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Fetal Growth Restriction: Molecular Mechanisms and Long-Term Outcomes

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Anatomy and Cell Biology

FETAL GROWTH RESTRICTION: MOLECULAR MECHANISMS AND LONG-TERM OUTCOMES

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ABSTRACT

Fetal undernutrition is a major factor in the pathophysiology of fetal growth restriction (FGR) in many species, including humans. Our hypothesis is that mild maternal nutrient restriction (MNR) in a mouse provides a clinically relevant model to study FGR mechanisms and long-term effects similar to humans and the developmental origins of health and disease (DOHaD) predisposition. A MNR mouse model of FGR was developed by feeding mothers 70% normal daily caloric intake during E6.5 to E18.5 gestation. Significant reduction in fetal weight and fetal liver and lung weights with less impact on brain weight resulted, similar to asymmetric human FGR. To determine autocrine/paracrine and endocrine factors and mechanisms underlying FGR, the insulin-like growth factor (IGF) and glucocorticoid systems were investigated in the liver, placenta and lungs at E18.5 and postnatally. At E18.5, MNR fetal livers increased the expression of growth inhibiting IGF binding proteins (IGFBPs), with corresponding increases in circulating levels. MNR males developed glucose intolerance six months postnatally with pancreatic morphology indicating pathophysiology of underlying glucose intolerance. Glucocorticoids stimulate maturation in fetal organs, however when elevated levels are prolonged, they restrict growth. To determine if glucocorticoids cause aberrant fetal lung maturation with postnatal pulmonary function impairment, we studied circulating glucocorticoids and glucocorticoid metabolizing enzyme 11-β hydroxysteroid dehydrogenase. At E18.5, maternal and fetal plasma corticosterone concentrations were higher in FGR, lung weight disproportionately reduced compared to fetus, fewer type II alveolar cells and decreased SP-A and SP-C expression. At one and three months postnatally, MNR female offsprings demonstrated impaired lung compliance. We

investigated placental morphology and function in MNR. At E18.5, MNR caused reduced placental efficiency with placental weight reduced disproportionately compared to the fetus. Placental stereology indicated decreased surface area for nutrient exchange and increased interhemal membrane thickness. Passive and faciliated diffusion was decreased, although secondary active amino acid transport was unchanged. Despite the increased placental IGF-2 mRNA expression, increased IGFBP mRNA expression suggests local sequestation of IGF-2, thus impeding placental growth. These studies demonstrated that mild MNR in a mouse caused asymmetric growth restriction similar to human FGR with gender-specific long-term impact on glucose intolerance (males) and pulmonary dysfunction (females); IGF and glucocorticoid systems may play prominent roles in the pathophysiology.

Keywords: Fetal growth restriction, Insulin-like growth factor system, Glucocorticoid system, developmental programming, fetal pancreas, fetal liver, fetal lung, placental efficiency, stereology, type 2 diabetes, surfactant proteins, pulmonary function

STATEMENT OF CO-AUTHORSHIP

Chapters 3 through 5 are research studies which are ready for submission to refereed journals. The individual contributions of each author are stated.

Chapter 3

Maternal Nutrient Restriction Alters Fetal Insulin and Insulin-Like Growth Factor Systems and Impairs Long-Term Metabolic Function

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Status: Submitted to Endocrinology

Experimental work was performed by C. Albion with assistance by S. Dixon with regards to animal care, qRT-PCR, IGF ELISAs tissue DNA and protein quantification; Daniel Belisle assisted with immunohistochemistry and ISH tissue analysis. N. Moda and M. Vijayan performed the liver enzyme function assays. Programs used to analyse the images were optimized by C. Albion and D. Belisle. The manuscript was written and revised by C. Albion, and reviewed by S. Dixon, D. Hill and V. Han.

Chapter 4

Pulmonary Consequences in the Offspring of Maternal Nutrient Restriction Pregnancies

Authors: Caroline Albion, Steven Dixon, K Yang, Fred Possmayer, Lynda McCaig, Ruud Veldhuizen, Victor Han

Status: Prepared for submission to Pediatric Research.

Experimental work was performed by C. Albion with assistance by S. Dixon with regards to animal care, qRT-PCR, blood glucocorticoid levels, tissue DNA and protein quantification; Daniel Belisle assisted with immunohistochemistry and surfactant protein Western Blots. K. Yang performed measurements of 11β-HSD1 enzyme assays, protein and mRNA expression. L. McCaig, S. Dixon and R. Veldhuizen performed the pulmonary function measurements. Programs used to analyse the images were optimized by C. Albion and D. Belisle. Consultation regarding experimental work and technique development was provided by F. Possmayer, R. Veldhuizen and V. Han. The manuscript was written and revised by C. Albion, and reviewed by V. Han.

Chapter 5

Changes in Placenta Structure and Function by Maternal Undernutrition in a Mouse Model of Fetal Growth Restriction

Authors: Caroline Albion, Steven Dixon, Daniel Belisle, Ting Lee, Timothy Regnault, Thomas Jansson, Victor Han

Status: Prepared for submission to Placenta

Experimental work was performed by C. Albion with assistance from S. Dixon with regards to animal care, qRT-PCR, and placental transport of radiolabelled solutes; Daniel Belisle assisted with stereology measurements of the mouse placenta and ISH imaging. Programs used to analyse the images were optimized by C. Albion and D. Belisle. Consultation regarding experimental work and technique development was provided by T. Regnault and T. Jansson. The manuscript was written and revised by C. Albion, and reviewed by P. Lala and V. Han.

This Thesis is dedicated to my Parents,

Douglas and Ursula Albion

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LIST OF ABBREVIATIONS

11ß-HSD1 ..... 11ß-Hydroxysteroid Dehydrogenase Type 1

11ß-HSD2 ..... 11ß-Hydroxysteroid Dehydrogenase Type 2

^{14}C ..... Carbon Isotope

^{35}S ..... Sulfur 35 Isotope

^{51}Cr ..... Chromium Isotope

ACTH ..... Adrenocorticotropic hormone

ALS ..... Acid Labile Subunit

ANOVA ..... Analysis of Variance

AUC ..... Area Under the Curve

BCA ..... Bicinchoninic Acid assay

BPD ..... Bronchopulmonary Dysplasia

CHO ..... Carbohydrates

CRH ..... Corticotrophin Releasing Hormone

D ..... Decidua

DAB ..... Diaminobenzidine

DAPI ..... 4',6-diamidino-2-phenylindole

DHT ..... Dihydrotestosterone

DOHaD ..... Developmental Origins of Adult Health and Disease

E ..... Embryonic Day in Mouse Gestation

ECM ..... Extracellular Matrix

EDTA ..... Ethylenediaminetetraacetic acid

EFW ..... Estimated Fetal Weight

ELISA ..... Enzyme-linked immunosorbent assay

ER ..... Estrogen Receptor

FC ..... Fetal Capillary

FGR ..... Fetal Growth Restriction

g ..... Gravity

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

GC ..... Glucocorticoids

GC ..... Glycogen Cell

GH ..... Growth Hormone

GLUT ..... Glucose Transporter

GR ..... Glucocorticoid Receptor

HPA ..... Hypothalamic-Pituitary-Adrenal Axis

IGF ..... Insulin-Like Growth Factor

IGF1R ..... Insulin-Like Growth Factor Type 1 Receptor

Igf2P0 ..... Placental Specific Promoter of Insuliln-Like Growth Factor 2 Isoform

IGF2R ..... Insulin-Like Growth Factor Type 2 Receptor

IGFBP ..... Insulin-Like Growth Factor Binding Protein

INSR ..... Insulin Receptor

IRS1 ..... Insulin Receptor Substrate 1

ISH ..... In situ Hybridization

IUGR ..... Intrauterine Growth Restriction

Jz ..... Junctional Zone

LP ..... Isocaloric Low (5-8%) Protein Diet

Lz ..... Labyrinth Zone

MBS ..... Maternal Blood Space

MeAIB ..... Methylaminoisobutyric

MNR ..... Maternal Nutrient Restriction

mSP-C ..... Mature Surfactant Protein C

MVM ..... Microvillous Membrane

NADP ..... Nicotinamide adenine dinucleotide phosphate

PAS ..... Periodic acid-Schiff

PBS ..... Phosphate Buffered Saline

PCNA ..... Proliferating Cell Nuclear Antigen

PCR ..... Polymerase Chain Reaction

PDVF ..... Polyvinylidene fluoride

Pdx-1 ..... Pancreatic and Duodenal Homeobox 1

PEPCK ..... Phosphoenolpyruvate Carboxykinase

proSP-C ..... Precursor Surfactant Protein C

qRT-PCR ..... Quantitative Real-Time Polymerase Chain Reaction

RA ..... Retinoic Acid

RDS ..... Respiratory Distress Syndrome

RT ..... Room Temperature

SDS-PAGE ..... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEM ..... Standard Error of the Mean

sFlt1 ..... Soluble Vascular Endothelial Growth Factor Receptor 1 Variant

SGA ..... Small for Gestational Age

SNAT ..... Sodium-Coupled Neutral Amino-Acid Family of Transporters

SP ..... Surfactant Protein

SpT ..... Spongiotrophoblast Cell

VEGF ..... Vascular Endothelial Growth Factor

VEGFR1 .....Vascular Endothelial Growth Factor Type 1 Receptor

VEGFR2 .....Vascular Endothelial Growth Factor Type 2 Receptor

β2M β-2-Microglobulin

CHAPTER I

LITERATURE REVIEW

1.1 FETAL GROWTH RESTRICTION IN HUMANS

1.1.1 Definitions, Types and Causes

Fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) is defined as a pregnancy in which the fetus fails to achieve his/her genetically determined growth potential or optimal growth. Optimal fetal growth is defined as birth weight that is achieved in the absence of fetal, maternal or placental factors that can exert a pathological effect on growth (1). Clinically, whether the fetus or newborn has achieved appropriate growth for gestation is inferred from gender-specific standardized growth charts. FGR is diagnosed if the weight is below the 10^{th} percentile for a given gestational age. During pregnancy, the diagnosis is made if the estimated fetal weight (EFW) has either fallen below the 10^{th} percentile or if it is on a downward trajectory on consecutive measurements indicating that FGR can be diagnosed even if the EFW is within the normal percentiles. Therefore, an accurate assessment of the gestational age of a pregnancy is critical for the diagnosis of FGR.

After prematurity, FGR is the leading cause of perinatal morbidity and mortality (2). The incidence of FGR is estimated to be approximately 10 percent in the general obstetric population (3), but as the incidence of high-risk pregnancies such as multiple gestations rises, so does the number of FGR pregnancies (4). The incidence of FGR varies among various populations and its prevalence increases with decreasing gestational age (i.e. premature births are more likely to be FGR) (5). FGR is a serious health concern

worldwide (6); approximately 10% of term infants in developed countries, and almost a quarter of term infants in developing countries are diagnosed with FGR (5). There is a greater perinatal mortality rate in FGR fetuses at all gestations – at term, fetuses of less than 2,500 g have a perinatal mortality rate that is 5 to 30 times greater than those at the 50th percentile, and it increases to 70 to 100 times higher in fetuses less than 1,500 g (2).

FGR results in the birth of an infant who is small for its gestational age (SGA). A widely used definition for SGA is a fetus whose estimated weight is below the 10th percentile for its gestational age (7, 8). Thus, FGR and SGA are not synonymous. FGR indicates a pregnancy associated with pathology, whereas, SGA refers to a newborn whose birth weight is below expected for the gestational age. The designation of a newborn as SGA does not distinguish him/her as pathologically growth restricted or constitutionally small. Constitutionally small infants have reached their appropriate growth potential. They are well proportioned and developmentally normal (2). In contrast, FGR results from pathological pregnancies and includes infants whose estimated weight is above or below the 10^{th} percentile but growth restricted relative to their growth potential. These fetuses are malnourished and dysmorphic (2). Approximately 70 percent of fetuses with a birth weight below the 10th percentile for gestational age are constitutionally small, whereas the remaining 30 percent, the FGR is pathological (2).

FGR infants may be symmetrically or asymmetrically growth restricted. Symmetric FGR occurs when the ponderal index (PI = birth weight [g] x 100/length [cm]) remains normal and all growth measurements of the body are proportional to body size (2). Reduction in growth in symmetric FGR begins early in gestation and may be caused by congenital infections, chromosomal abnormalities, skeletal abnormalities, fetal

alcohol syndrome, constitutional short stature and low socio-economic status (9). The decreased nutrient supply early in development can restrict growth in all organs (5). Since symmetric FGR fetuses appear similar to constitutionally small fetuses, it can be concluded that a fetus is constitutionally small only after a pathological process has been excluded. Generally, these infants are at low risk for neonatal complications following birth (10).

Asymmetric FGR results in a low ponderal index - birth length and head growth is normal, but birth weight is reduced (2). Asymmetic FGR is a late-onset growth failure usually beginning in the late second or third trimesters (9). This abnormal fetal growth is due to a reduction in the supply of nutrients to the fetus that limit glycogen and fat storage yet allow continued brain growth, due to preferential blood flow to the brain and away from organs such as the gut, liver, muscle and skin (11-16). Generally, these infants are at a higher risk for neonatal complications and death (10). Reductions to fetal nutrient supply are commonly caused by placental insufficiency, pre-eclampsia (and other maternal hypertensive syndromes), maternal renal disease, long-standing diabetes mellitus, smoking, and living at high altitude (9).

FGR may therefore be caused by maternal, placental or fetal factors, with approximately one-third attributed to genetics and the remainder attributed to a poor intrauterine environment (17). However, no underlying etiology can be identified in at least 40 percent of FGR infants (5). Other than mitigating known risk factors, such as treating maternal conditions and attempting to prevent high-risk pregnancies through education, there is currently no effective treatment for FGR (4).

1.1.2 Short-Term Consequences

The consequences of FGR during gestation are evident in the perinatal as well as adult life with elevated risk of chronic diseases. Fetal, neonatal and perinatal mortality is increased in FGR infants, rising abruptly when birth weight is below the 5th percentile (5). In utero, fetal undernutrition in FGR fetuses results in reduced glycogen content in skeletal muscle and liver due to lower fetal plasma glucose and insulin concentrations (5, 18). Concentrations of most essential amino acids are lower in umbilical cord blood of FGR fetuses compared to normal fetuses, due to decreased placental transfer and/or increased protein catabolism by fetus and placenta (19, 20). FGR fetuses with these metabolic derangements develop with less total body fat, lean mass and bone mineral content (21). At the time of birth, induction of labour and operative delivery occurs with greater frequency in FGR pregnancies. Following birth, FGR infants may suffer from impaired immune function, hypothermia due to increased heat loss and reduced heat production due to reduced subcutaneous fat, hypoglycemia from low intrauterine insulin concentrations resulting in lower glycogen stores, and viscous blood from intrauterine hypoxia inducing erythropoietin production (5). Many FGR infants are also born prematurely, and as a result, may have additional complications such as greater rates of neonatal death, necrotizing enterocolitis and respiratory distress syndrome (22).

1.1.3 Long-Term Consequences

FGR infants who survive the perinatal period are at increased risk for later morbidity and mortality, such as cardiovascular disease, obstructive pulmonary disease, type 2 diabetes mellitus, renal insufficiency and impaired reproductive function (7, 23)

(**TABLE 1.1**). Many of these associations of low birth weight to disease in adulthood have been demonstrated worldwide, in different cultures and countries of varying affluence (24). This suggests the importance of optimal development during early life to minimize future disease susceptibility.

It is generally thought that the fetus adapts to an inadequate maternal environment *in utero* with changes to its body structure, physiology and metabolism resulting in reduced fetal growth (48). Although these adaptations may be suitable to survive the intrauterine environment, the persistence of these prenatal adaptations postnatally may lead to dysfunction or disease (23). The presence of additional risk factors in adulthood (i.e. smoking, obesity and inactivity) can further increase the risk of disease (24).

Ninety percent of FGR infants experience catch-up growth, which generally occurs during the first two years of life and is most rapid in the first six months (7). It is thought that the fetus adapts its growth rate to survive the poor environment in utero, however when nutrition improves after birth, compensatory growth occurs. Catch up growth in humans has been associated with the development of obesity, chronic diseases and shortened lifespan (49). FGR neonates are born with less adipose tissue (21) and catch up growth likely promotes increased adiposity (50) as fat mass is accumulated much faster than muscle mass (7). A study of 8-year-old Indian children found that insulin resistance, lipid concentrations and blood pressure were inversely correlated with birth weight, and positively correlated with current ponderal index, such that the highest measurements for each of these parameters were seen in children with the lowest birth weights and highest current ponderal index (51). Similar findings have been demonstrated in studies from other countries (52-54). Usually, FGR infants show

TABLE 1.1 Long-term consequences in organ systems following fetal growth restriction.

ORGAN	DISEASE STATE	REF
Brain and Nervous System	Schizophrenia	(25)
	Impaired Learning and Cognition	(26)
	Neurological Disorders, Dementia	(27)
Lung	Bronchopulmonary Dysplasia	(28)
	Compromised Pulmonary Function	(29)
	Chronic Obstructive Airways Disease	(30)
	Asthma	(31)
Heart	Coronary Heart Disease	(32)
Blood vessels	Atherosclerosis	(33)
	Hypertension	(34)
Liver	Dyslipidemia	(35)
	Coagulation Disorders	(36)
	Insulin Resistance	(37)
Kidney	Renal Disease	(38)
	Renin Angiotensin System	(38)
Pancreas	Type 2 Diabetes Mellitus	(39)
Bone	Osteoporosis	(40)
Muscle	Insulin Resistance	(37)
Fat	Obesity	(41)
	Insulin Resistance	(37)
Endocrine System	Hypothalamic-Pituitary-Adrenal Axis	(42)
	Growth Hormone-IGF Axis	(43)
Reproductive system	Early Menarche/Adrenarche	(44)
	Polycystic Ovarian Syndrome	(45)
Immune System	Thyroid Autoantibodies	(46)
	IgE Concentrations	(47)

moderate insulin resistance during the catch-up growth period in the first 2 years of life (54, 55) and tend to remain insulin resistant as infants and children if catch up growth was achieved (53, 54).

Compensatory growth represents a dilemma, as ample nutrition has a positive effect on brain and neural development, and poor postnatal growth increases the risk of compromised neurodevelopment. The most important predictor of subnormal performance on standard psychological and intelligence tests in 18 year old Swedish males born SGA is the absence of catch-up growth (56). However, promoting rapid weight gain and fat deposition of FGR infants increases the risk of developing metabolic and cardiovascular diseases later in life. Breast-feeding results in a slower rate of weight gain during infancy (57) due to lower calorie and protein intakes and different patterns of insulin response to feeding; this may be protective against developing obesity (7). Relative undernutrition in early life is associated with diminished insulin resistance in adolescence; FGR infants fed ordinary formula or breast milk had fewer incidences of insulin resistance in adolescence than those fed with nutrient-enriched formula (58). These studies suggest that postnatal insulin resistance following FGR may be prevented by prohibiting calorie-dense formulas being fed to these infants.

1.2 MEDIATORS OF FETAL GROWTH AND LONG-TERM DISEASE

Factors mediating fetal growth have an important role also in determining adult health and susceptibility to disease. Manipulation of fetal hormone levels by exogenous administration, drug treatment, gene deletion or surgical ablation of endocrine glands have identified hormones essential for normal fetal growth and development (59). Of

these, insulin, insulin-like growth factors (IGFs) and glucocorticoids are particularly sensitive to changes in the intrauterine environment and respond to a wide range of metabolic, endocrine and neural stimuli (59). *In utero*, these hormones trigger key developmental events and control cellular availability of nutrients for fetal growth; they regulate rates of cell proliferation, apoptosis and differentiation in many fetal tissues (59). The ability of these hormones to respond to changes in the intrauterine environment may signal the availability of oxygen and nutrients to the developing fetal tissues to match the rate of fetal growth to the fetal nutrient supply (59). They may also regulate fetal growth by mediating changes in the placenta, and thereby controlling maternal resources allocated for fetal growth (60). Changes to the expression of insulin/IGF axis and glucocorticoid systems may persist after birth and are associated with the development of chronic adult diseases associated with low birth weight (42, 43). Abnormalities in the insulin/IGF axis have been observed in cardiovascular disease, diabetes and osteoporosis with low serum IGF levels (43). These observations have generated interest in the insulin/IGF axis as a putative mechanism to explain the associations between low birth weight and adult disease as the insulin/IGF axis may influence postnatal growth, insulin resistance, and consequently the risk of disease. Studies in humans and animals have correlated low birth weight and prenatal stress to altered hypothalamic-pituitary-adreanal (HPA) axis activity later in life (61). Glucocorticoids are released into circulation during maternal stress, with approximately 10% of maternal cortisol crossing the placenta to enter the fetal compartment (62). Therefore, increased maternal cortisol concentrations could contribute to a significant change to fetal glucocorticoid exposure during development, which could be compounded by reduced metabolism in the placenta before

entering the fetal circulation (61, 62). Human studies have found that maternal stress during mid-gestation reduces birth weight and head circumference (63) and glucocorticoid treatment during preterm labor is associated with alterations in insulin resistance (64).

1.2.1 Insulin and Insulin-like Growth Factors

Insulin and IGFs are structurally related peptides essential for optimal embryonic and fetal development. Fetal insulin deficiency caused by transient neonatal diabetes or pancreatic agenesis in humans causes uniform growth restriction with the majority of fetal tissues equally affected (59, 65). Fetal hyperinsulinaemia increases fetal fat deposition and weight gain (59, 65). Insulin may act to promote fetal growth by several mechanisms including supporting uptake and utilization of nutrients by insulin sensitive fetal tissues to cause cellular hypertrophy, direct mitogenic actions on cells or release of other growth factors (such as IGFs and their binding proteins) (65).

IGF-I and -II are polypeptides homologous to pro-insulin, both approximately 7.6 kDa (66). IGF-I and -II are essential to appropriate fetal growth and development, as deletion of *Igf1* or *Igf2* in mice results in a 40% decrease in birth weight compared to wild-type littermates. If both *Igf1* and *Igf2* genes are deleted, there is an additive growth retardation of 80% decrease in birth weight (**TABLE 1.2**). The importance of the IGF system during gestation has been confirmed in humans as case reports of *IGF1* gene mutations describe severe FGR, mental retardation and sensorineural deafness (4). Liver is the major site of IGF-I and IGF-II production in fetal and postnatal life, although almost all tissues express these peptides (59, 67), suggesting that local expression of IGF-

TABLE 1.2. Placental and embryonic/fetal phenotypes resulting from altered function of the IGF system in knockout and transgenic mice[1].

GENOTYPE	BW (% of WT)	FETAL PHENOTYPE	PLACENTAL PHENOTYPE	REF
KNOCKOUT MICE				
Igf1 $^{-/-}$	60	Small, poor postnatal growth	Normal size	(71, 72)
Igf2 $^{-/-}$	60	Small, normal postnatal growth	Size smaller; fewer cells; fewer glycogen cells.	(73)
Igf1r $^{-/-}$	45	Small, die after birth with respiratory distress	Normal size.	(71, 72)
Igf2r m-/p+	140	Large, late gestation demise, cardiomegaly, kinky tail	Larger; grows until term (WT complete at E17)	(74)
Igfbp2 $^{-/-}$	100	No fetal phenotype	Normal size	(74)
Igfbp4 $^{-/-}$	85-90	Not described	Not described	[Pintar]
Ins1 $^{-/-}$ *& Ins2* $^{-/-}$	78	Increased pancreatic islet size	Not described	(75)
Insr $^{-/-}$	90	Normal at birth, die within week	Not described	(75)
Igf2 paternal disomy	150	Large, organomegaly	Larger	(75)
TRANSGENIC MICE				
m*Igf2*	136	Heart, liver, kidney overgrowth. Increased pancreatic islet size	Increased size (25% of WT)	(75)
h*Igfbp1* (MT)[1]	100	No fetal phenotype	Not described	(76, 77)
h*Igfbp-1* (AFP)	83-92	18% reduction in fetal size	15% larger	(77)
h*Igfbp-1* (hIGFBP-1)	100	No fetal phenotype	Larger labyrinth; fewer glycogen cells; smaller decidual component	(76)
h*Igfbp-1* (AFP)	80	Liver, brain and kidneys disproportionately small	Size smaller (70% of WT)	(77)
m*Igfbp2* (CMV)	100	No fetal phenotype	Not described	(75)
h*Igfbp3* (MT)	100	Not described	Not described	(75)
h*Igfbp3* (PGK)	90	Not described	Not described	(78)
h*Igfbp3* (CMV)	90	Not described	Not described	(78)
h*Igfbp-5* (β-actin)	80	Fewer pancreatic β cells	Not described	(78)

BW = birth weight; WT = wild type; [1]promoter in parentheses.

I and IGF-II regulate fetal growth via autocrine or paracrine mechanisms (65). IGFs act at several stages of the cell cycle and affect cell proliferation, differentiation, maintenance, regeneration and apoptosis (66). They stimulate cell differentiation in fetal muscle, bone, brain and adrenal cells during late gestation in preparation for extrauterine life (60). They also have anabolic effects on fetal metabolism, stimulating glucose utilization and protein synthesis (60). IGFs may also influence growth and development as they have a major influence on the production of insulin (68). IGFs control pancreatic β cell mass and the capacity for insulin secretion in the fetus (68). Insulin up-regulates tissue expression and circulating concentrations of IGF-I, while IGF-I reduces circulating insulin levels in the fetus (60). IGFs begin expression in pre-implantation embryos, with IGF-II expression greater in fetal tissues than IGF-I during gestation in rodents and humans (4). *IGF-II* is imprinted and expressed only from the paternal allele in the placenta and fetal tissues excluding the brain, where it is expressed from both alleles (69). Following birth in humans, IGF-II expression becomes biallelic, (but this does not occur in rodents) and plasma levels of IGF-I increase rapidly as growth hormone (GH) stimulates its production (69). GH is the major regulator of growth postnatally, stimulating tissue production of IGFs and elevating circulating levels (69). However, in the fetus, pituitary GH has little or no role regulating growth, except in late third trimester growth when mild growth restriction occurs if GH is deficient (70).

Manipulation of the *Igf2* gene demonstrates the importance of promoter and tissue specific expression of this imprinted gene to placental and fetal growth and development. In *Igf2* null mice, the placenta is smaller (79), in contrast to *Igf1* null mice where placental size is not altered (72). However, IGF-I may alter placental nutrient transfer by

regulating the expression of placental glucose and amino acid transporters (80). Selectively mutating the placental promoter of a placental specific *Igf2* isoform (*Igf2P0*) results in a small placenta and FGR at term, however, all components of the murine placenta experience growth restriction earlier in gestation than the fetus (79, 81). This *Igf2* isoform contributes only 10% of all placental IGF-II mRNA (81), yet, it is vital for fetal and placental growth as no further reductions in placental growth are observed when all forms of *Igf2* are absent (82). IGF-II promotes many aspects of placental development - stimulating the migration and invasion of placental trophoblast cells during early placental development (83, 84), increasing surface area and reducing barrier thickness for solute exchange (82). IGF-II has been shown to affect expression of many nutrient transporters in human and murine placenta (85). In the *Igf2P0* null mouse, fetal tissues continue to express IGF-II, but not in the complete *Igf2* null (82). The continued expression of IGF-II from the fetal tissues appears to act as a fetal signal to the placenta to supply more substrates despite compromised placental growth as the *Igf2P0* placenta transfers more glucose and amino acids per gram than the wild-type placenta and up-regulates expression of glucose and amino acid transporters (59, 79, 82). This demonstrates that IGFs affect growth of fetal tissues not only directly but indirectly through changes in placental capacity to deliver nutrients to the fetus (59, 79, 82). In contrast, IGF-II overexpression either through relaxation of imprinting (74) or deletion of the *Igf2r* (86), leads to fetal and placental overgrowth. Overexpression of IGF-II in human infants also causes overgrowth as demonstrated in Beckwith-Weidemann syndrome (87).

Numerous studies, conducted within a range of birth weights, have found a positive relationship between fetal cord blood IGF-I and birth weight in normal term pregnancies as well as other parameters of size, namely birth length, ponderal index, placental weight, and head circumference (61). In pregnancies complicated by FGR, umbilical cord blood IGF-I is reduced (61). It is generally thought that IGF-II is not correlated to birth weight (61) but there may be a positive correlation between cord blood IGF-II and placental weight (88). Since plasma IGF-I concentrations are more responsive to glucose and oxygen levels than IGF-II levels in the fetus, IGF-I may act as a nutrient sensor that ensures fetal growth is coupled to nutrient supply, while IGF-II provides continuous drive to fetal growth (59).

1.2.2 IGF Receptors

IGF-I and -II promote growth primarily by binding to the insulin-like growth factor type 1 receptor (IGF1R), which is a transmembrane heterotetrameric ($\alpha 2\beta 2$) glycoprotein with extracellular ligand-binding and intracellular tyrosine kinase domains (89). The extracellular α-subunit of IGF1R binds IGF-I and IGF-II, though it has an affinity for IGF-I that is 15-20 times greater than for IGF-II (65, 90)**.** Following IGF binding, IGF1R activates insulin receptor substrate 1 (IRS1) and the AKT and MAP-kinase pathways to induce cellular proliferation and survival, resulting in general growth (4). Deletion of the *Igf1R*, which is responsible for the actions of both IGF-I and -II, causes a greater degree of growth retardation than deletion of either Igf gene. *Igf1r*$^{-/-}$ mice, at birth are only 45% of normal weight and die within minutes after birth, due to respiratory failure resulting from widespread muscle hypoplasia, which includes the

respiratory muscles (**TABLE 1.2**). Case reports of defects in *IGF1R* affect growth as patients present with pre- and post-natal growth retardation, mental retardation and behavioural abnormalities (4). The insulin-like growth factor type 2 receptor (IGF2R)/(mannose-6-phosphate receptor) is a single chain polypeptide with a high affinity for IGF-II, but an inability to bind IGF-I or insulin (71). IGF2R inhibits IGF-II action, eliminating excess IGF-II from the cellular environment (4) and has intracellular signaling functions (91). Although IGF-II functions primarily via IGF1R, it can also act through the insulin receptor (INSR) early in development prior to IGF1R expression (4). Insulin receptors are found in all fetal tissues and their binding affinity for insulin increases with progressing gestation (92), making the actions of fetal insulin most apparent late in the third trimester (65). INSR transcript undergoes alternative splicing producing splice variants of INSR (isoforms A and B); the INSR-A isoform displays high affinity for IGF-II (93). Hybrid receptors may form from dimerization of IGF1R and INSR hemireceptors, creating receptors with differential affinities for IGF-I, IGF-II and insulin (93). An IGF1R/INSR-A hybrid receptor binds IGF-I, IGF-II and insulin, while an IGF1R/INSR-B hybrid receptor binds IGF-I with high affinity, IGF-II with low affinity, but does not bind insulin (93).

1.2.3 IGF Binding Proteins (IGFBPs)

The expression of IGFs and IGF1R during gestation is ubiquitous, but expression of IGFBPs is precisely regulated by the gestational age and within each organ (65). This implies that the major level of control of the IGF axis in development is regulation of IGF bioavailability and actions by IGFBPs (65, 69, 94). IGFs are bound to one or more of the

six high affinity IGF binding proteins, IGFBP-1 through IGFBP-6, which serve as carrier proteins to extend the biological half-life and control IGF action by either interacting or competing with IGF1Rs (69, 94). IGFBP-1 and -2 contain an integrin-binding motif that allows binding to integrins on the cell surface and may potentiate IGF actions by facilitating the presentation of IGFs to the IGF1R (65, 66, 94). All IGFBPs except IGFBP-1 contain heparin-binding domains, which allow binding to sulfated glycosaminoglycans in the extracellular matrix (ECM) and cell membrane (65, 94). Insulin and IGF-I regulate the production of IGFBPs, which can both enhance and impede delivery of IGFs and insulin to IGF1R (60).

Of the IGF binding proteins, IGFBP-1 has been implicated to have a major role in inhibiting fetal growth by sequestering IGF-I and IGF-II, as its affinity for IGFs is greater than either of the IGF receptors (95). IGFBP-1 thus prevents the growth promoting actions of IGFs. IGFBP-1 exists in several phosphorylated forms, but it is the heavily phosphorylated isoforms that have the greatest affinity for IGF-I (96). Fetal serum contains large amounts of nonphosphorylated IGFBP-1 (97); plasma from pregnant mothers contains nonphosphorylated and less phosphorylated isoforms (98), while plasma from non-pregnant females contains highly phosphorylated forms (98). Dephosphorylation of IGFBP-1 during pregnancy may increase the bioavailability of IGF-I (96). Fetal serum IGFBP-1 concentrations are inversely correlated with birth weight (61). In FGR fetuses, the concentration of IGFBP-1 in fetal cord serum is significantly higher (61) with an increased proportion of phosphorylated IGFBP-1 (99), suggesting greater inhibition of IGF actions by IGFBP-1. IGFBP-1 may inhibit embryonic growth and development by binding to and impairing the activities of IGFs

causing FGR. IGFBP-1 is dynamically regulated in human plasma with levels varying more than 10-fold in response to changes in insulin and hypoxia, which regulate IGFBP-1 expression (95). IGFBP-3 circulates with IGF-I or IGF-II and the acid labile subunit (ALS) (4). It is the primary binding protein that extends the half-life of IGFs; acting as a reservoir for IGFs in the circulation (4). There is a positive correlation between IGFBP-3 and birth weight in preterm and term infants (61). IGFBP-3 levels fall in fetal cord serum of FGR infants (100).

Deletion of genes encoding the IGFs and their receptors causes obvious disruption to fetal growth and disruption of genes encoding IGFBPs or the acid labile subunit has little effect on fetal growth, due to compensation of the deleted IGFBP by the other IGFBPs or redundancy among IGFBPs (7) (**TABLE 1.2**). IGFBP-2,-4,-5, and -6 are present in low concentrations in fetal plasma (61). However, modest postnatal growth restriction had been documented in transgenic models of mice overexpressing IGFBP-1, IGFBP-2, or IGFBP-3 (101, 102) (**TABLE 1.2**).

1.2.4 Glucocorticoids

Glucocorticoids are essential for the development and maturation of fetal organs before birth, as elevated levels stimulate differentiation of a wide range of fetal tissues including liver, lungs, gut skeletal muscle and adipose tissue (103). They induce biochemical, morphological and functional changes in fetal tissues, which may have little or no function in fetal life but are essential for long-term adaptations to extrauterine life and survival (59). The ability of glucocorticoids to stimulate maturation of fetal organs, particularly the lung has been exploited clinically as a treatment for women at risk of

premature delivery to lower the risk of neonatal respiratory distress syndrome and its associated mortality (104, 105).

Glucocorticoids are beneficial to induce tissue maturation, however, they may also inhibit fetal growth. The actions of glucocorticoids are predominantly catabolic in utero (59). They restrict fetal tissue accretion in the fetus by activating endogenous glucose production and enhance proteolysis (103), reduce placental weight, with weight reduction in the placenta greater than that in the fetus (59) and interfere with expression of the IGF axis. Glucocorticoids affect expression of IGF-I and -II in the placenta and other fetal tissues (liver, skeletal muscle, and adrenal) in a tissue specific manner (59, 60) and suppress ß cell development and insulin content of pancreas (103). The rise in cortisol prior to term may be responsible for the transition to GH dependent production of endocrine IGF-I characteristic of adults (103, 106) and the transition from IGF-II to IGF-I as predominant growth regulatory IGF (106).

Due to the growth inhibitory actions of glucocorticoids in humans and many animal species, the surge of cortisol prior to term corresponds to the period when fetal weight remains constant (107). Fetal glucocorticoid levels rise with adverse intrauterine conditions, such as undernutrition and hypoxia, which may contribute to FGR (108, 109). Administration of glucocorticoids to the mother during late gestation leads to FGR in several species including humans, with the most significant reductions in fetal growth occurring in fetuses receiving multiple doses of antenatal glucocorticoids compared to a single dose before birth (105, 110-113). Total body weight and weight of most individual fetal tissues are proportionately reduced by these treatments (103), with animal models demonstrating decreased brain weight, neurological damage and placental lesions (61).

The degree of FGR depends on the dose and type of glucocorticoid used, frequency and route of administration, and sex and gestational age of fetus (59).

Glucocorticoids appear to act as a switch from fetal tissue accretion to tissue differentiation in preparation for delivery (59). If this switch occurs prematurely, due to glucocorticoid overexposure, the normal pattern of fetal growth may be altered with inappropriate tissue development for the gestational stage with long-term consequences for tissue function later in life (59).

1.2.5 11ß-Hydroxysteroid Dehydrogenase Enzymes (11ß-HSD)

In humans, two isoforms of 11ß-hydroxysteroid dehydrogenase (11ß-HSD1 and 11ß-HSD2) interconvert glucocorticoids with their inactive 11-keto metabolites (114). 11ß-HSD type 1 catalyzes the bi-directional interconversion of cortisol and cortisone, but acts primarily to convert cortisone to cortisol due to its higher affinity for cortisone (114). 11ß-HSD1 is found in the liver, adipose tissue, lung and testis, where it functions to increase the availability of glucocorticoids to the glucocorticoid receptor (GR) (114). The expression and activity of 11ß-HSD1 increases with advancing gestation, possibly to allow for the rise in cortisol concentration at term to regulate fetal maturation and activate pathways associated with labor (115). 11ß-HSD type 2 is a high affinity unidirectional enzyme, catalyzing only the dehydrogenase reaction, which converts active cortisol to inactive cortisone (114). In the placenta, 11ß-HSD2 primarily controls the passage of cortisol from mother to fetus (114). Maternal cortisol concentrations are 5-10 times higher than fetal cortisol concentrations, and 11ß-HSD2 protects the fetus from this high concentration of maternal glucocorticoids (62). Synthetic glucocorticoids, (such as

dexamethasone and betamethasone) are poorly metabolized by 11ß-HSD2 and therefore pass unaltered through the placenta and into the fetal circulation (42). There are conflicting results regarding whether the expression and/or activity of 11ß-HSD2 increases or decreases with advancing gestation (116); at term, approximately 75% of cortisol found in the fetus is of fetal origin, while all the cortisone in the fetus is of maternal origin, suggesting that placental 11ß-HSD2 is an effective glucocorticoid barrier as fetal cortisol is predominately derived from the fetal adrenal and not from the mother (116, 117). Birth weight is positively correlated to placental 11ß-HSD2 activity (61). Pregnancies complicated by FGR demonstrate a reduction in placental 11ß-HSD2 expression and activity (61) with reduced concentrations of umbilical cord vein cortisone, demonstrating less placental conversion of cortisol to cortisone (118). Furthermore, animal studies have implicated decreased 11ß-HSD2 activity and elevated levels of fetal glucocorticoids in developmental programming (42, 119, 120), demonstrating the importance of placental glucocorticoid metabolism in the regulation of fetal growth and long-term development of disease.

1.3 ANIMAL MODELS

1.3.1 Importance of Animal Models

Appropriate animal models that mimic FGR in humans are essential to understanding how the fetus adapts to the poor intrauterine environment and the development of complex multisystem diseases postnatally. Epidemiological research in humans only allows associations to be established between FGR and less invasive measurements such as growth factors in plasma, or non-invasive measurements such as

morphometric body measurements (121). Once similar associations are confirmed in an animal model, experiments may be conducted in animals that cannot be performed in humans to establish the pathophysiological mechanisms of FGR during gestation. It is possible to design protocols aimed at dissecting the relative contributions of nutrients, oxygenation, growth factors and genetics to the onset of FGR in a well-controlled research environment (121). In an animal model, it is feasible to utilize invasive techniques to sample tissue or blood during different stages of development (122). Additionally, it is possible to modify both prenatal and postnatal environmental conditions including nutrition, to determine mechanisms of disease and ultimately to design potential therapeutic interventions (122). Environmental manipulation is extremely important for studies elucidating the epigenetic mechanism of long-term programming, as postnatally, many factors in the environment can also affect disease risk, particularly during the period of compensatory growth. As previously described, other than mitigating known risk factors, such as treating maternal conditions and attempting to prevent high-risk pregnancies through education, there are no medical treatments for FGR (4). Animal models allow scientist to test which treatments may be effective or harmful to advances in the care of FGR infants.

1.3.2 Forms of Manipulation to induce Fetal Growth Restriction (FGR)

Experimental animal models that mimic the human clinical condition use manipulations in each the maternal, placental and fetal compartments (**TABLE 1.3**). Interventions to the mother to cause FGR include dietary regimes, where there is a

TABLE 1.3 Manipulations of species utilized to produce fetal growth restriction with postnatal consequences.

MANIPULATION	SPECIES	LONG-TERM DISEASE	REF
Maternal Undernutrition			
Calorie Deprivation	Rat	Hypertension, hypercholesterolemia, obesity, glucose intolerance	(125-127)
	Guinea Pig	Hypertension, insulin resistance, obesity	(128, 129)
	Sheep	Hypertension, altered HPA axis	(130, 131)
Protein Deprivation	Rat	Hypertension, glucose intolerance, insulin resistance	(132-134)
Iron Deficiency	Rat	Hypertension	(135)
Placental Insufficiency			
Increased litter size	Guinea Pig	Glucose intolerance, insulin deficiency	(128)
Restricted Blood Flow	Rat	Glucose intolerance, insulin deficiency and resistance	(136)
	Guinea Pig	Hypertension	(137)
Decreased Placental Size	Sheep	Hypertension, glucose intolerance	(138)
Glucocorticoid Exposure			
Maternal Dexamethasone	Rat	Hypertension, glucose intolerance, insulin resistance, obesity	(139-141)
	Guinea Pig	Altered HPA axis	(142)
	Sheep	Hypertension, insulin resistance, altered HPA axis	(143-145)
Placental 11B HSD2 Inhibition	Rat	Hypertension, glucose intolerance, insulin resistance	(146, 147)
Maternal Stress	Rat	Altered HPA axis	(148)
Gene Deletion			
IGF-I$^{-/-}$	Mouse	Infertile, Postnatal Growth Deficit	(149)
IGFR1$^{-/-}$	Mouse	Postnatal Growth Deficit	(150)
Plag1$^{-/-}$	Mouse	Reduced Fertility	(151)
IRS-1$^{-/-}$	Mouse	Insulin Resistance	(152)
Gcgr$^{-/-}$	Mouse	Altered Islet Development	(153)
TR4$^{-/-}$	Mouse	Reproduction & Maternal Behaviour Defects	(154)

Referenced from Vuguin (123) and Fowden et al. (123).

reduction in global caloric intake, macronutrients (e.g. low protein diets), or micronutrients (e.g. iron deficiency) or over-nutrition at various points during gestation.

Alternatively, FGR can be induced via the mother by pharmacological means, such as administering glucocorticoids, or manipulating the environment to induce maternal hyperthermia. Interfering with placental function by ligating the uterine blood vessels to restrict blood flow to the placenta and umbilical embolization or surgical removal of placental components is possible primarily in large animals such as sheep, to induce FGR (123, 124). The fetus itself may be manipulated for the development of FGR through infection (123), although with the advent of genetic manipulation, many gene-targeting experiments specifically in the fetus or placenta have produced FGR phenotypes during gestation and contributed significantly to knowledge of the genes and mechanisms necessary to sustain adequate fetal growth (121, 123).

1.3.3 Species Utilized for Fetal Growth Restriction (FGR) Research

There are many different experimental animal models of human FGR involving studies in rats, mice, guinea-pigs, sheep and non-human primates (124) (**TABLE 1.3**), with each species having benefits and disadvantages as models of human FGR (**TABLE 1.4**). Two species, sheep and rat, have been extensively studied for determination of fetal adaptations to an adverse intrauterine environment and long-term implications on adult health.

The pregnant sheep is a long-standing model for the study of placental-fetal interactions. Similar to humans, sheep have a long gestation period with organogenesis occurring early in gestation with the fetal endocrine, nervous, renal and cardiovascular

TABLE 1.4 Benefits and disadvantages of common species utilized to model human fetal growth restriction.

