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Instructions for use

Studies on the function of miR-124 during neurogenesis

in the medaka, *Oryzias latipes*

（メダカ神経発生過程における **miR-124** の機能に関する研究）

A DISSERATION
submitted to the Graduate School of Science, Hokkaido University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY

By YUMIKO KATO

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Contents

General Introduction

Vertebrate brains are extremely diverse and complex in their morphology, neural cells, and their neural networks. Nevertheless, they are composed of conserved structural elements (basically the forebrain, midbrain, and hindbrain) that were present in the common ancestor of vertebrates (Northcutt, 2002). Brain patterning is controlled by conserved developmental regulators, including transcription factors such as Hox, Otx, and Pax (Acampora *et al*., 2001; Lemons and McGinnis, 2006; Georgala *et al*., 2011). Noncoding regions of genomes are also now thought to contribute to brain diversity and complexity, because the ratio of noncoding regions to total genomic DNA correlates well with increasing biological complexity (Meisler, 2001; Taft *et al*., 2007).

MicroRNAs (miRNAs) are a group of small noncoding RNAs that are ~22 nucleotides (nt) in length. MiRNAs regulate gene expression at the post-transcriptional level by either repressing translation or promoting mRNA degradation (Bartel, 2009) (Fig. 0.1). Most miRNAs are located in intergenic and intragenic regions, and are transcribed as primary miRNA (pri-miRNA) including 5′cap and 3′poly (A)-tails by RNA polymerase II (Lee *et al.*, 2003). The pri-miRNA is cropped as stem-loop precursor miRNA (pre-miRNA) of ~60–100 nt long by the nuclear RNaseIII enzyme, Drosha/DGCR8. The pre-miRNA is then exported by Exportin-5 from the nucleus to the cytoplasm after cropping, and cleaved near its terminal loop by RNA III enzyme, Dicer/TRBP, to form ~22 nt long mature miRNA (mat-miRNA). The mat-miRNA is incorporated into Argonanute (Ago), forming the RNA-induced silencing complex (miRISC), and negatively control the target mRNA expression by binding to the 3′ untranslated region (UTR). The seed sequences between the second and the seventh positions in 5′ end of mat-miRNA are perfectly complementary to the target mRNA, which is essential for the binding of the miRNA to the mRNA (Mishima, 2011).

Many mammalian miRNAs are highly or specifically expressed in neural tissues and approximately 70% of experimentally detectable miRNAs are expressed in the brain (Cao *et al*., 2006), suggesting that miRNAs are essential for the nervous system. Giraldez *et al*. (2005) found that maternal-zygotic dicer mutants in zebrafish, in which all miRNA formation was blocked, led to defects in brain morphogenesis. In addition, some studies have reported the relationship between miRNA disparity and vertebrate complexity, including brain morphology (Heimberg *et al*., 2008; Somel *et al*., 2011).

Medaka (*Oryzias latipes*) is an excellent vertebrate model for molecular

embryology, because it has a relatively small genome (about 800 Mb), and a draft genomic analysis has been completed (Kasahara *et al.*, 2007). The medaka embryo is transparent and thus ideal for developmental genetic studies. The spatiotemporal expression and function of genes can, for example, be examined by reporter gene assay in developing embryos. Tani *et al.* (2009) found that miR-430 was expressed during early embryogenesis and could regulate expression of a reporter construct of the 3′UTR of *TDRD7*, a primordial germ-cell specific gene, *in vivo*. Some other miRNAs are also known to be expressed in medaka tissues, including the central nervous system (CNS) (Ason *et al.*, 2006). However, it has not been demonstrated that miRNA functions in the CNS development of medaka.

To clarify the function of miRNA in medaka neural development, I studied on a neuron-specific miRNA, miR-124. MiR-124 is well conserved between invertebrates and vertebrates, and expressed abundantly in the CNS. In Chapter 1, I characterized medaka miR-124 and examined their expression during embryogenesis. In Chapter 2, I identified the nucleotide and amino acid sequences of medaka polypyrimidine tract binding proteins (PTBPs), which are the candidate target genes in post-transcriptional regulation by miR-124. In Chapter 3, I performed GFP reporter assay to clarify whether miR-124 directly targets the 3′UTR of the medaka *Ptbp1a* and *Ptbp1b*.

From these studies, I suggested that miRNA is involved in post-transcriptional regulation of target mRNA in medaka neural development and multiple copies of a miRNA have different roles. It is useful for comparative analysis of miRNA function among vertebrates and will help to understand how differences in brain development, morphology, and function have evolved.

Figure
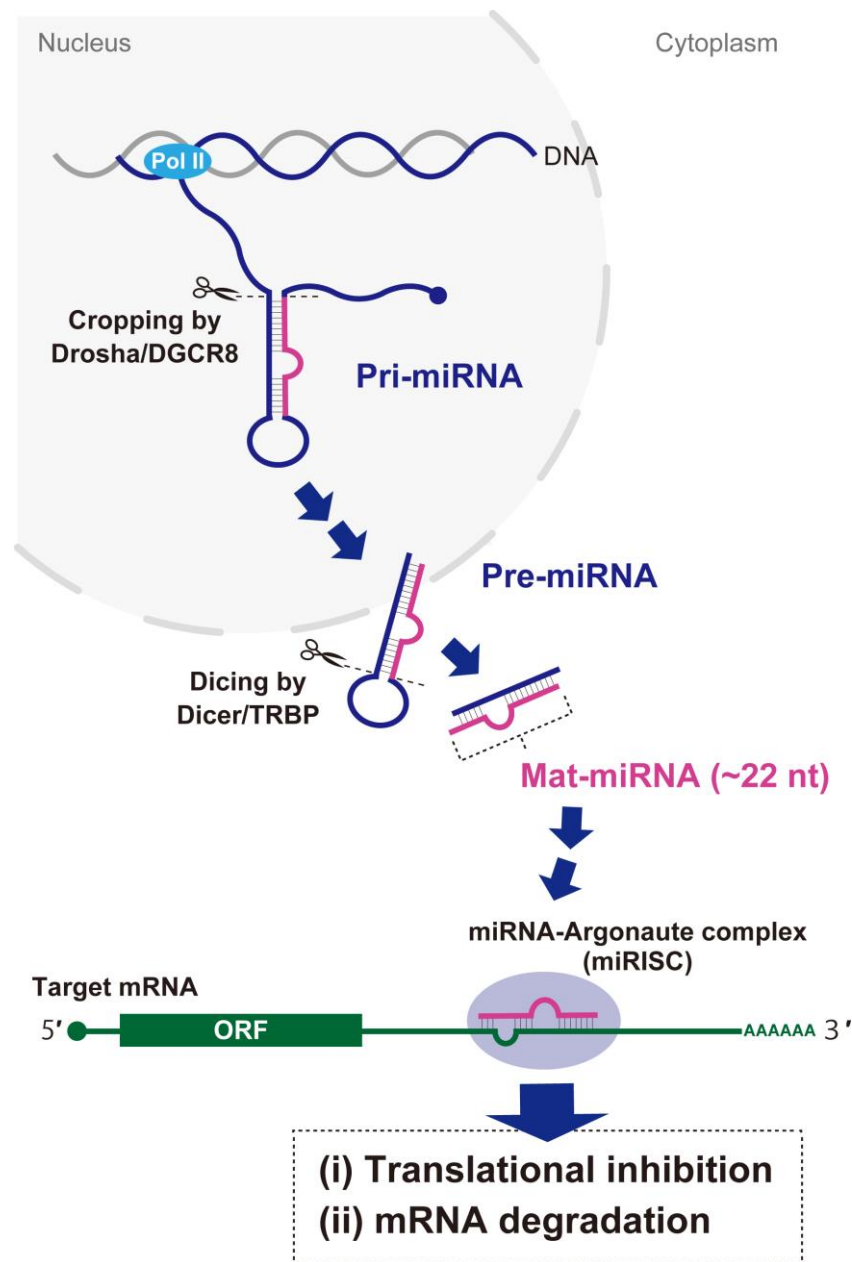
Fig. 0.1. MiRNA pathway. MiRNA is transcribed as a primary miRNA (pri-miRNA) by polymerase II (Pol II) and processed by Drosha/DGCR8 to generate precursor miRNA (pre-miRNA). Pre-miRNA is further processed by Dicer/TRBP to form mature miRNA (mat-miRNA) ~22 nucleotide (nt) long. Mat-miRNA is incorporated into miRNA-Argonaute complex (miRISC), which induces the post-transcriptional regulation of target miRNAs (Mishima, 2011).

Chapter 1

Genomic organization and expression of the medaka miR-124

1.1. Introduction

MiR-124 (also known as miR-124a and miR-124-3p) is highly conserved and is the most abundant miRNA in the central nervous system (CNS) during embryogenesis and later development in vertebrate species (Lagos-Quintana *et al*., 2002, Miska *et al*., 2004; Chen *et al*., 2005; Kloosterman *et al*., 2006). Deo *et al*. (2006) have reported a precise analysis of miR-124a expression by section *in-situ* hybridization of mouse brain. MiR-124a is expressed in the region of differentiating and mature neurons of the CNS in mouse embryo. On the other hand, miR-124a is expressed throughout most parts of the brain, including the cerebral cortex and hippocampus in the adult. In chick embryo, miR-124a is also expressed strongly in the brain, especially in the hindbrain, midbrain, lateral regions of the spinal cord and the pituitary rudiment (Darnell *et al*., 2006; Sweetman *et al*., 2006). MiR-124a has also been shown to be expressed in eye; it is detected strongly in most cells in the neural retina but not in the pigmented epithelium (RPE) (Deo *et al*., 2006; Frederikse *et al.*, 2006). In the embryo of *Xenopus laevis*, miR-124 expression is detectable after neurulation, and getting stronger in the entire CNS and the eyes (Qiu *et al.*, 2009). Hence, miR-124 is assumed to play a pivotal role in the CNS and the *in-vitro* and *in-vivo* functions of miR-124 have been studied in multiple species (Cao *et al*., 2007; Visvanathan *et al*., 2007; Qiu *et al*., 2009; Sanuki *et al*., 2011). However, a precise role of teleost miR-124 is still unexplained.

In Chapter 1, I described the sequences and expression pattern of miR-124 in medaka. I identified the five candidate miR-124 precursor sequences in medaka genome database. I examined the expression of the medaka miR-124 by northern blotting and *in-situ* hybridization with locked nucleic acid (LNA)-modified oligonucleotide probes, which increase sensitivity for the detection of very small RNA molecules. I found out that the medaka mat-miR-124 was specifically expressed in the CNS and in the eyes during embryogenesis similarly to other vertebrate species. To examine the miR-124 expression in more detail, I performed *in-situ* hybridization of transverse sections of the embryo and larva. This result indicated that miR-124 expression of medaka is most likely restricted in differentiated neuron. By RT-PCR and whole mount *in-situ* hybridization, I showed the possibility that the five medaka pri-miR-124s were expressed at different levels. These results suggest that the medaka miR-124 is involved in the neural development and that each homolog from the medaka pri-miR-124s might have spatiotemporally different roles in the embryo and adult tissues.

1.2. Materials and Methods

Animals

Adult and embryonic Japanese medaka, *Oryzias latipes* (orange-red variety), were maintained in the laboratory tanks at 27°C, with a photoperiod of 10 h dark/14 h light (Iwamatsu, 1978). Naturally spawned and fertilized eggs were collected and cultured in distilled water containing 0.6% methylene blue in Petri dish. To let embryos develop normally, it is necessary to remove attachment filament and separate eggs. Attachment filaments were cut with scissors holding eggs with forceps or removed by gently rolling eggs on tissue papers. Developmental stages herein are expressed in terms of days post-fertilization (dpf), are as defined by Iwamatsu (2004).

Dechorionation of embryos

For *in-situ* hybridization, embryos were dechorionated using hatching enzyme (Yamagami, 1972) provided by the National Bio Resource Project (NBRP) Medaka (Okazaki, Japan). Embryos at each stage were washed in distilled water once and rolled on sand paper (#1200, waterproof) with the forefinger to damage the outer surface until the filaments became invisible. After washing in 1× Iwamatsu′s balanced salt solution (BSS) pH 7.4 three times, embryos were placed in 1× BSS with 20mg/ml pronase (Calbiochem) and incubated for 30 min at 27°C. After washing in 1× BSS three times, embryos were placed in 1/2–1/5 hatching enzyme diluted with 1× BSS and incubated at 27°C for 30–90 min (until the embryos were dechorionated). After washing in 1× BSS three times, the dechorionated embryos were maintained in 1× BSS at 27°C. Pronase and hatching enzyme were kept on ice or at 4°C and repeatedly used until activity is lost (approximately 1–2 weeks).

Identification of miR-124 in the medaka genome

To identify miR-124 genes in the medaka genome, I carried out a BLAST search of the Ensembl Medaka genomic database (http://www.ensembl.org/Oryzias_latipes/Info/Index), using the zebrafish pre-miR-124 obtained from miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml) as the query sequence. In addition, I obtained sequences of miR-124 from various other animal species from the miRBase and Rfam (http://www.sanger.ac.uk/Software/Rfam/) databases and also used them to conduct BLAST searches for medaka genomic sequences in Ensembl Medaka. Candidate miR-124 genes identified were aligned by using CLUSTALW software (http://www.genome.jp/tools/clustalw/), and their secondary structures were predicted

with CentroidFold software (http://www.ncrna.org/centroidfold/).

LNA northern *blotting*

Northern blotting was performed with LNA -modified oligonucleotide probes labeled with digoxigenin (DIG) at the 3′ end (Thermo Fisher Scientific, Germany), as previously described (Tani *et al*., 2009). The nucleotide sequences of the LNA probes were as follows: ola-miR-124, TCGCATTCACCGCGTGCCTTAA; *O. latipes* U6 snRNA, AAAATGAGGAACGCTTCACGA (Tani *et al*., 2009).

Total RNA was isolated using ISOGEN (Nippongene). For each developmental stage, 3 µg of RNA was separated on 12% denaturing polyacrylamide gels and blotted onto a nylon membrane (Hybond-N+, GE Healthcare Life Sciences). Blots were prehybridized in hybridization buffer (50% formamide, 5× SSC, 2% blocking solution, 0.1% N-lauroyl sarcosine, 0.1% SDS, 200 µg/ml yeast tRNA) for 1h at 60°C. Blots were washed twice with 2× SSC containing 0.1% SDS and once with 1× SSC containing 0.1% SDS at room temperature for 15 min each, and once with 0.2 × SSC containing 0.1% SDS and 0.1× SSC containing 0.1% SDS at 65°C for 30 min each. After a brief wash with maleic acid buffer (0.15 M NaCl, 0.1 M maleic acid, pH 7.5), the blots were incubated in 1% blocking reagent (Roche) in maleic acid buffer for 30min, and then incubated in 1% blocking reagent containing anti-DIG-AP Fab fragment (Roche) at a concentration of 1/5000 for 30 min at room temperature. The blots were washed twice in maleic acid buffer containing 0.3% Tween20, followed by a brief wash in AP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl). Then, blots were incubated with CDP-star chemiluminescent (Roche) for 5 min at room temprature. Signals were captured on X-ray films or detected by LAS3000 (Fujifilm).

LNA in-situ hybridization

In-situ hybridization with the LNA probes was performed on medaka embryos as previously described (Tani *et al*., 2009). Dechorionated embryos at 5 dpf were fixed in 4% paraformaldehyde (PFA) in PBS and stored in 100% methanol. After rehydration, the embryos were bleached in 6% H_2O_2 in PBT (0.1% Tween20 in PBS) for 1h, and then treated with proteinase K (1 µg/ml for 0 dpf, 2 µg/ml for 1–2 dpf, and 2.5 µg/ml for 3 dpf embryos) in PBT at room temperature for 10 min. The embryos were refixed in 4% PFA, and transferred to hybridization buffer (50% formamide, 5× SSC, 0.1% Tween20, 50 µg/ml yeast tRNA, 50 µg/ml heparin). After pre-hybridization, the embryos were transferred to fresh hybridization buffer containing the same miR-124 LNA probe used for northern blotting, and were incubated at 45°C overnight.

Subsequently, the embryos were washed at 45°C three times in 50% formamide, 2× SSCT (SSC containing 0.1% Tween-20) for 30 min each and three times in 2× SSCT for 15 min each. After a brief wash with PBT, the embryos were blocked in 1% blocking reagent (Roche) in PBT at room temperature for 1h. Antibody reaction was carried out in 1% blocking reagent containing anti-DIG-AP Fab fragment (Roche) at a concentration of 1/5000 at 4°C overnight. The embryos were washed in blocking reagent in PBT four times for 30 min each time, incubated in staining buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 0.1% Tween20, 1 mM levamisole) for 10 min, and then stained in staining buffer containing 450 μg/ml NBT (Sigma) and 175 μg/ml BCIP (Sigma). Embryos were washed three times in PBT and profixed in 4% PFA. To obtain transverse sections, samples were embedded in Tissue-tek (Sakura Finetek) and frozen at –80°C. The frozen samples were cryosectioned at a thickness of 10 μm at –25°C. The sections were fixed in 4% PFA, and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine/HCl buffer (pH 8.0). Hybridization was conducted at 45°C for 16–18 h in hybridization buffer (50% formamide, 5× Denhardt's solution, 0.5 mg/ml yeast tRNA, 6×SSPE).

Hematoxylin-eosin staining

Larvae were fixed in Bouin's fluid (picric acid saturated aqueous solution: formalin: acetic acid = 15: 5: 1) at 45°C overnight. After dehydration with ethanol and xylene, the specimens were embedded in paraffin and sliced serially into 5 μm thich sections. The sections were stained with Delafield's hematoxylin and eosin (HE).

Semi-quantitative RT-PCR

To investigate whether all five loci detected for medaka miR-124 actually produce mat-miR-124, I performed semi-quantitative RT-PCR using primers (Table 1.1) corresponding to *pri-miR-124* homologs and total RNA extracted with Isogen (Nippongene, Tokyo) from whole, stage-34 embryos and from adult tissues. First-strand cDNA synthesis was performed with ReverTra Ace (Toyobo). *OlCA1* (AB525196) was used as a positive control (Kusakabe *et al*., 1999). PCR was conducted with Ex taq DNA polymerase (TaKaRa) and normalized amounts of template. PCR conditions were 30 cycles of 98°C for 10 sec, 60°C for 30 sec, and 72°C for 1 min, followed by 7 min at 72°C. In control reactions using the *OlCA1* primers, 20 PCR cycles were used. PCR products were electrophoresed on 1.5% agarose gel in TAE buffer, and then signals were detected after staining with ethidium bromide. Expression of *ola-miR-124* is

presented as expression relative to that of the internal control (relative expression), with values given as the mean ± SD of three independent experiments. Differences in expression among tissue sources were evaluated with Student's *t*-test.

In-situ hybridization with RNA probes

Whole mount *in-situ* hybridization with RNA probes was performed according to the protocol described by Inohaya *et al.* (1995). DIG-labeled RNA probes were synthesized from cDNA fragments containing medaka *pri-miR-124* sequences by PCR amplification with the primers (Table 1.1). The cDNA fragments were cloned into pBluescript II SK+ (Stratagene) and the plasmid DNA were linearized by cutting with *Xho*I or *Eco*RI, which were used for antisense or sense primers, respectively. RNA probes were transcribed using T3 or T7 RNA polymerase from using a DIG RNA labeling mix (Roche). The RNA probes were purified through ProbeQuant G-50 Micro Columns (GE Healthcare). Dechorionated embryos were fixed in 4% PFA in PBS and stored in MeOH. After rehydration, the embryos were bleached using 6% H_2O_2 in PBT for 1h, and were treated with proteinase K (1 μg/ml for 0 dpf, 2 μg/ml for 1–2 dpf, and 2.5 μg/ml for 3 dpf embryos) in PBT at room temperature for 10 min. The embryos were refixed in 4% PFA and transferred to hybridization buffer (50% formamide, 2× SSC, 0.1% Tween20, 50 μg/ml yeast tRNA, 50 μg/ml heparin) and incubated at 55 or 60°C for 1 h. The embryos were transferred to fresh hybridization buffer containing RNA probes and incubated at 55 or 60 °C overnight. Subsequently, the embryos were washed at 55 or 60 °C two times in 50% formamide, 2× SSCT for 30 min each, and at 37 °C two times in 2× SSCT for 15 min each. After a brief wash with PBT, the embryos were incubated at 37°C with 50 μg/ml RNaseA in PBT for 30 min to remove excess RNA probes. The embryos were washed once at 37°C in 2× SSCT for 10 min, once at 55 or 60 °C in 50% formamide, 2× SSC for 1 h, and two times at 55 or 60°C in 0.2× SSCT for 15 min each. After a brief wash with PBT, the embryos were blocked in 1% blocking reagent (Roche) in PBT at room temperature for 1h. Antibody reaction was carried out in 1% blocking reagent containing anti-DIG-AP Fab fragment (Roche) at a concentration of 1/5000 at 4°C overnight. The embryos were washed in blocking reagent in PBT four times for 30 min each time. The embryos were then incubated in staining buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 0.1% Tween20, 1 mM levamisole) for 10 min, and then stained in staining buffer containing 450 μg/ml NBT (Sigma) and 175 μg/ml BCIP (Sigma). Embryos were washed three times in PBT and profixed in 4% PFA.

1.3. Results

Analysis of medaka miR-124 sequences

I detected five candidates for medaka miR-124 (ola-miR-124) precursor genes that contained mat-miR-124 (miR-124-3p) sequences of 21 nt long (Table 1.2). Two of the five candidate genes (*ola-miR-124-1* and *ola-miR-124-2*) were distantly located on chromosome 7, and the others were located on different chromosomes. The genes were 59 to 104 nt long. Three of them had been registered in miRBase, as *ola-miR-124-1*(MI0019450) on chromosome 7, *ola-miR-124-2* (MI0019452) on chromosome 7, and *ola-miR-124-3* (MI0019533) on chromosome 20 (Li *et al*., 2010). I named the remaining two, *ola-miR-124-4* on chromosome 17 (ENSORLT00000026119) and *ola-miR-124-5* on chromosome 24 (ENSORLT00000026119). In the Ensembl medaka genome, *ola-miR-124-5* is located in the intron of a novel protein-coding gene; the other four candidate genes are located in intergenic regions. The pre-miR-124 sequences were all predicted to fold into a typical stem-loop structure, with the mature sequence on the 3′ side of the stem region (Fig. 1.1).

An alignment of miR-124 sequences from medaka and other, representative vertebrate species (Table 1.3) shows that mat-miR-124 is highly conserved in vertebrates (Fig. 1.2A). Teleosts have the three common types of pre-miR-124 found in mammals (miR-124a-1, miR-124a-2, and miR-124a-3) (Fig. 1.2B), and the primary region in miR-124a-1, which includes the loop region, is completely conserved among medaka, two other teleosts, and two mammals (human and mouse) (indicated by asterisks and heavy dashed lines in Fig. 1.2B). Medaka ola-miR-124-2 and -4 do not match the three mammalian pre-miR-124s because they lack the sequence corresponding to miR-124* (miR-124-5p) in the 5′ arm of the hairpin (underlined in the mammalian sequences in Fig. 1.2B).

MiR-124 expression during medaka embryogenesis

Northern blotting using LNA probes complementary to mature miR-124 revealed temporal changes in the expression of the 21-nt, single-stranded RNA, during medaka embryongenesis (Fig. 1.3A). No signal bands were detected from stage-8 or -18 embryos at 0 and 1 dpf, respectively, and only a weak signal band was detected from stage-25 embryos. The signals gradually intensified from stage-28 to -36 as embryogenesis proceeded. Pre-miR-124 (~80 nt) gave much weaker signals than

mat-miR-124 on the northern blot (Fig. 1.3A), indicating that pre-miR-124 is rapidly converted into mat-miR-124. Whole mount *in-situ* hybridization using the same probe as for northern blotting showed putative miR-124 expression in the brain and the eyes (Fig. 1.3B, C). In a transverse section of the embryonic eye, signal was evident in retina but not detected in the proliferative ciliary marginal zone (CMZ) and the optic nerve (Fig. 1.3E, F). I also investigated miR-124 expression of transverse sections of medaka larval brain (Fig. 1.4). Compared to HE staining, miR-124 was markedly expressed in perikarya of telencephalon, optic tectum, and cerebellum (Fig. 1.4).

