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Maternal undernutrition during pregnancy alters the epigenetic landscape and the expression of endothelial function genes in male progeny.

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Abstract

Recent studies point to the important role of *in utero* malnutrition in gene programming and in the development of vascular diseases. We hypothesize that maternal undernutrition affects vascular function in the offspring by promoting epigenetic changes that drive the differential expression of genes involved in endothelial function. To test this, we exposed mice to nutrient deprivation *in utero* and analyzed its effect on global DNA methylation and expression of endothelium specific genes in the pulmonary endothelium of the adult progeny. Mice were kept either on *AdLibitum* (AL) or *Caloric Restricted* (CR) diet during the second and third trimesters of gestation. Mice in the CR group received 65% of calories compared to mice in the AL diet group. Pulmonary endothelial cells were isolated from 6 weeks old male offspring mice (AL-F1 and CR-F1). The expression of genes in the pulmonary endothelium was analyzed using quantitative RT-PCR array and confirmed by qRT-PCR. Several genes including fibronectin 1 (Fn1) and plasminogen activator inhibitor 1 (Serpine 1) were upregulated in the endothelium of male CR-F1 mice, while the expression of genes involved in regulation of histone acetylation was significantly attenuated. At the same time, the global DNA methylation did not change in pulmonary endothelial cells of CR-F1 mice compared to AL-F1 mice. Overall, we found that maternal undernutrition during pregnancy affects the expression of genes involved in regulation of endothelial cell function in the pulmonary vasculature of male progeny, which could potentially promote pulmonary vascular remodeling.

Keywords

oxidative stress; transcription; maternal undernutrition; epigenetics; histone acetylation; DNA methylation

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1. Introduction

The important role of maternal diet and malnutrition on gene programming, in general, and on the development of vascular diseases, in particular, has become obvious only during the last two decades [1-3]. In humans, the low birth weight of newborn babies has been linked to hypertension as it was shown in the Dutch famine birth cohort study. This study indicated that those conceived during the Dutch famine (lasted for 5 months, average calorie intake dropped from 1800 per day to 600 per day) were of low birth weight and had higher risk of hypertension during adulthood [4, 5]. Thus, inadequate supply of nutrients during pregnancy may negatively influence the development of the fetus and increase the likelihood of diseases such as type 2 diabetes and hypertension in the progeny of affected mothers [6-8]. Moreover, an adverse intrauterine environment including uteroplacental vascular insufficiency, may result in intrauterine growth retardation (IUGR), which is associated with significantly greater risk of developing chronic cardiopulmonary diseases in adulthood [9, 10]. These epidemiological studies are supported by experimental animal models showing that nutrient deficiency during fetal development, which mimics the human IUGR phenotype, increased the incidence of hypoxic pulmonary hypertension and right ventricular hypertrophy [11]. Interestingly, administration of the histone deacetylase inhibitor trichostatin A (TSA) reversed changes in pulmonary DNA methylation and histone modifications induced by dietary restriction. In addition, a free radical scavenger significantly reduced the magnitude of pulmonary arterial hypertension (PAH) and vascular dysfunction observed, suggesting the importance of oxidative stress and reactive oxygen species in this process. Moreover, caloric restriction during neonatal development was found to predispose infants to other pathological conditions including asthma and allergic reactions. [12, 13]. Altogether, the above studies suggest a close relationship between availability of nutrients during *in utero* development and predisposition to a variety of diseases in adulthood. However, the precise molecular mechanisms that govern these correlations remain elusive.

