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Hypoxia inhibits lymphatic thoracic duct formation in zebrafish

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ABSTRACT

Hypoxia promotes blood vessel growth through up-regulation of pro-angiogenic pathways but its role on the lymphatic system remains unclear. The homeobox transcription factor *Prox1* is a master control gene for generating lymphatic endothelial cells (LECs) and is up-regulated by hypoxia-inducible factors in mammals. While vascular endothelial growth factor A (VEGFA) is critical for angiogenesis, VEGFC and its receptor VEGF receptor-3 (VEGFR-3) are essential for the initial sprouting and directed migration as well as for the subsequent survival of LECs. The aim of this study was to determine the effects of hypoxia on the development of the lymphatic system in zebrafish. Zebrafish embryos were obtained from Tg(SAGFF27C; UAS:GFP) animals carrying a lymphatic reporter gene coupled to green fluorescent protein (GFP). Exposure of 1-day old zebrafish embryos to hypoxic conditions (5% O2) for 24 h inhibited thoracic duct formation (−27%, $p < 0.0001$). Hypoxia inhibited the expression of pro-lymphangiogenic factors *prox1a*, *vegfc* and *vegfr-3*. This inhibition was relieved after re-oxygenation. On the other hand, hypoxia increased the expression of *vegfa*, a pro-angiogenic factor. In conclusion, hypoxia has opposite effects on vascular development in zebrafish, inhibiting the development of the lymphatic vascular system while promoting the development of the blood vascular system.

1. Introduction

The lymphatic system has an important function in fluid homeostasis, fat absorption and immune surveillance. In addition, the lymphatic system plays a critical role in the onset and progression of pathological conditions such as cancer, lymphoedema and inflammation [1–3]. Despite having such important functions, the lymphatic vasculature has been far less studied than the blood vascular system, partially due to the difficulty in identifying and visualizing lymphatic vessels.

In the past decade, significant progress has been made in lymphatic vessel detection which has led to a better understanding of lymphatic system development. In mice, it was demonstrated that the transcription factor *Sox18* and the chicken ovalbumin upstream promoter-transcription factor II (*Coup-TFII*) play essential roles in the expression of prospero homeobox 1 (*Prox1*) [4,5]. *Prox1* is required for lymphatic endothelial cell (LEC) progenitors generation which arise mainly from venous endothelial cells [6,7]. Vascular endothelial growth factor (VEGF) C is a major regulator of lymphatic development and binds to VEGF receptor 2 (VEGFR-2) and VEGFR-3. VEGFR-2 is expressed in lymphatic vessels but its role in lymphatic development is still controversial [8]. It is known that VEGFC/VEGFR-3 signaling induces the proliferation, migration and survival of LECs which lead to the formation of the lymphatic system. The origin of LECs and lymphatic factors is largely conserved in vertebrates [9].

The transparent zebrafish embryo has emerged as a powerful tool to investigate vascular development [10,11]. Several transgenic lines showing the expression of lymphatic-specific markers have been developed and are useful for the visualization of the formation and migration of lymphatic vessels *in vivo* [12–14].

Hypoxia has a major impact on the blood vascular system and occurs in physiological situations such as during embryonic development, as well as in pathological conditions such as cancer and ischemia [15]. Hypoxia-inducible factors (*HIFs*) are transcription factors induced by hypoxia which activate the expression of a broad range of genes. Some of these genes such as vascular endothelial growth factor A (*VEGFA*), trigger the outgrowth of new capillaries to ensure an appropriate delivery of O2 and nutrients to the cells [16].

Although the role of hypoxia on blood vessel growth during development and disease has been extensively investigated, its role

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on the lymphatic system is far less understood. We have examined the impact of hypoxia on the development of the lymphatic system in zebrafish.

2. Materials and methods

2.1. Maintenance of zebrafish and generation of embryos

Wild-type AB and the transgenic zebrafish line Tg(SAGFF27C; UAS:GFP) carrying a lymphatic reporter gene coupled to GFP was used in this study [13]. Husbandry was conducted in accordance with the current European and local ethical guidelines for use of laboratory animals. All fish were housed at 28 ± 0.5 °C on a 14:10 h light:dark cycle as previously described [17]. Male wild-type AB and female transgenic fish were crossed and their offspring were collected and raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.1% Methylene Blue, pH 7.4). 22 h after fertilization, the eggs expressing the GFP reporter gene were separated from the other eggs and used for stereomicroscopy or confocal imaging. Remaining eggs were used for gene expression analysis. One day-old eggs were dechorionated by pronase treatment (2 mg/mL in E3 medium) (Roche Diagnostics GmbH, Mannheim, Germany), distributed into 6-well plates, and placed in E3 medium supplemented with 0.2 mM of 1-phenyl-2-thiourea (Sigma-Aldrich, Bornem, Belgium) to reduce melanogenesis and allow a better visualization of the lymphatic system in the trunk. Plates were either incubated in a hypoxic chamber (5% oxygen) (Galaxy® 48R, New Brunswick, Thermo Fisher Scientific, Asse, Belgium) or a normoxic chamber (atmospheric air) at 28 °C. Half of the medium was replenished every day with fresh medium pre-incubated in hypoxic or normoxic conditions overnight.

