UNIVERSITY OF CALIFORNIA SAN DIEGO

Effect of folic acid on somite segmentation and neural tube closure

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Ashna Nisal is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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Ashna Nisal

Master of Science in Biology

University of California San Diego, 2024

Professor Joseph Gleeson, Chair
Professor Jill Wildonger, Co-Chair

Neural tube defects (NTDs) are developmental malformations caused by incomplete closure of the neural tube (NT), a precursor of brain and spinal cord during development. Sufficient periconceptional levels of folic acid (FA) through supplementation in humans significantly reduces the risk of NTDs. Nevertheless, the underlying mechanism by which FA alleviates the risk of NTDs still remains unknown. Multiple genes have been implicated in NTDs making it difficult to outline a specific mechanism that may cause NTD. FA plays critical roles in single carbon metabolism, required for production of nucleotides and methylation through the intermediary S-adenosyl methionine. We found that murine dams treated with low

periconceptional FA had embryos with fewer somites than normal FA condition. Therefore, we investigated how FA affects the rate of somite formation and how this in turn may affect NT closure. We differentiated mouse epiblast stem cells (mEPiSCs) to presomitic mesodermal (PSM) tissue that is known to segment and produce somites and monitored the expression pattern of segmentation clock marker gene HES7. Inhibiting FA pathway with methotrexate during the PSM induction decreased the amplitude of HES7 cycling. We found that treating these PSM cells with methionine increases the HES7 cycling amplitude. Thus, FA may exert its effect on NTD risk through regulation of key metabolites including methionine that could impact somitogenesis.

INTRODUCTION

The central nervous system, constituting the brain and spinal cord, is derived from a transient structure called the neural tube. During vertebrate embryonic development, a process called gastrulation results in formation of three germ layers known as endoderm, mesoderm, and ectoderm. A transient structure called notochord secretes signaling molecules that induce differentiation of ectodermal cells into neuroectoderm, which then forms a flat layer of cells called the neural plate (Elshazzly et al., 2024). Continued signaling cues provided by the notochord subsist neural plate differentiation, which is followed by neurulation wherein the lateral edges of the neural plate fold and form a tube-like structure called the neural tube. In humans, neurulation completes 28 days after fertilization (Kuwar Chhetri & Das, 2024). A closed neural tube then gives rise to various parts of the central nervous system. A failure in closure of the neural tube results in a neural tube defect (NTD) that can manifest as an external sac-like protrusion with or without the herniation of the neural tissue at the brain or spinal level (Avagliano et al., 2019).

In mammals, contact between the edges of the two lateral folds of the neural plate is initiated at multiple points along the rostro-caudal axis. However, neurulation in the cephalic (anterior) region is temporally more advanced than that in the caudal region (Avagliano et al., 2019). Therefore, the pathophysiology of each type of NTD differs depending upon the site where the neural tube fails to close completely. Closing failure at the posterior neuropore, which is located near closure site 5, leads to spina bifida, the least severe type of NTD. Similarly, failure to close at one of the anterior neuropores located near closure site 2 leads to anencephaly, a more severe type of NTD wherein the fetus is born with an open head and often leads to infant

mortality (Mai et al., 2019). NTD conditions where the closure sites near the anterior and posterior neuropore both fail to close is called Craniorachischisis and manifests as both brain and spinal cord having failed closure at birth – a combination of anencephaly and spina bifida (*Craniorachischisis - about the Disease*, 2023). Most types of NTDs either lead to a failed pregnancy or stillborn fetus in severe NTD types, and partial paralysis, lack of motor control or no symptoms in less severe NTD types (Kidane et al., 2023). In the United States, NTDs affect approximately 300,000 pregnancies each year and are a leading cause of infant mortality in most developed countries (Estevez-Ordonez et al., 2018)·(Greene et al., 2009).

Numerous studies since 1965 have suggested that folic acid (FA) may reduce risk of neural tube defects (Kidane et al., 2023). In 1991, the Medical Research Council of United Kingdom conducted a large randomized double-blinded trial in women with history of NTD pregnancies and showed that periconceptional folic acid supplementation of up to 4000µg daily had a significant protective effect against NTDs by preventing recurrence risk by 70% (Czeizel & Dudás, 1992). They found that the group treated with FA and other multivitamin supplementation had 72% reduced occurrences of NTDs, while the group treated with only the other multivitamins not including FA showed no significant protective effect. Numerous other NTD-FA studies have shown similar results, such as a large placebo-controlled population study conducted by Mills et. al in 2004 which reported 25-50% decrease in NTD prevalence post mandatory FA fortification (Mills & Signore, 2004). Despite such strong evidence of a protective effect exerted by FA in prevention of NTDs, the molecular mechanism underlying this relation between FA and NTDs is still unclear.

