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Effect of Maternal Food Restriction on Fetal Lung Lipid Differentiation Program

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Summary

Although “fetal programming” has been extensively studied in many organs, there is only limited information on pulmonary effects in the offspring following intrauterine growth restriction (IUGR). We aimed to determine the effects of nutrient restriction on the lung structure and lung lipid differentiation programs in offspring using an animal mode of maternal food restriction (MFR). We utilized a rodent model of 50% MFR from day 10 of gestation to term and then using lung morphology, Western blotting, Real Time- RT-PCR and oil red O staining, lung structure and development of the offspring were examined at postnatal days (p) 1, p21, and 9 months (9M). At postnatal day 1, MFR pups weighed significantly less compared to control pups, but at p21 and 9M, they weighed significantly more. However, lung weight, expressed as a percentage of body weight between the two groups was not different at all time-points examined. The MFR group had significantly decreased alveolar number and significantly increased septal thickness at p1 and 9M, indicating significantly altered lung structure in the MFR offspring. Furthermore, although at p1, compared to the control group, lung lipid accumulation was significantly decreased in the MFR group, at 9M, it was significantly increased. There were significant temporal changes in the Parathyroid Hormone-related Protein/Peroxisome Proliferator-Activated Receptor gamma signaling pathway and surfactant synthesis. We conclude that MFR alters fetal lung lipid differentiation programming and lung morphometry by affecting specific epithelial-mesenchymal signaling pathways, offering the possibility for specific interventions to overcome these effects.

Keywords

Maternal food restriction; fetal lung development; lipid differentiation program; fetal programming; PTHrP; PPAR γ

INTRODUCTION

It is abundantly clear that development and later function of many organs can be affected by environmental conditions such as fetal and early postnatal growth restriction^{1–4}. This phenomenon, termed “fetal programming” has been extensively studied in many organs, but there is only limited information on pulmonary affects in the offspring following intrauterine growth restriction (IUGR). Limited available epidemiological and experimental evidence points to persistent alterations in lung structure and function following restricted fetal and

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early postnatal growth. Intrauterine growth restriction has also been suggested to be an important risk factor for both early and late postnatal respiratory morbidity⁵⁻⁸.

Intrauterine growth restriction can result from a wide range of maternal, placental, or fetal factors that affect the intrauterine environment, including the delivery of nutrients to the fetus. Although specific pulmonary effects related to each of these specific causes that lead to IUGR are not known, a wide range of general cellular and molecular effects of IUGR on the developing lung has been described, including an overall reduction in lung weight, DNA or protein content⁹⁻¹¹, reduced surfactant content and activity¹²⁻¹⁴, impaired maturation of the alveolar type II (ATII) cells¹⁵, decreased alveolar formation^{6,16,17}, reduced alveolar surface area for gas exchange^{6,18,19}, an immature and thicker air-blood barrier, and thicker alveolar wall^{15,18}. However, the molecular mechanisms underlying these effects remain poorly understood and are likely to be complex.

Since lung development is determined by spatio-temporally specific alveolar epithelial-mesenchymal interactions, we hypothesized that IUGR would affect the key alveolar epithelial-mesenchymal signaling pathways that are essential for normal lung development. To study this hypothesis, we utilized a well established model of maternal food restriction (MFR) during gestation to produce IUGR. This model is associated with adult-onset obesity, diabetes, and hypertension^{20,21}. Whether there are pulmonary changes in the offspring in this model is not known. In addition to determining the effect of MFR on lung structure in the offspring, using this model we tested whether MFR affects Parathyroid Hormone-related Protein (PTHrP)/Peroxisome Proliferator-Activated Receptor (PPAR) γ -driven alveolar epithelial-mesenchymal signaling, which is central in driving the lung lipid differentiation program and in maintaining the lipogenic phenotype of the pulmonary mesenchyme that is essential for normal lung development and homeostasis²².

MATERIALS AND METHODS

Rat Model of Maternal Food Restriction

All studies were approved by the Animal Research Committee of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, and are in accordance with the American Association for the Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. First-time-pregnant Sprague Dawley rat dams (Charles River Laboratories, Inc, Hollister, CA) were housed in a facility with constant temperature and humidity and on a controlled 12 h light-12 h dark cycle. We utilized a previously described model of rat dam 50% food restriction during pregnancy with slight modifications^{20,23}. Briefly, at 10 days of gestation, dams were provided either an ad libitum diet of standard laboratory chow (LabDiet 5001, Brentwood, MO, USA: protein 23%, fat 4.5%, metabolizable energy 3030 kcal/kg) (Control group) or a 50% food restricted diet (MFR group), as determined by the quantification of the normal intake in the ad libitum fed rats. At day 1 after birth, the litters were separated by gender and body weights of individual pup recorded. To avoid an undue bias towards selecting heavier or lighter pups, the median body weight per litter per gender was determined and the 4 females closest to the median body weight for the litter were selected for the study. The pups were allowed to breast feed ad libitum. In order to eliminate the effect of MFR on lactation, offspring from food restricted dams were cross-fostered to rat dams fed ad libitum during pregnancy²⁴. Control group offspring were also cross fostered to control for the study design. At postnatal day 21 (p21), all offspring were weaned to an ad libitum diet and housed individually. Animals were sacrificed and lungs collected for further processing at postnatal day 1 (p1), p21, and 9 months (9M). One female littermate from each litter was sacrificed at each time-point examined, resulting in 6 females per group from 6 different litters for each time-point.

