Hypoxia-inducible myoglobin expression in nonmuscle tissues

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Myoglobin (Myg) is an oxygen-binding hemoprotein that is widely thought to be expressed exclusively in oxidative skeletal and cardiac myocytes, where it plays a key role in coping with chronic hypoxia. We now show in a hypoxia-tolerant fish model, that Myg is also expressed in a range of other tissues, including liver, gill, and brain. Moreover, expression of Myg transcript was substantially enhanced during chronic hypoxia, the fold-change induction being far greater in liver than muscle. By using 2D gel electrophoresis, we have confirmed that liver expresses a protein corresponding to the Myg-1 transcript and that it is significantly up-regulated during hypoxia. We have also discovered a second, unique Myg isoform, distinct from neuroglobin, which is expressed exclusively in the neural tissue but whose transcript expression was unaffected by environmental hypoxia. Both observations of nonmuscle expression and a brain-specific isoform are unprecedented, indicating that Myg may play a much wider role than previously understood and that Myg might function in the protection of tissues from deep hypoxia and ischemia as well as in reoxygenation and reperfusion injury.

globin expression | hypoxia adaptation | microarray | proteomics

Myoglobin (Myg) has been intensively studied for many years in relation to protein structure (1). Myg is widely thought to be exclusively expressed in oxidative myocytes of skeletal and cardiac muscle, where it plays a role in oxygen storage (2). Undoubtedly the richest source of Myg protein is in the muscles of mammals such as whales and seals that undertake extended breath-hold dives (3). However, Myg is also expressed in human oxidative muscle, and it has long been known that Myg expression is up-regulated in response to increased demand for oxygen or during hypobaric hypoxia (4).

The wider functional role of Myg is still under discussion, because the transgenic knockout in mouse does not give rise to the expected hypoxia-sensitive phenotype in adults because of the activation of alternative compensatory mechanisms (5). In addition to its well known role in oxygen storage and diffusion, Myg has also been implicated in protecting cell respiration from NO-inhibition (6) and in scavenging reactive O_2 species (ROS) (7). Given that ROS damage and NO synthesis occur more widely than in muscle and that some tissues are as metabolically active as muscle, some authorities have questioned why the tissue distribution of Myg is not wider (7). This question was addressed by the more recent discovery of other members of the hemoprotein superfamily that may undertake these various roles, namely neuroglobin, cytoglobin (8), and globinX (9), consistent with the evolution of ancient paralogues, which serve protective roles in tissues other than muscle. To date, however, members of the Myg subfamily have been restricted to muscle only.

The common carp, *Cyprinus carpio*, routinely experiences extreme environmental hypoxia in isolated ponds, to which it displays a series of cardiorespiratory and metabolic adaptations (10). Together with other members of the Cyprinidae, *C. carpio* has become a model for investigating fundamental mechanisms of hypoxia tolerance. We have undertaken a large-scale transcriptomic screen of genes responding to hypoxia exposure in a broad range of carp tissues by using cDNA microarrays. This study has unexpectedly revealed the expression of Myg-1 in several nonmuscle tissues that we have now characterized. Second, we show that Myg expression is up-regulated in some of these tissues after hypoxia treatment. Finally, in screening cDNA libraries from carp tissues, we have identified a previously uncharacterized Myg isoform called Myg-2, whose expression is restricted to the neural tissue.

Results

We have explored responses to chronic hypoxia in the common carp, *C. carpio*, by using postgenomic screening techniques (11, 12). EST characterization of a large collection of cDNAs from multiple carp tissues (see carpBASE, http://legr.liv.ac.uk) has identified cDNAs for a number of hemoproteins, including four hemoglobin isoforms, one cytoglobin isoform, and a Myg isoform that corresponded precisely to a known carp gene, here termed *Myg-1*. The sequences of all of these genes aligned closely with the corresponding known isoforms from fish homologues (Fig. 1*a*).

We have also discovered a second, Myg isoform, Myg-2 (GenBank accession no. DQ338464). Despite a search for similar sequences in dbEST, GenBank, Swissprot, and the current genomic sequences for zebrafish, fugu, and Tetraodon, we failed to find a single hit. Myg-2 shares 78% sequence identity with Myg-1 and diverges particularly in two domains between residue numbers 54–59 and 119–132 (Fig. 1*b*). The sequence differences were mapped onto the structure of tuna Myg (Fig. 2), showing that none of the variable positions between the two carp isoforms contacted the bound heme.

