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Knocking down 10-formyltetrahydrofolate dehydrogenase increased oxidative stress and impeded zebrafish embryogenesis by obstructing morphogenetic movement☆

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ABSTRACT

Background: Folate is an essential nutrient for cell survival and embryogenesis. 10-Formyltetrahydrofolate dehydrogenase (FDH) is the most abundant folate enzyme in folate-mediated one-carbon metabolism. 10-Formyltetrahydrofolate dehydrogenase converts 10-formyltetrahydrofolate to tetrahydrofolate and CO_2, the only pathway responsible for formate oxidation in methanol intoxication. 10-Formyltetrahydrofolate dehydrogenase has been considered a potential chemotherapeutic target because it was down-regulated in cancer cells. However, the normal physiological significance of 10-Formyltetrahydrofolate dehydrogenase is not completely understood, hampering the development of therapeutic drug/regimen targeting 10-Formyltetrahydrofolate dehydrogenase.

Methods: 10-Formyltetrahydrofolate dehydrogenase expression in zebrafish embryos was knocked-down using morpholino oligonucleotides. The morphological and biochemical characteristics of fdh morphants were examined using specific dye staining and whole-mount in-situ hybridization. Embryonic folate contents were determined by HPLC.

Results: The expression of 10-formyltetrahydrofolate dehydrogenase was consistent in whole embryos during early embryogenesis and became tissue-specific in later stages. Knocking-down fdh impeded morphogenetic movement and caused incorrect cardiac positioning, defective hematopoiesis, notochordmalformation and ultimate death of morphants. Obstructed F-actin polymerization and delayed epiboly were observed in fdh morphants. These abnormalities were reversed either by adding tetrahydrofolate or antioxidant or by co-injecting the mRNA encoding 10-formyltetrahydrofolate dehydrogenase N-terminal domain, supporting the anti-oxidative activity of 10-formyltetrahydrofolate dehydrogenase and the in vivo function of tetrahydrofolate conservation for 10-formyltetrahydrofolate dehydrogenase N-terminal domain.

Conclusions: 10-Formyltetrahydrofolate dehydrogenase functioned in conserving the unstable tetrahydrofolate and contributing to the intracellular anti-oxidative capacity of embryos, which was crucial in promoting proper cell migration during embryogenesis.

General significance: These newly reported tetrahydrofolate conserving and anti-oxidative activities of 10-formyltetrahydrofolate dehydrogenase shall be important for unraveling 10-formyltetrahydrofolate dehydrogenase biological significance and the drug development targeting 10-formyltetrahydrofolate dehydrogenase.

Abbreviations: FDH, 10-formyltetrahydrofolate dehydrogenase; THF, tetrahydrofolate; dpf, day-post-fertilization; hpf, hour-post-fertilization; MO, morpholino oligonucleotide; WISH, whole-mount in situ hybridization; FDH-N-ter, FDH N-terminal domain

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1. Introduction

Folate is a B vitamin essential for embryonic development and heavily involved in the metabolism of many fundamental biomolecules, including purines, choline, amino acids, neurotransmitters and S-adenosylmethionine (SAM), the major methyl donor for most intracellular methylation reactions. Insufficient folate has been associated with many congenital anomalies including heart and neural tube defects [1]. In cells, tetrahydrofolate carries a one-carbon unit at either the N5 and/or N10 positions, forming different folate adducts. These folate adducts participate in one-carbon metabolic (OCM) pathways and are interconverted via several redox and synthetic reactions catalyzed by several folate enzymes. Among all the folate enzymes participating in this pathway, 10-formyltetrahydrofolate dehydrogenase (10-formyltetrahydrofolate:$NADP^+$ oxidoreductase; FDH or ALDH1L1; E.C.1.5.1.6) is the most abundant one, comprising 1% of total soluble protein in the rabbit liver [2]. FDH catalyzes the conversion of 10-formyltetrahydrofolate (10-formyl-THF) to tetrahydrofolate (THF) and CO_2 (Eq. 1). Therefore, FDH was proposed to regulate the ratio of these two major intracellular reduced folate pools.

Mammalian FDH is a homotetramer consisting of four identical 99 kD subunits. Each subunit is composed of a large C-terminal domain and a small N-terminal domain connected by a linking peptide. The proper alignment to bring both N- and C-terminal domains in a correct orientation is required for 10-formyltetrahydrofolate dehydrogenase activity [3]. The large C-terminal domain contains a $NADP^+$ binding site and has aldehyde dehydrogenase activity (Eq. 2) [2,4]. This aldehyde dehydrogenase activity was not found in zebrafish FDH [5]. The small N-terminal domain contains a 10-formyl-THF binding site and catalyzes a 10-formyl-THF hydrolysis reaction (Eq. 3). The physiological significance of this hydrolase activity remains unclear. One proposal is that this $NADP^+$-independent hydrolase activity is to produce THF irrespective of the redox state (NADPH/$NADP^+$ ratio) in the cell [6]. On the N-terminal domain also resides a non-catalytic THF tight binding site, which partially overlaps with the substrate catalytic site [7]. Currently, the function of this non-catalytic site is unknown.