Tissue Preparation

Rat lungs were inflated *in situ* with 4% paraformaldehyde (PFA) in phosphate buffer (PBS) at a standard inflation pressure of 20 cm of H₂O. The trachea was ligated and the lung was removed and placed in 4% PFA for approximately 4 hours at 4°C. The lung was subsequently transferred to PBS containing 30% sucrose (w/v) until equilibrated (4°C). The lung tissue was either paraffin embedded or frozen in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA). Frozen sections were cut at 8-μm thickness and paraffin-embedded sections were cut at 5μm thickness. H & E staining was used for lung morphology.

Lung Morphology

An investigator unaware of the treatment group of each animal sample performed lung morphometry, which was objectively assessed by determining the alveolar count and alveolar septal thickness. For alveolar count determination, fifty randomly selected non-overlapping fields from sections obtained from twelve blocks (2 blocks/animal) from each treatment group were examined. Each field was viewed at 200-fold magnification, scanned with a digital camera and projected onto a video monitor. For each field, the number of alveoli was counted visually and is expressed per mm². Septal thickness was analyzed with AxioVision image analysis software (Carl Zeiss Microimaging Inc.). In brief, slides were examined at 100-fold magnification and septal thickness of at least 50 alveoli for each section was measured by drawing a straight line crossing the whole width of the septum perpendicular to its course. At least two sections from each pup were used, and the average septal thickness was then calculated for each treatment group.

Oil Red O (ORO) Staining

The slide mounted 5 μm lung tissue sections were fixed in 10% formalin, and then rapidly rinsed in distilled water. After draining the water off, the slides were immersed in 100% propylene glycol for 5 minutes x 2. Oil Red O was prepared by slowly dissolving it in propylene glycol (0.7 g/100 ml), while heating to 100°C, but not over 110°C, for a few minutes. At the same time, it was stirred constantly. This solution was then filtered (Whatman no. 2 filter paper) and cooled, and then filtered again. The slides were then dipped in Oil Red O for 7 minutes, with occasional agitation, following which they were dipped in 85% propylene glycol for 3 minutes. Subsequent to Oil Red O staining, the slides were rinsed in distilled water and then allowed to react with hematoxylin for 1 min, washed in water, and then mounted with glycerol.

Choline Incorporation into Saturated Phosphatidylcholine Assay

Incorporation of [*methyl*-³H]-choline chloride (NEN Dupont) into saturated phosphatidylcholine was determined in cultured explants as previously described²⁵. Briefly, freshly isolated lung explant cultures in Waymouth's medium in 6-well plates were incubated with 1 μCi/ml of [*methyl*-³H]-choline chloride for 4 hours. After incubation, explants were washed 3 times with ice cold phosphate buffered saline. The explants were thoroughly homogenized and the cellular lipids were extracted with chloroform and methanol (2:1). The organic phase was dried under a stream of nitrogen at 60°C, resuspended in 0.5 ml of carbon tetrachloride containing 3.5 mg of osmium tetroxide, and left at room temperature for 15 min. The reaction mixture was redried under nitrogen and resuspended in 70 μl of chloroform/methanol (9:1, vol/vol). The lipid extracts were transferred to Silica Gel plates (Kodak, Rochester, NY) and developed in a chloroform:methanol:water (65:25:4) solvent system. Pure dipalmitoyl phosphatidylcholine was used as the chromatographic standard. The developed plates were stained with bromothymol blue, blotted and vacuum-dried for 5 min at 90°C. Chromatogram spots

corresponding to the migration of saturated phosphatidylcholine were scraped from the plates and counted by liquid scintillation spectrometry. The amounts of [*methyl*-³H]-choline chloride incorporated into saturated phosphatidylcholine were expressed as disintegrations per min (dpm) per mg protein.