To investigate whether all five loci detected for medaka miR-124 actually produce mat-miR-124, I performed semi-quantitative RT-PCR using primers corresponding to pri-miR-124. The results indicate differential expression of medaka pri-miR-124s during embryogenesis and in adult tissues (Fig. 1.5A). No expression was detected for any of the pri-miR-124s in the adult liver. Pri-miR-124-3 was the most strongly expressed form, with moderate expression in stage-34 embryos and the adult brain, and significantly higher expression in the adult eye (Fig. 1.5B). In contrast, pri-miR-124-1, -2, and -5 were equivalently expressed in the embryo and adult eye and brain, within the limits of ability to detect differences. Pri-miR-124-1 showed slightly higher and more uniform expression in these tissue sources. Pri-miR-124-2 and -5 were expressed in all the three sources at levels ranging from low to very low but detectable. Pri-miR-124-4 was expressed at very low levels in the adult brain and eye, and no expression was detected in the embryo. Furthermore, I prepared antisense and sense RNA probes against pri-miR-124 for whole mount in-situ hybridization. Although by no means clear signals, the brains of 5-dpf embryos were stained with the antisense probes against pri-miR-124-1, -2, -3, and **-**5 (left in Fig. 1.6A–D). No signals were observed by using the sense probes (right in Fig. 1.6A–D). In Fig. 1.6B, the frontal zone of the cerebellum seemed to be specifically stained with antisense probe against pri-miR-124-2.

1.4. Discussion

I found that medaka, like other vertebrates studied to date, has multiple copies of genes transcribing pre-miR-124 (Table 1.2). The five copies of medaka miR-124 genes are separately located on different chromosomes, differing in nucleotide length, and can potentially generate functional miRNAs through conventional miRNA pathway by folding into stem-loop structures (Fig. 1.1). In contrast to medaka, zebrafish has six copies of miR-124 genes, according to miRBase. Although pre-miR-124 sequences are nearly identical between mammals and fishes, the multiple copies of genes transcribing pre-miRNAs in each vertebrate genome can be expected to have different but perhaps overlapping functions. Semi-quantitative RT-PCR results suggest that the five forms of medaka pri-miR-124s have different functions between the stage-34 embryo and the adult brain and eyes (Fig. 1.5). For example, *ola-miR-124-3* transcription is markedly elevated in the adult eye, whereas *ola-pri-miR-124-1*, *-2*, and *-5* appear to be equivalently transcribed in the embryo and adult eye and brain. Whole mount *in-situ* hybridization for pri-miR-124s also showed the possibility of functional diversity of the medaka miR-124 (Fig. 1.6). The three miR-9 genes in humans, on chromosomes 1, 5, and 15, are separately transcribed from different promoters and regulate the expression of different genes (Bazzoni *et al.*, 2009; Laneve *et al.*, 2010; Hsu *et al.*, 2009). Genomic organization and the number of gene copies are related to expression levels (Olena and Patton, 2009). In medaka and zebrafish, the miR-430 family has 16 and 72 copies, respectively, clustered on chromosome 4. The copies are expressed abundantly early in embryogenesis and function for a short period (Thatcher *et al.*, 2008; Tani *et al.*, 2009).

By *in-situ* hybridization, I showed that medaka mat-miR-124 is strongly expressed in the CNS and the eyes during embryogenesis and later development (Fig. 1.3B, C, E, and F), as has been reported for other vertebrates, including zebrafish, *Xenopus laevis*, chick, and mouse (Wienholds *et al.*, 2005; Cao *et al.*, 2007; Makeyev *et al.*, 2007; Qiu *et al.*, 2009). In the eyes, mat-miR-124 is expressed in the retina, whereas, unexpressed in the proliferative CMZ containing multipotent retinal progenitors (Fig. 1.3 E, F), suggesting that miR-124 is absent from proliferative cells and widely expressed in differentiated neural cells. These results were consistent with mouse and zebrafish miR-124 expression (Darnell *et al.*, 2006; Kapsimali *et al.*, 2007). MiR-124 is still abundantly expressed in the brain and eye of larva and adult (Fig. 1.4 and 1.5). In medaka, cell proliferation and differentiation continue in the brain after hatching (Alunni *et al.*, 2010). MiR-124 is thought to regulate adult neurogenesis in medaka.

Additionally, the northern blot analysis showed that medaka pre-miR-124 is

expeditiously processed into mat-miR-124, and that the amounts of the latter steadily and drastically increase as the embryogenesis progresses (Fig. 1.3A). The period (stages 25-36) over which I observed the increase in mat-miR-124 levels coincides with the development of the CNS in medaka, suggesting that the former may be involved in the latter. In stage 25 (the neural-tube stage), the five brain vesicles (telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon) are still developing (Ishikawa, 1997); in this stage, the mesencephalon expands dorsolaterally, forming a well-developed optic tectum covering the rostral half of the cerebellum (Kage *et al*., 2004). Starting in stage 26, cells proliferate extensively in the optic tectum and begin to migrate to the outer layer; cell differentiation begins at stage 30 (Nguyen *et al*., 1999). The basic structure of the medaka brain, including neural pathways, has completely formed by late embryonic stage 34. Considering the time course of medaka neurogenesis, the temporal expression pattern of medaka miR-124 suggests that it is essential for neural development.
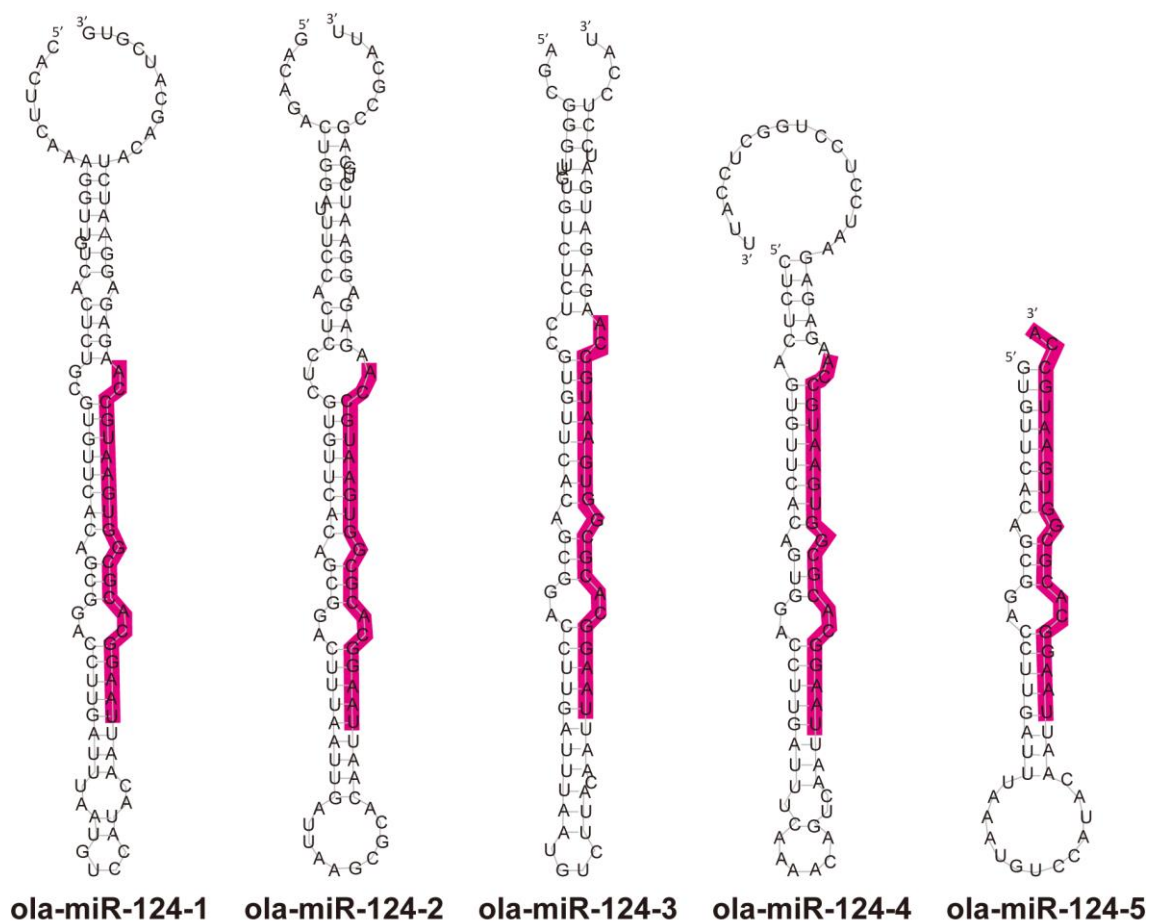
1.5. Figures and Tables

Fig. 1.1 Predicted secondary structures of medaka miR-124 precursors using CentroidFold. Five pre-miR-124 candidates are folded into typical stem-loop structure. Mat-miR-124 sequences are indicated in magenta shading.

A

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ola-miR-124   -UAAGGCACGCGGUGAAUGCCA-  21
dre-miR-124   -UAAGGCACGCGGUGAAUGCCAA  22
fru-miR-124   -UAAGGCACGCGGUGAAUGCCAA  22
xtr-miR-124   UUAAGGCACGCGGUGAAUGCCA-  22
mmu-miR-124   -UAAGGCACGCGGUGAAUGCC--  20
hsa-miR-124   -UAAGGCACGCGGUGAAUGCC--  20
              *********************
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B

