Early Embryonic Gene Expression Profiling of Zebrafish Prion Protein (Prp2) Morphants

Rasoul Nourizadeh-Lillabadi, Jacob Seilø Torgersen*, Olav Vestrheim, Melanie König, Peter Aleström, Mohasina Syed*
Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science, Oslo, Norway

Abstract

Background: The Prion protein (PRNP/Prp) plays a crucial role in transmissible spongiform encephalopathies (TSEs) like Creutzfeldt-Jakob disease (CJD), scrapie and mad cow disease. Notwithstanding the importance in human and animal disease, fundamental aspects of PRNP/Prp function and transmission remains unaccounted for.

Methodology/Principal Findings: The zebrafish (Danio rerio) genome contains three Prp encoding genes assigned prp1, prp2 and prp3. Currently, the second paralogue is believed to be the most similar to the mammalian PRNP gene in structure and function. Functional studies of the PRNP gene ortholog was addressed by prp2 morpholino (MO) knockdown experiments. Investigation of Prp2 depleted embryos revealed high mortality and apoptosis at 24 hours post fertilization (hpf) as well as impaired brain and neuronal development. In order to elucidate the underlying mechanisms, a genome-wide transcriptome analysis was carried out in viable 24 hpf morphants. The resulting changes in gene expression profiles revealed 249 differently expressed genes linked to biological processes like cell death, neurogenesis and embryonic development.

Conclusions/Significance: The current study contributes to the understanding of basic Prp functions and demonstrates that the zebrafish is an excellent model to address the role of Prp in vertebrates. The gene knockdown of prp2 indicates an essential biological function for the zebrafish ortholog with a morphant phenotype that suggests a neurodegenerative action and gene expression effects which are apoptosis related and affects gene networks controlling neurogenesis and embryonic development.

Introduction

The molecular mechanisms responsible for prion pathogenesis share similarities with a wider group of neurodegenerative disorders. The genetic tractability of zebrafish (Danio rerio) makes it a suitable model for addressing the fundamental molecular mechanisms underlying neurodegenerative disorders such as prion disease [1], Alzheimer disease [2] and Parkinson’s disease [3].

In the prion theory of Prusiner [1] transmissible spongiform encephalopathies are proposed to be caused by a proteinaceous infectious agent, a pathological variant of the normal cellular prion protein, PRNP scrapi (PRNPSc); also named PrPSc, which acts as a template for the conversion of normal cellular prion protein PRNP (also named PrPc) to PRNPSc. There are however, major unresolved issues in prion research relating to the more fundamental biological questions of the prion gene function and mechanisms for transmission of the disease. PRNP is a glycosylated glycosylphosphatidylinositol (GPI) anchored membrane protein, but its function, cellular distribution and polarized sorting in the cell remain to be definitively established. Also the functional relationship with its binding partner(s) remains unaccounted for. A list of PRNPSc candidate binding partner proteins together with the major proposed functions for PRNPSc itself has been summarized in a review by Westergard et al. [4], including protection against apoptosis and oxidative stress, copper ions uptake and binding, synaptic signal transduction and as linker to the extracellular matrix.

The PRNP gene is phylogenetically conserved, though teleost genomes harbor multiple prp paralogues due to extra genome duplication. Of the three zebrafish PRNP genes, prp3 is the most divergent variant [5], whereas prp1 and prp2 more closely resemble the mammalian variant with prp2 most likely being the closest ortholog [6]. Miesbauer et al. [7] demonstrated that PrP-related proteins from zebrafish are complexly glycosylated and contain a glycosylphosphatidylinositol anchor. It appears that the nomenclature for prion protein variants and the corresponding genes can be confusing (Table S1). In this paper we have chosen to use prp1, prp2, prp3 and Prp1, Prp2, Prp3 for the zebrafish genes and proteins respectively. For the mammalian homolog we denote the normal variant gene and protein PRNP and PRNP (PrPc)
respectively. The misfolded pathological PRNP scrapie variant is termed PRNP\textsuperscript{Sc} (PrP\textsuperscript{Sc}).

Knockout mice lacking the neuronal cell surface PRNP protein showed normal nervous system development and behavior [8]. On the contrary, pp1 mRNA knockdown in zebrafish resulted in a strong embryonal phenotype, characterized by absence of embryonic cell adhesion and arrested gastrulation [9]. Similarly, pp2 morphant zebrafish also developed a lethal phenotype but at a later larval stage at 7 days post-fertilization (dpf) [9]. In the same study it was also demonstrated that both zebrafish Prp and mouse PRNP mRNAs could rescue the knockdown phenotype, indicating an evolutionary conserved Prion protein function.

The apparent difference between the mouse gene knockout and zebrafish models suggests that important aspects of the Prion protein biology may be revealed in the zebrafish model, using morpholino (MO) mediated translation blocking [10]. Aiming at uncovering the function of zebrafish pp2 gene, two MOs (pp2-MO1 and pp2-MO2) for targeting the pp2 mRNA were designed and injected into one cell stage zebrafish embryos. The resulting phenotypes were characterized and microarray studies were conducted on 48 hpf zebrafish embryos microinjected with the most potent of the two MOs, prp2-MO2. In order to confirm the microarray data and demonstrate that both MOs showed the same specificity, quantitative real-time PCR (qRT-PCR) was performed on mRNA of selected genes from both prp2-MO1 and prp2-MO2 injected embryos.

