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# Pillar cell and erythrocyte localization of fugu $ET_A$ receptor and its implication

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**Abstract**

Endothelin, a vasoconstrictor peptide, plays important roles not only in the mammalian circulatory system but also in non-mammalian systems, such as the gill lamellar vascular network with complex structural characteristics. Here, we show that (i) the contraction of pillar cells that delimit the lamellar vasculature is controlled by endothelin through the type A endothelin receptor ($ET_A$) linked to the intracellular calcium signaling system and (ii) $ET_A$ receptor is also highly expressed on fugu erythrocytes, a hitherto unexpected finding. Database mining revealed the presence of five endothelin receptor (ETR) sequences in the fugu genome. By Northern blotting, cDNA cloning, and fura-2 monitoring, the branchial ETR subtype was shown to be $ET_A$ able to induce a $Ca^{2+}$ transit. Immunohistochemistry revealed its pillar cell and erythrocyte localization. These results suggest an endothelin/$ET_A$-mediated coordinated regulation of the pillar cell shape and erythrocyte membrane flexibility.


*Keywords:* Cardiomyocyte; Endothelin; Erythrocyte; Gill; Pillar cell

Since its discovery in 1988 as a powerful vasoconstrictor peptide [1], endothelin and its receptors have been studied intensively in mammals and it has been established that (i) the endothelin family of peptides consists of three structurally related members called endothelin-1 (ET-1), ET-2, and ET-3 [2], among which ET-1 being the predominant physiological isoform and (ii) there are two subtypes of ET receptors (ETRs), termed $ET_A$ and $ET_B$, which belong to the G protein-coupled receptor (GPCR) family [3]. Concerning vascular effects of ETs, the $ET_A$ has been established as the mediator of ET-induced constriction of the vascular smooth muscle with the highest affinity to ET-1, whereas $ET_B$ has a broad ligand specificity and mediates the release of endothelium-derived relaxing factors, such as nitric oxide and prostacyclin [3].

Compared to the mammalian ET system, relatively little is known about the molecular aspect of the non-mammalian ET system. But nevertheless, it also plays important roles in the control of non-mammalian physiological systems. Especially, ET regulation of specialized organs such as the gill, which are not present in mammals, is thought to deserve extensive clarification at the molecular level. Recently, comparative studies using ET have demonstrated that ET-1 affects the perfusion pathways in the gill vasculature by contracting the pillar cells [4,5] that are post-like cells with unique structural features: they possess a cylindrical cell body connecting two parallel sheets of respiratory epithelium, contain ET-sensitive contractile machinery, and delimit a network of vascular compartments within the lamellae of fish gills [6,7] (for the structure of lamellae, see Supplementary Fig. S1).

In the present study, through a combination of database mining, molecular cloning, second messenger assay, and immunohistochemistry, we demonstrated the presence of $ET_A$ receptor on the fugu pillar cells, which accounts for the major population of ETR in the gill. Another interesting finding, although unexpected, is the presence of relatively high levels of the $ET_A$ receptor on red blood cells (RBCs) of fugu, *Takifugu rubripes*. In contrast to

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non-nucleated mammalian RBCs, fish RBCs contain the nucleus. It seems, therefore, reasonable to assume that, in fish, $ET_A$ is synthesized in nucleated RBC and is expressed on its surface to respond to the circulating and locally generated ETs. Our data suggest that ETs regulate the gill microcirculation by altering the dynamic mechanical properties of not only pillar cells but also RBCs, which are circulating through the lamellar vasculature of the gill, a new concept in vascular biochemistry and physiology.

## Materials and methods

*Animals*. Fugu were reared and sacrificed as described previously [8]. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology.

*RNA isolation, cDNA cloning, and Norther blot analysis*. Various tissues were isolated and pooled from three fishes. Total RNA was extracted from the tissues with Isogen according to the manufacturer's manual (Nippon Gene, Japan). cDNA of *ETR* genes was amplified from total RNA by RT-PCR with gene-specific primers (Supplementary Table 1). The full-length of the cDNA was obtained by rapid amplification of cDNA ends using First Choice RLM-RACE kit (Ambion) according to the manufacturer's instruction. All the sequences were confirmed using ABI PRISM 310 genetic analyzer. Coding sequence of $trET_A$ (tr, *T. rubripes*) receptor was cloned into mammalian expression vector pcDNA3 (Invitrogen), and used for the ET-1-induced intracellular $Ca^{2+}$ mobilization experiment. Northern blot analyses were performed using total RNA (20 μg/lane) from various tissues of fugu as described previously [8].

