Original Research

Maternal chromium restriction modulates miRNA profiles related to lipid metabolism disorder in mice offspring

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Impact statement

For the first time, our study demonstrates important miRNA differences in the effect of maternal chromium restriction in offspring. These miRNAs may serve as ''bridges'' between the mother and the offspring by affecting the MAPK pathway.

Abstract

Increasing evidence shows that maternal nutrition status has a vital effect on offspring susceptibility to obesity. MicroRNAs are related to lipid metabolism processes. This study aimed to evaluate whether maternal chromium restriction could affect miRNA expression involved in lipid metabolism in offspring. Weaning C57BL/6J mice born from mothers fed with normal control diet or chromium-restricted diet were fed for 13 weeks. The adipose miRNA expression profile was analyzed by miRNA array analysis. At 16 weeks old, pups from dams fed with chromium-restricted diet exhibit higher body weight, fat weight, and serum TC, TG levels. Six miRNAs were identified as upregulated in the RC group compared with the CC group, whereas eight miRNAs were lower than the threshold level set in the RC group. In the validated target genes of these differentially expressed miRNA, the MAPK signaling pathway serves an important role in the influence of early life chromium-restricted diet on lipid metabolism through miRNA. Long-term programming on various specific miRNA and MAPK signaling pathway may be involved in maternal chromium restriction in the adipose of female offspring.

Keywords: microRNA, lipid metabolism, developmental programming, MAPK pathway, chromium restriction, nutrition

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Introduction

The nutrition status during the gestational and lactation period is a key time for both mothers and their offspring. Malnutrition during pregnancy could influent the mother's health and change the offspring's metabolic status.[1,2] This association may be regulated epigenetically via various changes, including expression of microRNAs (miRNAs) and variations in their target genes. Alterations in miRNA expression change the metabolic plasticity and physiological processes in response to early environment conditions.[3,4] MiRNA may represent a link between the prenatal, maternal nutrition and offspring metabolism status.

As an necessary element, chromium has important function on normal lipid metabolism.[5] Chromium deficiency subjects exhibit elevated triglycerides (TG), total cholesterol (TC) and decreased high density lipoproteins (HDL-C).[6] In many area, all around the world, the average chromium intake in adults does not meet the minimum suggested daily chromium intake (30 μg/day).[7,8] Particularly, because of the enhance of metabolic stress and the reduction of absorption ratio, pregnant and elderly individuals have high risk of chromium deficiency.[9,10,11] Feng *et al.*[12,13] found that chromium supplementation reduced serum lipid levels in type 2 diabetic rodents. A recent study showed that maternal chromium restriction induced obesity in rodent pups likely due to increased oxidase stress.[14] Our previous study highlighted the DNA methylation changes in MAPK signaling pathway and involved with lipid metabolism disorder in the mice pups from chromium-restricted mothers.[15]

Besides DNA methylation, miRNAs, which are small non-coding RNAs, represent a key governing factor in adipogenesis and lipid metabolism. For example, miR-33 controls cholesterol and lipid metabolism through regulating its target gene, sterol-regulatory element-binding protein (SREBP).[16] MiR-34a is an important switch of hepatic lipid homeostasis.[17] MiR-122 can also control cholesterol synthesis and lipoprotein secretion in the liver.[18]

In this study, we aimed to identify epigenetic mechanisms of fat metabolism dysregulation as a result of maternal chromium restriction using a mouse model.

Materials and methods

Animal treatments and diets

All animal experiment protocols were approved by the Animal Care Committee of Peking Union Medical Hospital (Permit Number: MC-07-6004), and all efforts were made to minimize suffering. Seven-week-old female and male C57BL mouse were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China, SCXK-2013-0107). According to our previous paper,[15] virgin female C57BL mice (18.2 ± 1.3 g, $n = 16$) were mated with male mice. Vaginal plug was checked to confirm the pregnancy. In gestation and lactation period, female mice were fed with either normal control diet (casein-based diet based on the American Institute of Nutrition AIN-93G diet) or chromium-restricted diet (only 90% reduction of chromium potassium sulfate from control diet, $n = 8$/group). The concentrations of chromium in the control diet and chromium-restriction diet are 1.19 and 0.14 mg chromium/kg diet, respectively (assessed by atomic absorption spectrometer (TAS986, Beijing Persee General Corporation, Beijing, China)). The integrant of AIN-93G diet is suitable for rodent normal growth and reproduction (including 1.19 mg chromium/kg diet). Ninety percent reduction of chromium content is the lowest chromium content in pregnant mice diet. All diets were produced by Research Diets (New Brunswick, NJ, USA). To make each pup get equal nutrition, new born pups were adjusted to three male and three female mice in each litter. At weaning (week 3), pups born from control dams were randomly assigned to normal control diet (CC) or chromium-restricted diet (CR); pups born from chromium-restricted diet dams were adopted by control (RC) and chromium-restriction diet (RR, $n = 8$/group, eight female pups from eight different litters). Because this study focused on the influence of prenatal chromium-restricted diet on offspring, RC group was designed to avoid the effect of offspring diet on the lipid metabolism. Because of the different phenotypic of maternal malnutrition on male and female offspring, this study exclusively focused on female offspring.[19] At 16 weeks of age, female mice ($n = 8$/group) were sacrificed. The blood sample was taken from overnight fasting mice (anesthetized by ketamine 100 mg/kg i.p., Pharmacia and Upjohn Ltd, Crawley, UK). Pup adipose tissue was immediately collected and stored at -80°C for further analysis. Figure 1 shows the experiment protocol.

