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RESEARCH ARTICLE

Two isoforms of aquaporin 2 responsive to hypertonic stress in the bottlenose dolphin

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ABSTRACT

This study investigated the expression of aquaporin 2 (AQP2) and its newly found alternatively spliced isoform (alternative AQP2) and the functions of these AQP2 isoforms in the cellular hyperosmotic tolerance in the bottlenose dolphin, *Tursiops truncatus*. mRNA sequencing revealed that alternative AQP2 lacks the fourth exon and instead has a longer third exon that includes a part of the original third intron. The portion of the third intron, now part of the coding region of alternative AQP2, is highly conserved among many species of the order Cetacea but not among terrestrial mammals. Semi-quantitative PCR revealed that AQP2 was expressed only in the kidney, similar to terrestrial mammals. In contrast, alternative AQP2 was expressed in all organs examined, with strong expression in the kidney. In cultured renal cells, expression of both AQP2 isoforms was upregulated by the addition to the medium of NaCl but not by the addition of mannitol, indicating that the expression of both isoforms is induced by hypersalinity. Treatment with small interfering RNA for both isoforms resulted in a decrease in cell viability in hypertonic medium (500 mOsm kg^{-1}) when compared with controls. These findings indicate that the expression of alternatively spliced AQP2 is ubiquitous in cetacean species, and it may be one of the molecules important for cellular osmotic tolerance throughout the body.

KEY WORDS: Cellular osmoregulation, Osmotic tolerance, Alternative splicing, Cetacea

INTRODUCTION

Cetaceans, i.e. whales and dolphins, are adapted to an aquatic life in seawater. The skin and digestive tracts are unavoidably involved in confronting osmotic stress. Plasma osmolality of cetaceans, approximately 310–360 mOsm kg^{-1}, is up to 75 mOsm kg^{-1} higher than that in terrestrial mammals, and their plasma Na^+ concentration would be indicative of dehydration in humans (Ortiz, 2001). In addition, their plasma osmolality fluctuates widely; for example, bottlenose dolphins can be tolerant against fluctuation in osmotic stress levels as their plasma osmolality is in the range of 319 to 358 mOsm kg^{-1} (Ridgway and Venn-Watson, 2010), in contrast to terrestrial mammals, in which internal fluid osmolality is rigidly controlled in a narrow range to prevent cellular damage. What are the osmoregulatory mechanisms that can facilitate resistance to such elevations and fluctuations in the osmotic and electrolyte content of extracellular fluid in cetaceans? Studies on this question have been non-existent so far.

Aquaporins (AQPs) are water channel proteins that confer the selective plasma membrane water permeability required for rapid and regulated physiological processes, such as secretion and reabsorption (Gomes et al., 2009). In terrestrial mammals, AQP2 expression is limited to the principal cells of the renal collecting duct, where it functions in the reabsorption of water from primitive urine by trafficking through cells from the endosomal compartments to the apical membrane under the control of vasopressin (Coleman et al., 2000; Fushimi et al., 1997; Nielsen et al., 1999). In the principal cells of the renal inner medulla, AQP2 expression is induced by hypertonicity (Hasler, 2009; Kasono et al., 2005; Storm et al., 2003). Osmotic tolerance is important for maintaining cellular shape in eukaryotes and is crucial in renal cells, which are confronted with high fluctuations in intra- and extracellular concentrations of urea and NaCl during antidiuresis and diuresis (Beck et al., 1998). Cells defend against severe osmotic stress by dramatically increasing and decreasing intracellular organic osmolyte concentrations (Neuhofer and Beck, 2006). In response, water molecules passively flow according to osmotic gradients to balance the osomolality between intra- and extracellular fluids. Under hypertonic conditions, tonicity-responsive enhancer binding protein (TonEBP/NFAT5) binds to the tonicity-responsive enhancer *cis* element located upstream of AQP2, upregulating AQP2 expression (Miyakawa et al., 1999). Increased AQP2 expression increases water flux across the plasma membrane, thereby increasing the water permeability of principal cells.

For these characteristics of AQP2 and in view of reports that AQP2 was positively selected during the secondary adaptation of cetacean species to the marine environment (Nery et al., 2013; Xu et al., 2013; Yim et al., 2014), for this study it was hypothesized that AQP2 could play some roles in cellular osmoregulation in cetaceans, who are living in a hypertonic environment in comparison to their body fluids. It was previously reported that AQP2 is distributed in the principal cells of the kidneys of the bottlenose dolphin and Baird's beaked whale, similar to terrestrial mammals (Suzuki et al., 2008). During the process of the present study, unique data were unexpectedly obtained from rapid amplification of cDNA ends (RACE)-PCR experiments, indicating alternative splicing of the AQP2 gene expressed in many organs of bottlenose dolphin.

Many genes undergo alternative splicing, resulting in proteome complexity by providing different protein isoforms from a single gene, and it sometimes provides a spark for evolution (Mironov et al., 1999; Modrek and Lee, 2002, 2003; Tarrío et al., 2008; Woodley and Valcarcel, 2002). Thus, alternative splicing of AQPs

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is assumed to occur as with other genes; however, only a few cases of alternative expression of AQP family proteins in mammals have been reported (Moe et al., 2008; Amiry-Moghaddam et al., 2005). To elucidate the biological implication of alternative splicing of AQP2, details on the expression of AQP2 and its alternatively spliced isoform (alternative AQP2) in the bottlenose dolphin, the function of the isoforms for cellular hyperosmotic/hyperosmolality tolerance, and the universality of alternative AQP2 expression in cetaceans were investigated in this study.

MATERIALS AND METHODS

All experiments were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology and the guidelines for animal experiments in the College of Bioresource Sciences, Nihon University.

Samples and tissue preparation

In January 2010, 19 organs were obtained from a male and a female bottlenose dolphin, *Tursiops truncatus* (Montagu 1821) (sample IDs: 10TH009 and 10TH014), under the cooperation of the scientific surveys by the National Research Institute of Far Seas Fisheries, Fisheries Research Agency of Japan. The organs were collected immediately post mortem. Samples were taken from each organ, and sub-samples were preserved in RNAlater (Ambion/Life Technologies, Carlsbad, CA, USA) at −20°C for RNA isolation and semi-quantitative analysis. Other sub-samples were frozen in liquid nitrogen to extract proteins for immunoblotting. For immunohistochemistry, tissues were cut into small blocks and fixed in 4% paraformaldehyde (Wako, Osaka, Japan). For the establishment of a dolphin renal cell line, the kidney of a female bottlenose dolphin that had been kept clinically healthy for more than 36 years but died in February 2013 at Izu Mito Sea Paradise, Shizuoka, Japan, was used. For genome analyses, tissues of six other cetacean species in five families were collected. Details on each specimen of the six species are shown in Table 1.

