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Histological demonstration of glucose transporters, fructose-1,6-bisphosphatase, and glycogen in gas gland cells of the swimbladder: Is a metabolic futile cycle operating?

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ABSTRACT

Luminal surface of the swimbladder is covered by gas gland epithelial cells and is responsible for inflating the swimbladder by generating O_2 from Root-effect hemoglobin that releases O_2 under acidic conditions. Acidification of blood is achieved by lactic acid secreted from gas gland cells, which are poor in mitochondria but rich in the glycolytic activity. The acidic conditions are locally maintained by a countercurrent capillary system called rete mirabile. To understand the regulation of anaerobic metabolism of glucose in the gas gland cells, we analyzed the glucose transporter expressed there and the fate of ATP generated by glycolysis. The latter is important because the ATP should be immediately consumed otherwise it strongly inhibits the glycolysis rendering the cells unable to produce lactic acid anymore. Expression analyses of glucose transporter (*glut*) genes in the swimbladder of fugu (*Takifugu rubripes*) by RT-PCR and *in situ* hybridization demonstrated that *glut1a* and *glut6* are expressed in gas gland cells. Immunohistochemical analyses of metabolic enzymes demonstrated that a gluconeogenesis enzyme fructose-1,6-bisphosphatase (Fbp1) and a glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Gapdh) are highly expressed in gas gland cells. The simultaneous catalyses of glycolysis and gluconeogenesis reactions suggest the presence of a futile cycle in gas gland cells to maintain the levels of ATP low and to generate heat that helps reduce the solubility of O_2.

1. Introduction

The gas gland of the swimbladder is composed of the gas gland cells and a tight bundle of arterial and venous capillaries called rete mirabile (Supplemental Fig. S1) [1–3]. The gas gland cells produce lactic acid from glucose by an anaerobic glycolysis and secrete the acidic metabolite into nearby capillaries where it lowers blood pH [4,5] and promotes dissociation of O_2 from Root-effect hemoglobins [6,7]. The O_2 released is then transferred from the bloodstream to the swimbladder lumen by passive diffusion across the capillary wall to inflate the bladder (Fig. 1I). The acidic condition and high partial pressure of O_2 in the capillary are locally maintained by back-diffusion and countercurrent concentration in the rete mirabile [4,8,9]. Reflecting its metabolic function and secretory nature, the gas gland cell has (i) a relatively large cytoplasm that contains glycogen-like deposits and very few mitochondria and (ii) numerous invaginations of basolateral membranes, called basal labyrinth, at the capillary interface [10–13].

While characterizing the metabolic properties of the swimbladder, we noticed a predominant expression of the fructose-1,6-bisphosphatase gene (*fbp*) in the swimbladder in the zebrafish database ZFIN [14]. This was surprising because the swimbladder is thought to be a tissue with a high glycolytic activity and lack fructose-1,6-bisphosphatase (Fbp), an enzyme involved in gluconeogenesis. This fact suggested two possibilities: (i) gluconeogenesis occurs in certain cells other than the gas gland cells or (ii) in the gas gland cell, Fbp forms an ATP-dependent metabolic futile cycle as shown below [15,16]. Gluconeogenesis in the gas gland cell seems unlikely considering that gluconeogenesis requires some

Abbreviations: DIG, digoxigenin; F6P, fructose 6-phosphate; Fbp, fructose-1,6-bisphosphatase; FBP, fructose-1,6-bisphosphate; Glut, glucose transporter protein; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; *glut*, glucose transporter gene; PBS, phosphate-buffered saline; PFA, paraformaldehyde; Pfk, phosphofructokinase; *sglt*, Na^+/glucose transporter gene.

