Parallel early development of zebrafish interrenal glands and pronephros: differential control by \textit{wt1} and \textit{ff1b}

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SUMMARY

Steroids are synthesized mainly from the adrenal cortex. Adrenal deficiencies are often associated with problems related to its development, which is not fully understood. To better understand adrenocortical development, we studied zebrafish because of the ease of embryo manipulation. The adrenocortical equivalent in zebrafish is called the interrenal, because it is embedded in the kidney. We find that interrenal development parallels that of the embryonic kidney (pronephros). Primordial interrenal cells first appear as bilateral intermediate mesoderm expressing \textit{ff1b} in a region ventral to the third somite. These cells then migrate toward the axial midline and fuse together. The pronephric primordia are \textit{wt1}-expressing cells located next to the interrenal. They also migrate to the axial midline and fuse to become glomeruli at later developmental stages. Our gene knockdown experiments indicate that \textit{wt1} is required for its initial restricted expression in pronephric primordia, pronephric cell migration and fusion. \textit{wt1} also appears to be involved in interrenal development and \textit{ff1b} expression. Similarly, \textit{ff1b} is required for interrenal differentiation and activation of the differentiated gene, \textit{cyp11a1}. Our results show that the zebrafish interrenal and pronephros are situated close together and go through parallel developmental processes but are governed by different signaling events.

Key words: Steroidogenesis, P450scc, Ultra-structure, side-chain cleavage enzyme, CYP11A1, SF1, Ad4BP, Adrenal gland, Head kidney, Kidney, Morphogenetic movement, SF1

INTRODUCTION

Corticosteroids are secreted from the adrenal cortex to control the homeostasis of glucose and electrolytes (Dallman et al., 1989; Jones and Bellamy, 1964). Engagement in the synthesis of corticosteroids, is a hallmark of adrenocortical genes such as \textit{CYP11A1} (\textit{P450scc}, \textit{scc}), \textit{CYP21} and \textit{3\beta-HSD}. The combined products of these genes constitute the differentiated functions of the adrenal cortex (Mesiano and Jaffe, 1997). Adrenal insufficiencies are mostly diseases caused by defects of steroidogenic genes. Congenital adrenal hyperplasia is a common inborn congenital disorder caused by mutations of the \textit{CYP21}, \textit{3\beta-HSD} and \textit{CYP11B1} genes (Chung, 1996; Rheume et al., 1995; White et al., 1994; and Speiser, 2000). Those affected suffer from insufficient corticosteroid secretion resulting in virilization and salt wasting. Knockout mice deficient in steroidogenic genes like \textit{Star} and \textit{P450scc} have been generated to investigate the molecular mechanism of adrenal insufficiencies (Caron et al., 1997; Hu et al., 2002; Ishii et al., 2002). Despite extensive investigation into the molecular bases of these diseases, very little is known about the early events leading to the formation of functional adrenal glands.

The origin of the adrenal gland is still controversial. It is thought to share the same origin as the kidney and gonads, derived from coelomic epithelium of the urogenital ridge and/or the underlying mesenchyme (Keegan and Hammer, 2002; Morohashi, 1997). \textit{WT1} (Wilms' tumor suppressor 1) is first expressed in the intermediate mesoderm lateral to the coelomic cavity and is crucial for urogenital ridge development (Armstrong et al., 1993). Mutations of \textit{WT1} cause WAGR, Denys-Drash, and Frasier syndromes that are associated with disorders of the kidney and gonad (Baird et al., 1992; Barbaux et al., 1997; Pelletier et al., 1991). The function of \textit{Wt1} in urogenital ridge development has been further evaluated in knockout mice that lack adrenal glands, kidneys and gonads (Kreidberg et al., 1993). Yet the function of \textit{Wt1} in the adrenal gland is still not clear, since \textit{Wt1} is not expressed in the developing adrenal gland (Armstrong et al., 1993). Adrenal size is greatly reduced in \textit{Wt1} knockout mice that are partially rescued by the human \textit{Wt1} gene (Moore et al., 1999). It indicates that \textit{Wt1} may play a role during early adrenal gland development, although the precise mechanism has not been clarified.

