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The Medaka FoxP2, a Homologue of Human Language Gene *FOXP2*, has a Diverged Structure and Function

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Forkhead box (Fox) genes are involved in organogenesis and cell differentiation. A mutation of *FOXP2* was discovered in patients with severe defects in speech and language. The medaka FoxP2 was cloned in order to clarify the molecular evolution and difference in the protein structure and function by comparing human/mouse and medaka genes. The result showed that medaka FoxP2 had a 73.7% homology to the human and mouse counterparts, and its zinc finger, leucine zipper and forkhead domain structures were conserved. However, medaka FoxP2 lacked a long polyglutamine repeat and had two insertions of unique amino acid sequences. FoxP2 expression was found in the epiphysis and retina, in addition to the midbrain and cerebellum. The transcriptional assay revealed that medaka FoxP2 showed a very weak repressive activity to the *CC10* promoter while mouse Foxp2 exhibited a strong repressive activity. Mutational analyses of medaka FoxP2 showed that the three amino acids of forkhead domain were responsible for the weak repressive activity. These results suggest that medaka FoxP2 may play a different function in the development of the medaka fish.

Key words: forkhead domain, FoxP2, language gene, medaka, molecular evolution.

Abbreviations: DAPI, 4,6-Diamidino-2-phenylindole; dpf, days post-fertilization; EST, expression sequence tag; HRP, horseradish peroxidase; hpf, hours post-fertilization; IPTG, isopropyl-β-d-thiogalactopyranoside; PBS, phosphate-buffered saline.

Forkhead box (FOX) proteins are transcriptional factors, which play important roles in organogenesis and cell differentiation (*1*, *2*). The Fox family is subdivided into several subclasses according to the degree of homology in the forkhead domain (*3*). The Fox genes exist throughout species such as yeast, fungi, fish and mammals. The roles of the Fox genes family in organisms have been studied and found to have pleiotropic functions in development and metabolism. For instance, *FOXC1* is important for eye development (*4*) while *FOXC2* is important in lymphatic vessel development (*5*). *Foxj1* is important for ciliogenesis and left–right axis determination in the body (*6*). *FOXP3* plays an important role in T-cell maturation (*7*).

The mutation in the forkhead domain of *FOXP2* causes severe language and speech problems (*8*). The investigation through three generations of a family (KE family) revealed a missense mutation at R553H which causes impaired linguistic and grammatical skills including difficulties in the control of complex face and mouth movement that leads to disruption of speech (*9*). An unrelated individual with the similar symptoms was found to have a chromosomal translocation at *FOXP2*. *FOXP2* was the first gene identified that was associated with language.

FoxP2 has been studied in several species and is conserved among vertebrates. Mouse Foxp2 and songbird FoxP2 are 99.6% and 99.0% identical, respectively, in their amino acid sequences to the human protein (*10*, *11*). Their expression are also similar in several regions of brain, such as the cerebral cortex, basal ganglion and cerebellum (*12*, *13*). Recent studies reveal that zebrafish and *Xenopus* also have FoxP2, and their expression patterns in development are similar to the other organisms (*14–16*). In the mouse, several Fox proteins including Foxp2 are expressed in the lung. The Fox family members such as Foxa1, Foxa2, Foxf1, Foxf2, Foxj1 and Foxp1 are expressed in a variety of different cells in the lung and distinctly regulate lung-specific genes (*17–20*). *In vitro* studies show that Foxp2 works as a transcriptional repressor on the mouse *CC10* promoter in the lung and could work as a hetero- or homo-dimer for the repression (*10*, *21*).

In this study, the medaka (*Oryzias latipes*) *FoxP2*, was cloned in order to characterize its expression and investigated its molecular function. The results showed that medaka FoxP2 had three conserved domain structures but lacked a long polyglutamine repeat and had two extra amino acids insertions. The expression pattern in the brain was similar to those in the other organisms except that medaka FoxP2 was expressed in a

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pineal body. As a transcription factor, medaka FoxP2 had a very weak repressive activity over the *CC10* promoter in comparison to mouse Foxp2. Finally, a three amino acid difference in the forkhead domain was found to be responsible for the weak repressive activity of medaka FoxP2.

MATERIALS AND METHODS

Cloning of Medaka FoxP2 cDNA and Production of Monoclonal Anti-medaka FoxP2 Antibody—To clone medaka FoxP2 cDNA, an EST made from medaka fish at various developmental stages at website (http://medaka.lab.nig.ac.jp/est_index.html) was searched and it revealed that the MF015DA027C04 clone was likely to be the medaka homologue of the mouse *Foxp2* gene. Sequencing of the clone showed that it was a true medaka homologue. Next, a medaka cDNA library constructed from total RNA extracted from whole medaka fishes at 3 days post-fertilization (dpf) was screened with the 5′ side 300 bp as a probe that yielded the 574 bp longer cDNA. Based on the fact that medaka fish genes are similar to fugu fish genes, the reverse transcriptase-polymerase chain reaction (RT-PCR) method was performed. Two oligonucleotides, 5′-ATGATGCAGGARTCNGYNACAGAG-3′ (R=A+G, Y=C+T, N=A+C+G+T) and 5′-GTCGTCGCAGATGTTCTCACA-3′, were used as primers. The sequencing of the PCR product showed that it contained the 5′ part. The complete cDNA contained an open reading frame of 775 amino acids.

To make the HA-tagged FoxP2 expression vector in cultured cells, PCR was used to amplify a complete cDNA with 5′-TTTGAATTCATGTACCCCTATGACGTGCCCGACTACGCCGTCATGATGCAGGAGTCTGTGACA-3′ and 5′-TCTGAATTCTCACTCCAGGTCTTCCGATAG-3′ as primers. The EcoRI-cut PCR product was ligated into the EcoRI-cut CX-N2 expression vector (*22*) and named CX-HA-FoxP2. Mouse L cells were transfected with the CX-HA-FoxP2 expression vector using the calcium phosphate precipitation method and cultured in DMEM containing 10% fetal calf serum (FCS) and G-418 (0.25 mg/ml). The surviving cells were cloned using a cylinder technique and the cloned cell lines were stained with rabbit anti-HA antibody (MBL, Nagoya, Japan) as the primary antibody and with the FITC-conjugated swine anti-rabbit IgG antibody as the second antibody. The HA-positive cells were used as the FoxP2 protein-expressing cells for immunostaining to assess the anti-medaka FoxP2 antibody-producing hybridoma clones.