By using cDNA microarrays containing 13,440 probes (13), including those for both Myg genes, we then explored transcript expression in carp exposed to hypoxia (0.8 mg O_2/liter) over an 8-day period at either 17°C or 30°C. These two temperatures constitute differing levels of hypoxic stress because of the differing metabolic demands at the two temperatures. For liver, this analysis revealed a large number of responding genes, the most prominent of which was *Myg* (see Table 1, which is published as supporting information on the PNAS web site). We have also detected *Myg* transcript expression in brain, skeletal muscle, and gill. In some tissues, *Myg* expression was hypoxia-

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Abbreviation: Myg, myoglobin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ338464). The microarray data files are available in the ArrayExpress database (accession no. E-MAXD-10).

See Commentary on page 2469.

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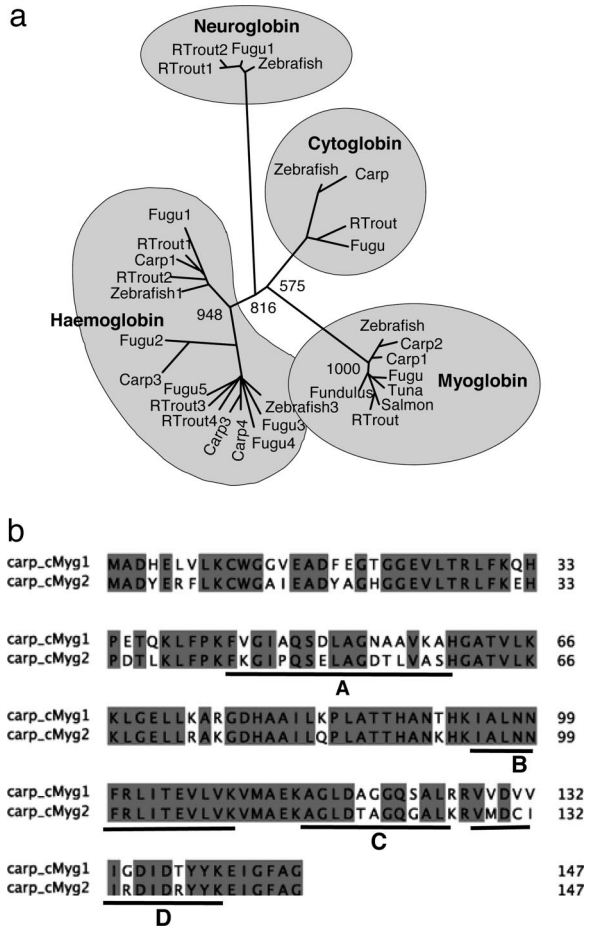

Fig. 1. Phylogenetic and sequence analysis of the two carp myoglobin isoforms. (*a*) Similarity analysis of the globin superfamily in fish. The source of gene sequences included in this analysis and methods used are listed in Table 2, which is published as supporting information on the PNAS web site. RTrout indicates rainbow trout. Bootstrapping confidence values (1,000 replicates) are shown for the major branches. In particular, the placing of both carp sequences reported here in the Myg group is supported at the highest bootstrapping level. (*b*) The amino acid alignment between carp MYG-1 and MYG-2 proteins with substituted residues indicated by a white background and the peptide fragments identified in MYG-1 by using Q-TOF-MS by the solid lines and letters A–D.

inducible; thus, in muscle, hypoxia was associated with a 4-fold increase over 8 days of exposure but only at the higher, more stressful temperature (Fig. 3*a*). For liver, we found a much stronger increase in expression at both temperatures (Fig. 3*b*). For gill, transcript amounts increased at both temperatures to a 5-fold increase on day 5 (Fig. 3*c*). By contrast, brain was unaffected at both temperatures (Fig. 3*d*), and cytoglobin showed no changes in transcript expression in all tissues (data not shown).

Because of the possibility of cross-hybridization between the two types of cDNA probe, we tested the tissue-specificity of *Myg* isoform expression by designing PCR primers capable of distinguishing between the two isoforms. We found that PCR products for *Myg-1* were generated against cDNA from heart, kidney, liver, and gill and, to a much lesser extent, in brain (Fig. 4). By contrast, *Myg-2* products were evident only in brain, which corresponds with the available ESTs for *Myg-2* being isolated from brain tissue. Therefore, we interpret the transcript expression profiles as corresponding to *Myg-1* for muscle, liver, and gill and both *Myg-1* and *Myg-2* for the brain.