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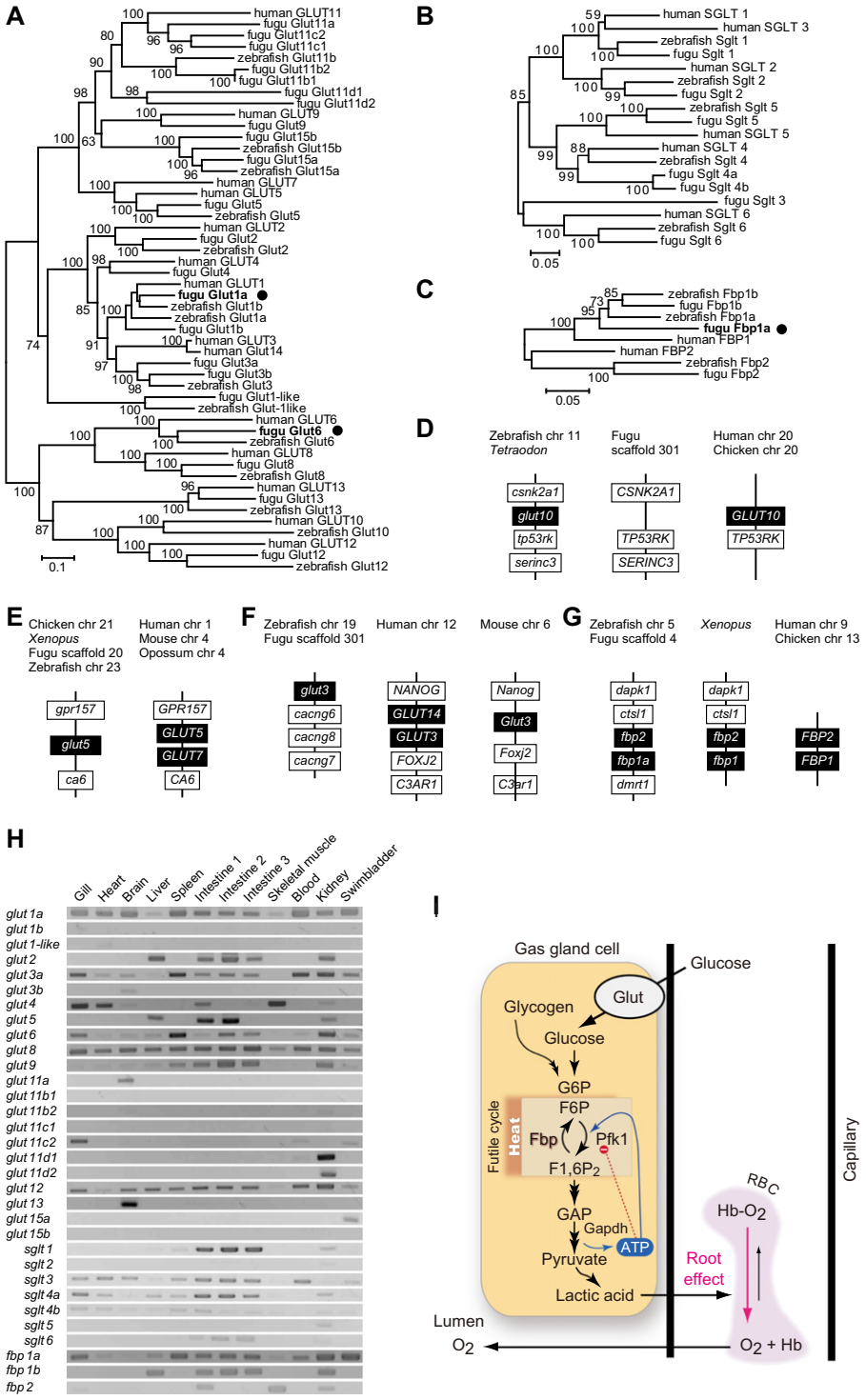

Fig. 1. Expression of Glut and Fbp in the fugu swimbladder and proposed model of metabolic futile cycle in gas gland cells. (A–C) Phylogenetic trees of fugu Glut, Sglt, and Fbp. Numbers indicate bootstrap values and the scale bars represent a genetic distance of amino-acid substitutions per site. Fugu Glut1a, Glut6, and Fbp1a are highlighted with black circles. For review of the Glut and Sglt families, see Uldry and Thorens [22]. (D–G) Synteny analysis of Gluts and Fbps. (H) Tissue-specific gene expression analysis for fugu *glut*, *sglt*, and *fbp* families by semiquantitative RT-PCR. Intestine 1, 2, and 3 represent the anterior, middle, and posterior parts of the intestine. (I) A metabolic futile cycle driven by phosphofructokinase 1 (Pfk1) and fructose-1,6-bisphosphatase (Fbp). The cycle consumes ATP that tends to inhibit the glycolytic pathway if allowed to accumulate as shown by a red no-entry sign; ATP is both a substrate and an allosteric inhibitor of Pfk1 whose activity is also modulated by F6P. Furthermore, heat generated by hydrolysis of ATP is very favorable to increase oxygen partial pressure by decreasing solubility of oxygen in water. Heat, as well as metabolites and gases, is recovered at rete mirabile. Acid-induced release of oxygen from hemoglobin (Root effect) is also shown. Glut, glucose transporter; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; $F1,6P_2$, fructose 1,6-bisphophate; GAP, glyceraldehyde 3-phosphate; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; RBC, red blood cell; Hb, hemoglobin; $Hb\text{-}O_2$, oxygenated hemoglobin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mitochondrial enzymes but the gas gland cell is very poor in mitochondria. If the latter is the case, the futile cycle is attractive for explaining how the gas gland cell avoids the inhibition of glycolysis by accumulated ATP and continue to generate lactic acid and how to generate heat that reduces the solubility of gas and helps inflate the swimbladder; the net reaction of the futile cycle

is consumption of ATP and generation of heat (see equations below and Fig. 1I).