To make a GST-fusion protein, PCR was performed to amplify a complete FoxP2 with 5′-TCTGGATCCGGCTTGTTGCAGACCGGCCA-3′ and 5′-TCTGAATTCTCACTCCAGGTCTTCCGATAG-3′ as primers. The BamHI and EcoRI-cut PCR product was inserted into the BamHI and EcoRI-cut pGEX-6P-1 vector (GE Healthcare) to make the GST-FoxP2 plasmid. The GST-fusion protein containing the C-terminal 80 amino acids (^{696}G-^{775}E) of the FoxP2 protein was produced in an *Escherichia coli*, BL21 strain, by adding 1 mM IPTG, extracted in PBS containing 1 mg/ml lysozyme and 1% Triton X-100 and purified on Glutathione Sepharose 4B (GE Healthcare). The purified GST-FoxP2 protein (100 μg) emulsified in complete Freund adjuvant (Difco) was injected into the footpads of a female WKY/Izm rat (SLC, Hamamatsu, Japan) at the age of 4 weeks. Three weeks later, the lymphocytes were prepared from the retroperitoneal lymph nodes and fused with the SP2/W myeloma cells. The cells cultured in the GIT medium (Wako Chemicals, Osaka, Japan) containing 10% FCS, 10% BM-Condimed H1 (Roche #11 088 947 001) and HAT (Gibco). The HA-FoxP2- transfected cell lines (described above) were immunostained with the culture supernatants as the primary antibody and then with FITC-conjugated rabbit anti-rat IgG antibody as the second antibody. The hybridoma cells in the positive wells were cloned twice by limiting dilution. The cloned hybridoma cells were injected into the pristane-pre-treated abdomen of male BALB/c nude mice. The ascites were collected from the mice and the antibodies were purified with Protein G Sepharose 4 Fast Flow (GE Healthcare).

Cloning of Mouse Foxp2 cDNA and Production of Monoclonal Anti-mouse Foxp2 Antibody—The first strand cDNAs were produced by reverse transcription using poly(A)$^+$ mouse lung RNA with oligo(dT)$_{12–18}$ as a primer and then amplified by PCR using 5′-GTCATGATGCAGGAATCTGCG-3′ and 5′-CGTTCTCATTCCAGGTCCTCAG-3′ as primers. The PCR product was subcloned into the pGEM-Teasy vector and the entire DNA was sequenced. The confirmed plasmid was named pGEM(t)Foxp2. To add an HA tag at the N-terminal, PCR was used to amplify pGEM(t)Foxp2 with 5′-TTTGTTTTCATGTACCCCTATGACGTGCCCGACTACGCCGTCATGATGCAGGAATCTGCG-3′ and 5′-CGTTCTCATTCCAGGTCCTCAG as primers and subcloned into pGEM-Teasy. After confirmation by sequencing, it was named pGEM(t)HA-Foxp2. The production of monoclonal anti-mouse Foxp2 antibody will be published elsewhere (Z. Yang *et al*., submitted for publication).

Whole Mount Immunostaining and Immunohistochemistry—For whole mount immunostaining, eggs at specific embryonic stages were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The chorions of the eggs were removed and kept in methanol at –20°C until use. The embryos were gradually re-hydrated with PBS/methanol and soaked in PBST (0.3% Triton X-100 in PBS). PBSMT (2% skimmed milk and 0.3% Triton X-100 in PBS) was used for blocking for 2 h at 4°C. Monoclonal anti-FoxP2 antibody was used as the primary antibody at a dilution of 1:500 in PBSMT at 4°C overnight. The samples were washed with PBSMT for several times at 4°C and incubated at room temperature for 2 h in the same solution. Next, horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody (DakoCytomation, Denmark) was applied in PBSMT at 1:500 dilution at 4°C overnight. After washing, DAB solution [0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.003% H_2O_2 and 50 mM Tris–HCl, pH 7.5] was used for staining.

For immunohistochemistry, the 3-month-old adult fish was immersed in Bouin's fixative at 4°C for several days until the bone got softer. After fixation, the whole fish was embedded in paraffin, and then the sections measuring 8 microns in width were sliced and placed

onto glass slides. The sections were deparaffinized and immersed in 0.5 mM EDTA and boiled by microwave. After cooling, the samples were treated with 0.3% H_2O_2 in methanol for 30 min. The samples were blocked with 10% goat serum in PBS for 10 min, and then incubated with the primary antibody (anti-FoxP2) at 1:500 dilution in PBS containing 10% goat serum at 4°C overnight. The primary antibody was washed by PBS, and HRP-conjugated anti-rat IgG antibody was added at 1:500 dilution as the secondary antibody, followed by incubation for 30 min at room temperature. After washing, DAB solution was then used for staining.

Construction of Mutant Medaka FoxP2 and Mouse Foxp2 Expression Vectors—All site-directed mutagenesis were performed using Mutant-Express Km Enzyme/Oligo Set (Takara #6090, Kyoto). EcoRI fragments of medaka FoxP2 and mouse Foxp2 were ligated into the EcoRI-cut pKF18k-2. The oligonucleotide 5′-CACCCCCTGTACGGC CATGGCGTGTGCAAGTGG-3′ was used for introduction of NcoI site in the medaka GHG sequence. The oligonucleotide 5′-CCTTTGCTAAGTAACCCGGGACTGA TCAATAACGC-3′ was used for introduction of SmaI site in the mouse NPG sequence. The oligonucleotide 5′-CATAAGACAGGCGATCATGGAGTCTTCTGACAGGC AGCTAACGCTCAACGAGA-3′ was used for three amino acids mutation in the medaka FoxP2 forkhead domain. The mutated DNAs were named medaka FoxP2 (NcoI), mouse Foxp2 (SmaI) and medaka FoxP2 (FH-mut), respectively. As a result, mouse Foxp2 and medaka FoxP2 (NcoI) have a NcoI site in the GHG sequence which is located between the N-terminal part and central part, and medaka FoxP2 and mouse Foxp2 (SmaI) have a SmaI site in the NPG sequence which is located between the central part and C-terminal part. For chimera protein constructions, the EcoRI/NcoI fragment of the N-terminal portion, the NcoI/SmaI fragment of the central portion and the SmaI/EcoRI fragment of the C-terminal portion were connected in all combinations (Fig. 5A).

The EcoRI fragments of medaka FoxP2, medaka FoxP2 (FH-mut) and mouse Foxp2 were ligated into the EcoRI-cut CX-N2 expression vector and named CX-HA-FoxP2, CX-HA-FoxP2 (FH-mut) and CX-HA-Foxp2, respectively. The EcoRI fragment of six chimera protein constructs were ligated into the EcoRI-cut CX-N2 expression vector, and named OEO, OEE, OOE, EEO, EOE and EOO.

Cell Culture and Measurement of Luciferase Activity—The *CC10* promoter DNA was obtained by PCR using a genomic DNA from HepG2 cells with 5′-GGTAAGGC CTGGGAATGGCTAAC-3′ and 5′-GGGTATGTGTGGG TGTGTGGC-3′ as primers and subcloned into pGEM-Teasy. The EcoRI fragment of pGEM (t) CC10-promoter was filled-in and inserted into the EcoRV-cut pGL4.10 (luc2) vector (Promega, WI) and named *CC10*-luc2. The Clara cell-like lung adenocarcinoma H441 cells were obtained from ATCC and cultured in RPMI1640 medium containing 10 mM HEPES, 2 mM glutamine and 10% FCS.

