Spatio-temporal expression of chromogranin A during zebrafish embryogenesis

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Abstract

Chromogranin A (CHGA), a protein participating in the biogenesis of dense core secretory granules in various neuroendocrine tissues, plays a critical role in the release of hormones/peptides and the pathogenesis of pheochromocytoma. However, little is known about the developmental origin of *CHGA*-expressing cells during embryogenesis. Here, we report the structural characterization and spatio-temporal expression pattern of zebrafish (*Danio rerio*) ortholog of mammalian *CHGA*. The earliest expression of *chga* transcripts was observed at 16 h post fertilization in the developing cranial ganglia as six distinct cellular masses arranged bilaterally as strings of beads in the dorsal root ganglia (DRG) precursors along the dorsal trunk. With development advancing, the *chga* transcripts were expressed abundantly in

diencephalon, mesencephalon, and rhombencephalon as well as in the DRG. Interestingly, double *in situ* hybridization assay of *chga* with genes expressed in pronephros (Wilms' tumor suppressor 1, *wt1*), adrenal cortex (side-chain cleavage enzyme, *scc*), and sympathoadrenal neuron/chromaffin cell (dopamine- β -hydroxylase, *dbh*), respectively, showed that the *chga*-expressing cells are spatially separated from *wt1*-, *scc*-, and *dbh*-positive cell populations during early embryonic development. The pronephros region does not express *chga* even up to 7 days post fertilization, while *chga* positive-staining cells bind in the brain and DRG, indicating that *chga* may play an important role in nervous system development during the early embryonic stages.

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Introduction

Chromogranin, a family of soluble, acidic proteins being co-secreted with resident hormones and biogenic amines, represents many major constituents in the secretory vesicle cores of virtually all neuroendocrine tissues. Over the past four decades, seven proteins of chromogranin family with distinct functions have been identified: chromogranin A, B, and C (also known as secretogranin II), and four other acidic secretory proteins, secretogranin III, IV, V, and VI. Chromogranin A (CHGA) was originally identified as a major soluble protein in the chromaffin granules of adrenal medullary four decades ago and has been extensively studied as to its structure, expression, and function (Taupenot *et al.* 2003). Both *in vitro* and *in vivo* experiments have demonstrated that the CHGA plays a critical role in a number of vital physio- and pathological processes, including the biogenesis of dense core secretory granules, immunity against microbes, neurodegenerative disorders, cardiovascular diseases (e.g., hypertension), and as

a potential marker for several endocrine tumors, such as pheochromocytoma, paraganglioma, gastrinoma, and insulinoma (Kim & Loh 2005).

Although the study of CHGA has been explored widely, the expression of *CHGA* at early stages of development has remained elusive because of the invisibility of the embryogenesis in mammals. Several animal models (chicken, bovine, and rat) have been employed for studying the developmental and anatomic distribution of *chga*-positive cells in these species. The existing results mainly indicate the expression of CHGA protein in adrenal medulla and gastro-entero-pancreatic system that abounds with various endocrine cells (Kent & Coupland 1989, Mahata *et al.* 1993, Wang *et al.* 1994, Totzauer *et al.* 1995, Kameda *et al.* 1998). The distribution of *Chga* transcripts in the rat brain has also been reported (Mahata *et al.* 1993).

The zebrafish has become an important vertebrate model for studying human development and endocrine diseases, because of its many unique advantages, such as a large number of embryos per clutch, rapid and external development,

and optical clarity, which allows for the direct visualization of organogenesis and cellular migration (McGonnell & Fowkes 2006). In this study, we investigated the zebrafish *chga*, which is the ortholog of the human counterpart, and performed whole-mount *in situ* hybridization (WISH) using the anti-sense *chga* probe to observe the spatio-temporal distribution of *chga*-positive cells during early development.

