AN ABSTRACT OF THE THESIS OF

Paulina M. Nguyen for the degree of Honors Baccalaureate of Science in General Science presented on May 9, 2012. Title: Effect of Perinatal Low Protein Diet on the Expression of Renal Organic Cation Transporter 2 and Novel Organic Cation Transporter 2 in Adult Rats.

Abstract approved:

_____

Ganesh Cherala

Studies have established that there is a greater risk for onset of adult metabolic syndrome disease such as diabetes mellitus, hypertension, hyperlipidemia, and obesity in subjects born with low birth weight (LBW). Undernourished fetuses make adaptions that result in structurally, morphologically, and functionally altered organs. In terms of drug pharmacokinetics, the alterations of organs that are associated with absorptions, distribution, metabolism, and excretion of drugs seen in LBW animals are significant. There is a possibility for LBW-induced susceptibility to adverse drug reactions or altered drug therapy which makes the study of renal drug transporters important for predicting drug dose and dosing regimen for optimal dosing pharmacotherapy. The purpose of this study is to evaluate the effect of LBW on the mRNA and protein expression of Organic Cation Transporter-2 (OCT2) and Novel Organic Cation Transporter -2 (OCTN2) in young (Day28 old), adolescent (Day65 old), and adult (Day150 old) rats. In this study, the maternal low protein diet (LPD) was used. OCT2 and OCTN2 mRNA levels were significantly altered in the LBW Day150 female offspring. Western blotting of various membrane fractions indicated slight differences in protein level expression of these transporters. Further studies can provide insights into how altered kidney development through LBW can affect drug pharmacokinetics.

Key words: organic cation transporters, pharmacokinetics
Corresponding e-mail address: nguyepau@onid.orst.edu

Effect of Perinatal Low Protein Diet on the Expression of Renal Organic Cation Transporter 2 and Novel Organic Cation Transporter 2 in Adult Rats

by

Paulina Nguyen

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the degree of

Honors Baccalaureate of Science in General Science (Honors Scholar)

Presented May 9, 2012
Commencement June 2012

Honors Baccalaureate of Science in General Science project of Paulina M. Nguyen presented on May 9, 2012

APPROVED:

_____

Mentor, Ganesh Cherala, representing Pharmacy

_____

Committee Member, Kevin Ahern, representing Biochemistry and Biophysics

_____

Committee Member, Indira Rajagopal, representing Biochemistry and Biophysics

_____

Dean, College of Pharmacy

_____

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Paulina M. Nguyen, Author

ACKNOWLEDGEMENTS

I would like to acknowledge the support of the following people, without whom this thesis would not be possible:

Dr. Ganesh Cherala, who has been there for me with advice and support all the time. No one could ask for a better mentor, adviser, or counselor.

Dr. Indira Rajagopal, who guided and encouraged me step-by-step from studying to doing summer research ever since I was still a freshman.

Dr. Kevin Ahern, who patiently spent time to guide and help me through the HHMI research program.

The OSU HHMI Summer Undergraduate Research Program.

My Honors College advisors, Rebekah Lancelin and LeeAnn Baker.

My Head Academic Advisor, Angela Austin Haney.

My loving parents, Triet and Kim Nguyen, for their continuous support and helping me both mentally and physically especially my mother – my role-model and guide, who has been with me every step of the way.

My lab colleagues, Jake Pearson and Barent DuBois.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Chapter I

Introduction

Epigenetics investigates heritable changes in gene expression occurring without changes in DNA sequence and in recent years, scientists have discovered that such heritable changes play a major role in human development like allowing genetically identical cells to develop different characteristics. Several epigenetic mechanisms, including DNA methylation or histone modifications can change genome function under exogenous influence. Most epigenetic studies have been limited to a cellular context or have examined the epigenetic effects of drugs or diet *in utero* where early environmental exposures on imprinted genes offers insight into the mechanisms affecting the fetal epigenome and subsequent adult disease susceptibility [1]. Research has shown that the effects of an animal's environment during adolescence can be passed down to future offspring and if such research proves to be applicable to humans, it would suggest that the impact of childhood education, environmental factors, and maternal diet could severely impact an individual's health and development in later life [2].

Epidemiologic studies support an early origin of adult human diseases with an association between low birth weight and a greater risk of coronary heart disease, hypertension, stroke, depression, type 2 diabetes, and osteoporosis in later life ([1], [2], [3], [4], [5], [6], [7], [8], [9]). The early origins of adult disease is at least partly rooted in the process of altered development ([2], [3], [10], [11]) where most human organ systems begin to develop early in gestation and do not become fully mature until weeks, months, or years after birth. Exposures such as under-nourishment, infection, and hormonal, drug, or toxin during critical periods of maturation result in permanent

alterations in the structure or function of specific organ systems, this process is commonly referred to as “fetal programming” or “developmental origins of health and disease” (DOHAD) ([3], [5], [6], [12]). The theory for correlation between the maternal nutrition and the birth-weight of the fetus is consistent with the fetal programming hypothesis that the maternal-placento supply of nutrition is not sufficient to sustain the fetal development in the womb [12].

The fetal programming hypothesis proposes that cardiovascular disease or non-insulin dependent diabetes in adults originate from adaptations that a fetus makes when it is malnourished [12]. Such adaptations may be vascular, metabolic or endocrine. These adaptations can permanently change the function and the structure of various organs throughout the life and well into adulthood since studies have shown over the last two decades that there has been a strong link between low birth weight and metabolic syndrome diseases (diabetes, hypertension, hyperlipidemia, and obesity) in adults [3]. The programming is an adaptive trait, since it is an attempt to establish phenotypes that meet the demands of later-life environment ([1], [3], [10], [11]). When the resulting phenotypes match the predicted later-life demands, the individual will remain healthy. However, when there is a high degree of mismatch, one’s adaptability to adult life challenges will be impeded and disease risk will be elevated. The latter scenario is more frequent today than in past decades since contemporary human life is greatly influenced by lifestyle choices such as low physical activity levels as well as a high fat and sodium diet, which often are in conflict with the aforementioned programmed adaptive changes made during early development.

There is research that suggests that alterations to internal cues for gene expression of various traits could subsequently induce fetal programming. The result of such fetal adaptations is low birth weight (LBW) individuals. LBW offspring can be produced in an animal model through the following mechanisms: maternal low protein diet, maternal caloric deprivation, maternal iron restriction, prenatal glucocorticoid exposure, and uterine ligation [13]. Maternal low protein diet (LPD) administration throughout gestation and lactation is a widely used animal model. These studies using this model suggest that there are morphological and physiological changes induced by adverse perinatal environment in various organs which include the kidney, an organ vital in the elimination of drugs from body [14].

LBW offspring tend to exhibit significant differences in organ morphology and functionality, specifically in the intestines, liver, and kidneys [15]. More specifically, it has been shown that there is a decrease in nephron number in rat LBW offspring [16]. A decrease in nephron number has been shown to result in decreased functionality of the kidney, thus providing an interesting field of research for how the ability of LBW offspring to eliminate endogenous and exogenous compounds from the body. See a pictorial representation of adaptations to low birth weight in Figure 1.

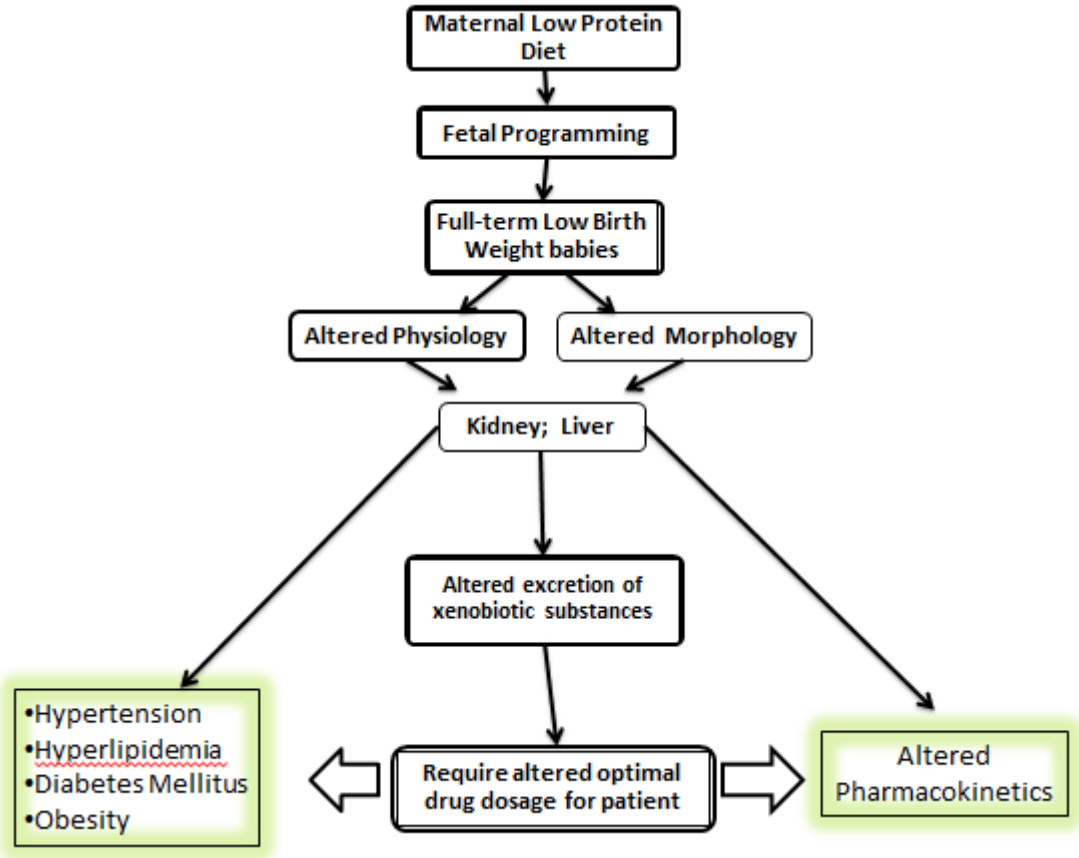

Figure 1. Schematic of the factors involved in the Low Birth Weight (LBW) model and the pharmacokinetic alterations associated with LBW.

