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Effect of maternal undernutrition on vascular expression of micro and messenger RNA in newborn and aging offspring

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Khorram O, Han G, Bagherpour R, Magee TR, Desai M, Ross MG, Chaudhri AA, Toloubeydokhti T, Pearce WJ. Effect of maternal undernutrition on vascular expression of micro and messenger RNA in newborn and aging offspring. *Am J Physiol Regul Integr Comp Physiol* 298: R1366–R1374, 2010. First published March 3, 2010; doi:10.1152/ajpregu.00704.2009.—The aim of this study was to test the hypothesis that maternal undernutrition (MUN) alters offspring vascular expression of micro-RNAs (miRNAs), which, in turn, could regulate the expression of a host of genes involved with angiogenesis and extracellular matrix remodeling. The expression of miRNA and mRNA in the same aortic specimens in 1-day-old (P1) and 12-mo-old offspring aortas of dams, which had 50% food restriction from gestation *day 10* to term, was determined by specific rat miRNA and DNA arrays. MUN significantly downregulated the expression of miRNAs 29c, 183, and 422b in the P1 group and 200a, 129, 215, and 200b in the 12-mo group, and upregulated the expression of miRNA 189 in the P1 group and 337 in the 12-mo group. The predicted target genes of the miRNAs altered in the two age groups fell into the categories of: 1) structural genes, such as collagen, elastin, and enzymes involved in ECM remodeling; and 2) angiogenic factors. MUN primarily altered the expression of mRNAs in the functional category of cell cycle/mitosis in the P1 group and anatomic structure and apoptosis in the 12-mo age group. Several of the predicted target genes of miRNAs altered in response to MUN were identified by the DNA array including integrin- β_1 in the P1 aortas and stearoyl-CoA desaturase-1 in the 12-mo age groups. These results are consistent with the hypothesis that MUN modulation of offspring gene expression may be mediated in part by a miRNA mechanism.

aorta; DNA array

Micro-RNAs (miRNAs) are short, noncoding RNA molecules of 20–25 nucleotides in length that regulate gene expression at the posttranscriptional level (2, 4) and play a significant role in the regulation of physiologic and pathologic processes. Several thousand miRNAs have been cloned and/or predicted and may regulate up to 90% of human protein-coding genes, primarily through translational repression, and, in some cases, mRNA degradation (9, 19, 35). The role of miRNAs in fetal development and postnatal maturation is largely unstudied, and their role in fetal programming of adult disease is unknown. It is well known that maternal undernutrition (MUN) alters fetal gene expression, and changes in transcription rates cannot explain all these effects. Therefore, we have hypothesized that MUN influences the expression of offspring miRNAs, which then would influence processing of key developmental pro-

teins, and this might represent a novel epigenetic mechanism for regulation of gene expression in the offspring.

Previously, we reported that MUN induced significant vascular remodeling of offspring vascular extracellular matrix (17, 18) and inhibited angiogenesis (16). miRNAs have been shown to play a role in the regulation of both of these processes, and therefore we sought to determine whether MUN alters miRNA expression, which, in turn, might influence the regulation of genes important in angiogenesis and ECM remodeling. Recent accumulating evidence indicates a significant role for miRNAs in the regulation of genes involved in angiogenesis (40) and vascular differentiation (7). In multiple species ranging from fish (12) to humans (20, 39), alterations in miRNA maturation produce significant defects in expression of VEGF and its receptors (45) and correspondingly, changes in blood vessel and capillary formation, which can be embryonically lethal (6). Collectively, these reports provide strong evidence for a role of miRNAs in regulating vasculogenesis, angiogenesis, and cardiovascular remodeling, and raise the possibility that these molecules may play a role in changes in vascular structure and function associated with MUN.

METHODS

Animals and tissue collection. The protocol for this study was approved by the Animal Use and Care Committee at La BioMed at the Harbor-University of California Los Angeles Medical Center Medical Center. First-time pregnant Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were housed in a facility with constant temperature and humidity and a controlled 12:12-h light-dark cycle. At 10 days of gestation, rats were provided either an ad libitum diet of standard laboratory chow (23% protein, 4.5% fat, 3,030 kcal/kg metabolizable energy; Lab Diet 5001; PMI Nutrition, Brentwood, MO) or 50% food-restricted diet determined by quantification of normal intake in the ad libitum-fed rats. The respective diets were given from 10 days of pregnancy to term. Maternal body weights and the food intake were recorded daily. At *day 1* after birth, all offspring from food-restricted and control rat dams were cross-fostered to rat dams fed ad libitum, and litter size was culled to four males and four females per dam. Offspring were weaned at 3 wk of age to ad libitum standardized laboratory chow. Animals were anesthetized under isofluroane gas. Thoracic aortas were dissected and cleaned of periaortic fat and snap frozen in liquid nitrogen and stored at -80°C . This vessel was chosen because, in our previous work, we have extensively characterized the effects of MUN on angiogenic genes and genes regulating ECM remodeling. For immunohistochemical studies, blood vessels were fixed in 4% paraformaldehyde for 24–48 h and then transferred to 70% ethanol. These conditions were previously shown to be optimum for immunohistochemical detection of proteins of interest. Arrays were used to examine three specimens from each dietary group, representing pooled RNA from four to six animals in

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the case of P1 offspring for each data point and two animals in the case of 12-mo-old offspring. Offspring tissues were obtained from six different dams in both age groups, and for pooling purposes, specimens were derived from different litters.