miR-124-1
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                  10        20        30        40        50        60        70        80
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-miR-124a-1  AGGCCUCUCUCUCCGUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCCAAGAAUGGGGCUG  85
mmu-miR-124a-1  AGGCCUCUCUCUCCGUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCCAAGAAUGGGGCUG  85
fru-miR-124-2   -CUGGUCUCUCUCCGUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCCAAGAGAG------  78
dre-miR-124-2   -CCUGCUUUUCUUCGUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCCAAGAGAGAUGGC-  83
ola-miR-124-5   --------------GUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCCA-----------  59
                              *****************************************************************
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miR-124-2
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                    10        20        30        40        50        60        70        80        90
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-miR-124a-2  -----AUCAAGAUUAGAGGCUCUG-CUCUCCGUGUUCACAGCGGACCUUGAUUUAAUGUCUAUACAAUUAAGGCACGCGGUGAAUGCCAAG  84
mmu-miR-124a-2  -----AUCAAGAUCAGAGACUCUG-CUCUCCGUGUUCACAGCGGACCUUGAUUUAAUGUCAUACAAUUAAGGCACGCGGUGAAUGCCAAG  84
fru-miR-124-1   ---------------GGUUGUGUCUCCGUGUUCACAGCGGACCUUGAUUUAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCCAAG  73
dre-miR-124-3   GGCUCUGUGGGAUUUCAGACUCUGGCUUUCCGUGUUCACAGCGGACCUUGAUUUAAUGUCUUACAAUUAAGGCACGCGGUGAAUGCCAAG  90
ola-miR-124-3   -------------AGCGGGUUGUGUCUCUCCGUGUUCACAGCGGACCUUGAUUUAAUGUCUUACAAUUAAGGCACGCGGUGAAUGCCAAG  77
                *   *  **  ****************************************** **** ****************************
```
```
                  100       110
         ....|....|....|....|....|..
hsa-miR-124a-2  AGCGGAGCCUACGGCUGCACUUGAA--  109
mmu-miR-124a-2  AGCGGAGCCUACGGCUGCACUUGAA--  109
fru-miR-124-1   AGAU-----------------------  77
dre-miR-124-3   AGCGGAGCCUUUUAACAUCAGCAGGCC  117
ola-miR-124-3   AGAUGAUCCUCCAU-------------  91
                **
```

miR-124-3
```
                    10        20        30        40        50        60        70        80        90
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-miR-124a-3  -----------------------UGAGGGCCC-CUCUGCGUGUUCACAGCGGACCUUGAUUUAAAUGUCUAUACAAUUAAGGCACGCGGUG  66
mmu-miR-124a-3  --------------------------------CUCUGCGUGUUCACAGCGGACCUUGAUUUUAAUGUCUAUACAAUUAAGGCACGCGGUG  57
fru-miR-124-3   -----------------------GGUUUGAGCUCUUUGUGUUCACAGUGGACCUUGAUUUAAAUUUCAAUACAAUUAAGGCACGCGGUG  65
dre-miR-124-5   GGGUUUUGCUCGUGCGUUCUUUUUGAGUUCUCGCUCUGCGUGUUCACAGCGGACCUUGAUUUAAUGUCCAUACAAUUAAGGCACGCGGUG  90
ola-miR-124-1   ----------------CACUUCAAAGGUUGUCACUCUGCGUGUUCACAGCGGACCUUGAUUUAAUGUCCAUACAAUUAAGGCACGCGGUG  74
                                ****  ********** **************** **  *************************
```
```
                  100       110       120
         ....|....|....|....|....|....|....|....
hsa-miR-124a-3  AAUGCCAAGAGAGGCGCCUCC------------------  87
mmu-miR-124a-3  AAUGCCAAGAG--------------------------  68
fru-miR-124-3   AAUGCCAAGAGAGAAGCC-------------------  83
dre-miR-124-5   AAUGCCAAGAGAAGAAUCUCUCCAGCAACGAGUUUGCGC  129
ola-miR-124-1   AAUGCCAAGAGAGGAAUCUACAGCAUCGUG--------  104
                **********
```

Fig. 1.2. Multiple alignments of verterbrate miR-124. **(A)** MiR-124 sequences of medaka (ola-) (Accession: MIMAT0022573, currently registered as miR-124-3p), zebrafish (dre-) (MIMAT0001819), takifugu (fru-) (MIMAT0002896), *Xenopus* (xtr-) (MIMAT0003683) mouse (mmu-) (MIMAT0000134, currently registered as miR-124-3p), and human (hsa-) (MIMAT0004591, currently registered as miR-124-3p) aligned by CLUSTALW algorithm. Seed regions are indicated in box. **(B)** Three types

of pre-miR-124 which are homologous to mammals existed in teleosts. Mature regions of pre-miR-124s are indicated in magenta letters. Mammalian pre-miR-124 included star sequences, mat-miR-124*, are indicated in black lines. Dashed black lines indicate loop regions in the secondary structures. Asterisks indicate nucleotides conserved in all the listed genes (A, B).

A

| | 0 | 1 | 2 | 3 | 4 | 6 dpf |
| stage | 8 | 18 | 25 | 28 | 32 | 36 |

80 nt –   pre-miR-124

21 nt –   mat-miR-124

U6

miR-124   miR-124

B   5 dpf   C   3 dpf

CE   MO   MO
OT   SC   CE   SC
                N   OT   N
DI   TE
TE   E
E   *   *

D

Cerebellum
(CE)   Medulla oblongata
(MO)

Optic tectum
(OT)

Telencephalon
(TE)   Spinal cord
(SC)
                        Notochord
                        (N)

                Diencephalon
                (DI)

Eye (E)
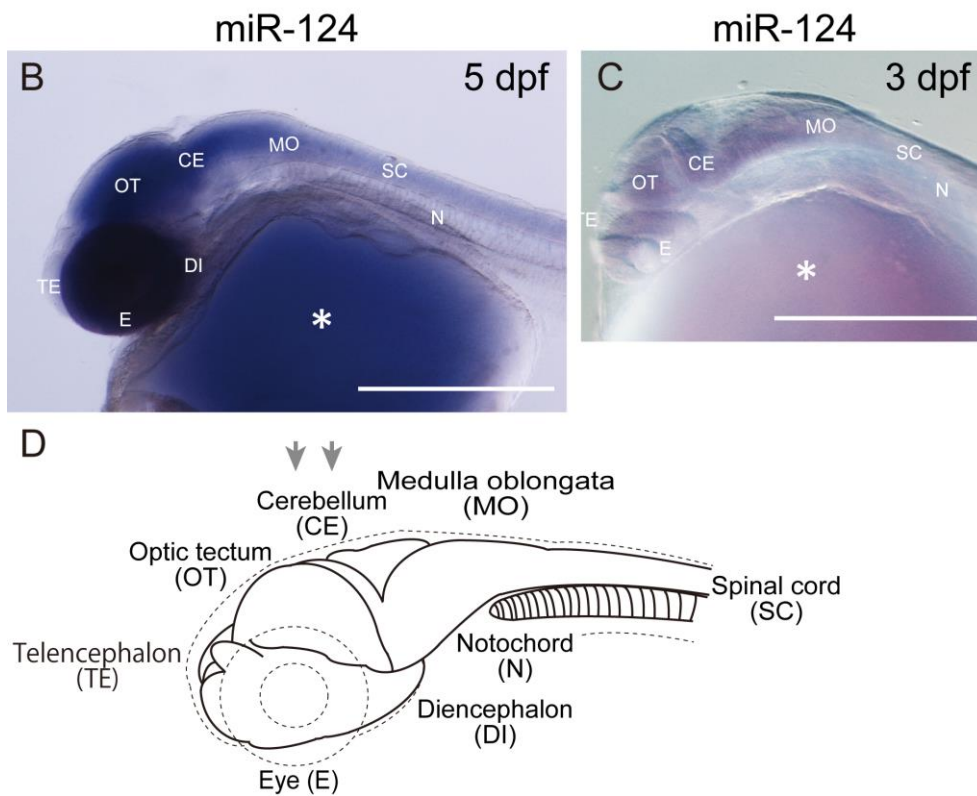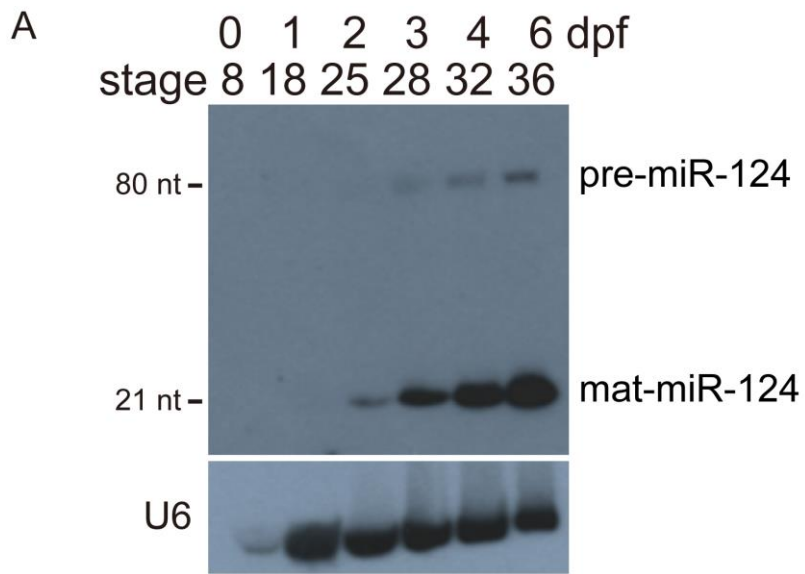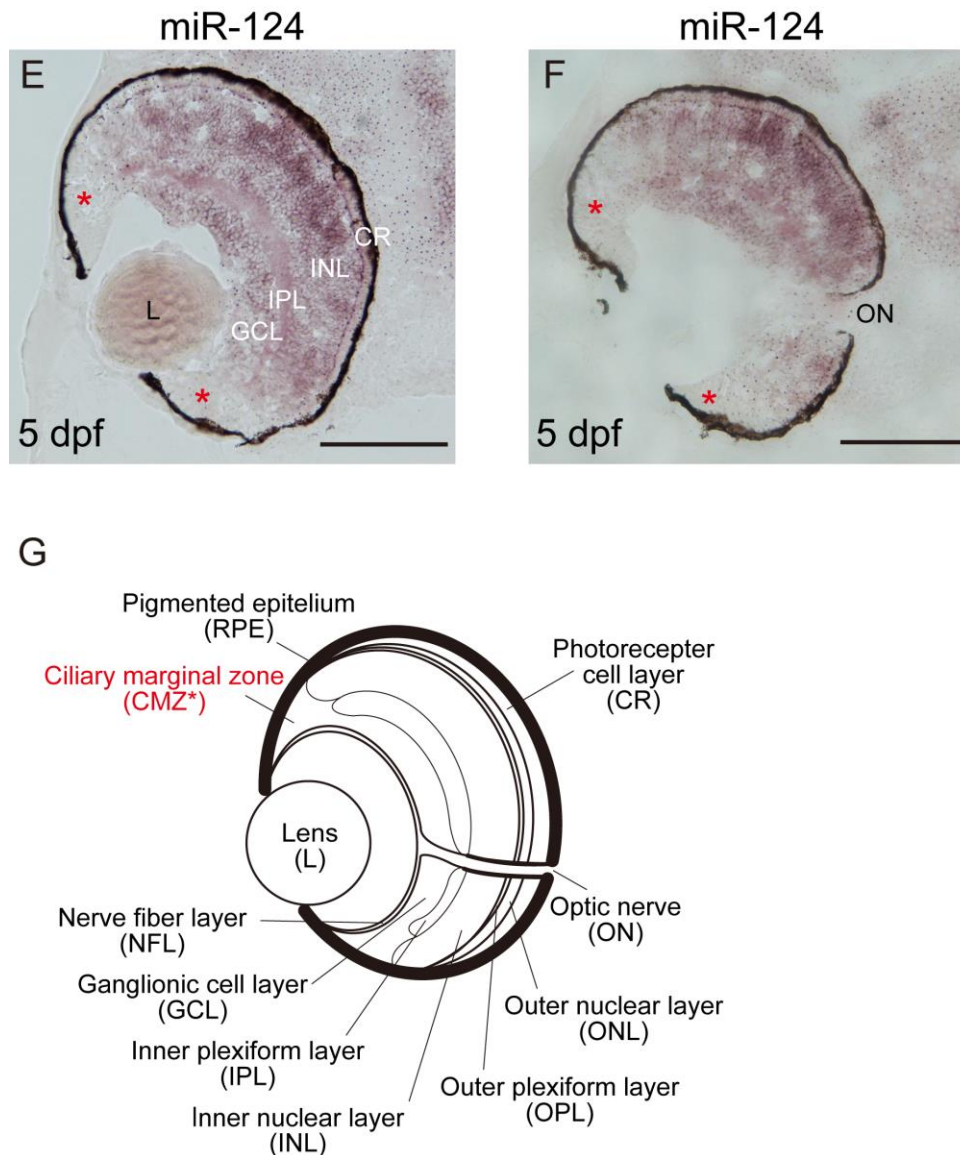
Fig. 1.3. Spatiotemporal expression of medaka miR-124. **(A)** Northern blotting of total RNA extracted from different developmental stage embryos. Each lane is loaded with 3 μg of total RNAs. The membrane was hybridized with LNA probes complementary to either ola-miR-124 or U6 snRNA. dpf, days post-fertilization. **(B, C)** Whole mount *in-situ* hybridization of 5- and 3-dpf embryo. The embryos were bleached using 6% H_2O_2. White asterisk indicates yolk sac. Scale bar = 500 μm. **(D)** Schematic illustration of medaka CNS in late-stage embryonic development (5 dpf). **(E, F)** Transversal section *in-situ* hybridization of embryonic eyes at 5-dpf. Scale bar = 100 μm. Red asterisks indicate CMZ. **(G)** Schematic illustration of medaka eye in late-stage embryonic development (5dpf). B, C, E, and F were hybridized with the miR-124 LNA probe used for northern blotting.
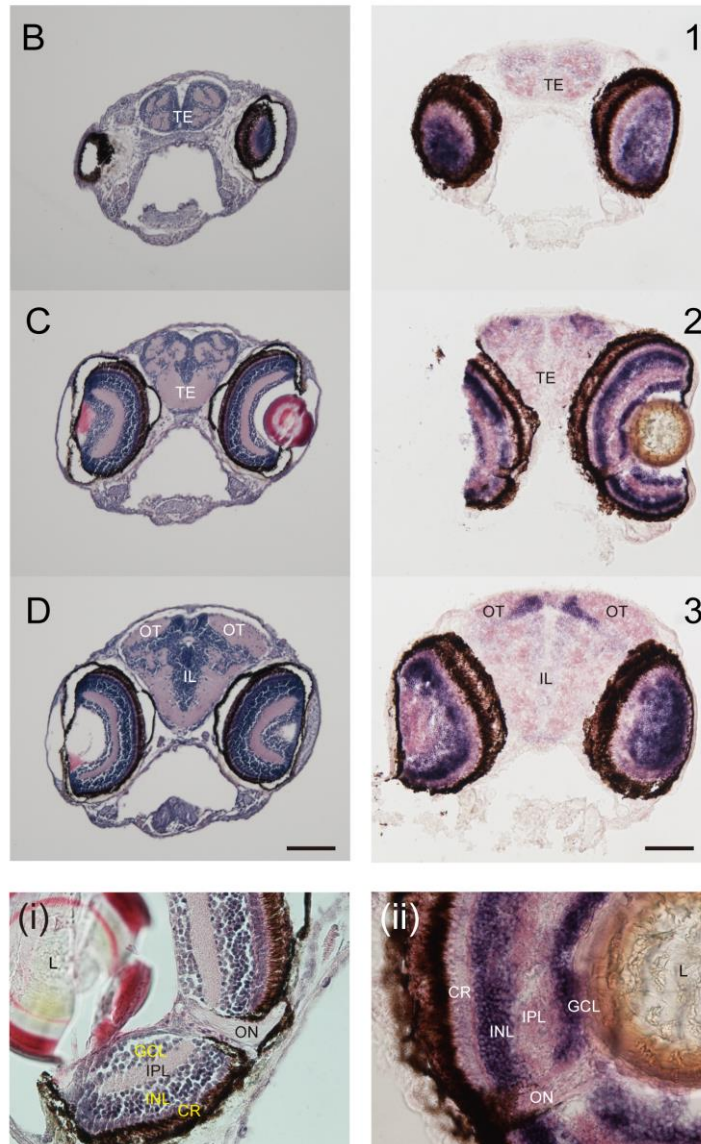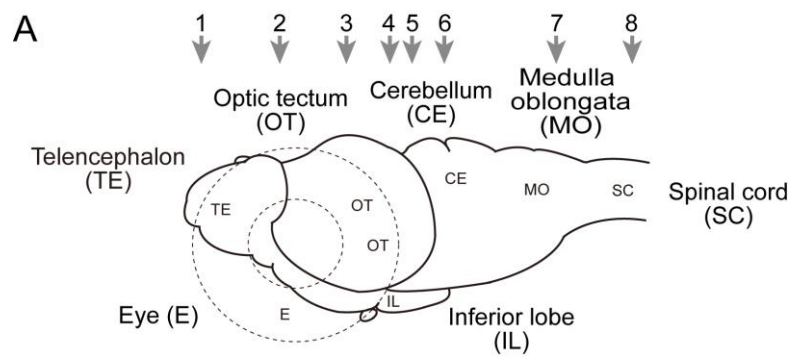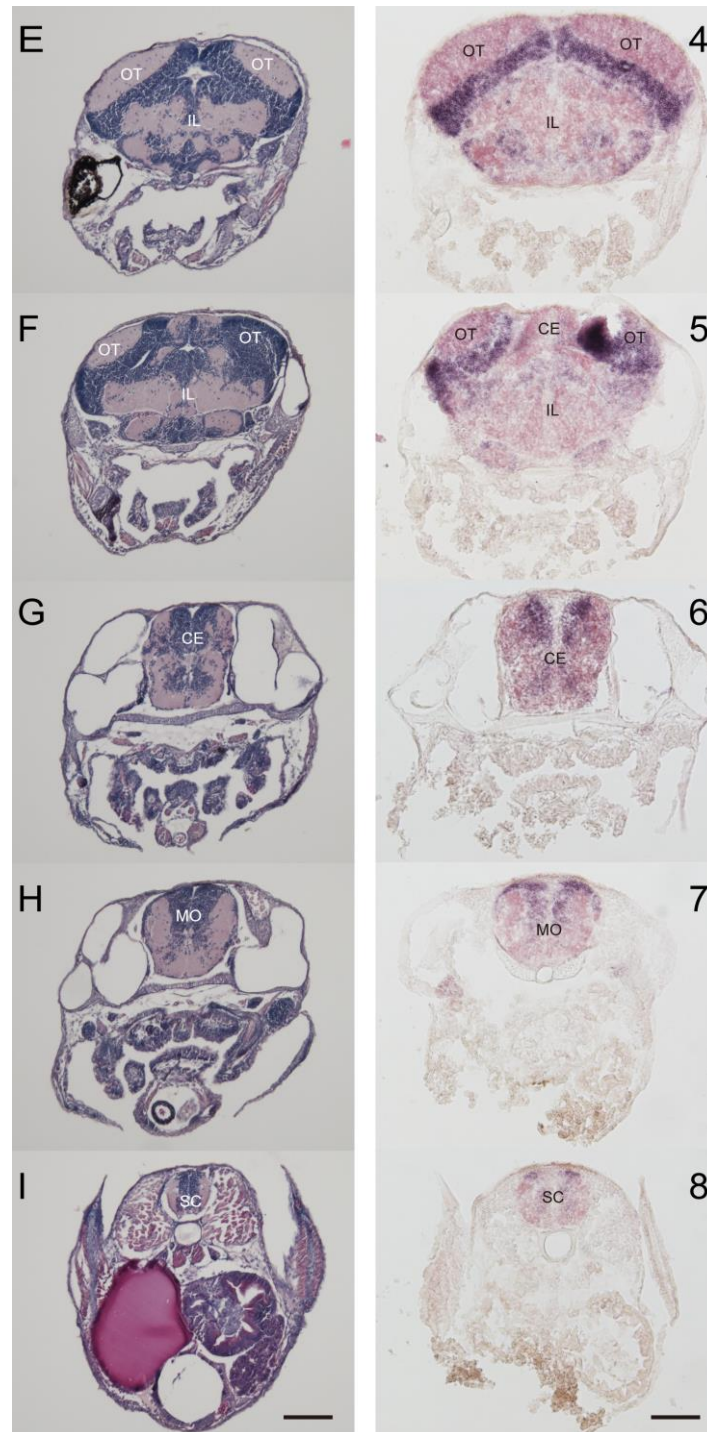
Fig. 1.4. **(A)** Schematic illustration of medaka CNS at larval stage. The numbers and arrows indicate the locus of sections in 1–8. **(B–I, 1–8)** HE staining and *in-situ* hybridization of transverseal sections of medaka brain at larval stage. The figures of the eyes are showed in (i) and (ii). Abbreviations of the eyes are described in Fig. 1.3.1–8 and (ii) were hybridized with the miR-124 LNA probe used for northern blotting. Scale bar = 100 μm.
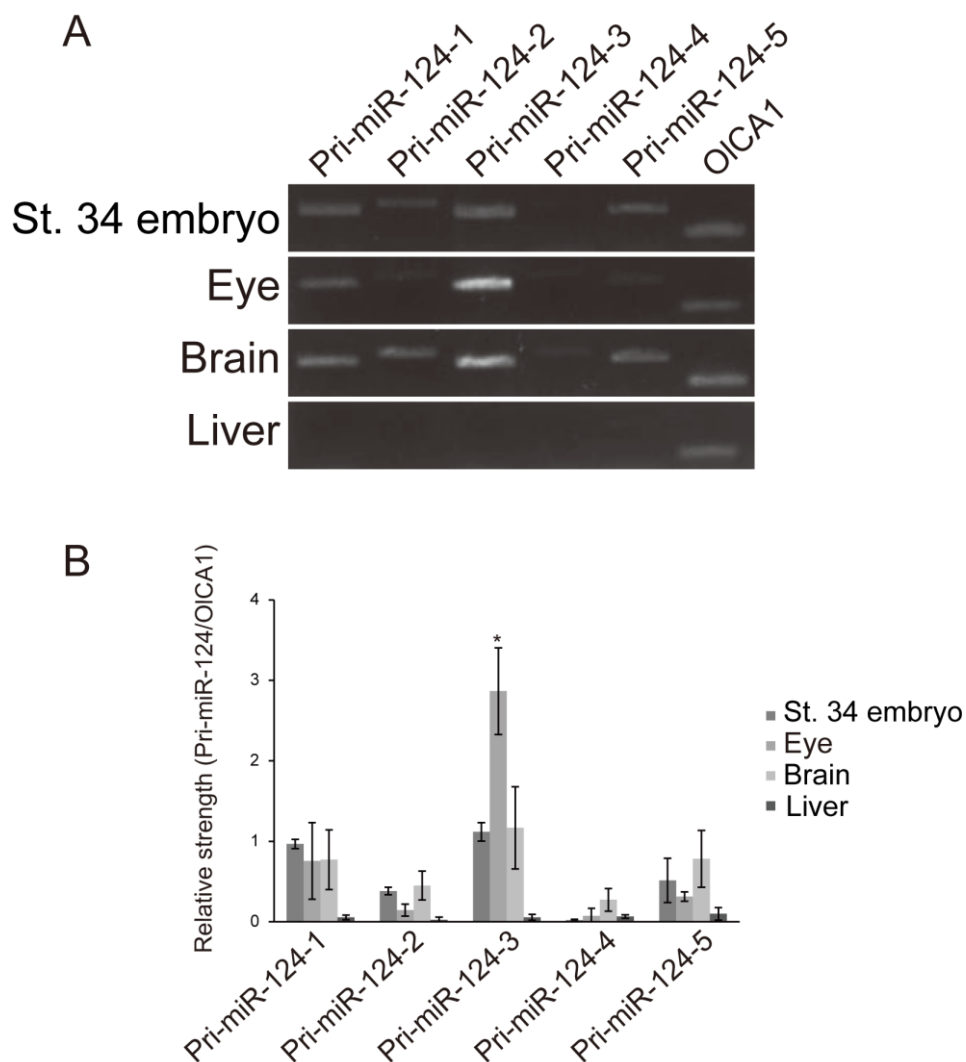
Fig. 1.5. Semi-quantitative RT-PCR using total RNA isolated from St. 34 embryos, adult brain, eye, and liver, for analyzing *pri-miR-124* expression. Relative strength of *pri-miR-124* expressions are compared with those of *OlCA1* as an internal control. In all experiments, values represent the means ± SD of three independent experiments. Asterisk indicates significant difference (P<0.05).
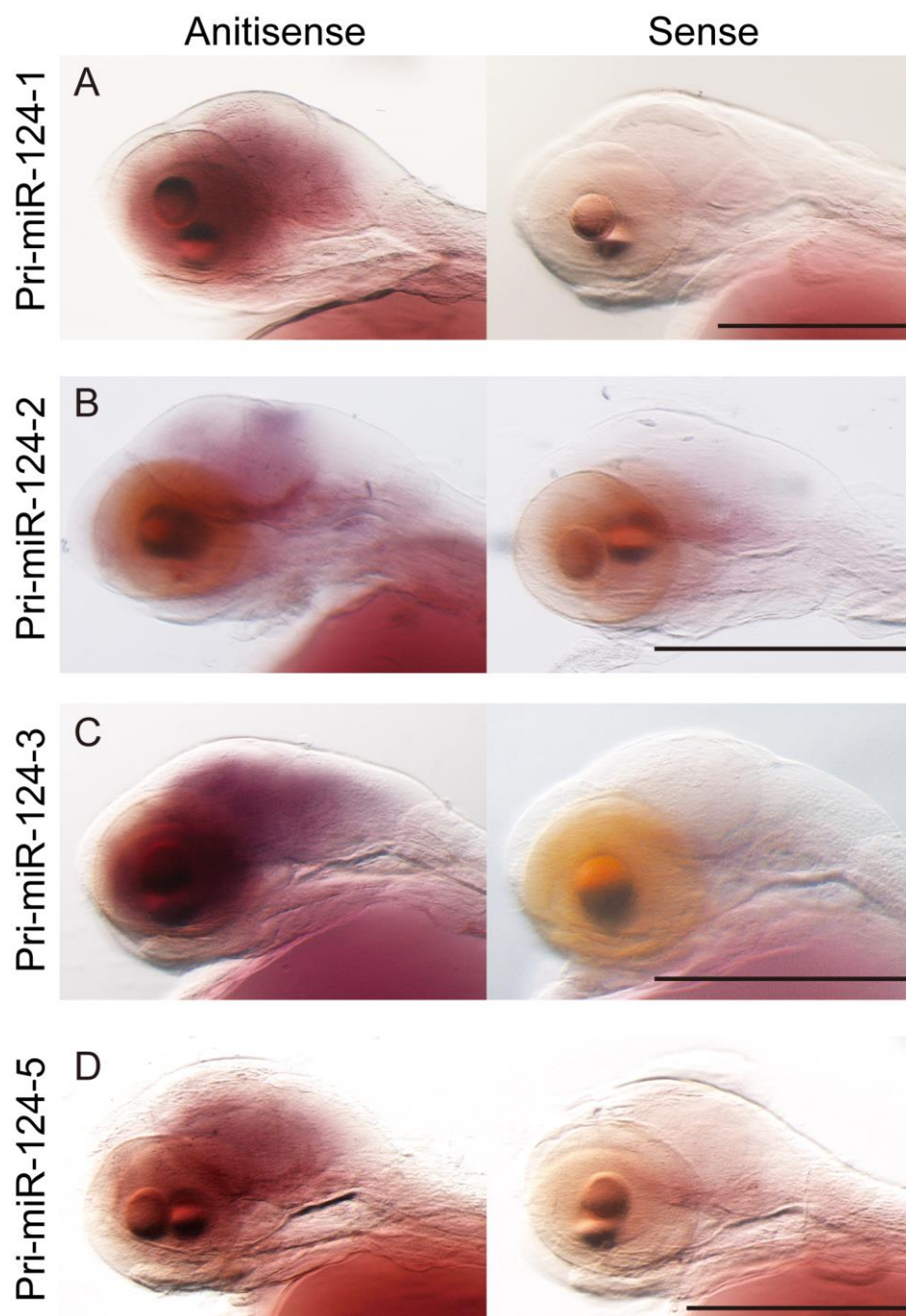
Fig. 1.6. Whole mount *in-situ* hybridization for the medaka pri-miR-124s. **(A–D)** Whole mount *in-situ* hybridization using digoxigenin-labeled antisense probe (left) and sense probe (right). Scale bar = 500 μm.

Table 1.1. Primers used in RT-PCR. Underlines indicate the restriction enzyme sites.

Gene		Nucleotide sequences	Size of amplified products (bp)
ola-pri-miR-124-1	Forward	TTTCTCGAGGCGTGTCTATGTACCCATGT	508
	Reverse	TGGGAATTCAAAGAACCTGCGCATTGCGT	
ola-pri-miR-124-2	Forward	GAGCTCGAGAAGCGTCAGAGAGATCTGGA	575
	Reverse	CTGGAATTCTGAGTGTCCGCTAAGGATGT	
ola-pri-miR-124-3	Forward	CTACTCGAGAACCACGATGGAAACTGCTC	484
	Reverse	TTTGAATTCATCGAGGCATGGAGGATCAT	
ola-pri-miR-124-4	Forward	CTGCTCGAGGCTGTGTCCTTGACATGTCA	566
	Reverse	TCAGAATTCAGCACCTAAAGCAGAGCCTA	
ola-pri-miR-124-5	Forword	GGTCTCGAGAAAGTGGATGGGAGGAAGCA	508
	Reverse	TCTGAATTCTGGTCCAACAGAGAACCACT	
OLCA1	Forward	CAGACACGTATTTGCCTCTG	312
	Reverse	CAAGTCGGAACACATGTGCA	

Table 1.2. Chromosomal locations and nucleotide sequences of miR-124 precursor genes in medaka. Mature regions of miR-124 are indicated in upper case. *, transcription products registered in miRBase (Accession numbers: MI0019450, MI0019452, MI0019533).

Gene	Position			Strand	Size	Precursor sequences
	Chr	Start	End			
ola-miR-124a-1	7	16234646	16234749	-1	104	cacuucaaagguugucacucugcguguucacagcggaccuugauuuaauguccauacaauU AAGGCACGCGGUGAAUGCCAAGAGAGGAaucuacagcaucgug*
ola-miR-124a-2	7	22012171	220122174	-1	104	gacagacuggauuuccacuccucguguucacagcggacuuuaauugauuaagcgcacaauU AAGGCACGCGGUGAAUGCCAagagaggaaucugcagccgcauu*
ola-miR-124a-3	20	15452264	15452354	-1	91	agcggguugugucucuccguguucacagcggaccuugauuuaaugucuuacaauUAAG GCACGCGGUGAAUGCCAagagaugauccuccau*
ola-miR-124a-4	17	122960177	12960263	1	87	cucucaguguucacaguggaccuugauuucaaaacagucaauUAAGGCACGCGGU GAAUGCCAagagagaauccuccuggcuccauu
ola-miR-124a-5	24	1126727	1126785	1	59	guguucacagcggaccuugauuuaaauguccauacaauUAAGGCACGCGGUGA AUGCCA

Table 1.3. Chromosomal locations and nucleotide sequences of miR-124 precursor genes in human (hsa-), mouse (mmu-), and zebrafish (dre-). All the sequences are registered in miRBase. Accession numbers: human (MI0000443, MI0000444, MI0000445); mouse (MI0000716, MI0000717, MI0000150); zebrafish (MI0001966, MI0001967, MI0001968, MI0001969, MI0001970, MI0001971). Mature regions of miR-124 are indicated in upper case.

| miRNA | Position | | | Strand | Size | Precursor sequences |
	Chr	Start	End			
hsa-miR-124a-1	8	9760898	9760892	-1	85	aggccucucucuccguguucacagcggaccuugauuuaaauguccauacaauUAAGGCACGCGGUGAAUGCCaagaauggggcug
hsa-miR-124a-2	8	65291706	65291814	1	109	aucaagauuagaggcucugcucuccguguucacagcggaccuugauuuaaugucauacaauUAAGGCACGCGGUGAAUGCCaagagcggagccuacggcugcacuugaa
hsa-miR-124a-3	20	61809852	61809938	1	87	ugagggccccucugcguguucacagcggaccuugauuuaaugucuauacaauuAAGGCACGCGGUGAAUGCCaagagaggcgccucc
mmu-miR-124a-1	14	65209494	65209578	1	85	aggccucucucuccguguucacagcggaccuugauuuaaauguccauacaauUAAGGCACGCGGUGAAUGCCaagaauggggcug
mmu-miR-124a-2	3	17695562	17695770	1	109	aucaagaucagagacucugcucuccguguucacagcggaccuugauuuaaugucauacaauUAAGGCACGCGGUGAAUGCCaagagcggagccuacggcugcacuugaa
mmu-miR-124a-3	2	180628745	180628812	1	68	cucugcguguucacagcggaccuugauuuaaugucuauacaauUAAGGCACGCGGUGAAUGCCaagag
dre-miR-124a-1	2	26793487	26793568	-1	82	ggcucucgcuguacguguucacaguggaccuugauuuauuguauuucaauUAAGGCACGCGGUGAAUGCCAAcagcacagcc
dre-miR-124a-2	20	38930240	38930322	-1	83	ccugcuuuucuucguguucacagcggaccuugauuuaaauguccauacaauUAAGGCACGCGGUGAAUGCCAAgagagauggc
dre-miR-124a-3	24	25063580	25063696	1	117	ggcucugugggauuucagacucuggcuuuccguguucacagcggaccuugauuuaaugucuuacaauUAAGGCACGCGGUGAAUGCCAAgagcggagccuuuuaacaucagcaggcc
dre-miR-124a-4	17	27261182	27261264	1	83	gguuuuugcucuuugcguguucacaguggaccuugauuuaauuucaauacaauUAAGGCACGCGGUGAAUGCCAAgagagaagcc
dre-miR-124a-5	23	17954131	17954259	1	129	ggguuuugcucgugcguucuuuuugaguucucgcucugcguguucacagcggaccuugauuuaauguccauacaauUAAGGCACGCGGUGAAUGCCAAgagaagaaucucuccagcaacgaguuugcgc
dre-miR-124a-6	8	23975705	23975817	-1	113	ggguggugacacaggcccgccacucugcguguucacggcggaccuugauuuaauauccauacaauUAAGGCACGCGGUGAAUGCCAAaccggagaggggucuuaaaacgacaa

Chapter 2

Sequences and expression of miR-124 target genes,

Polypyrimidine tract binding proteins

2.1. Introduction

The polypyrimidine tract binding protein 1 (PTBP1, also known as PTB or hnRNP I) is an RNA binding protein of 58 kDa. PTBP1 was first identified in HeLa cell nuclear extracts (Garcia-Blanco *et al*., 1989; Patton *et al*., 1991). As its name indicates, PTBP1 binds preferentially to polypyrimidine-rich (CU-rich) motifs. Previous studies showed that PTBP1 functions in multiple aspects of mRNA metabolism such as splicing regulation, polyadenylation, 3′-end formation, internal ribosomal entry site-mediated translation, and RNA localization and stability (Auweter and Allain, 2008; Keppetipola *et al*., 2012).

PTBP1 contains four RNA recognition motifs (RRMs) separated by three linkers. Each RRM has a common βαββαβ fold and is defined by two conserved RNA-protein complex (RNP) motifs (Oh *et al*., 1998). In alternative splicing regulation, PTBP1 binds to CU-rich motifs in precursor mRNA through RRMs (Oberstrass *et al*., 2005). PTBP1 is ubiquitously expressed at high levels in non-neuronal cells but is down-regulated in neuronal cells, thus allowing the inclusion of neuron-specific exons in mature mRNAs (Ashiya and Grabowski, 1997; Lillevälia *et al*., 2001). The reduced expression of PTBP1 coincides with an increased expression of its neuron-enriched homolog, PTBP2 (also known as nPTB or PTBLP) structurally and functionally similar to PTBP1 (Kikuchi, *et al*. 2000; Lillevälia *et al*., 2001). The mechanism called 'PTBP1/PTBP2 switch' controls neural differentiation through the post-transcriptional regulation by miR-124 (Coutinho-Mansfield *et al.*, 2007; Makeyev *et al.,* 2007) (Fig. 2.1). In non-neuronal cells, PTBP1 inhibits the neuron-specific alternative splicing of *Ptbp2* mRNA. In neuronal cells, miR-124 is expressed abundantly and binds to the 3′UTR of *Ptbp1* to suppress PTBP1 expression. As a result, PTBP2 is expressed and regulates neuron-specific alternative splicing.

In Chapter 2, I described the results of an analysis of the medaka *Ptbp* as a possible target gene by miR-124. A molecular phylogeny in *Ensembl* shows that medaka have two *Ptbp* genes occurred by teleost-specific gene duplication. The nucleotides and amino acids sequences suggested that the medaka PTBPs function as a RNA binding protein and they are able to be bound by miR-124 in the 3'UTR. In addition, whole mount *in-situ* hybridization showed that two paralogous genes, *Ptbp1a* and *Ptbp1b* are differently expressed in 5-dpf medaka embryo. These results indicated that the medaka *Ptbp1a* and *Ptbp1b* play different roles in embryogenesis and their expression are possible to be regulated by miR-124.

2.2. Materials and Methods

Animals

I used the same animals as described in Chapter 1.

Identification of medaka PTBP

cDNA clones inserted into pGCAP10 (olte56k20, FS527928; oleb24g01, DK120951; olea22l09, DK052475) and homologous to zebrafish *Ptbp1* or *Ptbp2* were obtained from the NBRP Medaka EST database, and their sequences were analyzed. Olte56k20 did not contain the entire 3′UTR of PTBP1a, and thus 3′RACE was performed using the TaKaRa 3′-Full RACE Core Set according to the manufacturer's protocol. Oleb24g01 encodes a *Ptbp1b* splicing isoform skipped an exon 34 nucleotide (nt) long, thus I cloned a cDNA coding for a complete protein by RT-PCR using total RNA isolated from stage 34 embryo as a template. PCR was conducted with Prime STAR DNA polymerase (TaKaRa). PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 2 min, followed by 7 min at 72°C. The primers were as follows: *Ptbp1b* (forward, CGT<u>GAATTC</u>CAATGGACGGCAGTGTCCAC; reverse, GTA<u>CTCGAG</u>TGTTGGAATGATCGGAGTGTA). Underlines indicate the restriction enzyme sites of *Eco*RI or *Xho*I . The PCR products were ligated in-frame to the *Eco*RI and *Xho*I site of pBluescript II SK (+) vector after digestion with *Eco*RI and *Xho*I.

Identified medaka *Ptbp* sequences were translated using ApE and BioEit software. Transcription and protein sequences of various animal species were obtained from *Ensembl* genome (http://uswest.ensembl.org/index.html). Those sequences were aligned using CLUSTALW software (http://www.genome.jp/tools/clustalw/). MiR-124 binding motifs (target sites) in the *Ptbp1* 3′UTR were predicted with RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html). To detect homology between medaka and other vertebrates, the target sites in the human, mouse, and zebrafish PTBP1 were predicted by using the two software, RNAhybrid and TargetScan (http://www.targetscan.org/vert_61/).

RT-PCR

The medaka *Ptbp* primers used for RT-PCR are described in Table 2.1. PCR was conducted using *Ex taq* DNA polymerase (TaKaRa) and normalized amounts of template. The PCR conditions were as follows: 25 cycles of 98°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by 7 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel in TAE buffer, and then signals were detected after

staining with ethidium bromide.

Detection of alternative splicing

   The medaka *Ptbp* primers were designed across the skipped exon (Fig. 2.2B; Table. 2.2). PCR was conducted using Ex taq DNA polymerase (TaKaRa) and normalized amounts of template. The PCR conditions were as follows: 30 cycles of 98°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by 7 min at 72°C. PCR products were electrophoresed in 10% polyacrylamide gel (acrylamide:bisacrylamide = 29:1) in TBE buffer, and then signals were detected after staining with ethidium bromide.

Whole mount in-situ hybridization with RNA probe

   cDNA fragments containing medaka PTBP1 sequences were amplified by PCR using the primers shown in Table 2.1. The cDNA fragments were cloned into pBluescript II SK+ (Stratagene) and the plasmid DNA were linearized by cutting with *XhoI* or *EcoRI*, which were used for antisense or sense primers, respectively. RNA probes were transcribed using T3 or T7 RNA polymerase by using a digoxigenin (DIG) RNA labeling mix (Roche). The RNA probes were purified through ProbeQuant G-50 Micro Columns (GE Healthcare). Whole mount *in-situ* hybridization was performed according to the protocol described by Inohaya (1995). Dechorionated embryos were fixed with 4% PFA in PBS and stored in MeOH. After rehydration, the embryos were bleached using 6% H_2O_2 in PBT (PBS containing 0.1% Tween20) for 1 h, and were treated with proteinase K (10 μg/ml for 5-dpf embryos) in PBT at room temperature for 10 min. The embryos were refixed in 4% PFA and transferred to hybridization buffer (50% formamide, 2× SSC, 0.1% Tween20, 50 μg/ml yeast tRNA, 50 μg/ml heparin) and incubated at 55 or 60°C for 1 h. The embryos were transferred to fresh hybridization buffer containing RNA probes and incubated at 55 or 60 °C overnight. Subsequently, the embryos were washed at 55 or 60 °C two times in 50% formamide, 2× SSCT for 30 min each and at 37 °C two times in 2× SSCT for 15 min each. After a brief wash with PBT, the embryos were incubated at 37°C with 50 μg/ml RNaseA in PBT for 30 min to remove excess RNA probes. The embryos were washed once at 37°C in 2× SSCT for 10 min, once at 55 or 60 °C in 50% formamide, 2× SSC for 1 h, and two times at 55 or 60°C in 0.2× SSCT for 15 min each. After a brief wash with PBT, the embryos were blocked in 1% blocking reagent (Roche) in PBT at room temperature for 1 h. Antibody reaction was carried out in 1% blocking reagent containing anti-DIG-AP Fab fragment (Roche) at a concentration of 1/5000 at 4°C overnight. The embryos were washed in

blocking reagent in PBT four times for 30 min each time. The embryos were then incubated in staining buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 0.1% Tween20, 1 mM levamisole) for 10 min, and then stained in staining buffer containing 450 μg/ml NBT (Sigma) and 175 μg/ml BCIP (Sigma). Embryos were washed three times in PBT and profixed in 4% PFA.

2.3. Results and Discussion

Analysis of medaka PTBP1 and PTBP2

According to *Ensembl* genome database, tetrapod genomes include one each *Ptbp1* and *Ptbp2*. On the other hand, two each paralogs are detected in the teleost genomes (Fig. 2.2A). In medaka genome, two *Ptbp1* (1 of 2, ENSORLG00000011430; 2 of 2, ENSORLG00000014019) are located on chromosome 22 and 4, respectively. One of *Ptbp2* (2 of 2, ENSORLG00000013557) is located on chromosome 17, but the other (1 of 2, ENSORLG00000019191) is detected in ultracontig236. The *Ensembl* database discloses incomplete cDNA sequences deduced from genomic data. I sequenced two paralogs, *Ptbp1a* (AB819423) and *Ptbp1b* (AB819425), using cDNA clones obtained from NBRP Medaka and isolated the 3′UTR of *Ptbp1a* (AB19424) by 3′RACE. These *Ptbp1a* cDNA sequences (AB819423, AB19424) are created by a third party annotation (BR001025). *Ptbp1b* (AB819425) encodes a splicing isoform skipped an exon, thus we cloned a cDNA coding for a complete protein (AB819426). Transcription product of another *Ptbp2* on ultracontig236 was not detected by RT-PCR although I tried to obtain the cDNA from stage-34 embryos (Fig. 2.2B). *Ptbp2* on ultracontig236 might be a pseudogene.

The identified medaka *Ptbp* cDNAs included an initiation codon, terminator codon, and polyadenylation signals (Figs. 2.3–2.5). The medaka *Ptbp1a* cDNA consisted of a 90-bp 5′UTR, a 1734-bp open reading frame (ORF), and a 2080-bp 3′UTR (Fig. 2.3). Oleb24g01cDNA coding medaka PTBP1b contains a 1083-bp coding DNA sequence (CDS), that is deleted a 34-nt sequence (red in Fig. 2.4) unlike the *Ensembl* sequence (ENSORLT00000014313). On the other hand, I obtained a 1620-bp coding DNA sequence (CDS) by RT-PCR using a cDNA from stage 34 embryo as a template, which includes the 34-nt sequence. Based on these sequences, the medaka *Ptbp1b* consisted of a 14-bp 5′UTR, a 1620-bp ORF, and a 3183-bp 3′UTR (Fig. 2.4). *Ptbp2* cDNA consisted of a 42-bp 5′UTR, a 1572-bp ORF and a 1060-bp 3′UTR (Fig. 2.5).

Oleb24g01cDNA coding the medaka PTBP1b seems to be an alternative spliced isoform. PTBPs produce several alternative spliced isoforms. Human PTBP1 has three main isoforms, PTB-1, 2, and 4, which differ by the insertion of 19 or 26 amino acids, respectively, between the second and third RNA recognition motif domains (Wollerton *et al.*, 2001). In addition, Human *Ptbp1* and *Ptbp2* mRNA are skipped exon 11 and exon 10, respectively, that induce NMD (Wollerton *et al.*, 2004; Rahman *et al.*, 2004). The medaka *Ptbp1b* deleted the 34-nt sequence is possible to introduce a frame

shift and a PTC (yellow in Fig. 2.4; Fig. 2.8A). The 34-nt sequence is probably located in exon 11 although the *Ensembl* sequence is incomplete. The equivalent 34-nt sequences were also detected in medaka *Ptbp1a* and *Ptbp2* (red in Figs. 2.3; Fig. 2.5). Both of the sequences correspond to exon 12 in comparison to the *Ensembl* sequences. They could introduce a frame shift and a PTC (yellow in Fig. 2.3 and Fig. 2.5) if a predicted alternative splicing exon were skipped. I detected the alternative splicing by RT-PCR using primers designed across the putative skipped exon (underlined in Figs. 2.3–2.5). RT-PCR showed two bands: a weak signal band below a strong one (Fig. 2.8B). The weak bands were thought to be produced by alternative splicing. These results suggested that medaka PTBPs have alternative splicing isoforms.

Next, I predicted protein sequences from the identified medaka *Ptbp* cDNA and compared to mammalian and zebrafish PTBPs (Figs. 2.6–2.7). Medaka PTBP1a, PTBP1b (including deleted 34-nt sequence), and PTBP2 consisted of 577, 539, and 523 amino acids (aa), respectively. Medaka PTBP1a and PTBP1b included bipartite NLSs, Gly-Thr-Lys-Arg and Lys-Lys-Phe-Lys. These sequences are contained in mammalian and zebrafish PTBP1. Medaka PTBP2 also included bipartite NLSs, Gly-Val-Lys-Arg and Lys-Lys-Gln-Arg. Lys-Lys-Gln-Arg is a canonical NLS contained in such as E6 oncoprotein (Le Roux and Moroianu, 2003). Medaka PTBP1a, PTBP1b and PTBP2 could have four RRMs including RNP1 and RNP2 as well as mammalian and zebrafish PTBPs (Figs. 2.6–2.7). Functional domains of PTBP proteins, such as NLSs and RRMs, were highly conserved in medaka PTBP, suggesting that medaka PTBP1 and PTBP2 might both function as a RNA binding protein similarly to that in mammals (Tables 2.3–2.4).

A computational analysis with RNAhybrid predicted potential miR-124 binding sites in the 3′UTR of both *Ptbp1a* and *Ptbp1b* (Fig. 2.9A). The binding sites are positioned at +546 to +572 and +1086 to +1115 in the *Ptbp1a* 3′UTR, +471 to +500 in the *Ptbp1b* 3′UTR, and +72 to +94 in the *Ptbp2* 3′UTR, respectively, counting the nucleotide following the stop codon as +1. Seed sequences from the second to the seventh positions at the 5′ end of miR-124 are perfectly complementary to the target mRNA (gray shading in Fig. 2.9A), which is essential for the miRNA to bind to the mRNA (Lewis *et al*., 2005). The seed sequence in *Ptbp2* is not perfectly matched and contains GU wobble, however, medaka *Ptbp1* and *Ptbp2* may possibly be bound by miR-124 in the 3′UTR. The miR-124 target sites of PTBP1 among human, mouse, zebrafish, and medaka are not seemingly conserved outside the seed sequences (Fig. 2.9B). The miR-124 target sites in *Lhx2* or *SCP1* 3′UTR are well conserved among at least mammals compared to *Ptbp1* (Visvanathan *et al*., 2007; Qiu *et al*., 2009). The

target sites in PTBP1 might have diverged, or been acquired in different way during vertebrate evolution, in sharp contrast to that the perfect complementarity to the seed sequences is necessary for the post-transcriptional regulation. By the RNAhybrid prediction, medaka PTBP1a has two target sites in the 3′UTR. In reference to Grimson *et al.* (2007), I used the forward site in the following assay in Chapter 3.

Expression of medaka Ptbp1a and Ptbp1b

To gain further information, I also examined the expression of medaka *Ptbp1a* and *Ptbp1b* by whole mount *in-situ* hybridization. Medaka *Ptbp1a* is expressed ubiquitously in 5-dpf embryo using antisense probe (Fig. 2.10A) similarly to mammals. On the other hand, *Ptbp1b* is specifically expressed in the otic vesicles in 5-dpf embryo using antisense probe (Fig. 2.10B). No signals were observed when the sense probes were used. In addition, RT-PCR showed the different expression patterns among stage-34 embryo, adult brain, and eye (Fig. 2.2B). Intrestingly, *Ptbp1b* is higher expressed in brain and eye than *Ptbp2*. Two PTBP1 paralogs occurred by teleost-specific gene duplication could have different functions and contribute to teleost characteristic structures. Further analysis of the expression of medaka PTBPs will reveal whether they have any specific function during embryogenesis.

2.4. Figures and Tables
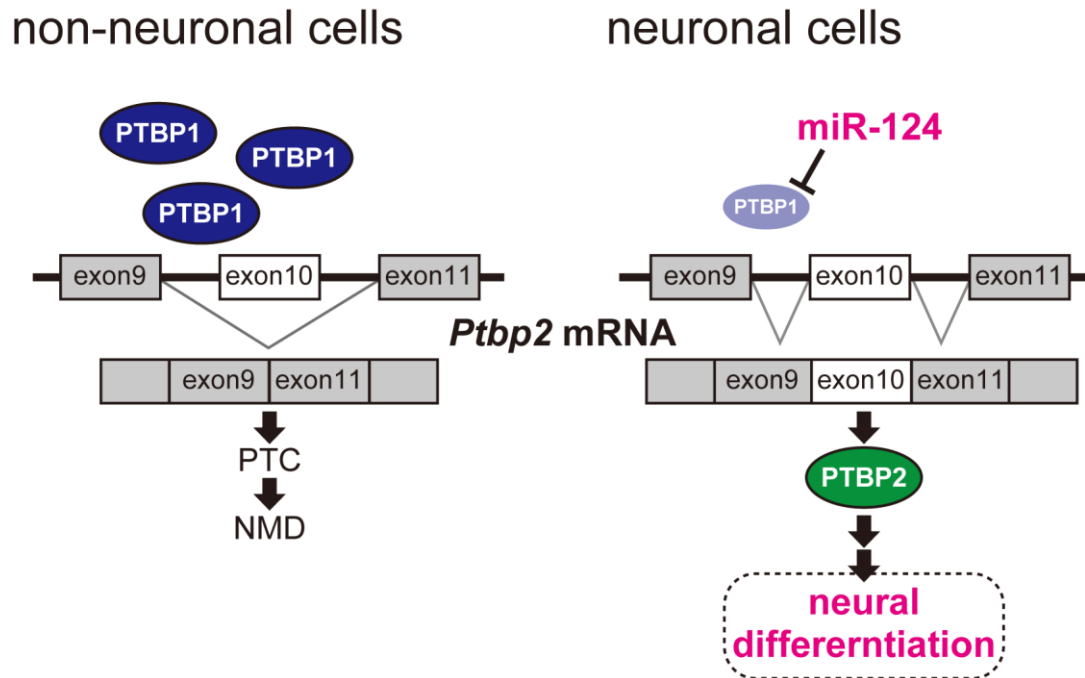
Fig. 2.1.   In non-neuronal cells, PTBP1 suppresses the alternative inclusion of exon 10 in *Ptbp2* mRNA. The exon 10 skipping introduces a frame shift and a premature termination codon (PTC), and PTBP2 is targeted to the nonsense-mediated mRNA decay (NMD) pathway at last. In neuronal cells, miR-124 inhibits the translation of PTBP1 by binding the 3′UTR of *Ptbp1*. Therefore, *Ptbp2* mRNA is translated to PTBP2, which leads to neural differentiation by regulating of neuron-specific alternative splicing (Coutinho-Mansfield et al., 2007; Makeyev et al., 2007).
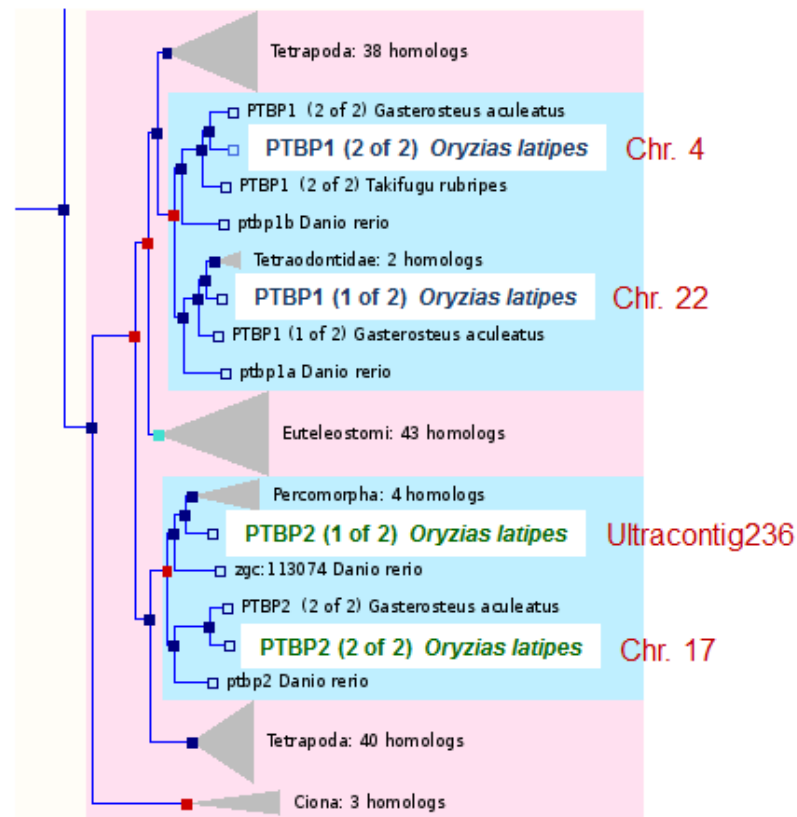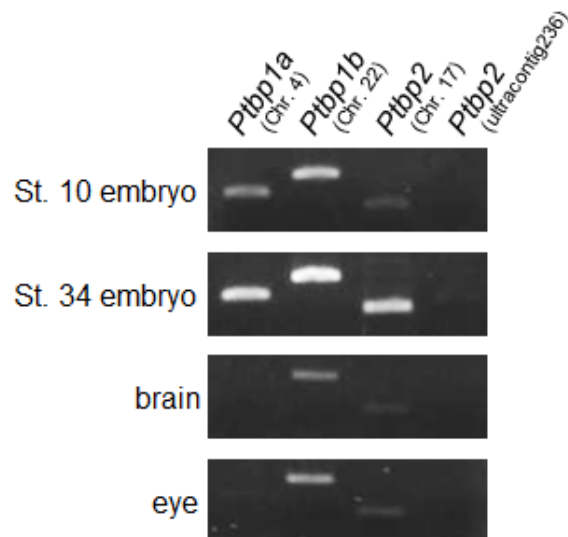
Fig. 2.2. Teleosts have two *Ptbp* genes. (**A**) A molecular phylogeny of *Ptbps* in *Ensembl* (ENSGT00550000074508), using maximum likelihood phylogenetic gene trees. (**B**) RT-PCR using total RNA isolated from stage 34 embryos at 5dpf, adult brain, eye, and liver, for analyzing the medaka *Ptbp1* and *Ptbp2* expression.

Medaka PTBP1a