**Results**

Optimization of the two MOs specific for the 5′ UTR of GenBank sequence AJ620614.1 (Fig. 1) injections revealed that 0.2 nM prp2-MO2 resulted in 71% mortality at 24 hpf and 74% at 48 hpf. In order to reduce the mortality, 0.15 nM prp2-MO2 was chosen because it led to a mortality rate below 50%. Phase contrast microscopy of Prp2 morphants revealed a clear phenotype with midbrain and hindbrain developmental defects as compared to the control group (Fig. 2 B–D). To further characterize the phenotypic effects, wholemount immunofluorescence was carried out using antibodies against Prp2 and HNK-1. Fluorescence microscopy analysis of the wild type embryos at 24 hpf unveiled low but significant levels of Prp2 proteins in the telencephalon, eye and trigeminal ganglion (Fig. 3A). A double staining of HNK-1 showed strong staining to the trigeminal ganglion and telencephalon, eye and trigeminal ganglion (Fig. 3A). Investigation at hpf unveiled low but significant levels of Prp2 proteins in the midbrain-forehead neurons in the head (Fig. 3C). The cluster of “embryonic development” and “nervous system development and function” (Fig. S1, Table 2). The cell death/apoptosis related gene cluster was the largest sub-cluster with a total of 32 genes, of which 5 were related to apoptosis of neural cells: TP53, TP73, and BAX (upregulated) and SNPR1 and SNCE (downregulated, Fig. 4A, Table S2). The cluster of “embryonic development” (Fig. S1, Table 2) contained 11 genes, of which IPA could join 9 to each other in a network of sub-clusters in which SHH was the only downregulated gene and BMP4 had the highest score of the upregulated (Fig. S2). Both SHH and TP53 were included in one of the sub-clusters directly involved in the developmental process of embryonic cell lines. Analysis of genes related to “nervous system development and function” (Fig. S1, Table 2) revealed 5 sub-functional clusters involved in the developmental processes.

Among these clusters, development of brain, forebrain, neuroepithelial cells, neurons and the central nervous system (CNS) were present (Fig. 4B). Here TP53, AKTIP and BMP4 (upregulated) with SHH (downregulated) play a central role with TP53 acting on CNS and BAX on neuron development (Fig. 4B). In the cluster “nervous system development and function”, IPA connected 13 of the differentially expressed genes to each other in a network of different sub-function clusters. The most prominent network was the sub-function cluster “neurogenesis”, which contained TP53, BMP4, MEF2C and BAX (upregulated) together with SOX11, GEFAP, ACHE and SHH (downregulated) (Fig. S2). Also, the “differentiation of neurogila” and “migration of neurons” harbored 5 genes each.

In order to explicitly investigate whether the differentially expressed genes in the prp2-MO2 injected embryos could be directly associated with prion gene (PRNP/pp2) functions, a customized IPA network with prion and prion associated genes was generated and found to include 18 upregulated and 13 downregulated genes, where the mammalian pp2 ortholog PRNP has a direct relation to MMP2 and an indirect relation to BAX and TP53 (Fig. 5). However, PRNP (pp2) itself and some of the other key molecules of the predicted network are not a part of the differentially expressed genes submitted from the microarray dataset (Fig. 5) but showed change in expression by qRT-PCR (Fig. 6; See below and discussion for further clarification). Validation of the microarray results by qRT-PCR showed a high Pearson’s correlation coefficient (0.990) between the microarray and qRT-PCR data (Fig. 6). Further, the corresponding

---

**Figure 1. Morpholino sequences.** Morpholino sequences (prp2-MO1 and prp2-MO2 sequences underlined) used for knockdown of the zebrafish Prp2 gene function.

doi:10.1371/journal.pone.0013573.g001
**Figure 2.** prp2-MO2 morphant 24 hpf larvae phenotypes. Larvae were immobilized in CyGEL prior to microscopy. 

A) Non-injected wt control. B–D) three individual morphants displaying defective midbrain and hindbrain development.

doi:10.1371/journal.pone.0013573.g002

**Figure 3.** Whole mount immunofluorescence analysis of 24 hpf wild type (A and B) and Prp2 morphants (C and D). Neuronal structures were visualized by the Zn12 antibody (Yellow), whereas Prp2 was detected with a rabbit anti serum (Red). 

A) In the wild type 24 hpf Prp2 is observed in the trigeminal ganglion and telencephalon, but also present in other neuronal tissue. 

B) Neuronal structures visualized by HNK-1 staining (Zn-12 antibody) in the same specimen as in A. 

C) Aberrant morphology of the trigeminal ganglion in Prp2 morphants (arrow) as well as reduced number of peripheral neurons visualized by HNK-1 staining. 

D) Non deconvolved single plane image of a morphant with condensed nuclei (inside stippled ring). Abbreviations: eye (e), telencephalon (t) and trigeminal ganglion (tg).

doi:10.1371/journal.pone.0013573.g003
qRT-PCR analysis of the prp2-MO1 morphant transcriptome, using the same 14 gene probe sets, resulted in a comparable pattern of gene expression as the above mentioned results (Pearson’s correlation coefficient 0.92). Hence, all of the qRT-PCR expression data strongly support the assumption of a gene specific action by the two prp2 specific MOs used. In addition, no change in prp1 gene expression was observed by qRT-PCR analysis of the control and/or the two Prp2 morphants (data not shown).