We hypothesize that severe undernutrition during the second and third trimesters of gestation promotes epigenetic changes in pulmonary endothelial cells that modify the expression of endothelium-specific genes, which in turn, enhance susceptibility to vascular remodeling. To test this, we set out to determine the effect of *in utero* exposure of mice to caloric restriction on epigenetic modifications in the nucleus of pulmonary endothelial cells. The rationale for the proposed work is that information gained about the role of maternal undernutrition on regulation of endothelium homeostasis by epigenetic mechanisms may unveil potential targets for intervention and suggest novel approaches to selectively alter the aberrant expression of genes in endothelial cells within remodeled pulmonary arteries. Consistent with the hypothesis, we found that while caloric restriction in adult animals did not change overall gene expression significantly, while similar malnutrition during *in utero* development significantly affected the expression of several genes involved in regulation of endothelial function and acetylation of histone residues.

2. Methods and materials

2.1. Experimental animals and animal care.

C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Louisville, and the care and handling of the animals were in accordance with National Institutes of Health guidelines.

2.2. Animal model.

In order to compare the homeostasis of the pulmonary endothelium in the setting of caloric restriction in adult mice versus caloric restriction during *in utero* development, two separate experiments were performed. In the first experiment, adult male mice 6-8 weeks old were exposed to caloric restriction for two weeks and euthanized by CO₂ asphyxiation followed by cardiac puncture at the end of the exposure. For this, mice were separated into two groups with 6 animals per group. The control group was fed by standard autoclavable Laboratory Rodent Diet 5010 (LabDiet, St. Louis, MO) *ad libitum* (AL). This diet provided 28.67% protein, 13.11% fat, 58.22% carbohydrates, and metabolizable energy 3.02 kcal/gm. Mice in calorie-restricted (CR) group were administered only 65% of the *ad libitum* intake using paired feeding (determined by the amount of food consumed by the control group).

In the second set of experiments, and to test the effect of caloric restriction during *in utero* development, pregnant female mice were exposed to a calorie-restricted diet during second and third trimesters of gestation. The control group was fed by standard autoclavable breeder diet chow 5021 (LabDiet, St. Louis, MO) *ad libitum* (AL). This diet provided 23.10% protein, 23.69% fat, 53.21% carbohydrates, and metabolizable energy 3.4 kcal/gm. Mice in the calorie-restricted (CR) group were administered only 65% of the *ad libitum* intake. Drinking water was provided *ad libitum* in all experimental groups. The average daily food intake (in grams) was measured under AL conditions in individual female or male mice. The mice were provided a known amount of food (~50 g), and the difference in chow weight was recorded the following day at approximately the same time. The quantity of chow given to the caloric restricted (CR) mice was adjusted every 2 days according to the intake of the corresponding AL mice. Female mice in both groups were kept on AL diet during first trimester of pregnancy. During the second and third trimesters, females were fed with CR diet until delivery. On the day of delivery, females were given AL diet to reduce the incidence of pup cannibalization. The F1 progeny from both groups was placed on a regular Laboratory Rodent Diet 5010 (LabDiet, St. Louis, MO) at the time of weaning. Mice were euthanized by CO₂ asphyxiation followed by cardiac puncture. We used 5-6 mice per group for F0 and F1 progeny for future studies. To avoid variability induced by the hormonal cycle in females, only male offspring were used for our study in the offspring mice.

2.3. Reagents.

Primers and probes for real-time PCR were obtained from Integrated DNA Technologies (Coralville, IA) and ThermoFisher Scientific (Waltham, MA). All other chemicals and enzymes were from Sigma Chemical Co. (St. Louis, MO), or Invitrogen (Carlsbad, CA).

2.4. Isolation of lung endothelial cells.

Lung microvascular endothelial cells were isolated using magnetic-activated cell sorting (MACS) technology as described previously [14, 15]. Briefly, murine lungs were perfused using sterile saline to remove blood cells and then minced into small pieces. Next, the lung tissue was digested using Lung Dissociation Kit and gentleMACS Dissociator (Mytenyi Biotech, Auburn, CA). Cells were passed through 70 μ m and 30 μ m filters and pulmonary endothelial cells were isolated using the CD146 MicroBeads mouse Kit (Mytenyi Biotech, Auburn, CA) according to manufacturer's protocol. Cells not binding to the CD146 column (pulmonary flow through cells) were also collected and used for further analysis.