In mammals, the adrenal cortex derives from the embryonic mesoderm, in contrast to the ectodermal origin of the medulla. The adrenal glands (with the cortex around the medulla) located at the upper poles of the kidneys at the late stage of embryogenesis, however, continue to develop for years after birth (Unsicker *et al.* 2005). In fish, things are quite different: in some species, the cortex and the medulla are separated, while in others, they are fused together. In the former ones, the adrenal cortex is located between two kidneys, with the neighboring medulla (with chromaffin cells forming small clusters) spreading toward and adjoining the sympathetic nerves and blood vessels; while in the latter ones, the two kinds of adrenal cells are embedded in the lymphoid-like hematopoietic cells of the head kidney, and this area is called 'pronephros' (Grassi Milano *et al.* 1997). Zebrafish, a kind of teleost, is confirmed to have an intermingled group of cells named as interrenal in the pronephros that produce both catecholamines (CAs) and steroid hormones in adults (Grassi Milano *et al.* 1997, Hsu *et al.* 2003). Although the anatomical positions of the kidneys, adrenal cortex, and medulla in adult fish are explored widely, their early developments remain unknown. Our study aims to find out the origin of *CHGA*-positive cells and the development of the chromaffin cells in embryogenesis. In zebrafish, it is found that the interrenal development parallels that of the embryonic kidney (pronephros; Hsu *et al.* 2003). The chromaffin cells and interrenal gland cells are mixed together, both of which are situated in the head kidney region of adult kidney (Grassi Milano *et al.* 1997, Hsu *et al.* 2003). The chromaffin cells contain vesicles with strong electron-dense granules under transmission electron (Hsu *et al.* 2003). In human and other species, *CHGA* is considered to be a relatively specific protein expressed in adrenal medulla chromaffin cells (Tischler *et al.* 2004), in zebrafish, side-chain cleavage enzyme (*sc*) and Wilms' tumor suppressor 1 (*wt1*) are the markers of interrenal (Hsu *et al.* 2003) and pronephros (Chai *et al.* 2003, Hsu *et al.* 2003) respectively. Therefore, it is intriguing to investigate the anatomic relationship between *CHGA*-expressing cells and the interrenal cells/pronephros during embryogenesis by double *in situ* hybridization assay.

Furthermore, in humans, dopamine- β -hydroxylase (DBH) and *CHGA*, both co-express in the chromaffin cells, are critical for CA biogenesis, storage, and release. According to the previous study, *dbh* is a marker of sympathoadrenal noradrenergic neurons/chromaffin cells in zebrafish (Reid *et al.* 1995), and we would also like to find out whether *dbh*- and *chga*-positive cells are of the same origin.

Materials and Methods

Fish culture and embryo collection

Zebrafish adults and embryos were maintained at 28.5 °C under standard conditions. Staging of embryos was carried out according to Kimmel *et al.* (1995). Embryos were cultured in 0.03% phenylthiourea (Sigma) solution starting at 12 hpf to eliminate pigmentation. Zebrafish for the experiment were maintained under the guidelines of the Chinese Council of Animal Care.

Bioinformatics analysis

The information of the *Danio rerio* *chga* protein sequence and that of the other species were obtained from the web sites <http://www.ncbi.nlm.nih.gov> and <http://www.ensembl.org>. The database of *D. rerio* *chga* genomic structure was retrieved from the web site <http://www.ncbi.nlm.nih.gov>. The human and *D. rerio* gene maps were constructed based on gene loci from LocusLink (<http://www.ncbi.nlm.nih.gov/LocusLink/>). The protein alignment and the bootstrap neighbor-joining phylogenetic tree were constructed with CLUSTAL X using the *D. rerio* *chga* amino acid sequence as an outgroup (bases on number per 500 duplicates).

Cloning of zebrafish *chga*

Reverse transcriptase PCR (RT-PCR) was performed using total RNA isolated from 7 dpf zebrafish embryos. Total RNA was prepared using TRIzol (Life Technologies, Inc.) followed by reverse transcription (RT) according to manufacturer's instruction (Promega). *chga* mRNA was amplified by La-Taq (TaKaRa, Otsu, Shiga, Japan) and the primers used were as follows: forward 5'- GGGAGTTCAAGCACTCCAAG and reverse 5'- TTCTGGAACCCAAAAACCA to obtain a cDNA fragment corresponding to the nucleotides 1049–1699 bp of the zebrafish *chga*. Reactions were carried out in a PTC-225 DNA Engine Tetrad (MJ Research Inc., Waltham, MA, USA). Denaturation was performed at 95 °C for 3 min., followed by 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 40 s for 30 cycles. The PCR products were loaded onto 1.2% agarose gels and visualized by ethidium bromide under u.v. light.