**Discussion**

In the current study, we have targeted prp2 mRNA translation using two different targeting MOs, prp2-MO1 and prp2-MO2. The former had a significantly weaker effect on mortality which may be attributed to its different binding site on the prp2 mRNA (Fig. 1). A difference in prp2-MO induced mortality compared to what Malaga-Trillo reported may also be explained by different morpholinos [9]. Quantification of gene expression in the morphants prp2-MO1 and prp2-MO2 by qRT-PCR (Fig. 6) implies that the observed changes in gene expression are reliable both with respect to technical quality of the microarray data per se and with respect to gene target specificity of the prp2-MO2 chosen for the microarray experiment. Investigations of the corresponding

| Category                              | P-value           | Molecules                                                                 |
|---------------------------------------|-------------------|---------------------------------------------------------------------------|
| **Cellular Development**              | 1.77E-05-9.52E-03 | SHH, BMP4, CTGF, TP73, AKTIP, SDC4, FOXD3, CD164, STMN1, HBB (includes EG:3043), HMGB1 (includes EG:3146), ASPM, ANXA1, CRB2, HNF4A, COL18A1, ECT2, RASGRF2, TP53, TNC, PTP4A3, BHLHB5, ACEH, MMP2, BAX, MLLT1, TYRP1, INSL1, PRKCI, CD9, ABC5C, MEF2D, IHH, ALDOA, MEFC2, NPM1 (includes EG:4869), HMGB3 |
| **Developmental Disorder**            | 2.15E-05-7.67E-03 | TP53, SHH, BMP4, SLCL31A1, DKK1, HSPB8, KAL1, ACEH, MMP2, BAX, COL1A2, PRKCI, LAMA4, CRYBA4, PHGDH, ACSL4, IHH |
| **Cell Death**                        | 2.39E-05-9.8E-03  | SHH, CTGF, BMP4, SLCL31A1, CCN1, TP73, AKTIP, HSPB8, SDC4, MDH1, MYLK, CCN1, EGLN1, STMN1, HMGB1 (includes EG:3146), ANXA1, ACSL4, IGFBP1, SN CB, COL18A1, RASGRF2, TP53, TNC, ACEH, MMP2, BAX, PRKCI, SWAP70, NDEL1, MCM2, CD9, ABC5C, MEF2D, S1PR1, ALDOA, IHH, MEFC2, NPM1 (includes EG:4869), HMGB3 |
| **Cellular Assembly and Organization**| 3.34E-05-8.36E-03 | CTGF, CKMT1B, SDC4, CBK, STMN1, HMGB1 (includes EG:3146), GFAP, HNF4A, COL18A1, TP53, TNC, CKN, PTP4A3, ACEH, MMP2, BAX, PRKCI, SWAP70, MCM2, CD9, LAMA4, H2AFX, S1PR1, GPM6A, NPM1 (includes EG:4869) |
| **Cardiovascular System Development and Function**| 4.76E-05-7.67E-03 | TP53, SHH, CTGF, BMP4, TP73, MMP2, COL1A2, EGLN1, LAMA4, S1PR1, IHH, MEFC2, COL1A1, ECT2 |
| **Gene Expression**                   | 5.06E-05-7.67E-03 | TP53, PRKCI, HMGB1 (includes EG:3146), TP73, MEF2D, ATF7IP, EIF5, MEFC2, BAX, NPM1 (includes EG:4869), HNF4A |
| **Cell Cycle**                        | 5.83E-05-9.15E-03 | TP53, SHH, BMP4, CCN1, TP73, BAX, CCNG1, SESN1, STMN1, MCM2, ANXA1, S1PR1, HNF4A, COL18A1, ECT2, NPM1 (includes EG:4869) |
| **Immunological Disease**             | 5.83E-05-8.41E-03 | TP53, SHH, SWAP70, CD9, HMGB1 (includes EG:3146), TP73, AKTIP, ANXA1, ACEH, BAX |
| **Embryonic Development**             | 1.74E-04-7.67E-03 | TP53, SHH, SLCL31A1, MMP2, ABC5C, PTP4A3, IHH, MMP2, BAX, HNF4A, COL1A1 |
| **Nervous System Development and Function**| 1.74E-04-8.23E-03 | TP53, SHH, BMP4, TP73, AKTIP, ACEH, BAX, STMN1, ECLN1, CD9, NDEL1, HMGB1 (includes EG:3146), LAMA4, PHGDH, S1PR1, IHH, COL1A1, ECT2 |
| **Organ Development**                 | 1.74E-04-8.36E-03 | TP53, SHH, BMP4, TP73, AKTIP, MMP2, BAX, FOXO3, TYRP1, ECLN1, STMN1, HMGB1 (includes EG:3043), S1PR1, PHGDH, IHH, MEFC2, IGFBP1 |
| **Neurological Disease**              | 5.74E-04-8.71E-03 | RNF10, SHH, CTGF, TNN12, HSPB8, SDC4, MDH1, EEF1G, TTUB22B, CBK, SPON1, STMN1, ASPM, PHGDH, LAMB1, AC5C, GFAP, SNCB, HNF4A, TP53, ACEH, MMP2, BAX, LRRN1, SLCL31A1, S1PR1, PDLDM1, UCK2, NPM1 (includes EG:4869) |
| **Organisinal Development**           | 6.42E-04-7.67E-03 | TP53, SHH, BMP4, SLCL31A1, TP73, PTP4A3, ACEH, MMP2, BAX, PRKCI, HMGB1 (includes EG:3146), SLCL1A1, IHH, IGFBP1, COL1A1 |
| **Cell Signaling**                    | 7.34E-04-4.17E-03 | TP53, CKM, ANXA1, CKMT1B, BAX, COL1A1 |

