The NADH Oxidase ENOX1, a Critical Mediator of Endothelial Cell Radiosensitization, Is Crucial for Vascular Development

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Abstract

ENOX1 is a highly conserved NADH oxidase that helps to regulate intracellular nicotinamide adenine dinucleotide levels in many cell types, including endothelial cells. Pharmacologic and RNA interference (RNAi)–mediated suppression of ENOX1 impairs surrogate markers of tumor angiogenesis/vasculogenesis, providing support for the concept that ENOX1 represents an antiangiogenic druggable target. However, direct genetic evidence that demonstrates a role for ENOX1 in vascular development is lacking. In this study, we exploited a zebrafish embryonic model of development to address this question. Whole-mount in situ hybridization coupled with immunofluorescence performed on zebrafish embryos demonstrate that enox1 message and translated protein are expressed in most tissues, and its expression is enriched in blood vessels and heart. Morpholino-mediated suppression of Enox1 in Tg(fli1-eGFP) and Tg(flk1-eGFP) zebrafish embryos significantly impairs the development of vasculature and blood circulation. Using in vivo multiphoton microscopy, we show that morpholino-mediated knockdown of enox1 increases NADH levels, consistent with loss of enzyme. VJ115 is a small-molecule inhibitor of Enox1’s oxidase activity shown to increase intracellular NADH in endothelial cells; we used VJ115 to determine if the oxidase activity was crucial for vascular development. We found that VJ115 suppressed vasculogenesis in Tg(fli1-eGFP) embryos and impaired circulation. Previously, it was shown that suppression of ENOX1 radiosensitizes proliferating tumor vasculature, a consequence of enhanced endothelial cell apoptosis. Thus, our current findings, coupled with previous research, support the hypothesis that ENOX1 represents a potential cancer therapy target, one that combines molecular targeting with cytotoxic sensitization.

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Introduction

Because of its critical role in promoting tumor growth, neovascularization represents a therapeutic pathway worthy of exploitation (1). However, targeting can be challenging because vascularization is not restricted to a single process. Tumors can use several processes, including sprouting angiogenesis, vasculogenesis, and/or vascular mimicry (2). This diversity of options may help to explain why current antiangiogenic monotherapy strategies can improve the duration of progression-free survival, but has not increased overall survival (3). Overcoming this problem requires a comprehensive understanding of the pathways that regulate vascularization.

Evolving research indicates that adaption of endothelial cell metabolism to the tumor microenvironment is a critical step in tumor vascularization (4). Proangiogenic tumor microenvironments can subject endothelial cells to conditions that require reprogramming from respiration to glycolytic ATP production (4). Rapidly changing endothelial cell bioenergetic demands may require a switch to glutamine anaplerosis (4). These, as well as important ribosylation and deacetylation pathways (5), are driven by NAD+ and NADH, whose levels are maintained by both redox recycling and biosynthesis. Failure to maintain nicotinamide adenine dinucleotide homeostasis can impair ATP production, polyADP ribosylation, and NAD+–dependent deacetylation. Although poorly understood, NAD+ has been shown to promote endothelial cell migration (6), whereas the NAD biosynthetic inhibitor FK866/K22.175 has been found to be antiangiogenic (7). Thus, one can conceptualize that NAD cofactors support vasculogenesis and angiogenesis (5), two important processes that contribute to tumor neovascularization.
ENOX1 is a NADH oxidase that regulates NAD homeostasis through oxidization of NADH to NAD⁺ (8). The gene is highly conserved in vertebrates (98% identity between *H. sapiens* and *M. musculus*; 72% identity between *H. sapiens* and *D. rerio*). Purified enzyme exhibits NADH oxidase and protein disulfide-thiol interchange activity, with a periodicity of 24 minutes (9). However, we have only been able to validate the presence of the oxidase activity in vivo (10). The enzyme resides at the ectosomal surface of the plasma membrane. Differential detergent fractionation studies reveal that ENOX1 is also present in the cytoskeletal compartment of endothelial cells (unpublished data). RNAi-mediated suppression of ENOX1 in human or mouse endothelial cells inhibits migration and the ability to form tubule-like structures in Matrigel (11). VJ115 is a (Z)-(+/−)-2-(1-benzylindol-3-ylmethylene)-1-azabicyclo[2.2.2]octan-3-ol that inhibits ENOX1’s NADH oxidase activity, and therefore significantly increases intracellular NADH (10, 11). VJ115 inhibits formation of tubule-like structures by endothelial cells in Matrigel and tumor cell–mediated neoangiogenesis, as assessed in a dorsal skinfold vascular window model (11). Administration of daily injections of VJ115 to mice with syngeneic Lewis lung carcinoma (LLC) xenografts produces the same degree of tumor growth delay as fractionated x-irradiation (11). We thus reasoned that ENOX1 represents a potential druggable target for the inhibition of angiogenesis/vasculogenesis.

