dax1 expression at 80 dph was significantly higher in the testis than at any other stage (p < 0.05), while expression remained low in the ovary. The expression of dax1 in mouse and chicken is associated with ovarian development [8, 23]. In contrast, the observed up-regulation of dax1 in carp testis in the current study suggests the involvement of dax1 in testicular development. However, the functions of dax1 are complex and remain unclear. Previous studies have also demonstrated that dax1 is involved in ion homeostasis and transport, lipid transport, and skeletal development [26]. Another recent study demonstrated that dax1 is required for maintaining
Table 3: Amino acid sequence identities between carp dax1 and other vertebrate dax1s.

|       | CC  | CA  | DR  | OM  | ON  | GR  | AM  | GG  | MM  | HS  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CC    | 100%|     |     |     |     |     |     |     |     |     |
| CA    | 94.50%| 100%|     |     |     |     |     |     |     |     |
| DR    | 92.70%| 87.90%| 100%|     |     |     |     |     |     |     |
| OM    | 63.10%| 56.60%| 62.80%| 100%|     |     |     |     |     |     |
| ON    | 60.00%| 54.20%| 60.80%| 73.40%| 100%|     |     |     |     |     |
| GR    | 59.90%| 56.40%| 60.50%| 54.00%| 53.70%| 100%|     |     |     |     |
| AM    | 64.50%| 57.60%| 65.20%| 58.70%| 61.10%| 61.60%| 100%|     |     |     |
| GG    | 66.10%| 62.20%| 66.70%| 60.90%| 61.60%| 65.90%| 80.20%| 100%|     |     |
| MM    | 55.00%| 50.10%| 55.70%| 52.40%| 52.30%| 55.30%| 61.90%| 66.50%| 100%|     |
| HS    | 59.20%| 54.90%| 59.70%| 54.60%| 52.40%| 58.10%| 68.70%| 73.30%| 75.50%| 100%|

GenBank accession numbers are given in the Figure 3. CC, Cyprinus carpio; CA, Carassius auratus ssp; DR, Danio rerio; OM, Oncorhynchus mykiss; ON, Oreochromis niloticus; GR, Glandirana rugosa; AM, Alligator mississippiensis; GG, Gallus gallus; MM, Mus musculus; HS, Homo sapiens.

Figure 3: Phylogenetic analysis of carp dax1 and other known dax1s in vertebrates. The scale bar indicates 0.05 expected amino acid substitutions per site.

Figure 4: QRT-PCR analysis of dax1 from various tissues of carp. (A), image of adult carp. (B), image of juvenile carp. G, gonad; H, heart; L, liver; K, kidney and B, brain. The values were calibrated with the internal control β-actin, *indicate statistically significant differences between the female and male (p < 0.05), **indicate statistically significant differences between the female and male (p < 0.01).

pluripotency of embryonic stem cells [27, 28]. It has also been proposed that dax1 maintains multipotent adrenocortical progenitor cells in an undifferentiated state [29]. It is generally known that males retain more germine stem cells for spermatogenesis than are retained in the ovary. In this study, the expression of dax1 in testis was higher than in the ovary, which could reflect a role for dax1 in maintaining pluripotency of germline stem cells in the testis,
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Figure 5: The mRNA expressions of *dax1* measured by QRT-PCR from blastocyst to 80 dph. The values were calibrated with the internal control β-actin. Each value is expressed as mean ± SEM (n = 15 for each value). * indicate statistically significant differences (p < 0.05).

whereas this function was not needed in the ovary during the same time period.

The expression of *dax1* in several non-mammalian vertebrates, including chicken, frog, *Pengze crucian* carp, and European sea bass [2, 3, 8] has been reported to be highest in the liver. In the current study, the highest level of expression of *dax1* was also observed in the liver. Interestingly, the expression of *dax1* in adult female liver was higher than in males. Vitellogenin, a protein which is incorporated into the yolk of oocytes during vitellogenesis, is synthesized in the liver [30]. Vitellogenesis and steroidogenesis are regulated by *Ad4BP/SF-1*, *ERα*, and *LRH-1*, which are also highly expressed in the liver of teleosts [31–33]. Suzuki demonstrated that *dax1* was able to interact with *Ad4BP/SF-1*, *ERα*, and *LRH-1* through the LXXLL-related motif [17]. Therefore, the higher level of expression of *dax1* in the liver of adult females may indicate an involvement in the accumulation of yolk during the formation of vitellogenic oocytes. In accordance with our findings, Meng also reported that Pengze crucian carp *dax1* might be involved in the regulation of vitellogenesis in the liver [9].

In conclusion, we have successfully cloned and characterized a full length carp *dax1* cDNA, and describe here the tissue-specific distribution of *dax1* expression. The expression pattern of the carp *dax1* gene suggests that this receptor is associated with embryogenesis and gonadal development. The information provided in the present study will broaden understanding of the molecular mechanisms affecting *dax1* in carp development and reproduction.

Figure 6: In situ hybridization of *dax1* in gonad of carp. (A), image of in situ hybridization of *dax1* in ovary of juveniles; arrowheads indicate *dax1* positive cytoplasm of provitellogenic oocytes. (B), *dax1* in testis of juveniles; arrowheads indicate *dax1* positive cytoplasm of germ cell, include spermatocyte. (C), *dax1* in ovary of adult carp; arrowheads indicate *dax1* positive cytoplasm of oocyte. (D), *dax1* in testis of adult carp; arrowheads indicate *dax1* positive cytoplasm of cytoplasm of germ cell. SC, spermatocytes; SP, spermatid; SZ, spermatozoa; Y, yolks; N, nucleus; C, cytoplasm; PO, provitellogenic oocytes; VO, vitellogenic oocytes.
Figure 7: Expression analysis of dax1 by whole-mount in situ hybridization. 
(A) blastula stage (2.5 hpf); (B) gastrulae stage (7 hpf); (C) neurula stage (13 hpf); (D) tailbud formed stage (24 hpf) lateral view, hybrid signals focused on nerviduct; (E) hatched larva stage (54 hpf), ventral view, arrowheads indicate the rostral diencephalon of carp; (F) hatched larva stage (54 hpf), dorsal view, arrow heads indicate dax1 positive two clusters of cells symmetric to the embryo body; (G) 5d post hatching, lateral view, L, liver; H, hindbrain. Arrow head shows obvious hybrid signals.

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