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Zebrafish as a model to study PTPs during development

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ABSTRACT

Protein-tyrosine phosphatases (PTPs) have important roles in signaling, but relatively little is known about their function *in vivo*. We are using the zebrafish as a model to study the function of PTPs at the organismal, cellular and molecular level. The zebrafish is an excellent experimental model for the analysis of gene function. We have developed methods to quantitatively study effects of PTP knockdown or expression of (mutant) PTPs, particularly with respect to gastrulation cell movements. Moreover, we have studied the phosphoproteome of zebrafish embryos. In this review, we will discuss methods to manipulate the zebrafish genome and techniques that we have developed to assess developmental defects during gastrulation and to assess differences in the phosphoproteome.

1. Introduction

Much is known about catalysis and regulation of PTPs [44]. However, to fully understand the function of PTPs, their role in whole organisms *in vivo* needs to be addressed. Several model organisms have been used to investigate the function of PTPs, including the invertebrates *Drosophila* and *Caenorhabditis.elegans* and vertebrate models, particularly the mouse. Many mouse knock-outs have been generated that provided important insight into the function of PTPs in development and conditional knock-outs have allowed the analysis of the function of PTPs in particular tissues [18,19]. Generation of mouse models can be laborious however, and *in vivo* imaging is difficult. We and others have used the zebrafish as a model for the analysis of PTP function, because of the many advantages that zebrafish embryos have to offer as an experimental system. Zebrafish are particularly amenable for genetics, intravital imaging and large scale approaches. In addition, we have recently developed a method to derive cell lines from single zebrafish embryos and tumors [10], which complements the wide range of experimental approaches in zebrafish. Here, we will review zebrafish as a model system in general, the genetic tools available, the approaches that we have used to assess PTP function in zebrafish, focusing on *in vivo* cell behavior and phosphoproteomics and we will give an outlook on approaches that may be used in the near future to assess PTP function *in vivo*.

2. Zebrafish as an experimental system

The zebrafish is an excellent model system that was initially used for large scale forward genetic screens [13,16]. Since then, zebrafish are increasingly being used to model human diseases [35]. Major advantages of the zebrafish as an experimental system are that large numbers of embryos can be obtained easily; 100–200 embryos per clutch, 1–2 clutches per week per adult zebrafish pair. The embryos develop quickly and outside the mother; after 1–2 days most organs have formed. The embryos are transparent, facilitating analysis of embryonic development by (time-lapse) microscopy. Many genetic mutants are available and transgenesis is feasible. More and more transgenic lines are becoming available, expressing fluorescent marker proteins under the control of specific promoters, which allows for intravital imaging. Overexpression of genes or proteins of interest can easily be achieved by micro-injection of synthetic mRNA encoding the protein of interest at the one-cell stage. In addition, transient knockdown of target proteins and target-selected gene inactivation are feasible in zebrafish (see below). Finally, chemical compounds can easily be administered to zebrafish embryos by simple addition to the aqueous embryo medium. Medium to large scale screens for bioactive compounds have been done using zebrafish development as read-out [11,38]. Taken together, the zebrafish is an ideal model system for analysis of gene function at the genetic, molecular and cellular level in whole organisms.

3. Zebrafish protein tyrosine phosphatases

The zebrafish genome sequence is available in public databases (http://www.ensembl.org/Danio_rerio/Info/Index) [20]. In general, orthologs of most human genes can be found in the zebrafish

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genome. In fact, some genes are duplicated in the zebrafish genome, due to a genome duplication in teleosts 320 million years ago [22,48]. In human, PTPs are divided in four classes i.e. classical and VH1-like dual specific protein phosphatases (DSPs) (class I), low molecular weight phosphatases (class II), Cdc25 phosphatases (class III) and Aspartic acid–based pTyr specific phosphatases (IV) [2]. Class I classical PTPs are further subdivided into receptor and non-receptor PTPs. Class I DSPs are divided into seven groups, namely: mitogen activated protein kinase (MAPK) phosphatases (MKPs), atypical DSPs, the slingshot phosphatases, PRLs, CDC14s, phosphatase and tensin homologues (PTENs) and myotubularins. Since we focus on classical PTPs and PTENs, these will be discussed in this review.

The zebrafish genome encodes 51 classical PTPs and all the subtypes that have been identified in the mammalian genomes are represented in the zebrafish genome [45] (Table 1). Fourteen PTP genes are duplicated in the zebrafish genome and whether these duplicated genes are all functional remains to be determined. Comparison of the PTP family in the genome of five distinct fish species led to the surprising discovery that *ptpn20*, which was supposed to encode little more than a PTP domain, actually encodes a large PTP with multiple functional domains, resembling PTP-BL. The human and mouse *ptpn20* genes have a similar structure as zebrafish *ptpn20*, and we confirmed this by reverse transcription PCR [46].

To unravel the function of PTPs during zebrafish development, we have compared the expression patterns of all PTP genes in zebrafish by whole mount *in situ* hybridization using standard protocols [43,45]. Most PTP genes are expressed throughout development with broad expression patterns early on, which become more restricted later in development [45]. Interestingly, some of the duplicated PTP genes have overlapping spatio-temporal expression patterns whereas others are mutually exclusive. It is likely that the function of the latter PTPs has diverged since their duplication. For gene specific expression data during embryonic development we refer to [45].