Isolation of full-length cDNA of the new aquaporin

Full-length cDNAs encoding the AQP2 isoforms were isolated using the method of Suzuki (2010). Briefly, total RNA was isolated from the kidney of the bottlenose dolphins using Isogen reagents (NipponGene, Chiyoda, Tokyo, Japan). After DNA degradation by deoxyribonuclease treatment, 5 µg of RNA was reverse transcribed to cDNA using the GeneRacer® kit for RLM-RACE (Invitrogen/Life Technologies). The AQP2 cDNA coding region was first amplified with primers (AQP2, FW1 and RV1; Table S1) using standard procedures to design gene-specific primers. 5′ and 3′ RACE PCR were carried out using the primers (each of AQP2-GRRV and alternative AQP2-GRRV was used with the 5′ primer for 5′ RACE; each of AQP2-GRFW3, AQP2-GRFW4, alternative AQP2-GRFW1 and alternative AQP2-GRFW2 was used with the 3′ primer for 3′ RACE; Table S1) according to the manufacturer's instructions. PCR products were size-fractionated, purified and subcloned into the pGEM T-Easy Vector System (Promega, Madison, WI, USA) followed by the TA cloning, and the nucleotide sequences were determined using a DNA sequencer. Transmembrane sites of alternative AQP2 were predicted using TMPred (http://ch.embnet.org/software?TMPRED_form.html).

Table 1. Samples for genomic PCR

Species	Sex	Origin
Minke whale *Balaenoptera acutorostrata*	M	Stranded in Shizuoka, Shizuoka, 4 Apr 2007
Dwarf sperm whale *Kogia sima*	F	Stranded in Honmoku, Kanagawa, 6 Nov 2008
Baird's beaked whale *Berardius bairdii*	M	Stranded in Arasaki, Kanagwa, 27 Jul 2005
Pseudo killer whale *Pseudorca crassidens*	M	Died in Hakkeijima Sea Paradise, 9 Sep 2009
Pacific white-sided dolphin *Lagenorhynchus obliquidens*	M	Stranded in Chita, Aichi, 16 May 2007
Finless porpoise *Neophocaena phocaenoides*	F	Stranded in Hokota, Ibaraki, 6 Nov 2005

Preparation of constructs

Each region containing the full open reading frame (ORF) of dolphin AQP2 and alternative AQP2 was amplified by reverse transcription PCR (RT-PCR) using kidney cDNA and specific primers (AQP2-FW2 and -RV2 for AQP2, AQP2-FW2 and alternative AQP2-RV2 for alternative AQP2; Table S1). The PCR products were each cloned into the pGEM T-Easy Vector system (Promega), and the plasmids were purified via transformation in JM109 (NipponGene). Fragments containing the ORF were cut from the plasmid using EcoRI (Takara, Kusatsu, Shiga, Japan) and subsequently ligated into the 3Z vector (Promega) linearized by the same enzyme. Using the plasmid obtained, capped AQP2 and alternative AQP2 cRNAs with a poly-A tail were transcribed *in vitro* using the mMessage mMachine SP6 kit (Ambion/Life Technologies) after digestion with NaeI (Takara) for AQP2 and BtsI (Takara) for alternative AQP2.

Water permeability tests

Water permeability tests were performed as reported previously (Suzuki, 2010). Briefly, *Xenopus laevis* oocytes were defolliculated with collagenase I and microinjected with 10 ng of the synthesized cRNA or 50 nl of distilled water as a control and incubated at 18°C for 48 h in modified Barth's saline (MBS). After incubation, oocytes were transferred from 200 mOsm kg^{-1} H_2O to 70 mOsm kg^{-1} H_2O in MBS diluted with distilled water. A photograph of the oocytes silhouette was taken every 20 s for up to 2 min or until the time of oocyte rupture. The oocyte volume was calculated from its cross-sectional area. The osmotic water permeability (P_f) was calculated according to Preston et al. (1992). To test sensitivities of the aquaporins to $HgCl_2$, which blocks the path for water molecules of aquaporins, the effects of mercuric chloride were examined by incubating oocytes in MBS containing 3 µmol l^{-1} $HgCl_2$ for 15 min before the test. Significant differences in P_f values were determined by one-way ANOVA, followed by the Steel–Dwass multiple comparison method.

Determination of AQP2 and alternative AQP2 tissue expression

To determine the tissue expression of AQP2 and alternative AQP2, semi-quantitative RT-PCR was performed for each gene. Total RNA was isolated from tissue samples of 19 organs (brain, pituitary, epidermis, muscle, heart, kidney, adrenal gland, testis, ovary, placenta, forestomach, main stomach, pyloric stomach, small intestine, large intestine, liver, pancreas, spleen and lung) of the male bottlenose dolphin. A total RNA sample (5 µg) obtained from each organ was reverse transcribed into cDNA using the Ready-to-Go kit (Promega). RT-PCR of AQP2 and alternative AQP2 was conducted using the cDNAs and gene-specific primer sets (AQP2-

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FW1 and -RV1 for AQP2, alternative AQP2-FW and -RV1 for alternative AQP2; Table S1). Primers for amplification of each AQP2 were constructed to include the full ORF, and those for GAPDH were described by Beineke et al. (2004). The sequences of PCR products were confirmed via TA cloning.

Immunohistochemistry and immunoblotting

Immunohistochemical localization of AQP2 and alternative AQP2 in the kidney of the dolphin was determined using the method previously described (Suzuki et al., 2008). Alternative AQP2 distribution was also detected in another 15 organs (epidermis, esophagus, forestomach, main stomach, pyloric stomach, small intestine, liver, pancreas, spleen, adrenal gland, ovary, lung, muscle, heart and brain). Briefly, 5 µm paraffin sections were prepared, then after deparaffinization, the sections were soaked in 0.6% H_2O_2 in methanol for 30 min to inactivate the endogenous peroxidase. After washing and blocking in 10% non-immune goat serum for 30 min, sections were incubated in a 1:1000 dilution (0.8 µg ml^{-1} for AQP2 and 6.9 µg ml^{-1} for alternative AQP2) of each antibody or rabbit IgG (2 µg ml^{-1}) overnight at 4°C. After washing, the sections were incubated with HRP-labeled polymer anti-rabbit IgG (Dako EnVision+ System; Agilent Technologies, Santa Clara, CA, USA) for 30 min and washed with PBS. The sections exposed to antibodies were reacted with 0.02% diaminobenzidine tetrahydrochloride (DAB, Wako) in PBS with 0.02% H_2O_2 for colorization. Localization of AQP2 and alternative AQP2 in *Xenopus* oocytes injected with each cRNA was determined by fluorescent immunostaining using 1:5000 dilutions of goat anti-rabbit IgG (H+L) secondary antibody (Alexa Fluor 594 conjugate; Invitrogen/Life Technologies) instead of the HRP-labeled polymer.

Immunoblots were visualized using the Vectastain *Elite* ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the method reported previously (Suzuki et al., 2008). Briefly, frozen dolphin kidney tissue was thawed and homogenized in buffer containing a protease inhibitor. The homogenate was centrifuged and the supernatant was mixed with an equal volume of 2× sample buffer and heated. The fraction was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 5–20% polyacrylamide gel, and separated proteins were electrotransferred to a polyvinylidene difluoride membrane. After washing and blocking, the membrane was incubated with 1:1000 dilutions of each antibody overnight at 4°C. Following washing, the membrane was incubated with biotinylated anti-rabbit IgG. After washing, the blot was incubated with the ABC mixture and visualized using DAB.

