Low Vitamin B12 in Pregnancy Is Associated With Adipose-Derived Circulating miRs Targeting PPARγ and Insulin Resistance

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Context: Low vitamin B12 during pregnancy is associated with higher maternal obesity, insulin resistance (IR), and gestational diabetes mellitus. B12 is a key cofactor in one-carbon metabolism.

Objective: We hypothesize that B12 plays a role in epigenetic regulation by altering circulating microRNAs (miRs) during adipocyte differentiation and results in an adverse metabolic phenotype.

Design, Settings, and Main Outcome Measure: Human preadipocyte cell line (Chub-S7) was differentiated in various B12 concentrations: control (500 nM), low B12 (0.15 nM), and no B12 (0 nM). Maternal blood samples (n = 91) and subcutaneous adipose tissue (SAT) (n = 42) were collected at delivery. Serum B12, folate, lipids, plasma one-carbon metabolites, miR profiling, miR expression, and gene expression were measured.

Results: Our *in vitro* model demonstrated that adipocytes in B12-deficient conditions accumulated more lipids, had higher triglyceride levels, and increased gene expression of adipogenesis and lipogenesis. MiR array screening revealed differential expression of 133 miRs involving several metabolic pathways (adjusted $P < 0.05$). Altered miR expressions were observed in 12 miRs related to adipocyte differentiation and function in adipocytes. Validation of these data in pregnant women with low B12 confirmed increased expression of adipogenic and lipogenic genes and altered miRs in SAT and altered levels of 11 of the 12 miRs in circulation. After adjustment for other possible confounders, multiple regression analysis revealed an independent association of B12 with body mass index (β: −0.264; 95% confidence interval, −0.469 to −0.058; $P = 0.013$) and was mediated by four circulating miRs targeting peroxisome proliferator–activated receptor γ and IR.

Conclusions: Low B12 levels in pregnancy alter adipose-derived circulating miRs, which may mediate an adipogenic and IR phenotype, leading to obesity. (*J Clin Endocrinol Metab* 102: 4200–4209, 2017)

Maternal obesity is a major public health concern, and its prevalence has doubled in the past two decades. In the United Kingdom (1) and the United States (2), 27% of women of childbearing age are overweight and 20% to 32% are obese. Maternal obesity is characterized by the presence of an excessive amount of adipose tissue (AT) and has adverse effects on maternal health and the developing fetus, predisposing them to cardiometabolic

Abbreviations: ACACA, acetyl CoA carboxylase; AT, adipose tissue; BMI, body mass index; CEBPα, CCAAT/enhancer–binding protein α; FASN, fatty acid synthase; IR, insulin resistance; miR, microRNA; q-RT-PCR, quantitative real-time polymerase chain reaction; PPARγ, peroxisome proliferator–activated receptor γ; RXRα, retinoic acid X receptor α; SAM, S-adenosyl-methionine; SAT, subcutaneous adipose tissue; T2D, type 2 diabetes.

disease later in life (3). AT development involves two distinct processes: adipogenesis (increased adipocyte number) and lipogenesis (increased accumulation of lipids) (4). In humans, adipogenesis occurs predominantly in the prenatal and postnatal periods and is set during childhood and adolescence (5). However, during childbearing years the rate of adipocyte generation gradually decreases. Any dietary or environmental changes that disturb the balance between adipogenesis and lipogenesis can result in increase in adipocyte size and accumulation of lipids, both known to increase insulin resistance (IR) (6). Therefore, understanding adipocyte biology during this period will elucidate the effect of adiposity on maternal and child health.

B12 deficiency in pregnant women is increasingly common (7) and has been shown to be associated with higher body mass index (BMI) in many studies (8), as well as IR (9), gestational diabetes, and type 2 diabetes (T2D) in later life (10). An animal study demonstrated (11) that a B12-restricted diet resulted in higher adiposity, adipocytokines, dyslipidemia, and adverse gestational outcomes. Although biochemical plausibility has been postulated, the exact mechanisms of this link between B12 and BMI are not known (12). B12 is needed for the synthesis of methionine, the precursor of *S*-adenosyl-methionine (SAM), a key methyl donor for DNA methylation (12). DNA methylation is involved in the functioning of genes and depends on the supply of methyl groups by methyl-donors such as B12 from the diet (13). Evidence from two independent US cohorts demonstrated that the methylation variant of a transcription factor HIF3A (rs3826795) exhibited opposite effects on weight change in response to low and high B vitamin intakes (14). We have shown that low B12 is associated with hypomethylation of cholesterol transcription factor SREBF1. Our experiments with methylation inhibitors also showed that there may be other epigenetic mechanisms involved (15). Thus, it is plausible that deficiency in B12 might influence methylation patterns in the DNA as well as other epigenetic modulators such as microRNAs (miRs), which regulate gene expression (13).

Recently, much attention has been given to other regulators of AT development, such as miRs. MiRs are epigenetic mediators that control adipocyte differentiation, which when perturbed can potentially result in an unhealthy metabolic phenotype (16, 17), such as dyslipidemia, hypertension, IR, and possibly elevated risk of developing T2D. Maternal diet-induced obesity can program AT and modulate miRs during fat cell development (3, 13). In addition, circulating miRs have shown to be altered in gestational obesity (18) and in adults with different degrees of obesity and T2D (16, 19).

