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Identification of possible downstream genes required for the extension of peripheral axons in primary sensory neurons

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ABSTRACT

The LIM-homeodomain transcription factor Islet2a establishes neuronal identity in the developing nervous system. Our previous study showed that Islet2a function is crucial for extending peripheral axons of sensory neurons in zebrafish embryo. Overexpressing a dominant-negative form of Islet2a significantly reduced peripheral axon extension in zebrafish sensory neurons, implicating Islet2a in the gene regulation required for neurite formation or proper axon growth in developing sensory neurons. Based on this, we conducted systematic screening to isolate genes regulated by Islet2a and affecting the development of axon growth in embryonic zebrafish sensory neurons. The 26 genes selected included some encoding factors involved in neuronal differentiation, axon growth, cellular signaling, and structural integrity of neurons, as well as genes whose functions are not fully determined. We chose four representative candidates as possible Islet2a downstream functional targets (*simplet*, *tppp*, *tusc5* and *tmem59l*) and analyzed their respective mRNA expressions in dominant-negative Islet2a-expressing embryos. They are not reported the involvement of axonal extension or their functions in neural cells. Finally, knockdown of these genes suggested their direct actual involvement in the extension of peripheral axons in sensory neurons.

1. Introduction

Islet2a (Isl2a) is a LIM-homeodomain (LIM-HD) transcription factor expressed in post-mitotic neurons to specify motor neuron identity in the developing spinal cord [1–3]. LIM-HD proteins contain N-terminal tandem repeats of cysteine-rich regions called the LIM domain and a DNA-binding homeodomain [4]. The LIM domains bind to Ldb-1 protein (also called the nuclear LIM interactor, NLI) [5] and Lhx-3 to form a protein complex that mediates the transcription of specific mRNA [6]. During neurogenesis, this complex synergistically induces transcription from HB9 promoters to promote motor neuron differentiation [6]. In zebrafish, Isl2a is expressed in the primary sensory neurons and subsets of the primary motor neurons [2]. In a previous study we showed that overexpressing the LIM domains of Isl2a could inhibit the binding to Ldb-1 and thus functionally repress Isl2a [7]. As a result, the peripheral axons of zebrafish primary sensory neurons did not extend normally, whereas the central axons remained intact [7]. We therefore hypothesized that factors transcribed by Isl2a signaling might be involved in the extension of peripheral axons, and proposed a model whereby genes transcribed by Isl2a might determine why two axons extend and/or how the polarized growth of axons is achieved.

To address our hypothesis, we needed to first identify molecules whose expression patterns are affected by functional blockage of Isl2a. Accordingly, we developed a transgenic fish model in which GFP expression is specifically activated in sensory neurons to enable their efficient identification and recovery [8,9]. The trigeminal neurons were dissected out and collected using fine glass capillary under a dissecting microscope. Next we pooled an annotated cDNA library of interest and manually selected clones thought to represent rare transcripts that had not been studied in neurons to test their expression in sensory neurons. Finally, we compared gene expression patterns between control and LIM^{Isl2a}-overexpressing embryos. By these relatively laborious procedures, we could isolate novel genes expressed in sensory neurons and examine changes in mRNA expression induced by the functional depression of Isl2a.

2. Materials and methods

2.1. Fish maintenance

Wild-type and transgenic zebrafish were maintained in our laboratory as described elsewhere [10]. Embryos were incubated

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Abbreviations: MO, morpholino antisense oligonucleotide; cDNA, complementary DNA.

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at 28.5 °C and developmental stages were identified based on Kimmel et al. [11].

2.2. Construction of a sensory neuron cDNA library using trigeminal ganglion

GFP-expressing trigeminal neurons were dissected from Tg(*SSX-isl1:GFP, rw0145*) transgenic fish [8,9] embryos using a glass microcapillary. mRNAs were purified and converted into cDNAs to construct a library using the Creator™ SMART™ cDNA Library Construction Kit (Clontech). cDNA sequence was determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems) and further analyzed by a similarity search in NCBI using the BLASTX algorithm.

2.3. Injection of antisense morpholino oligonucleotides

Antisense morpholino oligonucleotides (MOs) (Gene Tools) against *simplet, tppp, tusc5*, and *tmem59l* were designed to block protein translation of the respective genes. MO sequences were as follows: *simplet*-MO: CAACACACATCTTTGCCACGGTCCA, *tppp*-MO: TATTTCACCCGAACCCTCAGCCATG, *tusc5*-MO: TCCGTATCTGTG TTTACTGCCATTG, *tmem59l*-MO: ACCCTGCCGCCGAACTGGAGCATCT. MOs were dissolved in double-distilled water and microinjected by air pressure into 1 to 2-cell-stage zebrafish embryos as described previously [7].

