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Inhibition of proteolysis in histiotrophic nutrition pathways alters DNA methylation and one-carbon metabolism in the organogenesis-stage rat conceptus

Karilyn E. Sant, Dana C. Dolinoy, Muna S. Nahar, and Craig Harris*

Department of Environmental Health Sciences, University of Michigan, Ann Arbor, Michigan, 48109

Abstract

Epigenetic modifications, including DNA methylation, contribute to the transcriptional regulation of developmental genes that control growth and differentiation during embryogenesis. The methyl donor, S-adenosylmethionine (SAM) is biosynthesized from methionine and ATP by methionine adenosyltransferase 2a (Mat2a) in the one-carbon (C₁) metabolism pathway. SAM biosynthesis requires a steady supply of nutrients, vitamins, and cofactors obtained by the developing conceptus through histiotrophic nutrition pathways (HNPs). The visceral yolk sac (VYS) captures proteins and their substrate cargos by receptor-mediated endocytosis (RME) and degrades them using lysosomal proteases. We hypothesize that leupeptin, a protease inhibitor, reduces the availability of methionine and C₁ substrates, restricting SAM biosynthesis, and altering patterns of DNA methylation. Rat conceptuses were exposed to 50 and 100 μM leupeptin in whole embryo culture (WEC) for periods of 26 h from gestational day (GD) 10 or 6 h on GD11. after 6 h on GD11, the 100 μM leupeptin treatment significantly decreased methionine in embryo (EMB) and VYS, reduced Mat2a protein levels, and inhibited Mat2a specific activity; all of which produced a significant 52% reduction of SAM in the VYS. The 50 and 100 μM leupeptin treatments significantly decreased global methylation levels by 6–9% in EMB and 11–15% in VYS following both 6 and 26 h exposure periods. This study demonstrates that HNP disruption alters C₁ activity and significantly reduces global DNA methylation during organogenesis. Because epigenetic reprogramming is crucial for normal differentiation and growth, these findings suggest a possible mechanism through which nutrients and environmental factors may alter early developmental regulation.

Words

DNA methylation; one-carbon metabolism; histiotrophic nutrition; embryonic development; methionine; S-adenosylmethionine; developmental regulation; visceral yolk sac

*Correspondence should be addressed to: Craig Harris, Address: Toxicology Program, Department of Environmental Health Sciences, University of Michigan, 1415 Washington Heights, Ann Arbor, Michigan 48109-2029, Tel: (734) 936-3397, charris@umich.edu.

INTRODUCTION

Cell differentiation that gives rise to form and function during embryogenesis is achieved through a highly coordinated and integrated program of cell signaling and gene expression. Both embryonic and extra-embryonic cells within the conceptus contain identical genetic codes from which variable gene expression is programmed epigenetically in spatial and temporal patterns. DNA methylation is an important process involved in the epigenetic regulation of gene expression along with chromatin modification and non-coding RNAs. DNA hypermethylation is generally associated with gene silencing while hypomethylation is associated with transcriptional activation [1, 2]. Cells within the very early preimplantation embryo (EMB) exist in a globally demethylated state, and then undergo remethylation in a cell- and gene-selective manner to establish specific differentiation characteristics as embryogenesis progresses [3, 4]. In mature cells, a large proportion of the genome becomes methylated and is, therefore, not expressed [1]. Regulatory regions in promoter sequences of actively expressed genes remain hypomethylated and enriched in open-chromatin marks to remain transcriptionally poised to respond to a wide variety of signals and stimuli [5]. While the roles of several chemicals and dietary components have been characterized in the disruption of these processes [6, 7], very little is known about the mechanisms through which specific nutrients and other environmental or developmental stress factors interact to modify the epigenome during differentiation and embryonic development.

Alterations in maternal diet during the preimplantation and fetal phases of development have been shown to reduce the conceptual availability of whole protein, amino acids (methionine), folate, choline, and cobalamin (Vitamin B₁₂) and are also known to elicit changes in the epigenome through reprogramming of DNA methylation patterns [8]. These dietary nutrients are the source of precursors, vitamins, and cofactors required for one-carbon (C₁) metabolism, the process that culminates in S-adenosylmethionine (SAM) biosynthesis. SAM is the universal methyl donor for DNA methylation, and is generated in its final form from L-methionine and ATP by the enzymatic activity of methionine adenosyltransferase 2a (Mat2a). C₁ precursors must be constantly supplied to the conceptus suggesting that maternal diet and micronutrient bioavailability are crucial for proper epigenetic regulation of developmental processes.

Embryonic and fetal nutrition play a critical role in growth, differentiation, and developmental plasticity [9]. During organogenesis, the early postimplantation-stage mammalian conceptus is physically isolated from direct access to maternal micronutrients, as the placenta is not yet fully functional. Thus, the organogenesis-stage conceptus obtains very few nutrients through transcellular transport and must rely on the receptor-mediated endocytosis (RME) or pinocytosis of proteins and nutrients via the visceral yolk sac (VYS) endothelium [10–13]. Proteolytic degradation of bulk maternal protein in lysosomes of trophoblast-derived tissues provides the conceptus with the majority of amino acids necessary for *de novo* protein biosynthesis [14–17]. In addition to the bulk proteins, RME provides access for other substrates, vitamins, and cofactors, which enter cells by the same pathways bound to specific carrier proteins. The RME, proteolysis, and processing of nutrients, termed histiotrophic nutrition pathways (HNPs), comprise the primary route of

nutrition during organogenesis. HNPs are, therefore, also responsible for the maintenance of C₁ metabolism during organogenesis (Figure 1).