Taking these observations together, we hypothesize that low B12 levels during pregnancy may affect the AT development due to altered adipose-derived circulating miRs resulting in a metabolic phenotype. In this study, we aimed to investigate (1) the effects of B12 deficiency on adipogenesis and lipogenesis in human adipocyte cell line (Chub-S7), (2) investigate the effects of B12 deficiency on the miR profile in differentiated Chub-S7 and their secretion, and (3) validate the miRs identified in Chub-S7 with the levels of miRs in subcutaneous adipose tissue (SAT) and circulating miRs in the serum of pregnant women with low B12 levels.

Materials and Methods

The methods of clinical data collection and *in vitro* and *in vivo* experiments are detailed in Supplemental Methods. They are articulated in brief in the following paragraphs.

Differentiation of human preadipocyte cell line (Chub-S7)

The normal culture medium of adipocytes (DMEM/F12, Cat#11039-Gibco, Waltham, MA, USA) contains 500 nM of B12. Green *et al.* (20) cultured 3T3 adipocytes in B12 concentrations (0 to 500 nM) and showed that the accumulation of odd-chain fatty acids and methylmalonic acid (tissue marker of B12 deficiency) occurs in B12-deficient conditions, which were prevented by supplementation with 500 nM B12. Our previous study also showed that the methylation potential was optimal at 500 nM of B12 (15, 20). Therefore, we chose similar conditions [control (500 nM), low B12 (0.15 nM), and no B12 (0 nM)] for our *in vitro* experiments.

Study population

A cross-sectional study was conducted in the University Hospital Coventry and Warwickshire, Coventry, UK. Fasting maternal blood samples (n = 91) and SAT (n = 42) were collected at the time of cesarean section (21).

Lipid accumulation

In adipocytes, cellular lipid accumulation was determined by oil red O staining, and triglycerides were determined in cell lysates according to the manufacturer's protocol (Abcam, Cambridge, UK).

Quantitative real-time polymerase chain reaction of messenger RNA

RNA isolation and quantitative real-time polymerase chain reaction (q-RT-PCR) from Chub-S7 and human SAT were performed (15).

Locked nucleic acid–based miR array profiling

miR profiling of RNA from Chub-S7 differentiated in low B12 were performed with locked nucleic acid miRCURY arrays (Exiqon, Vedbaek, Denmark).

Bioinformatic analysis of miRs

To examine which metabolic and signaling pathways were affected, we used a bioinformatics prediction database: Bioconductor (R) package miRNAtap, which has prediction algorithms, such as DIANA (22), MiRanda (23), PicTar (24), and TargetScan (25). To further determine functional relationships

of the miRs, we constructed an integrated regulatory network of miR–gene–pathways by using Cytoscape software.

q-RT-PCR of miR

miRCURY locked nucleic acid miR PCR system (Exiqon) was used to assess the miRs in Chub-S7 and SAT and the circulating miRs in conditioned media and maternal serum.

Analytical determinations

Serum B12, folate, cholesterol, triglycerides, high-density lipoprotein cholesterol (21), and plasma one-carbon metabolites (SAM, *S*-adenosyl-homocysteine, methionine, homocysteine, methylmalonic acid) were determined by methods as described in Supplemental Methods (26).

Statistical analysis

Continuous data were reported as mean $\pm$ standard deviation. *In vitro* data were presented as mean $\pm$ standard error of the mean for at least six independent experiments to ensure reproducibility. Student *t* test was used for comparison of groups, all tests were two-sided, and $P < 0.05$ was considered statistically significant. Where appropriate, clinical data were log-transformed before correlation and regression analyses. All analyses were performed in SPSS Statistics version 21 (IBM Corp., Armonk, NY).

Results

In vitro study of B12 deficiency effects on human adipocyte cell line (Chub-S7)

Effects of B12 deficiency on adipogenesis and lipogenesis

To evaluate the effect of B12 deficiency in adipocytes and the underlying mechanism, we differentiated Chub-S7

in B12-deficient conditions. As shown in Fig. 1A–1E, we demonstrated increased accumulation of lipid droplets, and the triglyceride content in adipocytes differentiated in B12-deficient conditions. We found that through the process of differentiation of adipogenesis, B12-deficient conditions increased the gene expression of key transcriptional regulators of adipogenic differentiation such as peroxisome proliferator–activated receptor γ (PPARγ) within 48 hours and CCAAT/enhancer–binding protein α (CEBPα) after 6 days, and the levels remained significantly higher for the rest of the differentiation time course (14 days) (Supplemental Fig. 1), indicating that low B12 directly affects adipogenesis. Then we showed that at day 14, in addition to gene expression of PPARγ and CEBPα, the nuclear receptor retinoic acid X receptor α (RXRα) that heterodimerizes with PPARγ to regulate lipid metabolism was upregulated. Similarly, gene expression of lipogenic enzymes such as fatty acid synthase (FASN) and acetyl CoA carboxylase (ACACA) and the lipid-coating protein (perilipin) were also increased in adipocytes with B12-deficient levels (Fig. 1F, 1G). These findings suggest that low B12 levels in adipocytes might induce adipogenesis and lipogenesis.