I. Significance of Renal Secretion

Renal secretion of organic electrolytes of broadly diverse chemical structures plays a critical role in limiting the body's exposure to toxic compounds of exogenous and endogenous origin (including a wide array of compounds of clinical importance). Renal excretion is facilitated by glomerular filtration and carrier-mediated pathways mediated by transporters in the renal proximal tubule. This process is essential to an individual's well-being because if the renal excretion is suppressed or dampened somehow, this may increase the exposure of the body to potentially dangerous and toxic substances. The kidney goes through the process of eliminating various compounds using drug

transporters. These transporters are important transmembrane proteins that mediate the cellular entry and exit of a wide range of substrates throughout the body and thereby play important roles in human physiology, pharmacology, pathology, and toxicology. The regulation of transporter activity in response to endogenous and exogenous signals may occur at various levels, such as transcription, mRNA stability, translation and various post transcriptional levels (glycosylation, phosphorylation, protein-protein interaction) that involved a change in substrate affinity and membrane trafficking. Such varying regulatory mechanisms may correspond to various biological signals. Some of the renal transporters include organic anion transporters (OATs or OATPs) and organic cation transporters (OCTs) [17].

Organic transporters in various tissues help to determine the final distribution levels of substrates between the extracellular and intracellular space. Indirect modification of the transporter levels in a body can cause changes in the functional activity of these transporters that would result in impaired drug distribution and renal secretion. Renal OCTs play a significant role in the elimination of a wide variety of organic cations, including endogenous compounds such as monoamine neurotransmitter [18] and creatinine, drugs like cimetidine [19], metformin [18], amantadine [20], and cisplatin [20] and xenobiotics such as Tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium (MPP^+) [21].

At present, at least six distinct OCT isoforms have been cloned from various tissues. These are the electrogenic organic cation transporter (OCT1-3) and electroneutral organic cation novel-type transporter (OCTN1-3). Several of the identified OCTs have been shown to be expressed in a variety of organs but are most prominent in the kidney,

liver, and small intestine. In the kidney, organic cations may be ultra-filtrated in the glomeruli or secreted in renal proximal tubules. Secretion of organic cations occurs mainly in proximal tubules as shown in Fig 3. In human kidney, OCT2 and OCT3 are supposed to be important for basolateral uptake of organic cation substance ([22], [23]).

In 1996, Okuda et al. isolated rat OCT2 from a rat kidney cDNA library (54). rOCT2 mRNA was detected predominantly in the kidney but not in liver and lung or intestine. Immuno-localization of rOCT2 indicated that it was localized to the basolateral membrane of the renal proximal tubules. OCT2 are predicted to encode proteins of 593, 555, and 554 amino acids in length for the rat, human and pig, respectively. OCT2 is mainly expressed in the kidney, but has also been found in human placenta, rat thymus, rat choroid plexus, and neurons of the human OCT2 [24]. The expression of rOCT2 is dependent on the sex [24]. Studies of their expression revealed that OCT2 is expressed mostly in the kidney ([25], [26], [27]).

RT-PCR on isolated tubule segments from the kidneys confirmed the tissue distribution of rat OCT2 (as well as mouse OCT2) is in the cortex and medulla within the superficial and juxtamedullary proximal straight and convoluted tubules along with medullary thick ascending limbs, distal convoluted tubules, and cortical collecting ducts of the rat kidney (see Figure 2). However, rOCT2 was found to not be expressed in the liver ([25], [24], [28], [29]).

OCTN2 appears to involve transport of select substrates and Na+-co-transporter, OCTN2 involves the secondary active reabsorption of the zwitterions, carnitine, and related compounds. OCTN2 has been shown to be expressed in the luminal membrane of proximal tubule cells [29]. OCTN2-mediated reabsorption of carnitine could serve as a

driving force to support electroneutral luminal efflux of TEA. Studies involved with measuring TEA clearance in the juvenile visceral steatosis (JVS) mouse, a mutant strain with an inherited systemic carnitine deficiency, suggests that OCTN2 may play a quantitatively significant role in the secretion of a wide array of OCs [30].

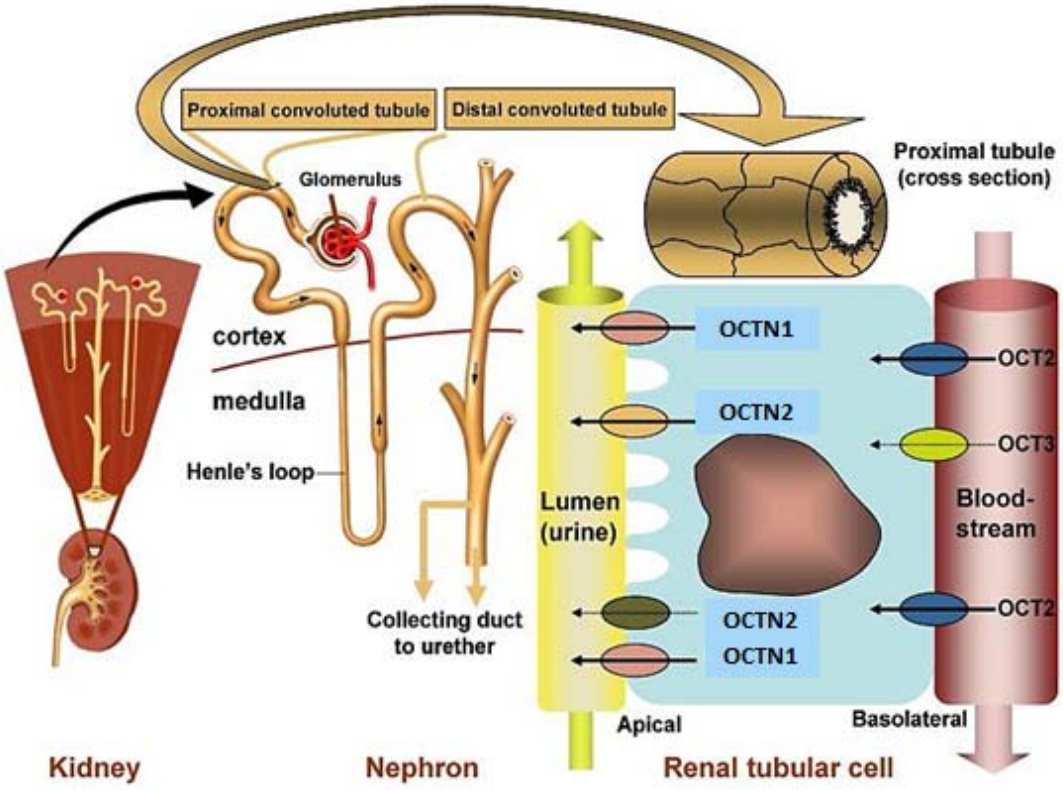

Figure 2. Diagram of basolateral and luminal uptake and efflux transport in renal tubule cell (B Hagenbuch, 2009). Illustrates proximal tubule transport of Organic Cation Transporters (OCT) and Novel Organic Cation Transporters (OCTN)

Drug handling and efficacy can be greatly affected when serious diseases and pathological conditions, such as diabetes, are shown to be associated to diminished organic drug transporter clearance. Due to the morphological alteration of organs relevant to drug pharmacokinetics (such as the kidneys) as a consequence of LBW, the question of

whether LBW affects the transport of pharmacological compounds becomes significant since altered drug transport is directly related to the adverse drug reactions and inefficient pharmacological responses. My hypothesis is that the ability of LBW offspring to eliminate various drugs or medications could be diminished, resulting in the need for optimization in medication dosage for LBW individuals because an adverse perinatal environment may leave a long term permanent effect on the expression of OCT2 and OCTN2 in the nephrons since these two transporters have been shown to be expressed predominantly in the kidney and are associated with the treatment and understanding of diabetes and obesity respectively.

Chapter II

Materials and Methods

Materials and Methods:

2.1 Diets

Modified versions of the AIN76A purified diet (Control), and the corresponding isocaloric low protein formulation (LPD) were purchased in pellet form from Purina Test Diets (Richmond, IN). The purified control and LPD diets contained 19% and 8% crude protein in the form of casein, respectively. Detailed compositions of both diets are available in the literature [14].

2.2 Experiment with dams

The study was approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University, Portland, OR. Virgin female Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were mated by housing one male rat with two female rats. Day 1 of pregnancy was assigned upon observation of sperm in the daily morning vaginal smears, at which time the rats were randomly assigned to the two diet groups. Each group consisted of 5-7 pregnant rats, and these rats received their assigned diet throughout pregnancy and lactation (Figure 3).