miRNA expression profiling. Total RNA was extracted from aortas using Trizol (Invitrogen) with their quantity and quality determined using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Agilent Bio-analyzer (Agilent Technologies, Foster City, CA), respectively. The isolated miRNAs were 3'-end labeled with Cy3 using the mirVana miRNA Array Labeling Kit (Ambion, Austin, TX) and the Post-Labeling Reactive Dye Kit (Amersham Bioscience, Pittsburgh, PA). miRNA profiling was performed by Ocean Ridge Biosciences (Jupiter, FL) by using custom-developed microarrays containing 237 NCode-2 35–44-mer rat miRNA oligonucleotide probes manufactured by Invitrogen and spotted in duplicate. At the time of analysis, these miRNAs represented an entire coverage of Sanger mirBASE version 9.0 content. After hybridization, the arrays were scanned using a GenePix 4000A array scanner (Axon Instruments, Union City, CA). The intensity of each oligo probe was averaged among triplicate spots and then normalized and analyzed using GeneSpring 7.0 Software (Silicon Genetics, Redwood City, CA). Normalization was performed by expressing each miRNA abundance relative to control miRNA (Ambion) added to each sample. Threshold and 95th percentiles of negative controls (TPT95) were calculated based on the hybridization signal from negative control probes, including mismatch and shuffled control probes and nonconserved *Caenorhabditis elegans* probes. The two base pair mismatch probes demonstrated a signal below or at TPT95 on all arrays. The computational algorithms TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>), miRDB (<http://mirdb.org/>), and miRanda (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) were utilized for identifying predicted target genes of miRNAs.

DNA microarray. Total RNA isolated from the same tissues was also subjected to large-scale transcript profiling using RatRef-12 Expression BeadChip representing 22,524 probes for a total of 22,228 rat genes selected primarily from the NCBI RefSeq database (Release 16; Illumina, San Diego, CA). The arrays were utilized in accordance with the manufacturer's instructions. Following amplification and cDNA synthesis, 5 µg of purified cDNA was reverse transcribed and labeled with the TotalPrep RNA Labeling Kit using biotinylated-UTP (Ambion, Austin, TX). Hybridization was carried out in Illumina IntelliHyb chambers at 58°C for 18.5 h, followed by washing and staining. The signal was developed by staining with Cy3-streptavidin. The BeadChip was scanned on a high-resolution Illumina BeadArray reader.

The expression values were background subtracted and globally normalized using BeadStudio version 1.5.1.3 (Illumina), and probes with differential scores of ≥ 13 were independently removed from each cohort. The transformed expression values were subjected to unsupervised and supervised learning and statistical analysis in "R" programming as previously described (41). Gene expression values having a statistical significance of $P \leq 0.05$ (ANOVA, Tukey test) were selected and subjected to a 1.5-fold cutoff change (statistically significant) either positively or negatively. These values were subjected to functional annotation and visualization using the Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov>) (DAVID) software.

RT-PCR chain reaction. Using 2 µg of total RNA isolated from the above samples, complimentary DNA was generated using Taqman reverse transcription reagent. The newly synthesized cDNA was used for PCR performed in 96-well optical reaction plates by using cDNA equivalent to 100 ng RNA in a 50-µl reaction volume containing 1× Taqman Universal Master Mix; optimized concentrations of FAM-labeled probe and specific forward and reverse primer were selected from Assay on Demand (Applied Biosystems). Real-time PCR was carried out using Applied Biosystems 7300 Fast Real-Time PCR System at 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 40 cycles. The miRNAs and mRNAs expression values were analyzed using the comparative method (34) following transformation and normalization to RU6B and 18S rRNA expression, respectively, according to the manufacturer's guidelines; the threshold cycle was set within the exponential phase of the PCR and converted into fold change based on a doubling of PCR product in each PCR cycle.

Morphometry. Histologic analysis for elastin was performed using Van Gieson staining and images analyzed as previously described in detail (17). To avoid variation in staining conditions, all specimens from each age group were stained simultaneously. The slides were analyzed by the same investigator blinded to the treatment groups under the same magnification and light intensity. The area of staining and staining intensity were quantified by image analysis by using the Image Pro 4.1 software (Media Cybernetics) coupled to an Olympus BHS microscope/spot RT digital camera. The images were calibrated for background intensity and converted to gray scale. The concentration of the immune reactive antigen was determined using integrated optical density; these values were proportional to the unweighted average optical density per area. Integrated optical density of four sections of the aorta ($\times 20$ images) were analyzed, and mean values were obtained for the analysis.

Western blot analysis. Western blot analysis for AT₁-receptor protein, which was identified on the 12-mo age group DNA array and was determined to be significantly altered, was performed using a monoclonal antibody (Santa Cruz Labs, Santa Cruz, CA). We used 7.5% polyacrylamide gel and 5% milk buffer as a blocking agent. GAPDH was used as an internal control. Details of the procedure have been previously published (16).

Statistical analysis. The results are expressed as means \pm SE and were statistically analyzed using Student's *t*-test for comparison of two groups and ANOVA followed by Tukey's test for multiple comparisons after verification of normal distribution. For nonnormally distributed data, the Mann-Whitney *U*-test or Kruskal-Wallis multiple-comparison test was used. $P < 0.05$ was considered significant.