*In silico analyses*. Hydropathy profile was determined by the Kyte and Doolittle method using Genetyx-MAC software (Genetyx, Japan). The post-translational modifications were predicted by NetPhos 2.0 server [9]. Multiple alignment of the amino acid sequence of $trET_A$ with other relevant gene products was performed using Genetyx-MAC program. For the phylogenetic tree, sequences were aligned with Clustal W software [10], and the tree was constructed by neighbor-joining method [11] with 2000 bootstrap replicates using MEGA version 3.1 software [12]. Synteny of the neighboring genes of the $ET_A$ was investigated by comparing predicted genes of the scaffold 17 with the human chromosome 4. Fugu scaffold and human chromosomal information was collected using the fugu genome database (JGI, http://genome.jgi-psf.org/Takru4) and Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/), respectively.

*Mammalian cell culture*. COS7 cells were seeded into 24-well glass bottom plate (Iwaki, Japan), and transiently transfected either with pcDNA3-$trET_A$ or mock vector using Lipofectamine-2000 (Invitrogen) as per supplier's manual. Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin (Invitrogen) at 37 °C in 5% $CO_2$. At 36 h post-transfection, cells were processed for ligand-induced intracellular $Ca^{2+}$ measurement experiments.

*Measurements of $trET_A$-mediated intracellular $Ca^{2+}$ mobilization*. Cells were incubated with 3 μM fura-2 AM (Dojindo Inc., Japan) in DMEM for 45 min at 37 °C. Fura-2 AM-loaded cells were incubated with DMEM for 1 h at 37 °C for recovery. For antagonism experiments, cells were parallely treated with DMEM containing 50–250 nM of either BQ123 (Sigma) or BQ788 (Sigma) at 37 °C for 1 h. Cells were washed twice with PBS containing 0.1% BSA and subjected to fluorescence analyses with the addition of ET-1 (Peptide Institute, Japan) at 1 nM final concentration. The fluorescence at 510 nm elicited by excitation at 340 or 380 nm was measured using a Nikon Eclipse TE 2000-U microscope equipped with a ORCA-ER camera (Hamamatsu Photonics, Japan) and an image processing software (Aquacosmos; Hamamatsu Photonics, Japan). The F340/F380 ratio was measured to estimate the intracellular $Ca^{2+}$ concentration.

*Antibody production, purification, and absorption*. To prepare recombinant protein as antigen, cDNA fragments encoding the cytosolic C-terminal of $ET_A$ receptor (amino acid residues 401–468) was cloned into bacteria expression vector pGEX-4T-1 (GE healthcare), and the glutathione *S*-transferase (GST)-fusion protein was expressed as described previously [8]. The expressed protein was purified from the supernatant of cell lysate by GST affinity resin (Amersham Bioscience) according to the manufacturer's manual and dialyzed against saline (0.9% w/v NaCl) at 4 °C. Polyclonal antibodies to the purified proteins were raised in Japanese White rabbits as described previously [8]. The antibody was purified using Affi-Gel 10 (Bio-Rad) as per supplier's protocol. The purified antibody was eluted with 0.1 M glycine buffer, pH 3, and neutralized to pH 7.5 with 1 M Tris buffer, pH 9. The eluted antibody was finally dialyzed against PBS at 4 °C. The purified antibody (500 μl) was absorbed with the antigen (500 μg) for 16 h at 4 °C with continuous mixing, and was used as control for the immunohistochemical experiments.

