Int. J. Dev. Biol. 53: 517-524 (2009)
doi: 10.1387/ijdb.082651dv

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

A critical role for myoglobin in zebrafish development

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ABSTRACT The globin family, including hemoglobin, myoglobin, neuroglobin and cytoglobin, plays an important role in oxygen storage and delivery. Myoglobin has been shown to be necessary for cardiac function during development, but no information is currently available on the developmental regulation of *myoglobin* gene expression during embryogenesis. In this study, we used whole mount *in situ* hybridization to visualize *myoglobin* mRNA expression during zebrafish development. Our results show for the first time the spatial and temporal gene expression pattern of myoglobin during embryogenesis. *Myoglobin* was expressed as a maternal RNA and ubiquitous expression was observed until the end of gastrulation. At later stages of development, we discovered novel expression domains for *myoglobin*, including several non-muscular ones. Environmental stresses, like low oxygen tension (hypoxia) can lead to a developmental delay in zebrafish embryos. We show here that hypoxic stress induces *myoglobin* expression in skeletal muscle cells of anterior somites and in the dorsal aorta of zebrafish larvae. Finally, we analyzed the role of myoglobins in development by targeted gene knock-down. Silencing myoglobin in zebrafish embryos with gene-specific morpholinos led to a dose dependent curvature, vascular defects, enlarged pericardia and reduction of the gut. In conclusion, our results indicate that myoglobin plays a crucial role in zebrafish development and is important for angiogenesis and gut development.

KEY WORDS: *development, zebrafish, hypoxia, heart development, vascularization, angiogenesis*

Introduction

The globin family in vertebrates consists of four types of globins, hemoglobin, myoglobin (Mb), neuroglobin and cytoglobin which differ in structure, tissue distribution and function (Pesce *et al.*, 2002). Globins are small respiratory proteins that reversibly bind O_2 by means of an iron-containing porphyrin ring. They are widely distributed through all three kingdoms of life and can be found from bacteria to eukaryotes (Vinogradov *et al.*, 2006).

Mb contributes to intracellular oxygen storage and facilitates transcellular diffusion of oxygen (Flogel *et al.*, 2004, Ordway and Garry, 2004, Suzuki and Imai, 1998, Takahashi and Doi, 1998, Wittenberg and Wittenberg, 2003).

The importance of Mb for mammalian development has been demonstrated by studies of Mb deficient mice. Initially it was shown that mice without myoglobin, are fertile and exhibit normal exercise capacity and a normal ventilatory response to low oxygen levels (hypoxia) (Garry *et al.*, 1998). A later study, however showed that the majority of myoglobin deficient mice die in utero at midgestation with signs of cardiac failure (Meeson *et al.*, 2001). It was shown that Mb is necessary for cardiac function during development. However, some animals showed adaptive responses which could fully compensate for the defect in cellular oxygen transport (Godecke *et al.*, 1999, Meeson *et al.*, 2001).

In adult animals, Mb is typically expressed in skeletal and cardiac muscle tissues, where continuous oxygen flow is required for high activity of aerobic metabolism (Iwanami *et al.*, 2006, Lee-de Groot *et al.*, 1998). Mb expression studies were mainly focused on cardiac and skeletal muscle tissue. Recent microarray studies have shown that Mb is also expressed in a range of other tissues, e.g. in the gills of adult zebrafish (van der Meer *et al.*, 2005) as well as in the liver, gills and brain of adult carp (Fraser *et al.*, 2006).

Still little is known about the expression pattern of Mb during vertebrate development. Longo *et al.* (1973) investigated Mb

Abbreviations used in this paper: dpf, days post fertilization; Mb, myoglobin; MBT, mid-blastula transition; MO, morpholino.

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Accepted: 15th July 2008. *Published online:* 2 April 2009.

ISSN: Online 1696-3547, Print 0214-6282

expression in embryonic tissue samples of human and sheep cardiac and skeletal muscle and found that Mb protein accumulates in cardiac muscle early in fetal development. However, Mb protein expression did not appear in skeletal muscle until late in gestation. Weller *et al.* also investigated embryonic tissue samples from mouse and human cardiac and skeletal muscles and found that Mb was expressed in embryonic mouse cardiac and skeletal muscle after 14 days of gestation and at 10 weeks of gestation in human skeletal muscle (Weller *et al.*, 1986).

We describe here the temporal and spatial gene expression patterns of Mb during zebrafish development and identify novel expression domains of this important member of the globin family. We further show that Mb knock down leads to severe defects in developing embryos and that hypoxic stress induces Mb expression.

Results

Genomic organization of zebrafish myoglobin *and phylogenetic analysis of myoglobins*

The zebrafish Mb gene had been initially described by Madden *et al.* (Madden *et al.*, 2004). We have cloned here a zebrafish Mb cDNA in order to study its expression pattern during embryogenesis. The derived amino acid (Aa) sequence was identical to a previously described zebrafish Mb clone (GenBank Acc. Number: BC065862). We have manually annotated the zebrafish Mb gene, which is located on Chromosome 1 at location 64,311,869-64,314,844 in the current release (Zv6) of the zebrafish genome project (Ensemble gene ID: ENSDARG00000031952; http://www.ensembl.org/index.html).