[1] A reaction in glycolysis catalyzed by phosphofructokinase 1 (Pfk1):
ATP + fructose-6-phosphate
→ fructose-1,6-bisphosphate + ADP.
[2] A reaction in gluconeogenesis catalyzed by fructose-1,6-bisphosphatase (Fbp):
Fructose-1,6-bisphosphate + H_2O
→ fructose-6-phosphate + Pi.
[3] The overall reaction:
ATP + H_2O → ADP + Pi + Heat

We therefore decided to determine the cells expressing Fbp by immunohistochemistry. In this report, we describe the metabolism of glucose in the gas gland cell, including its glycogen-rich nature and glucose transporter (Glut), with special emphasis on the presence of a futile cycle.

2. Materials and methods

2.1. Animals

Torafugu (*Takifugu rubripes*) was purchased from a local dealer, and kept in artificial seawater (Rei-sea, Japan) at 20 °C before use. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology.

2.2. Isolation of torafugu tissues total RNAs and cDNA synthesis

Torafugu tissues were isolated after anesthesia with 0.1% ethyl *m*-aminobenzoate in seawater. RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan). Total RNA (5 μg) from each tissue was used for cDNA synthesis with Superscript III (Invitrogen, Carlsbad, CA, USA) to obtain 20 μl of cDNA solution. Synthesized cDNA was diluted to 160 μl with distilled water.

2.3. Database search and construction of phylogenetic trees

We obtained sequences for human and zebrafish genes with Ensembl genome browser (http://www.ensembl.org/index.html). Collected protein sequences were aligned with CLUSTALW phylogenetic analysis service (http://www.ddbj.nig.ac.jp/search/top-e.html) and phylogenetic trees were constructed with MEGA4 software (http://www.megasoftware.net/), based on Neighbor-Joining method with 1000 bootstrap replicates. Synteny analysis was performed with Ensembl genome browser.

2.4. Semiquantitative reverse transcription (RT)-PCR

Semiquantitative RT-PCR was performed with GoTaq Green Master Mix (Promega, Madison, WI, USA) by using the primers listed in Supplemental Table S1. Each reaction mix contained 6.25 μl of GoTaq Green Master Mix, 1.5 μl of each primer (10 μm), 0.5 μl of tissue cDNA solution and 3.25 μl of nuclease-free water. PCR condition was 27 cycles for denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 40 s). After PCR reaction, 5 μl from each reaction mix was electrophoresed on a 1.5% agarose gel in Tris–HCl/acetic acid/EDTA buffer. The gel was stained with 0.5 μg ml^{-1} ethidium bromide for 30 min, and fluorescent image was analyzed with Kodak Image Station 2000R (Eastman Kodak, Rochester, NY, USA).

2.5. Transmission electron microscopy

Swimbladders were fixed with 4% (w/v) paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.1 M phosphate buffer, and processed by standard procedures including tannic acid staining. Ultrathin sections were examined with an electron microscope (H-7500, Hitachi, Tokyo, Japan).

2.6. In situ hybridization

Swimbladders were perfused and fixed with 10% phosphate-buffered neutral formalin. Harvested swimbladders were embedded in paraffin and sectioned to 6 μm thickness. *In situ* hybridization histochemistry was performed essentially as described previously [17]. Briefly, a 339-bp fragment of *glut1a* cDNA (nucleotides 67–405) and a 605-bp fragment of *glut6* cDNA (nucleotides 209–813) were subcloned into pGEM-T vector (Promega) and used as templates for preparation of digoxigenin (DIG)-labeled cRNA probes (Roche Diagnostics, Mannheim, Germany). Sections were hybridized with DIG-labeled cRNA probes at 60 °C for 16 h, and washed several times at 60 °C. The bound label was detected with NBT/BCIP solution, and observed with a microscope and AxioCam HRc camera (Carl Zeiss, Germany).

2.7. Antibody production and specificity

Polyclonal antisera were made in rabbits by immunizing with a keyhole limpet hemocyanin (KLH)-conjugated synthetic peptides corresponding to parts of Glut6 (amino acid residues 52–65 and 218–231). Antibody specificity was established by staining of COS7 cells exogenously expressing fugu Glut6 proteins as previously described [18]. COS7 cells were transfected with a p3xFLAG-CMV10 expression vector containing the Glut6-coding sequences in its EcoRV site using Lipofectamine LTX (Invitrogen). The cells were fixed and stained with anti-Glut6 (1:1000 dilution) and anti-FLAG M2 (1:1000, Sigma–Aldrich, St. Louis, MO, USA) antibodies, and Alexa Fluor-labeled secondary antibodies (Invitrogen) as previously described [18]. Fluorescence images were obtained with a laser confocal microscope (TCS-SPE; Leica, Wetzlar, Germany) using LAS AF software (Leica).