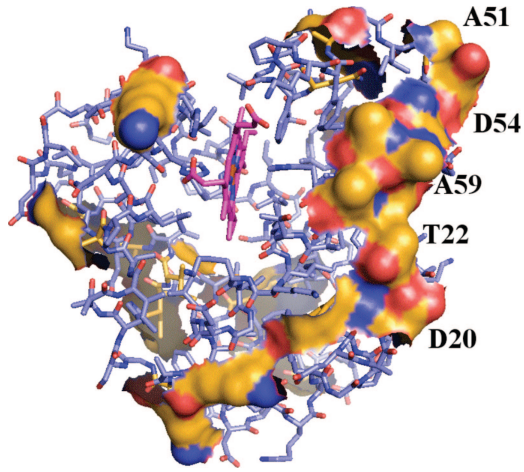

Fig. 2. Diagram (made by using the program PYMOL), illustrating the surface formed by the residues substituted between carp MYG-1 and MYG-2 proteins superimposed on the crystal structure for tuna Myg (PDB code 1myt) (29). Selected tuna myoglobin residues are labeled as in the crystal structure. Tuna Myg shares 73% sequence identity with cMyg-1 and 67% with cMyg-2. The bound heme is shown with pink sticks in the center of the protein molecule.

Given that transcripts may not be translated, we sought evidence for Myg protein expression (MYG) in nonmuscle tissues by using proteomic techniques. Initially, we separated protein extracts of liver from hypoxia-treated carp on 2D PAGE and, with predictions of protein size and pI in conjunction with mass spectrometry, located the spot containing the MYG protein corresponding to the *Myg-1* isoform (Fig. 5*a*). This spot on digestion and sequence analysis by MS/MS generated four long peptide sequences that aligned perfectly against the predicted MYG-1 isoform (see Fig. 1*b*). We then undertook relative quantification of this spot by densitometry of silver-stained gels of liver extracts from five carp specimens for both control and hypoxia-treated groups. The details in Fig. 5*a* indicate the consensus differences in gel pattern, and Fig. 5*b* shows a 2.8-fold increase in relative amounts of protein in the livers of 5-day, hypoxia-treated carp (Student's *t* test, $P < 0.05$). We also used an alternative quantification technique, namely iTRAQ (14), which directly compares the abundance of peptide fragments between control and 5-day-treated carp liver by using amine-specific peptide-tagging reagents. Again, this technique showed a 2.3-fold difference (Fig. 5*c*, $P < 0.05$).

Discussion

The liver plays a key role in the hypoxia-adaptation of the common carp, primarily through the up-regulation of anaerobic metabolism (15, 16). We now provide another potentially important hypoxia-adaptive response in this tissue, namely the substantial up-regulation of MYG-1 protein expression in liver and other nonmuscle tissues through a transcriptional mechanism. Interestingly, gill showed induced responses at both 17°C and 30°C, whereas brain and muscle showed induction only at the higher temperature. This result might indicate increased sensitivity of gill tissue to hypoxic disturbance, despite the fact that the gill would be exposed to the highest environmental PO_2s.

The expression of a liver Myg is unlikely to be unique to carp or even to members of the Cyprinidae family, because an EST clone that aligns to Myg has been isolated from zebrafish kidney and another from the liver of the minnow *Fundulus* (http://genomics.rsmas.miami.edu/funnybase). Also, *Myg* transcript expression has recently been detected in zebrafish gills as part of a wider oligoarray screen (17), although the implications were not explored. Even in the human, nonzero levels of *Myg* tran-