2.3 Experiments with offspring

Upon birth, pups were sexed, and litter size was noted. All litters were randomly culled to 12 pups (6 male and 6 female) on the day of birth and further randomly culled to 8 pups (4 males and 4 females) on day 4 after birth. Offspring from both groups were

weaned on day 28 after birth and were housed in isosexual groups according to perinatal diet treatment. Pups from litters in both groups were weaned onto a nonpurified diet. It is therefore important to note that different dietary treatments were administered only during gestation and lactation. One male and female offspring from each litter in both groups were randomly chosen and sacrificed using CO_2 asphyxiation on Day 28, 65, and 150 after birth. Various organs including kidneys were collected from all sacrificed animals, weighed, snap-frozen in liquid nitrogen and stored at -80°C.

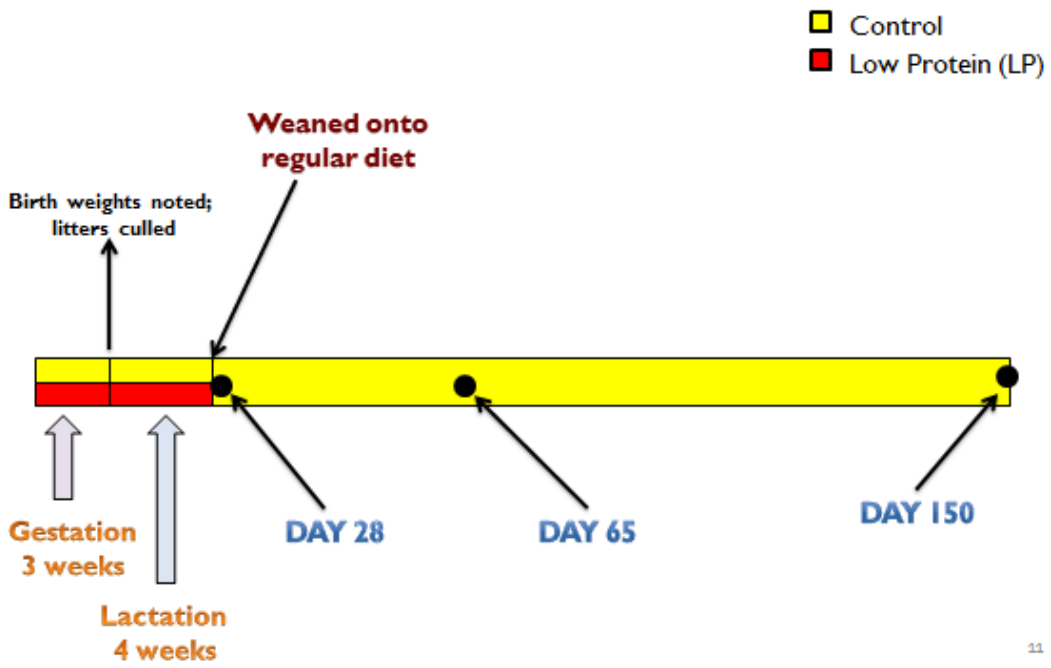

Figure 3. Pictorial representation of the study design.

I. RT (q)-PCR

RNA isolation:

Total RNA was extracted from 100-200 mg of cryogenized whole kidney tissue. The tissue was homogenized using a mortar and pestle over liquid nitrogen and RNA was isolated using the TRIZOL method. All care was taken to ensure the samples remained

frozen until completely homogenized with the TRIZOL to prevent RNA degradation. Purity and concentration of the product were quantified from absorbance ratios of 260/230 and 260/280, respectively. Ideal values for these ratios were considered to be 1.8 ± ~0.3. Measurements were obtained using the Nanodrop 1000. RNA was stored at -80˚C until cDNA synthesis.

cDNA synthesis:

cDNA was synthesized using the iScript cDNA synthesis kit from BioRad. Briefly, 1 µg of total RNA was added to a reaction solution containing 4 µL iScript reaction mix, 1 µL iScript reverse transcriptase, and 14 µL nuclease free water, for a total reaction volume of 20 µL. The reaction was incubated in the BioRad MyiQ thermocycler with temperatures as follows: 25˚C for 5 min, 42˚C for 30 min, and 85˚C for 5 min.

RT-PCR:

BioRad iQ SYBR Green supermix was used for PCR reaction. Briefly, 10 µL of cDNA was added to a reaction mixture containing 25 µL iQ SYBR Green supermix and 7.5 µL of each forward and reverse primers [250 nM concentration] for a total final volume of 50 µL. The reaction was incubated in the BioRad MyiQ Thermocycler with reaction temperatures as follows: initial denaturation for 95˚C for 5 min, 95˚C for 10 seconds followed by 59˚C for 30 seconds. This reaction was repeated for 40 cycles of qPCR. The messenger RNA for renal OCTs was quantified using real time reverse transcription polymerase chain reaction (RT-PCR) in the iQ5 Optical System (Bio-Rad Laboratories, Munich, Germany). The primers used: OCT2 forward primer (5’TCCATGTGTGACATTGGCGGCA-3’), OCT2 reverse primer (3’CGACAAGGCCAACCACAGCAAA-5’); OCTN2 forward primer

(5'GCCAGCATCATCCCCAATGGCTTTAC-3'), OCTN2 reverse primer (3'CCTTCATCTCCAACGGGATGCTGTG-5') (Invitrogen). The initial amount of target mRNA in each sample was estimated from the experimental cycle threshold (C_t) value with a standard curve generated using known amounts of standard plasmid DNA. The C_t value is the cycle number at which the fluorescence signal from SYBR green, which binds to increasing amounts of DNA product, crosses the arbitrary threshold for each sample. β-actin mRNA was used as an internal standard to ensure accurate comparison of expression of the gene of interest between different samples. As a standard/reference gene, β-actin is a gene that is a stably expressed gene in most tissue samples.

Calculations:

The efficiency (E) of primer pairs was determined using a 10x serial dilution of a control cDNA sample [13]. The slope of the relationship between Log transformation of dilution factor and cycle threshold (Ct) values at each dilution factor was calculated. The 'E' of each primer pair was then calculated as 10^(-1/slope). Fold differences in the expression of a reference gene between groups were calculated as $E^{(\Delta Ct_{(control-LPD)})}$. Theoretical effects on target gene expression were calculated by the method adapted from Pfaffl method [17].

II. Western Blot

Sample Preparation:

Crude membrane fractions were prepared as previously described [31]. The resulting mitochondrial and microsomal pellets were suspended in pH 7.4 buffer [50 mM Tris and 0.25 M sucrose], and stored at -80˚C. Protein concentrations were determined

with the Bradford protein assay (Bio-Rad Laboratories, Munich, Germany) using bovine serum albumin as the standard.

Gel Electrophoresis:

Proteins were subjected to gel electrophoresis and transferred to nitrocellulose filters (Bio-Rad Laboratories, Munich, Germany). Rabbit anti-rat antibodies for rat OCT2 was obtained from Alpha Diagnostic International (San Antonio, Texas). The antibodies were diluted to a concentration of 1:1500 with a 1:1 mixture of 1x phosphate buffer saline (PBS) and LiCor Biosciences (Lincoln, Nebraska) Odyssey® Blocking Buffer. Goat anti-rabbit polyclonal secondary antibodies were obtained from LiCor Biosciences, with IRDye® in the ranges of both 680nm and 800nm. β-actin was used as a loading control, as a standard housekeeping protein, β-actin is a protein that is stably expressed in most samples.

Twenty μg of total protein was resolved on an SDS-polyacrylamide gel (7% stacking, 10% resolving) at 150V for 60 minutes and transferred to a nitrocellulose membrane at 100V for 60 minutes. The membrane was briefly washed with TBS-T (1x TBS with 0.5% of 10% Tween). The filters were blocked for 1 hour at room temperature with a mixture of TBS-T (100 mM Tris pH 7.4, Tween 20) with Odyssey Blocking Buffer. Incubation with the affinity-purified polyclonal antibody against rat OCT2 (dilution of 1:15000) was performed at 4°C overnight (in 1:1 Odyssey Blocking Buffer/ 1X PBS). The membranes were briefly washed the next day with TBS-T for one hour with goat-anti-rabbit in 1X PBS/Odyssey Blocking Buffer dilution mixture, followed by four washes with 1X TBS before drying in preparation for imaging. Antibody binding

was visualized with the LiCor Biosciences Odyssey® imaging system, and images were quantified using NIH ImageJ software.

To demonstrate that the band observed was the OCT2 band, primary antibody inhibition was done using a blocking peptide as described: 10 µl of 1µg/µl OCT2 primary antibody and 10µl of 1µg/µl OCT2 blocking peptide were mixed, gently vortexed for 10 sec, and incubated at room temperature for 4 hours, as per manufacturer's protocol. This solution was diluted in 1:1 solution of 1x PBS and Odyssey® Blocking Buffer, and incubated with the membrane at room temperature for 1 hour before using for primary antibody wash step, as described in the previous paragraph.

For OCTN2, rabbit anti-Mouse antibodies for rat OCTN2 were obtained from Alpha Diagnostic International (San Antonio, Texas) and diluted to a concentration of 1:1500 as described previously for the OCT2 antibodies. Goat anti-rabbit polyclonal secondary antibodies were obtained from LiCor Biosciences, with IRDye® in the ranges of both 680nm and 800nm. Due to the nonspecific binding of OCTN2 antibodies to β-actin protein at the molecular weight of actin, a positive control (a sample known to express OCTN2 protein) was used as a loading control in place of β-actin.