2.5. LDL-DyLight 549 uptake assay.

Eluted pulmonary endothelial cells (PEC) and pulmonary flow through cells (PFTC) were plated on plastic dishes and allowed to attach and grow for 24-48 hours. LDL-DyLight 549 (Cayman, Ann Arbor, MI) was added in serum-free medium at a dilution of 1:100 and incubated for 3 hours in CO₂ incubator at 37 °C in the dark. After washing with PBS, the degree of LDL uptake was determined using a microscope with filter capable of measuring excitation and emission wavelengths 540 nm and 570 nm, respectively.

2.6. qRT-PCR array.

The RT² Profiler™ PCR Array Mouse Endothelial Cell Biology was performed using 1 μ g of cDNA according to the manufacturer's instructions (SABiosciences-QuiaGen, Valencia, CA). Total RNA from pulmonary endothelial (PE) cells was purified using RNAqueous-Micro Kit (Applied Biosystems, Foster City, CA). An additional genomic DNA elimination reaction and complementary DNA (cDNA) synthesis were performed on the RNA samples using the SABiosciences RT First Strand Kit. The PCR reactions were run on a StepOnePlus Real-Time PCR Detection System (Applied Biosystems, Foster City, CA) using the following cycling parameters: one cycle of 95°C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Product purity was verified by melting curve analysis. The

Ct value for each gene was determined by dividing the gene Ct value by the mean Ct value of three housekeeping genes. The 2^{-Ct} method was used to analyze data and the change in gene expression levels were reported as fold change.

2.7. Quantitative RT-PCR.

Total RNA was prepared from purified PEC and PFTC cells using RNAqueous-Micro Kit (Applied Biosystems, Foster City, CA). The synthesis of single stranded DNA from RNA was performed using SuperScript First-Strand Synthesis System for RT-PCR and random hexamers (Invitrogen, Carlsbad, CA), according to the protocol provided by manufacturer. To quantitate the abundance of gene-specific mRNAs, quantitative PCR was undertaken using the StepOnePlus Real-Time PCR Detection System (Applied Biosystems, Foster City, CA) and an SYBR® Green Master Mix. The PCR cycles were 95 °C for 3 min, then 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The nucleotide sequences of primers used for qRT-PCR are presented in Table 1. PCR assays were run in triplicate, and gene expression was normalized to P-Actin mRNA levels.

2.8. Global genomic DNA methylation.

Genomic DNA from mouse lung or isolated PEC and PFTC was purified using gDNA purification kit (Zymo, Irvin, CA). All purified genomic DNA had A260/280 ratio of 1.5 or higher. Analysis of genomic DNA methylation was performed using 5-mC DNA ELISA Kit (Zymo, Irvin, CA) according to the protocol provided by the manufacturer.

2.9. Statistical analyses.

All values are presented as means $\pm$ SD of data from n independent experiments, as indicated in the figure legends. Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Point, Richmond, CA, USA). The statistical significance of differences was determined by t-test. A P-value of <0.05 indicated statistically significant differences. Poststudy power analyses were performed with a sample power of at least 80% at a confidence level of 95%.

3. Results

3.1. Caloric restriction regimen and characterization of pulmonary endothelial cells.

To investigate the effect of maternal caloric restriction on gene expression in pulmonary endothelial cells, pregnant female mice were exposed to AL and CR diets during second and third trimesters of pregnancy as described in Figure 1. F1 male progeny mice were euthanized, as previously described, 6 weeks after birth and pulmonary endothelial cells were isolated using magnetic MACS technology. Isolated pulmonary endothelial cells were characterized by their ability to uptake the modified low density lipoproteins (LDL-DyLights 495). Isolated PEC showed intense staining, while cells that did not bind CD-146 affinity column (PFTC) were unable to uptake acetylated LDL (Figure 2A). In addition, we analyzed the expression of several endothelium specific genes in isolated cells including endothelium specific nitric oxide synthase (eNOS or Nos3), platelet endothelial cell adhesion molecule (Pecam1/CD31) and melanoma cell adhesion molecule (Mcam/CD146). Again, isolated cells showed significantly higher expression of endothelium-specific genes compared to the flow through cells (Figure 2B). These data confirm that endothelial cells were isolated from the pool of lung cells with a yield of more than 80%.

