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SYSTEMATIC IDENTIFICATION OF FACTORS IN ZEBRAFISH REGULATING THE EARLY MIDBRAIN AND CEREBELLAR DEVELOPMENT BY ODERED DIFFERENTIAL DISPLAY AND CAGED mRNA TECHNOLOGY

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1. ABSTRACT

During brain development, various transcription factors are activated in the regional specific manner and define the identities characteristic to individual regions, and many of such factors have been identified in the vertebrate brain, by taking advantage of the structural and functional conservation of them with the invertebrate counterparts. However, it is still largely unknown why individual transcription factors can define the final morphology and function of the tissues expressing these factors because of the lack of knowledge on which genes are actually up- or down-regulated as downstream targets of individual transcription factors. In this review, we introduce novel technologies which we have invented or improved as a part of our endeavor to identify and functionally analyze the downstream target genes of Isle-3 which are involved in development of the midbrain and the midbrain/hindbrain boundary region in zebrafish embryos. Our strategy and technologies can be applied to analyzing the downstream genes of any other transcription factors.

2. THE RECIPROCAL INTERACTION BETWEEN THE MIDBRAIN AND MIDBRAIN-HINDBRAIN BOUNDARY (MHB)

In the developing brain, the region at the border between the midbrain and the hindbrain forms a characteristic fold (isthmus), and is specially termed as the midbrain/hindbrain boundary (MHB) (Figure 1A). This tissue will later give rise to the cerebellum (Figure 1B). The molecular mechanisms underlying the establishment and the development of MHB have been vigorously studied (1-3). In late gastrulation, the presumptive MHB is specified at the border between two adjacent regions that express two distinct transcription factors, Otx2 and Gbx2; *Otx2* is expressed in the midbrain, whereas *Gbx2* is expressed in the hindbrain. In the presumptive MHB of zebrafish embryos, expression of the transcription factors Pax2.1 and Engrailed2 (Eng2) and the signaling molecules Wnt1 and FGF8 is initiated at around 10 hours post-fertilization (hpf), *i.e.* the bud stage (4).

In later stages, it has been proposed that the expression of these factors is maintained by reciprocal inductive signaling between the MHB and the presumptive tectal region of the midbrain, in which they positively and reciprocally regulate each others expression levels, and maintain the structural and molecular identities of these brain regions (5,6). The MHB tissue transplanted in the caudal forebrain induced *Wnt1* expression in the neighboring host tissue and transformed it into an ectopic midbrain, suggesting that MHB induces midbrain differentiation (7,8). Recent data have suggested that FGF8 intrinsically expressed in MHB may act as a signaling molecule mediating this induction (9). Presence of inductive signaling in the opposite direction, *i.e.* from the midbrain MHB, has also been suggested. Although *Wnt1* is not expressed in MHB in normal mouse development, *Wnt1* mutant mice lack both of the midbrain and the cerebellum, a derivative of MHB (10-12). In such mice, *En1* expression is initiated normally but is lost later, demonstrating that *Wnt1* expression in the midbrain is necessary for the maintenance of the *En1* expression. Recent data have further suggested that the signaling mediated by Wnt1 regulates development of the midbrain and MHB region mainly by activating *En*