Antibodies

Anti-AQP2 antibody was affinity-purified anti-rabbit antibody against a synthetic peptide corresponding to the 15 carboxyl-terminal amino acid residues of rat AQP2 (Alomone Laboratories, Jerusalem, Israel). Anti-alternative AQP2 antibody was raised using antigen elaborated from the specific amino acid sequence at the position 204–222 (MAETSLPSPPPETHLAGR). Rabbits were immunized with the antigen conjugated with keyhole limpet hemocyanin and serum was retrieved. Antibody was purified from serum using protein A.

Effects of hyperosmotic environment on expression of the AQP2 isoforms in bottlenose dolphin renal cells

Cell culture

Dolphin primary renal cells were isolated from the kidney of a female bottlenose dolphin that died at Izu Mito Sea Paradise. Isolated cells were maintained in RPMI 1640 (Wako) supplemented with 15% cosmic calf serum (HyClone; Thermo Fisher Scientific, Provo, UT, USA), 1% non-essential amino acids (Gibco, Long Island, NY, USA), 1 mmol l^{-1} sodium pyruvate (Gibco) and ZellShield (Minerva Biolabs, Berlin, Germany). After four passages, an expression plasmid DNA encoding the large T antigen origin-defective simian virus 40 (SV40) was transfected into the renal cells using Lipofectamine 2000 (Invitrogen/Life Technologies). Transfected cells were selected by addition of G418 and maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 100 U ml^{-1} streptomycin and 100 mg ml^{-1} penicillin. The established renal cells were designated as Dol KT1 cells.

NaCl and mannitol treatment

Dol KT1 cells were cultured in 6-well plates until reaching approximately 90% confluence. NaCl or mannitol was added to the medium to 350 or 500 mOsm kg^{-1} H_2O, respectively. After incubation for 24 h in a CO_2 incubator at 37°C, cells were lysed in 1 ml of Isogen (Nippon Gene).

Real-time PCR

Total cellular RNA was extracted using Isogen according to the manufacturer's instructions. cDNA was synthesized using 1 µg of total RNA with the High-capacity RNA-to-cDNA kit (Life Technologies). Real-time PCR was performed using the cDNA mixed with 0.3 µmol l^{-1} primers and Quantitect SYBR Green Mix (Qiagen, KJ, Venlo, The Netherlands) using Rotor-Gene Q (Qiagen). Primer sequences used for real-time PCR of GAPDH, AQP2 and alternative AQP2 are shown in Table S1 (AQP2-FW3 and -RV3 for AQP2, AQP2-FW3 and alternative AQP2-RV3 for alternative AQP2, and GAPDH-FW and -RV for GAPDH).

RT-PCR to assess tonicity-enhancer binding protein expression

To ascertain TonEBP expression in the cells, RT-PCR was conducted using the standard procedure with the synthesized cDNA and specific primers to amplify its full-length ORF (TonEBP FW1-RV1; Table S1). Changes in TonEBP mRNA expression by addition of NaCl to medium to 350 and 500 mOsm kg^{-1} H_2O were also tested by semi-quantitative RT-PCR using the cDNAs above and another set of primers (FW2-RV2; Table S1).

Statistics

Significant differences in mRNA quantities were determined by one-way ANOVA, followed by Scheffe's paired comparison test.

RNA interference of AQP2 and alternative AQP2 expression

Effect of knockdown in hyperosmotic medium

siRNAs were designed to correspond to positions unique to AQP2 and alternative AQP2 mRNAs, as shown in Table S2; the siRNAs were then synthesized (Hokkaido System Science Co., Sapporo, Hokkaido, Japan). Eighty percent confluent Dol-KT1 cells were prepared in DMEM with 10% fetal bovine serum in 6-well plates, and 2.5 µg of siRNA for each of AQP2, alternative AQP2 and negative control siRNA (Allstar Negative Control siRNA, Qiagen) were transfected into the cells using Lipofectamine 3000 (Invitrogen/Life Technologies) in Opti-MEM (Invitrogen/Life Technologies). After 72 h incubation to complete the siRNA transfection, NaCl was added to the medium to 500 mOsm kg^{-1} H_2O. After 48 h of incubation in the hypertonic medium, cells were photographed and tested for viability using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions. Luminescence intensity of each well,

representing ATP content, was measured by a luminometer. Numbers of cells in photographs were also counted to compare the cell survival rate.

Knockdown efficiency test

To confirm siRNA transfection, cells were co-transfected with psiCHECK-1 vector (Promega), ligated to the ORF of GAPDH, AQP2 and alternativeAQP2 cDNA, and 2.5 µg of each siRNA into 90% confluent Dol-KT1 cells. After 72 h incubation, luciferase activity was evaluated using the *Renilla* Luciferase Assay System (Promega), comparing the activity with or without transfection of each siRNA.

Statistics

Significant differences in luminescience intensities, cell numbers and luciferase activity were determined by one-way ANOVA, followed by Scheffe's paired comparison test.

Genomic analyses

To determine whether the alternative AQP2 can be expressed in multiple species, the sequence of the region comprising the third exon and the following intron up to the stop codon of the alternative AQP2 mRNA was determined in seven cetacean species representing five families (Balaenidae: Minke whale, *Balaenoptera acutorostrata*; Kogiidae: dwarf sperm whale, *Kogia sima*; Ziphiidae: Baird's beaked whale, *Berardius bairdii*; Delphinidae: bottlenose dolphin, Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, and pseudo killer whale, *Pseudorca crassidens*; and Phocoenidae: finless porpoise, *Neophocaena phocaenoides*). Tissue samples were collected post mortem from captive or stranded animals (Table 1), and genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega). PCR was performed to amplify AQP2 using specific primers (AQP2-FW1 and AQP2-RV1; Table S1). Via TA subcloning, the regions comprising intron 2 to exon 4 were sequenced.

The sequence in the dolphin was aligned using mVISTA (Frazer et al., 2004) for cetaceans and their closely related ungulates (cow, *Bos taurus*, Ensembl: ENSBTAG00000008374; bison, *Bison bison*, NW_011494955; chiru, *Pantholops hodgsonii*, NW_005816316; sheep, *Ovis aries*, Ensembl: ENSOARG00000018180; pig, *Sus scrofa*, ENSSSCG00000021193; camel, *Camelus bactrianus*, XM_010964262). To compare the substitution rate, mVISTA was also run for four families in Ruminantia (cow, bison, chiru and sheep) that diverged 31.6 million years ago, almost at the same as cetaceans (32.3 million years ago), using the sequence in cow as a reference.