Effects of B12 deficiency on epigenetic regulation

To assess the effect of B12 on epigenetic regulation, we treated the adipocytes with B12 in the presence of a methylation inhibitor (5-aza-2-deoxycytidine). Gene expression of adipogenic regulators (PPARγ, CEBPα, RXRα), lipogenesis (FASN, ACACA), and lipid-coating
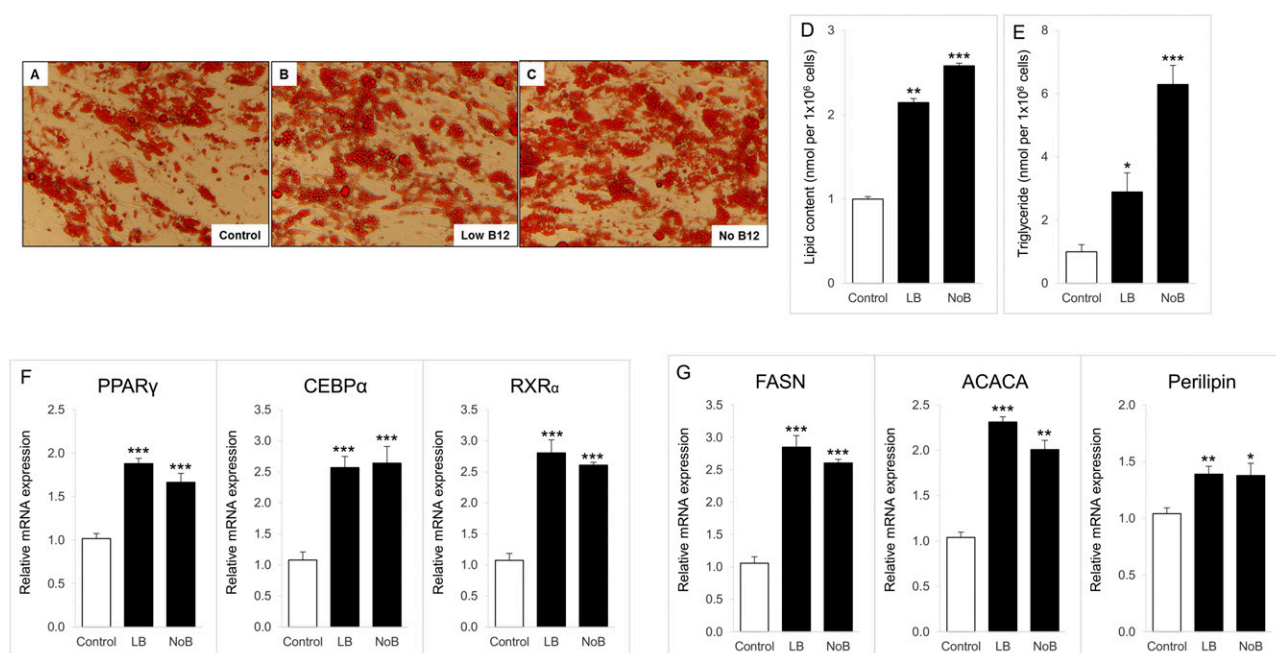

Figure 1. (A–C) Oil red O staining of adipocytes in (A) control, (B) low B12, and (C) no B12 (lipid droplets stained as red). (D and E) B12-deficient conditions increase (D) lipid accumulation and (E) triglycerides in human adipocytes. (F and G) Low B12 increases gene expression of (F) adipogenic regulators and (G) lipogenesis in Chub-S7. All experiments were performed as n = 6. LB, low B12; NoB, no B12. Values are mean $\pm$ standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; *P* value compared with control.

protein (perilipin) were increased, similar to low B12 (Supplemental Fig. 2). It is clear from these findings that methylation inhibition (or hypomethylation) alone may not be the only mechanism involved in these alterations, and this led us to further explore other epigenetic mechanisms such as miRs.

Effects of B12 deficiency on miR profiling in Chub-S7

To investigate the effect of B12 on miR, we assessed expression of >2042 human miRs annotated in miR-Base 18.0 by using miR microarrays in adipocytes differentiated with low B12. The miR profiling detected 560 mature human miRs, of which 133 miRs (23.8%) were differentially expressed (adjusted $P < 0.05$). The two-way hierarchical clustering analysis showed 97 miRs were significantly downregulated and 36 miRs were upregulated in adipocytes cultured in low B12 ($P < 0.01$) (Fig. 2A). These findings show that low B12 alters miR levels. We then carried out the pathway analysis involved with these aberrant miR expressions.

MiR targets and biological pathway prediction

To further identify and validate the biological roles of the aberrant miRs, analyses were carried out with

