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Involvement of Prop1 homeobox gene in the early development of fish pituitary gland

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ABSTRACT

When mutated in mammals, *paired-like* homeobox *Prop1* gene produces highly variable pituitary phenotypes with impaired regulation of *Pit1* and eventually defective synthesis of Pit1-regulated pituitary hormones. Here we have identified fish *prop1* orthologs, confirmed their pituitary-specific expression, and blocked the splicing of zebrafish *prop1* transcripts using morpholino oligonucleotides. Very early steps of the gland formation seemed unaffected based on morphology and expression of early placodal marker *pitx*. Prop1 knock-down reduced the expression of *pit1*, *prl* (prolactin) and *gh* (growth hormone), as expected if the function of Prop1 is conserved throughout vertebrates. Less expectedly, *lim3* was down regulated. This gene is expressed from early stages of vertebrate pituitary development but is not known to be Prop1-dependent. *In situ* hybridizations on *prop1* morphants using probes for the pan pituitary gene *pitx3* and for the hormone gene markers *prl*, *gh* and *tshβ*, revealed abnormal shape, growth and cellular organization of the developed adenohypophysis. Strikingly, the effects of *prop1* knock-down on adenohypophysis morphology and gene expression were gradually reversed during late development, despite persistent splice-blocking of transcripts. Therefore, *prop1* function appears to be conserved between mammals and fish, at least for the mediation of hormonal cell type differentiation via *pit1*, but the existence of other fish-specific pathways downstream of *prop1* are suggested by our observations.

1. Introduction

Important knowledge of the mechanisms of pituitary gland development has accumulated during the last 10 years from molecular and genetic studies involving spontaneous or induced mutants [37]. The anterior part of the gland (adenohypophysis) is formed from the Rathke's pouch, and at least six major hormone-producing cell types differentiate: lactotropes producing prolactin (PRL), somatotropes producing growth hormone (GH), thyrotropes producing thyroid stimulating hormone (TSH), gonadotropes producing gonadotropins (LH and FSH), and corticotropes and melanotropes producing proopiomelanocortin (POMC). Adenohypophysis development proceeds under the control of dorsal and ventral signaling molecules and a number of transcription factors including several homeodomain proteins such as Pitx, Anf/Hesx1/Rpx, Lhx3/Lhx4, Pit1 and Prop1 [10,53]. The pituitary gland organization and function are globally conserved in vertebrates [26,36], and the crucial role of Pit1 in the specification of hormonal cell types as well as in the expression of hormone genes has been recognized in several studies [29,15,4,34].

The mechanisms of *Pit1* expression appear to involve auto-regulation and a positive *cis*-regulation by Prop1 [41,45,13]. Additional *cis*-regulation by the homeodomain protein Atbf1 has been recently revealed [38]. Mutant analysis in fish has not yet uncovered the regulatory networks to which Pit1 belongs, including direct *pit1* regulators. We decided to implement a candidate gene approach of *pit1* regulation, and first searched for fish orthologs of mammalian *prop1*. *Prop1* is a member of the *paired-like* class of homeobox genes which was first identified by positional cloning of the mouse *Ames dwarf* (*df*) locus [45] and then characterized in other mammals [51]. Studies in mice pinpointed Prop1 as an upstream regulator of *pit1* [2,45], therefore involved in the ontogenesis of Pit1-dependent somatotropes, lactotropes, and caudal thyrotrope lineages [19].

The results of prop1 misexpression on pit1 and Pit1-dependent hormone genes have been somewhat controversial. Indeed, in distinct studies of df/df mutant mice, pit1 gene expression was shown

Abbreviations: Prop1, prophet of pit; τ_s, tau-somite units; ORF, open reading frame; RT, reverse transcription; RACE, rapid amplification of cDNA ends; hpf, hours post fertilization; aa, amino acid; MO, morpholino; cRNA, capped RNA; ISH, *in situ* hybridization; ef1A, elongation factor 1.

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to be either suppressed [2], or initiated with subsequent cell lineage specification but abnormal proliferation of somatotropes, thyrotropes and lactotropes [18,20]. Prop1−/− knock-out mutants were found to lack somatotrope and lactotrope cells, but had few thyrotrope cells and showed retarded gonadotrope lineage development [33]. Finally, in cell culture experiments, Prop1 alone failed to strongly induce pit1 expression [45].

Here we cloned the salmon and zebrafish orthologs of the mammalian *prop1* gene and found that their expression is pituitary-specific. We then knocked down *prop1* expression in zebrafish using morpholino antisense oligonucleotides and monitored the expression of *pit1*, of genes encoding adenohypophyseal hormones like *prl*, *gh* and *tshβ*, as well as of *lim3* and *pitx* genes which are normally expressed during early adenohypophysis development [22,25,27,3]. Overall, the results support the idea that the function of *prop1* is at least partly conserved in fish, though not necessarily involving strictly identical pathways.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio*) fertilized eggs were obtained from a wild-type strain in the Sars zebrafish animal resource (Bergen), according to a protocol developed in our laboratory (http://www.sars.no/facilities/fishRaisingprotocol.doc). Embryos were staged according to Kimmel et al. [28]. For ISH studies, pigmentation of embryos/larvae was prevented by adding 0.003% phenylthiourea to the E3 medium. Atlantic salmon (*Salmo salar*) fertilized eggs were collected at Voss salmon hatchery (Voss, Norway) at 140, 177 and 190 τ_s of embryonic development [23].