```
   1 TGGCCTCCGTCTGTTCAGCCATTTTGTAAGAAAATCAAGCAAGTCTGCCGCCCTACCTTCGTCTCAGACCTGCAGTTTTTTTCTTGTGCA  90
  91 ATGGACGGCCGCCTAGACGCCACTGAGTTGTACCCCCTGGGATCCGGCTACGTCCCTGAAATCGAAAGTGTCCATGACATATCGGTTGGC 180
   1  M  D  G  R  L  D  A  T  E  L  Y  P  L  G  S  G  Y  V  P  E  I  E  S  V  H  D  I  S  V  G   30

 181 ACAAAGAGGGGATCCGACGAACTCTTTTCTTCCTGCATAACAAACGGACCCTATATCATGAACTCAGCCAATGGCAACGACAGCAAAAAA 270
  31  T  K  R  G  S  D  E  L  F  S  S  C  I  T  N  G  P  Y  I  M  N  S  A  N  G  N  D  S  K  K   60

 271 TTCAAAGGTGACGTCCGCAGCCCTGGTGTGCCCTCACGTGTTGTCCATGTTCGCAAGCTGCCCAACGACATCAATGAGGCGGAGGTGATA 360
  61  F  K  G  D  V  R  S  P  G  V  P  S  R  V  V  H  V  R  K  L  P  N  D  I  N  E  A  E  V  I   90

 361 AGCCTGGGACTGCCTTTTGGGAAAGTCACCAATCTGCTCATGTTGAAAGGAAAAAACCAGGCATTCTTGGAGTTTGGTAGTGAGGAGTGT 450
  91  S  L  G  L  P  F  G  K  V  T  N  L  L  M  L  K  G  K  N  Q  A  F  L  E  F  G  S  E  E  C  120

 451 GCTCAGACGATGGTGAGCTACTACTCCTCCGTCACCCCTGTCATCAGGAACCCACCCCATTTACATGCAGTACTCCACCCATAAGGAACTC 540
 121  A  Q  T  M  V  S  Y  Y  S  S  V  T  P  V  I  R  N  H  P  I  Y  M  Q  Y  S  T  H  K  E  L  150

 541 AAGACAGACAACTCTCCTAACCAAGTGCGTGCTCAAGCAGCTCTGCAGGCAGTCAATGCCCTCCATGGAAGTGGGATGGGAAGCATGGCC 630
 151  K  T  D  N  S  P  N  Q  V  R  A  Q  A  A  L  Q  A  V  N  A  L  H  G  S  G  M  G  S  M  A  180

 631 ATTTCTGCAGAAGCTGCTAGCATGGGAGGAGCAGCCAGCCAGAGTCCTGTGCTCAGAGTTATTGTTGAGAACCTCTTCTACCCGGTGACC 720
 181  I  S  A  E  A  A  S  M  G  G  A  A  S  Q  S  P  V  L  R  V  I  V  E  N  L  F  Y  P  V  T  210

 721 CTGGATGTGCTGCACCAGATCTTTTCAAAGTTTGGTACTGTGTTGAAGATCATCACTTTCACCAAGAACAACCAGTTTCAGGCTCTGATT 810
 211  L  D  V  L  H  Q  I  F  S  K  F  G  T  V  L  K  I  I  T  F  T  K  N  N  Q  F  Q  A  L  I  240

 811 CAGTACGCTGACGCCATGACTGCTCAGCACGCTAAACTGTCACTGGACGGCCAGAACATCTACAATGCTTGTTGTACCCTGAGAATCAGC 900
 241  Q  Y  A  D  A  M  T  A  Q  H  A  K  L  S  L  D  G  Q  N  I  Y  N  A  C  C  T  L  R  I  S  270

 901 TTCTCCAAGCTCACCAGCCTCAACGTCAAATACAACAATGACAAAAGCAGGGATTACACCCGCCCAGACCTTCCCACTGCCGACTCGCAG 990
 271  F  S  K  L  T  S  L  N  V  K  Y  N  N  D  K  S  R  D  Y  T  R  P  D  L  P  T  A  D  S  Q  300

 991 CCCTCTCTTGACCACCAGGCGATGGCAGCTGCTGCGTTTGGGCCAGGAATAATCTCAGCCTCGCCCTACGGTGGAGCTCATGCTTTCCCC 1080
 301  P  S  L  D  H  Q  A  M  A  A  A  A  F  G  P  G  I  I  S  A  S  P  Y  G  G  A  H  A  F  P  330

1081 CCCACCTTTACTTTCCAGCAAGCAGGTCTGTCTATGCAGGGCATTCCCAGTGCTCTAGCATCCCTGGGTGTGGGACACTCTGGCATGGCG 1170
 331  P  T  F  T  F  Q  Q  A  G  L  S  M  Q  G  I  P  S  A  L  A  S  L  G  V  G  H  S  G  M  A  360

1171 GCTGCTGCAGCTGCAGCAGCAGCTACCAGACTTGGCCTGTCAGGACTTGGTGTACAGGGGGACACAACGTCTTACTGGTCAGCAATCTG 1260
 361  A  A  A  A  A  A  A  A  T  R  L  G  L  S  G  L  G  A  T  G  G  H  N  V  L  L  V  S  N  L  390

1261 AACCCTGAGAGCGTTACGCCACACTGCCTCTTTATTCTTTTCGGTGTGTACGGCGATGTGATGAGAGTCAAGATCCTGTTCAACAAAAAG 1350
 391  N  P  E  S  V  T  P  H  C  L  F  I  L  F  G  V  Y  G  D  V  M  R  V  K  I  L  F  N  K  K  420

1351 GAAAATGCTTTGGTCCAGATGTCTGATGGCACGCAGGCTCAACTCTCCATGAGCCACCTGAATGGCCAGCGTCTTCACGGCAGAGCGATT 1440
 421  E  N  A  L  V  Q  M  S  D  G  T  Q  A  Q  L  S  M  S  H  L  N  G  Q  R  L  H  G  R  A  I  450

1441 CGCGTGACTTTGTCAAAGCACACAACAGTGCAGCTGCCCAGAGAAGGTCATGAGGACCAGGGCCTCACCAAGGATTACAGCAACTCCCCC 1530
 451  R  V  T  L  S  K  H  T  T  V  Q  L  P  R  E  G  H  E  D  Q  G  L  T  K  D  Y  S  N  S  P  480

1531 CTGCATCGCTTCAAGAAGCCTGGCTCAAAAAACTACTCCAACATCTTCCCACCCTCTGCGACGCTCCACCTCTCCAACATCCCGCCTTCT 1620
 481  L  H  R  F  K  K  P  G  S  K  N  Y  S  N  I  F  P  P  S  A  T  L  H  L  S  N  I  P  P  S  510

1621 GTGATGGAGGACGACCTCAGGAGGCTATTCGCCAGCACAGGAGCCACAGTCAAGGCCTTCAAGTTCTTTCAGAAGGACCGCAAGATGGCT 1710
 511  V  M  E  D  D  L  R  R  L  F  A  S  T  G  A  T  V  K  A  F  K  F  F  Q  K  D  R  K  M  A  540