Leupeptin, a naturally occurring inhibitor of proteolytic degradation, reduces amino acid and micronutrient availability for cellular processes such as protein synthesis, glutathione (GSH) biosynthesis, and C₁ metabolism [18] (Figure 1). Treatment with leupeptin mimics nutritional deficiency in the conceptus, and has been shown to decrease the activity of HNPs [10]. Because substrates obtained via HNPs such as methionine, folate, choline, and vitamin B₁₂ are required for SAM biosynthesis, leupeptin treatment, during organogenesis, may be used as a model to study developmental outcomes associated with nutritional deficiencies. While several studies have begun to investigate the epigenetic and morphological consequences of these nutritional deficiencies during embryonic development, the role of HNPs in these outcomes has yet to be characterized [8]. This study examines a unique window of developmental susceptibility where alterations in HNP lead to compromised C₁ metabolism and a reduction of global DNA methylation during organogenesis. Within this context, we propose to test the hypothesis that leupeptin, a protease inhibitor, disrupts HNP functions in the organogenesis-stage conceptus, reducing the availability of methionine required for biosynthesis of SAM by the C₁ pathway and, thereby, altering patterns of DNA methylation.

MATERIALS AND METHODS

Chemicals and Reagents

Leupeptin hemisulfate salt and deuterated isotopic standards (Homocysteine-*d*₈ and N,N-dimethyl-*d*₆-glycine HCl) for mass spectrometry quantification of C₁ components were purchased from Sigma (St. Louis, MO). L-methionine (methyl-*d*₃) and D,L-cysteine (3,3-*d*₂) were purchased from Cambridge Isotope Laboratories (Andover, MA). S-Adenosyl-L-methionine-*d*₃ (S-methyl-*d*₃) tetra (*p*-Toluenesulfonate) Salt was from C/D/N Isotopes (Pointe-Claire, Quebec, CA). All other reagents and chemicals were purchased from standard vendors and were of the appropriate purity for culture and analytical applications. Antibodies for immunoblots were purchased from Abcam (Cambridge, MA) [Anti-Mat2a antibody (Rabbit polyclonal) - ab77471] and Santa Cruz Biotechnology (Santa Cruz, CA) [Actin antibody (Goat polyclonal) - sc-1615; Alkaline phosphatase secondary antibody (donkey anti-goat) - sc-2022; Alkaline phosphatase secondary antibody (chicken-anti-rabbit)- sc-2959].

Whole Embryo Culture (WEC)

Female Sprague-Dawley rats were time-mated and obtained from Charles River (Portage, MI). Females were checked for positive pregnancy by vaginal smear on the morning following copulation. The morning of a sperm-positive result was designated as gestational day (GD) 0. Animals were kept on a 12 h-12 h light/dark cycle, and were given food and water *ad libitum*. On the day of each experiment dams were anesthetized, exsanguinated, and sacrificed as previously described [19]. Conceptuses were explanted on GD10, prepared for culture, and placed into 60 ml culture bottles containing 10 ml of control media. Two different leupeptin exposure protocols were used, both starting with conceptuses explanted

into culture on GD10. The first group designated as “26 h” was exposed to 50 μ M leupeptin by direct addition to the culture medium on the morning of explant and were assessed on GD11 after a total of 26 h in culture. In the second group, designated as “6 h”, conceptuses were cultured for 20 h in control media from GD10 and then treated with 100 μ M leupeptin on GD11, beginning after the 95% O₂/5% CO₂ gas change, for a total treatment duration of 6 h. Conceptuses from four to six different litters were age-matched based on extent of neural tube closure and degree of axial rotation at the time of tissue collection and randomized across treatment groups to remove age and litter bias. Control media contained 5 ml heat-inactivated rat serum and 5ml 1X Hanks’ Balanced Salt Solution (HBSS), supplemented with 43 μ l of penicillin-streptomycin (10,000 U/ml). No more than ten conceptuses were cultured in each bottle. To optimize growth, bottles were gassed on GD10 after explantation with 20% O₂/5% CO₂ and on the morning of GD11 with 95% O₂/5% CO₂. Culture bottles were incubated at 37°C in a roller apparatus throughout the culture period as described previously [10, 19, 20].

Exposure and Sample Collection

Leupeptin was suspended in double-distilled water and added to each culture bottle at the time of exposure to yield a 50 μ M final concentration in the culture medium for 26 h exposures or a 100 μ M final concentration for 6 h exposures. These concentrations were optimized for stage of embryonic development and duration of culture based upon morphology, growth parameters, viability, and the ability to inhibit proteolysis in WEC [10, 21]. Whole embryo culture of rat conceptuses for 26 h in concentrations of leupeptin up to 100 μ M, beginning on GD 10, maintains complete viability but reduces total EMB and VYS protein content. The 26 h exposures of 50 μ M leupeptin result in viable, but much smaller, embryos with few gross anatomical defects but with a high incidence of avascular VYS, anophthalmia, and some other defects of the optic field. No defects or growth restrictions were seen in the 6 h exposures under any condition. At the conclusion of the exposure, conceptuses were removed from the culture bottles and washed 2X in HBSS. The VYS and embryo were manually separated for each conceptus using watchmaker’s forceps.

VYS and EMB samples for DNA isolation were pooled in duplicate, snap frozen, and stored at –74°C. Samples to be assayed for Mat2a activity were pooled in triplicate and placed in 250 μ l of reaction mixture composed of 50mM tris ethanesulfonic acid (TES), 50 mM KCl, 15mM MgCl₂, and 0.3mM EDTA prior to beginning the Mat2a activity protocol [22]. Samples for analysis of C₁ components were collected in 100 μ l of reducing agent, consisting of 200mM dithiothreitol and 100mM NaOH in water. EMB and VYS tissues collected for immunoblotting were placed into 50 μ l of RIPA buffer, briefly sonicated, and stored at –80°C until their use in the immunoblot protocol.

