Development 130, 2107-2116

doi:10.1242/dev.00427

Parallel early development of zebrafish interrenal glands and pronephros: differential control by *wt1* and *ff1b*

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Accepted 30 January 2003

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 *ff1b* in a region ventral to the third somite. These cells then migrate toward the axial midline and fuse together. The pronephric primordia are *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 *wt1* is required for its initial restricted expression in pronephric primordia, pronephric cell migration and fusion. *wt1* also appears to be involved in interrenal development and *ff1b* expression. Similarly, *ff1b* is required for interrenal differentiation and activation of the differentiated gene, *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 *CYP11A1* (*P450scc, scc*), *CYP21* and *3β-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 *CYP21*, *3β-HSD* and *CYP11B1* genes (Chung, 1996; Rheaume et al., 1995; White et al., 1994; White and Speiser, 2000). Those affected suffer from insufficient corticosteroid secretion resulting in virilization and salt wasting. Knockout mice deficient in steroidogenic genes like *Star* and *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). *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 *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 *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 *Wt1* in the adrenal gland is still not clear, since *Wt1* is not expressed in the developing adrenal gland (Armstrong et al., 1993). Adrenal size is greatly reduced in *Wt1* knockout mice that are partially rescued by the human *WT1* gene (Moore et al., 1999). It indicates that *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 *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 *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 *flh* 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^{b16} embryos, absent notochord in $flh^{ni(s)}$ embryos and U-shaped somites in syu^{tq252} 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 immunofluoresence

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* (*Xho*I/T7), *3β-HSD* (*Xba*I/T7), *ff1b* (*Not*I/SP6), *wt1* (*Bam*HI/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 Axiovert 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 $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes, pH 7.6) and injected into the yolk of 1-4 cell embryos. The morpholino sequences are as follows: *ff1b*: AATCCTCATCTGCTCTGAAGTCCAT, *wt1*: TGAGGTCACGAACATCAGAACCCAT. *ff1a*: CTGACTCGACTTTAGGCAGCATGAC. *cyp17* sense: CAGTTGAATATAGCTGACAATGGT.

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 (OsO_4) 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 10% 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 (*SCC*/2.3*lacZ*; mtP*SCC*2.3/*lacZ*) 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