SF1, also termed Ad4BP or NR5A1, is an Ftz-F1 member of the nuclear receptor superfamily (Morohashi and Omura, 1996; Parker et al., 2002). It is not only essential for the activation of several steroidogenic enzymes including \textit{scc} (Guo et al., 1994; Rice et al., 1991), but is also the earliest gene that can be detected in the adrenal-gonadal primordium. An \textit{SF1} heterozygous mutation causes adrenal insufficiency and XY
sex reversal (Achermann et al., 1999). In Sf1 (Nr5a1) knockout mice, the adrenal and gonadal primordia arise initially, but regress later through apoptosis (Ikeda et al., 1995; Luo et al., 1995). Moreover, Sf1 can cause embryonic stem cells to differentiate into the steroidogenic cell type (Crawford et al., 1997). These observations indicate that Sf1 functions at multiple levels to control differentiation of the endocrine lineage. However, the mechanism of Sf1 action for endocrine lineage determination has not been fully elucidated.

One of the reasons for the lack of understanding of adrenal gland organogenesis is the difficulty in studying mammalian embryogenesis. To circumvent this problem, in our study we used zebrafish, a teleost, since zebrafish embryos are amenable to molecular manipulation and genetic dissection (Briggs, 2002; Penberthy et al., 2002). The adrenal cortex homologue in teleost is called the interrenal gland, because together with chromaffin cells (counterpart of adrenal medulla), it is embedded inside the anterior part of the kidney (Chester Jones and Mosley, 1980). Although interrenal glands in some species of teleosts have been identified by histological methods (Grassi Milano et al., 1997; Rocha et al., 2001), very few molecular studies have been carried out with regard to their differentiation and gene expression.

In this report, we have identified the interrenal gland in zebrafish using molecular probes and morphological studies. We characterized the morphogenetic movements of pronephric and interrenal primordia during early embryogenesis. We used the antisense morpholino knockdown strategy to show that wt1 is important for the development of both pronephric and interrenal primordia. The differentiation of the interrenal primordia is also controlled by ff1b, probably through direct activation of the scc gene. In addition, the morphogenetic movement of the interrenal gland is abnormal in ff1b and oep mutant embryos, which are defective in midline signaling. This is the first detailed report of early interrenal differentiation and morphogenesis; it provides a mechanistic view of these processes controlled by the wt1 and ff1b genes.

**MATERIALS AND METHODS**

**Zebrafish stock**

Zebrafish were maintained at 28.5°C. Mutant embryos were phenotypically identified under a dissecting microscope by the eye fusion in oep m134 and cyc m60 embryos, absent notochord in ff1b m60 embryos and U-shaped somites in sry m232 and smu b577 embryos.

**Generation of SCC antibodies**

The fragment of zebrafish scc cDNA from 299 bp to 1020 bp, encoding amino acids 100-340, was subcloned into pET-29 vector. The SCC recombinant protein was purified as described previously (Hu and Chung, 1990). About 200 μg of the gel-purified recombinant protein were injected into rabbit subcutaneously. The same amount of booster was given 21 days later. Antiserum collected 9 days after the second booster was used throughout the study.

**Whole-mount in situ hybridization and immunofluorescence**

Whole-mount in situ hybridization was performed using digoxigenin- or fluorescein-labeled antisense RNA probes and alkaline phosphatase-conjugated secondary antibodies, as described previously (Chiang et al., 2001). The following templates were linearized and transcribed to make antisense RNA probes: scc (XhoI/T7), 3β-HSD (XbaI/T7), ff1b (NotI/SP6), wt1 (BamHI/T7). Double in situ hybridization was carried out as described previously (Jowett, 2001). For immunohistochemistry, hybridized embryos were incubated with primary antibody (polyclonal rabbit anti-zebrafish SCC antibody, 1:5000) for 1 hour at room temperature. After washing, they were incubated with secondary antibody (horse anti-rabbit IgG FITC antibody, 1: 5000, Chemicon, Temecula, CA, USA) for 1 hour, washed well, and mounted. The intermediate mesoderm containing primordial interrenal and pronephros was dissected from embryos using needles before photographs were taken under an Olympus BX50 microscope. For analysis of the double in situ hybridization, digoxigenin-labeled wt1 signals were captured using transmitted light, and fluorescein-labeled ff1b signals, stained by Fast Red, were captured with an Argon 543-nm laser connected to a Zeiss Axiocamt 100M microscope equipped with LSM510 (Carl Zeiss Inc, Germany). The images were merged using the Release 2.5 software.

