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ACE2 orthologues in non-mammalian vertebrates (*Danio*, *Gallus*, *Fugu*, *Tetraodon* and *Xenopus*)

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Abstract

Angiotensin-converting enzyme 2 (ACE2), a newly identified member in the renin–angiotensin system (RAS), acts as a negative regulator of ACE. It is mainly expressed in cardiac blood vessels and the tubular epithelia of kidneys and abnormal expression has been implicated in diabetes, hypertension and heart failure. The mechanism and physiological function of this zinc metallopeptidase in mammals are not yet fully understood. Non-mammalian vertebrate models offer attractive and simple alternatives that could facilitate the exploration of ACE2 function. In this paper we report the *in silico* analysis of *Ace2* genes from the *Gallus* (chicken), *Xenopus* (frog), *Fugu* and *Tetraodon* (pufferfish) genome assembly databases, and from the *Danio* (zebrafish) cDNA library. Exon ambiguities of *Danio* and *Xenopus Ace2s* were resolved by RT-PCR and 3'RACE. Analyses of the exon–intron structures, alignment, phylogeny and hydrophilicity plots, together with the conserved synteny among these vertebrates, support the orthologous relationship between mammalian and non-mammalian ACE2s. The putative promoters of *Ace2* from human, *Tetraodon* and *Xenopus tropicalis* drove the expression of enhanced green fluorescent protein (EGFP) specifically in the heart tissue of transgenic *Xenopus* thus making it a suitable model for future functional genomic studies. Additionally, the search for conserved cis-elements resulted in the discovery of WGATAR motifs in all the putative *Ace2* promoters from 7 different animals, suggesting a possible role of GATA family transcriptional factors in regulating the expression of *Ace2*.

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1. Introduction

The renin–angiotensin system (RAS) plays a key role in the regulation of cardiovascular and renal functions. Renin stimulates

the formation of angiotensin I (AngI) from angiotensinogen and angiotensin-converting enzyme (ACE) removes a dipeptide from the C-terminus of the decapeptide AngI to yield the octapeptide angiotensin II (AngII). The latter serves as a potent vasoconstrictor that elevates blood pressure. Since it was described as a 'hypertensin-converting enzyme' in 1956, the zinc metallopeptidase ACE, also a carboxydipeptidase, has been a prime target for the treatment of hypertension (Skeggs et al., 1956). Many ACE inhibitors and receptor blockers have been used to control blood pressure and other cardiovascular conditions (Menard and Patchett, 2001). About half a century later, a zinc metallo-carboxymonopeptidase became the first human ACE homologue identified (Donoghue et al., 2000) and it acts as a counter-balance against ACE in the RAS. By removing a single amino acid residue from the C-terminus of AngII, angiotensin-converting enzyme 2 (ACE2) converts AngII to Ang 1–7, a vasodilator that decreases blood pressure. ACE has two active catalytic domains and

Abbreviations: BLAST, basic local alignment search tool; cDNA, complementary DNA; CHO, chinese hamster ovary; EGFP, enhanced green fluorescent protein; Gb, giga base pairs; Mb, mega base pairs; nt, nucleotide; NCBI, national center for biotechnology information; NHS, Nance–Horan syndrome; ORF, open reading frame; RAS, renin–angiotensin system; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region(s).

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contains two HEXXH+E zinc binding motifs whereas ACE2 has only one for its mono-peptidase activity. In contrast to the widely distributed ACE in tissues, the expression of ACE2 is more restricted, being found mainly on the endothelial cells of the arteries, arterioles and venules in kidney and heart (Donoghue et al., 2000; Tipnis et al., 2000; Oudit et al., 2003). ACE2 is also expressed in the renal tubular epithelium, the vascular smooth muscle cells of the intrarenal arteries, coronary blood vessels (Donoghue et al., 2000) and adult Leydig cells of the testis (Douglas et al., 2004).

From studies of kidney and heart disease caused by unregulated expression of ACE2, it was suggested that ACE2 has a renoprotective role in diabetes as both *Ace2* mRNA and protein levels were decreased by ~ 50% in diabetic renal tubules. This reduction could be prevented by ACE inhibitor therapy (Burrell et al., 2004). Severe impairment in myocardial contractility, with accompanying higher AngII levels, has been observed in ACE2 null mice, and this cardiac defect was rescued completely by genetic ablation (double knockouts) of ACE (Crackower et al., 2002; Oudit et al., 2003). The antagonistic relationship between ACE and ACE2 modulates the balance between AngII (vasopressor) and Ang 1–7 (vasodilator), which plays a significant role in regulating renal and cardiovascular functions. ACE2 has recently been identified as the functional receptor of severe acute respiratory syndrome (SARS) coronavirus (CoV) (Li et al., 2003) and its expression was located on the surface of lung alveolar epithelial cells and enterocytes of the small intestine (Hamming et al., 2004). SARS-CoV infections and the spike (S) protein reduce the expression of ACE2, and injection of the S protein into mice exacerbates acute lung failure in vivo (Kuba et al., 2005). Furthermore, ACE2 and the AngII type 2 receptor protect mice from severe acute lung injury whereas other components of the RAS (including ACE, AngII, and AngII type 1a receptor) promote disease pathogenesis (Imai et al., 2005). These findings provide a possible therapeutic role for ACE2 in acute lung injury affecting millions of people worldwide every year. As the receptor of SARS-CoV, ACE2 was shown to mediate the binding between a Vero E6–ACE2 expressing cell line and the recombinant S protein expressed on the surface of CHO cells, even under high stringency washing conditions (Chou et al., 2005).

Overexpression of ACE2 in the hearts of transgenic mice results in lethal ventricular arrhythmia and subsequent heart failure. This has been shown to be associated with the disruption of gap junction formation (Donoghue et al., 2003). The high occurrence of sudden death of transgenic mice correlates with the expression levels of ACE2. For ACE2 to play the appropriate physiological role in RAS and in the maintenance of properly functioning heart or kidney, the expression requires not only the correct level but also the tissue specificity. According to the World Health Organization predictions cardiovascular diseases will be the leading cause of death by the year 2020, thus it is imperative to include ACE2 as a potential target for developing therapies to control such diseases. Currently, mouse and rat models are available for the study of ACE2, however non-mammalian vertebrates such as frog, zebrafish (Lohr and Yost, 2000) and chicken are particularly useful in the studies for dissecting heart

development at early stages. Frog heart contains two atria and a single ventricle, and is a good tool for studying congenital heart diseases. In this paper, we describe a comparison between ACE2 sequences of chicken, frog, pufferfish and zebrafish. We also show the expression of EGFP in transgenic *Xenopus* hearts, as driven by *Ace2* promoters from human, *Tetraodon* and *Xenopus*.