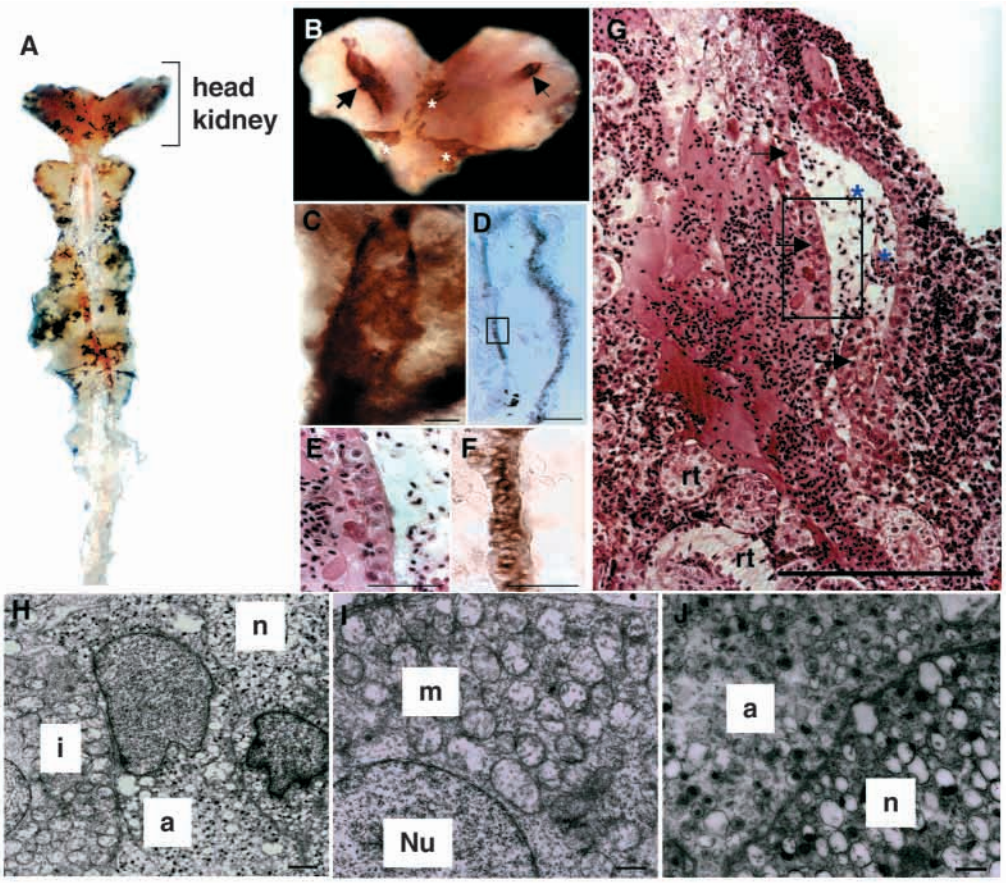

Fig. 1. The structure and ultrastructure of adult zebrafish head kidney. (A) The zebrafish head kidney is located in the anterior part of kidney. (B) In situ hybridization of *scc*, which identifies the interrenal gland of the head kidney (arrows, ventral view). Asterisks indicate pigment. (C) Higher magnification of the interrenal gland of the right lobe. (D) A section of the gland. (E,F) Higher magnification of the boxed regions in G and D, respectively. (G) Interrenal epithelial cells (arrows) lie adjacent to a blood vessel (asterisks). rt, renal tube. (H) Transmission electron micrographs showing interrenal epithelial cells (i) associated with adrenaline (a) and noradrenaline (n) chromaffin cells. (I) The interrenal epithelial cell has many mitochondria (m). Nu, nucleus. (J) The adrenaline cell (a) contains electron-lucent vesicles and the noradrenaline cell (n) contains vesicles with strong electron-dense granules. Scale bars; 10 μm (C,D,G), 5 μm (E,F), 1.1 μm (H), 0.6 μm (I), and 0.4 μm (J).

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

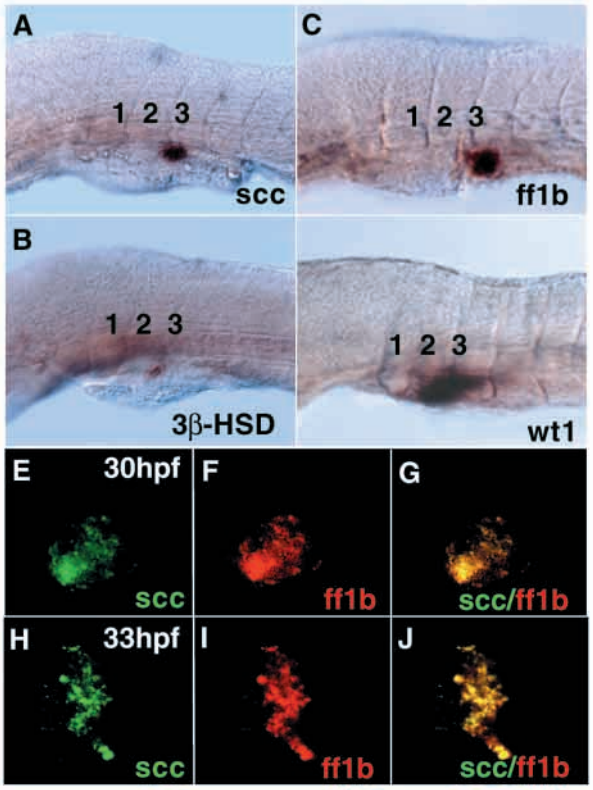

Fig. 2. Marker gene expression in the interrenal primordium. In situ hyridization showing expression of *scc* (A), *3β-HSD* (B) and *ff1b* (C) in the region ventral to the third somite and *wt1* (D) expression in a wider region ventral to the second and third somites at 36 hpf. (E-J) Co-expression of *ff1b* and *scc* at 30 (E-G) and 33 hpf (H-J). Green indicates *scc* expression, red indicates *ff1b* expression and yellow indicates the merged signal of *scc* and *ff1b*.