Western Blot Analysis—The lysates of transfected H441cells were separated by electrophoresis on 8% SDS–polyacrylamide gel under reducing conditions. The proteins were loaded according to their protein concentration and normalized by transfection efficiencies of the cotransfected Renilla luciferase plasmid (10 ng). The proteins were transferred onto a nylon membrane and were incubated with rabbit anti-HA antibody followed by HRP-conjugated anti-rabbit IgG antibody. Finally, the protein bands were detected using the chemiluminescent ECL kit (Perkin-Elmer Life sciences Inc.).

Fish—The medaka fish strain Cab (*23*) was maintained with 14 h/10 h day–night cycle at 28°C. Eggs were kept in medaka Ringer's solution (0.65% NaCl, 0.04% KCl, 0.011% $CaCl_2$, 0.01% $MgSO_4$, 0.01% $NaHCO_3$ and 0.0001% methylene blue) at 28°C.

RESULTS

Cloning of Medaka FoxP2 Gene—Since human, chimpanzee, mouse and songbird *FoxP2* genes are highly conserved, the medaka EST sequences were searched to find sequences with homology to the mouse Foxp2 nucleotide sequence at the medaka website (http://medaka.lab.nig.ac.jp/est_index.html) and one candidate clone was found. The sequencing of the clone showed that it was a true medaka homologue of mouse *Foxp2* gene, but it lacked the sequence coding for the N-terminal portion of the protein. The complete cDNA was obtained by two methods described in the MATERIALS AND METHODS section. The entire medaka FoxP2 sequence has been deposited in the GenBank (accession number EU143691).

The medaka FoxP2 protein was predicted to be 775-amino acid long (Fig. 1). The comparison of the FoxP2 protein to mouse Foxp2 protein showed a 73.7% homology with conservation of a zinc finger, a leucine zipper and a forkhead domain (Fig. 1B). The most prominent differences in medaka Foxp2 were a lack of a 40-glutamine repeat which is present in mouse and human, and insertions of an extra 16 amino acids (N-terminal) and 58 amino acids (C-terminal). Recently, zebrafish FoxP2 was cloned (*14*, *15*). Interestingly, it also lacked a 40-glutamine repeat and also had a 15-amino acid insertion at the C-terminal portion (Fig. 1A and B). The arginine residue (^{553}R) that is mutated in patients with defects in language and speech was conserved in medaka and zebrafish.

Production of Monoclonal Anti-medaka FoxP2 Antibody—In order to determine the temporal and spatial expression of medaka FoxP2 in developmental stages, rat monoclonal anti-medaka FoxP2 antibodies, Z3.1 and Z1.1, were made. Since both antibodies showed the same results, data using antibody Z3.1 is shown hereafter. First, the cell extracts from the untransfected and FoxP2 expression vector-transfected H441 cells were subjected to a western blot analysis with anti-medaka FoxP2 antibody. The antibody recognized the FoxP2 protein only in the FoxP2-expressing cells, but not in the untransfected cells (Fig. 2A). Next, the untransfected and FoxP2 expression vector-transfected H441 cells were stained with the antibody. Immunofluorescent microscopy showed that FoxP2 was localized in the nucleus (Fig. 2B). These confirmed that the monoclonal antibodies specifically recognized FoxP2.

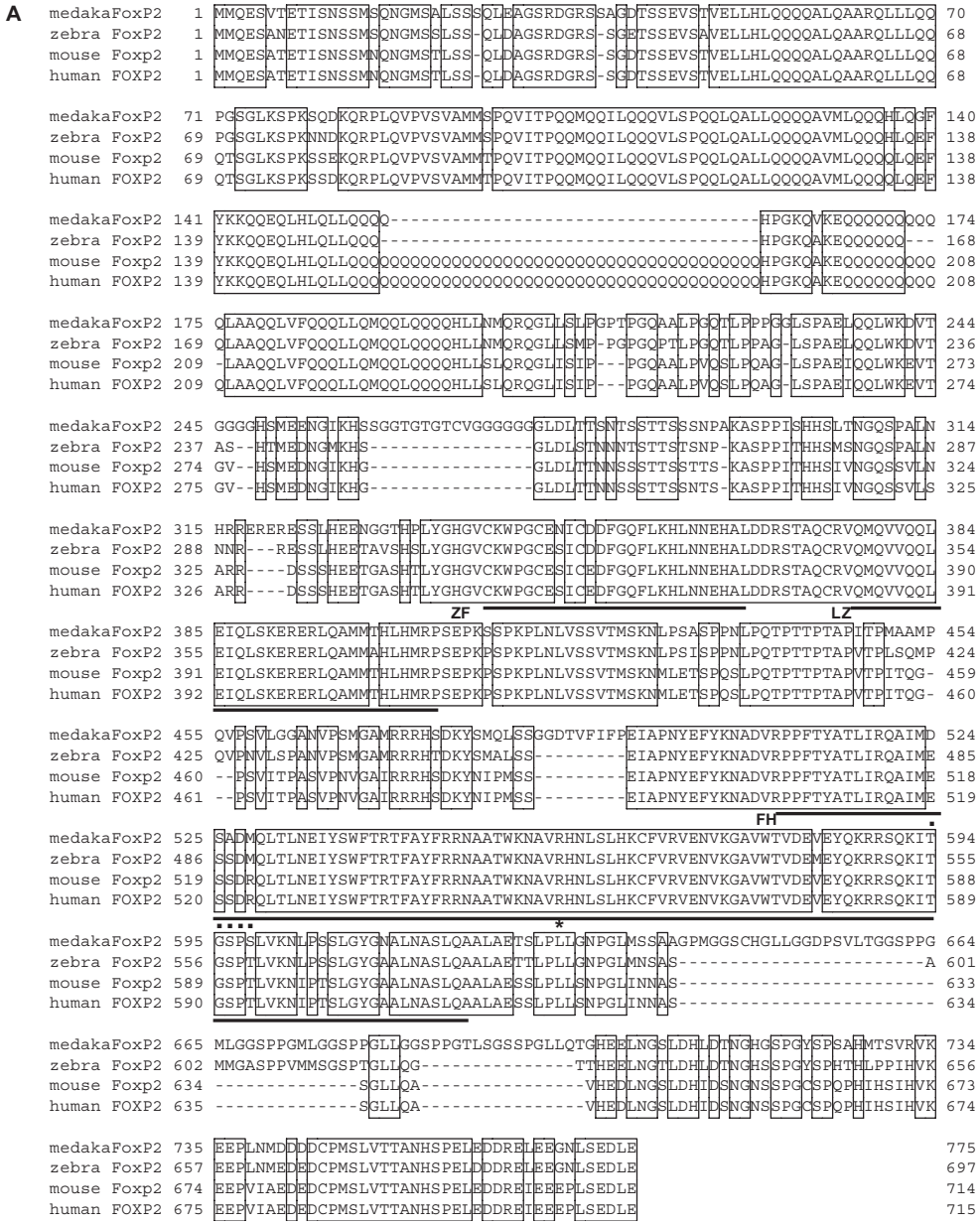

Fig. 1. **Amino acid sequence of FoxP2 protein.** (A) Amino acid alignment of medaka fish, zebrafish, mouse and human FoxP2. Conserved amino acid sequences are enclosed in boxes. A zinc finger structure, a leucine zipper and a forkhead domain are underlined with the headings, ZF, LZ and FH, respectively. The DSDAM sequence has underlying dots and R553 is indicated by an underlying asterisk. (B) A schematic presentation of FoxP2 proteins of human, mouse, zebrafish and medaka fish. Polyglutamine repeat (Blue), zinc finger (red), leucine zipper (green) and forkhead (yellow) domains are conserved. Black boxes of zebrafish and medaka fish show extra amino acid sequences different from the other orthologues. The patients with severe defects in language and speech have a missense mutation at R553H.