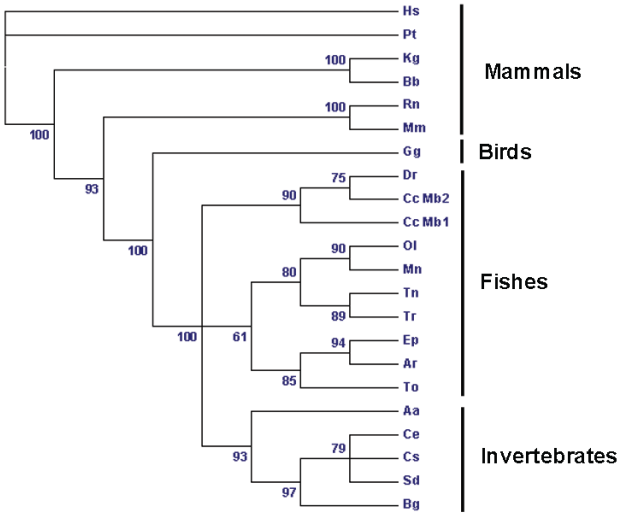


Fig. 1. Phylogenetic analysis of myoglobins (Mbs). *Shown is the phylogenetic analysis of Mbs based on aminoacid sequences of 16 different vertebrate and 5 invertebrate species. Abbreviations used and accession numbers (GenBank and Ensembl): Hs:* Homo Sapiens *(Human) (CU013116); Pt:* Pan troglodytes *(Chimpanzee)* (*ENSPTRG00000023553); Kg:* Kogia breviceps *(Pygmy Sperm whale) (AB271147); Bb:* Balaenoptera borealis *(Sei whale) (AB271151); Rn:* Rattus norwegicus *(Rat) (NM_021588); Mm:* Mus musculus *(Mouse) (NM_013593); Gg:* Gallus gallus *(Chicken) (ENSGALG00000012541); Dr:* Danio rerio *(Zebrafish) (BC065862); Cc:* Cyprinus carpio *(Common Carp) (DQ338464 Isoform 2) (P02204 Isoform 1); Ol:* Oryzias latipes (*Japanese* Medaka) (*ENSORLG00000004130)*; Mn: Makaira nigricans *(Blue Marlin) (AF291833); Tn:* Tetraodon nigroviridis (*Spotted green pufferfish) (AJ628044);* Tr: Takifugu rubripes (*Japanese pufferfish): Ep:* Euthynnus pelamis *(Oceanic Bonito) (AF291837); Ar:* Auxis rochei *(Fregatte Makarel) (AB154423); To:* Thunnus obesus *(Bigeye tuna) (AB104433).*

TABLE 1

MYOGLOBIN EXPRESSION DOMAINS IN THE DEVELOPING ZEBRAFISH

Embryonic stage	Expression domain
prim-6	eye, otic vesicle, gut
long-pec	lens, retina, optic chiasm, otic vesicle, heart, intermandibularis posterior, middle cerebral vein, cardinal vein, epidermis, gut, liver, pectoral fin bud, telencephalon, diencephalon
4 dpf	eye, telencephalon, midbrain, hindbrain, otic vesicle, mandible, cephalic musculature, branchial arches, heart, pectoral fin buds, intestine, liver, epidermis, apical epidermis, peridermis, fin rays, floorplate, roofplate, cardinal vein, intersegmental vessels
5 dpf	lens, retina, ganglion cell layer, inner nuclear layer, optic chiasm, telencephalon, meckel's cartilage, basihyal, ceratohyal, palatoquadrate, otic vesicle, sensory epithelium, midbrain, notochord, somites, pectoral fin bud, liver, pancreas, intestine

Only a single zebrafish Mb gene was found to be encoded in the zebrafish genome. This is in contrast to the common carp for which two Mb forms have been described (Fraser *et al.*, 2006). A detailed analysis of Mb intron exon structures in plants and animals can be found elsewhere (Dixon and Pohajdak, 1992).

We used protein sequences from different Mb homologues of 16 different vertebrate and 5 invertebrate species to perform a phylogenetic analysis (Fig. 1B). The results with high Bayesian support values reveal that whale myoglobin branches of after the rodents. A phylogeny of whale Mbs had been previously described (Iwanami *et al.*, 2006). The unrooted phylogenetic tree in Figure 1 shows that, among the proteins analyzed, the closest homolog to zebrafish Mb was Mb2 of carp.

Gene expression pattern of myoglobin *during zebrafish development*

In this study, we have characterized the temporal and spatial expression patterns of Mb during early zebrafish development. Zebrafish embryos were staged according to Kimmel (Kimmel *et al.*, 1995). Table 1 shows an overview of myoglobin expression domains during development. We found that Mb is present as a maternal RNA (Fig. 2A and B). It is ubiquitously expressed during gastrulation (at the shield stage in midgastrulation; Fig.2 C) and at the end of gastrulation (at tail bud stage; Fig. 2 D).

Distinct expression domains were observed at later stages of development. In prim-6 embryos, Mb is expressed in the eye, the otic vesicle and the developing gut (Fig. 2F). Embryos at the long pec stage showed Mb expression in the eye (in the lens and retina), the metencephalic vein, the otic vesicle, the liver and in the gut. Staining was also detected in the epidermis and in the caudal vein (Fig. 2 E,H).