Mat2a Specific Activity

Mat2a activity was quantified in EMB and VYS crude homogenates based on the measured rates of new SAM biosynthesis from methionine and ATP. EMBs and VYSs were manually separated, placed into 250 μ l of TES reaction buffer, and snap frozen until incubation and processing. Samples were thawed, gently sonicated, and allowed to equilibrate by incubation at 37°C for 10 min. After that time, 20 μ M L-methionine and 5mM ATP were added to the

incubation mixture and allowed to react for 1 h at 37°C. Sodium acetate buffer (0.1M) and 10% perchloric acid were added to the incubation mixture to terminate the reaction. The samples were then centrifuged at $10,500 \times g$ for 2 min, the supernatant was removed to a new tube, and samples were processed for SAM determination as described above. Protein pellets were solubilized in 0.25M sodium hydroxide and total protein was determined using the bichinchoninic acid (BCA) procedure (Pierce, Rockford, IL). Activities are expressed as $\mu\text{mol SAM synthesized/min}$, and data was normalized to protein values (mg) for determination of specific activities.

RNA Isolation and qPCR

RNA was isolated from EMB and VYS using the RNeasy Mini Kit (Qiagen, Valencia, CA) as performed on a QIAcube (Qiagen). RNA was converted into cDNA using the RT² First Strand Kit (SABiosciences, Valencia, CA). Commercially available qPCR primers for *Mat2a*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and housekeeping gene *ActB* (SABiosciences) were used to detect changes in gene expression. cDNA, primers, and SYBR Green master mix (Qiagen) were added onto a 384-well plate, with each gene being run in triplicate for each sample. PCR was performed at the University of Michigan Microarray Core Facility using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Controls without template and controls without reverse transcriptase were performed for each sample to ensure run quality. Results were normalized to actin expression for each respective tissue and treatment.

Western/Immunoblotting

Samples were thawed and sonicated in RIPA buffer. Protein concentrations were determined by BCA assay. 30 μg of protein per sample were loaded and separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked overnight with TBST-milk at 4°C prior to a 2 h incubation with anti-Mat2a rabbit polyclonal antibody (Abcam, Cambridge, MA) at a concentration of 1 $\mu\text{g/ml}$. The membrane was then washed with TBST prior to 2 h incubation with alkaline phosphatase (AP)-conjugated chicken anti-rabbit IgG (Santa Cruz, Santa Cruz, CA) at a concentration of 1 $\mu\text{g/ml}$. Secondary antibody fluorescence was developed following reaction with ECF Substrate (GE Healthcare Life Sciences, Piscataway, NJ) and visualized with a Fujifilm FLA-5000 Imager (Valhalla, NY). Because Mat2a and actin have similar molecular weights, the membrane was cleared using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), washed twice with PBS, and washed twice with TBST. The membrane was then incubated for 2 h with anti-actin goat polyclonal antibody (Santa Cruz) at a concentration of 1 $\mu\text{g/ml}$. The membrane was washed with TBST and incubated for 2 h with AP-conjugated donkey anti-goat IgG (Santa Cruz) at a concentration of 1 $\mu\text{g/ml}$. Signals were visualized as above and densitometric measurements were taken for both the Mat2a and actin images using Image Reader software (Fujifilm). Mat2a/actin ratios were calculated from densitometry data for all control and leupeptin-treated samples. Relative ratios from the normalized control and treated values were calculated to determine percent change between treatment groups.

C₁ Component Quantitation

Quantitation of SAM, total cysteine, methionine, homocysteine (Hcy), and dimethylglycine (DMG) was performed using an LC-MS/MS method for a TSQ Quantum (Thermo Scientific, Rockford, IL) coupled with a Waters 2695 Separations Module outfitted with a Luna C18(2) 5 μ m 250 $\times$ 4.6 mm column and 4 mm C18 guard column (Phenomenex, Torrance, CA) and recorded using Xcalibur software (Thermo Scientific). Sample preparation was done using a modified version of the protocol outlined by Weaving [23]. Briefly, samples were sonicated and incubated in the reducing agent at room temperature for 15 min. At this time, isotopic standards for each analyte were added at concentrations of 500 ng/ml SAM, 200 ng/ml DMG, 500 ng/ml cysteine, 10 ng/ml Hcy, and 1000 ng/ml methionine. To suspend the C₁ components, 500 μ l of a mixture containing 0.1% formic acid and 0.05% trifluoroacetic acid in water was added to each vial. Vials were centrifuged at 9000 $\times$ g for 2 min, and the supernatant was transferred to an HPLC vial for analysis.

Mobile phases were prepared, with buffer A being comprised of water with 0.1% formic acid and buffer B comprised of acetonitrile with 0.1% formic acid. An instrument method was programmed to start at 100% A, linearly transition to 90% A and 10% B over 10 minutes, return to 100% A after 15 seconds, and hold there to equilibrate to a total of 30 minutes. Flow rate was set to 0.5 mL/min, and the injection volume was set at 10 μ L. *m/z* transitions were optimized for detection of analytes and isotopic standards as follows: 399.1-249.9 (SAM), 402.1-249.9 (S-adenosylmethionine-*d*₃), 122.0-76.0 (cysteine), 124.0-78.0 (cysteine-*d*₂), 150.0-104.0 (methionine), 153.0-107.0 (methionine-*d*₃), 136.0-90.0 (Hcy), 140.0-94.0 (homocysteine-*d*₈), 104.0-58.0 (DMG), and 110.0-64.0 (N,N-dimethyl-*d*₆-glycine HCl). All parameters were optimized by direct infusion into the MS and tuning. Analyte concentrations were calculated by determining the ratio of analyte peaks to the known isotopic peak concentrations. Because leupeptin may alter cellular protein pools, data was normalized using the control protein concentrations.