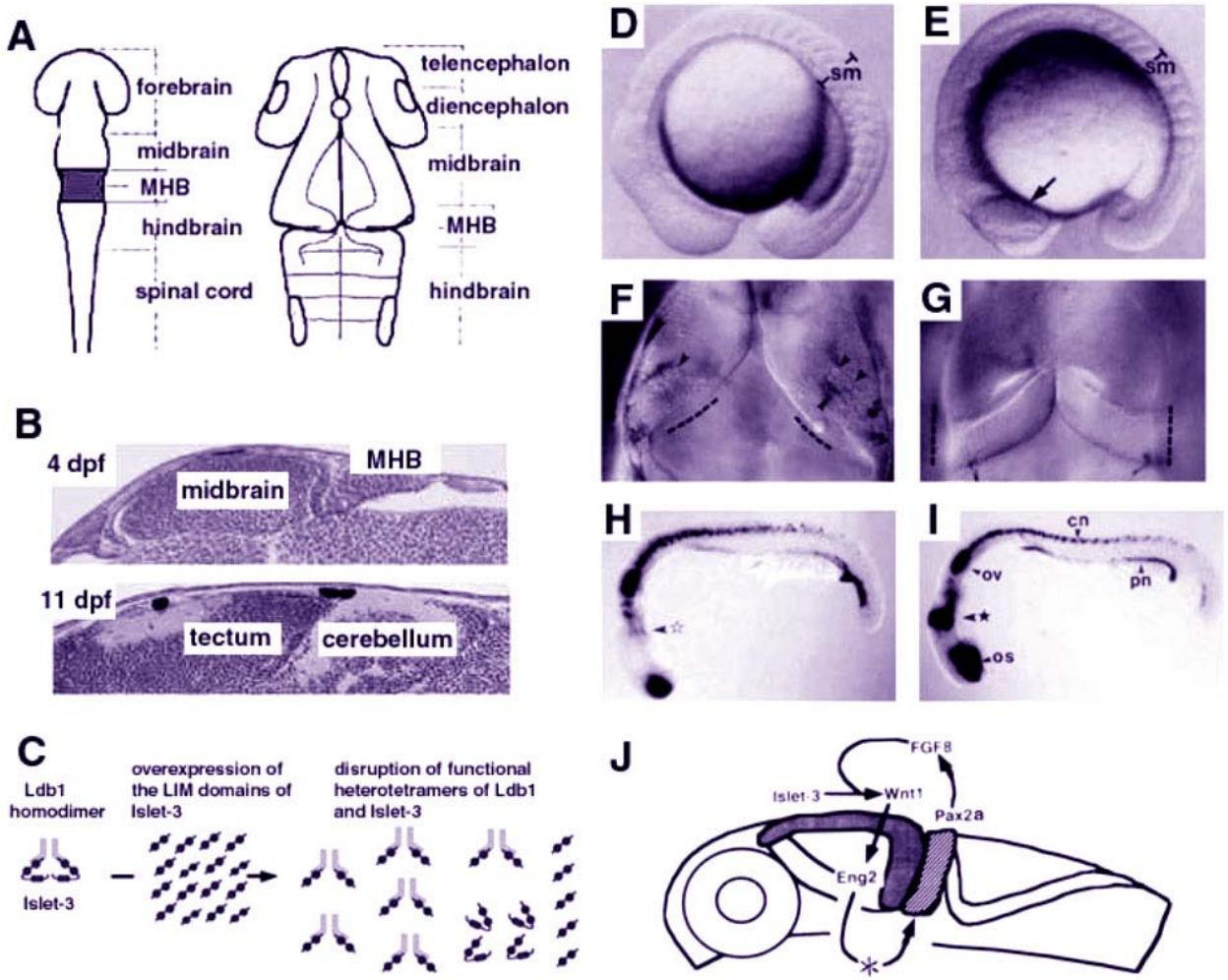

Figure 1. Involvement of Islet-3 in the reciprocal interaction between the midbrain and MHB in embryonic zebrafish. (A) Development of zebraifsh brain from 14 hours post-fertilization (hpf) through 24 hpf. (B) Development of the zebrafish midbrain and MHB respectively into tectum and cerebellum from 4 days post-fertilization (dpf) through 11 dpf. (C) Schematic illustration of the mechanism for the functional repression of Islet-3 by overexpression of the LIM domains (LIMIsl-3) of Islet-3. (D, E) Loss of the optic vesicles (arrow) in the 15.5-hpf embryo overexpressing LIMIsl-3 (D) compared to the normal embryo (E). sm, somites. (F, G) Loss of the isthmic fold (broken lines) around MHB in the 32-hpf embryo overexpressing LIMIsl-3 (F) compared to the normal embryo (G). (H, I) Loss of the pax2a expression around MHB (asterisk) in the 20-hpf embryo overexpressing LIMIsl-3 (H) compared to the normal embryo (I). os, optic stalk; ov, otic vesicle; cn, commissural neurons; pn, pronephros. (J) A possible role for Islet-3 in the reciprocal interaction between the midbrain and MHB. Induction of one molecule by the other is symbolized by an arrow connecting the involved two molecules. Wnt1 expression in the caudal midbrain and Pax2a expression in MHB are indicated by shade and hatch, respectively. A molecule (asterisk) that is released from the midbrain and mediates inductive signaling for the maintenance of pax2a expression in MHB has not been identified.

transcription, and that En protein then activates a putative midbrain signal which promotes normal development of the midbrain and MHB region (13).