SPECIES	ADVANTAGES	DISADVANTAGES
Non-Human Primates	Similarity to Humans Large Species Similar Placenta Non-litter bearing	Expensive Housing Long Lifespan Ethical Considerations
Sheep	Large Species Prenatal Nervous & Endocrine Maturation Non-litter bearing	Expensive Housing Long Lifespan Different Placenta Structure
Guinea Pig	Prenatal Nervous & Endocrine Maturation Similar placenta to humans	Few studies performed Litter bearing
Rat	Short Life-Span Similar Placenta Extensively Studied Model	Litter bearing Postnatal Nervous & Endocrine Maturation
Mouse	Short Life-Span Genetic Manipulation	Litter-Bearing Postnatal Nervous & Endocrine Maturation

Referenced from Vuguin (123) and Armitage et al. (155)

systems relatively mature by late gestation and responsive to intrauterine insults (156-158). The long period of gestation allows fetal adaptations to be studied in acute and chronic intrauterine insults (124). Several experimental approaches have been used in sheep to mimic human FGR caused by a range of factors, including maternal nutrient restriction, administration of glucocorticoids (GC), maternal overnutrition in the pregnant adolescent ewe and experimental induction of maternal hyperthermia (106, 123, 124, 155) (**TABLE 1.3**). The benefit of the sheep model is the large size of the organism, which allows surgical manipulation of the placenta including the removal of most of the endometrial caruncles from the non-pregnant uterus prior to conception, ligation of the umbilical artery or embolization of the placenta (124) (**TABLE 1.4**). These surgical models target undernutrition of the fetus and placenta as well as hypoxia, which models placental insufficiency in humans (124) (**TABLE 1.4**). The large size of the sheep fetus allows catheters and other instrumentation to measure changes in blood gases, hormones and cardiovascular parameters in response to placental dysfunction (122) (**TABLE 1.4**). Disadvantages of the sheep models are the different structure of the placenta and the long periods of gestation and lifespan (155) (**TABLE 1.4**). The long lifespan makes it difficult and expensive to monitor long-term developmental programming effects (155). All sheep models are capable of inducing FGR, but the degree of growth restriction is variable depending on the breed, maternal nutrition and intervention used (123).

Most studies are performed in rodents as they have a short gestation period and are excellent for follow-up studies to determine adult health outcomes after FGR because of their short life-span (159). Furthermore, molecular mechanisms can be dissected

relatively easily given the genetic information available for the mouse and rat (123) (**TABLE 1.4**).

Many models of nutrient manipulation, including high fat diets (160, 161), total caloric restriction, and iron deficiency (**TABLE 1.4**) have been performed in rats and demonstrate long-term sequelae. A low protein diet in pregnant rats is the most extensively studied model of early growth restriction (106, 123, 124, 155, 159) (**TABLE 1.4**). Rat dams are fed a control diet of standard 20% protein or an isocaloric low (5-8%) protein (LP) diet during pregnancy and lactation (159). At mid-pregnancy, glucose and insulin levels are significantly lower in LP mothers compared to controls (162). Litter size does not differ between groups, but pups born to LP mothers have reduced birth weight of approximately 15 percent (159). The LP rat model of FGR is an important model for studying developmental programming as many organ systems have demonstrated to be affected in adulthood in a similar manner as characterized in humans (**TABLE 1.1** and **TABLE 1.5**).

The caveats of the LP diet model are the variations of diet used to establish FGR, as the composition of the diet elicits different programming effects (155). The Southampton and the Hope farm diets are low protein diets most commonly used in animal models of fetal programming. The Southampton diet programs adult hypertension (176) and the Hope farm diet programs insulin resistance (177). Methionine is a major component of the Southampton diet, and is in excess compared to other amino acids within the diet (123, 155). Methionine may cause elevated circulating homocystine levels, a known risk factor for cardiovascular disease, whereas the Hope farm diet contains cardioprotective polysaturated fatty acids (123, 155). The clinical relevance of

this model system may be limited to those who lack adequate protein intake either for economical or cultural reasons (123). Global undernutrition may provide a more relevant model, as placental insufficiency or maternal undernutrition, results in fetal undernutrition of all nutrients.

TABLE 1.5 Long-term consequences in organ systems following maternal low protein in rat dams.

ORGAN	DISEASE STATE	REF
Brain and Nervous System	Physical & Behavioural Deficits	(163)
	Lower Intelligence	(164)
Lung	Altered Pulmonary Mechanics	(165)
Heart	Cardiac Dysfunction	(166)
Blood Vessels	Endothelial Dysfunction	(155)
	Hypertension	(167)
Liver	Dyslipidemia	(155)
	Insulin Resistance	(159)
Kidney	Fewer Kidney Nephrons	(168)
	Renin-Angiotensin System	(169)
Pancreas	Type 2 Diabetes Mellitus	(159)
Bone	Reduced Adult Bone Mass	(170)
Muscle	Insulin Resistance	(159)
Fat	Obesity	N/A
	Insulin Resistance	(159)
Endocrine System	Hypothalamic-Pituitary-Adrenal Axis	(171)
	Growth Hormone-IGF Axis	(172)
Reproductive System	Females: Delayed Sexual Maturation & Premature Aging of Reproductive Function	(173)
	Males: Delayed Sexual Maturation & Low Sperm Counts	(174)
Immune System	Impaired Acute Phase Immune Response	(175)
	Impaired Cytokine Production	(175)

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CHAPTER 2

THESIS INTRODUCTION AND RATIONALE

2.1 MOUSE AS THE ANIMAL MODEL OF CHOICE

Clinicians taking care of pregnant women, fetuses and newborns have benefited from translational knowledge obtained from FGR studies in several experimental animal species (rodents, guinea pigs, sheep, non-human primates), each making unique contributions to our understanding of the physiology of normal growth and pathophysiology of abnormal growth. The majority of FGR studies (approximately 75%) are performed on rodents. As mammals, the mouse and rat are similar to humans, sharing a similar genome, biochemical pathways, tissue systems and organs, and physiology. However, only a small portion (~ 10%) of rodent studies use the mouse as a model system, mainly because of the genetic variations of the mouse, challenges in animal care and maintenance when pregnancy is perturbed, and inconsistencies in pregnancy and fetal outcomes compared to the rat. The mouse and rat share many common characteristics which make them ideal models for studying fetal growth restriction and its long-term outcomes. Both share a placental structure (hemochorial) similar to humans with many cell types of identical structure and function. The short life-span of the rat and mouse allows changes to tissue structure and physiology resulting from *in utero* insults to be monitored over the short course of life-span and is ideal for studying pathophysiological mechanisms at tissue, cellular and molecular levels of short and long term impacts on the phenotype and disease processes in multiple organs and systems (developmental programming or developmental origins of adult health and disease - DOHaD). Mice and rats are litter-bearing animals allowing studies of variations among fetuses (such as

gender studies) in a similar *in utero* environment in the same mother. However, the most important advantage of creating a mouse model of fetal growth restriction is the availability of genetically manipulated mouse models to conduct subsequent studies to further delineate potential relevance of a single gene or use genetic manipulation of overexpression or deletion of a single gene identified in FGR studies. Gene targeting and transgenic experiments have lead to considerable advances in the knowledge of genes responsible for placental and fetal growth, however, maternal and fetal responses to *in utero* insults have been lacking due to unavailability of information on the impacts in normal wild type animals. Advances in understanding epigenetics, alterations to the epigenome by one of the most important components of the developing embryo/fetal environment, the nutritional environment, and the potential epigenetic mechanisms that may be responsible for long-term impact on the expression of crucial genes in critical cells of the manipulated animals may delineate the mechanisms of DOHaD.

2.2 POTENTIAL BENEFITS OF THE MODEL

Our laboratory has developed a mouse model that consistently and reproducibly induces FGR by a mild maternal nutrient reduction (MNR) of 70% of normal daily total caloric and nutrient intake during 6.5 to 18.5 days of gestation. We believe that this type of nutrient restriction is most relevant clinically. It creates fetal undernutrition, which is a common pathophysiology underlying the majority of FGR in humans, in both developed and developing countries. In developed countries, fetal undernutrition is caused by placental insufficiency, whereas in developing countries, it is caused by maternal under- or mal-nutrition. Therefore, it represents the fetal and placental adaptation to maternal

undernutrition during pregnancy, relevant particularly to developing countries, and fetal adaptation to placental insufficiency, relevant to developed countries. Importantly, identification of specific genes or pathways in fetal adaptation will allow future genetic manipulation studies to delineate tissue/cellular mechanisms in fetal growth restriction or long-term adverse outcomes.

2.3 OVERALL HYPOTHESIS

The **overall hypothesis** of this thesis is that mild maternal total caloric and nutrient restriction in a mouse leads to adaptation of placenta and fetal tissues and organs to maintain fetal survival resulting in fetal growth restriction similar to human FGR, and changes in the genome that ultimately results in endocrine/metabolic and cardiorespiratory disorders in childhood and adulthood (DOHaD). This model will provide an ideal model for mechanistic studies of fetal growth and developmental programming (DOHaD), particularly epigenetic mechanisms for further studies.

2.4 RATIONALE FOR ORGANS STUDIED

The first thesis chapter addresses the hypothesis that *global maternal caloric restriction is a* ***clinically relevant model*** *to study alterations in fetal growth and long-term developmental programming, and is similar to human FGR and predisposition to DOHaD*. To support this hypothesis, we determined changes to placental, fetal and fetal organ weights to investigate how fetal growth is affected by total caloric and nutrient restriction and whether it represents a symmetric or asymmetric form of FGR. Changes to the IGF system in fetal circulation as well as changes in placental and fetal tissues were

assessed as major autocrine/paracrine and endocrine regulators of fetal growth. Alterations to fetal liver structure and function were assessed as the liver is the major organ that is impacted in FGR and may be responsible for some of the metabolic/endocrine outcomes of DOHaD, and is the main organ that produces circulating IGFs and IGFBPs. Finally, this chapter will describe whether this mouse model, which does not subject postnatal animals to overnutrition unlike other models, develops glucose intolerance one of the outcomes of DOHaD.

The second thesis chapter addresses the *hypothesis that global maternal caloric restriction* ***disrupts normal fetal lung development*** *and compromises postnatal pulmonary function*. Asymmetric FGR is associated with higher incidences of neonatal morbidity and mortality; some may be the result of respiratory complications. Organs commonly affected by FGR are the liver and lung, and these organs typically weigh less in proportion to the weight of the fetus. Developmental programming of the pulmonary system in FGR has been reported in humans but little research has been done due to a lack of appropriate animal models. Our mouse model may be suitable to study lung development during FGR and the mechanisms which compromise long-term lung function. To investigate our hypothesis, fetal lung structure, including lung weights, architecture and surfactant proteins were analyzed as well as alterations to circulating glucocorticoid levels and tissue glucocorticoid metabolism. Postnatal pulmonary function were measured prior to and following puberty to determine if fetal undernutrition permanently affects postnatal lung function.

The third chapter of the thesis focuses on the placenta and hypothesizes that global maternal caloric restriction ***leads to adaptation of the placenta to provide***

nutrient and substrates *to the fetus and its failure contributes to FGR.* The placenta forms the interface between the maternal and fetal environments. During maternal nutrient restriction, placental structure was analyzed in terms of weight, volume, variations to cell types, and dimensions of the vasculature. Changes to function were analyzed by determining the placental transport of EDTA, glucose and methylaminoisobutyric (MeAIB). Finally, alteration to the expression of the IGF system was assessed, to determine the paracrine system that could be responsible for changes in structure and function.

This thesis aims to demonstrate the impact and adaptation of maternal nutrient restriction in a murine model to gain insights into the pathophysiological mechanisms of fetal growth restriction in humans.

CHAPTER 3

MATERNAL NUTRIENT RESTRICTION ALTERS FETAL INSULIN AND INSULIN-LIKE GROWTH FACTOR SYSTEMS AND IMPAIRS LONG-TERM METABOLIC FUNCTION

Manuscript submitted to Endocrinology

3.1 INTRODUCTION

Fetal growth restriction (FGR) in pregnancy occurs when the fetus fails to achieve his/her genetically determined growth potential or optimal growth due to fetal, placental or maternal factors. A small for gestational age (SGA) newborn is the outcome (1). FGR affects 3-10% of human pregnancies and is associated with high perinatal mortality and morbidity (2, 3). In developed countries, placental insufficiency is the major etiology, causing fetal undernutrition by impaired transfer of nutrients from mother to fetus despite normal maternal nutrition. In developing countries, inadequate maternal nutrition is the major etiology impairing availability of nutrients to the fetus. Fetal growth restriction can be reproduced in experimental animals by maternal undernutrition, and is utilized to study the impact of fetal undernutrition in the fetus and placenta (4). The mouse is important to study the pathophysiology of FGR, since it is a mammalian species with

similar genetic makeup, biochemical pathways, cell types and physiology to humans (4). The mouse is also amenable to genetic manipulation, and knockout or transgenic models of genes of interest can be generated for mechanistic studies.

Fetal and placental growth is regulated by many growth factors; the insulin-like growth factor (IGF) system is the principal growth factor. It regulates cell proliferation, differentiation, migration, and aggregation during development by endocrine and autocrine/paracrine mechanisms (5). The IGF system includes two growth factors, IGF-I and IGF-II, two cell surface receptors, IGF-IR and IGF-IIR, and six IGF-binding proteins. Biologic effects of IGF-I and II are mediated by binding to IGF-IR and IGF-II binding to the insulin receptor A (INSRA) isoform (6). The INSRA isoform expression is elevated during gestation and mediates cell mitogenesis (6, 7), as opposed to the insulin receptor B isoform which is highly expressed postnatally and mediates the metabolic effects of insulin with little affinity for IGF-II (6, 7). Six high-affinity IGF binding proteins (IGFBP-1 to -6) regulate the availability of IGF peptides to developing fetal tissues, protecting them from degradation in circulation and modulating binding of IGF peptides to their receptors (8). The IGF peptides are synthesized by a wide variety of cells in the fetus and placenta with the paracrine IGFBPs determining the cells targeted for IGF actions (5). Expression of the IGF system is coupled to the supply of nutrients to ensure fetal growth is adequate for the amount of nutrients available (5). Alterations in fetal nutrient supply can change the expression of this system in a tissue specific manner to regulate overall fetal growth and development of individual fetal organs.

Developmental alterations in organ structure may affect organ function immediately after birth, and ongoing effects on function leading to increased

predisposition to disease later in life. Adults who were fetal growth restricted in utero are at greater risk for development of diseases such as hypertension, coronary heart disease, stroke and type-2 diabetes as adults (9). This association has led to the concept of the "fetal origins of adult disease", which hypothesizes that suboptimal growth during fetal life in response to undernutrition or other stresses causes fetal organ systems to adapt and develop in a way that predisposes them to the development of adulthood diseases (10).

Several models of FGR use knockout and transgenic mice in which components of IGF system genes have been manipulated (11-15). The role of the IGF system in FGR has not been previously characterized in the mouse. To determine if the impact of fetal undernutrition on fetal growth in the mouse is similar to FGR in humans, we generated a maternal nutrient restricted (MNR) mouse model of FGR in which mothers were fed 80%, 70% or 60% normal daily caloric intake during pregnancy. We studied growth of organ systems during fetal development by determining changes in fetal organ weights, and tissue-specific IGF-system expression. Specific structural changes in the liver, which is the major source of the endocrine IGF system, were analyzed. Since male FGR mice developed glucose intolerance as adults, changes to insulin and glucagon expression in the pancreas were investigated during fetal life and postnatally.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Dietary Restriction Protocol

Protocols on Animal Care were approved by the University Council on Animal Care at the University of Western Ontario (appendix), and maintained in accordance with guidelines of the Canadian Council for Animal Care. Ten week old virgin CD-1 females

and stud males were obtained from Charles River Laboratories (Montreal, PQ, Canada). Mice were housed in the Lawson Health Research Institute Vivarium (London ON, Canada) with a 12:12 light:dark cycle and water provided *ad libitum*. Female mice were housed singly with stud males in late afternoon and checked the following morning for a vaginal plug, indicating embryonic day 0.5 (E0.5). Females were housed individually and fed grain-based dustless precision pellets (#F0173, Bio-Serv, Frenchtown NJ, USA) containing 20.7% protein, 4.0% fat, 6.7% ash, 59.6% carbohydrates, 4.0% fiber and 5.0% moisture *ad libitum* using feeding tubes with cage clips (Bio-Serv, Frenchtown NJ, USA) until E6.5. Feeds for control mice were pre-weighed each morning and remaining pellets weighed at the same time each day to establish average daily feed intake during pregnancy. MNR pregnant mice from E6.5 were fed daily either 80% (n = 8 litters), 70% (n = 9 litters) or 60% (n = 7 litters) by weight of average daily intake of control mice (n = 24). Mice were euthanized at E18.5 by carbon dioxide inhalation. Fetal tissues and placentae, trimmed of membranes and yolk sac, were dissected, weighed, and pooled by litter before flash freezing in liquid nitrogen. Maternal and pooled fetal blood was obtained using heparinized capillary tubes (Fisher Scientific, Unionville ON, Canada).

Weight data was analyzed for differences between MNR and control groups using one-way ANOVA followed by Tukey's multiple comparisons test. Non-parametric data was analyzed using Kruskal-Wallis and Dunn's post-hoc tests.

3.2.2 Insulin and Insulin-Like Growth Factor (IGF) ELISAs

Plasma insulin was measured in six month old adult males at 0, 60 and 120 minute timepoints during a glucose tolerance test using Ultra Sensitive Mouse Insulin

ELISA Kit (Crystal Chem Inc., Downers Grove, IL) (**Appendix 1.1.1**). Plasma insulin of E18.5 mothers and their respective litter was quantified using the multiplexed bone metabolism mouse panel 1A biomarker immunoassay kit according to manufacturers' instruction (Milli*plex*, Millipore Corp, MA). A Bio-Plex™ 200 readout system was used (Bio-Rad Laboratories, Hercules, CA), utilizing Luminex® xMAP™ fluorescent bead-based technology (Luminex Corp., Austin, TX). Levels were calculated from standard curves using Bio-Plex Manager software (v.4.1.1, Bio-Rad). IGF system peptides were measured in maternal and fetal plasma using ELISA kits as per manufacturers' instructions from AssayPro (St. Charles, MO) for IGF-I, Insight Genomics (Falls Church,VA) for IGFBP-1, ALPCO (Salem, NH) for IGFBP-2 and R&D Systems (Minneapolis, MN) for IGFBP-3.

3.2.3 Glucose Tolerance Testing

Blood glucose was measured with a Contour glucometer (Bayer Healthcare, Toronto ON). Thirteen control and nine 70% MNR males as well as ten control and ten 70% MNR females were tested for glucose tolerance at 6 months of age. A bolus of glucose (2 mg/g weight) was given intraperitoneally following an overnight fast. Blood samples taken every 30 min for 2 hours during the test. Resulting blood glucose levels during the 30 min intervals were plotted and the area under the curve was calculated and compared between groups using a Mann-Whitney statistical test.

3.2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA from flash frozen control and 70% MNR fetal tissues were extracted using Trizol (Invitrogen, Burlington, ON) and purified with RNAeasy Mini Kit (Sigma, Maryland, USA), following manufacturer's instructions.

The relative abundance of IGF-I and -II and IGFBP-1, -2, and -3 mRNAs was determined using qRT-PCR with the Taqman MGB probe method (FAM dye-labeled) in an ABI PRISM 7900HT system (Applied Biosystems, Foster City, CA) with Sequence Detection System, Version 2.2.1 (Applied Biosystems). Loading controls were GAPDH mRNA for the lung and β-2-microglobulin (β2M) in remaining fetal tissues (**TABLE 3.1**). PCR assays were run in triplicate using 100 ng RNA in 10 µl Taqman One-Step RT-PCR Master Mix (#4309169, Applied Biosystems). One-step PCR cycling was carried out as follows: 48□C, 30 min for 1 cycle; 95□C, 10 min for 1 cycle; 95□C, 15 sec; 60□C, 1 min for 40 cycles. Criteria for amplicon acceptance was an $r^2 > 95\%$ and slope between -3.1 and -3.6 (with difference between target gene and endogenous control gene of no more than 0.2).

3.2.5 Protein and DNA quantification

Fetal livers were pooled for each litter, homogenized with protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany) in phosphate buffer. For protein quantification, a sample aliquot was syringed three times and centrifuged at 13,000 x g for 1 hr. Triplicate samples were quantified using bicinchoninic acid (BCA) assay (Pierce; Rockford, IL). DNA quantification was performed by fluorometric DNA assay. An aliquot was centrifuged at 4000 x g for 5 min. Supernatant was diluted with 2

TABLE 3.1 Catalog information for Primers and Probes from Applied Biosystems for real time RT-PCR

Primer Name	Catalog #
IGF-I	Mm00439561_m1
IGFBP-1	Mm00515154_m1
IGFBP-2	Mm00492632_m1
IGFBP-3	Mm00515156_m1
GAPDH	Mm99999915_m1
β2-Macroglobulin	Mm0437764_m1
IGF-II	5'-GTGGCATCGTGGAAGAGTGC-3' 5'-GTCCGGAAGTACGGCCTGAG-3' Probe: 5'-GACCTGGCCCTCCTGGAGACATAC-3'

mM EDTA and Hoechst 33342 dye (Molecular Probes; Eugene, OR) was added to samples. Duplicate measurements were obtained by fluorescence spectrophotometry (Fluoroskan Ascent FL, ThermoLabsystems; Milford, MA), and quantified against a standard curve generated with salmon testis DNA (Sigma-Aldrich; Oakville, ON).

3.2.6 Liver Enzyme Activity Measurements

Fetal and adult male liver tissues (approximately 50 mg) were homogenized (Ultra Turrax; IKA Works, Wilmington, NC) followed by sonication (Microson, NY) in 50 mM Tris buffer with protease inhibitor (Roche, Mannheim, Germany) (16). Homogenates were prepared for enzyme measurement as previously described. The enzymes and assay conditions (found to give optimal activities) used have been described before for Glucokinase (EC 2.7.1.2) (17) and Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) (16).

3.2.7 Tissue Preparation

Fetal mouse livers were harvested at E18.5 and fixed in 4% paraformaldehyde for 48 hrs. Whole mouse embryos were harvested at E18.5, immersed in 4% paraformaldehyde for 24 hrs, bisected, and immersed in 4% paraformaldehyde for an additional 24 hrs. Adult mouse pancreases were harvested at 6 months of age, fixed in 4% paraformaldehyde for 24 hrs. Tissue specimens were washed 3 times in PBS over 3 days and preserved in 70% ethanol before embedded in paraffin. E18.5 fetal livers were

sectioned at 3 μm, with remaining tissues sectioned at 5 μm and mounted onto Superfrost® slides (Fisher Scientific, Fairlawn, NJ).

3.2.8 In situ Hybridization (ISH)

Fetal liver sections were utilized for analysis of IGF-I, IGF-II, IGFBP-1, -2 and -3 mRNA expression by *in situ* hybridization using ^{35}S-labeled antisense riboprobes as described previously (21). The ^{35}S-labeled antisense and sense cRNA probes were generated from cDNA (**TABLE 3.2**) using the Promega Riboprobe Combination System SP6/T7 kit (Promega Corp., Madison, WI) and ^{35}S-UTP (Perkin Elmer, Waltham, MA). *In situ* hybridization was performed as previously described (21) (**Appendix 1.1.2**). Slides were viewed under dark and bright fields, using a Carl Zeiss Axio Imager Z1 with photomicrographs obtained at 400× magnification using AxioCamHR3 and AxioVision Release 4.7.2 software.

3.2.9 Immunofluorescence

Sections were deparaffinized and rehydrated in a descending series of ethanol solutions. Tissue sections were blocked with Background Sniper Blocking Reagent (Biocare Medical, Concord, CA) for 10 min prior to application of primary antibodies; ter-119 and PCNA to liver sections at RT for 1 hour (**TABLE 3.3**). Tissue sections were washed, incubated for 30 min at RT with appropriate Alexa Fluor 568-conjugated IgG secondary antibodies (1:200; Molecular Probes Inc., Eugene OR), counterstained with pre-mixed DAPI (Molecular Probes Inc.) and coverslipped with ProLong® Gold

TABLE 3.2 cDNA Vectors for generating cRNA probes for in situ hybridization of IGF system mRNAs.

AntiSense Probe	Target (gi #)	Size (bp)	Restriction Site AS	S	Vector	Origin	Ref
mIgf-1	33612736	271	XhoI	EcoRV	pBluescript LION	imaGenes	-
mIgf-2	170172554	468	EcoR I	Sst I	pBluescript KS	Dr. Graeme Bell	(18)
rIgfbp-1	6981079	1477	Kpln	XhoI	pGEM 7Zf	Matt Rechler	(19)
rIgfbp-2	148747420	585	HindIII	EcoR I	pGEM 3z	Matt Rechler	(19)
rIgfbp-3	6981083	905	EcoRI	SmaI	pGEM 3z	Dr. Adrian H. Herington	(20)

The nucleotide sequence homology between rat and mouse IGFBP-1 to -3 cDNAs is 90-94%.

TABLE 3.3 Primary Antibodies used for Immunohistochemistry and Immunofluoresence.

Antibody	Host	Catalogue #	Dilution	Source
IGF-I	Rabbit	#RB-9240-P	1:10	Labvision Corp., Fremont, CA
IGF-II	Rabbit	PAAL1	1:15	GroPep, Adelaide SA, Australia
IGFBP-1	Goat	Sc-6072	1:400	Santa Cruz Biotech Inc., Santa Cruz, CA
IGFBP-2	Rabbit	#06-107	1:750	Upstate Biotech Inc., Lake Placid, NY
IGFBP-3	Rabbit	DSL-R00534	1:750	Diagnostic Systems Laboratories, Inc., Webster, TX
Ter-119	Rat	116201	1:50	BioLegend, Inc., San Diego, CA
PCNA	Goat	Sc-9857	1:500	Santa Cruz Biotech Inc., Santa Cruz, CA

AntiFade Reagent (Molecular Probes Inc.). Omission of primary antibody controlled for autofluorescence and nonspecific binding. Fluorescent images were captured and analyzed using a Carl Zeiss Axio Imager Z1 microscope equipped with AxioCamMR3 camera and AxioVision Release 4.7.2 software. To determine the proportion of liver composed of hematopoietic cells in 8 control and 8 MNR fetuses, the number of cells expressing ter119 per total number of cells were counted in 12 random fields of view at 400× magnification. The same technique was used to determine the proportion of proliferating cell nuclear antigen (PCNA) positive cells in the liver.

At E18.5, eight control and seven 70% MNR mouse embryos and six month old adult mouse pancreas from thirteen control and seven 70% MNR animals were sectioned at 5 μm and every 10^{th} section was collected for analysis of β-cell mass. Sections were deparaffinized as above then rinsed in PBS before applying Universal Sniper Blocking Agent (Biocare Medical, Concord, CA) for 10 min. Mouse anti-insulin (1:1000; Sigma-Aldrich, St. Louis, MO) and Rabbit anti-glucagon (1:200; Dako, Capinteria, CA) was applied to sections for 1 h at RT. The slides were washed in PBS three times for 5 min and incubated for 30 min at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG diluted 1:200 (Invitrogen) for insulin and Alexa Fluor 568-conjugated Goat anti-rabbit at 1:200 for glucagon (Invitrogen). Pancreases were counterstained with DAPI as above. Fluorescent labeling was visualized and photographed using a Carl Zeiss Axio Imager Z1 microscope equipped with AxioCamMR3 camera and AxioVision Release 4.7.2 software. In embryos, the percentage of pancreas area stained for insulin and glucagon was measured. In six month old adult pancreases, β-cell mass was calculated by measuring area of the pancreas stained with insulin and dividing by total area occupied

by pancreatic tissue. This ratio was multiplied by the total mass of the pancreas dissected from the mouse. Alpha-cell mass was calculated by measuring the area of the pancreas stained with glucagon and dividing by the total area occupied by pancreatic tissue and multiplying by the weight of the pancreas.

3.2.10 Histochemistry

Apoptotic cells were detected using an Apoptag Kit from Chemicon International (Millipore, Billerica, MA), as per the manufacturer's instructions. To determine the percentage of apoptotic cells in the liver of eight control and eight MNR fetuses, the number of diaminobenzidine (DAB) positive cells were counted in 12 random fields of view per total number of nuclei at 400× magnification (**Appendix 1.1.3**).

To determine the proportion of glycogen in eight control and eight MNR livers, Periodic acid-Schiff (PAS) staining was performed using PISSARRO-PAS Stain Kit (Biocare Medical, Walnut Creek, CA) according to manufacturer's instructions. The area of PAS staining in 12 random fields of view was determined at 400× magnification (**Appendix 1.1.4**).

3.2.11 Statistical Analysis

All data was expressed as mean ± SEM. Parameters measured in control and 70% MNR animals were compared with Student's non-paired two-tail *t* test. A Mann-Whitney test was performed to determine fold changes in 70% MNR litters for qRT-PCR. Significance was set at $P < 0.05$.

3.3 RESULTS

3.3.1 Effects of MNR on the fetus and fetal organ weights

To determine if our maternal undernutrition regimen induces a phenotype similar to human fetal growth restriction, fetal organ weights at E18.5 were determined for each treatment group. Fetal weights were significantly less in each MNR regimen compared to controls (**TABLE 3.4**). The 70% MNR fetuses demonstrated the most consistent and significant organ weight reduction with exception of the heart, kidney and brain; this demonstrates an asymmetric phenotype of FGR (**TABLE 3.4**). In 80% MNR fetuses, the placenta and brain weights were spared with no significant reduction compared to controls, while remaining tissues were significantly reduced (**TABLE 3.4**). In the 60% MNR, the litter size was significantly reduced but fetal weight was maintained. In 60% MNR fetuses, organ weights were comparable to 80% MNR, with the exception of the liver (**TABLE 3.4**). Therefore, for the remainder of studies 70% MNR was used.

3.3.2 Effects of FGR on the IGF system

3.3.2.1 Endocrine IGF system

Endocrine levels of the IGF system were determined in fetal and maternal blood to determine if similar changes in our mouse undernutrition model are similar to changes to endocrine levels of the IGF system documented in human pregnancies afflicited with fetal growth restriction. In maternal plasma of 70% MNR, IGF-I and IGFBP-3 concentrations were significantly decreased (**Fig. 3.1**; IGF-I: control 56 ± 12 ng/mL vs. MNR 26 ± 4 ng/mL; $P < 0.05$; IGFBP-3: control 1048 ± 70 ng/mL vs. MNR 586 ± 84

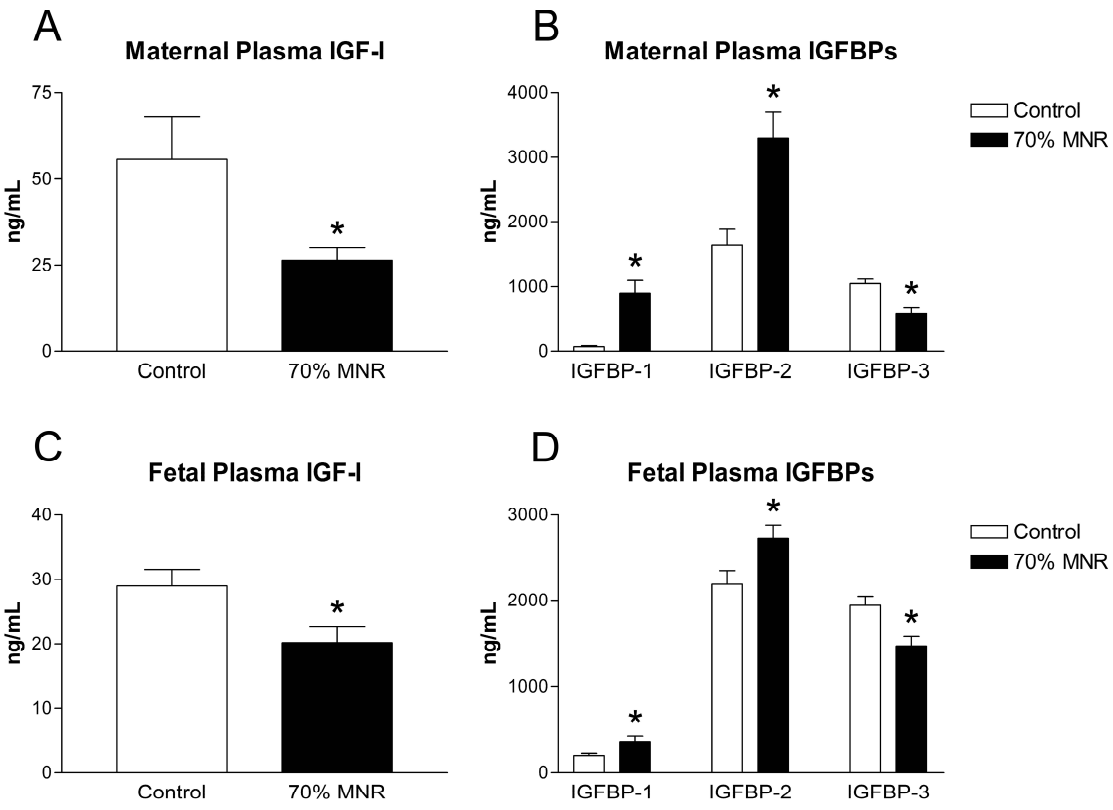
TABLE 3.4 Effect of Maternal Nutrient Restriction beginning at E 6.5 of pregnancy on placental and fetal tissue weights at E 18.5.

	Control (n=24)	**80% MNR (n=8)**	**70% MNR (n=9)**	**60% MNR (n=7)**
Fetus	1397 ± 17	1149 ± 64[a]	894 ± 52[a,b]	967 ± 63[a,b]
Placenta	95 ± 3	91 ± 5	72 ± 3[a,b]	78 ± 4[a]
Heart	8 ± 1	6 ± 1[a]	5 ± 1[a]	5 ± 1[a]
Lung	51 ± 1	39 ± 3[a]	29 ± 2[a,b]	32 ± 2[a]
Liver	91 ± 2	69 ± 5[a]	46 ± 3[a,b]	50 ± 3[a,b]
Gut	81 ± 1	67 ± 4[a]	48 ± 4[a,b]	55 ± 5[a]
Kidney	12 ± 1	9 ± 1[a]	7 ± 1[a]	8 ± 1[a]
Brain	69 ± 1	64 ± 3	56 ± 2[a]	58 ± 3[a]
Carcass	977 ± 12	801 ± 4[a]	644 ± 38[a,b]	673 ± 50[a]
Litter Size	13 ± 1	11 ± 1	11 ± 1	10 ± 1[a]

Pregnant mothers were fed ad libitum (control) or 80%, 70% or 60% MNR beginning at E 6.5. At E 18.5, mothers were sacrificed and placenta and fetal tissues were weighed for each fetus for a mean litter value. The result, litter mean ±SEM for 'N' number of litters, is expressed in mg.

Superscripts ([a] = different from control ($P < 0.05$); [b] = different from 80% MNR ($P < 0.05$); [c] = different from 70% MNR ($P < 0.05$) among treatment groups indicate a significant difference in weight by one-way ANOVA ($P < 0.05$), followed by Tukey's multiple comparisons test.

Figure 3.1 The effect of maternal nutrient restriction on circulating concentrations of the IGF system in the maternal and fetal plasma at E18.5. (**A**) Maternal plasma IGF-I concentrations (mean ± SEM) were 56 ± 12 ng/mL in controls (N=8) and 26 ± 4 ng/mL in 70% MNR (N=8) (P<0.05). IGF-I concentrations were 55% lower in 70% MNR mothers (P < 0.05). (**B**) Maternal plasma concentrations (mean ± SEM) of IGFBP-1, -2 and -3 in controls (N=8) and 70% MNR (N=8). In 70% MNR mothers, the IGFBP-1 concentration of 899 ± 201 ng/mL (vs 68 ± 17 ng/mL in controls) and IGFBP-2 concentration of 3,299 ± 432 ng/mL (vs 1648 ± 253 ng/mL in controls) were significantly higher (both P < 0.005), while IGFBP-3 concentration of 586 ± 84 ng/mL (vs 1048 ± 70 ng/mL in controls) was significantly lower (P < 0.001). (**C**) Fetal plasma IGF-I concentrations (mean ± SEM) were 29 ± 3 ng/mL in controls (N=8) and 20 ± 2 ng/mL in 70% MNR (N=8). IGF-I concentrations were 30% lower in 70% MNR fetuses (P < 0.05). (**D**) Fetal plasma concentrations (mean ± SEM) of IGFBP-1, -2 and -3 in controls (N=8) and 70% MNR (N=8). In 70% MNR fetuses, the IGFBP-1 concentration of 362 ± 70 ng/mL (vs 197 ± 25 ng/mL in controls) and IGFBP-2 concentration of 2719 ± 170 ng/mL (vs 2194 ± 169 ng/mL in controls) were significantly higher (both P < 0.05), while IGFBP-3 concentration of 1475 ± 112 ng/mL (vs 1944 ± 111 ng/mL in controls) was significantly lower (P < 0.01).


A
Maternal Plasma IGF-I
ng/mL
75
50
25
0
Control
70% MNR
B
Maternal Plasma IGFBPs
ng/mL
4000
3000
2000
1000
0
IGFBP-1
IGFBP-2
IGFBP-3
Control
70% MNR
C
Fetal Plasma IGF-I
ng/mL
40
30
20
10
0
Control
70% MNR
D
Fetal Plasma IGFBPs
ng/mL
3000
2000
1000
0
IGFBP-1
IGFBP-2
IGFBP-3
Control
70% MNR

ng/mL; $P < 0.001$), whereas IGFBP-1 and IGFBP-2 concentrations were significantly increased (**Fig. 3.1**; IGFBP-1: control 68 ± 17 ng/mL vs. MNR 899 ± 201 ng/mL; $P < 0.005$; IGFBP-2: control 1648 ± 253 ng/mL vs. MNR 3299 ± 432 ng/mL; $P < 0.005$). In fetal plasma, changes to concentrations of the IGF system peptides were similar to that of maternal plasma with lower IGF-I and IGFBP-3 concentrations and higher IGFBP-1 and IGFBP-2 concentrations in MNR fetuses compared to controls (**Fig. 3.1**; IGF-I: control 29 ± 3 ng/mL vs. MNR 20 ± 2 ng/mL; $P < 0.05$; IGFBP-3: control 1944 ± 111 ng/mL vs. MNR 1475 ± 112 ng/mL; $P < 0.01$; IGFBP-1: control 197 ± 25 ng/mL vs. MNR 362 ± 70 ng/mL; $P < 0.05$; IGFBP-2: control 2194 ± 169 ng/mL vs. MNR 2719 ± 170 ng/mL; $P < 0.05$). The ratio of IGF-I to IGFBP-3 was similar between control and 70% MNR in both fetal and maternal plasma. Assays for IGF-II levels in plasma are unavailable for reliable estimates in the small volumes of mouse plasma.

3.3.2.2 IGF system mRNAs in fetal tissues and placentae

The expression levels of mRNAs encoding the IGF system in different fetal organs/tissues were performed to determine autocrine/paracrine changes of the system that correspond with changes in organ/tissue growth, and the organs/tissues that are responsible for the changes in fetal endocrine levels of the IGF system. IGF-I, -II, and IGFBP-1,-2,-3 mRNA levels were determined in heart, lung, liver, brain, carcass and placenta in control and 70% MNR litters using qRT-PCR. The liver demonstrated greatest response to MNR with significantly increased levels of IGFBP-1, IGFBP-2, and IGFBP-3 mRNAs (**TABLE 3.5**). Placenta, lung and brain demonstrated an increase in IGFBP-3 mRNA levels (**TABLE 3.5**). The placenta was the only organ that showed a

TABLE 3.5 Effect of 70% Maternal Nutrient Restriction beginning at E 6.5 of pregnancy on Insulin-Like Growth Factor (IGF) System mRNA levels at E 18.5 in placental and fetal tissues.

	IGF-I	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3
Placenta	0.53 ± 0.12*	1.11 ± 0.07	0.64 ± 0.20	1.10 ± 0.16	1.76 ± 0.12*
Heart	0.58 ± 0.08	1.04 ± 0.09	-	-	1.24 ± 0.13
Lung	0.71 ± 0.11	0.93 ± 0.09	1.85 ± 0.80	0.97 ± 0.21	2.22 ± 0.51*
Liver	0.70 ± 0.10	1.04 ± 0.11	5.10 ± 1.86*	1.86 ± 0.33*	1.29 ± 0.08*
Brain	0.80 ± 0.09	0.88 ± 0.09	2.29 ± 0.50	0.90 ± 0.08	1.48 ± 0.13*
Carcass	0.76 ± 0.06	1.14 ± 0.10	-	1.60 ± 0.20	1.45 ± 0.15

Pregnant mothers were fed ad libitum (control) or 70% MNR beginning at E 6.5. At E 18.5 mothers were sacrificed and placenta and fetal tissues collected and pooled by litter. The IGF system mRNA levels were measured by quantitative real-time PCR and corrected for the internal housekeeping gene, β2 macroglobulin. The relative mRNA levels of each tissue of 70% MNR group (N = 6 litters) are expressed relative to those of control group (N = 6 litters), which are assigned a value of one.

* Indicates a significant fold-change between control and 70% MNR groups by Mann-Whitney Test ($P < 0.05$).

significant reduction in IGF-I mRNA level. In most fetal tissues, IGF-I mRNA levels were relatively lower, with no change in IGF-II mRNA levels (**TABLE 3.5**). At E11.5, the liver is the major source of hematopoietic cells during gestation as it is populated by hematopoietic stem cells and provides a developmental niche for the expansion, maturation and enucleation of primitive erythroid cells (22). Late in development, from approximately E16 onwards, the site of hematopoiesis shifts gradullay to the bone marrow, which is the definitive site where hematopoietic stem cells and progenitors of the adult hematopoietic system reside (22). In situ hybridization demonstrated IGF-I and -II and IGFBP-1, -2 and -3 mRNAs to be expressed in hepatocytes and not in hematopoietic cells (**Fig. 3.2** and **Fig. 3.3**) (**Appendix Fig. 1.1, Fig 1.2** and **Fig. 1.3**).

3.3.3 Effects of FGR on liver

Fetal liver was demonstrated to be significantly affected by maternal undernutrition in terms of weight and IGF system expression. Analysis of liver structure was determined by immunohistochemistry of liver cell types at E18.5 (**Fig. 3.4**) and analysis of liver function by measurement of hepatocyte enzyme activity. Immunohistochemistry of the liver showed that IGFs and IGFBPs were localized in hepatocytes and not in hematopoietic cells. The percentage of hematopoietic cells as determined by ter-119 immunoreactivity was not different between MNR and controls, indicating that the proportion of hepatocytes and hematopoietic cells was maintained in FGR fetal livers and IGFBP expression increased in each hepatocyte during MNR (**Fig. 3.5A**). Glycogen staining in MNR liver was significantly reduced compared to controls

Figure 3.2 Insulin-Like Growth Factor (IGF) System mRNA expression in fetal livers of control and 70% MNR at E 18.5. Autoradiography of in situ hybridization of IGF-I (**A, B**), IGF-II (**C, D**) in control (**A, C**) and 70% MNR (**B, D**). Images are captured by darkfield microscopy. White grains indicate location of IGF mRNA. Open arrows indicate representative hematopoietic cell clusters; closed arrows indicate representative hepatocytes. Note the hepatocytes of the control livers are filled with glycogen. *Scale bars*, 100 μm.