Bioconductor (R) package miRNAtap for target gene prediction. The number of target genes for significantly differentially expressed miR varied from 22 target genes (miR-146a/miR-377) to 994 target genes (miR-23c), with a median number of 344 target genes (Fig. 2B). The union set of all predicted target genes of 133 differentially expressed miRs was analyzed *via* the hypergeometric statistical test to significantly enrich pathways with pathway definitions taken from the Kyoto Encyclopedia of Genes and Genomes database for biological processes and Recon 2 for metabolic processes. Pathway enrichment analysis resulted in significant ($P < 0.01$) enrichment of genes that were related to the regulation of metabolic processes such as lipid, amino acid, nucleotide, transport, and glycan metabolism (Fig. 2C). Enrichment of biological processes revealed that these miRs were involved in signaling pathways particularly related to IR, developmental biology, immunity and inflammation (Fig. 2D). Interestingly, enrichment analysis indicated that these miRs were involved in classic metabolic and adipocyte differentiation pathways, such as the insulin signaling, Wnt signaling, adipocytokine signaling, peroxisome proliferator–activated receptor signaling, phosphatidylinositol signaling, and triacylglycerol synthesis (Fig. 2C).
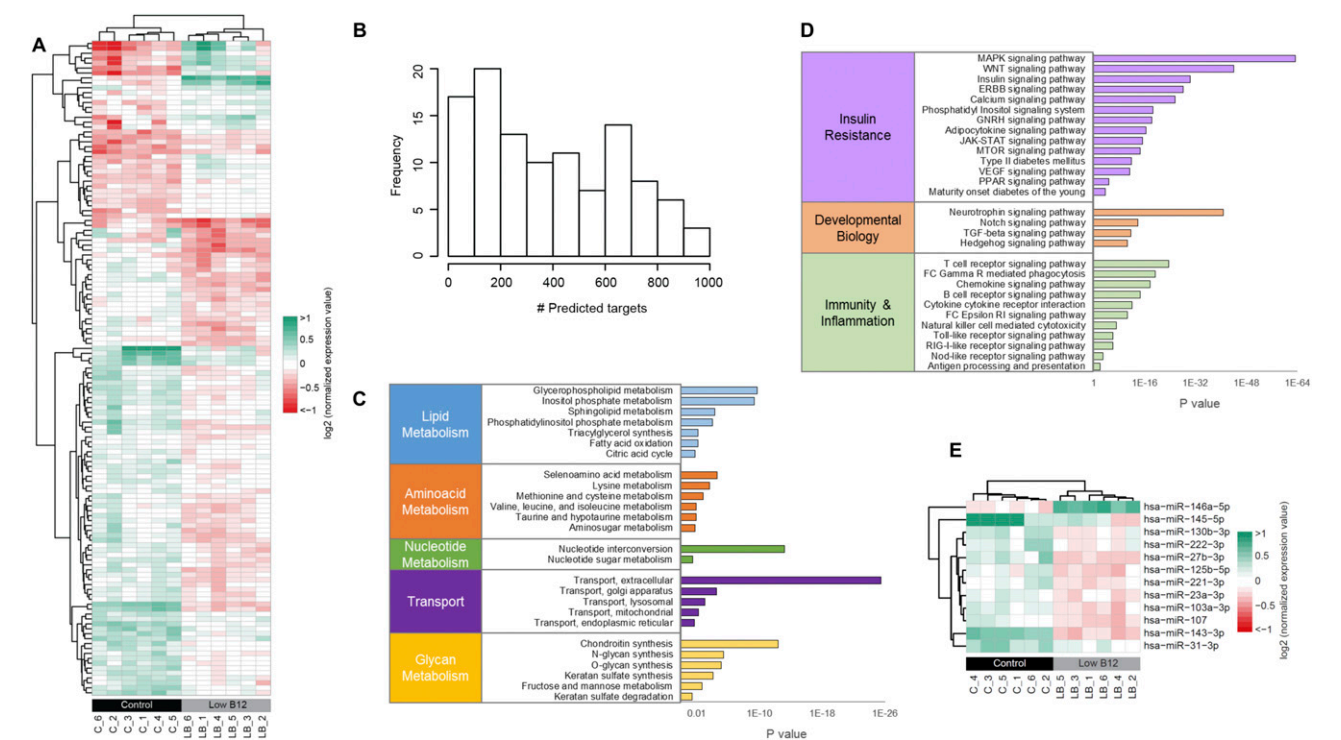

Figure 2. (A) Hierarchical clustering of 133 differentially expressed miRs in adipocytes differentiated *in vitro* with low B12 levels. Control, n = 6; low B12, n = 6. (B) Number of predicted target genes for 133 differentially expressed miRs that varied from 22 targets (miR-146a and miR-377) to 994 targets (miR-23c), with a median number of 344 targets. (C) Enrichment analysis for metabolic pathways *via* Recon 2; $P < 0.01$. (D) Enrichment analysis for signaling pathways *via* the Kyoto Encyclopedia of Genes and Genomes database; $P < 0.01$. (E) Hierarchical clustering of 12 selected miRs related to adipocyte differentiation and function; adjusted $P < 0.05$. Red represents expression level below mean (downregulated), and green represents expression level above mean (upregulated); adjusted $P < 0.05$. C, control; LB, low B12.

Effects of B12 deficiency on miR expression in Chub-S7 and its secretion

To confirm the differences observed in miR array screening, miRs with significant changes in expression level and the putative target genes associated with adipocyte differentiation and function were selected (Fig. 2E) and validated by q-RT-PCR analysis. The 12 miRs chosen for validation were the following: 3 targeting PPARγ (miR-27b, miR-23a, miR-130b), 1 targeting CEBPα (miR-31), 6 targeting adipocyte differentiation (miR-143, miR-145, miR-146a, miR-221, miR-222, miR-125b) and 2 involved in IR pathways (miR-103a, miR-107) (Fig. 3A). q-RT-PCR analysis confirmed that

