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**Title:** Maternal intermittent fasting deteriorates offspring metabolism via suppression of hepatic mTORC1 signaling

**Running Title:** maternal IF and offspring metabolism

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**Abstract**

The metabolic benefits of intermittent fasting (IF) have been well-recognized. However, limited studies have examined the relationship between long-term maternal IF before pregnancy and offspring health. In this study, a C57BL/6J mouse model of long-term IF before pregnancy was established: 4-week-old female mice were subjected to alternate-day fasting for 12 weeks and resumed normal diet after mating. Female mice in control group were fed ad libitum. Offspring mice were weaned at 6 weeks of age and fed normal chow diet or 60% high fat diet. The effects of long-term pre-pregnancy IF on offspring metabolism and its underlying mechanism were examined. We found that neonatal IF offspring weighted significantly less relevant to control mice. This difference gradually disappeared as a result of catch-up growth. In the IF offspring, adipose tissue mass was significantly increased. This alteration was associated with a considerable deterioration in glucose tolerance. No significant difference in food intake was observed. Further, lipid deposition, as well as triglyceride contents in the liver were greatly increased. Maternal IF significantly decreased levels of DNA methyltransferase in the liver of offspring. DNA methylation modifications of molecules associated with the mTORC1 signaling pathway were significantly altered, leading to the significant inhibition of mTORC1 signaling. Overexpression of S6K1 activated hepatic mTORC1 signaling, and reversed the metabolic dysfunction in IF offspring. In conclusion, long-term pre-pregnancy IF increases hepatic steatosis, adiposity, as well as impairs glucose

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metabolism in adult offspring. This occurs through DNA methylation-dependent suppression of hepatic mTORC1 signaling activity.

**Keywords**

maternal nutrition; intermittent fasting; hepatic steatosis; adiposity; DNA methylation

**Abbreviations:**

IF, intermittent fasting; DOHaD, Developmental Origins of Health and Disease; AL, ad libitum; DNMT, DNA methyltransferase; BAT, brown adipose tissue; DMSO, dimethyl sulfoxide; eWAT, epididymal white adipose tissue; rWAT, retroperitoneal fat; HFD, high-fat diet; mTOR, mechanistic target of rapamycin; NCD, normal chow diet; PPAR, peroxisome proliferator-activated receptor; S6K1, ribosomal protein S6 kinase beta-1; sWAT, subcutaneous white adipose tissue; TSC1, tuberous sclerosis 1; UCP1, uncoupling protein 1; WAT, white adipose tissue; RYGB, Roux-en-Y Gastric Bypass.

## Introduction

Due to its metabolic benefit, calorie restriction is becoming a popular approach for weight reduction. Among all calorie restriction methods, intermittent fasting (IF) is accomplished by fasting completely or partially for a period of time followed by a regular meal. Because of its being easy to implement, IF is becoming popular(1-3). There are three main methods for IF: alternate-day fasting, time-restricted eating and

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periodic fasting (5:2 diet)(4). Experimental studies in rodents have shown that IF can significantly improve many physiological functions, and delay the progression of chronic metabolic diseases and a variety of age-related neurological disorders(1, 5-7). Human epidemiological studies have also shown that IF can significantly reduce body weight, leading to a decrement in risk for diabetes and cardiovascular disease(8, 9). The clinical trial by Stekovic et.al in which IF was applied to a medium-sized human cohort for a long period of time has showed that IF is more tolerable than sustained calorie restriction to improve cardiovascular system and body composition(10). More importantly, IF has been demonstrated to be safe to practice in healthy non-obese adults for more than 6 months(11). Metabolic transformation of fed-fast cycle induced by IF significantly enhances the anti-stress and protective capacity of cells, which promotes cell repair, induces autophagy and optimizes stress responses(12-14).

The impact of long-term IF before pregnancy on offspring metabolism remains largely unknown. A series of evidence has indicated that proper nutritional status before and during pregnancy is critical for maternal health and fetal development(1, 15). Due to the special metabolic needs of placenta, fetal tissue, blood volume and brain development during pregnancy, maternal nutrition has a considerable impact on fetal health. Maternal overnutrition/obesity or undernourishment increases the risk of developing metabolic disease in adult offspring(16, 17). The " Developmental Origins of Health and Disease (DOHaD)" theory highlights the link between early fetal nutrition and adult health(18, 19). According to this theory, poor intrauterine nutrition

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renders foetation adaptive responses to enhance viability. The development, structure, and function of important tissues or organs of the fetus such as thymus, skeletal muscle, lungs, pancreas, and kidneys may experience long-term irreversible changes, that is, re-programming. If the postnatal environment does not match the intrauterine environment, "mismatch" and poor adaptability increase the risk of chronic non-communicable disease of the adult offspring(20). Consistently, epidemiological studies of Ramadan fasting have indicated that maternal intermittent fasting impairs fetal growth(21). Further mechanistic studies have revealed that most fetal phenotypic programming in the DOHaD hypothesis is not accompanied by genotypic changes. Changes in the expression of these genes are primarily due to epigenetic modifications such as DNA methylation, histone modifications, and non-coding RNA modifications(22).