*RBC enrichment*. Blood was collected from the anesthetized animal with a heparinized syringe directly from the heart, and diluted to 30% in an ice-cold PBS with 5 U sodium heparin/ml. Cells were precipitated by centrifugation at 2000*g* for 5 min. Supernatants and buffy coats were removed by slow aspiration. The mid layer of the pelleted cells were resuspended in an ice-cold PBS containing 2% paraformaldehyde (PFA) and repelleted by centrifugation at 2000*g* for 5 min. After removal of the supernatant and the top layers by aspiration, the mid layer was collected, washed again in ice-cold PBS, and smeared on the 3-aminopropyltrieth-oxysilane (APS)-coated glass slides (Matsunami Glass, Japan), air-dried for 10 min, and processed for immunohistochemistry.

*Immunohistochemistry*. Fugu were perfused with an ice-cold PBS, pH 7.4, for 2 min through intra-arterial infusion and subsequently fixed by perfusion with 2% w/v PFA for 10 min. The fixed gills and hearts were excised, and the tissue sections were prepared as described previously [8]. Sections were processed for peroxidase-substrate staining, using 750 times dilution of the purified antibody and 400 times dilution of the preabsorbed antibody, with Vectastain Elite ABC kit (Vector Laboratories Inc.) as per supplier's protocol. For fluorescence immunohistochemistry, slides were treated with 0.1% v/v Triton X-100 in PBS for 7 min, washed gently with PBS, and incubated with 3% v/v FBS for 1 h at room temperature. After the blocking, they were incubated for 16 h at 4 °C within the FBS containing the absorbed and purified antibody at 1:30 and 1:50 dilutions, respectively. Then the slides were rinsed with PBS, and incubated for 1 h at room temperature in a cocktail mix consisting of Alexa Flour 488-conjugated anti-rabbit IgG (1 μg/ml, Invitrogen) with Hoechst 33342 (100 ng/ml, Invitrogen). For the gill sections, tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma) was also used at a 0.1 μg/ml concentration. Slides were processed, and images were taken as described previously [8].

## Results

### *Identification and molecular properties of ETR expressed in fugu gill*

A database search revealed the presence of 5 fugu ETR homologs of the human ETR genes in scaffolds 17, 103, 163, 263, and 376 of the *T. rubripes* genome (JGI, http://genome.jgi-psf.org/Takru4), which were tentatively named trETR(scaffold 17), trETR(scaffold 103), trETR(scaffold 163), trETR(scaffold 263), and trETR(scaffold 376), respectively. Primers were designed based on these sequences, and partial cDNA sequences were obtained by RT-PCR using mRNA preparations from the fugu heart, and confirmed by sequencing.

As the original objective of this study was to find the ETR(s) that is expressed in pillar cells, we first determined the tissue distributions of the five candidate clones by Northern blot analyses (Fig. 1). trETR(scaffold 17) transcript was found relatively highly expressed in multiple tissues including the gill, heart, kidney, and blood cells, but