FA is an essential vitamin that is crucial for DNA synthesis and methylation. FA derivate 5-methyltetrahydrofolate (5-CH_3THF) donates a methyl group to S-adenosylmethionine, which

is an important methyl donor to most DNA and histone methyltransferases that regulate DNA methylation and regulate gene expression (Williams & Schalinske, 2007). Its derivative 5,10-methylenetetrahydrofolate (5,10-CH_2THF) also aids in the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to form 2′-deoxythymidine-5′-monophosphate (dTMP, thymidylate), which is a rate limiting step in the formation of nucleotide thymidine (Chu & Gollerkeri, 2002). FA deficiency in adults is linked to folate deficiency anemia, infertility, and dementia, while it is also strongly associated with severe neural tube defects such as spina bifida and anencephaly when insufficient during pregnancy.

Several genes have been implicated in NTD that when mutated might lead to NTD (Mauch & Schoenwolf, 2001). To investigate the causes of NTDs in humans, our lab has assembled a large cohort of NTD cases, stratified based upon FA exposure history. While analysis is still ongoing, we have published two manuscripts this year that supports a contribution of de novo mutations to risk of NTDs (Vong et al., 2024)·(Ha et al., 2024). With the aim of understanding the role of FA during normal development, we investigated how varying periconceptional FA supplementation at the parts-per-million (ppm) levels in female wildtype inbred mice alters developmental processes during neurulation. To standardize the age of the embryo, we quantified the number of somites in E10.5 embryos and surprisingly observed that the low (0ppm) FA condition embryos had significantly fewer somites than the basal (2ppm FA) condition. Based on these preliminary results, we hypothesized that FA may reduce the speed of somitogenesis (process of formation of somites), which may in turn play a role in neural tube closure and NTDs.

Neural tube closure occurs approximately at the same time as somite segmentation and the two developmental processes share close temporal and spatial proximity (Copp & Greene,

2013). During vertebrate embryonic development, paraxial mesoderm tissue neighboring the neural plate tissue rhythmically segments to form bilateral pairs of epithelial tissue blocks called somites in a process known as somitogenesis. Somites are transient tissue blocks that give rise to the vertebral column, ribs, skeletal muscles, and subcutaneous tissues over the course of development (Kageyama et al., 2010). The precise number of somites formed in humans is not known, however, mice are known to have approximately 60 somites that form by around day E14 (Wong et al., 2015). The number of somites formed during development are different across species. Somites form periodically in a pairwise fashion around both sides of the neural tube/grove (parallel to it) in an anterior-posterior axis (Cook et al., 2017). The somites near the anterior part or head of the embryo form first, followed by the ones near the posterior part which form last. The time period after which every subsequent pair of somites is formed following the previous is called segmentation period. *Hes7* is an oscillatory gene that marks the somite segmentation cycle and provides the rhythmic nature due to a negative feedback loop self-regulating its expression. *Hes7* interacts with a network of oscillating genes driven by *Wnt* and *Notch* signaling which together contribute to the periodicity of the segmentation clock (Pourquié, 2003).

To understand how FA deficiency affects embryonic development at a transcriptomic level during neural tube closure, corresponding to embryonic time point 10.5 (E10.5) in mice, a former lab member treated groups of female mice with FA deficient, and counted umber of somites to normalize the age of embryos across groups. Surprisingly, they observed that embryos in low FA group had significantly fewer somites (Supplementary figure 1). This suggested that FA could impact the rate of somitogenesis but did not exclude a possibility that the conception time of embryos could have been varied due to overnight mating, leading to the observed

variance in embryo age. Somites are formed every 2-3 hours in mice; therefore, the variance of 14-16 hours could lead to a large difference in the number of somites observed at E10.5 after overnight mating. However, this observation helped us hypothesize that FA deficiency is reucing the rate of somite formation.