At 4 days post fertilization (dpf) strong expression was observed at the anteriormost portion of the head. Staining was found in the eye, the telencephalon, the midbrain and hindbrain, the otic vesicle, the cephalic musculature, the mandible and the branchial arches. Strong expression was detected in the heart and fin rays. Expression was also observed in the epidermis, the peridermis and the apical epidermis of the pectoral fin buds. Additionally, in embryos at 4 days of development Mb was expressed in the intestine, the

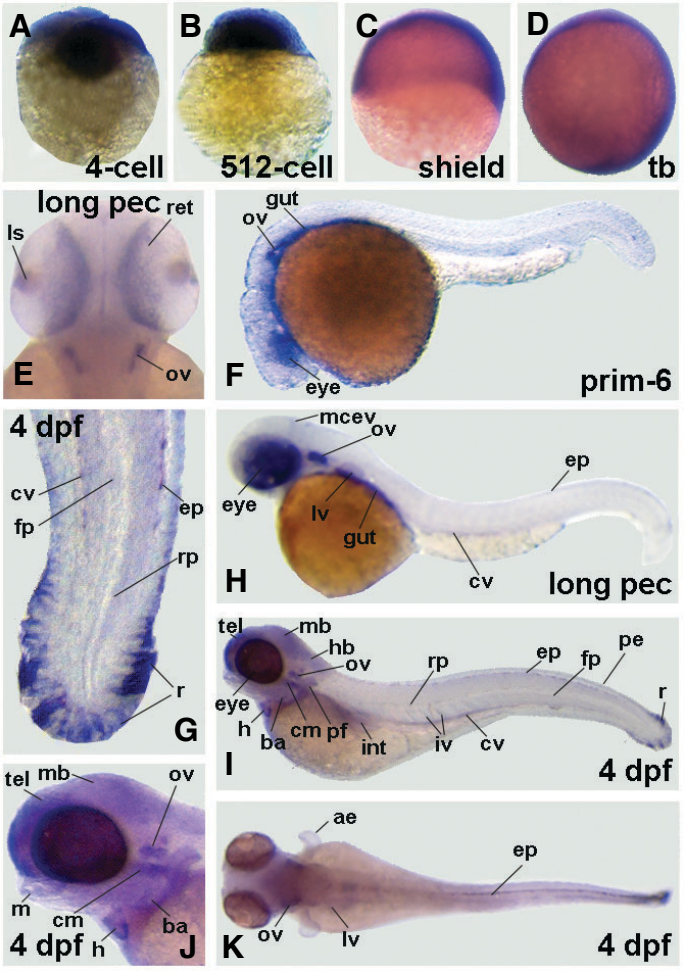


Fig. 2. Gene expression pattern of *myoglobin* during zebrafish development. *The early embryos shown are during cleavage period at the 4-cell stage* **(A)** *followed by the 512-cell stage* **(B)** *shortly before mid blastula transition (MBT). Mid gastrulation is represented by shield stage embryos* **(C)** *and the end of gastrulation by embryos at tail bud stage* **(D)**. *Later stages shown during the pharyngula period are: prim-6* **(F)** *and long pec stages* **(E,H)**. *The early larval period is represented by embryos at 4 dpf* **(G-K)**. *Abbreviations used are: ae, apical epidermis; ba, branchial arches; cm, cephalic musculature; cv, caudal vein; ep, epidermis; fp, floorplate; h, heart; int, intestine; iv, intersegmental vessels; l, liver; ls, lens; m, mandibulae; mb, midbrain; mecv, midencephalic vein; ov, otic vesicle; pe, peridermis; pf, pectoral fin bud; r, fin rays; ret, retina and rp, roofplate.*

liver, the floorplate, the roofplate, the caudal vein and in intersegmental vessels (Fig. 2 G, I, J and K).

To further characterize the gene expression pattern of Mb during zebrafish development, we investigated expression domains in sections of long pec and 5 dpf embryos (Fig. 3). Sections of embryos at long pec stage and 5 dpf showed distinct expression patterns for Mb. At long pec stage Mb expression was found in the lens, the retina, the optic chiasm, the telencephalon and the diencephalon (Fig.3 A). In more posterior sections, staining was restricted to the epidermis and pectoral fin buds of embryos at the long pec stage (Fig. 3 B and C). In embryos at 5 dpf, Mb was detected in the telencephalon, the telencephalon, the lens, the ganglion cell layer, the inner nuclear layer, the optic chiasm, the meckel's cartilage, the basihyal, ceratohyal and the palatoquadrate (Fig.3 D, E and F). More posterior parts of embryos at 5 dpf showed expression in the otic vesicle, the sensory epithelium, the liver, the pancreas and the intestine. Weaker expression was also found in the midbrain, the notochord and the somites (Fig. 3 G, H, I).