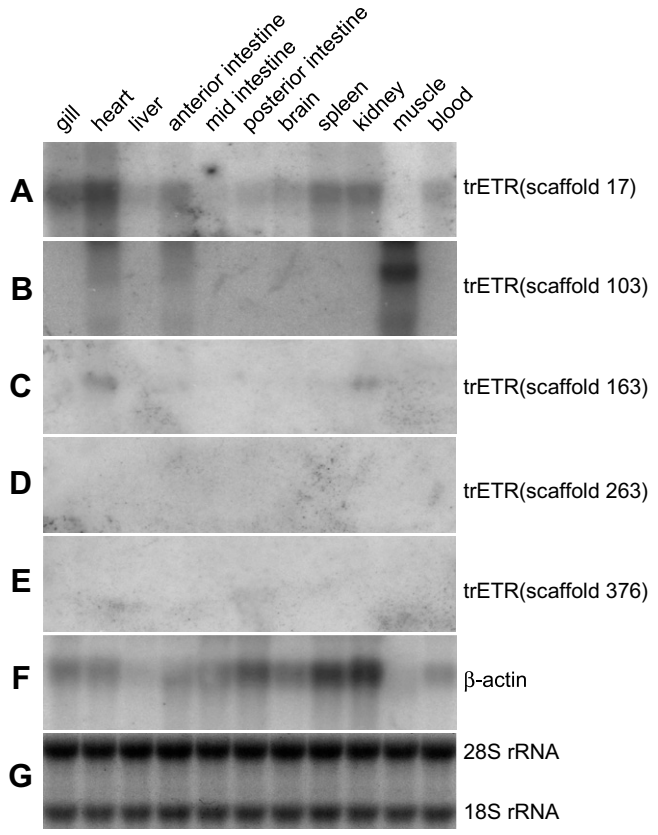


Fig. 1. Tissue distribution of five endothelin receptors in fugu. Northern blot analyses were performed using 20 μg of total RNA preparations from the gill, heart, liver, anterior intestine, mid intestine, posterior intestine, brain, spleen, kidney, muscle, and blood cells. Five putative ET receptors identified by a database mining are denoted by their scaffold numbers. β-Actin was used as internal control. 28S and 18S ribosomal RNA are also shown as indicators of equal loading and RNA integrity. A representative data set from triplicate experiments is shown.

not in the skeletal muscle (Fig. 1A). In contrast, trETR(scaffold 103) is highly expressed in the skeletal muscle (Fig. 1B), and faintly in the heart and anterior intestine. The transcript of trETR(scaffold 163) was found moderately expressed in the heart and kidney (C). Expression of messages of trETR(scaffold 263) and trETR(scaffold 376) was not detected in any of the tissues examined (Fig. 1D and E). As trETR(scaffold 17) was the only clone expressed in the gill, we chose it for further characterization and obtained the full-length of its cDNA sequence using a gill total RNA preparation. The sequence, encoding a 468-amino acid residue protein, was submitted to the DDBJ database with the Accession No. AB289620.

Fig. 2A shows the gene structure of trETR(scaffold 17). Comparison with the cDNA sequence indicated that the gene is transcribed from an ~8.2-kb segment of fugu genome scaffold 17 and is composed of 9 exons. The start and stop codons reside in exon 3 and exon 9, respectively.

A hydropathy profile analysis (Fig. 2B) showed that trETR(scaffold 17) consists of a hydrophobic presequence (putative signal peptide), an N-terminal extracellular domain, seven transmembrane (TM) spans and a C-terminal cytoplasmic tail, which are the trademark of the GPCR superfamily. Two putative N-linked glycosylation sites are present in the extracellular region and two potential protein kinase C phosphorylation sites, in the C-terminal cytosolic tail (Fig. 2C).

### *Identification of trETR(scaffold 17) as $ET_A$ by phylogeny, synteny, and sequence similarity analyses*

Phylogenetic analysis (Fig. 2D) revealed that trETR(scaffold 17) is grouped in the $ET_A$ receptor subclass, and hence we named it $trET_A$. It was also found that trETR(scaffold 263) is grouped in the $ET_B$ subclass. trETR(scaffold 103), trETR(scaffold 163), and trETR(scaffold 376) seem to be non-mammalian paralogs as they are positioned in separate branches of the phylogenetic tree.

In the phylogenetic tree, trETR(scaffold 376) is apparently closer to the mammalian $ET_A$ subgroup than trETR(scaffold 17). However, a synteny analysis established the orthologous relationship between the mammalian $ET_A$ and trETR(scaffold 17) (Fig. 2E). A high degree of conservation of gene order between neighboring genes is seen between the human $ET_A$ gene on chromosome 4 and the trETR gene on scaffold 17. We therefore hereafter term trETR(scaffold 17) as $trET_A$.

Multiple sequence alignment revealed that the TM-spans and relevant interlinking regions of $ET_A$ are highly conserved among the fish and mammalian receptors except the 4th TM (Supplementary Fig. S2). The extracellular N-terminal and cytosolic C-terminal extreme regions are relatively variable among the species.

### *Functional characterization of $trET_A$*

To determine whether the cloned $trET_A$ is functionally active, we transiently expressed $trET_A$ in COS7 cells and examined its link to intracellular calcium signaling using fura-2 AM. The transfected cells exhibited a rapid and transient elevation of intracellular calcium concentration in response to 1 nM ET-1 (Fig. 3A). No such transient intracellular $Ca^{2+}$ mobilization was observed in the mock transfected cells (Fig. 3B). The $Ca^{2+}$ transition was not inhibited with BQ123 and BQ788 (Fig. 3C and D), which are specific antagonists for mammalian $ET_A$ and $ET_B$, respectively, suggesting that $trET_A$ is different from mammalian $ET_A$ in the structure of the antagonist-binding site.