In the present study, we examined the effects of long-term pre-pregnancy IF on offspring metabolism using the mouse model of fasting intermittently for 12 weeks prior to mating. We reported here that long-term IF before pregnancy increases hepatic steatosis, adiposity, as well as impairs glucose metabolism in adult offspring.

## Materials and Methods

1. Reagents

5-Aza-CdR and DMSO were from Sigma (St Louis, MO, USA). Rabbit anti-phospho-mTOR (ser235/236, 1:1000), rabbit anti-mTOR, rabbit anti-phospho-Raptor

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(ser792, 1:1000), rabbit anti-Raptor, rabbit anti-phospho-S6 (ser235/236, 1:1000), rabbit anti-S6, and mouse anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, 1:2000). Rabbit anti-PPARγ, rabbit anti-SCD1, rabbit anti-ACADVL, rabbit anti-PPARδ and rabbit anti-DNMT1 antibodies were obtained from ABclonal (Wuhan, China, 1:500). Rabbit anti-DNMT3b antibody was from Abcam (Cambridge, MA, 1:500).

2. Animals

Four-week-old C57BL/6J female mice and male mice were purchased from the Experimental Animal Center at Peking University Health Science Center and maintained at the Experimental Animal Center at Peking University Health Science Center. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8023, revised 1978). Mice were fed ad libitum unless indicated otherwise.

3. Maternal IF feeding

Maternal IF feeding model was established as follows: After acclimatizing to the vivarium for 5 days, the experimental diet was started. In order to enhance the maternal effect of intermittent fasting, we started dietary intervention in mice at 4 weeks of age. 4-week-old female mice were subjected to intermittent fasting (M-IF) for 12 weeks and resumed normal diet after mating. Female mice in the control group were fed ad libitum (M-AL). The presence of vaginal plugs on female mice after

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overnight mating was designated as embryonic day 0.5 of gestation (E0.5). Intermittent fasting (IF) was achieved by ad libitum feeding for 24 hours followed by fasting for 24 hours. Normal chow diet (10 kcal% fat, D12450H, Research Diets Inc., New Brunswick, NJ) was weighted and provided during the dark period. All food was removed at the beginning of the dark period the next day to calculate food consumption. This feeding scheme eliminates interference of the circadian rhythm and feeding cycle(23).

Preliminary experiments have shown that IF offspring develop relatively slowly and have increased mortality after weaning at 4-week-old. Therefore, in subsequent experiments, both male and female offspring were weaned at 6 weeks of age and fed either normal chow diet (NCD,10 kcal% fat, D12450H, Research Diets Inc., New Brunswick, NJ) or 60% high fat diet (HFD, 60 kcal% fat, D12492), respectively for 12 weeks (Fig. S1A).

4. Measurement of activity and energy expenditure

Metabolic cage measurements were performed by the indirect calorimetry as previously described(24). Briefly, mice were individually housed in metabolic chambers maintained at 20-22°C with a 12-hour/12-hour light-dark cycle, lit at 7:00 and extinguished at 19:00. Metabolic measurements (oxygen consumption, respiratory exchange rate, exercise activity, energy consumption) were continuously obtained using the CLAMS from Columbus Instruments (Columbus, OH). During this

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period, the mice were fed ad libitum. Fat and lean mass were measured by NMR (Echo Medical Systems, Houston, TX).

5. Tissue sample preparation

Mice were anesthetized with 1% pentobarbital sodium, then blood drawn from the heart. Appropriate amount of tissues was harvested from liver, pancreas, subcutaneous WAT, epididymal WAT and perirenal WAT. These tissues were fixed with 4% paraformaldehyde, extracted RNA or protein with RNA trip or RIPA lysis buffer respectively. The rest of the tissue was wrapped in aluminum foil, placed in liquid nitrogen for rapid freezing, and stored at -80°C.

6. Western blot analysis and quantitative RT-PCR

Proteins were separated by an SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. The membrane was incubated in TBST with 5% skim milk at room temperature for 1 hour, then incubated with the primary antibody overnight at 4°C. IRDye-labeled secondary antibodies were used to detect specific reactions and visualized with the Odyssey infrared imaging system.

The extracted total RNA was subjected to reverse transcription and real-time quantitative PCR as described previously(25). Table S1 shows the primer sequences used.

7. Histological analysis

Tissues fixed with 4% paraformaldehyde were embedded in paraffin, sectioned and baked at 60°C for 30 minutes before H&E staining.

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Liver tissues were fixed with 4% paraformaldehyde, embedded in OCT, sectioned and stored at -80°C until used for oil red O staining. Frozen sections of the liver were stained with Oil Red O working solution for 60 minutes, then counterstained with hematoxylin for 5-10 minutes and rinsed with running water for 60 minutes. Staining signal was observed with Leica microscope.