Antibody binding was visualized with the LiCor Biosciences Odyssey® imaging system, and images were quantified using NIH ImageJ software. OCTN2 band verification was done by primary antibody inhibition using a blocking peptide in the procedure described above. For the blocking peptide step, 10 µl of 1µg/µl OCTN2 primary antibody and 10µl of 1µg/µl OCTN2 blocking peptide were mixed, gently vortexed for 10 sec, and incubated at room temperature for 4 hours, as per manufacturer's protocol. This solution was diluted in 1:1 solution of 1x PBS and Odyssey® Blocking

Buffer, and incubated with the membrane at room temperature for 1 hour before using for primary antibody wash step.

Band densities of OCT2 and OCTN2 for LPD and control samples were normalized to β-actin or positive control band densities, respectively in order to determine protein expression of OCT2 and OCTN2.

III. Data Analysis

Data are expressed as means ± S.D. Data were analyzed statistically using the unpaired Student *t* test. Data were considered significantly different at values of $P<0.05$.

Chapter III

Results

I. Efficiencies of primers were acceptable.

A primer efficiency (E) experiment was done to insure that the annealing efficiency of the primers was acceptable, as inefficient priming can affect the amount of amplified product. The E of the primer pairs was calculated via a serial dilution as described in the methods section above. OCT2, OCTN2, and β-actin primer pairs were found to be acceptably efficient, with E being 96.4% for β-actin, 98.2% for OCT2 and 99.5% for OCTN2. For the two genes of interest and the internal standard, the efficiency of the primers was slightly less than 100% consequently, the amount of DNA increased by a factor of 1.9278 for β-actin, 1.9639 for OCT2, and 1.9890 for OCTN2 at the end of each PCR cycle. The above E values were employed in estimating fold differences in gene expression described below.

II. Significant differences in mRNA expression of OCT2 and OCTN2 were only observed in Day 150 females.

RT-PCR was used to compare mRNA expression of OCT2 and OCTN2 between the LPD and control animals. The fold differences in mRNA expression of OCT2 and OCTN2 on Day 28, 65, and 150 for both sexes are shown in Tables 1 and 2. In Day 28 old male and female offspring, there were no significant differences in the mRNA expression of OCT2 and OCTN2 between the two groups. Similarly, no significant differences in the mRNA expression of both transporters were found in Day 65 old male and female offspring. In Day 150 old male offspring, there were no significant differences in the expression of OCT2 or OCTN2. However, in the female offspring, the

mRNA expression of OCT2 in LPD females significantly decreased by 27-fold. Furthermore, the expression of OCTN2 increased significantly by 41 fold (Figure 4). A summary of the changes are depicted in a pictorial format in Figure 4.

Table 1: mRNA expression of renal organic cation transporter 2 (OCT2)

Sex	Age	Fold Difference	SD
Male	Day 28	-2.504	0.6820
	Day 65	-3.482	0.6628
	Day 150	1.057	1.120
Female	Day 28	1.091	0.7307
	Day 65	2.128	1.018
	Day 150	-27.64	4.409

Table 2: mRNA expression of renal novel organic cation transporter 2 (OCTN2)

Sex	Age	Fold Difference	SD
Male	Day 28	-2.252	1.187
	Day 65	1.801	1.762
	Day 150	3.265	2.190
Female	Day 28	1.392	1.458
	Day 65	1.080	2.129
	Day 150	41.25	0.6880

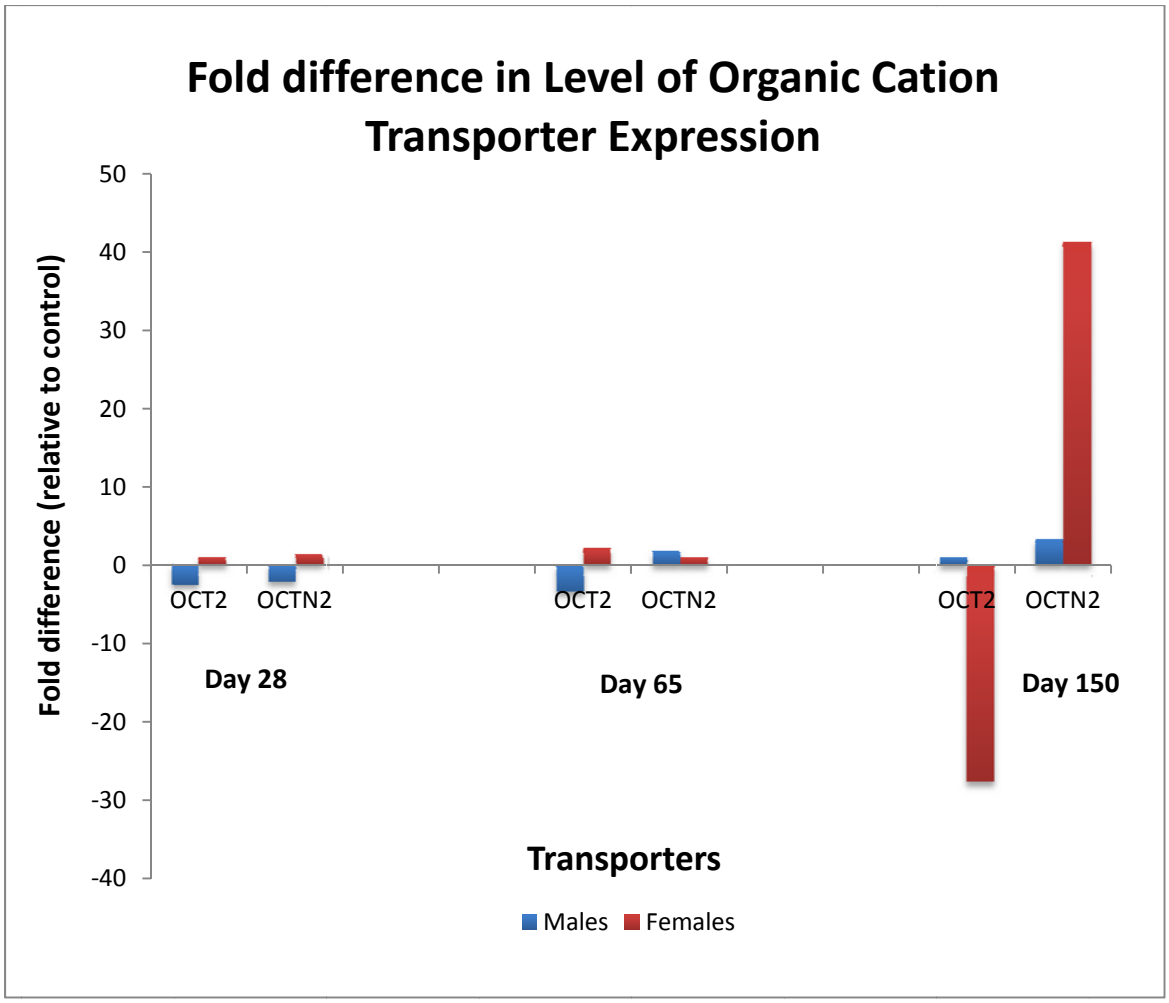

Figure 4: mRNA expression of renal organic cation transporter 2 (OCT2) and renal novel organic cation transporter 2 (OCTN2).

III. No significant differences were seen in the protein expression of OCT2 and OCTN2 for either sex

Western blotting of the OCT2 and OCTN2 proteins on microsomal fractions was carried out to compare protein expression of OCT2 and OCTN2 between the LPD and control animals. A representative image of OCT2 western blot was shown in Figure 5 and graphical representation was shown in Figure 6 for males and females. Based on the blocking peptide experiment results, in which the blocking peptide should displace the

OCT2 protein thus indicating which band was the OCT2 protein, we have quantified the top band closer to 60-70 kDa (~66kDa) as the OCT2 band ([32], [33]). For males, the OCT2 expression showed a slight decrease of 0.744 fold in Day 150 old. For females, a 1.38 fold increase was observed in Day 150 old (Table 3). None of the fold differences were statistically significant ($p<0.05$).

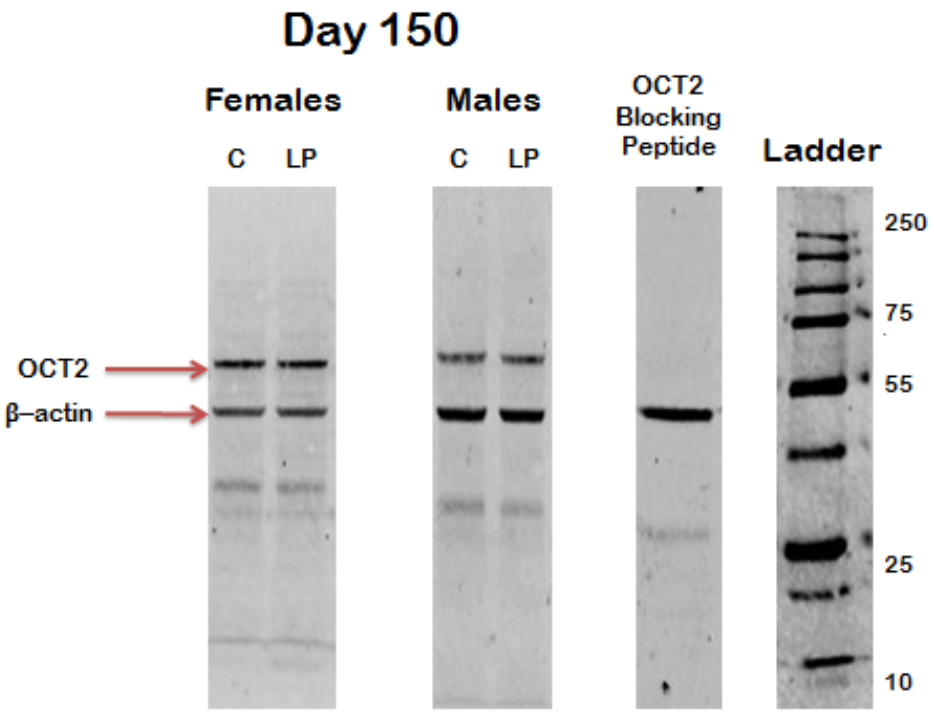

Figure 5. Images of western blotting of microsomal fraction for Day 150 samples. OCT2 and β-actin bands are labeled on left and relative position is conserved throughout the images. Molecular weight marker shown on far right with numerical values in kilodaltons. LP=Low Protein. C=Control.