RESULTS

Full-length cDNAs of two AQP2 isoforms were isolated from the kidney of the bottlenose dolphin

The structures of AQP2 and full-length AQP2 and alternative AQP2 mRNA are presented in Fig. 1. The gene comprises four exons and three introns, and comparison of the AQP2 and alternative AQP2 sequences with the genomic DNA of bottlenose dolphin AQP2 (ENSTTRG00000001003, GeneScaffold 1570) revealed that alternative AQP2 has a longer third exon, where the 3′ sequence corresponds to the third intron, and it also lacks the fourth exon. The alternative AQP2 mRNA sequence comprises 988 bp, including a 744 bp ORF encoding 247 amino acids (DDBJ accession no. LC053642); AQP2 cDNA comprises 1411 bp, including a 916 bp ORF encoding 271 amino acids (DDBJ accession no. LC053641). Both of the AQP2 isoforms have two asparagine–proline–alanine (NPA) boxes (Fig. 1B). The alternative AQP2 lacks the phosphorylation sites (serine 256 and 261) present in the C terminus of AQP2, which are responsible for vasopressin-stimulated trafficking to the plasma membrane (Hoffert et al., 2006). Six segments, amino acid positions 12–32, 37–57, 86–106, 130–150, 157–177 and 227–247, in alternative AQP2 were predicted as transmembrane sequences by hydrophybicity predicted transmembrane sites (Fig. 2C).

Two AQP2 isoforms demonstrate water permeability

The water permeability of *Xenopus* oocytes injected with bottlenose dolphin AQP2 cRNA, alternative AQP2 cRNA or water (control) was determined to test the aquaporin properties. The swelling rate of oocytes expressing AQP2 was 1.2 times that of controls (Fig. 2). The water permeability of oocytes expressing alternative AQP2 was higher than that of controls but lower than that of oocytes expressing AQP2. Treatment with 3 µmol l^{-1} $HgCl_2$ significantly decreased the water permeability of oocytes expressing both isoforms (Fig. 2A,B). The expression of each protein was confirmed by fluorescent immunostaining (Fig. 2C). Fluorescence was concentrated on the plasma membrane in oocytes expressing AQP2, but in both the plasma membrane and intracellular compartments in those expressing alternative AQP2.

AQP2 is expressed only in the kidney, but alternative AQP2 is ubiquitously expressed in bottlenose dolphin organs

Semi-quantitative RT-PCR was used to investigate the expression of AQP2 and alternative AQP2 in 19 organs. As shown in Fig. 3A, the PCR product for AQP2 (813 bp) was observed only in the kidney. The PCR product for alternative AQP2 (915 bp) was present in all 19 organs tested, with the most intense band appearing in the kidney.

AQP2 and alternative AQP2 were localized in a different distribution in the kidney

The tissue distribution of the two AQP2 isoforms in the bottlenose dolphin kidney was examined using immunohistochemical analysis of paraffin sections (Fig. 3B). Intense positive staining was observed in the renal medulla, particularly in the papilla, for both AQP2 isoforms. AQP2 was detected in the apical membrane of the principal cells of the renal collecting duct. Intense positive staining for alternative AQP2 was observed in the principal cells of the duct and transitional cells of the renal papilla; only weak staining was observed in other cells. Intense bands were observed at the anticipated positions (28.9 and 25.7 kDa, respectively) in immunoblotting with both anti-AQP2 and anti-alternative AQP2 antibodies. Light, smeared bands presumed to be glycosylated forms of each protein were observed above the intense bands (Fig. 3B).

Transcriptional activity of AQP2 and alternative AQP2 were increased in renal cells exposed to hypersaline medium

To investigate mRNA expression of AQP2 and alternative AQP2 in response to elevated NaCl levels or increased osmolality, NaCl or mannitol was added to the medium with cultured bottlenose dolphin renal cells. AQP2 mRNA substantially increased (478±59%; Fig. 4A) as did alternative AQP2 mRNA (481±49%) under elevated levels of NaCl (500 mOsm kg^{-1} medium; Fig. 4B). Alternative AQP2 mRNA expression tended to increase under mildly increased levels of NaCl (350 mOsm kg^{-1} medium; 315±101% of control) but without statistical significance (P=0.15). Hyperosmolality induced by the addition of mannitol did not change the mRNA expression of either AQP2 isoform. RT-PCR was performed to confirm TonEBP/NFAT5 expression in the renal cells, revealing amplification of the full-length 4605 bp ORF (Fig. 4C) with a sequence consistent with the registered sequence (XM_004312069). TonEBP mRNA expression was increased by

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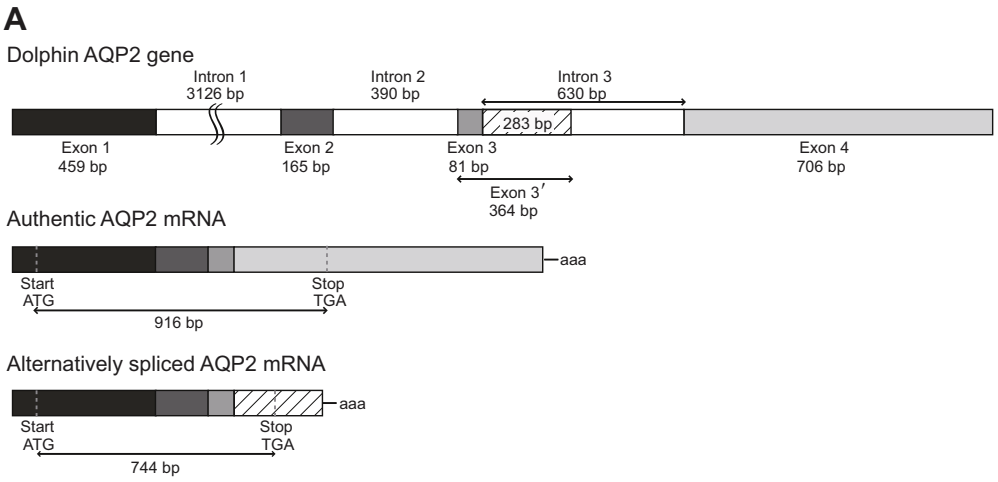