### *Pillar cell and RBC localization of $trET_A$*

Immunohistochemistry with affinity-purified antibody showed that $trET_A$ receptor is located in the pillar cells of the fugu gill (Fig. 4A and B). Regions other than the pillar cells were not stained, indicating that the pillar cell is the major site of $ET_A$ expression in the gill. Unexpectedly, when we stained non-perfused gill sections with the anti-$trET_A$ antibody, a strong staining of RBCs was observed (Fig. 4D); thrombocytes, as identified by F-actin staining,

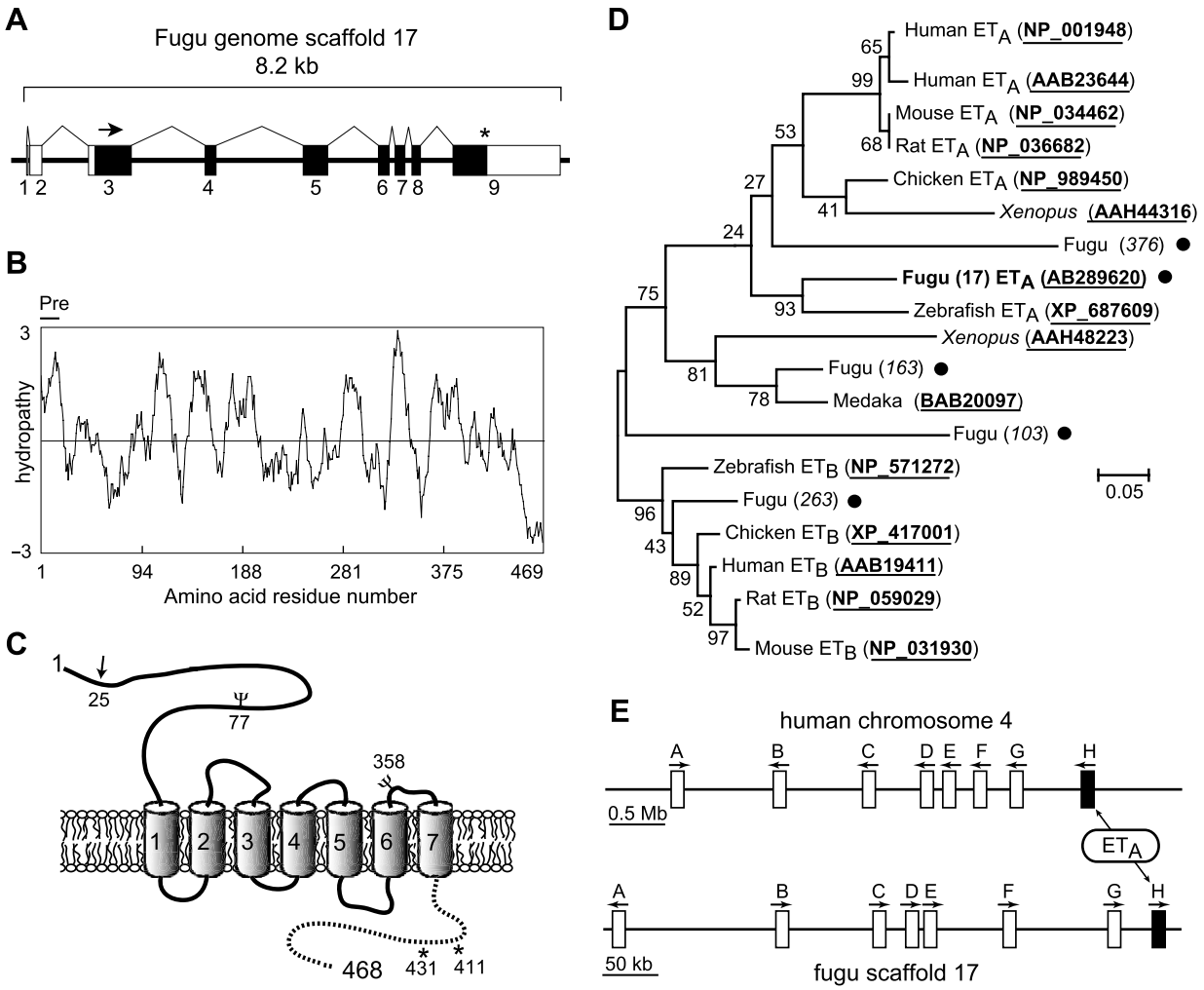


Fig. 2. Structural features of fugu $ET_A$ receptor. (A) Exon–intron organization of the fugu $ET_A$ receptor gene. Black and white boxes represent coding and non-coding regions, respectively. Black line represents the fugu genome sequence. Start and stop codons are indicated with an arrow and an asterisk, respectively. (B) Hydropathy profile of fugu $ET_A$ receptor. Pre, pre-sequence. (C) Predicted topology of fugu $ET_A$. The seven transmembrane spans are indicated with 1–7. ↓, putative signal peptide cleavage site; Ψ, putative N-linked glycosylation sites; ∗, potential PKC phosphorylation sites. C-terminal dotted region was used as antigen. (D) Phylogenetic relationship of ETR. Phylogenetic analyses using the amino acid sequences of ETRs of human, fugu, zebrafish, medaka, *Xenopus*, rat, mouse, and chicken were constructed using the neighbor-joining method, and the bootstrap values (2000 replicates) are shown on each branch. Accession numbers are given in brackets. Fugu sequences are indicated with filled circles (●). Numbers in parenthesis for the fugu sequences indicate the relevant fugu genome scaffolds. $ET_A$ and $ET_B$ indicate endothelin receptor A type and B type, respectively. Scale bar represents genetic distance of 0.05 amino