Triglyceride Uptake Assay

The method used to quantitate triglyceride uptake by fetal rat lung explants has previously been described. Briefly, culture medium was replaced with DMEM containing 20% adult rat serum mixed with [³H]triolein (5μCi/ml). The explants were incubated at 37°C in 5% CO₂-air balance for 4 h. At the termination of the incubation, the medium was decanted, the explants were rinsed twice with 1 ml of ice-cold PBS, and the tissue thoroughly homogenized. An aliquot of the tissue suspension was taken for protein assay, and the remaining tissue suspension was extracted for neutral lipid content.

Protein Expression by Western Blotting

Western blotting was performed according to previously described methods²⁵. The specific primary antibodies used included PPARγ (1:2,000, Alexis Biochemicals, San Diego, CA), SP-B and SP-C (1:2,000 for each, Chemicon, Temecula, CA), cholinephosphate cytidyltransferase-α (CCT-α) (1:2,000, a kind gift from Dr. Mallampalli, University of Iowa, Iowa), Adipocyte Differentiation Related Protein (1:3,000, a kind gift from Dr. Constantine Londos, NIDDK), PTHrP (1:500, Santa Cruz, CA), and PTHrP receptor (1:100, Upstate, Temecula, CA).

Real Time RT-PCR

In brief, samples were collected, treated with RNA Later preservative (Ambion/Applied Biosystems, Foster City, CA), and total RNA was isolated using the RNAqueous-4PCR kit (Ambion). RNA was DNase-treated and quantitated by absorbance using a nanodrop spectrophotometer (Nanodrop Instruments, Wilmington, DE). Integrity of RNA was assessed from the visual appearance of the ethidium bromide-stained ribosomal bands following fractionation on a 1.2% (wt/vol) agarose-formaldehyde gel and quantitated by absorbance at 260 nm. 1 μg of total RNA was reverse-transcribed into single-stranded cDNA using the TaqMan Gold RT-PCR Kit at 50°C for 30 min in a total volume of 20 μls. The PCR reaction mix consisted of 1 μl of 10-fold diluted cDNA, PCR Gold DNA polymerase reagent mix, and optimized for forward and reverse gene specific primers (900 nMs each) with a gene-specific probe (250 nM, FAM dye label). All primer and probe sets were purchased as pre-designed or, if needed, custom designed TaqMan Gene Expression Assays (Applied Biosystems). Real-Time PCR reactions were run in triplicate on 384 well plates using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Reactions proceeded by activation of DNA polymerase at 95°C for 10 min followed by 38 PCR cycles of denaturing at 95°C for 15 sec and annealing/extension at 60°C for 1min. Normalization control was the 18S ribosomal RNA TaqMan Gene Expression Assay. Data were analyzed to select a threshold level of fluorescence that was in the linear phase of the PCR product accumulation [the threshold cycle (C_T) for that reaction]. The C_T value for 18S was subtracted from the C_T value of the gene to obtain a delta C_T (ΔC_T) value. The relative fold-change for each gene was calculated using the ΔΔC_T method (21). Results were expressed as the mean ± SE and considered significant at p < 0.05. RT PCR probes used included-Rat PPARγ: 5' TGATATCGACCAGCTGAACC and 3' TGGCGAACAGCTGAGAGGAC; Rat ADRP: 5' GAACAAAGGTCCTCATTATGG and 3' ACAGTGATGAAGCCTGCTC, Rat Surfactant Protein-B: 5' TACACAGTACTTCTACTAGATG and 3' ATAGGCTGTTCCTGTTGTTCC, Rat αSMA: 5' CGCAAATATTCTGTCTGGATCG and 3' TCACAGTTGTGTGCTAGAGACA; rat Sterol Regulatory Element Binding Protein

(SREBP1c): 5' CAAGTGCTGCAGGAACTGA and 3' CATGGCCTTGTCATGGAAC; rat Lipoprotein Lipase (LPL): 5' ACAGGTGCAATTCCAAGGAG and 3' CTTTCAGCCACTGTGCCATA; rat Fatty Acid Synthase (FAS): 5' AGAGGCCAGTGCATTAAGGA and 3' AAACACGCCCTCTTGCTTTA; rat Hormone-sensitive Lipase (HSL): 5' CCTCAAAGTCAAACCCTCCA and 3' CATTGTGCGTAAATCCATGC, and rat 18s: 5' TTAAGCCATGCATGTCTAAGTAC and 3' TGTTATTTTTCGTCACCTCC.

Statistics

Unpaired t-test was used to analyze the experimental data. A p value of $p < 0.05$ was considered to indicate statistically significant differences between the MFR and control groups at each time-point examined. Results are expressed as means $\pm$ SE.