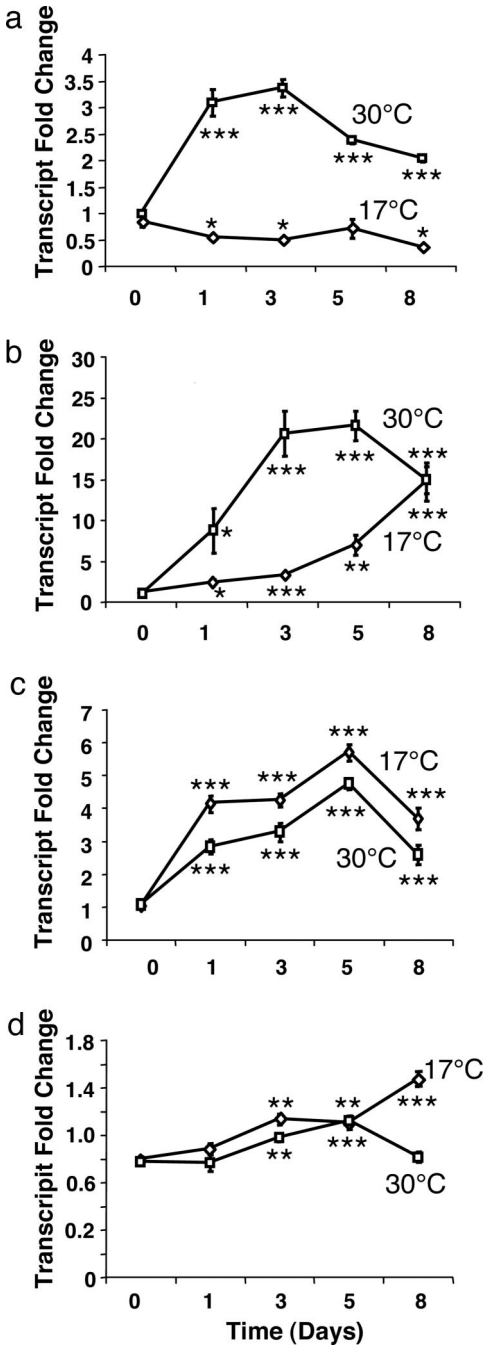

Fig. 3. Changes in transcript expression for Myg-1 in muscle (*a*), liver (*b*), gill (*c*), and brain (*d*) during hypoxia exposure over an 8-day period at either 17°C or 30°C. Values and bars represent the mean (±SEM), calculated relative to the mean of the normoxia values measured on day 0 ($n = 5$). Statistical differences of each time point relative to time 0 were calculated by using Student's *t* test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.005$. Please note the different ordinate scales for each of the four graphs.

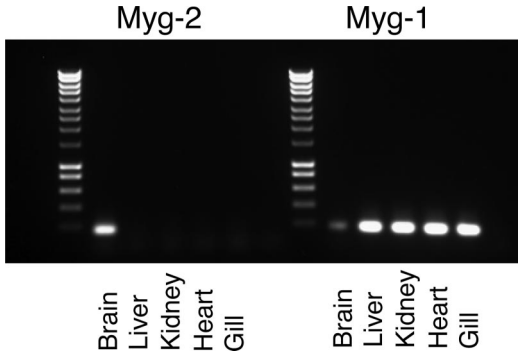

Fig. 4. PCR products amplified from Myg-1 and Myg-2 transcripts in tissues of hypoxia-treated carp by using isoform-specific primers (see *Methods*). Samples were analyzed by Tris-acetate-EDTA agarose gel electrophoresis to confirm the presence of the expected 183-bp product.

script have been detected on Affymetrix arrays in prostate, liver, and pancreatic tissue (Genecard, http://genecards.bcgsc.bc.ca). Furthermore, *Myg* SAGE tags have been discovered in prostate and *Myg* ESTs in thymus, liver, prostate, and lung.

An important question is whether the nonmuscle expression of this gene is biologically meaningful; does such expression truly represent expression within different cell types within the liver, gill, or brain, or might expression occur in smooth muscle or endothelial cells within the tissue microvasculature (18), or does the expression merely represent slippage of transcriptional control within tissue cells? In the liver of hypoxic carp, Myg constitutes up to 1.5% of the protein extract applied to the 2D gels and revealed by silver staining (see Fig. 5), which we calculate to be equivalent to 1.2 mg of Myg per g of wet tissue weight. This value exceeds that of cardiac muscle of Antarctic fish species (0.4–0.7 mg/g) (19) but is somewhat lower than that in the heart of the temperate *Hemitripterus americanus* (2.3 mg/g) (20). Thus, although the very low levels of Myg transcript in nonmuscle human tissues noted above may result from slippage, carp liver expresses protein amounts that compare with the "classical" tissue location for Myg, namely oxidative muscle fibers. This finding, together with the clearly regulated and tissue-specific pattern of Myg expression during hypoxia, supports the argument for a physiologically meaningful role.