acid substitutions per site. (E) Conserved synteny between human chromosome 4q31.22 and fugu scaffold 17. Open boxes and arrows represent apparent relative gene loci and their directions, respectively. Location of the $ET_AR$ gene is shown with closed box and are labeled. Other relevant genes are: (A) inositol polyphosphate-4-phosphatase (INPP4B); (B) SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 (SMARCA5); (C) hedgehog interacting protein (HHIP); (D) SMAD1-BRARE mothers against decapentaplegic homolog 1 (SMAD1); (E) methylmalonic aciduria (cobalamin deficiency) type A (MMAA); (F) LSM6 homolog, U6 small nuclear RNA associated protein (LSM6); (G) POU domain, class 4, transcription factor 2 (POU domain), and (H) endothelin receptor type A ($ET_A$), respectively.

were not stained (Fig. 4D, asterisks). RBC fractions, purified from fugu blood samples by centrifugation, also exhibited positive staining of the periphery of RBCs (Fig. 4E). This staining of RBCs is consistent with the result of Northern blot analysis, which demonstrated the presence of $trET_A$ mRNA in blood samples (Fig. 1A). To firmly establish the reliability of our immunohistochemical approach, we performed immunohistochemistry on fugu heart sections as a positive control; expression of $ET_A$ on cardiomyocytes has been well-demonstrated in mammals [13,14]. In the heart sections, $trET_A$ protein was found expressed in the myocardial cells (ML) but not in the bulbus arteriosus (BA) and bulboventricular valve (BV) (Fig. 4G and H). Preabsorbed antibody did not produce any stain or gave weak background staining, and worked as control for the specificity of the antiserum (Fig. 4C, F, and I).

## Discussion

Previously, the branchial pillar cell was thought to be a static structure just supporting the thin sac-like structure of