RESULTS

FA deficiency delays production of somites *in vitro.*

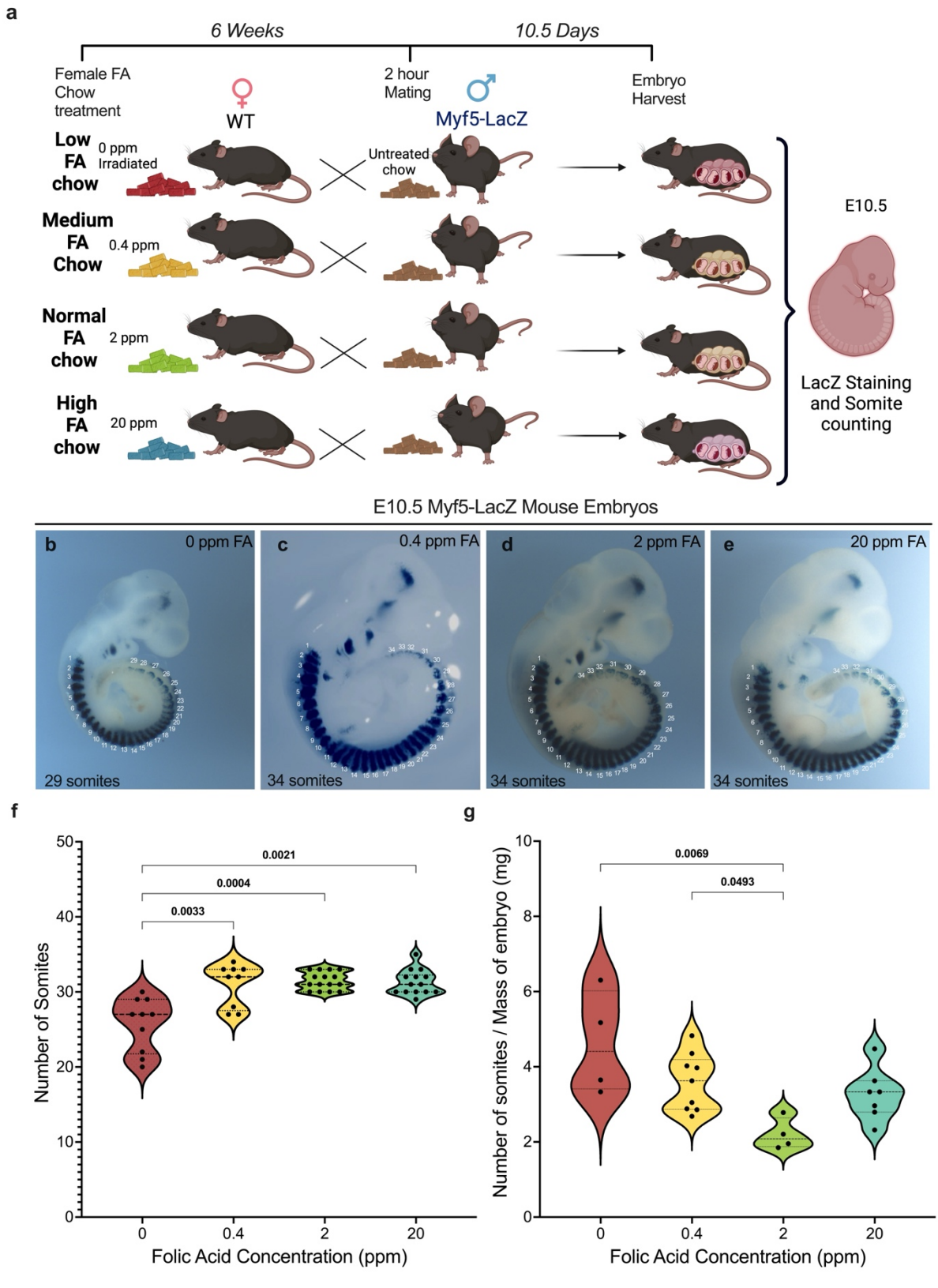
To investigate how varying levels of FA affect development I treated 4 week old female mice with 0ppm, 0.4ppm, 2ppm and 20ppm FA for 6 weeks prior to conception. To understand how FA affects developmental processes such as somitogenesis at a precise embryonic age, I restricted the mouse mating time to 2 hours, followed by checking for copulation plugs, and harvesting the embryos at E10.5 (Fig. 1a). The 0.4ppm FA treatment condition was an additional dose included to identify if an intermediary FA dose leads to an intermediate somite number or an overall developmental phenotype. To better quantify somite number, the cross was performed with male carriers of the Myf-LacZ gene (strain #018626), which marks the somites at E10.5 with LacZ expression but does not impact somitogenesis.

We observed that embryos from 0ppm group (median 27±5.75 somites) had significantly fewer somites than 2ppm (32±2 somites) and 20ppm (31±2 somites) embryos (Kruskal-Wallis p=0.0004 0ppm vs. 2ppm) (Fig 1b-f). Intermediary 0.4ppm group had same median number of somites (32±5 somites) as 2ppm group, however showed greater variance in number of somites and embryo size observed within a litter. To evaluate whether the position of the embryo in the uterus correlated with embryo size, we compared embryo weight with position relative to the midline of the uterus, but found no clear correlation (data not shown). This suggests that FA can impact somitogenesis even when controlling for mating time and position within the uterus.

To investigate whether the difference in number of somites is a result of global developmental delay or a segmentation specific defect, we compared the ratio of embryo somite number to embryo wet mass across the four treatment groups. If reduction in somite number in deficient groups was a result of global developmental delay and smaller embryo size, we would

expect the somite number to scale with the mass and the overall ratio to not change. We observed that 0ppm and 0.4ppm groups had a significantly higher ratio of somite number to embryo mass (4.41±1.90 and 3.63±1.14 respectively) than 2ppm (2.08±0.42) condition (Kruskal-Wallis p=0.0069 and 0.0493 respectively). However, since we observed that the somite to mass ratio is not consistent across the FA deficient across the 0ppm, 0.4ppm and 2ppm groups, it is likely that the developmental defect is specific to somitogenesis.