**Morpholino injection**

Morpholino oligonucleotides (Gene Tools, Corvallis, OR, USA) were dissolved in water at a concentration of 10 μg/μl. The stock solution was diluted to working concentrations of 0.5-3 μg/μl in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM Hepes, pH 7.6) and injected into the yolk of 1-4 cell embryos. The morpholino sequences are as follows: ff1b: AATCTCTGTGCTTTAGATGCAAT; wt1: TGAGGTCACAGACATCA-GAACCAAT; ff1a: CTGACTCGACCTTACAGGACATGAC. cyp17 sense: CAGTGTAAATAGCTGCAATGGT.

**Electron microscopy**

Zebrafish embryos were processed for electron microscopy as described previously (Drummond et al., 1998). Embryos were fixed in 1% glutaraldehyde at 4°C overnight, washed, then fixed in 2% osmium tetroxide (OsO4) at RT for 2 hours. They were then dehydrated with serial concentrations of acetone, infiltrated serially with solutions of embedding reagent and polymerized at 70°C. Sections of 0.2 μm were cut and examined using a Zeiss EM109 electron microscope. All the reagents were purchased from Electron Microscopy Science (Washington, PA, USA).

**Transfection**

Transfection was performed in H1299 cells cultured in 60-mm plates in RPMI 1640 medium plus 10% FBS and 1% antibiotics. The lacZ reporter gene under the control of the wild-type human SCC promoter or human SCC promoter with a mutation in the proximal SF1 binding site (SCC2.3lacZ; mtPSCC2.3lacZ) were constructed previously (Hu et al., 1999; Hu et al., 2001). The ff1b cDNA was cloned into pcDNA3 vector under the control of the CMV-IE promoter. The transfected DNA included 5 μg expression vector and 2 μg reporters (1 μg lacZ reporter and 1 μg luciferase reporter pGL2 as internal control). Cells were lysed and assayed as previously described (Hu et al., 1999). β-Galactosidase activity was normalized against the internal control luciferase. The activity of SCC/lacZ reporter alone was set as 100. Data represent mean±s.e.m. of four independent experiments.

**RESULTS**

The zebrafish interrenal gland

The zebrafish head kidney consists of fused bilateral lobes located in the anterior part of the kidney (Fig. 1A). The interrenal gland is located within the head kidney of many species of teleost fishes for the synthesis of steroid hormones (Grassi Milano et al., 1997). To identify the location of the interrenal gland in zebrafish, we dissected out the adult head kidney and hybridized it with cyp11a1 (scc) that was
previously cloned in our laboratory (Hsu et al., 2002; Lai et al., 1998). The scc gene can be used as a marker for steroidogenic tissues, such as interrenal glands (Hu et al., 2001a; Simpson, 1979). We found that interrenal glands (scc-expressing cells) are located in both lobes of the head kidney, but the right gland is larger than the left one (Fig. 1B). The interrenal cells are arranged as layers of epithelial cells in association with the posterior cardinal vein (Fig. 1C-G). The chromaffin cells are interposed with interrenal epithelial cells (Fig. 1H).

Ultrastructural analysis showed that interrenal epithelial cells contained many mitochondria with tubulovesicular cristae (Fig. 1I). Unlike mammalian adrenocortical cells, interrenal cells do not contain lipid droplets. Two types of chromaffin cells were identified (Fig. 1J). (1) The noradrenaline type (n), which contain heterogeneous vesicles with electron-dense granules located asymmetrically within the vesicular membrane. (2) The adrenaline type (a), in which the vesicles are smaller and contain homogenous electron-lucent granules that are separated from the vesicular membrane by a visible halo.