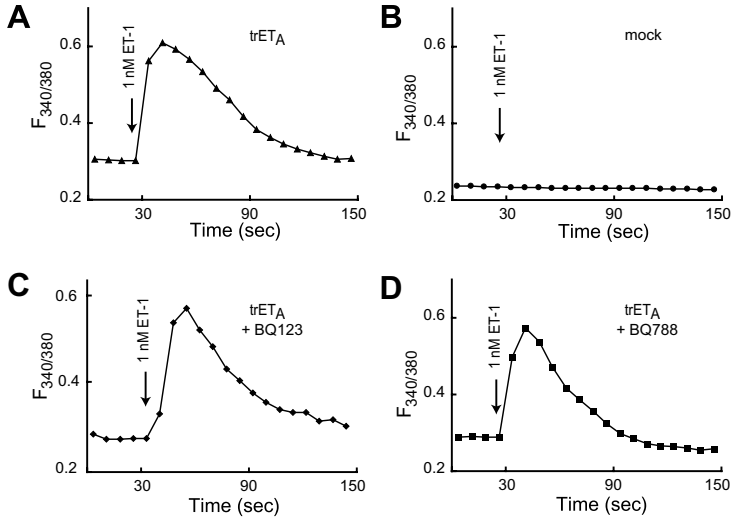


Fig. 3. ET-1-induced mobilization of intracellular $Ca^{2+}$ by $trET_A$ in COS7 cells. Transiently transfected COS7 cells were loaded with the calcium indicator fura-2 AM (3 μM) for 1 h at 37 °C in serum-free DMEM. Cells were washed twice with PBS containing 0.1% BSA. ET-1 was added at a 1 nM final concentration, and the intracellular $Ca^{2+}$ was measured. Experiments were performed with three wells (total $3 \times 6$ cells were measured) for each sample. The experiments were duplicated and a representative data set is shown. (A) pcDNA3-$trET_A$ transfected cells; (B) mock transfected cells; (C) pcDNA3-$trET_A$ transfected cells were incubated with 50 nM BQ123 for 1 h; (D) pcDNA3-$trET_A$ transfected cells were incubated with 50 nM BQ788 for 1 h. Arrows indicate the point of ET-1 addition.