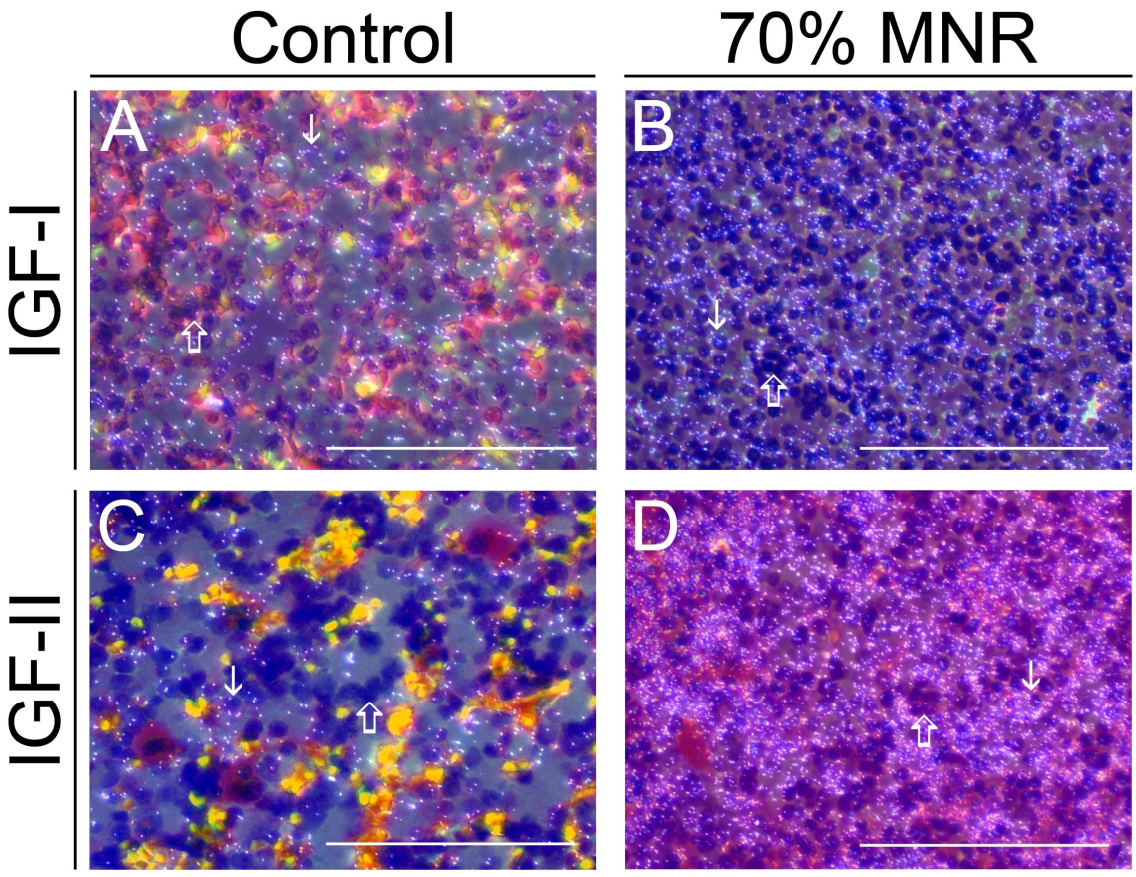

Control
70% MNR
IGF-I
IGF-II
A
B
C
D

Figure 3.3 Insulin-Like Growth Factor (IGF) System mRNA expression in fetal livers of control and 70% MNR at E 18.5. Autoradiography of in situ hybridization of IGFBP-1 (**A, B**), IGFBP-2 (**C, D**), IGFBP-3 (**E, F**) in control (**A, C, E**) and 70% MNR (**B, D, F**). Images are captured by darkfield microscopy. White grains indicate location of IGF mRNA. Open arrows indicate representative hematopoietic cell clusters; closed arrows indicate representative hepatocytes. Note the hepatocytes of the control livers are filled with glycogen. *Scale bars*, 100 μm.

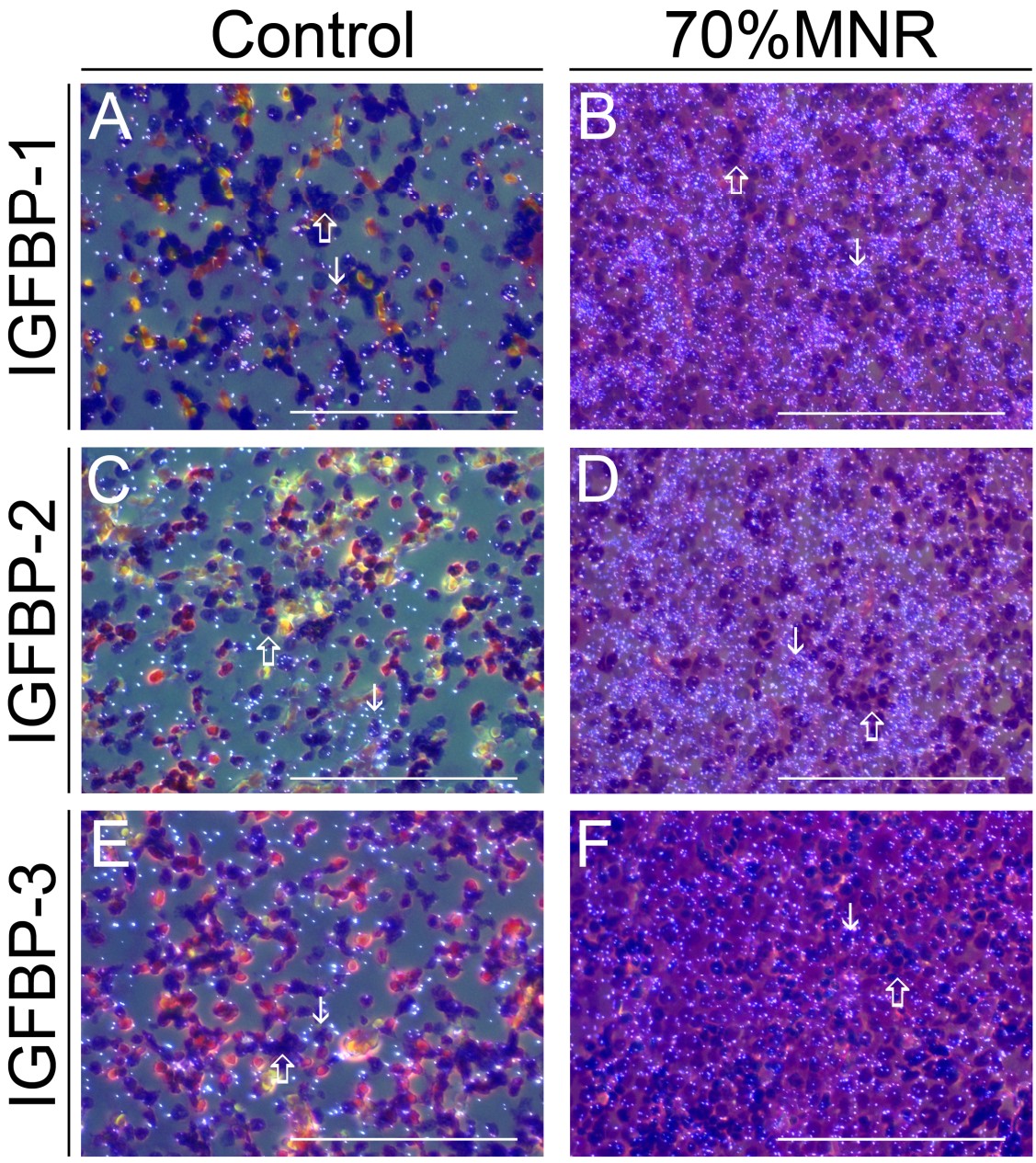

Control
70%MNR
IGFBP-1
IGFBP-2
IGFBP-3
A
B
C
D
E
F

Figure 3.4 Representative photomicrographs of changes to fetal liver at E 18.5 following 70% MNR. Cells positive for hematopoietic marker (**A** and **B**), ter119, in fetal livers of control (**A**) and 70% MNR (**B**). Glycogen (PAS) stained hepatic cells (**C** and **D**) in the control (**C**) and 70% MNR group (**D**). Cells positive for PCNA (**E** and **F**) in the control (**E**) and 70% MNR group (**F**). Apoptotic cells (**G** and **H**) in control (**G**) and 70% MNR (**H**). Arrows indicate positive markers. *Scale bars*, 100 μm.

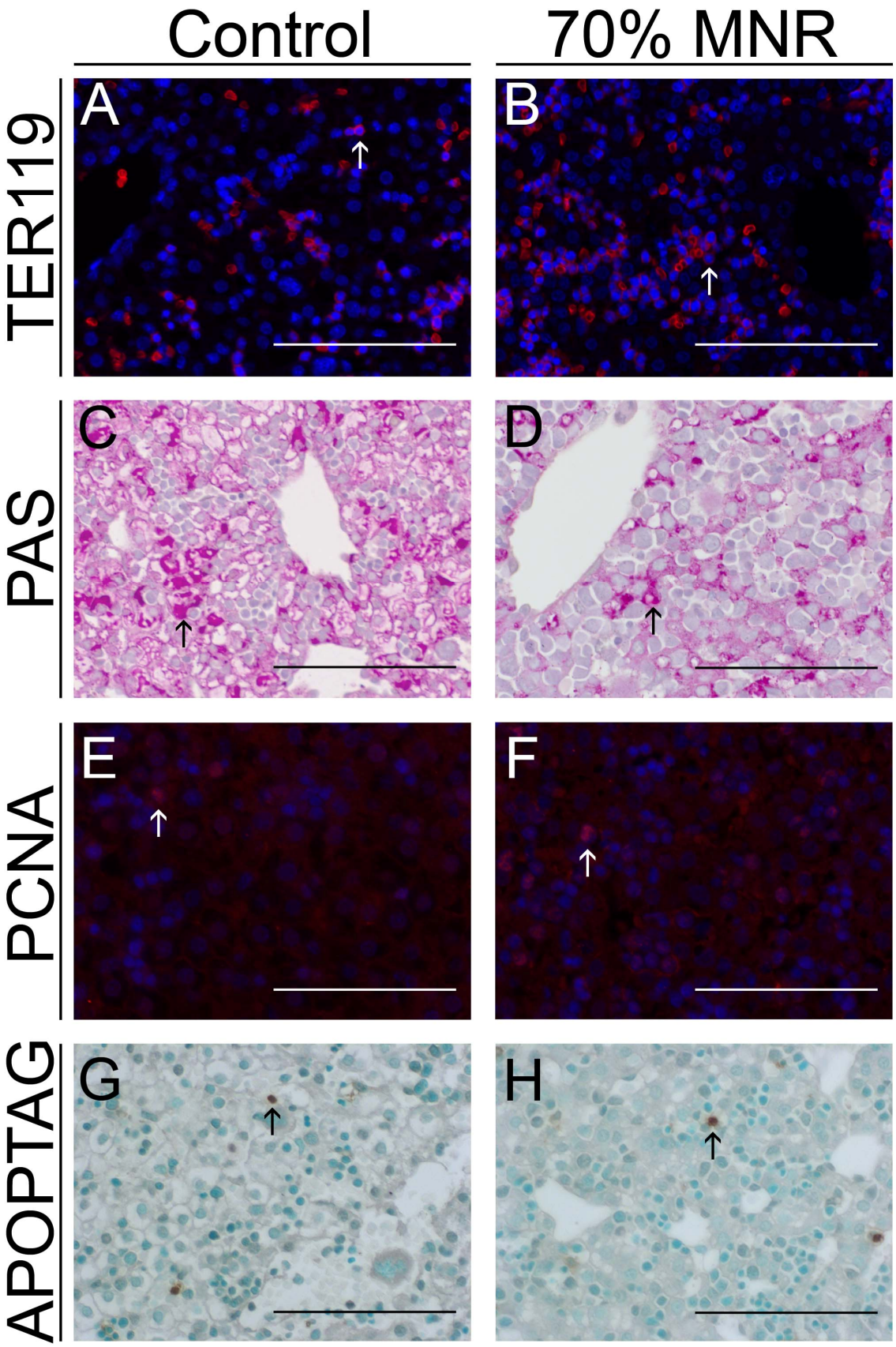
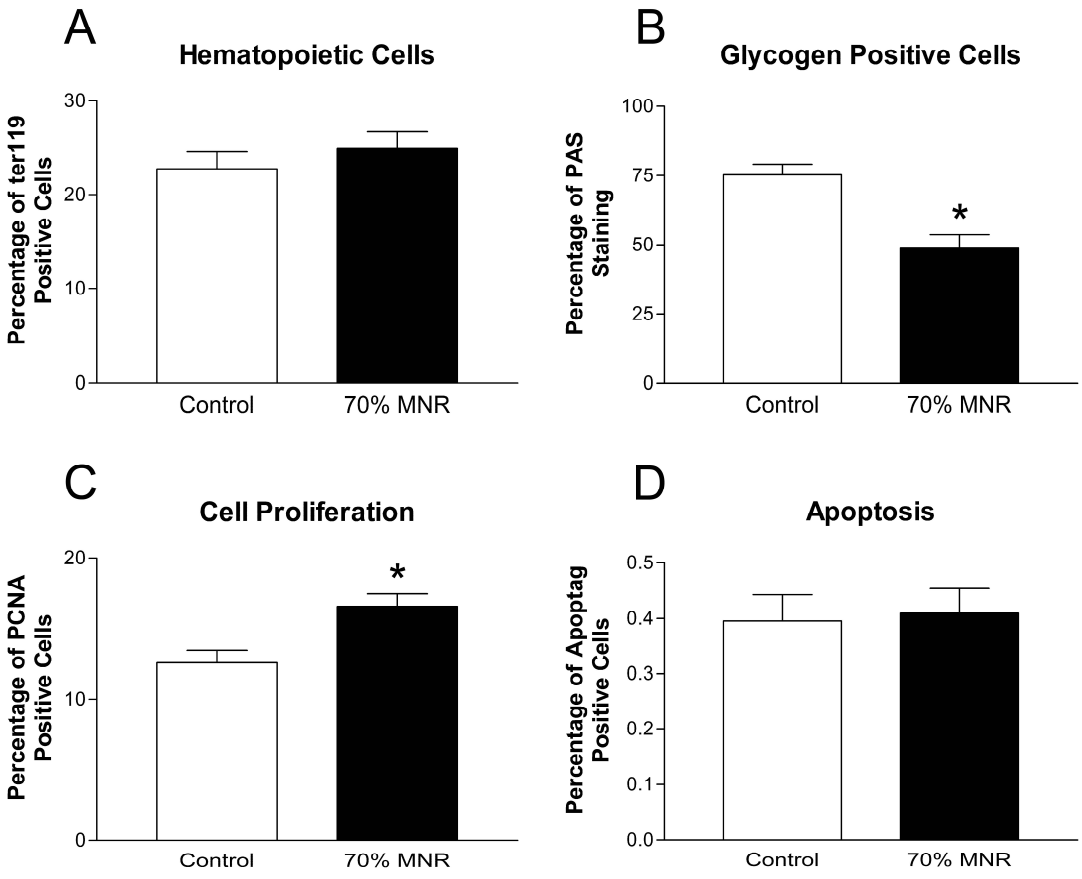

Control
70% MNR
TER119
PAS
PCNA
APOPTAG
A
B
C
D
E
F
G
H

Figure 3.5 Changes to fetal liver cells at E 18.5 following 70% MNR. (**A**) Cells positive for hematopoietic marker, ter119, (mean ± SEM) in fetal livers of control (N=8) and 70% MNR (N=8) showed no differences. (**B**) Percentage of glycogen (PAS) stained hepatic cells (mean ± SEM) in the 70% MNR group of 49 ± 5% (vs 75 ± 4% in control) was significantly reduced ($P < 0.01$). (**C**) Percentage of cells positive for PCNA (mean ± SEM) in the 70% MNR group of 17 ± 0.9% (vs 13 ± 0.9% in controls) was significantly increased ($P < 0.01$). (**D**) Percentage of apoptotic cells (mean ± SEM) demonstrated no differences.

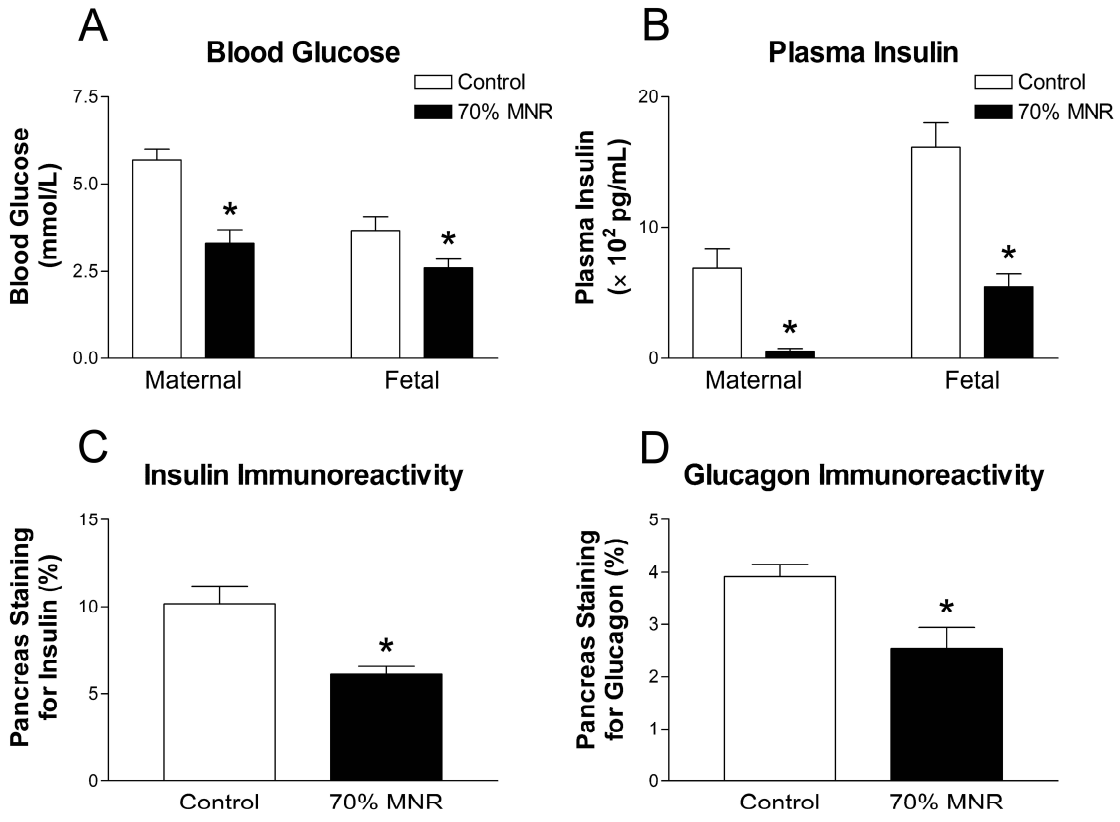

A
Hematopoietic Cells
Percentage of ter119 Positive Cells
30
20
10
0
Control
70% MNR
B
Glycogen Positive Cells
Percentage of PAS Staining
100
75
50
25
0
*
Control
70% MNR
C
Cell Proliferation
Percentage of PCNA Positive Cells
20
10
0
*
Control
70% MNR
D
Apoptosis
Percentage of Apoptag Positive Cells
0.5
0.4
0.3
0.2
0.1
0.0
Control
70% MNR

(**Fig. 3.5B**; control 75 ± 4% *vs.* MNR 49 ± 5%; P < 0.001). Livers in MNR had a greater percentage of cells expressing the cell proliferation marker, PCNA, (**Fig. 3.5C**; control 13 ± 0.9% *vs.* MNR 17 ± 0.9%; P < 0.01), with no change in percentage of apoptotic cells (**Fig 3.5D**). Liver DNA and protein content (μg/mg wet weight) were not significantly different between groups (**Appendix TABLE 1.1** and **1.2**). Activity of phosphoenolpyruvate carboxykinase (PEPCK) and glucokinase were not different between control and 70% MNR fetal livers at E18.5. However, at six months, adult males demonstrated glucokinase (control 1.98 ± 0.09 μmol/min/g *vs.* MNR 1.47 ± 0.06 μmol/min/g; P < 0.0005) activity was significantly lower in 70% MNR group, while PEPCK activity (control 0.039 ± 0.012 μmol/min/g *vs.* MNR 0.073 ± 0.009 μmol/min/g; P < 0.05) was significantly increased in the 70% MNR group.

3.3.4 Glucose Intolerance

At E18.5 fetal levels of glucose and insulin were measured to determine the exposure of fetal tissues to this important substrate and growth factor, respectively. At E18.5, both maternal (**Fig. 3.6A**; control 5.7 ± 0.3 mmol/L *vs.* MNR 3.3 ± 0.3 mmol/L; P < 0.001) and fetal blood glucose levels (**Fig. 3.6A**; control 3.7 ± 0.3 mmol/L *vs.* MNR 2.6 ± 0.2 mmol/L; P < 0.05) were significantly lower in the 70% MNR animals compared to controls, with a reduction in plasma insulin in both mother (**Fig. 3.6B**; control 692 ± 147 pg/mL *vs.* MNR 56 ± 19 pg/mL; P < 0.005) and fetus (**Fig. 3.6B**; control 1615 ± 188 pg/mL *vs.* MNR 546 ± 104 pg/mL; P < 0.001). Due to the reduction in fetal blood glucose and insulin levels with maternal undernutrition, we determined if there were structural changes to the fetal endocrine pancreas at E18.5. Tissue sections of whole

Figure 3.6 Changes to fetal endocrine pancreas structure at E 18.5 following 70% MNR. (**A**) Blood glucose levels (mean ± SEM) of control (N=12) and 70% MNR (N=9) mothers and control (N=8) and 70% MNR (N=8) litters. The blood glucose concentration, in 70% MNR mothers of 3.3 ± 0.3 mmol/L (vs 5.7 ± 0.3 mmol/L in controls) and 70% MNR fetuses of 2.6 ± 0.2 mmol/L (vs 3.7 ± 0.3 mmol/L in controls) were significantly lower ($P < 0.0001$) and ($P < 0.05$), respectively. (**B**) Plasma insulin concentrations (mean ± SEM) of control (N=6) and 70% MNR (N=6) mothers and control (N=6) and 70% MNR (N=6) litters. The plasma insulin concentration, in 70% MNR mothers of 56 ± 19 pg/mL (vs 692 ± 147 pg/mL in controls) and 70% MNR fetuses of 546 ± 104 pg/mL (vs 1615 ± 188 pg/mL in controls) were significantly reduced ($P < 0.005$) and ($P < 0.001$), respectively. (**C**) Percentage of pancreas area staining positive for insulin (mean ± SEM) in control (N=8) and 70% MNR (N=7) fetuses. Insulin staining in the 70% MNR group of 6.2 ± 0.5 % (vs 10.2 ± 1.1% in controls) was significantly lower ($P < 0.01$). (**D**) Percentage of pancreas area staining positive for glucagon (mean ± SEM) in control (N=8) and 70% MNR (N=7) fetuses. Glucagon staining the in the 70% MNR group of 2.5 ± 0.4% (vs 3.9 ± 0.2% in controls) was significantly less ($P < 0.01$).


A
Blood Glucose
Control
70% MNR
Blood Glucose (mmol/L)
7.5
5.0
2.5
0.0
*
*
Maternal
Fetal
B
Plasma Insulin
Control
70% MNR
Plasma Insulin (× 10² pg/mL)
20
10
0
*
*
Maternal
Fetal
C
Insulin Immunoreactivity
Pancreas Staining for Insulin (%)
15
10
5
0
*
Control
70% MNR
D
Glucagon Immunoreactivity
Pancreas Staining for Glucagon (%)
5
4
3
2
1
0
*
Control
70% MNR

embryos were used to determine insulin and glucagon immunoreactivity, and expressed as percentage of the pancreas occupied by β- and α-cells respectively. MNR embryos demonstrated a significantly reduced insulin immunoreactivity (**Fig. 3.6C**; control 10.2 ± 1.1 % *vs.* MNR 6.2 ± 0.5 %; $P < 0.01$), suggesting fewer beta cells in the fetal pancreas. A similar reduction was observed in glucagon immunoreactivity (**Fig. 3.6D**; control 3.9 ± 0.2 % *vs.* MNR 2.5 ± 0.4 %; $P < 0.01$). These findings indicate that MNR impeded development of both types of endocrine cells in fetal pancreas.

Studies of glucose tolerance were followed after birth to determine if there were long-term consequences to glucose metabolism following maternal undernutrition during gestation. At six months, adult male mice demonstrated glucose intolerance with a significantly increased area under the curve (AUC) for glucose (**Fig. 3.7C**; control 660 ± 61 *vs.* MNR 1287 ± 128; $P < 0.0001$). There was no significant difference in body weights between controls and MNR (control 50.1 ± 5.0 g *vs.* MNR 51.8 ± 5.7 g). Pancreatic weights were not different between the two groups (control 331 ± 70 mg *vs.* MNR 286 ± 50 mg). Fasting glucose at time 0 of the glucose tolerance test was not different between the two groups, however, each subsequent 30 min time point demonstrated significantly higher blood glucose levels (**Fig. 3.7A**). Insulin levels measured at 60 min and 120 min during the glucose tolerance test were not significantly different (**Fig. 3.7F**). MNR females did not show glucose intolerance at six months (**Fig. 3.7B** and **D**).

Due to the changes to glucose tolerance at 6 months, the structure of the male endocrine pancreas were studied. At this time point, the β-cell mass of adult male MNR mice was significantly decreased (**Fig. 3.8A**; control 9.1 ± 1.6 mg *vs.* MNR 2.8 ± 1.1 mg;

Figure 3.7 Glucose tolerance testing at six months postnatal. (**A**) Blood glucose levels (mean ± SEM) of control (N=13) and 70% MNR (N=9) male mice during glucose tolerance testing. Blood glucose levels were higher in the 70% MNR males from 30 to 120 minutes. (**B**) Blood glucose levels (mean ± SEM) of control (N=10) and 70% MNR (N=10) female mice during glucose tolerance testing, demonstrated no differences between groups. (**C**) Area under the curve (AUC) of the glucose tolerance test in 70% MNR male mice of 1287 ± 128 (vs 660 ± 61 in controls) was significantly elevated ($P < 0.0001$). (**D**) Area under the curve (AUC) of the glucose tolerance test in 70% MNR female mice demonstrated no differences between groups. (**E**) Plasma insulin concentration (mean ± SEM) of male mice during glucose tolerance testing, demonstrated no differences between groups.

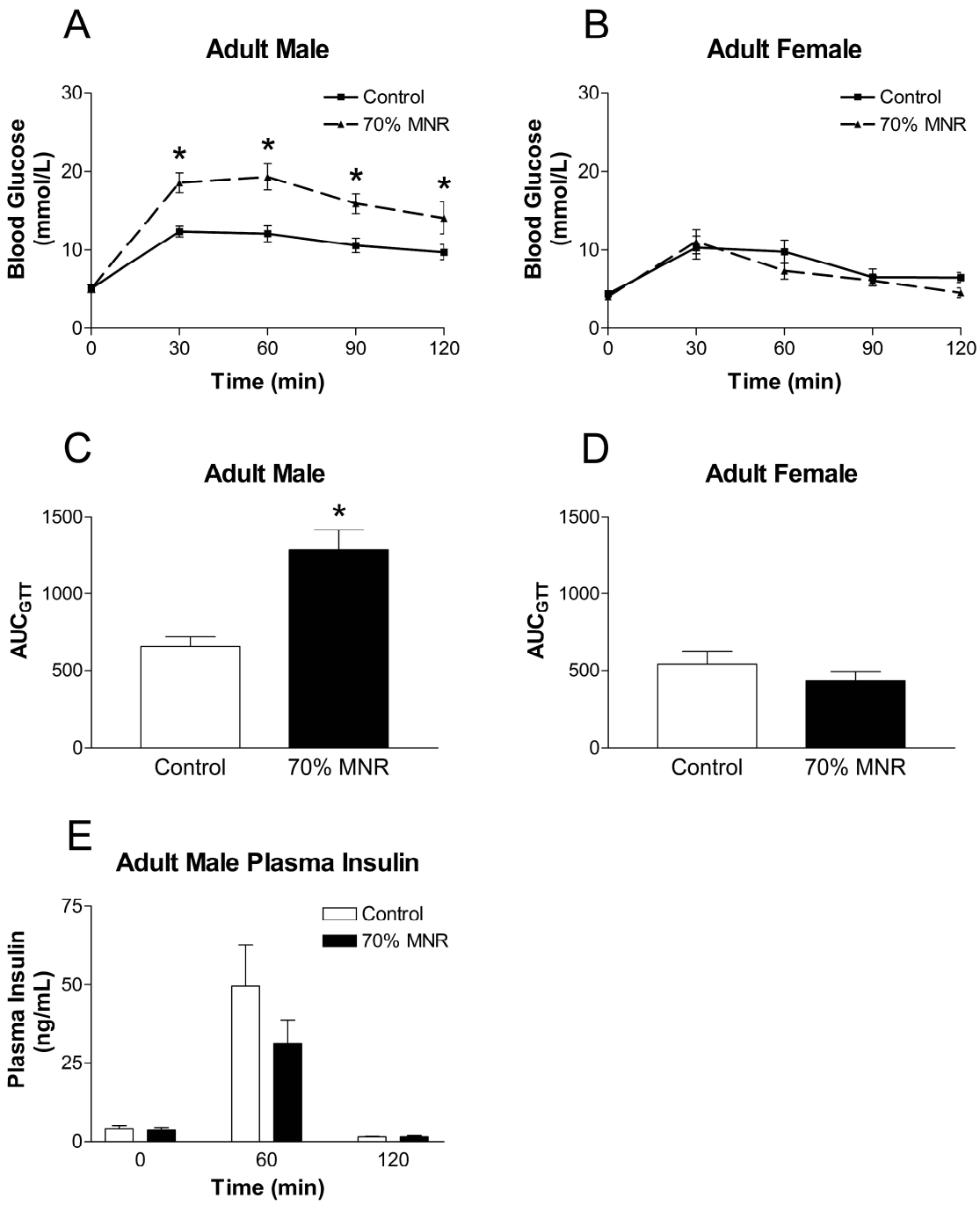
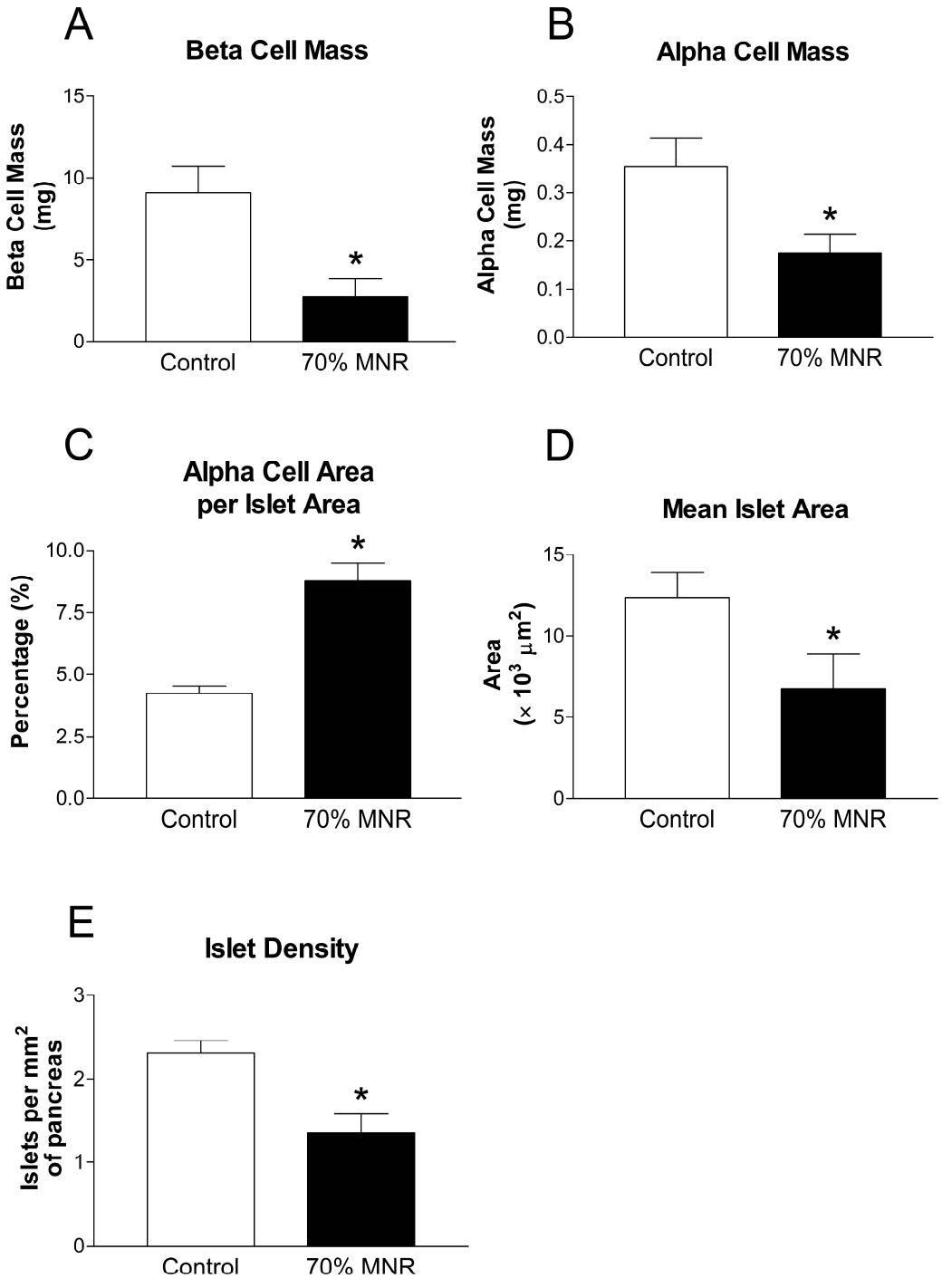

A
Adult Male
Control
70% MNR
Blood Glucose (mmol/L)
Time (min)
B
Adult Female
Control
70% MNR
Blood Glucose (mmol/L)
Time (min)
C
Adult Male
AUCGTT
Control
70% MNR
D
Adult Female
AUCGTT
Control
70% MNR
E
Adult Male Plasma Insulin
Control
70% MNR
Plasma Insulin (ng/mL)
Time (min)

Figure 3.8 Adult male endocrine pancreas structure at six months postnatally, following 70% MNR in utero. (**A**) Beta cell mass (mean ± SEM) in control (N=13) and 70% MNR (N=7) males. Beta cell mass in 70% MNR males of 2.8 ± 1.1 mg (vs 9.1 ± 1.6 mg in controls) was significantly reduced ($P < 0.05$). (**B**) Alpha cell mass (mean ± SEM) in control (N=13) and 70% MNR (N=7) males. Alpha cell mass in 70% MNR males of 0.18 ± 0.04 mg (vs 0.35 ± 0.06 mg in controls) was significantly reduced ($P < 0.05$). (**C**) Alpha cell area per islet area (mean ± SEM) in control (N=13) and 70% MNR (N=7) males. Alpha cell mass in 70% MNR males of 0.18 ± 0.04 mg (vs 0.35 ± 0.06 mg in controls) was significantly reduced ($P < 0.05$). (**D**) Area of islets (mean ± SEM) in control (N=13) and 70% MNR (N=7) males. Mean islet area in 70% MNR males of 6,753 ± 2,123 μm^2 (vs 12,373 ± 1,536 μm^2 in controls) was significantly reduced ($P < 0.05$). (**E**) Density of islets (mean ± SEM) per area of pancreas in control (N=13) and 70% MNR (N=7) males. Islet density in 70% MNR males of 1.4 ± 0.2 islets/mm^2 (vs 2.3 ± 0.1 islets/mm^2 in controls) was significantly reduced ($P < 0.005$).


A
Beta Cell Mass
Beta Cell Mass (mg)
15
10
5
0
*
Control
70% MNR
B
Alpha Cell Mass
Alpha Cell Mass (mg)
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
70% MNR
C
Alpha Cell Area per Islet Area
Percentage (%)
10.0
7.5
5.0
2.5
0.0
*
Control
70% MNR
D
Mean Islet Area
Area (× 10³ μm²)
15
10
5
0
*
Control
70% MNR
E
Islet Density
Islets per mm² of pancreas
3
2
1
0
*
Control
70% MNR

$P < 0.05$). The α-cell mass was also reduced (**Fig. 3.8B**; control 0.35 ± 0.06 mg *vs.* MNR 0.18 ± 0.04 mg; $P < 0.05$). Interestingly, the proportion of islet area staining for glucagon was greater in 70% MNR male mice (**Fig. 3.8C**; control 4.2 ± 0.3 % *vs.* MNR 8.8 ± 2.0 %; $P < 0.01$). In MNR male pancreases, the density of islets (**Fig. 3.8E**; control 2.3 ± 0.1 islets/mm^2 *vs.* MNR 1.4 ± 0.2 islets/mm^2; $P < 0.005$), and mean area of islets were significantly reduced (**Fig. 3.8D**; control 12,373 ± 1,536 μm^2 *vs.* MNR 6,753 ± 2,123; $P < 0.05$). The size of each α-cell and β-cell were not significantly different between the two groups, suggesting that the reduction in α- and β-cell mass was due to fewer α- and β-cells in 70% MNR males. Total pancreatic insulin content was not different between groups. This suggests MNR caused fewer endocrine cells to differentiate during development and beta cells were more affected than alpha cells. This reduction in endocrine cells carried through to adulthood contributing to development of glucose intolerance as adults.

3.4 DISCUSSION

This study showed that MNR in mice from E6.5 to E18.5 led to a reproducible FGR phenotype. MNR of 70% of normal caloric intake was the best model as it was most consistent without changing litter size and represents asymmetric human FGR. Increasing severity of MNR did not further reduce birth weight with 60% MNR. In MNR of 80%, fetal and organ weights (except for brain and placenta) were slightly less than controls. In 70% MNR, weights were significantly lower than controls and 80% MNR mice. However, in 60% MNR, fetal weights were lower than controls but comparable to 80% MNR. This could be due to a reduction in litter size, which would increase limited

nutrient supply to surviving fetuses. Thus, the 70% MNR was chosen for further detailed studies. This model led to findings of asymmetrical FGR with relative sparing of the brain, likely due to blood flow redistribution from less critical organs (liver, lung and gut) to more critical organs (brain, adrenals and heart) (23-27). Asymmetrical FGR occurs in humans when an insult, such as uteroplacental insufficiency or malnutrition, occurs in late second to third trimester (27, 28). Fetal and organ weights are simple measurements of growth, however they do not provide a true indication of how organ development has been affected by factors restricting fetal growth.

The concentrations of IGF system proteins in fetal and maternal circulation in human FGR have been described previously (10). In most reports, circulating IGF-I and IGFBP-3 concentrations are decreased, whereas IGFBP-1 concentrations are increased, and IGF-II concentrations are variably altered. Tissues of origin and causes of these changes have not been determined in human subjects. Studies of blood and tissues in the mouse model should provide insight into these important questions. In 70% MNR fetuses, fetal weights were reduced without significant changes in tissue IGF-I mRNA levels except the placenta. IGF-I mRNA levels were lower in most organs. The reduction of circulating IGF-I may be due to overall changes in tissues and reflect fetal response due to reduction in nutrient availability and a signal to decrease growth. It also suggests that circulating IGF-I in the fetus may be contributed not only by the liver as in growing animals or adults (29-31) but also by other tissues. The circulating levels of IGFBP-3 were reduced in 70% MNR fetuses similar to that of human FGR (10). Circulating IGFBP-3 is coupled to IGF-I and is usually decreased in human FGR (10), as IGF-I regulates its expression (32). IGFBP-3 mRNA expression was increased in the majority

of tissues, suggesting the importance of the autocrine/paracrine IGFBP-3 actions in FGR. Null mutation of *Igf1* or *Igf2* leads to a 40% reduction in fetal weight (11, 12) and *Igf2* null mice have smaller placentae and smaller fetuses, indicating its importance in placental and fetal development (11, 33-35). Human fetal serum IGF-II levels are not consistently correlated with birth weight, and do not tend to change with FGR (10). Therefore, it was not unexpected that MNR fetuses did not demonstrate changes to IGF-II transcript expression.

Despite lack of change in hepatic IGF-I or IGF-II mRNA levels, the mouse fetus may have responded to MNR and restricted growth by enhancing levels of hepatic IGFBPs to inhibit IGF actions. Transgenic mouse models over-expressing IGFBPs demonstrate a FGR phenotype, with organ-specific changes in weight and function (13). Transgenic mice over-expressing human IGFBP-1 in the fetal liver are 18% smaller at birth, with relative sparing of placental weight (15). Elevated IGFBP-1 serum levels are negatively correlated to birth weight (10), and IGFBP-1 sequesters IGF-I and –II with greater affinity than either receptor, preventing either protein from signaling fetal growth (36). IGFBP-2 mRNA expression was increased in the livers of MNR fetuses, leading to increased circulating levels. The significant increase in circulating IGFBP-1 and IGFBP-2 concentrations during MNR in the mouse mimics elevated circulating levels during human FGR and may contribute to the pathophysiology (37, 38). Studies of IGFBP-2 implicate this role as being primarily inhibitory to IGF actions, as transgenic IGFBP-2 mice demonstrate mild growth restriction and IGFBP-2 in vitro inhibited IGF-II mediated cell proliferation (39, 40). Our mouse model indicates that the source of increased

circulating IGFBPs is the liver suggesting that a similar pathophysiology occurs in humans.

Elevated IGFBPs in MNR livers may explain the reduction of glycogen despite elevated levels of glucocorticoids in fetal circulation, which have previously been demonstrated to enhance glycogen deposition prior to birth (41). Recently, IGF-II has been proposed to be a major regulator of fetal hepatic glycogen synthesis (7). *Igf2* knockout mice demonstrate significantly less glycogen stores which correlated with a reduction in glycogen synthase, the rate-limiting enzyme in glycogen synthesis, despite normal levels of circulating insulin (42). This suggests the importance of IGF-II to fetal metabolism and that the reduction in liver glycogen in MNR fetuses may be due to a reduction in IGF-II action, by elevating hepatic IGFBPs. Increased proliferation of liver cells supports an immature liver in MNR fetuses, as fewer hepatocytes are differentiated to express enzymes for glycogen synthesis and hepatocyte precursor cells continue to proliferate.

The elevation of circulating and tissue IGFBP expression may lead to impaired growth and development of the fetal pancreas. β-cell proliferation, differentiation and survival is dependent on IGF expression (43, 44). The elevated fetal IGFBPs may inhibit IGF action on the proliferation and maturation of β-cells (44). Decreases in total IGF availability may exist when apoptosis is high (43, 44). This is believed to be responsible for the wave of β-cell apoptosis in the neonatal rat observed 2 weeks postnatally and coincides with diminished pancreatic expression of IGF-II within the islets (45). A maternal low protein diet causes a decreased rate of fetal β-cell replication with increased incidence of apoptosis, which is associated with reduced expression of IGF-II within

islets (46). Therefore, the fetal availability of IGF-II may determine the number and function of adult β-cells. Similarly, altered expression of IGF or IGF binding proteins associated with FGR may cause a population of β-cells that are not equipped to adequately handle postnatal metabolic challenges (44). Transgenic IGFBP-1 mice were hyperglycemic, hyperinsulinemic and glucose intolerant with increased relative pancreatic weight. Pancreatic islets were significantly larger and more numerous, although pancreatic insulin content was reduce. Hyperglycemia in these mice may be explained by IGFBP-1 inhibiting the hypoglycemic effect of IGFs and the impaired pancreatic function may be a consequence of persistent hyperglycemia, which would also explain the insulin depletion (40). Therefore the intrauterine environment of hypoglycemia, low IGF levels with elevated IGFBPs may alter the development of the endocrine pancreas and its ultimate structure and function.

At six months, FGR male mice developed impaired glucose tolerance although the insulin secretion was similar to control male mice. FGR mice showed catchup growth and weight that was similar to control mice at weaning and six months postnatally. There was a reduction in both α- and β-cell mass, however β-cell mass showed a greater reduction as evident by α-cells contributing to a greater proportion of islets. Similar changes in α-cells in islets have been demonstrated in human populations (47) and baboon models of type II diabetes (48) and may contribute to glucose intolerance (48, 49). Several factors may contribute to reduction in β-cells following MNR during gestation. Low glucose levels may impede expression of Pdx-1, an inducer of early endocrine lineage cells in β-cells (44, 50). MNR during embryogenesis leads to low fetal glucose levels to limit Pdx-1 expression and the recruitment of endocrine cell precursors

(43), as demonstrated by reduced α- and β-cells mass at E18.5. This may cause FGR infants to have limited β-cell mass as adults and predispose them to diabetes. During catch up growth, high glucose levels along with elevated saturated fatty acids, lipoproteins, and leptin may overwhelm β-cells inducing apoptosis and/or necrosis (51). High glucose concentrations in early postnatal life may play a critical role, as this has been shown to stimulate β-cell apoptosis (51, 52). Increased β-cell apoptosis has been observed in humans with type 2 diabetes (53). The decrease in number of islets in pancreases of FGR males may be due to impaired development of pancreatic progenitor endocrine cells by MNR during gestation. The reduction in β-cell mass at six months in FGR males may result from reduced expression of IGFs or increased expression of IGFBPs during gestation and metabolic stress of catchup growth in FGR pups. Although pancreatic islet morphology was different in MNR males, plasma insulin levels were not different from controls during the glucose tolerance test. This suggests that there was insulin insensitivity. Glucokinase is an important hepatic "glucose sensor" as its activity to phosphorylate glucose to glucose-6-phosphate increases with elevated concentrations of blood glucose and insulin (54). In contrast, PEPCK catalyzes the rate-controlling step in gluconeogenesis, which allows cells to synthesize glucose from non-carbohydrate precursors. Insulin inhibits PEPCK expression and activity (55). Disruption of glucokinase activity (56-58) or overexpression of PEPCK (59, 60) demonstrates clinical signs consistent with type II diabetes mellitus in mice and humans (61, 62). Analysis of hepatic glucokinase and PEPCK activity demonstrates that despite adequate insulin release, glucokinase activity is low while PEPCK activity remains elevated in MNR males. This suggests that insulin may be ineffective at enhancing the activity of

glucokinase and suppressing the activity of PEPCK during glucose challenge and hence glucose continues to be released into the blood and is unable to be taken up into the hepatocytes. This may contribute to insulin insensitivity.