Whole-mount mRNA *in situ* hybridization and double *in situ* hybridization

The *chga* cDNA fragment was cloned into the PCSII+ vector, BamH1 was used to digest the plasmid and T7 RNA polymerase was used for the synthesis of either digoxigenin- or fluorescein-labeled zebrafish *chga* RNA anti-sense probes. As a control, sense RNA was synthesized by Sp6 RNA polymerase. Plasmids for *sc* and *dbh* were linearized with *Xho*I and *Nco*I respectively, and transcribed with Sp6 polymerase. *Wt1* plasmid was linearized with *Not* I and transcribed with

T7 polymerase. The *scc* plasmid and *dbh* plasmid were kindly provided by Prof. B C Chung (Academia Sinica, Taipei, Taiwan) and Dr V Korzh (Institute of Molecular and Cell Biology, Singapore) respectively. All of the constructs were verified by restriction analysis and nucleotides sequence analysis.

WISH and double *in situ* hybridization were performed by the protocols according to previous study (Song *et al.* 2004). Embryos were collected from timed matings, raised at 28.5 °C, and carefully staged before fixing overnight at 4 °C in 4% paraformaldehyde (PFA) in PBS. Digoxigenin- or fluorescein-labeled anti-sense RNA and sense RNA probes were generated for *chga*, *wt1*, *scc*, and *dbh*. Probes were detected using anti-digoxigenin or anti-fluorescein antibodies conjugated to alkaline phosphatase (Roche), followed by incubation with 5-bromo 4-chloro 3-indolyl phosphate and nitro blue tetrazolium or Fast Red.

For analysis of the double *in situ* hybridization, digoxigenin-labeled signals were captured using transmitted light and fluorescein-labeled signals were captured using an Argon 543-nm laser. Double *in situ* hybridization was carried out as

described previously (Jowett 2001). Stained embryos were postfixed in 4% PFA and washed for 15 min twice in PBST (0.1% Triton X-100 in PBS). This was followed by tissue clarification in 50% glycerol in PBS. Embryos were mounted on glass slides and photographed under stereo microscopy (NIKON) and inverted microscope (LEICA).

Results

Structural characterization of the zebrafish Chga

Sequence of *D. rerio* Chga protein encodes 362 amino acids, and it shares 40% similarities with human CHGA, the percentages of similarities of *D. rerio*chga and each species are 31%, 40%, 38%, 40%, and 36% with *Macaca mulatta*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, and *Xenopus tropicalis* respectively (Fig. 1A). *D. rerio*chga has a conserved phylogenetic relationship among species (mentioned before) (Fig. 1B). The genomic structures of both the *D. rerio*chga gene (25 773 bp) and that of human (12 144 bp) are composed of eight exons and seven introns (Fig. 1C).

Figure 1 Homology analysis of the zebrafish Chga. (A) The amino acid sequence of the zebrafish Chga is aligned with the CHGA amino acid sequence of the *Homo sapiens*, *Macaca mulatta*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, and *Xenopus tropicalis*. Converted sequences are colored with four levels from primary red to secondary blue, to tertiary green and base white. (B) Phylogenetic analysis of the CHGA amino sequences among species. The branches are drawn so that their lengths are proportional to the evolutionary distance along that branch. The scale relates the length of a branch to the distance. (C) Genomic structure of zebrafish *chga* and human *CHGA*. The blue boxes indicate the 5'-untranslated region (5' UTR, left) and 3'-UTR (right), the red boxes and the lines between red boxes denote exons and introns respectively. (D) Comparison of syntenic relationships of the CHGA on human chromosome 14 (chr.14) and zebrafish chromosome 20 (chr.20).

In humans, there are two genes in the vicinity of *CHGA* locus: *KCNK13* and *ITPK1*, which together define a 3.0 Mb genomic region on chromosome 14q 31–32 (Fig. 1D, left). Each of the *D. rerio* homologs (*kenk13*, *chga*, and *itpk1*) is located in the region of *D. rerio* chromosome 20 (Fig. 1D, right).