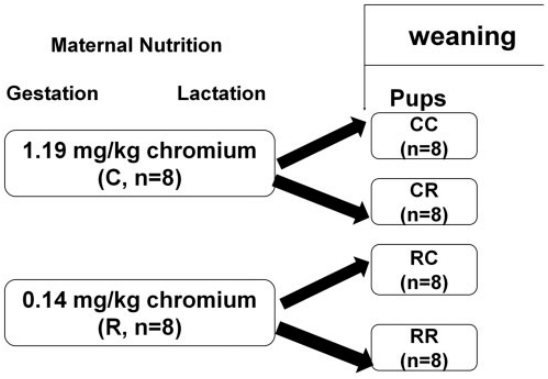

Figure 1 Schematic representation of the feeding protocol for different groups

Analysis of serum chromium levels

Serum was collected from the mothers at weaning and offspring at 16 weeks old to measure chromium levels. The serum chromium concentration was measured by atomic absorption spectrometer (Atomic Absorption Spectrophotometer, Hitachi, Japan) as previously described.[15]

Measurements of food intake and body weight

Body weight was recorded at birthday, 3 weeks and 16 weeks of age in offspring. Food intake was calculated at 16 weeks from pre-weighed food portions dispensed from the food hopper. Food intake measurements represent the average 24-h food intake calculated over the stated time period.

Measurement of adiposity index

At 16 weeks of age, mice were sacrificed, and fat pads were collected and weighed. The adiposity index (AI) was determined as follows.[20]

$$\mathbf{AI} = \frac{\textit{sums of retroperitoneal, mesenteric and ovarian fat masses}}{\textit{body weight}}.$$

Measurement of serum biochemistry parameters

At 16 weeks of age, serum lipid levels were measured by an enzyme end-point method (Roche Diagnostics, GmbH, Mannheim, Germany).

RNA extraction and Reverse Transcription of miRNA

Total RNA isolated from fat in RC and CC groups was extracted by mirVana™ RNA Isolation Kit (Ambion, SanPaulo, SP, Brazil). cDNA was synthesized using TaqMan miRNA Reverse Transcription kit (Life Technologies, Rockville, MA, USA) as previously described.[21]

miRNA array hybridization

To elucidate the influence of maternal chromium restriction on miRNA expression in pup adipose, miRNA expression levels in RC and CC group were assessed by Affymetrix Multispecies miRNA 4.0 Array (Affymetrix, Santa Clara, CA, USA). This array fully covers all known rodent miRNA (557 unique miRNAs, based on miRBase version 20.0, released in Jun 2013, http://www.mirbse.org). After subtracting the background, the data were normalized using a LOWESS filter (Affymetrix, Santa Clara, CA, USA). A heat map was constructed by TIGR MeV (MultiExperiment Viewer) software (http://www.tm4.org/mev.html).[22] The validated gene targets for differentially expressed miRNA by maternal chromium restriction in offspring liver were searched using the MiRTarBase

Database version 6.0 (http:// http://mirtarbase.mbc.nctu.edu.tw/, released Sep 2015).[23]

miRNA real-time PCR

To validate miRNAs regulated by chromium restriction in dams identified by arrays, we performed qPCR analysis of eight differentially expressed miRNAs. U6 was used as a reference control for normalization. Real-time PCR reactions were conducted with ABI 7700 (Applied Biosystems, Foster City, CA, USA) as previously described.[21]

miRNA target prediction

miRTarBase database 6.0 (mirtarbase.mbc.nctu.tw, released in Sep 2015) was used to seek the validated target genes of differentially expressed miRNAs.[23]