1711 CTGATCCAGATGGGCTCGGTGGAGGAGGCCATCGAGTGCCTCATCGAGTTCCACAACCACGACCTGGGGGAGAACCATCATCTTCGAGTG 1800
 541  L  I  Q  M  G  S  V  E  E  A  I  E  C  L  I  E  F  H  N  H  D  L  G  E  N  H  H  L  R  V  570

1801 TCCTTCTCCAAGTCCACCATCTGAGTGCCGCTCCCTCCACCCTTCAAACCGTTTCCACCCGAATGTTACCGATATTAGCTTGTGGGCGTC 1890
 571  S  F  S  K  S  T  I  *                                                                   577

1891 GCCCTAAAATGTTTTGTTCCTTTTTTGAAGAATGTTGTTTCTTTAATCAGATTTATGAAAACCCTTTTTCTGGTCAAACCAATACAAGTG 1980
1981 TAAAAAACCCTTTTTGTCTGATTTACCAGTATTTTAGGGGAGAGACACCTGCGCAACTTTAGTTTGAGTGTTTGGCCACGAGTACAAAGC 2070
2071 CCTGAACTCAGACTCAGAACGAGTCAACAAGTCTGTTTTCCGTAGTGTTGGTTTACTCTGAACGTTAACCAACATTTGCTCAGGAATTCA 2160
2161 CACAAATTTCTATTATTTTTGCCAAATTAGCAAGATGCATTTCTGTAACTAAACTTTTTTTTTTTTTTTTTTTTCAACTAATTTTATAA 2250
2251 CTGAATTCCATAGAAAGGACAACATTCTTCAGAAAAAGTGTTTTAGAGATGGCGTCCCGCCCCAAACTGTCCGTCAGACCAATCCATAG 2340
2341 AGCGGCCTCCAGCAGACGCCGCTGCTGCGGTGGCGCTCACGTTCCCGTGTGCCTTTTGGAAAGCCTTTCCATTCCTTCAGGCCAGTCA 2430
2431 GAAGCTGCTTTGACAGATCGACTCCTCAAAACATCCTCGTTTTGTTCTCGACATCCAAACCACTATCGCCTGTTTTACTTTTTTACCAGC 2520
2521 TAGTCTTAAGCCTTTTTGGCAGCGTTGCTCTGGATTCAGAAACATTCTGCCAAAACATTTTCTATTCACCAGCTTTTTTTTTCTTTTTTA 2610
2611 CAACAATTTTCTTTGTATTTACGCTTATTTAATCATTTTCTTAAATACACAACAATTTTCTTTGTATTTACGCAACTCAGACTTTTAAAA 2700
2701 CAATATCTTCGCTCTTGTTTTGTTTTTTTGGAGGCGCGGCGCTGTACTCAGCCTTCCCATGTCAGCTTCATGACGTCCTGCCGGGACTAG 2790
2791 GGGCTTATTTAACCATATTATGTTTCTATGTGTACAACTATATATTCATGTACGTTTTCTTTTAAAGTTAGAATTCACGTGTATCTCTGT 2880
2881 TGAACTTAACTGATGCCTCTACTTAGCGGCTGGTGTTCATGGCTAACGTAGTGCCTTGACTTTTTTAATCTTAGGTTGAAAGTAGCCCAG 2970
2971 TTAGCTGGCGTTGTTTACCGTAACCCCTTTTGTCGTTTTGTTTGTTTTTCATTAATGTATCATTCTGAAAAGTTGCCTTTGCTTTTGATA 3060
3061 CGCTGTGAGATCCACAGGAGGAAAGGCCAACACACAAAGATCTTGACCTTTACATTTTAGAGGGGCAGCGGCGCCGTCACGACAAACTC 3150
3151 CCACAGGTCAGGGGGGCACGTTCGTGCACTTTTCCTCGGCGTAAAGCCTGAGTCCATCACACCCGAGTCTTAATTGTCGACACCTTCCGT 3240
3241 TGTGCCTCACGCACCAGTTTCCTGTAAACTACTCTGCAGTAGTTCTGACCAGAAACGGATACTGTTTGTATTTTTGTAGAACTCTTATG 3330
3331 CACGACCGCAAAGACGGTTGATGCTGTTGAGGACCAAAGTTTGCTGGGTTTTTTTTTTTTCTTAAGTTCCTGGTTCAACATTCCCTGCAGTT 3420
3421 CTGCCCAAAACACTTTGGGTTTTGTGTTCCTCCGTGCTTTTGGGGTAACGAAGTTATTACTGCTTTTTGTTTCCCTTTTTCAGATCTTGG 3510
3511 TAGAGATGGCATGTGGATGAATCCTTAATCCAGCACAGTGCTAGCTGTGAAGTTGACTGTAGAAGATTCTATGCCTTTCAGTTTCGTTGA 3600
3601 AACTTTTTATTTTCTCTTTCTTCTTTTTTTTATAGGGGGGCGGGGTAAGGAAAACGTAAGAAGCGGGGTTGATGGTTGTATATATTCATG 3690
3691 TTTTTGTACCCCAAAATAATTTGGATTATACCCCCTTTTACGTTCAGAACATGTATATTTTGATAAACGGGGCAGACATATGAAAAAAAA 3780
3781 AACTTTTGTAACACAAAGACATTAAATCTATTTGAACACAGGAGGGTGTCTGTATGTGCCCCCCCCATGCCATTCTGTTCTGCATCTTCT 3870
3871 GTCGCCTTCAATAAATCTTTGTGTTTTTCTTCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3960
3961 AAAAAA 3966
```

Fig. 2.3. Nucleotide sequences of *Ptbp1a* and the deduced amino acid sequences of the putative encode protein. Open boxes indicate nucleotides corresponding to the translation initiation codon AUG and termination codon UGA. The premature terminator codon in alternative splicing is indicated in yellow shading. The putative target sites by miR-124 are indicated in green. The polyadenylation signals are indicated by orange letters. The primers used in the analysis of alternative splicing are underlined.

Medaka PTBP1b

```
  1                                                        TTCCGTGTTGTGCA  14
 15 ATGGACGGCAGTGTCCACCAAGATTTAACAGTTGGTACTAAGAGAGGATCTGACGAACTTTTATCCAGCGTCTCCAACGGCCCGTATATC 104
  1  M  D  G  S  V  H  Q  D  L  T  V  G  T  K  R  G  S  D  E  L  L  S  S  V  S  N  G  P  Y  I  30

105 ATGAACACCACAGCCAATGGCAATGACAGCAAGAAGTTCAAAGGTGACTTAAGGGGTCCCAACGTGCCGTCCAGGGTCCTGCACATACGC 194
 31  M  N  T  T  A  N  G  N  D  S  K  K  F  K  G  D  L  R  G  P  N  V  P  S  R  V  L  H  I  R  60

195 AAGCTTCCCATCGACATCACAGAGACCGAGGTCATCAGCCTCGGCCTGCCTTTCGGCGACGTCACCAACCTGCTGATGCTGAAAGCCAAA 284
 61  K  L  P  I  D  I  T  E  T  E  V  I  S  L  G  L  P  F  G  D  V  T  N  L  L  M  L  K  A  K  90

285 AACCAGGCCTTCTTGGAGATGAACTCGGAGGAAGCGGCGCAGAACATGGTGGGTTATTACTCCACAGTGATGCCGATTATCCGACAACAA 374
 91  N  Q  A  F  L  E  M  N  S  E  E  A  A  Q  N  M  V  G  Y  Y  S  T  V  M  P  I  I  R  Q  Q  120

375 CCAGTCTTTGTCCAGTTCTCCAACCACAAGGAGCTGAAAACTGACAACTCCCCCAACCAAGAGAGGGCCCAGGCGGCTCTGCGGGCCCTG 464
121  P  V  F  V  Q  F  S  N  H  K  E  L  K  T  D  N  S  P  N  Q  E  R  A  Q  A  A  L  R  A  L  150

465 AGCTCGTCTCATGTGGACACTGCGGCGCTGGTGGTGGCTCCGAGCTCCGTCCTGAGGGTGGTGGTGGAGAATCTGCTGTATCCCGTCTCC 554
151  S  S  S  H  V  D  T  A  A  L  V  V  A  P  S  S  V  L  R  V  V  V  E  N  L  L  Y  P  V  S  180

555 TTGGACGCACTGTGCCAGATCTTTTCAAAGTTCGGCACTGTGCTGAGGATCATCGTTTTCACCAAGAACAACCAGTTCCAGGCTCTGCTG 644
181  L  D  A  L  C  Q  I  F  S  K  F  G  T  V  L  R  I  I  V  F  T  K  N  N  Q  F  Q  A  L  L  210

645 CAGTATGCTGAAGGGGCGTCGGCTCAGGCGGCCAAAGTGTCTCTGGATGGGCAGAACATCTACAACGGCTGCTGCACTCTGAGGATCAGC 734
211  Q  Y  A  E  G  A  S  A  Q  A  A  K  V  S  L  D  G  Q  N  I  Y  N  G  C  C  T  L  R  I  S  240

735 TTCTCCAAGCTGACGACCCTCAACGTGAAATACAACAACGACAAGAGCCGCGACTTCACCAGGCCGGACCTGCCGTCCGGAGACGGCCAG 824
241  F  S  K  L  T  T  L  N  V  K  Y  N  N  D  K  S  R  D  F  T  R  P  D  L  P  S  G  D  G  Q  270

825 CCCACGATGGAGCACCAAGCCATGGCCACAGCCTTCGGTCCTGGGCATCATCTCTGCTTCACCGTACACGGGAGCAACTCATGCTTTCCCA 914
271  P  T  M  E  H  Q  A  M  A  T  A  F  G  P  G  I  I  S  A  S  P  Y  T  G  A  T  H  A  F  P  300

915 CCAGCCTTTGCTCTCCAGCCAGTCTCCTCCCCCTATGCAGGCCTGACGATGTCCGGTCTGCCCGGAACGCTGGCCTCTCTGACGCTCCCT 1004
301  P  A  F  A  L  Q  P  V  S  S  P  Y  A  G  L  T  M  S  G  L  P  G  T  L  A  S  L  T  L  P  330

1005 GCTGGGGCCAGGCTGGGATTCCCAGCCATCCCTGCAGGACACTCCGTCGTGCTGGTCAGCAACCTTAACGCTGAGAGAGTTACGCCCCAC 1094
331  A  G  A  R  L  G  F  P  A  I  P  A  G  H  S  V  V  L  V  S  N  L  N  A  E  R  V  T  P  H  360

1095 TGCCTCTTTATTCTCTTCGGTGTTTATGGCGACGTGATGCGAGTGAAGATTCTCTTCAACAAGAAGGAGAACGCTCTGGTTCAGATGAGC 1184
361  C  L  F  I  L  F  G  V  Y  G  D  V  M  R  V  K  I  L  F  N  K  K  E  N  A  L  V  Q  M  S  390

1185 GACAGCACCCAGGCTCAGCTAGCGATGAGCCATCTGAATGGTCAGCGGCTGCATGGGAGGCCTCTTCGCATCACTTTGTCCAAACACACG 1274
391  D  S  T  Q  A  Q  L  A  M  S  H  L  N  G  Q  R  L  H  G  R  P  L  R  I  T  L  S  K  H  T  420

1275 AGTGTGCAGCTCCCCCGGGAAGGTCACGAGGACCAGGGTCTGACCAAAGACTACAGCAGCTCCCCCCTGCACCGCTTCAAAAAGCCTGGA 1364
421  S  V  Q  L  P  R  E  G  H  E  D  Q  G  L  T  K  D  Y  S  S  S  P  L  H  R  F  K  K  P  G  450

1365 TCTAAGAACTATTCCAACATTTTTCCACCGTCGGCCACCTTGCACCTTTCCAACATCCCCCCCTCTGTGGTTGAAGATGATCTAAAGATG 1454
451  S  K  N  Y  S  N  I  F  P  P  S  A  T  L  H  L  S  N  I  P  P  S  V  V  E  D  D  L  K  M  480

1455 CTGTTTTCCAGTTCAGGAGCTGTGGTTAAAGCCTTCAAGTTCTTTCAGAAGGACCATAAAATGGCTCTGATCCAGATGGGCTCTGTGGAG 1544
481  L  F  S  S  S  G  A  V  V  K  A  F  K  F  F  Q  K  D  H  K  M  A  L  I  Q  M  G  S  V  E  510

1545 GAGGCCATTGAGTGTCTCATCCAGCTCCACAACCACGACCTGGGAGAGAACCACCACCTGCGGGTTTCCTTCTCAAAGTCCTCTATCTGA 1634
511  E  A  I  E  C  L  I  Q  L  H  N  H  D  L  G  E  N  H  H  L  R  V  S  F  S  K  S  S  I  *  540

1635 GCCTTCGACTCGCCGGTCAGATCCTGGTCAGGGAGCGGACCGGATGGCACCCACTACACTCCGATCATTCCAACAAGCGTCTACAGAAAC 1724
1725 TACCATGAAGTCTGATGTAACCTTAAATTATTGTTTTTAAATTAATTGATTCATTTTCAGAGGTCAGGTATTCTTGAGAAAATTCCCAGG 1814
1815 TTTTCCTCTGAAGTCCGGATCTGGAGGAATCCGGGTTTTTAGGATTTTTCAAAATGTAGCCACAAGATTGTTAGAATACACTCTCAAATC 1904
1905 AGTCTCAGCCTTGCTATAAACATTTTTTTAATCTCTTTCTTGCCGTCGGACCTTAAAAAACCAGACTCCTGGGGTCTGTTGTCCTCAGCT 1994
1995 CTCACTTAAATCTTAAAATTCGAATTTCACACATTTCAAACATTTAAACTCAGATTACGGTTCTCCTGCTCCTGATCCTCCTGCTGTAGC 2084
2085 TTTCACCTGTTCATGATGCCGTGGCTTTATCAAAGGACCCCTGTGCCTTAATTCTGAAGTCGCAGTGACTTCCTCTTTAACTGAAAAGCC 2174
2175 TCTGTGAATTGGCAGGTTTACAGTTGGTAAAGGTTTACTGTAGAGATGTATTACAGATATATCCATATTTTAAGTGTATTTTATTGGTGG 2264
2265 TGTTGTCAGTGCTTGTTAGTGTTTTGTACTGCTGGTCATAGAGTGAAACACGTGAACTGTTATAGAGGGTTTGCTGGATTTTTAGCCGTA 2354
2355 GAACTTAATTATTTCTTTTTCTTTTTTTTTCTTTAACTTCTTTATTTCCACTTATGCAATCATGTTGTTATCATTTATCCTAGCATTATT 2444
2445 TTAATCATTCCTAATTTTTCCTTTTTCTTGCTTTGATGGTTTCCTCTGAGAAACCCCATGGAATGTGTCGTTAGCTTGTCTGTCGCCTTG 2534
2535 AACGGGTTNTATTTATACGTTTTGTGATCCACTACAGCTGAAACCAAAAATGGCCTTCTAGATGCAAACAAATCTGCCTGCCTCAGGTTTC 2624
2625 TGGTCTAGCGGACCAGAAAAAGGATTTGTGGACTGGTTCAGGCTCCTGTGGGAATGTTTAGCGATTTCTCGACTGGATCCAGCCCATCGG 2714
2715 TGCAACTTCAGTGAGGGGAGAGAGAAAAAACAAAACAAAAAGAGTATGTGGAGTGCCTTCAAAGCAGCACTGAGTTGTAGATCAGACATG 2804
2805 CATTCCTCTTTGTTTGGACAGGGGGAGGACGCCCCACAGCAAACCAGAAGACACTGGTTTGGTTTGTTAAATAGTTATGATTAAGCGGAC 2894
2895 ATTCCAGCCCCTCAGAGATCCTCCCCGCCTTCAAAACCCAAGATTCTACAGTGAGGACGACACTTTCCTCTGACTGAACTGCTGATTCTG 2984
2985 CAACCGTGATGCTTTCAGGGCACCTGGGAAGACTTTTTCAAAATAAAGTGCTACAGCACACACTTATTTGGATTTAAAAGTGAACTTTAA 3074
3075 ATATGGCTCTAAAGTTTGGAGCAACAAATTGGATCTAAAATAACTGCAGAGTTGGCATCCCAAAAATCTAGTATGCATCCAGCATTCATG 3164
3165 GACTTTGATGTCCAGATTAGATTCAACTACAAAGACAGTTAAGTTTAGTTTAACCTTGATGCTTGTAAGAGTTTTTGTCCTCATTTTATA 3254
3255 TTTCTATTATATTTTTTATTATATGAACGTTGACTGGGGTCTCAACCTAGTATCCCAGCTTGAGACGAAGACAGGCCAGCAGACTAGGTT 3344
3345 TGATCAATTTAATGCTTATTCCCCCAGCAGATTAACTCATCACGTTCCAGTCGGTGCTCAACCATTGATTGCTGAACGGTTACATTGAA 3434
3435 AGCTAAACTTAAAGCCACTGACTGGATGCATTTCTGCATGTAGCTTTGACGCTATAATTGAAGCCAACAGTTTTGTAGTTGTGATTGACC 3524
3525 TTGTACACCAACTTTGACAAAGCATGTTGGATTGGATCATAAACCCTGCCAAATTGGTAAACCACATTCTAATTTATAATCAAACATTAA 3614
3615 ATCCATATTTGAGTCTTCTAGTCTAGTATAACTTTATGAACACCAATTAATTTGCCGTTTTTTTTTTTAACATTCTAATATGTTTCTTAA 3704
3705 ACGGTTATATTAAATGTGTAACTAATAGTAAAAACCGGTAATCTCATTGTGTAATCGGTTTGAGATTAGACATTAGACGAATTTCTCTTT 3794
3795 TTAATCAATTTAAATCATCTAAAGTACTTTTACGTAACTCATTTTTCAACAAACCCCATTAATTTCTTATTAGGTCAGAAAAAAAGTTA 3884
3885 TGTACTCCAGAAATACGGTAGGCATTACTTTTGTTGATTTTTGTGTTTATAGATACGAAAAAATAATTTATGTTAACATTGTGACTTAAC 3974
3975 ATTCAAGGAGAGCTTTAGCCTATTGAAACTAATAATTCATATCATTACATGAATCAATAAAACATTTTCTGCAGCAAATAAATCCCTTT 4064
4065 TTCCAGGTGTACTTGAACGCATCACGGTCTTTCTGTGCCGGATGAGGCAGGTATGGCATCGGTCTGTGGCAGGAGGGAGGGGAGGTTGGT 4154
4155 CAAGTGTATATACATGTTTTATACACGGTTGTCTGAGGTGATTATTGGTTTTATATCCGATGAAACTTGTATATTTTGATTAAAAGTGTG 4244
4245 AAACCGGTTCTGAGCCTTACACTGTTTGTGTCACAAAGAAAAAAAAAAACAGATTAAATAACTTTTTATCTAAAAAAAAAAAAAAAAAAA 4334
4335 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 4424
4425 AAAAAAAA                                                                                    4433
```

Fig. 2.4. Nucleotide sequences of *Ptbp1b* and the deduced amino acid sequences of the putative encode protein. Open boxes indicate nucleotides corresponding to the translation initiation codon AUG and termination codon UGA. The premature terminator codon in alternative splicing is indicated in yellow shading. The putative target sites by miR-124 are indicated by green letters. The polyadenylation signals are indicated by orange letters. The primers used in the analysis of alternative splicing are underlined.

Medaka PTBP2

```
1                                          GGTGCGAAAATATCAGCAGACAGAAGAGGACAAAGAGCTGCA 42
43  ATGGACGGTGATGTTGCAGTTGGTGTGAAGAGAGGCTCAGATGAGCTCAACATGTACAGCAGCAGCCCCAGCGCCATGACCGCAAACGGA 132
1   M  D  G  D  V  A  V  G  V  K  R  G  S  D  E  L  N  M  Y  S  S  S  P  S  A  M  T  A  N  G   30

133 AGCGACAGTAAGAAGCAGCGCCTCGACGAGTCCCCTCCCTCCAGAGTCCTTCACATCAGAAAACTTCCCAATGAAGTGACGGAGACCGAA 222
31  S  D  S  K  K  Q  R  L  D  E  S  P  P  S  R  V  L  H  I  R  K  L  P  N  E  V  T  E  T  E   60

223 GTCATCGCTCTGGGCCTGCCGTTTGGAAAGGTCACCAACATATTGATGCTGAAGGGGAAAAATCAGGCTTTTCTTGAATTGGGCACAGAG 312
61  V  I  A  L  G  L  P  F  G  K  V  T  N  I  L  M  L  K  G  K  N  Q  A  F  L  E  L  G  T  E   90

313 GAAGCAGCTATTACTATGGTCAACTACTACACAGCTGTCACACCTCAGGTCAGAAACACGCCAGTCTTCATCCAGTATTCAAACCACAAG 402
91  E  A  A  I  T  M  V  N  Y  Y  T  A  V  T  P  Q  V  R  N  T  P  V  F  I  Q  Y  S  N  H  K   120

403 GAATTGAAAACAGATTCAGCTCTGAACCAGAGAGCCCAGGCAGTTCTGCAGGCGGTGTCAGCCGTTCAGGATGGCAGCTCTCCATCCTCT 492
121 E  L  K  T  D  S  A  L  N  Q  R  A  Q  A  V  L  Q  A  V  S  A  V  Q  D  G  S  S  P  S  S   150

493 GACCCAGGAGTTTTGGAGCTGGCTCCGCCCCCCAGTCCCGTCCTGCGAATCATCATCGACAACATGTTTTATCCCGTCACACTGGACGTT 582
151 D  P  G  V  L  E  L  A  P  P  P  S  P  V  L  R  I  I  I  D  N  M  F  Y  P  V  T  L  D  V   180

583 TTACAACAGATCTTCAGCAAGTTCGGGACCGTGATGAAGATCATAACTTTCACCAAGAACAACCAGTTTCAGGCTCTGCTGCAGTTCAGT 672
181 L  Q  Q  I  F  S  K  F  G  T  V  M  K  I  I  T  F  T  K  N  N  Q  F  Q  A  L  L  Q  F  S   210

673 GATCCCGTCAATGCACAGCAGGCTAAACTGTCTTTAGATGGACAAAATATCTACAATTCCTGCTGTACTCTTCGGATAGACTTCAGTAAA 762
211 D  P  V  N  A  Q  Q  A  K  L  S  L  D  G  Q  N  I  Y  N  S  C  C  T  L  R  I  D  F  S  K   240

763 CTAGTCAACCTCAACGTCAAATACAACAACGATAAGAGTCGGGATTACACCAGACCTGACCTCCCGACAGGAGACGGAGATTCAGCCAAC 852
241 L  V  N  L  N  V  K  Y  N  N  D  K  S  R  D  Y  T  R  P  D  L  P  T  G  D  G  D  S  A  N   270

853 AAGGATCATTCTCTGCTGGGAACCCCATCTGGAGCGCTCGCCTCCTACTCCAGTGGAGGAAGTTATTCATCGTCTCTGTCGCTCTCACAG 942
271 K  D  H  S  L  L  G  T  P  S  G  A  L  A  S  Y  S  S  G  G  S  Y  S  S  S  L  S  L  S  Q   300

943 GGTGGAGGAGCCATCAGTCCTCTGAGCGCCGCAGCGGCGGCGGCGGCAGCTGCAGGTCGTGTGGCTCTGTCCGGGTCTGGCGTGTCCGGG 1032
301 G  G  G  A  I  S  P  L  S  A  A  A  A  A  A  A  A  G  R  V  A  L  S  G  S  G  V  S  G   330

1033 GTCCTGCTGGCGTCCAACCTGAACGAAGAGATGGTCACGCCTCAAAGTCTCTTTACCCTCTTCGGGGTCTATGGGGACGTGCAGAGGGTG 1122
331 V  L  L  A  S  N  L  N  E  E  M  V  T  P  Q  S  L  F  T  L  F  G  V  Y  G  D  V  Q  R  V   360

1123 AAGATCCTCTACAACAAGAAGGACAGCGCTCTGATCCAACTTTCTGACGGGAACCAGGCTCAGCTCGCCATGAGTCACCTGAACGGTCAA 1212
361 K  I  L  Y  N  K  K  D  S  A  L  I  Q  L  S  D  G  N  Q  A  Q  L  A  M  S  H  L  N  G  Q   390