In summary, 70% MNR in mice caused consistent FGR without impacting on litter size with an asymmetrical FGR phenotype similar to that observed in human FGR due to uteroplacental insufficiency. The livers were significantly smaller in MNR mainly due to reduction in glycogen stores without impacting hematopoiesis. MNR also increased the synthesis of IGFBPs, primarily IGFBP-1 and IGFBP-2, that would lead to reduced IGF actions by sequestering bioavailable IGFs and/or inhibiting IGF actions at the tissue level. The tissue IGFBP-3 biosynthesis was increased in the majority of tissues. During pregnancy, 70% MNR is associated with hypoinsulinemia and hypoglycemia in both maternal and fetal circulation, with a reduction in the proportion of α- and β-cells in fetal pancreatic islets. Deficits in islet endocrine cells continued postnatally and persisted at six months of age. FGR males demonstrated impaired glucose tolerance. Therefore, changes to insulin and IGF endocrine systems due to MNR during gestation may be associated with neonatal morbidity as well as predisposition to metabolic diseases in childhood or adulthood.

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CHAPTER 4

PULMONARY CONSEQUENCES IN THE OFFSPRING OF MATERNAL NUTRIENT RESTRICTION PREGNANCIES

Manuscript prepared for Pediatric Research

4.1 INTRODUCTION

Suboptimal nutrition during fetal life can induce immediate and long-term alterations in the formation and function of many organs, including the lung (1). Fetal growth restriction (FGR) is a decrease in fetal growth that prevents a newborn from obtaining its optimal growth. Clinically, it is defined as a pregnancy in which estimated fetal weight is below the 10^{th} percentile for gestational age (2) resulting in a small for gestational age newborn with birth weight less than 10^{th} percentile for gestational age. Fetal growth restriction can have maternal, fetal or placental causes, however, the major pathophysiological condition that underlies most clinically relevant FGR is fetal undernutrition. In developed countries, placental insufficiency is the major etiology and it causes fetal undernutrition by impaired transfer of nutrients between mother and fetus due to placental disease despite an adequate supply in maternal circulation. In developing countries, poor maternal nutrition is the major etiology. FGR is associated with many adverse outcomes in multiple organ systems including the lungs, leading to poor respiratory function at all stages of postnatal life (3-7). Infants born prematurely are at

increased risk of lung diseases such as respiratory distress syndrome and bronchopulmonary dysplasia and fetal undernutrition may increase the severity and incidence of bronchopulmonary dysplasia (BPD) (8). Bronchopulmonary dysplasia is a disease observed primarily in very low birth weight (< 1 kg) preterm newborns (< 28 weeks) (9). The lungs of BPD are characterized by extreme immaturity with fewer and larger alveoli, thickened, fibrotic alveolar walls and decreased dysmorphic capillaries (10). These structural changes are believed to reflect an arrest in lung development (9) with long term consequences (11, 12). Advances in treatment, including antenatal steroids, postnatal exogenous surfactant and newer ventilation techniques for neonates, have improved survival of increasingly premature infants (11, 12). Antenatal glucocorticoids improve outcomes in premature infants by accelerating maturation of type II alveolar epithelial cells and the components of surfactant they produce.

FGR is an independent risk factor for the development of BPD suggesting that factors that control fetal somatic growth may have a significant impact on vulnerability to lung injury (13-16). The benefit of antenatal steroids to preterm fetuses who are FGR is questionable (9, 17-19), as FGR infants already have endogenously elevated glucocorticoid levels (17). In animal models, the administration of high dose glucocorticoids in newborns is used to recreate BPD (20, 21). High levels of glucocorticoids are known to inhibit cell proliferation by inhibiting DNA and protein synthesis, and this may contribute to the developmental arrest of lungs seen in infants and rodent models with BPD (20, 21). In human pregnancy, maternal cortisol concentrations are higher than in the fetus (17). This difference is maintained by 11β-hydroxysteroid dehydrogenase (11β-HSD) in the placenta, which controls the passage of cortisol from

mother to fetus. Two isoforms of 11β-HSD interconvert glucocorticoids with their inactive metabolites. 11β-HSD1 catalyzes bi-directional interconversion of cortisol and cortisone, but acts primarily as a reductase, converting inactive cortisone to active cortisol. 11β-HSD2 is a high-affinity unidirectional enzyme, catalyzing only the dehydrogenase reaction of cortisol into cortisone. Glucocorticoids are potent factors that influence tissue maturation in the fetus. However, both absence and excess of glucocorticoids are detrimental to normal fetal development. The intricate control of glucocorticoid action within fetal tissues by 11β-HSDs plays a critical role in normal fetal maturation, however FGR results when their control is disrupted. Alterations in the glucocorticoid hormone levels, glucocorticoid receptors and/or metabolizing enzymes have been implicated in developmental programming or developmental origins of health and disease. High levels of steroids to induce lung maturation in preterm FGR babies may harm rather than benefit, but the mechanism of how this occurs remains to be established.

Developmental programming of the pulmonary system in FGR has been reported in humans (1) but has been poorly studied due to lack of appropriate animal models. Our mouse model of FGR is potentially suitable to study lung development during FGR and the mechanisms compromising long-term pulmonary function. To make comparable, clinically relevant observations of expression changes of the glucocorticoid system during fetal undernutrition and their impact on fetal growth, we generated a mouse model of FGR by maternal nutrient restriction (MNR) in which mothers were fed 70% of normal daily caloric intake during pregnancy (Chapter 3). We studied lung growth and development by determining overall changes to weight and structure, and lung maturation by surfactant protein expression. Postnatal measurements of lung mechanics were

performed to determine the impact of MNR on postnatal lung function. Our overall hypothesis is that fetal undernutrition compromises fetal lung development with long-term adverse pulmonary function and elevated levels of fetal glucocorticoids contribute to this pathology.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Dietary Restriction Protocol

Animal protocols were approved by the University Council on Animal Care of the University of Western Ontario and maintained in accordance with guidelines of the Canadian Council for Animal Care. Ten week-old virgin CD-1 females and stud males were obtained from Charles River Laboratories (Montreal, PQ, Canada). Mice were housed in standard shoe box cages in the Victoria Research Laboratories Vivarium (London ON, Canada) with a 12:12 light:dark cycle with water provided *ad libitum*. Female mice were housed singly with stud males in late afternoon and checked the following morning for presence of a vaginal plug, indicating embryonic day 0.5 (E0.5). Females were then housed individually and fed using feeding tubes with cage clips (Bio-Serv, Frenchtown NJ, USA) and grain-based dustless precision pellets (#F0173, Bio-Serv, Frenchtown NJ, USA) containing 20.7% protein, 4.0% fat, 6.7% ash, 59.6% carbohydrates, 4.0% fiber and 5.0% moisture ad libitum until E6.5. Feeds for control mice were pre-weighed each morning and remaining pellets weighed at the same time next day to establish average daily feed intake during pregnancy. From E6.5, mice were fed daily 70% (n = 9) by weight of average daily intake of control mice (n = 24). Mice were euthanized at E18.5 by carbon dioxide inhalation. Fetal tissues and lung were

dissected, weighed, and pooled by litter before flash freezing in liquid nitrogen. Individual maternal and pooled fetal bloods were obtained using heparinized capillary tubes (Fisher Scientific, Unionville ON, Canada). For newborn blood collection, pups were decapitated at 9AM on postnatal days 1 and 3 and blood was collected in the same manner as maternal and fetal blood.

4.2.2 Quantitative real-time PCR (qRT-PCR)

Total RNA from flash frozen control and 70% MNR fetal tissues were extracted using Trizol (Invitrogen, Burlington, ON) and purified with RNAeasy Mini Kit (Sigma, Maryland, USA), following the manufacturer's instructions.

SYBR green qRT-PCR was used to study mRNA expression in fetal lungs of SP-A, -B, -C and -D and endogenous control gene, ribosomal protein L7 (RL7), and glucocorticoid receptor (GR), 11β-HSD1 and 28S endogenous control gene. Using reagents from Invitrogen (Burlington, ON), 1 μg of RNA was reversed transcribed (RT) using Superscript III and 500 ng of Oligo(dT) with 20U RNaseOUT in 21.5 μl final volume, followed by digestion of template RNA with 2U RNase H. Reactions were incubated at 70□C, 10 min; 42□C, 2 min; 70□C, 15 min; 4□C, 10 min and 37□C, 20 min. Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) was used as recommended with 200 μM of primers for SPs and RL7 and 600 μM for all others (Invitrogen; **TABLE 4.1**), mixed with 100 ng RT product in a total volume of 10 μl in triplicate. Thermal cycling conditions for surfactant protein (SP) and RL7 mRNA were 95□C, 10 min; 95□C, 20 sec; 60□C, 20 sec; 72□C, 30 sec for 40 cycles. For 11β-HSD

1, GR and 28S, the conditions were as previously described (23). qRT-PCR data was divided by

TABLE 4.1 Primer Sequences for SYBR Green Quantitative Real-Time PCR.

Primer Name	Primer Pair Sequence	Fragment Size (bp)	Ref
SP-A	5'-ATCAAGTGCAATGGGACAGA-3' 5'-CTTTCTCCAGGCTCTCCCTT-3'	230	-
SP-B	5'-GGCCTCACACTCAGGACTTC-3' 5'-GGACAAGGCCACAGACTAGC-3'	255	-
SP-C	5'-CTCCACACCCACCTCTAAGC-3' 5'-GTTTCTACCGACCCTGTGGA-3'	161	-
SP-D	5'-CCATGCTTGTCCTGCTTGTA-3' 5'-GTCCAGGTTCTCCAGCAGAG-3'	273	-
RL7	5'-GGAGCTCATCTATGAGAAGGC-3' 5'-AAGACGAAGGAGCTGCAGAAC-3'	202	-
GR	5'-GGAAGTTAATATTTGCCAATGGAC-3' 5'-CGCAGAAACCTTGACTGTAGC-3'	150	(22)
28 S	5'- TTGAAAATCCGGGGGAGAG-3' 5'-ACATTGTTCCAACATGCCAG-3'	99	(22)
11B HSD 1	5'-GTGTCTCGCTGCCTTGAACTC-3' 5'-TTTCCCGCCTTGACAATAAATT-3'	101	(22)

endogenous control values and each litter was divided by the control mean, to determine fold-change in mRNA expression.

4.2.3 Tissue Preparation

Fetal mouse lungs were harvested at E18.5, fixed in 4% paraformaldehyde for 48 hrs, and washed 3 times in PBS over 3 days. Tissue were embedded in paraffin, sectioned at 3 μm and mounted onto Superfrost® slides (Fisher Scientific, Fairlawn, NJ).

4.2.4 Immunofluorescence

Sections were deparaffinized and rehydrated in a descending series of ethanol solutions. Tissue sections were blocked with Background Sniper Blocking Reagent (Biocare Medical, Concord, CA) for 10 min prior to application of mouse anti-SP-B to lung sections at RT for 1 hr (**TABLE 4.2**). Tissue sections were washed and incubated for 30 min at RT with appropriate Alexa Fluor 568-conjugated IgG secondary antibody (1:200; Molecular Probes Inc., Eugene OR), counterstained with pre-mixed DAPI (Molecular Probes Inc.) and coverslipped with ProLong® Gold AntiFade Reagent (Molecular Probes Inc.). Omission of primary antibody served as controls for autofluorescence and nonspecific binding. Fluorescent images were captured and analyzed using a Carl Zeiss Axio Imager Z1 microscope equipped with AxioCamMR3 camera and AxioVision Release 4.7.2 software. To determine the proportion of lung composed of type 2 alveolar cells in eight control and six MNR lungs, the number of cells

TABLE 4.2 Primary Antibodies used for Immunoflurescence (IF) and Western Blotting (WB).

Antibody	Host	Catalogue #	Dilution		Source
			IF	WB	
SP-A	Rabbit	-	-	1:1000	gift from Dr. McCormick
SP-B	Mouse	-	1:200	1:1000	gift from Dr. Possmayer/Suzuki
proSP-C	Rabbit	WRAB-SPC	-	1:1000	Seven Hills, Cincinnati, OH
mSP-C	Rabbit	WRAB-MSPC	-	1:1000	Seven Hills, Cincinnati, OH
SP-D	Mouse	WMAB-1A10A9	-	1:1000	Seven Hills, Cincinnati, OH
11β-HSD1	Goat	AF3397	-	1:2000	R&D Systems Inc., Minneapolis, MN
Pan-actin	Mouse	#MS-1295	-	1:7000	Thermo Fisher Scientific, Fermont, CA
GAPDH	Rabbit	IMG-5567	-	1:5000	Imgenix, San Diego, CA

expressing SP-B per total number of cells were counted in 12 random fields of view at 400x magnification.

4.2.5 Lung Morphometry

Fetal lungs were collected from eight control and six MNR fetuses and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, and post fixed with 1% osmium tetroxide in 0.1 M phosphate buffer and dehydrated followed by transition through propylene oxide. Tissue samples were embedded in Polybed©/ araldite and cured at 65□C overnight. One micron sections were stained with toluidine blue. Total Lung Volume was determined by volume displacement (24). Sixteen random fields of view were taken with 200x magnification using ImagePro Plus 4.5 (Media Cybernetics, Bethesda, MD). The area occupied by airspace was traced and expressed as a fraction of the total field of view. This fraction was multiplied by the volume of the lung to determine absolute volume of airspace in the E18.5 mouse lung. Areas occupied by airways and blood vessels were counted separately and excluded from calculation.

Pooled fetal lungs from each litter were homogenized with protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany) in phosphate buffer. For protein quantification, a sample aliquot was syringed three times and centrifuged at 13,000 x g for 1 hr. Triplicate samples were quantified using BCA assay (Pierce; Rockford, IL). DNA quantification was performed by fluorometric DNA assay. An aliquot was centrifuged at 4000 x g for 5 min. The supernatant was diluted with 2 mM EDTA and Hoechst 33342 dye added (Molecular Probes; Eugene, OR). Duplicate measurements were obtained by fluorescence spectrophotometry (Fluoroskan Ascent FL,

ThermoLab Systems, Milford, MA), and quantified against a standard curve generated with salmon testis DNA (Sigma-Aldrich, Oakville, ON).

4.2.6 Corticosterone and ACTH ELISAs

Plasma corticosterone was measured using the AssayMax Corticosterone ELISA Kit (AssayPro, St. Charles, MO) as per manufacturer's instructions (**Appendix 1.1.1**). Plasma ACTH was quantified using a multiplexed bone metabolism mouse panel 1A biomarker immunoassay kit according to the manufacturer's instruction (Milliplex, Millipore Corp, MA). A Bio-Plex™ 200 readout System was used (Bio-Rad Laboratories, Hercules, CA), which utilizes Luminex® xMAP™ fluorescent bead-based technology (Luminex Corp., Austin, TX). Levels were automatically calculated from standard curves using Bio-Plex Manager software (v.4.1.1, Bio-Rad).

4.2.7 Western Blotting

Surfactant protein expression was determined in the membrane fraction of fetal lungs. Membrane fractions were prepared from frozen tissue, homogenized in cold lysis buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4 with protease inhibitor cocktail) and centrifuged at 12,000 x g for 10 min. The supernatant was centrifuged at 100,000 x g for 1 hr with the pellet resuspended in PBS. For 11β-HSD1, lung and placenta were prepared as previously described (25), and homogenates were used for Western blotting and enzyme activity assays. Protein content of lung and placental homogenates was determined by BCA assay (Pierce, Rockford, IL).

Western blotting was performed using 10-20 μg of total protein loaded into a 12.5% SDS-PAGE gel, and electrophoresis performed at constant 100 V for 2 hrs. Protein was transferred to PVDF membrane for 1.5 hrs at constant 350 mAmp. After transfer, membranes were blocked with 5% skim milk in TBS for 1 hr at RT, then incubated with primary antibody overnight at 4□C (**TABLE 4.2**). After washing, membranes were incubated with the appropriate peroxidase-labeled secondary antibody for 1 hr, washed and bands were visualized with Western Lighting Enhanced chemiluminescence detection reagents (Perkin-Elmer, Boston, MA). Blots were stripped and reprobed for mouse anti-pan-actin as a loading control for SPs and rabbit anti-GAPDH for 11β-HSD1. Blot analysis was performed by densitometry using Alpha Imager and FluoroChem 8800 software (Alpha Innotech Corporation, San Leandro, CA). Bands of interest were normalized to loading control and values for each litter normalized to control mean, in order to determine fold-change in protein expression.

4.2.8 11β-HSD1 Dehydrogenase Activity Assay

11β-HSD1 dehydrogenase activities in placenta and lung homogenates were determined as previously described (25), with the following modifications. The conversion assay tubes contained tissue homogenate (0.1-0.2 g protein), 100,000 dpm [^{3}H]-corticosterone, and final concentrations of non-radioactive corticosterone and cofactor NADP at 1 and 500 μM, respectively.

4.2.9 FlexiVent Measurements of Lung Mechanics

Mice were weighed and injected with a Euthanyl dose of 110mg/kg. The mouse was exsanguinated by abdominal incision and locating and cutting abdominal blood vessels when the mouse was adequatedly anaesthetized as determined by toe pinch. The neck was exposed and dissected with a small incision made in the trachea. An 18G metal endotracheal tube was inserted and ligated with surgical silk. The endotracheal tube was attached to the ventilator of the flexiVent system (Scireq, Montreal, Canada), with the mouse supine and thoracic cavity opened with connective tissues dissected away to determine changes to lung mechanics irrespective of tension contributed by the chest wall. The flexiVent was used to measure lung mechanics, including lung compliance (distensibility), lung resistance (obstruction of flow in relation to time), airway resistance (including both the large central and smaller peripheral airways), tissue damping (tissue resistance), tissue elastance, and tissue hysterisivity (ratio of the lungs ability to dissipate and conserve energy). The flexiVent technique involves 2 different perturbations, with the first determining whole-lung dynamic compliance and lung resistance by fitting the linear single-compartment model using multiple linear regression. The second perturbation distinguished central versus peripheral lung mechanics, reflecting the contributions of airways versus peripheral tissue, respectively. These measurements, which included airway resistance, tissue damping, tissue elastance, and tissue hysterisivity, were obtained using the forced oscillation technique that fits the constant phase model to input impedence.

4.2.10 Statistical Analysis

All data are expressed as mean ± SEM. Parameters measured in control and 70% MNR animals were compared with Student's non-paired two-tail t test. A Mann-Whitney test was performed to determine significantly different fold changes in the 70% MNR litters for Western blot densitometry and qRT-PCR. Significance was set at $P < 0.05$.

4.3 RESULTS

4.3.1 Effects of Fetal Growth restriction on Fetal Organ Weights

Measurements of fetus and lung weights were recorded to determine the impact of maternal undernutrition. Fetal weights were significantly reduced in the MNR group (**Fig. 4.1A**; Control: 1,396 ± 25 mg vs. MNR: 894 ± 52 mg; $P < 0.0001$). Lung weights were significantly reduced in the MNR group (**Fig. 4.1B**; Control: 52 ± 2 mg vs. MNR: 29 ± 2 mg; $P < 0.0001$). Lung weights were disproportionately smaller compared to fetal weights in 70% MNR fetuses (**Fig. 4.1C**; control 3.70 ± 0.06, 70% MNR 3.15 ± 0.12, all values $\times 10^{-2}$; $P < 0.0005$).

4.3.2 Effects of FGR on Lung Structure

Fetal lung structure was analyzed at E18.5 to determine alterations during maternal undernutrition. Fetal lung volume was significantly less in 70% MNR (**Fig. 4.2A**; control 33.5 ± 1.8 mm^3 vs. MNR 25.3 ± 1.0 mm^3; $P < 0.005$), with less lung parenchyma volume (**Fig. 4.2A**; control: 21.3 ± 1.2 mm^3 vs. MNR 15.7 ± 1.6 mm^3; $P < 0.05$) however, airspace volume was maintained. The proportion of type 2 alveolar cells was significantly reduced in MNR lungs compared to controls (**Fig. 2B**; control 12.2 ±

Figure 4.1 The effect of maternal nutrient restriction on fetal weights at E18.5. (**A**) Fetal weight (mean ± SEM) in control (N=14) and 70% MNR (N=9) litters. In the 70% MNR fetuses, fetal weight of 894 ± 52 mg (vs 1,396 ± 25 mg in controls) was significantly less ($P < 0.001$). (**B**) Fetal lung weight (mean ± SEM) in control (N=14) and 70% MNR (N=9) litters. In the 70% MNR fetuses, lung weight of 29 ± 2 mg (vs 52 ± 2 mg in controls) was significantly reduced ($P < 0.001$). (**C**) Ratio of fetal lung weight to fetal body weight (mean ± SEM) in control (N=14) and 70% MNR (N=9) litters. In the 70% MNR litters, the ratio of $3.15 \pm 0.12 \times 10^{-2}$ (vs $3.70 \pm 0.06 \times 10^{-2}$ in controls) was significantly reduced ($P < 0.0005$).

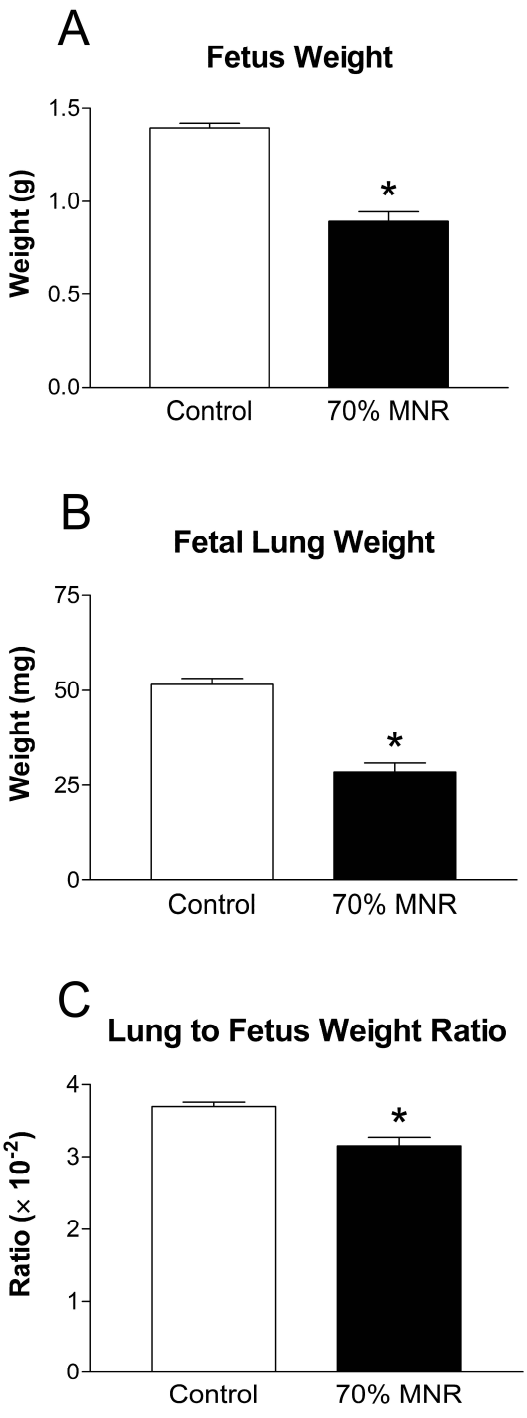
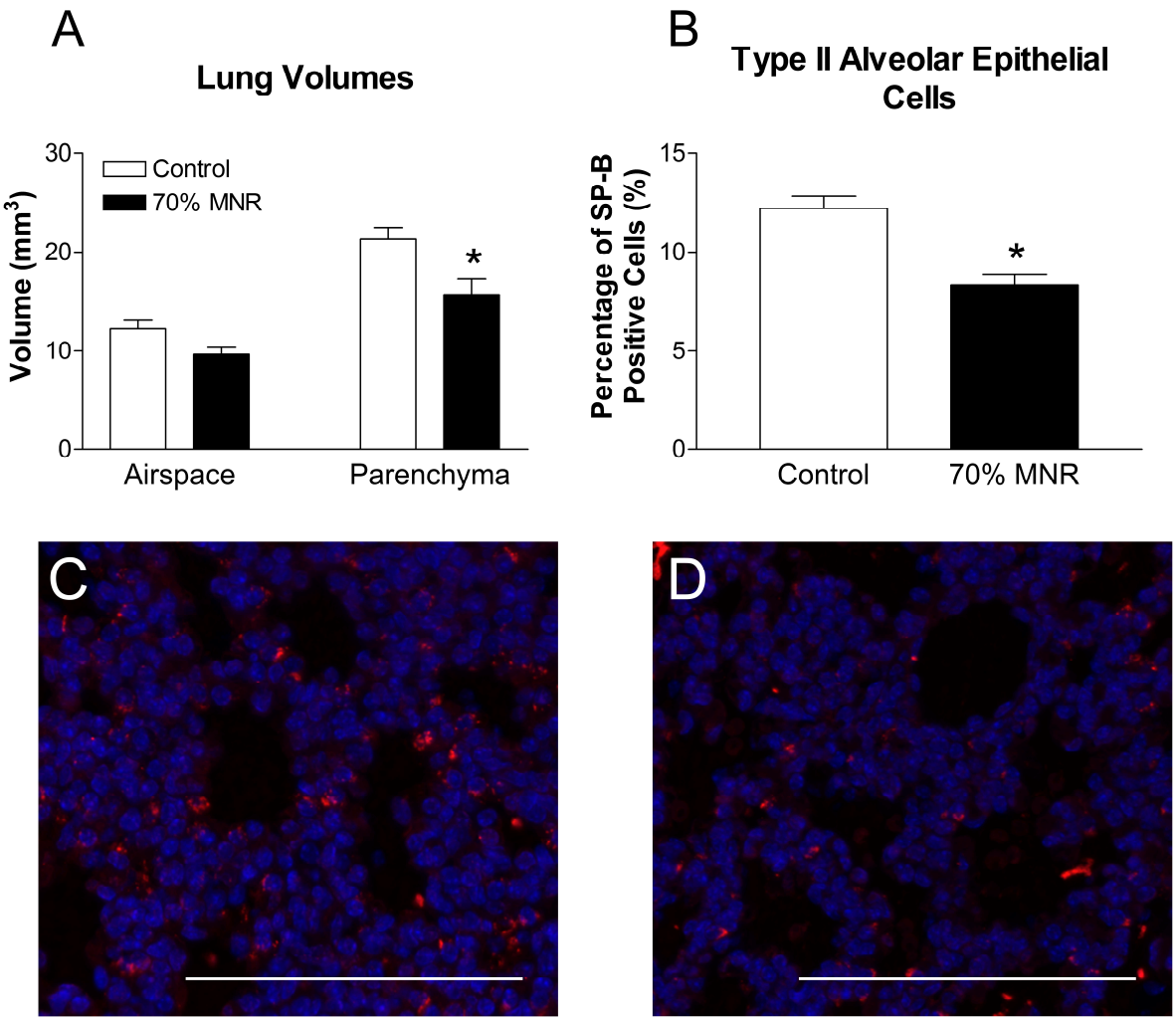

A
Fetus Weight
Weight (g)
1.5
1.0
0.5
0.0
*
Control
70% MNR
B
Fetal Lung Weight
Weight (mg)
75
50
25
0
*
Control
70% MNR
C
Lung to Fetus Weight Ratio
Ratio (× 10-2)
4
3
2
1
0
*
Control
70% MNR

Figure 4.2 The effect of maternal nutrient restriction on fetal lung structure at E18.5. (**A**) Lung airspace and parenchyma volume (mean ± SEM) in control (N=8) and 70% MNR (N=6) fetuses. In the 70% MNR fetuses, parenchyma volume of 15.7 ± 1.6 mm^3 (vs 21.3 ± 1.2 mm^3 in controls) was significantly less ($P < 0.05$), with no change to airspace volume. (**B**) Percentage of type II alveolar cells (mean ± SEM) in control (N=8) and 70% MNR (N=6) fetal lungs. In the 70% MNR fetuses, the percentage of type II cells of 8.3 ± 0.6 % (vs 12.2 ± 0.6 % in controls) was significantly reduced ($P < 0.001$). (**C**) Representative fluorescent photomicrograph of control lung with SP-B staining. (**D**) Representative fluorescent photomicrograph of 70% MNR lung with SP-B staining. *Scale bars*, 100 μm.


A
Lung Volumes
Volume (mm3)
30
20
10
0
Control
70% MNR
*
Airspace
Parenchyma
B
Type II Alveolar Epithelial Cells
Percentage of SP-B Positive Cells (%)
15
10
5
0
*
Control
70% MNR
C
D

0.6% vs. MNR 8.3 ± 0.6%; $P < 0.001$) with no change in DNA and protein content (μg/mg wet weight; **Appendix TABLE 1.1** and **1.2**). Type 2 alveolar cells produce the components of surfactant, important for increasing lung compliance during respiration postnatally. Surfactant proteins were analyzed to determine expression changes during maternal undernutrition. MNR lungs had lower levels of SP-A and -B mRNA with no changes in other SPs (**Fig. 4.3A**; fold change in MNR: SP-A: 0.57 ± 0.04; SP-B: 0.56 ± 0.10; both $P < 0.05$). Western blotting for proteins demonstrated significantly lower levels of SP-A and precursor and mature forms of SP-C (**Fig. 4.3B and C**; fold change of SP-A in MNR: 0.55 ± 0.10; $P < 0.05$; proSP-C: 0.48 ± 0.07; $P < 0.001$; mSP-C: 0.62 ± 0.08; $P < 0.05$).

4.3.3 Effect of FGR on the glucocorticoid system

Glucocorticoid levels are known to be an important regulartor of lung development during gestation; levels were measured at E18.5 to determine changes to expression during maternal undernutrition. Plasma corticosterone levels, the major glucocorticoid in rodents, were measured in mothers and their litters at E18.5. Plasma levels were significantly higher by 5-fold in 70% MNR mothers and their litters (**Fig. 4.4A**; Mothers: control 150 ± 69 ng/mL vs. MNR 757 ± 195 ng/mL; $P < 0.05$; Fetus: control 182 ± 21 ng/mL vs. MNR 435 ± 74 ng/mL; $P < 0.01$). Corticosterone was measured in neonates at 1 and 3 postnatal days, with plasma levels normalizing to control levels (**Fig. 4.4B**). At E18.5, ACTH levels in 70% MNR maternal plasma were significantly increased (**Fig. 4.4C**; control 5 ± 1 pg/mL vs. MNR 15 ± 4 pg/mL; $P <$

Figure 4.3 The effect of maternal nutrient restriction on fetal surfactant protein expression at E18.5. (**A**) Fold change of surfactant protein (SP) transcript levels (mean ± SEM) in control (N=6) and 70% MNR (N=6) litters. In the 70% MNR litters, the fold change of SP-A of 0.57 ± 0.04 (vs 1.00 ± 0.08 in controls) and SP-B of 0.56 ± 0.10 mg (vs 1.00 ± 0.16 in controls) were significantly reduced (both $P < 0.05$). (**B**) Representative Western Blots for SP-A, SP-C (precursor (proSP-C) and mature forms (mSP-C)), SP-D and SP-B and action loading control in pooled fetal lung homogenates of mothers fed either control (C) or 70% MNR (N) during gestation. (**C**) Fold change of surfactant protein (SP) levels (mean ± SEM) in control (N=7) and 70% MNR (N=7) litters by densitometry. In the 70% MNR litters, the fold change of SP-A of 0.55 ± 0.06 (vs 1.00 ± 0.10 in controls), the precursor form of SP-C of 0.48 ± 0.07 (vs 1.00 ± 0.15 in controls) and the mature form of SP-C of 0.62 ± 0.08 (vs 1.00 ± 0.11 in controls) were all significantly lower ($P < 0.05$, $P < 0.001$ and $P < 0.05$, respectively).

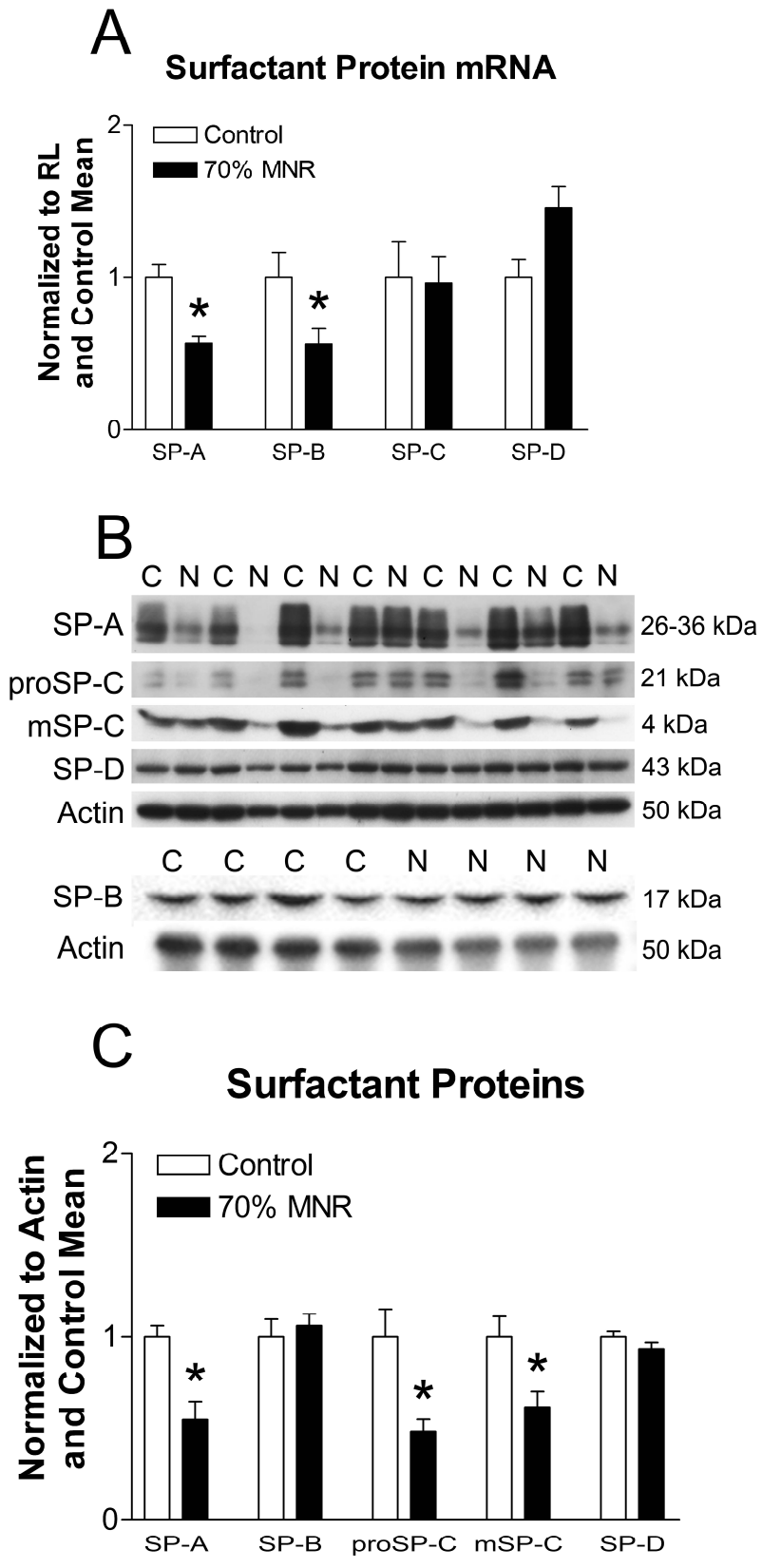
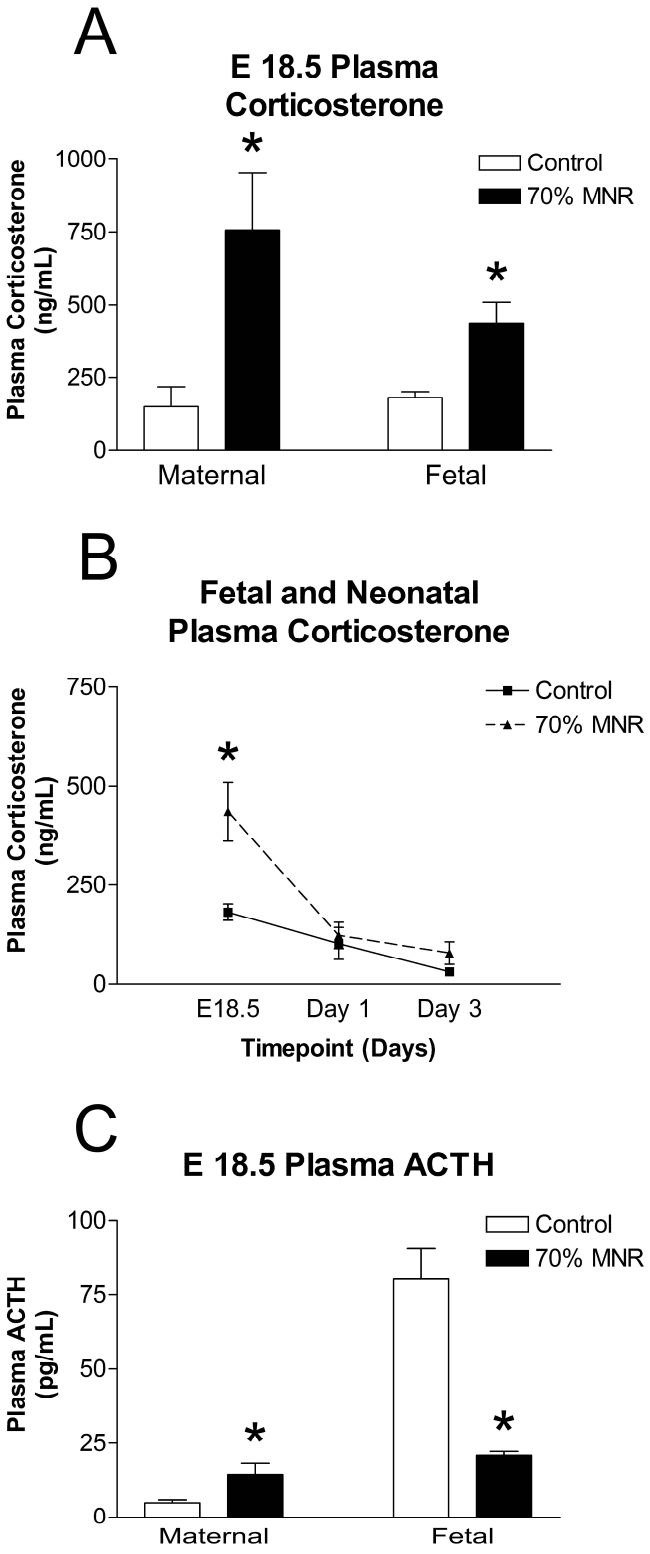

A
Surfactant Protein mRNA
Normalized to RL and Control Mean
Control
70% MNR
2
1
0
*
*
SP-A
SP-B
SP-C
SP-D
B
C N C N C N C N C N C N C N
SP-A
26-36 kDa
proSP-C
21 kDa
mSP-C
4 kDa
SP-D
43 kDa
Actin
50 kDa
C C C C N N N N
SP-B
17 kDa
Actin
50 kDa
C
Surfactant Proteins
Normalized to Actin and Control Mean
Control
70% MNR
2
1
0
*
*
*
SP-A
SP-B
proSP-C
mSP-C
SP-D

Figure 4.4 The effect of maternal nutrient restriction on circulating concentrations of corticosterone and ACTH in maternal and fetal at E18.5 and in neonatal plasma 1 and 3 days following birth. (**A**) Maternal plasma corticosterone concentration (mean ± SEM) was 150 ± 69 ng/mL in controls (N=6) and 757 ± 195 ng/mL in 70% MNR (N=6). Corticosterone concentration was 5 times higher in 70% MNR mothers ($P < 0.05$). Fetal plasma corticosterone concentration (mean ± SEM) was 182 ± 21 ng/mL in controls (N=6) and 435 ± 74 ng/mL in 70% MNR (N=6). Corticosterone concentration was 2.4 times higher in 70% MNR fetuses ($P < 0.01$). (**B**) Neonatal plasma corticosterone concentration (mean ± SEM) was not different between control (N=7) and 70% MNR (N=5) neonates at 1 and 3 days following birth. (**C**) Maternal plasma ACTH concentration (mean ± SEM) was 4.9 ± 1.0 pg/mL in controls (N=5) and 14.5 ± 3.7 pg/mL in 70% MNR (N=6). ACTH concentration was 3 times higher in 70% MNR mothers ($P < 0.05$). Fetal plasma ACTH concentration (mean ± SEM) was 80.2 ± 10.3 pg/mL in controls (N=6) and 20.9 ± 1.3 pg/mL in 70% MNR (N=6). ACTH concentration was 4 times less in 70% MNR fetuses ($P < 0.01$).


A
E 18.5 Plasma Corticosterone
Plasma Corticosterone (ng/mL)
1000
750
500
250
0
Control
70% MNR
*
*
Maternal
Fetal
B
Fetal and Neonatal Plasma Corticosterone
Plasma Corticosterone (ng/mL)
750
500
250
0
Control
70% MNR
*
E18.5
Day 1
Day 3
Timepoint (Days)
C
E 18.5 Plasma ACTH
Plasma ACTH (pg/mL)
100
75
50
25
0
Control
70% MNR
*
*
Maternal
Fetal

0.05), but significantly reduced in MNR fetal plasma (**Fig. 4.4C**; control 80 ± 10 pg/mL vs. MNR 21 ± 1 pg/mL; $P < 0.0005$).

Regardless of elevated endocrine levels of glucocorticoids in the MNR fetuses, it is ultimately the expression of and activity of glucocorticoid metabolizing enzymes in the tissues and expression if the glucocorticoid receptor that determines the effect on tissue structure. In the 70% MNR, placental 11β-HSD1 dehydrogenase protein expression and activity was reduced (**Fig. 4.5**; MNR Protein fold change 0.75 ± 0.09; $P < 0.05$; Activity: control 7.23 ± 0.45 pmol/min/mg protein vs. MNR 6.00 ± 0.27 pmol/min/mg protein; $P < 0.05$) with no change in mRNA expression (fold change in MNR: 1.48 ± 0.25; $P > 0.05$). No changes in the expression of GR (MNR mRNA fold change: 1.04 ± 0.29; $P > 0.05$) or 11β-HSD1 dehydrogenase activity (MNR mRNA fold change: 1.07 ± 0.16; MNR Protein fold change 0.87 ± 0.08; MNR activity fold change 0.87 ± 0.12; all $P > 0.05$) were demonstrated in MNR fetal lung.

4.3.4 Postnatal Pulmonary Function

The Flexivent system was used to measure lung function at one and three months after birth to determine the longterm postnatal consequences of maternal undernutrition during gestataion. There were no differences in body weight between the control and experimental animals at one and three months postnatally. The combined cohort of males and females demonstrated significantly increased lung (**TABLE 4.3**; control 1.07 ± 0.07 cm H_2O·s/mL vs. MNR 1.48 ± 0.16 cm H_2O·s/mL; $P<0.05$) and airway resistance (**TABLE 4.3**; control 0.42 ± 0.05 cm H_2O·s/mL vs. MNR 0.54 ± 0.03 cm H_2O·s/mL; $P<0.05$) with an increase in tissue elastance (control 68.1 ± 2.9 cm H_2O/mL vs. MNR

Figure 4.5 The effect of maternal nutrient restriction on placental 11β-HSD1 expression and activity. (**A**) Fold change of Placental 11β-HSD1 mRNA transcript expression (mean ± SEM) in control (N=6) and 70% MNR (N=6) litters did not differ between groups. (**B**) Fold change of placental 11β-HSD1 protein expression (mean ± SEM) in control (N=9) and 70% MNR (N=7) litters by densitometry. In the 70% MNR litters, the fold change of 11β-HSD1 of 0.75 ± 0.09 (vs 1.00 ± 0.07 in controls) was significantly reduced (both $P < 0.05$). (**C**) Placental 11β-HSD1 protein activity (mean ± SEM) in control (N=9) and 70% MNR (N=7) litters. In the 70% MNR litters, the activity of 11β-HSD1 of 6.00 ± 0.27 pmol/min/mg protein (vs 7.23 ± 0.45 in controls) was significantly reduced (both $P < 0.05$).