Table 3: Protein expression of renal organic cation transporter 2 (OCT2).

	Control	LP	Fold Difference
Males	0.0107 ± 0.0049	0.00797 ± 0.0044	0.744
Females	0.0165 ± 0.0199	0.0228 ± 0.0230	1.38

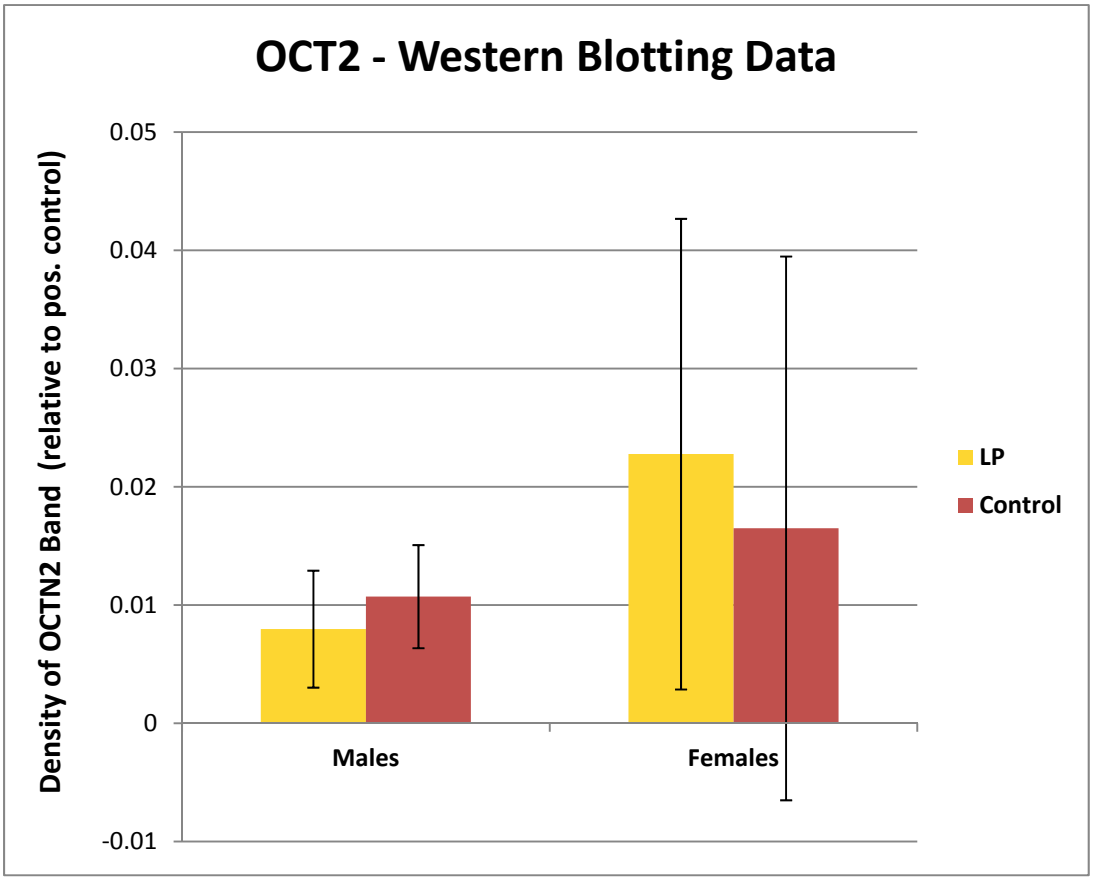

Figure 6. Graphical representation of protein expression of renal organic cation transporter 2 (OCT2).

A representative image of the OCTN2 western blot is shown in Figure 8 and graphical representation was shown in Figure 9 for males and females. The top dark band around 63 kDa corresponding to expected molecular weight of rat renal OCTN2 [34] was quantified. OCTN2 protein expression increased by 1.011 for males and increased by 1.139 in the females for Day 150 old (Table 4). None of the fold differences were statistically significant ($p<0.05$).

Western blotting experiments were done on the microsomal cellular fraction for OCT2 and the mitochondrial cellular fraction for OCTN2. The use of two different cellular fractions was necessary because when western blotting was done on the microsomal cellular fraction to study OCTN2, no protein bands at the molecular weight of OCTN2 appeared as seen in Figure 4. On the other hand, when western blotting was done on the mitochondrial cellular fraction to study OCTN2, there were protein bands at the molecular weight of OCTN2 protein (Figure 7). This is why the “mitochondrial fraction” was used to determine OCTN2 expression while the “microsomal fraction” was used in western blotting to determine OCT2 expression.

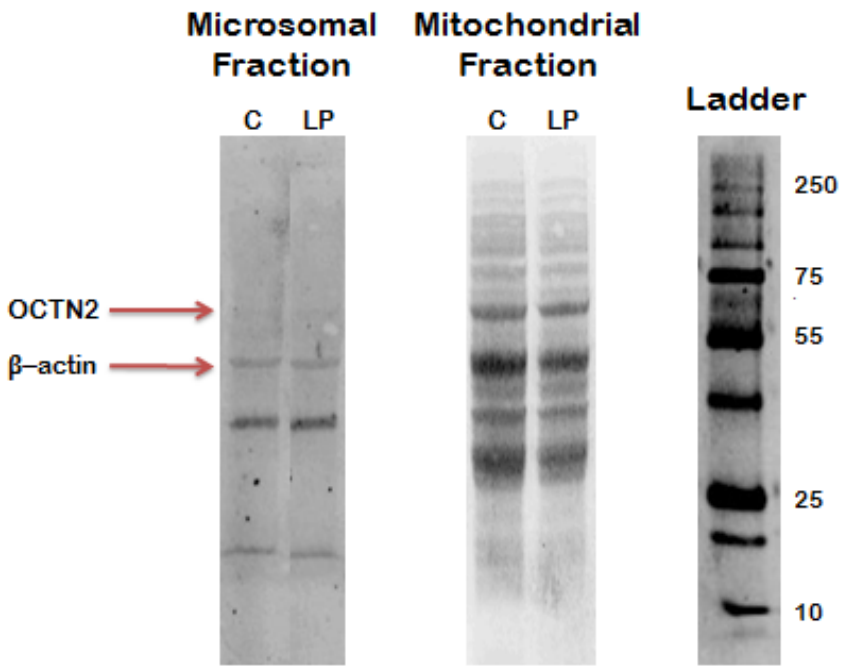

Figure 7. Images of western blotting of microsomal and mitochondrial fraction for Day 150 samples. OCTN2 and β-actin bands are labeled on left and relative position is conserved throughout the images. Molecular weight marker shown on far right with numerical values in kilodaltons (kDa). LP=Low Protein. C=Control.

The mitochondrial cellular fraction, containing mitochondria and plasma membranes, yielded various presumed OCTN2 bands making quantitation difficult, as seen in Figure 8. OCTN2 band quantification was based on normalization of the ~63kDa band with a similar band in the positive sample.

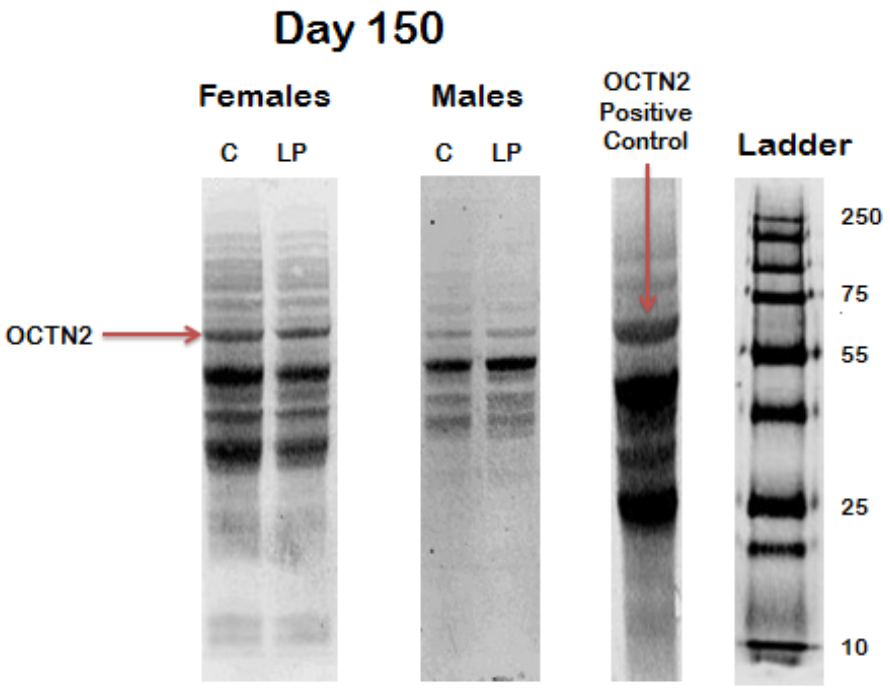

Figure 8. Images of western blotting of mitochondrial fraction for Day 150 males and females. OCTN2 and positive control bands are labeled and relative position is conserved throughout the images. Molecular weight marker shown on far right with numerical values in kilodaltons (kDa). LP=Low Protein. C=Control.