Expression of Medaka FoxP2—The expression pattern of medaka FoxP2 was determined. First, whole mount immunostaining was used with developing embryos at different stages. A strong expression was detected at the neural tube of early somitic stage embryo, which continued until the late somite stages (Fig. 3A and B). At 3dpf, the expression was decreased in the neural tube, and the expression was found at the otolith and midbrain (Fig. 3C). As development proceeded, FoxP2 was expressed in the whole brain and two layers of the retina (Fig. 3D).

Whole mount immunostaining of the adult brain showed a very complex pattern (Fig. 3E). So section immunohistochemistry was performed to observe medaka FoxP2 spatial expressions of the adult brain. In the adult anterior forebrain, FoxP2 was expressed at the medialis of the dorsal telencephalis and nervus terminalis (Fig. 3F). In the section of the posterior forebrain,

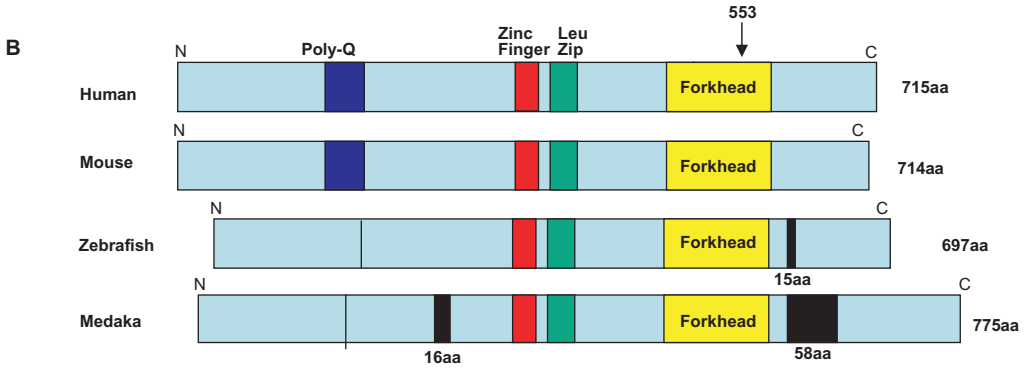

Fig. 1. **Continued.**

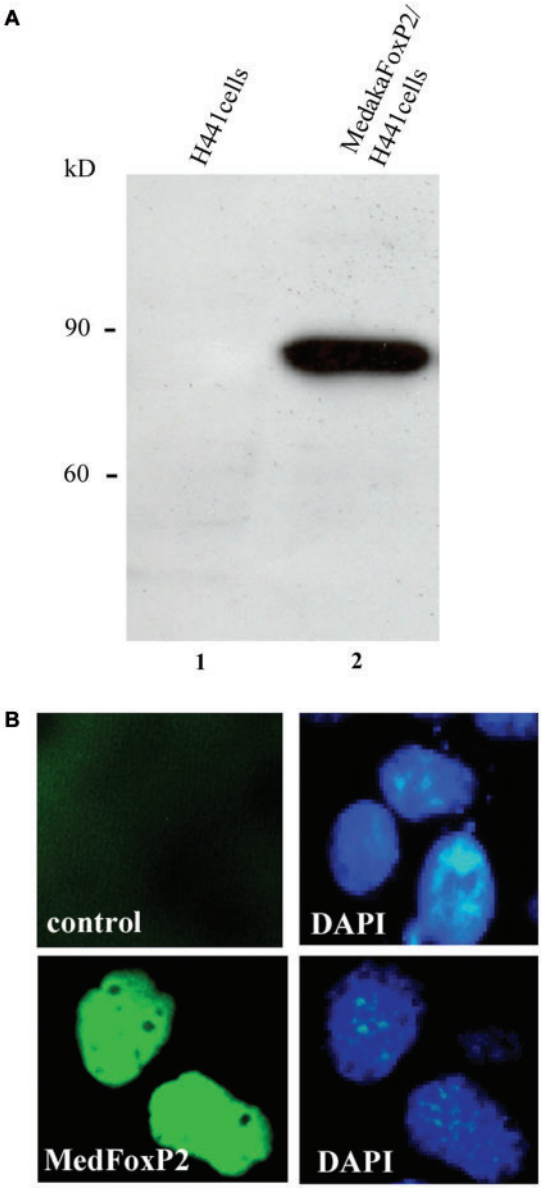

Fig. 2. **Monoclonal anti-medaka FoxP2 antibody recognized the FoxP2 protein localized in the nucleus.** (A) A western blot analysis with anti-medaka FoxP2 antibody. The cell extracts (20 μg) from the untransfected H441 cells (lane 1) and the CX-HA-FoxP2 expression vector-transfected H441 cells (lane 2) were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to a nylon membrane. (B) Immunofluorescent staining with anti-medaka FoxP2 antibody. The H441 cells were transfected with the empty expression vector (upper panels) and the CX-HA-FoxP2 expression vector (lower panels). The cells were fixed, incubated with anti-FoxP2 antibody and then stained with FITC-conjugated anti-rat IgG antibody (left panels). They were also stained with DAPI to identify the nucleus (right panels).

moderate signals were detected in the nucleus preopticus parvocellularis and optic stalk (Fig. 3G). A very strong expression was observed at the epiphysis, which is believed to contain photoreceptors present among fish (*25*) (Fig. 3H). A layer of optic tectum [outer most layer of periventricular grey zone 3 (PGZ3)] expressed FoxP2 in the midbrain (Fig. 3I). A strong signal was observed in the thalamus and hypothalamus (Fig. 3H–J, arrowheads). In the hindbrain, FoxP2 was expressed in the Purkinje cells (Fig. 3J, an arrow). FoxP2 signals were detected in two layers of the adult retina (Fig. 3L) as observed in the embryonic retina (Fig. 3D). Interestingly, we observed the FoxP2 was expressed in a portion of cells at the inner nuclear layer and ganglion cell layer (Fig. 3L, inlet). In contrast, immunohistochemistry of the mouse retina using monoclonal anti-mouse Foxp2 antibody showed mouse Foxp2 to be expressed in nearly all cells at both layers (Fig. 3K, inlet).