Spatial and temporal expression of chga during early embryonic development

The expression pattern of *chga* transcripts in the developing zebrafish embryos was examined by WISH with digoxigenin-labeled anti-sense RNA as a probe. Throughout two-cell stage to 12 hpf, no signals were detected (Fig. 2A), indicating that the *chga* transcripts were not expressed maternally. At 16 hpf, *chga* transcripts were first detected faintly in two specific regions; one region was in the vicinity of the otic placode, and the other in the dorsal side of trunk region (Fig. 2B and C). In the vicinity of the otic placode, three pairs of cellular clusters were detected bilaterally along the midline, which mark the positions of the trigeminal placode/ganglion, the statoacoustic ganglion, and the posterior lateral line placode/ganglion respectively (Fig. 2B). The expression of *chga* in the trunk was observed as two strings of beads bilaterally (Fig. 2C), and these *chga*-positive cells were reminiscent of the dorsal root ganglia (DRG) precursors.

At 18 and 24 hpf, the statoacoustic ganglion, the posterior lateral line placode/ganglion, and the newly formed anterior line placode expressed *chga* transcripts, while the expression in the trigeminal placode/ganglion was not detectable (Fig. 2D, F and G). In the trunk region, the *chga*-expressing DRG precursors became more detectable, distributing distinctly at the ventrolateral edge of the spinal cord in each of the somatic segments (Fig. 2E and F).

At 35 hpf, the anterior line placode developed into the anterodorsal, the media, and the anteroventral lateral line ganglia (Liu *et al.* 2006). The *chga* expression signals were detected in anteroventral lateral line ganglia, besides, statoacoustic ganglion, and posterior lateral line ganglion. The signals in the DRG became stronger (Fig. 2H and I).

In 48 hpf embryos, the anterodorsal, the anteroventral lateral line ganglia, and statoacoustic ganglion as well as posterior lateral line ganglion expressed the *chga* transcripts (Fig. 2K). However, the signals in the DRG were too weak to be seen (Fig. 2J).

WISH using the probe of sense *chga* mRNA was performed as a negative control; no positive signal was detected at any stage of embryogenesis (Fig. 2L).

Positional relationship of developmental expression of CHGA related to other interrenal-, pronephros-, and sympathoadrenal neuron/chromaffin cells expressing genes

As a result, the interrenal primordia were marked by *sec* on both sides of the notochord in the third somite at 24 hpf (Fig. 3A), then assembled toward the right of the notochord at

Figure 2 Spatio-temporal expression of *chga* in embryogenesis. (A) Lateral views of the whole-mount embryos from 0–75 to 12 hpf. (B and D) Lateral, dorsal view, and higher magnification in 16 and 18 hpf embryos. (C and E) The dorsal view and the magnification lateral view of the trunk. (F, H, and J) Lateral views of the whole-mount embryo, the dorsal head, and the trunk (insets) at 24, 35, and 48 hpf respectively. (G) Higher magnification of the hindbrain (boxed part in Fig. 2F). (I) Higher magnification of the head and the trunk (boxed part in Fig. 2H). (K) Higher magnification of the head (boxed part in Fig. 2J) with different focuses. (L) WISH using sense *chga* mRNA probe as negative control at several stages. t, trigeminal placode/ganglion; a, anterior line placode; op, otic placode; p, posterolateral line placode/ganglion; DRG, dorsal root ganglia; sa, statoacoustic ganglion; c, cerebellum; di, diencephalon; h, hindbrain; av, anteroventral lateral line ganglion; ad, anterodorsal lateral line ganglion. The white arrow refers to the signals out of the focus; the otic placode (op) and the otic vesicle (ov) are outlined with dashed lines. LC, locus coeruleus; AAN, arch-associated neurons; chr, chromaffin cell; ent, enteric neurons; sym, sympathetic neurons; MC, medulla catecholaminergic cluster; Me, mesencephalon; Rha, rhombencephalon anterior part; Di, diencephalon.

Figure 3 The distributions of the *chga*- and *scc*-expressing cells in embryogenesis. (A, B, and C) The double staining of *chga* (red) with *scc* (dark blue) at 24, 30, and 48 hpf. From top to bottom, the lateral view (anterior facing left), the dorsal view (anterior facing left), the dorsal view under the TITC (tetraethylrhodamine isothiocyanate) filter, and the negative control with sense mRNA *chga* and *scc* probes. Arrows indicate the cranial ganglia and arrowheads indicate the interrenal.