The identification of zebrafish interrenal primordium

To understand the structure and development of the zebrafish interrenal gland, we identified the interrenal primordium by detecting expression of interrenal marker genes, such as scc and 3β-HSD. Our in situ hybridization showed that scc and 3β-HSD are expressed in a region ventral to the third somite in 36 hpf (hours post fertilization) embryos (Fig. 2A,B). Another gene, ff1b, which is a member of the Ftz-F1 nuclear receptor family (Chai and Chan, 2000), is also expressed in the same region (Fig. 2C). Since the interrenal is located within the head kidney, we examined the location of the primordial kidney (pronephros) in fish embryos by hybridization with a pronephric marker, wt1 (Serluca and Fishman, 2001). Fig. 2D shows that wt1 is present in a wider region, ventral to both the second and third somites.

We used immunofluorescence to detect SCC protein expression. SCC expression overlaps with ff1b transcripts in the interrenal primordia, both at 30 and 33 hpf (Fig. 2E-J). This result confirmed that ff1b-expressing cells constitute the interrenal primordium where steroid hormones are produced.

Histological analysis of the interrenal gland

We examined histological sections of interrenal cells to further understand their morphological details. The interrenal primordium is located in a region caudal to the glomerulus (data not shown) and ventral to the notochord (Fig. 3A). At 3 days post fertilization (dpf), interrenal cells that express ff1b are already enclosed by a capsule like structure, indicating that it is a distinct organ, although it does not show epithelial characteristics, such as columnar cell shape and organized cell arrangement (Fig. 3B,C). Electron micrographs show the presence of many mitochondria inside the interrenal primordial cells (Fig. 3D). Higher magnification shows that these mitochondria contain tubulo-vesicular cristae (Fig. 3E), which are typical for cells engaged in active steroidogenesis (Farkash et al., 1986). It indicates that these cells have already acquired...
steroidogenic potential. At 3 dpf, and at 5 dpf (data not shown), we did not find any epithelial characteristics in the interrenal primordium, nor was it associated with blood vessels. This indicates that although the interrenal primordium appears at 20-22 hpf (Fig. 4F,K), interrenal gland organogenesis is so slow that it is incomplete at 5 dpf.

The morphogenetic movement of interrenal primordial cells

In order to understand the morphogenesis of the interrenal gland in greater detail, especially with respect to the development of the pronephros, we assessed \( wt1 \) and \( ff1b \) expression in pronephric and interrenal primordia, respectively, at different time points, by double in situ hybridization. The resulting data are summarized as cartoons (Fig. 4A-E). At 20-22 hpf, \( wt1 \) is expressed in intermediate mesoderm bilateral to the notochord and ventral to the second and third somites; some \( wt1 \) expression domains appear to express \( ff1b \) (Fig. 4F,K). At 24 hpf, the \( ff1b \) expression domains have increased and are separated from the \( wt1 \)-expressing cells (Fig. 4G,L). Around 30 hpf, the bilateral \( ff1b \)-positive cells are fused together and located slightly to the right of the notochord (left from the ventral view). The \( wt1 \)-expressing cells remain at the same bilateral position (Fig. 4H,M), but start to move to the axial midline at 33 hpf (Fig. 4I,N), and differentiate into podocytes, forming glomeruli, at around 40-44 hpf (Drummond et al., 1998). At 3 dpf, the \( ff1b \) expression domains expand further and are distributed on both sides of the notochord again (Fig. 4J,O).

Analysis of migration of interrenal primordial cells in mutants defective in midline signaling

The notochord and floor plate are midline structures important for patterning associated cells, such as axial and paraxial
mesoderm, and neuroectoderm (Brand et al., 1996; Halpern et al., 1997). The interrenal primordium is located close to the notochord. To determine whether its development could also be affected by midline signaling, we followed the morphogenetic development of interrenal primordia in mutants lacking midline structures, by labeling them with scc. At 36 hpf, the normal interrenal primordium has fused into a group of cells near the midline (Fig. 5A). In the flh mutant embryo, which lacks a notochord (Halpern et al., 1995), the interrenal primordia are formed but never fuse together; they remain at their original bilateral locations (Fig. 5D). Similarly, in oep (one-eyed pinhead) mutants, which lack the floor plate and endoderm (Schier et al., 1997), the interrenal primordia cannot fuse; moreover, they move to ectopic bilateral locations (Fig. 5E).