3.2. Global DNA methylation in lung endothelial cells did not change after CR diet.

Having confirmed the phenotype of the isolated endothelial cells, we analyzed the effect of CR diet on DNA methylation in endothelial cells we measured global methylation of CpG sites in the genomic DNA of male progenies. No significant differences in global DNA methylation were observed in the pulmonary endothelial cells of F0 (submitted to caloric restriction as adults) and F1 (submitted to caloric restriction while *in utero*) progeny of mice (Figure 3B). However, global genomic DNA methylation in isolated pulmonary endothelial cells was significantly higher compared to the pooled lung cells (Figure 3C). These data indicated that caloric restriction during second and third trimester of pregnancy did not change the global DNA methylation. They also confirm that endothelial cells have significantly higher number of methylated CpG sites compared to the pool of other lung cells.

3.3. Differential expression of endothelium-specific genes in mice exposed to AL or CR diets during intrauterine development.

We then set out to investigate the effect of CR diet during pregnancy on regulation of endothelial-specific genes in the offspring. For this, we performed qRT-PCR array that analyzed the expression of 80 genes involved in regulation of endothelial function. We found that only fibronectin 1 (Fn1) and P-selectin glycoprotein ligand 1 (Selplg1) gene expression were upregulated 1.5 fold in pulmonary endothelial cells of CR-F1 mice while the expression of a number of other genes was slightly downregulated (Figure 4A and B). We confirmed an upregulated expression of Fn1 and Selplg1 genes using qRT-PCR, while downregulation of Sod1, Pf4, Bax and F3 genes was not supported by qRT-PCR analysis (Figure 4B). Thus, our data indicate that at least two genes involved in regulation of endothelial homeostasis were significantly upregulated in pulmonary endothelium of CR-F1 mice.

3.4. Expression of genes regulating endothelial function, inflammation and oxidative stress.

Next, we analyzed the differences in expression of genes involved in regulation of endothelial vascular responsiveness and oxidative stress. In particular, the expression of the major antioxidant gene, extracellular superoxide dismutase (Sod3) and the major pro-oxidant gene, NADPH oxidase 4 (Nox4), were compared in AL and CR mice. The expression of these genes did not change significantly; the same as was observed for the expression of endothelial nitric oxide synthase (Nos3). However, we confirmed that the expression of the extracellular matrix protein fibronectin 1 was upregulated in the offspring of CR mice (Figure 5B). Similar upregulation in the mRNA levels was detected for the pro-inflammatory gene Serpine 1 (Figure 5B). In contrast, the expression of these genes did not change in pulmonary endothelial cells of CR-F0 mice that were kept on CR diet during adulthood (Figure 5A).

3.5. Expression of genes involved in protein acetylation.

Afterwards, we analyzed the effect of CR during prenatal development on expression of genes regulating protein acetylation. Despite our inability to find differences in the global methylation of genomic DNA, we detected a significant downregulation of genes that are involved in maintaining the acetylation status of histones and other cellular proteins. Specifically, the expression of histone deacetylases HDAC1 and HDAC8 were attenuated in the endothelium of CR-F1 mice (Figure 6B). Interestingly, the expression of genes involved in acetylation of histones, histone acetyl transferases Nat8 and Kat2A, were also attenuated in the progeny of CR- F1 exposed mice (Figure 6B), while their expression was not affected in the endothelium of CR- F0 mice that were exposed to caloric restriction during their adult life (Figure 6A). The differences in mRNA levels did not correlate well with detected protein concentrations or histone acetylation (Figure 7). These results indicate that CR during intrauterine development might significantly affect the expression of genes involved in epigenetic regulation and the control of endothelial cell function.