Unbolded molecules are upregulated and bolded molecules are downregulated. doi:10.1371/journal.pone.0013573.t002
prp1 mRNA levels by qRT-PCR showed unaltered levels of this paralogue in the morphants, which suggests that the Prp1 and 2 proteins have gained different main functions in zebrafish. The lethal effect of prp2 knockdown is in agreement with the proposal of an apoptosis-protective role of the PRNPC protein [11], as also confirmed by hyperdense nuclei observed in DAP1 stained morphants (Fig. 3D). The 24 hpf prp2-MO2 morphant transcriptome (Table S2 and S3) also reveals a cluster of 39 genes involved in cell death, of which 32 were in the sub-cluster of apoptosis (Fig. S1 and 4A). Cyclin G1 (CCNG1) the gene with the highest level of upregulated expression in our experiment is known to control TP53 and TP73 which are involved in cell cycle control and induction of apoptosis [12], two processes which are augmented in the present study. Furthermore, TP53 and TP73 together with S1PR, BAX and SNCB directly influence apoptosis of cortical neurons, neuroepithelial, neuroblastoma, neural...

Figure 4. IPA cluster analysis of significantly differentially expressed genes. IPA cluster analysis of significantly differentially expressed genes for 24 hpf zebrafish prp2-MO2 morphant embryos. The clusters reveal genes involved in (A) cell death and apoptosis with functions focused on apoptosis in neural cells and (B) genes involved in nervous system development. Red color indicates up- and green downregulation. doi:10.1371/journal.pone.0013573.g004
Figure 5. **A network for connecting differentially expressed genes.** A network for connecting differentially expressed genes from zebrafish 24 hpf prp2-MO2 morphant embryos was identified by IPA in which PRNP, APP, heparin and EP300 occupy a central role. The uncolored protein symbols, including PRNP, are added by IPA to fill network gaps and are not among the analyzed dataset of differentially expressed genes. Red color indicates up- and green downregulation. A significant prp2 mRNA down-regulation was experimentally demonstrated by qRT-PCR (see Fig. 6).

doi:10.1371/journal.pone.0013573.g005

Figure 6. **Comparison between microarray data and qRT-PCR data.** Microarray data (blue staples), qRT-PCR of RNA from 24 hpf prp2-MO2 injected embryos (red staples) and 24 hpf prp2-MO1 injected embryos (yellow staples) are presented as fold change of expression of 14 genes. Mean fold change (± SD) for qRT-PCR are based on triplicate embryo pools.

doi:10.1371/journal.pone.0013573.g006
precursor and nervous tissue cells (Fig. 4A). The altered expressions of these genes with their role in apoptosis of neural cells [13, 14] support a putative role for Prp2 in the control of apoptosis of neural cells in zebrafish development. For example, the SNCAβ protein have been reported to decrease apoptosis of TSM1 neurons [13] and SIRP-null mice show a dramatic increase in apoptosis with a decrease in mitosis in the developing nervous system [14].

Both the observed phenotype morphology (Fig. 2B and C) and impaired neurogenesis of the trigeminal ganglion and peripheral neurons (Fig. 3C and D) of the morphants can be correlated to the observed changes in gene expression, clustered to functions associated with embryo development and development of brain, forebrain and central nervous system (Fig. 4B and S2). Two of these key molecules, sonic hedgehog (SHH) and bone morphogenic protein 4 (BMP4), were both downregulated. SHH acts as a signal molecule in many tissues including the midline structures in the brain, spinal cord and thalamus by the zona limitans intrathalamicus [15–17]. SHH also has a reported ability to act on axonal guidance [18] as well as on neurogenesis where it influences proliferation of neuroblasts and granule cell precursors [19, 20] (Fig. S2). Similar to SHH, BMP4 is involved in many biological processes including negative control of neurogenesis in olfactory epithelial cells and in brain/forebrain development [21]. However, since both SHH and BMP4 are involved in many biological processes, exemplified by their presence in 11 and 9 respectively of the 14 IPA defined biological function clusters of this study (Table 2), further investigations are needed to pinpoint more specific effect-relationships. In addition to SHH and BMP4, several other genes associated with the processes of neurogenesis, SOX11, GEFAP and ACHE, were downregulated in prp2 knockdown embryos (Fig. S2). SOX11 is suggested to function in the developing nervous system while lack of glial fibrillary acidic protein (GFAP) gene function in knock-out mice increases the outgrowth of axons from neurons of the spinal cord [22] (GFAP is central in an IPA cluster related to neurological disorders. See below and Fig. S3). ACHE has been reported to be essential for normal dendrite and axon formation in hippocampal neurons and may function in excitatory synapse development, plasticity and remodeling [22–24]. The present microarray and qRT-PCR analyses demonstrate an upregulation of the BAX gene, which is a pro-apoptotic protein required for neurotrophin-deprived neuronal apoptosis [25]. Overall, prp2 knockdown in zebrafish embryos suggests impaired neurogenesis (Fig.’s 2, 3 and 4B) which is in concert with the proposed function of IPA cluster analysis could link all the 5 genes to neurological disorders (Fig. S3). CD9 and HSPB8 included in the IPA apoptosis cluster (Fig. 4A). When comparing each mouse study alone against the sCJD data [29, 30] the overlapping gene numbers are 11–14 which is not far from the 4–5 for the zebrafish-sCJD or zebrafish-mouse, taken into consideration the fact that only 120 of 249 of the zebrafish genes have known mammalian homologs (Table 1). With this background it is fair to state that the zebrafish prp2 morphants provide a good model to shed light on the normal biological function of the prion protein. Our gene expression study is based on depleting the mammalian prion protein ortholog from the developing zebrafish embryo and has been able to link the zebrafish prp2 gene to biological processes including cell death, embryo development and neurogenesis. Although not many genes overlap when comparing 3 mammalian scrapie or zebrafish mouse, taken into consideration the fact that only 120 of 249 of the zebrafish genes have known mammalian homologs (Table 1). Many genes associated with these processes have been uncovered but still a more distinct mechanism of action for Prp2 remains to be found. We conclude that the data from this study, together with the paper of Malaga-Trillo et al. [9], place zebrafish as a highly relevant model to address the still largely unanswered questions of the molecular mechanisms underlying prion protein function, aggregation and the corresponding disease pathology.