4. Genetic tools to study PTP function in zebrafish

PTP function has been studied in zebrafish by transient, morpholino-mediated knockdown, in genetic loss-of-function mutants and by expression of exogenous PTP genes and mutants thereof.

4.1. Morpholinos

Transient knockdown of target proteins by microinjection of morpholinos is widely used to study gene function [29,337]. Morpholinos are either directed at the start ATG to block translation of the target protein or at splice sites to block splicing of the target RNA (Fig. 1). Morpholino-mediated knockdown of Pez (*ptpn14*) results in developmental defects in the heart and somites, which may at least in part be mediated by impaired TGFβ3 signaling-dependent epithelial to mesenchymal transition [52]. Knockdown of PTPσ results in accumulation of synaptic vesicles in the axon terminals of olfactory sensory neurons [8]. PTPσ is related to the LAR receptor tyrosine phosphatases and these guide peripheral sensory axons to the skin in zebrafish embryos [50]. Ve-ptp is required for vascular integrity, due to its role in adherens junctions where it regulates VEGFR-dependent VE cadherin phosphorylation and cell polarity [6,17]. Ptpro is structurally related to Ve-ptp and is required for cerebellar formation [32]. Knockdown of Shp1 (*ptpn6*) hyperactivates the innate immune system [26]. All of the studies above used transient morpholino-mediated knockdown of the PTP target proteins. To study the function of all classical PTPs in development, we have designed two non-overlapping morpholinos against each classical PTP gene in the zebrafish genome and assessed their effects on early development by micro-injection at the one-cell stage. Not all pairs of morpholinos induced the same defects, suggesting off-target effects [47]. We have pursued some knockdowns in detail (see below) and rescue of the morpholino-induced defects by co-expression of the respective target RNAs is taken as good evidence that the observed defects are not merely off-target effects.

4.2. Target selected gene inactivation

Off-target effects of morpholinos are a concern and reverse genetics approaches have been developed to generate genetic mutants of target genes. Both mutagenesis and viral insertions are commonly used to disrupt target genes. Many loss-of-function mutations have been identified in PTP genes (www.zfin.org) (Table 1). Mutagenesis-based gene inactivation makes use of reagents such as N-ethyl-N-nitrosourea (ENU) to generate random single nucleotide polymorphisms (SNPs) in adult males. Subsequently, offspring is generated and mutations are identified in target genes by SNP detection or sequencing. Target selected gene inactivation by random mutagenesis and resequencing of the gene of interest in a large number of F1 mutants has led to the identification of nonsense mutations in more than 200 target genes [51] (www.zfin.org). An ongoing project makes use of high-throughput sequencing and is aimed at disrupting every gene in the zebrafish genome. So far potentially disruptive mutations have been identified in 38% of the zebrafish genes [28].

One of the first genes that were disrupted by the original target selected gene inactivation method was zebrafish *pten*. PTEN is a prominent member of the PTP superfamily, even though its catalytic activity is directed at lipids, rather than phosphotyrosine. *PTEN* is one of the most frequently mutated tumor suppressor genes in human cancer and is essential for mammalian development. We identified an early stop in the *pten* gene and much to our surprise, homozygous fish were viable. However, unlike the human genome, the zebrafish genome encodes two *pten* genes, *ptena* and *ptenb*, prompting us to disrupt the second *pten* gene too. Zebrafish embryos without functional Pten ($ptena^{-/-}ptenb^{-/-}$) display various hyperplastic/dysplastic defects and are embryonic lethal [14]. Genetic mutants that lack functional Ptena or Ptenb are viable and fertile, indicating that Ptena and Ptenb have at least partially redundant functions. Yet, adult fish that retain only a single wild type *pten* allele ($ptena^{+/-}ptenb^{-/-}$ and $ptena^{-/-}ptenb^{+/-}$) develop hemangiosarcomas, endothelial tumors [9]. These findings illustrate that the duplication of genes in teleosts can be used to study gene function in a way that is not possible in mammalian systems. Future analysis of the *pten* mutants will provide new insights into Pten function *in vivo*.

4.3. Gene targeting

Specific gene inactivation by directing nuclease activity to target genes has been developed successfully for zebrafish as well. Zinc Finger Nuclease (ZFN) technology has been used [12,36] and more recently, Transcription Activator Like Effector Nuclease (TALEN) technology appears even more successful [5]. Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated systems (Cas) technology has also been applied to inactivate genes in zebrafish [21] and holds much promise for future inactivation of target genes. All nuclease technologies are based on the generation of double-stranded breaks in the target gene, which are repaired by endogenous DNA repair mechanisms, particularly non-homologous end-joining (NHEJ). NHEJ frequently makes mistakes, leading to short deletions and these actually result in frame shifts and hence inactivation of the target genes. In principle, a template can be provided exogenously for DNA repair by homologous recombination, facilitating the introduction of (disease-associated)