1213 AAGGTTTTTGGTAAAGTCATGAGAGTGACGCTGTCAAAGCATCAAACAGTCGCTTTGCCTCGAGAAGGACTGGATGACCAGCTGTTAACT 1302
391 K  V  F  G  K  V  M  R  V  T  L  S  K  H  Q  T  V  A  L  P  R  E  G  L  D  D  Q  L  L  T   420

1303 AAAGACTTTGCCGGCTCTCCACTCCATCGCTTCAAGAAGCCTGGGTCCAAAAACTTCCAGAATATTTTCCCTCCGTCTGCAACGCTTCAC 1392
421 K  D  F  A  G  S  P  L  H  R  F  K  K  P  G  S  K  N  F  Q  N  I  F  P  P  S  A  T  L  H   450

1393 CTCTCCAACATCAGGGAGGGAGTGGGAGAGGAAGATCTGCGTCTTCTGTTTTCTAACAGTGGAGGAGCGGTCAAAGCCTTCAAGTTCTTC 1482
451 L  S  N  I  R  E  G  V  G  E  E  D  L  R  L  L  F  S  N  S  G  G  A  V  K  A  F  K  F  F   480

1483 CAGGACCGTAAAATGGCTTTGATCCAGATGTCTTCAGTAGAGGAAGCCATCCAGGCTCTGATGGACCTTCACAACTACGACATGGGAGGA 1572
481 Q  D  R  K  M  A  L  I  Q  M  S  S  V  E  E  A  I  Q  A  L  M  D  L  H  N  Y  D  M  G  G   510

1573 AATCATCATCTTAAAGTTTCCTTCTCCAAGTCCACCATCTAATCCAGACCCGAAACTCCATCAAACCATTTACACTTTTCTGTGAATAGT 1662
511 N  H  H  L  K  V  S  F  S  K  S  T  I  *

1663 CTTCAGGTTAGGCACGACCACGGGGCAGATTCCACCACGTTTCTTATCTTTGTTTCTCTTAGAGGCGGCCGCTGTCCACAGGCGGCTCTG 1752
1753 CAAACGAAACACCAGCTAAAACGTCTCTCATCTCTATTTGAAGCACAAATGCAGAAGTGCTGCTCAGCTTCAGGACTCCAGAGCCGAATG 1842
1843 TTTTCCTAAGTGATGCTCTTCATTCAGAAGAGACTGGGAGCGGATGTTGTGTTGCTGCAAAATCGCTGTTTTGAAAATGTGCATTGCTCA 1932
1933 AAGAGAAGCTGCTCTGTTCGGGTTCTTCTGCTGCGTTTGAAGGTCTTCTGGCTTCTTAATTCACCCGTCAGAAGGATTTGAAAACGACTC 2022
2023 CAAGTTCCTGAGCAAATGTCTGTATTAAGCTCCTGTTTTTCCTTTAATCGTTTGAAGAAAAAAGGTCAGTGGTTGAGTTTGGTGCCTAGT 2112
2113 CCATTCTACAAACATTCAGAAGTAGTTATTGTGATCAAATCAACAGCTGGCCGTGTAAATAGTGAAAATGTTTGGGCACCTCTAGTGTGT 2202
2203 TTTTTTTCTTCTCTTGNGAACACTCGGTTACGTTTAGATACTGCTTTTTAAAAAAAAAAAAGACTAACTGTTTTTAATATTGAAGCAAAAA 2292
2293 AGTTGTTTGTTTTTGGGGGGGTCATTCATTATTTTCCTTTAAAACAAACACATATTTAAATTTTATTTTTACCTTTTCTCACAACTTT 2382
2383 AAAAAAATCTAGCATCATTGAAAAAAAATAACTTAAAATTTTTTAAATTTTTTTGTCTTAAAGAAATGTTCCTTTTTTCTTTTACTAA 2472
2473 ATGTTTCTGGATTTTGAATTTTCTGTTAAAGAAGAAATCCTTTCTTTTTCAGTCCTTTAACACACATTACTGCCCTTTGAATTTGTGCTC 2562
2563 TTTTCTTTTCCTTTAATATGTGAAATGAGGCCTGAGAAGAATTACGTCATGTCTGTTCTAGTGTTTATGATTATTGTTTTTGCAGCAAAT 2652
2653 AAACTTTGGTCTGACTGATCTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2739
```

Fig. 2.5. Nucleotide sequences of *Ptbp2* and the deduced amino acid sequences of the putative encode protein. Open boxes indicate the nucleotides corresponding to the translation initiation codon AUG and termination codon UAA. The premature terminator codon in alternative splicing is indicated in yellow shading. The putative target sites by miR-124 are indicated in green. The polyadenylation signals are indicated by orange letters. The primers used in the analysis of alternative splicing are underlined.

```
                    10        20        30        40        50        60        70
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1    1  MDG------------------IVPDIAVGTKRGSDELFSTCVTNGPFIMSSNSASAANGNDSKKFKGDS   51
mmu-PTBP1    1  MDG------------------IVPDIAVGTKRGSDELFSTCVSNGPFIMSS-SASAANGNDSKKFKGDN   50
dre-PTBP1a   1  MDGRLET-DLYPLGSSYVTEIDSVHDITVGTKRGSDELFSSCISNGPFIMSSNTEEAANGNDSKKFKGDI   66
dre-PTBP1b   1  MDGRLET-ELYPMGSSYA-ELDVVHDIAVGTKRGSDELFS-CVTSGPFIMSS----AANGNDSKKFKGDI   63
ola-PTBP1a   1  MDGRLDATELYPLGSGYVPEIESVHDISVGTKRGSDELFSSCITNGPFIMNS-----ANGNDSKKFKGDV   65
ola-PTBP1b   1  MDG------------SVHQDLTVGTKRGSDELLLS-SVSNGPFIMNT----TANGNDSKKFKGDL   47

                    80        90        100       110       120       130       140
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1   52  RSAGVPSRVIHIRKLPIDVTEGEVISLGLPFGKVTNLLMLKGKNQAFIEMNTEEAANTMVNYYTSVTFVL  121
mmu-PTBP1   51  RSAGVPSRVIHVRKLPSDVTEGEVISLGLPFGKVTNLLMLKGKNQAFLEMNTEEAANTMVNYYTSVTFVL  120
dre-PTBP1a  67  RSPGIPSRVIHVRKLPNDINEAEVISLGLPFGKVTNLLMLKGKNQAFLEMNTEESAQTMVSYYSSVTFVI  136
dre-PTBP1b  64  RSPAVPSRVIHVRKLPNDINEAEVIALGLPFGKVTNLLMLKGKNQAFLEMNSEEAAQTMVIYYSSVTFVI  133
ola-PTBP1a  66  RSPGVPSRVVHVRKLPNDINEAEVISLGLPFGKVTNLLMLKGKNQAFLEFGSEECAQTMVSYYSSVTFVI  135
ola-PTBP1b  48  RGPNVPSRVLHIRRKLPIDITETEVISLGLPFGGVTNLLMLKAKNQAFLEMNSEEAAQNMVGYYSTVMFII  117

                    150       160       170       180       190       200       210
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1  122  RGQPIYIQFSNHKELKTDSSPNQARAQAALQAVNSVQ---SGNLALAASAAAVDAGMAMAGQSFVLRIIV  188
mmu-PTBP1  121  RGQPIYIQFSNHKELKTDSSPNQARAQAALQAVNSVQ---SGNLALAASAAAVDAGMAMAGQSFVLRIIV  187
dre-PTBP1a 137  RNHPIFMQYSNHKELKTDNSPNQVRAQAALQAVNAVQ---TGGMSLAAIDG-----AGMGSQSFVLRVIV  198
dre-PTBP1b 134  RNHPIFMQYSNHKELKTDNSPNQVRAQAALQAVNAVQ---TGSLTLGGVDP-----SGMTGPSFVLRVIV  195
ola-PTBP1a 136  RNHPIYMQYSTHKELKTDNSPNQVRAQAALQAVNALHGSGMGSMAISAEAASM---GGAASQSFVLRVIV  202
ola-PTBP1b 118  RQQFVFQFSNHKELKTDNSPNQERAQAALRALSSSH----VDTAAL------------VVAPSSVLRVVV  172

                    220       230       240       250       260       270       280
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1  189  ENLFYPVTLDVLHQIFSKFGTVLKIITFTKNNQFQALLQYADPVSAQHARLSLDGQNIYNACCTLRIDFS  258
mmu-PTBP1  188  ENLFYPVTLDVLHQIFSKFGTVLKIITFTKNNQFQALLQYADPVSAQHARLSLDGQNIYNACCTLRIDFS  257
dre-PTBP1a 199  ENLFYPVTLDVLHQIFSKFGTVLKIITFTKNNQFQALVQYSDGMTAQHARLSLDGQNIYNACCTLRISFS  268
dre-PTBP1b 196  ENLFYPVTLDVLHQIFSKFGTVLKVITFTKNNQFQALLQFTDGLTAQHARLALDGQNIYNGCCTLRISFS  265
ola-PTBP1a 203  ENLFYPVTLDVLHQIFSKFGTVLKIITFTKNNQFQALIQYADAMTAQHARLSLDGQNIYNACCTLRISFS  272
ola-PTBP1b 173  ENLLYPVSLDALCQIFSKFGTVLRIIVFTKNNQFQALIQYAEGASAQHARVSLDGQNIYNGCCTLRISFS  242

                    290       300       310       320       330       340       350
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1  259  KLTSLNVKYNNDKSRDYTRPDLPSGDSQPSLDQT--MAAAFGAPGIISASFYA-GAG-FPPTFAIPQAAG  324
mmu-PTBP1  258  KLTSLNVKYNNDKSRDYTRPDLPSGDSQPSLDQT--MAAAFGAPGIMSASFYA-GAG-FPPTFAIPQAAG  323
dre-PTBP1a 269  KLTSLNVKYNNDKSRDYTRPDLPTGDSQPSFDAQTMAAAALTAPGIISASFYA-GAHGFPQAFAIQQAAG  337
dre-PTBP1b 266  KLTSLNVKYNNDKSRDYTRPDLPTGDSQPALEHH-AVAAAFAAPGIISAAPYA-SAHAFPPAFAIQQAG-  332
ola-PTBP1a 273  KLTSLNVKYNNDKSRDYTRPDLPTADSQPSLDHQAMAAAAFG-PGIISASPYG-GAHAFPPTFTFQQAG-  339
ola-PTBP1b 243  KLTFLNVKYNNDKSRDFTRPDLPSGDGQPTMEHQ-AMAFAFG-PGIISASPYTGATHAFPPAFALQPVSS  310

                    360       370       380       390       400       410       420
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1  324  ----LSVPNVHG-ALAPLAIPS----AAAAAAAAGRIAIPGLAGAG-NSVLLVSNLNPERVTPQSLFILF  384
mmu-PTBP1  323  ----LSVPNVHG-ALAPLAIPS----AAAAAAAS-RIAIPGLAGAG-NSVLLVSNLNPERVTPQSLFILF  382
dre-PTBP1a 337  ----LSLQGMPAGALASLSVPG-AAAAAAAAAAAGRLGLSGLTAAG-NSVLLVSNLNPESVTPQCLFILF  401
dre-PTBP1b 332  ----LTIPGVPG-ALASLTIPG-AAAAAAAS---RLGFPTLTGT--HCVMLVSNLNPERWTPHCLFILF  391
ola-PTBP1a 339  ----LSMQGIPS-ALASLGVGHSGMAAAAAAAAATRLGLSGLGATGGHNVLLVSNLNPTPHCLFILF  404
ola-PTBP1b 311  PYAGLTMSGLPG-TLASLTLPA-----------GARLGFPAIPAG--HSVVLVSNLNAERWTPHCLFILF  366

                    430       440       450       460       470       480       490
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1  385  GVYGDVQRVKILFNKKENALVQMADGNQAQLAMSHLNGHKLHGKPIRITLSKHQNVQLPREGQEDQGLTK  454
mmu-PTBP1  383  GVYGDVQRVKILFNKKENALVQMADGSQAQLAMSHLNGHKLHGKSVRIITLSKHQSVQLPREGQEDQGLTK  452
dre-PTBP1a 402  GVYGDVMRVKILFNKKENALVQMSDGTQAQLAMSHLNGQKLYGKALRATLSKHITVQLPREGHEDQGLTK  471
dre-PTBP1b 392  GVYGDVLRVKIMFNKKENALVQMSDGTQAQLAMSHLNGQKLYGRPLRITLSKHITVQMPREGHEDQGLTK  461
ola-PTBP1a 405  GVYGDVMRVKILFNKKENALVQMSDGTQAQLSMSHLNGQRLHGRAIRVTLSKHITVQLPREGHEDQGLTK  474
ola-PTBP1b 367  GVYGDVMRVKILFNKKENALVQMSDSTQAQLAMSHLNGQRLHGRPLRITLSKHTSVQLPREGHEDQGLTK  436

                    500       510       520       530       540       550       560
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|

hsa-PTBP1  455  DYGNSPLHRFKKPGSKNFQNIFPPSATLHLSNIPPSVSEEDLKVLFSSNGGVVRGFKFFQKDRKMALIQM  524
mmu-PTBP1  453  DYGSSPLHRFKKPGSKNFQNIFPPSATLHLSNIPPSVSEEDLKSLFSSNGGVVKGFKFFQKDRKMALIQM  522
dre-PTBP1a 472  DYSNSPLHRFKKPGSKNYSNIFPPSSTLHLSNIPPSVTEDDLRGLFLSSGAVVKAFKFFQKDRKMALIQL  541
dre-PTBP1b 462  DYSNSPLHRFKKPGSKNYSNIFPPSATLHLSNIPPSVVEDDLKLLFGSSGALVRNFKFFQKDRKMALIQL  531
ola-PTBP1a 475  DYSNSPLHRFKKPGSKNYSNIFPPSATLHLSNIPPSVMEDDLRRLFASIGATVKAFKFFQKDRKMALIQM  544
ola-PTBP1b 437  DYSSSPLHRFKKPGSKNYSNIFPPSATLHLSNIPPSVVEDDLKMLFSSSGAVVKAFKFFQKDHKMALIQM  506

                    570       580       590
            ....|....|....|....|....|....|....|

hsa-PTBP1  525  GSVEEAVQALIDLHNHDLGENHHLRVSFSKSTI  557
mmu-PTBP1  523  GSVEEAVQALIELHNHDLGENHHLRVSFSKSTI  555
dre-PTBP1a 542  ASVEEAIESLIQFHNHDLGENHHLRVSFSKSTI  574
dre-PTBP1b 532  SSVEEAIESLIQFHNHDLGENHHLRVSFSKSTI  564
ola-PTBP1a 545  GSVEEAIECLIEFHNHDLGENHHLRVSFSKSTI  577
ola-PTBP1b 507  GSVEEAIECLIQLHNHDLGENHHLRVSFSKSSI  539
```
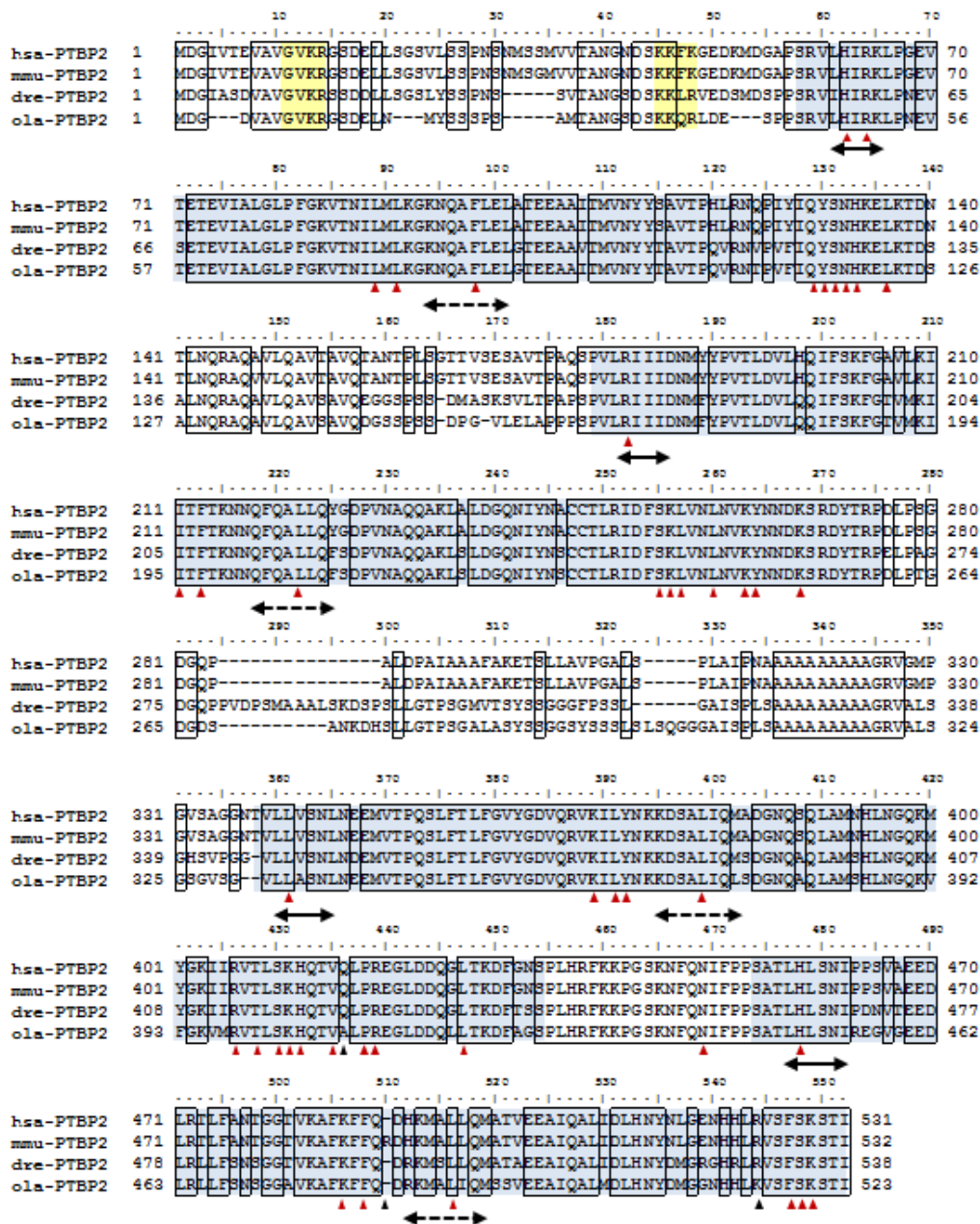
Fig. 2.6. Alignment of PTBP1 amino-acid sequences among human (hsa-PTBP1; NP_002810), mouse (mmu-PTBP1; NP_001070831), zebrafish (dre-PTBP1a and 1b; NP_001018313 and NP_001116126), and medaka (ola-PTBP1a and 1b). The regions of identical amino acid residues are outlined. The positions of bipartite nuclear localization signals (NLSs) at the N terminus are indicated by light yellow shading. Light blue shading indicates the four RNA recognition motifs (RRMs). Solid lines and dash lines with arrows indicate RNP1 and RNP2, respectively. Arrowheads indicate RNA interacting residues.

```
              10         20         30         40         50         60         70
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2  1  MDGIVTEVAVGVKRGSDELLSGSVLSSPNSNMSSMVVTANGNDSKKFKGEDKMDGAPSRVLHIRKLPGEV  70
mmu-PTBP2  1  MDGIVTEVAVGVKRGSDELLSGSVLSSPNSNMSGMVVTANGNDSKKFKGEDKMDGAPSRVLHIRKLPGEV  70
dre-PTBP2  1  MDGIASDVAVGVKRSSDDLLSGSLYSSPNS-----SVTANGSDSKKLRVEDSMDSPPSRVIHIRKLPNEV  65
ola-PTBP2  1  MDG---DVAVGVKRGSDPLN---MYSSSPS-----AMTANGSDSKKQRLDE---SPPSRVLHIRKLPNEV  56

              80         90        100        110        120        130        140
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2  71 TETEVIALGLPFGKVTNILMLKGKNQAFLELATEEAAITMVNYYSAVTPHLRNQFIYIQYSNHKELKTDN 140
mmu-PTBP2  71 TETEVIALGLPFGKVTNILMLKGKNQAFLELATEEAAITMVNYYSAVTPHLRNQFIYIQYSNHKELKTDN 140
dre-PTBP2  66 SETEVIALGLPFGKVTNILMLKGKNQAFLELGTEEAAVTMVNYYTAVTFQVRNYFVFIQYSNHKELKTDS 135
ola-PTBP2  57 TETEVIALGLPFGKVTNILMLKGKNQAFLELGTEEAAITMVNYYTAVTFQVRNIFVFIQYSNHKELKTDS 126

             150        160        170        180        190        200        210
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2 141 TLNQRAQAVLQAVTAVQTANTPLSGTTVSESAVTPAQSPVLRIIIDNMYYPVTLDVLHQIFSKFGAVLKI 210
mmu-PTBP2 141 TLNQRAQVVLQAVTAVQTANTPLSGTTVSESAVTPAQSPVLRIIIDNMYYPVTLDVLHQIFSKFGAVLKI 210
dre-PTBP2 136 ALNQRAQAVLQAVSAVQEGGSPSS-DMASKSVLTPAFSPVLRIIIDNMFYPVTLDVLQQIFSKFGIVMKI 204
ola-PTBP2 127 ALNQRAQAVLQAVSAVQDGSSPSS-DPG-VLELAPPPSPVLRIIIDNMFYPVTLDVLQQIFSKFGTVMKI 194

             220        230        240        250        260        270        280
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2 211 ITFTKNNQFQALLQYGDPVNAQQAKLALDGQNIYNACCTLRIDFSKLVNLNVKYNNDKSRDYTRPDLPSG 280
mmu-PTBP2 211 ITFTKNNQFQALLQYGDPVNAQQAKLALDGQNIYNACCTLRIDFSKLVNLNVKYNNDKSRDYTRPDLPSG 280
dre-PTBP2 205 ITFTKNNQFQALLQFSDPVNAQQAKLSLDGQNIYNSCCTLRIDFSKLVNLNVKYNNDKSRDYTRPELPAG 274
ola-PTBP2 195 ITFTKNNQFQALLQFSDPVNAQQAKLSLDGQNIYNSCCTLRIDFSKLVNLNVKYNNDKSRDYTRPDLPTG 264

             290        300        310        320        330        340        350
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2 281 DGQP---------------ALDPAIAAAFAKETSLLAVPGALS-----PLAIPNAAAAAAAAAGRVGMP 330
mmu-PTBP2 281 DGQPPVDPSMAAALSKDSPSLLGTPSGMVTSYSSGGGFPSSL-----GAISPLSAAAAAAAAAGRVALS 338
dre-PTBP2 275 DGQPPVDPSMAAALSKDSPSLLGTPSGMVTSYSSGGGFPSSL-----GAISPLSAAAAAAAAAGRVALS 338
ola-PTBP2 265 DGQS---------ANKDHSLLGTPSGALASYSSGSYSSSLSLSQGGGAISPLSAAAAAAAAAGRVALS 324

             360        370        380        390        400        410        420
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2 331 GVSAGGNTVLLVSNLNEEMVTPQSLFTLFGVYGDVQRVKILYNKKDSALIQMADGNQSQLAMNHLNGQKM 400
mmu-PTBP2 331 GVSAGGNTVLLVSNLNEEMVTPQSLFTLFGVYGDVQRVKILYNKKDSALIQMADGNQSQLAMNHLNGQKM 400
dre-PTBP2 339 GHSVPGG-VLLVSNLNDEMVTPQSLFTLFGVYGDVQRVKILYNKKDSALIQMSDGNQAQLAMSHLNGQKM 407
ola-PTBP2 325 SSGVSG--VLLASNLNEEMVTPQSLFTLFGVYGDVQRVKILYNKKDSALIQLSDGNQAQLAMSHLNGQKV 392

             430        440        450        460        470        480        490
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
hsa-PTBP2 401 YGKIIRVTLSKHQTVQLPREGLDDQGLTKDFGNSPLHRFKKPGSKNFQNIFPPSATLHLSNIPPSVAEED 470
mmu-PTBP2 401 YGKIIRVTLSKHQTVQLPREGLDDQGLTKDFGNSPLHRFKKPGSKNFQNIFPPSATLHLSNIPPSVAEED 470
dre-PTBP2 408 YGKIIRVTLSKHQTVQLPREGLDDQGLTKDFTSSPLHRFKKPGSKNFQNIFPPSATLHLSNIPDNVTEED 477
ola-PTBP2 393 FGKVMRVTLSKHQTVALPREGLDDQLLTKDFAGSPLHRFKKPGSKNFQNIFPPSATLHLSNIREGVGEED 462