these 12 miRs were significantly altered in adipocytes, and 9 miRs were significantly altered in the condition media (except miR-130b, miR-103a, and miR-107) (Fig. 3B). These results show that low B12 alters adipose-derived miRs related to adipocyte differentiation and function. To further confirm whether altered miRs in response to low B12 could be a putative epigenetic mechanism, we validated these 12 miRs in adipocytes with B12 in the presence of a methylation inhibitor. We found that nine miRs in adipocytes and eight secreted miRs in conditioned media were altered, similar to low B12 (Supplemental Figs. 3 and 4). Here it is evident that in addition to methylation inhibition, other epigenetic
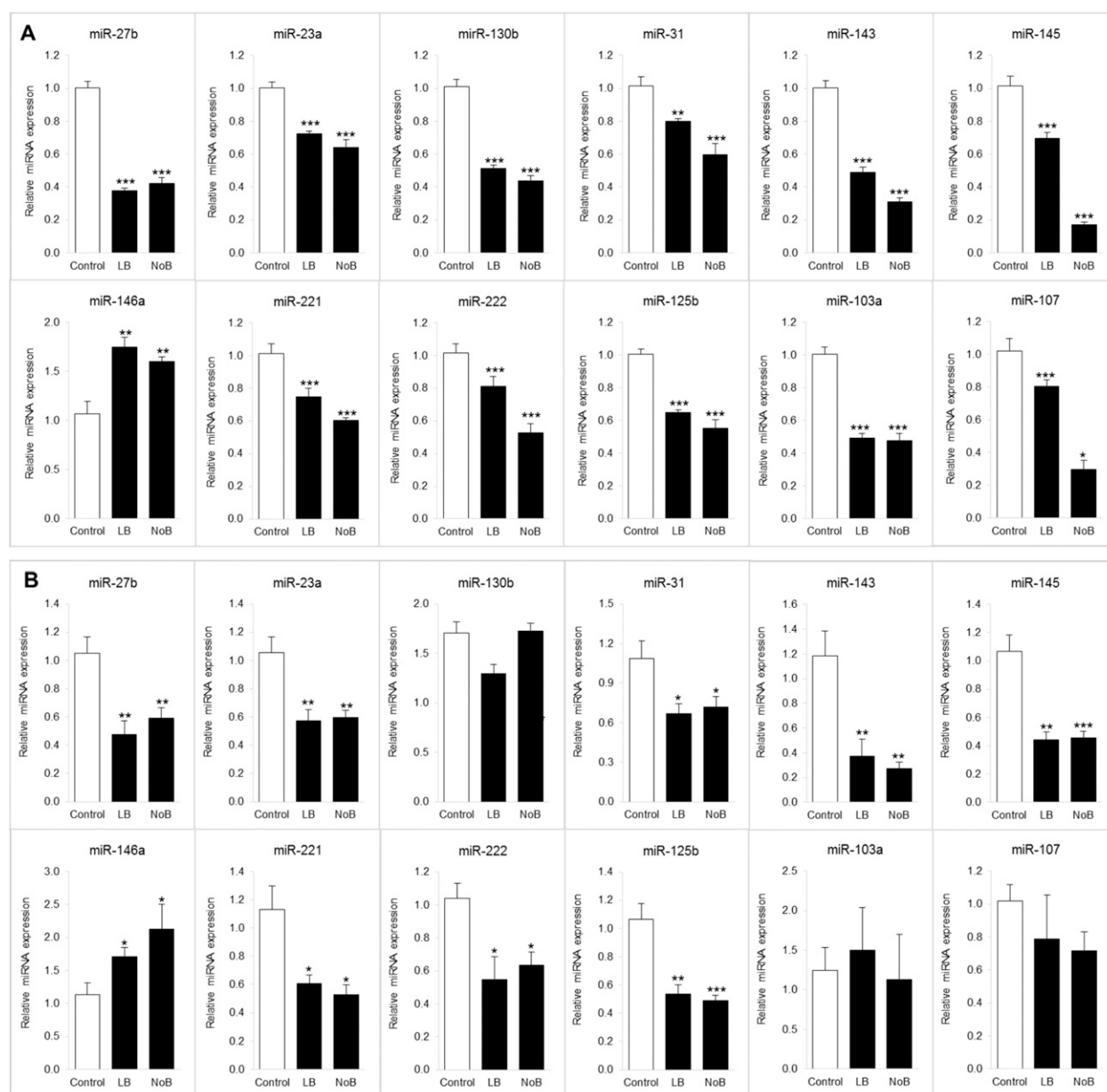

Figure 3. (A) Low B12 alters miRs related to adipocyte differentiation and function in Chub-S7. (B) Low B12 alters secreted miRs related to adipocyte differentiation and function in conditioned media from Chub-S7. All experiments were performed as n = 6; LB, low B12; NoB, no B12. Values are mean ± standard error of the mean. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. P value compared with control.

mechanisms such as miRs are involved in B12-deficient conditions.

Study on pregnant women with low B12 levels

One-carbon metabolites in pregnant women

The clinical characteristics of the study population are shown in Supplemental Table 1. Reduced methylation potential was observed in women with low B12 status, such as lower SAM and methionine (Supplemental Table 1). These results indicate that low B12 disturbs one-carbon metabolism alters the levels of SAM, the methyl donor that may reduce the effect of methylation of DNA and other epigenetic regulators (miRs).

Adipogenic and lipogenic gene expression in human maternal SAT

To further evaluate the tissue-specific effect of B12 on human SAT, gene expression of adipogenesis and lipogenesis were compared in SAT of pregnant women with low B12 levels and with normal B12 levels. We demonstrated that the gene expression of adipogenic regulators (PPARγ, CEBPα, RXRα), lipogenesis (FASN, ACACA), and lipid-coating protein (perilipin) (Fig. 4) was upregulated in maternal SAT with low B12, similar to the observation seen in Chub-S7 (Fig. 1F and 1G). These findings suggest that low B12 levels in pregnant women might increase adipogenesis and lipogenesis.