2. Materials and methods

2.1. Identification of non-mammalian vertebrates *Ace2* genes

Human ACE2 protein sequence (NCBI accession no. AF291820) was used to BLAST the *Fugu* database <http://fugu.biology.qmul.ac.uk/blast> using the “tblastn” algorithm, and partially identified *Fugu Ace2* coding sequence was used to BLAST search at <http://www.ncbi.nlm.nih.gov/BLAST> for *Tetraodon Ace2*. Full length *Ace2* genes of *Fugu* and *Tetraodon* were completed by using the program GENESCAN (<http://genes.mit.edu/GENSCAN.html>) to locate coding sequences from the genomic DNA sequences. The incomplete *Danio rerio Ace2* mRNA (NCBI accession no. BC085667) was completed by sequencing the RT-PCR fragments of the 13th and 18th exons. The partial *Gallus gallus* and *Xenopus tropicalis Ace2* gene sequences were found by BLAST searches against the database of University of California, Santa Cruz Genome Bioinformatics at <http://genome.ucsc.edu> using the human protein sequence. The chicken was completed in the same way as the pufferfish genes, however, exon 18 of *Xenopus Ace2* was confirmed by 3'RACE.

2.2. *Danio* and *Xenopus* mRNA preparation, RT-PCR and 3' RACE

The Zebrafish *Ace2* 13th exon was RT-PCR amplified from RNA mix of 4 to 10-hpf (hours post fertilization) Zebrafish using Qiagen One-Step RT-PCR Kit (Valencia, CA). Primers 5'-CATGGAGTGGTTGAAGGAGG-3' and 5'-ATGTCATTGCGTTCAGGTG-3' were used and the RT-PCR was performed according to manufacturer's instructions. Fragments of 156 bp were extracted from the gel (Qiagen Qiaquick Gel Extraction kit), and cloned using Invitrogen (Carlsbad, CA) TOPO TA Cloning Kit and then sequenced. The PCR primer pair: 5'-TAGAAGCTTCGCCAGAATATCCTTTAAATTTG-3' and 5'-TAGGGTACCTTACAGTCCTGTTGTTCAATG-3' was used to clone the 409 bp fragment (containing the 18th exon of *Danio Ace2*) in a similar manner.

An adult male *X. tropicalis* was sacrificed and its heart was surgically removed for RNA extraction using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The purified total RNA was reverse transcribed into cDNA using Superscript RT (Gibco BRL, Grand Island, NY) and anchor primer, 5'-TTGACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTT-3'. The cDNA was the template for a normal 1st round PCR with forward primer 5'-CTATTGTTGCGGTAGCTGCC-3' and anchor primer as the reverse primer. The PCR was carried out at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min and a final extension of 68 °C for 5 min. For the 2nd round nested PCR, the forward primer 5'-GGTGCCGTAGCTGCCATAA-3'

and the reverse primer 5'-CCTTGACCACGCGTATC-GATGTCGTTTT-3' were used to amplify the 3' RACE fragment. TOPO cloning was employed to clone fragments around 1.1 kb for sequencing.

2.3. Sequencing analysis, alignment, phylogenetics and hydrophilicity plot

Human and mouse *Ace2* sequences (protein [mouse ACE2 NCBI accession no. AAH26801], cDNA [human NCBI accession no. BC039902 and mouse NCBI accession no. BC026801] and genomic DNA [Supplementary data 1]) were retrieved from the NCBI database. Genomic DNA and cDNA of *Ace2* from human, mouse, chicken, frog and pufferfish were analyzed for comparing the exon–intron organization. Alignments of the ACE2 protein sequences of all animals were generated using the program ClustalW. The phylogenetic analysis of ACE2s and ACEs were obtained using the program MegAlign (DNASTar, Madison, WI) with the default ClustalW parameters. The accession nos. of ACEs are CAG04404 (*Tetraodon*), Q10751 (chicken), AAR03504 (human) and P09470 (mouse). The hydrophilicity plots of the ACE2 sequences were analyzed using DNASTar Protean with the default Kyte and Doolittle hydrophilicity parameters.

2.4. Construction of *Ace2* promoter–EGFP constructs for transgenic *Xenopus*

The intergenic region between *Tetraodon Ace2* and *Nhs* as well as the 5'UTR of human (0.6 kb) and *Xenopus* (0.5 kb) were used as putative *Ace2* promoters. They were amplified by PCR using *Tetraodon* genomic DNA (isolated from green spotted puffer), human genomic DNA (isolated from ATCC CCL-171 MRC-5 cell line) and *Xenopus* genomic DNA (isolated from *X. tropicalis*) as templates. The following PCR primer pairs: 5'-GCGGATCCCGTGCACGCCAGAAATAACG-3' and 5'-GCGGATCCCTCAGCTCCGTCTGAACAC-3' were used for amplifying the 2.8 kb intergenic region of *Tetraodon*, 5'-GCGGTACCCAGACCGAGACTCAGTCTC-3' plus 5'-GCGGATCCCGTCCCTGTGAGCCAAGATC-3' were for the amplification of human 0.6 kb putative promoter and 5'-GCGGTACCAGGTTGAACACATCAGTCAGC-3' with 5'-GCGGATCCCTTACTTCTGAGCCGAGG-3' were for *Xenopus* 0.5 kb putative promoter. All fragments were TOPO-cloned and checked by sequencing, and then inserted into pEGFP-1 (Clontech, Palo Alto, CA) at the *Bam*HI site for the *Tetraodon* fragment or *Kpn*I and *Bam*HI sites for human and *Xenopus* putative promoters. *Xho*I and *Bgl*II restriction enzymes were used to obtain the DNA fragments from all constructs, each fragment containing the *Ace2* putative promoter with the reporter EGFP and the polyadenylation signal. Fragments purified from the gel by Qiagen Qiaquick Gel Extraction kit were microinjected into *Xenopus laevis* eggs.

2.5. Microinjection

A modified restriction enzyme-mediated integration was used to generate transgenic *X. laevis* tadpoles (Kroll and Amaya, 1996;

Sparrow et al., 2000). Briefly, unfertilized eggs were squeezed from female frog primed with HCG (human chorionic gonadotropin; Sigma-Aldrich, St. Louis, MO) and dejellied in 2% L-cysteine (W/V) in 1× MMR (100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂ and 5 mmol/L HEPES at pH 7.5). Dejellied eggs were transferred to 60 mm Petri-dish coated with 2% solidified agarose and filled with 0.4× MMR + 6% Ficoll 400. Meanwhile, 2.5 μL of sperm nuclei (stock concentration of 1 × 10⁵/μL) and 2.5 μL of linearized DNA (use 100 ng of DNA for every 3 kb) were mixed and left at room temperature for 10 min. The sperm nuclei and DNA mix were then diluted with 495 μL Sperm Dilution Buffer. Injections were carried out with a 60 μM ID needle and at a constant pump rate of 0.7 μL/min (Harvard Apparatus DHD 2000 Infusion). Injected eggs were incubated at 16 °C for 3 h. Normal developed 4-cells stage embryos were selected and cultured in 0.1× MMR + 6% Ficoll 400 + 50 μg/mL gentamycin at 16 °C. These embryos are cultured until the 15–20th stage before transferring to 0.1× MMR buffer containing 50 μg/mL gentamycin and left at room temp. Successfully transgene tadpoles can be identified by EGFP production under a fluorescent microscope and recorded by digital camera.