Vascular smooth muscle cells do appear to express Myg (18). although the level of expression within smooth muscle cells and its precise cellular location remains uncharacterized. However, although we cannot exclude a smooth muscle component in nonmuscle tissues, it seems unlikely to account for more than a small proportion of what is an appreciable level of MYG protein expression, at least in liver. Moreover, we have observed major differences in transcript responses among the four tissue samples with respect to both fold-change and temperature. This tissue-specificity argues against a common mechanism, such as induction in arteriolar smooth muscle, although we cannot exclude the tissues being different simply because of their different positions in the circulatory loop and their different metabolic activities. Clarifying this point must await more precise definition of the cellular site(s) of protein expression in MYG-positive tissues when a suitable anti-carp MYG antibody becomes available. Also, understanding the physiological role of MYG expression, particularly in the brain and retina, must await analysis of the effects of experimentally induced or ablated MYG expression on tolerance of hypoxia, reoxygenation, or reperfusion injury. Interestingly, Nitta *et al.* (21) have recently shown that the virally induced expression of Myg in rat liver significantly reduced the extent of ischemia-reperfusion injury. This important result demonstrates that, under some circumstances, Myg can act in roles other than as an oxygen store and may be a crucial agent for protection against the damaging effects of both oxygen deprivation and its subsequent restoration.

To our knowledge, the discovery of the carp *Myg-2* gene is the first time that two Myg isoforms have been identified within a single genome. Both genes are clearly members of the Myg subfamily, and we show that the amino acid substitutions in Myg-2 relative to Myg-1 are not located in or close to the

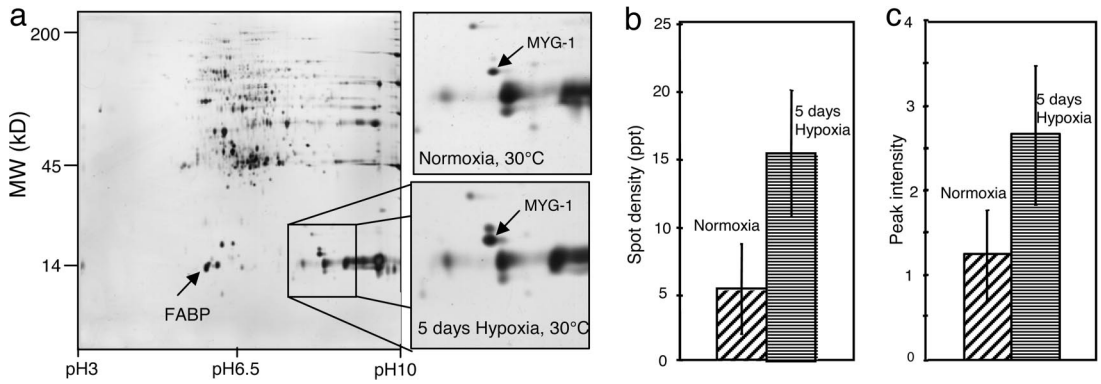

Fig. 5. Proteomic identification of MYG-1 in carp liver. (*a*) The whole 2D gel and the two details to the right provide the consensus ($n = 5$) for the region of the Myg spot for both normoxic and hypoxia-treated carp. (*b* and *c*) The quantitative difference in expression of MYG-1 protein in extracts from normoxic and 5-day-hypoxia-treated carp by using silver-stained densitometry of 2D gels (*b*) ($n = 4$) and by using the iTRAQ technique (*c*) ($n = 3$), respectively. For *b*, values (mean ± SEM) represent fractional density of the MYG spot relative to the densitometric value for all spots detected on the entire gel, expressed as parts per thousand (ppt), whereas, in *c*, values (mean ± SEM) represent intensities of mass spectrometric peaks derived from fragments obtained from normoxia- and hypoxia-treated specimens. For both *b* and *c*, the values for normoxia- and hypoxia-treatment samples were at a <0.05 level of significance (Student's *t* test). The spot corresponding to an abundant liver protein, fatty acid-binding protein (FABP) is indicated for comparison.

heme-containing ligand-binding site. Instead, they are spread over the surface of the protein, mainly in loops where they might either affect the conformational flexibility of the critical hinge region of the protein or, alternatively, form a different interface with an as yet unidentified protein or multiprotein complex. Distinguishing between these options will require detailed functional analysis.