Real time PCR for target genes of differentially expressed miRNAs

For the validation of miRNA target genes in the MAPK pathway, real-time PCR experiments were performed using the SYBR Green method on ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described.[21] Primers were obtained from Applied Biosystems (Supplementary Table S1). The signal of the housekeeping gene *CypA* (cytochrome P450 A) was used to normalize the result. Comparative Ct method was used to compare the relative quantification between different groups.

miRNA target genes pathway analysis

To fully interpret the biological meaning of target genes, we analyzed the gene ontology (GO) classification and Kyoto encyclopedia of genes and genomes (KEGG) pathways of the target genes, by using DAVID (Data-base for annotation, visualization and integrated discovery) software (http://david.abcc.ncifcrf.gov/).[24] The miRNA and mRNA interaction was analyzed using Cytoscape software (http://www.cytoscape.org).[25]

Statistical analysis

All results are shown as the mean ± standard deviation (SD). Statistical analyses were calculated with two-way ANOVA followed by Tukey's *post hoc* test, unpaired Student's *t* test, and Fisher's exact test. $P < 0.05$ was set as statistical significance. GraphPad Prism Software version 5.0 (San Diego, CA, USA) was used for data analysis.

Results

Mother

As expected, C57BL female mice fed a chromium-restriction diet exhibited lower serum chromium content compared with the normal control mice at weaning (0.32 ± 0.02 ng/mL vs. 0.92 ± 0.12 ng/mL, $P < 0.01$).

Offspring

Serum chromium. The CR (0.23 ± 0.03 ng/mL) and RR groups (0.17 ± 0.02 ng/mL) had lower serum chromium levels compared with the CC groups (0.83 ± 0.09 ng/mL, $P < 0.01$), while the RC group (0.79 ± 0.07 ng/mL) had comparable serum chromium level with the CC group ($P > 0.05$) at 16 weeks.

Body weight in offspring at birth, 3 weeks and 16 weeks. Birth and three-week-old weight of the offspring was comparable between the C and R groups. At 16 weeks of age, body weight in the RR (28.43 ± 3.21 g) and RC groups (26.37 ± 2.14 g) was increased compared with the CC group (20.37 ± 1.84 g, $P < 0.05$), while the CR group (21.26 ± 1.46 g) had similar body weight with the CC group ($P > 0.05$).

Food intake and fat pad weight. Consistent with the increased body weight, RR (6.71 ± 0.46 g) and RC (6.43 ± 0.36 g) offspring exhibited increased fat pad weight than that in CC group (4.63 ± 0.43 g, $P < 0.05$). Moreover, the CR group had increased fat pad weight ($P < 0.05$). However, the food intake was comparable among the CC, CR, RC, and RR groups.

Serum lipid profile levels. Serum TC levels were increased in the RC group compared with the CC group (1.83 ± 0.15 mmol/L vs. 1.25 ± 0.08 mmol/L, $P < 0.05$), while the CR group (1.38 ± 0.11 mmol/L) and RR group (1.43 ± 0.16 mmol/L) had similar TC levels with CC group ($P < 0.05$). Serum TG levels were increased in the RC (1.04 ± 0.08 mmol/L), CR (1.25 ± 0.06 mmol/L), and RR (1.21 ± 0.08 mmol/L) groups compared with the CC groups (0.57 ± 0.03 mmol/L, $P < 0.05$). Serum LDL-C and HDL-C levels were comparable among groups.

MiRNA expression profiles. Differentially expressed miRNAs in the RC group were filtered by volcano plot, compared with CC group (fold change ≥ 2 and *P*-value <0.05). Six miRNAs (mmu-miR-25-5p, mmu-miR-291b-5p, mmu-miR-504-3p, mmu-miR-1947-3p, mmu-miR-7014-5p, and mmu-miR-7025-5p) were identified as upregulated ($\geq$2-fold) in the RC group compared with the CC group, whereas eight miRNAs (mmu-miR-149-5p, mmu-miR-151-5p, mmu-miR-324-3p, mmu-miR-338-5p, mmu-miR-351-5p, mmu-miR-181b-5p, mmu-miR-125b-1-3p, and mmu-miR-674-5p) were less than the threshold level (0.5-fold) set in the RC group (Supplementary Table S2, Figure 2). Then, hierarchical clustering of the 14 miRNAs was performed. These differentially expressed miRNAs clearly distinguished the RC samples from the CC sample (Figure 3).

Validation of microarray results by real time PCR. To confirm the array results, all differentially expressed (DE) miRNAs were subject to further validation using real-time PCR. Among the 14 miRNAs, all of them were differentially expressed between the RC and CC groups ($P < 0.05$, Figure 4). The results agreed with the array data.