30 and 48 hpf (Fig. 3B and C), as described previously (Hsu *et al.* 2003). The pronephric primordia marked by *wt1* distributed on both sides of the notochord straddling over the second and third somites at 24 and 30 hpf (Fig. 4A and B), and

Figure 4 The distributions of the *chga*- and *wt1*-expressing cells in embryogenesis. (A, B, and C) The double staining of *chga* (dark blue) with *wt1* (red) at 24, 30, and 48 hpf. From top to bottom, the lateral view (anterior facing left), the dorsal view (anterior facing left), the dorsal view under the TITC filter, and the negative control with sense mRNA *chga* and *wt1* probes. Arrows indicate the cranial ganglia and the DRG arrowheads indicate the pronephros.

became spatially closer at 48 hpf (Fig. 4C). The *chga*-expressing cells were distributed in the cranial ganglia and the DRG at 24–48 hpf. Thus, our results revealed that the *chga*-positive cells did not co-express with either the *scc*- or *wt1*-positive cells. In other words, there are no *chga* expressed in the interrenal/pronephros during the early embryonic stages. The *dbh*-expressing cells were detected in locus coeruleus (LC) and arch-associated neurons (AAN), and they did not co-localize with the *chga*-expressing cells (Fig. 5).

Since we did not witness the co-expression of *chga* with *dbh*, *scc*, and *wt1* at 48 hpf, we further evaluated the co-localization at later stages (3, 4, and 7 dpf). At 3 and 4 dpf, the cranial ganglia (marked by *chga*) remained the same as observed at 48 hpf; however, *chga* transcripts were undetectable in the DRG (Fig. 6A and E). The *dbh*-labeled cells expressed in several areas: LC, medulla catecholaminergic cluster (AAN), cervical sympathetic neurons (sym), medulla catecholaminergic cluster (MC), and enteric neurons (ent). Some *dbh*-expressing cells detected in the vicinity of pronephros were

Figure 5 The distributions of the *chga*- and *dbh*-expressing cells in embryogenesis. (A, B, and C) The double staining of *chga* (red) with *dbh* (dark blue) at 24, 30, and 48 hpf. From top to bottom the lateral view (anterior facing left), the dorsal view (anterior facing left), the dorsal view under the TITC filter (C gives the oblique view from lateral shown making the staining clear), and the negative control with sense mRNA *chga* and *dbh* probes. LC, locus coeruleus; AAN, arch-associated neurons.

Figure 6 The distributions of *chga*, *dbh*, *wt1*, and *scc* at 3, 4, and 7 dpf in zebrafish. The *chga*, *dbh*, *wt1*, and *scc* expressions in 3 dpf (A–D), 4 dpf (F–I), and 7 dpf (K–M) embryos. Lateral view and dorsal view in each panel. The dorsal view of the whole-mount embryo and the dorsal trunk (inset) labeled by *chga* are added in (K). WISH using sense *chga* probe as negative control (E, J, and N).

considered to be chromaffin cells (Chai *et al.* 2003; Fig. 6B and F). The bilateral *wt1*-positive pronephros have fused together to become a single cellular cluster (Fig. 6C and G). The interrenal gland cells (marked by *scc*, Fig. 6D and H) remained the same as observed at 48 hpf. At 7 dpf, it is intriguing to find that abundant *chga* transcripts were detected in the diencephalon, mesencephalon and anterior rhombencephalon, and the DRG (Fig. 6I). The *dbh*-labeled cells were concentrated in the LC, medulla catecholaminergic cluster (MC), enteric neurons (ent), and chromaffin cells (Fig. 6J). The *wt1* marked the region of the pronephros diverging into two symmetrical cellular clusters separated by the midline (Fig. 6K). The WISH using sense *chga* probe was performed as negative control (Fig. 6E, J and N).

Discussion

We have obtained the cDNA sequences encoding the *chga* of zebrafish (*D. rerio*) from the web site (<http://www.ncbi.nlm.nih.gov>).

Based on conserved gene structure, phylogenetic analysis, and syntenic relationship, we identified the ortholog of mammalian *CHGA* gene in zebrafish. There were several studies on the early expression of *CHGA* in other animals. *CHGA* protein was found in chromaffin cells through all stages in the developing porcine and bovine adrenal medulla (Wang *et al.* 1994, Totzauer *et al.* 1995). *CHGA* and its derived peptides were also detected in the gastro-entero-pancreatic system of rat and porcine (Kent & Coupland 1989), as well as in the secretory-specific cells of the hypophyseal pars tuberalis of chicken (Kameda *et al.* 1998). At high levels of *Chga*, mRNAs have been already selectively traced in the intermediate cortex at gestational day 16 in the rat brain (Mahata *et al.* 1993). To our knowledge, our study is the first report of the spatio-temporal expression of *chga* in fish. As mentioned above, the zebrafish has the advantage of being able to trace the spatio-temporal expression of genes during early embryo development. We used the method of WISH to investigate the dynamic expression pattern during