Materials and Methods

Zebrafish and prp2-MO microinjections

Newly fertilized embryos were obtained from zebrafish reared in Alestrom Zebrafish Lab http://zebrafish.no/ (http://zebrafish.no) according to husbandry standards as described in Alestrom Lab Standard Operating Procedures. As an AAALAC (http://www.aaalac.org) accredited laboratory, all activities in the unit are subject for evaluation by an IACUC. In all of the experiments presented, only zebrafish embryos of age up to 24 hpf were used.
For titration of dose response, two MOs specific for the 5’UTR of GenBank sequence AJ620614.1 (Fig. 1) were injected in single cell embryos at the following concentrations: 0.1, 0.15, 0.5 and 1mM. The mortality rate for negative control embryos (non-injected and mock injected with 1 x Daniecu solution) was below 20% (data not shown).

Method for immobilizing embryos and microscopy

Prp2 morphants larvae were embedded in CryoGEL Sustain™ (Biostatus Limited, Leicestershire, UK) and investigated for phenotype using a Nikon AZ 100 fitted with Nikon Digital Sight DS K1 camera. Morphants and wild type embryo were fixed in 4% PFA and transferred to methanol for immunofluorescence.

Whole mount immunofluorescence

Wild type and morphant embryos were dechorinated and fixed in 4% PFA and then dehydrated in 70% methanol. Fluorescent immunohistochemistry was carried out on rehydrated and permeabilized embryos (10 min 1% Triton X-100, Sigma) after a 2 hr blocking in 5% dry milk dissolved in 1 x Phosphate Buffered Saline Tween 20 (PBST; 1 x PBS, 0.1% Tween). Antibodies applied included the HNK-1 specific Zn12 [31] (Developmental studies Hybridoma Bank, IA, USA) and a rabbit serum against Prp2 [32]. The former was used at a 200 fold dilution in 2% dry milk with 1% DMISO in 1 x PBST, whereas the latter was diluted 100 x. After over night incubation at 4°C with the primary antibodies, the embryos were washed thoroughly in 1 x PBST and incubated with Alexa conjugated secondary antibodies diluted 200 x (Invitrogen, Ca, USA) for 2 hours. Final 1 x PBST washes was carried out before glycerol mounting and microscopy. All images were captured using a Zeiss Axioplan Z1 fitted with the Apotome system for structured illumination microscopy. The images were post processed deconvolved and visualized in 3D using Zeiss Axiovision software.

RNA isolation

Total RNA from injected control (1 x Daniecu solution) and MO injected 24 hpf zebrafish embryo was isolated for microarray and qRT-PCR analysis. Briefly, pools of 50 embryos were treated with 1 mL of Trizol reagent (Invitrogen, CA, U.S.A). Homogenization was carried out using MagNA Lyser Beads (Roche Diagnostics Gmbh, Mannheim, Germany) and total RNA isolation was performed according to the Trizol manufacturer (Invitrogen), followed by a 15 min DNaseI treatment (Qiagen Hilden, Germany) at 25°C. Further RNA purification was conducted using RNAeasy mini kit (Qiagen). After purification, the samples were eluted in 50 μL of RNase-free water and aliquoted for microarray and qRT-PCR analyses. RNA yield and integrity were determined using a NanoDrop ND-1000 instrument (NanoDrop Technologies, DE, U.S.A) and Agilent 2100 bioanalyzer (Agilent Technologies, CA, U.S.A) respectively. None of the samples showed signs of degradation or impurities (260/280 and 260/290 >1.8, RIN >8.0).

Microarray strategy

The total RNA from zebrafish embryo morphant pools was compared to total RNA from Daniecu-injected control pools. The replicate embryo pools were analyzed in two sets of dye-swaps making a total of four hybridizations. Normalized microarray datasets did not result in significantly altered gene expression for zebrafish housekeeping gene β-actin2 (172 probes in our arrays). Like other microarray gene expression profiling studies, we see a reproducible high correlation between microarray and qPCR data [33].

Data deposition

Microarray data have been submitted to the European Bioinformatics Institutes Array Express accessible through experiment accession number E-MEXP-2365.