2.4. Histological analysis of zebrafish embryos

Whole-mount *in situ* hybridization and immunohistochemistry were carried out as described previously [7]. Axons were immunostained using an anti-acetylated α-tubulin antibody (Sigma) followed by the appropriate secondary antibody and diaminobenzidine reaction [12]. Embryos were then flat-mounted in glycerol and imaged by differential interference contrast on an Axioplan2 microscope (Zeiss) fitted with an AxioCam digital camera (Zeiss). Branching of peripheral axons was evaluated by counting the number of crossing points of the peripheral branches with the ventral edge of the spinal cord in the 8th–17th somite. Statistical differences were evaluated by using the unpaired *t*-test and PRISM 5 software (Graph Pad Software).

3. Results and discussion

3.1. Screening for genes transcriptionally regulated by Isl2a in zebrafish sensory neurons

We conducted a systematic screen in zebrafish for molecules regulated by Isl2a (Fig. 1). First, we created a sensory neuron-derived cDNA library from transgenic fish embryos expressing GFP specifically under control of the sensory neuron-specific enhancer of *islet-1* [8,9]. We determined the cDNA sequence of 3188 individual clones followed by a similarity search in NCBI using the BLASTX algorithm and identified 139 genes not present in the zebrafish EST database and not reported in any species at the time of experiment. Next, we confirmed that 44 of the 139 genes showed mRNA expression in sensory neurons using 24 h post-fertilization (hpf) zebrafish embryos. Of these, 26 genes showed changed mRNA expression with overexpression of the dominant-negative form of Isl2a (Supplementary Table 1) [7], with all but one showing reduced or diminished mRNA expression and the remaining gene showing increased expression (Supplementary Table 1). These 26 genes were therefore regarded as possible downstream targets of Isl2a.

Our screening revealed several genes already reported to function in neuronal cells. For example, clone 578 is *drg11*, a paired homeodomain transcription factor expressed in sensory neurons and required for the projection of cutaneous sensory afferent fibers [13], while clone 4557 is *fez1*, a homolog of *unc-76* required for normal axon growth and fasciculation in nematode [14]. Several genes have also been implicated in maintaining the structural integrity of neuronal processes such as clone 5884, coding for *map1b*, which plays a role in enhancing microtubule assembly in axons and extension rate in developing neurons [15,16]. Finally, clone 4055 is *beta-thymosin*, an actin-binding protein that functions in axonal extension in zebrafish embryonic brain. Antisense-mediated knockdown of *beta-thymosin* caused a defect in midbrain–hindbrain boundary formation as well as neuronal process formation [17]. These data suggested that the phenotype observed in Isl2a functional repression could result from altered expression of genes regulating axon extension, neuronal differentiation, or neuronal survival.

3.2. simplet, tppp, tusc5, and tmem59l clones are possible Islet2a downstream genes

We finally obtained 26 clones showing changes in mRNA expression in the LIM^{Isl2a}-overexpressing embryos, with several already implicated in axon extension or the developing nervous system. This suggested that our screening could be effective to isolate genes involved in inhibiting axon extension in sensory neurons with LIM^{Isl2a} overexpression. Next, we selected four genes from our screening that have no reported function in axon growth to identify novel genes involved in Isl2a-dependent axon extension.

The first such candidate gene, *simplet*, is homologous to human *fam53b* [18]. This gene encodes no functional protein domains and its exact molecular function has not been determined. *simplet* was mainly expressed in the central nervous system and also in muscle cells. Knocking down of *simplet* caused delayed epiboly formation and reduced body size, suggesting a role in cell proliferation [18]. Another report implicated *simplet* in fin regeneration of zebrafish [19]. In the 24 hpf zebrafish embryo studied herein, *simplet* was expressed in trigeminal neurons, cranial ganglion cells, and a subset of hindbrain neurons (Fig. 2A). In spinal cord, *simplet* transcripts were found in Rohon–Beard (RB) sensory neurons (Fig. 2B), and in Isl2a dominant-negative embryos, the mRNA expression of *simplet* was significantly reduced in both spinal cord and trigeminal neurons (Fig. 2E and F).

The second novel gene candidate is *tppp*, which is homologous to human *tubulin polymerization promoting protein (tppp)*. This protein was isolated from bovine brain [20] and later found to promote polymerization or stabilization of microtubule [21,22]. In wild-type zebrafish embryo, the *tppp* was expressed in trigeminal neurons (Fig. 2C), spinal neurons including RB neurons, and primary motor neurons (Fig. 2D). In non-neural tissue, the *tppp* transcript was found in intestine (Fig. 2D). In contrast, the Isl2a dominant-negative embryos showed no *tppp* expression in any tissue or cell type (Fig. 2G and H).

The third gene selected for study was *tusc5*, a homologue of the human tumor suppressor candidate 5 protein (*tusc5*) gene. *tusc5* expression is missing in lung cancer patients due to a chromosomal deletion [23]. This gene codes for the CD225 domain-containing protein, which has been associated with interferon-induced cell growth suppression [24]. *tusc5* is expressed in adipose tissue and somatosensory neurons, it is downregulated by cold stimulation, and its expression increases with adipocyte maturation and augmented PPAR gamma signaling. [25,26]. In zebrafish embryos, the *tusc5* gene was expressed in trigeminal and RB neurons as well as hindbrain neurons (Fig. 2I and J), and the Isl2a dominant-negative embryos showed slightly reduced mRNA