Influence of hypoxia on myoglobin *gene expression in zebrafish embryos*

In this study we were interested in the effects of hypoxia on Mb gene expression during zebrafish development. We exposed zebrafish embryos to two hypoxic conditions (20% air saturation: $5x10^{-5}$ mol/l or 1.6 mg/l O_2 and 10% air saturation: $2.5x10^{-5}$ mol/l or 0.8 mg/l O_2). Control zebrafish were kept under normoxic conditions (100% air saturation: $2.5x10^{-4}$ mol/l or 8 mg/l O_2). We observed that severe hypoxic conditions (10%) led to increased Mb expression in the dorsal aorta and in skeletal muscles of anterior somites in early embryos (Fig. 4). This was observed in whole mount embryos (Fig. 4D) and in sections (Fig. 4E). Incubation in 20% air saturated water did not lead to a similar induction (Fig.4C). We confirmed these results by quantitative RT-PCR and observed a 1.7 fold induction of Mb (SD +/- 0.06) expression in 48 hpf embryos exposed to 10% hypoxia (Fig. 4 F).

Effects of myoglobin *knock down on zebrafish development*

To better understand the regulatory and developmental functions of Mb, we injected gene-specific morpholinos (MO) into zebrafish embryos and surveyed the phenotypic changes at

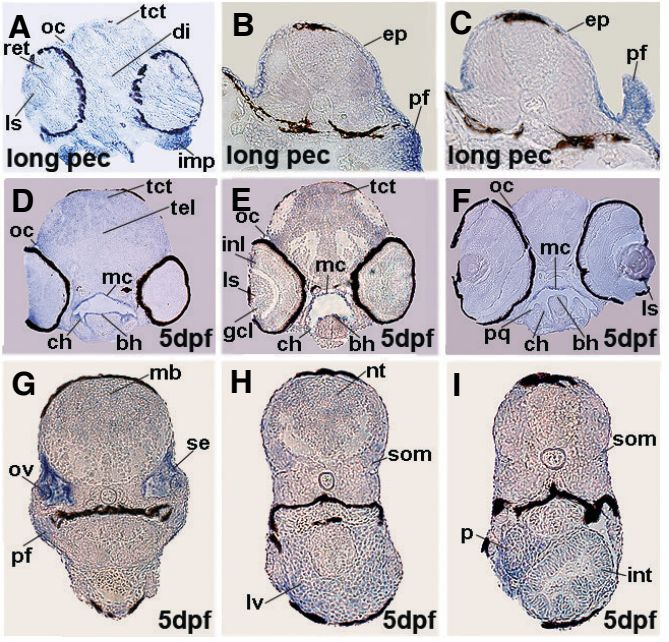
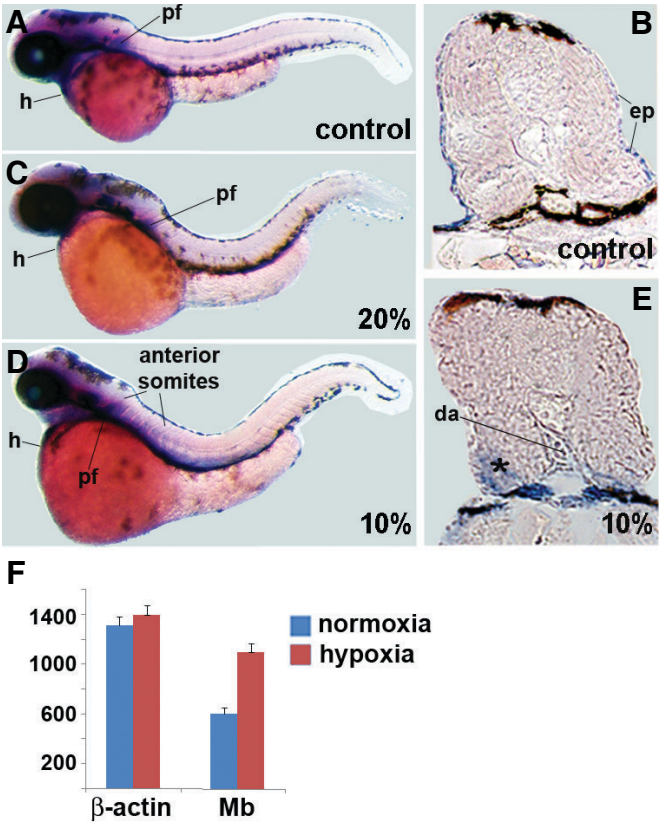


Fig. 3. Gene expression domains of *myoglobin* during zebrafish development in sections of long pec and 5dpf embryos. *Shown are sections of whole mount* in situ *hybridized embryos at the indicated stages. Abbreviations used are: basihyal (bh), ceratohyal (ch), diencephalon (di), epidermis (ep), ganglion cell layer (gcl), inner nuclear layer (inl), intermandibularis posterior (imp), intestine (int), liver (lv), meckel's cartilage (mc), midbrain (mb), notochord (nt), optic chiasm (oc), otic vesicle (ov), palatoquadrate (pq), pancreas (p), sensory epithelium (se), somites (som) and telencephalon (tel).*

different stages of development (Fig. 5 and Table 2). We observed a dose dependent effect on the curvature of embryos. A low dose of Mb-MO (1ng) led to a minimal increase in the number of curved embryos (1.3%) compared to control injected embryos (Table 2). Higher Mb-MO doses (2ng and 4ng) led to over 30% embryos with a curved body. We have categorized the extent of curvature into mild and severe (Fig. 5). More severely curved embryos were found when a high dose of Mb-MO (4ng) was used compared to the lower Mb-MO dose (2ng) (Table 2). Slight differences to control embryos were visible at the 14 somite stage but curving was apparent only at 24 hpf (Fig. 5). Both concentrations of Mb-MO also induced an enlarged pericardium in injected embryos (Fig. 5 and Table 2), which was not shown after injection of the control MO. We further observed an increase in lethality over time in the Mb knock down embryos.