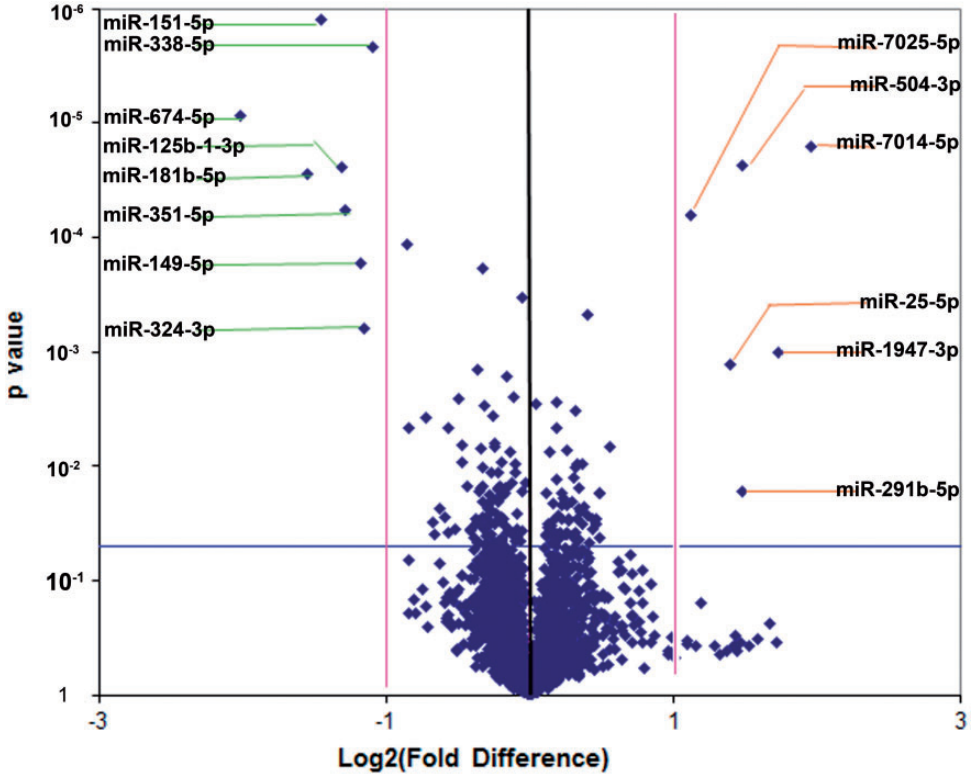

Figure 2 Differentially expressed miRNAs identified in the microarray. Fourteen miRNA passed the volcano plot filtering. Volcano plots are a useful tool for visualizing differential expression patterns between two groups (RC vs. CC). The vertical lines correspond to 2-fold up-and down-regulation, and the horizontal line represents a *P*-value of 0.05. (A color version of this figure is available in the online journal.)

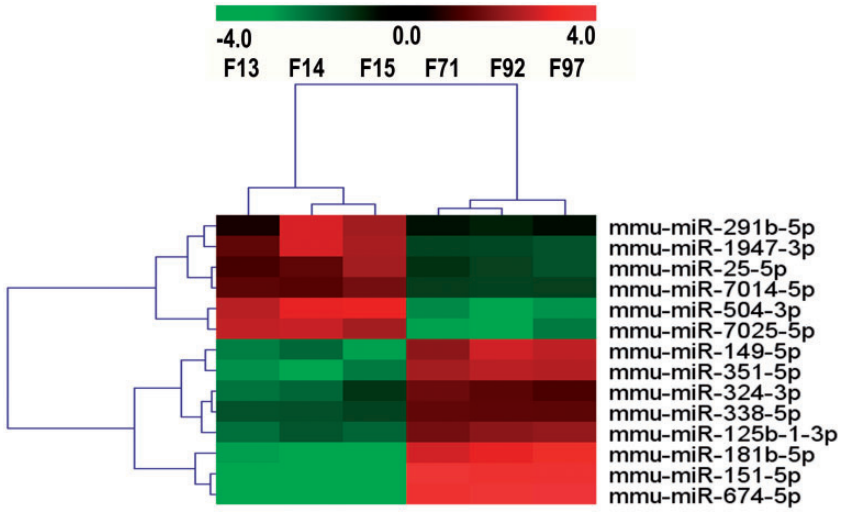

Figure 3 MiRNA profiles differentiate RC group from CC group. Samples consist of three mice from each group. Both down-regulated (green) and up-regulated (red) miRNAs were identified in RC group. F13, F14, F15 belong to RC group; F71, F92, F97 belong to CC group

Microarray-base gene ontology analysis of different expression miRNAs. In miRTarBase database, only nine DE miRNAs have experimentally validated target genes. To explore the function of 9 DE miRNAs among the two groups, we obtained the 929 experimentally validated miRNA targets using miRNA database (Supplementary Table S3). Then, we studied the biological function of validated target genes. The validated miRNA targets were classified into several biological functions (Supplementary Table S4). Biological processes affected by targets genes include phosphorus metabolic, phosphate metabolic (Figure 5). Figure 6 presents an miRNA-mRNA interaction network using Cytoscape.