Fig. 4. Morphogenetic movement of the interrenal gland from 20 hpf to 3 dpf. (A-E) Cartoons showing development of pronephric (black) and interrenal (red) primordia; ventral view. The notochord is represented in light blue; the second and third somites are shown as dark blue rectangles. (F-O) wt1 (black) and ff1b (red) double in situ hybridization; (F-I) lateral view, (K-O) ventral view. (A,F,K) At 20-22 hpf, the wt-expressing cells lie on both sides of the notochord and some regions are also labeled with ff1b. (B,G,L) At 24 hpf, the ff1b-expressing cells are separated from the wt1-expressing cells on both sides of the notochord. (C,H,M) At 30 hpf, the wt1-expressing cells are still separate, whereas the ff1b-expressing cells have assembled towards the right of the notochord (left when viewed ventrally). (D,I,N) At 33 hpf, the wt1-expressing cells start to fuse. (E,J,O) At 3 dpf, the wt1 cells occupy the midline, while the ff1b-expressing cells continue to proliferate and redistribute on both sides of the notochord.

Fig. 5. Migration of the interrenal primordium in mutants defective in midline signaling. The internal primordia are labeled by scc. A group of fused interrenal primordial cells are located slightly to the right of the notochord (left in this ventral view) in wild-type (wt; A), syu (B), smu (C), and cyc (F) embryos. In the flh (D) and oep (E) embryos, the two groups of interrenal primordial cells do not fuse.

One of the major signals coming from the midline structure is sonic hedgehog (SHH). We therefore examined whether SHH affects interrenal morphogenesis. In embryos defective in the shh pathway, syu (sonic you) (Schauerte et al., 1998) and smu (slow muscle omitted) (Barresi et al., 2000), the location of the interrenal primordium is not affected (Fig. 5B,C). This indicates that migration and fusion of interrenal primordial cells do not require SHH signaling. The morphogenetic movement is also normal in the cyc (Cyclops) mutant, which is defective in Nodal-related signaling (Rebagliati et al., 1998) (Fig. 5F).

In all these mutants, scc transcription is not affected. This indicates that the signals that are important for the morphogenetic movement of interrenal cells are not essential for its differentiation.

Restricted wt1 expression in the intermediate mesoderm is important for early pronephric and interrenal development

The expression of wt1 is restricted to the second and third somites once the pronephros begin to differentiate (Fig. 4) (Drummond et al., 1998). We examined the role of wt1 in the developing interrenal gland by knocking down wt1 with an antisense morpholino (mo). We also used two unrelated morpholino oligos as controls: antisense ff1a and sense cyp17 (Tables 1 and 2). Although these embryos were not visibly different from the wild-type embryos at 24 hpf, many of them developed edema at 5 dpf, and some of them died (Tables 1 and 2). We examined pronephric and interrenal primordia, identifiable by wt1 and ff1b expression, respectively. At 24 hpf, compared to the wild-type embryos (Fig. 6A) and ff1a morphants (Fig. 6B and Fig. 7B), wt1 morphants have less restricted pronephric primordia; and the size of the interrenal primordia is significantly reduced (Fig. 6C). At 36 hpf, the pronephric primordia of wild-type embryos (Fig. 6D) and ff1a morphants (Fig. 6E) are already partly fused and at 2 dpf glomeruli are formed (Fig. 6G,H). Yet in wt1 morphants, the morphogenetic movement of pronephric primordia is inhibited and their sizes are reduced (Fig. 6F,I). The interrenal
primordium is also reduced in size. In addition, \( ff1b \) expression in the interrenal primordia also appears to be reduced.