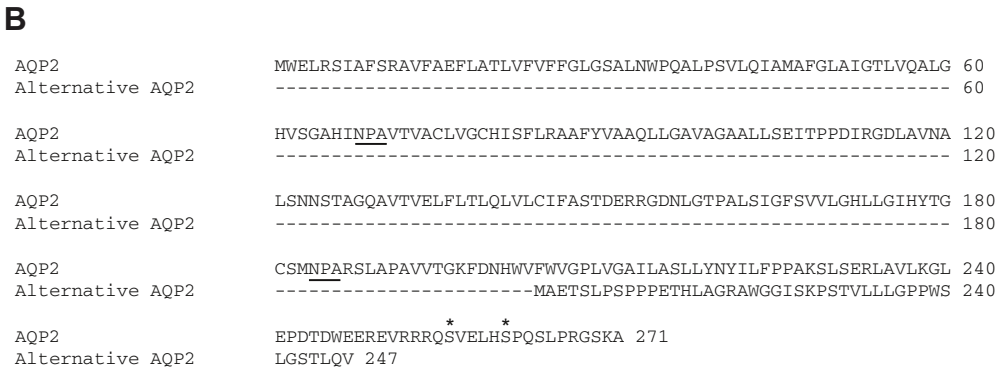

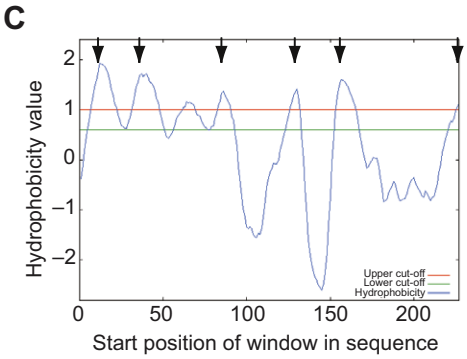

Fig. 1. Splicing pattern of AQP2 in the bottlenose dolphin. (A) Structures of AQP2 gene, AQP2 mRNA and alternative AQP2 mRNA. Filled bars represent exons. Authentic AQP2 mRNA comprises exons 1, 2, 3 and 4 (total, 1411 bp; ORF, 916 bp), and alternative AQP2 mRNA contains exons 1, 2 and x3′ (total, 988 bp; ORF, 744 bp). (B) Alignment of the deduced AQP2 and alternative AQP2 amino acid sequences. Hyphen indicates the same residue as that in AQP2. Underlines indicate the asparagine–proline–alanine motifs highly conserved in aquaporin family members. Asterisks indicate serine residues that are phosphorylation sites responsible for AQP2 trafficking to the plasma membrane. (C) Kyte and Doolittle hydropathy profile of the dolphin alternative AQP2 protein. Arrows indicates the locations of the transmembrane domain.

addition of NaCl to the medium (146±12% and 277±7% in 350 and 500 mOsm kg^{-1} H_2O, respectively; Fig. 4C).

RNA interference of AQP2 and alternative AQP2 resulted in cell death in hypersaline medium

To reveal the function of each AQP2 in cellular osmotic tolerance, ATP content was measured to determine cell viability upon exposure to medium containing increased levels of NaCl for 48 h on each line of AQP2 knockdown cells. In the medium with elevated NaCl levels, ATP content was decreased to 44.4±2.1% for AQP2 knockdown cells and to 24.1±0.6% for alternative AQP2 knockdown cells compared with that of the cells in normal medium (Fig. 5A). In contrast, cell viabilities of siRNA-free and negative control groups did not change significantly (93.4±0.6% and 87.9±1.4%, respectively). Numbers of knockdown cells were significantly decreased by NaCl addition (448±32 cells cm^{-2} in control, 160±31 cells cm^{-2} in AQP2 siRNA, 136±13 cells cm^{-2} in alternative AQP2 mRNA; $P<0.001$). The cells transfected with AQP2 and alternative AQP2 siRNAs shrank severely when exposed to medium with increased NaCl levels (Fig. S2).

To check the siRNA knockdown efficiency, luciferase assays were performed using cells transfected with the psiCHECK vector ligated to cDNA of GAPDH, AQP2 or alternative AQP2 with or without co-transfection of each siRNA (Fig. 5B). In all groups, the luminescence intensity was suppressed to very low levels by siRNA (GAPDH, 8.4±0.7%; AQP2, 28.2±10.6%; alternative AQP2, 13.2±3.3%) compared with controls without siRNA.

The coding region of alternative AQP2 is highly conserved only in Cetacea

The sequence through the third exon to the stop codon of alternative AQP2 (219 bp) in AQP2 genes of seven cetacean species was

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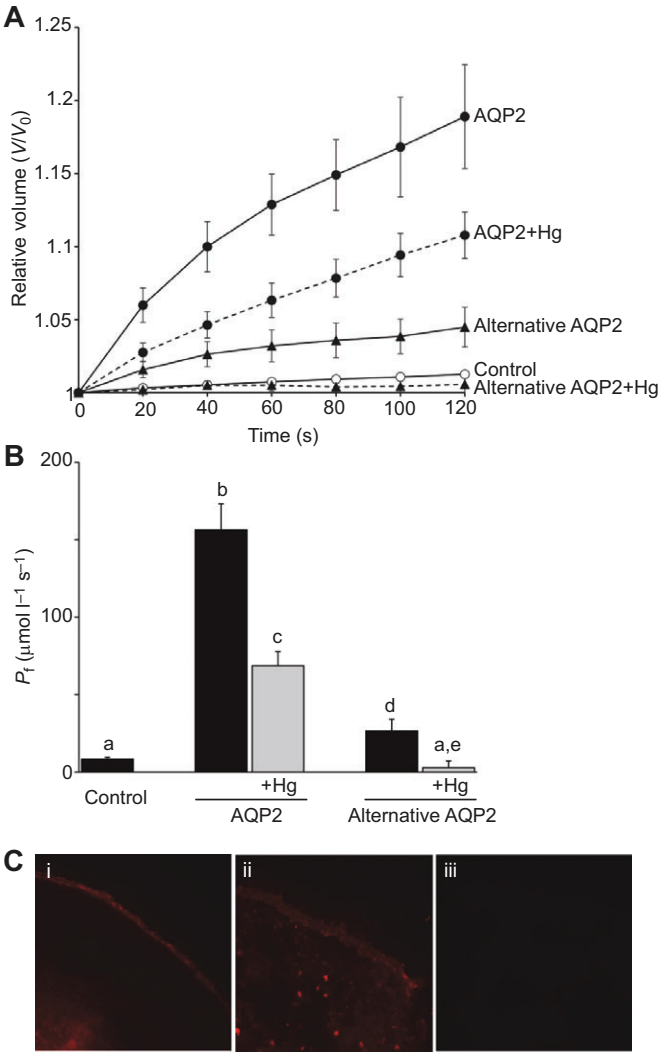

Fig. 2. Water permeability of *Xenopus laevis* oocytes injected with cRNA of AQP2 or alternative AQP2 from the bottlenose dolphin. (A) Osmotic oocyte volume (V) change in oocytes injected with water (control), dolphin AQP2 cRNA, dolphin alternative AQP2 or 0.03 mmol l^{-1} l^{-1}–$HgCl_2$ (+Hg). (B) Water permeability of oocytes (mean±s.e.m.) in each treatment group. Values marked with different letters are significantly different from each other (ab, ac, bd, be, ce: P<0.01; ad, bc, cd, de: P<0.05). (C) Fluorescent immunostaining of oocytes injected with cDNA of AQP2 (i) or alternative AQP2 (ii) and control (iii).

determined to check for the universality and possibility of alternative AQP2 expression. As shown in Fig. 6A and Fig. S3, the region is quite highly conserved among the species, with neither inserts nor deletions. The sequences in the homologous region of the intron in Ruminantia with a similar divergent time to Cetacea are not highly conserved (Fig. 6A). The corresponding regions in Artiodactyla are not similar to those of the cetacean AQP2, with several insertions and deletions, indicating that alternative AQP2 is highly unlikely to be expressed in the same form in those mammals (Fig. 6B).