Our findings that Mb is expressed in the caudal vein led us to further investigate a role of Mb in vascularization and angiogenesis. In order to test this, we injected Mb-MO) in a transgenic zebrafish line, Tg:fli1/eGFP (Lawson and Weinstein, 2002). The Tg:fli1/eGFP embryos express the green fluorescence protein under an early endothelial promotor and therefore exhibit a fluorescent green vasculature. Our results using laser scanning confocal microscopy showed that Mb knock down leads to angiogenesis defects (Fig. 6 and Table 3). Vascular defects were induced by Mb-MO injections in a dose



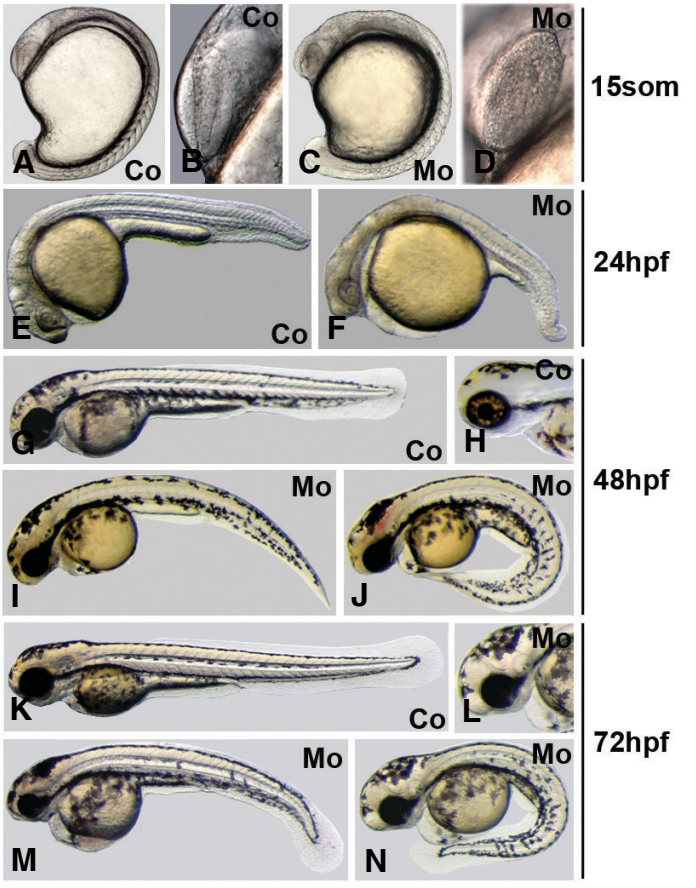


Fig. 4 (Left). Influence of hypoxia on *myoglobin (Mb)* gene expression in zebrafish embryos. *Zebrafish embryos were exposed to two different oxygen concentrations (10% and 20% air saturated). Controls were kept at 100% air saturated. Embryos were fixed at 48 hpf for the control* **(A)** *and the 20% air saturated water embryos* **(C)** *and at 4 dpf for the 10% air saturated water embryos* **(E)** *(which were strongly delayed in development). Fixed embryos were subjected to whole mount* in situ *hybridization. Blue and purple staining indicates* myoglobin *expression. Similar results were seen in five different specimen and staining in the anterior somites was never observed in control or 20% air saturated water embryos. Staining in the pectoral fins (pf) and in the heart (h) is indicated for all embryos and anterior somites and the dorsal aorta (da) are marked in the 10% air saturated water embryos. In addition, shown are sections of the anterior somite area of control embryos* **(B)** *and embryos exposed to 10% air saturated water* **(D)**. *The latter shows stronger staining in regions of the anterior somites supporting the results of the whole mount pictures. Skeletal muscles are indicated with an asterisk and staining in the dorsal aorta (da)* **(D)**. *The quantitative RT-PCR results for 48 hpf embryos under normoxic conditions or exposed to hypoxia (10%) are shown in* **(F)**. *Relative expression of* myoglobin *is given based on normalization to β-actin. A standard curve for β-actin was included in the experiments. Data represents two independent experiments each done in triplicates. In 48 hpf embryos, 10% hypoxia led to a 1.7 fold induction of Mb normalized to actin (SD+/- 0.06).*

Fig. 5 (Right). Effects of microinjection of *myoglobin* gene-specific Mo on zebrafish development. *Shown are stereoscope pictures and DIC microscopy pictures of embryos and larvae at different stages of development. Embryos were injected at the one-cell stage with either a control Mo or the Mb specific MO (examples shown were injected with Mb-MO (2 ng/embryo* **(C,D,F,I and M)** *or 4 ng/embryo* **(J,L and N)** *or control MO (4 ng) were used as indicated).*