RESULTS

General

At postnatal day 1, MFR pups were significantly lighter (6.1 ± 0.3 g, $p < 0.05$, $n=6$) compared to control pups (7.0 ± 0.3 g, $n=6$). However, at p21 (49 ± 3 vs. 41 ± 3 g, $p < 0.05$, MFR vs. control, $n=6$) and 9M (410 ± 18 vs. 340 ± 20 g, $p < 0.05$, MFR vs. control, $n=6$), they weighed significantly more. Similarly, the wet lung weights of the MFR offspring were lower at p1 (0.1172 ± 0.01 g, $p < 0.05$, $n=6$) compared to control pups (0.134 ± 0.01 g, $n=6$), but higher at p21 (0.48 ± 0.03 vs. 0.37 ± 0.02 , MFR vs. control, $p < 0.05$, $n=6$) and 9M (1.58 ± 0.07 vs. 1.39 ± 0.06 g, $p < 0.05$, MFR vs. control, $n=6$). The lung weights expressed as a percentage of body weight between the two groups was not different at all time-points examined ($p > 0.05$, $n=6$).

Effect of MFR on Lung Morphometry

On morphometric analysis of lungs at p1 and 9M, the MFR group had significantly decreased alveolar number and increased septal thickness (Fig. 1, $p < 0.05$, MFR vs. control, $n=6$), indicating significantly altered lung structure in the MFR offspring. Compared to the control group, at p1, the lung lipid accumulation was significantly decreased in the MFR group (Fig. 2A and Fig. 2B). However, in contrast, at 9M it was significantly increased in the MFR group (Fig. 2C and Fig. 2D).

Effect of MFR on PTHrP/PPAR γ signaling pathway

In the MFR offspring, there were significant temporal changes in the expression of PTHrP and PTHrP receptor. In the whole lung lysates, by Western hybridization, compared to the control group, in the MFR group there was a significant decrease in PTHrP expression at p1, with no significant change at p21, followed by a significant decrease at 9M (Fig. 3A). This was accompanied by a significant increase in whole lung PTHrP receptor expression at d1, without any significant change at p21, and a significant decrease at 9M ($*=p < 0.05$, MFR vs. control, $n=4$). PPAR γ , a key target of the PTHrP/PTHrP receptor interaction in alveolar interstitial fibroblasts, showed a trend towards a decrease at p1, a significant decrease at p21, and a significant increase at 9M at both the mRNA and protein levels ($p < 0.05$ vs. controls, $n=4$, Fig. 3B). Consistent with changes in PPAR γ expression, we found significant compensatory changes in PPAR γ target genes. For example, SREBP 1c, which induces the lipolytic enzyme LPL, FAS, the key enzyme involved in fatty acid synthesis, and HSL, an intracellular lipolytic enzyme that acts on stored triglyceride, and releases free fatty acids from the adipocyte, expression were all significantly increased at p1, decreased at p21, and again increased at 9M ($*=p < 0.05$, MFR vs. control, $n=4$, Fig. 3B). Adipocyte Differentiation-Related Protein, a gene necessary for lipid uptake and storage, was also

significantly increased at p1, decreased at p21, and again increased at 9M at both the mRNA and protein levels (*= $p<0.05$, MFR vs. control, $n=4$, Fig. 3C).

Effect of MFR on Surfactant Phospholipid Synthesis

Since surfactant synthesis is the functional end-product of the lung adipogenic differentiation program, we next examined the effect of MFR on surfactant phospholipid synthesis. For this, [^3H]triolein uptake and [^3H]choline incorporation into saturated phosphatidylcholine, two key functional indicators of surfactant phospholipid synthesis by the lung, were determined using lung explants obtained from different treatment groups. These data were also complemented by CCT- α expression, the rate-limiting enzyme for surfactant phospholipid synthesis. Compared to the control group, in the MFR group there was a significant increase in both [^3H]triolein uptake and [^3H]choline incorporation into saturated phosphatidylcholine at p1, with no significant change at p21, followed by a significant decrease at 9M (*= $p<0.05$, MFR vs. control, $n=6$, Fig. 4A). Consistent with these data, there was a significant increase in CCT- α expression at p1, with no significant change at p21, followed by a significant decrease at 9M (*= $p<0.05$, MFR vs. control, $n=6$, Fig. 4B).

DISCUSSION

In a well established model of fetal programming, we examined the effects of MFR during pregnancy on offspring lung structure and on the lung adipogenic differentiation program. Consistent with the previous description of this model, at p1 the MFR pups were lighter compared to the control pups, but at p21 and 9M they were significantly heavier. However, lung weight, expressed as a percentage of body weight between the two groups was not different at all time-points examined. The MFR group had significantly decreased alveolar number and significantly increased septal thickness at p1 and 9M, indicating a markedly altered lung structure in the MFR offspring. Although at p1, compared to the control group, lung lipid accumulation appeared to be decreased in the MFR group, at 9M it was conspicuously increased. Furthermore, there were significant temporal changes in the PTHrP/PPAR γ signaling pathway, a key determinant of the lung adipogenic differentiation program, and normal lung development and homeostasis.