DNA Isolation

Genomic DNA was isolated from EMB and VYS tissue using a phenol-chloroform extraction procedure. Briefly, thawed samples were homogenized in 540 μ l of buffer ATL (Qiagen) and digested overnight at 50°C after addition of 60 μ l proteinase K (Qiagen). Approximately 12 μ l of 100 mg/ μ l RNase A (Qiagen) was added to the mixture before repeated extractions from phenol-chloroform isoamyl alcohol in phase-lock gel tubes (5 PRIME), followed by a chloroform-only wash. Finally, samples were precipitated in ethanol and dried prior to storage in Tris-EDTA buffer until use.

LUMA Assay

The LUMinometric Methylation Assay (LUMA) was used to measure global methylation of EMB and VYS DNA [24–26]. Methylation-sensitive and -insensitive enzymatic digestion of 300ng genomic DNA at CCGG sites was completed using HpaII and MspI enzymes (Invitrogen), respectively. Cleavage with EcoRI (Invitrogen) was also completed as an internal control. Annealing buffer (Qiagen) was added after digestion, and the products were analyzed using the PyroMark™ Q96 MD system (Qiagen). GTGTACATGTGTG was used as the dispensation order to eliminate background. The MspI/HpaII ratios were calculated

relative to the EcoRI control, and the percent methylation of each sample was calculated using the equation: $1 - [(HpaII/EcoRI)/(MspI/EcoRI)] \times 100$. Samples were run in triplicate to account for within sample variation.

Mat2a promoter methylation

Sodium bisulfite conversion of EMB and VYS DNA was completed utilizing the EpiTect[®] Bisulfite Kit (Qiagen) and subsequent cleanup was accomplished using the QIAcube platform (Qiagen). PCR and pyrosequencing primers were designed using the PyroMark Assay Design 2.0 Software (Qiagen). The assay was designed against the reverse bisulfite-converted strand, and primer sequences and sequence for analysis are listed in Table 1. Within the sequence to analyze, the letter 'R' represents a site where a purine (either A or G) is interrogated, giving complementation to either cytosine or thymine. The primer amplicon falls within a CpG island on the *Mat2a* promoter upstream of the transcriptional start site. Of the eight sites investigated for CG methylation using this pyrosequencing assay, site 7 interrogates methylation at the GC box required for binding of the transcriptional factor, Sp1 (Figure 2). The region of interest was amplified using the bisulfite-converted DNA (2 µl), HotStarTaq master mix (Qiagen), reverse primer (0.5 pmol), and a biotinylated forward primer (0.5 pmol) in a PCR reaction with an annealing temperature of 54°C. Amplified products were verified using gel electrophoresis and analyzed in duplicate using the PyroMark[™] Q96 MD System (Qiagen) and a predetermined sequence-to-analyze run (Table 1). Two samples were excluded from the VYS leupeptin 26 h exposure group due to SNPs in the *Mat2a* locus.

Statistical Analyses

Values presented are means $\pm$ SD. Statistical significance was determined using unpaired two-tailed student t-tests, and a confidence level of 95%. Statistical outliers were removed using a threshold of 1.5 times the inter-quartile range outside of the first and third quartile values.

RESULTS

Mat2a specific activity

Mat2a enzyme specific activities (µmol SAM/min/mg protein) were determined in EMB and VYS based on SAM biosynthesis from methionine and ATP and were normalized to total protein concentrations (Figure 3). Mat2a specific activities were almost identical ($p=0.991$) in control EMB (3.06 ± 0.42) and VYS (3.07 ± 1.32). Following leupeptin treatment, Mat2a specific activities decreased significantly after 6 h in EMB (2.17 ± 0.48) by 29% ($p=0.022$). Similarly, leupeptin treatment in the VYS (0.08 ± 0.02) reduced Mat2a specific activity by 97% ($p=0.007$).

Mat2a and DNA Methyltransferase (Dnmt) expression

Mat2a expression in EMB (1.34 ± 0.05) was significantly higher than *Mat2a* expression in VYS (1.20 ± 0.10 ; $p=0.020$) (Figure 4). No significant changes in *Mat2a* expression due to leupeptin treatment were produced in EMB (1.30 ± 0.11 ; $p=0.429$) nor in VYS (1.24 ± 0.13 ;

$p=0.580$). No changes were observed in *Dnmt1*, *Dnmt3a*, or *Dnmt3b* expression due to leupeptin treatment, nor did expression differ by tissue for any gene ($p>0.1$).

Mat2a and total protein quantification

Leupeptin treatment during the 6 h incubation on GD 11 produced a $15.7\% \pm 0.1$ decrease in Mat2a protein concentration in the EMB, and a $59.2\% \pm 0.1$ decrease in the VYS based on densitometry from the immunoblots (Figure 5A,5B). Mat2a densitometry results were normalized to the actin values from the same column of the gel.

Total protein values, as determined by the BCA assay, were increased by 79.3% in the VYS and 18.4% in the EMB following 6 h leupeptin treatment (Figure 5C). The total protein effects of leupeptin-treatment significantly differed by tissue ($p=0.001$), and the increase in the VYS was statistically significant ($p<0.001$).