3. ROLE OF ISLET-3 IN THE MHB DEVELOPMENT IN EMBRYONIC ZEBRAFISH

Zebrafish, *Danio rerio,* is widely used as a model organism for investigation of genetic control in early morphogenesis (14), because their transparent bodies make it easy to observe development of tissues and neurons *in vivo*. Islet-3 is a LIM/homeodomain-type transcription factor which is initially expressed ubiquitously, and is then gradually restricted to the optic vesicles and the presumptive tectal region of the midbrain in the central nervous system between 20 and 24 hours (hr) after fertilization in embryonic zebrafish (15).

The LIM/homeodomain-type transcription factors are involved in various aspects of neural specification. The LIM/homeodomain proteins are characterized by an N-terminal tandem repeat of two cysteine-rich metal binding domains (the LIM domains). These are thought to mediate

the assembly of protein complexes necessary for regulating gene expression (16). One family of the proteins that interact with the LIM domains is the LIM domain-binding (Ldb) proteins [also termed Nuclear LIM interactor (NLI) or co-factors of LIM/homeodomain proteins (CLIM)] (17-19). Ldb proteins have two modular domains: an N-terminal dimerization domain and a C-terminal LIM-interacting domain. Thus, Ldb proteins can assemble a complex in which two LIM/homeodomain proteins are bridged by an Ldb dimer, and are expected to mediate the synergistic actions of LIM/homeodomain proteins. This model has lead to the prediction that overexpression of only the LIM domains of any LIM/homeodomain proteins would displace LIM/homeodomain proteins from the Ldb dimers, and disrupt the synergistic actions of LIM/homeodomain proteins (Figure 1C).

In fact, overexpression of a protein (LIM^{Isl-3}) consisting of only the LIM domains of Islet-3 specifically prevented formation of the optic vesicles and severely impaired development of the midbrain/hindbrain boundary (MHB), which is the primordium of the cerebellum (20) (Figure 1D-G). Since the defects were all rescued by simultaneous overexpression of Islet-3, but not by overexpression of any other LIM/homeodomain proteins, the defects in the optic vesicles and MHB development induced by overexpression of LIM^{Isl-3} were attributed mainly to functional repression of Islet-3. In our experiment, even when Islet-3 function is repressed in this way, the expression of marker genes for the MHB, such as *wnt1*, *eng2*, and *pax2a*, was normally initiated at around 10 hpf, the early somitogenetic period. However, they are downregulated between 14-20 hpf, the mid-somitogenetic period when it is thought that the gene expression in the MHB start to depend on the reciprocal inductive signaling between the MHB and the midbrain (Figure 1H, I). Therefore, Islet-3 is implicated in maintenance of this reciprocal signaling (Figure 1J).

4. IDENTIFICATION OF DOWNSTREAM TARGET GENES OF ISLET-3 USING ODERED DIFFERENTIAL DISPLAY

We searched for genes that may be either down- or up-regulated by Islet-3 signaling pathway by comparing gene expression profiles in the midbrain-MHB region of normal embryos with those of the Islet-3-repressed embryos using a specially modified version of differential display method termed Ordered Differential Display (ODD) in combination with *in situ* hybridization analysis (21). ODD is a powerful and sophisticated method for the comparison of gene expression profiles (22,23) (Figure 2A, B). In ODD, double stranded cDNA is synthesized using oligo-dT-containing T-primer, and is digested with the restriction enzyme *Rsa*I, which recognizes a 4-nucleotide sequence and produces blunt-ended fragments. Following ligation of an adaptor to the digested fragments, the pools of 3'-most cDNA fragments (basic sample) are selectively amplified using a combination of adaptor-specific primer and T-primer under conditions in which the amplification of digested cDNA fragments with an adaptor sequence on both ends is suppressed (24,25). When displaying cDNA ragments, unlike conventional Differential Display (DD), which utilizes arbitrary primers (26,27), the basic samples are further amplified with adaptor- and T-primer-specific extended primers (AdE2-primers and TE-primers, respectively) that possess two extended bases at their 3'-ends, which contribute to the amplification of a specific subset of the basic sample. Use of these adaptor-specific primers facilitates highly reproducible results with few false-positive signals. In addition, less than 1 μg of total RNA is sufficient for a whole series of ODD reactions, a major advantage when studying tiny tissue samples such as those from one region of the developing zebrafish brain.