8. Glucose metabolism

Oral glucose tolerance tests: After fasting for 16 hours, mice were orally administered 3 g/kg body weight of glucose. Blood was collected from the tail tip at 0, 15, 30, 60, 90, and 120 minutes after glucose administration. And glucose concentration was immediately measured.

Insulin tolerance test: After fasting for 6 hours, mice were injected intraperitoneally with insulin (0.75 U / kg). Blood was drawn from an incision at the tail tip 0, 30, 60, 90, and 120 minutes after insulin injection, and glucose concentration measured immediately.

9. Measurement of liver and plasma triglycerides

Liver tissue was homogenized with 1 mL of a mixture of chloroform and methanol and left overnight at 4°C. The homogenate was mixed with 200ul distilled water, and centrifuged at 3000 rpm for 10 min at 4 °C. The lower phase was collected, vacuum dried and dissolved in 5% Triton X-100 for lipid measurement. Levels of liver and plasma triglycerides were measured according to the instructions of manufacturer.

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10. Cell Culture

The LO2 human liver cell line and AML12 mouse hepatic cell line, purchased from American-Type Culture Collection (ATCC, Rockville, MD, USA), was cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at a humidified incubator containing 5% $CO_2$ with humidified atmosphere at 37°C. The hepatocyte cell lines (LO2 and AML12) in passage ranges of 5-15 were seeded in separate 6-well plates with a density of $10^6$ cells per well and grew to approximately 80% confluency for experimentation.

11. Whole genome bisulfite sequencing (WGBS)

Four mice were randomly selected from the NH and IH groups. Liver tissue was subjected to genome-wide DNA methylation analysis based on an online random number generator (www.graphpad.com/quickcalcs/randomize1/). The WGBS was conducted by Novgene (Tianjin, China). A detailed explanation of this method is described by Zhao et al (26).

The differentially methylated region (DMR) was identified by DSS analysis software (GA, USA) (27-29). The DMR-related genes in this article are genes whose genome or promoter region overlaps with DMR.

12. Statistical analysis

Using Prism software for graphing and statistics, the experimental results were shown as mean±SEM. Differences between two groups were analyzed using the Student's unpaired t-test. One-way ANOVA was used for comparisons between

multiple groups, followed by a Tukey post-hoc test. $P<0.05$ denotes significant difference.

**Results**

1. Effects of intermittent fasting on maternal metabolism

Although food intake in the maternal intermittent fasting mice (M-IF) increased significantly on the day of feeding (Fig. S1B), cumulative food intake was significantly lower relative to the M-AL animals (Fig. S1C). Body weight of M-IF mice was also lower (Fig. S1D). Random blood glucose on the day of fasting showed a significant reduction in the M-IF mice. After resuming ad libitum feeding upon mating, the random blood glucose levels gradually increased to levels of those animals in the M-AL group (Fig. S1E). Further, glucose tolerance was improved in the M-IF mice on gestation day 4.5 (E4.5), E12.5 and E18.5 (Fig. S1F-H). However, the glucose tolerance of M-IF mice was not remarkably different from that of the control group on postnatal day 10(P10) and P21 (Fig. S1I-J). Circulating triglyceride levels were measured by blood sample harvested from the medial eye angle of mice. The results showed that plasma triglyceride levels were significantly reduced in M-IF mice on E18.5 and P10 (Fig. S1K-N). All these results suggest a beneficial effect of IF on maternal metabolism.

2. Maternal intermittent fasting reduces placenta and neonatal weight

At E18.5, embryos and placenta were harvested and weighed. Fetal weight in the maternal IF group was slightly reduced, but not statistically significant (Fig. 1A).



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Placental weight was lower than in the control group on E18.5 (Fig. 1A). Consistently, neonatal offspring from IF dams lost weight at birth (P0) (Fig. 1B). There was no significant difference in body length (Fig. 1B and Fig. 1C). There was no notable difference in litter size on P1 and P21 (at weaning) between mothers fed different diets (Fig. 1D).

3. Maternal intermittent fasting increases adiposity and glucose tolerance in offspring via a mechanism independent of food intake

Maternal IF reduced birth weight in male offspring relative to controls. This alteration gradually became insignificant as the offspring grew up (Fig. 2A and Fig. 2B). Weight changes in female offspring were similar to those in males (Fig. S2A and Fig. S2B). There was no difference in fat mass between the male offspring of dam fed ad libium (O-AL) and those fasting intermittently (O-IF) under NCD condition (Fig. 2C). Despite of the negligible change in body weight, fat mass demonstrated a significant increase in O-IF male offspring fed HFD (Fig. 2D). Glucose tolerance was substantially deteriorated in O-IF male offspring fed either NCD or HFD (Fig. 2E and Fig. 2F). For O-IF female offspring, glucose tolerance did not change with NCD feeding, but was impaired in mice fed HFD (Fig. S2C and Fig. S2D). In addition, no significant difference in food intake was observed (Fig. 2G and Fig. 2H), suggesting a mechanism independent of food intake.