4. Discussion

This study investigated the effects of maternal undernutrition on differential expression of genes in the pulmonary vascular endothelium of male offspring. We found that maternal caloric restriction during the second and third trimesters of pregnancy resulted in significant upregulation of fibronectin 1, Selp1g and Serpin-1 genes in the lung endothelium of the male offspring. In addition, several genes that regulate acetylation status of histones were significantly downregulated in the male progeny of mothers exposed to the caloric restriction. None of these changes was detected in the control animals exposed to caloric malnutrition during adulthood. Therefore, based on the observations made, we accept our working hypothesis that maternal undernutrition influences endothelial homeostasis in the lung of male progeny.

This work was prompted by studies implicating caloric restriction during pregnancy in the development of endothelial dysfunction and hypertension in the adult offspring [16, 17]. Moreover, inadequate nutrition during pregnancy was found to increase the likelihood of pulmonary hypertension by changing the levels of histone acetylation and the expression of genes involved in endothelial function [11, 18, 19]. Note that we used male mice in our studies to eliminate the effect of the mouse reproductive cycle on DNA methylation and gene expression. Moreover, several studies indicated that only male progeny exposed to CR during gestation showed significant changes in energy homeostasis, insulin resistance and fat deposition [20, 21]. Another study, focusing on male rats, described hypertension and fat accumulation in the offspring of caloric restricted dams that were assigned to hypercaloric diet throughout adulthood [22].

Recent studies indicated that pulmonary vascular dysfunction in the adult progeny of females that were calorie restricted during pregnancy was attributed to several epigenetic mechanisms including DNA methylation [11]. However, we found no significant differences in total methylation of CpG sites in endothelial cells derived from mice exposed to CR diet during adulthood or in mice exposed to CR diet during intrauterine development. The effect of CR on global and gene specific DNA methylation had been studied previously with mixed results. For example, caloric restriction during pregnancy increased global DNA methylation in the rat lungs of F1 progeny compared to the rats that were kept on complete diet [11]. In that study, however, the authors analyzed DNA methylation in the whole lung tissue of rats, while our experiments were performed in isolated mouse pulmonary endothelial cells. Differences in cell-type and animal species might explain the observed discrepancies. In addition, while we were unable to detect differences in global DNA methylation, we did not evaluate the methylation status of CpG sites at the promoters of differentially expressed genes. This is relevant because others found that the expression of Glut3 gene in the placenta of caloric restricted pregnant mice decreased by 50% when compared to controls and was associated with a two-fold increase in methylation frequency of the Glut3 promoter [23]. In addition, it has been shown that prenatal undernutrition can permanently alter DNA methylation in the sperm of adult offspring, however, these changes did not transfer into late-gestation somatic tissues of the subsequent generations [24]. In this regard, it is interesting that significant differences in the expression of genes responsible for endothelial homeostasis and epigenetic regulation were observed only in mice exposed to caloric

deprivation *in utero*, but not in adult mice. Others previously described differential effects of maternal undernutrition or overnutrition on insulin sensitivity, glucose tolerance, and liver lipid metabolism in male progeny [25-27]. Overall, these published results and our own data suggest that the memory of an embryonic nutritional insult can be carried into adulthood and even throughout generations. The molecular mechanisms and adaptation benefits of these changes are not clear at this time, but most likely include epigenetic changes such as DNA methylation and histone modifications. This adaptation (or maladaptation) mechanism triggered by environmental stressors might be flexible allowing reversion to former phenotypes if food supplies become normal.