Mouse Foxp2 has been reported to be expressed in the lungs and intestine (*10*). FoxP2 was expressed in the medaka intestine (Fig. 3N). Medaka fish have gills instead of lungs. However, no FoxP2 expression was observed in the gills (Fig. 3M). In addition, the FoxP2 expression was observed in the ovary and spinal cord (data not shown).

Molecular Characterization of Medaka FoxP2 Protein— The N-terminal and the C-terminal portions of medaka FoxP2 were very different from those of mouse Foxp2. So the transcriptional activity of medaka FoxP2 was determined. Unfortunately, the target genes of mouse Foxp2 in the brain were not known, as is the case with the medaka FoxP2. It was previously reported that Foxp2 inhibits the *CC10* gene promoter in the H441 cells, so this system was used to study the transcriptional activity of medaka FoxP2 (*10*). HA-tagged expression vectors were made for mouse Foxp2 and Medaka

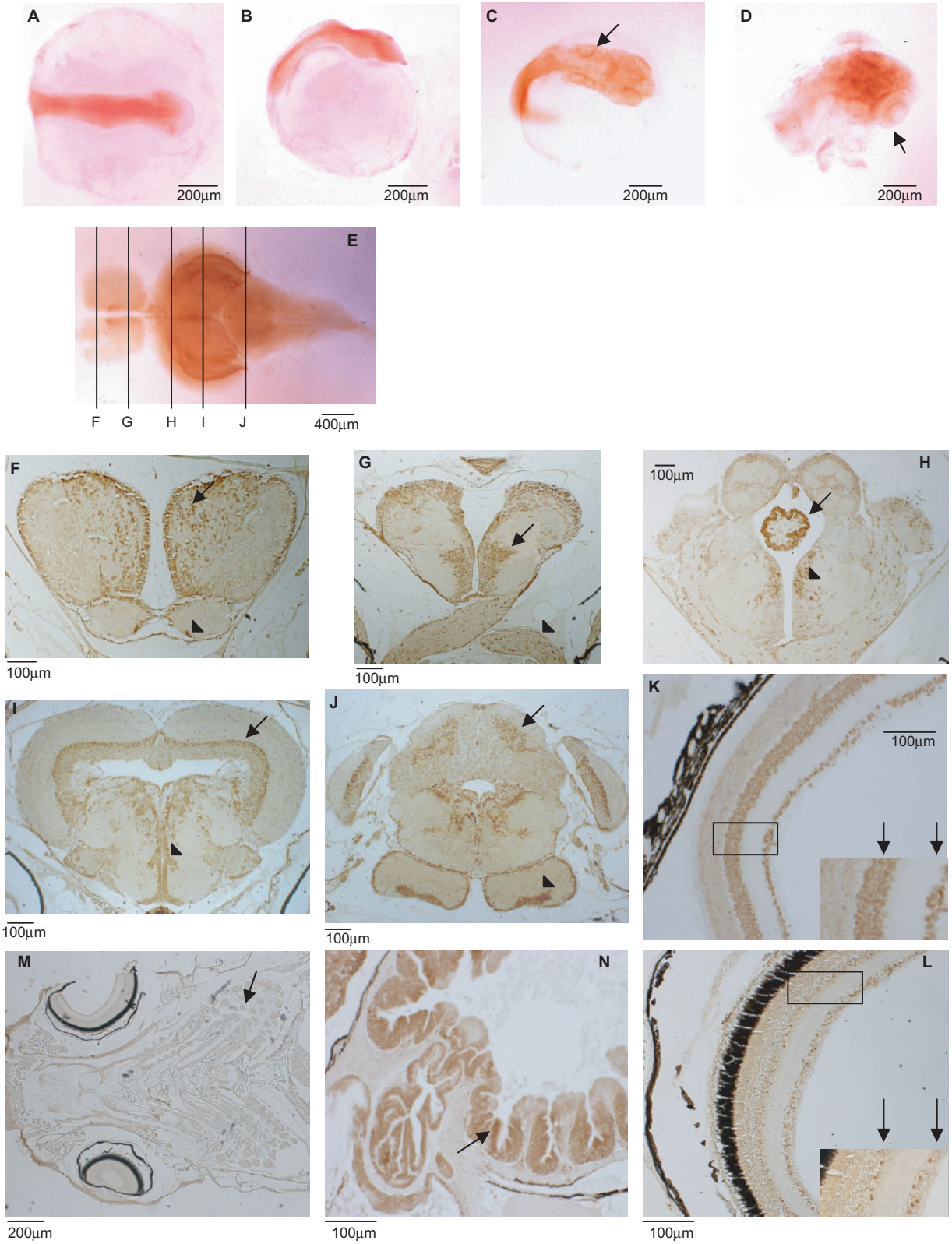

Fig. 3. **Expression of medaka FoxP2 in the developing embryo and adult fish.** (A–D) Whole mount immunostaining of FoxP2 expressions during medaka development. A, Dorsal view at 2 dpf. The strong expression of FoxP2 is detected through the neural tube. Right side is anterior. B, Lateral view at 2 dpf. Right side is anterior. C, Dorsal view at 3 dpf. Otolithes (an arrow) express FoxP2. D, Dorsal view at 6 dpf. In addition to strong expression in the midbrain, FoxP2 is detected at the retina (an arrow). (E) FoxP2 immunostaining of adult medaka fish brain (dorsal view). The brain was dissected from 3-month-old medaka fish, fixed in a Bouine's solution and processed to whole mount immunostaining with anti-FoxP2 antibody. (F–J) FoxP2

FoxP2 (Fig. 4A, upper two constructs). When the expression vector was cotransfected with *CC10*-luc2 reporter gene, the mouse Foxp2 dramatically inhibited the luciferase activity in a dose-dependent manner (Fig. 4B, filled diamond). In contrast, the medaka FoxP2 showed a very weak repression on the *CC10* promoter (Fig. 4B, shaded square). A western blot analysis showed that the expressed proteins had the expected sizes (Fig. 4C, lanes 2 and 3).