RESULTS

The expression profile of the significantly altered miRNAs and some of their predicted target genes in P1 and 12-mo-old offspring aortas are shown in Tables 1 and 2, respectively. Out of the 248 miRNAs on the array, 173 rat miRNAs profiled were detectable in the aorta. Statistical analysis showed one miRNA (miRNA 189) was significantly expressed more in the P1 MUN aortas, and three that were significantly expressed less (miR-29c, miR-183, miR-422b) compared with age-matched controls (Table 1); in the 12-mo aortas, four miRNAs

Table 1. miRNAs showing statistically significant change in expression and their predicted target genes in 1-day-old group (P1) aortas

miRNA	Fold Change	P Values	Predicted Target Genes
29c	2.6	0.02	COL 5A3, COL 1A1, COL4A5, Col 11A1, HAS3, ADAM 18, 19 and 9, BCL2L2, VEGFA, Eln
183	2.3	0.01	LRP6, PTPN4, FGF9, EPHA4, THSB3, NRP2, MBNL1, VEGFD, Integrin beta 1
422b	1.2	0.02	GRB-2, TBL1XR1, SULF1, PDE7B, BCL2L2, XPO5
189	0.5	0.02	STK3, H1F3 Alpha, RGPR, NCOA3, HDAC4

(miR-200a, miR-200b, miR-129, and miR-215) were significantly downregulated in the 12-mo MUN aortas and one miRNA (miR-337) was upregulated (Table 2). The predicted target genes of miRNAs altered by MUN in the two age groups included 1) structural genes, such as collagen, procollagen, protocadherins, and elastin; 2) enzymes involved in ECM remodeling and synthesis (ADAM, matrix metalloproteinases, hyaluronidase synthase-3); 3) angiogenic genes such as ephrin, neuropilin, thrombospondin, VEGF, FGF and its receptor, TGF-related genes; and 4) genes regulating cell proliferation, such as BCL2, and signaling factors (MAP kinase, STAT, NCOA3).

There was a striking difference in the expression profile of miRNAs in the two age groups studied, which was independent of the in utero nutritional history of the offspring (Table 3). The level of expression of miRNAs influenced by postnatal maturation is listed in Table 3 in descending order of magnitude of change in expression levels. Comparison of the two age groups shows that the level of expression of many miRNAs were significantly higher in the P1 group compared with the 12-mo group. The predicted target genes for miRNAs that showed the greatest change in expression during development are shown in Table 4.

The results of transcript profiling, as determined by a rat-specific DNA array and organized into functional categories using DAVID bioinformatic analysis, are shown in Tables 5 and 6 for the P1 and 12-mo groups, respectively. The complete list of mRNAs showing significant change in expression is shown in the Supplemental Tables S1–S4 (Supplemental data for this article is available online at the *American Journal of Physiology–Regulatory, Integrative and Comparative Physiology* website.). In P1 aortas, 221 mRNAs were statistically significantly upregulated (127 were > 1.5-fold) and 125 were downregulated (91 > 1.5-fold) in controls compared with MUN aortas. In contrast to the P1 aortas, in the 12-mo aortas 338 mRNAs were expressed significantly more (240 > 1.5-fold) in control aortas compared with MUN aortas, and only 30 mRNAs (29 > 1.5-fold) were expressed more in MUN aortas compared with controls. There was little overlap between the mRNAs that commonly showed a change in expression in the two age groups in response to maternal undernutrition. Only three mRNAs were upregulated significantly in the MUN groups in both the P1 and 12-mo age groups (C11orf8h, LOC313778, LOC498945), and three mRNAs were downregulated (Prpsap1, LOC500216, LOC501549). Two mRNAs were upregulated in the P1 group but downregulated in the 12-mo age group (LOC498682, Rn.46489). In the P1 group, out of the 319 DAVID IDs, 175 genes could not be clustered into any functional category; in the 12-mo groups, out of 344 DAVID IDs, 149 genes could not be clustered into any functional group. As demonstrated in Table 5, in the P1 group genes

Table 3. Changes in miRNA induced by postnatal maturation

miRNA Species			
Group 1: 12-mo > P1 for Control			
29c	29b	193	34a
189	29a	195	
497	140	664	
Group 2: 12-mo < P1 for Control			
431	369-5p	200c	370
134	322	200a	20b
541	300	539	19a
409-3p	376a	487b	335
433	543	329	323
381	382	18	130b
299	183	1	17
127	376b	342	122a
542-3p	154	429	205
409-5p	379	141	333
351	377	206	
494	298	137	
337	200b	350	
503	449	181c	

Shown are the miRNA species that changed significantly and by 1.5-fold or more as a result of postnatal maturation. The miRNAs are listed in decreasing order of the magnitude of fold change as read from top to bottom in each column beginning with the left-most column.

regulating cell cycle/mitosis and DNA replication had the highest enrichment score (greatest representation), whereas in the 12-mo group genes regulating anatomic structure/development and apoptosis had the highest enrichment scores. There was a significant overlap of genes that fell in multiple functional categories in both age groups. In the angiogenic functional category, VEGFD, fibroblast growth factor binding protein (FGFBP), p-selectin, MMP14, and thrombospondin 2 were significantly altered in the P1 group, whereas in the 12-mo group CTGF, FGF2, AT-receptor 1b, integrin- α_v , and TNF receptor 12 were altered (Tables 5 and 6). Each age group had functional categories that were unique to it. In the case of the P1 group, these categories included DNA modification with constituent genes, such as DNA methyl transferase-1, and steroid metabolism with such members as HSD1 and HSD7. In the 12-mo group, a number of genes fell into the category of sulfotransferases with constituents, such as heparan sulfate 3-O sulfotransferase-1. Notable genes showing a decrease in expression in the P1 MUN aortas were FGFBP-1, HSD1, and Arginase-1. Genes showing an increase in expression in P1 MUN aortas were thrombospondin-1 and -2, collagen 5A1, ADAM, p-selectin and Notch3. In the 12-mo aortas, specific genes of interest with decreased expression in MUN aortas

Table 2. micro-RNAs (miRNAs) showing statistically significant change in expression and their predicted target genes in 12-mo-old group aortas

miRNA	Fold Change	P Values	Predicted Target Genes
200a	4	0.02	Zeb1, Hsbst2, Thbd, IRS2, B3GNT1 and 2, STAT5, PCDH subtypes, Has2, TGFB2, TGFA, Sept7
129	2.3	0.03	PCDH19, Cib2, SOX4, SMAD1, MAP4K4, ADAM10, CALPN2, VCAN
215	2.5	0.05	Serp7A, EREG, F2r, BMP5, VCAM1, LOXL2
200b	1.7	0.005	Zeb1, Scd1, Fn1, DUSP1, Matr3, OLR1, NOG, EphA1, Sema 3f, Casp2
337	0.2	0.01	Rybp, Adam 13, STAT2, FGFRL1, Sept6, IRS1, SEMA6A, COL11A2, PDGFRB, PANX3, TUFT1

Fold change [control/maternal nutrition (MUN)].