We identified 6 cDNAs that were specifically expressed around the MHB, 12 cDNAs specifically expressed in the tectum, and 3 cDNAs expressed in neural crest cells. Four novel clones were specifically expressed in the MHB (21) (Figure 2C-F).

5. DEVELOPMENT OF CAGED mRNA TECHNOLOGY FOR ACCESSING THE FUNCTIONS OF DOWNSTREAM TARGET GENES

One powerful approach for studying gene functions is a gain-of-function approach by ectopic expression of genes in a temporal- and spatial-specific manner. However, there is still no simple and effective system for the temporal and/or spatial control of gene expression in zebrafish. One promising approach is mRNA-caging, in which mRNA is inactivated by covalent attachment of a photo-removable protecting group (caging group), and then reactivated by photo-illumination with light of a specific wavelength. RNA caging was first achieved by the site specific modification of the 2'-hydroxyl nucleophile in the substrate RNA of the hammerhead ribozyme with a caging functionality, O-(2-nitrobenzyl) caging group (28). Susceptibility of the substrate RNA to hammerhead-catalyzed cleavage reaction was abolished by this modification, but recovered rapidly and efficiently after removal of this group by photo-illumination (308 nm, 10 J/ cm^2) with excimer laser. Another pioneering attempt to achieve spatio-temporal control of gene expression has been made by caging DNA with the 1-(4, 5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) group (29). HeLa cells trasfected with the plasmid which encoded GFP and was caged with this agent showed reduced levels (0~25 %) of GFP expression compared with the cells transfected with the intact plasmid, and exposure of these cells to UV light (365 nm, 0.25~0.5 J/ cm^2) doubled the expression level.

To apply mRNA caging technology for controlling gene expression at spatially and temporally high resolution in rapidly developing zebrafish embryos which have been injected with caged mRNA at the one-cell stage, we developed a novel RNA caging system that uses the caging agent 6-bromo-4-diazomethyl-7-hydroxycoumarin (Bhc-diazo) (30). Bhc-caged mRNA loses almost all translational activity, but illumination with 350 through 365 nm ultraviolet (UV) light removed Bhc from caged mRNA, resulting in recovery of translational activity (Figure 3A). Bhc can be removed from RNA by photo-illumination with a low level of energy (100 mJ/cm^2). This advantage has enabled the precise control of expression of genes in live zebrafish embryos by photo-illumination without giving a critical damage to the tissue.

