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Zinc finger MYND-type containing 8 promotes tumour angiogenesis via induction of vascular endothelial growth factor-A expression

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ABSTRACT

Zinc finger, MYND-type containing 8 (ZMYND8) encodes a receptor for activated C-kinase protein. Here, we report that ZMYND8 promotes angiogenesis in prostate cancer xenografts in zebrafish, as well as tube formation in human umbilical vascular endothelial cell (HUVEC) cultures. Using transcriptome analyses, we found upregulation of ZMYND8 expression in both zebrafish prostate cancer xenografts and prostate cancer samples from patients. In vitro and in vivo ZMYND8 knockdown suppressed angiogenesis, whereas ZMYND8 overexpression enhanced angiogenesis. Notably, ZMYND8 induced *vegfa* mRNA expression selectively in prostate cancer xenografts. Integrated analysis of human and zebrafish transcriptomes, which identified *ZMYND8*, might be a powerful strategy to determine also other molecular targets for inhibiting prostate cancer progression.

1. Introduction

Because tumour-associated angiogenesis is crucial for solid malignancies including prostate cancer, inhibition of tumour neovascularization and/or destruction of tumour vasculature might maintain tumours in a dormant state or, in combination with cytotoxic therapies, may potentiate tumour shrinkage. Molecular targeted agents (mainly antagonists of vascular endothelial growth factor [VEGF] pathways) have been developed to block proangiogenic signal transduction. For example, bevacizumab, a humanized antibody against VEGF-A, was the first antiangiogenic agent to be approved for treatment of several advanced cancers [1]. Furthermore, sunitinib, a small molecule that blocks intracellular VEGF, KIT, Flt3, and platelet-derived growth factor receptors, regulates angiogenesis and cell growth, and has been approved for treatment of advanced renal cell cancer and malignant gastrointestinal stromal tumours [2]. However, there are still a limited number of target molecules to inhibit cancer progression including tumour angiogenesis and metastasis. To overcome this limitation, several approaches based on screening and omics technologies together with animal modelling have been developed for target discovery and validation.

Various animal models have been established to investigate angiogenic processes and identify proangiogenic and antiangiogenic compounds. Over the last few decades, the zebrafish as a disease model has emerged as a high throughput and cost effective alternative to other animal models, which has been used to assess the efficacy and toxicity of several chemical compounds [3,4]. In cancer research, xenografts of various cancer cell lines induce neovascularization in zebrafish [5–8]. Furthermore, a high degree of conservation of the pathways involved in tumourigenesis, angiogenesis, and metastasis has been proven in zebrafish and mammals [9,10]. Zebrafish also present powerful imaging solutions through in vivo fluorescent labelling of desired organs such as the vasculature system in combination with their body wall transparency [11]. Because the zebrafish is exploitable for genome-wide, loss-of-function analyses, we used human cancer-xenografted zebrafish in

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combination with DNA microarray analysis and morpholino antisense oligonucleotide (MO) knockdown, and found a new therapeutic target, zinc-finger MYND-type containing 8 (zmynd8), for inhibition of tumour angiogenesis.

2. Materials and methods

An expanded Methods section is available in the online-only Data Supplement.

2.1. Ethics approval

This study was approved by the Ethics Committee of Mie University. The procedures were performed according to the Japanese animal welfare regulation 'Act on Welfare and Management of Animals' (Ministry of Environment, Japan) and complied with international guidelines.

2.2. Chemicals

LY317615 and Ly333531 were purchased from Selleck Chemicals (Houston, TX, USA) and Tocris Bioscience (Bristol, UK), respectively. Stock solutions (10 mM) were prepared by dissolving the chemicals in dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO, USA). For anaesthesia, 100 ppm 2-phenoxyethanol (2-PE; Tokyo Kasei, Tokyo, Japan) was diluted in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$).

2.3. Zebrafish

The care and breeding of zebrafish followed previously described protocols [12]. Because of the transparency of their bodies, which facilitates in vivo monitoring of tumour angiogenesis, *nacre/fli1:egfp* zebrafish obtained by cross-breeding *nacre* mutants [13] and *fli1:egfp* transgenic zebrafish [11] were used in the experiments. At 3 days prior to xenotransplantation, individual female zebrafish were placed in mating tanks with males. The next morning, mating was initiated by light stimuli, followed by collection of the resulting fertilized eggs. The eggs were incubated in E3 medium at 28 °C. Preceding xenotransplantation, 48 h post-fertilization (hpf) embryos were dechorionated using a 2 mg/ml pronase solution (Roche Diagnostics, Mannheim, Germany) as described previously [12]. After dechorionation, the embryos were anaesthetized by immersion in 100 ppm 2-PE. Then, the embryos were arrayed onto an embryo-holding sheet for xenotransplantation procedures.