Table 4. Predicted target genes of miRNAs that are most affected in expression by development

miRNA	Fold Change	Predicted Target Genes
431	26	DMN, Mtap1b, Adam 18, Fgl2, Adcy7
134	18	Pex13, Tcf21, PLD1, Chsy1, CUL5, IL-7, VEGFA, Agrt1l
541	14	USp15, StK3, Anxa1, FGF1
433	10	Sema6a, Enth, CCar1, MapK8, ApoF
409-3p	10	Notch1 induced protein, USp33, Cntn4, Zeb1, Matr3
381	9.8	Arid4b, Stx12, PdpN, Ccnt2, Jag2, CO111A1
299	7.5	Sema3d, Usp9x, Ppm11, E1n, MapK8
127	7.4	Cisd1, Sept7, Cntn4
494	6.3	Pdcl3, Sept9, Fgfr2, CyclinT2, COL12A1
337	6.3	(See Table 2)
351	6.1	Slitrk6, ItgA7, STAT3, Bmf
29c	-5.9	(See Table 1)
189	-4.6	(See Table 1)
497	-3.4	Spn1, Rai2, Ccnt2, MapK8, COL24A1, VEGFA, SgK1
29b	-3.4	COL4A5, COL9A1, COL3A1, COL5A2, Serpin5, E1n, Igf1, VEGFA, PCDH subtypes
29a	-1.9	COL4A5, COL9A1, COL3A1, Eln, IGFA1, DUSP2, PCDH subtypes
140	-1.5	FGF9, SnX2, Sept2, PDGFRA, Lrp4, VEGFA, VEGFD

Fold changes (P1/12 mo) are comparison of P1 levels of miRNA in both control and MUN as compared with 12-mo-old group expression. Positive numbers signify greater expression in P1 as compared with 12-mo-old group levels and negative numbers signify the opposite.

were integrin- α_D and integrin- α_5 , serpine1, angiotensin II receptor type 1, hyaluronidase, ADAM, FGF2, annexin A1. Only a few genes were upregulated in the 12-mo MUN aortas including IL-17 β and stearoyl-CoA desaturase 1 (SCD1).

An analysis was performed to determine which predicted target genes of miRNAs that changed in response to MUN could also be identified by the DNA array to show the expected up- or downregulation, and these genes are listed in Table 7.

The miRDB and PICTAR were mainly used for sources of the predicted miRNAs. In the P1 offspring, miRNAs 29c and 183 were upregulated in expression in MUN, and therefore their predicted target genes would be expected to be downregulated. The integrin- β_1 gene met this criterion showing decreased expression by the DNA array. In the 12-mo group SCD1 mRNA, a target of miRNA 200b upregulated in the MUN group, showed a decreased expression by the DNA array.

Table 5. Functional categories of genes altered by maternal undernutrition in the P1 offspring aortas as determined by DAVID bioinformatic analysis

Function	Number of Genes	Enrichment Score	Sample Genes
Cell cycle/mitosis	32	5.7	Cyclin E, Igf2, VEGFD, Stathmin 1, Polymerase delta 1, integrin beta 1
DNA replication	12	3.3	Geminin, Thymidylate Kinase, PCNA, Replication factor C
Extracellular region	49	3.2	Notch gene homolog 3, Neuregulin 1, Tissue factor pathway inhibitor, Afamin, laminin, Procollagen Type XII, Thrombospondin 1 and 2
Cytoskeleton	53	3.1	Actin-like 7A, Tubulin beta 2, Kinesin-like 7, Paxillin
Isomerase	7	2.6	Gal-4 epimerase, isopentyl-diphosphate delta isomerase, HSD-1
DNA repair/response to stress	40	2.3	Exonuclease 1, cytochrome 2, Thymidylate synthase, Trefoil factor 1, arginase 1, PCNA
Cell nucleus/organelle	66	2.2	Transporin 1, Aurora Kinase b, Nucleoporin 107, FGFBP-1, Wilmstumor 1, p-selectin, Nurim
Microtubule	12	2.1	Tubulin beta 2, Stathmin 1, Kinesin-like 7, Kinetochore associated 1
Vascular development/angiogenesis	9	1.8	MMP14, EGF-like domain 7, Thrombospondin 2, VEGFD, FGFBP, p-selectin, myosin heavy polypeptide9
Cell motility	13	1.7	MMP14, p-selectin, laminin, stathmin, Paxillin, ret proto-oncogene
Metabolism/transcription	124	1.7	Gal-4-epimerase, Aurora Kinase b, Neuregulin 1, Exonuclease 1, Gata-binding protein 6, Replication factor C, contactin1
Steroid biosynthesis	15	1.6	Squalene epoxidase, mevalonate decarboxylase, HSD7, HSD1
EGF	6	1.5	Neuregulin 1, p-selectin, fibulin2, EGF-like domain7 and 3
Chromatin	10	1.4	DNA methyl transferase 1, Kinesin 22, Replication factor C
Anatomic structure	54	1.3	Notch 3, Wilmstumor 1, laminin, lymphotoxin b, Igf2, Thrombospondin 2
Morphogenesis/ECM	10	1.3	Integrin beta 1, Procol type 12, MMP14, Col Type5, Adam12 and 14
Signal transduction	10	1.2	Ras p 21 protein activator 3, rho-guanine nucleotide exchange factor7, Integrin beta 1
Proteolysis	17	1.2	MMP 14, Dipeptidylpeptidase 3, carboxypeptidase, Adam 12, 14, and 1
Cell adhesion	15	1.2	Contactin1, Mucosal vascular addressin, p-selectin, Co11-Type 5, laminin, Procol type 12
Pur/Pyr metabolism	5	1.1	Polymerase delta1, DNA primase, Thymidylate synthase
DNA modification	3	0.9	Aurara kinase b, contactin1, DNA methyltransferase1
Growth factor activity	6	0.8	Igf2, Trefoil factor 1, Neuregulin 1, VEGFD
Protein kinase cascade	10	0.6	STAT4, Paxillin, MAP kinase Kinase 6
Cell death	12	0.5	Apoptosis-assoc. speck protein, Fc receptor Ig6, TNF-5 induced protein, macrophage migration inhibitory factor, Protein Tyrosinphosphatase Type 6

