Folic acid exerts dose-dependent biphasic effects on cardiac development of zebrafish embryos

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Abstract

Folic acid, one of the 13 essential vitamins, plays an important role in cardiovascular development. Mutations in folic acid synthesis gene 5,10-methylenetetrahydrofolate reductase (MTHFR) is significantly associated with the occurrence of congenital heart disease. However, the mechanisms underlying the regulation of cardiac development by mthfr gene are poorly understood. Here, we exposed zebrafish embryos to excessive folate or folate metabolism inhibitors. And we established a knock-out mutant of mthfr gene in zebrafish by using CRISPR/Cas9. The zebrafish embryos of insufficient or excessive folic acid, and *mthfr*$^{-/-}$ mutant all gave rise to early pericardial edema and cardiac defect at 3 days post fertilization(dpf). Furthermore, the folic acid treated embryos showed abnormal movement at 5dpf. The expression levels of cardiac marker genes *hand2*, *gata4* and *nppa* changed in the abnormality of folate metabolism embryos and *mthfr*$^{-/-}$ mutant, and there is evidence that they are related to the change of methylation level caused by the change of folate metabolism. In conclusion, our study provides a novel model for the in-depth study of *MTHFR* gene and folate metabolism. And our results reveal that folic acid has a dose-dependent biphasic effect on early cardiac development.

Key words: *mthfr*; folic acid; heart development; zebrafish; CRISPR/Cas9

Introduction

Folic acid plays a vital role in cardiovascular development as it is an important vitamin necessary for methylation reaction, nucleotide synthesis and maintaining homocysteine at non-toxic level. Insufficient folate metabolism will lead to methionine circulation obstruction and Hyperhomocysteinemia (Hhcy) . Hhcy is an independent risk factor for congenital heart disease (CHD) . Some studies have shown that folic acid deficiency can also affect development of brain, liver and other organs, leading to the occurrence of various diseases . In the world, taking folic acid supplementation during pregnancy to prevent the occurrence of various congenital diseases has become a consensus. But there are still 15-20% of pregnant women due to the combined use of diet and folic acid supplements, resulting in 1-4 times the dose of excessive folic acid supplementation . Therefore, it is very important to evaluate the effects of folic acid deficiency and excess on organ development.

The metabolism of folic acid includes the transformation of exogenous folic acid into 5-methyltetrahydrofolate (5-MTHF) and the methylation of 5-MTHF into Hcys . As a carrier of one carbon unit, folic acid mediates the transfer of one carbon unit in the form of coenzyme in the process of amino acid metabolism and mutual transformation between methionine and Hcys ; These reports indicate that folic acid plays an important role in the early development of biological organs, especially in the development of neural tube. However, it is still unclear whether the excessive and deficient folate will affect the development of heart and the mechanism of folate affecting the development of organs including heart.

Results

1. Folic acid has biphasic effects on early heart development in zebrafish

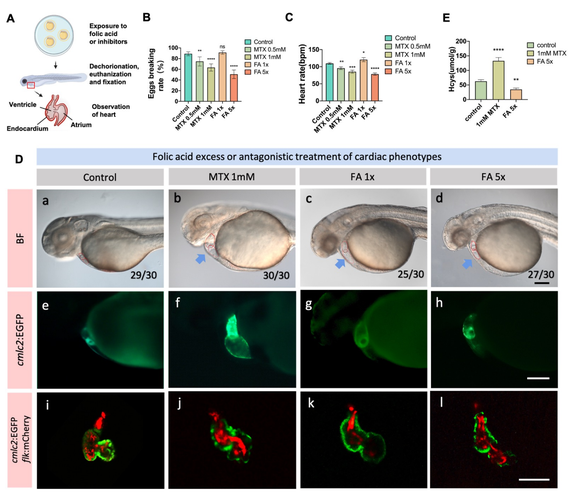

Figure 1 Folate excess and folate deficiency can lead to different degrees of abnormal development of cardiac physiological function and abnormal metabolism of homocysteine.
(A) Treatment of zebrafish embryos with folic acid and folic acid inhibitors. (B) The rate of membrane rupture of zebrafish embryos treated with folic acid and folic acid inhibitor was statistically analyzed. (C) The rate of membrane rupture of zebrafish embryos treated with folic acid and folic acid inhibitor was statistically analyzed. (D) The phenotypes of zebrafish 3dpf embryonic heart after different concentrations of folic acid and MTX inhibited folate metabolism pathway were demonstrated (E) High homocysteine content in zebrafish of different groups.