$$\text{10-formyl-THF} + NADP^+ \rightarrow \text{THF} + CO_2 + \text{NADPH} \quad (1)$$

$$\text{Propanal} + NADP^+ \rightarrow \text{propanoate} + \text{NADPH} \quad (2)$$

$$\text{10-formyl-THF} \rightarrow \text{THF} + \text{formate} \quad (3)$$

The physiological significance of FDH is not completely understood. The clinical importance of FDH was first recognized in methanol detoxification, in which FDH is responsible for the ultimate removal of formate [8]. However, this could not be considered an adequate reason for FDH to exist in such a high quantity, leaving the biological significance of FDH under normal physiological conditions an unanswered question. Sequestering unstable THF for storage and regulating free THF availability is one of the functions postulated for FDH [9,10]. The specific expression of FDH in the radial glia at the midline of neural tube during murine embryogenesis suggested a role for FDH in neural tube formation, although loss-of-function FDH mutations did not impair neural tube closure [11]. Homozygous mice lacking FDH appeared completely normal but exhibited a reproductive deficiency, which was attributed to a possible increase of fetal lethality [12]. Recently, overexpressing FDH has been shown to evoke metabolic stress and cause apoptosis in cancer cells, shedding light on the possibility of FDH being a chemotherapeutic target [13–15]. Nevertheless, the biological significance of FDH remains incompletely understood, especially under normal physiological condition, hampering the development of therapeutic drug/regimen targeting FDH.

Zebrafish has been a prominent vertebrate model for biological and medical research. Considering its features of transparent embryos, external and rapid development and high throughput, zebrafish could serve as a valuable complementary in vivo platform for folate-related studies. Several zebrafish folate enzymes, including FDH, had been characterized and shown to resemble mammalian orthologs, supporting the use of zebrafish to model folate-mediated one-carbon metabolism [5, 16–21]. In this study, we report the spatially specific but temporally constant expression of FDH during embryogenesis. We examined the biological significance of FDH in develo\ping embryos using knock-down strategies. The potential physiological function of FDH is also discussed.

2. Materials and methods

2.1. Materials

The SMART™ RACE amplification kit was purchased from Clontech, Inc. (California, US). Enzymes used for cloning were purchased from New England BioLabs, Inc. (Maryland, US). The HPLC gel filtration column Alltech ProSphere SEC, 250 HR, S-200 (4.6 mm × 30.0 cm) was purchased from Alltech (Illinois, US). 10-Formyl-5,8-dideazafolate-ω-Aminohexyl-Sepharose 4B affinity column was prepared as previously described [2]. Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology Inc. (California, US). Folates were generous gifts from Dr. Moser (Merck Eprova AG, Switzerland). The AB strain zebrafish embryo was a generous gift from Dr. Hans Georg Frohnhöfer (Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany). The cRNA expression vector pcGlobin2 was a gift from Dr. Myungchull Rhee (Chungnam National University, Daejeon, Korea). All other chemicals, including coenzymes, buffers, amino acids, and antibiotics, were purchased from Sigma-Aldrich Chemical Co. (Montana, US). The *Escherichia coli* strains and vectors used for cloning and protein expression have been described previously [5].

2.2. Fish care and sample preparation for Western blot and RT-PCR analysis

Zebrafish (*Danio rerio*, AB strain) were bred and maintained in a 14–10 h light–dark diurnal cycle following the standard procedure [22]. Embryos were staged according to Kimmel et al. [23]. The animal studies and all procedures for handling zebrafish and embryos, including breeding and maintenance of fish and sample collection, were approved by Affidavit of Approval of Animal Use Protocol of National Cheng Kung University (IACUC Approval NO. 96062). Embryos and tissues obtained from adult zebrafish were prepared for Western blot analysis and RT-PCR to determine cytosolic FDH (CFDH) levels and distribution as previously described [18]. The dominant yolk proteins in embryos before 24-hpf were removed for Western blot analysis to avoid interference [24]. The purified rabbit anti-zebrafish cytosolic FDH-N antibody was used at 1:500 dilutions. Rabbit anti-actin antibody was used as a loading control. The primer pairs used for RT-PCR were as follows: 5′-CGCTGAGC ATATGAGGGTGGTG-3′(forward) and 5′-GGTATAGACTGCTCCCGAG-3′(reverse) for zebrafish CFDH (570-bp fragment) and 5′-AGACATCA AGGAGAAGCTGTG-3′(forward) and 5′-TCCAGACGGAGTATTTAC-3′(reverse) for β-actin (391-bp fragment) as a control for the RNA isolation and reverse-transcription.

2.3. Whole mount F-actin staining

Embryos were fixed overnight in 4% paraformaldehyde at 4 °C. Embryos were washed in 0.1% PBSTx and dechorionated. They were then incubated for 1 h in 0.5% PBSTx, followed by 4-hour incubation in blocking buffer (PBS, 1% BSA, 10% goat serum, 1% DMSO, 0.1% Triton X100). Embryos were then incubated overnight at 4 °C in blocking solution containing phalloidin or primary antibody. Alexa 488 phalloidin (molecular probe) was used at 1:400 dilutions. Images were observed under a multi-photon confocal microscope (Olympus) and acquired with the software FluoView FV1000.