Out of 319 DAVID IDs, 175 genes could not be clustered in any category. Enrichment score is statistically determined to point the relative importance of a particular pathway based on the expression level.

Table 6. Functional categories of genes altered by maternal undernutrition in 12-mo-old offspring aortas as determined by DAVID bioinformatic analysis

Function	Number of Genes	Enrichment Score	Sample Genes
Anatomic structure/ development	74	5.5	TGF beta induced transcript, CTGF, compliment component 1, NADPH oxidase 4, BMP receptor Type1A, FGF2, Angioteusin receptor 1b, clusterin, Decorin, Calpain-2
Apoptosis	18	2.9	Serine/thrkinase 3, clusterin, annexinA1, apoptosis caspase activion inhibiting inhibitor, rhob gene, secreted phosphoprotein1
Regulation of cell process	59	2.7	Cxxcfinger4, CTGF, Annexin A1, integrin alpha v, FGF2, Kruppel-like factor15, STAT3
Cell adhesion	20	2.5	CTGF, Osteomodulin, rhob gene, integrin alpha dc and alpha1
ECM-receptor	5	2.3	Syndecan4, secreted phosphoprotein1, integrin alpha5, integrin alpha v
Cell growth/interaction	8	2.2	CTGF PKC delta binding protein, FGF2, Diphteria toxin alpha 1 receptor
Receptor binding	17	2.2	TNF receptor member 11b, IL-17b, syndecan, Thrombospondin 1, Annexin A1
Cell motility	15	2.2	TNF receptor member 12a, Serum response factor, STAT 3, Destrin, Vimentin, integrin alpha 1 and v
Cell morphogenesis	20	2.1	NADPH oxidase 4, FGF2, Clusterin, CTGF, Reticulon 4 receptor, p21 activated kinase 1
Protein kinase binding	7	2.1	Syndecan4, rashomologe, PKC binding protein, Kinase interacting substance 220
GAG/heparin binding	6	1.9	CTGF, FGF2, Dermatan sulphate proteoglycan 3, Thrombospondin 1
ECM	6	1.9	Lumican, decorin, CTGF, osteomodulin, Thrombospondin 1, dentin matrix protein 1
Phospholipid binding	9	1.9	Hyaluronidase 2, Reticulon 4 receptor, Syndecan 4
Ion binding	54	1.8	Scd1, Annexin A1, cxxcfinger 4, Adenylate cyclase 5, calpain 2
Vascular development/angiogenesis	9	1.8	CTGF, Serine proteinase inhibitor (clade e and f), FGF2, rhob gene, Angiotensin receptor1b, integrin alpha v, TNF receptor 12a
Tissue remodeling	6	1.4	CTGF, NADPH oxidase4, secreted phosphoprotein 1, TNF receptor 11b
Extracellular region	40	1.4	IL17b, RNAase a family 4, Prostaglandin-endoperoxide synthase1, lumican, protease serum 23, Fibulin1, proteoglycan 3
Cell junction	10	1.4	Nexilin, Claudin 19, gap junction channel protein alpha 5
Signal transduction	58	1.2	Cxxcfinger 4, Tensin, ADP-ribosylation factor 3, syndecan binding protein, AT ₁ - receptor, integrin alpha d
Sulfotransferase	3	1.1	Heparan sulphate3-0-sulfotransferase1, Estrogen sulfotransferase, sulphotranferase family-1a
Serpine	3	1.0	Serine proteinase inhibitor (clade f, member 1; dade b member 6; clade e member 1)
Cell proliferation	15	1.0	NAPDPH oxidase 4, Clusterin, FGF2, AnnexinA1, BMP receptor1A, integrin alpa v
Response to stress/inflammation	24	0.8	STAT3, NADPH oxidase4, IL17B, AnnexinA1, compliment factor H, Compliment component1
EGF	3	0.5	Hyaluronidose 2, Diphtoria toxin receptor, prostaglandin-endoperoxide synthase1

Out of 344 DAVID IDs, 142 genes could not be clustered in any category.

Although few of the predicted targets of the significantly altered miRNAs were identified by the DNA array, many of the genes detected by the DNA array to change in expression had significant functional similarity to the predicted target genes of the miRNAs, an example of which is miRNA29c, which has several collagen subtypes as predicted targets.