C₁ Component Concentrations

C₁ component concentrations (μg) were quantified for each sample to determine whether leupeptin inhibition of protease activities in the VYS results in depletion of cellular substrates for C₁ metabolism in the EMB and VYS after a 6 h exposure (Table 2). Control EMB SAM concentrations (1.22 ± 0.30 ; $n=6$) were significantly greater than control VYS concentrations (0.21 ± 0.06 ; $n=6$; $p<0.001$). Although SAM concentrations were decreased in EMB due to leupeptin treatment, variability was high in the leupeptin-treated samples. For this reason, no statistically significant difference was observed between control EMB and leupeptin-treated EMB (0.75 ± 0.81 ; $n=6$; $p=0.237$). In the VYS, leupeptin-treatment significantly reduced SAM concentrations compared to control concentrations (0.10 ± 0.07 ; $p=0.012$).

Methionine concentrations in control EMB (1.74 ± 0.58 ; $n=6$) were significantly higher than control VYS methionine concentrations (0.67 ± 0.34 ; $n=6$; $p=0.003$) (Table 2). Leupeptin treatment significantly reduced methionine concentrations in EMB (0.24 ± 0.10 ; $n=6$; $p=0.001$), as well as in the VYS (0.17 ± 0.01 ; $n=4$; $p=0.015$). Cysteine concentrations in control EMB (6.37 ± 2.18 ; $n=6$) and control VYS did not significantly differ (4.81 ± 1.55 ; $n=5$; $p=0.211$). Leupeptin treatment significantly reduced cysteine concentrations in EMB (2.67 ± 0.81 ; $n=6$; $p=0.003$). Leupeptin treatment yielded a 56% increase in cysteine concentrations in VYS, though this change was not statistically significant (7.52 ± 2.59 ; $n=6$; $p=0.071$).

Control EMB Hcy concentrations (0.46 ± 0.04 ; $n=5$) and control VYS concentrations did not significantly differ (0.57 ± 0.20 ; $n=6$; $p=0.258$). Leupeptin treatment yielded no significant effect on Hcy in EMB (0.47 ± 0.90 ; $n=5$; $p=0.998$), nor in VYS (0.54 ± 0.12 ; $n=6$; $p=0.738$). DMG concentrations were below the limit of detection in VYS for this method, so no results can be reported on this trend. In EMB, leupeptin-treated EMB (0.11 ± 0.03 ; $n=6$) had higher DMG concentrations than controls, though this change was not statistically significant (0.09 ± 0.03 ; $n=6$; $p=0.196$).

Global methylation levels

Global DNA methylation status was quantified in the conceptus using the LUMA assay. Embryos and VYSs were assayed separately on GD11 with and without leupeptin treatment as described for each of the 26 h and 6 h exposure protocols. Control EMB DNA was found to be 75.63 ± 3.30 and 72.42 ± 3.29 percent methylated in the 6 h and 26 h protocols, respectively (Figure 6). These differences were not significant. Untreated VYS controls were methylated at a significantly lower rate than their corresponding embryos at 57.94 ± 8.20 and 53.44 ± 6.64 percent, respectively for the 6 h ($p < 0.001$) and 26 h ($p < 0.001$) protocols. Leupeptin treatment significantly reduced embryo methylation to 71.00 ± 3.71 percent after 6 h exposure ($p = 0.009$) in the EMB and to 50.58 ± 6.55 ($p = 0.05$) in the VYS compared to untreated controls (Figure 6A). Leupeptin treatment for 26 h in the VYS reduced the global methylation percentage to 66.00 ± 5.13 percent in the EMB ($p = 0.007$) and to 45.45 ± 9.45 percent in the VYS ($p = 0.05$) (Figure 6B).

Mat2a promoter methylation

Because *Mat2a* contains several CpG sites flanking a Sp1 transcription factor binding site, it was important to identify whether changes in histiotrophic nutrition may indirectly affect genome-wide epigenetic regulation via hypermethylation of the *Mat2a* promoter and decreased *Mat2a* expression and activity. After disruption of endocytosis, no significant changes in methylation at CpG sites in the *Mat2a* promoter were observed (Figure 7). *Mat2a* exhibited low methylation levels at this locus across all tissues, exposures, and exposure durations (Figure 7A,7B). The individual CpG site 7 (*****Figure 7C,7D) showed increased methylation levels compared to other sites in the *Mat2a* locus, but did not reveal significant methylation effects by tissue type or exposure group.

DISCUSSION

Genetics play a large role in instructing phenotypic outcomes during development, but these regulatory controls are complemented by nutrients, environmental factors, and physical insults that inform the epigenetic patterning required for differentiation and the establishment of terminal cell fates and functions. Because the placenta is not yet formed or fully functional during the early postimplantation phases of development, histiotrophic uptake of maternal proteins and their cargos, as facilitated by RME, represents the primary source of nutrient supply during organogenesis. While well established in rodent models, the relevant importance of histiotrophic processes in human embryonic development has been debated. Even though significant anatomical differences exist between the maternal-conceptual interface and the duration of gestation of rodents and humans, recent evidence also strongly supports a significant role for HNP in mediating nutrient uptake in the human conceptus until the time when the placenta becomes fully functional at the end of the first trimester [27, 28]. Nutrients, in the form of vitamins, precursors and cofactors that are important for C₁ metabolism and other cellular processes, are believed to be obtained exclusively through HNP by uptake across maternal-conceptual interfaces such as the syncytiotrophoblast and VYS during the organogenesis stage of embryonic development [14, 15, 29]. Bulk proteins are captured into vesicles, fused with lysosomes in these tissues and degraded by cysteine proteases to provide amino acids for embryonic biosynthesis.

Leupeptin inhibition of lysosomal proteolysis in the VYS during early organogenesis prevents the degradation of maternal proteins, thus, limiting the availability of methionine and other components required for biosynthesis of SAM in the embryonic C₁ pathways. Our hypothesis states that interruption of HNPs should decrease SAM biosynthesis and its cellular concentrations due to the reduced supply of substrates and precursors required for C₁ metabolism and, as a result, affect the overall extent of DNA methylation required to sustain normal epigenetic programming.