TABLE 2

MORPHOLINO INJECTIONS IN ZEBRAFISH EMBRYOS

		24hpf			48hpf			72h		
Morpholino [amount]	Injected	Total (%survival)	Curved phenotype (mild/severe)	Enlarged Pericardium (curved)	Total (%survival)	Curved phenotype (mild/severe)	Enlarged Pericardium (curved)	Total (%survival)	Curved phenotype (mild/severe)	Enlarged Pericardium (curved))
Control [4ng]	766	722 (94%)	0	0	690 (90%)	0	0	686 (90%)	0	0
Mb [1ng]	85	79 (93%)	1 (1/0) 1.3%	1 (1) 1.3%	76 (89%)	0	0	73 (86%)	0	0
Mb [2ng]	630	548 (87%)	215 (142/73) 39.2%	266 (215) 49%	485 (77%)	135 (90/45) 27.8%	186 (135) 38.3%	446 (71%)	108 (71/37) 24.2%	134 (108) 30.0%
Mb [4ng]	706	636 (90%)	235 (78/157) 36.9%	233 (233) 36.6%	566 (80%)	174 (57/117) 30.7%	180 (172) 31.8%	506 (72%)	115 (45/70) 22.7%	120(114) 23.7%

dependent manner (Table 3). In the Mb-MO injected embryos, we observed an impaired formation of dorsal longitudinal anastomotic vessels (DLAV) with several of these connective vessels missing (Fig. 6).

Our expression study also showed that Mb is strongly expressed in the gut of the developing zebrafish embryos. To test the hypothesis that Mb plays a role in gut development, we used a gut-GFP transgenic zebrafish line, which expresses GFP under the EF-1α promotor (Tg:ef-1α/eGFP) (Field *et al.*, 2003).

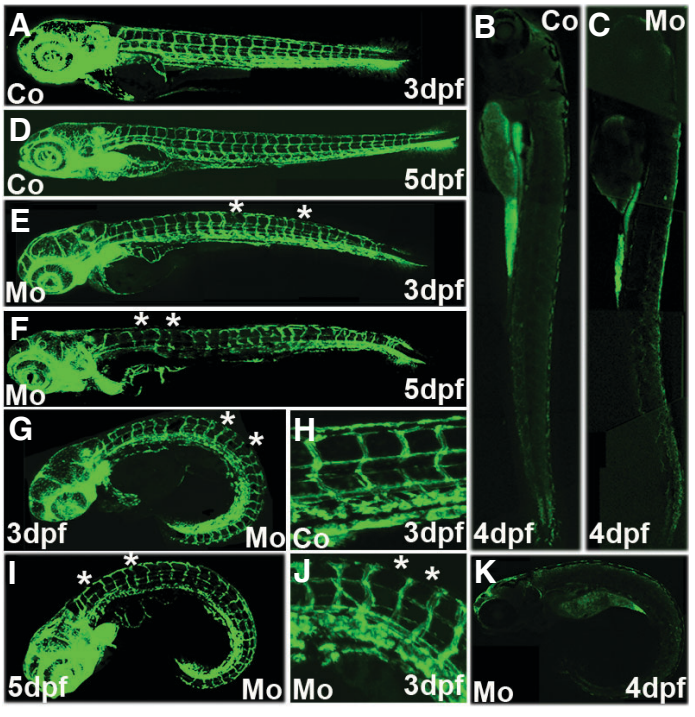


Fig. 6. Effects of microinjection of *myoglobin* gene-specific morpholinos in Tg:fli1/eGFP and Gut/GFP transgenic fish. *Tg:fli1/eGFP embryos* **(A, D-J)** *and Gut/GFP (Tg:ef-1α/eGFP)* **(B,C,K)** *were injected with 4 ng/embryo of either Control Morpholino (Co) or Mb specific Morpholino (Mo) as indicated. Zebrafish larvae at three different time points are shown: 3 dpf, 4 dpf and 5 dpf. The fluorescent gut is not well visible before 4 dpf. All pictures show confocal images of representative examples for control and knock down embryos. Higher magnification images* **(H,J)** *give examples for a control* **(H)** *and an Mb knock down* **(J)** *embryo, respectively. An asterisk is used to mark representative sites of vascular defects* **(E-G, I,J)** *(not all defects are marked).*

The gut-GFP embryos exhibit a green fluorescent gut, which initially becomes visible at 72hpf. The Mb-MO injected embryos did show a reduced size of the gut in over 30% of cases (Table 3 and Fig. 6). These findings indicate that Mb could play a role in gut development.

Discussion

Although Mb is, like most of the globins, a very well studied molecule, there is very limited amount of information available on its expression and function during vertebrate embryogenesis. Longo *et al.* (1973) had compared the Mb protein levels in different vertebrate embryos but focused their study on muscle tissue extracts (Longo *et al.*, 1973). Another study using northern blot analysis focused also solely on muscle tissues and described embryonic gene expression in RNA preparations of cardiac and skeletal muscle (Weller *et al.*, 1986). No *in situ* data on spatial and/or temporal expression of Mb gene expression in vertebrates has been published up to date.