Zebrafish embryos represent a transparent and genetically tractable model for the study of angiogenesis and vasculogenesis. The dorsal aorta and posterior cardinal vein are first formed by vasculogenesis. Then, secondary vessels, such as intersegmental vessels, are formed by classic sprouting angiogenesis (2). Herein, we used two transgenic zebrafish embryonic models, *Tg(fli1:egfp)* and *Tg(ki1:egfp)*, to establish a role for ENOX1 in vascularization. We then demonstrate that pharmacologic targeting of the oxidase inhibits vascularization and blood flow.

**Materials and Methods**

**Zebrafish stocks and growth conditions**

Zebrafish strains *ab*, *Tg(fli1:egfp)*, and *Tg(ki1:egfp)* were raised in Vanderbilt’s Zebrafish facility. Briefly, all fish were grown at 28°C under standard conditions according to the policies and procedures of the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

**Morpholinos**

Morpholino phosphorodiamidate oligonucleotides that targeted two separate intron–exon boundary sequences of *enox1* were designed by Gene Tools. The sequences were as follows: M01, 5′-AGATCTGGGCAATCCCCAAAAAATGC-3′ and M02, 5′-TAATCTGCTCAACTTTTGTTGCTAA-3′. Gene Tools control nonsilencing morpholino sequence was 5′-CCTCTTACCTCAGTACATTATA-3′. Morpholinos were dissolved in distilled water and 4 ng was injected into one-cell stage embryos. Injection of control morpholinos did not produce a phenotype when compared with uninjected embryos (data not shown).

**In vivo multiphoton microscopy of NADH**

Microscopy was performed as described previously (12). A custom built, commercial multiphoton fluorescence microscope (Prairie Technologies) was used to acquire autofluorescence images of NADH with a ×40 water-immersion objective [1.15 numerical aperture (NA)]. A titanium:sapphire laser (Coherent Inc.) provided excitation light at 750 nm with an average power at the sample of 7.5 to 7.8 mW. A GaAsP PMT (H7422P-40; Hamamatsu) was used to detect emitted photons through a 400- to 480-nm bandpass filter. A pixel dwell time of 4.8 µs was used for scanning each 256 × 256-pixel image. Each image was captured and averaged eight times to reduce noise. For analysis, NADH fluorescence images were set to a threshold to remove background and nuclear fluorescence. The average NADH fluorescence intensity per cell was computed with ImageJ software.

**Whole-mount in situ hybridization**

Wild-type embryos were grown to the desired developmental stage in 0.2 mmol/L phenylthiourea to inhibit pigmentation. The embryos were fixed overnight in 4% paraformaldehyde (PFA) followed by dehydration in increasing concentrations of methanol, stage-dependent treatment with proteinase K and hybridization with Dig-labeled *enox1* antisense RNA probe. Antisense probes were generated with T7 RNA polymerase from a *HindIII*-linearized vector (Open Biosystems; clone id, EXELIXIS853870) containing an approximately 700 bp fragment of *enox1*.

**Enox1 antibody**

In collaboration with the Vanderbilt Antibody Core and Covance Inc., a custom ENOX1 antibody was raised by injecting rabbits with antigen conjugated with ENOX1 specific peptide [H]-CKEEQSHTQALLKVLQEQLKGTK-[NH2] (10).

**Immunofluorescence**

Embryos were fixed in 4% PFA at 4°C overnight and then treated in 30% sucrose at 4°C, followed by embedding in OCT medium and sectioning. Zebrafish cryosections were incubated with 1:300 diluted primary antibody against Enox1 followed by 1:300 Alexa Fluor 555 fluorescently conjugated secondary antibody (Molecular Probes). DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear counterstaining. Confocal images were taken with an Olympus FV-1000 inverted confocal microscope (Vanderbilt Cell Imaging Shared Resource).

**Quantitative real-time PCR**

Total RNA was extracted from 50 embryos at different embryonic developmental stages and cDNA synthesis and real-time PCR using SYBR Green Supermix (Bio-Rad) was performed. The following primers were used to amplify *enox1*: F, 5′-TTGGGCTTCTTGGAGATC-3′ and R, 5′-GGCAGTCGACATTAATCT-3′. All experiments were performed in triplicate.