PTHrP/PPAR γ signaling has been shown to be essential for normal alveolarization. The central significance of PTHrP-driven signaling in lung development is clear from the fact that PTHrP is expressed in the embryonic endoderm²⁶, its receptor is present on the adipothelial mesoderm²⁷, and most importantly, PTHrP knock-out causes a stage-specific inhibition of fetal lung development and arrested alveolarization²⁸. During lung development, under the influence of sonic hedgehog, the developing endoderm expresses PTHrP and its receptor on the adjoining mesenchyme. PTHrP binding to its receptor on the mesenchyme activates the PKA pathway, which actively down-regulates the default Wingless/Int pathway and up-regulates the adipogenic pathway through a key nuclear transcription factor, PPAR γ , and its down-stream regulatory genes, such as SREBP1c, LPL, FAS, HSL, ADRP, and leptin^{29,30}. PTHrP signaling is necessary for normal lung development and function at the time of birth^{28,30}. The PTHrP/PPAR γ paracrine signaling pathway stimulates leptin production by the lipofibroblast^{29,31}. Leptin secreted by the mesenchyme acts on its cognate receptor on the ATII cell, stimulating both surfactant phospholipid and protein synthesis²⁹, thereby highlighting the role of epithelially-derived PTHrP and mesenchymally-derived leptin in lung alveolar development and homeostasis³². The significance of alveolar epithelial-mesenchymal paracrine interactions through PTHrP and leptin is also reinforced by the hypoplastic lung in leptin deficient ob/ob mouse. Though the mechanism of this phenotype has not been precisely determined, some of the respiratory changes can be rescued with exogenous leptin^{33,34}.

Though it remains unclear whether the lack of any specific nutritional component (s) contributes to the altered lung structure, in the rat model utilized by us, the MFR lung was characterized by a significant decrease in alveolar number and thickened septa by 9 months of age, which are characteristic of chronic lung disease due to surfactant deficiency³⁵. The pattern of changes in the lung PTHrP/PTHrP receptor pathway is commensurate with the lung's initial, *i.e.*, at p1 and p21, compensatory response for decreased PTHrP/PTHrP receptor signaling, which later, *i.e.*, at 9M, fails, therefore accounting for surfactant deficiency despite increased expression of PPAR γ and its down-stream target genes. The causal role of surfactant deficiency in developmental chronic lung disease has been validated by the decrease in bronchopulmonary dysplasia with surfactant replacement, providing an argument for repeated treatment with exogenous surfactant³⁶. Since surfactant production is necessary for normal alveolar structure and function, and decreased surfactant activity causes alveolar remodeling and fibrosis typified by decreased alveolar number and alveolar wall thickening, we speculate that chronic surfactant deficiency could at least partially account for the alveolar changes observed in the MFR lung. If this is the case, we speculate that this phenotype can be rescued with exogenous surfactant treatment. Alternatively, exogenous supplementation with PTHrP and/or a PPAR γ agonist might be helpful, since stabilizing the PTHrP/PPAR γ signaling pathway is likely to prevent surfactant deficiency from occurring in the first place. In this regard, administration of these interventions postnatally from p1 to p21 might be critical since in the rat this is the period of most active alveolarization.

Previous studies suggest that the timing, type, and severity of fetal growth restriction determine the effects on postnatal lung structure. For example, in an ovine model, fetal growth restriction during the sacular-alveolar stages of lung development resulted in a significant reduction in alveolar number at 8 weeks after term birth, which persisted into adulthood¹⁶. However, in another study, 20 days of fetal growth restriction due to umbilico-placental embolization did not cause lung hypoplasia, but did result in an increase in lung weight adjusted for body weight accompanied by alterations in the DNA/protein ratio of lung tissue³⁷. Other studies using placental restriction to produce IUGR in fetal sheep have also produced conflicting data, finding either no effect on lung weight at term, or finding lung hypoplasia³⁸⁻⁴⁰. Although the type, severity, and timing of nutrient restriction during development seem to determine the effects on lung structure, overall the lung appears to have only a limited ability to recover from early compromised development, which therefore can permanently impair lung structure⁴¹⁻⁴⁴. This is also supported by our data where we observe abnormal lung structure at 9M despite normal nutritional intake postnatally following intrauterine exposure to MFR.