Insulin tolerance test showed that insulin sensitivity of O-IF group was not significantly different from that of the O-AL group, whether under NCD or HFD

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feeding (Fig. S3A and Fig. S3B). We thus analyzed the insulin release in offspring. Insulin release levels of O-IF group remained unaltered under NCD condition (Fig. S3C). However, glucose-stimulated insulin release levels in O-IF group were higher than those in the control group under HFD condition (Fig. S3D). Consistent with this observation, the morphology of pancreas showed that the number and area of islets of the O-IF group was significantly higher than that of the O-AL group under HFD condition (Fig. S3E). These results indicate that offspring of the IF group may have hyperinsulinemia. The liver also plays an important role in the regulation of glucose homeostasis. However, there were no significant changes in the gene expression of key enzymes involved in glycogen metabolism, glycolysis, and gluconeogenesis in the liver of O-IF group, regardless of NCD or HFD feeding (Fig. S3G and Fig. S3H). These results suggest that the deterioration of glucose metabolism in the O-IF group may rely on alteration in insulin release rather than changes in insulin sensitivity, and hepatic glucose metabolism.

4. Maternal intermittent fasting renders offspring prone to HFD induced adiposity

Under the NCD feeding condition, there was no significant difference in the white adipose tissue mass between the O-IF male group and O-AL male group (Fig. 3A-3C). However, under the HFD feeding condition, the weights of subcutaneous fat (sWAT) (Fig. 3A), retroperitoneal fat (rWAT) (Fig. 3B), and epididymal fat (eWAT) (Fig. 3C) in the O-IF male group were significantly increased relevant to the O-AL control group. H&E staining showed that adipocyte sizes in O-IF group were larger

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than those in O-AL group under HFD conditions (Fig. 3D-F). Changes in white adipose tissue mass were not apparent in O-IF female group, except for an increase in rWAT weight under HFD condition (Fig. S2E-G).

However, levels of genes critical for browning and adipogenesis remained largely unaltered in O-IF mice fed either NCD (Fig. S4A-C) or HFD (Fig. S4D-F).

5. Maternal intermittent fasting increases energy metabolism and activity in offspring

As shown in Fig.4A and 4B, O-IF male mice fed either NCD or HFD demonstrated an increment of spontaneous motility. Also increased were energy and oxygen consumption in O-IF male mice fed NCD (Fig. 4C and 4D) or fed HFD (Fig. 4F and 4G). Interestingly, the respiratory quotient was significantly reduced (Fig. 4E and 4H), indicating an increase in lipid utilization.

6. Maternal intermittent fasting deteriorates liver steatosis via stimulation of lipogenesis

We next examined the liver, a major organ critical for lipid metabolism. Both hepatic weight and triglyceride content were significantly increased in O-IF male mice fed either NCD or HFD (Fig. 5A and Fig. 5B). Plasma levels of triglyceride was significantly increased only in the male offspring fed HFD (Fig. 5C). As shown in Fig.5D, lipid deposition was significantly increased in the liver of O-IF male mice fed either NCD or HFD. For female offspring, the hepatic weight of the O-IF mice was higher than that in the control group under NCD condition (Fig. S2H). However,

changes in hepatic and circulating triglyceride content were not significant (Fig. S2I and Fig. S2J). We thus focused our study on male offspring in the following experiments.

In the O-IF male mice fed NCD, the mRNA levels of hepatic genes relevant to lipid metabolism such as *Pparγ* and *Cd36* were significantly increased, whereas levels of *Acadvl* reduced (Fig. 5E). In the O-IF animals fed HFD, levels of lipogenesis-related gene (*Scd1*, *Pparγ*) were significantly increased (Fig. 5F). In addition, hepatic β-oxidation-related genes (*Acadv, Acadvl*, *Atgl* and *Pparδ*) demonstrated substantial decrement (Fig. 5F). No change in hepatic lipid transport-related genes was observed (Fig. 5F). Consistently, protein levels of lipogenesis-related genes such as PPARγ and SCD1 were significantly increased in O-IF mice fed HFD (Fig. 5G). No significant change was observed for in the protein levels of hepatic genes relevant to β-oxidation (Fig 5H). These results indicate that maternal IF may increase hepatic lipid deposition in the offspring primarily by increasing lipid synthesis.