**Western blotting**

Embryos at different developmental stages were deyolked by scraping them off using a sterile syringe, and cell lysates were
obtained using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (Sigma). The egg yolk was removed before homogenization. Protein concentration was measured using Bio-Rad protein assay reagent. Standard Western blotting protocol was followed using Enox1 antibody. Twenty-five embryos were used for protein extraction for each time point.

Methods for fluorescence-activated cell sorting (FACS) and RNA Seq library preparation, sequencing, and transcriptome analysis are available as Supplementary Methods.

Results and Discussion

We generated an antibody to ENOX1 (10) that detects human ENOX1, murine Enox1, and zebrafish Enox1, whereas preimmune sera do not (Supplementary Fig. S1A and S1B). The ability to detect human ENOX1 using the ENOX1 antibody was validated using ENOX1 short hairpin RNA (shRNA; Supplementary Fig. S1C). Morpholinos, designed against two different regions in the enox1 gene and injected at the one-cell stage significantly suppressed expression of enox1 mRNA, as determined by quantitative real-time PCR (qRT-PCR) performed 48 hours post fertilization (hpf; \( P < 0.005 \), Student t test). Immunoblotting validated morpholino-mediated suppression of zebrafish Enox1. Representative data are shown in Supplementary Fig. S1D and S1E.

We evaluated Enox1 expression in zebrafish embryos. Whole-mount in situ hybridization using an enox1 antisense RNA probe to exons 1 to 6 revealed expression of enox1 mRNA in longitudinal axial vessels (i.e., dorsal aorta/posterior cardinal vein), 24 hpf, and in heart 48 hpf (Fig. 1A and B). Confocal immunofluorescent imaging of Enox1, 72 hpf, revealed expression of Enox1 in a number of tissues (Fig. 1C), including heart (Fig. 1C), tail, and chondrocytes (Supplementary Fig. S2).

The transcription factor \( \beta l 1 \) is one of the earliest and well-validated indicators of vasculature development (13). We made a single-cell suspension from 24 hpf \( Tg(Fli1:GFP) \) embryos, immunostained the cells with Enox1 antibody, and quantified the number of cells that expressed both eGFP and Enox1 immunofluorescence by FACS. The analysis indicated that more than 90% of Fli1-GFP–expressing cells coexpressed Enox1 (Fig. 1D).

We used multiphoton fluorescence microscopy, which records intrinsic NADH fluorescence intensity in live cells (12), to determine NADH levels (Fig. 2). Whole-mount in situ hybridization (Fig. 1), RT-PCR, and immunoblotting (data not shown) established that Enox1 was expressed 24 hpf, a time where optical clarity is excellent. Two areas in each embryo’s tail were analyzed. Suppression of Enox1 was accompanied by increased NADH levels in enox1 morphants compared with control (\( P < 0.05 \), rank-sum test; Fig. 2B), a result that can be attributed to loss of Enox1 oxidase activity.

Whole-transcriptome sequencing of 20 enox1 morphant and 20 control embryos was undertaken at 48 hpf. Expression of 867 transcripts in enox1 morphants exhibited a log2 fold change (\( P < 0.05 \), cutoff at 5% false discovery rate as a threshold; Supplementary Dataset S1). The PANTHER 8.1 classification system (14) mapped 549 of the 608

![Figure 1. Expression of Enox1 in zebrafish embryos. A and B, in situ hybridization of enox1 antisense probe at the longitudinal axial vessels (A) and heart (B). C, confocal imaging of Enox1 Alexa 488 green at 72 hpf. D, single-cell suspensions of 24 hpf \( Tg(Fli1:GFP) \) embryonic cells analyzed by FACS for GFP fluorescence and/or Enox1 Rhodamine Red immunofluorescence. Y, yolk; H, heart.](image-url)
downregulated transcripts; 43% (236) were assigned to GO:0008152, metabolic processing (Supplementary Dataset S2). Of the 259 upregulated transcripts, 224 were mapped and 53% (119) were assigned to GO:0008152 (Supplementary Dataset S3). This analysis suggests that Enox1 activity supports metabolic processes.