2.8. Section immunohistochemistry

Swimbladders were fixed with ice-cold 2% PFA in PBS for 2 h, and sections (6 μm) were prepared with a cryostat as described previously [18]. The sections were permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated with 5% fetal bovine serum in PBS for 1 h. Primary-antibody reactions for the following antibodies were performed at room temperature for 16 h: (i) cultured medium of hybridoma containing anti-glycogen monoclonal antibody (IgM isotype, 1:1 dilution) [19], (ii) anti-FBP monoclonal antibody (IgG isotype, 1:200, Abnova, Taipei, Taiwan), (iii) anti-GAPDH monoclonal antibody (IgM isotype, 1:100, Sigma), and (iv) anti-Glut6 rabbit polyclonal antiserum (1:1000). After washing with PBS, sections were incubated in a mixture of Alexa-594-labeled concanavalin A (50 μg ml^{-1}), Hoechst 33342 (100 ng ml^{-1}) and either Alexa488-labeled secondary antibodies (Invitrogen). Sections were analyzed with a confocal microscope.

3. Results

3.1. Histological demonstration of glycogen in gas gland cells

Previous electron microscopic examinations demonstrated the presence of numerous glycogen-like granules in the gas gland cells

that are poor in subcellular organelles including mitochondria [10–13]. We therefore first wanted to confirm this glycogen-rich nature of the gas gland cells also in fugu. As in other fish species, electron microscopic observations demonstrated the presence of many glycogen granule-like structures in the fugu gas gland cells that are poor in mitochondria (Fig. 2D). We next prepared frozen sections of the swimbladder and stained them with anti-glycogen monoclonal antibody. Fig. 2A–C shows glycogen-rich cells reacted with the antibody. Gas gland cells were quite strongly stained indicating that gas gland cells are the major site of glycogen storage in fugu swimbladder.

3.2. Identification of glucose transporters expressed in swimbladder by RT-PCR

We first searched for fugu homologs of glucose transporter genes in the fugu genome database, and found 22 genes for facilitative glucose transporter (*glut* or *slc2*) and seven genes for Na^+/glucose cotransporters (*sglt* or *slc5*). Their phylogenetic relationship to human and zebrafish counterparts [20] is shown in Fig. 1A–B based on their predicted amino acid sequences. Fugu lacks the *glut7*, *glut10*, and *glut14* genes, and zebrafish lacks *glut4*, *glut7*, *glut9*, and *glut14*. *glut7* is similar to *glut5*, located next to *glut5* in mammalian chromosomes, but lacked in the chromosomes of fishes, *Xenopus tropicalis*, and birds (Fig. 1E). *glut10* is lacked in the genome database of fugu but present in those of *Tetraodon*, stickleback, and zebrafish (Fig. 1D). *glut14* is highly similar to *glut3*, located next to *glut3* in the chromosomes of human and some closely related species, but lacked in the genome databases of the other mammals, fishes, *X. tropicalis*, and birds (Fig. 1F). *glut4* and *glut9* are lacked in the genome database of zebrafish, but present in those of other fishes and tetrapods.

We next performed RT-PCR to determine the types of glucose transporters relatively highly expressed in the fugu swimbladder using mRNA preparations from a variety of tissues including the swimbladder. Primers used for PCR amplification were designed based on the fugu genome sequence information [21]. Among 22 *glut* genes and seven *sglt* genes, seven *glut* genes (*glut 1a*, *3a*, *6*, *8*, *11c2*, *12*, and *15a*) were found to be relatively highly expressed, but the *sglt* genes were only weakly expressed in the swimbladder (Fig. 1H). We therefore focused on the expression of the *glut* genes.

3.3. Identification of glucose transporters expressed in gas gland cells

To reduce the number of candidate clones expressed in gas gland cells, we next carried out *in situ* hybridization histochemistry. Paraffin sections of fugu swimbladder were hybridized with digoxigenin (DIG)-labeled antisense cRNA probes for mRNAs of *glut1a*, *3a*, *6*, *8*, *11c2*, *12*, and *15a*. Under high stringency conditions, *glut1a* and *glut6* probes gave positive signals in the gas gland (Fig. 2E and G). No significant hybridization was observed with the sense probes (Fig. 2F and H). Full-length cDNAs coding for fugu Glut1a and Glut6 were obtained by 5′- and 3′-RACE and the corresponding amino acid sequences were deduced (Supplementary Fig. S2).