In the HNP model, leupeptin should cause a decrease in C₁ precursors available for methylation processes. Table 2 shows changes in methionine and total cysteine in both the EMB and the VYS, although the magnitude of these changes differs by tissue. Methionine is significantly decreased due to leupeptin treatment in both the EMB and VYS, as would be expected from disruption of HNPs. Total cysteine, on the other hand, decreased in the EMB and increased in the VYS. Decreases in total cysteine in the EMB can be explained by inhibition of source protein degradation in the VYS. Increases in total cysteine in the VYS may be related to changes in the dynamic balance of cysteine transport and utilization that occur in biosynthesis pathways, such as incorporation into GSH. Hcy and DMG (C₁ metabolic products) remain unchanged following treatment and exhibit no tissue differences in cellular concentrations.

The disruption of HNPs and altered methionine and cysteine in the EMB and VYS are accompanied by an approximate 52% decrease in SAM in the VYS and a 39% decrease in SAM in the EMB. The significant decrease in SAM in the VYS is expected based on the reduction of methionine available for synthesis. A change in the EMB was noted, but was not significant, due to the high variability of SAM in the EMB after leupeptin treatment. This suggests that leupeptin treatment introduces a degree of instability in the embryo, likely resulting in disruption of multiple cellular processes. The difference in SAM concentrations between EMB and VYS is also notable. SAM concentrations in the EMB are nearly six times those found in the VYS.

In this study, we have identified several C₁ metabolism-related outcomes that are affected by the disruption of HNPs and the attenuated provision of nutrients to the developing conceptus. SAM is the critical final product of C₁ metabolism and is essential for its role in for epigenetic programming. Variations in several other C₁ substrates and cofactors that contribute to SAM biosynthesis (folate, choline, vitamin B₁₂) have also been associated with malformations and abnormal developmental outcomes. The conceptual bioavailability of these substrates and cofactors as a function of maternal diet and changes in their concentrations due to chemicals and environmental factors has yet to be characterized. In future studies we hope to determine whether depletion of these substrates required by the C₁ pathway occurs due to exposure *in utero* and whether dietary supplementation of C₁ metabolic precursors may be a potential intervention against developmental growth deficiencies and embryotoxicity.

The enzyme Mat2a is responsible for the final catalytic step in SAM biosynthesis, combining adenosine from ATP with methionine. Lack of sufficient precursors could limit SAM biosynthesis as proposed, but other direct effects on the enzyme itself cannot be ruled

out. Six-hour GD11 exposure to leupeptin in WEC produced a significant reduction in Mat2a specific activity in both the EMB and the VYS, which is consistent with observed decreases in SAM concentrations. Mat2a specific activities (Figure 3) were normalized to total sample protein according to accepted protocols. Because of leupeptin's inhibition of proteolytic activity in the VYS and the continued endocytotic uptake of maternal proteins, total protein in the VYS was significantly increased over that normally found in the untreated VYS (Figure 5C). This results in a decrease in Mat2a protein to total protein ratio. Because this affects the specific activity calculation, Mat2a specific activity was also calculated using control protein values for normalization (Supplemental Figure 1). The reductions in Mat2a specific activity in both the EMB and VYS remained statistically significant, again, supporting the decreases in SAM concentrations.

Mat2a expression was unchanged in both the EMB and the VYS after treatment, and DNA methylation was extremely low in the CpG island flanking the Sp1 regulatory region of the *Mat2a* promoter. The *Mat2a* promoter region was found to be virtually un-methylated, indicating any further decreases in methylation due to the leupeptin-induced reductions in SAM availability are, therefore, not expected. The lack of significant alterations in *Mat2a* expression and methylation can be expected, as the product of this gene plays a very important functional role in the cell in catalyzing the final step of SAM biosynthesis and would, therefore, be expected to be constitutively expressed. Regulation is likely to be mediated through cellular sensors that may not be easily induced or suppressed. As the universal methyl donor in cells, SAM availability is critical for methylation processes in epigenetic programming such as for DNA and histone methylation. Because methylation is a semi-permanent modification, methylation would not be a likely regulatory mechanism for *Mat2a* expression.

Immunoblot data suggest reduced Mat2a protein concentrations in both the embryo and VYS due to leupeptin treatment, with a more profound decrease in Mat2a in the VYS. However, these changes may not be entirely accurate due to the significant leupeptin-induced differences in relative total protein concentrations in the EMB and VYS. Decreased proteolysis due to leupeptin treatment increases the total cellular protein content, mostly contained in endocytotic vesicles, and reduces the relative proportion of intrinsic proteins and enzymes active in cellular processes. Thus, this decrease in Mat2a abundance in the embryo and VYS may be partly artifactual due to these altered protein proportions.

Because both Mat2a specific activity and protein concentration decrease with inhibition of HNPs, it is important to consider the regulation of the Mat2a protein. It is possible that redox signaling may play a role in Mat2a regulation. Oxidative modifications to the cysteine-150 residue of the Mat2a peptide has been shown to lead to a loss of activity and dissociation of the tetrameric complex, likely because the residue falls within the Mat2a ATP binding site [30]. Thus, it is possible that oxidative stress may be able to decrease the catalytic activity of Mat2a by a directed post-translational modification. Preliminary evidence from our laboratory suggests that attenuation of HNP activity leads to decreases in GSH biosynthesis and a shift to more oxidizing conditions in the conceptus (Harris *et al.*, unpublished results). Direct regulation of Mat2a may also explain changes in specific activity and protein abundance. Degradation of the Mat2a protein by compensatory protease

activity may be increased due to leupeptin treatment. It is also possible that the Mat2a protein has a high turnover rate. If the Mat2a protein has fast rates of both biosynthesis and degradation, disruption of HNPs would decrease the amount of amino acids needed for new protein synthesis and shift the balance in favor of lower Mat2a concentrations. Ultimately, these regulatory changes may lead to altered Mat2a function and cellular abundance.