Confirmation studies were performed for both miRNA and mRNA data. Real-time RT-PCR showed a significant decline in the expression of miRNA 29c in MUN aortas in two age groups, namely 3-wk-old and 12-mo-old offspring, and a trend toward a decrease in the P1 offspring (Fig. 1A). One of the genes regulated by miRNA 29c is elastin. We examined the expression of elastin by histological analysis and image analysis in aortas obtained from P1 and 12-mo-old offspring (Fig. 1B). Based on the decline in expression of miRNA 29c, the expectation would be to detect increased elastin mRNA and protein expression. As expected, the expression of elastin in MUN aortas from both groups was elevated. We also confirmed the changes in the DNA array for thrombospondin 1 involved in inhibition of angiogenesis and the AT₁-receptor involved in the regulation of vascular tone (Fig. 1C). As demonstrated in Fig. 1D, there was confirmation of the array data by real-time RT-PCR with an increase in expression of thrombospondin-1 mRNA in P1 MUN aortas and decreased AT₁-receptor protein expression as determined by Western blot analysis in 12-mo MUN aortas.

DISCUSSION

This study profiled the expression of all known rat miRNAs and their associated mRNAs in vascular specimens from the offspring of dams undernourished during gestation. The results revealed an altered expression of only a few miRNAs but a large number of mRNAs in both age groups. The target genes of miRNAs whose expression was altered as a result of MUN included genes regulating extracellular matrix remodeling, angiogenesis, cell proliferation, apoptosis, and cell signaling in both age groups. Most prominently, MUN inhibited the expression of miRNA29c in both P1 and 12-mo-old offspring. This miRNA predominantly regulates the components of ECM, such as collagen and elastin, which we have shown be influenced significantly by MUN (17).

Our data provide new support for the hypothesis that the expression profiles of certain miRNAs and mRNAs change significantly during development independently of in utero nutritional environment. The target genes of miRNAs down-regulated by development related primarily to cell structure and cytoskeletal organization (Dmn, Ina, Mtap16), regulation of cell division and apoptosis (Anxa1, Pdcl3, Sema 3d, cyclin 2), regulation of transcription and translation (eif4e, Arid), and mediators of vascular growth (FGF, FGF receptor, VEGF). In contrast, miRNAs upregulated during development were associated mainly with target genes functionally associated with

Table 7. Genes identified to be significantly altered by maternal undernutrition and also found to be a predicted target of the miRNAs that were significantly altered in expression in P1- and 12-mo-old offspring

miRNA	Accession ID	Gene	Fold Change
<i>1-Day-Old-Offspring</i>			
29c	NM017022	Integrin beta 1 (fibronectin receptor)	0.7
	NM001004215.1	Peptidyl prolyl isomerase c	0.7
	NM001008297	Similar to DNA segment, Ch14, era to doi 449, expressed similar to hypothetical protein	1.5
183	XM 574501	(LOC499213) mRNA	1.4
	NM 001004276	COMM domain containing 10	1.3
189	NM 017022.1	Integrin beta 1	0.7
378		None	—
<i>12-Month-Old Offspring</i>			
129	NM017116	Calpain 2	1.3
200a	NM181087	Cytochrome p450, family 26	1.7
	XM574724	Fk506 binding protein 5	
200b	NXM030838	Solute carrier organic anion transporter family (1a5)	
	XM219925	Ankyrin repeat domain 15	1.3
	NM203493	Dentin matrix protein 1	1.5
	XM343782	Similar to mospd2 protein	1.9
	NM139192	Stearyl-coenzyme A desaturase 1	0.3
337	NM012870	TNF receptor superfamily, member 11b (osteoprotegerin)	
215	XM57598	Heat shock protein 8	1.7
	NM031735	Serine/threonine kinase 3	1.3

Values < 1 indicate lower expression in MUN. Fold changes are control/MUN.

ECM components, including collagens and elastin, but they also influenced genes involved in cell growth and division (Rai2, cyclin) and vascular growth factors (VEGFA, FGF9, PDGFR, IGF1). Of particular importance was the finding that two of the developmentally regulated miRNAs (29c and 189) were also influenced by MUN in the P1 offspring, which supports the hypothesis that these miRNAs are involved in the patterns of fetal vascular programming associated with undernutrition.

Our findings that MUN altered the expression of only a small number of miRNAs, but influenced expression of many mRNAs could be due to several factors. First, miRNA and mRNA expression and turnover are regulated very differently, particularly during vascular programming. Second, miRNAs regulate target gene expression at the posttranscriptional level, which implies that changes in protein abundance, function, and location can modulate the regulatory effects of miRNAs during vascular programming. Third, the currently postulated targets for each known miRNA involve considerable uncertainty and may not predict all actual target genes affected (37). Fourth, several different miRNAs may target the same gene through either additive or competitive influences. Fifth, not all genes are regulated by miRNAs, and thus miRNA-independent mechanisms may mediate some effects of MUN. Sixth, a single miRNA may target a different combination of genes in different cell types, or in the same cell type at different stages of the cell cycle. Correspondingly, inhibition of mRNA would be expected only in the cell type in which the miRNA is expressed (10). Such effects might be obscured in whole tissue homogenates that reveal only averaged relations between the miRNA and mRNA across all cell types in any given homogenate. Given these considerations, a global analysis of the miRNAs and mRNAs regulating particular pathways or physiologic processes is a more useful initial approach than a focus on a

specific gene. In support of this approach, other studies that have simultaneously analyzed miRNA and mRNA in pathologies, such as acute myeloid leukemia, have also found only weak associations between miRNAs and changes in their target mRNAs at the transcriptional level (15).