2.4. DU145-Kusabira orange (KOr) cell xenotransplantation

DU145 human prostate cancer cells were obtained from the RIKEN Cell Bank (Tokyo, Japan), and DU145 cells stably expressing Kusabira orange (KOr) (DU145-KOr cells) were established as described in Supplementary Methods. DU145-KOr xenotransplantation was conducted according to a previous report [8] with some modification. DU145-KOr cells (1×10^6 cells) were suspended in 30 μl Matrigel (BD Biosciences, San Jose, CA, USA) at 4 °C. The glass needles used to inject the cells were prepared from GD-1 glass capillaries (Narishige, Tokyo, Japan) using a PP-830 gravity puller (Narishige) and finely polished with an EG-44 microforge (Narishige). The avascular region of the yolk sac was then injected with 10 nl of the cell suspension containing 100–200 cells using a glass needle and FemtoJet injection system (Eppendorf, Hamburg, Germany). The xenografted zebrafish were subsequently incubated in E3 medium at 32 °C. At 48 hpi, 80–90% of the injected zebrafish exhibited tumours in their yolk sac.

2.5. Imaging of xenotransplants in zebrafish

Zebrafish were anesthetised with 100 ppm 2-PE. Images were then captured under a MZ16F stereoscopic microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DP71 digital camera (Olympus, Tokyo, Japan). For fluorescence imaging, we used GFP2 (for fli1:egfp) and DsRed (for KOr) filters. Tumour cell proliferation was calculated by the ratio of the KOr fluorescence intensity in the tumour area with ImageJ software (National Institutes of Health, Bethesda, MD, USA) as described previously [14].

2.6. cDNA synthesis and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from DU145-xenografted zebrafish at 48 h post-implantation (hpi) using Trizol reagent (Life Technologies, Carlsbad, CA, USA) in combination with a cleanup protocol (RNeasy Mini Kit; Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 200 ng total RNA using a Super Script III cDNA synthesis kit (Life Technologies) and random primers (Life Technologies). qRT-PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and a 7300 real-time PCR system (Applied Biosystems) as recommended by the manufacturer. The target gene was amplified using the primers shown in Table S1. Data were normalized to the mRNA level of beta-2-microglobulin (human B2M, NM_004048; zebrafish b2m, NM_001159768).

2.7. DNA microarrays

Total RNA was extracted from DU145-xenografted zebrafish at 48 hpi with or without tumour angiogenesis as described above. The samples for each condition were obtained from three independent experiments. Each experimental group included 5–10 xenografts. Detailed methods for the DNA microarray analysis are described in Supplementary Methods.

2.8. MOs

Based on the cDNA sequences in GenBank for zebrafish zmynd8 (XM_687794) and vascular endothelial growth factor aa (vegfaa) (NM_131498), MOs against zfZMYND8 (zfZMYND8 MO) and zfVEGFAa (zfVEGFAa MO) were synthesized by Gene Tools (Philomath, OR, USA). For the negative control, we used a control MO (human β-globin mutant sequence; GeneTools). The MO sequences are shown in Table S1. Each MO was injected into the yolk sac (48 hpf) with or without DU145-KOr cells using a fine glass needle connected to the FemtoJet automatic injector. To confirm the MO distribution, we injected lissamine-conjugated control MO (GeneTools) into 48 hpf embryos (Fig. S1). One hundred picoliters of 100 μM MO in water was injected in accordance with a previous report [15].

2.9. ZMYND8 mRNA synthesis and rescue

pCMV-SPORT6.1 carrying full-length human ZMYND8 cDNA was obtained from Open Biosystems (Huntsville, AL, USA) and subcloned into a pTnT vector (Promega, Madison, WI, USA). The full-length human ZMYND8 cDNA was then amplified by PCR, and the PCR products were used as templates for RNA synthesis. ZMYND8 mRNA was synthesized using a mMessage mMachine transcription kit for T7 RNA polymerase (Ambion, Austin, TX, USA). For the rescue experiment, a solution containing 0.5 ng/nl ZMYND8 mRNA and 8 ng zfZMYND8-MO was injected into 48 hpf embryos with xenotransplants.