Epigenetic modifications, like DNA methylation, are crucial for normal development. The disruption of HNPs and resulting decrease in C₁ components such as SAM may lead to a decrease in the substrates available for methylation processes and/or changes in methylation and demethylation enzymes required for modifications. Expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* was unchanged in both the EMB and VYS. Because these are the enzymes directly involved in the placement of methyl groups on the DNA, it is suggested that regulation of DNA methylation in this case does not occur at the transcriptional level. It is likely that these enzymes are constitutively expressed during this period of development, as epigenetic patterning is being established in tissues.

Using the LUMA assay to measure global DNA methylation at CCGG sites, it was shown that EMB and VYS differ significantly with regards to their baseline percentage of global methylation. Average methylation values in the EMB approached 72%, while VYS values were found to be about 58%. The EMB and VYS tissues differ with respect to their relative states of terminal differentiation, cellular homogeneity, imprinting patterns, and embryonic germ line of origin, all of which have been previously noted as being important factors for differential methylation status [31]. It is quite possible that differences of similar magnitude may also exist within the EMB proper based on the high degree of cell and tissue heterogeneity present during this stage of development, but the details of spatial methylation patterns are not known.

Along with significant differences in basal levels of global methylation, for each of the treatment protocols, leupeptin produced a significant additional reduction in DNA methylation in EMBs from 6–9% and in the VYS from 11–15%. The ability of leupeptin to significantly reduce global methylation levels in WEC after only 6 h of treatment is remarkable. Of interest is the observation that leupeptin exposure for periods of time, much longer than 6 h, did not produce any additional changes in the percentage decrease in global DNA methylation levels. This suggests that the full range of methylation-related events occurs rapidly in the conceptus and that a threshold of change exists, beyond which no additional demethylation will occur. Several different factors could be responsible for the decrease in global methylation levels including (a) a reduction in the developmentally programmed and ongoing DNA re-methylation events aided by insufficient SAM availability; (b) the inability of the conceptus to maintain established DNA methylation marks, including those that are replicated through successive cell generations via enzymatic DNMT1 maintenance activity; and (c) the demethylation of existing DNA methylation marks. Further investigations into the regulation of DNMT function by environmental and biochemical factors will be required to understand this potential mechanism of susceptibility. Because epigenetic programming during this stage of development is crucial for differentiation, normal tissue patterning, and growth, significant modifications of the global epigenome would be expected to exert their effects on embryonic viability and the

elicitation of morphological defects. Based on the significant decreases in global methylation observed, however, it is reasonable to expect that methylated regulatory sites throughout the epigenome may be subject to demethylation. Thus, epigenome-wide DNA methylation studies will be necessary to fully identify the subset of regulatory regions that are sensitive to epigenetic change during this phase of development.

To summarize, decreased amounts of C₁ precursors lead to decreased SAM and global DNA methylation. It is likely that Mat2a plays a large role in the regulation of these methylation processes, as SAM concentrations mimic Mat2a activity. It is unknown at this time how other changes in HNPs, such as disruption of RME, may also alter these processes. Because RME is responsible for the uptake of C₁ substrates such as folate, choline, betaine, and B vitamins at this stage of development, modifications to the endocytotic potential of the VYS may be able to produce detrimental epigenetic effects in the developing EMB. Future investigation into the relationships between toxicant-induced changes in these processes and the nutrient environment of the conceptus is necessary to understand the pathways by which developmental exposures may contribute to abnormal epigenetic programming.

In conclusion, this study identifies a window of susceptibility during which the developing epigenome may be affected by nutrients and environmental factors during organogenesis. Disruption of HNPs leads to decreased availability of C₁ components, reduced Mat2a activity, and decreased global DNA methylation in the VYS and embryo. All of these changes indicate that histiotrophic nutrition is crucial for epigenomic programming in the early organogenesis-stage conceptus. Tissue-specific differences between EMB and VYS were evident throughout this study, corresponding with divergence in degrees of differentiation and homogeneity. Environmental hindrance of HNPs may epigenetically alter gene expression in the conceptus, and these changes may be detrimental throughout the life course. Further investigation into the specific pathways and products being altered by dietary factors and chemical exposures, namely HNPs and C₁ metabolism, which lead to developmental abnormalities and predisposition to disease throughout the life-course is crucial to understanding the overlaying role of embryonic nutrition during development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Histiotrophic nutrition pathways (HNPs) influence C_1 metabolism, SAM biosynthesis, and DNA methylation

HNPs are the primary mechanism of nutrient supply during early organogenesis.

Histiotrophic functions include receptor-mediated endocytosis (RME) and proteolysis of endogenous nutrient proteins taken up through the VYS brush border. After proteolysis, resulting amino acids are made available for cellular functions such as protein biosynthesis and C_1 metabolism. Leupeptin treatment inhibits proteolysis, limits the availability of critical substrates through pathways (marked with an X), and results in limited biosynthesis of biomolecules (down arrows). This figure summarizes our hypothesis, showing the reduction of precursors to pathways, through which SAM, the primary methyl donor for DNA (and other molecule) methylation, is generated. The lack of SAM will limit DNA methylation and result in the observed global DNA hypomethylation.