Glut1 transports glucose across the plasma membrane of various mammalian cells. However, the role of Glut6 and its presence in the plasma membrane have not been established yet [22]. To demonstrate membrane localization of Glut6 in gas gland cells, we developed antiserum against Glut6. In gas gland cells, plasma membrane was stained by anti-Glut6 antiserum (Fig. 2I) but not by preimmune serum (Fig. 2O). The specificity of the antiserum was confirmed by immunocytochemical analysis of COS7 cells exogenously expressing FLAG-tagged Glut6 (Supplemental Fig. S3).

3.4. Expression of Fbp1a in gas gland cells

Database mining identified three genes for fugu Fbps. A phylogenetic analysis of predicted amino acid sequences demonstrated that the three fugu Fbps are orthologs of zebrafish Fbp1a, Fbp1b, and Fbp2 (Fig. 1C). Fish *Fbp1a* and tetrapod *Fbp1* are located next to fish and tetrapod *Fbp2*, respectively, in the chromosomes (Fig. 1G).

Tissue distribution analyses of fugu *fbp* mRNAs by RT-PCR showed that *fbp1a* is highly expressed in the swimbladder (Fig. 1H). These results indicated that the high expression of *fbp* in the swimbladder is conserved between zebrafish and fugu but the Fbp subtype in the swimbladder is different among species (Fbp1a in adult fugu and Fbp2 in zebrafish larva).

To identify cell types that express Fbp in the swimbladder at high levels, frozen sections of fugu swimbladder were stained with antibody to Fbp. Strong signal for Fbp was observed only in gas gland cells (Fig. 2J, M and L). This staining pattern is quite similar to that of Gapdh (Fig. 2K, N and L), suggesting coexistence of Fbp and Gapdh in gas gland cells. Although both antibodies were obtained from commercial sources and raised against human antigens, they are expected to crossreact with the fugu counterparts reflecting high sequence similarities (Supplemental Fig. S4).

4. Discussion

In the present study, in an attempt to characterize the functions of the gas gland of swimbladder at the molecular level, we determined the subtype and location of the glucose transporters that have been supposed to be highly expressed in the glucose-demanding gas gland cells. *In situ* hybridization indicated the expression of *glut1a* and *glut6* in the gas gland cells (Fig. 2E–H). Recently, Tseng et al. [23] have determined the Michaelis–Menten constants (K_m) of zebrafish Glut1a, Glut6, and Glut13.1 to be 3.4 ± 0.2, 17.5 ± 2.6, and 1.6 ± 0.4 mM, respectively. The K_m of zebrafish Glut1a and Glut6 for glucose are quite similar to those of human GLUT1 (K_m = 3 mM) and GLUT2 (K_m = 17 mM), respectively. Because the amino-acid sequences of Glut1a and Glut6 are well conserved among zebrafish and fugu, it is highly expected that the fugu orthologs exhibit similar functions. If combined with the fact that the gas gland cells can store a substantial amount of glycogen (Fig. 2A, C and D), the presence of multiple types of glucose transporters in the same gas gland cells can be interpreted as follows: when blood glucose level is low, high-affinity Glut1a mediates glucose absorption; and when blood glucose level is high, low-affinity Glut6 takes up glucose for storage as glycogen.

Fructose-1,6-bisphosphatase (Fbp) is an enzyme that converts fructose-1,6-bisphosphate (FBP) to fructose 6-phosphate (F6P) in gluconeogenesis, which is the reverse reaction of the glycolytic step catalyzed by phosphofructokinase (Pfk; see Section 1 and Fig. 1I). High expression of Fbp in gas gland cells was unexpected since the gas gland has been thought to be a tissue with an exceptionally high glycolytic activity and there has been no report describing Fbp in the swimbladder. In the present study, however, immunostaining demonstrated that gas gland cells express high levels of a glycolytic enzyme (Gapdh) and a gluconeogenic enzyme (Fbp) (Fig. 2J–N). Simply, it may seem natural to assume that Fbp is involved in gluconeogenesis. However, it is inconceivable that gas gland cells synthesize glucose by gluconeogenesis because of the following reasons: in gluconeogenesis, pyruvate is converted to oxaloacetate in mitochondria, but the gas gland cells contain only few mitochondria [24]. Furthermore, NADH, required for gluconeogenesis, is also mainly supplied by mitochondria [24]. As a role of Fbp in mitochondrion-poor cells, we propose that Fbp is involved in a futile cycle for generating heat in expense of ATP as described below.