2.6. Identification of potential transcription factor binding sites in *Ace2* gene promoter

One kilobase 5'UTR sequences upstream of the ATG codon of 7 vertebrate *Ace2* genes (human, mouse, chicken, *Xenopus*, *Fugu*, *Tetraodon* and zebrafish) were searched at <http://www.cbrc.jp/research/db/TFSEARCH> for transcription factor binding sites.

3. Results

3.1. Non-mammalian *Ace2* genes

Fugu scaffold 2251 (GenBank accession no. CAAB01002251.1) containing *Fugu rubripes Ace2* was BLAST identified using the human ACE2 protein sequence. To confirm the finding, we checked human *Ace2* neighbor genes for *Fugu* homologs which were located in the same scaffold using tblastn. A Nance–Horan Syndrome (*Nhs*) gene (present at the human *Ace2* locus) homologue was also found in *Fugu* scaffold 2251. The gene was connected to *Fugu Ace2* in a head-to-head orientation without any genes in the intergenic region. *Ace2* and *Nhs* genes of *Tetraodon nigroviridis* were found in chromosome 5 (NCBI accession no. CAAE01014581) in the same orientation as *Fugu* genes. Reference pufferfish ACE2 sequences were used to assist in the identification of homolog genes from chicken, frog and zebrafish. The full length ACE2 sequences of *Fugu* (803 amino acids, see Supplementary data 1), *Tetraodon* (803 a.a.; Supplementary data 1) and *Gallus* (808 a.a.; Supplementary data 1) were identified in silico from available databases. Since teleosts are known to contain 2 copies of several single-copy mammalian genes due to a whole-genome duplication early during the evolution of teleosts (Christoffels et al., 2004), we searched the *Fugu* and *Tetraodon* genomes using the identified ACE2 protein sequences to determine whether there is a second copy. However, our search indicated that

Table 1
Exon–intron structures

	Exons (coding sequences)				Introns (intronic regions)					
	h ^a <i>Ace2</i> and m <i>Ace2</i>	g <i>Ace2</i>	x <i>Ace2</i>	f <i>Ace2</i> and t <i>Ace2</i>	h <i>Ace2</i>	m <i>Ace2</i>	g <i>Ace2</i>	x <i>Ace2</i>	f <i>Ace2</i>	t <i>Ace2</i>
1	186	183	189	195	5722	12,203	2220	21,076	86	86
2	159^b	159	159	159	2522	3297	1785	679	100	91
3	94	94	94	94	372	1257	571	1901	335	315
4	144	144	144	144	2256	2762	456	939	132	150
5	113	113	113	116	1485	1402	681	628	75	79
6	106	109	109	106	2180	1538	708	292	353	511
7	98	98	89	89	4084	3782	1428	583	102	88
8	170	170	170	173	2905	1609	867	531	611	601
9	227	227	227	227	2278	2558	604	1037	348	260
10	145	145	145	145	2200	1561	1604	1312	126	103
11	99	99	99	99	1043	1088	668	169	152	95
12	123	123	123	123	404	428	319	493	148	86
13	173	173	179	179	1270	1492	704	820	796	867
14	59	59	62	59	2468	2887	3951	4212	127	129
15	101	101	101	98	1356	2065	951	1385	88	95
16	117	117	111	117	2034	2925	1197	290	126	95
17	195	195	195	195	2010	1665	609	1210	151	113
18	106	115	268	91						
TBP^c	2,415	2,424	2,577	2,409	36,589	44,519	19,323	37,557	3856	3764

The number of nucleotides in each exon and intron are shown.

^a *Ace2*s are prefixed by h (human), m (mouse), g (chicken), x (*Xenopus*), f (*Fugu*) and t (*Tetraodon*).

^b High-lighted nucleotide numbers indicating the exon among all *Ace2*s are same.

^c Total base pairs.

ACE2 is a single copy gene in these teleosts, as are some other genes such as the *Fugu* erythropoietin gene (Chou et al., 2004). Incomplete *Danio* ACE2 protein sequence was obtained using

BLAST (blastp) at NCBI with the *Fugu* ACE2 protein sequence. It was described as a 785 amino acid protein with unknown function (NCBI accession no. AAH85667). When

Ace2 – *Nhs* loci

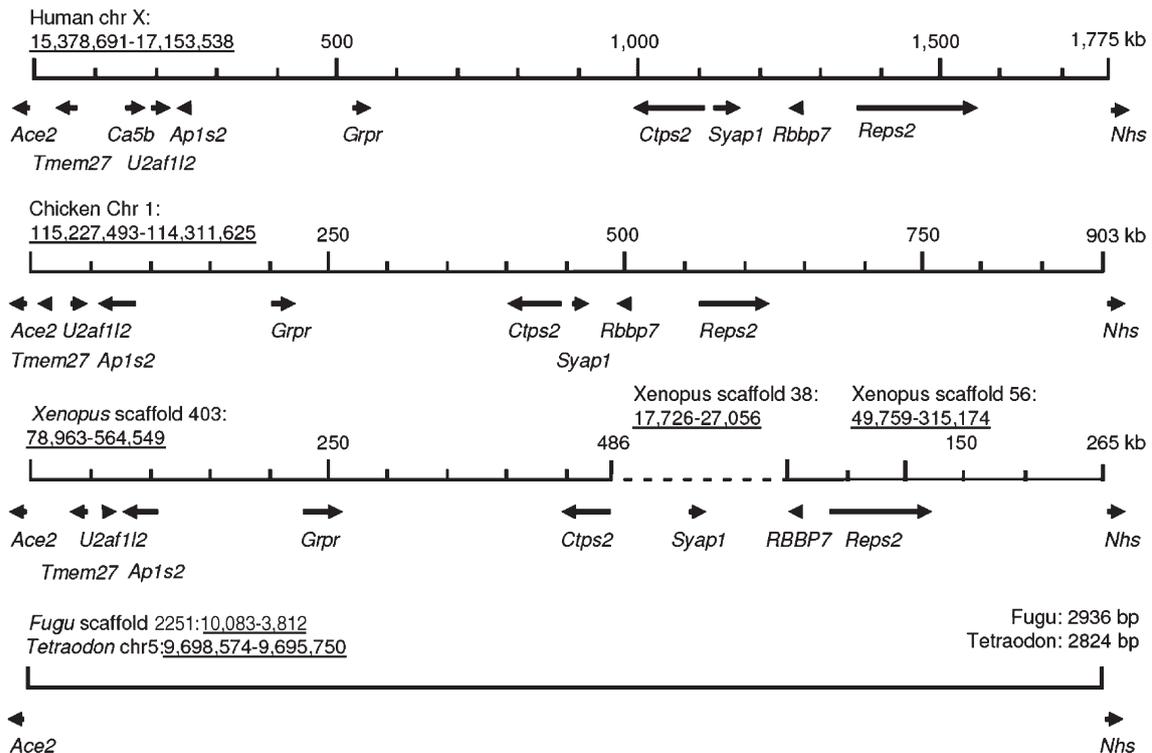


Fig. 1. *Ace2*–*Nhs* loci of human (chromosome [chr] X), chicken (chr 1), *Xenopus* (scaffold 403, 38 and 56), *Fugu* (scaffold 2251) and *Tetraodon* (chr 5). Genes and their orientations are shown as arrows. The dashed line in *Xenopus* locus indicates the discontinuity and distinguishes the three scaffolds. The gene orders were obtained from the UCSC Genome Browsers (<http://genome.ucsc.edu>). Locus positions in the chromosome or scaffold are underlined.