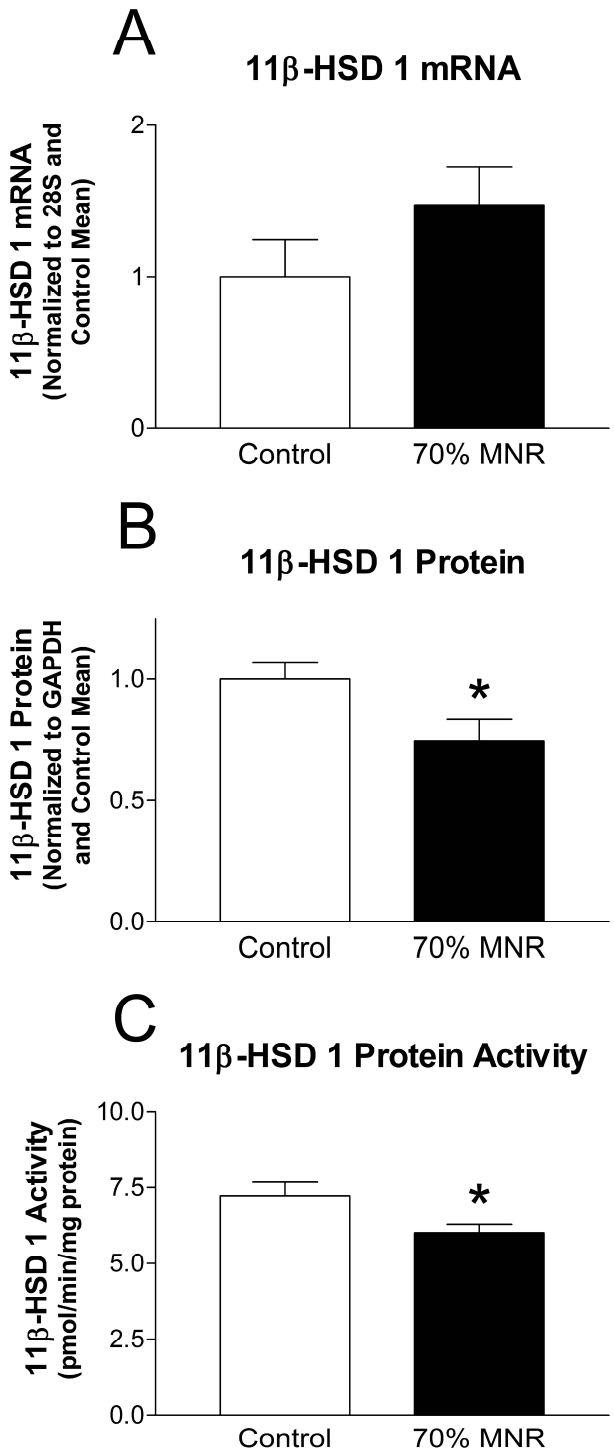

A
11β-HSD 1 mRNA
11β-HSD 1 mRNA (Normalized to 28S and Control Mean)
2
1
0
Control
70% MNR
B
11β-HSD 1 Protein
11β-HSD 1 Protein (Normalized to GAPDH and Control Mean)
1.0
0.5
0.0
*
Control
70% MNR
C
11β-HSD 1 Protein Activity
11β-HSD 1 Activity (pmol/min/mg protein)
10.0
7.5
5.0
2.5
0.0
*
Control
70% MNR

TABLE 4.3 Lung function measurements in control and maternal nutrient restricted mice at one and three months postnatally.

One Month Postnatal	ALL		MALE		FEMALE	
	Control	70% MNR	Control	70% MNR	Control	70% MNR
Resistance ($cmH_2O \cdot s/mL$)	1.07 ± 0.07	1.48 ± 0.16*	1.15 ± 0.11	1.20 ± 0.17	0.99 ± 0.08	1.77 ± 2.11**
Compliance (mL/cmH_2O)	0.014 ± 0.001	0.012 ± 0.001	0.014 ± 0.001	0.014 ± 0.001	0.014 ± 0.001	0.010 ± 0.001***
Airway Resistance ($cmH_2O \cdot s/mL$)	0.42 ± 0.05	0.54 ± 0.03*	0.47 ± 0.09	0.50 ± 0.04	0.37 ± 0.03	0.59 ± 0.06**
Inertance ($cmH_2O \cdot s \cdot s/mL$)	0.0015 ± 0.0003	0.0022 ± 0.0004	0.0012 ± 0.0005	0.0013 ± 0.0005	0.0019 ± 0.0001	0.0032 ± 0.0005*
Tissue Damping (cmH_2O/mL)	10.5 ± 1.0	14.4 ± 1.8	11.7 ± 1.7	11.3 ± 1.6	9.3 ± 0.8	17.6 ± 2.7*
Three Months Postnatal						
	ALL		MALE		FEMALE	
	Control	70% MNR	Control	70% MNR	Control	70% MNR
Resistance ($cmH_2O \cdot s/mL$)	0.46 ± 0.02	0.63 ± 0.03***	0.46 ± 0.04	0.62 ± 0.07	0.45 ± 0.03	0.65 ± 0.04**
Compliance (mL/cmH_2O)	0.026 ± 0.001	0.021 ± 0.001*	0.023 ± 0.002	0.023 ± 0.003	0.030 ± 0.001	0.019 ± 0.001***
Airway Resistance ($cmH_2O \cdot s/mL$)	0.20 ± 0.01	0.30 ± 0.03*	0.20 ± 0.02	0.30 ± 0.08	0.21 ± 0.03	0.30 ± 0.02
Inertance ($cmH_2O \cdot s \cdot s/mL$)	0.0009 ± 0.0001	0.0010 ± 0.0001	0.0007 ± 0.0001	0.0009 ± 0.0002	0.0012 ± 0.0002	0.0012 ± 0.0001
Tissue Damping (cmH_2O/mL)	4.6 ± 0.2	6.2 ± 0.3***	4.6 ± 0.3	5.9 ± 0.7	4.6 ± 0.2	6.5 ± 0.3***

Pregnant mothers were fed ad libitum (control) or 70% MNR from E 6.5. Following birth, 70% MNR pups were cross-fostered to control mothers and fed ad libitum after weaning. The SCIREQ flexiVent system measured lung function (mean ± SEM) at 1 and 3 months postnatally. 'All' compasses both male and female genders of the cohort. 'Male' and 'female' is analysis of cohort members separately by gender.

Significant differences between control and 70% MNR among the entire cohort, male and female gender is indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$

89.0 ± 8.1 cm H_2O/mL; P<0.05). However, once the cohort was divided by gender, the MNR males did not demonstrate any significant differences in pulmonary function from the control males. At three months the increase in lung resistance in 70% MNR females was maintained, however the increase in airway resistance had resolved. Male MNR mice did not demonstrate any differences in lung function measurements at three months (**TABLE 4.3**).

4.4 DISCUSSION

Elevated glucocorticoid level is important in preparing the fetus for birth and postnatal life, as it stimulates differentiation and maturation of organ systems. This has led to its administration to mothers with threatened preterm labour prior to 34 weeks gestation and has resulted in greatly improve neonatal outcomes. However, repeated dosing with antenatal glucocorticoids has been shown to cause FGR in animal models and possibly humans (26-28) as glucocorticoids can inhibit DNA replication and protein synthesis (29). Prolonged and elevated fetal glucocorticoid levels may inhibit the growth and development of glucocorticoid sensitive tissues such as the lung. In this study, we demonstrated that MNR significantly reduced fetal lung weights more than the fetal weight as the ratio of lung weight to fetal weight was significantly reduced. Despite the significantly lower weight of the MNR fetal lung, the proportion of cells and proteins per milligram of wet lung weight was not different between groups. Therefore the size and protein content of MNR lung cells were not different from controls, but the overall reduction in the number of MNR cells in the lung contributed to the reduced MNR lung weight. Our MNR growth restricted neonates from FGR pregnancies were born at term

and did not demonstrate respiratory difficulties after birth, therefore it is not an appropropriate model for bronchopulmonary dysplasia (BPD). However, our MNR mouse model may be a valuable model to study the changes to lung structure during FGR and the long-term consequences of impaired lung function postnatally, as there are currently no adequate models to study this organ system following FGR.

The 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes in the placenta have been implicated as one of the key components in the pathogenesis of FGR due to its primary function in maintaining the glucocorticoid barrier, buffering the fetus from high maternal glucocorticoid levels by metabolizing corticosterone in the placenta and in fetal tissues. Dysfunction of these enzymes can lead to the elevated fetal glucocorticoid levels causing FGR. In human fetal growth, the activity of 11β-HSD2 is positively correlated to birth weight, with its activity reduced in FGR pregnancies (17). In the mouse after E16.5, 11β-HSD2 expression declines and is no longer detectable in the fetal component of the mouse placenta, the labyrinth, or the fetal lung (22). In placenta from FGR mice, the expression of 11β-HSD1, which can act as a reductase or dehydrogenase, is high at E18.5 in the labyrinth layer and in vitro acts predominantly as a dehydrogenase (25). In the 70% MNR placenta, 11β-HSD1 protein expression and dehydrogenase activity were reduced, which is similar to 11β-HSD2 levels and activity reported in human FGR. With elevated corticosterone levels in the mother and reduced corticosterone metabolism in the placenta, more maternal corticosterone can enter the 70% MNR fetuses to impair fetal growth. Subsequently, high fetal corticosterone levels exert negative feedback on fetal pituitary ACTH release and inhibit the activity of the fetal HPA axis prior to parturition. Therefore, during pregnancy, the FGR fetus loses the ability to regulate its own plasma

levels of corticosterone due to the transfer of maternal corticosterone. Once the FGR fetus is born, the ability to control its circulating levels of corticosterone is reestablished. Despite the elevated levels of corticosterone in the fetal plasma, the expression of glucocorticoid receptor (GR) and the expression and activity of 11β-HSD1 did not change to compensate for the elevated plasma levels of glucocorticoids. Thus, the fetal lung was not protected from the effects of greatly elevated plasma corticosteroids in FGR.

Mouse models with genetic disruption of the corticotrophin releasing hormone (CRH) (30) or glucocorticoid receptor (GR) (31) demonstrate fetal lungs characterized by loss of cellularity and thinning of saccules and cell differentiation, indicating that glucocorticoids are essential to achieve anatomic maturation. Conversely, highly elevated glucocorticoids in the fetus impair lung development. In 70% MNR fetuses in which circulating levels of corticosterone were highly elevated, the lungs showed the volume of airspace maintained with less parenchymal volume. These changes in lung structure together with a disproportionately smaller lungs compared to the fetus support the growing evidence that FGR leads to developmental alterations in lung structure that impair lung function at birth and possibly postnatally. In a cohort of carunclectomized sheep, FGR fetuses in late gestation demonstrated lung growth sparing, with an increased number of air spaces and reduction in gas exchange surface density (32). Postnatal FGR lambs at 8 weeks had evidence of increased blood-air barrier thickness, likely due to edema and/or deposition of increased amounts of extracellular matrix, which may permanently reduce lung compliance (1). A similar long-term functional consequence of FGR was demonstrated in our mouse model of female FGR adults.

Our study, in combination with other FGR models demonstrating aberrant surfactant protein expression, questions the benefit of administering exogenous glucocorticoids to mothers with FGR in which circulating levels are already increased in both mother and fetus. Sheep fetuses that are growth restricted by carunclectomy demonstrated SP-A and -B gene expression was inversely related to plasma cortisol concentration with reduced lung SP-A, -B and –C protein and mRNA expression (33). Maternal undernutrition in rats impaired surface tension-lowering properties of fetal lung extracts and slowed prenatal and postnatal maturation of alveolar type II cells in offspring (34). A hypoxic preterm mouse model of FGR also demonstrated lower levels of SP-A, -B and -C mRNA (35). Thus, our data further supports previous reports that FGR alters surfactant and the expression of surfactant proteins during gestation. Additional exogenous glucocorticoids given to mothers with FGR may not enhance the development of the surfactant system, but suppress/inhibit the surfactant system by enhanced endogenous corticosteroid exposure of the fetus. The most intriguing finding in our study was the reduction of SP-A at both the mRNA and protein levels in our 70% MNR fetal lungs. Maternal protein restriction in rats has previously demonstrated a reduction in fetal lung SP-A during gestation (36, 37). Synthesis of SP-A *in vitro* has a biphasic response to glucocorticoids (38); at low glucocorticoid concentrations, SP-A expression is enhanced, but at high concentrations, it is inhibited. Recently, Islam and Mendelson reported that the glucocorticoid receptor inhibits SP-A gene expression in human type II cells and this inhibition can be enhanced by glucocorticoid treatment. The mechanism involves the induction of histone deacetylases with increased binding to the SP-A promoter to cause a closed chromatin structure that blocks gene expression (39). Changes to the epigenetic

gene regulation of SP-A may have long lasting effects, which could program SP-A expression beyond gestation and into postnatal life.

A large number of randomized controlled trials have demonstrated that prenatal glucocorticoid treatment reduced the risk of respiratory distress syndrome (RDS) in premature human infants, generally between 28 and 32 weeks gestation (40), with greater effect observed in females. The presence of surfactant in amniotic fluid of males appears two weeks later than in females (41), with male amniotic fluid cortisol concentrations significantly lower than females (41). This reduction in cortisol does not seem to be due to cortisol deficiency, since exogenous glucocorticoid administration failed to correct the male deficit in lung maturation (42, 43). In rodents, fetal lung growth peaks earlier in females than males (44) with more epithelial cells and lamellar bodies per type II alveolar cell in female fetal rat lung than male (45). Female fetal rat, mouse, rabbit, sheep and human lungs begin synthesis of surfactant phospholipid earlier than male fetuses (46-48), however, surfactant levels are equivalent between genders at term (49).

Androgens have been implicated to act as antagonists to glucocorticoids (49). Exogenous androgen treatment stimulates tissue and cell growth, by stimulating mitosis of type II cells and fibroblasts in cell culture, while blocking their differentiation (44). Pregnant rabbits treated with dihydrotestosterone (DHT) showed delayed spontaneous and dexamethasone-stimulated surfactant production by both male and female fetuses (44, 50, 51), while treatment of fetal rabbits with the antiandrogen Flutamide inhibited the delay in surfactant production by raising the male surfactant phospholipid values to be equivalent to females, with no effect on the females (50). A dose-response relationship is noted between surfactant production and levels of androgen the fetuses were exposed to

as a result of their position *in utero* in litter-bearing rodents (50). Among males, there were no siginificant differences in surfactant production, whereas all levels were low compared to females (50). In females, those *in utero* placed between two females had the highest surfactant level, those females between two males had the lowest levels, and those with only one male neighbor had intermediate levels (50). Androgens and estrogens have been shown to exert inhibitory and stimulatory effects, respectively (52). Maternal administration of estrogen accelerates lung maturation and stimulates surfactant production in the fetal rabbit and rat (53, 54); with prenatal estrogen deprivation impairing alveolar formation and fluid clearance in neonatal piglets (55). Adult female rodents have more and smaller alveoli than males and the formation and maintenance is dependent on estrogens (56) and the estrogen receptor (ER) α and ERβ (57) as genetic deletion of ERα or ERβ decreases the number and increases the size of alveoli in mice with changes more prominent in females than males (57, 58). Therefore, it is possible that androgens in male MNR mice may protect the male lungs from prolonged, elevated levels of corticosterone, and therefore no long term impact on lung mechanics was detectable at one or three months postnatally. The converse was likely true for females. We expected the changes in lung mechanics of FGR females at 1 month to be reversed when tested at 3 months, due to the initiation of puberty and the stimulatory effects of estrogen on lung development and maintenance. However, the deficit in lung function remained unchanged in the FGR female cohort at 3 months, this suggests the insult from MNR *in utero* persists despite the positive effects of circulating estrogen.

The influence of gender and sex hormones lung development are particularly significant for preterm infants (49). The origin of gender-based differences may

ultimately lie at the chromosomal level, with the role of sex chromosomes in lung development to be elucidated. Surfactant production in male and female chick embryos, in which sex karyotype is reversed such that if the homogametic sex (i.e. ZZ) is male, male surfactant production increases earlier than in females with males responsive earlier to treatment with glucocorticoids *in ovo* than females (59). Therefore, regardless of sex hormones, it may ultimately be the interaction of elevated corticosterone or MNR with the sex chromosome complement in females may be responsible for the postnatal consequences of compromised lung mechanics. The heterogametic sex is at a disadvantage for the timing of lung development and suggests a biologic advantage in having two complete sex chromosomes (49). However, during FGR, this may become a disadvantage.

In summary, during FGR, maternal glucocorticoids enter the fetal circulation due to both high levels in the mother and reduced metabolism in the placenta. This causes the FGR fetus to be exposed to prolonged elevated levels of glucocorticoid, which may inhibit optimal development of glucocorticoid sensitive tissues, such as the lung. The elevated corticosterone and the other endocrine disturbances that occur during MNR were associated with reduced volumes of lung parenchyma with no change in airspace volume and reduction of surfactant protein-A, -C and possibly -B expression. These structural and biochemical changes may be associated with neonatal morbidity or predisposition to diseases in childhood or adulthood. Measurement of pulmonary mechanics in the offsprings at one and three months postnatally demonstrated a reduction in lung function, only in females. This study questions the benefit of administering antenatal

glucocorticoids to preterm FGR infants, as our study suggests that this may have deleterious effects on lung development both in short term as well as long term.

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CHAPTER 5

CHANGES IN PLACENTAL STRUCTURE AND FUNCTION BY MATERNAL UNDERNUTRITION IN A MOUSE MODEL OF FETAL GROWTH RESTRICTION

Manuscript prepared for Placenta

5.1 INTRODUCTION

The placenta forms an important interface between the mother and fetus, adapting nutrient supply to the demands of the fetus while buffering changes in the maternal compartment. The ability of the placenta to adjust to the needs of the fetus depends on size, morphology, blood flow and transporter abundance of the placenta in conjunction with the synthesis and metabolism of nutrients and hormones by the placenta (1). If the placental supply of nutrients is compromised, the placenta adapts to enhance nutrient transport for maintaining fetal growth as demonstrated in humans (2, 3) and animal models (4), such that placental efficiency (grams of fetus supported per gram of placenta) increases.

If the placenta fails to adapt and meet the required nutrient transfer to the fetus for growth, fetal growth restriction (FGR), may ensue. FGR is defined as a decrease in fetal growth rate that prevents a newborn from obtaining his or her optimal growth potential. FGR affects 3-10% of human pregnancies, is associated with high perinatal mortality and

morbidity (5, 6), and is associated with an increase risk of diseases in adulthood. FGR can be reproduced in experimental animals by maternal undernutrition, and is used to study pathophysiology in the fetus and placenta during FGR (7). The mouse is a valuable animal model for FGR research since it is a mammalian species with genetic makeup, biochemical pathways, cell types and physiology similar to humans (7). In particular, the mouse, which is amenable to genetic manipulation, has a hemochorial placenta that is functionally similar to humans, with many cell types having equivalent properties (8). The fetal compartment of a mouse placenta consists of the labyrinth zone, which contains fetal and maternal blood vessels for nutrient exchange, and the junctional zone, which stores glycogen and produces hormones. The junctional zone rests on the decidualized endometrium of the uterus.

Insulin-like growth factor-II (IGF-II) is a major placental regulator of nutrient transfer to the fetus, placental growth and development. Placental efficiency increases with *Igf2* gene deletion selectively in the mouse placenta (*Igf2P0*); at E16, more glucose and amino acids are transferred to the fetus per gram of placenta with upregulation of specific transporters in the placenta (9). These changes are not present when *Igf2* is deleted in all fetal tissues, including the placenta (*Igf2* null); there is a disproportionate decrease in labyrinth zone with hypoplasia and disturbances in cell differentiation in the junctional zone (10). Both forms of *Igf2* deletion demonstrate reduced placental surface area and increased barrier thickness, with changes more severe in the *Igf2* null (10). IGF-II production in the fetal compartment is presumed to be a genetic signal to the placenta to enhance fetal growth, as the *Igf2* null placenta fails to increase its efficiency when fetal IGF-II is removed (11).

IGF-II is an important member of the IGF system during embryonic and fetal development, and is an important mediator of placental structure and function (8); other members of this system can alter the activity of IGF-II. The IGF system includes IGF-I and IGF-II signaling peptides, two cell surface receptors, IGF-1R and IGF-2R, and six binding proteins, IGFBP-1 to IGFBP-6. The majority of effects of IGF-I and -II are mediated by binding to IGF-1R. IGF-II binding to the insulin receptor A (INSRA) isoform also mediates cell mitogenesis as this isoform is highly expressed during gestation (12, 13), as opposed to the insulin receptor B isoform which is highly expressed postnatally and mediates the metabolic effects of insulin with little affinity for IGF-II (12, 13). In contrast, IGF-2R binds only IGF-II and clears IGF-II from the plasma, decreasing its bioavailability. Six high-affinity IGF binding proteins regulate the availability of IGF-I and -II to fetal tissues, protecting them from degradation in circulation and inhibiting IGF-I and -II from binding to their receptors (14). The IGF-I and -II peptides are produced widely by the fetus and placenta, and it is believed that the paracrine production of IGFBPs determines which cell types will be targeted for the growth promoting actions of IGFs (15).

The insulin-like growth factor (IGF) system is the principal regulator of normal fetal and placental growth as it directs cell proliferation, differentiation, migration, and aggregation during gestation (15). Many transgenic and knockout mice targeting this system have demonstrated aberrant fetal and/or placental growth and development. The IGF system is present in most fetal tissues and its expression is coupled to nutrient supply to ensure that fetal growth is adequate relative to the amount of nutrients available (15). Alterations in fetal nutrient supply can change the expression of this system in a tissue

specific manner to regulate not only overall fetal growth, but the growth and development of individual fetal organs and placenta.

This study was designed to investigate how maternal nutrient restriction (MNR) affects the structure and function of the murine placenta. The tissue morphology and expression of IGF system mRNAs were examined in the placenta of control and MNR litters at 18.5 days of pregnancy as mouse fetuses undergo rapid growth. Based on previous studies, we hypothesized that reduction of maternal nutrient supply would cause the placenta to maintain an adequate supply of nutrients to sustain fetal growth via up-regulation of IGF system genes.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Dietary Restriction Protocol

Protocols on Animal Care were approved by the University Council on Animal Care at the University of Western Ontario, in accordance with guidelines of the Canadian Council for Animal Care. Ten week-old virgin CD-1 females and stud males were obtained from Charles River Laboratories (Montreal, PQ, Canada). Mice were housed at the Lawson Health Research Institute Vivarium (London ON, Canada) with 12:12 light:dark cycle and water provided *ad libitum*. Female mice were housed singly with stud males in late afternoon and checked the following morning for vaginal plugs, indicating embryonic day 0.5 (E0.5). Females were housed individually and fed grain-based dustless precision pellets (#F0173, Bio-Serv, Frenchtown NJ, USA) containing 20.7% protein, 4.0% fat, 6.7% ash, 59.6% carbohydrates, 4.0% fiber and 5.0% moisture *ad libitum* using feeding tubes with cage clips (Bio-Serv, Frenchtown NJ, USA) until

E6.5. Feeds for control mice were pre-weighed each morning and remaining pellets weighed at same time each day to establish average daily feed intake during pregnancy. From E6.5, MNR mice (n = 21) were fed daily 70% by weight of the average daily intake of control mice (n = 14). Mice were euthanized at E18.5 by carbon dioxide inhalation. Fetuses were removed and weighed, placentae trimmed of membranes and yolk sac were dissected, weighed, and flash frozen in liquid nitrogen and stored at -80□C.

5.2.2 Stereology

Stereology was performed as outlined previously (16) with minor modifications. Weights were recorded for all randomly selected placentae from six litters for each group. Placentae were sectioned at 5 μm thickness and stained with hematoxylin and eosin.

Cross sections of mouse placentae were imaged using 5× objective of a Carl Zeiss Axio Imager Z1 and AxioCamHR3. AxioVision, Release 4.7.2 Software was used to combine individual fields of view enabling a complete view of tissue section. StereoInvestigator Software (MicroBrightField Bioscience, Inc., Williston, VT) was used for all stereology measurements. Using the Cavalieri Estimator probe (MicroBrightField Bioscience, Inc., Williston, VT), a 100 μm grid was superimposed over the cross section and two different markers used to count points falling on the Junctional Zone and Labyrinth Zone of the mouse placenta. (The absolute placental volume is defined as the sum of the junctional and labyrinth zone volumes.) Shrinkage during the paraffin-embedding process was accounted for as previously described (16).

Resin sections 1 μm thick at the placental midline were used to resolve the labyrinth vasculature in detail. A 63× oil objective lens on the Carl Zeiss Axio Imager Z1

and AxioCamHR3 was used to randomly capture 12 fields of view within the labyrinth zone, and to determine the volume densities, surface densities, length densities and interhemal membrane thickness as previously described (16).

Theoretical diffusion capacity was calculated by dividing mean surface area of the fetal and maternal interhemal membrane by its thickness then multiplying by the Krogh diffusion coefficient for oxygen, as described previously (16). Specific diffusion capacity is an estimate of oxygen diffusion in terms of fetal requirements, obtained by theoretical diffusion capacity per mg of fetal weight.

5.2.3 Quantitative Real-Time PCR

Total RNA from flash frozen control and MNR placentae, pooled by litter, were extracted using Trizol (Invitrogen, Burlington, ON) and purified with the RNAeasy Mini Kit (Sigma, Maryland, USA), following the manufacturer's instructions.

SYBR Green qRT-PCR was used to study mRNA expression of glucose transporters, Glut-1 and -3, System A sodium-dependent neutral amino acid transporters isoforms, Slc38a1, -2 and -4, and System L amino acid transporter isoforms, Lat-1 and-2. Using reagents from Invitrogen, 1 μg of RNA from the placenta was reverse transcribed (RT) using Superscript III and 500 ng of Oligo(dT) with 20 U RNaseOUT in a 21.5 μl final volume, followed by digestion of the template RNA with 2 U RNase H. Reactions were incubated at 70□C, 10 min; 42□C, 2 min; 70□C, 15 min; 4□C, 10 min and 37□C, 20 min. Specific primers (**TABLE 5.1**) were purchased from Invitrogen. Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) was used as recommended with 200 μM of primers, mixed with 100 ng RT product in a total volume of 10 μl in triplicate.

TABLE 5.1 Primer Sequences for SYBR Green quantitative Real-Time PCR.

Primer Name	Primer Pair Sequence	Fragment Size (bp)	Reference
Glut-1	CCAGCTGGGAATCGTCGTT (F) CAAGTCTGCATTGCCCATGAT (R)	76	(17)
Glut-3	CTCTTCAGGTCACCCAACTACGT (F) CCGCGTCCTTGAAGATTCC (R)	121	(17)
Lat-1	GCTGTGGACTTCGGGAACTACC (F) CCACACACAGCCAGTTGAAGAA (R)	291	AY162432/ GI:27227810
Lat-2	CTCTTCACATGCCTCTCCAC (F) TACACGACCACACACATC (R)	379	AY162433/ GI:27227860
Slc38A1	TGCAGAACACTGGGGCGAT (F) GTGTAGCCAAGATACCCCAGG (R)	195	NM_134086/ GI:142381438
Slc38A3	CGAATCATGCCCACTGACAA (F) AACCGCAGCGAAACAAAGG (R)	71	(18)
Slc38A4	AGGGTGCTGAGAAATGCCAA (F) GACCTCAGGGTGGCAGACAA (R)	100	(18)

Thermal cycling conditions were 50□C, 2 min; 95□C, 20 sec; 60□C, 20 sec; 72□C, 30 sec for 40 cycles. A Mann-Whitney test was performed to determine if the fold-change of MNR litters was significantly different from controls.

5.2.4 Microvillous Membrane (MVM) Extraction

Pooled placentae from each litter were trimmed of fetal membranes and umbilical cords, chopped into small pieces and rinsed three times in HT saline (1.8 g NaCl in 10 mM HEPES-Tris). Placentae were homogenized in Buffer D (250 mM sucrose in 10 mM HEPES-Tris) for 30 sec at 15,000 rpm. A homogenate sample was reserved for alkaline phosphatase enrichment to assess final MVM extract purity. The homogenate was centrifuged at 3,000× g for 10 min at 4□C to remove nuclei and large debris. Resulting supernatant was centrifuged at 14,000× g for 30 min to pellet the crude membrane fraction of the homogenate and resuspended in Buffer D with magnesium chloride to isolate the MVM fraction. Precipitates were discarded following a 2,500× g spin for 10 min at 4□C and the MVM fraction in the supernatant concentrated at 100,000× g. Protein concentration was determined by BCA assay. Purity of MVM extract was assessed by MVM/homogenate alkaline phosphatase enrichment ratio, and was found to be 16 ± 2 fold (n = 14).

5.2.5 Western Blots

Glucose transporter isoforms GLUT1 and GLUT3 protein levels were analyzed in placental MVM extract using Western blotting (**TABLE 5.2**). In brief, 15 µg of total

TABLE 5.2 Primary Antibodies used for Immunohistochemistry (IHC) and Western Blotting (WB).

Antibody	Host	Catalogue #	Dilution		Source
			IHC	WB	
IGF-I	Rabbit	#RB-9240-P	1:10	-	Labvision Corp., Fremont, CA
IGF-II	Rabbit	PAAL1	1:15	-	GroPep, Adelaide SA, Australia
IGFBP-1	Goat	Sc-6072	1:400	-	Santa Cruz Biotech Inc., Santa Cruz, CA
IGFBP-2	Rabbit	#06-107	1:750	-	Upstate Biotech Inc., Lake Placid, NY
IGFBP-3	Rabbit	DSL-R00534	1:750	-	Diagnostic Systems Laboratories, Inc., Webster, TX
GLUT1	Rabbit	07-1401	-	1:4000	Millipore, Temecula, CA, USA
GLUT3	Rabbit	AB1344	-	1:2000	Millipore, Temecula, CA, USA
Pan-actin	Mouse	#MS-1295	-	1:7000	Thermo Fisher Scientific, Fermont, CA

protein was loaded onto 10% SDS-PAGE gels, and electrophoresis was performed at constant 100 V for 2 h. Protein was transferred onto PVDF membranes for 1.5 h at constant 350 mAmp and blocked with 5% Carnation non-fat powdered milk in TBS for 1 h at RT. Membranes were incubated with primary antibodies overnight at 4□C, washed, and incubated with appropriate secondary peroxidase-labeled IgG for 1 h. After washing, bands were visualized using Western Lighting Enhanced Chemiluminescence (ECL) detection reagents (Perkin-Elmer, Boston, MA). Blots were stripped and reprobed for pan-actin as a loading control. Analysis was performed by densitometry using an Alpha Imager and FluoroChem 8800 software (Alpha Innotech Corporation, San Leandro, CA). A Mann-Whitney test was performed to determine if the fold-change in MNR litters was significantly different from controls.

5.2.6 Tissue Preparation

Placentae were harvested at E18.5, fixed in 4% paraformaldehyde for 48 h, bisected, then washed 3 times in PBS over 3 days. Tissue specimens were embedded in paraffin, sectioned at 5 μm and mounted onto Superfrost® slides (Fisher Scientific, Fairlawn, NJ).

5.2.7 Immunohistochemistry

Sections were deparaffinized and rehydrated in descending series of ethanol solutions. Endogenous peroxidase was quenched with 3% H_2O_2, blocked using Avidin and Biotin kit (Biocare Medical, Concord, CA) and the Background Sniper Universal Blocking Reagent (Biocare Medical) as per manufacturer's instructions. Tissue sections

were incubated with primary antibodies against IGF-I, -II and IGFBP-1, -2 and -3 overnight at 4□C (**TABLE 5.2**). Slides were incubated for 30 min at RT with appropriate secondary IgG. After washing, sections were incubated for 30 min at RT in peroxidase-conjugated avidin–biotin–complex (Vectastain Elite ABC kit; Vector Laboratories Inc, Burlingame, CA) and DAB (Cardassian Diaminobenzidine Chromogen Kit; Biocare Medical, Walnut Creek, CA) was applied. Tissue sections were counterstained with methyl green (DakoCytomation Inc., Carpinteria, CA). Preabsorbed negative controls were performed with each antibody.

5.2.8 In situ Hybridization (ISH)

Placenta sections from control and MNR groups were analyzed for IGF-I, IGF-II, IGFBP-1, IGFBP-2 and IGFBP-3 mRNA expression by *in situ* hybridization using ^{35}S-labelled antisense riboprobes as described (8). The ^{35}S-labelled antisense and sense cRNA probes were generated from cDNAs (**TABLE 5.3**) using a Promega Riboprobe Combination System SP6/T7 kit (Promega Corp., Madison, WI) and ^{35}S-UTP (Perkin Elmer, Waltham, MA). *In situ* hybridization was performed as previously outlined (8) (**Appendix 1.1.2**). The slides were viewed under both dark- and bright fields, using a Carl Zeiss Axio Imager Z1 and appropriate photomicrographs were obtained at 400× magnifications using AxioCamHR3 and AxioVision Release 4.7.2 software.

Quantification was performed on eight control and eight MNR placentae. For the IGF system, 12 random fields of view were taken in the labyrinth zone of the placenta from each animal. Using Carl Zeiss Automeasure Plus Software, IGF-I, IGF-II, IGFBP-2 and IGFBP-3 mRNA abundance per cell was quantified by counting total grains and

TABLE 5.3 cDNA Vectors for generating cRNA probes for insitu hybridization of Igf System RNA.

AntiSense Probe	Target (gi #)	Size (bp)	Restriction Site AS	S	Vector	Origin	Ref
Plp-I	23820972	800	NotI	BamHI	pCR®II-TOPO	Dr. James Cross (University of Calgary)	(19)
mIgf-1	33612736	271	XhoI	EcoRV	pBluescript LION	imaGenes (Berlin, Germany)	-
mIgf-2	170172554	468	EcoR I	Sst I	pBluescript KS	Dr. Graeme Bell (University of Chicago)	(20)
rIgfbp-1	6981079	1477	Kpln	XhoI	pGEM 7Zf	Matt Rechler	(21)
rIgfbp-2	148747420	585	HindIII	EcoR I	pGEM 3z	Matt Rechler	(21)
rIgfbp-3	6981083	905	EcoRI	SmaI	pGEM 3z	Dr. Adrian H. Herington (University of Melbourne)	(22)

The nucleotide sequence homology between rat and mouse IGFBP-1 to -3 cDNAs is 90-94%.

dividing by number of cells, in each field of view at 400× magnification. For Plp-1, a cross-section of the midline placenta was generated using 10× objective and combined in AxioVision Release 4.7.2 software. Cells in junctional zone were manually counted and those expressing Plp-1 were classified as spongiotrophoblast cells (19).

5.2.9 Placental Transport Assays of Radiolabeled Solutes

Pregnant mice at E19 were anesthetized by intraperitoneal injection of 0.03 mL/10 g of ketamine and xylazine at concentrations of 4 mg/mL and 1 mg/mL, respectively. A neck incision was made, and jugular vein exposed. A 100 µl bolus of PBS containing 3.5 µCi ^{14}C-methyl-aminoisobutyric acid (MeAIB) or 3.5µCi ^{14}C-methyl-D-glucose with 70 µCi ^{51}Cr-EDTA was injected into the jugular vein. Four minutes after injection, animals were killed and pups removed by hysterectomy. Fetuses, placentae and 100 µl maternal blood were placed in 4 ml of Scintisafe and beta or gamma radiation counted. Radioactive counts in each fetus were expressed relative to counts in maternal blood sample and used to calculate the amount of radioisotope transferred per gram of placenta or per gram of fetus for each feto-placental unit in the litter. Average values for each litter were normalized to the mean of the control group to express the resulting fold change in solute transport.

The fetal accumulation of radioisotope expressed relative to placental gives a relative measure of placental transfer of solute. The fetal accumulation of radioisotope expressed relative to fetal weight gives a relative measure of the amount of solute received by the fetus. Data for placental transfer assays were collected from four control

and eight MNR litters for ^{51}Cr-EDTA; eight control and 12 MNR litters for ^{14}C-methyl-D-glucose; six control and seven MNR litters for ^{14}C-MeAIB.

5.2.10 Statistics

All data are expressed as mean ± SEM. Parameters in control and MNR animals were compared with Student's non-paired two-tailed *t* test, unless stated otherwise. For radioactive solute studies, *t* test was performed prior to normalization to the control mean. Significance was set at $P < 0.05$.

5.3 RESULTS

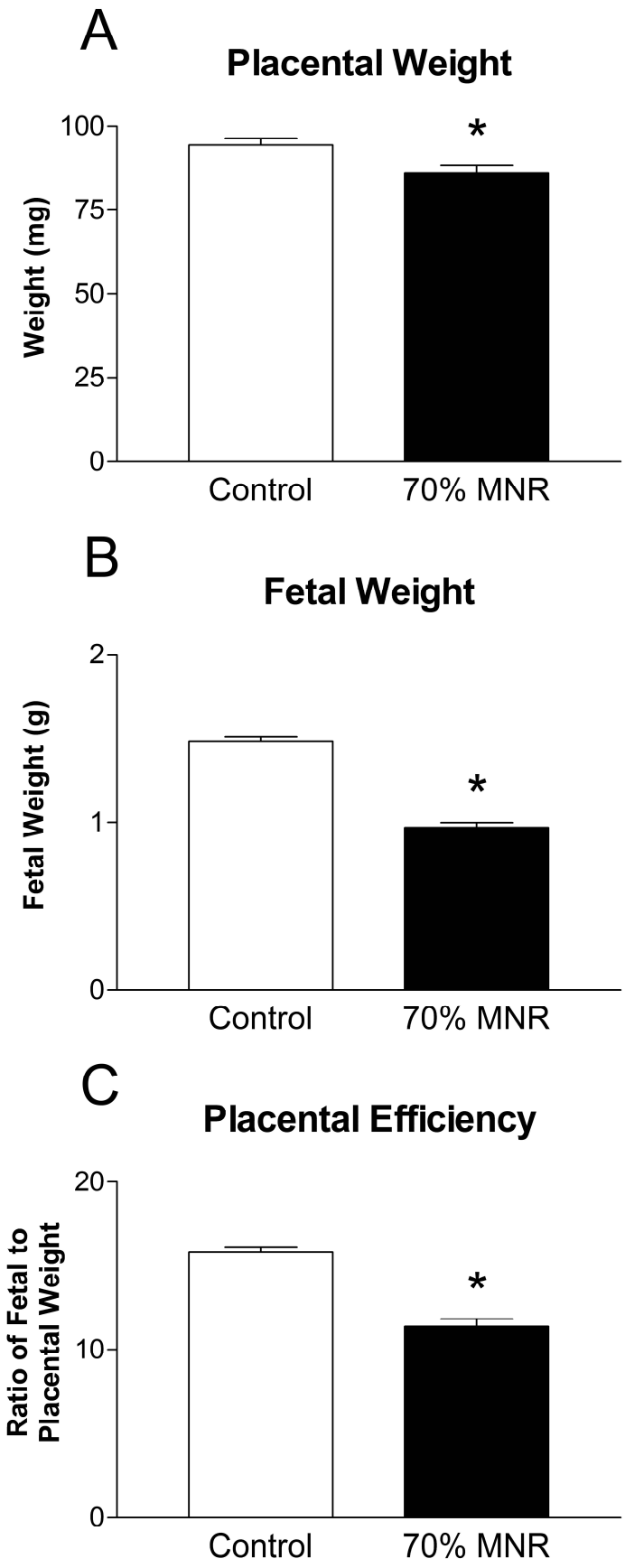
5.3.1 Fetal and Placental Weights at E 18.5

Placental and fetal weights were measured at E18.5 to determine the effect of maternal undernutrition during gestation. The MNR group demonstrated significantly reduced placental weights (**Fig. 5.1A**; Control: 94 ± 2 mg *vs.* MNR: 86 ± 2 mg; $P < 0.05$), fetal weights (**Fig. 5.1B**; Control: 1482 ± 27 mg *vs.* MNR: 969 ± 30 mg; $P < 0.001$) and placental efficiency (**Fig. 5.1C**; Control 15 ± 1 *vs.* MNR 11 ± 1: $P < 0.001$) compared to control.

5.3.2 Absolute Volumes

Whole placental weight was reduced during maternal undernutrition, however this does not indicate how the structure of the placenta adapted to a reduced supply of maternal substrates. Stereology was employed to measure alterations to placental

Figure 5.1 The effect of 70% maternal nutrient restriction on fetal and placental weight and placental efficiency at E18.5. (**A**) Placental weight (mean ± SEM) in control (N=14) and 70% MNR (N=21) litters. In 70% MNR litters, the placental weight of 86 ± 2 mg (vs 94 ± 2 mg in controls) was significantly lower ($P < 0.05$). (**B**) Fetal weight (mean ± SEM) in control (N=14) and 70% MNR (N=21) litters. In 70% MNR litters, the fetal weight of 969 ± 30 mg (vs 1482 ± 27 mg in controls) was significantly less ($P < 0.001$). (**C**) Placental efficiency (mean ± SEM) in control (N=14) and 70% MNR (N=21) litters. In 70% MNR litters, placental efficiency of 11 ± 1 (vs 15 ± 1 in controls) was significantly reduced ($P < 0.001$).


A
Placental Weight
Weight (mg)
100
75
50
25
0
*
Control
70% MNR
B
Fetal Weight
Fetal Weight (g)
2
1
0
*
Control
70% MNR
C
Placental Efficiency
Ratio of Fetal to Placental Weight
20
10
0
*
Control
70% MNR

structure following maternal undernutrition. The absolute volume, defined in our study as combined junctional and labyrinth zone volume, was significantly less in the MNR group (**Fig. 5.2A**; Control: 0.129 ± 0.008 *vs.* MNR: 0.081 ± 0.005 cm^3; $P < 0.0005$), with both zones significantly reduced (**Fig. 5.2A**; LZ, Control: 0.081 ± 0.005 *vs.* MNR: 0.058 ± 0.003 cm^3, $P < 0.005$; JZ, Control: 0.048 ± 0.004 *vs.* MNR: 0.022 ± 0.003 cm^3, $P < 0.0005$). The junctional zone contributed significantly less to absolute volume in MNR placenta (**Fig. 5.2B**; Control: 37 ± 2% *vs.* MNR: 27 ± 2%; $P < 0.005$).

5.3.3 Junctional Zone Study

Stereology indicated a reduction in the volume of the junctional zone; further studies were performed to determine changes to the cell population residing in this zone. The junctional zone contained fewer total cells in the MNR group compared to control (**Fig. 5.3A**; Control: 4375 ± 232 *vs.* MNR: 2411 ± 184; $P < 0.00005$), with both glycogen cells (GC) and spongiotrophoblast cells (SpT) significantly reduced (**Fig. 5.3B**; SpT, Control: 2883 ± 159 *vs.* MNR: 1874 ± 138, $P < 0.0005$; GC, Control: 1492 ± 193 *vs.* MNR: 537 ± 93, $P < 0.001$). Proportion of glycogen cells was significantly less in the MNR group (**Fig. 5.3C**; Control: 33 ± 3% *vs.* MNR: 22 ± 3% mg; $P < 0.05$).

5.3.4 Labyrinth Volumes

Stereology measurements indicated that the volume of the labyrinth zone was significantly reduced following maternal undernutrition, but its volume was relatively spared at the expense of the volume of the junctional zone. Further stereology

Figure 5.2 The effect of 70% maternal nutrient restriction on placental volumes and the proportion of Junctional and Labyrinth Zones of the placenta at E18.5. (**A**) Representative cross-section at placental midline of control placenta. (**B**) Representative cross-section at placental midline of 70% MNR placenta. (**C**) Placental volume (mean ± SEM) in control (N=6) and 70% MNR (N=7) placentas. In 70% MNR placenta, the placental volume of 0.081 ± 0.005 cm3 (vs 0.129 ± 0.008 cm3 in control) was significantly reduced ($P < 0.0005$), as well as the volume of the labyrinth of 0.058 ± 0.003 cm3 (vs 0.081 ± 0.005 cm3 in controls) and junctional zone 0.022 ± 0.003 cm3 (vs 0.048 ± 0.004 cm3 in controls) were both significantly reduced ($P < 0.005$ and $P < 0.0005$, respectively). (**D**) Proportion of labyrinth (mean ± SEM) and junctional zone (mean ± SEM) in control (N=6) and 70% MNR (N=7) placentas. In the 70% MNR placenta, the proportion of the labyrinth of 73 ± 2% (vs 63 ± 2% in controls) was significantly increased at the expense of the junctional zone of 27 ± 2% (vs 37 ± 2% in controls) ($P < 0.005$).