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*-expessing 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

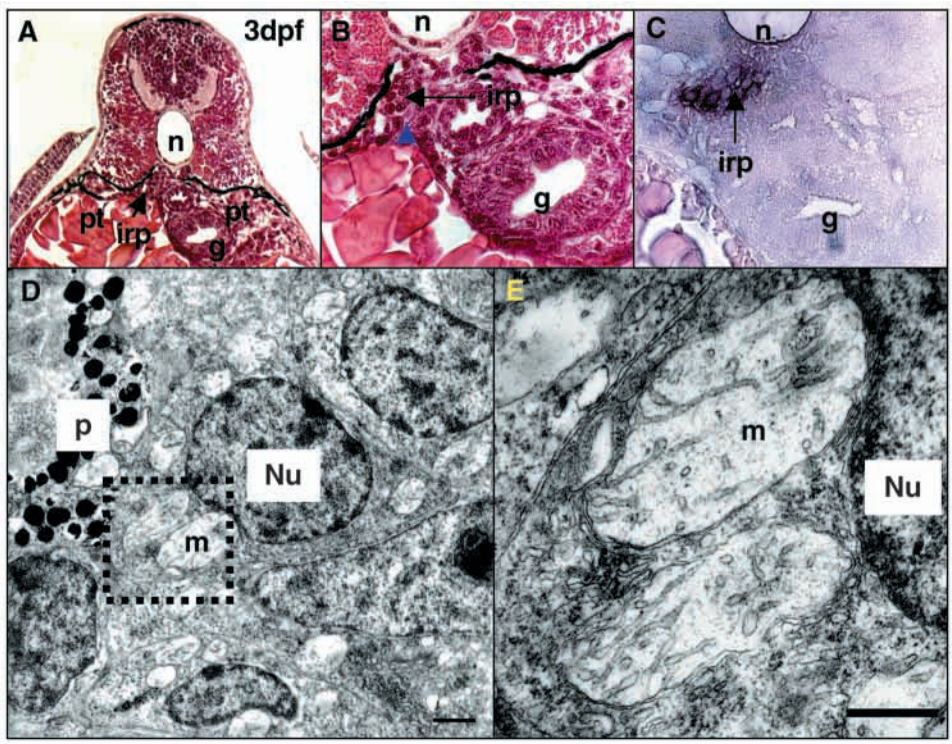

Fig. 3. Histological analysis of the interrenal primordium at 3 dpf. (A-C) Interrenal primordium (irp) is located ventral to the notochord (n) and dorsal to the gut (g). (A,B) Hematoxylin and Eosin staining and (C) hybridization of the cross sections of interrenal primordium with *ff1b*. (B,C) Higher magnification of A. The blue arrowhead indicates the capsule like structure. (D) The interrenal primordial cells have many mitochondria in their cytoplasm. (E) Higher magnification of the dashed square in D. g, gut; m, mitochondria; n, notochord; Nu, nucleus; p, pigment; pt, pronephric duct. Scale bar: 1.1 μm (D), 0.25 μm (E).

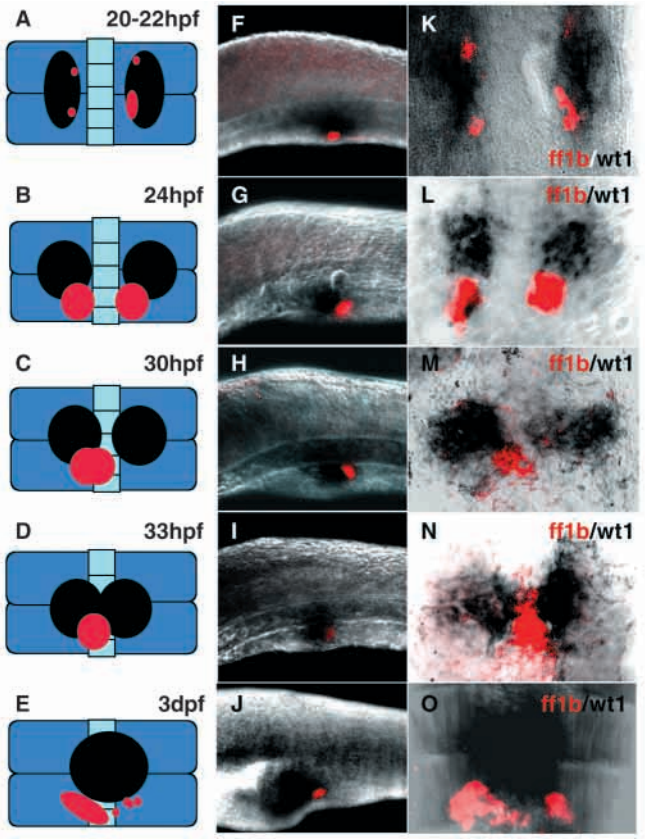

Fig. 4. Morphogenetic movement of the interrenal gland from 20 hpf to 3 dpf. (A-E) Cartoons showing development of pronephric (black) and interrenal (red) primordia; ventral view. The notochord is represented in light blue; the second and third somites are shown as dark blue rectangles. (F-O) *wt1* (black) and *ff1b* (red) double in situ hybridization; (F-J) 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.