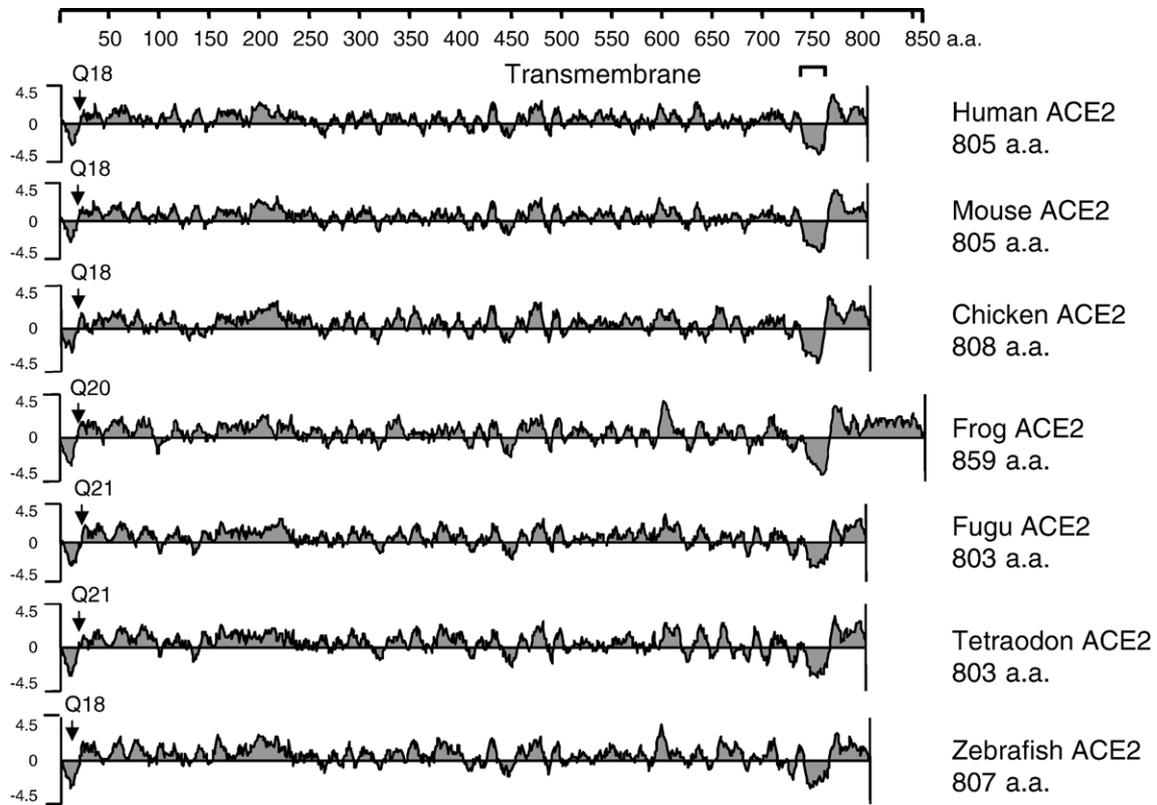


Fig. 2. The hydrophilicity plots for ACE2s from human, mouse, chicken, *Xenopus*, *Fugu*, *Tetraodon* and zebrafish generated using Kyte and Doolittle hydrophilicity parameters. The location of the N-terminal amino acid (a.a.) residue of the mature ACE2s is indicated by arrows. \square denotes the transmembrane domains.

this sequence was aligned with human and pufferfish ACE2s, it was found to contain 11 extra a.a. residues at the end of the 13th exon. Also, exon 18 was absent from the sequence. Primers were designed according to the *Danio* cDNA sequence (NCBI accession no. BC085667) for RT-PCR, and PCR products from exons 13 and 18 (based on the alignment) were sequenced. The 33 nucleotides (coding for the 11 a.a. peptide) in the BC085667

sequence and a G at the end of the 17th exon were absent in our sequence data. The extra G causes a frame shift that results in a premature stop signal, 3 codons downstream of exon 17. The full-length *Danio* ACE2 sequence was then identified as an 807 a.a. (Supplementary Data 1) polypeptide. The partial sequence of frog ACE2 was found in the *X. tropicalis* scaffold 403 by BLAST, and GENESCAN was used to identify the 18th

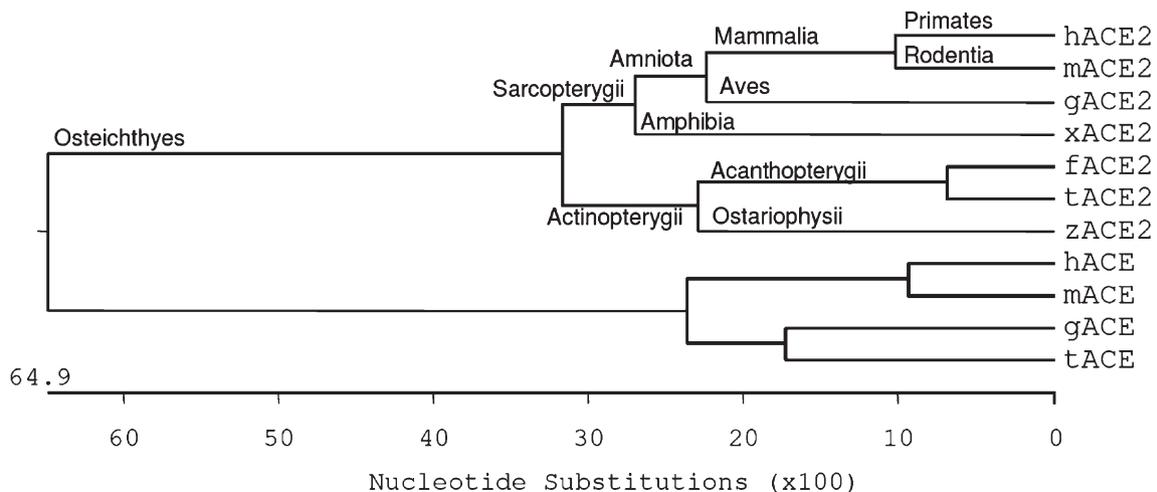


Fig. 3. The phylogenetic analysis of the ACE2 protein sequences, obtained using the program MegAlign with the default ClustalW parameters. The source of ACE2s and ACEs are prefixed by h (human), m (mouse), g (chicken), x (*Xenopus*), f (*Fugu*), t (*Tetraodon*) and z (zebrafish).

exon which contains 162 additional nucleotides compared to the 18th exon of human *Ace2* (Table 1). To confirm the sequence, we designed 3'RACE primers based on exon 17. We found that the polyadenylation signal was 680 bp downstream of the stop codon, and the frog *Ace2* encodes 859 a.a. (Supplementary Data 1) residues with an extra 54 a.a. (compared to human ACE2) located in the cytoplasmic domain.