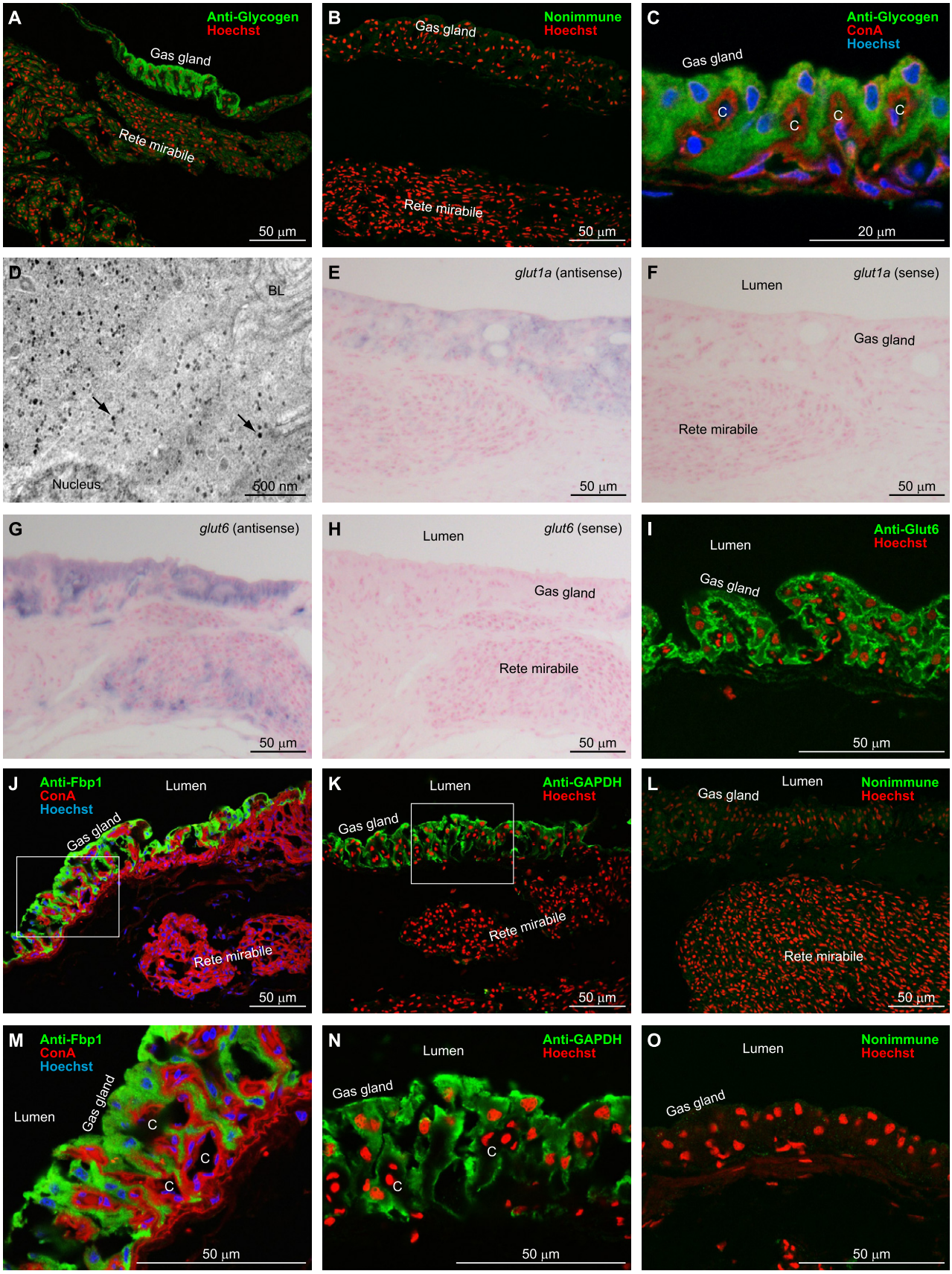

Fig. 2. Histological demonstration of glycogen, Gluts, Fpb1, and Gapdh in gas gland cells. (A–C) Frozen sections of fugu swimbladder stained with anti-glycogen monoclonal antibody or control serum. In C, typical capillaries are indicated by c. (D) Transmission electron microscopy of a gas gland cell showing glycogen-like granules in the cytosol. A part of nucleus and a part of basal labyrinth (BL) are also shown. Arrows indicate typical glycogen-like granules. Basal labyrinth is extensive infoldings of the basal plasma membrane. (E–H) *In situ* hybridization analysis for cellular distribution of *glut1a* and *glut6* mRNAs in the fugu swimbaldder. (I–O) Frozen sections of fugu swimbladder stained with anti-Glut6, anti-Fbp1, anti-Gapdh, or control serum. In M and N, typical capillaries are marked by c.