Linear RNA amplification, target labeling and hybridization

One μg total RNA was linearly amplified and labeled, using an Amino Allyl MessageAmp™ II aRNA amplification kit (Ambion TX, U.S.A). Five μg of the resulting amplified RNA (aRNA) from the MO-treated and control groups were labeled either with Cy3-dUTP or Cy5-dUTP (GE Healthcare CT, U.S.A). The labeled targets were examined for amplification yield and incorporation efficiency by measuring the aRNA concentration at 260 nm, Cy3 incorporation at 550 nm and Cy5 incorporation at 650 nm using a Nanodrop ND-1000. One to 5 μg of each labeled target aRNA were mixed with 9 μL of 25 x fragmentation buffer (Agilent Technologies) and the final volume adjusted to 225 μL with RNase free H2O followed by incubation for 30 min at 60°C. The hybridization solution was prepared by adding 220.5 μL of 2 x hybridization buffer (Agilent) and 4.5 μL (10 μg/μL) sonicated herring sperm DNA (Promega WI, U.S.A) to the labeled target aRNA. Microarray slides were pre-hybridized at 42°C, 60 min using 0.1% Bovine Serum Albumin Fraction V, 5 x SSC and 0.1% SDS. Hybridization was performed at 60°C for 16 hours using Agilent gasket slides G2534-60003, hybridization chamber and oven according to the manufacturer (Agilent Technologies). Microarray slides were then washed 3 x 5 min in 0.5 x SSC and 0.01% SDS with the first wash at 42°C and the next two at room temperature. Finally, slides were washed 3 times at room temperature with 0.06 x SSC and dried immediately by centrifuging at 1000 rpm for 1 minute.

Zebrafish oligonucleotide library and microarray construction

The Zebrafish OligoLibrary™ (Compugen, Ontario, Canada) was used as composed of 16,399 (16k) 65-mer oligonucleotide probes, originally representing 16,228 clusters predicted to be representing unique genes plus 171 positive control probes. All probe sequences originally representing 16,228 clusters predicted to be representing unique genes plus 171 positive control probes. All probe sequences were selected from the 3’ UTR in order to cover a maximum number of splice variants. The oligonucleotides were printed on CMT UltraGAPS slides at the Norwegian Microarray Consortium (NCM, www.microarray.no ) with a Microgrid II (BioRobotics, Oslo). Probe annotation and information about Unigene ID, Unigene description, Gene symbol, GO ontology and human and mouse homologue ID and descriptions were obtained with the Unigene build release, using the Genome institute of Singapore Unigene & Gene Ontology Annotation Tool Genome institute of Singapore [http://giscompute.gis.a-star.edu.sg/~govind/unigene_db/]. GenBank accession numbers were used as a query. In the present study annotations were made using Danio rerio Build #107-38.

Pathway analysis and functional profiling

For pathway and biological function analysis of significantly differentially expressed genes, the Ingenuity Pathway Analysis (IPA) program was used. The results were analyzed using the Unigene & Gene Ontology Annotation Tool for identification of mammalian (human, mouse and rat) orthologs before IPA software analysis. The output results comprise gene networks, pathways and functional clusters, where given scores and p-values are based on numbers of uploaded genes in the cluster or network and the size of cluster or network in the IPA knowledge database.
Fisher’s exact test was used to determine the statistical significance and the likelihood of random clustering to a pathway. Scores of 2 or higher have at least a 99% likelihood of not being generated by chance alone. Mammalian orthologs from IPA are displayed using human homolog identifiers whereas zebrafish genes and proteins or higher have at least a 99% likelihood of not being generated by chance alone. Mammalian orthologs from IPA are displayed using human homolog identifiers whereas zebrafish genes and proteins are annotated according to standard zebrafish nomenclature guidelines (Table S1).

Quantitative real-time PCR
The accuracy of the microarray results were validated by analyzing 14 differentially expressed genes by qRT-PCR using the same samples of total RNA. The intron spanning qRT-PCR primers were designed using “Universal Probe Library Assay Design Center/ProbeFinder version 2.40” (http://www.roche-applied-science.com/sis/rtpcr/upl/adp.jsp). Primer sequences are summarized in Table 3. Table cDNAs were produced from 1 μg total RNA using Superscript III Reverse Transcriptase according to manufacturer conditions were: 95°C for 10min followed by 40 cycles of 10 s at 60°C and 6 s at 72°C. Melting curve analysis and agarose gel electrophoresis was carried out to confirm PCR products. Data analysis with crossing point (Cp) was determined by use of the maximum-second-derivative function in the LightCycler® Software version 4.0 (Roche). Relative gene expression levels were obtained using beta-actin as the internal reference gene and efficiency correction with external standards. The mean gene expression values for each group (exposed versus control) were compared and p-values calculated using unpaired Student’s t-test.