DISCUSSION

This study revealed the expression of two AQP2 isoforms in bottlenose dolphin. One is what might be considered to be normal AQP2 in non-cetacean species, and is expressed only in the kidney and has high water permeability, similar to AQP2 of other mammals. The other is alternatively spliced AQP2 expressed in multiple organs with lower P_f values. Almost all instances of alternative splicing result from the use of one or more than four basic modules: alternative 5′ splice-site choice, alternative 3′ splice-site choice, cassette-exon inclusion or skipping, or intron retention (Keren et al., 2010; Nilsen and Graveley, 2010). The dolphin alternative AQP2 reported in this study is the result of intron retention.

Analyses of AQP2 in six cetacean species, in the families Balaenidae, Kogiidae, Ziphiidae, Delphinidae and Phocoenidae, indicate that the same pattern of alternative AQP2 splicing is probably present in a wide range of cetacean species. However, AQP2 in closely related artiodactyl species, such as pig, cow, sheep, camel and other terrestrial mammals, do not have the base sequence corresponding to the alternatively spliced region in the third intron of cetaceans. Besides, the region of concern is not very well conserved among Ruminantia, which diverged later than the order Cetacea. Thus, the newly acquired exon region is uniquely conserved in the cetacean lineage. Conservation of a specific alternative splicing pattern throughout evolutionary history provides strong evidence of biological function (Mironov et al., 1999). Herein, alternative AQP2 is likely functional and it must have been acquired quite early in the evolution of modern cetaceans and highly conserved during evolution.

Immunohistochemistry revealed different distribution patterns of AQP2 and alternative AQP2. In the kidney, AQP2 was observed at the apical membrane of the collecting duct, whereas alternative AQP2 was observed in a variety of structures. Alternative AQP2 protein was also detected in many organs (Fig. S1) and these data are coincident with the results of RT-PCR, suggesting that alternative AQP2 may be ubiquitously localized in the dolphin body. In the renal collecting duct, alternative AQP2 was found in both the cytoplasm and the plasma membrane, while AQP2 was restricted to the plasma membrane, as observed in forced expression of AQP2 isoforms in *Xenopus* oocytes. This difference in isoform distribution may be related to differences in their amino acid sequences. The deduced amino acid sequence of alternative AQP2 lacks phosphorylation sites at the C terminus of AQP2 responsible for trafficking to the (apical) membrane (Hoffert et al., 2006). The lack of these sites in dolphin alternative AQP2 may impair the trafficking efficiency of the protein to the plasma membrane, leading to lower water permeability and freedom from the control of vasopressin. These data suggest that alternative AQP2 had lower levels of water flux through plasma membrane than normal AQP2 and it would be less effective at osmotic water regulation on the plasma membrane as well as on the membrane of organelles. Further investigations, especially on the details of the intracellular distribution, are necessary to unveil the specific function of alternative AQP2.

Expression of both AQP2 isoforms was induced by hypertonicity due to additional NaCl. The renal medullary cells are constantly bathed in a hypertonic interstitial solution of varying concentrations that is required for concentrating urine. AQP2 is a molecule that responds to this hypertonic environment (Kwon et al., 2009). The total amount of AQP2 in epithelial cells of the renal collecting duct is tightly regulated by hormones such as vasopressin. AQP2 expression is also induced by hypertonicity (Hasler, 2009; Kasono et al., 2005; Storm et al., 2003), and this response may occur via TonEBP, a transcription factor that may play an osmosensitive role in cell regulation during hypertonic stress (Miyakawa et al., 1999). Under hypertonic conditions, TonEBP is expressed and binds to the tonicity-responsive enhancer *cis* element upstream of AQP2 to initiate its expression (Miyakawa et al., 1999). TonEBP also

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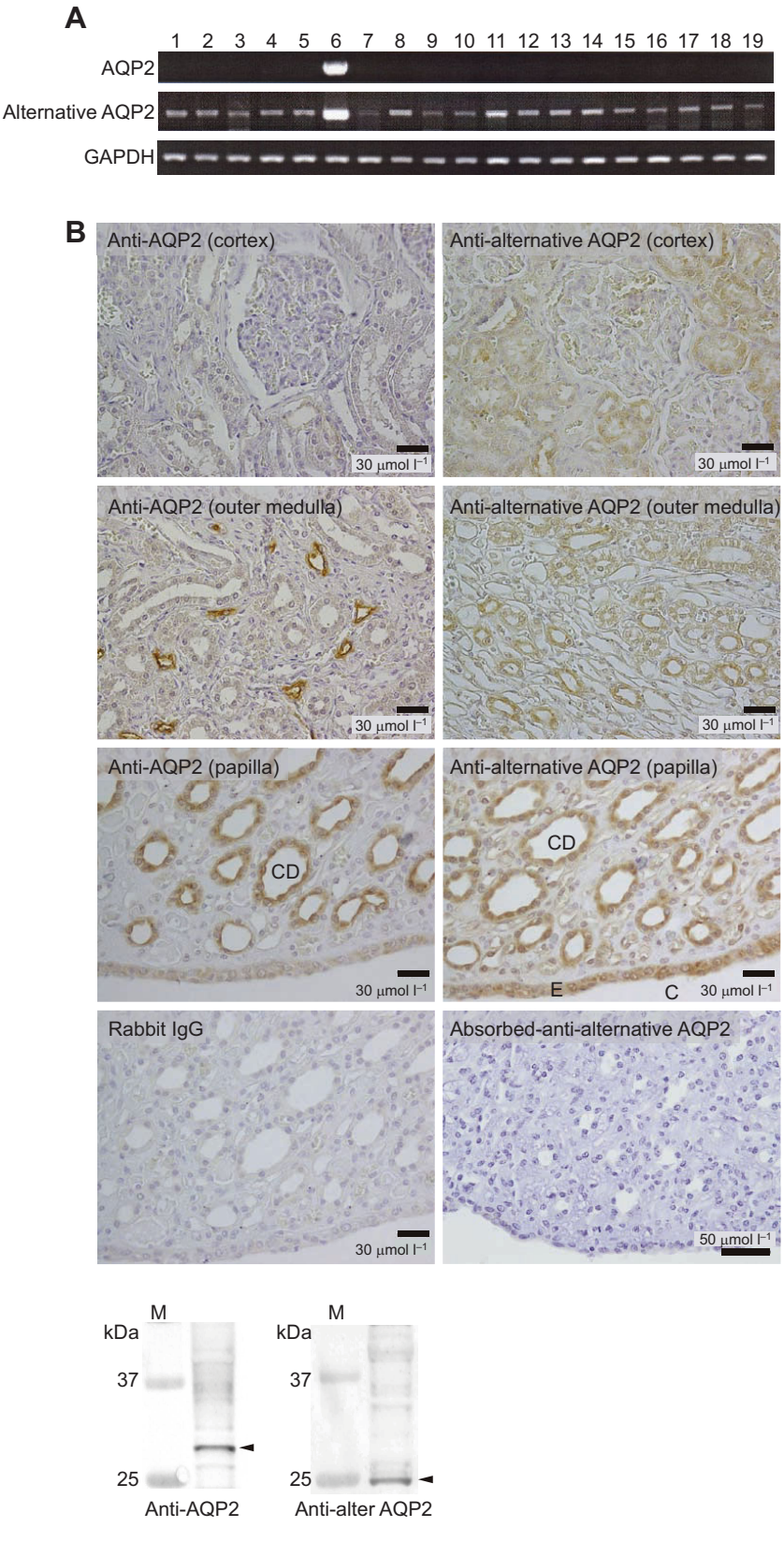