7. Maternal intermittent fasting suppresses hepatic mTORC1 signaling in offspring

Significant decrease in protein levels of hepatic DNA methyltransferases Dnmt1 and Dnmt3b were detected in the O-IF mice fed HFD (Fig. 6A). Interestingly, whole genome bisulfite sequencing revealed that DNA methylation modifications of molecules relevant to the mTOR signaling pathway were substantially altered. Among them, DMR-related genes PI3K (*Pik3ca*), PTEN (*Pten)*, PKC (*Prkca*), RSK (*Rps6ka6*), and mTOR (*Mtor*) were hypomethylated, whereas eIF4B (*Eif4b*) and



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Raptor (*Rptor*) were hypermethylated (Fig. 6B). Consequently, the hepatic mTOR signaling activity was significantly inhibited in the O-IF mice fed either NCD (Fig. 6C) or HFD (Fig. 6D). However, the pancreatic mTOR signaling pathway was not significantly altered. (Fig. S3F).

To determine whether decrease of hepatic DNA methyltransferases (DNMT) contributes to inhibition of the mTOR signaling pathway, LO2 cells, a human liver cell line, was treated with various concentrations of the DNA methylation inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR) for 72 hours. As shown in Fig. 6E, DNMT1 protein expression was significantly suppressed by 5-Aza-CdR. This alteration was associated with a significant reduction in mTOR signaling activity evidenced by decreased phosphorylation of ribosome S6, a downstream effector of the mTORC1 signaling pathway (Fig. 6E). Similar results were observed in the AML12 cells, a mouse hepatic cell line (Fig. 6F). Together, these results suggest that maternal IF before pregnancy may suppress hepatic mTORC1 signaling via a DNA methylation-dependent mechanism.

8. Activation of offspring hepatic mTORC1 restores the metabolic phenotype induced by maternal intermittent fasting

The observation that hepatic mTORC1 signaling is inhibited by maternal IF prompted us to examine whether activation of hepatic mTORC1 signaling in offspring could rescue its metabolic phenotype. Hydrodynamic tail vein technique is an effective method for delivering a drug primarily to the liver by rapidly injecting a

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large amount of fluid into the blood vessel (30). Administration of the ribosomal protein S6 kinase beta-1 (S6K1, the mTOR downstream effector) adenovirus (Ad-S6K1) via tail vein injection for 2 consecutive weeks significantly increased the phosphorylation levels of the ribosome S6 and mTOR levels, indicating activation of the hepatic mTOR signaling (Fig. 7A). Activation of S6K typically results in increase of gene transcription and translation, leading to the upregulation of cell growth (31). As a consequence, we observed the increment of mTOR levels induced by overexpression of S6K. This approach did not alter the mTOR signaling pathway in pancreas (Fig. S5A). Administration of Ad-S6K1 ($10^9$ pfu) through the tail vein substantially reduced body weight in both O-AL and O-IF mice fed HFD (Fig. 7B). The deteriorated glucose tolerance in the O-IF mice was significantly reversed by over-expression of S6K1 (Fig. 7C). The increase in levels of hepatic and circulating triglyceride in O-IF mice was significantly reversed to the levels observed for the O-AL animals (Fig. 7D and Fig. 7E). Hepatic ballooning and steatosis were substantially improved (Fig. 7F). Liver weight and the mRNA levels of genes relevant to lipogenesis, lipid transport and β-oxidation remained largely unaltered (Fig. S5B-E). Similarly, HFD-induced adiposity in O-IF mice was significantly reduced (Fig. 7G-Fig. 7I). Furthermore, the increase in adipocyte size was significantly attenuated. A left shift in adipocyte size histogram was observed for sWAT (Fig. 7J), rWAT (Fig. 7K), and eWAT (Fig. 7L).

**Discussion**

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Maternal nutrition not only affects the early growth and development of the fetus, but also plays an important role in the development of non-communicable diseases in the offspring after adulthood. Our studies reveal that long term maternal intermittent fasting renders offspring prone to liver steatosis, adiposity and glucose intolerance despite of its metabolic benefit for the dam. This conclusion is supported by following observations: 1) After 12 weeks of intermittent fasting, female mice demonstrate a significant reduction in food intake and body weight, decreased circulating triglyceride levels, as well improvement in glucose tolerance relevant to control animals fed ad libitum; 2) Maternal IF before mating results in placental and neonatal weight loss; 3) Despite of the negligible change in body weight, both subcutaneous and visceral fat mass show a significant increase in adult IF offspring fed HFD; Also increased is the adipocyte size; 4) Lipid deposition, as well as triglyceride content in the liver and circulation are increased in offspring of maternal IF; 5) Reduced levels of hepatic DNA methyltransferases in IF offspring is associated with inhibition of the hepatic mTORC1 signaling; 6) Activation of hepatic mTORC1 signaling by tail intravenous injection of Ad-S6K1 reverses maternal IF-induced liver steatosis in the progeny and further improved metabolic phenotype of offspring.