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).

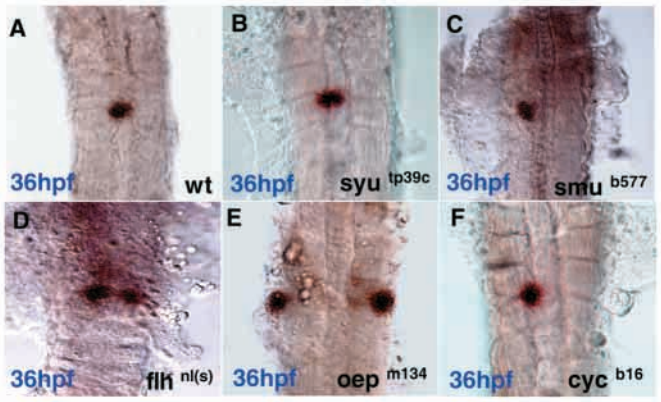

Fig. 5. Migration of the interrenal primordium in mutants defective in midline signaling. The internal primordia are labeled by *scc*. A group of fused interrenal primordial cells are located slightly to the right of the notochord (left in this ventral view) in wild-type (wt; A), *syu* (B), *smu* (C), 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 primordum 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

Table 1. Phenotypes of *wt1* and *ff1b* morphants

Morpholino treatment	Total number	Number with visible defects at 24 hpf* (% total)	Number with edema at 5 dpf	Number dead at 5 dpf	Number of normal embryos
wt1 (6 ng)	311	0	230 (74%)	22 (7%)	20 (6%)
ff1b (9 ng)	303	254 (84%)	240 (79%)	22 (7%)	23 (7%)
ff1a (9 ng)	313	0	0 (0%)	24 (8%)	275 (88%)
cyp17 sense (9 ng)	228	0	0 (0%)	36 (16%)	184 (81%)
none	204	0	5 (2%)	12 (6%)	187 (92%)

*The defects are thinner yolk extension and delayed yolk absorption at 24 hpf.

Table 2. Defects of the pronephric primordium (PP) and interrenal primordium (IRP)

	Number of embryos with defect/total number				
Morpholino treatment	Expanded *wt1* expression at 24 hpf	No PP migration at 36 hpf	No PP fusion at 2 dpf	No IRP at 3 dpf	Few IRP at 3 dpf
wt1 (6 ng)	60/70	30/61	49/60		
ff1b (9 ng)				36/52	14/52
ff1a (9 ng)	0/22	0/17	0/25	0/25	0/25
cyp17 sense (9 ng)	0/16	0/14	0/20	0/25	0/25
none	0/20	0/20	0/22	0/24	0/24

wt1 morphants were collected and hybridized with *wt1* and *ff1b* at different times. *ff1b* morphants with thinner yolk extension and delayed yolk absorption were collected at 3 dpf and hybridized with *ff1b*. The number of embryos with a particular defect compared with the total number of embryos in that group was counted.