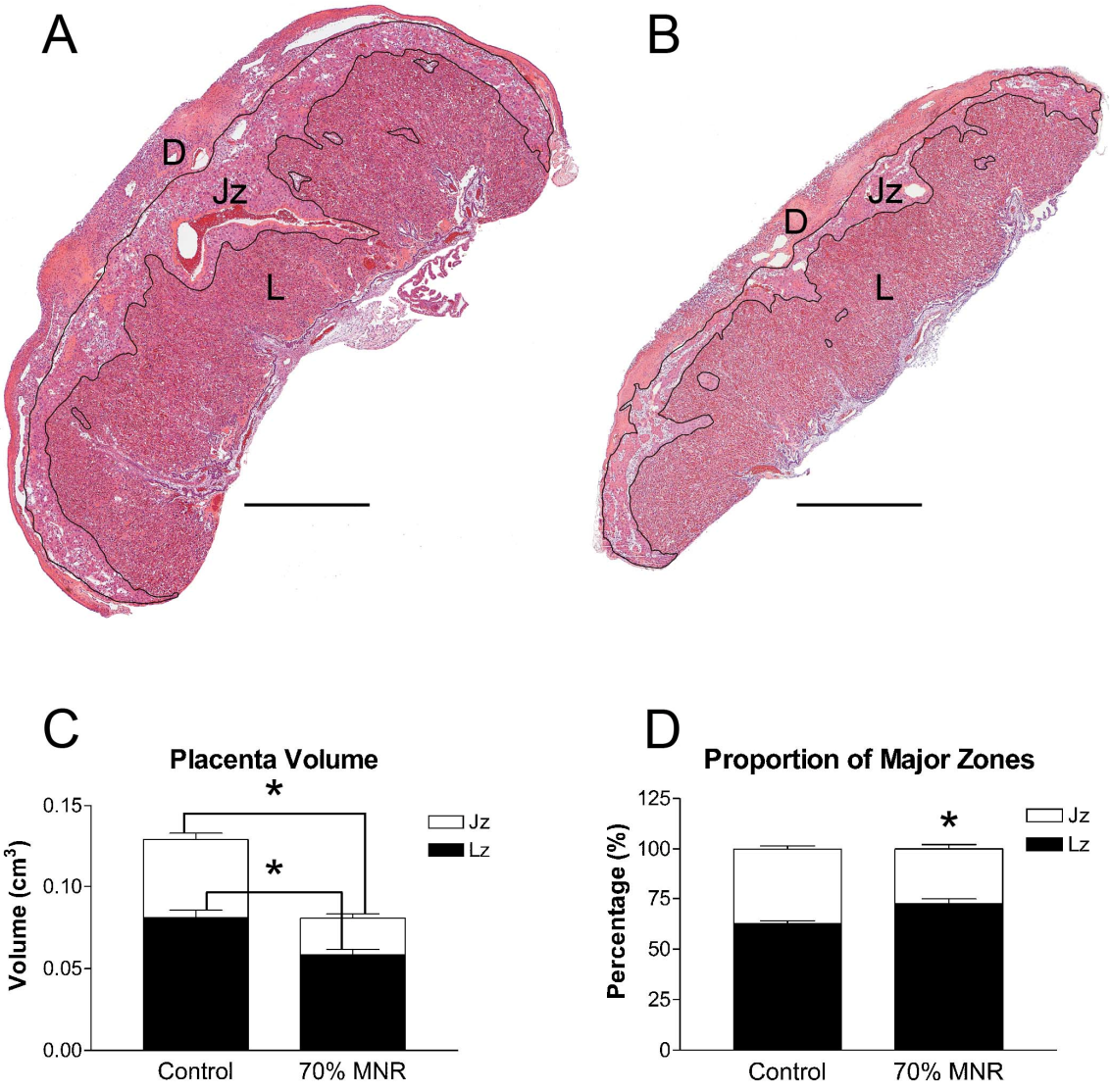
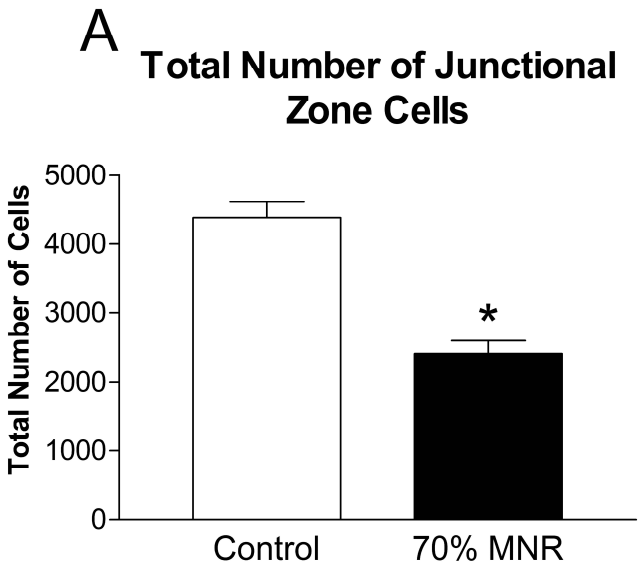
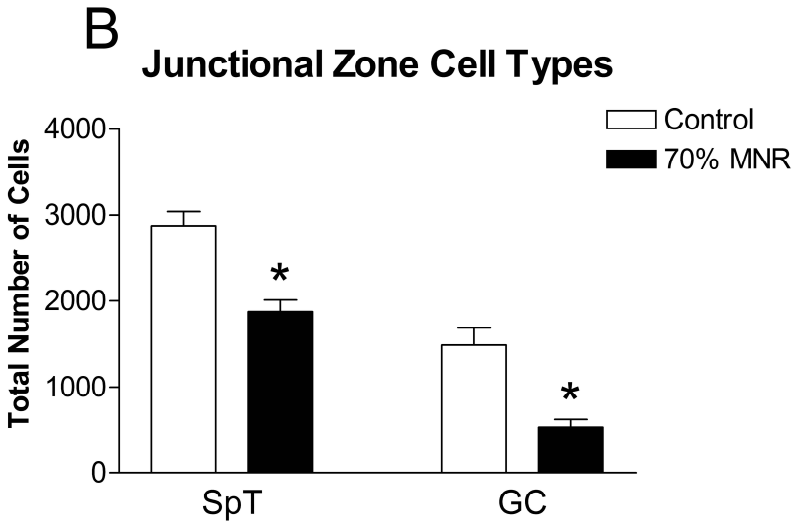
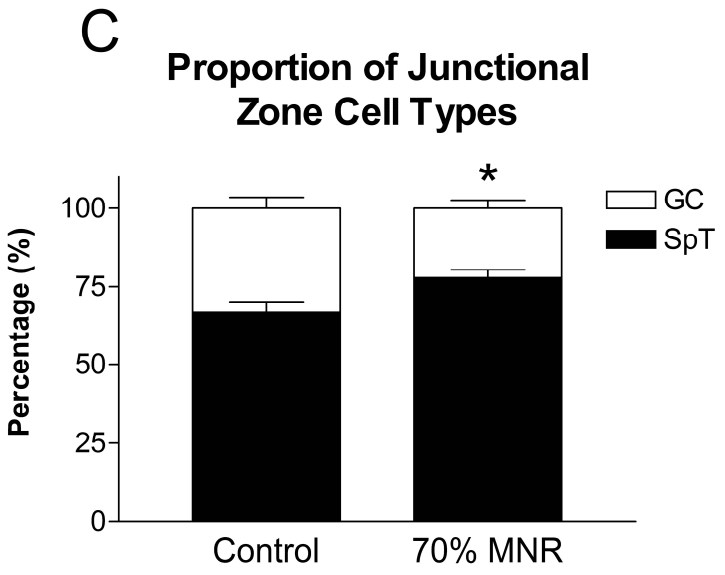

A
D
Jz
L
B
D
Jz
L
C
Placenta Volume
Volume (cm3)
0.15
0.10
0.05
0.00
Jz
Lz
Control
70% MNR
D
Proportion of Major Zones
Percentage (%)
125
100
75
50
25
0
Jz
Lz
Control
70% MNR

Figure 5.3 The effect of maternal nutrient restriction on total cell numbers and the proportion of difference cell types in the junctional zone of the placenta at E18.5. (**A**) Total number of cells in the junctional zone (mean ± SEM) at the placental midline in control (N=7) and 70% MNR (N=7) placentas. In the 70% MNR placentas, the total number of cells of 2411 ± 184 (vs 4375 ± 232 in controls) was significantly reduced ($P < 0.00005$). (**B**) Cell counts (mean ± SEM) of spongiotrophoblasts (SpT) and glycogen cells (GC) at the placental midline in control (N=7) and 70% MNR (N=7) placentas. In the 70% MNR placentas, the number of spongiotrophoblast cells of 1874 ± 138 (vs 2883 ± 159 in controls) and glycogen cells of 537 ± 93 (vs 1492 ± 193 in control) were significantly reduced ($P < 0.005$ and $P < 0.001$, respectively). (**C**) Percentage (mean ± SEM) of spongiotrophoblast and glycogen cells in the junctional zone at the placental midline in control (N=7) and 70% MNR (N=7) placentas. In the 70% MNR placentas, the percentage of the glycogen cells of 22 ± 3% (vs 33 ± 3% in controls) in the junctional zone was significantly reduced ($P < 0.05$).


A
Total Number of Junctional Zone Cells
Total Number of Cells
5000
4000
3000
2000
1000
0
*
Control
70% MNR


B
Junctional Zone Cell Types
Control
70% MNR
Total Number of Cells
4000
3000
2000
1000
0
*
*
SpT
GC

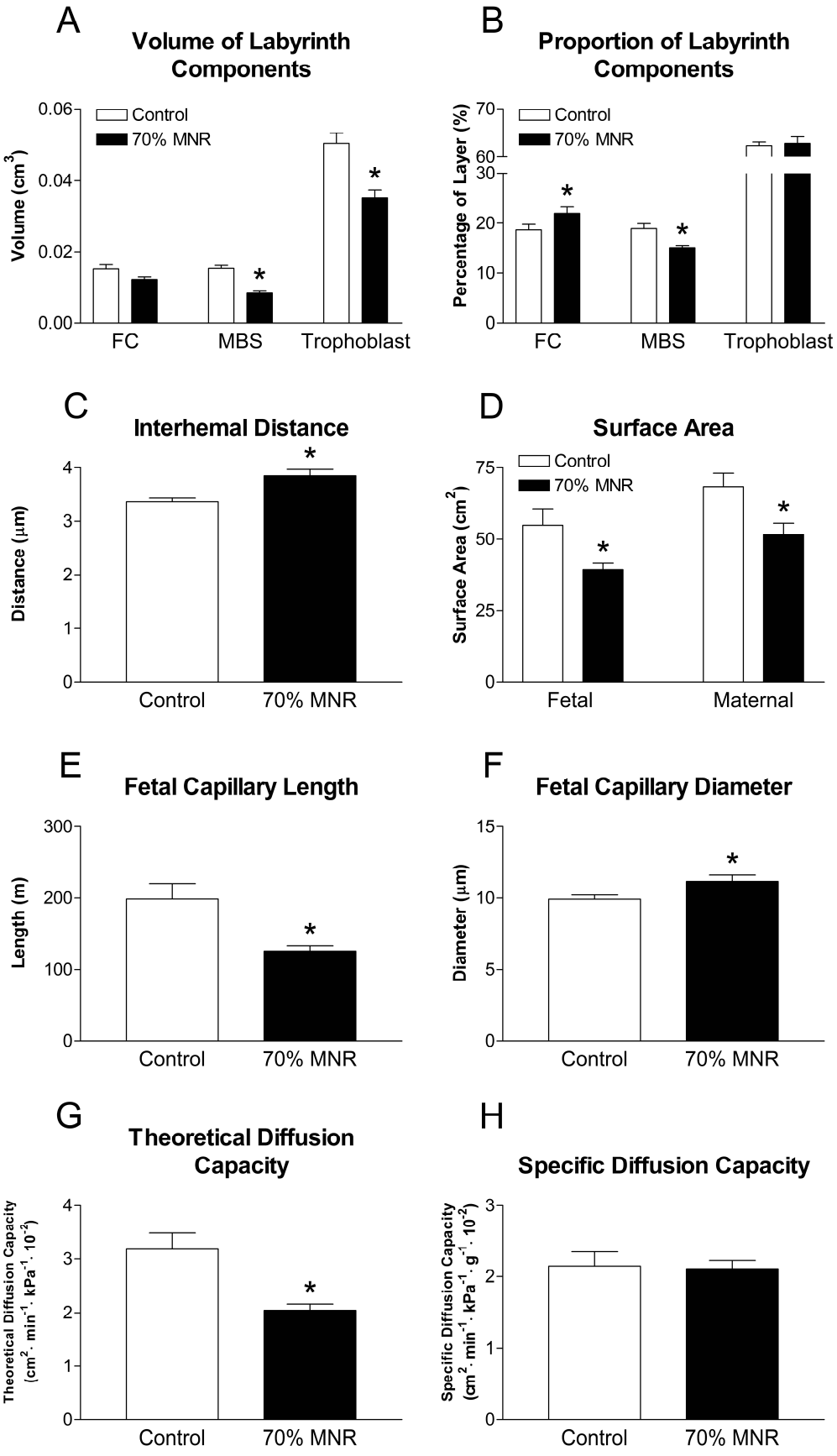

C
Proportion of Junctional Zone Cell Types
GC
SpT
Percentage (%)
100
75
50
25
0
*
Control
70% MNR

measurements were performed to determine subtle changes to the maternal and fetal vasculature of the labyrinth zone, responsible for substrate exchange between the two circulations. Labyrinth volume composed of fetal capillaries was not significantly different between groups, while volume of maternal blood spaces and trophoblast were significantly reduced (**Fig. 5.4A**; MBS, Control: 0.015 ± 0.001 *vs.* MNR: 0.008 ± 0.001 cm^3, $P < 0.001$; Trophoblast, Control: 0.050 ± 0.003 *vs.* MNR: 0.035 ± 0.002 cm^3, $P < 0.005$). Therefore, the percentage of labyrinth composed of fetal capillaries was significantly increased (**Fig. 5.4B**; Control: 19 ± 1% *vs.* MNR: 22 ± 1%, $P < 0.05$), the maternal blood space was significantly reduced (**Fig. 5.4B**; Control: 19 ± 1% *vs.* MNR: 15 ± 1%, $P < 0.005$) and the trophoblast was not different. MNR placenta had a significant increase in interhemal distance (**Fig. 5.4C**; Control: 3.4 ± 0.1 μm *vs.* MNR: 3.9 ± 0.1 μm; $P < 0.005$). The fetal capillary surface area and maternal surface area were significantly reduced in the MNR group (**Fig. 5.4D**; FC, Control: 54.9 ± 5.7 *vs.* MNR: 39.4 ± 2.2 m^2, $P < 0.05$; MBS, Control: 68.5 ± 4.6 *vs.* MNR: 51.7 ± 3.9 m^2, $P < 0.05$). Length of fetal capillaries in MNR placenta was significantly less (**Fig. 5.4E**; Control: 198.6 ± 21.9 m *vs.* MNR: 126.1 ± 7.4 m, $P < 0.05$) and diameter was significantly increased (Fig. 5.4F; Control: 9.9 ± 0.3 μm vs. MNR: 11.2 ± 0.4 μm, $P < 0.05$).

5.3.5 Theoretical Diffusional Capacity

Measurements performed by stereology of the maternal and fetal vasculature in the labyrinth zone allowed estimation of the theoretical diffusion capacity of the control and MNR placentas. Theoretical diffusion capacity was significantly reduced in MNR animals (**Fig. 5.4G**; Control: 0.032 ± 0.003 *vs.* MNR: 0.020 ± 0.01, $P < 0.01$), however

Figure 5.4 The effect of maternal nutrient restriction on dimensions of fetal capillaries and diffusion capacity in the labyrinth zone of the placenta at E 18.5. (**A**) Volume (mean ± SEM) of fetal capillaries (FC), maternal blood space (MBS), and trophoblast in control (N=6) and 70% MNR (N=6) placentas. In the 70% MNR placentas, the volume of the MBS of 0.008 ± 0.001 cm^3 (vs 0.015 ± 0.001 cm^3 in controls) and trophoblast of 0.035 ± 0.002 cm^3 (vs 0.050 ± 0.003 cm^3) were significantly reduced ($P < 0.001$ and $P < 0.005$, respectively) with no difference in FC volume. (**B**) Percentage (mean ± SEM) of labyrinth composed of FC, MBS, and trophoblast in control (N=6) and 70% MNR (N=6) placentas. In the 70% MNR placentas, the percentage of FC volume of 22 ± 1% (vs 19 ± 1% in controls) was significantly increased ($P < 0.05$) and the percentage of MBS volume of 15 ± 1% (vs 19 ± 1% in controls) was significantly reduced ($P < 0.005$), with no difference in the percentage of trophoblast volume. (**C**) Interhemal distance (mean ± SEM) in control (N=6) and 70% MNR (N=6) placentas. In the 70% MNR placentas, interhemal distance of 3.9 ± 0.1 μm (vs 3.4 ± 0.1 μm in controls) was significantly increased ($P < 0.005$). (**D**) Surface area (mean ± SEM) of FC and MBS in control (N=6) and 70% MNR (N=6) placentas. In 70% MNR placentas, surface area of the FC of 39.4 ± 2.2 m^2 (vs 54.9 ± 5.7 m^2 in controls) and MBS of 51.7 ± 3.9 m^2 (vs 68.5 ± 4.6 m^2 in controls) were significantly reduced (both $P < 0.05$). (**E**) Length (mean ± SEM) of FC in control (N=6) and 70% MNR (N=6) placentas. In the 70% MNR placentas, FC length of 126.1 ± 7.4 m (vs 198.6 ± 21.9 m in controls) was significantly reduced ($P < 0.05$). (**F**) Diameter (mean ± SEM) of FC in control (N=6) and 70% MNR (N=6) placentas. In the 70% MNR placentas, FC diameter of 11.2 ± 0.4 μm (vs 9.9 ± 0.3 μm in controls) was significantly increased ($P < 0.05$). (**G**) Theoretical diffusion capacity (mean ± SEM) in control (N=6) and 70% MNR (N=6) placentas. In the 70% MNR placentas, the theoretical diffusion capacity of 0.020 ± 0.01 $cm^2 \cdot min^{-1} \cdot kPa^{-1} \cdot 10^{-2}$ (vs 0.032 ± 0.003 $cm^2 \cdot min^{-1} \cdot kPa^{-1} \cdot 10^{-2}$ in controls) was significantly reduced ($P < 0.01$). (**H**) Specific diffusion capacity (mean ± SEM) in control (N=6) and 70% MNR (N=6) placentas, was not different between groups.


A
Volume of Labyrinth Components
Volume (cm3)
0.06
0.04
0.02
0.00
Control
70% MNR
*
*
FC
MBS
Trophoblast
B
Proportion of Labyrinth Components
Percentage of Layer (%)
70
60
30
20
10
0
Control
70% MNR
*
*
FC
MBS
Trophoblast
C
Interhemal Distance
Distance (μm)
4
3
2
1
0
*
Control
70% MNR
D
Surface Area
Surface Area (cm2)
75
50
25
0
Control
70% MNR
*
*
Fetal
Maternal
E
Fetal Capillary Length
Length (m)
300
200
100
0
*
Control
70% MNR
F
Fetal Capillary Diameter
Diameter (μm)
15
10
5
0
*
Control
70% MNR
G
Theoretical Diffusion Capacity
Theoretical Diffusion Capacity (cm2. min-1. kPa-1. 10-2)
4
3
2
1
0
*
Control
70% MNR
H
Specific Diffusion Capacity
Specific Diffusion Capacity (cm2. min-1. kPa-1. g-1. 10-2)
3
2
1
0
Control
70% MNR

when expressed in weight as grams of fetus, the diffusion capacity was appropriate for weight of the fetus, with no difference between groups (**Fig. 5.4H**).

5.3.6 IGF System

The IGF system has been implicated to have a significant role in promoting development and function of the murine placenta (8). The expression of the system was analyzed specifically in the labyrinth zone during maternal undernurition. MNR placentae demonstrated significantly increased mRNA expression of IGF-II (**Fig. 5.5 E&G**; Control: 10 ± 2 *vs.* MNR: 19 ± 3 grains/cell, $P < 0.05$), IGFBP-2 (**Fig. 5.5 M&O**; Control: 23 ± 1 *vs.* MNR: 40 ± 4 grains/cell, $P < 0.001$) and IGFBP-3 (**Fig. 5.5 Q&S**; Control: 21 ± 1 *vs.* MNR: 41 ± 2 grains/cell, $P < 0.001$), with no significant differences in IGF-I expression. IGFBP-1 expression was localized to the yolk sac with strong expression in the cuboidal epithelium lining, the non-vascular spaces in the allantoic plate and appeared greater in MNR placenta though not quantified.

5.3.7 Transporter Studies

Stereology allowed measurement of placental structure, but did not indicate changes to the placental function of nutrient transport. Transfer studies of radiolabelled substrates from the maternal to fetal circulation were performed to measure the function of the placenta during maternal undernutrition. Analysis of solute transfer per gram of placenta for each radiolabelled substrate demonstrated no significant differences between control and MNR groups for MeAIB. However transport of EDTA (**Fig. 5.6A**; MNR 0.05

Figure 5.5 The effect of maternal nutrient restriction on placental IGF System Expression at E18.5. In situ hybridization of IGF-I (**A** and **C**), IGF-II (**E** and **G**), IGFBP-1 (**I** and **K**), IGFBP-2 (**M** and **O**), and IGFBP-3 (**Q** and **S**) of control (**A, E, I, M** and **Q**) and 70% MNR (**C, G, K, O** and **S**) placentas. Immunohistochemistry of IGF-I (**B** and **D**), IGF-2 (**F** and **H**), IGFBP-1 (**J** and **L**), IGFBP-2 (**N** and **P**), and IGFBP-3 (**R** and **T**) of control (**B, F, J, N** and **R**) and 70% MNR (**D, H, L, P** and **T**) placentas. (**U**) Counts of IGF System mRNA grains per cell (mean ± SEM) in the labyrinth from control (N=8) and 70% MNR (N=8) placentas. In the 70% MNR placentas, transcript expression of IGF-II of 19 ± 3 (vs 10 ± 2 in controls), IGFBP-2 of 40 ± 4 (vs 23 ± 1 in controls) and IGFBP-3 of 41 ± 2 (vs 21 ± 1 in controls) were significantly elevated ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively). *Bars*, 100 μm.

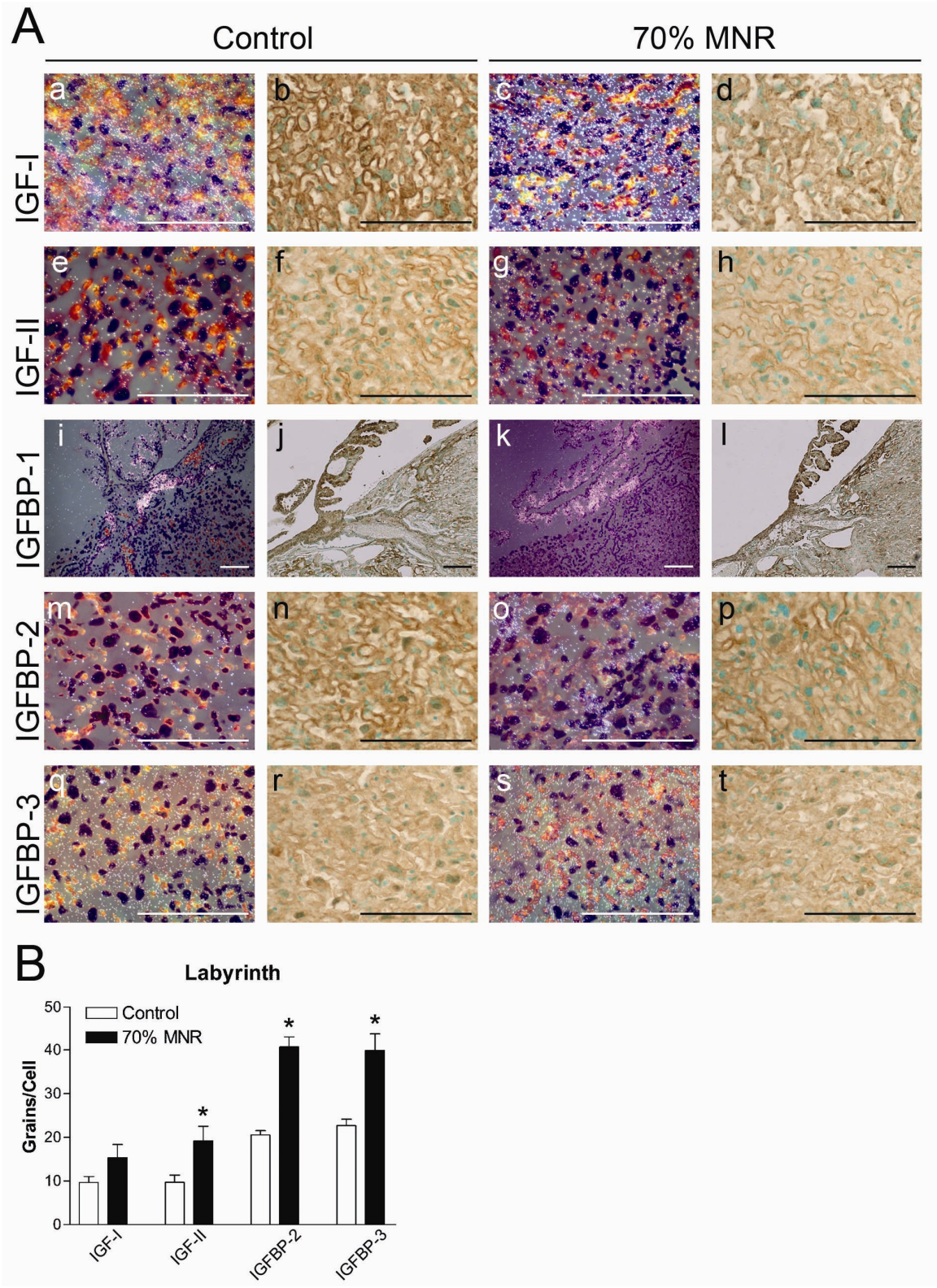
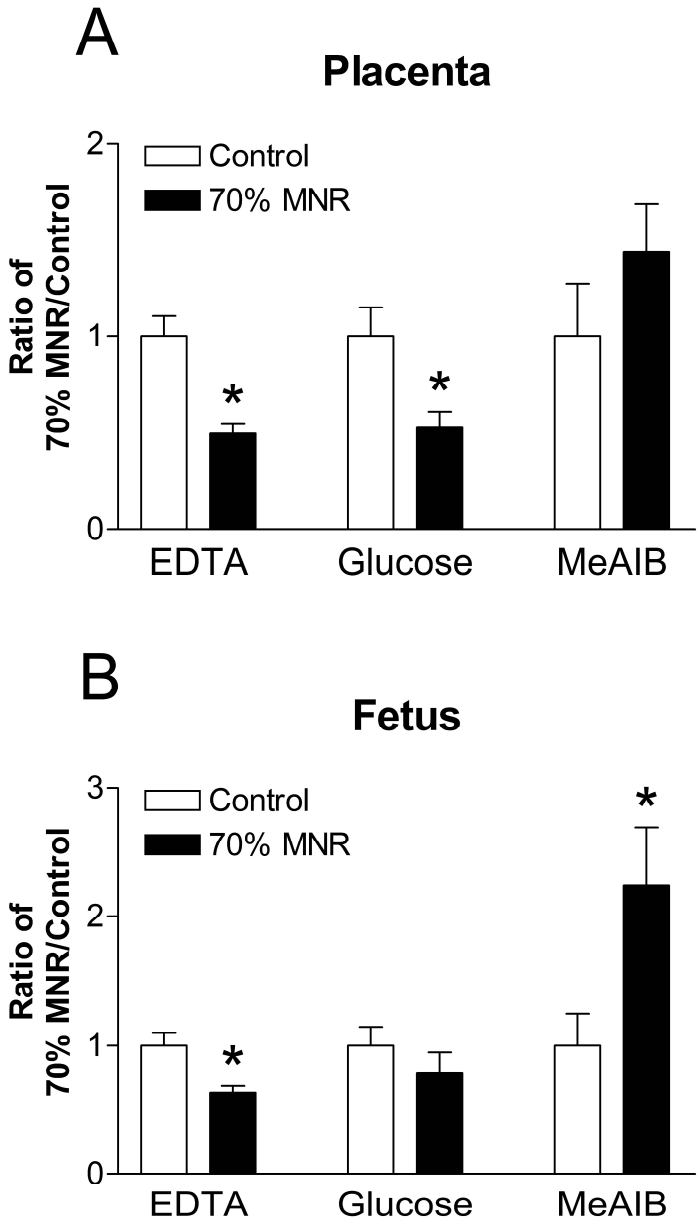

A
Control
70% MNR
IGF-I
IGF-II
IGFBP-1
IGFBP-2
IGFBP-3
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
B
Labyrinth
Control
70% MNR
Grains/Cell
50
40
30
20
10
0
*
*
*
IGF-I
IGF-II
IGFBP-2
IGFBP-3

Figure 5.6 The effect of maternal nutrient restriction on placental transfer of solutes at E18.5. (**A**) Fold change of EDTA, glucose and MeAIB transfer to fetus per gram of placenta (mean ± SEM) in control (N =4, 8, 6, respectively) and 70% MNR (N=8, 12, 7, respectively) litters. In the 70% MNR placentas, the transfer of EDTA of 0.49 ± 0.05 (vs 1.00 ± 0.10 in controls) and glucose of 0.52 ± 0.08 (vs 1.00 ± 0.15 in controls) was significantly reduced ($P < 0.001$ and $P < 0.01$, respectively). (**B**) Fold change of EDTA, glucose and MeAIB transfer to fetus per gram of fetus (mean ± SEM) in control (N =4, 8, 6, respectively) and 70% MNR (N=8, 12, 7, respectively) litters. In the 70% MNR fetuses, the accumulation of EDTA of 0.63 ± 0.05 (vs 1.00 ± 0.10 in controls) was significantly reduced ($P < 0.005$), but MeAIB accumulation per gram of fetus of 2.24 ± 0.46 (vs 1.00 ± 0.25 in controls) was significantly increased ($P < 0.05$).


A
Placenta
Control
70% MNR
Ratio of
70% MNR/Control
2
1
0
*
*
EDTA
Glucose
MeAIB
B
Fetus
Control
70% MNR
Ratio of
70% MNR/Control
3
2
1
0
*
*
EDTA
Glucose
MeAIB

fold change 0.49 ± 0.05, P < 0.001) and glucose (**Fig. 5.6A**; MNR 0.52 ± 0.08 fold change, P < 0.01) was significantly reduced in MNR group. When normalized to fetal weight, the fetus received less EDTA per gram than controls (**Fig. 5.6B**; MNR fold change 0.63 ± 0.05, P < 0.005), however more MeAIB (**Fig. 5.6B**; MNR fold change 1.44 ± 0.25, P < 0.05), with no significant differences in glucose. No significant differences were demonstrated in mRNA expression of Glut-1 and -3, Lat-1 and-2, and Slc38a1, -2 and -4 and at the protein level of GLUT-1 and GLUT-3 in the microvillous membrane (MVM) (**Appendix Fig. 1.4**).

5.4 DISCUSSION

This study demonstrated that 70% MNR adversely affects the structure and function of the placenta to adequately supply nutrients to the growing fetus. In contrast to previous studies (2-4), we showed that a relatively mild reduction of maternal nutrient supply (70% of controls), caused a reduction in placental efficiency and impairment of solute transport that was inefficient relative to the weight of the fetus. Previously, we have shown that the 70% MNR mouse model of FGR demonstrated the most severe asymmetrical FGR phenotype when compared to 80% and 60% MNR (Chapter 3). Coan et al., recently published a placental study in a 80% MNR mouse model, which according to our previous data, may not be severe enough to induce a consistent FGR phenotype, as many fetal weight parameters were similar to control (23).

Fetal weight was reduced to a greater extent than placental weight, suggesting that during MNR, the placenta was relatively spared compared to the fetus as maternal nutrient supply was utilized to support placental metabolism, growth and development

first. In a previous study, we demonstrated that transgenic mice overexpressing hepatic hIGFBP-1 were growth-restricted at birth with increased placental weight and reduced placental efficiency near term similar to the current MNR model (24). The placental changes may be the direct effects of IGFBP-1 on placental growth or may be compensatory changes designed to increase passage of nutrients to the growth-restricted fetus (24). Previously, we demonstrated increased IGFBP-1 expression in MNR fetal liver, and hence elevated circulating IGFBP-1 may be a fetal distress signal to the placenta to increase growth to supply more nutrients to the fetus [submitted]. Placental weight that was proportionately greater relative to fetal weight may be a compensatory mechanism to increase nutrient supply *in utero*, but may increase the long-term risk of disease as a high placental to birth weight ratio has been associated with elevated blood pressure and increased prevalence of coronary heart disease and impaired glucose tolerance (25-27).

To further study MNR placental structure, the volume of each placental layer and changes to the cellular composition were analyzed. The labyrinth zone contains fetal capillaries (FC) and maternal blood spaces (MBS) that are separated by two layers of syncytiotrophoblast and cytotrophoblast epithelium as well as fetal capillary endothelium and its supporting mesenchyme (16). The junctional zone is composed of three principal cell types, the spongiotrophoblasts, glycogen cells and secondary giant cells that rest on the decidualized endometrium of the uterus (16). Significant reductions in both placental zones were demonstrated, but the junctional zone was disproportionately reduced compared to the labyrinth zone, suggesting that the labyrinth was spared to maintain survival of the fetus and placenta. The junctional zone is the least understood of the

placental layers, however, it is critically important for fetal survival as demonstrated by embryonic lethality of the *Mash2* knockout mouse, which lacked a junctional zone (28). Analysis of the junctional zone demonstrated reductions of both spongiotrophoblast and glycogen cells, however, there was a greater decline in the population of glycogen cells. These junctional zone changes are similar to those described by Lopez et al. where glycogen cells were disproportionately reduced in the placenta of *Igf2* null mice (29). Previous studies utilized glycogen vacuoles to identify glycogen cells and could not differentiate whether glycogen cell reduction is due to less glycogen synthesis or disturbed glycogen cell differentiation. Using Plp-1 mRNA as a specific molecular identifier of glycogen cells, we demonstrated less glycogen cell differentiation during MNR (19). The function of the glycogen cell is unclear, but the stored glycogen may be used as an energy source for the placenta itself or may be stored to support fetal growth, as there are high demands on maternal resources at term (30). The lack of glycogen cells in our MNR model supports the theory of glycogen being stored to support fetal growth, as at E18.5, fetal growth was compromised more than placental growth.

The absolute volume of the labyrinth zone was significantly reduced in the MNR placenta, with the vasculature demonstrating fine differences in structure. The absolute volume of fetal capillaries was spared, but at the expense of volume reductions in the maternal blood spaces and placenta trophoblast and interstitium. The relative increase in the proportion of MNR labyrinth composed of fetal capillary suggests that the fetal circulatory component was attempting to compensate by vasodilatation to increase blood flow and extract more nutrients from the mother. In contrast, the reduction of the maternal blood space volume may be a maternal response to limit nutrient transfer to the

fetus by constricting blood flow through the placenta. Our results support the changes to the placenta seen in MNR guinea pigs (31) and are consistent with stereological studies of human FGR placentae (32, 33). Reduced surface area for exchange and increased barrier thickness between maternal and fetal circulations suggest an impedance to transfer of solutes across the placenta with FGR.

Simple diffusion of EDTA was significantly reduced in keeping with the stereology data. Glucose transport was significantly reduced per gram of placenta, but was appropriate per gram of fetus. Glucose is an important substrate for growth in late gestation as fetal weight is correlated specifically to the supply of glucose. Our data demonstrating no changes to mRNA and protein levels with MNR are consistent with human studies of FGR placentae (34, 35). System A is a ubiquitous Na+-dependent transport system used by small neutral amino acids (glutamine, glycine, and serine) and consists of three different isoforms SNAT-1 (ATA-1), SNAT-2 (ATA-2) and SNAT-4 (ATA-3) in human and rodent placenta (36). MeAIB is a System A specific substrate, particularly for SNAT-2 (36). The placental transport of MeAIB per gram of placenta was not different between groups with no change in SNAT mRNA expression. However, significantly more MeAIB accumulated in growth restricted MNR fetuses indicating a potential mechanism to increase fetal growth or energy stores prior to birth. The reduced permeability of EDTA and increased MeAIB accumulation in the fetus may be complementary, such that reduced passive permeability of MNR placenta may lower the back flux of small molecules (e.g. EDTA) down their concentration gradient from fetal to maternal circulation (10) and result in increased net flux of actively transported amino acids across the MNR placenta to the fetus and accumulation of MeAIB (10).

Analysis of the insulin-like growth factor system expression in the labyrinth layer suggests an intriguing paracrine mechanism by which the placenta altered growth and differentiation in the event of MNR. The cellular sites of expression of the respective mRNAs of the IGF system were similar to those previously described (8). IGF-II mRNA was highly expressed in the labyrinth layer of the MNR placenta compared to the control and is consistent with several previous studies which reported increased placental expression of IGF-II mRNA in human (37, 38) and animal models of FGR (39, 40). IGF-II gene transcription may be increased in an attempt to compensate for growth restriction, as IGF-II over-expression caused by relaxation of imprinting (41) or disruption of the IGF-2R clearance receptor lead to placentomegaly (42). Alternatively, increased expression may be an attempt by the placenta to overcome the inhibitory actions of elevated IGFBP expression. Our model supports the latter hypothesis. IGFBP-2 mRNA was highly expressed in the labyrinth layer in the MNR placenta, specifically in the trophoblast and capillary endothelium. Although IGFBP-2 actions on the placenta is yet unknown, its expression tends to be increased in fetal circulation of FGR humans and animal models, and suggests to be a causal factor of decreased growth (43). IGFBP-2 may have specific effects on the placenta as it has a higher affinity for IGF-II over IGF-I, as IGF-II is the main promoter of growth and differentiation of the placenta. IGFBP-3 was localized to the mesenchyme of the labyrinth and its expression was increased in MNR placentae. Similarly, the function of IGFBP-3 in the placenta is unknown, but it may suppress IGF effects as shown in placental fibroblasts (44). Many of the morphological changes in the MNR labyrinth and junctional zones are similar to those seen in the *Igf2* null mutant mouse placentae (10), and overall suggest an impairment of

IGF-II action in the MNR placenta. Thus, resultant phenotype of the placenta in MNR indicates that despite increased expression of IGF-II mRNA, the concomitant increase of IGFBPs may inhibit IGF-II actions on placental development

In conclusion, our MNR mouse model of FGR demonstrated many changes to placental vasculature that have been documented in human cases of FGR. Our model demonstrates a failure of the placenta to adapt to MNR, as placental efficiency in terms of weight is reduced while the specific transport of substrates crossing the placenta by diffusion, facilitated diffusion and active transport do not correspond to the amounts necessary to sustain fetal growth. Although the role of IGF-II has been demonstrated to be an important regulator of placental development and function, the role of its main regulators, the IGFBPs, have yet to be established in the pathophysiology of FGR and the placenta. Our model demonstrated important changes in placental IGFBP expression and may serve as an important model system to determine the interaction of IGFBPs on IGF-II placental action during FGR with subsequent changes in placental structure and function.

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CHAPTER 6

THESIS DISCUSSION

6.1 THE MODEL

Our model of fetal growth restriction (FGR) in mice demonstrated many of the phenotypic characteristics of human FGR. The variations in fetal morphometry indicated that the mouse model was consistent with an asymmetric form of FGR (1). The primary regulators of fetal growth, the IGF and glucocorticoid systems, demonstrated expression changes consistent with those seen in human FGR neonates. The circulating IGF-1 and IGFBP-3 were decreased, and IGFBP-1 elevated (2). Tissue expression of murine IGF system mRNAs were consistent with humans as the liver being the main site of production during gestation for these peptides (3). The glucocorticoid system demonstrated elevated circulating levels of corticosterone (4, 5) in the fetus with decreased dehydrogenase metabolism to inactive metabolites in the placenta as seen in human FGR (2). Maternal total caloric restriction in the mouse caused similar changes to organ structure compared to those demonstrated in human FGR fetuses. In the liver, glycogen storage was reduced (6); in the lung, airspaces were enlarged consistent with bronchopulmonary dysplasia following human FGR complicating prematurity (7-12); and in the placenta, the vascular structure demonstrated reduced surface area of fetal capillaries and maternal blood space, with increased thickness of the exchange barrier between the maternal and fetal circulations. These changes correspond to vascular changes in human FGR placentas uncomplicated by pre-eclampsia (13). Our mouse model showed evidence of catch-up growth and developmental programming of chronic diseases in adulthood in a gender specific manner. The catch-up growth the in MNR

mouse offspring was completed by three weeks postnatally and MNR adults remained the same weight as controls by six months postnatally. The accelerated accretion of mass in our MNR postnatal animals occurred without subjecting the pups to high fat diets (14) or abundant calories postnatally (15) and long-term disease occurred despite having the same food allocation as the control pups. Evidence of developmental programming occurred in our male FGR mice that demonstrated postnatal glucose intolerance, whereas female FGR mice demonstrated impairment in postnatal lung function.

The important advantage of a mouse model of FGR is the ability to utilize genetic manipulation (knockout or transgenic) to address phenotypic or functional role of specific genes to delineate mechanistic contribution *in vivo*. Epigenetic alterations to DNA are hypothesized to be an important mechanism responsible for developmental programming of fetal tissues following FGR (16-18). The IGF and the glucocorticoid systems are important in these epigenetic changes. Components of the IGF system, IGF-II in humans, and IGF-II and IGF2R in the mouse, are imprinted *in utero* (19). Persistent epigenetic changes to IGF-II have been demonstrated in humans following maternal malnutrition (20) and genetic alteration of IGF-II (21) or IGF2R (22, 23) in mice demonstrated alterations in placental and fetal growth. *In utero*, glucocorticoids can alter DNA methylation, particularly at the GR promoter (18), which may persist after birth to alter adult gene expression (18). Glucocorticoid overexposure during late gestation in rats has been linked to demethylation of the GR promoter and increased GR gene expression in the kidneys of offspring during adulthood, contributing to the development of hypertension (18, 24). However, little is known about the effects of hormones on histone modifications during gestation (18); it is likely that changes in the fetal endocrine

environment through maternal dietary manipulation and placental insufficiency lead to histone modifications (18, 24-26), as has been demonstrated in adult tissues (27). A mouse model of FGR with long-term compromised function of the pancreas and lung, which are highly sensitive to alterations of fetal IGF expression and elevated glucocorticoid exposure, provides an opportunity to determine the epigenetic alterations and their contribution to the pathophysiology of developmental programming.

Despite the similarities our FGR mouse model has to human FGR, the mouse model has some disadvantages. A major limitation of the rodent model in fetal development research is that they are born with a relatively immature central nervous system and endocrine/paracrine systems that continue to mature during the weaning period (28). This may be an important reason why we were not able to demonstrate long term neurologic or neurobehavioral adverse outcomes in our model. Rodents are also litter-bearing, which may question their relevance to human pregnancy. In the first thesis chapter, the mouse model appeared to reduce its litter size with increasing severity of maternal nutrient restriction, allowing the potentially more hardy fetuses to survive with less severe growth restriction. Humans do not have a similar mechanism of reducing the severity of FGR. Despite the entire litter of maternally nutrient restricted fetuses and placentae developing in the same uterus, the supply of nutrients to the fetus may be variable (29), as blood flow may be preferentially diverted to some fetuses at the expense of others, possibly due to the position in the uterine horn (30). This caveat was addressed in the thesis by collecting tissues from all fetuses in the litter and combining them into a single sample, to give an overall indication of tissue changes. However, this technique precluded the ability to detect fetal sex differences in tissue expression. A caveat of our

mouse model, which does not occur in the rat, is the lack of 11β-HSD2 beginning at E16 (31). In rats and humans, 11β-HSD2 continues to be expressed until term (32) and changes to expression or activity have been demonstrated to contribute to the severity of FGR (2). In our initial hypothesis, that IGFs and glucocorticoids are the primary regulators of fetal growth, we anticipated globally altered gene expression of these systems in each fetal tissue during maternal nutrient restriction. However, each tissue responded variably to maternal undernutrition. In the IGF system, some tissues demonstrated no changes to any component of the IGF system assayed (e.g. heart and carcass), other tissues elevated the expression of binding proteins (e.g. lung, liver and brain), while other tissues reduced the expression of IGF-I and increased the expression of binding proteins (e.g. placenta). Similar varied responses to maternal undernutrition were demonstrated in the glucocorticoid system, circulating glucocorticoids were elevated in the maternal and fetal plasma. However, the dehydrogenase activity of 11β-HSD1 was reduced in the placenta, but did not change in the lung; nor did the expression of the glucocorticoid receptor change in these tissues. This indicates that there was no global tissue response to hormonal changes with substrate reductions that occurred during fetal undernutrition. Each fetal tissue had a unique response and mechanism that adapted to maternal undernutrition to preserve fetal viability. Despite these differences, our mouse model provides a good representation of human FGR to be used for studies involving fetal programming, gender differences and disruption to fetal vasculature.