Proper nutritional status before and during pregnancy is critical for maternal health and fetal development(32). Maternal overnutrition or undernourishment may increase the risk of metabolic disease in adult offspring(33). According to the "Developmental Origins of Health and Disease (DOHaD)" theory, exposure to a poor

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intrauterine nutritional environment may reprogram the development, structure, and function of important tissues or organs of the fetus(34). If the postnatal environment does not match the intrauterine environment, "mismatch" and poor adaptability may increase the risk of chronic non-communicable disease in the adult offspring(35). Our studies provide direct evidence supporting this concept. Despite of its metabolic benefit for the mother dam, long term intermittent fasting before pregnancy increases the risk of metabolic dysfunction in the adult offspring. Substant reduction in placental and neonatal weight suggests a significant impact on the development of offspring by the long term maternal intermittent fasting even though dam resumes normal diet from the beginning of mating. The body weight difference is gradually vanishing, indicating a catch-up growth in the offspring. Interestingly, the catch-up increase in body weight appears to be due to the lipid deposition in fat tissue and liver, especially under HFD feeding condition. In the liver, lipid accumulation is firstly detected in the maternal IF offspring fed NCD, then becomes more severe in animals fed HFD. In the fat tissue, increase of fat mass becomes obvious only in offspring fed HFD. The increased lipid accumulation is independent of food intake and energy expenditure. Food intake in the maternal IF offspring is largely unaltered, whereas energy expenditure demonstrates a significant increase. All these observations indicate that lipid metabolic dysfunction may be initiated in liver. We thus focused on the hepatic mechanism underlying the lipid metabolic dysfunction in the maternal IF offspring.

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Analysis of DNA methylation revealed a reduced protein level of DNA methyltransferases (DNMT1 and DNMT3b). This alteration was associated with the substantial alteration in hepatic DNA methylation of molecules relevant to mTORC1 signaling. This observation suggests that DNA methylation-dependent modulation of hepatic mTORC1 may contribute to the dysfunction of lipid metabolism. Evidence supporting this concept includes: (1) Maternal IF induces a profound alteration in the DNA methylation of mTORC1-relevant molecules in the offspring liver; (2) Alteration in DNA methylation is associated with a reduction in hepatic mTORC1 activity evidenced by decrease of S6 phosphorylation; (3) Suppression of DNMT activity reduces mTORC1 in hepatocytes; (4) Activation of hepatic mTORC1 activity by overexpression of S6K1 rescues the metabolic dysfunction in the maternal IF offspring. Our study thus reveals a DNA methylation-dependent regulation of hepatic mTORC1 signaling, whose activity is critical for the liver steatosis and adiposity in maternal IF offspring. Consistently, study by Chen et al. has shown that increased expression of DNMT1 in diabetic nephropathy leads to activation of the mTORC1 signaling pathway(36). In addition, reduction of DNMT1 by decitabine, a potent DNA methyltransferase inhibitor, decreases the mTORC1 activity in mice with acute rejection of renal transplantation(37).

As a critical pathway linking the energy supply with cell functions, mTORC1 has been proposed as a major modulator in hepatic lipid metabolism. Activation of mTORC1 increases hepatic de novo lipogenesis by the SREBP-dependent mechanism

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(38). Inhibition of mTORC1 attenuates the upregulation of SREBP-1c and genes crucial for lipogenesis, leading to subsequent amelioration of hypercholesterolemia and hepatic steatosis induced by high-fat and high-cholesterol diets(39). In contrast to these reports, activation of mTORC1 by deletion of the tuberous sclerosis complex 1 (TSC1), an upstream inhibitor of mTOR, reduces lipid accumulation(40). On the other hand, suppression of mTORC1 by rapamycin increases the content of free fatty acids and triglycerides in the liver(41). Reasons underlying these conflicting results remain largely unknown, but may be attributed to the distinct spatial and temporal activation of mTORC1 signaling in different models. Our study suggests that maternal IF suppresses hepatic mTORC1 activity, leading to subsequent liver steatosis. However, it is worth noting that our study could not rule out that the impact of long-term energy surplus on the hepatic mTOR signaling may also contribute to the lipid accumulation in the liver of offspring although this potential is unlikely in our model. Long-term energy surplus typically increases mTOR signaling in liver, which is different from our observation of decreased mTOR signaling in offspring of IF dam. Again, this different result indicates the complexity of spatiotemporal mTOR in the control of hepatic lipid accumulation.

One of another interesting findings is that activation of mTOR signaling in the liver is associated with impaired glucose tolerance. In contrast, previous studies have suggested a beneficial effect of mTOR-S6K1 signaling suppression on liver glucose metabolism (42). For example, the activity of mTORC1/S6K1 is typically increased

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in obese rodent liver, resulting in IRS 1 degradation and liver insulin resistance(43). Reasons underlying the difference between these observations may be attributed to the impairment of lipid metabolism in liver observed in the maternal IF offspring. Also possible is the distinct spatiotemporal alteration in mTOR signaling as described above. Consistently, Houde et al. have reported that long-term inhibition of mTOR in rats enhances liver gluconeogenesis(44).