Table 4: Protein expression of renal novel organic cation transporter 2 (OCTN2).

	Control	**LP**	**Fold Difference**
Males	0.6864 ± 0.0795	0.6941 ± 0.1164	1.011
Females	0.6852 ± 0.0966	0.7805 ± 0.0411	1.139

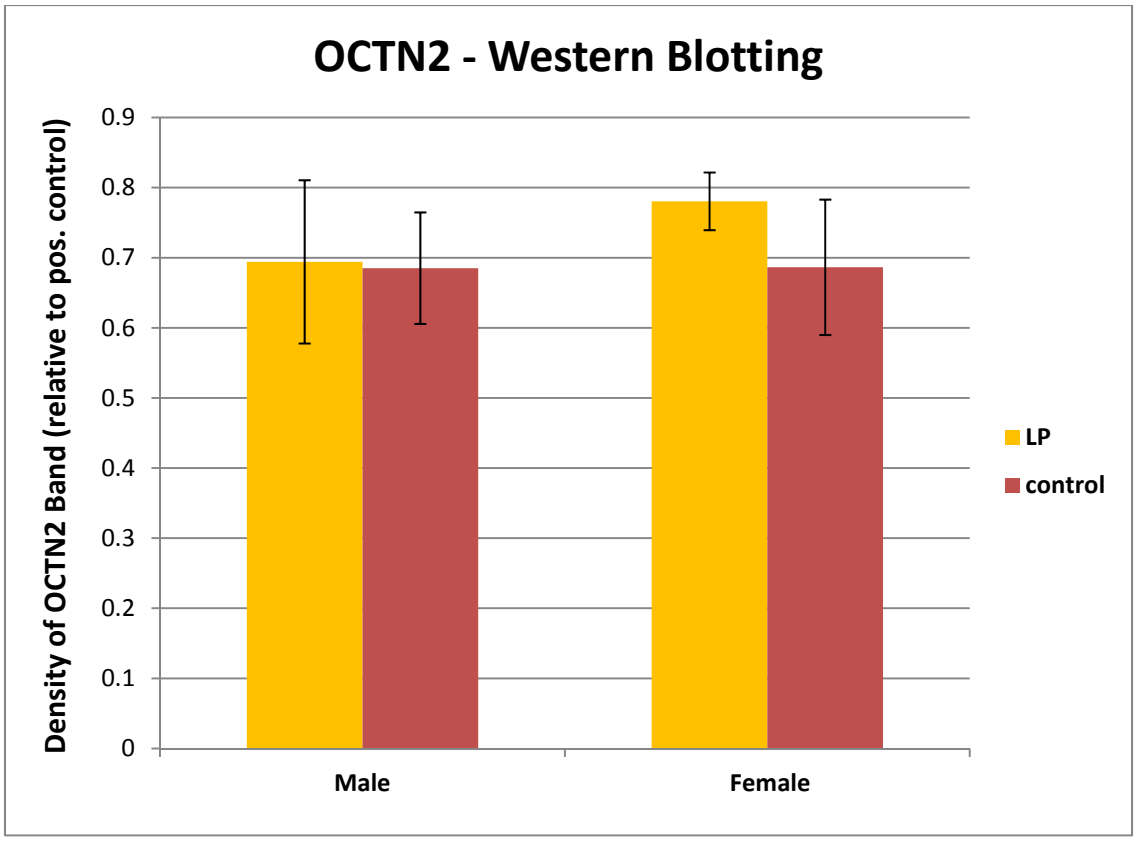

Figure 9. Graphical representation of protein expression of renal novel organic cation transporter 2 (OCTN2).

Chapter IV

Discussion

My hypothesis is that a morphological alteration to fetal kidney development, due to poor environmental conditions for the mother, would result in LBW progeny with altered expression of renal OCT2 and OCTN2 throughout life. This hypothesis is based on the findings that link higher incidence of chronic adult-onset diseases with LBW [14]. My predictions were that there is altered mRNA expression of OCT2 and OCTN2, with corresponding alterations in the protein expression of OCT2 and OCTN2 in the LPD treatment group compared to the mRNA and protein expression of these two transporters in the control group. The hypothesis was tested by measuring expression of two important renal drug transporters, OCT2 and OCTN2, at mRNA and protein levels using RT-PCR and western blotting, respectively.

Consistent with my hypothesis, OCT2 and OCTN2 mRNA levels were significantly altered in the LPD Day150 female offspring, but not the male offspring. Further, these alterations were limited to female Day 150-old offspring alone. This finding suggests that an adverse perinatal environment leaves a long-term permanent effect on the expression of OCT2 in a sex-dependent fashion. This is probably mediated by sex-hormones in which testosterone up-regulates expression of OCT2 and estrogen down-regulates expression of OCT2 [24]. One study showed that the treatment of both male and female rats with testosterone significantly increased the mRNA and protein expression levels of OCT2 in the kidney while estradiol treatment moderately decreased the expression of OCT2 [35]. Furthermore, it has also been shown that mid-aged rats (~120 day old) have elevated levels of serum estradiol [36] which supports the suggestion

that OCT2 mRNA expression is down-regulated by estrogen, where the 27-fold decrease in OCT2 mRNA expression was only seen in the Day 150-old females.

In terms of protein expression, there were no changes in protein expression even though significant differences in mRNA expression of OCT2 and OCTN2 were observed, therefore my hypothesis is that there would be an alteration to protein expression of renal OCT2 or OCTN2 may not be supported by the protein expression data. However, the fold differences in protein expression data were not statistically significant (Figure 6 & 9). The discordance between mRNA and protein expression data could be attributable to multiple factors. My ability to correlate mRNA and protein expression data may have been compromised by the large variability in protein expression data (Figure 6 & 8).

A possible explanation for the presence of OCT2 and OCTN2 in different cellular fractions is due to the localization of the OCT2 and OCTN2 transporters on the proximal tubule. As mentioned previously, the OCT2 protein is located on the basolateral membrane of the renal proximal convoluted tubule whereas OCTN2 protein is located on the apical (brush-border) membrane of the tubule. The brush-border membrane is denser than the basolateral membrane, so when the proximal tubule cells were being broken apart during preparation of crude membrane fractions, the brush-border membranes containing the OCTN2 protein would separate out of solution and into the pellet known as the “mitochondrial fraction”. On the other hand, the less dense basolateral membrane containing the OCT2 protein would stay in solution and become part of the supernatant; the supernatant was further separated spun at higher centrifugal force to pellet the “microsomal fraction”.

Ultimately, there were significant differences in the expression of OCT2 and OCTN2 in LBW offspring based on the collected data at the mRNA level. It is notable that the Day 150 females expressed a ~27-fold decrease in OCT2 mRNA and a ~41-fold increase in OCTN2 mRNA. If the data for protein expression is taken at face value then there were no observed differences in OCT2 and OCTN2 expression at the protein level, however this data was not statistically significant therefore no specific conclusions can be made with this data. The apparent discrepancy between the mRNA levels of OCT2 and OCTN2, and the protein levels of these transporters indicates that there is a need to examine the regulation of OCT2 and OCTN2 more closely. In addition, better isolation of cell membrane fractions for western blot experiments may be necessary.

In a study done by Urakami et. al. (2000), immunoblot analysis of OCT2 protein showed that treatment of male and female rats with testosterone produced significant increases in OCT2 protein expression, whereas administration of estradiol resulted in a slight decrease in the OCT2 protein expression in males but not in females. Those alterations to OCT2 protein expression corresponded with their data on mRNA expression of OCT2 in male and female rats being increased or decreased by treatment of testosterone or estradiol, respectively. Therefore, Urakami et. al. concluded that "the modulation of OCT2 protein expression by hormone treatment appeared to be due to regulation of its mRNA expression" [2]. The regulation of OCT2 by the overall balance of testosterone and estradiol contribute to differences in OCT2 expression between males and females [3]. The findings of these studies suggest that the pathway for regulation of OCT2 protein expression by the internal hormonal regulation OCT2 mRNA expression in LBW animals is altered somehow. Based on the Urakami et. Al. (2000) study, a possible

explanation for the 27-fold decrease in mRNA expression for OCT2 in the Day 150 females of the LPD group is that the Day 150 LPD females have higher levels of estradiol compared to females of the control group. Further studies need to be done to measure the hormone levels in LPD animals to confirm this theory. If the Day 150 females from the LPD group are shown to have higher estradiol levels compared to the control group then that will be further support for the finding of this study which showed that there is a 27-fold decrease observed in OCT2 mRNA expression for that group.

Diet, hormones, and social and lifestyle factors have all been shown to influence the genotype and might exert extreme effects on many aspects of child and adult health, and susceptibility to disease in later life ([38], [4], [5]). Environmentally induced epigenetic adaptations occurring at crucial stages of life could potentially change behavior, disease susceptibility, and long-term survival [38]. Different environments are able to alter gene expression and change an individual's phenotype by modifying the epigenome [37]. Epigenome is defined as alterations to genomic patterns that are characteristic of an organism, where these patterns can occur by modifying gene expression without altering the underlying DNA sequence [36]. Defects in the epigenome are known to lead to disease and these defects include changes in the density of DNA methylation, incorrect histone modifications, or altered function of chromatin-modifying proteins that can lead to abnormal gene expression (Figure 10).