6.2 COMMON ETIOLOGY/PATHOPHYSIOLOGY

In the organ systems studied, common features that may provide clues to the overall pathophysiology of FGR are noted. Two themes that arise across the organ systems studied in the thesis are the disruption FGR causes to the fetal vasculature and the sexual dimorphism that occurs in organ function in adulthood following FGR.

6.2.1 Importance of Fetal Vasculature

Abnormal vascular development has been implicated in many organs affected by FGR. During FGR, an adaptive response alters the circulation to divert nutrient rich blood away from the trunk (compromising the liver, muscle, gut and lung) to sustain brain and heart metabolism (33-39). The loss of blood flow to trunk organs during development may permanently compromise circulation to that organ and its function (40). Low perfusion could result in vascular remodeling, oxidative stress, apoptosis, and proliferation of vascular smooth muscle, which contribute to the development of cardiovascular disease (40). Furthermore, abnormal vascularization has been detected in the pancreas, in the lung (41, 42), and the cardiovascular system following FGR (43). Therefore, disruption to the fetal vasculature may be a common pathophysiology in the long-term consequences of fetal programming.

The primary system mediating vessel growth is the vascular endothelial growth factor (VEGF) system. VEGFs are crucial regulators of vascular development during embryogenesis (vasculogenesis) and blood vessel formation (angiogenesis) in the adult (44). Five VEGF ligands, which occur in several different splice variants and processed forms have been identified (44). VEGF binds with high affinity to transmembrane

tyrosine kinase receptors, VEGFR1 and VEGFR2, which are expressed on vascular endothelium (44). VEGFR2 is the dominant receptor involved in vasculogenesis and angiogenesis as it signals endothelial cell proliferation, migration, and survival (44). Negative regulation of VEGFR2 is exerted, at least in part, by an alternatively spliced soluble VEGFR1 variant that binds to VEGF and prevents VEGF binding to VEGFR2 (44). In the placenta, elevated production of the soluble VEGFR1 variant (sFlt1) and consequent immobilization of VEGF and decreased VEGFR2 signaling is implicated in preclampsia (45). Preeclampsia causes placental insufficiency, which in turn leads to fetal undernutrition. However, if elevated sFlt1 production is associated with preclampsia and altered placental vasculature, it is possible that fetal tissues may also be producing elevated sFlt1 levels. Regardless of the fetal nutrition status, the possible disturbance to the fetal vasculature throughout the body due to less VEGF may cause the development of small and immature organs characteristic of FGR.

Glucocorticoids and IGFs have been demonstrated to influence the expression of the VEGF system. Glucocorticoids inhibit blood vessel development and VEGF expression (46-49). Deletion of fetal 11β-HSD2 and exposure of the fetus and placenta to high levels of maternal glucocorticoids result in reduced fetal and placental growth at E18, with impaired capillary development and reduced VEGF mRNA in the labyrinth zone of the placenta (50). Increased placental glucocorticoid exposure may inhibit VEGF expression which is a key factor for placental vascular remodeling (51, 52). In contrast, insulin and IGFs potentiate and increase the expression of the VEGF system. IGF-I may also be permissive to VEGF action, as VEGF can stimulate endothelial cell proliferation only if there is sufficient IGF-I (53). IGFs can directly stimulate the proliferation of

endothelial cells and blood vessel formation (54) and insulin is considered to be a growth factor for endothelial cells (55). Therefore, low insulin and IGF levels with elevated glucocorticoid levels during FGR may be responsible for reduced VEGF and vascularization of fetal organs with stunted tissue growth and development.

The clinical importance of appropriate expression of growth factors mediating angiogenesis is supported by the reduced mRNA and protein expression of vascular endothelial growth factor (VEGF) and its receptors in lung tissue from infants with BPD (56-58). Reduced expression of VEGF signaling components in BPD suggests that the disruption of pulmonary vasculature may be an underlying cause of poor alveolarization (56-58). Specifically, inhibiting the VEGFR2 receptor by pharmacological approaches during the neonatal period of alveolarization in rats, caused hypoplastic lungs with a reduction in capillaries and distended underdeveloped alveoli (59). Conversely, many animal models using adverse lung stimuli to disrupt alveolarization demonstrate reduced expression of VEGFR2, which may contribute to inhibition of alveolar formation (60-62). The mechanism by which VEGFR2 expression is reduced in these models is unknown, but appears to be related to the elevated exposure of fetal lungs to glucocorticoids.

The islet cells of the endocrine pancreas highly express VEGF, which likely maintains the dense network of islet microvessels (63-65). VEGFR2 is expressed by the endothelial cells and pancreatic ductal cells during fetal development (66). VEGF is mitogenic for the pancreatic ductal epithelium (which contain ß cell precursors) (67), and the blood vessels provide inductive signals for endocrine pancreas development (68). This implicates VEGF as having a potential role in organogenesis of the endocrine pancreas and its population of islets (69). FGR rat fetuses of mothers fed an 8% low

protein diet throughout pregnancy developed small islets due to less islet cell proliferation and more apoptosis with impaired insulin secretion (70). LP fetuses had reduced islet blood vessel density, with fewer islet cells producing VEGF and VEGFR2 (70). However, the pancreatic duct cells had enhanced expression of VEGFR2, suggesting upregulation of VEGFR2 production in duct cells in response to the fewer islet cells induced by LP (70). This mechanism for enhancing islet neogenesis was insufficient as VEGF production by LP endocrine cells was too low (70).

Normalizing appropriate VEGF expression in fetal tissues during organ development may be an important key to acquiring normal tissue structure and function, as well as to offset the development of disease in adulthood. Understanding the interaction of the VEGF system with the common mediators of tissue growth and development, such as the glucocorticoid and IGF system, may help uncover important clues to the impairment to fetal organs that occur during FGR.

6.2.2 Gender Differences of Long-Term Disease

Sex specific differences in disease prevalence are known to occur in the human population. Generally, the effects of poor fetal growth are more obvious in males following FGR (71-73). Our mouse model may be an important model system for studying gender differences to disease following FGR. Our model demonstrated sex-specific changes in glucose metabolism and lung function in postnatal life, with implications in determining fetal response to stress, antenatal glucocorticoid treatment and developmental programming.

In utero, it is known that the IGF and glucocorticoid system demonstrates gender differences. Despite no difference in fetal cord blood cortisol levels, female fetuses have reduced 11β-HSD2 activity compared to male fetuses (74), suggesting placental glucocorticoid metabolism differs according to fetal gender. Similar gender differences have been observed in the mouse placenta (75) and human kidney (76). Differences in glucocorticoid metabolism between genders may contribute to reduced sensitivity of male fetuses to the effects of glucocorticoids; as prenatal glucocorticoid treatment is less effective in preventing respiratory distress syndrome in male fetuses compared to females (77). The mechanisms for each gender to cope with glucocorticoid exposure may explain the resulting differences in fetal programming in males and females. The IGF axis is known to have gender differences in expression. IGF-II (78), IGFBP-1 (79) and growth hormone (GH) (80) concentrations are higher from the umbilical cord serum in male neonates than those in females. However, cord plasma IGF-I and IGFBP-3 are higher in female neonates than in males (79, 80). Glucocorticoids and IGFs may be involved in the development of chronic diseases such as insulin resistance in adulthood following FGR (3, 32) as they may alter the structure and function of the pancreas (81), liver (82) and lung (83, 84) during development, and components of the IGF and glucocorticoid systems are expressed differently between genders. Gender-specific expression changes in the glucocorticoid and IGF system during FGR may determine sex specific adaptations to fetal growth via these mediators, and establish mechanisms for the differential incidence of long-term disease risk between genders.

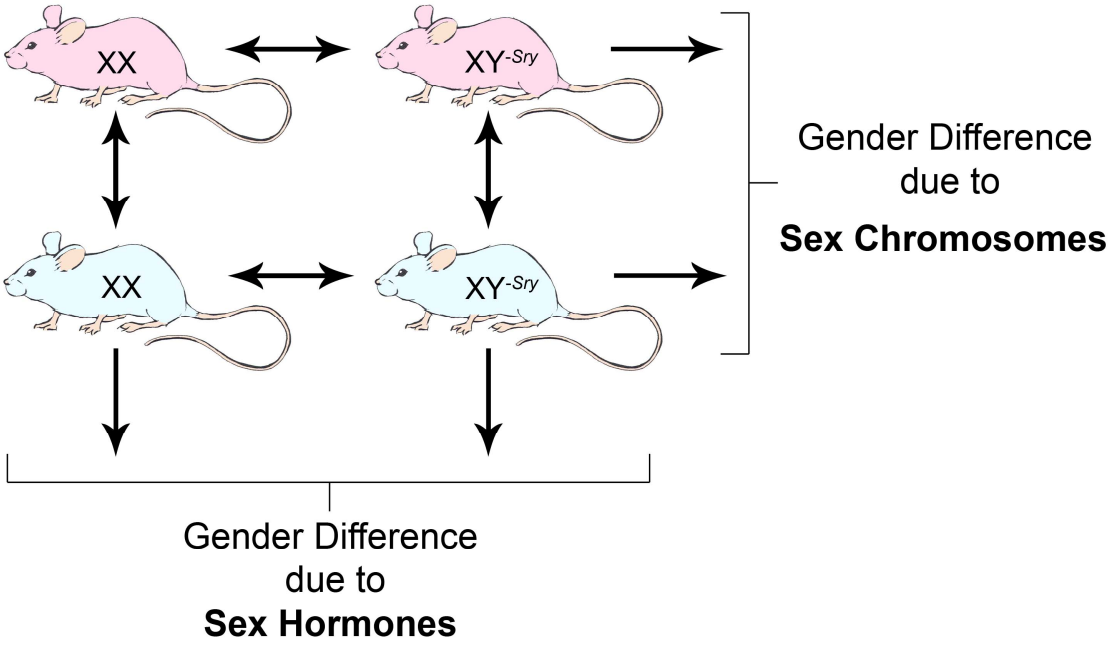
6.3 FUTURE DIRECTIONS

6.3.1 Four Core Genotype Mouse Model

Many of the differences in the incidences of disease across genders, such as hypertension (85), autoimmune disease (86), alcohol abuse (87), which were previously attributed to sex hormones may be due to the presence or absence of Y chromosome or additional X chromosome. Genetic female (e.g. XX) and genetic male (e.g. XY) cells have a different number of X and Y genes (88). These genomic differences may cause sex differences in which cells function, both in sex specific organs and organs throughout in the body (88). Recently, Arnold et al. developed the four core genotype model in which sex chromosome complement (XX vs. XY) is unrelated to the gonadal sex in mice (89). These mice were produced by a two-step genetic manipulation: the *Sry* gene (which determines the presence of testes) was deleted from the Y chromosome (90), and a functional *Sry* transgene was inserted onto an autosome, driven by its own promoter (91). Therefore, testes determination was transferred from the Y chromosome to an autosome; the Y chromosome and the number of X chromosomes were no longer correlated with gonadal sex (89). The four genotypes are XX gonadal males or females, and XY gonadal males or females (**Fig. 6.1**). The model allows determination of the differences in phenotype caused by sex chromosome complement (XX vs. XY), different effects of ovarian and testicular hormones, and the interactive effects of sex chromosomes and gonadal hormones (89). The four core genotype model would allow dissection of the etiology of dimorphic disease prevalence following FGR.

Maternal nutrient restriction in the fore core genotype model, to compare the development of insulin resistance between XX and XY females, as well as between XX

Figure 6.1 Four Core Genotype Model. Four core genotype mice are a result of breeding a XX female with a XY^{-Sry} male that has the *Sry* gene on an autosome chromosome. Pink mice represent phenotypic females, with female gonads and their associated secretions. Blue mice represent phenotypic males with male gonads and their associated secretion as a result of the *Sry* gene on an autosome chromosome. Genotypic sex chromosome complement is represented by the chromosomes depicted in the centre of the mouse. Doubled headed arrows indicate comparisons made between genders with single headed arrows indicating the relevance of the comparison. Y^{-Sry} is a Y chromosome with the *Sry* gene deleted. Adapted from Arnold et al. (89)


XX
XY-Sry
XX
XY-Sry
Gender Difference
due to
Sex Chromosomes
Gender Difference
due to
Sex Hormones

and XY males, would determine if insulin resistance was due to the sex chromosome compliment inherited by the cells. If the XY males and the XY females developed insulin resistance, this may be due to the presence of the Y chromosome or absence of the additional X chromosome (89) (**Fig. 6.1**). This may also occur in the etiology for compromised pulmonary function in fetal growth restricted females. If the development of insulin resistance or reduced pulmonary compliance is determined to be a result of sex chromosomes, other mouse strains, which vary in the number (or segments) of X and Y chromosomes, will be needed to determine whether developmental programming is caused by X or Y genes (89). Since epigenetics has been implicated as a mechanism for long-term programming of adult diseases following FGR, and if sex chromosomes are also implicated, imprinted X genes may be further studied. In females, the XX complement inherits the genomic imprint of both maternal and paternal X chromosomes, however in females with an XY sex chromosome compliment, only the maternal X chromosome imprint is expressed (89). Comparisons between the XX and XY females may give clues to the imprinted genes involved. Mosaicism may be implicated as a mechanism for gender differences; mosaicism will cause XX mice to express alleles of the maternal X chromosome in half their cells and alleles from the paternal X chromosomes in the other half (89). The greater diversity of X-linked genes in XX offspring could protect against environmental challenges more than in XY offspring (92).

Alternatively, our maternal nutrient restriction regime can be inflicted on the four core genotype model and the development of long-term diseases between XX males and XY females can be compared. If insulin resistance is detectable in males with an XX chromosome complement and if insulin resistance is not detectable in the XY females,

the development of insulin resistance may rely on the secretion of gonadal hormones (89) (**Fig. 6.1**). FGR has been implicated in altering production of sex hormones as well as sexual development (33, 34). Therefore, maternal nutrientrestriction in the four core genotype model may verify the role of gonadal hormones in the development of insulin resistance or decreased lung compliance following FGR.

Utilization of the four core genotype model in conjunction with our maternal nutrient restriction regime may determine an understanding of why adult diseases are more prevalent in one gender following FGR.

6.3.2 Maternal Supplements to Normalize Fetal Vasculature

Targeting components mediating fetal vasculature development may be essential for mitigating the severity of postnatal complications following FGR. The VEGF system in particular is central for vascular and tissue development and most importantly, seems amendable to maternal nutrient supplementation.

6.3.2.1 Maternal Supplementation of Taurine

Taurine was one of the amino acids most reduced in fetal serum in the LP diet model (70). It is a sulfur-containing amino acid, highly concentrated in the islets (93); it stimulates insulin release by fetal ß cells in vitro (94). Taurine supplementation in vivo and in vitro can reverse many of the impairments caused by a low protein diet during fetal and neonatal life. Establishing taurine to typical levels in the fetal blood circulation by supplementing LP mothers during gestation normalized fetal insulin secretion, islet and ß cell mass, apoptotic and low proliferative rates of the pancreatic endocrine cells

(95, 96). Taurine may have normalized the endocrine pancreas by restoring the number islet cells expressing VEGF and VEGFR2 and reversing the reduced blood vessel volume density and number of fetal islets induced by the LP diet (70, 96). However, VEGFR2 expression in the pancreatic duct cells remained elevated (70). This study suggests that taurine normalizes or enhances the expression of VEGF and VEGFR2 in islet cells (70) and prevents vascular changes to the islets during a LP maternal diet (70). The mechanism in which taurine affects VEGF and VEGFR2 expression is not understood, however, taurine may increase VEGF expression and stimulate islet vascularization, which in turn controls ß cells in the fetal endocrine pancreas (70).

Alternatively, taurine may protect endothelial cells from apoptosis due to cytotoxic stimuli, high glucose or IL-2 mediated cell injury (97-99). The supplementation of taurine to an inappropriate maternal diet may reduce the need for taurine production from cysteine. To produce taurine, cysteine must be processed through the transulfuration pathway, which also produces homocysteine byproducts that have adverse effects on endothelial cell function (70, 100). Taurine enhances growth factor production, as supplementation to LP dams during gestation restored normal IGF-II expression in fetal islet cells (95), which may be stimulatory factors of endothelial cell proliferation and blood vessel formation (53, 54).

6.3.2.2 Maternal Supplementation of Retinoic Acid (RA)

Retinoic acid (RA) supplementation in postnatal animal models has demonstrated promising results to reverse the effects of adverse stimuli on lung development and enhance the formation of alveoli. The ability of retinoic acid (RA) to induce alveolar

regeneration represents a potential means for preventing or reversing the loss of gas-exchange surface area in the lung (101). Rats treated with elastase to destroy the alveoli greatly reduced the surface area for gas exchange (102). Administration of all-trans-RA for 12 days restored the size and surface area of alveoli (102). Similar results using RA have been obtained in other animal models with restoration of the gas-exchange surface area (103-108). Dexamethasone treatment in newborn rodents has been used to generate reduced gas-exchange surface areas, with enlarged and fewer alveoli (107, 108). Treatment of 4 week old rats with all-trans-RA induced a partial recovery and in 6 week old mice a full recovery was obtained (107, 108). Human BPD has many characteristics of dexamethasone treated newborn rodents and it has been associated with low serum RA levels (109). Postnatally, three weekly doses with RA is one of few interventions found to significantly reduce incidence of BPD in extremely low birthweight infants (50, 110) and may have therapeutic implications in adults with emphysema (28, 101). The mechanism by which RA induces alveoli formation and antagonizes the effects of glucocorticoids is unknown. Affymetrix oligonucleotide arrays were used to identify gene expression changes in the neonatal mouse lung following treatment with dexamethasone to reduce alveolarization (111). Dexamethasone reduces VEGFR2 expression by 2.1 fold, which may contribute to loss of alveolar septae (111). Lung VEGFR2 expression is elevated 1.2 fold by all-trans-RA and decreased only 1.3 fold in lungs treated with all-trans-RA plus dexamethasone (111). *In vitro* studies have demonstrated RA enhances angiogenesis and VEGFR2 expression, but the mechanism remains unknown (29). These studies suggest RA may reverse the effects of dexamethasone on alveolarization by increasing the expression of VEGFR2.

Taurine and retinoic acid supplementation demonstrate that a single nutrient may restore normal vascular cues during development and potentially restore organ function. Studies that supplement low calorie or low protein maternal diets with these nutrients should consider studying the effects of supplementation on various fetal organs and determine if vascularization is restored. Studying common fetal tissue responses to maternal undernutrition and their restoration with nutrient supplements may indicate mechanisms and pathways disrupted during FGR and may identify common targets among fetal organs to restore function and prevent adverse long-term fetal programming.

6.4 CONCLUSIONS

In conclusion, our mouse model using maternal nutrient restriction closely mimics the adaptations in the endocrine/paracrine system regulating fetal growth, tissue structure and function demonstrated in human FGR. This thesis demonstrates the impact and adaptation of maternal nutrient restriction on a murine model and provides insight into the pathophysiological mechanisms of FGR in humans. This model is clinically relevant, as the nutrient deficient environment of the fetus during gestation caused a hormonal milieu not conducive to optimal fetal growth and development, consisting of elevated circulating glucocorticoids and reduced bioavailability of IGFs. This caused growth alterations similar to asymmetric FGR and long-term programming of insulin resistance in males, consistent with observations in other animal models and humans. The lung was adversely affected during gestation in a nutrient deficient environment as it developed proportionately smaller to the size of the fetus with alterations to its structure. Importantly, the gestational changes in the lung may lead to compromised postnatal pulmonary function in females. The placenta failed to adapt to maternal nutrient restriction by increasing the efficiency by which it transports nutrients and substrates to the fetus, regardless of reduced overall placental weight and volume. This failure to adapt may implicate the placenta as contributing to the pathophysiology to fetal growth restriction.

There are many benefits and disadvantages to using a mouse as a model of FGR. The potential benefit of genetic manipulation during this period in science when advances into the understanding of epigenetic alterations and the long term implications has great benefit to neonatal health management and approaches to chronic diseases in adulthood.

This mouse model also raises many important questions regarding gender and long-term disease risk following FGR, as well as the impact on vasculature in all FGR organs, as potential areas to study and manipulate for understanding the mechanisms involved in FGR and long-term disease risk. This mouse model of FGR may assist in identifying new targets and mechanisms that occur in fetal tissues to adapt fetal growth during periods of a compromised intrauterine environment and develop new techniques to mitigate changes in fetal tissues to restore normal development prior to birth and long-term disease risk.

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APPENDIX

1.1 ADDITIONAL METHODS

1.1.1 Insulin, Corticosterone and ACTH ELISA

Plasma insulin was measured in mothers and E18.5 fetuses and six month adult males using a Crystal Chem Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL). In brief, a standard curve was generated with two fold dilutions. Five micro litres of mother, fetal or adult male plasma sample was added per well with 95 µl of diluent and were incubated for 2 hrs at room temperature. The microplate was washed and 100 µl of horse radish peroxidase-conjugated anti-insulin was added to each well and incubated for 30 min. The microplated was washed before adding 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and incubating for 40 min. Stop solution was added to each well and the absorbance of colour reaction was read a wavelength of 450 nm in a plate reader. The intra- and interassay coefficients of variation were $\leq$ 10.0% and $\leq$ 10.0% respectively, with a minimum detectable dose of insulin of 0.10 ng/ml.

Plasma corticosterone was measured in mothers and E18.5 fetuses using an AssayMax Corticosterone ELISA Kit (AssayPro, St. Charles, MO). In brief, a standard curve was generated with four fold dilutions. Twenty-five micro litres of mother or fetal plasma sample was added per well and 25 µl of biotinylated corticosterone was added immediately afterwards and the solutions were incubated for 2 hrs at room temperature. The microplate was washed and 50 µl of Streptavidin-Peroxidase to each well and incubated for 30 min. The microplated was washed before adding 50 µl of Chromogen

Substrate and incubating for 10 min. Stop solution was added to each well and the absorbance of colour reaction was read a wavelength of 450 nm in a plate reader. The intra- and interassay coefficients of variation were 5.0% and 7.0% respectively, with a minimum detectable dose of Corticosterone of 40 pg/ml.

Plasma ACTH was quantfied in maternal and pooled fetal mouse plasma using multiplexed bone metabolism mouse panel 1A biomarker immunoassay kits (cat. No. MBN1A-41K) according to manufacturers' instruction (Milli*plex*, Millipore Corp, MA). A Bio-Plex^{TM} 200 readout System was used (Bio-Rad Laboratories, Hercules, CA), which utilizes Luminex® xMAP^{TM} fluorescent bead-based technology (Luminex Corp., Austin, TX). Levels were automatically calculated from standard curves using Bio-Plex Manager software (v.4.1.1, Bio-Rad).

1.1.2 In situ Hybridization Protocol

Liver and placental sections from control and maternal NR groups were analyzed for Igf-1, Igf-2, Igfbp-1, Igfbp-2 and Igfbp-3 mRNA expression by *in situ* hybridization using ^{35}S-labelled antisense riboprobes with the ^{35}S-labelled antisense and sense cRNA probes were generated from the cDNAs (listed in **Tables 3.2** and **Table 5.3**) using a Promega Riboprobe Combination System SP6/T7 kit (Promega Corp., Madison, WI) and ^{35}S-UTP (Perkin Elmer, Waltham, MA).

Tissue sections, mounted on Superfrost® slides (Fisher Scientific, Fairlawn, NJ), were deparaffinized, rehydrated in descending ethanol series (l00%, 90%, and 70%), and incubated in the following solutions: 0.2% Triton-X 100 in PBS for 1 hr, proteinase-K

(0.2 U/ml) for 30 min at 37 □C and acetic anhydride (0.25%) in triethanolamine buffer (Sigma, St. Louis, MO) for 10 min.

The sections were then dehydrated in ascending ethanol series and prehybridized with prehybridization buffer (0.3 M NaC1, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 X Denhardt's solution, 500 μg/ml yeast transfer RNA, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, 100 mM dithiothrietol, and 50% formamide) at 45 □C for 2 hrs.

Following prehybridization, tissue sections were hybridized overnight at 45 °C–65 °C with ^{35}S-labelled antisense riboprobes specific for each IGF or PLP-I mRNA (radiolabeled to a specific activity of $\geq l0^9$ cpm/ml and used at a concentration of 10 X $l0^6$ cpm/ml) in 1 x hybridization buffer (prehybridization buffer with 10% dextran sulfate) overnight at 55 °C. Excess probe was washed twice with 2X SSC with 10 mM DTT and the prehybridization buffer was applied to the slides for 10 min at the same temperature as the probe hybridization temperature. Tissue sections were treated with RNAse (10 mg/250 mL) to cleave unhybridized probe. Tissue sections then underwent washes in decreasing salt buffers (2.0× SSC to 0.1× SSC) at specific temperatures for each probe.

Tissue sections were then dehydrated in ascending ethanol series and exposed to x-ray film (XAR, Kodak) for 24 hrs. The duration for exposure to photoemulsion was judged by the autoradiographic intensity of the sections on x-ray film. The slides were coated with photoemulsion (NTB-3 nuclear track emulsion, Eastman Kodak, Rochester, NY) and exposed at 4 °C for 1-16 weeks, under dessicating conditions.

The photoemulsion was developed with a D-19 developer (Kodak), fixed, stained with Harris's hematoxylin and eosin, and mounted with Permount® (Fisher Scientific). The specificity of in situ hybridization was demonstrated by the absence of hybridization

signal when adjacent tissue sections were subjected to identical in situ hybridization procedures with radiolabeled sense RNA probes.

The slides were viewed under both dark- and brightfields, using a Carl Zeiss Axio Imager Z1 (Zeiss, City, Country) and appropriate photomicrographs were obtained at 40× magnifications using AxioCamHR3 and the AxioVision Release 4.7.2 software.

1.1.3 Apotag Staining for Apoptotic Cells

Liver tissue sections were prepared and deparaffinized in decreasing concentrations of ethanol. Apoptotic cells were detected using an Apoptag Kit from Chemicon International. Briefly, tissue sections were then immersed in 0.4 U/mL of Proteinase K in PBS for 20 min at room temperature, then washed four times in deionized water. Slides were quenched in 3% H_2O_2 in methanol for 10 min at room temperature and then washed in deionized water. Slides were then incubated in equilibration buffer for 1 hr, before incubating with TdT enzyme at a dilution of 1:3 at 37 □C in a humidified chamber for 1 hr. The reaction was stopped with Stop/Wash Buffer and slides were washed in deionized water. Anti-D.I.G conjugate was applied to the slides and incubated at room temperature for 30 min in a humidified chamber. DAB was used to detect the enzymatic reaction product and tissue was counterstained with Methyl Green (DakoCytomation Inc., Carpinteria, CA). Specificity of the staining product was verified by generating a positive control slide by inititally incubating the liver sections in DNAse 1 (Invitrogen) in TBS/MgSO4 for 30 min in a humidified chamber at 37 ‘C. Negative Control slides were generated by omitting the TdT enzyme incubation step.

1.1.4 Periodic Acid Schiff (PAS) Stain

To determine the glycogen content present in the liver of the control and 70% NR fetuses, PAS staining was performed using the PISSARRO-PAS Stain Kit (Biocare Medical, Walnut Creek, CA). Liver tissue sections were prepared and deparaffinized in decreasing concentrations of ethanol. Tissue sections were oxidized in PISSARRO Periodic Acid Solution for 10 min then rinsed four times in distilled water. Liver sections were then placed in PISSARRO Schiff's Reagent for 15 min. Slides were counterstained with CAT Hematoxylin then mounted with Permount and coverslipped.

1.2 ADDITIONAL RESULTS

TABLE 1.1 Effect of Maternal Nutrient Restriction beginning at E 6.5 of pregnancy on placental and fetal tissue DNA content at E 18.5.

	Control (n=10)	**70% MNR (n=10)**
Placenta	1.19 ± 0.10	0.67 ± 0.08*
Lung	6.93 ± 0.38	7.10 ± 0.91
Liver	3.69 ± 0.23	3.00 ± 0.39
Brain	4.40 ± 0.36	4.06 ± 0.34

Pregnant mothers were fed ad libitum (control) or 70% MNR beginning at E 6.5. At E 18.5, mothers were sacrificed and placenta and fetal tissues were weighed for each fetus for a mean litter value. The result, litter mean ± SEM for 'N' number of litters, is expressed in μg/mg.

Significant differences from control (*$P < 0.05$).

TABLE 1.2 Effect of Maternal Nutrient Restriction beginning at E 6.5 of pregnancy on placental and fetal tissue protein content at E 18.5.

	Control (n=10)	**70% MNR (n=10)**
Placenta	33.54 ± 1.41	30.12 ± 2.38
Lung	62.05 ± 7.00	72.22 ± 8.81
Liver	52.87 ± 6.31	68.00 ± 10.77
Brain	68.96 ± 10.31	50.92 ± 5.50

Pregnant mothers were fed ad libitum (control) or 70% MNR beginning at E 6.5. At E 18.5, mothers were sacrificed and placenta and fetal tissues were weighed for each fetus for a mean litter value. The result, litter mean ± SEM for 'N' number of litters, is expressed in μg/mg.

Significant differences from control (*$P < 0.05$).

Figure 1.1 Insulin-Like Growth Factor (IGF) System mRNA expression in livers of control fetuses at E 18.5. AntiSense controls of autoradiography in situ hybridization of IGF-I (**A**), IGF-II (**C**) and IGFBP-2 (**E**) and sense controls of IGF-I (**B**), IGF-II (**D**) and IGFBP-2 (**F**). Images are captured by darkfield microscopy and white grains indicate the location of mRNA signal. *Scale bars*, 100 μm.

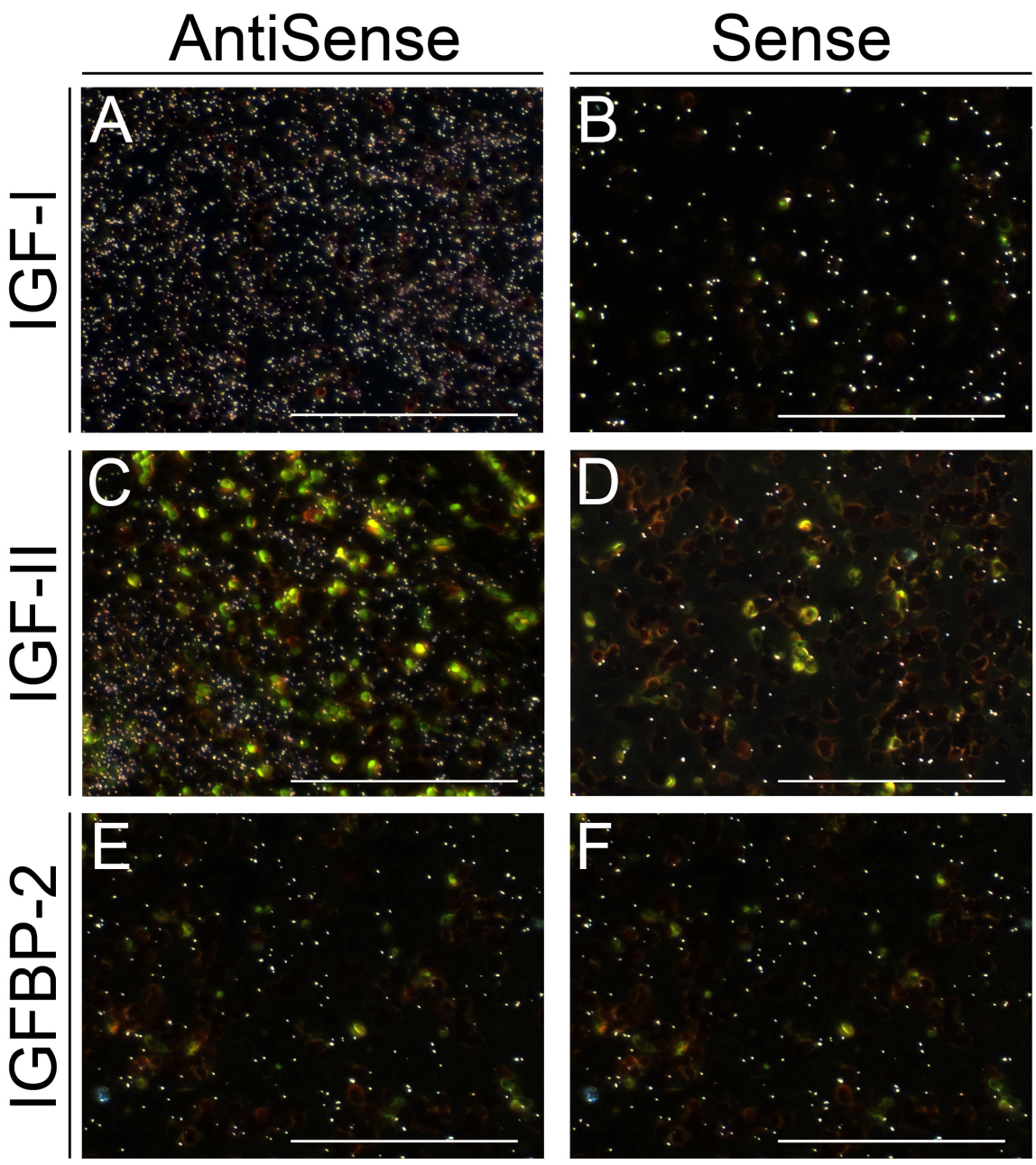

AntiSense
Sense
IGF-I
IGF-II
IGFBP-2
A
B
C
D
E
F

Figure 1.2 Insulin-Like Growth Factor (IGF) System protein expression in fetal livers of control and 70% MNR at E 18.5. Immunohistochemistry of IGF-I (**A, B**), IGF-II (**C, D**) in control (**A, C**) and 70% MNR (**B, D**). Images are captured by darkfield microscopy. White grains indicate location of IGF mRNA. Open arrows indicate representative hematopoietic cell clusters; closed arrows indicate representative hepatocytes. Note the hepatocytes of the control livers are filled with glycogen. *Scale bars*, 100 μm.

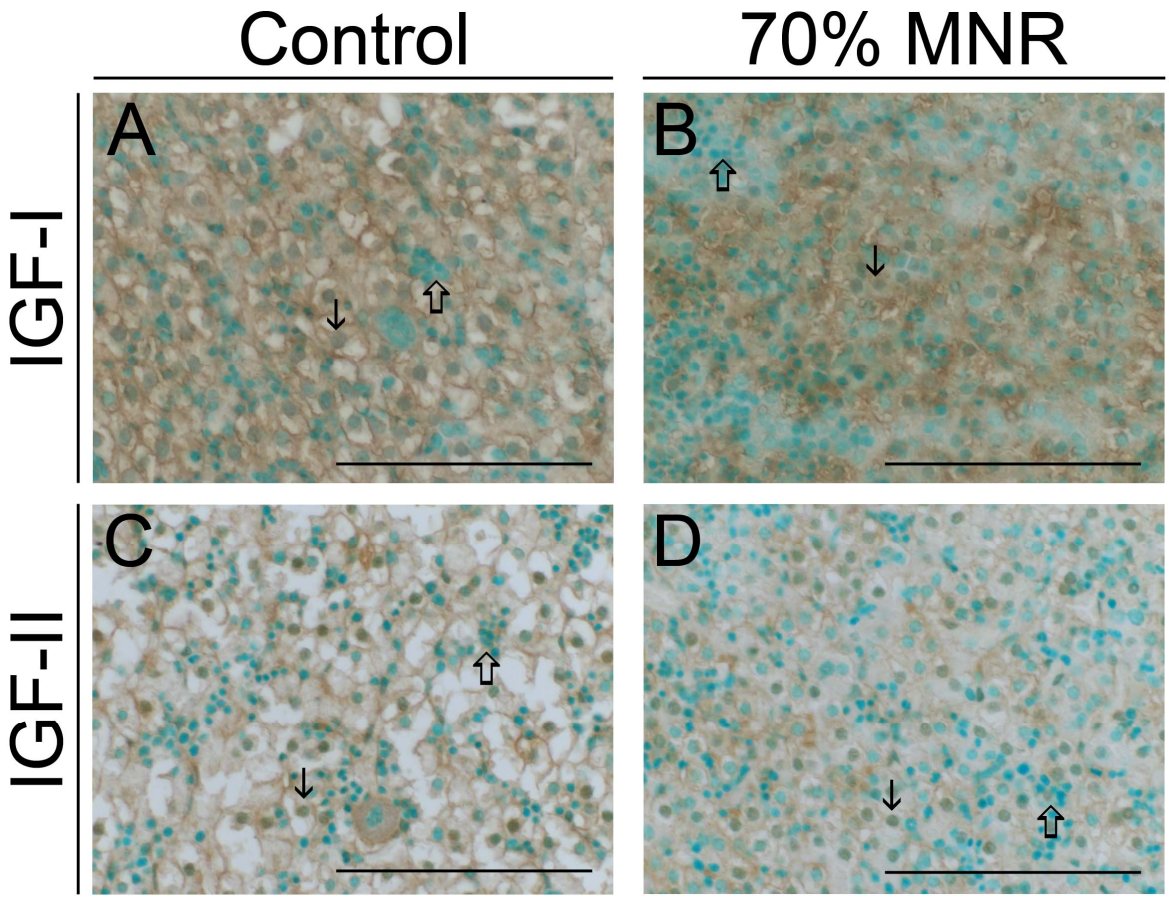

Control
70% MNR
IGF-I
IGF-II
A
B
C
D

Figure 1.3 Insulin-Like Growth Factor (IGF) System protein expression in fetal livers of control and 70% MNR at E 18.5. Immunohistochemistry of IGFBP-1 (**A, B**), IGFBP-2 (**C, D**), IGFBP-3 (**E, F**) in control (**A, C, E**) and 70% MNR (**B, D, F**). Images are captured by brightfield microscopy. DAB staining indicates location of IGFBP proteinA. Open arrows indicate representative hematopoietic cell clusters; closed arrows indicate representative hepatocytes. Note the hepatocytes of the control livers are filled with glycogen. *Scale bars*, 100 μm.

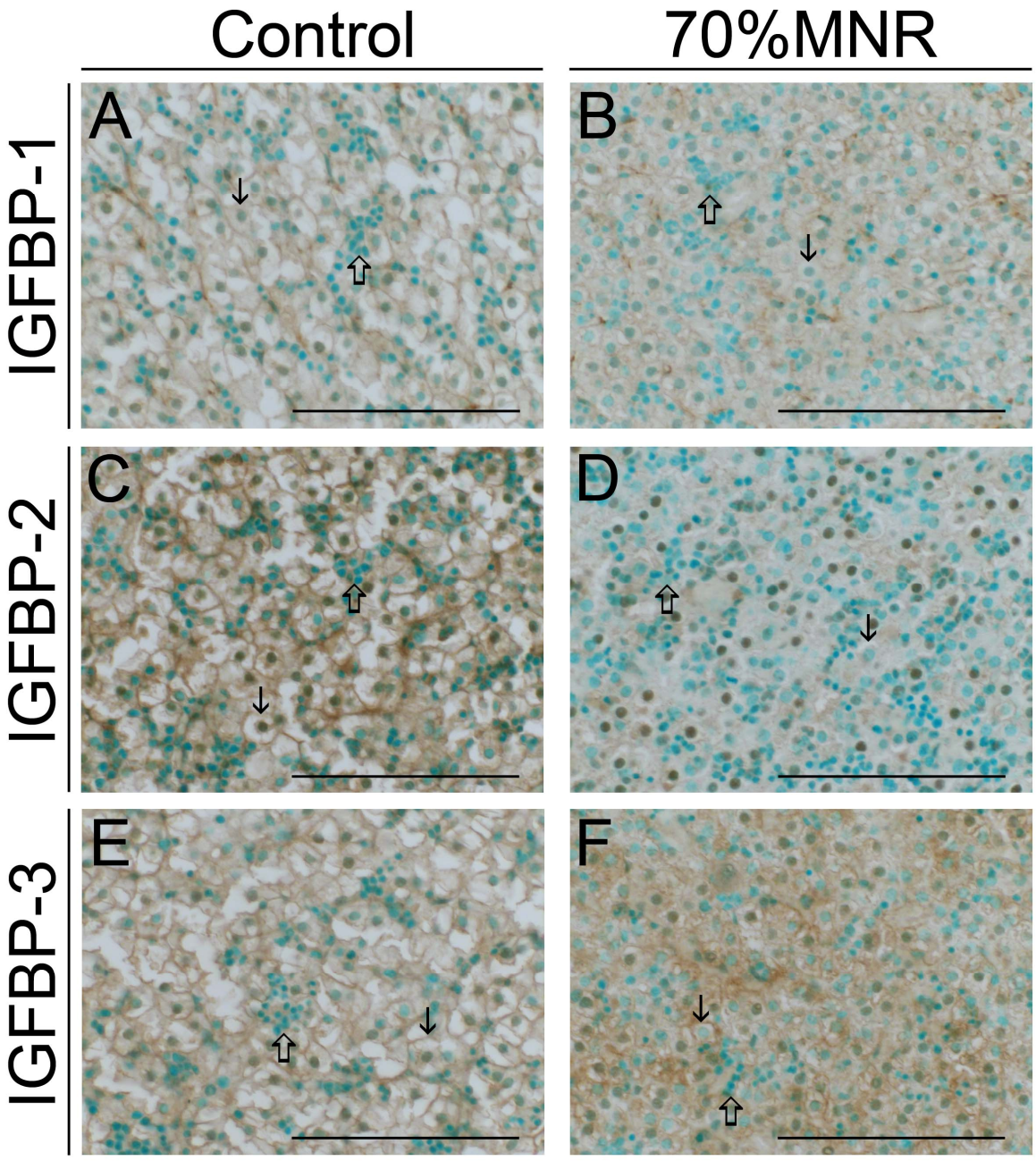
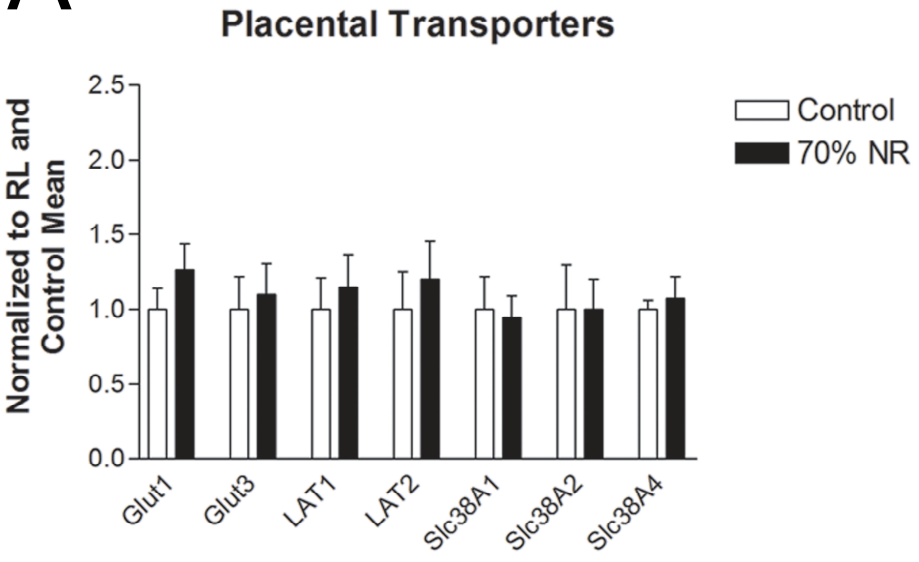
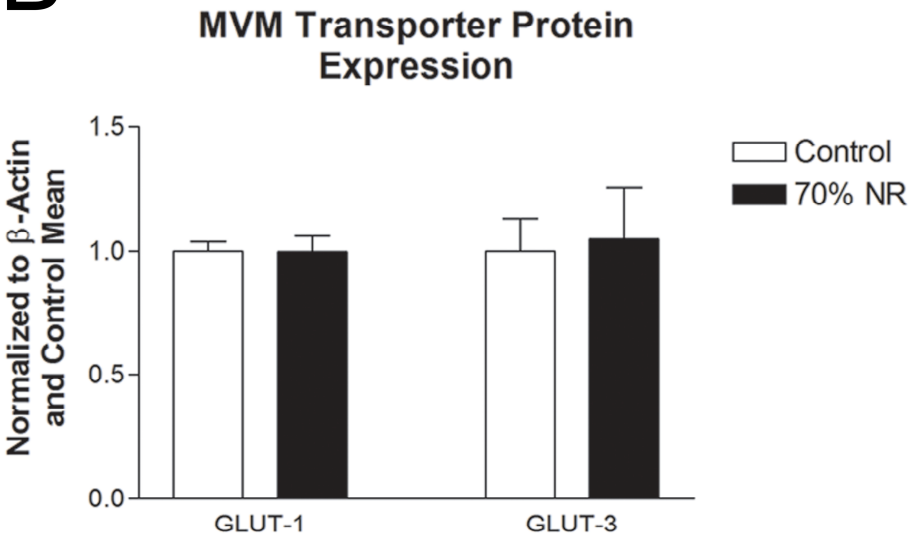

Control
70%MNR
IGFBP-1
IGFBP-2
IGFBP-3
A
B
C
D
E
F

Figure 1.4 Nutrient transporter expression in placenta of control and 70% MNR at E 18.5. (**A**) Expression of nutrient transporter mRNA measured by quantitative Real-Time PCR ($P > 0.05$). (**B**) Expression of nutrient transporter protein determined by Western Blotting and measured by densitometry ($P > 0.05$).