### \( ff1b \) affects the size of interrenal primordia and the expression of the \( scc \) gene

To study the function of \( ff1b \) in the interrenal gland, we used an antisense morpholino oligo to block its translation and to label the interrenal primordia with \( ff1b \) and \( scc \) by double in situ hybridization (Fig. 7A,C,D,F). We also used an antisense \( ff1a \) morpholino oligo as a control (Fig. 7B,E,H). Both \( ff1a \) and \( ff1b \) are FTZ-F1 family members with high sequence homology; therefore \( ff1a \) should be the best control for \( ff1b \) morpholino study. The size of the interrenal primordia is reduced in \( ff1b \) morphants at 24 hpf, and the right side (left

![Fig. 6. Knockdown of \( wtl \) by morpholino affects early development of both the pronephros and interrenal glands. The pronephric and interrenal primordia of wild-type (A,D,G) and \( wtl \) morphants (mo; C,F,I) are labeled with \( wtl \) (black) and \( ff1b \) (red) (ventral view). (B,E,H) The pronephric primordia of \( ff1a \) morphants are labeled with \( wtl \) (brown). (A-C) 24 hpf, (D-F) 36 hpf, (G-I) 2 dpf. The pronephric primordia in C are more spread out than in A and B. The pronephric primordia in F1 are smaller and unfused. The sizes of interrenal primordia and \( ff1b \) expression in \( wtl \) morphants (C,F,I) are also reduced.](image)

![Fig. 7. Disruption of \( ff1b \) expression by antisense morpholinos reduces interrenal primordia and decreases \( ff1b/scc \) expression. The interrenal primordia of the wild-type embryos (A,D,G, \( ff1a \) morphants (B,E,H) and \( ff1b \) morphants (C,F,I) are labeled with \( ff1b \) (brown color) and also (in A,C,D,F) \( scc \) (red color). (A-F) Ventral view, (G-I) side view. The sizes of the interrenal primordia (arrows) and the expression of \( ff1b \) and \( scc \) are reduced in \( ff1b \) morphants at 24 hpf (C), 33 hpf (F) and 3 dpf (I), while the hypothalamic region, marked by \( ff1b \) (arrowheads) becomes larger (I).](image)
from the ventral view) is more affected than the left side (Fig. 7A-C). The interrenal primordial cells fuse normally at 33 hpf, although with fewer cells than in the wild-type and ff1a morphants, and the levels of scc and ff1b transcripts are also lower (Fig. 7D-F). At 3 dpf, the interrenal primordia in most ff1b morphants is gone (Fig. 7G-I), although some morphants still retain some interrenal primordia (Tables 1 and 2).

The reduction in cell differentiation and gene expression appears to be specific only for the interrenal primordia, as the ff1b morpholino has an opposite effect on the hypothalamus; the ff1b staining in the hypothalamus of the ff1b morphants is stronger and covers a larger area than that of the wild type (Fig. 7E,F). In addition, wtil expression in pronephric primordia is not affected in ff1b morphants (data not shown).

**ff1b can directly activate SCC transcription**

Fig. 7 shows that ff1b is important for interrenal differentiation and scc expression. We questioned whether ff1b can activate scc transcription directly, as the mammalian counterpart of ff1b, SF1, activates SCC gene expression by recognizing functional SF1-binding sites on its promoter (Hu et al., 2001). In order to examine the transcriptional activity of zebrafish ff1b, we co-transfected ff1b and the lacZ reporter gene driven by 2.3 kb of the human SCC promoter with or without a mutation at its SF1-binding site into H1299 cells (Fig. 8). When transiently co-transfected with the wild-type SCC promoter, ff1b induced the promoter activity to more than eightfold that of the control. Mutation of the proximal SF1-binding site resulted in a threefold reduction of the transcriptional activity (Fig. 8). Hence, ff1b can directly drive human SCC transcription through its SF1-binding sequence.

**DISCUSSION**

In this report, we have characterized zebrafish interrenal gland development, which is tightly coupled to pronephric development. Interrenal and pronephric primordial cells are both initially differentiated from lateral intermediate mesoderm in the region ventral to the third somite, with the pronephros covering a wider area. Both groups of differentiated lateral cells then undergo medial migration followed by fusion. The wtil gene regulates the development of both the interrenal and pronephros, but through different mechanisms. Pronephric cell condensation, migration and morphogenesis all appear to involve wtil, which although involved in the differentiation, does not affect the morphogenetic movement of interrenal cells. In addition, ff1b controls the differentiation of interrenal cells and directly activates transcription of the scc gene, which is a hallmark of steroid synthesis. We also found that the morphogenetic movement of interrenal cells is disrupted in the flh and oep mutants, which are defective in midline structures.