It is important to point out some key differences in findings of this study from that of a previously published study by Desai et al²³. In contrast to the findings of no difference in wet lung weight between the control and MFR groups at p21 and a significant decrease in lung weight/body weight ratio at 9M in the MFR group by Desai et al, we found a significant increase in the wet lung weight at p21 in the MFR group and no change in lung weight/body weight ratio at 9M between the two groups. It is likely that these differences are due to the differences in the selection criteria used for litter size culling in the two studies. In the early study by Desai et al, the litter size culling was done arbitrarily. Since then, the culling of litter size has been refined and in the current study, pups for the study were selected based on the median weight of the litter. At day after birth, the litters were separated by gender and body weights of individual pup recorded. The median body weight per litter per gender was determined and the 4 females closest to the median body weight for the litter were selected for this study. This modification was necessary in order to avoid undue bias of selecting pups that were heavy or lighter. We feel that this may in turn have impacted the growth differences in the two studies. Indeed in the current study, the mean

body weights of control pups at p21 is 41g versus 45g reported by Desai et al.²³ Similar is the case at 9 months of age. It can be argued that finding permanent molecular and structural differences in lungs of the control and MFR groups despite no changes in wet lung/body weight ratio at 9M suggests the specificity of these effects in the MFR lungs.

Overall, this is the first study that links the altered lung structure in MFR offspring to alterations in a specific epithelial-mesenchymal signaling pathway that is known to be essential for normal lung development, suggesting the possibility of a targeted preventive/therapeutic intervention. It will also be interesting to determine if supplementation with specific nutritional components in face of general MFR will prevent the molecular and structural changes in the MFR lung observed by us.

References

1. Barker DJ. The developmental origins of chronic adult disease. *Acta Paediatr Suppl* 2004;93(446): 26–33. [PubMed: 15702667]
2. Ravelli AC, van der Meulen JH, Osmond C, Barker DJ, Bleker OP. Infant feeding and adult glucose tolerance, lipid profile, blood pressure, and obesity. *Arch Dis Child* 2000;82(3):248–252. [PubMed: 10685933]
3. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition* 2004;20(1):63–68. [PubMed: 14698016]
4. Kallapur SG, Ikegami M. Physiological consequences of intrauterine insults. *Paediatr Respir Rev* 2006;7(2):110–116. [PubMed: 16765296]
5. Tyson JE, Kennedy K, Broyles S, Rosenfeld CR. The small for gestational age infant: accelerated or delayed pulmonary maturation? Increased or decreased survival? *Pediatrics* 1995;95(4):534–538. [PubMed: 7700754]
6. Lipsett J, Tamblyn M, Madigan K, Roberts P, Cool JC, Runciman SI, McMillen IC, Robinson J, Owens JA. Restricted fetal growth and lung development: a morphometric analysis of pulmonary structure. *Pediatr Pulmonol* 2006;41(12):1138–1145. [PubMed: 17063475]
7. Kramer BW. Antenatal inflammation and lung injury: prenatal origin of neonatal disease. *J Perinatol* 2008;28(Suppl 1):S21–27. [PubMed: 18446173]
8. Maritz GS, Morley CJ, Harding R. Early developmental origins of impaired lung structure and function. *Early Hum Dev* 2005;81(9):763–771. [PubMed: 16081227]
9. Winick M, Noble A. Cellular response in rats during malnutrition at various ages. *J Nutr* 1966;89(3):300–306. [PubMed: 5913937]
10. Sahebajami H, MacGee J. Effects of starvation on lung mechanics and biochemistry in young and old rats. *J Appl Physiol* 1985;58(3):778–784. [PubMed: 3156843]
11. Cock ML, Albuquerque CA, Joyce BJ, Hooper SB, Harding R. Effects of intrauterine growth restriction on lung liquid dynamics and lung development in fetal sheep. *Am J Obstet Gynecol* 2001;184(2):209–216. [PubMed: 11174504]
12. Lin Y, Lechner AJ. Surfactant content and type II cell development in fetal guinea pig lungs during prenatal starvation. *Pediatr Res* 1991;29(3):288–291. [PubMed: 2034477]
13. Chen CM, Wang LF, Su B. Effects of maternal undernutrition during late gestation on the lung surfactant system and morphometry in rats. *Pediatr Res* 2004;56(3):329–335. [PubMed: 15201404]
14. Faridy EE. Effect of maternal malnutrition on surface activity of fetal lungs in rats. *J Appl Physiol* 1975;39(4):535–540. [PubMed: 1242720]
15. Curle DC, Adamson IY. Retarded development of neonatal rat lung by maternal malnutrition. *J Histochem Cytochem* 1978;26(5):401–408. [PubMed: 659840]
16. Maritz GS, Cock ML, Louey S, Suzuki K, Harding R. Fetal growth restriction has long-term effects on postnatal lung structure in sheep. *Pediatr Res* 2004;55(2):287–295. [PubMed: 14630984]