Microarray-based KEGG pathway analysis of different expression miRNAs. Using DAVID, we found the target

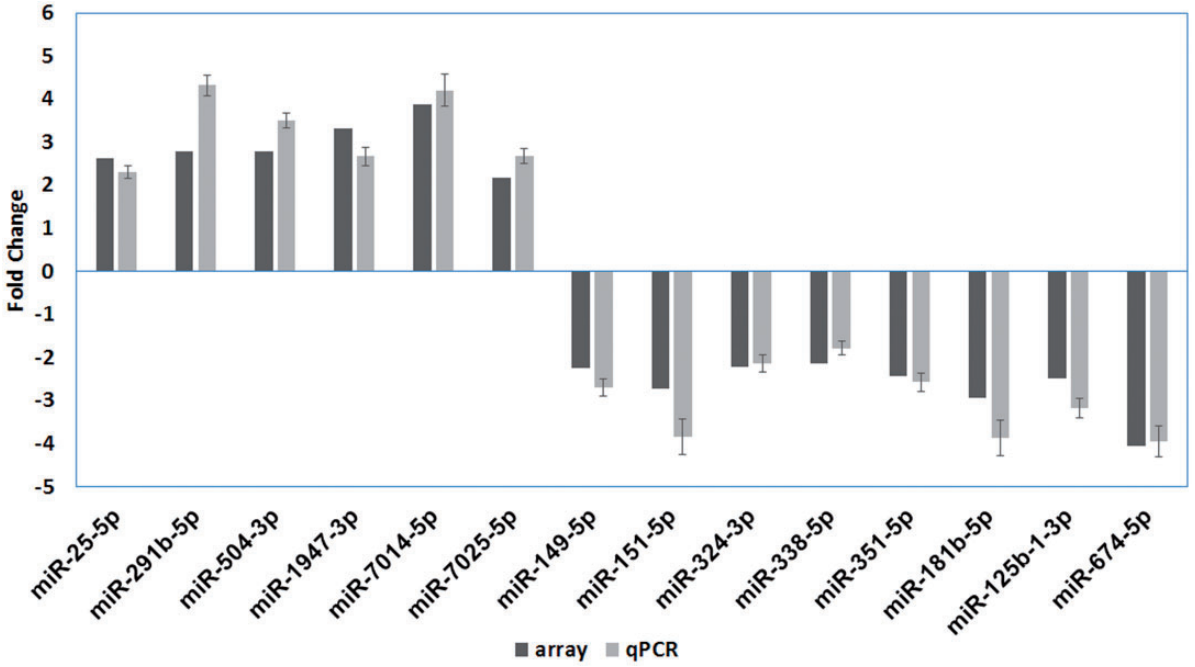

Figure 4 Differential miRNA expression in gene array and qPCR validation

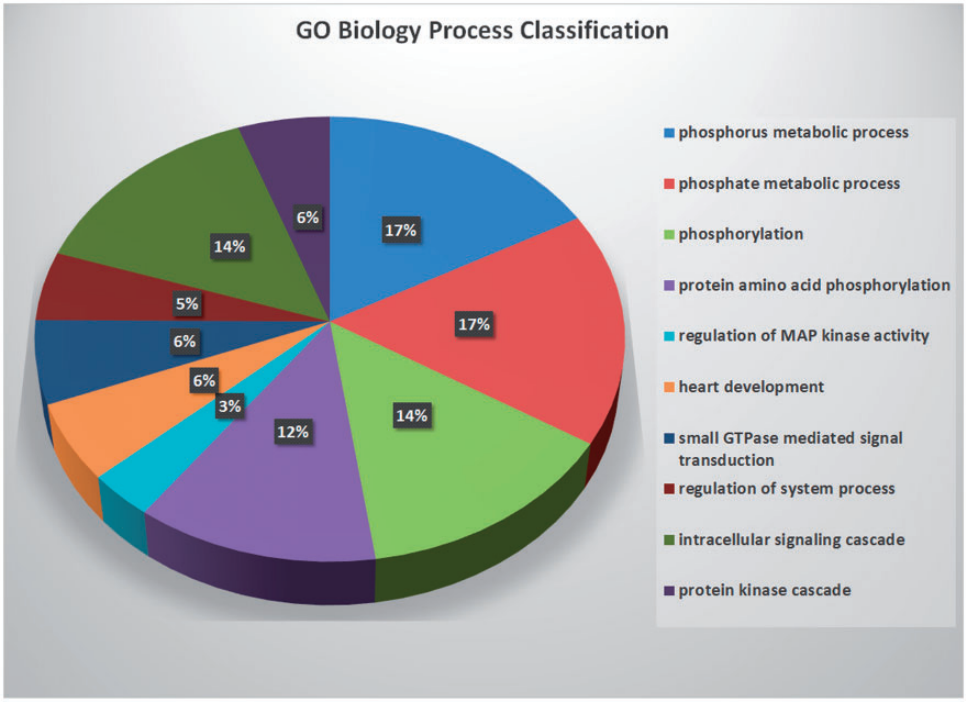

Figure 5 MiRNA target significant GO biology process

genes of DE miRNAs clustered into the neurotrophin signaling pathway, pancreatic cancer, colorectal cancer, chronic myeloid leukemia, ErbB signaling pathway, MAPK signaling pathway, chemokine signaling pathway, axon guidance, and apoptosis ($P < 0.0001$, Supplementary Table S5).