Validation of miR expression in human maternal SAT

Next, we attempted to validate the differential miR expression identified in Chub-S7 to human maternal SAT. Expression levels of all 12 miRs were significantly upregulated or downregulated in SAT from pregnant women with low B12, similar to the observation seen in Chub-S7 (Fig. 5A).
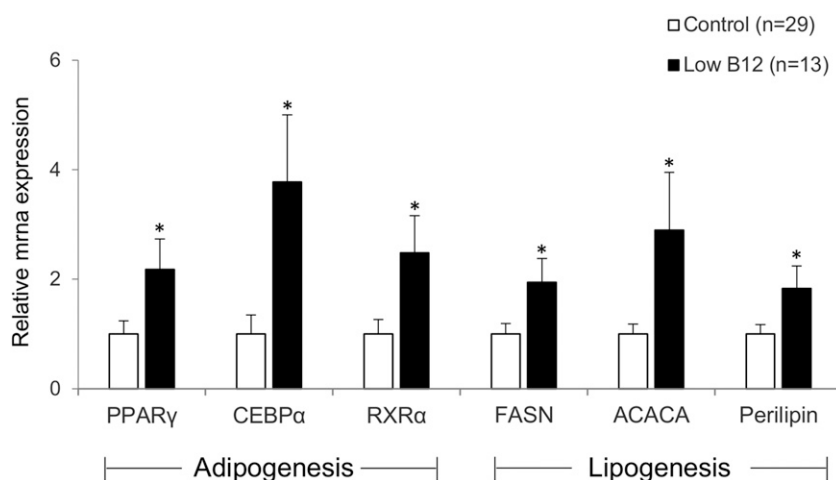
Validation of circulating miRs in serum from pregnant women

Similarly, we validated the adipose-derived miR expression observed in conditioned media from Chub-S7 to serum from pregnant women. We observed that 11 of the 12 miRs were significantly altered in the circulation, except 1 (miR-31, not detected) in pregnant women with low B12 levels (Fig. 5B). Interestingly, we also observed that 6 of the 11 miRs from serum correlated significantly with miRs from SAT, and 9 of the 12 miRs from the conditioned media correlated significantly with the miRs from the Chub-S7 (Supplemental Table 2). These results reveal that these circulating miRs are adipose-derived and are altered by low B12.

Association of circulating B12 and circulating miRs with obesity

To further explore the relation between circulating B12 and miRs with obesity, we performed the following correlation analyses. Circulating B12 was inversely correlated with maternal BMI ($r = -0.292$; $P = 0.007$) and positively with seven circulating miRs: miR-27b ($r = 0.390$; $P = 0.001$), miR-103a ($r = 0.344$; $P = 0.004$), miR-107 ($r = 0.387$; $P = 0.001$), miR-125b ($r = 0.311$; $P = 0.010$), miR-23a ($r = 0.323$; $P = 0.007$), miR-221 ($r = 0.274$; $P = 0.026$) and miR-222 ($r = 0.400$; $P = 0.001$). To further investigate whether the circulating B12 and miRs independently contribute to BMI, multiple regression analysis was carried out. Circulating B12 and four circulating miRs (miR-27b, miR-23a, miR-103a, miR-107) were independently associated with BMI after adjustment for likely confounders (age, parity, smoking, insulin, glucose, and supplement use) (B12, $\beta = -0.264$, $P = 0.013$; miR-27b, $\beta = -0.250$, $P = 0.041$; miR-23a, $\beta = -0.271$, $P = 0.026$; miR-103a, $\beta = -0.226$, $P = 0.049$; miR-107, $\beta = -0.228$, $P = 0.041$).

We also confirmed that when we excluded the subjects with gestational diabetes and low B12 on insulin or metformin therapy (n = 2), the association of B12 and miRs with BMI remained the same, suggesting no effect of associated therapy on BMI (data not shown). However, in multiple regression analysis, the association of B12 with BMI became nonsignificant after further adjustment for these four circulating miRs, thereby highlighting a mediating role of circulating miRs between B12 and BMI (Table 1).

To further study the function of these four circulating miRs in metabolic pathways, we constructed a miR–gene–pathway network for these
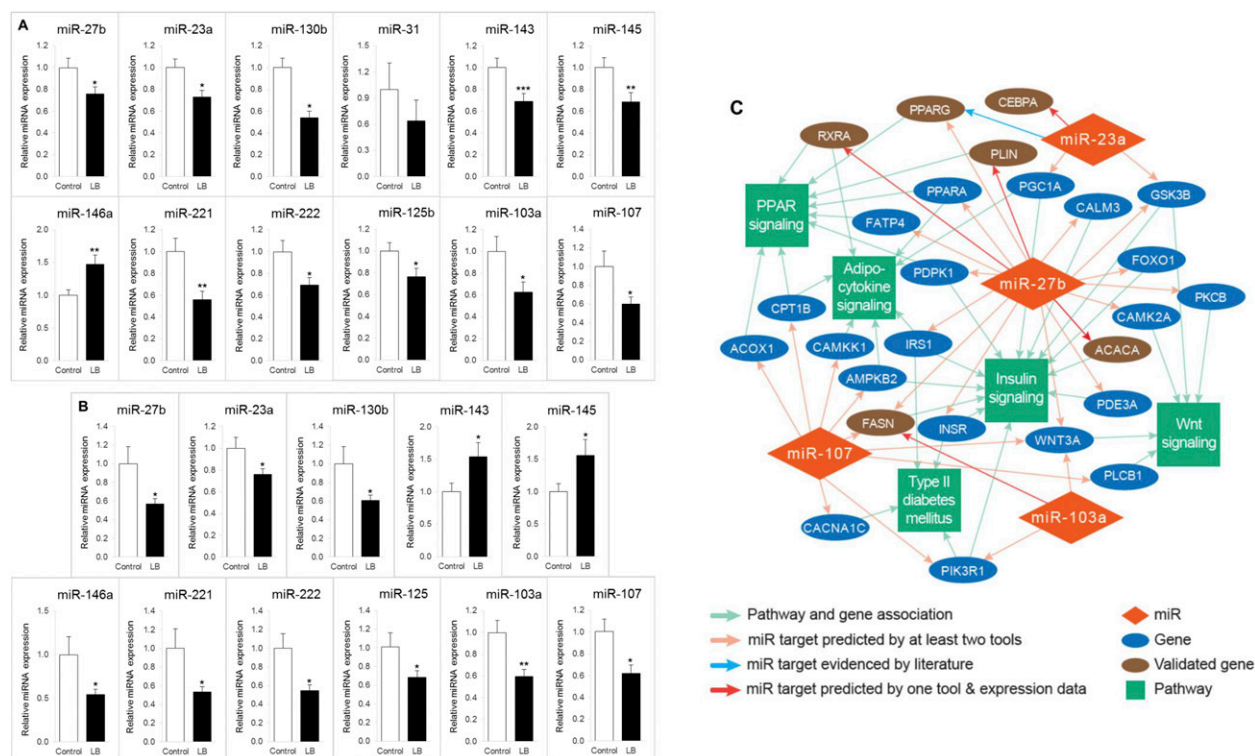