The cardiac development of zebrafish was observed at 36 hpf. The results showed that folic acid inhibitor could make the pericardial cavity of zebrafish embryos swell and the ventricles expand in different degrees. Confocal images showed that zebrafish heart looping was abnormal after different gradients of folic acid and MTX folic acid antagonist treatment. At the same time, the structure of cardiac chamber also changed, and the thickness of myocardial wall also changed more and more obviously with the change of treatment dose.

2.CRISPR/Cas9 mediated *mthfr* gene knock-out model in zebrafish

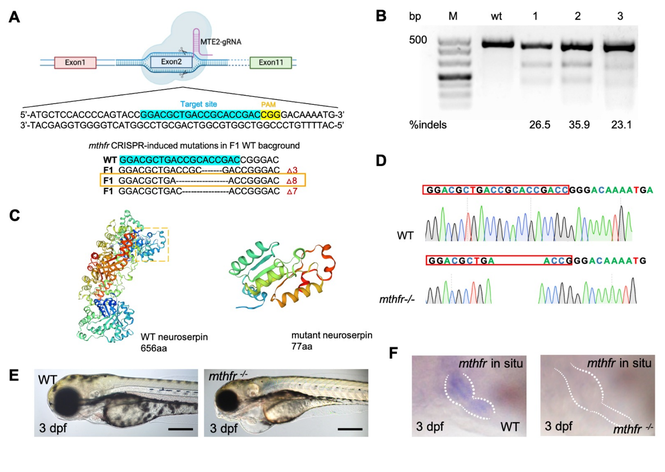

Figure 2 Generation of *mthfr* mutant using the CRISPR/Cas9 system.
(A) Design of sgRNA target in exon 2 of zebrafish mthfr gene. The mutation type of code shift selected by orange frame is selected finally. (B) Two short DNA fragments were obtained by T7E1 digestion. In the control group, 437 bp fragment was amplified from wild-type embryos with the same set of primers. (C) The predicted truncation of Mthfr protein. (D) Sequencing map of homozygous zebrafish adults. (E) Bright-field views of *mthfr*$^{-/-}$ zebrafish showed Pericardial enlargement, ventricular enlargement and abnormal heart looping. Scale bars: 500μm. (F) The results of in situ hybridization showed that the expression of mthfr gene in homozygous mutant zebrafish was changed.

We constructed the mthfr gene knock-out mutant by using CRISPR/Cas9 in zebrafish. *mthfr* gene was knocked out by a specific sgRNA target on exon 2. Microinjection of mthfr sgRNA binding Cas9 protein into the fertilized single-cell embryos was performed. One fragment was amplified with primers spanning the exon 2 gRNA sequence of *mthfr,* and two shorter DNA fragments were obtained by T7E1 digestion. In the control group, a 437 bp fragment was amplified from wild-type embryos. Compared with wild-type zebrafish embryos, *mthfr* $^{-/-}$ mutant zebrafish embryos have obvious abnormal cardiac development. Pericardial enlargement, ventricular enlargement and abnormal heart looping were found. In situ hybridization showed that *mthfr* gene was expressed at 3dpf wild-type zebrafish heart. The expression of mthfr gene decreased significantly in *mthfr* $^{-/-}$ mutant.