It is conceivable that Pfk and Fbp constitute a futile cycle by catalyzing the forward and reverse reactions of the phosphorylation of F6P (see Section 1), whose net effect is the hydrolysis of ATP and the generation of heat. This outcome is critically important for the gas gland cells to synthesize lactic acid because the process is strongly inhibited if ATP is accumulated. Two glycolytic enzymes, Pfk and pyruvate kinase, are inhibited by higher physiological concentration of ATP via the allosteric binding sites, and the inhibition by ATP is promoted by low pH [25–28]. Another merit of the futile cycle is the generation of heat that helps to reduce the solubility of oxygen and hence to increase its partial pressure (P_{O_2}), which in turn helps to maintain a P_{O_2} gradient down which O_2 diffuses into the lumen of swimbladder. Since the solubility of O_2 in blood plasma becomes less not only at increasing temperature but also at increased salinity (salting-out effect), the lactic acid secreted from the gas gland cells helps to increase P_{O_2} by two ways: (i) releasing O_2 from Root-effect hemoglobins and (ii) reducing solubility of O_2 by its salting-out effect.

Metabolic futile cycles have been demonstrated in muscles and adipose tissues of various animals. For example, the futile cycle involving Fbp and Pfk is used by bumble bees to produce heat in flight muscles and warm up their bodies considerably at low ambient temperatures [16]. Another example is a futile cycle of triglyceride breakdown and resynthesis from glycerol and free fatty acids, which has been demonstrated to be inducible in adipocytes by treating with thiazolidinediones, a class of drugs for diabetes mellitus type 2 [29]. The metabolic Fbp/Pfk futile cycle reported here may deserve a comment concerning its link with rete mirabile, a countercurrent exchange system of capillary vessels. In teleost and elasmobranch fishes, heat conservation by rete mirabile is known in the brain, eye, and muscle of lamnid sharks, tuna, and swordfish [30–32]. In the fugu swimbladder, the heat that is generated and being transported out of the site of generation may be constantly transferred back through the rete mirabile so as to maintain the temperature of the gas gland higher than other areas of the body.

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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.2011.12.006.