3.2. Exon–intron structure of *Ace2* genes

We determined the exon–intron structure of the chicken, frog and pufferfish *Ace2* genes by mapping the cDNAs (Supplementary data 1) to their respective genomic sequence. The consensus GT and AG splice donor and acceptor sequences used for the mappings are suitable for all the genes in Table 1. All of the vertebrate *Ace2* genes contain 18 exons and 17 introns, similar to the mammalian *Ace2*s. The lengths of all corresponding exons of *Ace2* are identical between human and mouse and between *Fugu* and *Tetraodon*. Among all the *Ace2*s shown in Table 1, exons 2, 3, 4, 9, 10, 11, 12 and 17 are conserved in length, whereas the lengths of exons 1 and 18 are different in all *Ace2*s. The *Xenopus* has the longest *Ace2* with 2477 bp (Supplementary data 1) and the shortest 2409 bp are found in the pufferfish (Supplementary data 1). Pufferfishes are known for their compact genomes, their genes are densely packed with short intergenic and intronic sequences (Venkatesh et al., 2000). The gene density in genomes of human (3 Gb), mouse (3 Gb), chicken (1.2 Gb), frog (1.7 Gb), *Fugu* (365 Mb; Aparicio et al., 2002) and *Tetraodon* (350 Mb; Crollius et al., 2000) can be correlated with the size of the *Ace2* intronic regions shown in Table 1, except for the larger frog *Ace2* gene.

3.3. Conserved synteny between *Ace2* genes from different vertebrates

We found that *Nhs* and *Ace2* genes are arranged with head-to-head juxtaposition in both *Fugu* and *Tetraodon* genomic sequences. The intergenic regions between them in the *Fugu* and *Tetraodon* genomes are 2.9 and 2.8 kb respectively. This *Ace2*–*Nhs* locus was used to study the synteny relationship among the loci in human, chicken, frog and pufferfishes. Excluding the *Ace2* and *Nhs* genes, there are 9 other genes in the human locus (see <http://genome.ucsc.edu> and also for the identification of other loci) within a 1775 kb fragment in chromosome X (Fig. 1). The chicken locus contains 8 human orthologs (*Tmem27*, *U2af112*, *Ap1s2*, *Grpr*, *Ctps2*, *Syap1*, *Rbbp7* and *Reps2*) except the gene *Ca5b*, and the fragment spans 903 kb in chromosome 1. The locus of *X. tropicalis* is separated in 3 parts according to the available information from the genome assembly program. We found 3 scaffolds (403, 38 and 56) that contain the 8 chicken orthologs as shown in Fig. 1. There are other genes downstream of *Ctps2* in scaffold 403, and genes in both flanking regions of *Syap1* in scaffold 38 as well as upstream of *Rbbp7* in scaffold 56. The locus is either discontinuous in the *Xenopus* genome or forms a much bigger fragment. The *Tetraodon* *Ace2* locus is in chromosome 5 while the location of the *Fugu* locus is currently unknown.