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

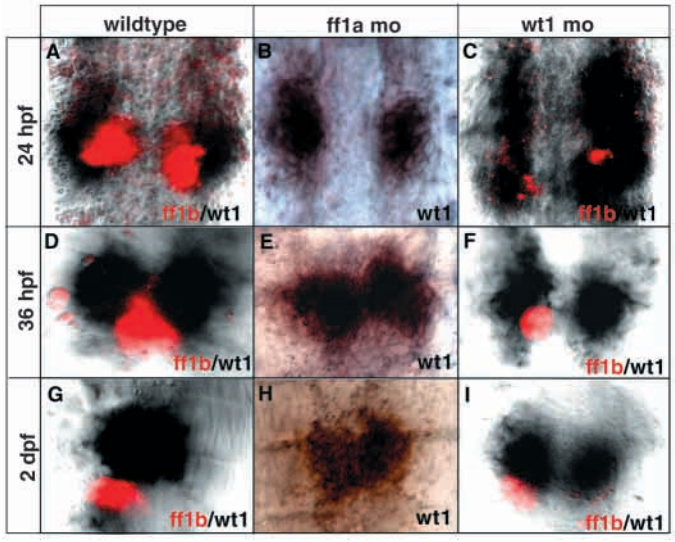

Fig. 6. Knockdown of *wt1* by morpholino affects early development of both the pronephros and interrenal glands. The pronephric and interrenal primordia of wild-type (A,D,G) and *wt1* morphants (mo; C,F,I) are labeled with *wt1* (black) and *ff1b* (red) (ventral view). (B,E,H) The pronephric primordia of *ff1a* morphants are labeled with *wt1* (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 F,I are smaller and unfused. The sizes of interrenal primordia and *ff1b* expression in *wt1* morphants (C,F,I) are also reduced.

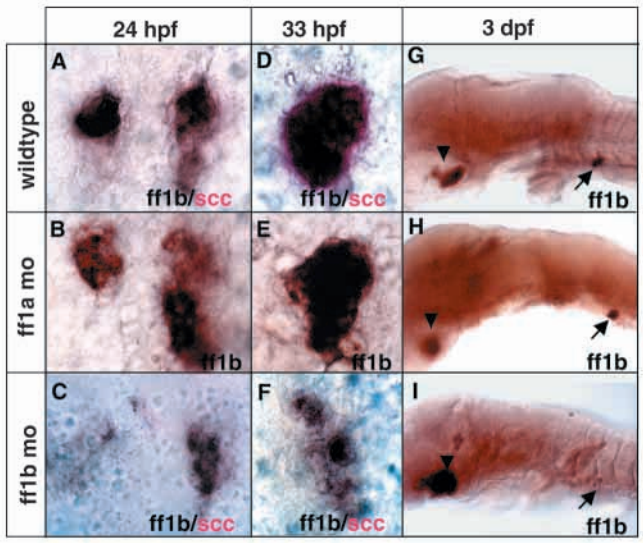

Fig. 7. Disruption of *ff1b* expression by antisense morpholinos reduces interrenal primordia and decreases *ff1b/scc* expression. The interrenal primordia of the wild-type embryos (A,D,G), *ff1a* morphants (B,E,H) and *ff1b* morphants (C,F,I) are labeled with *ff1b* (brown color) and also (in A,C,D,F) *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).

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, *wt1* 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 *wt1* gene regulates the development of both the interrenal and pronephros, but through different mechanisms. Pronephric cell condensation, migration and morphogenesis all appear to involve *wt1*, 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.

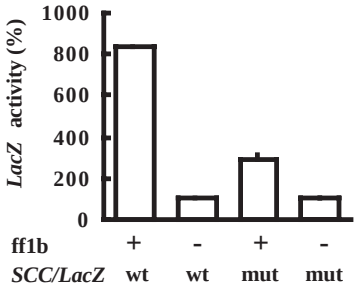

Fig. 8. *ff1b* can activate human *SCC* transcription directly. Zebrafish *ff1b* was transiently co-transfected into H1299 cells with either wild-type or mutated *SCC/lacZ* reporter. The activity of the mutated *SCC/lacZ* reporter alone was set as 100.

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 and 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 (NR5 A1). Although *ff1b* was previously classified as *nr5a4* (Chai and Chan, 2000), it is

probably more appropriate to call it *nr5a1* based on the functional studies described in this paper.

In *ff1b* morphants, *ff1b* expression is decreased relative to the wild-type and this could be due to a decreased interrenal population or decreased *ff1b* expression in the interrenal primordium. However, the expression of *ff1b* in the hypothalamic region is increased (Fig. 7F). It appears that *ff1b* can control its own gene expression. Depending on the site of *ff1b* 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 *ff1b*-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. *ff1b*-expressing cells were not found in the gonads at early stages, although we did detect *ff1b* 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 *ff1b* expression and smaller interrenal primordia. WT1 appears to be a determining factor for the differentiation of interrenal and *ff1b* 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 (Nachtigal 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 interrenal 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.

We thank Sue-Ping Lee for assistance in EM work, S. K. Tong, M. W. Kuo and J. C. Chen for the construction of plasmids and over-expression of Scc protein, Chai Chou and Chan Woon Khiong for the *ff1b* cDNA and communication of unpublished data, Monte Westerfield and Vladimir Korzh for help in setting up the fish stock. This work was supported by grants NSC91-2611-B-001-002 from the National Science Council, and Academia Sinica, Republic of China.

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