Figure 1: FA deficiency significantly reduces the number of somites formed at E10.5. (a) FA mouse mating experimental design. (b-e) Images of E10.5 embryos from different treatment groups stained for LacZ as a marker of somites with number of somites highlighted in white. (f) Median number of somites ± interquartile range (IQR) observed across various FA treatment group embryos, Kruskal-Wallis p-values. (g) Median of ratio of number of somites to wet mass of embryos (mg) observed across various FA treatment group embryos, Kruskal-Wallis test.


a
6 Weeks
10.5 Days
Female FA Chow treatment
WT
2 hour Mating
Myf5-LacZ
Embryo Harvest
Low FA chow
0 ppm Irradiated
Untreated chow
Medium FA Chow
0.4 ppm
Normal FA chow
2 ppm
High FA chow
20 ppm
E10.5
LacZ Staining and Somite counting
E10.5 Myf5-LacZ Mouse Embryos
b
0 ppm FA
29 somites
c
0.4 ppm FA
34 somites
d
2 ppm FA
34 somites
e
20 ppm FA
34 somites
f
Number of Somites
0.0021
0.0004
0.0033
Folic Acid Concentration (ppm)
g
Number of somites / Mass of embryo (mg)
0.0069
0.0493
Folic Acid Concentration (ppm)

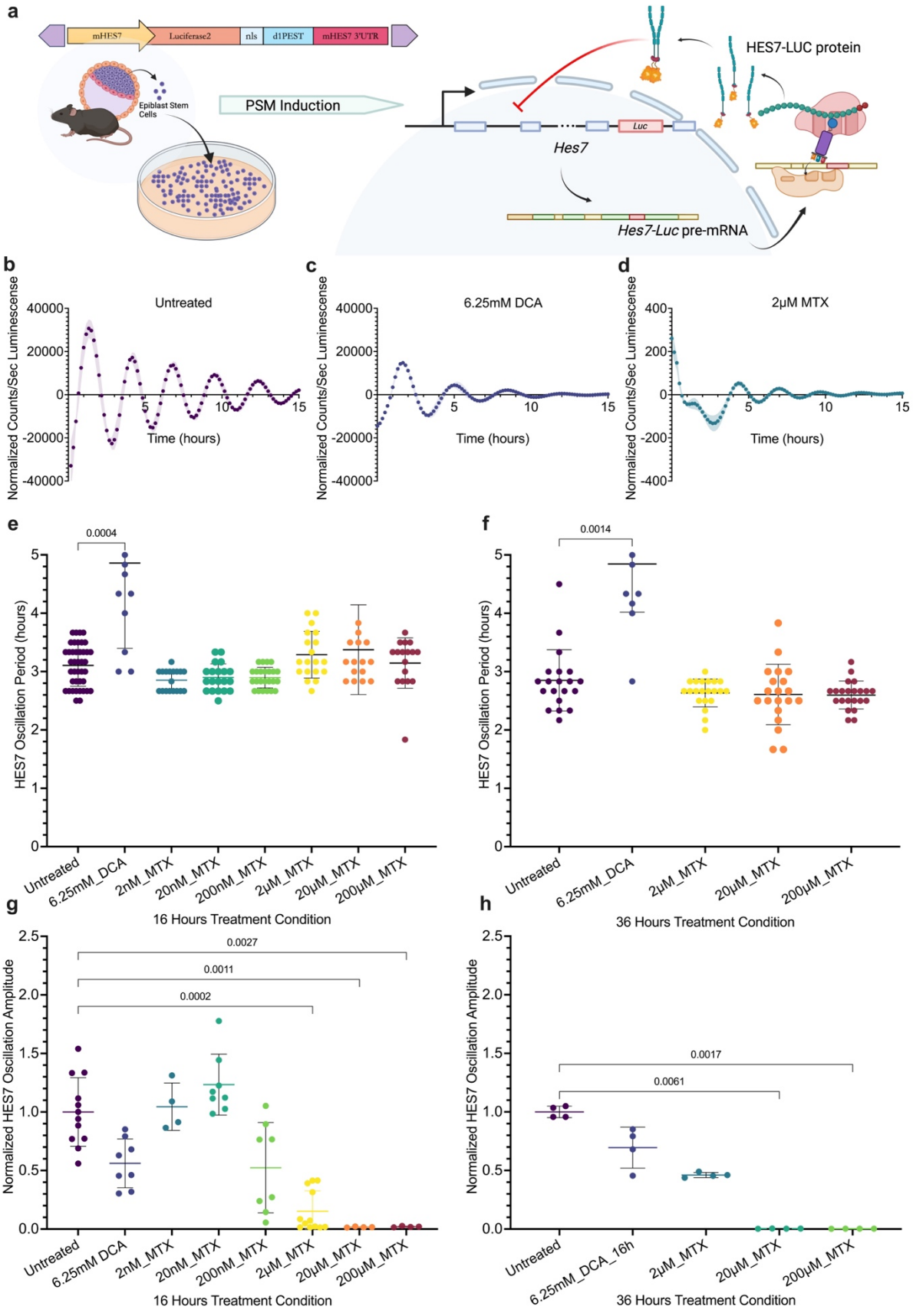
FA pathway blocker Methotrexate dampens the somite clock *in vitro*.

To understand the FA deficiency induced segmentation defect, we adapted a previously established 2D *in vitro* segmentation model comprise of presomitic mesodermal tissue (PSM) cells, which are known to differentiate and produce somites *in vivo* (Diaz-Cuadros et al., 2020),(Matsuda et al., 2020). We differentiated mouse epiblast stem cells expressing luciferase reporter linked to segmentation clock marker gene *Hes7* to PSM cells and treated them with FA inhibitor methotrexate (MTX). MTX competitively inhibits the conversion to FA to dihydrofolate reductase (DHFR) thereby blocking the production of tetrahydrofolate (THF), the bioactive form of FA (Wentzel et al., 2005). *Hes7* is a marker gene with a cyclic expression pattern (Fig. 2a) that corresponds to formation of new somite at each peak caused by increased expression of HES7. Since low FA leads to fewer somites observed in embryos, we hypothesized that blocking FA with MTX would lead to an increased period of segmentation leading to an increased period of HES7 oscillation, which may result in decreased speed of somite production.