Fig. 3. Expression of mRNA for AQP2 and alternative AQP2 in the bottlenose dolphin. (A) Semi-quantitative RT-PCR for AQP2 and alternative AQP2 with cDNAs from 19 organs. Numbers represent organs as follows: 1, brain; 2, pituitary; 3, epidermis; 4, muscle; 5, heart; 6, kidney; 7, adrenal gland; 8, testis; 9, ovary; 10, placenta; 11, fore stomach; 12, main stomach; 13, pyloric stomach; 14, small intestine; 15, large intestine; 16, liver; 17, pancreas; 18, spleen; and 19, lung. Lengths of the RT-PCR products are 913, 813 and 228 bp for AQP2, alternative AQP2 and GAPDH, respectively. (B) Immunohistochemistry for AQP2 and alternative AQP2 in the renal cortex, outer medulla and papilla, and immunoblotting for the aquaporins in the renal medulla. Rabbit IgG was used instead of antibodies for a negative control. To be certain of the specificity of the antibody for alternative AQP2, an antibody absorption treatment was carried out, using peptide antigen. C, renal calix; CD, collecting duct; E, transitional epithelium; M, molecular marker.

participates in compatible osmolyte accumulation by upregulating relevant genes (Coleman et al., 2000; Miyakawa et al., 1998; Rim et al., 1998). The DNA sequence of the TonEBP binding site in the AQP2 promoter is GGAAA (Halterman et al., 2012); the bottlenose dolphin AQP2 proximal promoter includes this sequence at −139 and −327 bp upstream of the start codon (turTru1: GeneScaffold_1570, Ensembl Genome Blowser). The TonEBP gene is present in the bottlenose dolphin (ENSTTRG00000010017), and we confirmed that TonEBP mRNA is expressed and upregulated by addition of NaCl in dolphin renal cells. Collectively, these observations indicate

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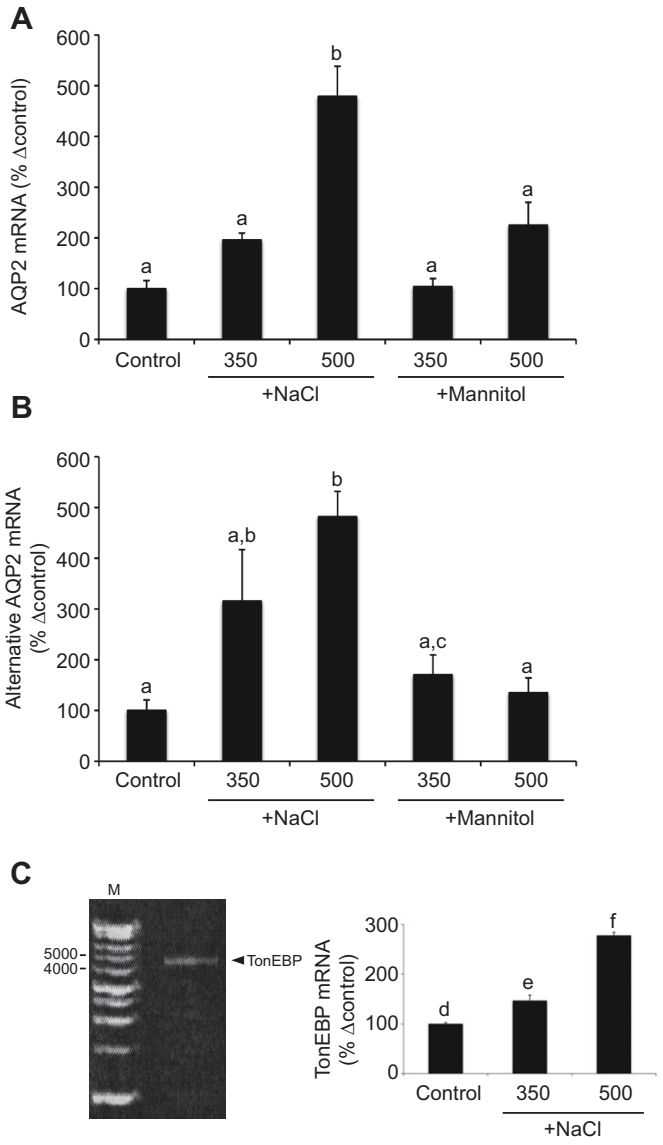

Fig. 4. Effects of medium with additional NaCl or mannitol on the mRNA expression of AQP2 isoforms in cultured renal cells of bottlenose dolphin. Expression of (A) AQP2 mRNA and (B) alternative AQP2 mRNA. NaCl or mannitol was added to the culture to increase the extracellular osmolality to 350 or 500 mOsm kg^{-1}. Control used normal medium (255 mOsm kg^{-1}). Values are presented as % change in mRNA quantity from the control. (C) Amplification of the full-length ORF of TonEBP using cDNA from cultured renal cells and effects of NaCl addition on its mRNA expression. Values marked with different letters are significantly different from each other (ab, df, ef: $P<0.01$; bc, de: $P<0.05$). M, molecular marker.