Next, the portion of medaka FoxP2 responsible for its weak repressive activity was determined. Nucleotide sequences for medaka FoxP2 and mouse Foxp2 were mutated to create NcoI and SmaI sites at the corresponding nucleotide positions, respectively, without any changes of the amino acids (Fig. 5A). DNA fragments with EcoRI/NcoI, NcoI/SmaI and SmaI/EcoRI sites were obtained and combined to make the six chimera proteins (Fig. 5A, lower part). The central part within the NcoI and the SmaI sites has a zinc finger, a leucine zipper and a forkhead domain. Two wild-type and six chimera protein expression vectors were transfected into H441 cells and the luciferase activity for the *CC10* promoter was measured (Fig. 5B). Surprisingly, the constructs that had the mouse central part showed a strong repressive activity as did the wild-type mouse Foxp2 construct and those that had the medaka central part exhibited a weak repressive activity similar to the wild-type medaka FoxP2 construct (Fig. 5B). This indicates that the repressive activity is determined by the central part of the protein. The sizes of chimera proteins were confirmed by western blotting (Fig. 5C). The results showed that the proteins with the expected sizes were produced. Unexpectedly, the amounts of protein that had the mouse C-terminal part were larger than those of protein that had the medaka C-terminal part. It is possible that since medaka fish live at lower temperatures, the production of medaka protein at 37°C might thus cause an instability of medaka FoxP2. However, the amount of medaka FoxP2 in culture at 28°C was similar to that at 37°C (data not shown). These results indicate that the amount of protein cannot account for the repressive activity in Fig. 5.

To further analyse the importance of the central part for the repressive activity, medaka and mouse forkhead domains were compared. The results showed that there was a three-amino acid difference between medaka FoxP2 (DSADM) and mouse Foxp2 (ESSDR) within the forkhead domain. These three amino acids of medaka protein were mutated into the mouse type (Fig. 4A, lower construct). When the medaka forkhead mutant (FH-mut) was transfected into H441 cells, it repressed the luciferase activity of the *CC10* promoter as the wild-type mouse Foxp2 did (Fig. 4B, shaded triangle). This result indicates that a difference in three amino acids is a determinant for a weak repressive activity of medaka FoxP2.

DISCUSSION

FoxP2 protein has been studied in several types of organisms, but its molecular role has not yet clearly elucidated (*10*–*16*). Comparison of the orthologues could give an insight of the molecular evolution and functional difference of the protein. The medaka FoxP2 was cloned and analysed to compare with different species such as human, mouse and zebrafish. Similarities and differences were found in the protein structures, expression patterns and transcriptional activities.

In the amino acid sequence of medaka FoxP2, notable differences from the mammalians included a deletion of a long glutamine repeat and insertions of extra amino acids. Other teleosts, such as fugu (presumptive) and zebrafish, also lack a long glutamine repeat and have extra amino acid insertions (*14*, *15*). However, FoxP2 proteins among the teleosts were relatively different while those in the terrestrial organisms are similar to each other. For example, the homology between medaka and zebrafish was 80%, and that between medaka and fugu fish was 73.5% while the homologies of the mammalian FoxP2 are over 99%. The songbird, which separated from mammalians at a very early evolutionary point, shows a strong homology to its mammalian counterparts (*24*). Interestingly, *Xenopus* FoxP2 also has the long glutamine repeat and is 95% identical to human FOXP2 (*16*). In summary, the conservational level of FoxP2 in amphibians lies between the aquatic and terrestrial organisms.

The expression of medaka FoxP2 was similar to that of other organisms. During embryonic development, the expressions were dynamic. At the early somite stage, it was diffusely expressed in the entire neural tube. Thereafter, as the development proceeded, the expression was more localized to specific areas, such as the midbrain, otolith and retina. The FoxP2 expression in the adult medaka brain more closely resembled to that in the adult mammalian brain. FoxP2 was expressed in the thalamus, hypothalamus, tectum, retina, ventral

immunostaining of brain sections at several sites. A whole adult medaka fish was fixed and embedded in paraffin. The section samples corresponding to the planes shown in E were processed to immunohistochemistry with anti-FoxP2 antibody. (F) Forebrain. FoxP2 immunoreactivity is observed at the area medialis of dorsalis telencephali (an arrow) and the nervus terminalis (an arrowhead). (G) Posterior forebrain. The signals are detected in the nucleus preopticus parvocellularis anterioris (an arrow) and optic stalk (an arrowhead). (H) Anterior midbrain. FoxP2 immunoreactivity is observed at the epiphysis (an arrow) and nucleus ventromedialis thalami (an arrowhead). (I) Middle midbrain. The signals are detected in the optic tectum (an arrow) and thalami (an arrowhead). (J) Hindbrain. FoxP2 immunoreactivity is observed in the Purkinje cells (stratum gangilonare; an arrow) and hypothalamus periventricularis dorsalis (an arrowhead). (K) Foxp2 immunostaining of adult mouse retina. An eyeball was dissected from an adult mouse, fixed and embedded in paraffin. The section was processed to immunohistochemistry. An inlet shows the magnified picture marked in the box. Foxp2 immunoreactivity is observed in the inner nuclear layer and layer of the ganglion cells (arrows). (L) FoxP2 immunostaining of adult medaka retina. A whole adult medaka fish was fixed and embedded in paraffin. The section of an eye was processed to immunohistochemistry. An inlet shows the magnified picture marked in the box. A part of cells in the inner nuclear layer and layer of the ganglion cells shows immunoreactivity (arrows). (M and N) FoxP2 immunostaining of gills and gut. The paraffin section was immunostained with anti-FoxP2 antibody. Immunoreactivity was not detected in the gills (an arrow) (M), but detected in the gut (an arrow) (N).