             500        510        520        530        540        550
        ....|....|....|....|....|....|....|....|....|....|....|....|....|..
hsa-PTBP2 471 LRTLFANTGGTVKAFKFFQ-DHKMALLQMATVEEAIQALIDLHNYNLGENHHLRVSFSKSTI 531
mmu-PTBP2 471 LRTLFANTGGTVKAFKFFQRDHKMALLQMATVEEAIQALIDLHNYNLGENHHLRVSFSKSTI 532
dre-PTBP2 478 LRLLFSNSGGTVKAFKFFQ-DRKMSLLQMATAEEAIQALIDLHNYDMSRGHRLRVSFSKSTI 538
ola-PTBP2 463 LRLLFSNSGGAVKAFKFFQ-DRKMALIQMSSVEEAIQALMDLHNYDMGGNHHLKVSFSKSTI 523
```

Fig. 2.7. Alignment of PTBP2 amino-acid sequences among human (hsa-PTBP2; NP_067013), mouse (mmu-PTBP2; NP_062423), zebrafish (dre-PTBP2; NP_001093477), and medaka (ola-PTBP2). The regions of identical amino acid residues are outlined. The positions of bipartite nuclear localization signals (NLSs) at the N terminus indicated light yellow shading. Light blue shading indicates the four RNA recognition motifs (RRMs). Solid lines and dash lines with arrows indicate RNP1 and RNP2, respectively. Arrowheads indicate RNA interacting residues.
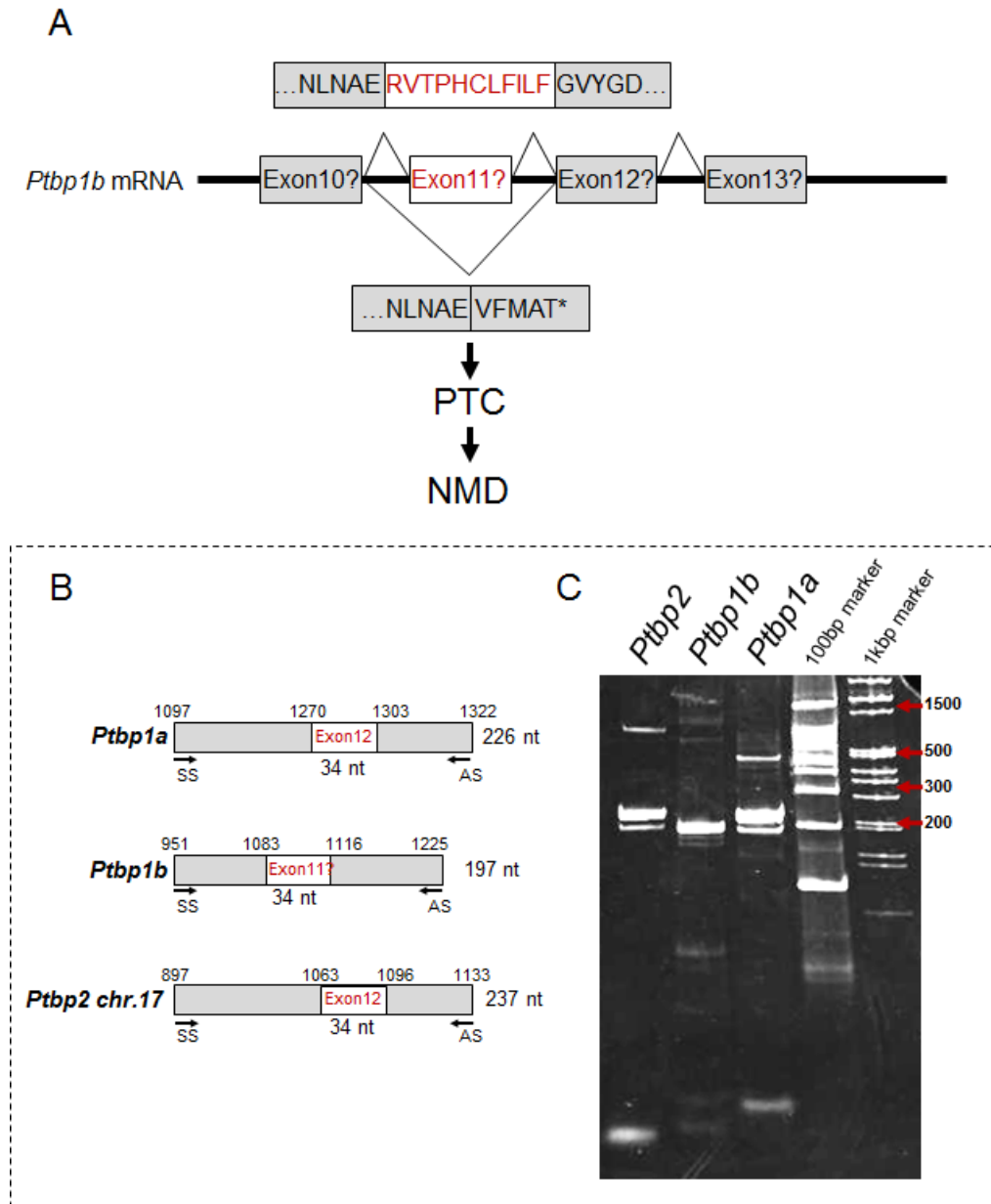
Fig. 2.8. Alternative splicing of *Ptbp* mRNAs. **(A)** Schematic illustration of alternative splicing of medaka *Ptbp1b* mRNA. Skipping exon 11 (the number of exon is uncertain) introduces PTC (premature terminator codon) and occurs NMD (nonsense-mediated mRNA decay). **(B)** Schematic illustration of the positions and size of putative skipped exons (34 nt) and PCR products. Black arrows indicate sense (SS) and antisense (AS) primers used in (C). **(C)** Detection of alternative splicing product by RT-PCR. Red arrows indicate the size of marker.

A

RRM1 RRM2 RRM3 RRM4

5' ——— 3'

PTBP1a-ts

(i)
```
5' C            CGUUC                    3'
   UGGCG UCA      CCGUGUGCCUUU
   ACCGU AGU      GGCGCACGGAAU
3' A     A                               5'
```

(ii)
```
5' C         G  UAACGUA         A 3'
   UGGUGUUCAU GC      GUGCCUUG
   ACCGUAAGUG CG      CACGGAAU
3'            G                  5'
```

PTBP1b-ts

```
5' G    UUUA    AAGGAC  C          A 3'
   UGGC    UCA      CC UGUGCCUUA
   ACCG    AGU      GG GCACGGAAU
3' A    UA                C          5'
```

PTBP2-ts

```
5' G    GAUUC    A   U    U 3'
   GGCA      CACC CGU UCUUA
   CCGU      GUGG GCA GGAAU
3' AA    AA       C   C     5'
```

B