To investigate how MTX affects HES7 oscillation period, we treated epiblast derived mouse PSM cells with varying concentrations of MTX and observed HES7 oscillation period and amplitude (Fig 2 b, c). We used previously described pyruvate dehydrogenase kinase (PDK) inhibitor dichloroacetic acid (DCA) that is known to increase HES7 oscillation period as our positive control (Fig 2c). Treatment with up to 200μM MTX did not lead to significant changes in HES7-LUC segmentation period, even after prolonged 36h MTX treatment (Fig 2e, f). However, we did observe that MTX treatment does significantly affect the amplitude of HES7-LUC oscillation (Fig 2g, h). Small concentrations 2nM – 200nM of MTX slightly increased (not statistically significant) the HES7 oscillation amplitude, however, treatment with 2μM or greater

MTX concentration significantly decreased the oscillation amplitude. We therefore show that 2μM MTX reduces *Hes7* expression without affecting the segmentation period.

Figure 2: MTX treatment significantly reduces amplitude of Hes7 cycling in vitro.
(a) Schematic of 2D *in vitro* culture system used and summary of HES7 oscillatory expression. (b) HES7 oscillations for epiblast stem cells differentiated to PSM cells in untreated, (c) DCA treated, and (d) 2μM MTX treated conditions. (e, f) Period and (g, h) amplitude of HES7 oscillations in hours across various MTX concentrations, treated for 16 hours and 36 hours, respectively. Kruskal Wallis p-values denoted on graphs.


a
mHES7
Luciferase2
nls
d1PEST
mHES7 3'UTR
Epiblast Stem Cells
PSM Induction
HES7-LUC protein
Luc
Hes7
Hes7-Luc pre-mRNA
b
Untreated
c
6.25mM DCA
d
2μM MTX
Normalized Counts/Sec Luminescence
Time (hours)
e
f
HES7 Oscillation Period (hours)
0.0004
0.0014
Untreated
6.25mM_DCA
2nM_MTX
20nM_MTX
200nM_MTX
2μM_MTX
20μM_MTX
200μM_MTX
16 Hours Treatment Condition
36 Hours Treatment Condition
g
h
Normalized HES7 Oscillation Amplitude
0.0027
0.0011
0.0002
0.0017
0.0061
6.25mM_DCA_16h

Methionine treatment increases amplitude of HES7 oscillation *in vitro.*

The two main pathways associated with FA are DNA methylation and DNA synthesis. FA promotes production of S-adenosylmethionine (SAM) by aiding conversion of homocysteine to methionine (MTH), which then helps with the production of SAM. SAM is an important methyl donor to many methyltransferases. Since we observed changes in expression levels of HES7 upon MTX treatment, we hypothesized that the observed reduction in HES7 expression could a result of impaired SAM production. We therefore treated the MTX PSM cells with MTH investigate whether MTH can rescue the reduced amplitude caused by MTX treatment. We observed that MTH treatment increases HES7 cycling amplitude in a dose dependent manner. (Fig. 3a).