Among the miRNAs influenced by MUN, miR-29C may be the most significant functionally, as this miRNA targets the expression of genes involved in two fundamental processes involved in vascular programming: angiogenesis (16) and ECM remodeling (17). Among the predicted target genes for miR-29C are components of the ECM, including collagens and elastin along with the enzymes involved in their synthesis and remodeling. Targets for miR-29C also include angiogenic genes, such as VEGF and integrin $\alpha_5\beta_1$, a suppressor of angiogenesis and known to be upregulated by hypoxia (26). Other angiogenic targets include ADAM, a disintegrin and metalloproteinase with a thrombospondin motif that binds and sequesters VEGFA, thereby inhibiting endothelial cell proliferation (25). ADAM enzymes are also involved in ECM remodeling, endothelial cell invasion, and vascular sprout formation (38), as well as vascular smooth muscle migration (43). We have also previously reported that MUN stimulates vascular collagen deposition in a pattern consistent with development of vascular fibrosis (17). Consistent with this finding, miRNA29 can also contribute to cardiac fibrosis (42), and to ECM remodeling in cancer (36). Given these findings, gene-based loss and gain of functions studies offer a promising direction for future studies of this important miRNA.

MUN upregulated only one miRNA in the P1 age group, miRNA-189. The predicted target of miRNA-189 includes at least four main genes: 1) serine/threonine kinase 3 (STK3); 2) HIF3 α , a basic helix-loop-helix PAS protein that activates hypoxia-responsive genes (23); 3) regucalcin gene promoter region-related protein (RGPR), a transcription factor that binds

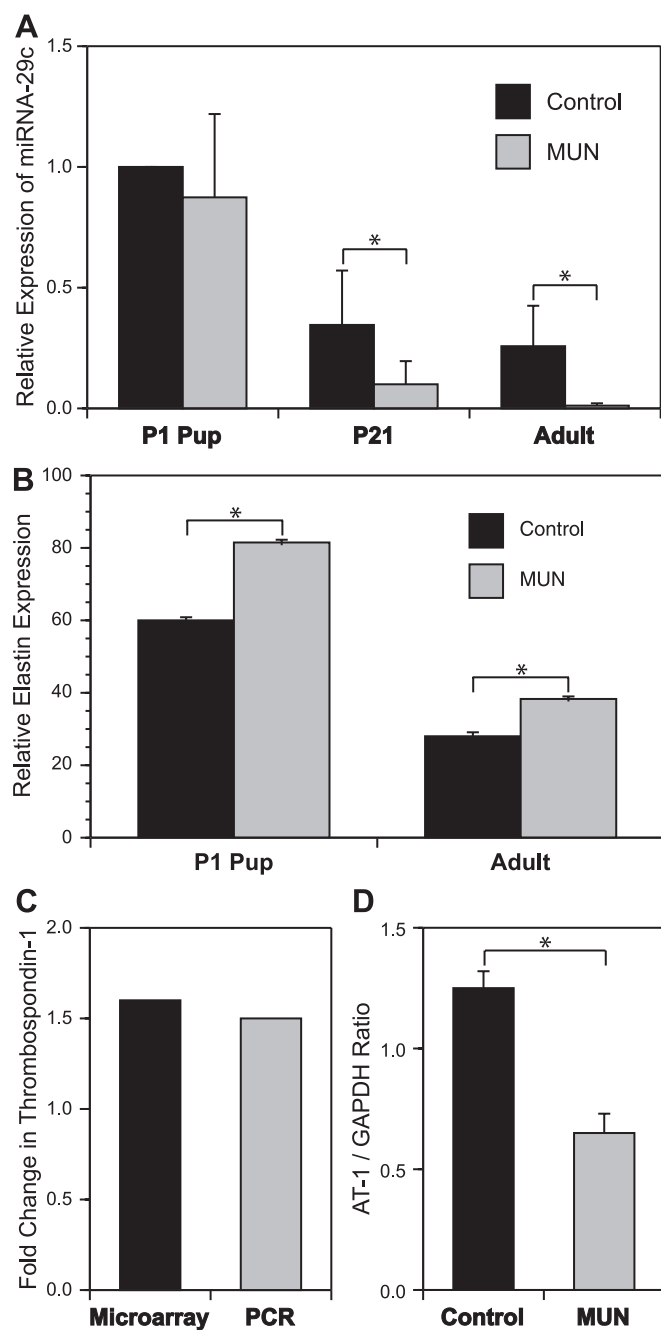


Fig. 1. *A*: expression of miRNA 29c at 3 different age groups (P1, 1 day old; P21, 3 wk old; adult, 12 mo old) as determined by real-time RT-PCR. Differences were determined by ANOVA, Tukey test at $*P < 0.05$ ($n = 4-6$ /group). *B*: elastin expression in P1 (left) and 12-mo aortas (right) as determined by Van Gieson staining and image analysis. Relative expression is %integrated optical density; $n = 5$ derived from different litters; $*P < 0.05$ for P1 and $P < 0.01$ for adult group. *C*: expression of thrombospondin-1 by real-time RT-PCR compared with microarray analysis. Bars show the fold increase in expression in maternal undernutrition (MUN) aortas compared with controls in P1 aortas. *D*: summary bar plot derived from Western blot analysis demonstrating the expression of AT₁-receptor protein relative to GAPDH in 12-mo control and MUN aortas. $*P < 0.01$.

to the regucalcin gene and controls apoptosis (44); and 4) nuclear receptor coactivator 3 (NCOA3), a transcriptional coactivator protein that has intrinsic histone acetyltransferase activity and acylates histones making downstream DNA more acces-

sible to transcription (3). The array of functions affected by miRNA-189 suggests diverse effects on numerous genes through actions on DNA-binding proteins with secondary influences with the promoter regions of multiple different genes.