DE miRNAs affect the MAPK pathway. Among the involved pathways, we identified eight DE miRNAs affected the MAPK pathway (Supplementary Table S5). In particular, numerous target genes of miR-149-5p and miR-324-3p are in the MAPK pathway.

Real time PCR. The expression of mitogen-activated protein kinase 7 (*Map3k7*), mitogen-activated protein kinase 14 (*Mapk14*), activating transcription factor 2 (*Atf2*) and peroxisome proliferator-activated receptor gamma (*Pparg*)

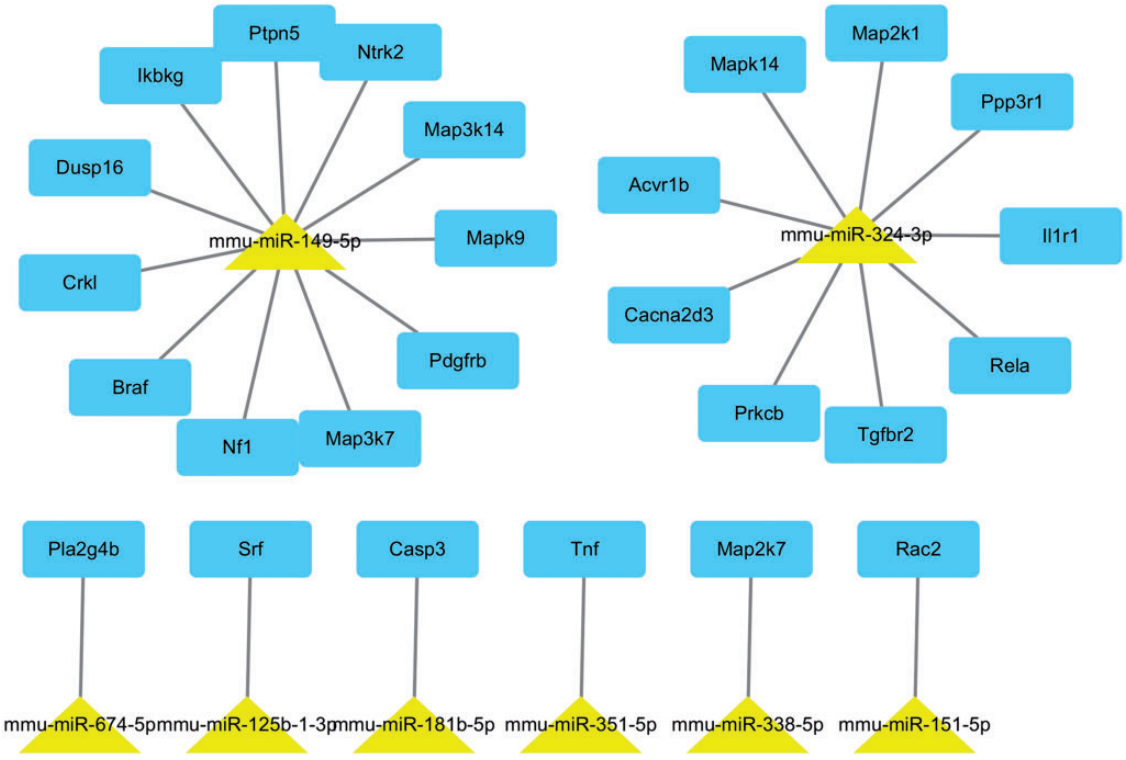

Figure 6 The network of target genes of the eight miRNAs involved in MAPK pathway. (A color version of this figure is available in the online journal.)