Table 3. qRT-PCR primers (5’-3’) used to validate microarray and beta-actin.

| GenBank | Symbol | Unigene Descp | Forward Primer | Reverse Primer |
|---------|--------|---------------|----------------|----------------|
| AI959372 | ccng1 | Cyclin G1 | agttgacagcagctgtag | aacagcgccgcttcgtaa |
| U60804  | Tps3  | Tumor protein p53 | ctggaaagtctccagagc | ttgggccttcacattc |
| BB891654 | bax   | Bcl2-associated X protein | cgaggtacgcttctgtag | gttgacagcagcttgaa |
| BB870836 | senl  | Strerin 1 | gcttcaggagctacacaa | cacagctggtagctg |
| AI350383 | hbae3 | Hemoglobin alpha embryonic-3 | atccgggtgagagcttt | gggagagttcttgagactt |
| U14590  | ascl1b | Achaete-scute complex-like 1b (Drosophila) | ccagggagggcttcagagc | caatcctgtagctgtaaac |
| BG302998 | ald4h | Similar to Aldehyde dehydrogenase 9 family, member A1 like 1 | aaacacacagctgctgg | gttgacagcagcttg |
| A1964216 | rps9  | Ribosomal protein S9 | gggtgacagcagcttg | gggtgacagcagcttgtaa |
| BB693186 | hbael | Hemoglobin alpha embryonic-1 | aaactcatcttcacagactg | gggactgtacacacatct |
| AF082662 | hbbe1 | Hemoglobin beta embryonic-1 | tctccagctatgctgcaacct | tgaatcctgtagctgtaaac |
| AF116539 | iclp2 | Invariant chain-like protein 2 | agtcttggctctttcttcct | ttccagcctttctcttct |
| BB480762 | gpm6aa | Similar to Glycoprotein M6A | aacagggagacctggaa | ttgacagcagcttacatc |
| AF180888 | pvalb2 | Parvalbumin 2 | gccaacagcgccttgg | ttcctcggagtccttgaa |
| AY438684.1 | prp2 | Prion protein 2 | caatccggcagagagcagac | caagagctcagccagacatag |
| AY438683.1 | prp1 | Prion protein 1 | gctttcctctctctcttggt | gccctcttctctctcttggt |
| AF025305.1 | beta-actin | | | |

DOI:10.1371/journal.pone.0013573.s003

Supporting Information

Figure S1 IPA generated functional clusters of differentially expressed genes. IPA generated functional clusters of differentially expressed genes in 24 hpf zebrafish prp2 morphant (prp2-MO2) embryos. All clusters have p-values <0.05 and are ranked by decreasing p-values. Corresponding p-values are illustrated in Table 3. The numbers in the pie diagram corresponds to the numbers of genes with significantly different expression in each cluster. The clusters appear in the same order in the diagram as in the ID pane. Found at: doi:10.1371/journal.pone.0013573.s001 (0.24 MB TIF)

Figure S2 IPA cluster analyses of significant differentially expressed genes. IPA cluster analyses of significant differentially expressed genes for 24 hpf zebrafish Prp2 morphant embryos. The cluster reveals genes involved in nervous system function. Red color indicates up- and green downregulation. Found at: doi:10.1371/journal.pone.0013573.s002 (2.60 MB TIF)
Figure S3 Comparative analysis of differentially expressed genes. Comparative analysis of differentially expressed genes between the prp2-MO2 morphant and MO-injected zebrafish embryos. The functional clustering by IPA indicates a central role for these genes in neurological disorders: Huntington disease, Parkinson disease, retinopathy, Alexander disease, motor neuron disease, hydrocephalus and hypertrophy of astrocytes. Found at: doi:10.1371/journal.pone.0013573.s003 (0.42 MB TIF)

Table S1 Nomenclature and relationship between prion protein genes and proteins. The arguments for prp2/Prp2 being the mammalian PrNP/PrP homolog is summarized. To simplify reading we have used the old conventional names (in bold) in the article and in Table S1. Found at: doi:10.1371/journal.pone.0013573.s004 (0.04 MB DOC)

Table S2 Fold change of differentially expressed genes mapped by IPA in 24 hpf prp2-MO2 MO-injected zebrafish embryos. Found at: doi:10.1371/journal.pone.0013573.s005 (0.15 MB DOC)

Table S3 Fold change of differentially expressed genes which were not mapped by IPA in 24 hpf prp2-MO2 injected zebrafish embryos. These genes are annotated with zebrafish gene symbols obtained from the Genome Institute in Singapore. Found at: doi:10.1371/journal.pone.0013573.s006 (0.15 MB DOC)