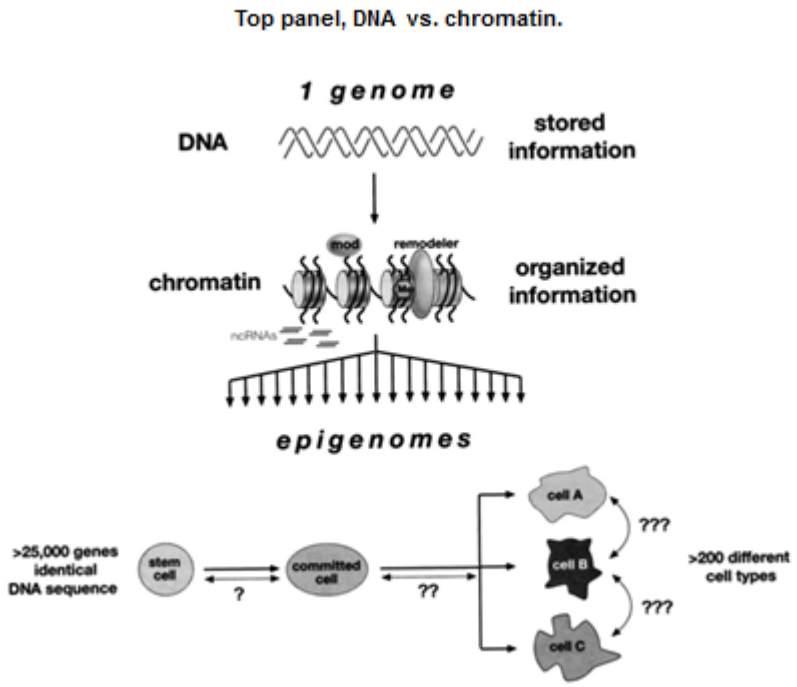

Figure 10. Epigenome diversification.

Epigenetic mechanisms result in stable regulation of gene expression without alterations to the DNA sequence and trigger initiation and maintenance of cell-specific transcription. The precise control of transcription is achieved by modulating the chromatin structure and three-dimensional organization of the nuclear architecture and genome. Epigenetic modulation or modification of gene expression is possible, for example, when maternal nutrition can influence gene expression of the growing fetus *in utero* and in the developing progeny after its birth ([41], [42], [43]), and DNA methylation can be prenatally manipulated by hormonal stimuli [44]. Therefore, "specific environmental factors, the aging process, and the actions of sex hormones may influence the chromatin modifying enzymes" ([45], [46]) and these effects can accumulate with increasing age. The variability of epigenetic modifications makes it possible for all these above-mentioned influences to alter existing DNA methylation patterns, create new

histone marks, or modify the chromatin structure during a particular time frame, sometimes in a sex-specific manner ([47], [48]).

Epigenetic modifications such as histone modifications have been proposed as a plausible link between the alterations in gene expression, environment, and disease susceptibilities [36]. When the amino-terminal tails of all eight core histones protrude through the DNA and are exposed on the nucleosome surface, these tails are subject to many enzyme-catalyzed modifications of specific amino-acid side chains, such as acetylation of lysines, methylation of lysines and arginines, and phosphorylation of serines and threonines. Histone tail modifications are put in place by modifying and demodifying enzymes. The activity of these enzymes can be modulated by environmental and intrinsic signals. Modifications may function as short-term, on-going processes (such as transcription, DNA replication and repair) and in more long-term functions (determination of chromatin conformation like heterochromatin formation), all of which can be used as heritable markers that both predict and are necessary for future changes in transcription [16]. However, this does not mean that a single type of histone posttranslational modification dictates a single outcome. Instead, a combination and enrichment of histone posttranslational modifications define different chromatin formations with specific functional outcomes. Chromatin has a crucial role in the inheritance of transcriptional regulation but it is still unclear how histone modifications are reproduced after DNA replication and are inherited from generation to generation ([37], [49]).

The epigenetic changes to DNA, like histone modifications, may be involved in generating both short-term and long-term outcomes to mRNA expression of renal rat

OCT2 and OCTN2. The LPD treatment group may have some internal cues that caused changes in histone modifications affecting which parts of DNA sequences are expressed. Thus this gives a possible explanation for the differences seen in OCT2 and OCTN2 mRNA expression between the LPD and control groups. The epigenetic modifications made by the LBW offspring in response to the maternal LPD may be maintained in later life so that the mRNA expression changes that underlie the fetal programming would contribute to the onset of metabolic syndrome disease in later life.

On the other hand, epigenetic changes to gene expression do not account for the lack of corresponding protein expression changes in OCT2 and OCTN2 protein expression for the LPD and control groups. One possible explanation is that there are some posttranscriptional, translational or posttranslational modifications that cause the OCT2 and OCTN2 protein made by the LPD animals to be more stable. The relative rates of cellular protein synthesis and protein degradation determine the amount of protein present at any point in time. Usually, transcriptional regulation determines the concentrations of specific proteins expressed within cells or the amounts of key enzymes and regulatory proteins, such as transcription factors, are controlled through selective protein degradation [50]. Alterations to the posttranslational modification of proteins in the LPD animals could have resulted in decreased rate of proteolysis as well as modifications to the amino acid sequence of the OCT2 or OCTN2 proteins. This could lead to less degradation of these specific transporter proteins in the LPD group resulting in accumulation of OCT2 or OCTN2 protein regardless of the decreased mRNA expression of these two transporters, which can account for the lack of correlation between protein and mRNA expression of OCT2 and OCTN2. Further studies using

different methods besides western blotting to determine OCT2 and OCTN2 protein expression can be done to determine the physiological effects of the fold differences in mRNA expression for OCT2 and OCTN2.

Chapter V

Conclusion

It has been suggested that the baby receives a forecast of the environment it will encounter after birth from its mother and modifies its metabolism, whole body physiology, and growth trajectory appropriately to maximize its chances of survival postnatally [49]. In other words, the fetus senses maternally transmitted environmental cues, such as undernutrition, during prenatal and early postnatal life. These adaptations that the fetus makes can become harmful if the conditions after birth are not the same as the ones encountered prenatally where the environment after birth can be adequate or deprived causing "mismatch" between the fetus's former prenatal environment and its eventual mature environment then the risk of metabolic disease is enhanced (see Figure 10 for a schematic).

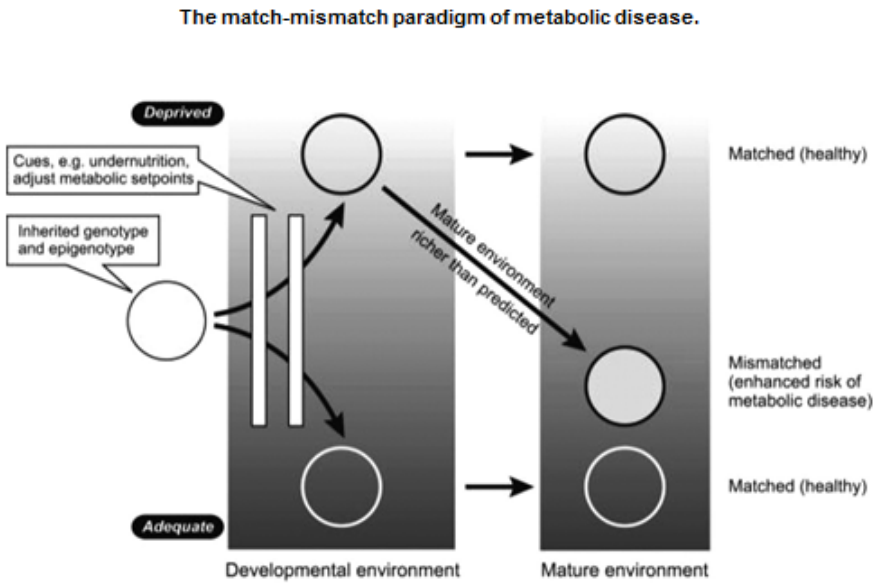

Figure 11. Schematic of mismatch between the fetus's environment in the womb and postnatal environmental conditions.

There is evidence that epigenetic marks can be modulated by environmental factors, are heritable, and perpetuate gene-expression changes that underlie programming (Figure 11). The evidence suggests that epigenetic alterations, including DNA methylation and histone modifications, are transmitted transgenerationally. DNA methylation and histone modifications serve to stably alter the gene expression pattern in cells such that cells can "remember where they have been" or decrease gene expression which lets the cells be able to differentiate into specific functions based on “previous experience” with a similar environment ([3]). The traditional view of epigenetic modifications as static on/off switches that control gene expression is altered by the idea that these modifications are dynamic so that some environmental factors or cues (i.e. histone modification) can act on the epigenetic machinery to alter gene expression.