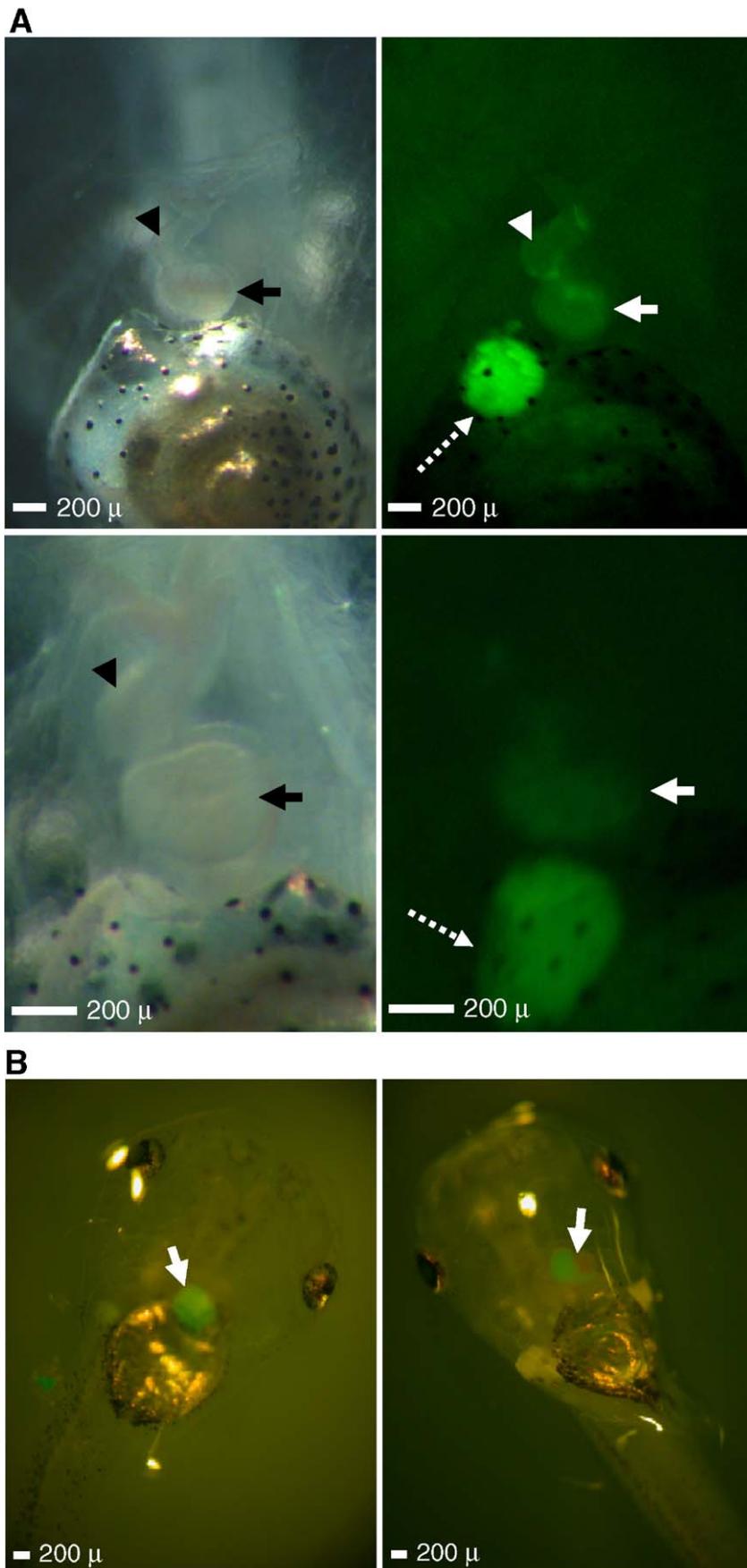
3.4. Comparisons of ACE2 sequences

Supplementary Data 2 presents the alignment of ACE2s from human, mouse, chicken, frog and teleosts (*Fugu*, *Tetraodon* and zebrafish). ACE2 is a zinc metallo-carboxymonopeptidase, and it is distinct from ACE as it carries only a single catalytic domain to perform the monopeptidase activity. As seen from the alignment, the HEXXH+E zinc binding consensus motif was found to be conserved in exon 9 of all mammalian and non-mammalian *Ace2* genes. Among all 7 ACE2s, *Fugu* and *Tetraodon* share the highest sequence identity of 88% while the human and mouse proteins are slightly lower (82%). The identities between human and non-mammalian ACE2s are from 56% (pufferfish) to 66% (chicken), between pufferfish and the other 5 ACE2s are around 53% to 57% (frog, human, mouse and chicken) and 65% (zebrafish). Although the identities among ACE2s varies from 53% to 88%, the hydrophilicity plots of all 7 ACE2s are very similar (Fig. 2), with the exception of the *Xenopus* ACE2, which has a larger cytoplasmic domain. Highly hydrophobic a.a. residues at the N-termini make up the signal peptides of ACE2s and the N-terminal a.a. residue of the mature proteins is indicated. The transmembrane domains are located at the hydrophobic region around a.a. residue 750, and the rest of the downstream sequence forms the small cytoplasmic domain.

Primates (*Homo*), Rodentia (*Mus*), Aves (*Gallus*), Amphibia (*Xenopus*), Ostariophysii (*Danio*) and Acanthopterygii (*Fugu* and *Tetraodon*) are represented by important animal models studied today that have evolved from the Osteichthyes (Bony fishes) lineage. We used ACE2 protein sequences from these 7 animal models together with ACE sequences of human, mouse, chicken and *Tetraodon* to analyze the phylogeny. The phylogenetic tree in Fig. 3 shows the evolutionary relationship of the ACE2 sequences from divergent animals. As expected from orthologous sequences, the phylogram supported the standard classification of the vertebrates (including the frog ACE2 with its large cytoplasmic domain).

3.5. *Ace2* putative promoters are tissue specifically active in the heart of transgenic frogs

ACE2 expression in the testis, kidney and heart had been demonstrated using Northern blot analysis (Donoghue et al., 2000). To analyze tissue specificity, we used the putative promoter of *Tetraodon* and human *Ace2* to drive the expression of EGFP in transgenic frogs. In addition, to confirm the *Ace2* of *X. tropicalis* (regarding the larger cytoplasmic domain) we checked the promoter tissue specificity as well. All three constructs were linearized and mixed with *X. laevis* sperm nuclei before microinjection into the eggs. After 5 days, around 10% of transgenic tadpoles expressed EGFP on the surface of heart ventricle or truncus arteriosus when visualized with a fluorescent microscope (Fig. 4 A and B). From our observation, for all constructs, the heart of transgenic frogs is the only tissue expressing EGFP. However, the nonspecific fluorescence from the gastrointestinal tracts (Fig. 4A) might mask expression in other tissues. The nonspecific fluorescence is also present in



-640 to -230 5'UTR consensus **WGATAR** (T/AGATAA/G; C/TTATCT/A) sequences

hAce2	ATCGCGCCACTGTGCTCCAGCCTGGGCGACAGACCCGAGACTCAGTCTCAAAAAAAAAAAAAAAAAAAAAAAGTATGATCAGAAAT	-558
mAce2	GGTGGTTGTGGTGCACACCTTTAATCCCAGCAGTCAAGGAGGAGGAAAGCAGATCTCTTGAGTTTGAGGCTAGCCCTAGTC	-558
gAce2	TAAGGATTTAAGTTAAGTTAGGTCACCTACACCCAGAGGCTACGATCCCACCTATGTCCTTGCTCCCTTGCTCCCTTACACCT	-558
xAce2	TTGCTCTTACTCGGTCACTTACAATAATTCAGGCTCTTCAAGCCCTCTGTGGAAGGCTTATATGGAAACGCAAGTGCCTT	-558
fAce2	TGCCTTTGTGTTGATGATTTGAATGAATTT TTATCA CAACTTTAGGACT TTATCT GTATTCTGACTTGCCATTTTTTAAGCTGTTGG	-558
tAce2	ATTTCT TTATCA CAACTTTAGGCTTT TTATCA GTCTCTGACTTGCCATTTTTTAAGATATCGGAAAACCAAGTAACTTGGAC AGAA	-558
zAce2	CTATCA CGCAGTATTATTTAATAATTTTAAAGGTGAATTTAACTCTGAAAGTCTCTGAGCTATAGGCT TTATCTTTGATAA AGAA	-560
hAce2	ATGAATTCATAAAGACAAGGGCAAAGTCAATGATTTTGAAGGGAAAAATGTTGCCAAGTAGAGAGTTTCTGGGAATATGAT	-476
mAce2	TACAGAGGGAGTTCAGGACAGCCAAAGCTACAGAGAGGAACTGTCTAAAAACACCAAGAAAGAGAGAAAGGAGAGAGGGAG	-476
gAce2	TTAAAAATTTAATTTTGTATGCCCTACTCATTTTGAAAATGAGTTTTCAAAGCCTTTTAAAAACACGGACCTGAACATCCAAC	-476
xAce2	TCGTCTTCTGCTTAAGCTTTAGAACATTTAAAATGTAATCAATAGGTTGAACACATCAGTCAGCGGGGAGCAAAAGAAA	-476
fAce2	AAAACACAGGTAACCACT AGATAA AGATGGGCTGGGAATATGTTGTTTTCCAT GTATAA CTTTTTTAATTTATTTT	-476
tAce2	TAA GACTGAGAACTCTGTTGCTTCTGTTTCCACCATCAGTTGTTGATTTTGTATTTTGTAAATTAATTTGATGGAT	-476
zAce2	TCTACTTCAAGCCTTCAAGTCACTGTTATTTATGCTTTTATAGCCCTACATTTATGTTTGCATGTGATTTCCAGTCCCTCCTCAAC	-478
hAce2	CTTGAATAAAAAATAAATGTGAGATAACCTATTAATGAAATGTTCTGAAAACCATACAAACACCAACAT TTATCT TCATGATC	-394
mAce2	AGGATGGATAGCTTAT GTATA GAATTTGTGAGAAAAGGCTATAAGTTCCAATATGTTGCCATGATTTCTAAGCTTAGCCCTT	-394
gAce2	CAGGTACCCTATGTCAGGCTATGGTAATTTCTTCTGCTGGGAAATAAGGTCATGGCTTGGTTCAGTGAATTTTCTA	-394
xAce2	TCAATGCCTTATATGCCTCTTTATGGAGACAAACAAACGCTCTATAATTTCCAGAAAGCAGTGCATAGTCTGCCACATAA	-394
fAce2	TTAGTATTTAATAATGCTGCTATTTATAGTTTAAAGTAAATCAAGTTCCATATAAAGAAAGACCCAT TTATCT CCTAGGTAACA	-394
tAce2	TTTCATATGAGCTACAGTATTCGCTCAAAGCATGTTCCGTCAGCTGAAAAGAAACGGTCCGAGTATGTTTTCCAGAACCTC	-394
zAce2	TGAATTCACAGGTGCTCTTCCACAAAGAAAATAATTTAGTGTGTTTTTCTTATTTCCATGTATGTTTTCCAGTAGCCAT	-396
hAce2	CCTAGTTCTAGACCTCTTGGTCACTGTAAAATATAACATTTTCCGTTGATCTTTAACAGCTTTCTAGGAAAATATTAACC	-312
mAce2	TCTGTATAGTAAAATCATAGTACACCCCTCCTCCAGTGTATCTTTAACAGCTTTTAAAGAAACATATAACTAAATGTCC	-312
gAce2	AATATTTTTACAGCT TTATCA CCAGCCTGTTAAAGGATCAAAGATTTCTGAGCTACCAAAGACACCAGCAGGCTAG AGATAA	-312
xAce2	TTCCCTTTTATGTTGTTGTCGCGAAACCTTTAGGCTCTGTTATTAACCCCAAAATCCAAAGTTCCAGAGTGAACCGAACCAT	-312
fAce2	GGATTTGTCAAGAGGACATTTGAAAAGAACATATTAAGTATGTTGCTGACTAGAACTCTACTGTATTTGGCATTGTTACGCATTA	-312
tAce2	TGTAACCTTCAGCTGAAAAGAAAAGCTTAAAAAGACAAGCCTTTCAATTTAGCAGTGGTGACAG ACTATCA GTTTTGGCAG	-312
zAce2	ACAACCTGTTTACCTTCTATAAAAACCTCAGATTTGTTCAAATAAACTGTATTTAAAAAAGTACAGTTTAAACACTTTTAAACA	-314
hAce2	AAAAGTACCGGTTTTGATTTGGCCATAAAATGTTAGCAAGGCTAAGGTTTTCTAGGATTTAAGATAAATGCTTTTATTTAGTTTACTT	-230
mAce2	AGGTTTTGATTTGGCCATAAAATGTTAGCAAGGCTAAGGTTTTCTAGGATTTAAGATAAATGCTTTTATTTAGTTTACTT	-230
gAce2	GTGACGAGCATGATCATTTGACAGCACACTTGGGAAACATTAACATAAAAGTAAAGGCTTTTATAAGAAGGCCATAAAATGGT	-230
xAce2	CAGTCAT TTATCA GTGTCATCCGATTTGCAGGTAATGGAGACTACGACCCCTGCCTGAAAATGCTTTTACATTCATTTATGCAA	-230
fAce2	TTTTCTTATATTTCTTATATTTCCATGGCCCTTTAATTTATCAGCTTTAATTAATCCTCCTTAATCATCCTCTATATAAA	-230
tAce2	TTCTGCATTAATTTCACTTGAATATATTTGTCAGCGCTTTTATTTTATGCTTTAATTT TTATCA CTTTATCTTAAAAAGCAA	-230
zAce2	ACATGACTTTACTTTTAAACAATGACTGATTTTCATCAACATCTAATTTCTACTACTTGCAGTGCTCACACAGTGAAAGTC	-232