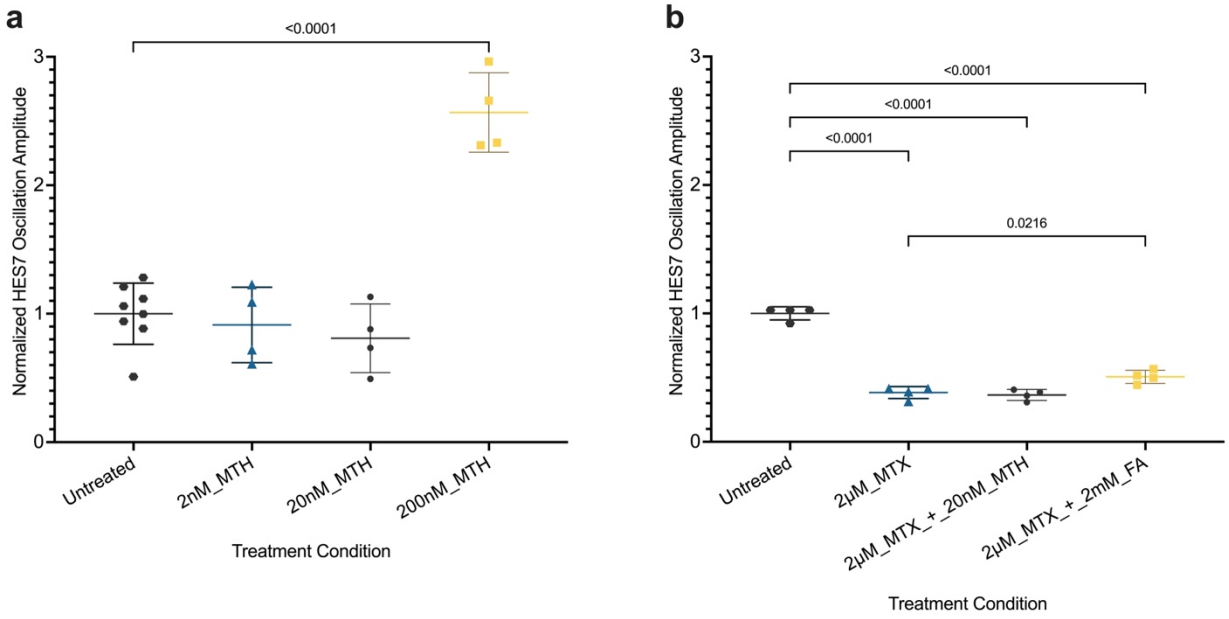


Figure 3: MTH treatment increases Hes7 cycling amplitude. (a) HES7 cycling amplitude in response to various concentrations of MTH treatment. (b) HES7 cycling amplitude in response to cells treated with 2μM MTX and with 20nM MTH and 2mM FA. Kruskal Wallis p-values denoted on graphs.

DISCUSSION

Here we show that periconceptional dietary FA deficiency in mice leads to reduced somite number in embryos at the time of neural tube closure. Although the link between FA and somite segmentation has not been extensively studied, some publications report that diabetic rats that are known to have higher incidence of NTDs have significantly fewer somites than normal rats and that FA supplementation rescues this deficit (Wentzel et al., 2005). This highlights an important link between FA and somitogenesis, suggesting that FA deficiency might disrupt the segmentation clock or other key regulatory mechanisms involved in somite formation. Consequently, we observe that this disruption leads to delayed development of somites, which may thereby increase the risk of NTDs. Our findings emphasize the critical role of FA in early embryogenesis, particularly in processes that are tightly regulated, such as somite formation. These results also support the broader understanding of FA's importance in preventing congenital malformations and underscore the necessity of adequate FA intake during the periconceptional period. Further research is needed to uncover the molecular mechanisms by which FA influences somitogenesis and how this may interplay with the etiology of NTDs.

Okamura et. al report that inhibiting methylation increases the period of somite-clock and HES7 oscillations *in vivo* as observed during *in vivo* live imaging of HES7-luciferase mice (Fustin et al., 2020). Based on our *in vivo* somite quantification results indicating decreased somite number in response to FA deficiency, we hypothesized that we would observe an increased period of HES7 cycling in our *in vitro* experiments. However, we did not observe any significant changes in HES7-Luc period in our 2D PSM culture. Instead, we observed that MTX decreases the amplitude of HES7 oscillations. Furthermore, we found that treatment with MTH, a FA-SAM pathway intermediate, increases the amplitude of HES7 cycling. These findings

suggest that while folate deficiency and methylation inhibition might influence Hes7 cycle period and resultant number of somites *in vivo*, *in vitro* culture of isolated PSM cells does not exhibit similar effects on HES7 cycling. One possible reason for this discrepancy could be that the change in period observed *in vivo* is dependent on interaction between multiple cell types that are not present in the *in vitro* culture.

In future, we plan to further validate the effect of MTX and FA deficiency on SAM levels in vivo using LC-MS/MS. HES7 amplitude rescue with MTH suggests that the observed reduction in somite number could be due to an underlying methylation defect. We also plan to investigate the molecular mechanism by which FA contributes to regulating the speed of the somite clock.

METHODS

Animals

Myf5-Tm1Pas/J (strain #:018626) and wildtype C57BL/6J (strain #:000664) lines were acquired from the Jackson Laboratory. Animals were genotypes using common forward: 5'TCT CCC TCT CTG CTG AAT CC3'; mutant reverse: 5'GGC CTC TTC GCT ATT ACG C3'; wildtype reverse: 5'CAC GTG CTC CTC ATC GTC T3' primers.

Breeding

Wildtype females of age 6 weeks were treated with folic acid chow (either 0ppm, 0.4ppm, 2ppm or 20ppm) for 6 weeks. The females were mated with LacZ +/- males for 2 hours, separated and checked for presence of seminal plugs. Females with plugs were harvested at embryonic day 10.5.

LacZ Staining

E10.5 embryos were harvested under a dissection scope, removing the embryo from the uterine sac and Reichert's membrane using dissection microforceps on cold 1x phosphate buffered saline (PBS). The Reichert's membrane was used to genotype the embryo by PCR (see animals section in methods for list of primers). LacZ heterozygous embryos were fixed in a freshly made fixative solution (2% paraformaldehyde [Millipore Sigma, #8187151000], 0.2% glutaraldehyde [Millipore Sigma, # G7776], 0.02% Na Deoxycholate [Millipore Sigma, #D6750], 0.01% NP-40 [Thermo Scientific, #85124] and 1x PBS) for 12 minutes. Following fixation, embryos were rinsed twice in ice cold 1x PBS and incubated in permeabilization solution (0.02% Na Deoxycholate and 0.01% NP-40 in 1x PBS) overnight shaking gently at 4°C.