References

[1] R. Fange, Physiology of the swimbladder, Physiol. Rev. 46 (1966) 299–322.
[2] B. Pelster, PH regulation and swimbladder function in fish, Respir. Physiol. Neurobiol. 144 (2004) 179–190.
[3] M. Berenbrink, Historical reconstructions of evolving physiological complexity: O_2 secretion in the eye and swimbladder of fishes, J. Exp. Biol. 210 (2007) 1641–1652.
[4] J.B. Steen, The physiology of the swimbladder in the eel *Anguilla vulgaris*. III. The mechanism of gas secretion, Acta Physiol. Scand. 59 (1963) 221–241.
[5] B. Pelster, Blood acidification in the swimbladder: mechanisms and metabolic pathways, Braz. J. Med. Biol. Res. 28 (1995) 1133–1138.
[6] R.W. Root, The respiratory function of the blood of marine fishes, Biol. Bull. 61 (1931) 427–456.
[7] M. Berenbrink, P. Koldkjaer, O. Kepp, A.R. Cossins, Evolution of oxygen secretion in fishes and the emergence of a complex physiological system, Science 307 (2005) 1752–1757.
[8] H. Kobayashi, B. Pelster, P. Scheid, Water and lactate movement in the swimbladder of the eel, *Anguilla anguilla*, Respir. Physiol. 78 (1989) 45–57.
[9] H. Kobayashi, B. Pelster, P. Scheid, CO_2 back-diffusion in the rete aids O_2 secretion in the swimbladder of the eel, Respir. Physiol. 79 (1990) 231–242.
[10] A. Jasinski, W. Kilarski, On the fine structure of the gas gland in some fishes, Z Zellforsch. Mikrosk. Anat. 102 (1969) 333–356.
[11] D.E. Copeland, Fine structural study of gas secretion in the physoclistous swim bladder of *Fundulus heteroclitus* and *Gadus callarias* and in the euphysoclistous swim bladder of *Opsanus tau*, Z Zellforsch. Mikrosk. Anat. 93 (1969) 305–331.
[12] A. Jasinski, W. Kilarski, Gas secreting cells in the rear chamber of the swimbladder of the pond-loach, *Misgurnus fossilis* L. An electron microscopic study, Z Zellforsch. Mikrosk. Anat. 134 (1972) 273–282.
[13] C. Prem, W. Salvenmoser, J. Wurtz, B. Pelster, Swim bladder gas gland cells produce surfactant: in vivo and in culture, Am. J. Physiol. Regul. Integr. Comp. Physiol. 279 (2000) R2336–R2343.
[14] J. Sprague, L. Bayraktaroglu, Y. Bradford, T. Conlin, N. Dunn, D. Fashena, K. Frazer, M. Haendel, D.G. Howe, J. Knight, P. Mani, S.A. Moxon, C. Pich, S. Ramachandran, K. Schaper, E. Segerdell, X. Shao, A. Singer, P. Song, B. Sprunger, C.E. Van Slyke, M. Westerfield, The Zebrafish Information Network: the zebrafish model organism database provides expanded support for genotypes and phenotypes, Nucleic Acids Res. 36 (2008) D768–D772.
[15] R.A. Challiss, J.R. Arch, E.A. Newsholme, The rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate in skeletal muscle, Biochem. J. 221 (1984) 153–161.
[16] E.A. Newsholme, B. Crabtree, S.J. Higgins, S.D. Thornton, C. Start, The activities of fructose diphosphatase in flight muscles from the bumble-bee and the role of this enzyme in heat generation, Biochem. J. 128 (1972) 89–97.
[17] K. Saito, N. Nakamura, Y. Ito, K. Hoshijima, M. Esaki, B. Zhao, S. Hirose, Identification of zebrafish Fxyd11a protein that is highly expressed in ion-transporting epithelium of the gill and skin and its possible role in ion homeostasis, Front. Physiol. 1:129 (2010) 1–13.
[18] A. Kato, T. Muro, Y. Kimura, S. Li, Z. Islam, M. Ogoshi, H. Doi, S. Hirose, Differential expression of Na^+–Cl^- cotransporter and Na^+–K^+–Cl^- cotransporter 2 in the distal nephrons of euryhaline and seawater pufferfishes, Am. J. Physiol. Regul. Integr. Comp. Physiol. 300 (2011) R284–R297.
[19] O. Baba, Production of monoclonal antibody that recognizes glycogen and its application for immunohistochemistry, Kokubyo Gakkai Zasshi (J. Stomatological Soc., Jpn.) 60 (1993) 264–287.
[20] Y.C. Tseng, R.D. Chen, J.R. Lee, S.T. Liu, S.J. Lee, P.P. Hwang, Specific expression and regulation of glucose transporters in zebrafish ionocytes, Am. J. Physiol. Regul. Integr. Comp. Physiol. 297 (2009) R275–R290.
[21] S. Aparicio, J. Chapman, E. Stupka, N. Putnam, J.M. Chia, P. Dehal, A. Christoffels, S. Rash, S. Hoon, A. Smit, M.D. Gelpke, J. Roach, T. Oh, I.Y. Ho, M. Wong, C. Detter, F. Verhoef, P. Predki, A. Tay, S. Lucas, P. Richardson, S.F. Smith, M.S. Clark, Y.J. Edwards, N. Doggett, A. Zharkikh, S.V. Tavtigian, D. Pruss, M. Barnstead, C. Evans, H. Baden, J. Powell, G. Glusman, L. Rowen, L. Hood, Y.H. Tan, G. Elgar, T. Hawkins, B. Venkatesh, D. Rokhsar, S. Brenner, Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*, Science 297 (2002) 1301–1310.
[22] M. Uldry, B. Thorens, The SLC2 family of facilitated hexose and polyol transporters, Pflugers Arch. 447 (2004) 480–489.
[23] Y.C. Tseng, J.R. Lee, S.J. Lee, P.P. Hwang, Functional analysis of the glucose transporters-1a, -6, and -13.1 expressed by zebrafish epithelial cells, Am. J. Physiol. Regul. Integr. Comp. Physiol. 300 (2011) R321–R329.
[24] H.G. Hers, L. Hue, Gluconeogenesis and related aspects of glycolysis, Annu. Rev. Biochem. 52 (1983) 617–653.
[25] G.E. Staal, G. Jansen, D. Roos, Pyruvate kinase, the "high ATP syndrome", J. Clin. Invest. 74 (1984) 231–235.
[26] G.P. Dobson, E. Yamamoto, P.W. Hochachka, Phosphofructokinase control in muscle: nature and reversal of pH-dependent ATP inhibition, Am. J. Physiol. 250 (1986) R71–R76.
[27] S. Mazurek, H. Grimm, C.B. Boschek, P. Vaupel, E. Eigenbrodt, Pyruvate kinase type M2: a crossroad in the tumor metabolome, Br. J. Nutr. 1 (Suppl. 87) (2002) S23–S29.
[28] G. Wegener, U. Krause, Different modes of activating phosphofructokinase, a key regulatory enzyme of glycolysis, in working vertebrate muscle, Biochem. Soc. Trans. 30 (2002) 264–270.
[29] H.P. Guan, Y. Li, M.V. Jensen, C.B. Newgard, C.M. Steppan, M.A. Lazar, A futile metabolic cycle activated in adipocytes by antidiabetic agents, Nat. Med. 8 (2002) 1122–1128.
[30] B.A. Block, F.G. Carey, Warm brain and eye temperatures in sharks, J. Comp. Physiol. B. 156 (1985) 229–236.
[31] F.G. Carey, J.M. Teal, J.W. Kanwishe, K.D. Lawson, J.S. Beckett, Warm-bodied Fish, Amer. Zool. 11 (1971) 137–145.
[32] K.A. Fritsches, R.W. Brill, E.J. Warrant, Warm eyes provide superior vision in swordfishes, Curr. Biol. 15 (2005) 55–58.