3. Folic acid deficiency and excessive supplement lead to the changes of heart related genes

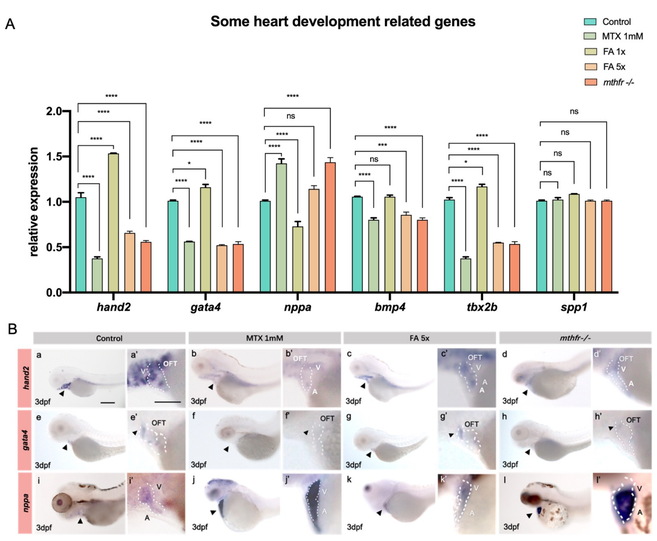

Figure 3 Abnormal folate metabolism results in changes of zebrafish embryo heart development gene expression.
(A) Results of Q-PCR for *hand2, nkx2.5, gata4, bmp4, tbx2b, spp1, hoxb1a, npp*a in the embryonic heart of zebrafish in each group. (B) In situ hybridization showed the expression of *hand2, gata4, nppa* in different groups of 3dpf zebrafish embryonic heart. A: atria V: ventricle OFT: outflow tract. n=3. Scale bars: 200μm.

The expressions of *hand2, gata4, nppa, bmp4, tbx2b, sp*p1 in the heart of zebrafish embryos at 3dpf were detected semi quantitatively by Q-PCR. Compared with the untreated control group, the changes of folate supplement and the expression of genes related to heart development in *mthfr* $^{-/-}$ mutant embryos were different .

According to the results of Q-PCR and the previous abnormal phenotype of heart, selected *hand2, gata*4 and *nppa* as the key genes of heart development and indicators for in situ hybridization. The expression of *hand2* in zebrafish embryonic heart was decreased in folic acid antagonistic treatment group, folic acid excess supplement group and *mthfr* $^{-/-}$ mutation group. In wild-type zebrafish embryos, *gata4* was strongly expressed in cardiac outflow tract (OFT) and AVC . Similarly, the expression of *gata4* gene decreased with the metabolism of folic acid In zebrafish embryonic heart, *nppa* gene expression was up-regulated with the decrease of folate metabolism.

4. The abnormal methylation of *hand2* and *gata4* were caused by folate metabolism changes

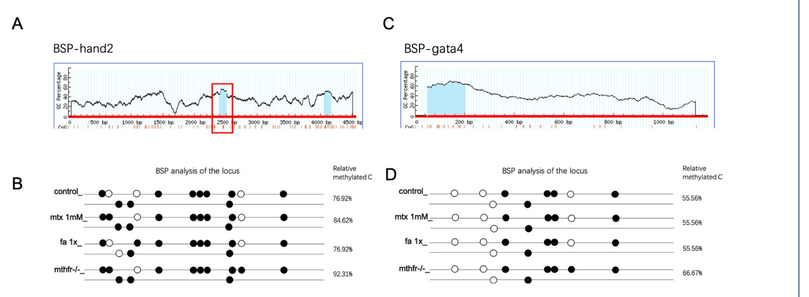

Figure 4. Effects of folic acid on partial gene methylation in zebrafish embryos.
(A) The CpG island of hand2 gene for BSP experiment was predicted and selected. (B) Zebrafish embryos with different folate metabolism groups BSP analysis of the locus of *hand2*. (C) The CpG island of gata4 gene for BSP experiment was predicted and selected. (D) Zebrafish embryos with different folate metabolism groups BSP analysis of the locus of *gata4*.

The results showed that folate inhibition and abnormal folate metabolism caused by *mthfr* $^{-/-}$ mutation could increase the methylation level of *hand2* gene.

Conclusion

We constructed a novel *mthfr* zebrafish mutation model and found the first time that insufficient or excessive folic acid intake can lead to changes in gene expression during early cardiac development of zebrafish embryos. Abnormal folate metabolism can lead to abnormal metabolism of Hcys and abnormal methylation of some genes in zebrafish. These results will increase the risk of all kinds of congenital diseases, including congenital heart disease. It has been proved that excessive folic acid supplementation has a negative effect on the early development of organisms. Folic acid has a dose-dependent biphasic effect on organism development.

Folic acid has biphasic effects on early heart development in zebrafish. The differential expression of cardiac development related genes is related to folate. Abnormal folate metabolism changes the methylation level of *hand2* and *gata4* promoters.

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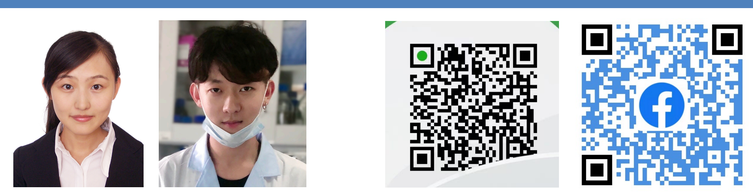

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