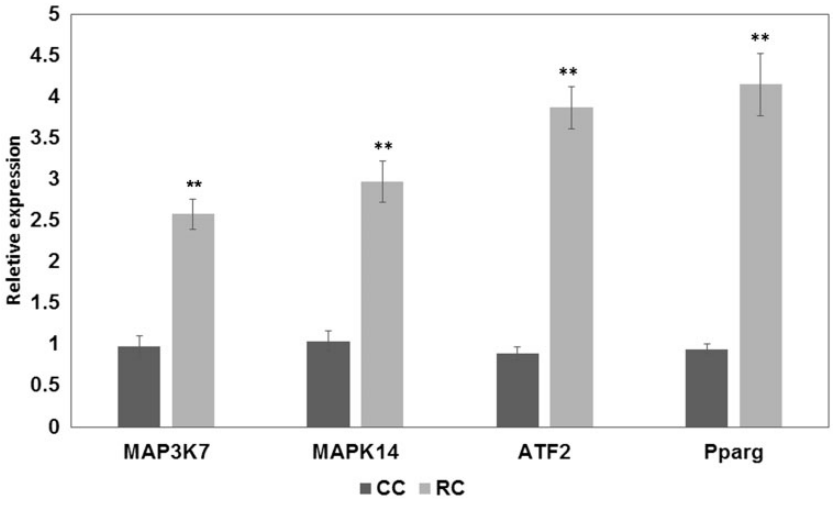

Figure 7 Q-PCR result of target genes of DE miRNAs and downstream molecular in MAPK pathway. $^{**}P < 0.01$ vs. CC group

increased in the RC group compared with the CC group ($P < 0.01$, Figure 7).

Discussion

Although body weight at birth and three weeks from mice born to chromium-restricted mothers did not change, in this study, the body weight of pup mice from chromium-restricted mothers increased at 16 week of age. Other maternal undernutrition animal model also reported this result. Maternal vitamin B_{12} deficiency reduced pup birth weight. However, pup body weight increased after three months of age.[26]

In this study, chromium restriction (CR group) could increase the fat pad in mice. Tinkov *et al.*[27] found that high-fat fed rats have lower chromium content in adipose tissue. And significant inverse correlation was observed for the chromium in adipose tissue and adipose tissue mass. In addition, maternal chromium restriction (RC group) increased irreversibly the fat pad weight of offspring at

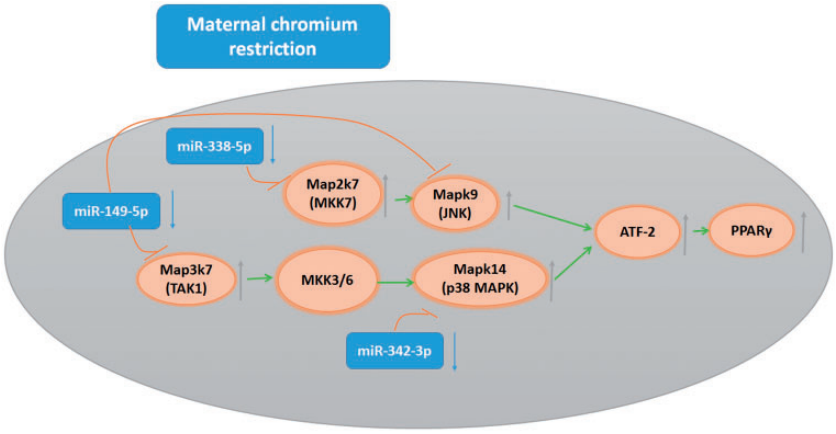

Figure 8 MicroRNAs involved in the effect of maternal chromium restriction in offspring. Red blunted arrows represent inhibition; green arrows represent activation. *Map3k7*: mitogen-activated protein kinase 7; *Mspk14*: mitogen-activated protein kinase 14; *Atf2*: activating transcription factor 2; *Pparg*: peroxisome proliferator activated receptor gamma; MKK3/6: mitogen-activated protein kinase 3/6; TAK1: TGFβ-activated kinase 1; ATF-2: activating transcription factor-2; JNK: c-Jun N-terminal kinase