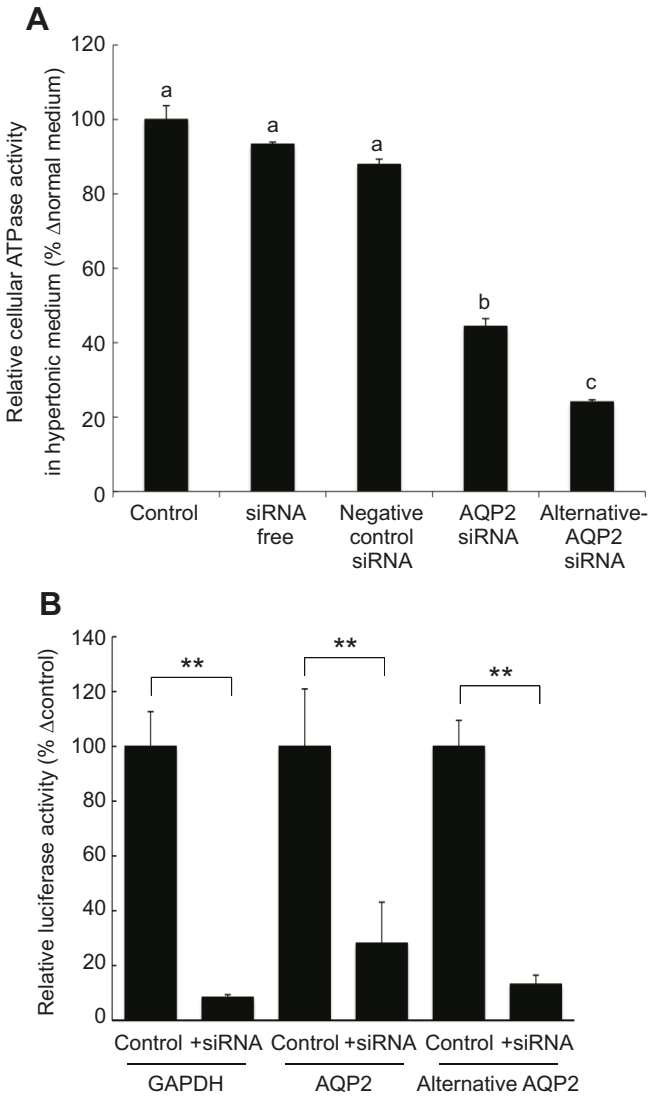

Fig. 5. Effects of siRNA knockdown of AQP2 and alternative AQP2 in cultured bottlenose dolphin renal cells. (A) Viability of cells (control, siRNA free, universal negative control siRNA, AQP2 siRNA or alternative AQP2 siRNA) after NaCl addition as assayed by cellular ATP contents. Values are presented as % change in ATP contents (mean±s.e.m.) from that of controls. siRNA free indicates cells treated with Lipofectamine 3000 without siRNA. (B) Relative luciferase activity is shown in cells transfected with GAPDH, AQP2 or alternative AQP2 siRNA. Values are presented as % change (mean±s.e.m.) in luminescence intensity from that of controls. Values marked with different letters and asterisks are differed significantly from each other (ab, ac: $P<0.0001$; bc, **: $P<0.01$).

that transcription of the dolphin AQP2 isoforms is induced by hypertonicity due to increased NaCl via TonEBP, as in other mammals. In addition, the fact that dolphin renal cells in control group survived in medium with increased NaCl levels but cells with siRNA knockdown for both of the dolphin AQP2 isoforms could not survive suggest that AQP2 and alternative AQP2 may play roles in cellular osmotic regulation.

The high degree of alternative AQP2 sequence conservation among members of the order Cetacea, its ubiquitous expression in different body tissues and its hypertonicity-responsive expression suggest that this alternatively spliced protein plays a role in cellular osmoregulation throughout the body of this group of aquatic mammals living in hypertonic environments. Several studies have recently reported that AQP2 was positively selected for during the secondary adaptation in cetacean species, suggesting that the evolution of the gene was an important event in the development of enhanced capacity for water reabsorption in the renal tubules (Nery et al., 2013; Xu et al., 2013; Yim et al., 2014). Extensive information can be obtained from genome-scale analyses, providing insight into the nature and extent of selective pressures that contributed to the evolution of a particular group. However, the physiological functions of each molecule, especially on unanticipated products such as alternatively spliced proteins, could be overlooked by genomic analyses in certain instances. Our findings reaffirm that the inferences on physiological mechanisms and the functional relevance of each

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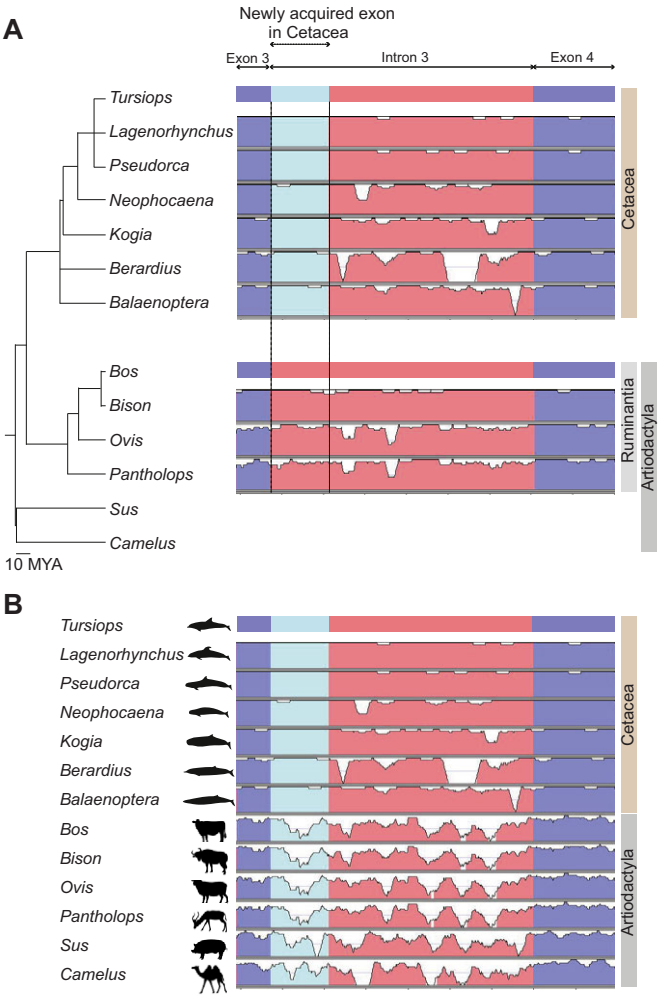

Fig. 6. Alignment of the region through the third intron of AQP2 of cetacean and artiodactyl species using mVISTA. (A) Alignments of Cetacea (upper) and Ruminantia (lower) with the bottlenose dolphin (*Tursiops*) sequence and that of cow (*Bos*) as a reference, respectively, are shown with a phylogenic tree. (B) Alignment among cetacean and artiodactyl species with the sequence in bottlenose dolphin as a reference. The blue, light blue and pink columns in AQP2 of the bottlenose dolphin/cow indicate authentic exons, the newly acquired exon in Cetacea, and the intron, respectively. The height of the shading represents the percent identity between two sequences. The areas between the two dashed lines in A indicate the corresponding region in AQP2 of other mammals.

molecule should be confirmed by investigative approaches based on functional studies.

CONCLUSIONS

This study revealed that (1) two alternatively spliced isoforms of aquaporin 2 (AQP2 and alternative AQP2) are expressed in the bottlenose dolphin, (2) both isoforms are upregulated by hypertonicity due to increased NaCl, (3) knockdown of the isoforms resulted in cell death in high NaCl hypertonic medium, (4) alternative AQP2 is expressed in every organ examined, in contrast to normal AQP2, which is expressed only in the kidney, and (5) the alternative splicing most probably occurs only in cetacean species. These results suggest that cetaceans may have utilized the abilities of AQP2, which is responsive to hypertonicity and functions in the kidney (in which intra- and extracellular concentrations of urea and NaCl fluctuate during antidiuresis and diuresis), in the whole body as an antidote to the hypertonic external medium when they radiated back into the marine environment. Further detailed studies on the intracellular function of alternative AQP2 will offer interesting suggestions on the physiological adaptation of cetaceans to aquatic life.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.S. conceived the study, conducted the field work, interpreted results, and drafted, revised and edited the manuscript. M.S., H.W., T.I., T.S., Y.I., K.Y. and K.K. processed samples and analyzed data. All authors approved the final version of the manuscript.

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Data availability

DNA Data Bank of Japan accession numbers for AQP2 and alternative AQP2 in bottlenose dolphin are LC053641 (http://getentry.ddbj.nig.ac.jp/getentry/na/LC053641) and LC053642 (http://getentry.ddbj.nig.ac.jp/getentry/na/LC053642), respectively.

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.132811/-/DC1

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