This *Myg2* isoform might be linked to the proposed whole-genome duplication event in carp within the last 12–15 million years (22) that has resulted in duplications in other key environmentally sensitive genes (23). If so, then this gene is likely to be limited to a restricted clade within the Cyprinidae, including *Cyprinus* and perhaps members of the anoxia-tolerant genus *Carassius* (crucian carp and goldfish), which also possess duplicated genomes. The brain-specific expression of carp *Myg-2* points to promoter divergence in addition to divergence of the coding sequence, and this divergence might have developed after duplication through subfunctionalization of the ancestral promoter (24). In human brain and retina, neuroglobin appears to undertake roles attributed in muscle to Myg (25), and, in the retina, neuroglobin is expressed at the same high levels as observed for Myg in muscle. This very high level of expression is perhaps more consistent with a role in oxygen storage and diffusion than in the more enzymatic roles of handling reactive O_2 species or NO. We have confirmed the existence of neuroglobin transcripts in carp brain by using PCR based on consensus fish neuroglobin motifs (data not shown). The existence of Myg-2 in carp neural tissue seems, therefore, to augment rather than replace neuroglobin function.

In summary, we reveal the expression of the carp Myg-1 in some nonmuscle tissues, some of which display hypoxia-inducible expression. We also show the existence of a second isoform, Myg-2, located exclusively in brain but which is not hypoxia-inducible. These unprecedented observations may account in part for the carp's hypoxia-tolerant physiology. Because nonmuscle Myg may also occur in the zebrafish, the phenomenon may well be more widespread, and the widely assumed muscle-only expression of Myg should not now be taken for granted. This interesting outcome justifies the use of open-screening approaches in revealing unexpected functional responses.

Methods

Animals and Hypoxia Exposure. Common carp were acclimated to either 17°C or 30°C for a 2-month period before hypoxia exposure. On day 0, the oxygen level was lowered to 0.8 mg/liter over a period of 3 hours, constituting a hypoxic stress of 8% and 10% normoxia at 17°C and 30°C, respectively. We sampled tissues from five to eight fish (1000–1100 h) on days 0, 1, 3, 5, and 8 after onset of hypoxia. Skeletal muscle was excised epaxially adjacent to the dorsal fin.

Microarray Experiment. For a full description of the array construction, cDNA sequencing, and annotation, refer to Gracey *et al.* (13). For the experiments reported here, we assessed a total of 366 microarrays for two temperatures (17°C and 30°C), five time points, five tissues (brain, heart, muscle, liver, and gill), all sampled with 5-fold biological replication and with dye-swap. The data were subjected to quality control analysis as described in ref. 13), normalized, and assessed statistically by using a popular signal-to-noise microarray statistic that uses permutation of the sample labels to control for the false-discovery rate associated with multiple testing procedures (26, 27). The raw microarray data files are available at ArrayExpress (accession no. E-MAXD-10).

MYG-1 Protein Analysis. Liver was homogenized in buffer [40 mM Tris base plus complete protease-inhibitor mixture (Roche Diagnostics)] and the homogenate centrifuged (15,000 × *g*, 4°C, 30 min). Supernatant proteins were precipitated by trichloroacetic acid-acetone, and resuspended in 320 μl of rehydration buffer [8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% ASB14 detergent, 0.2% biolytes, and 22 mM DTT]. Isoelectric focusing on pH 3–10 linear strips was carried out at 250 V for 15 min, 10 kV for 3 h, and a linear ramp 10–60 kV/h. Strips were equilibrated (Bio-Rad Literature Bulletin no. 4006166, www.biorad.com) and proteins resolved on 12.5% polyacrylamide gels at 100 V for 1 h and 200 V for 5 h at 15°C. Gels were silver-stained (28) and scanned, and data were analyzed by using the program PDQUEST V7.1 (Bio-Rad). For relative quantification, the measured density for the MYG-1 spot has been expressed relative to the sum of all detectable spots on the gel. Means were compared by using Student's *t* test. Protein from four replicate fish from normoxic and 5-day-hypoxia-treated carp were each analyzed on triplicate gels in two batches, each loaded with 150 μg of protein. Spots in the predicted location for Myg were excised in-gel and digested with trypsin, and the extracted peptides were analyzed by nano-LC (Ultimate) MS/MS (Q-TOF micro, Waters). Proteins

were identified by using the programs MASCOT and MASSLYNX PEPSEQ (Waters), searching against databases by using the FastS algorithm. For relative quantification by iTRAQ, the 10- to 30-kDa subfraction was first isolated by using spin concentrators, and the Myg in this subproteome was then analyzed by the iTRAQ MS/MS technique (http://appliedbiosystems.com/pebiodocs/04350831). The quantitative values generated were verified by using external protein standard mixtures.