References
1. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science 216: 134–144.
2. Newman M, Musgrave IF, Lardelli M (2007) Alzheimer disease: amyloidogenesis, the presenilins and animal models. Biochim Biophys Acta 1772: 285–297.
3. Breitad S, Allen G, Ingham PW, Bandmann O (2007) p53-dependent neuronal cell death in a D1-jdeficient zebrafish model of Parkinson’s disease. J Neurochem 109: 1626–1635.
4. Westergard L, Christensen IM, Harris DA (2007) The cellular prion protein (PrP(C)) its physiological function and role in disease. Biochim Biophys Acta 1772: 629–644.
5. Cotto E, Andre M, Forgue J, Fleurby HJ, Babin PJ (2005) Molecular characterization, phylogenetic relationships, and developmental expression patterns of prion genes in zebrafish (Danio rerio). FEBS J 272: 500–513.
6. Rivera-Milla E, Oidtmann B, Panagiotidis CH, Baier M, Sklaviadis T, et al. (2006) Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. FASEB J 20: 317–319.
7. Miesbauer M, Bamte R, Riemer C, Oldtmann B, Winkelhofer KF, et al. (2006) Prion protein-related proteins from zebrafish are complex glycosylated and contain a glycosylphosphatidylinositol anchor. Biochim Biophys Res Commum 341: 218–224.
8. Bueler H, Fischer M, Lang Y, Blaehnann H, Lipp HP, et al. (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP.
9. Malaga-Titus E, Sola GP, Schrock Y, Geiss C, Lanza L, et al. (2009) Regulation of embryonic cell adhesion by the prion protein 2. PLoS Biol 7: e55.
10. Naevius C, Ekker SC (2000) Effective targeted gene ‘knockdown’ in zebrafish. Nat Genet 26: 216–220.
11. Aigner A, Baumann F, Bremer J (2008) The prion’s elusive raison for being. 2. Annu Rev Neurosci 31: 489–477.
12. Jost CA, Marin MC, Kaelin WG, Jr. (1997) p73 is a simian [correction of human] p53-related protein that can induce apoptosis. 3. Nature 390: 191–194.
13. da Costa CA, Masliah E, Cheek F (2003) Beta-synuclein displays an antiapoptotic p53-dependent phenotype and protects neurons from 6-hydroxydopamine-induced caspase 3 activation: cross-talk with alpha-synuclein and implication for Parkinson’s disease. 1. J Biol Chem 278: 37330–37335.
14. Mizugishi K, Yamashita T, Oliveira A, Miller GP, Spiegel S, et al. (2005) Essential role for spingosine kinases in neural and vascular development. 8. Mol Cell Biol 25: 11113–11121.
15. Herzog W, Zeng X, Lelé Z, Sonntag C, Ting JW, et al. (2003) Adenohypophysial formation in the zebrafish and its dependence on sonic hedgehog. 1. Dev Biol 254: 36–49.
16. Lewis KE, Eisen JS (2001) Hedgehog signalling is required for primary motoroneuron induction in zebrafish. 5. Development 128: 3485–3495.
17. Schlopp S, Wolf O, Brand M, Lammens A (2006) Hedgehog signalling from the zona limitans intrathalamica orchestrates patterning of the zebrafish diencephalon. Development 133: 855–864.
18. Charron F, Stein E, Jeng J, McMahon AP, Tessier-Lavigne M (2005) The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. 1. Cell 113: 11–23.
19. Dahnane M, Altaba A (1999) Sonic hedgehog regulates the growth and patterning of the cerebellum. Development 126: 3099–3100.
20. Dahnane N, Sanchez P, Gitton Y, Palma V, Sun T, Beyna M, et al. (2001) The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. 1. Development 128: 5201–5212.
21. Shou J, Rin PC, Calof AL (1999) BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor. 2. Nat Neurosci 2: 339–345.
22. Menet V, Prieto M, Privat A, Ribotta M (2005) Axonal plasticity and functional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. 1. Proc Natl Acad Sci U S A 102: 9899–9904.
23. Jay P, Kloe C, Marsollier C, Faviaux S, Hardelin JP, Koopman P, et al. (1995) The human SOX11 gene: cloning, chromosomal assignment and tissue expression. Genesis 29: 541–545.
24. Oliveira S, Rodriguez-Llurralde D, Henley JM (2003) Acetylcholinesterase promotes neurite elongation, synaptic formation, and surface expression of AMPA receptors in hippocampal neurons. 1. Mol Cell Neurosci 23: 96–106.
25. Deckwerth TL, Elliott JL, Knudson CM, Johnson EM, Jr., Snider WD, et al. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17: 401–411.
26. Steele AD, Erney JG, Oezdiner PH, Lindeque S, Macklis JD (2006) Prion protein (PrP) positively regulates neural precursor proliferation during development and adult mammalian neurogenesis. 1. Proc Natl Acad Sci U S A 103: 3416–3421.
27. Santuccione A, Sytnyk V, Leschynskyi I, Schachner M (2005) Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. 1. J Cell Biol 166: 341–354.
28. Xiao M, Windfeld O, Westen M, Neumann M, Zerr I, et al. (2005) Cerebral Gene Expression Profiles in Sporadic Creutzfeldt-Jakob Disease. Ann Neurol 58: 242–257.
29. Hwang D, Lee IY, Yoo H, Gehlenborg N, Cho J-H, et al. (2009) A systems approach to prion disease. Molecular Systems Biology 5: Article number 252; doi:10.1038/msb.2009.48.
30. Sorenson G, Medina S, Parchaiah D, Phillipson C, Robertson C, et al. (2008) Comprehensive transcriptional profiling of prion infection in mouse models reveals networks of responsive genes. BMC Genomics 9: 114.
31. Metcalfe WK, Myers PZ, Tverravarn B, Bass MB, Kimmel CB (1999) Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. Development Oct; 110(2): 491–504.
32. Salta E, Panagiotidou C, Telious K, Petrakis S, Eleftheriadis E, et al. (2009) Evaluation of the Possible Transmission of BSE and Scrapie to Gilthead Sea Bream (Sparus aurata). PLoS ONE, Volume 4, Issue 7, e6175.

33. Nourizadeh-Lillebøld R, Lyche JL, Almaas C, Stavik B, Moe Sj, et al. (2009) Identification of genetic regulation in liver and testis associated with developmental and reproductive effects in male Zebrafish exposed to natural mixtures of POPs. J Toxicol Environ Health A 72: 112–30.

34. Smyth GK, Speed T (2003) Normalization of cDNA microarray data. Methods 31: 265–273.

35. Smyth GK, Michaud J, Scott HS (2005) Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21: 2067–2075.

36. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. 30. Stat Appl Genet Mol Biol 3: Article3.

37. Smyth GK, Speed T (2003) Normalization of cDNA microarray data. Methods 31: 265–273.

38. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological) 57: 289–300.