Fig. 5. Seven *Ace2* ATG codon upstream sequences from around nt -640 to -230 are shown. Sequences shown in bold are potential GATA binding sites. The source of *Ace2*s are prefixed by h (human), m (mouse), g (*Gallus*), x (*Xenopus*), f (*Fugu*), t (*Tetraodon*) and z (zebrafish).

wild type frogs. A movie of a beating fluorescent heart from a transgenic frog (using human 0.6 kb putative *Ace2* promoter to drive the expression of EGFP) is presented as Supplementary data 3.

3.6. *In silico* prediction of the cis-acting motif for transcriptional factor GATA

Since the promoters of human and pufferfish *Ace2* genes were found to drive expression in the heart of transgenic *Xenopus* (Fig. 4), we then compared the promoter sequences of *Ace2* genes from 7 vertebrates to search for transcriptional factor binding sites that might be responsible for this tissue specific expression. The DNA sequences of 7 putative *Ace2* promoters (1 kb upstream of ATG codon) were analyzed at <http://www.cbrc.jp/research/db/TFSEARCH>, and found to contain a conserved cis-element WGATAR which may serve as GATA transcription factor binding site (Ikonomi et al., 2000). The conserved motif WGATAR (T/AGATAA/G or reverse complement sequences C/TTATCT/A) was found between nt -640 and -230 (where the A of the ATG codon is +1) in all the 7 *Ace2* promoters (Fig. 5). The total number of this specific motif was found to vary between species, 1 in human, mouse and *Xenopus*, 2 in chicken, 3 in zebrafish, 5 in *Fugu* and 6 in *Tetraodon*. These conserved motifs may provide possible

targets for one or more of the GATA family transcriptional factors.

4. Discussion

In humans, it is predicted that cardiovascular diseases will be the most common cause of death worldwide by 2020. Our body uses the RAS to regulate systemic blood pressure, thereby maintaining stable total blood circulation. However, chronic high blood pressure results in hypertension which is the main cause of cardiovascular diseases. Since the discovery of ACE involvement in the production of the vasopressor AngII, numerous studies have focused on finding either ACE inhibitors or AngII receptor blockers as a means to counteract hypertension. ACE2, an antagonist of ACE in RAS, inactivates AngII to reduce the blood pressure, especially in heart arteries. To augment our understanding of the overall function of ACE2, we compared the structure of *Ace2* genes from various non-mammalian vertebrate genomes. We searched the *Fugu* (Aparicio et al., 2002) and *Tetraodon* (Jaillon et al., 2004) genome assembly databases because they are well documented. Together with *Ace2*s from pufferfishes, we also identified the gene from other selected vertebrates (chicken, frog and zebrafish) that are important in evolutionary and developmental studies.

Fig. 4. *Ace2* promoters drive the tissue specific expression of EGFP on the heart surface of transgenic *Xenopus*. (A) The expression was driven by 0.5 kb *Xenopus Ace2* promoter region (lower panels) and 0.6 kb human *Ace2* promoter region (upper panels). EGFP was visualized using a fluorescent microscope. The left panels were photos taken under brightfield and the right panels were taken under settings for EGFP fluorescence. The ventricle is shown by arrows and the truncus arteriosus by arrow heads. Dashed arrows indicate nonspecific fluorescence from GI tracts. (B) Fluorescenced hearts of transgenic *Xenopus* using the putative promoter of *Tetraodon* are shown and indicated by arrows. Photos were taken under filtered UV and dimmed light for presenting the fluorescenced heart in entire tadpoles.