```
 319  --------------------UGGCUUCCUUGUGCCUUAA-  hsa-PTBP1-ts
 239  ---------------AACAUAGCCGCUUUCUGUGCCUUA--  mmu-PTBP1-ts
 402  ----------------CCACAACAAGCAUUUGUGCCUUU--  dre-PTBP1a-ts
 257  -----AGGCCUUAGAUUAUAUUUGUACCAUGUGUGCCUUA--  dre-PTBP1b-ts (i)
1124  ---------------UUUGAAAUCUCACAAGUGCCUUU--  dre-PTBP1b-ts (ii)
 546  ------------GUGGCGCUCACGUUCCCGUGUGCCUUU--  ola-PTBP1a-ts (i)
1086  ----------CUGGUGUUCAUGGCUAACGUAGUGCCUUGA-  ola-PTBP1a-ts (ii)
 471  ---------GUGGCUUUAUCAAAGGACCCCUGUGCCUUA--  ola-PTBP1b-ts
```

Fig. 2.9. Schematic illustration of medaka *Ptbp1* mRNA (at top). The open reading frame (ORF) is indicated by a shaded bar, and the 5′ and 3′ UTR at both ends by black lines. The regions of the four RNA recognition motifs (RRM1–4) are indicated by darker shading. The MiR-124 target site (ts) in the 3′ UTR is indicated in white. In the dashed box below, the miR-124 target sites in the *Ptbp1a* and *Ptbp1b* 3′UTRs (PTBP1a-ts, PTBP1b-ts) are aligned in complementary fashion with the mature ola-miR-124 sequence, with imperfect base pairing indicated by offset nucleotides. The

seed sequence in ola-miR-124 and the complementary portion of each *Ptbp1* 3′UTR are boxed and shaded. Bases substituted in the mutated miR-124 ts (PTBP1a-mtts, PTBP1b-mtts) are underlined (Fig. 3.2 in Chapter 3).
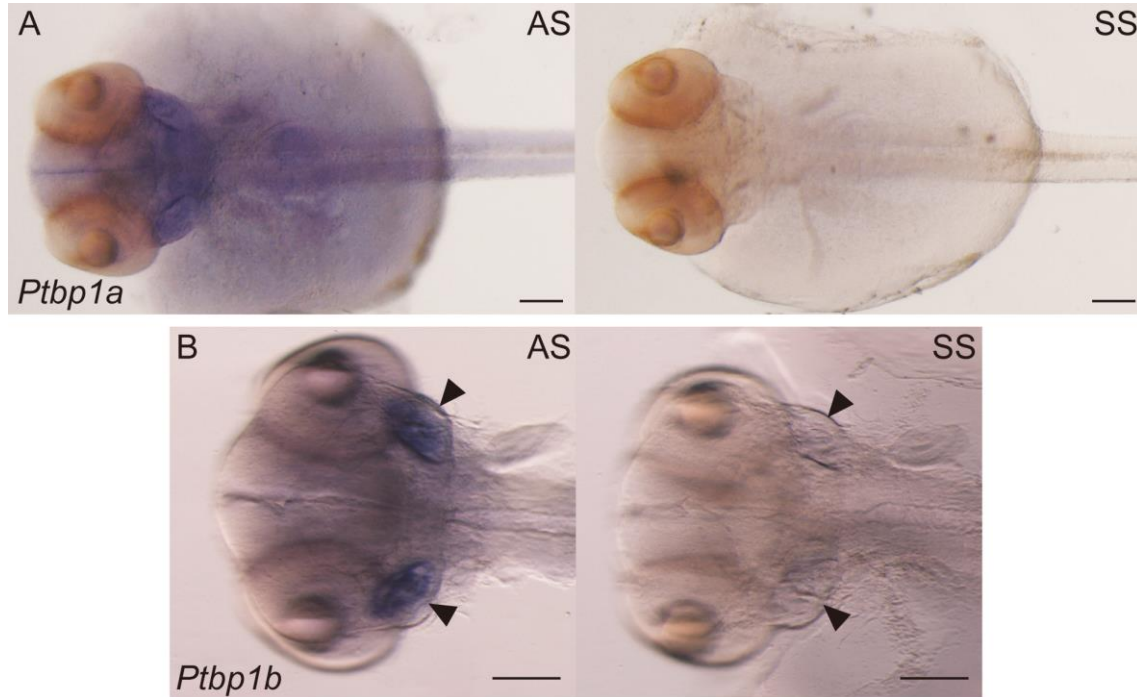
Fig. 2.10.   Medaka *Ptbp1a* and *Ptbp1b* are differently expressed in 5-dpf embryos. **(A, B)** Whole mount *in-situ* hybridization using digoxigenin-labeled *Ptbp1a* (A) or P*TBP1b* (B) RNA probes. The embryos were bleached using 6% H_2O_2. Scale bar = 100 μm. AA, antisense probe; SS, sense probe. Black arrowheads indicate otic vesicles.

Table 2.1. Primers used in RT-PCR. Underlines indicate the restriction enzyme sites.

Gene		Nucleotide sequences	Size of amplified products (bp)
Ptbp1a	Forward	CAACTCGAGTCAGGCTCTGATTCAGTACG	533
	Reverse	TCAGAATTCCGTACACACCGAAAAGAATA	
Ptbp1b	Forward	TGACTCGAGTCTCAACCTAGTATCCCAGC	633
	Reverse	CAAGAATTCTGCCTACCGTATTTCTGGAG	

Table 2.2. Primers used for detection of alternative splicing.

Gene		Nucleotide sequences	Size of amplified products (bp)
Ptbp1a	Forward	AGCAAGCAGGTCTGTCTATG	226
	Reverse	ATCACATCGCCGTACACAC	(192)
Ptbp1b	Forward	TATGCAGGCCTGACGATGT	197
	Reverse	ATCTTCACTCGCATCACGTC	(163)
Ptbp2	Forward	CTACTCCAGTGGAGGAAGTT	237
	Reverse	TAGAGGATCTTCACCCTCTG	(203)

Table 2.3. Amino acid sequence identity to ola-PTBP1a (top row) and ola-PTBP1b (bottom row).

Animal	length (aa)	Amino acid sequence identity (%)				
		Full	RRM1	RRM2	RRM3	RRM4
Human	557	73	76	94	83	82
	556	69	77	81	82	85
Mouse	574	73	76	94	81	85
	564	69	74	81	83	86
Zebrafish	577	85	91	97	93	87
	539	73	78	80	89	87

Table 2.4.   Amino acid sequence identity to ola-PTBP2.

Animal	Length (aa)	Amino acid sequence identity (%)				
		Full	RRM1	RRM2	RRM3	RRM4
Human	531	74	89	92	86	78
Mouse	532	73	89	92	86	77
Zebrafish	538	82	95	100	89	81

Chapter 3

Post-transcriptional regulation by miR-124

in medaka embryogenesis

3.1. Introduction

In Chapter 2, I showed that medaka has the homologous genes of *polypyrimidine tract binding protein1* (*Ptbp1*) reported as a target gene in post-transcriptional regulation by mouse miR-124 (Makeyev *et al*., 2007). The 3′UTR of medaka *Ptbp1* contains predicted binding motifs (target sites) for miR-124. MiR-124 target sequence is also conserved in a chordate *Ciona intestinali*s ortholog of PTBP1. Chen *et al.* (2011) demonstrated that miR-124 can regulate a number of non-neuronal genes in *Ciona intestinali*s. They carried out *in-vivo* sensor assay using the RFP-expressing transgenes containing the 3′UTR target genes, which were driven by an epidermis-specific enhancer of the *Ciona* EpiB gene. They showed that *Ciona* PTBP1 is down-regulated in the ectopic miR-124-expressing embryo. They also indicated that the endogenous miR-124 down-regulates the *Ciona* muscle specifier *Macho-1*.

In Chapter 3, in order to know the function of medaka miR-124 during development, I attempted a GFP reporter assay, in which binding of miR-124 to the target 3'UTR is directly tested in the developing medaka embryo. I synthesized reporter constructs of the miR-124 target sites and the entire 3′UTR of the medaka *Ptbp1a* and *Ptbp1b*. These constructs were injected into 1-cell stage embryos along with the miR-124 duplex and/or miR-124 morpholino oligonucleotides (MO). These results indicated that the miR-124 down-regulates PTBP1 expression at post-transcriptional level by binding to the target 3′UTR in developing medaka embryos.

3.2. Materials and Methods

Animals

        I used the same animals as described in Chapter 1.

Preparation of miR-124 target reporter constructs

        MiR-124 binding motifs (target sites) in the *Ptbp1* 3′UTR were predicted with RNAhybrid software (Fig. 2.9A in Chapter 2). Forward and reverse oligonucleotide fragments for the miR-124 target site (miR-124ts) and mutated miR-124ts (Fig. 2.9A in Chapter 2) connected a *Sal*I site at the 5′ end and *Xho*I-*Xba*I sites at the 3′ end (Table 3.1). They were annealed by lowering the temperature from 75°C to 20°C and were ligated in-frame to the *Xho*I-*Xba*I sites of the pCS2+-GFP vector (Rupp *et al.*, 1994). The cloned vector was cut with *Xho*I and *Xba*I, and then the annealed oligonucleotides were ligated into it. Repeating the same process, three miR-124 target sites in the pCS2+-GFP vector were obtained (Mishima *et al.*, 2006). cDNA fragments of the medaka PTBP1 3′UTR were amplified by PCR with primers (Table 3.2). These fragments were ligated in-frame to the *Xho*I-*Xba*I sites or *EcoR*I-*Xbo*I sites of the pCS2+-GFP vector. The plasmid DNAs were linearized by cutting with *ASP*718 and were transcribed *in vitro* by using the mMessage mMachine SP6 Kit (Ambion). RNA constructs were purified through ProbeQuant G-50 Micro Columns (GE Healthcare).

Microinjection of miR-124 target reporter constructs

        Microinjection into medaka embryos was carried out as described in Kusakabe *et al.* (1999). Fertilized eggs just after spawning were placed in 10 mM reduced glutathione in CAPS buffer and kept at 4°C to be arrested development. Attachment filaments of eggs were cut with scissors holding with forceps before injection. For coinjection, GFP and DsRed reporter RNA constructs were diluted at 0.1 μg/μl each containing 0.1% phenol red. The sequences of miRNA duplex (Sigma) and morpholino oligonucleotides (Genetools) are described in Table 3.1 and 3.3. In the reporter assay, at least 50 embryos (average 100 embryos) were injected with each set of reporter constructs. The experimentally assayed embryos were compared with each other in terms of the strength of the GFP signals observed at the same level of DsRed signals (positive control). To quantify the relative strength of GFP/DsRed signals, images under each fluorescence channel were captured with same exposure time and objective by using the software Leica AF6000. The mean ± SD were calculated for 16 DsRed-expressed embryos.

LNA northern blotting

Northern blotting with LNA probes was performed as described in Chapter 1. Total RNA samples isolated from 5-dpf embryos injected 1.6 mM miR-124 MO, Std control MO, or uninjected embryos. Expression of *ola-miR-124* is presented as relative strength to that of the internal control, U6 snRNA.

3.3. Results

I performed a GFP reporter assay to clarify whether miR-124 controls PTBP1 expression by binding to the target 3′UTR in developing medaka embryos. For this assay, I designed RNA constructs containing the GFP coding region fused to three copies of the miR-124 target site found in the 3′UTR (GFP:PTBP1a-ts and GFP:PTBP1b-ts) (Fig. 3.1A) and to the *Ptbp1a* or *Ptbp1b* 3'UTR (GFP:PTBP1a 3'UTR and PTBP1b 3′UTR) (Fig. 3.3A). I synthesized these reporter mRNAs *in vitro* and injected them into 1-cell stage embryos along with the miR-124 duplex or mutated miR-124 duplex. I coinjected DsRed mRNA as a positive internal control. At 1 dpf, I compared GFP signals observed at the same level of DsRed signals (Table 3.4). The pattern of the GFP/DsRed expression was never changed in each group, while there was a little difference of the intensity, stronger or weaker, among embryos.

When GFP: PTBP1a-ts or PTBP1b-ts mRNA was coinjected with the miR-124 duplex, GFP expression was considerably lower (Fig. 3.1B, D, top row) than in embryos coinjected with the mutated miR-124 duplex (Fig. 3.1C, E, top row). Strong DsRed signals were observed in embryos coinjected with either the miR-124 or mutated miR-124 duplex (Fig. 3.1B–E, bottom row).

To verify the direct binding of miR-124 to the target site, I coinjected a morpholino oligonucleotide against mat-miR-124 (miR-124 MO) along with the three copies target sites reporter mRNAs (Fig. 3.1F, H). Northern blotting showed that the miR-124 MO lowered mat-miR-124 expression (Fig. 3.2D); mat-miR-124 expression in embryos injected with 1.6 mM miR-124 MO was lower than in embryos injected with 1.6 mM control MO or uninjected. Embryos coinjected with GFP:PTBP1-ts mRNA, the miR-124 duplex, and the miR-124 MO showed GFP expression at 1 dpf (Fig. 3.1F, H); the miR-124 MO rescued the inhibition of GFP expression by the miR-124 duplex. In contrast, embryos coinjected with the miR-124 duplex and control MO did not express GFP (Fig. 3.1G, I).

I also examined GFP reporter mRNA fused with the mutated miR-124 target sites (GFP:PTBP1a-mtts, GFP:PTBP1b-mtts) (Fig. 3.2A). Two nucleotides were changed in the mutated copies (underlined in Fig. 2.9A in Chapter 2), so that they lost perfect complementarity to the miR-124 seed sequences. In 1-dpf embryos coinjected with the miR-124 duplex and the GFP:PTBP1a-mtts or GFP:PTBP1b-mtts duplex, GFP expression did not decrease (Fig. 3.2B, C).

Furthermore, I coinjected with mRNA of GFP fused to the *Ptbp1a* or *Ptbp1b*

3′UTR (GFP:PTBP1a 3′UTR and PTBP1b 3′UTR) and miR-124 duplex (Fig. 3.3A). When GFP:PTBP1a 3'UTR or PTBP1b 3'UTR mRNA was coinjected with the miR-124 duplex, GFP expression was considerably lower (Fig. 3.3B, D, top row) than in embryos coinjected with the mutated miR-124 duplex (Fig. 3.3C, E, top row). These results were consistent with the GFP/DsRed expression in the assay with the three copies of the target sites (Fig. 3.1B–E). The quantification of the signals in PTBP1b reporter assay was shown in Fig. 3.4. Exogenous miR-124 decreased 60–70% GFP expression compared to coinjection with mutated duplex, and miR-124 MO almost completely rescued the expression.

3.4. Discussion

The GFP reporter assay clearly showed that exogenous miR-124 negatively regulates translation of the target gene in the medaka embryo (Figs. 3.1–3.4). I performed the reporter assay in reference to Giraldez *et al.* (2005) and Mishima *et al* (2006). Three copies of the target sites recapitulated the down-regulation of the reporter gene with the nanos1 3′UTR by miR-430 in zebrafish (Mishima *et al*., 2006). In the present study, three copies were again sufficient to observe the regulation of reporter gene expression. This result effectively confirmed that the reporter assay system is applicable to developing medaka embryos. Moreover, medaka PTBP1a mRNA actually has two putative target sites by the prediction with RNA hybrid. Although I did not test with the other target site and with one or two copies of each target site, the reporter assay with three target sites clearly suggested that the miR-124 target sites are needed in the post-transcriptional regulation of PTBP1. The assay with the PTBP1 3′UTR fused to GFP showed consistent results with the GFP/DsRed expression in the assay with the three copies of the target sites (Fig. 3.3).

When only GFP:PTBP1 3′UTR or GFP:PTBP1-ts mRNA was injected, GFP expression did not decrease (data not shown). However, injected GFP and DsRed were already expressed in the gastrula, whereas a marked increase in miR-124 was first observed in stage 28 (Fig. 1.3A in Chapter 1). In a similar case, Tani *et al.* (2009) found that endogenous miR-430 inhibits expression of a reporter construct of the 3′UTR of *TDRD7* in medaka embryos, and medaka miR-430 is abundantly expressed around the late blastula stage. It is thus difficult to assess the influence of endogenous miR-124 in the GFP reporter assay.

From the reporter assays, I concluded that medaka miR-124 acts as a post-transcriptional regulator of PTBP1 expression during embryogenesis, and that the target sites in the medaka PTBP1 3′UTR are essential for this regulation.

3.5. Figures and Tables
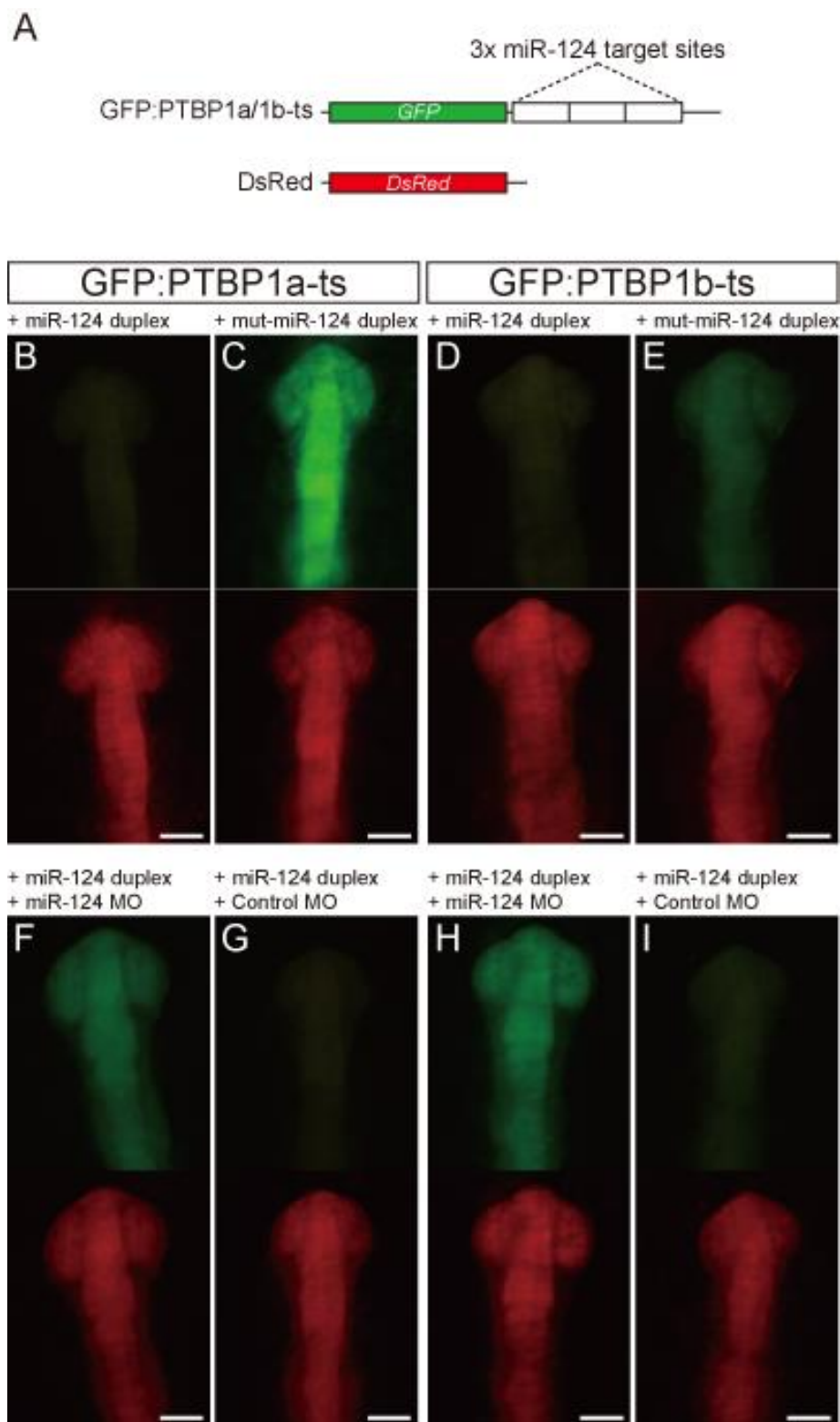
Fig. 3.1. **(A)** Schematic illustration of the reporter RNA constructs used in reporter assay. Normal miR-124 target sites in the 3′UTR of *Ptbp1a* and *Ptbp1b* were fused to the GFP coding region (GFP:PTBP1a/1b-ts). **(B–I)** Fluorescence light micrographs of 1-dpf embryos injected with reporter RNA (upper row) or showing control DsRed expression (lower row). Scale bars, 100 μM. MiR-124 duplex, duplex of mature miR-124 sequences; mut-miR-124, duplex of mutated mature miR-124 sequences; miR-124 MO, a morpholino oligonucleotide against mat-miR-124; control MO, a standard control morpholino oligonucleotide.
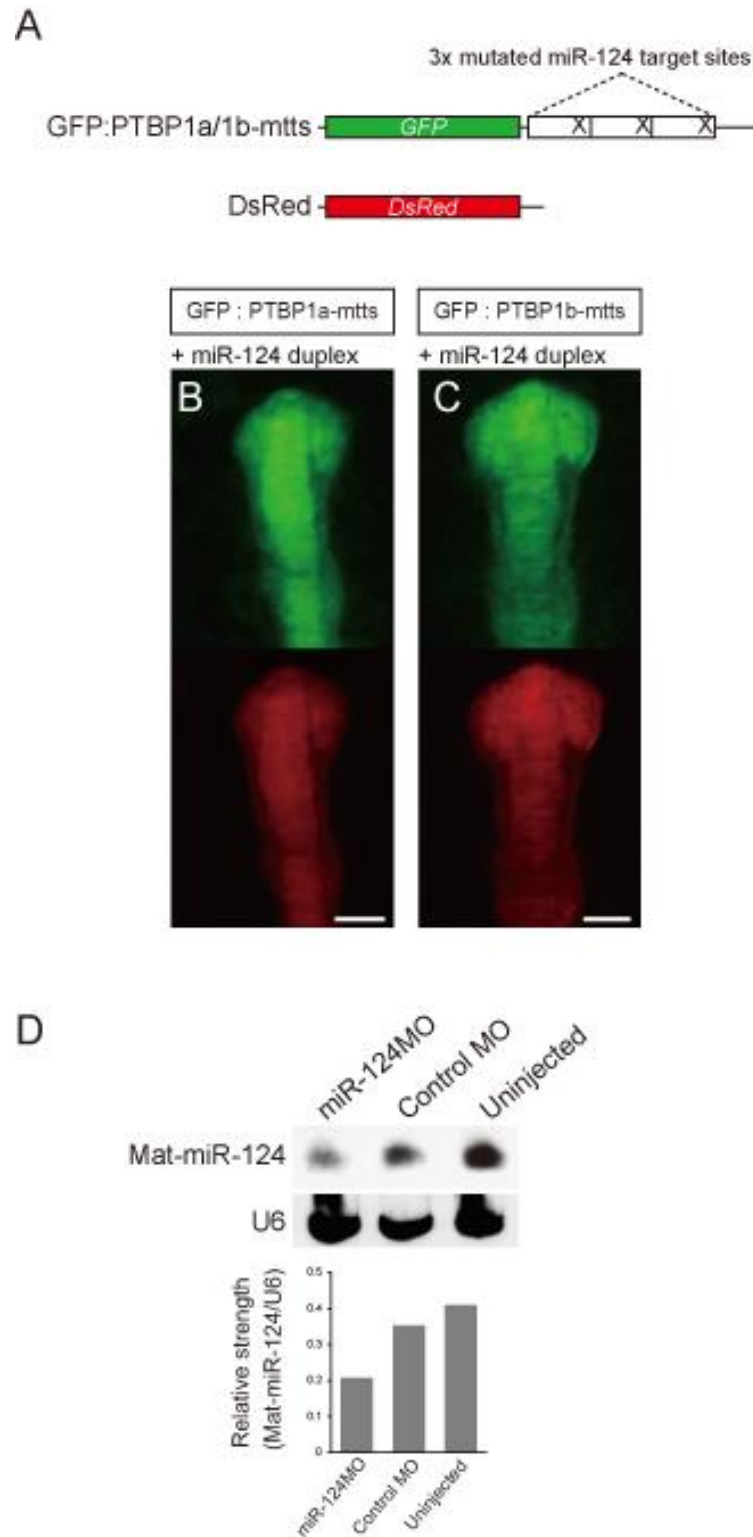
Fig. 3.2. **(A)** Schematic illustration of the reporter RNA constructs used in reporter assay. Mutated miR-124 target sites in the 3′UTR of *Ptbp1a* and *Ptbp1b* were fused to the GFP coding region (GFP:PTBP1a/1b-mtts). **(B–C)** Fluorescence light micrographs

of 1-dpf embryos injected with reporter RNA (upper row) or showing control DsRed expression (lower row). miR-124 duplex, a duplex of mature miR-124 sequences. Scale bar, 100 μm. **(D) (Above)** Northern blots of total RNA from embryos, with the membrane hybridized with the same LNA probes as in Fig. 3 to detect mat-miR-124 expression (top row), or with a probe to detect U6 snRNA (bottom row). Left lane (miR-124 MO), an miR-124 knockdown embryo injected with a morpholino oligonucleotide (1.6 mM) against mat-miR-124; center lane (Control MO), embryo injected with 1.6 mM control MO; right lane, uninjected embryo. **(Below)** Histogram showing the strength of mat-miR-124 expression relative to that of the internal control, U6 snRNA.
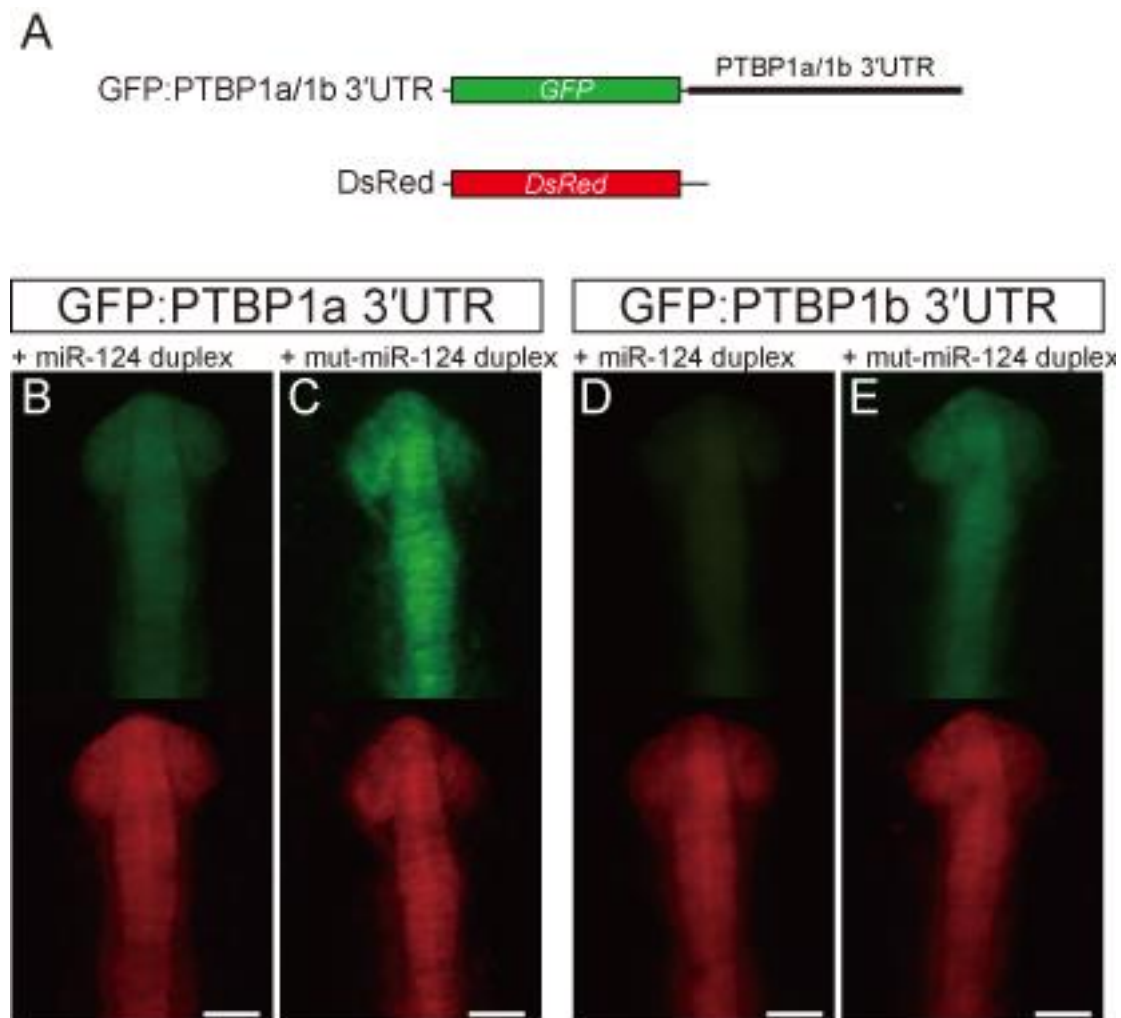
Fig. 3.3. **(A)** Schematic illustration of the reporter RNA constructs injected. The entire 3′UTR of *Ptbp1a* and *Ptbp1b* were fused to the GFP coding region (GFP:PTBP1a/1b 3′UTR). **(B–E)** Fluorescence light micrographs of 1-dpf embryos injected with reporter RNA (upper row) or showing control DsRed expression (lower row). Scale bars, 100 μM. MiR-124 duplex, duplex of mature miR-124 sequences; mut-miR-124, duplex of mutated mature miR-124 sequences.
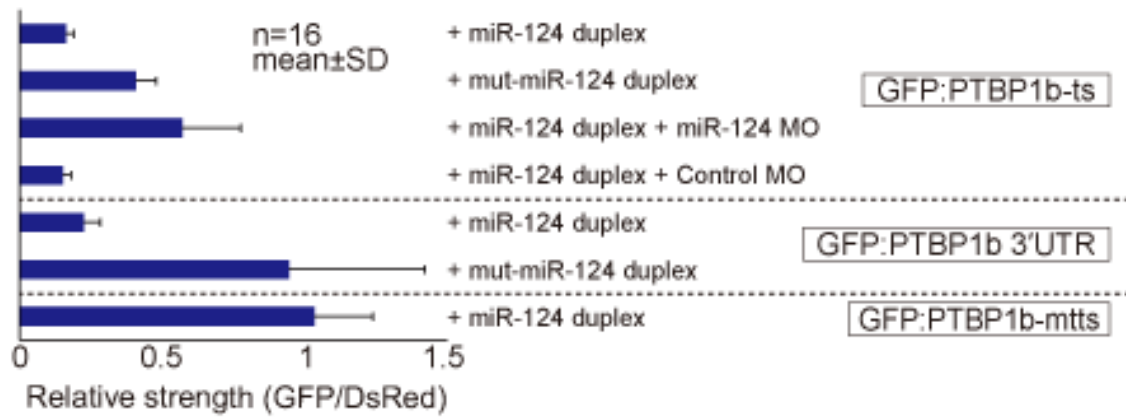
Fig. 3.4. Histogram showing the strength of GFP expression relative to that of the positive control, DsRed. Each bar and error line represents the mean ± SD of 16 DsRed-expressed embryos.

Table 3.1. Oligonucleotide fragments used for reporter assay. Underlines indicate the restriction enzyme sites.

Constructs		Nucleotide sequences
PTBP1a ts	Forward	<u>TCGAC</u>GTGGCGCTCACGTTCCCGTGTGCCTTT<u>CTCGAG</u>CCT
	Reverse	<u>CTAGA</u>GG<u>CTCGAG</u>AAAGGCACACGGGAACGTGAGCGCCACG
PTBP1b ts	Forward	<u>TCGAC</u>GTGGCTTTATCAAAGGACCCCTGTGCCTTA<u>CTCGAG</u>CCT
	Reverse	<u>CTAGA</u>GG<u>CTCGAG</u>TAAGGCACAGGGGTCCTTTGATAAAGCCACG
PTBP1a mtts	Forward	<u>TCGAC</u>GTGGCGCTCACGTTCCCGTGTGCGATT<u>CTCGAG</u>CCT
	Reverse	<u>CTAGA</u>GG<u>CTCGAG</u>AATCGCACACGGGAACGTGAGCGCCACG
PTBP1b mtts	Forward	<u>TCGAC</u>GTGGCTTTATCAAAGGACCCCTGTGCGATA<u>CTCGAG</u>CCT
	Reverse	<u>CTAGA</u>GG<u>CTCGAG</u>TATCGCACAGGGGTCCTTTGATAAAGCCACG
miR-124 duplex	Forward	UAAGGCACGCGGUGAAUGCdCdA
	Reverse	GUGUUCACAGUGGACCUUGdTdT
mutated miR-124	Forward	UAACCCACGCGGUGAAUGCdCdA
duplex	Reverse	GCAUUCACCGCGUGGGUAAdTdT

Table 3.2. Primers used for reporter assay. Underlines indicate the restriction enzyme sites.

Gene		Nucleotide sequences	Size of amplified products (bp)
Ptbp1a	Forward	CCA<u>CTCGAG</u>TCGAGTGTCCTTCTCCAAGT	2056
	Reverse	GGC<u>TCTAGA</u>AGACACCCTCCTGTGTTCAA	
Ptbp1b	Forward	TCT<u>GAATTC</u>GCCTTCGACTCGCCGGTCA	2473
	Reverse	AGA<u>CTCGAG</u>GTGATGCGTTCAAGTACACC	

Table 3.3. Morpholino oligonucleotides (MO) used in reporter assay.

MO	Nucleotide sequences
miR-124 MO	CTGTTGCATTCACCGCGTGCCTTA
Std control MO	CCTCTTACCTCAGTTACATTTATA

Table 3.4. The number of GFP expressed or not expressed embryos (GFP+ or GFP-) / total number of injected embryos in GFP reporter assy. PTBP1 ts, miR-124 target sites in 3' UTR of *Ptbp1a* and *Ptbp1b* fused to the GFP coding region. miR-124 MO, a morpholino oligonucleotide against mat-miR-124. Control MO, a standard contpl morpholino oligonucleotide. miR-124 duplex, duplex of mature miR-124 sequences. Mut-miR-124, duplex of mutated mature miR-124 sequences.

	Construction injected	PTBP 1a			PTBP 1b		
		total	GFP+	GFP-	total	GFP+	GFP-
i	PTBP1 ts + miR-124 duplex	119	0	70	153	0	92
ii	PTBP1 ts + mut-miR-124 duplex	75	41	0	155	99	0
iii	PTBP1 ts + miR-124 duplex +miR-124 MO	126	86	0	101	68	0
iv	PTBP1 ts + miR-124 duplex + Control MO	103	0	72	118	0	62
v	PTBP1 ts + miR-124 MO	59	27	0	52	30	0
vi	PTBP1 mtts + miR-124 duplex	116	69	0	103	60	0
vii	PTBP1 3'UTR + miR-124 duplex	57	0	36	90	0	55
viii	PTBP1 3'UTR + mut-miR-124 duplex	55	43	0	112	54	0

General Discussion

The present studies revealed that medaka miR-124 is specifically and abundantly expressed in the CNS, most likely restricted in differentiated neuron, and is involved in post-transcriptional regulation of target genes in neural development (Fig. 4.1), suggesting that miR-124 plays an important role in development of the CNS in medaka, as well as in other vertebrates.

Most miRNAs are well conserved between invertebrates and vertebrates. They are expressed in a tissue-specific manner, however, the timing and location of miRNA expression is not strictly conserved and the differences in expression are associated with changes in miRNA copy number, genomic context, or both, between species (Ason *et al.*, 2006). To reveal whether the distinct spatio-temporal expression patterns of pri-miR-124 are strictly regulated and contribute to differences in vertebrate miR-124 function, I will examine the expression in detail by section *in-situ* hybridization. Additionally, the sequence analysis of full-length *ola-pri-miR-124* genes is necessary for further analysis. Besides, promoter regions are typically located on 3000–5000 bp upstream sequences of coding sequences in medaka. Transgenic medaka lines using the promoter region fused to GFP coding sequence are possible to reproduce the expression of *pri-miR-124s*.

In knockdown and overexpression experiments on miR-124 by using the morpholinos and duplex, I obtained exhibited malformations of the brain and eyes in some of the embryos, although most embryos did not show obvious and invariable phenotypic effects (data not shown). In *Xenopus laevis*, down-regulation of miR-124a has no obvious effect on neural morphogenesis, whereas overexpression of miR-124a causes malformations in the optic nerve and optic cup (Qiu *et al.*, 2009; Liu *et al.*, 2011). MiRNA has a general role in 'fine-tuning' neural gene expression (Schratt, 2009; Vreugdenhil and Berezikov, 2010), hence medaka miR-124 might control the translation at too fine a level to result in obviously abnormal phenotypes upon disruption.

On the other hand, a knockout mutant for pri-miR-124a-containing gene ($Rncr3^{-/-}$ mice) exhibited abnormalities in the CNS, including small brain size, aberrant growth of dentate granule cell axons, and retinal cone death (Sanuki *et al.*, 2011). $Rncr3^{-/-}$ mice maintained 20–60% of miR-124a expression in some tissues, whereas, considerable loss of miR-124a expression was observed in the dentate gyrus. *Ola-pri-miR-124-3* is a widely and strongly expressed form of medaka pre-miR-124s. The knockout mutant for *ola-pri-miR-124-3* containing gene is possible to disclose the

medaka miR-124 function. Furthermore, many different types of brain-associated mutnants are avairable in medaka.

Vertebrate miR-124s regulate multiple gene expression. In *Xenopus*, miR-124 targets *Lhx2* and *NeuroD1*, both of which are transcription factors for neural differentiation (Qiu *et al.*, 2009; Liu *et al.*, 2011). In chick, miR-124 suppresses the anti-neural factor *SCP1* (Visvanathan *et al.*, 2007). In mouse, *REST*, *Sox9*, *BAF53a*, *Lhx2* and *Ptbp1* are reported as miR-124tartgets (Conaco *et al.*, 2006; Makeyev *et al.*, 2007; Cheng *et al.*, 2009; Yoo *et al.*, 2009). These target genes are associated with neurogenesis in multiple aspects, and some homologous genes are conserved in medaka genome. I need comprehensive analysis of candidate target genes by medaka miR-124 to investigate the sameness and differences among various vertebrate. MiRNA/mRNA interaction may allow the CNS complexity and diversity. These studies will provide us with invaluable insights to understand the complex vertebrate neurogenesis.

It is argued that miRNA disparity contributes to vertebrate complexity (Heimberg *et al.*, 2008; Somel *et al.*, 2011), while a large number of studies have reported links between alterations of miRNA homeostasis and pathologic conditions such as cancer, heart diseases, and neurodegeneration (Martino *et al.*, 2009). Disruption of miRNA expression induces many human diseases (Kumar *et al.*, 2007). MiRNA research will provide new insight into the underlying mechanisms of neurogenesis and will be required for drug discovery and therapy of neurodegenerative disease.

Figure
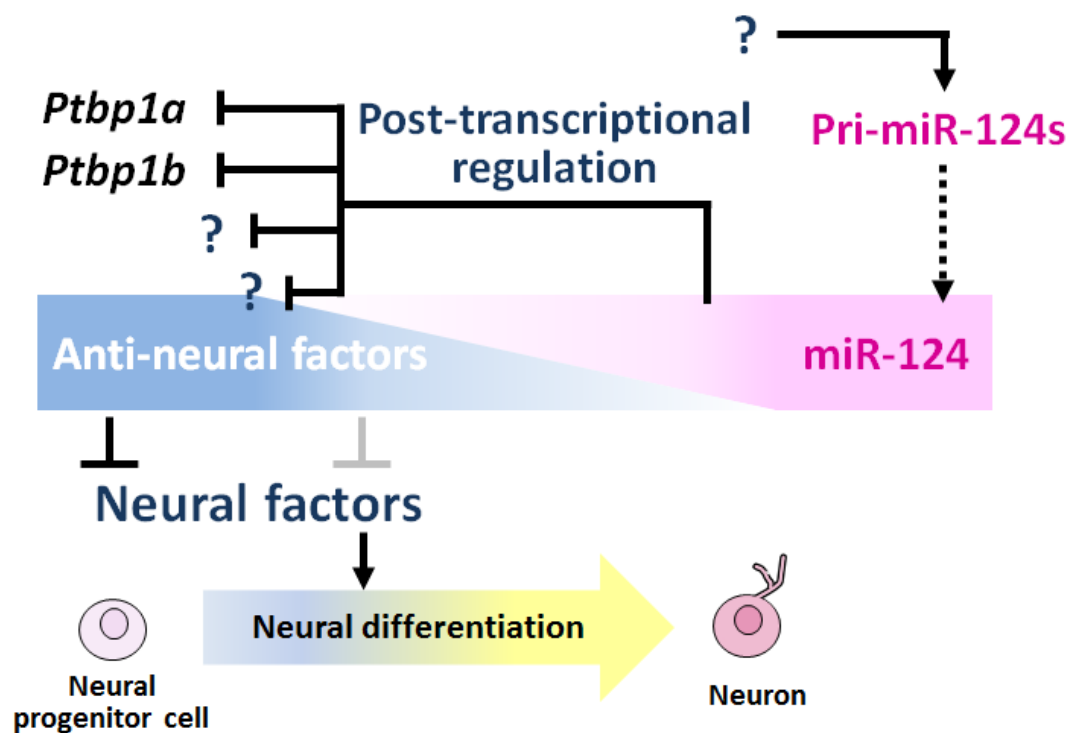
Fig. 4.1. MiR-124 regulates the translation of different anti-neural factors in the embryo and adult tissues and promotes neural differentiation.

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