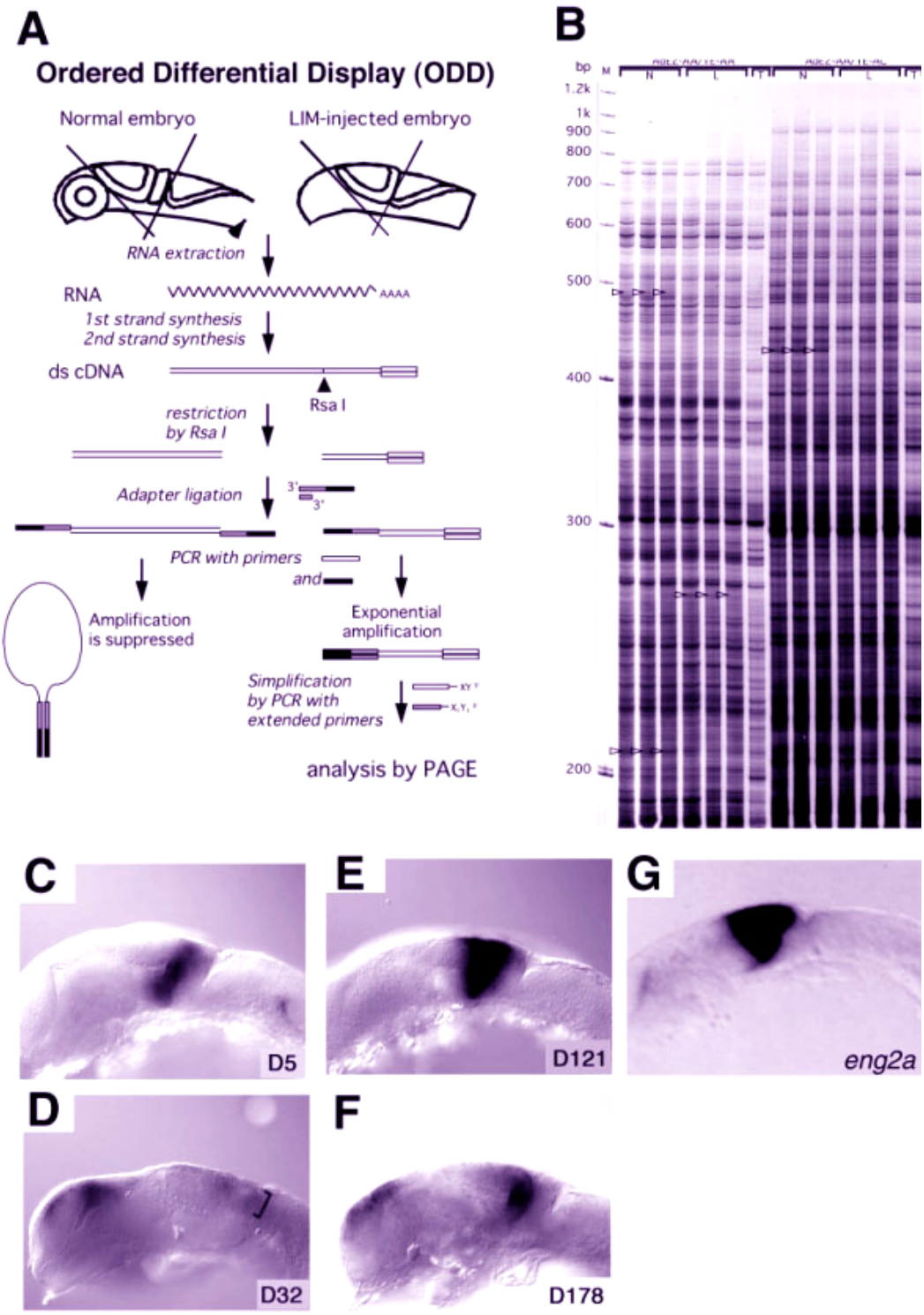

Figure 2. Searching for the downstream target genes of Islet-3 by ODD. (A) Schematic illustration of ODD. (B) Examples of ODD data for two different combinations of primer sets. N, the samples amplified from the normal midbrain and MHB; L, the samples amplified from the midbrain and MHB of the embryos overexpressing $LIM^{Isl\text{-}3}$. Three independently amplified samples were run next to each other from the same tissue. T, the sample amplified from the tail as a control. The fragments which are present only either of the normal or $LIM^{Isl\text{-}3}$-overexpressing tissues are indicated by arrowheads. (C-F) Expression patterns of four novel possible downstream target genes of Islet-3. (G) Expression pattern of eng2a for comparison.