To determine the potential effects of caloric restriction on homeostasis of the pulmonary endothelium, we analyzed the expression of approximately 80 genes involved in regulation of endothelial function. Only two genes were upregulated in CR-F1 endothelial cells: fibronectin (Fn1) and p-selectin glycoprotein ligand 1 (Selp1g). The Selp1g gene, also known as P-selectin glycoprotein ligand-1 (PSGL-1) or CD162, is involved in adhesion of leukocytes and neutrophils to the endothelium by interacting with P-selectins. Inhibition of Selp1g attenuated vascular inflammation, capillary trapping of leukocytes and improved microvascular perfusion in the lung of mice following streptococcal infection [28] or induced abdominal sepsis [29]. Thus, elevated expression of Selp1g in the pulmonary endothelium of CR exposed mice might predispose the pulmonary microvasculature to increased thrombosis and inflammation, thereby leading to the development of pulmonary hypertension.

We were intrigued by the observed upregulation of fibronectin gene expression in CR-derived endothelial cells. Endothelial-secreted fibronectin is involved in the maintenance of extracellular matrix, which is important for the integrity of endothelial permeability and responsiveness to a variety of external and internal stimuli. Extracellular matrix fibronectin may also initiate signaling mechanisms that mediate dilation in small resistance arterioles [30]. Interestingly, elevated expression of fibronectin in the lung fibroblasts of old mice has been suggested as a marker of aging [31, 32]. Moreover, elevated levels of fibronectin were detected in the blood of mothers with recurring preeclampsia pointing out to potential endothelial dysfunction [33].

Finally, we also observed attenuated expression of genes that regulate the acetylation status of histones. Epigenetic mechanisms including acetylation/deacetylation of histones play important roles in the homeostasis of the pulmonary vasculature. The inadequate supply of nutrients to the developing embryo can change the epigenetic landscape in pulmonary cells, which in turn, predisposes the pulmonary vasculature to abnormal angiogenesis and pathological remodeling in response to external insults. This might suggest new targets for intervention. For example, restrictive diets during pregnancy increased the systolic pulmonary artery pressure in the offspring due to endothelial vascular dysfunction [11]. These changes were alleviated by pretreatment of animals with HDAC inhibitor butyrate indicating an important role of histone acetylation in the transmission of pulmonary vascular abnormalities to the progeny. Our data provide additional evidence that nutrient restriction during pregnancy due to famine or preeclampsia can result in abnormal expression of genes that regulate the acetylation status of histones in the pulmonary endothelium. It has been

shown that the acetylation of histones at the endothelin-1 gene promoter is affected in pulmonary vascular endothelial cells isolated from rats with intrauterine growth retardation [19]. Interestingly, similar attenuation of Hdac1 and Kat2A gene expression was observed in the liver of 5 weeks old mice exposed *in utero* to a maternal high fat diet, while no changes in expression of these genes were observed in fetal hepatic tissues [34]. These data indicate that *in utero* exposure to either maternal reduced caloric intake or maternal high fat diet produces long-lasting effects on epigenetic regulation of gene expression.

The current study has several limitations. First, only one regimen of maternal caloric restriction was used; therefore, it is not clear if this is an optimal exposure to produce effects on pulmonary endothelial homeostasis. Second, while reported caloric restriction leads to significant weight loss in adult animals, the extent of malnutrition of pups *in utero* was not confirmed experimentally. Third, the differences in mRNA levels did not correlate well with detected protein concentrations or histone acetylation (Figure 7). It is possible that the induction of mRNA is not associated with protein production. This might explain why the overall structure of the lung and vasculature is not changed. However, this does not detract from the fact that the pulmonary endothelium might be “primed” for rapid production of such genes after insult. Finally, in our study, we used a maternal undernutrition model where pregnant dams were restricted of rodent regular diet intake without supplementing their requirements in vitamins and micronutrients. Therefore, it is possible that observed changes in vascular homeostasis can be attributed not only to caloric restriction, but to the deficit of vitamins as well.