RT-PCR. Isoform-specific PCR primers for Myg-1 and Myg-2 were designed to two variable regions between the Myg isoforms. Forward, N-terminal primers were designed to the DNA sequences encoding the N-terminal peptides MADHELV (Myg-1; atggccgatcacgaactggtt) and MADYERF (Myg-2; atggctgattacgagcggttt). Reverse primers were designed to the antisense DNA encoding the peptide sequences between positions 54 and 61, NAAVKAHG (Myg-1; gccgtgggccttcaccgctgcgtt) and DTLVASHG (Myg-2; accgtgggacgccaccaacgtgtc). Oligo-dT-primed synthesis from 3 μg of total RNA from each tissue of hypoxia-exposed carp was performed by using Superscript II (Invitrogen) according to the manufacturer's instructions. First-strand cDNA (1 μl; 1/20th of the total sample) served as template in a 50-μl PCR using 0.3 μl of *Taq* polymerase (5 units/μl; Bioline) and supplied 10× buffer, together with the individual isoform-specific primers (0.5 μM each), dNTPs (0.2 mmol/liter), and $MgCl_2$ (1.5 mmol/liter). Thermocycling included one cycle at 95°C for 2 min, followed by 27 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 1 min 30 s and, finally, one extended polymerization step at 72°C for 4 min.

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Corrections

IMMUNOLOGY. For the article "Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development," by Marc A. Gavin, Troy R. Torgerson, Evan Houston, Paul deRoos, William Y. Ho, Asbjørg Stray-Pedersen, Elizabeth L. Ocheltree, Philip D. Greenberg, Hans D. Ochs, and Alexander Y. Rudensky, which appeared in issue 17, April 25, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 6659–6664; first published April 14, 2006; 10.1073/pnas.0509484103), the authors note the incorrect placement of the α in Fig. 1*B*. In addition, the position of Fig. 1*E* was incorrect. The authors also note the omission of the γ after each instance of "IFN-" in Fig. 3. The corrected figures and their legends appear below. These errors do not alter the conclusions of the article.

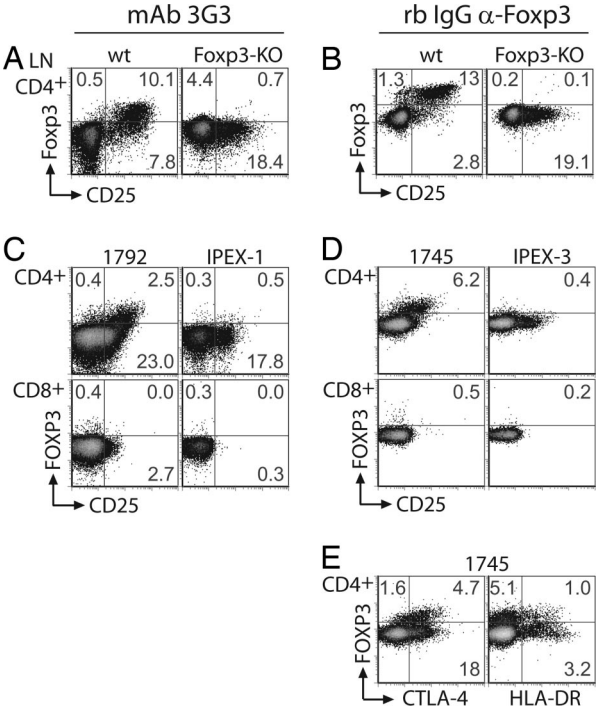

Fig. 1. Flow cytometric detection of Foxp3 in murine and human cells. (*A* and *B*) Normal or Foxp3-deficient mouse lymph node cells were stained for Foxp3 and cell-surface markers by using digoxigenin-conjugated mAb 3G3 (*A*) or Foxp3-specific rabbit antibody (*B*). $CD4^+$ gated lymphocytes are shown. (*C–E*) Normal (1792 and 1745) or FOXP3-deficient (IPEX) PBMC were stained for FOXP3 and lymphocyte markers by using digoxigenin-conjugated mAb 3G3 (*C*) or digoxigenin-conjugated Foxp3-specific rabbit antibody (*D* and *E*). Both $CD4^+$ and $CD8^+$ gated lymphocytes are shown. Additional IPEX-1 PBMC were not available for subsequent analysis with rabbit antibody. High background staining of $Foxp3^-$ cells is a consequence of the three-step staining procedure.