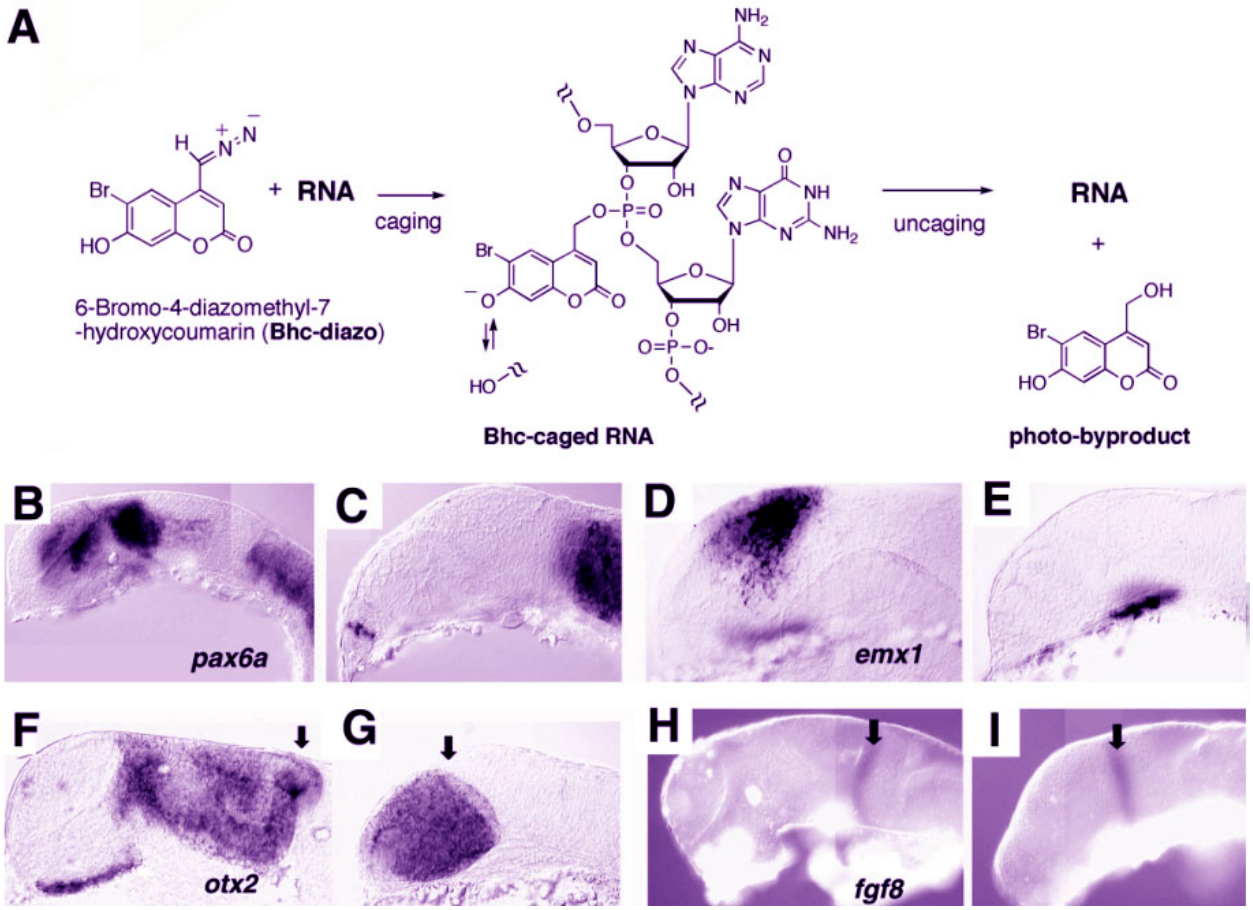

Figure 3. Ectopic overexpression of eng2a in the head region of embryonic zebrafish using caged mRNA technology. (A) Schematic illustration for inactivation of mRNA by reaction with Bhc-diazo and activation of caged mRNA by photo-illumination. (B-I) Anterior shift of the regions expressing the marker genes specific to the forebrain (pax6), telencephalon (*emx1*), the midbrain and diencephalons (*otx2*), and MHB (*fgf8*) in 22-hpf embryos (B, D, F, H) by ectopic expression of *eng2a* in the head region using caged mRNA technology compared to normal embryos (C, E, G, I). The arrows indicate the positions corresponding to MHB.

We used this mRNA caging system to examine the effects of ectopic expression of the homeodomain transcription factor Engrailed2a (Eng2a). Eng2a is predominantly expressed in the triangular region around MHB including the neighboring caudal midbrain and rostral hindbrain from an early stage (13 hpf) of embryonic development (Figure 2G).

The *pax6a*-positive region in the forebrain showed a dramatic reduction in size and shifted anteriorly (Figure 3B, C). We further examined the expression patterns of four other marker genes; *emx1*, a dorsal telencephalic marker, *otx2*, a dorsal midbrain marker whose caudal limit of expression shows a sharp edge near the caudal end of the midbrain, and *pax2a* and *fgf8*, markers of the MHB. In most of the severely affected embryos, the expression of *emx1* in the telencephalon was completely lost (Figure 3D, E). The *otx2*-positive region dramatically shifted rostrally, the anterior limit of which reached the most anterior tip of the brain (Figure 3F, G). Expression of both *fgf8* and *pax2a* was detected in the sharp narrow stripes in the MHB as in normal embryos, but had apparently shifted rostrally (Figure 3H, I). Our results demonstrate that *eng2a* can prevent the cells from taking the fates of the telencephalon as well as the diencephalons.