In short, we demonstrated that maternal undernutrition during the second and third trimesters increased the expression of two genes important for regulation of fibrogenesis and inflammation in the pulmonary endothelium of affected male progeny. These changes did not appear related to changes in global DNA methylation in CR endothelial cells. However, the expression of several histone deacetylases and histone acetyltransferases were significantly attenuated in caloric restricted progeny. Our results suggest that the expression of genes affecting endothelial homeostasis was significantly altered following maternal undernutrition during pregnancy, thereby predisposing the lung to the development of vascular abnormalities later in the life.

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Abbreviations

AL	<i>Ad libitum</i>
CR	Caloric restricted
IUGR	Intrauterine growth retardation
Fn1	Fibronectin 1

Selp1g	p-selectin platelet ligand
Serpine 1	plasminogen activator inhibitor 1
Nox4	NADPD oxidase 4
Nos3	Nitric oxide synthase, endothelial
Sod3	Superoxide dismutase, extracellular
Peacam-1	Platelet and endothelial cell adhesion molecule 1
HDAC	Histone deacetylase
Acat1	Acetyl-coenzyme A acetyltransferase 1
Nat8	N-acetyltransferase 8
Kat2A	K(lysine) acetyltransferase 2A
CD146	Cluster of differentiation 146
PEC	Pulmonary endothelial cells
PFTC	Pulmonary flow through cells

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Figure 1. Schematic depiction of study design.

A, Pregnant mice were kept on *AdLibitum* (AL) or Caloric Restricted (CR) diet during the second and third trimesters of pregnancy. Pulmonary endothelial cells (PEC) were isolated from male mice exposed to AL or CR diets-(FO) and from male offspring (F1) at 8 weeks of age; **B**, Caloric restriction of mice during neonatal development. As depicted in panel A, pregnant females were kept on AL diet during first trimester and on CR diet during second and third trimester of pregnancy.

Figure 2. Characterization of pulmonary endothelial cells (PEC).

A, PEC were isolated as described in “Methods and materials”. PEC or cells that did not bind to the affinity resin “pulmonary flow through cells” (PFTC) were incubated with Ac-LDL-Dylight-594 for 30 min, washed and analyzed under fluorescent microscope. Only PEC showed uptake of fluorescent acetylated LDL; **B**, the expression of endothelial markers NOS3, CD146 and Pecam1 were analyzed in purified cells using qRT-PCR. Gene mRNA levels were normalized to β -actin expression. The results are shown as means $\pm$ SD, * $p < 0.001$ when compared to corresponding control, t-test, $n = 6$.

Figure 3. Changes in mouse weight and genomic DNA methylation status.

A, The body weight was recorded in male mice that were kept for two weeks on AL or CR diets (F0) and in male offspring that were exposed to the AL and CR diets during the 2nd and 3rd trimesters of pregnancy (F1). **B**, Genomic DNA methylation was analyzed in pulmonary endothelial cells (PEC) derived from male F0 or F1 mice; **C**, Comparison of genomic DNA methylation between PEC and pulmonary flow through affinity column cells (PFTC). The results are shown as means $\pm$ SD, * p <0.05 when compared to corresponding control, t -test, n =5-6 per group.

A

B

Gene Name	Fold Change	Ct (ALD-F1)	Description
Fn1	1.49	26.2	Fibronectin 1
Selplg	1.47	27.3	Selectin, platelet (p-selectin) ligand
Sod1	0.44	24.6	Superoxide dismutase 1
Pf4	0.22	26.5	Platelet factor 4
Bax	0.48	25.5	Bcl2-associated X protein
F3	0.46	28.1	Coagulation factor III

Figure 4. Changes in expression of genes related to endothelial function in F1 progeny of mothers kept on CR during pregnancy. Lung endothelial cells were purified from F1 male progeny of C57BL6 females that were kept on AL or CR diets during pregnancy. Analysis of gene expression was performed using RT-PCR array consisting of 80 genes involved in regulation of endothelial function (Panel A). Six genes showed differential regulation. Two genes were upregulated and four genes were downregulated (Panel B).