the serial development stages and obtained a relatively integrated picture of the *chga* expression pattern. Our result showed that, *chga*-expressing cells distributed in cranial ganglia and DRG during embryogenesis (Fig. 2) and then spread throughout in the brain and specifically in DRG (Fig. 6A). It has been already proven that the cranial ganglia derives from neural crest (Stewart *et al.* 2006), our result suggested the possibility that the *chga*-expression cells were derived from the neural crest cells and imply a possible role for *chga* in nervous system patterning and organogenesis.

From the previous studies, *Chga* has been proven to induce an activated phenotype in microglial cells that lead to neuronal apoptosis by using cultured rodent microglia (Ciesielski-Treska *et al.* 1998, Ulrich *et al.* 2002). Besides, *chga* transcription and gene expression inhibited cell growth in the anterior pituitary-derived cell lines and decreased the rate of tumor growth in severe combined immunodeficiency disease mice (Stilling *et al.* 2005). The mutant null mice for the *Chga* were viable and fertile and had no obvious developmental abnormalities, and their neural and endocrine functions are not grossly impaired. The authors contribute this to the compensation of the other granin family members (Hendy *et al.* 2006). Anyway, the specific expression of *chga* in zebrafish nervous system may give us clues to pay attention to relationship between the *chga* and nerve development.

As well known, CHGA is named for its first discovery in the chromaffin cells of adrenal medulla. The chromaffin cells are confirmed to localize in the pronephros of adult zebrafish together with the interrenal cells (Hsu *et al.* 2003). However, our double whole-mount *in situ* hybridization results indicated that *chga* did not co-localize with either *wt1*-expressing pronephros or *scc*-expressing interrenal during early zebrafish embryogenesis (Figs 3 and 4). Besides, the results also suggested that the *chga*-expressing cells and the *dbh*-expressing cells might have different developmental origins (Fig. 5), and there were no *chga* transcripts detected within the pronephros by 7 dpf (Fig. 6).

It has been shown that the protein products of three genes, tyrosine hydroxylase (*th*), *dbh*, and phenylethanolamine-n-methyltransferase (*pnmt*), are required for CA synthesis, and *pnmt* is the most specific enzyme for converting the norepinephrine to epinephrine in the chromaffin cells of adrenal medulla (Wong 2003). Unfortunately, we are unable to detect any *pnmt*-expressing cells in the embryos using our current WISH protocols (Supplementary Figure 1, see Supplementary data in the online of version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol198/issue3/>). Furthermore, no morphologically recognized chromaffin cells were found in the Giemsa-stained serial sections of 7 dpf embryos (data not shown). However, the RT-PCR results did show that the *pnmt* transcripts can be detected in the 7 dpf embryos, although the levels of expression were relatively low compared with *th* and *dbh* (Supplementary Figure 2, see Supplementary data in the online of version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol198/issue3/>).