In the aortas from 12 mo olds, four miRNAs were significantly downregulated in the MUN offspring. As observed in the P1 offspring, many of the target genes of the affected miRNAs were related to regulation of the extracellular matrix and angiogenesis. Importantly, changes in the expression of some of these predicted genes were confirmed by the DNA array. Two of the miRNAs suppressed by MUN belonged to the "200" family that regulates a large number of genes in several important categories: 1) genes involved with proteoglycan synthesis such as β -1,3-glucuronyltransferase, (B3GAT) and hyaluronon synthase (HAS 2); 2) thrombomodulin (Thbd), which is an integral endothelial cell membrane protein and acts as a cofactor in the thrombin-induced activation of protein C (24); 3) several different protocadherins; and 4) angiogenic genes, such as ephrin A1 (eph A1), and semaphorin 3f (Sema 3f) (21). One of the predicted targets of miRNA 200b is SCD1, which was also identified by the DNA array as one of the few genes that were upregulated by MUN in the 12-mo-old aortas. SCD1 catalyzes the biosynthesis of monounsaturated fatty acids, thereby affecting lipid composition and fluidity of the cell membrane. Mice deficient in SCD1 exhibit cell membranes that are very rigid (27), and shear stress induces the expression of this enzyme in endothelial cells, possibly providing a protective response to atherosclerosis (31). Other angiogenic target genes of miRNA 200a include Zeb1, a two-handed zinc finger homeobox transcription factor that negatively regulates angiogenesis (13, 14), and epiregulin (32). Some of the predicted targets of miRNA-200, such as lysyl oxidase-like 3, are important in synthesis of ECM components. This enzyme encodes an extracellular copper-dependent amine oxidase that catalyses the first step in the formation of cross links in collagens and elastin (22), and vesreiscan, an extracellular secreted matrix protein and member of the large chondroitin sulfate proteoglycan family (29). Collectively, the broad range of effects of the miRNA-200 family clearly suggests great potential to modulate both vascular structure and function in the 12-mo-old animals, where changes in miRNA-200 were still prominent 1 yr after birth.

Although MUN influenced the expression of only a small number of different miRNAs, the expression profiles of many different mRNAs were altered by MUN. One potential reason for this finding is that the MUN may have influences on mRNAs independently of effects on miRNAs. The general profile of genes affected by MUN in the P1 aorta suggested activation of ECM remodeling via increased expression of ADAM, NOTCH, and MMPs, whereas in the aortas from 12-mo-olds, MUN suppressed many of these same mRNAs including ADAM, hyaluronidase, and serpine genes, which are ECM proteases. In the P1 offspring, MUN-induced changes in the expression of multiple mRNAs of genes involved in angiogenesis are consistent with our previous findings (16) and those from others (30) that indicated reduced angiogenesis in these offspring. Among the angiogenic genes identified by our array in the MUN-treated P1 aortas, FGFBP-1 was inhibited > 300-fold. The product of FGFBP-1 gene promotes angiogenesis through the activation and mobilization of stored FGF, which is bound by heparin sulfate glycans (1, 5). In addition, simul-

taneous inhibition of expression of neuregulin, another proangiogenic factor (33), together with increased expression of thrombospondin 1, a major inhibitor of angiogenesis (8), suggests that MUN influences angiogenesis in the newborn MUN offspring by altering the balance between proangiogenic and angiostatic influences (16).

In the 12-mo-old offspring, MUN primarily influenced the expression of gene families related to structural development with additional effects on genes governing apoptosis. Important genes that were downregulated included AT₁-receptor mRNA, which was confirmed by Western blot analysis. This was an unexpected finding because the MUN animals were hypertensive, which would suggest a possible upregulation of the AT₁-receptor expression in the 12-mo-old MUN vessels. Telomere dysfunction has been associated with hypertension in other studies (11), and our results were consistent with the possibility that MUN downregulated telomerase, the enzyme necessary with telomere capping. This could potentially represent a mechanism for the development of hypertension in the older MUN offspring. This intriguing possibility is an excellent candidate for further investigation.

Judging from the published literature, our study is the first to comprehensively profile the effects of MUN on the vascular expression of miRNAs and mRNAs in neonatal and mature offspring. The changes observed in miRNA and mRNA expression in the aorta may not be applicable to resistance vessels and may vary among different vascular compartments. A recent study by Yates et al. (46) examined the hepatic mRNA profile of a select battery of genes involved in atherosclerosis in the adult offspring of ApoE3 Leiden mice exposed to protein undernutrition in utero. This group showed suppression of SRBP-1 and altered expression of the LDL receptor superfamily. Nijland et al. (28) have also reported on the effect of maternal nutrient restriction on the offspring renal mRNA profile with decreased expression of genes involved in DNA/RNA and protein biosynthesis. Without a doubt, the effects of MUN are highly tissue specific and depend heavily on the model used. Nonetheless, the data presented here strongly indicate that MUN influences multiple species of miRNA with consequences for many more types of mRNA in rat aortas, and that these effects change during postnatal development but can still be detected for at least a year after birth.

Perspectives and Significance

This study demonstrates that miRNAs are subject to regulation by maternal nutritional cues and thereby may represent a novel epigenetic mechanism. MUN altered the expression of only a select few species of miRNA, but many of these influenced proteins governing transcriptional regulation and apoptosis, which may help explain why MUN affected the mRNA for many different genes, as revealed by our novel global survey approach. More directly, the miRNAs most potently affected by MUN had multiple targets among the genes that regulate angiogenesis and ECM remodeling. Together, these effects of MUN on miRNA suggest an important mechanism whereby maternal nutritional status can lead to vascular programming of the fetus. Future studies of these mechanisms offer great promise for improved understanding of both the genes governing vascular development and the roles played by miRNAs in these processes.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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