Figure 4. Gene expression of adipogenic regulators and lipogenesis in human maternal SAT. Values are mean ± standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; P value compared with control.

Figure 5. (A) MiR expression in a subset of human SAT. Control, n = 17; low B12, n = 13. (B) Circulating miR expression in a subset of serum from pregnant women with low B12 levels. Control, n = 38; low B12, n = 34. Values are mean ± standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; P value compared with control. (C) MiR–gene–pathway regulatory network shows the network of the five pathways related to IR and the six validated genes regulated by at least one miR each. MiR, genes, and pathways are represented by nodes such as orange diamonds (miRs), green squares (pathways), blue ovals (genes), and brown ovals (validated genes regulated by at least one miR each). Green arrows represent the relationship between gene and pathway, orange arrows represent the relationship between miR and gene predicted by at least two tools, red arrows represent the relationship between miR and gene predicted by one tool and validated gene, and blue arrows represent the relationship between miR and gene evidenced by literature. LB, low B12.

four miRs with their known or predicted target genes and annotated pathways. Figure 5C shows that the network of these four miRs shared the targets related to five metabolic pathways related to IR (PPARγ, adipocytokine, insulin, Wnt, and T2D) and the six validated genes uniquely regulated by at least one miR each (PPARγ gene as a predicted target of miR-27b and miR-23a; CEBPα as predicted target of miR-23a; FASN as predicted target of miR-107, miR-27b, and miR-103a; and RXRα, ACACA, and PLIN as predicted targets of miR-27b). Therefore, our findings strongly indicate that these four miRs could regulate the adipogenic and lipogenic genes and may mediate an obesity and IR effect of low B12 status.

Discussion

Our study shows that B12 deficiency in human adipocytes changes tissue-specific miRs and circulating miRs and leads to an adverse metabolic phenotype. Here we have demonstrated that B12 deficiency in adipocytes caused excess accumulation of lipids, increased the expression of genes that regulate adipogenesis and lipogenesis, and resulted in aberrant expression of miRs

involved in key metabolic pathways, such as PPARγ and IR. These were first demonstrated in human adipocyte cell line and then validated in human SAT. In addition, our clinical study findings indicate that the association between low B12 and obesity appeared to be mediated by adipose-derived circulating miRs targeting these pathways.

AT development is associated with both increasing adipocyte cell numbers and their ability to accumulate lipids (4, 6). We observed that low B12, both *in vitro* (adipocytes) and *in vivo* (AT at the time of childbirth), caused increased adipogenesis (PPARγ, CEBPα, RXRα) and lipogenesis (FASN, ACACA), indicating that low B12 affects the two distinct processes of AT development. Similar findings were observed in rats, where B12 deficiency resulted in differential expression of peroxisome proliferator–activated receptor signaling pathways (27) and higher activities of hepatic FASN and ACACA (28). We have previously shown in adipocytes that low B12 conditions caused hypomethylation of cholesterol transcription factor SREBF1 (15). It is known that SREBF1 induces PPARγ and regulates genes necessary for lipogenesis (29).

Table 1.   Multiple Regression Analysis of Maternal B12 With BMI

Maternal Variable (SDS)	BMI		
	β	95% Confidence Interval	P
		Model 1	
B12	−0.264	(−0.469, −0.058)	0.013
miR-27b	−0.250	(−0.488, −0.011)	0.041
miR-23a	−0.271	(−0.508, −0.034)	0.026
miR-103a	−0.226	(−0.452, −0.001)	0.049
miR-107	−0.228	(−0.446, −0.009)	0.041
B12		Model 2	
	−0.219	(−0.523, −0.084)	0.153
B12		Model 3	
	−0.225	(−0.500, 0.049)	0.106
B12		Model 4	
	−0.217	(−0.519, −0.084)	0.154
B12		Model 5	
	−0.211	(−0.523, 0.101)	0.181

Maternal variables (n = 91) and circulating miRs (n = 72, measured in a subset of pregnant women) were log transformed for statistical comparisons. β represents SDS change in the dependent variable per SDS change in the independent variable. Model 1: Maternal age, parity, folate supplement use, smoking, insulin, and glucose; Model 2: Model 1 + miR-27b; Model 3: Model 1 + miR-23a; Model 4: Model 1 + miR-103a; Model 5: Model 1 + miR-107. SDS, standard deviation score.