17. Wignarajah D, Cock ML, Pinkerton KE, Harding R. Influence of intrauterine growth restriction on airway development in fetal and postnatal sheep. *Pediatr Res* 2002;51(6):681–688. [PubMed: 12032261]
18. Das RM. The effects of intermittent starvation on lung development in suckling rats. *Am J Pathol* 1984;117(2):326–332. [PubMed: 6496657]
19. Massaro D, Teich N, Maxwell S, Massaro GD, Whitney P. Postnatal development of alveoli. Regulation and evidence for a critical period in rats. *J Clin Invest* 1985;76(4):1297–1305. [PubMed: 4056033]
20. Desai M, Gayle D, Babu J, Ross MG. Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. *Am J Physiol Regul Integr Comp Physiol* 2005;288(1):R91–96. [PubMed: 15297266]
21. Novak DA, Desai M, Ross MG. Gestational programming of offspring obesity/hypertension. *J Matern Fetal Neonatal Med* 2006;19(10):591–599. [PubMed: 17118732]
22. Torday JS, Rehan VK. Developmental cell/molecular biologic approach to the etiology and treatment of bronchopulmonary dysplasia. *Pediatr Res* 2007;62(1):2–7. [PubMed: 17515838]
23. Desai M, Gayle D, Babu J, Ross MG. Permanent reduction in heart and kidney organ growth in offspring of undernourished rat dams. *Am J Obstet Gynecol* 2005;193(3 Pt 2):1224–1232. [PubMed: 16157142]
24. Toste FP, de Moura EG, Lisboa PC, Fagundes AT, de Oliveira E, Passos MC. Neonatal leptin treatment programmes leptin hypothalamic resistance and intermediary metabolic parameters in adult rats. *Br J Nutr* 2006;95(4):830–837. [PubMed: 16571164]
25. Rehan VK, Wang Y, Sugano S, Santos J, Patel S, Sakurai R, Boros LG, Lee WP, Torday JS. In utero nicotine exposure alters fetal rat lung alveolar type II cell proliferation, differentiation, and metabolism. *Am J Physiol Lung Cell Mol Physiol* 2007;292(1):L323–333. [PubMed: 17215434]
26. Hastings RH, Duong H, Burton DW, Deftos LJ. Alveolar epithelial cells express and secrete parathyroid hormone-related protein. *Am J Respir Cell Mol Biol* 1994;11(6):701–706. [PubMed: 7946399]
27. Lee K, Deeds JD, Segre GV. Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* 1995;136(2):453–463. [PubMed: 7835276]
28. Rubin LP, Kovacs CS, De Paepe ME, Tsai SW, Torday JS, Kronenberg HM. Arrested pulmonary alveolar cytodifferentiation and defective surfactant synthesis in mice missing the gene for parathyroid hormone-related protein. *Dev Dyn* 2004;230(2):278–289. [PubMed: 15162506]
29. Torday JS, Rehan VK. Stretch-stimulated surfactant synthesis is coordinated by the paracrine actions of PTHrP and leptin. *Am J Physiol Lung Cell Mol Physiol* 2002;283(1):L130–135. [PubMed: 12060569]
30. Torday JS, Rehan VK. Up-regulation of fetal rat lung parathyroid hormone-related protein gene regulatory network down-regulates the Sonic Hedgehog/Wnt/betacatenin gene regulatory network. *Pediatr Res* 2006;60(4):382–388. [PubMed: 16940239]
31. Torday JS, Sun H, Wang L, Torres E, Sunday ME, Rubin LP. Leptin mediates the parathyroid hormone-related protein paracrine stimulation of fetal lung maturation. *Am J Physiol Lung Cell Mol Physiol* 2002;282(3):L405–410. [PubMed: 11839533]
32. Smith BT. Lung maturation in the fetal rat: acceleration by injection of fibroblast-pneumonocyte factor. *Science* 1979;204(4397):1094–1095. [PubMed: 582216]
33. Weiss ST, Shore S. Obesity and asthma: directions for research. *Am J Respir Crit Care Med* 2004;169(8):963–968. [PubMed: 14742299]
34. Tankersley CG, O'Donnell C, Daoud MJ, Watchko JF, Mitzner W, Schwartz A, Smith P. Leptin attenuates respiratory complications associated with the obese phenotype. *J Appl Physiol* 1998;85(6):2261–2269. [PubMed: 9843551]
35. Glasser SW, Detmer EA, Ikegami M, Na CL, Stahlman MT, Whitsett JA. Pneumonitis and emphysema in sp-C gene targeted mice. *J Biol Chem* 2003;278(16):14291–14298. [PubMed: 12519727]