6. COMBINED USE OF CAGED mRNA TECHNOLOGY AND GENE KNOCKDOWN BY INJECTION OF ANTISENSE MORPHOLINO OLIGONUCLEOTIDE COULD REVEAL EPISTATIC RELATIONSHIP OF GENES.

Recently, a 'knockdown' technology using antisense morpholino oligonucleotide (AMO) was developed to facilitate direct assignment of functions to the genes. As compared to the gene targeting technology using the embryonic stem cells of mouse, translational repression by injection of AMO specific to the transcript of the gene of our interest into the early-stage zebrafish embryos is by far time- and cost-saving.

Among the candidates of the downstream target genes of Islet-3 is a D121 gene which is expressed highly

specifically around MHB, and shows the expression pattern highly similar to that of *eng2a* (21) (Figure 2E, G). Injection of AMO against the region around the translation initiation codon of D121 specifically caused the severe defect in development of MHB (unpublished data) which is highly similar to the defect observed in the embryos co-injected with the cocktails of AMOs against the transcripts of *eng2a* and *eng3* (32). Therefore, it is intriguing whether D121 is acting as downstream of *eng2a* and/or *eng3*. It would be possible to answer such questions by combined use of caged mRNA technology and the injection of AMO. If the activation of the D121 gene around the MHB by activation of caged D121 mRNA could rescue the development of this region in the embryo injected with AMOs against *eng2a* and *eng3*, the D121 gene would be interpreted to be acting as downstream of the *eng2a* and/or *eng3*.

7. FUTURE PROSPECTS

As recent advance in systematically compiling information on sequences of the whole genome and the expressed sequence tags (ESTs) with expression patterns of individual genes has made identification of novel genes easier than ever, classifying the genes according to the genetic cascades in which they are specifically involved and revealing the epigenetic relationships among the genes within the same genetic cascade is becoming more and more important for the systematic understanding of the genetic networks. Two novel technologies, ODD and caged mRNA technology, together with the AMO injection present us with the capability to systematically approach this problem.

Recently, it has become possible to label the specific subset of tissues or cells with Green Fluorescent Protein under control of the tissue specific cis-acting regulatory element in live transgenic animals (33). Since ODD requires such a small amount of total RNA to perform profiling the gene expression patterns, it would be possible to apply this technology to analyze the gene expression pattern within a specific-subset of GFP-positive cells to identify the genes the expression patterns of which are specifically affected within the GFP-positive cells.

Compared with other previously known caging agents such as nitrobenzyl- and DMNPE-caged molecules, Bhc-caged molecules are several-fold more sensitive to UV and ~30-fold more sensitive to photolysis by pairs of coincident infrared photons (34). Therefore two-photon infrared laser photolysis should permit even better three-dimensional spatial localization, greater penetration into scattering or absorbing tissues, and reduced possibility of photodamage to neighboring cells, thus allowing the activation of caged mRNA in the highly restricted region or cells of developing embryos.

Since AMO does not carry the phosphate moiety in its backbone, it is not possible to cage it with Bhc-diazo for conditional induction of its antisense activity. Peptide Nucleic Acids (PNA) are DNA analogs in which the nucleosides are attached to an N-(2-aminoethyl)glycine backbone instead of to deoxyribose, as in DNA (35). However, poor water solubility and a tendency to self-aggregate have limited the utility of traditional PNAs. These shortcomings have been recently overcome by developing negatively charged modified PNAs termed "gripNA"s. gripNAs have been shown to be highly effective in gene silencing experiments in both mammalian cells and in zebrafish (36,37). Since gripNA has the phosphate moiety in its backbone, caging of this compound with Bhc-diazo may be possible. This would make the conditional gene knockdown much easier than with the currently available sophisticated technology in which deletion of the target gene is induced by Cre-recombinase mediated homologous recombination between the loxP sites (38).

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