2.2. Fluorescence imaging

Embryos were anesthetized with tricaine (0.016%) (Sigma Aldrich) and embedded in the thermo-reversible mounting gel Cygel (Biostatus Limited, Leicestershire, UK) on a coverslip. The trunk lymphatic network was visualized at 5 day olds by stereomicroscopy (Leica, Howald, LU). Thoracic duct length and embryo size were measured blindly and manually on images obtained from the stereomicroscope using Image J software.

2.3. Gene expression analysis

Total RNA was extracted using TriReagent® (Sigma Aldrich) and the RNeasy® micro kit (Qiagen, Hilden, Germany). Potential contaminating genomic DNA was digested by DNase I treatment (Qiagen). One μg of total RNA was reverse-transcribed using the Superscript® II Reverse Transcriptase (Invitrogen, Merelbeke, Belgium). PCR primers were designed using the Beacon Designer software (Premier Biosoft, Palo Alto, USA) and were chosen to encompass an intron, when possible. Primers are shown in Table 1. PCR was performed using the CFX96 apparatus and the IQ™ SYBR® Green Supermix (Biorad, Nazareth, Belgium). 1/10 dilutions of cDNA were used for PCR. PCR conditions were as follows: 3 min at 95 °C, 30 s at 95 °C and 1 min annealing (40-fold). Optimal annealing temperature was determined for each primer pair. Melting point analysis was obtained after 80 cycles for 10 s from 55 °C to 95 °C. Elongation factor 1 alpha (*eef1a1*) was chosen as a housekeeping gene for normalization [18]. Expression levels were calculated by the relative quantification method (ΔΔCt) using the CFX Manager 2.1 software (BioRad) which takes into account primer pair efficiency.

2.4. Data analysis

Results are expressed as mean ± SD. Analysis was performed with Prism 5 (GraphPad software. Inc, San Diego, CA, US). Comparisons between two groups were performed with two-tailed paired *t*-test for data with a normal distribution and Mann-Whitney test for non-normally distributed data. Normality was determined using the Shapiro-Wilk test. A P value < 0.05 was considered significant.

3. Results

3.1. Hypoxia delays thoracic duct formation

One day-old embryos were incubated under hypoxic conditions (5% oxygen) for 24 h and returned to normoxia (atmospheric air) until the embryos were 5 days old (Fig. 1A), a time at which the lymphatic system in the trunk is normally fully developed. Control embryos were maintained under normoxic conditions for 5 days. Exposure to hypoxia for 24 h caused a 27% reduction in length of the thoracic duct compared to embryos grown under normoxia (Fig. 1B&C). Hypoxia did not affect embryo size at 5 days of development (Fig. 1D).

3.2. Expression of lymphangiogenic factors are down-regulated by hypoxia

To decipher the molecular modifications which occur under hypoxia, we assessed the expression levels of major actors of lymphatic development after 24 h of hypoxia (Fig. 2A). Hypoxia significantly decreased the expression levels of the two *prox1* zebrafish orthologs: *prox1a* and *prox1b* by 65% and by 77%, respectively (Fig. 2B&C). In addition, *vegfc* and its receptors *vegfr-3* were also significantly down-regulated in the hypoxic condition (Fig. 2D&E). These findings suggest that features of lymphatic development such as LEC specification, proliferation and migration could be affected by hypoxia.

3.3. Unlike lymphangiogenic factors, hypoxia increases the expression of angiogenic factors

To verify that the well-described pro-angiogenic effect of

Table 1
Quantitative PCR primers.

Gene	Accession number	Forward primer	Reverse primer	Annealing temperature (°C)
eef1α1	NM_001101	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	58
prox1a	NM_131405	AAGATGGCACAATAACAG	GATTCATGGCACTAAGAA	56
prox1b	NM_001171587	AACACCGCCATATAACAC	GAATCCATAGCCTCCTTT	60
vegfaa	NM_001110349	AAATGATGTGATTCCCTTC	GATACTCCTGGATGATGT	54
vegfab	NM_001044855	TGGAGATTAAGAGATTGA	GTTGTAGAGTGTGAATAG	56
vegfc	NM_205734	CTGGAGTAATCACTTATG	TATTCTTGTCTAGGTAATG	56
vegfr-3	NM_130945	AAATACATCCCAGTCAAA	GTTATCTTTAAACCAGGAC	56

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