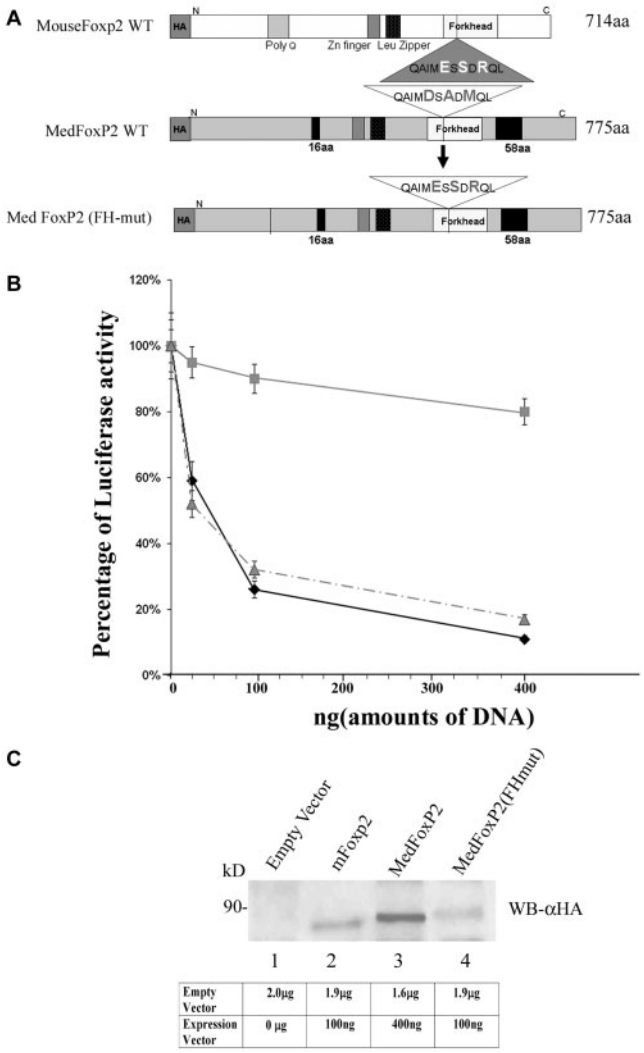

Fig. 4. **Medaka FoxP2 protein shows a weak repression.** (A) A schematic representation of the wild-type mouse Foxp2, wild-type medaka FoxP2 and medaka *FoxP2* forkhead mutant. (B) Transcriptional repressive activity of medaka FoxP2 and mouse Foxp2. Five hundred nanograms of *CC10*-luc2 plasmid and various amounts of CX-HA-Foxp2, CX-HA-FoxP2 and CX-HA-FoxP2 (FH-mut) expression vectors were cotransfected into H441 cells. Forty-eight hours after transfection, cells were harvested and the luciferase activity was measured. The transfection efficiencies were normalized using 10 ng of Renilla luciferase (GL4.7 hR-luc) plasmid. Data are expressed as percentages of control luciferase activity and represent mean ± SD. (C) A western blot analysis of proteins. Normalized amount of proteins were loaded and subjected to SDS–polyacrylamide gel electrophoresis and transferred to a nylon membrane. The transferred membrane was incubated with polyclonal anti-HA antibody.

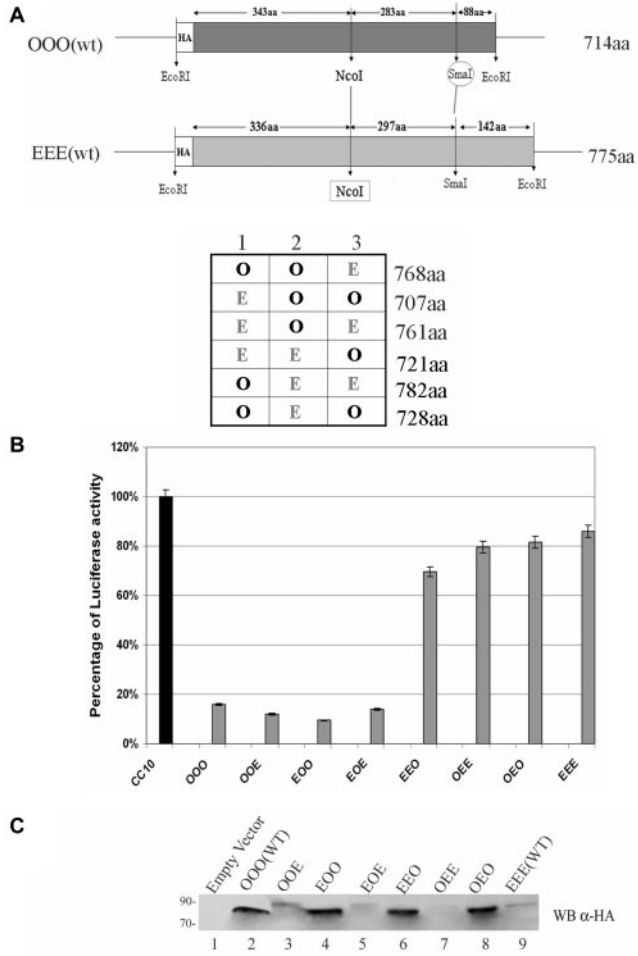

Fig. 5. **Transcriptional activity of medaka-mouse chimera proteins.** (A) A schematic representation of chimera proteins. Circled SmaI site in OOO wild-type mouse Foxp2 cDNA (wt) and boxed NcoI site in EEE wild-type medaka FoxP2 cDNA (wt) were created using site-directed mutagenesis. Table shows various chimera proteins and their expected amino acid numbers. (B) Transcriptional repressive activity of medaka-mouse chimera proteins. H441 cells were cotransfected with 500 ng of *CC10*-luc2 reporter plasmid and 400 ng of chimera protein expression vectors. Forty-eight hours after transfection, cells were harvested and the luciferase activity was measured. Transfection efficiencies were normalized using 10 ng of Renilla luciferase (GL4.7 hR-luc) plasmid. The data are expressed as percentages of control luciferase activity and represent mean ± SD. (C) A western blot analysis of chimera proteins with anti-HA antibody. Normalized amounts of proteins were loaded.

telencephalon and Purkinje cells in the adult medaka brain; these expressions are also found in human, mouse and zebrafish (*12*, *13*, *15*).

There were some differences in medaka FoxP2 expression in comparison to the other orthologues. Medaka FoxP2 was expressed strongly at the epiphysis. The fish epiphysis has photoreceptors (*25*). In addition, medaka FoxP2 expression was found in retina ganglion cells. However, FoxP2 was expressed all of the cells in the epiphysis including the photoreceptors while it is expressed only partially in ganglion cells in the retina. This implicates that it is a different constituent of photoreceptors between the pineal body and retina. The expressions in the retina were different in medaka and mouse. Mouse Foxp2 is expressed in all of the cells of inner nuclear layer and ganglion layer, whereas medaka FoxP2 was expressed in only a portion of the cells in both layers. Medaka FoxP2 did not show expressions in the heart, kidney and spleen in either embryo or adult unlike mouse Foxp2 and human *FOXP2*. Mouse Foxp2 is expressed in the lung, but medaka

FoxP2 was not expressed in the gills. Those observations suggest that medaka FoxP2 may act differently from mammalian FoxP2.

A comparison of medaka and zebrafish FoxP2 shows their similarities and differences. The similarities are a lack of a polyglutamine repeat and conservation of a zinc finger, a leucine zipper and a forkhead domain. A difference is that medaka FoxP2 has two longer amino acid insertions while zebrafish FoxP2 has one short amino acid insertion. Regarding the expression patterns, both medaka and zebrafish FoxP2 show similar temporal and spatial expressions in the embryo and adult as mentioned above. However, there are differences between the medaka and zebrafish FoxP2 expression patterns. In the beginning, zebrafish FoxP2 starts to be expressed diffusely entire embryo at 10 hpf (*14*), but medaka FoxP2 was first seen in the neural tube at the early somite stage. This phenomenon may be the result of different developmental speeds of both organisms, which thus makes it difficult to detect the early expression. In the adult, medaka FoxP2 was strongly expressed in the pineal body (Fig. 3) while zebrafish FoxP2 is not detected in the pineal body (*15*). Similarly, our study on mouse showed no expression in the pineal body (data not shown).