We were particularly interested in the identifying non-muscle related expression domains for Mb. Mb expression during development was found in several organs e.g. brain, pancreas, liver

TABLE 3

MORPHOLINO INJECTIONS IN TG:FLI1/EGFP AND TG:EF-1α/EGFP ZEBRAFISH EMBRYOS

Tg:fli1/eGFP			
Morpholino [amount]	Total	Vascular defects	Curved phenotype (mild/severe)
Control [4ng]	150	0	0/0
Mb [1ng]	71	3 (4.2%)	0/0
Mb [2ng]	207	53 (25.6%)	27/15
Mb [4ng]	121	48 (31.4%)	16/29
Tg:ef1α/eGFP			
Morpholino [amount]	Total	Gut defects	Curved phenotype (mild/severe)
Control [4ng]	200	0	0/0
Mb [1ng]	80	0	0/0
Mb [2ng]	112	34 (30.3%)	18/14
Mb [4ng]	120	44 (36.6%)	10/30

and gut. It is possible that Mb is involved in organogenesis and plays important roles during embryonic development other than related to muscle development. Our findings show that Mb knock down leads to morphological and anatomical defects. We observed severe bending of the embryos. Since injected embryos did not show any visible defects at the end of gastrulation, and at the tail bud stage (data not shown), the curvature of the embryo takes place at a later time point of development. Therefore, defects during gastrulation e.g. imperfect convergent extension movements can be ruled out as a cause for the curvature. A plausible explanation for the observed bending is a defect in muscle function and/or development. This muscle defect could be either directly related to the lack of Mb expression in the muscle cells, or indirectly by a limited oxygen supply to muscle cells due to vascular defects.

In this study, we observed vascular defects in Mb knock down embryos. There are no indications that absence of Mb is mechanistically involved in angiogenesis, but lack of Mb could affect vascularization indirectly. In addition to its role in oxygen transport, in mouse heart Mb has been shown to dioxygenate nitrogen oxide (NO) and reduce levels of hydrogen peroxide as well as superoxide (Flogel *et al.*, 2004, Flogel *et al.*, 2001). Lack of Mb in knock down embryos might lead to reduced scavenging of NO, causing locally elevated levels. This could in turn cause impaired vascularization, as has been described in the chick embryo chorioallantoic membrane assay and in cultured human umbilical vein endothelial cells (Cartwright *et al.*, 2000, Pipili-Synetos *et al.*, 1994). Substantial evidence also indicates that NO is positively related to angiogenesis in embryonic and mature tissues (Al-Ani *et al.*, 2006, Namba *et al.*, 2003, Nath *et al.*, 2004, Papapetropoulos *et al.*, 1997). The roles of Mb in regulating NO levels in the developing zebrafish and in vascularization remain to be determined.

Another way by which Mb knock down could lead to vascular defects is that the smooth muscle cells, surrounding the endothelial cells in blood vessels do not develop properly, due to lack of Mb. In human and chicken, Mb has been detected in smooth muscle of uterus, intestine and rectus tissues (Qiu, 1998, Schuder, 1979). Here, we detect weak Mb expression along the caudal vein, which is likely to be in the smooth muscle cells of these veins. The presences of Mb in smooth muscle cells suggests that it may play a role in oxygen delivery to the core of these cells and/or in NO induced vasodilation. However, Mb could also prevent excessively high levels of hydrogen peroxide, superoxide and/or NO and thereby protect smooth muscle cells from structural damage.

In zebrafish embryos, an enlarged pericardium, which is usually induced by an edema, is an indication for either a heart and/or a circulation defect. Earlier results in Mb deficient mice showed, that the majority of embryos lacking Mb die in utero at midgestation with signs of cardiac failure (Meeson *et al.*, 2001). We found here strong Mb expression in the developing zebrafish heart. Taken together, this could suggest that cardiac defects are responsible for the enlarged pericardia we found in Mb knock down embryos. Another possibility is that the strong curvature and/or the vessel defects lead to circulatory interruptions in the embryo and caused the edema. Finally, all of these three alternatives could contribute to some extend to the enlargement of the pericardium and the observed increase in lethality over time observed in Mb knock down embryos. Similarly, the reduced gut found in the Mb knock down embryos could be a consequence of the vascularization defects and/or the severe bending of the embryos. It is possible that Mb is expressed in smooth muscle cells in the gut and this might be important for proper gut development.

The question is raised whether knock down of Mb in cardiac myocytes caused cardiac failure prior to impaired angiogenesis. If so, the compromised cardiac function will cause a reduced flow of erythroblasts and as a consequence a lower shear stress in the circulation system. Recently, hemodynamic force has been shown to be necessary for vascular remodeling of the mouse yolk sac (Lucitti *et al.*, 2007). We hypothesize that the vascular defects observed in Mb-MO are due to elevated NO signaling and/or cardiac dysfunction.

Recently, it has been shown that severe hypoxic conditions lead to a developmental delay in zebrafish embryos"(Kajimura *et al.*, 2005, Padilla and Roth, 2001). In adult zebrafish, we and others showed that hypoxia leads to increased Mb expression in different tissues (Roesner *et al.*, 2006, van der Meer *et al.*, 2005). Similar results were found in the liver of adult carp (Fraser *et al.*, 2006). Moreover, endurance swim training of zebrafish embryos led to enhanced Mb expression in skeletal muscle (van der Meulen *et al.*, 2006). All these previous studies suggested a possible regulation of Mb expression by hypoxia in zebrafish embryos. Indeed our findings show that Mb expression is induced in developing embryos exposed to severe hypoxia. This could point to a potential adaptive response to hypoxia during embryonic development in order to regulate diverse processes (i.e. cellular oxygenation and NO metabolisms) (Riggs, 2006). Further research is warranted to identify a possible mechanism and the exact function of this regulation.