**Comparison of interrenal gland and adrenal cortex**

The interrenal gland is the major site of steroid synthesis in most teleosts (Grassi Milano et al., 1997), as is the adrenal cortex in mammals (Keegan and Hammer, 2002). Interrenal and adrenocortical cells both express steroidogenic genes, such as scc and 3β-HSD (Keegan and Hammer, 2002). We showed that the zebrafish interrenal gland is embedded in the head kidney, forming multiple epithelial layers interposed with two different types of chromaffin cells in association with blood vessels. The structure of the mammalian adrenal gland is quite different. It is a distinct organ with two distinct layers, cortex and medulla, situated near the kidney (Keegan and Hammer, 2002). The adrenal cortex contains three distinct functional layers, outer zona glomerulosa, center zona fasciculata and inner zona reticularis, each with a distinct cell morphology. Large quantities of blood vessels pass through the zona fasciculata and zona reticularis. The cytoplasm of both interrenal and adrenocortical cells contains a number of mitochondria, which are characteristic of steroidogenic cells. Adrenocortical cells also accumulate oil droplets as a result of their steroidogenic activities, yet this property does not seem to be typical. We never found oil droplets in the interrenal cells of zebrafish, nor were they detected in the interrenal cells of a neotropical fish, Brycon gephalus (Rocha et al., 2001); however, they were observed in the fathead minnow, Pimephales promelas (Yoakim and Grizzle, 1980).

**Ff1b is a functional homologue of SF1 in mammals**

Interrenal differentiation and scc gene expression are controlled by the transcriptional activator Ff1b, which has similar functions to mammalian SF1. Ff1b and SF1 are both transcriptional activators that directly activate scc gene expression (Hu et al., 2001). Ff1b is expressed in the interrenal, gonads and hypothalamus (Fig. 7 and our unpublished results); mammalian SF1 is also expressed in similar regions, plus the pituitary. When knocking down ff1b function with the use of an antisense morpholino oligo, the interrenal primordium was not maintained and disappeared around 3 dpf. This is similar to the situation in SF1 knockout mice (Lala et al., 1995; Luo et al., 1995), which do not have adrenal glands. Ff1b and SF1 are both members of the Ftz-F1 nuclear receptor in the NR5A family (Nuclear Receptors Nomenclature Committee, 1999). We and others have cloned four Ftz-F1 genes in zebrafish, termed ff1a, ff1b, ff1c and ff1d (Lin et al., 2000; Chai and Chan, 2000) (W. K. Chan, personal communication and M. W. Kuo, W. C. Lee, W. K. Chan, J. Postlethwait and B.-C.C., unpublished). Our phylogenetic analysis and the current functional studies indicate that Ff1b is probably the zebrafish orthologue of mammalian SF1 (NR5A1). Although ff1b was previously classified as nr5a4 (Chai and Chan, 2000), it is
probably more appropriate to call it nr5al based on the functional studies described in this paper.

In fflb morphants, fflb expression is decreased relative to the wild-type and this could be due to a decreased interrenal population or decreased fflb expression in the interrenal primordium. However, the expression of fflb in the hypothalamic region is increased (Fig. 7F). It appears that fflb can control its own gene expression. Depending on the site of fflb expression, the control can be positive, as in the interrenal, or negative, as in the hypothalamus. Similarly, mammalian SF1 can also be positively regulated in the adrenal gland through an autoregulatory loop (Nomura et al., 1996).

The organogenesis of the interrenal gland and adrenal cortex

The zebrafish interrenal gland and mammalian adrenal cortex are two functionally similar entities that are structurally different (Mesiano and Jaffe, 1997). There are also differences in their ontogeny. The mouse adrenal gland is derived from the urogenital ridge, which is characterized by the expression of SF1. Two different populations of SF1-expressing cells later differentiate into adrenal and gonadal primordia, but do not contribute to the kidney (Morohashi, 1997). Zebrafish fflb-expressing cells appear in a region ventral to the third somites (similar to the location in mice), these cells will form the interrenal gland at later developmental stages. fflb-expressing cells were not found in the gonads at early stages, although we did detect fflb expression in a region close to the bilateral gonads at 4 dpf (data not shown).