Figure 5. Expression of genes involved in regulation of endothelial function and oxidative stress. Relative mRNA levels of corresponding genes were determined using qRT-PCR and normalized to β -actin expression. The gene expression was analyzed in lung endothelial cells that were isolated from male mice that were kept for two weeks on AL or CR diets (pane A, F0) and in male offspring that were exposed to the AL and CR diet during 2-nd and 3-rd trimester of pregnancy (panel B, F1). The results are shown as means $\pm$ SD, * $p < 0.05$ when compared to AL cells using t-test, $n = 6$ per group.

Figure 6. Expression of genes involved in epigenetic regulation.

Relative mRNA levels of corresponding genes were determined using qRT-PCR and normalized to β -actin expression. The gene expression was analyzed in lung endothelial cells that were isolated from male mice that were kept for two weeks on AL or CR diet (pane A, F0) and in male offspring that were exposed to the AL and CR diet during 2-nd and 3-rd trimester of pregnancy (panel B, F1). The results are shown as means $\pm$ SD, * $p < 0.05$ when compared to AL cells using t -test, $n = 6$ per group.

Fig. 7 -

Western blot of whole cell lysate from PECs isolated from AL-F1 and ER-F1 mice. The samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted with antifibronectin, anti-acetylated Lys9 of H3 histone, and anti- β -actin antibodies. The same protein amount was loaded into each well.

Table 1.

Primer sequences for quantitative RT-PCR

Gene Name	Gene Bank Number	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon size (bp)
CD146	NM_023061	CACGTCACCTCCCATGATGA	CACGACATTGGCTTGAATAGTTG	126 bp
Peacam-1	NM_008816	AGAGACGGTCTTGTCGAGT	TACTGGGCTTCGAGAGCATT	152 bp
Nos3	NM_008713	AAGACAAGGCAGCGGTGGAA	GCAGGGGACAGGAAATAGTT	232 bp
Selp1g	NM_009151	CAAGTGTCTGGCAGTGTGGA	ATGGTACCGTGCCCAACAGA	102 bp
Sod3	NM_011435	GGCAACTCAGAGGCTCTTC	GTCAGCAAGCCGTAGAACAAGA	137 bp
Nox4	NM_015760	TTAAACACCTCTGCCTGCTC	GATGCTCTGCTTAAACACAATCC	139 bp
Sod1	NM_011434	AGGCTGTACCAGTGCAGG	GTCTCCTGAGAGTGAGATCACA	163 bp
Fn1	NM_001276409	GACTGTACTTGTCTAGGCGAAG	GTTTCCTCGGTTGTCCTTCT	129 bp
PAI-1	NM_008871	CTCCGCCCTACCAACATCT	CAGGCATGCCCAACTTCTCC	150 bp
HDAC1	NM_008228	AGTCTGTTACTACTACGACGGG	TGAGCAGCAAATTGTGAGTCAT	101 bp
HDAC2	NM_008229	GGAGGAGGCTACACAATCCG	TCTGGAGTGTTCTGGTTTGTCA	173 bp
HDAC3	NM_010411	CACCAAGAGCCTTGATGCCTT	GCAGCTCCAGGATACCAATTACT	230 bp
HDAC8	NM_027382	ACTATTGCCGGAGATCCAATGT	CCTCCTAAAATCAGAGTTGCCAG	107 bp
Acat1	NM_144784	CAGGAAGTAAGATGCCTGGAAC	TTCACCCCTTGGATGACATT	228 bp
Nat8	NM_023455	ATGGCTTCTTTTCGCATCCG	GGAAGGCAGTGGGTATGTGC	100 bp
Kat2A	NM_020004	AAGGCCAATGAAACCTGCAAG	CTCACAGCTACGGCACAACCTC	117 bp
Actin beta	NM_007393	ACAGCTTCTTTGCAGCTCCT	CCATCACACCCTGGTGCCTA	186 bp