2.2. Cloning of fish prop1

To clone homeobox pituitary genes from salmon, a 120 bp probe covering the homeodomain was generated by PCR using degenerate primers (F) 5′ACCATCACCGCCAAICAICTGGA and (R) 5′CGATTCT GRAACCANACYTG. The probe was verified by sequencing, labeled and used to screen an Atlantic salmon pituitary cDNA lambda gt10 library [31], following standard procedures [5]. The 1119 nt insert was subcloned in pGEM3zf (Promega) and sequenced. The salmon *prop1* ORF was then used as a query against the salmon genome database by means of the blastn software to deduce intron/exon boundaries (scaffolds Gnl|ti|2270282399 and gnl|ti|22650 29643; http://codgenome.no/blast/FastaFile.cgi?file= laks-genom).

Zebrafish *prop1* ORF and 5′ UTR regions were obtained by RT-PCR and RACE method according to the manufacturer's protocol (Marathon™ cDNA Amplification Kit, Clontech; see Table 1S for primer pair sequences). Products were analyzed by 1.2% agarose gel electrophoresis, purified using the QIAquick gel extraction kit (Qiagen), cloned into pGEM-T Easy vector (Promega) and sequenced. A total of six zebrafish cDNA clones containing full length ORFs were analyzed. The resulting sequences were compared to the zebrafish genome in the Ensembl database (http://www.ensembl.org/Multi/blastview) to deduce the intron/exon structure of the gene. cDNA sequences generated during the course of this study have been submitted to GenBank databases (EU848298; EF693961).

2.3. Phylogenetic analysis

Sequences were aligned with CLUSTAL X V1.81 program [46], or using MUSCLE (v3.7) configured for highest accuracy [17]. Phylogenetic trees were constructed by neighbor joining (NJ), parsimony and maximum likelihood methods (PAUP and TREE-PUZZLE softwares; PhyML program (v3.0 aLRT) [42,12].

2.4. Injections of morpholino oligonucleotides and synthetic RNA

MO oligonucleotide was purchased from Gene Tools LLC, and brought to 1 mM concentration (~8 mg ml^{-1}) as recommended. For injections, the stock solution was diluted to 240 μM in 1× Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 5 mM HEPES, pH 7.6). The injected volume of MO solution (or MO/cRNA mixture) was estimated by measuring the diameter of a droplet injected into mineral oil, using a micrometric objective (0.01 mm divisions). Each embryo of one cell-stage received near the yolk and cytoplasm interface 14 ng of *prop1* MO, either alone or co-injected with the same amount of MO with 15, 100–120 or 400 pg of sense cRNA (for rescue experiments). Wild type siblings used as controls received 1× Danieau. Injections were performed using a gas-driven microinjector apparatus (Picospritzer® II, (Parker instrumentation).

2.5. cRNA synthesis

Zebrafish *prop1* ORF including 168 bps of the 5′ UTR was RT-PCR-amplified using primers containing a recognition site for restriction endonucleases *BamHI* and *XbaI* (Table 1S). After digestion with these enzymes, the product was ligated into the pCS2+ vector (Clontech). Plasmids were linearized with *NotI* restriction endonucleases and used to generate *in vitro* synthetic sense RNA with mMESSAGE mMACHINE SP6 kit (Ambion).

2.6. Plastic and vibratome sectioning

Specimens for plastic sections were fixed in 4% paraformaldehyde and embedded in glycomethacrylate resin following the instructions provided by the supplier (Technovit 7100, Heraeus, Kulzer GmbH). Semi-thin sections (2 μm) were prepared using an ultracut UCT microtome (Leica) and stained with Lee's Methylene blue-basic fuchsin [6]. For vibratome sections, embryos were embedded in Gelatin/Albumin mixture as described by Hadrys et al. [24]. Sections from stained embryos were cut at 20 μm thicknesses, collected on glass slides and covered with 75% Glycerol in PBS to be photographed the same day.

2.7. In situ hybridization

Salmon and zebrafish embryos were fixed in 4% paraformaldehyde (PBS 0.1 M, pH 7.4) at 4 °C for 2–3 days. For ISH on cryostat sections, embryos were incubated overnight at 4 °C in 25% sucrose in PBS prior to embedding in OTC tissue tek® (Sakura OTC-Compound, Miles, USA). 10–15 μm transversal sections of embryo brains were collected on frozen Superfrost Plus glass slides (Merck, Germany) by using a Leica CM 1850 cryostat. ISH on sections and whole-mount was carried out as described previously by Ebbesson et al. [16] and Schulte-Merker et al. [43], respectively. Primer pairs for ISH detection of *pitx1*, *pitx2a*, *pitx2c*, *pitx3*, *lim3*, *prl*, *gh* and *tshβ* genes are listed in Table 1S. Representative sections were photographed with Leica M420/DMLB microscopes connected to CoolSNAP-Pro camera (Media, Cybernetics, USA).

2.8. Real-time quantitative PCR

Total RNA was extracted from control and *prop1* MO-injected zebrafish embryos with Trizol reagent, according to the manufacturer's protocol (Invitrogen). The integrity of the RNA was checked by electrophoresis on a formaldehyde agarose gel. cDNA was synthesized with random hexamers (Amersham), and oligo$(dT)_{12-18}$