Limitation for this study also exists. Firstly, plasma levels of triglyceride were significant reduced in O-IF HFD relevant to O-IF NCD. Consistent with our observation, previous studies by Spitler KM et al. have shown a lower levels of plasma triglyceride in HFD mice relevant to NCD animals(45). These observations indicate that increment of lipid accumulation in liver may not result in a hypertriglyceridemia, especially in the early stage of liver steatosis. Secondly, IF was begun in 4 weeks old dam. This may have more profound effects on offspring and may not be applicable to human being. Third, female mice were mated immediately after IF. Further experiments should explore whether similar metabolic dysfunction will also occur to their offspring if female mice would be mated a few weeks or months after IF.

In summary, our finding demonstrates that long term intermittent fasting before pregnancy renders the offspring prone to liver steatosis and adiposity. This occurs through the DNA methylation-dependent suppression of hepatic mTOR signaling (Fig. 8). Our study thus suggests that women planning for pregnancy should avoid

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prolong intermittent fasting because of its adverse effects on fetal development and offspring metabolic homeostasis.

**Study Limitations:**

1. Our study focuses on changes in hepatic DNA methylation in adult IF offspring rather than the fetal liver. Epigenetic modifications in the early fetal liver should be further elucidated in subsequent research.
2. Alteration in mTOR signaling in IF offspring may not be the sole change induced by maternal IF.
3. Our study provides limited insight into the mechanism underlying the impaired glucose tolerance in IF offspring. Further studies will explore whether maternal IF alters the gut microbiota and enteroendocrine hormones in the IF offspring.

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**Author contributions**

Conceptualization, Wenzhen Yin, Yue Yin and Weizhen Zhang; methodology, Wenzhen Yin and Yuan Liang; formal analysis, Wenzhen Yin, Yuan Liang, Yue Yin, and Weizhen Zhang; investigation, Wenzhen Yin, Lijun Sun, Yuan Liang, Chao Luo, Tiange Feng, and Yunhua Zhang; writing—original draft preparation, Wenzhen Yin; writing—review and editing, Wenzhen Yin, Lijun Sun, Yuan Liang , Yue Yin, and Weizhen Zhang; visualization, Wenzhen Yin, and Yuan Liang; supervision, Yue Yin,

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**Conflict of interest statement**

The authors declare no conflicts of interest.

**Data Availability Statement**

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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## Figure legends

**Figure 1. Effects of maternal intermittent fasting on the placenta and neonatal weight**

C57BL/6J female mice were weaned at the age of 4 weeks, randomly divided into ad libitum feeding and intermittent fasting groups, and fed NCD for 12 weeks. Before mating, all animals were resumed ad libitum feeding. The presence of vaginal plugs on female mice after overnight mating was designated as embryonic day 0.5 of gestation (E0.5). M-AL: mother dam fed ad libitum. M-IF: maternal intermittent fasting. O-AL and O-IF denote offspring of mice fed ad libitum and intermittently fasting respectively. Value was expressed as mean±SEM.

(A) Fetal and placental weights in female mice at E18.5 (n = 3-5 dams/group, n=6 to 9 embryos per dam, *P<0.05 versus M-AL);

(B) Body weight and body length of neonatal offspring (n=14 and 13 for O-AL and O-IF respectively, *P<0.05 versus O-AL);

(C) Representative images of neonatal mice;

(D) The pups were counted on postnatal day 1 (P1) and P21 (n=5 dams/group).

**Figure 2. Effects of maternal intermittent fasting on the adiposity, glucose tolerance and food intake of the offspring**

Offspring were weaned at 6 weeks of age and fed either NCD or HFD respectively for 12 weeks. Metabolic measurement was performed in offspring at the age of 15 weeks. Value was expressed as mean±SEM. *P<0.05 versus O-AL NCD, $^{\#}$P<0.05 versus O-AL HFD.

(A, B) Body weight of offspring fed NCD and HFD. (n=10, 9, 7 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively)

(C, D) Body fat mass of offspring fed NCD and HFD. (n=5, 5, 8 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively)

(E, F) Glucose tolerance of offspring fed NCD and HFD. (n=10, 9, 7 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively)

(G, H) Cumulative food intake of offspring fed NCD and HFD. (n=10, 9, 7 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively)

**Figure 3. Maternal intermittent fasting renders offspring prone to HFD induced adiposity**

Offspring were weaned at 6 weeks of age and fed either NCD or HFD respectively for 12 weeks. 18-week-old male offspring were sacrificed and, fat tissues harvested. Dam fed ad libitum was used as control. Value was expressed as mean±SEM. n=10, 9, 7 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively. *P<0.05 versus O-AL NCD, $^{\#}$P<0.05 versus O-AL HFD. Shown are the fat mass of subcutaneous white adipose tissue (sWAT, A), perirenal WAT (rWAT, B), and epididymal WAT (eWAT, C). And representative histology and histogram of adipocyte size of sWAT (D), rWAT (E), and eWAT (F) are also shown. About 150–300 adipocytes were analysed for each slide.