The embryos were then stained using freshly made staining solution (5mM K-Ferricyanide [Millipore Sigma, #P8131], 5mM K-Ferrocyanide [Millipore Sigma, #P9381], 2mM $MgCl_2$ [Millipore Sigma, #M8266], 0.02% Na Deoxycholate, 0.01% NP-40 and 1mg/ml X-gal [Promega, #V3941] in 1x PBS, warmed to 50°C then filtered using 0.45µM PVDF filter [Millipore Sigma, #SLHVR33RS]) overnight at 30°C with gentle rocking. Stained embryos were rinsed twice in 1x PBS and washed in permeabilization solution for 15 minutes followed by overnight fixation in 4% paraformaldehyde. The fixed embryos were then stored in 1x PBS containing 0.03% Sodium azide [Millipore Sigma, #S2002] at 4°C.

Stem Cell Cultures

Previously published Hes7-Luc-mEPiSC cell line was a generous gift from Dr. Ebisuya, et al. and was established as an in-vitro model for inducing PSM and monitoring segmentation activity through Hes7 expression pattern in their 2020 publication. Hes7-Luc-mEPiSCs were maintained on fibronectin [Millipore Sigma, #F1141] coated plates (2µg/cm2) in mEPiSC growth media which constituted 15% Knockout serum [Gibco, #10828-028] in DMEM/HamF-12 [Gibco, #10565-018] supplemented with 0.1mM non-essential amino acids [Gibco, #11140-050], 0.1mM 2-Mercapthanol [Millipore Sigma, #M3148], 20ng/ml Activin A[R&D Systems, #338-AC-010/CF], 10ng/ml bFGF [PeproTech, #450-33] and 2.5µM IWR-1-endo [Gibco, #10828-028]. The media was supplemented with 1µM Y-27632 Rock Inhibitor [Stemcell Technologies, #72307] when passaging.

PSM Induction

Cells were passaged using mEPiSC growth media (-) IWR-1-endo (+) Y-27632 and seeded onto fibronectin coated 35mm dished [Corning, #430165] at a density of 5,200 cells/cm^2 – day 0. For days 1, 2 and 3 chemically defined media (CDMi) was used as a base media). CDMi contained 1:1 mixture of F-12 nutrient mix and Isocove's Modified Dulbecco's Media (IMDM) [Sigma Aldrich, #I3390] supplemented with 450µM 1-Thioglycerol [Sigma Aldrich, #M1753], 15µg/ml Apo-transferrin [Sigma Aldrich, #T1147], 5mg/ml Bovine Serum Albumin [Sigma Aldrich, #A8531, 1% CD Lipid Concentrate [Gibco, #11905031, 50µg/ml Penicillin/Streptomycin [Gibco, #15140122] and 7µg/ml rh-Insulin [Millipore Sigma, #15140122]. On day 1, CDMi media containing 10µM SB431542 [Cellagen Technology, #C7243-5], 250nM LDN-193189 [Sigma Aldrich, #SML0559], 10µM CHIR99021 [Sigma Aldrich, #SML1046] and 20ng/ml bFGF in CDMi On Day 2, media was changed to CDMi supplemented with 10µM SB431542, 250nM LDN-193189, 3µM CHIR99021 and 20ng/ml bFGF. On day 3 the 200µM Luciferin [Thermo Scientific, #88291] substrate was added to the media (same media composition as day 2) and cells were imaged using a luminometer for 24 hours.

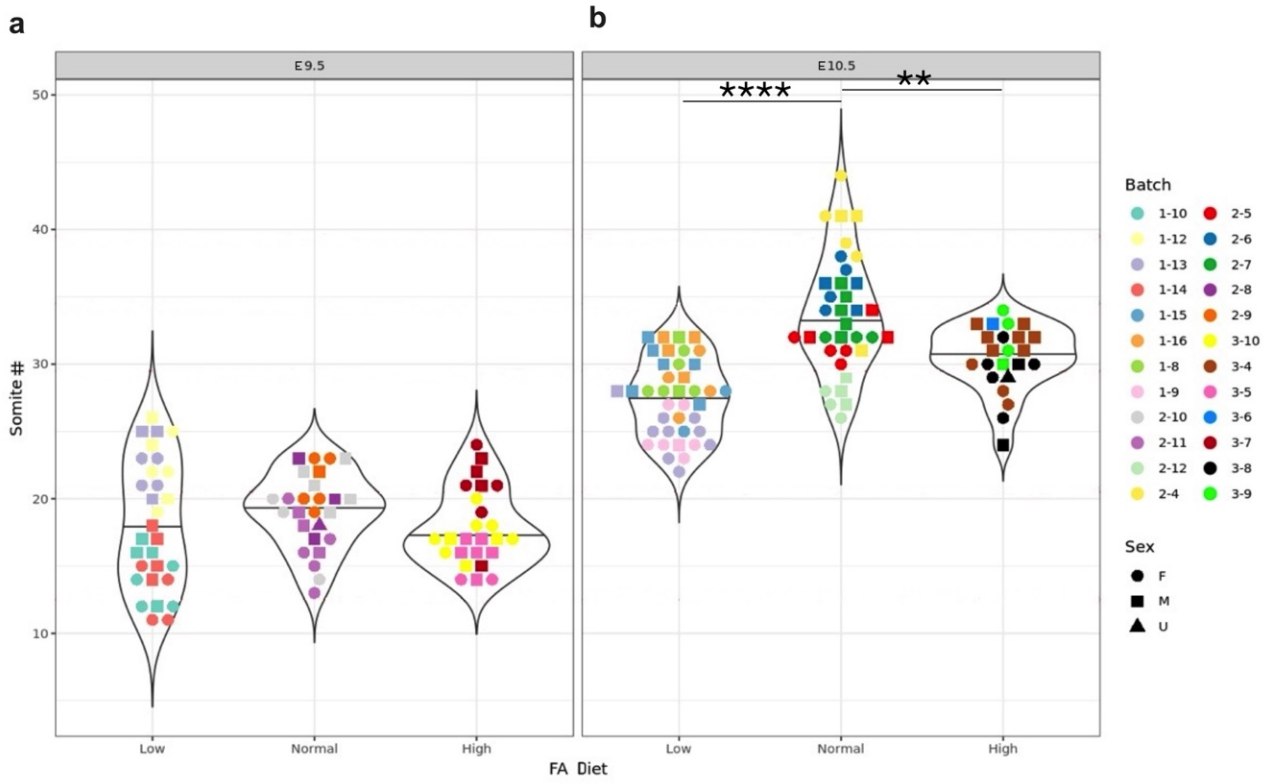
Recording Hes7-Luciferase expression activity