A


Placental Transporters
Normalized to RL and Control Mean
2.5
2.0
1.5
1.0
0.5
0.0
Control
70% NR
Glut1
Glut3
LAT1
LAT2
Slc38A1
Slc38A2
Slc38A4

B


MVM Transporter Protein Expression
Normalized to β-Actin and Control Mean
1.5
1.0
0.5
0.0
Control
70% NR
GLUT-1
GLUT-3

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1.3 ANIMAL USE PROTOCOL

For New, Full Renewal (5th Year) or Pilot Animal Use Projects

STEP 1: Initial electronic form submission will undergo a pre-submission review.

Please complete Sections A to I using your computer, then submit via email to aus@uwo.ca

STEP 2: Following pre-submission review, the AUS Administrator will email suggested revisions to be included in a revised protocol.

STEP 3: Resubmission of revised protocol including: electronic resubmission to aus@uwo.ca AND 15 double sided hard copies including signed authorization pages, [Sect. H-OHS Schedule 6 & Sect. I], with supporting documents to Rm. 510, MSB, Attn. AUS.

WARNING: Some computers lose data when a form 'unlock' is performed. Therefore, save this form often using varying save titles (i.e. '1', '2') while progressing through each section of this form.

A. INVESTIGATOR/GRANT/PROJECT INFORMATION - *Mandatory Completion Required*

Investigator Contact Information		
Investigator Name Dr. Victor Han	**Department** CHRI	**Institution** UWO
Office Address VRL A5-107	**Lab Address** A5 Pod 1	**Email Address** Victor.Han@sjhc.london.on.ca
Office Telephone x58306	**Lab Telephone** x55070/x55067	**Fax Number** x58186

Protocol & Grant Titles/Support Documentation		
Application Type: *Pick One Only* ☒ **New** ☐ **Pilot** ☐ **Full Renewal**	**If Full Renewal, Previous Protocol #**	**Proposed Start Date:** *mm/dd/yy* 08/01/06 **Proposed End Date:** *mm/dd/yy* 07/31/11
Project Title Animal Models and Mechanisms of Fetal Growth Restriction		
Grant Title *(if different from above)* Fetal Growth Restriction: Mechanisms and Outcomes		
Supporting Documentation Indicate 1 to 3 related publications supporting this proposed project. A website or attachment is acceptable.	Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. 2004 Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol. 561(Pt 2):355-77.	
	Cetin I, Foidart JM, Miozzo M, Raun T, Jansson T, Tsatsaris V, Reik W, Cross J, Hauguel-de-Mouzon S, Illsley N, Kingdom J, Huppertz B. 2004 Fetal growth restriction: a workshop report. Placenta. 25(8-9):753-757.	
	Wlodek ME, Westcott KT, O'dowd R, Senuto A, Wassef L, Moritz KM, Moseley JM. 2005 Uteroplacental restriction in the rat impairs fetal growth in association with alterations in placental growth factors including PTHrP. : Am J Physiol Regul Integr Comp Physiol. [Epub ahead of print]	

Funding Details			
Funding Source Grant **Name:** CIHR	**Source Grant #** HPG-78881 *OR* **Applied for** ☐	**Does this source conduct peer review?** *Pick One* ☒ Yes ☐ No ☐ Unsure	**Granting agency requires submission confirmation** ☒ Yes ☐ No **If *Yes,* Due Date:** *mm/dd/yy* 05/02/05

Purpose of Animal Use			
Describe the purpose of your use of animals using the following check boxes & drop-downs			
Purpose of Animal Use http://www.uwo.ca/animal/website/AUS/Content/Forms.htm	**Project Type** *Pick all that apply*	**Project Purpose** *Pick all that apply*	*Complete Related Section*
1-Fundamental Research	☐ **Acute** Non-recovery Surgery, Euthanasia for Tissue Collection	☒ **Research**	
CCAC Category of Invasiveness http://www.uwo.ca/animal/website/AUS/Content/Forms.htm		☒ **Breeding for Research**	F. 6
Choose maximum level within this study	☒ **Chronic** Long Term (i.e. nutrition), Recovery Study (i.e. post surgery)	☐ **Teaching**	H. Add. 1
C		☐ **Wildlife Study**	H. Add. 2
Check All Species Used Within this Protocol	☐ Bird ☐ Cat ☐ Dog ☐ Fish ☐ Frog ☒ Guinea Pig ☐ Hamster ☒ Mouse ☐ NHP ☐ Pig ☐ Rabbit ☒ Rat ☐ Squirrel ☐ Sheep ☐ Other, *explain*		

Major Study Elements Checklist
What are the major events involving animals in this project?
In addition to all **mandatory** sections, complete all specified sections beside each checked element

Pick all that apply	*Complete Related Section*	*Pick all that apply*	*Complete Related Section*
☒ **Agent Use – Non Hazardous** ☒ Drugs ☐ Other	*F.2*	☒ **Euthanasia**	*F. 1*
☐ **Agent Use -** ☐ Hazardous ☐ Biological	*F.2 & H. Ad.3*	☐ **Fasting**	*F. 3 & 7*
☒ **Anaesthesia**	*F.2, 3 & 5*	☐ **Genetic Mutation,** *i.e. transgenic, knockout*	*F. 8*
☐ **Antibody Production**	*F. 2 – 5, H. Ad. 3 – Sched. 3*	☒ **Injections**	*F. 2*
☐ **Behavioural Study**	*F. 5*	☒ **Model Creation**	*F. 9*
☒ **Breeding**	*F. 6*	☒ **Observation / Monitoring**	*F. 5*
☐ **Capture**	*F. 5*	☐ **Radiation –** i.e. *CT, MRI, PET, X-ray*	*F.2& H Ad.3*
☐ **Collection -** ☐ **Blood**	*F. 7*	☒ **Surgery – Recovery**	*F. 2 ,3,5*
☒ **Collection -** ☒ **Tissue** ☐ **Other,** *i.e. urine*	*F. 9*	☒ **Surgery – Non-recovery**	*F. 2 & 3*
☐ **Diagnostic Imaging**	*F. 9 & H. Ad. 3*	☐ **Treatment**	*F. 5*
☒ **Dietary Manipulation**	*F. 5*		

B. PROJECT LAY SUMMARY - *Mandatory Completion Required*

Describe your project concisely in lay terms at a Grade 9 level using 40 words or less per question – Avoid technical and scientific terms –
The Project Lay Summary is a CCAC required element to ensure committee lay member comprehension.
1. Project's Purpose Develop animal models of Fetal Growth Restriction (FGR) using dietary restrictions and uterine artery ligation
2. Expected Benefit FGR is associated with higher fetal morbidity and mortality with other diseases in later life linked to this condition
3. Reason for Using Animals Mechanistic studies investigating the molecular events inthe fetus and placenta can be studied only in animal models
4. Reason for Using Species Mice--gene targeting and transgenic techniques available; Rats--uterine artery ligation and dietary experiments previously established Guinea Pigs--prenatal developers unlike mice and rats

C. RESEARCH STAFF & THEIR TRAINING REQUIREMENTS – *Omitted from Thesis*

D. PROJECT OVERVIEW - *Mandatory Completion Required*

Provide a BRIEF overview of your project.
Use Section **E.** for **Animal Number Justification**, and **F.** & **G.** for **Experimental Details**
1. Rationale The intrauterine environment is the predominate factor that regulates the development and function of the placenta, which (under conditions of hypoxia and/or poor nutrition) lead to fetal morbidity and mortality. There is also evidence linking FGR to postnatal diseases such as diabetes and high blood pressure, suggesting a fetal programming effect
2. Hypothesis Animal models of FGR generated by maternal nutrient restriction and/or uteroplacental restriction are relevant to human FGR and its outcomes, and are robust models to study the mechanisms that underlie the pathophysiology of FGR and developmental programming
3. Objective(s) To generate animal models of FGR that are relevant to the human. This in turn will allow us to determine the structural and functional changes in placentae and to also identify epigenetic changes either in candidate genes or globally in the whole genome
4. Approach/Research Plan – ***Brief*** *Summary only* FGR will be developed by two methodologies--by uterine artery ligation and by maternal dietary manipulation (global nutrient restriction)

E. ANIMAL NUMBER JUSTIFICATION BY EXPERIMENTAL GROUP– *TWO OPTIONS* –

Mandatory Completion Required

i. Describe possible replacement, refinement and/or reduction alternatives to animal use, and offer justification if these are not to be employed, or a description of efforts to find such alternatives.	
Replacement--the focus of this study is the pathophysiology of FGR, from which the placenta is the key organ in the disease process. Thus we have chosen rodents as they have the physiology required but are considered a lower evolutionary species. Refinement--the model of uterine artery ligation has been demonstrated successfully for rats and guinea pigs; dietary restriction has been demonstrated successfully for all three rodent species Reduction--ANOVA will be used for the majority of the analyses and thus each component of the study has been assigned a number to reach statistical significance	
ii. Indicate how you have determined your animal numbers by breaking their use down into experimental groups including controls – Label each group with a numeric identifier, i.e. 1, 2, 3	
OR Option 2 – Use the following table to describe animal number per experimental group justification – If more than 4 groups are involved, reopen this file, then complete for additional groups, providing a different numeric Group ID # for each	
Experimental Group ID # MGNR	**←Use Experimental Group ID # (column directly left) to assign group to details in Sections F & G, right hand column**
Name of Experimental Group &/or Species or Strain	Mouse Global Nutrient Restriction
Total Animal Numbers per Experimental Group	60
Subgroup Animal Numbers	n=10 for each subgroup (100% 70% PR) per strain
Other Animal Number Related Detail	Males required for breeding Extra females may be required for cases of premature delivery or failure to conceive

F. EXPERIMENTAL DETAILS - *Mandatory Completion Required*

Using the groups you have indicated in Section E., provide details of all experiments, events & procedures involving animals in each of the groups.
Use the right-hand column to connect the 'event' with the specific group(s) identified in Section E.

F. 1. Animal Use Endpoints – *Mandatory Completion for All Projects*

"*In experiments involving animals, any actual or potential pain, distress, or discomfort should be minimized or alleviated by choosing the earliest endpoint that is compatible with the scientific objectives of the research. Selection of this endpoint by the investigator should involve consultation with the lab animal vet and the animal care committee.*" CCAC General Guideline						**Identify Section E Experimental Group ID #s Involving This Element**
For SOP detail go to http://www.uwo.ca/animal/website/VS/Content/SOPs.htm						
Experimental Endpoints	Mice--e18.5 Rats--e21.5 Guinea Pigs--e58					☒ All *Or* ID #s
Euthanasia SOPs	*Pick all that will be followed* ☒ #320 Euthanasia ☒ #321-Criteria for Early Euthanasia-Rodents	☐ #322-Criteria for Early Euthanasia-Non-Rodents ☐ Other Criteria, *please explain below*				☒ All *Or* ID #s
	If 'Other Criteria', explain					
Euthanasia Method	**Pick All Euthanasia Methods That Will Be Followed & Provide Euthanasia Related Agent Use Detail Below**					
	☐ Animals Not Euthanized ☒ Barbiturate Overdose ☐ CO2 ☐ Decapitation under Anaesthesia	**Agent Name** Sodium Pentobarbital	**Dose** 110 mg/kg	**Route** IP	**Volume** 0.5 ml	☐ All *Or* ID #s GPUAL GPGNR

	☒ *Decapitation with No Anaesthesia* ☐ Cervical Dislocation with Anaesthesia ☐ *Cervical Dislocation with No Anaesthesia* ☒ Other Method(s), Anaesthetized - *explain* ☐ *Other Method(s), Awake* - *explain*	**If 'Other Method(s)', explain** CO2 followed by exsanguination of dams for optimal blood collection in mice and rats Pups from guinea pig dams will be given additional sodium pentobarbital directly if not sufficient from dam, followed by exsanguination	☒ All *Or* ID #s
Scientific Justification	**If Method Type Has An Asterisk *, Provide Scientific Justification** This method is to be performed only on unborn fetuses extracted from the uterus of euthanized dam for mice and rats		☒ All *Or* ID #s

F. 2. Agent Use	**Does this agent have potential to cause pain or pronounced debilitation?**	**Identify Section E. Experimental Group ID #s Involving This Agent**
☒ **No Agent Use** *(Go to F 3.)*		
NON HAZARDOUS AGENTS INCLUDING DRUGS		

F. 3. Anaesthesia or Surgery or Recovery Projects			**Identify Section E. Experimental Group ID #s Involving This Element**
☐ **No Surgery or Anaesthesia or Recovery Elements** *(Go to F 4.)*			
Fasting Involved?	☐ Yes ☒ No	If Yes, provide justification & duration detail	☐ All *or* ID #s RUAL & GPUAL
Post Op Care	**Care Person(s)** Steven Dixon Animal Technician to be hired for WVB animals	**Contact #** x55067/473-5057 TBA	☐ All *or* ID #s RUAL & GPUAL
SOPs	**Pick all that will be followed**		

	☒ 330-Post-operative Care-Rodent ☐ 331-Post-operative/Post-Anaesthetic Care-Level 1 ☐ 332-Post-operative/Post-Anaesthetic Care-Level 2 ☐ 333-Post-operative/Post-Anaesthetic Care-Level 3		☐ 334-Post-operative/Post-Anaesthetic Care-Level 4 ☒ 343-Surgical Prep/Rodent/Recovery Surgery ☐ 350-Rodent Anaesthesia/Halothane/Induction Chamber ☐ Other, please explain	☐ All *or* ID #s RUAL & GPUAL
Surgery or Procedure Location	**Institution** WVB	**Room #** TBA	☐ Overnight Surgery/Recovery in non-approved area. If clicked, provide scientific justification:	☐ All *or* ID #s RUAL & GPUAL
Recovery Location	**Institution** WVB	**Room #** TBA	**When will animals be returned to the main facility?** Upon full recovery	☐ All *or* ID #s RUAL & GPUAL
Other Related Detail				

F. 4. Antibody Production

☒ **No Antibody Production** *(Go to F.5.)*

F. 5. Monitoring or Chronic Projects – i.e. Post-op, Feed, Drug and/or Disease Induction Studies		**Identify Section E. Experimental Group ID #s Involving This Element**
☐ **No Monitoring or Chronic Elements** *(Go to F 6.)*		
Monitoring Criteria	*Pick all that will be followed* ☐ 100-Monitoring/Tumour Growth in Rodents ☒ 310-Holding Period Post Admission ☐ Other, *please explain*	☒ All *or* ID #s

Monitoring Type	☒ **Weight** ☒ **Food Intake** ☒ **Behaviour** ☐ **Urine Output** ☒ **Other**, *explain* Muscle mass of mothers on nutrient restriction		☒ All *or* ID #s
Monitoring Frequency	**Pick all that apply to this project:** ☐ Hourly ☐ Twice Daily ☒ Daily ☐ Weekly ☐ Other, *please explain*	**If other, or multiple frequencies chosen, please provide detail:**	☒ All *or* ID #s
Monitoring Personnel	**Care Person(s)** Steven Dixon Animal Technician for WVB TBA	**Contact #** x55067, 473-5057	☒ All *or* ID #s
Monitoring Records	**Will animal monitoring records be kept with the animal?** ☐ **Yes** *or* ☒ **No**	**If 'No', identify monitoring record location** LHSC-VRL A7-144 TBA WVB	☒ All *or* ID #s
Other Related Monitoring Detail			

F. 6 Breeding

☐ **No breeding elements** *(Go to F.7)*

I. Indicate Breeding Type	☒ Breeding for research within this protocol ☐ Breeding for research NOT within this protocol
Complete for All Breeding Elements	
II. Provide justification for maintaining a breeding colony	Breeding only for experimental use; no continued colonies required
III. Provide justification for breeding numbers *Quote animal numbers within Section G. Animal Requirements*	Males required for breeding (n=10 for each species) Extra females may be required for cases of premature delivery or failure to conceive 60 females for mice and rats, but 120 Guinea pig females required due to smaller litter size
IV. List procedures used in the breeding colony	
V. Number estimation and use of surplus	Surplus Number Estimate:

animals *(those not required for experimental programs, or retired breeders)*	Surplus Use:
VI. Breeding colony location, *if different from research housing.*	
VII. Research associates directly involved in the care of animals in this breeding colony	Name: Contact #: Email: Name: Contact #: Email:
Breeding for External Protocols Only	
VIII. List External Protocol Researcher(s)	
IX. List External Protocol Numbers	

F. 7. Blood Collection	**Identify Section E. Experimental Group ID #s Involving This Element**
☒ **No Blood Collection** *(Go to F.8.)*	

F. 8 Genetically Altered Animal Information
☒ **No Genetically Altered Animals Used** *(Go to F. 9.)*

F. 9. Procedural Description - *Mandatory Completion for All Projects*			
Procedure Number	**Minus the details presented in Section F. 1. through F. 8, provide a detailed procedural description of all animal-related events**	**Does this procedure have potential to cause pain or pronounced debilitation?**	**Identify Section E. Experimental Group ID #s Involving This Procedure**
1	Female mice will be bred with male mice and presence of a vaginal plug will be marked as gestational day 0.5. Mice will be randomly assigned to one of 3 groups--100% (control), protein restricted diet (8.1%), or 70% Nutrition (starting on gestational day 6.5). Food consumption will be monitored daily using special diet and feeders obtained from Bio-Serv to determine the average daily intake of food for each gestational day. Mice in the Protein Restricted group will be allowed to eat the special diet ad libitum. On gestational day 18.5 mice will be euthanized for tissue collection from fetuses. This procedure is performed in both strains (CD-1 and c57bl6) to identify strain specific results.	☐ **Yes** ☒ **No**	☐ All *or* ID#s MGNR

G. ANIMAL REQUIREMENTS - *Mandatory Completion Required*

Detail all animal requirements including, species, strain, age/weight, sex, housing, dietary and animal total details for the Upcoming Year Only

Use bottom row of this section to add more detail to any column

Researcher must provide scientific justification for overnight animal housing in non-approved areas, i.e. labs, & receive AUS approval:

Use Section F. 3 for surgery related housing & bottom of this section for all other housing justification

Species	Strain &/or Other Species Detail For Genetic Mutation Use F. 8 For Details	Age or Weight	Sex	Animal Housing (See Drop-downs for complete listing) Abbreviations: CBM=Centre for Brain & Mind HSACF=Health Sciences Animal Care Facility LHRI=Lawson Health Research Institute, St. Joe's LHSC-UC=University Campus LHSC-SS=South Street LHSC-VRL=Victoria Research Lab LRCP=London Regional Cancer Program RACF= Robarts Barrier Facility REB= Robarts Experimental Barrier WVB=West Valley Bldg					Special Dietary Details Complete only if Standard diet is not adequate	1 Year Total Animal Numbers Required
				Housing Location	Use/Lab Location	Room #	If Specialized Housing Required, i.e. barrier, lab, Field Study, Other Provide Details	Used in Breeding ?		
Mouse	cd-1	8 wks	Female	LHSC-VRL	Same as Housing	A7-143		Yes	Details below	60
Mouse	cd-1	8 wks	Male	LHSC-VRL	Same as Housing	A7-143		Yes		10
Mouse	c57bl6	8 wks	Female	LHSC-VRL	Same as Housing	A7-143		Yes	Details below	60
Mouse	c57bl6	8 wks	Male	LHSC-VRL	Same as Housing	A7-143		Yes		10
Non-approved Housing Justification										
Other Animal	Specially formulated feeds and feeders obtained from Bio-Serv to facilitate accurate measurements of									

Requirement Detail	daily food intake of control groups for GNR groups Vitamin C supplementation will be provided in the drinking water for guinea pigs

H. ADDENDA

Addendum 1 – Teaching ONLY	☒ **No Teaching Elements**

Addendum 2 – Wildlife Study ONLY	☒ **No Wildlife Study Elements**

Addendum 3 - Hazardous &/or Biological Agent Use ONLY – Occupational Health and Safety	
☒ **No Hazardous &/or Biological Agent Use**	
University of Western Ontario **Animal Use Protocol Safety Form**	For further information, please contact J. Stanley - jstanle2@uwo.ca
Protocol Title: Animal Models and Mechanisms of Fetal Growth Restriction	Approved: June 10, 2003 *OHS Use Only* **Permit Number Issued**:

Approvals - Please Sign & Date Below

Principal Investigator	**Protocol Title**	**Date**
Print Name V. Han **Signature**	Animal Models and Mechanisms of Fetal Growth Restriction	*mm/dd/yy* 06/15/06

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1.4 MAJOR MODIFICATION PROTOCOL FORM

I. DECLARATION

1. **I believe that the proposed animal use conforms to my stated objectives, will advance knowledge and will employ the best methods on the smallest number of animals to obtain valid information.**
2. **I believe that, wherever possible, all procedures having the potential to cause pain or stress have been refined and/or reduced to minimize animal discomfort.**
3. **I confirm that the experimental method accurately describes ALL the proposed animal use. I accept responsibility for procedures performed on animals in this project. All procedures will be carried out by, or under the guidance of trained and competent personnel using recognized techniques.**
4. **All animals in this project will be used in compliance with the regulations of The Animals for Research Act of the province of Ontario, the guidelines of the Canadian Council on Animal Care and the policies and procedures of the University of Western Ontario Council on Animal Care.**
5. **I am aware that the data provided in this protocol will be entered into the Animal Research Protocol Management System and submitted to the Canadian Council on Animal Care.**
6. **I will ensure that any individual, who will perform any procedure(s) as described in this protocol, will complete all related mandatory training and will be familiar with the contents of this document.**

Authorization - *Please Sign & Date Below*	
Project Title - *Please repeat title used on page 1* Fetal Growth Restriction: Mechanisms and Outcomes	
Principal Investigator or Course Director **Print Name -** Dr. Victor Han **Signature -**	**Date** *mm/dd/yy* 06/15/06

AUS Office Use Only **APPROVAL OF ANIMAL USE SUBCOMMITTEE**	
Signature of Reviewing Veterinarian - **Date -** *mm/dd/yy* / /	**Signature of AUS Chair - Date -** *mm/dd/yy* / /

A. INVESTIGATOR DECLARATION – *Mandatory Completion*	
I. All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, "The Animals for Research Act," of the Province of Ontario.	
II. I confirm that this *Animal Use Protocol* accurately represents the proposed animal use.	
III. I accept responsibility for procedures performed on animals in this project.	
IV. I will ensure that any individual who will perform any animal-related procedure(s) within this protocol will complete all related mandatory training AND will be made familiar with the contents of this document.	
1. **I support the above declaration - YES ☒ Today's Date *mm/dd/yy*: 03/10/09**	
2. By checking 'YES' in this section, I authorize the submission of this form and its electronic delivery to aus@uwo.ca YES ☒ NO ☐	
3. I authorize Steven Dixon (PI Designate) to submit this form and to receive a copy of authorization via email on my behalf. Today's Date (*mm/dd/yy*)**: 03/10/09**	
AUS APPROVAL - *AUS Office Use Only -*	
Veterinary Authorization by Click Here **Authorization Date** *(mm/dd/yy)* **Signature:**	**AUS Committee Signature:**

B. PROTOCOL / INVESTIGATOR / FUNDING INFORMATION - *Mandatory Completion*		
B.1. PROTOCOL INFORMATION		
Protocol Number: 2006-095-08	**Protocol Title:** Animal Models and Mechanisms of Fetal Growth Restriction	
B.2. INVESTIGATOR CONTACT INFORMATION		
Principal Investigator Full Name: Dr. Victor Han	**Email Address:** vhan@uwo.ca	**Office / Lab Phone:** 58306
B.3. FUNDING SOURCE INFORMATION		
☒ **NO NEW/CHANGES TO Funding Information (Go to C.)**		

C. PROTOCOL OVERVIEW – CHANGES AND/OR ADDITIONS TO THE *ANIMAL USE PROTOCOL*
Mandatory Completion

1.1 Please provide justification for proposed changes outlined in this Protocol Modification

The placenta is a key component regulating the growth of the fetus in utero. This modification is designed to measure which mechanisms of transplacental transport are being affected (simple diffusion, facilitated diffusion and active transport).

1.2. IDENTIFY ALL CHANGES AND/OR ADDITIONS TO THE ORIGINAL *ANIMAL USE PROTOCOL* USING THIS CHECKLIST

Species Type	Use Type *Pick All That Apply*	Category of Invasiveness (CI)	CHECK ALL CHANGES AND/OR ADDITIONS PER SPECIES & COMPLETE IDENTIFIED SECTIONS			
			Procedure Type	Section Required	Procedure Type	Section Required
Mouse	☐ Chronic ☒ Acute	C	☐ Adverse Consequences ☐ Agent Use – Non-Hazards ☒ Agent Use – Hazardous ☒ Anesthesia ☐ Animal Requirements ☐ Antibody Production ☐ Behavioural	F L.1.1 L.1.2 & 9 L.2 G L.3 E L.4	☐ Genotyping ☐ Glossary of Terms ☐ Groups ☒ Location – Housing & Use ☐ Imaging ☐ Model Creation ☐ Monitoring	E D D H L.1.2 & 9 E J A

			☐ Blood Collection ☐ Breeding ☐ Collection - Other Tissue ☐ Dietary Manipulation ☐ Euthanasia ☐ Facility Transfer ☐ Funding - New ☐ Genetically Altered Animals	L.5 E E I H.4 B L.6	☐ PI Designate Authorization ☒ Procedures ☐ Protocol Transfer ☐ Staff & Their Training ☒ Surgical Procedures ☐ Teaching & Training ☐ Title – Protocol / Funding ☐ Wildlife Field Study	E G.3 K L.2 L.7 B L.8

D. LAY SUMMARY, GLOSSARY OF TERMS, SCIENTIFIC OBJECTIVES & PROCEDURAL OVERVIEW

D.2. GLOSSARY OF TERMS & SPECIALIZED EQUIPMENT

☒ **No NEW / CHANGES TO Glossary of Terms and/or Specialized Equipment and/or Group Flow Charts and/or Timelines**

E. PROCEDURAL OUTLINE – *Mandatory Completion*

☐ **NO NEW/CHANGES TO Procedural Outline (Go to F.)**

Provide a concise description of the procedural events experienced by the animals in each experiment. The intent is to chronologically order, name and briefly describe the procedural events that animals of each experimental cohort will experience. The species-specific events should be presented numerically in chronological number. Indicate the experimental groups experiencing each procedural event and evaluate the procedure-specific potential to cause pain.

Procedure Number	SPECIES & Sect. D 5.1 Group ID#	Potential To Cause Pain or Distress	PROCEDURE NAME	Short Description of the Experimental Procedure
1	Mouse ID# MGNR	☐ Yes ☒ No	Radiotracer infusion	Pregnant mice are anaesthetized with midazolam and ketamine/xylazine. After exposing maternal jugular vein, 1.3 kBq of a radiotracer in 100ul of saline (0.9% w/v) is injected into the vein. Pregnant mum is then euthanized by cervical dislocation 2 minutes after injection. Pups are removed from the mother and euthanized by

				decapitation. Tissues are then collected from the pups for further analysis.

F. ADVERSE CONSEQUENCES - *Mandatory Completion*

☒ **NO NEW/CHANGES TO Adverse Consequences (Go to G.)**

G. 4-YEAR TOTAL ANIMAL REQUIREMENTS – *Mandatory Completion*

☒ **NO NEW/CHANGES TO 4-Year Animal Requirements (Go to H.)**

G.3. PROTOCOL TRANSFER OF PREVIOUSLY USED ANIMALS – *Complete if Sect. G.2. Animal Source = Protocol Transfer-Citywide involving previously used animals (See Instruction Pages iii & iv)*

☒ **NO NEW/CHANGES TO Protocol Transfers - *Go to H.***

H. ANIMAL HOUSING & USE LOCATION REQUIREMENTS – *Mandatory Completion*

☒ **NO NEW/CHANGES TO Animal Housing & Use Location Requirements - *Go to I.***

☒ **NO NEW/CHANGES TO Citywide &/or External Facility Animal Transfers - *Go to I.***

I. EUTHANASIA INCLUDING CRITERIA FOR EARLY EUTHANASIA & EXPERIMENTAL ENDPOINTS

Mandatory Completion

☒ **NO NEW/CHANGES TO Euthanasia Including Criteria for Early Euthanasia & Experimental Endpoints - *Go to J.***

J. MONITORING – *Mandatory Completion*

☒ **NO NEW/CHANGES TO Monitoring - *Go to K.***

K. RESEARCH STAFF & THEIR TRAINING REQUIREMENTS – *Mandatory Completion*
Complete for ALL Staff Working Within This Specific Protocol

☒ **NO NEW/CHANGES TO Research Staff & Their Training Requirements - *Go to L.***

L. ADDENDA – *Complete as Indicated within Section C.2.*

L.1. AGENT / DRUG USE – NON-HAZARDOUS & HAZARDOUS

☐ **NO NEW/CHANGES TO Agent / Drug Use - *Go to L.2***

L.1.1. AGENT USE – NON-HAZARDOUS ONLY

☒ **NO NEW/CHANGES TO Non-Hazardous Agent / Drug Use - *Go to L.1.2***

L.1.2. AGENT USE – HAZARDOUS

☐ **NO NEW/CHANGES TO Hazardous Agent / Drug Use - *Go to L.2***

Provide detailed hazardous agent (Biological, Recombinant DNA, Viral Vectors, Chemical, Nuclear, Radiation, Laser, MRI, X-Ray Devices, Hazardous Drug, Hazardous Species, and Hazardous Hormone) use per species AND complete Addendum L.9.

SPECIES & SECT. D 5.1 GROUP ID#	HAZARDOUS AGENT TYPE	HAZARDOUS AGENT NAME	PAIN or DISTRESS POTENTIAL	DOSE	ROUTE	VOLUME	FREQUENCY	PERMIT REGISTRY #
Mouse **ID#** MGNR	Nuclear Substa If Other,	14C-Inulin	☐ Yes ☒ No ☐ Uncertain	1.3 kBq	IV	100ul	Once	HAN1003rev2
As Above **ID#**	Nuclear Substa If Other,	14C-methyl-D-glucose	☐ Yes ☒ No ☐ Uncertain	1.3 kBq	IV	100ul	Once	HAN1003rev2

As Above **ID#**	Nuclear Substa If Other,	14C-MeAIB	☐ Yes ☒ No ☐ Uncertain	1.3 kBq	IV	100ul	Once	HAN1003rev2

L.2. ANESTHESIA OR SURGERY OR RECOVERY PROJECTS

☐ **NO NEW/CHANGES TO Anesthesia, Surgery or Recovery - *Go to L.3***

Provide detailed anesthesia, surgery and/or recovery information per species. Ensure procedural outline from pre-op through to post-op including timelines, monitoring, drug administration, wound closure & suture removal. If surgery and/or anesthetic procedural outlines were previously detailed within Sect. E, provide Sect. E Row # as reference rather than repeating detail here.
See Instructions Pages for SOP Titles.

SPECIES & SECT D.5 GROUP ID#	SURGICAL AND/OR ANESTHETIC TYPE & NAME	OUTLINE SURGERY Pre-op to Post-op Timelines, Wound Closure Suture Removal, *or* Provide Sect. E Row #	FASTING ? IF 'YES', DURATION	ANESTHETIC TYPE & NAME	OUTLINE ANESTHETIC REGIME Depth, Duration, Frequency, Monitoring, *or* Provide Sect. E Row #	POST-OP CARE SOPs FOLLOWED	SURGICAL & RECOVERY LOCATIONS Buildings & Room #s
Mouse **ID#** MGNR	**Type:** Non-Recovery **Name:**	☒ SOP 343 & **Surgical Outline:** Sect E Row 1	☐ Yes ☒ No **Duration:**	**Type:** Injectible **Agent Name:** Midazolam + Ketamine/Xylazine	☐ SOP 350 & **Anesthetic Regime:**	☐ SOP 330 ☐ SOP 331 ☐ SOP 332 ☐ SOP 333 ☐ SOP 334	**Surgery – Bldg & Rm#** VRL A5-108 **Recovery – Bldg & Rm#** N/A

L.3. ANTIBODY PRODUCTION

☒ **NO NEW/CHANGES TO Antibody Production - *Go to L.4***

L.4. BLOOD COLLECTION

☒ **NO NEW/CHANGES TO Blood Collection - *Go to L.5***

L.5. BREEDING

☒ **NO NEW/CHANGES TO Breeding** - ***Go to L.6***

L.6. GENETICALLY ALTERED ANIMAL REQUIREMENTS

☒ **NO NEW/CHANGES TO Genetically Altered Animal Requirements** - ***Go to L.7***

L.7. TEACHING

☒ **NO NEW/CHANGES TO Teaching** - ***Go to L.8***

L.8. WILDLIFE FIELD STUDY

☒ **NO NEW/CHANGES TO Wildlife Study** - ***Go to L.9***

L.9. OCCUPATIONAL HEALTH & SAFETY FORM – *Complete for all Hazardous Protocol Elements*

☐ **NO NEW/CHANGES TO Occupational Health & Safety Form – *FORM IS COMPLETE***

9.1. HAZARD ASSESSMENT FOR WORK WITH MICROORGANISMS OR BIOLOGICAL AGENTS – Schedule 1

☒ **NO NEW/CHANGES TO Microorganisms or Biological Agents** - ***Go to L.9.2***

9.2. HAZARD ASSESSMENT FOR USE OF RECOMBINANT DNA OR VIRAL VECTORS DIRECTLY INTO ANIMALS – Schedule 2

☒ **NO NEW/CHANGES TO Recombinant DNA or Viral Vectors Directly Into Animals** - ***Go to L.9.3***

9.3. HAZARD ASSESSMENT FOR WORK WITH HAZARDOUS CHEMICALS and/or DRUGS – Schedule 3

☒ **NO NEW/CHANGES TO Hazardous Chemicals and/or Drugs** - ***Go to L.9.4***

9.4. HAZARD ASSESSMENT FOR WORK WITH NUCLEAR SUBSTANCES AND RADIATION / IMAGING DEVICES – Schedule 4

☐ **NO NEW/CHANGES TO work with Nuclear Substance and/or Radiation / Imaging Devices – *FORM IS COMPLETE***

Will nuclear substances and/or radiation devices be administered to live animals? ☐ Yes ☐ No
If 'Yes', complete this section. If 'No' FORM IS COMPLETE.
For containment level and other biosafety information, go to: www.uwo.ca/humanresources/biosafety

- All procedures must follow the *Radiation Safety* Manual, available on the website: www.uwo.ca/humanresources/facultystaff/h_and_s/h_and_sindex.htm
- Carcasses must be disposed of per the radioactive waste protocol for the institution/facility.
- All procedures must comply with the *Nuclear Safety and Control Act*, *Canadian Nuclear Safety Commission Regulations* and license issued to the institution/facility. See www.nuclearsafety.gc.ca/engl

For more information, contact the Radiation Safety Officer for your institution/facility where the work is performed.

9.4.1. Nuclear Substance and/or Radiation / Imaging Devices Information - Please list and provide information for each hazardous nuclear substance and/or radiation / imaging device below.

INFORMATION REQUIRED	SUBSTANCE / DEVICE 1	SUBSTANCE / DEVICE 2	SUBSTANCE / DEVICE 3	SUBSTANCE / DEVICE 4	SUBSTANCE / DEVICE 5
4.1.1. **Name** Nuclear Substance and/or Radiation Device	14C-inulin	14C-methyl-D-glucose	14C-MeAIB		
4.1.2. **Type**	Nuclear Substance	Nuclear Substance	Nuclear Substance	Click Here	Click Here
If Type = 'Nuclear Substance' Complete the Following:					
4.1.2.1. Radioisotope name &/or chemical form	Carbon-14	Carbon-14	Carbon-14		
4.1.2.2. Dose to the animal.	1.3 kBq	1.3 kBq	1.3 kBq		
4.1.2.3. Will the radioisotope-	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☐ No	☐ Yes ☐ No

contaminated animal be returning alive for housing in the Animal Quarters?					

9.4.2. Risk Control Measures – List the control measures required to eliminate or further minimize the risk.

ACTIVITY/HAZARD	RISK CONTROL MEASURES/ACTION
4.2.1 List all **preventative measures** to be taken to minimize the risk of exposure to RESEARCH STAFF handling the material	☐ No Anticipated Risk to Humans ☒ Safety Glasses ☒ Gloves ☒ Lab coat or equivalent ☐ Fumehood Use ☐ Other, *please specify*:
4.2.2. List all **preventative measures** to be taken to minimize the risk of exposure to ANIMAL FACILITY STAFF handling the material.	☒ No Anticipated Risk to Humans ☐ Safety Glasses ☐ Gloves ☐ Lab coat or equivalent ☐ Fumehood Use ☐ Other, *please specify*:

9.4.3 Training Requirements

- All Personnel must be trained in **Radiation Safety** prior to doing work on the protocol.
- All personnel must be trained to safely use the equipment specified, e.g. MRI, CT Scanner, X-ray machine and 3b or 4 Lasers
- CT Scanner and/or X-ray equipment users require **X-Ray Safety** training
- Class 3b and/or class 4 laser users require **Laser Safety** training

Training – List Completed Training for All Personnel Involved With Nuclear Substance and/or Radiation Devices

NAME	TRAINING TYPE 1	TRAINING DATE	TRAINING TYPE 2	TRAINING DATE	TRAINING TYPE 3	TRAINING DATE
Steven Dixon	Radiation Safety Other:	Jan 9/09	Click Here Other:		Click Here Other:	
Caroline Albion	Radiation Safety Other:	Oct 2/08	Click Here Other:		Click Here Other:	

9.4.4 Safety Officer Review: To be completed by safety officer associated with Section 9.4 only – Return by email to aus@uwo.ca

Pick One

☐ I authorize the contents of Section 9.4 – Schedule 4 with no further instructions or comments, *or,*
☐ I do not authorize the contents of Section 9.4 – Schedule 4 due to , *or,*
☐ I authorize the contents of Section 9.4 – Schedule 4 with the following instructions/comments: .

CURRICULUM VITAE

CAROLINE DIANE ALBION

Post Secondary Education and Degrees:

Doctor of Medicine **2006-Present**
Schulich School of Medicine and Dentistry
The University of Western Ontario, London, Ontario, Canada

PhD **2006-Present**
Anatomy and Cell Biology
The University of Western Ontario, London, Ontario, Canada
PhD Thesis: *Fetal Growth Restriction: Molecular Mechanisms and Long-Term Impact*

Bachelor of Medical Sciences **2002-2006**
Honor's Specialization in Pathology and Toxicology with Distinction
The University of Western Ontario, London, Ontario, Canada

Awards and Accomplishments:

MD-PhD Scholarship **2006-2012**
Canadian Institute for Health Research

Western Graduate Research Scholarship **2006-2010**
The University of Western Ontario, London, Ontario, Canada

Schulich Graduate Scholarship **2006-2010**
Schulich School of Medicine and Dentistry
The University of Western Ontario, London, Ontario, Canada

Gold Medal for Pathology and Toxicology and **2006**
Scholar's Electives Program
The University of Western Ontario, London, Ontario, Canada

Scholar's Electives Program Scholarship **2005-2006**
The University of Western Ontario, London, Ontario, Canada

In-Course Parent's Fund Scholarship **2003-2004**
The University of Western Ontario, London, Ontario, Canada

Frederick N. Lewis Award **2005**
Schulich School of Medicine and Dentistry
The University of Western Ontario, London, Ontario, Canada

Faculty Association Scholarship **2004-2005**
The University of Western Ontario, London, Ontario, Canada

In-Course Scholarship **2003-2004**
The University of Western Ontario, London, Ontario, Canada

Western Scholarship of Excellence, Entrance Scholarship **2002**
The University of Western Ontario, London, Ontario, Canada

Harvey F. Sullivan Scholarship **2002**
London Health Sciences Centre, London, Ontario, Canada

Related Work Experience:

Summer Research Training Program **2007-2008**
Schulich School of Medicine and Dentistry
The University of Western Ontario, London, Ontario, Canada

Department of Pediatrics Summer Student Researcher **2006**
London Health Sciences Centre, London, Ontario, Canada
The University of Western Ontario, London, Ontario, Canada

Canadian Surgical Technologies and Advanced Robotics (CSTAR), Research Assistant **2003-2005**
London Health Sciences Centre, London, Ontario, Canada
The University of Western Ontario, London, Ontario, Canada

McLaughlin Vascular Research Lab Assistant **2002**
London Health Sciences Centre, London, Ontario, Canada
The University of Western Ontario, London, Ontario, Canada

Contributions to Research:

Publications (Refereed):

Caroline Albion, Michael J. Shkrum, James Cairns. Contributing Factors to Methadone Related Deaths in Ontario. **American Journal of Forensic Medicine and Pathology.** 2010 Dec; 31(4):313-319.

Bob Kiaii, R. Scott McClure, Peter Stewart, Reiza Rayman, Stuart A Swinamer, Yoshiro Suematsu, Stephanie Fox, Jennifer Higgins, **Caroline Albion**, William J Kostuk, David Almond, Kumar Sridhar, Patrick Teefy, George Jablonsky, Pantelis Diamantouros, Wojciech B Dobkowski, Philip Jones, Daniel Bainbridge, Ivan Iglesias, John Murkin, Davy Cheng, Richard J Novick. Simultaneous Integrated Coronary Artery Revascularization With Long Term Angiographic Follow-Up. **J Thorac Cardiovasc Surg.** 2008 Sep;136(3):702-8.

Amit Badhwar, Aurelia Bihari, Alison A. Dungey, Jeffrey R. Scott, **Caroline D. Albion**, Thomas L. Forbes, Kenneth A. Harris and Richard F. Potter. Protective Mechanisms During Ischemic Tolerance in Skeletal Muscle. **Free Radical Biology and Medicine.** Volume 36, Issue 3, 1 February 2004, Pages 371-379.

Submitted Publications:

Caroline Albion, Steven Dixon S, Daniel Belisle, Nita Modi, Matt Vijayan, David Hill, Victor Han. Maternal Nutrient Restriction Alters Fetal Insulin and Insulin-like Growth Factor Systems and Impairs Long-Term Metabolic Function. **Submitted to Endocrinology.**

Oral Conference Presentations (Refereed):

Maternal Nutrient Restriction Impedes Murine Fetal Lung Development and Impairs Postnatal Lung Function. Presented at: **The Fetal and Neonatal Physiology Society Conference,** Winchester, England. July 2010.

Poster Conference Presentations (Referred):

Fetal Growth Restriction (FGR) by Maternal Nutrient Restriction (MNR) leads to Glucose Intolerance in Adulthood. C. Albion, S. Dixon, D. Belisle, D. Hill, V. Han
Presented at: **The Fetal and Neonatal Physiology Society Conference,** Winchester, England. July 2010.

Effects of Maternal Nutrient Restriction on Placental Phenotype and Insulin-like Growth Factor System and Glucocorticoid Metabolism System Expression. C. Albion, S. Dixon, K. Nygard, C. Reid, K. Yang and V. Han. Presented at: **The Endocrine Society Conference,** Washington, D.C. June 2009.