The organogenesis of zebrafish interrenal and mammalian adrenal glands are both quite slow. Mouse SF1 is expressed in the urogenital ridge at E9; steroidogenic genes like scc begin to be expressed in the adrenal primordium at embryonic day 11 (E11). Then neural crest cells migrate into the adrenal gland at E12-14. The medulla becomes separated from the cortex at birth, and the organogenesis is complete only at sexual maturity (Keegan and Hammer, 2002). The zebrafish interrenal primordium first appears at 20-22 hpf (Fig. 4F,K) and begins to express scc, producing steroids around 24 hpf (Fig. 7A). The interrenal primordium is surrounded by a capsule-like structure at 3 dpf (Fig. 3), but does not have any epithelial characteristics, or surround a blood vessel, even at 5 dpf. These observations indicate that both adrenocortical and interrenal cells have the ability to produce steroid hormones, although the organogenetic process is not completed.

Function of wt1 in the parallel development of the interrenal and pronephros

The zebrafish interrenal is located within the head kidney; its development also parallels embryonic kidney (pronephros) development. Zebrafish pronephric primordial cells are first characterized by the expression of wt1; a subset of these wt1-expressing cells appears to be the interrenal primordium (Fig. 4F,K). The interrenal primordial cells are separated from, but located close to, the pronephric primordium cells at 24 hpf (Fig. 4G,L). Both cell types then undergo central migration followed by fusion. The interrenal cells fuse at 30 hpf (Fig. 4H,M), and branch out into two separate groups at 3 dpf (Fig. 4J,O). The pronephric cells fuse into glomeruli at 40-44 hpf (Drummond et al., 1998) and stay close to the interrenal throughout all the developmental processes.

The transcription factor WT1 controls the development of both the interrenals and pronephros, but through different mechanisms. In our knockdown experiments, reduced WT1 levels resulted in decreased fflb expression and smaller interrenal primordia. WT1 appears to be a determining factor for the differentiation of interrenal and fflb gene expression. This situation is analogous to that in mammals, in which Wt1 has been shown to activate the SF1 gene directly and to regulate adrenal development (Nachtegaal et al., 1998; Wilhelm and Englert, 2002).

Reduced wt1 expression, however, results in its expanded expression domains in the anterior at 24 hpf (Fig. 6A,B). This is followed by the inability of the pronephric cells to migrate toward the midline and to fuse into glomeruli at later developmental stages. WT1 appears to affect the morphogenesis of the pronephros at multiple steps. However, the detailed molecular mechanisms controlling restricted distribution of wt1-expressing intermediate mesoderm still remain unknown. The organogenesis of the pronephros and interrenal glands needs further investigation.

Mammalian WT1 is expressed in the kidney, gonad and urogenital ridge, but not in the developing adrenal gland (Armstrong et al., 1993). The Wt1 knockout mice lack kidneys, gonads and adrenal glands (Kreidberg et al., 1993). The metanephric blastema of Wt1 null mice is unable to condense and proliferate upon proper induction. A function of Wt1 in adrenal development has also been shown by reduced adrenal size in partially rescued Wt1 null mice (Moore et al., 1999). These results indicate that mouse and zebrafish wt1 share similar functions at similar steps of kidney and adrenal (interrenal) developments.

The morphogenetic movements of pronephros and interrenal may be controlled by different signals from the axial midline

The morphogenetic movement of interrenal cells is affected in flh and oep mutants, but not in the cyc mutant or in mutants defective in the shh signaling pathway. This indicates that selective signals, defective in flh and oep mutants, are important for interrenal development. The mechanisms of midline signaling that affect interrenal migration still need further investigation.

Contrary to that of interrenal cells, morphogenetic movement of pronephric cells is affected in flh, syu and yot mutant embryos (Majumdar and Drummond, 2000). It appears that interenal and pronephric cells receive different signals as migratory cues. The pronephric cells are influenced by the shh pathway, but interenal cells are not, although the migrations of both cell types are affected by flh. These observations indicate that although both the pronephros and interrenals are derived from intermediate mesoderm and are located close to each other, their morphogeneses are regulated differently.

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