36. Merrill JD, Ballard RA, Cnaan A, Hibbs AM, Godinez RI, Godinez MH, Truog WE, Ballard PL. Dysfunction of pulmonary surfactant in chronically ventilated premature infants. *Pediatr Res* 2004;56(6):918–926. [PubMed: 15496605]
37. Joyce BJ, Louey S, Davey MG, Cock ML, Hooper SB, Harding R. Compromised respiratory function in postnatal lambs after placental insufficiency and intrauterine growth restriction. *Pediatr Res* 2001;50(5):641–649. [PubMed: 11641461]
38. Robinson JS, Kingston EJ, Jones CT, Thorburn GD. Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. *J Dev Physiol* 1979;1(5):379–398. [PubMed: 45373]
39. Rees S, Ng J, Dickson K, Nicholas T, Harding R. Growth retardation and the development of the respiratory system in fetal sheep. *Early Hum Dev* 1991;26(1):13–27. [PubMed: 1914984]
40. Maloney JE, Bowes G, Brodecky V, Dennett X, Wilkinson M, Walker A. Function of the future respiratory system in the growth retarded fetal sheep. *J Dev Physiol* 1982;4(5):279–297. [PubMed: 6891708]
41. Symonds ME, Bird JA, Clarke L, Gate JJ, Lomax MA. Nutrition, temperature and homeostasis during perinatal development. *Exp Physiol* 1995;80(6):907–940. [PubMed: 8962708]
42. Kotecha S. Lung growth: implications for the newborn infant. *Arch Dis Child Fetal Neonatal Ed* 2000;82(1):F69–74. [PubMed: 10634846]
43. Harding R. Sustained alterations in postnatal respiratory function following sub-optimal intrauterine conditions. *Reprod Fertil Dev* 1995;7(3):431–441. [PubMed: 8606954]
44. Cock ML, Camm EJ, Louey S, Joyce BJ, Harding R. Postnatal outcomes in term and preterm lambs following fetal growth restriction. *Clin Exp Pharmacol Physiol* 2001;28(11):931–937. [PubMed: 11703400]

Fig. 1. Maternal Food Restriction (FR) alters lung structure in the offspring

Food restriction in pregnant rat dams from 10 days gestation until term resulted in a significant decrease in alveolar number (Fig. 1B) ($p < 0.05$ vs.. control, $n=4$) and a significant increase in alveolar septal thickness (Fig 1C) ($p < 0.05$ vs.. control, $n=4$) upon morphometric analysis of lung samples from pups sacrificed at p1 and 9M. Lungs were fixed at a pressure of 20 cm of water. Representative tissue sections from p1 and 9M (Fig. 1A) are shown [magnification: 10X; Insets at magnification 20x showing alveolar size (arrows) and septal thickness (arrowheads) are included].

Figure 2. Maternal Food Restriction alters lung lipid accumulation in the offspring

Compared to the control group, at p1, the lung lipid accumulation (detected by oil Red O staining and shown in white arrows) was significantly decreased in the MFR group (Fig. 2A and Fig. 2B). However, in contrast, at 9M, it was significantly increased in the MFR group (Fig. 2C and Fig. 2D). Magnification: 40x.

Figure 3. Maternal Food Restriction (FR) alters PTHrP/PPAR γ signaling in offspring lung

By Western hybridization of the whole lung lysates, there was a significant decrease in PTHrP expression at d1, no change at p21, and a significant decrease at 9M, which was accompanied by a significant increase in PTHrP receptor expression at d1, no change at p21, and a significant decrease at 9M (Fig. 3A, $p < 0.05$ vs. control, $n = 4$). PPAR γ expression was significantly decreased at p21 and increased at 9M, at both the mRNA (RT-PCR) and protein (Western hybridization) levels (Fig 3B). The expression of SREBP1c, Lipoprotein Lipase (LPL), Fatty Acid Synthase (FAS), and Hormone-Sensitive Lipase (HSL) by RT-PCR and Adipocyte Differentiation-Related Protein (ADRP) by RT-PCR and Western hybridization increased significantly at d1, decreased at p21, and again increased at 9M ($p < 0.05$, MFR vs. control, $n = 4$, Fig. 3B and 3C, respectively).

Figure 4. Maternal Food Restriction (MFR) alters lung surfactant phospholipid synthesis

In the MFR lungs, there was a significant increase in both [^3H]tri-olein uptake and [^3H]choline incorporation into saturated phosphatidylcholine at p1, no significant change at p21, and a significant decrease at 9M ($^*p < 0.05$, MFR vs. control, $n=4$, Fig. 4A). Similarly, cholinephosphate cytidyltransferase- α (CCT- α) expression increased significantly at p1, with no significant change at p21, followed by a significant decrease at 9M ($^*p < 0.05$, MFR vs. control, $n=4$, Fig. 4B).