The sizes of *Ace2* genes in different animals provide a good example in demonstrating correlation between the genome size and the size of intronic regions (Table 1). With the exception of the mouse and *Xenopus* genes, decrease in *Ace2* gene density is due to the extraordinary large first intron. Ten out of eighteen *Ace2* exons show length variations in the six animals, however the exon boundaries in all six genes are identical as indicated by arrows in Supplementary data 2. Although the zebrafish genomic *Ace2* sequence has not yet been completed, it is reasonable to predict that the exon boundaries of zebrafish *Ace2* are similar to those of the other *Ace2*s. Disulfide bond formations between cysteine residues are important for the functions of many proteins. We found seven cysteine residues conserved in all ACE2s (Supplementary data 2) suggesting that they may be responsible for maintaining the conformation, stability (Maiti and Surewicz, 2001) or biological activities (Guo et al., 2004). ACE2 residues Arg 273 (critical for substrate binding), His 345 and His 505, involved in catalysis, as proven by site-directed mutagenesis (Guy et al., 2005), are conserved in all 7 genes. Glycosylation of mammalian ACEs is heterogeneous and differs in terms of extent of glycosylation, sites of glycosylation and structures of attached oligosaccharide units (Ripka et al., 1993). Rabbit testicular ACE (tACE) has 5 potential N-glycosylation sites and the first 2 sites are sufficient and essential for normal synthesis and processing of the active enzyme (Sadhukhan and Sen, 1996), whereas deglycosylated human tACE retains full enzymatic activity (Yu et al., 1997). We scanned the potential N-glycosylation sites from the 7 ACE2 sequences and found 7 sites in human ACE2, 5 in mouse ACE2, 11 in chicken ACE2, 3 in *Xenopus* ACE2, 11 in *Fugu* ACE2, 9 in *Tetraodon* ACE2 and 4 in zebrafish ACE2. There was only one site in human ACE2 (Asn53), which was conserved in other ACE2s except that of zebrafish, thus suggesting an important functional role for the putative N-glycosylation at this site. With the recent SARS pandemic and a possible future bird-flu pandemic looming, the transmission of pathogens from animals to human has received intense scientific attention. A study of the possible transmission of SARS-CoV in domestic poultry suggests that domestic poultry is unlikely to have been the reservoir, or associated with dissemination, of SARS-CoV (Swayne et al., 2004). However, is it possible that birds can become a reservoir? We tried to answer this question by studying the ACE2 receptor binding domain. It has been reported that amino acid residues 31, 41, 353, 355 and 357 of mammalian ACE2 are responsible for the binding with SARS-CoV S protein (Li et al., 2005). Comparing these binding sites from human, palm civet and chicken ACE2s showed that only residue 31 is variable (Lys in human, Thr in palm civet, and Glu in chicken). Interestingly, where palm civet ACE2 efficiently binds the S protein from SARS-CoVs isolated during the 2002–2003 SARS outbreak, the much less severe 2003–2004 outbreak and from the palm civet, human ACE2 could only bind efficiently to the S protein from the earlier human outbreak (2002–2003). This probably indicates that residue 31 plays an important role in the binding between ACE2 and SARS-CoV S protein. Therefore, since chicken ACE2 contains a Glu at position 31 (Supplementary data 2), we hypothesize that this may be an obstacle in the binding between SARS-CoV S protein and chicken ACE2.

The synteny of *Ace2–Nhs* loci was highly conserved between mammalian and bird, although their evolution diverged from Amniota 300 million years ago. Amniota and Amphibia have diverged from Sarcopterygii for 350 million years, and as a result of evolution the locus of Amphibia could either contain many other genes or have been rearranged. This was deduced from the 3 *Xenopus* scaffolds shown in Fig. 1, which have other genes in the flanking regions. Although there are gaps in the *X. tropicalis* genome assembly, the syntenies of genes between *Ace2* and *Nhs* appear to be conserved. After 450 million years of evolution the loci are quite different between Actinopterygii and Sarcopterygii, i.e. there was no gene in the intergenic region between *Ace2* and *Nhs* in pufferfish (Actinopterygii) whereas at least 8 genes were in the region of Sarcopterygii. It is unknown whether genomic rearrangement removed genes from the fish loci or whether they were inserted into Sarcopterygii locus. An ongoing genome project (Venkatesh et al., 2005) for a small genome (1200 Mb) cartilaginous fish (elephant fish), the ancestor of Sarcopterygii and Actinopterygii, may provide answers to this question.

In order to locate the promoters of human and *Xenopus* *Ace2*, we ligated the 1.2 kb (human) and 1 kb (*Xenopus*) 5'UTRs upstream of the reporter EGFP. Both constructs drove the expression of EGFP in the heart of transgenic *Xenopus* and in transfected Vero E6 cells (data not shown). The reduced promoter sizes of 0.6 kb (human) and 0.5 kb (*Xenopus*) still maintains tissue specificity. The 2.8 kb fragment from the intergenic region between *Tetraodon* *Ace2* and *Nhs* genes is able to drive the EGFP expression too, as in the case of the human and *Xenopus* promoters. NHS is an X-linked disorder characterized by congenital cataracts, dental anomalies, dysmorphic features, and, in some cases, mental retardation (Burdon et al., 2003), therefore the gene may be expressed during eye, teeth and brain developments. *Nhs* and *Ace2* genes are in a head-to-head orientation in the *Tetraodon* locus and expression driven by *Nhs* promoter was expected to be in the eyes and brain regions. However, we were unable to detect the reporter in transgenic *Xenopus* using the reverse orientation of the 2.8 kb fragment. In mammals, the kidney is another main organ expressing ACE2, but in our transgenic studies, the reporter was clearly absent from the kidney. The expression could only be observed on the heart surface, which may correspond to the expression in endothelial cells of the arteries, arterioles and venules (Oudit et al., 2003). As reported previously, detection methods with higher sensitivity such as quantitative RT-PCR (Harmer et al., 2002) could be used to identify gene expression in other tissues. Even so, the expected site of significant expression levels should still be the heart tissue. We also observed that the expression of all three constructs was shifting from ventricle to the truncus arteriosus or the entire heart but very seldom to atria alone. Transgenic frog lines are being established, and these may be useful for the secondary screening of drugs that target the up-regulation of ACE2 expression in heart blood vessels. Although the transgenic studies have shown tissue specificity and some potential GATA transcription factor binding sites have been found in the putative promoters of *Ace2* genes in 7 different vertebrates, the identification of tissue specific elements requires further studies, either using cellular transfection with

suitable heart endothelial cell lines or using transgenic animal models with mutated promoters. Since previous studies have implicated members of the GATA family in endothelial tissue development (GATA-2/3), as well as cardiac function (GATA-4/5/6; Jiang and Evans, 1996), we speculated that the GATA motifs identified in this study could be crucial for the transcription of *Ace2* in cardiac tissues. The conceptual amino acid sequence of exon 18 of *Xenopus* ACE2 was aligned with 6 other counterparts using all three reading frames. However, only the ORF encoding for the large cytoplasmic domain showed significant identity. To determine whether the larger cytoplasmic domain observed in *X. tropicalis* is typical of amphibian ACE2, the gene from *X. laevis* may be useful for additional characterization. In this paper we report five ACE2s from non-mammalian vertebrates. Several lines of evidence, such as identical exon–intron structure, conserved synteny, sequence identity, similar protein primary structure, sequences along the phylogeny and tissue specificity of putative promoters, showed that all the identified *Ace2* genes were the orthologs of mammalian *Ace2*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.03.010.

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