Our *in vitro* experiments showed that adipocytes in low B12 conditions displayed increased lipid accumulation. Because B12 is a key micronutrient essential for functioning of most tissues, similar processes may also happen in hepatocytes. If such dysregulation of lipid occurs in hepatocytes, it is plausible that this might contribute to higher circulating lipids, an observation seen in women with low B12 (16), who had lower methylation potential (Supplemental Table 1), and in mice fed with a B12-restricted (11, 28) or methyl-deficient diet (30). Interestingly, their pups also exhibited a dyslipidemic profile, an observation previously reported by us from this clinical cohort that lower maternal B12 was associated with lower high-density lipoprotein in the cord blood (21). Whether this effect is due to adverse epigenetic programming requires additional longitudinal, mechanistic, and interventional studies.

Nutrient imbalance can cause epigenetic modifications through several mechanisms including DNA methylation, histone modification, chromatin remodeling, and changes in the expressions of small and long noncoding RNAs such as miRs (13). DNA methylation and histone modification have been studied extensively, and a number of recent studies have explored the mechanistic aspects of miRs on regulation of protein-coding genes. However, there is no study demonstrating the effects of micronutrient deficiency on miR expression, especially in SAT. In this study, we report a comprehensive database of differential expression patterns of 133 miRs in adipocytes

differentiated with low B12. Pathway enrichment analysis of miR array data revealed that these miRs were involved in several metabolic pathways including adipocyte biology and IR, such as insulin signaling, Wnt, adipocytokine, PPAR, phosphatidylinositol, and triacylglycerol synthesis pathways. Here we report 12 miRs related to adipocyte differentiation and function that have been associated with obesity. Our *in vitro* adipocyte experiments showed that these miRs were significantly altered in B12-deficient conditions. These findings were replicated in SAT from pregnant women with low B12 levels. Previous studies have shown overexpression or knockdown of miR-27b (31), miR-23a (32), and miR-130b (33, 34) as important regulators of adipogenesis by targeting PPARγ, and these miRs are downregulated in AT from obese subjects with or without diabetes. Studies in AT from obese women have shown that miR-31 downregulates CEBPα expression at both the transcriptional and translational levels (35). In addition, miR-143 (17), miR-145 (36), miR-146a (37), miR-221 (16, 17), miR-222 (16, 17), and miR-125b (16, 17) have been shown to exhibit a role in adipocyte differentiation and were significantly altered in morbidly obese patients, prepubertal children, and the AT of obese mouse models. Furthermore, the role of miRNA-103a and miR-107 (17) in IR has been shown in rodent T2D models and in 3T3-L1 adipocytes. Thus, we showed that low B12 causes aberrant miR expression in SAT and their association with adipogenesis and obesity. Our findings support similar observations of altered tissue-specific miR expression in human placenta exposed to low folate (38) and 3T3-L1 adipocytes exposed to vitamin A (39).

In addition to these tissue-level changes, we observed that the secretion of the adipose-derived miRs was significantly altered in adipocytes differentiated *in vitro* in B12-deficient conditions and correspondingly in the circulation in pregnant women with low B12. The tissue expressions of the miRs and circulating miRs also correlated with each other (Supplemental Table 2), indicating that the primary source of these miRs could be the SAT. However, 3 of the 12 miRs (miR-143, miR-145, miR-146a) and the correlation of miR-145 with human SAT were in the opposite direction. Because the effects of low B12 status can be global, other tissues such as liver, muscle, or placenta could also contribute to the circulating levels, which may explain this observation. It is also possible that these miRs were induced during adipogenesis but are downregulated in the obese state, consistent with previous studies (17). Circulating miRs provide a possible mechanism for crosstalk between tissues. If these aberrant miRs are transferred across the placenta, they may cause adverse epigenetic changes in the tissues of the offspring and predispose them to

metabolic disorders in later life (40). Future studies are needed to prove these speculations.

Finally, multiple regression analyses revealed that the circulating B12 and four of the circulating miRs (miR-27b, miR-23a, miR-103a, and miR-107) were independently associated with BMI, after adjustment for other possible confounders. Further regression analysis showed that the inverse association between B12 and BMI was reduced when adjusted for these four miRs. This suggests that the link between B12 and BMI may be partly mediated through these miRs (Table 1). Other studies (8, 10, 15, 21) have shown strong inverse associations between B12 and BMI. However, this study demonstrates that these miRNAs regulating adipogenesis may play a causal role. On the contrary, although our clinical data showed the association of B12 with miRs targeting IR, it did not show with homeostatic model assessment of IR. This result might reflect the sample size, or it is possible that these pregnant women will develop IR in the future, because obesity usually precedes IR. If these findings are replicated in longitudinal studies, these miRs may represent early pregnancy biomarkers for IR in women with low B12 levels.

In summary, our study identified that B12 deficiency in pregnancy is independently associated with adipose-derived circulating miRs, which are known to affect PPARγ and IR pathways. This study provides insight that these adipose-derived circulating miRs can act as a mode of cell signaling molecules that may predispose metabolic disorders to both mothers and offspring in later life. Thus identification of B12-induced epigenetic signatures could provide a unique opportunity to study predictive miR biomarkers and future therapeutic targets for obesity.

Acknowledgments

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