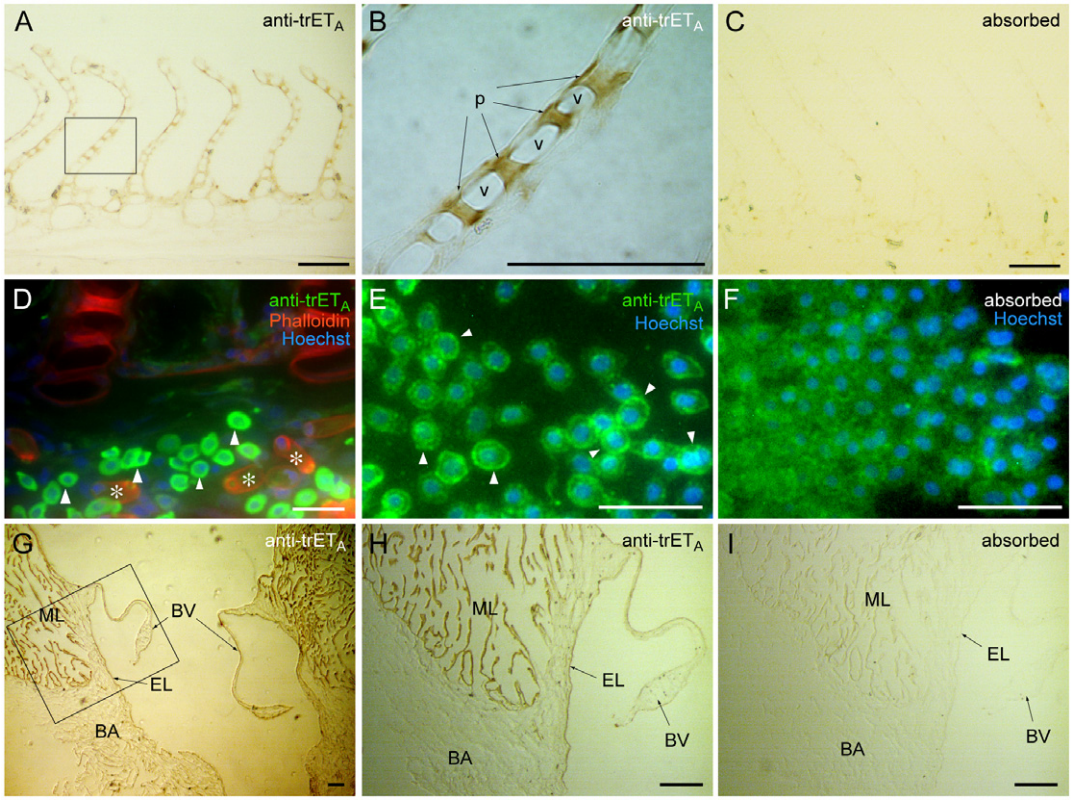


Fig. 4. Immunohistochemical localization of $ET_A$ receptor in gill, erythrocytes, and heart of *Takifugu*. (A–C) Sagittal sections of the gill stained with purified anti-fugu $ET_A$ receptor antibody (A and B). Boxed region of (A) is enlarged in (B). p, pillar cell; v, vascular space. (D) A sagittal section of non-perfused gill filament, containing blood cells, which was stained with anti-$trET_A$ (green) and phalloidin (red; a fluorescent dye for actin fibers). Arrowheads point to red blood cells, and asterisks point to thrombocytes. Thrombocytes were identified by their moderate levels of actin staining (red) and elongated shape. (E,F) Red blood cells were enriched by centrifugation and stained with anti-$trET_AR$ and absorbed control serum. Cell nuclei were visualized with the Hoechst 33342 (D–F; blue). (G–I) Sagittal sections of adult heart showing $ET_AR$ protein localization in the myocardial layer, endocardial layer, bulbus arteriosus, and bulboventricular valve leaflet. Selected area of (G) is enlarged in (H). ML, myocardial layer; EL, endocardial layer; BA, bulbus arteriosus; BV, bulboventricular valve leaflet. Absorbed antibody did not stain the tissues and served as the control (C, F, and I). Scale bars, 10 μm.

lamellae (Supplementary Fig. S1). Recently, however, its contractile nature has been demonstrated by epi-illuminating microscopy [5] and by the presence of contractile molecular machinery [4,15], which is expected to be sensitive to ET. In the present study, we provided definitive evidence for the presence of $ET_A$ endothelin receptor on fugu pillar cells, which provokes an increase in intracellular concentrations of $Ca^{2+}$ when activated by ET-1. The pillar cell localization is consistent with our previous autoradiographic visualization of $^{125}$I-ET-1-binding sites in the trout gill [16]. The presence of functional $ET_A$ receptor on the pillar cells suggests that ET-1 is involved in the regulation of blood flow through the vascular network of lamellae (i.e., redistribution of blood flow from the pillar cell region to the marginal channels of the lamellae) by changing the pillar cell height. This regulation is essential for achieving respiratory gas exchange at the minimum loss of homeostatic balance in body fluid.

We previously suggested the presence of high levels of $ET_A$-like endothelin receptor in the rainbow trout gill based on its ligand specificity, namely a high affinity for ET-1 but a low affinity for ET-3 [16]. At that time, we could not conclude that the receptor is $ET_A$ since it exhibited a low affinity for both $ET_A$ antagonist (BQ123) and $ET_B$ antagonist (BQ3020). The fugu $ET_A$ receptor characterized here also showed negligible affinities for both BQ123 and BQ788 even at 250 nM concentration (data not shown), suggesting that the antagonists which are specific for the mammalian receptor subtypes cannot be used as tools to investigate fish endothelin receptors. In support of this notion, considerable sequence variations in the N-terminal extracellular region, TM4, and cytosolic C-terminal tail between the teleost and mammalian $ET_A$ has been found.

A RBC must squeeze through narrow vascular spaces without rupturing its membrane. Deformability of RBC and its endurance to repeated shape changes have been well established [17,18]. It is therefore tempting to speculate that the endothelin $ET_A$ receptor, highly expressed on fugu RBC, is involved in the regulation of elasticity and flexibility of RBC plasma membrane and helps its smooth passage through the narrow thin space of gill lamellae in a coordinated fashion with pillar cell contraction. In this context, it would be noteworthy that fish RBC, unlike mammalian RBC, is nucleated and has the ability to synthesize the components of the endothelin signaling system including $ET_A$. Even in mammalian non-nucleated RBC, it has been demonstrated that the deformability of RBC is positively influenced by the addition of ET [19]. The unexpected finding of this study (i.e., relatively dense localization of $ET_A$ on fugu RBC) may open a new avenue for investigating the regulation of circulation through narrow blood capillaries.

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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.bbrc.2007.01.128.

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