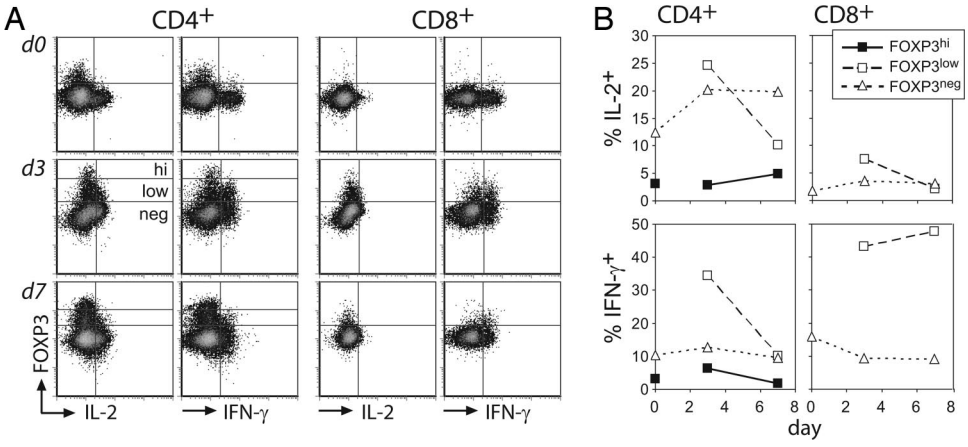

Fig. 3. Induced FOXP3 does not suppress IL-2 or IFN-γ synthesis. (*A*) Freshly isolated or stimulated (100 ng/ml anti-CD3) total PBMC from donor 1745 were incubated with PMA, ionomycin, and monensin and stained for FOXP3 (rabbit IgG-digoxigenin), IL-2, IFN-γ, and surface markers as described in *Materials and Methods*. (*B*) The percentage of cytokine-expressing cells among $FOXP3^{high}$, $FOXP3^{low}$, or $FOXP3^-$ cells is plotted. The distinction between high and low FOXP3 expression was not made for $CD4^+$ cells and $CD8^+$ cells on day 0. Data are representative of three separate experiments.

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PHYSIOLOGY. For the article "Hypoxia-inducible myoglobin expression in nonmuscle tissues," by Jane Fraser, Luciane Vieira de Mello, Deborah Ward, Huw H. Rees, Daryl R. Williams, Yongchang Fang, Andrew Brass, Andrew Y. Gracey, and Andrew R. Cossins, which appeared in issue 8, February 21, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 2977–2981; first published February 9, 2006; 10.1073/pnas.0508270103), the authors note that in the author line, the affiliations for Luciane Vieira de Mello, Yongchang Fang, and Andrew Brass appeared incorrectly, due to a printer's error. In addition, the name Yongchang Fang should have appeared as Yongxiang Fang. The online version has been corrected. The corrected author and affiliation lines and the original author footnotes appear below.

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GENETICS. For the article "Long-range multilocus haplotype phasing of the MHC," by Zhen Guo, Leroy Hood, Mari Malkki, and Effie W. Petersdorf, which appeared in issue 18, May 2, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 6964–6969; first published April 21, 2006; 10.1073/pnas.0602286103), the authors note that a grant was incorrectly cited. In line 5 of the Acknowledgments, "National Institutes of Health Grants AI18029" should have read "National Institutes of Health Grants CA18029."

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GENETICS. For the article "Insights into TOR function and rapamycin response: Chemical genomic profiling by using a high-density cell array method," by Michael W. Xie, Fulai Jin, Heejun Hwang, Seungmin Hwang, Vikram Anand, Mara C. Duncan, and Jing Huang, which appeared in issue 20, May 17, 2005, of *Proc. Natl. Acad. Sci. USA* (**102,** 7215–7220; first published May 9, 2005; 10.1073/pnas.0500297102), the authors wish to add a reference to a paper by C. W. Xu (1), who employed a microarrayer to fabricate bacterial and yeast cell microarrays on nitrocellulose membrane.

1. Xu, C. W. (2002) *Genome Res.* **12,** 482–486.

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