Taken together, the results suggested that the zebrafish may have chromaffin cells, but can not be detected by *pnmt* WISH method likely due to either the scarcity of chromaffin cells or the low levels of *pnmt* transcripts during early embryogenesis. Besides, the failure to detect the *pnmt* transcripts in *dbh*-expressing cells may be attributed to their functional immaturity during early embryogenesis, which warrants further study.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Chai C, Liu YW & Chan WK 2003 Ff1b is required for the development of steroidogenic component of the zebrafish interrenal organ. *Developmental Biology* **260** 226–244.
- Ciesielski-Treska J, Ulrich G, Taupenot L, Chasserot-Golaz S, Corti A, Aunis D & Bader MF 1998 Chromogranin A induces a neurotoxic phenotype in brain microglial cells. *Journal of Biological Chemistry* **273** 14339–14346.
- Grassi Milano E, Basari F & Chimenti C 1997 Adrenocortical and adrenomedullary homologs in eight species of adult and developing teleosts: morphology, histology, and immunohistochemistry. *General and Comparative Endocrinology* **108** 483–496.
- Hendy GN, Li T, Girard M, Feldstein RC, Mulay S, Desjardins R, Day R, Karaplis AC, Tremblay ML & Canaff L 2006 Targeted ablation of the chromogranin a (*Chga*) gene: normal neuroendocrine dense-core secretory granules and increased expression of other granins. *Molecular Endocrinology* **20** 1935–1947.
- Hsu HJ, Lin G & Chung BC 2003 Parallel early development of zebrafish interrenal glands and pronephros: differential control by *wt1* and *ff1b*. *Development* **130** 2107–2116.
- Jowett T 2001 Double *in situ* hybridization techniques in zebrafish. *Methods* **23** 345–358.
- Kameda Y, Miura M & Ohno S 1998 Localization and development of chromogranin A and luteinizing hormone immunoreactivities in the secretory-specific cells of the hypophyseal pars tuberalis of the chicken. *Histochemistry and Cell Biology* **109** 211–222.
- Kent C & Coupland RE 1989 Localisation of chromogranin A and B, met-enkephalin-arg6-gly7-leu8 and PGP9.5-like immunoreactivity in the developing and adult rat adrenal medulla and extra-adrenal chromaffin tissue. *Journal of Anatomy* **166** 213–225.
- Kim T & Loh YP 2005 Chromogranin A: a surprising link between granule biogenesis and hypertension. *Journal of Clinical Investigation* **115** 1711–1713.

- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B & Schilling TF 1995 Stages of embryonic development of the zebrafish. *Developmental Dynamics* **203** 253–310.
- Liu Q, Liu B, Wilson AL & Rostedt J 2006 Cadherin-6 message expression in the nervous system of developing zebrafish. *Developmental Dynamics* **235** 272–278.
- Mahata M, Mahata SK, Fischer-Colbrie R & Winkler H 1993 Ontogenic development and distribution of mRNAs of chromogranin A and B, secretogranin II, p65 and synaptin/synaptophysin in rat brain. *Brain Research. Developmental Brain Research* **76** 43–58.
- McGonnell IM & Fowkes RC 2006 Fishing for gene function – endocrine modelling in the zebrafish. *Journal of Endocrinology* **189** 425–439.
- Reid SG, Fritsche R & Jonsson AC 1995 Immunohistochemical localization of bioactive peptides and amines associated with the chromaffin tissue of five species of fish. *Cell and Tissue Research* **280** 499–512.
- Song HD, Sun XJ, Deng M, Zhang GW, Zhou Y, Wu XY, Sheng Y, Chen Y, Ruan Z, Jiang CL *et al.* 2004 Hematopoietic gene expression profile in zebrafish kidney marrow. *PNAS* **101** 16240–16245.
- Stewart RA, Arduini BL, Berghmans S, George RE, Kanki JP, Henion PD & Look AT 2006 Zebrafish *foxd3* is selectively required for neural crest specification, migration and survival. *Developmental Biology* **292** 174–188.
- Stilling GA, Bayliss JM, Jin L, Zhang H & Lloyd RV 2005 Chromogranin A transcription and gene expression in Folliculostellate (TtT/GF) cells inhibit cell growth. *Endocrine Pathology* **16** 173–186.
- Taupenot L, Harper KL & O'Connor DT 2003 The chromogranin–secretogranin family. *New England Journal of Medicine* **348** 1134–1149.
- Tischler AS, Powers JF & Alroy J 2004 Animal models of pheochromocytoma. *Histology and Histopathology* **19** 883–895.
- Totzauer I, Amselgruber W, Sinowatz F & Gratzl M 1995 Early expression of chromogranin A and tyrosine hydroxylase during prenatal development of the bovine adrenal gland. *Anatomy and Embryology* **191** 139–143.
- Ulrich G, Ciesielski-Treska J, Taupenot L & Bader MF 2002 Chromogranin A-activated microglial cells induce neuronal apoptosis. *Annals of the New York Academy of Sciences* **971** 560–562.
- Unsicker K, Huber K, Schutz G & Kalcheim C 2005 The chromaffin cell and its development. *Neurochemistry Research* **30** 921–925.
- Wang JM, De Ridder EF, De Potter WP & Weyns AL 1994 Localization of neurokinin A and chromogranin A immunoreactivity in the developing porcine adrenal medulla. *Histochemical Journal* **26** 431–436.
- Wong DL 2003 Why is the adrenal adrenergic? *Endocrine Pathology* **14** 25–36.

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