Figure 2. Sequence of the 5' flanking region of the rat *Mat2a* gene

The forward (biotinylated) and reverse primers bind to the bisulfite-converted sequence of the italicized region above, producing a 201 bp pyrosequencing amplicon highlighted in the box. A sequencing primer anneals to the TATA box region and extends in the opposite direction to interrogate eight individual CpG sites for methylation surrounding the Sp1 transcription factor binding GC box.

Figure 3. Mat2a specific activity after 6-hour leupeptin exposure

Mat2a specific activities are decreased by leupeptin exposure in both EMB and VYS. Mat2a activity is similar in the EMB (n=4) and VYS (n=5) under control conditions. Specific activity is significantly reduced by leupeptin treatment in the EMB (n=5; p=0.022) and in the VYS (n=5; p=0.007). **indicates p<0.01; *indicates p<0.05

Figure 4. Mat2a, DNMT1, DNMT3a, and DNMT3b gene expression after 6-hour leupeptin treatment

Mat2a gene expression was significantly higher in the EMB (n=5) than the VYS (n=6; $p=0.02$). No statistically significant changes were noted between control (n=5) and leupeptin-treated (n=6) EMB for any of the gene targets tested ($p>0.1$). Likewise, no significant changes occurred in the control (n=6) or leupeptin-treated (n=6) VYS samples ($p>0.1$) for all targets.

Figure 5. Mat2a protein concentrations and total protein content after 6-hour leupeptin treatment

Densitometric readings following Western blotting (**A**) revealed a significant effect difference between EMB (n=3) and VYS (n=3; $p=0.001$), indicating that leupeptin treatment produced a more significant decrease in the Mat2a protein in the VYS (–59%) than the EMB (–16%) (**B**). However, the accumulation of extrinsic proteins due to leupeptin treatment also results in an 18% increase in total protein in the EMB and 79% in the VYS (**C**). *indicates $p=0.001$, **indicates $p<0.001$

Figure 6. Global percent methylation for EMB and VYS tissues after 6-hour or 26-hour exposure determined by LUMA

EMB percent methylation is significantly higher than VYS methylation across all tissues ($p < 0.001$). **(A)** Control EMB ($n=10$) was significantly higher than leupeptin EMB ($n=10$) after 6-hour exposure ($p=0.009$). Control VYS ($n=11$) methylation was significantly higher than leupeptin VYS ($n=8$; $p=0.05$). **(B)** Control EMB ($n=9$) was significantly higher than leupeptin EMB ($n=8$) after 26-hour exposure ($p=0.007$). Control VYS ($n=10$) was higher than leupeptin VYS ($n=8$; $p=0.05$). **(A&B)** Control EMB and leupeptin EMB both showed significant decreases in percent methylation between 6- and 26-hour exposure ($p=0.05$ and 0.03 , respectively). VYS showed no significant change over time. **indicates $p < 0.01$; *indicates $p < 0.05$

Figure 7. *Mat2a* promoter methylation levels determined by Pyrosequencing

The *Mat2a* promoter exhibits low methylation levels surrounding the Sp1 binding site in both the EMB and VYS after 6-hour or 26-hour leupeptin exposure. **(A)** No significant difference was detected between control EMB (n=10) or leupeptin EMB (n=10) after 6-hour exposure, or between control VYS (n=10) or leupeptin VYS (n=9). **(B)** Likewise, there was no significant difference between control EMB (n=8) and leupeptin EMB (n=8) after 26-hour exposure, or between control VYS (n=8) and leupeptin VYS (n=6). **(C)** Although individual CpG site 7 methylation is above average for the *Mat2a* locus assayed, no significant changes in methylation were apparent for tissues after 6-hour leupeptin exposure. **(D)** No statistically significant changes in CpG site 7 methylation were detected after 26-hour leupeptin exposure.

Table 1PCR and Pyrosequencing Primers (5' to 3') and Analyzed Sequence for the *Mat2a* Promoter

Primer Type	Primer Sequence	Melting Temp (°C)
Forward-Biotinylated	GTTATTAGGATGATTAATTATTGTTAGG	54.5
Reverse	ACCCAATATTTATAAAACAACCTCC	56.3
Sequencing	CAATATTATAAAACAACCTCC	40.8
<i>Sequence to Analyze</i>	CRCRCRCRCCCRACCTCRAACCCCRCCCTCRACRTCRCRCRCCAATACRCCACRAACAAACRAAT	

Table 2

Leupeptin-induced effects on bioavailability (μg) of one-carbon metabolism components concentrations in embryo and visceral yolk sac

Component	Control	Leupeptin-treated
S-adenosylmethionine		
EMB	1.22 ± 0.30	0.75 ± 0.81
VYS	0.21 ± 0.06^a	0.10 ± 0.07^c
Methionine		
EMB	1.74 ± 0.58	0.24 ± 0.10^b
VYS	0.67 ± 0.34^a	0.17 ± 0.01^c
Cysteine (total)		
EMB	6.37 ± 2.18	2.67 ± 0.81^b
VYS	4.81 ± 1.55	7.52 ± 2.59
Homocysteine		
EMB	0.46 ± 0.04	0.47 ± 0.90
VYS	0.57 ± 0.20	0.54 ± 0.12
Dimethylglycine		
EMB	0.09 ± 0.03	0.11 ± 0.03
VYS	ND	ND

^a Indicates a significant difference between control EMB and control VYS ($p < 0.05$);

^b indicates a significant difference between control EMB and leupeptin-treated EMB ($p < 0.05$);

^c indicates a significant difference between control VYS and leupeptin-treated VYS ($p < 0.05$).

'ND' signifies that no data was collected due to results falling below the limit of detection.