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**Figure 4. Maternal intermittent fasting increases energy metabolism and activity in offspring**

Offspring were weaned at 6 weeks of age and fed either NCD or HFD respectively for 12 weeks. Energy expenditure and locomotor activity in 16-week-old male offspring were measured by indirect calorimetry during a 12-hour light-dark cycle, as described in the Methods section. Value was expressed as mean±SEM. n=5, 5, 8 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively. *P<0.05 versus O-AL NCD, #P<0.05 versus O-AL HFD. Shown are the locomotor activity (A, B), energy expenditure (C, F), oxygen consumption (D, G), and respiratory quotient (E, H) in offspring fed NCD or HFD.

**Figure 5. Maternal intermittent fasting deteriorates liver steatosis via stimulation of lipogenesis**

Offspring were weaned at 6 weeks of age and fed either NCD or HFD respectively for 12 weeks. Liver was harvested from maternal IF offspring at 18 weeks of age. mRNA was analyzed by qPCR and normalized by geometric mean expression of the housekeeping genes: Hprt, Rpl32A, and Tbp. β-actin was used as loading control for Western blot analysis. Value was expressed as mean±SEM. n=10, 8, 8 and 11 for O-AL NCD, O-IF NCD, O-AL HFD, and O-IF HFD respectively. * and # denote P<0.05 versus O-AL NCD and O-AL HFD respectively.

(A) Liver weight.

(B) Hepatic triglyceride content.

(C) Plasma levels of triglyceride. * denotes P<0.05 for NCD vs. HFD.

(D) H&E staining and Oil Red O of liver tissue.

(E) mRNA levels of hepatic genes relevant to lipogenesis, lipid transport, and β-oxidation in offspring fed NCD.

(F) mRNA levels of hepatic genes relevant to lipogenesis, lipid transport, and β-oxidation in mice fed HFD.

(G) Protein expression levels and quantitative results of hepatic PPARγ and SCD1 in offspring fed HFD.

(H) Protein expression levels and quantitative results of hepatic ACADVL and PPARδ in offspring fed HFD.

**Figure 6. Maternal intermittent fasting suppresses hepatic mTORC1 signaling in offspring via DNA methylation-dependent mechanism**

Offspring were weaned at 6 weeks of age and fed either NCD or HFD for 12 weeks. Liver was harvested from maternal IF offspring at 18 weeks of age.

(A) Protein expression levels and quantitative results of hepatic DNMT1 and DNMT3b in offspring fed HFD. n=5 for O-AL HFD, n=5 for O-IF HFD.

(B) DMR-related genes and their methylation levels in the hepatic mTOR signaling pathway. n=4 for O-AL HFD, n=4 for O-IF HFD. The areaStat of these genes were -308.939686, -602.5387445, -350.5120276, -22.12913296, -165.4081901, 53.16119031 and 261.904098, respectively.

(C) Protein expression levels and quantitative results of hepatic total and phosphorylated mTOR molecules in offspring fed NCD. n=5 for each group.

(D) Protein expression levels and quantitative results of hepatic mTOR signaling pathway molecules in offspring fed HFD. n=5 for O-AL HFD, n=5 for O-IF HFD.

(E, F) Protein expression and quantification results of intracellular DNMT1 and mTOR signaling pathway molecules in LO2 cells (E) and AML12 cells (F) treated with various concentrations of 5-Aza-CdR for 72 hours. β-actin was used as loading control. Value was expressed as mean±SEM. n=3. # denotes $P<0.05$ versus DMSO control.

**Figure 7. Activation of offspring hepatic mTORC1 restores the metabolic phenotype induced by maternal intermittent fasting**

Animals were grouped and treated as described in Figure 1. Offspring were fed with HFD for 12 weeks, then injected with GFP or S6K1 adenovirus via the tail vein for 2 consecutive weeks. Two weeks later, metabolic phenotypes were analyzed. Value was expressed as mean±SEM. n=4 for each group. Ad-GFP was used as a control. *$P<0.05$ versus O-AL GFP and #$P<0.05$ versus O-IF GFP.

(A)Protein expression levels and quantitative results of hepatic mTOR signaling pathway molecules. β-actin was used as loading control.

(B) Body weight of offspring.

(C) Glucose tolerance.

(D) Hepatic triglyceride content.

(E) Plasma levels of triglyceride.

(F) H&E staining and Oil Red O of liver tissue.

(G-I) Weight of sWAT, rWAT and eWAT.

(J-L) H&E staining and histogram of adipocyte sizes. About 150–300 adipocytes were analysed for each slide.

**Figure 8. Prolong maternal intermittent fasting before pregnancy increases the risk of metabolic disorders in offspring**

Prolonged intermittent fasting prior to pregnancy increases the risk of metabolic disorders in offspring. This occurs by suppression of hepatic mTORC1 signaling, which is likely dependent on the DNA methylation mechanism.

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