Hes7 cycling activity observed through luciferase expression pattern was recorded using Actimetrics LumiCycle luminometer and the raw data was extracted through Actimetrics LumiCycle app. The extracted data was filtered and analyzed using Python, Microsoft Excel and MATLAB.

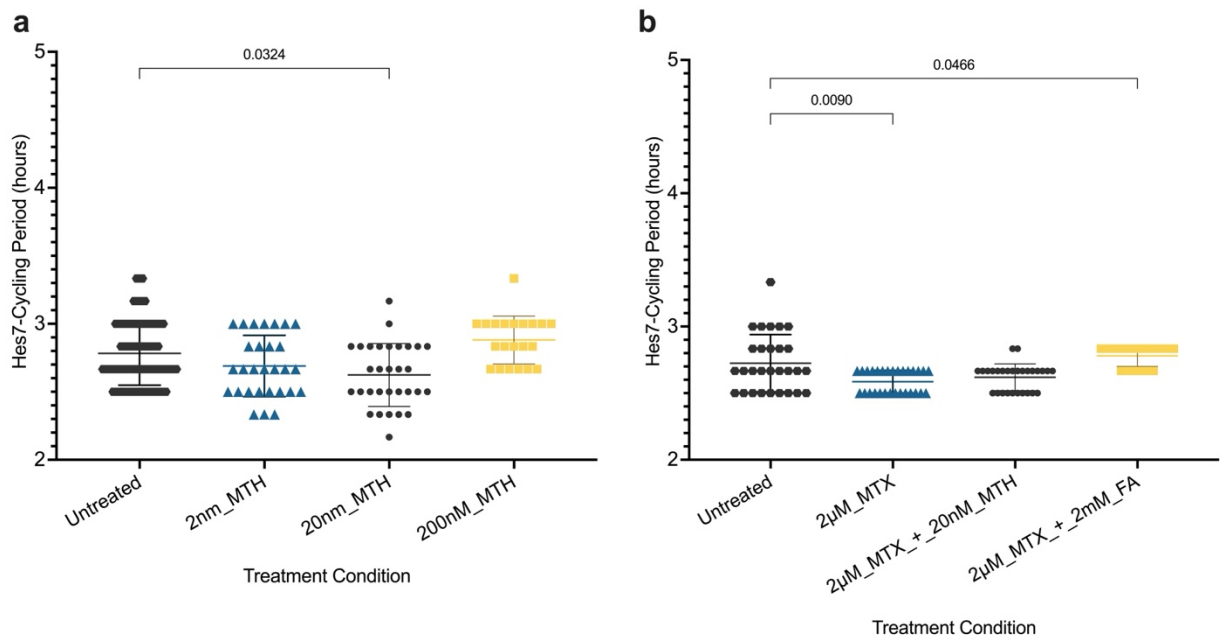
Segmentation period calculation

The segmentation period was calculated using the peak finder function in Python by measuring the distance between two adjacent peaks. The average period was calculated by averaging the first 3 periods of each replicate curve for a given condition.

SUPPLEMENTARY FIGURES



Supplementary Figure 1: Somite number counts for C57BL/6J embryos with 0ppm (low), 2ppm (normal) and 20ppm (high) periconceptional FA treatment observed at E9.5 (S1a) and E10.5 (S1b).



Supplementary Figure 2: MTH treatment has no effect on Hes7 oscillation period. HES7 oscillation period in response to various concentrations of (a) MTH treatment and (b) 2μM MTX treated cells rescued with different concentrations of MTH and FA. Kruskal Wallis p-values denoted on graphs.

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