In summary, we have identified here novel non-muscular expression domains for the Mb gene and showed that zebrafish development is critically dependent on Mb expression.

Materials and Methods

Animal care, handling and hypoxia treatment

Zebrafish were handled in compliance with local animal care regulations and standard protocols. Fish were kept at 28°C in aquaria with standard day/night light cycles. The developing embryos were kept at 28°C under two hypoxic conditions (20% air saturation: 1.6 mg/l O_2 and 10%: 0.8 mg/l O_2). A normoxic control group (100% air saturation: 8 mg/l O_2) was kept in identical aquaria of 100 liters. The oxygen level on the hypoxia group was kept constant by a controller (Applicon) connected to an oxygen electrode and solenoid valve in line with an air diffuser. The oxygen concentration level in the tank was kept constant by adding oxygen via the diffuser and thereby compensating the oxygen consumption of the embryos.

Cloning of zebrafish myoglobin

Whole zebrafish RNA was obtained from liquid nitrogen frozen adult fish through Trizol® (GIBCOBRL, Life technologies) extraction according to the manufacturer's instructions. The complete open reading frames were amplified by RT-PCR, using a SuperScriptIII kit (Invitrogen) in concert with flanking primers based on Mb sequences. The primers used were: 5' tcttcacagaggacaaacacc 3' (forward) and 5' cgctttatttatgactcccattt 3' (reverse).

Sequence alignment and phylogenetic analysis

The amino acid alignment of the zebrafish Mb gene was made using ClustalX"(Thompson *et al.*, 1997) and subsequently processed and

manually refined using Genedoc software (http://www.psc.edu/biomed/genedoc/). The phylogenetic tree was generated employing MrBayes (Huelsenbeck and Ronquist, 2001) version 3.1. The number of generations was set at 5000000, sampled every 100th generation, while 1250 trees were discarded as burnin. The consensus tree and Bayesian numbers were saved in a final figure with Treeview (Page, 1996).

Whole mount in situ *hybridization and sectioning of whole mount embryos*

The *in situ* hybridization procedure was modified from the Thisse protocol (Thisse *et al*., 1993) and has been previously described (te Velthuis *et al*., 2007). An antisense oligonucleotide probe corresponding to the full length zebrafish Mb coding sequence of 444 bp was used in the experiments. After *in situ* hybridization, embryos were fixed overnight in 4% PFA in PBS, at 4°C. They were subsequently dehydrated through an alcohol series: 70%, 80%, 90% and 3x 100% ethanol, 1h each. Next, they were transferred into Xylene, 3x 30 min. Finally, the embryos were transferred into paraffin, 3x 2h. 8 μm sections were cut with a Reichter-Jung Ultracut micrometer (Leica). Pictures were taken in Axioplan 2 microscope (Carl Zeiss) using AxioVision 4 software.

RNA isolation and quantitative RT-PCR

RNA preparation was described before (Ott *et al*., 2007) and a pool of 100 embryos was used per isolation. For Real-time quantitative RT-PCR the Roche Master SYBR Green kit was used. The annealing and synthesis temperature was 55°C alternating with 96°C for 45 cycles. Dissociation protocols were used to measure melting curves and control for unspecific signals from the primers. We used 100 ng of total RNA per reaction. A standard curve for β-actin using 1, 5, 10, 100, and 500 ng of total RNA was used for normalization. Samples were measured in the Roche LightCycler. The Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design primers for short amplicons between 50 and 100 bases using. Primers used for zebrafish β-Actin were previously described (Marques *et al*., 2008). The primer sequences for zebrafish myoglobin were: Forward: 5-tcaaaccactggccaataca-3 and Reverse: 5- agttgttgagggccactttg-3.

Targeted gene knock-down with myoglobin *morpholinos*

A Mb antisense morpholino oligonucleotides (Mb-MO) was designed (Gene Tools). The Mb-MO sequence is:
5'-CAGAACCAGATCATGATCAGCCATC-3'

The Mo was dissolved in water to a 4mM stock solution which was stored at -80°C. The stock of the respective Mo was thawed and diluted in Danieau's solution (58 mmol/L NaCl, 7 mmol/L KCl, 0.4 mmol/L MgSO4, 6.0 mmol/L Ca(NO3)2, and 5.0 mmol/L HEPES pH 7.6) to the working condition of which 1nl was injected into the yolk of 1- to 2-cell stage embryos. For the experiments, a standard control morpholino was used with the sequence: 5 2 CCTCTTACCTCAGTTACAATTTATA 3 2. This oligo has no target and was shown to have no significant biological activity (Gene Tools, LLC).

Acknowledgements

We are grateful to the Weinberg and Stainier Labs for creating and making the Fli1-GFP and the Gut-GFP transgenic lines available. We further like to thank David de Witt for animal care.

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