16 weeks. Venu *et al.*[28] reported that maternal Mg restriction increased pup body adipose percentage, and reduced lean body and fat-free mass at postnatal 90 days. Berends *et al.*[29] found that pups from protein-restricted diet mothers had higher adipocytes at 22 days and 3 months. Maternal zinc deficiency increased body adipose ratio in offspring at six months of age.[30]

Moreover, chromium restriction (CR group) increased serum TG level in mice. Previous studies showed that chromium supplementation had beneficial effect on serum TG in T2DM patients,[9,31,32] hyperlipidemic subjects[33] and in high-sugar diet rodents.[34] Importantly, maternal chromium restriction (RC group) increased irreversibly serum TC and TG at 16 weeks. Venu *et al.*[28] found that maternal Mg restriction increased pup plasma TC levels compared with control pups at 90 days. Bringhenti *et al.*[35] found that low-protein diet increase serum TC levels in offspring at 3 month.

By using miRNA array, this study showed that maternal chromium restriction (RC group vs. CC group) could downregulate miR-149 in offspring adipose. Mohamed *et al.*[36] found miR-149 was highly downregulated in skeletal muscle of obese mice. Poly(ADP-ribose) polymerase-2 (PARP-2) is a validated target gene of miR-149. MiR-149 inhibits the expression of PARP-2, thus reducing the mitochondrial function and biogenesis by inhibiting the sirtuin-1 (SIRT-1)/peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) pathway. In miR-149-overexpressing myotubes, mitochondria respiration with complex 1-linked substrates was significantly increased.[36]

Moreover, maternal chromium restriction downregulates miR-338-5p in offspring adipose. High-fat diet mice, pre-diabetic (6 weeks of age) and diabetic (14–20 weeks of age) db/db mice have increased miR-338-3p levels in islets.[37,38] Reduced miR-338-3p levels are related to compensatory β cell mass hypertrophy.

In addition, maternal chromium restriction downregulates miR-181b-5p in offspring adipose. The miR-181 family consists of miR-181a, miR-181b, miR-181c, and miR-181d. MiR-181a, miR-181b, and miR-181-d levels are low in obese subject monocytes. Weight loss normalizes their expression to the normal levels.[39]

Interestingly, miRNA target gene pathway enrichment analysis showed that the MAPK pathway was highly ranked. We found that maternal chromium restriction downregulated miR-149-5p, miR-338-5p and miR-324-3p in offspring adipose. Their targets include *Map3k7*, *Mapk9*, *Map2k7*, and *Mapk14*, which all belong to the MAPK pathway.

The mitogen-activated protein kinase (MAPK) family consists of the following three main members: extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases. Upon several different signals stimulation, MAPK kinases (mitogen-activated protein kinase 3/6 (MKK3/6)) can activate P38MAPK.[40] Upon stimulation, MKK3 or MKK6 is activated by upstream MAPKK kinase (TGFβ-activated kinase 1 (TAK1)).[41] Increasing evidence has revealed TAK1-P38MAPK pathway.[42,43] P38MAPK and TAK1 are encoded by the *Mapk14* and *Map3k7* genes in mice, respectively. Interestingly, *Mapk14* and *Map3k7* are target genes of miR-324-3p and miR-149-5p. P38MAPK and JNK activate PPARγ2 through activating transcription factor-2 (ATF-2).[44,45] PPARγ2 is an important mediator in adipocyte differentiation.[46] In this study, we also found that the expression of *Atp2* and *PPARγ2* in pup adipose tissue was enhanced by maternal chromium restriction. A previous study found that bisphenol A (BPA) can increase red lipid droplets and TG levels in 3T3-L1 cells through activating p38/MAPK.[47] A P38 inhibitor reduces obesity in mice fed a high-fat diet.[44] Our results shows that maternal chromium restriction downregulates miR-324-3p, miR-338-5p and miR-149-5p, thus upregulates Map3k7, Map2k7, and Mapk14, and activates the p38MAPK pathway and JNK pathway. There effects disrupt lipid metabolism (Figure 8).

In conclusion, for the first time, our study demonstrates miR-149-5p, miR-338-5p and miR-324-3p reduced in female offspring explored to maternal chromium restriction. These miRNAs may serve as ''bridges'' between the mother and

the offspring by affecting the MAPK pathway. However, these hypotheses need to be tested in future. These key miRNAs may aid in the identification of new therapies to treat the maternal undernutrition effect in offspring.

Authors' contributions: XH X designed the experiments, contributed reagents and materials; QZ, JZ, TW, CJ Q, ZX W and XJ W conducted the experiments; MY, ML and FP analyzed the data; QZ wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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