As the transcription factor Fli1 is a well-characterized endothelial marker (15), we examined flk-1 promoter–driven eGFP vascular expression in control, MO1, and MO2 Tg(fli1:eGFP) embryos. Seventy-five percent of morphants did not express discernible eGFP expression ($P < 0.001$, Fisher exact test; see Fig 3A for example). The remaining 25% of the enox1 morphants exhibited a partial loss of vasculature, defined as embryos that expressed longitudinal axial vessels but failed to develop intersegmental arteries and veins (see Supplementary Fig. S3A and Supplementary Videos for example). The zebrafish flk-1 gene encodes a receptor tyrosine kinase that was used as second marker of vasculogenesis. Examination of flk-1 promoter–driven eGFP vascular expression at 48 hpf in control and enox1 Tg(flk1:eGFP) morphants indicated that 45% of the morphants did not have discernible eGFP expression ($P < 0.001$, Fisher exact test). The remainder exhibited a partial loss, as defined earlier (Fig. 3B). Nomarski Interference contrast microscopy was used to assess morphology (Supplementary Fig. S3B). The presence of intersegmental vessels in control embryos (blue arrows) is diminished in enox1 morphants (red arrow). Differential interference contrast microscopy (DIC) images illustrate the presence of pericardial oedema, a finding that is consistent with the morphologic defects found in VEGF-A morphant zebrafish (15). These data and previous research that demonstrated that Eno1 shRNA suppressed mammalian endothelial cell proliferation and migration (11) suggest that Eno1 plays a critical role in vasculature development. However, Eno1 is expressed in multiple tissues and the morpholinos are not endothelial specific. Thus, we cannot rule out a role for Eno1-mediated paracrine signaling.

Video microscopy was used to obtain further evidence that Eno1 is crucial for vascular function. Movies comparing circulation in trunk/tail of control and enox1 morphants are provided as Supplementary Video S1 and S2 and demonstrate significant reduction in circulation in enox1-depleted animals.

Expression of transcription factors and membrane receptors critical for vascular development (16), such as etvc, gata2a, fli1a, fli1b, or flk1 were not affected by loss of Eno1 ($P > 0.05$; Supplementary Dataset S4). However, the PANTHER analysis indicated that reprogramming in enox1 morphants resulted in upregulation of the antiproliferation

Figure 2. Intracellular NADH is increased in enox1 morphants compared with control embryos. A, representative Nomarski interference contrast microscopy image of 24 hpf zebrafish embryo. Two areas of interest are noted. B, in vivo multiphoton microscopy was used to measure NADH fluorescence at a depth of 20 micrometers in area 1 and 2 ($n = 6$ embryos, 3 fields per embryo). * $P < 0.05$.

Figure 3. Loss of fli1- or flk1 promoter–driven eGFP vascular expression in enox1 morphants. A, vascular eGFP expression at 72 hpf in control ($n = 201$) or enox1 morphant (MO1, $n = 207$) Tg(fli1:eGFP) embryos. B, vascular eGFP expression at 48 hpf in control ($n = 201$) or enox1 morphant (MO1, $n = 209$) Tg(flk1:eGFP) embryos. Y, yolk. White arrowheads, expression of eGFP in intersegmental vessels.
inhibit vascular development in exposure of HUVECs to VJ115 suppressed expression of structures in Matrigel (11). Proteomic profiling revealed that expression of proangiogenic proteins ENSDARG00000056795, PAI-1 (increased 4.67-fold, \( P = 0.0015 \); ref. 17), as well as repression of antiangiogenic proteins ENSDARG0000033655, Stathmin (decreased 5.8-fold, \( P = 0.028 \); ref. 18) and ENSDARG0000060597, Phosphatidylinositol-3-kinase (decreased 4-fold, \( P = 0.0064 \); Supplementary Dataset S1; ref. 19). Although the exact mechanism by which Enox1 contributes to vasculogenesis is not yet known, the PANTHER analysis suggests the hypothesis that suppression of Enox1 impacts multiple pathways, which in aggregate impair vasculogenesis and angiogenesis.

VJ115 is a small-molecule inhibitor of ENOX1’s NADH oxidase activity. The compound produces significant radiation sensitization of LLC and HT29 xenografts (11). VJ115 also inhibits LLC-induced neangioinvasion in a mouse window model (11) and phenocopies ENOX1 RNAi effects with regard to inhibiting human umbilical vein endothelial cell (HUVEC) migration and inhibiting formation of tubule-like aggregates (11) and phenocopies ENOX1 RNAi effects with regard to inhibiting human umbilical vein endothelial cell (HUVEC) migration and inhibiting formation of tubule-like

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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