Fox protein is a transcription factor that has target genes to regulate. Target genes of *FoxP2* in the brain remain to be elucidated. It was reported that mouse Foxp2 represses the Clara cell-specific *CC10* gene (*10*, *21*). This system was applied to the medaka FoxP2 to compare the transcriptional activity to mouse Foxp2. Wild-type medaka FoxP2 inhibited the *CC10* promoter weakly. This study showed the mouse central part containing a leucine zipper, a zinc finger and a forkhead domain was important for the strong repression. In addition, the three amino acid change in the forkhead domain was crucial for the strong repression. Medaka-type sequence (DSADM) is found only in medaka while human-type sequence (ESSDR) is found in human, chimpanzee, mouse, songbird and *Xenopus*. Corresponding sequences of zebrafish and fugu are ESSDM and DSAEH, respectively. Recently, three-dimensional structure of forkhead domain of human FOXP2 was determined (*26*). The critical region (ESSDR versus DSADM) is located on the turn between helix1 and helix2 structures. Since this region is located at the reverse side from DNA-binding regions, it may change the structure of the forkhead domain itself or the other domain interacting with co-repressor proteins. The three-dimensional structure of forkhead domain in the FH-mut protein is very interesting.

There is a possibility that FoxP2 can be used as a protein with a different function in different species and different tissues. To clarify the roles of FoxP2 in the tissues and species, the target genes in each tissue and each species must be identified to determine whether FoxP2 plays distinct roles on the distinct target genes. In addition, in order to elucidate the molecular mechanism controlling speech and language, a sophisticated model animal and an evolutional analytical method must therefore be developed.

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REFERENCES

1. Carlsson, P. and Mahlapuu, M. (2002) Forkhead transcription factors: key players in development and metabolism. *Dev. Biol.* **250**, 1–23
2. Miura, N., Wang, T., and Tamakoshi, T. (2004) Introduction to Forkhead (Fox) Genes, in *Inborn Errors of Development? Molecular Basis of Clinical Disorders of Morphogenesis* (Epstein, C.J., Erickson, R.P., and Wynshaw-Boris, A., eds.), pp. 664–670, Oxford University Press, San Francisco
3. Kaestner, K.H., Knochel, W., and Martinez, D.E. (2000) Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev.* **14**, 142–146
4. Nishimura, D.Y., Swiderski, R.E., Alward, W.L.M., Searby, C.C., Patil, S.R., Benner, S.R., Kanis, A.B., Gastier, J.M., Stone, E.M., and Sheffield, V.C. (1998) The forkhead transcription factor gene *FKHL7* is responsible for glaucoma phenotypes which map to 6p25. *Nat. Genet.* **19**, 140–147
5. Petrova, T.V., Karpanen, T., Norrmen, C., Mellor, R., Tamakoshi, T., Finegold, D., Ferrell, R., Kerjaschki, D., Mortimer, P., Yla-Herttuala, S., Miura, N., and Alitalo, K. (2004) Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. *Nat. Med.* **10**, 974–981
6. Tamakoshi, T., Itakura, T., Chandra, A., Uezato, T., Yang, Z., Xue, X-D., Wang, B., Hackett, T., Yokoyama, T., and Miura, N. (2006) Roles of the Foxj1 and Inv genes in the left-right determination of internal organs in mice. *Biochem. Biophys. Res. Commun.* **339**, 932–938
7. Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27**, 68–73
8. Lai, C.S., Fisher, S.E., Hurst, J.A., Vargha-Khadem, F., and Monaco, A.P. (2001) A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature* **413**, 519–523
9. Vargha-Khadem, F., Watkins, K.E., Price, C.J., Ashuburner, J., Alcock, K.J., Connelly, A., Frackowiak, R.S., Friston, K.J., Pembrey, M.E., Mishikin, M., Gadian, D.G., and Passinham, R.E. (1998) Neural basis of an inherited speech and language disorder. *Proc. Natl Acad. Sci. USA* **95**, 12695–12700
10. Shu, W., Yang, H., Zhang, L., Lu, M.M., and Morrisey, E.E. (2001) Characterization of a new subfamily of winged-helix/forkhead (Fox) genes that are expressed in the lung and act as transcriptional repressors. *J. Biol. Chem.* **276**, 27488–27497
11. Haesler, S., Wada, K., Nshdejan, A., Morrisey, E.E., Lints, T., Jarvis, E.D., and Scharff, C. (2004) FoxP2 expression in avian vocal learners and non-learners. *J. Neurosci.* **31**, 3164–3175
12. Lai, C.S., Gerrelli, D., Monaco, A.P., Fisher, S.E., and Copp, A.J. (2003) FOXP2 expression during brain development coincides with adult sites of pathology in a severe speech and language disorder. *Brain* **126**, 2455–2462
13. Teramitsu, I., Kudo, L.C., London, S.E., Geschwind, D.H., and White, S.A. (2004) Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. *J. Neurosci.* **24**, 3152–3163

14. Bonkowsky, J.L. and Chein, C.B. (2005) Molecular cloning and developmental expression of foxP2 in zebrafish. *Dev. Dyn.* **234**, 740–746
15. Shah, R., Mdeina-Martinez, O., Chu, L.F., Samaco, R.C., and Jamrich, M. (2006) Expression of FoxP2 during zebrafish development and in the adult brain. *Int. J. Dev. Biol.* **50**, 435–438
16. Schon, C., Wochnik, A., Rossner, A., Donow, C., and Knochel, W. (2006) The FoxP subclass in Xenopus laevis development. *Dev. Genes Evol.* **216**, 641–646
17. Monaghan, A.P., Kaestner, K.H., Grau, E., and Shutz, G. (1993) Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chorda-mesoderm and neuroectoderm. *Development* **119**, 567–578
18. Aitola, M., Carlsson, P., Mahlapuu, M., Enerback, S., and Pelto-Huikko, M. (2000) Forkhead transcription factor FoxF2 is expressed in mesodermal tissues involved in epithelio-mesenchymal interactions. *Dev. Dyn.* **218**, 136–149
19. Tichelaar, J.W., Wert, S.E., Costa, R.H., Kimura, S., and Whitsett, J.A. (1999) HNF-3/forkhead homologue-4 (HFH-4) is expressed in ciliated epithelial cells in the developing mouse lung. *J. Histochem. Cytochem.* **47**, 823–832
20. Zhou, L., Lim, L., Costa, R.H., and Whitsett, J.A. (1996) Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J. Histochem. Cytochem.* **44**, 1183–1193
21. Li, S., Weidenfeld, J., and Morrisey, E.E. (2004) Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. *Mol. Cell. Biol.* **24**, 809–822
22. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–200
23. Loosli, F., Koster, R.W., Carl, M., Kuhnlein, R., Henrich, T., Mucke, M., Krone, A., and Wittbodt, J. (2000) A genetic screen for mutations affecting embryonic development in medaka fish (*Oryzias latipes*). *Mech. Dev.* **97**, 133–139
24. Webb, D.M. and Zhang, J. (2005) FoxP2 in song-learning birds and vocal-learning mammals. *J. Hered.* **96**, 212–216
25. Meissl, H. (1997) Photic regulation of pineal function. Analogies between retinal and pineal photoreception, Bio. *Cell* **89**, 549–554
26. Stroud, J.C., Wu, Y., Bates, D.L., Han, A., Nowick, K., Paabo, S., Tong, H., and Chen, L. (2006) Structure of the forkhead domain of FOXP2 bound to DNA. *Structure* **14**, 159–166