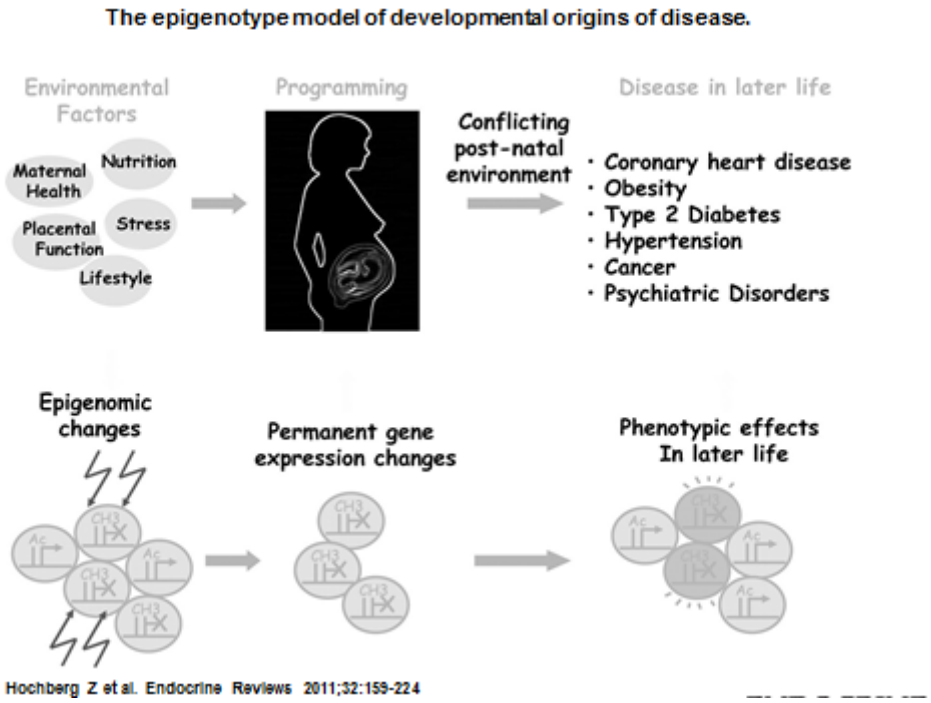

Figure 12. Pictoral description of epigenomic changes being linked with disease.

In this study, many fold changes in the mRNA expression of OCT2 and OCTN2 were seen especially in females of the oldest age group. The sex-specific findings could be attributed to the regulation of drug transporter proteins by sex hormones. We speculate that the profiles of sex hormones are altered in LPD offspring, especially female offspring, leading to the observed sex-specific alterations in the current study. Other researchers have conclusively stated that estrogen and testosterone levels were elevated and depressed, respectively, in adult animals that experienced adverse perinatal environment [35]. The data on their protein expression is inconclusive and needs further experimentation. However, the significant fold differences in mRNA expression of OCT2 and OCTN2 for the Day 150-old females are an important finding of the current study.

The "mismatch" between the LBW individual's prenatal environment and mature adult conditions has been linked to onset of metabolic syndrome diseases such as obesity, type II diabetes, insulin resistance, cardiovascular and atherosclerotic disease, dyslipidemia, and cognitive and behavioral disorders as mentioned previously. From a pharmacological perspective, those LBW individuals are more likely to seek pharmaceutical interventions in order to manage their chronic diseases. However, this study suggests that there is a possibility that these people are likely to get suboptimal pharmacotherapy due to the possible permanent effects of an adverse perinatal environment on pharmacokinetics because of alterations in expression of drug transporters in organs essential to drug metabolism and excretion.

REFERENCES

[1] W. J. J. R. Dolinoy DC, "Epigenetic gene regulation: linking early developmental environment to adult disease," *Reproductive Toxicology,* p. 23:297–307, 2007.

[2] M. W. Gillman, " Developmental Origins of Health and Disease," *New England Journal of Medicine,* p. 353(17): 1848–1850, 2005, October 27.

[3] L. K. B. G. G. P. H. M. Godfrey KM, "Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease.," *Pediatric Research,* p. 61:5R–10R., 2007.

[4] O. S. Fernandez-Twinn DS, "Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome," *Physiology & Behavior ,* p. 88:234–43., 2006.

[5] O. C. S. S. W. G. Barker DJ, "The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life," *BMJ (formerly British Medical Journal),* p. 306:422–6, 1993.

[6] H. M. Gluckman PD, "The developmental origins of the metabolic syndrome," *Trends in Endocrinology & Metabolism,* p. 15:183–7, 2004.

[7] V. D. M. J. M. R. O. C. B. D. H. C. B. O. Ravelli AC, "Glucose tolerance in adults after prenatal exposure to famine," *Lancet ,* p. 351:173–7., 1998.

[8] A. N. K. R. S. H. D. I. S. T. C. C. Dennison EM, "Birthweight, vitamin D receptor genotype and the programming of osteoporosis," *Pediatric Perinatal Epidemiology,* p. 15:211–9, 2001.

[9] S. H. R. I. O. C. B. D. Thompson C, " Birth weight and the risk of depressive disorder in late life," *British Journal of Psychiatry,* p. 179:450–5, 2001.

[10] B. D. C.-B. T. D. D. D. B. F. R. G. P. G. K. K. T. L. M. M. J. M. N. M. P. S. H. S. S. Bateson P, "Developmental plasticity and human health," *Nature,* p. 430:419–21, 2004.

[11] H. M. Gluckman PD, " Living with the past: evolution, development, and patterns of disease," *Science ,* p. 305:1733–6, 2004.

[12] B. Hocher, "The advanced fetal programming hypothesis," pp. 16:1298-1299, 2001.

[13] S. E. Ozane, "Metabolic programming in animals, type 2 diabetes," *Br Med Bull ,* pp. 143-152, 2001.

[14] B. D. Osmond C, "Fetal, Infant, and Childhood Growth Are Predictors of Coronary Heart Disease, Diabetes, and Hypertension in Adult Men and Women," *Environ Health Perspect,* pp. 108:545-553, 2000.

[15] S. P. D. M. C. R. D. H. C. N. l. R. A. G. J. P. G. T. C. a. B. R. A. Burns, " Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation.," *J. Clin. Invest.,* vol. 100, pp. 1768-1774, 1997.

[16] M. B. J. a. B. J. Zimanyi, "Nephron Number in the Offspring of Rats Fed a Low Protein Diet During Pregnancy," *Image Anal Sterol ,* vol. 19, pp. 219-222., 2000.

[17] N. a. E. H. Anzai, "Drug transport in the kidney," in *Drug transporters Molecular*

characterization and role in drug disposition, ed. , New Jersey, John Wiley & sons, Inc., 2007, pp. 463-493.

[18] I. T. J.-I. N. K. S. A. O. M. S. Y. S. a. A. T. Hikaru Yabuuchi, " Novel Membrane Transporter OCTN1 Mediates Multispecific, Bidirectional, and pH-Dependent Transport of Organic Cations," *JPET - Journal of Pharmacology and Experimental Therapeutics,* vol. 289, pp. 768-773, 1999.

[19] W. SH., "Role of organic cation transporters in the renal handling of therapeutic agents and xenobiotics," *Toxicol Appl Pharmacol ,* pp. 204: 309-319, 2005.

[20] C. N. H. D. L. T. a. K. C. Slitt AL, "Tissue distribution and renal developmental changes in rat organic cation transporter mRNA levels," *Drug Metab Dispos ,* pp. 30: 212-219, 2002.

[21] G. V. W. X. H. J. S. P. Y. P. F. T. H. N. Tang N, "(1999) Mutation of OCTN2, an organic cation/carnitine transporter, in primary carnitine deficiency," *Hum Mol Genet ,* p. 655–660, 1999.

[22] M. T. H. J. C. I. P. H. S. E. Pietig G, "(2001) Properties and regulation of organic cation transport in freshly isolated human proximal tubules," *J Biol Chem ,* p. 33741–33746, 2001.

[23] M. T. M. R. A. S. O. H. a. M. Y. Otsuka M, "A human transporter protein that mediates the final excretion step for toxic organic cations," *Proc Natl Acad Sci USA ,* p. 17923–17928, 2005.

[24] E. K. Z. X. C. N. S. D. a. D. W. Wright SH, "Functional map of TEA transport activity in isolated rabbit renal proximal tubules," *Am J Physiol,* pp. F442-F451, 2004.

[25] Y. J. G. V. L. N. .. Wang Y, "(1999) Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency," *Proc Natl Acad Sci USA ,* p. 2356–2360, 1999.

[26] N. H. N. Y. S. K. K. M. Lu K, "(1998) A missense mutation of mouse OCTN2, a sodium-dependent carnitine cotransporter, in the juvenile visceral steatosis mouse," *Biochem Biophys Res Commun ,* p. 590–594, 1998.

[27] I. T. J.-i. N. H. N. N. H. A. O. Y. S. M. S. a. A. T. Rikiya Ohashi, "Molecular and Physiological Evidence for Multifunctionality of Carnitine/Organic Cation Transporter OCTN2," *Molecular Pharmacology,* vol. 59, pp. 358-366, 2001.

[28] O. M. M. S. S. H. a. I. K. Urakami Y, " Functional characteristics and membrane localization of rat multispecific organic cation transporters, OCT1 and OCT2, mediating tubular secretion of cationic drugs," *J Pharmacol Exp Ther ,* p. 800–805, 1998.

[29] M. K. a. A. ND., "Cloning of the mouse organic cation transporter 2 gene, Slc22a2, from an enhancer-trap transgene integration locus," *Mamm Genome,* p. 218–224, 1999.

[30] S. B. a. D. A. Cherala G, "Two low protein diets differentially affect food consumption and reproductive performance in pregnant and lactating rats and long-term growth in their offspring," *J Nutr,* p. 2827–2833, 2006.

[31] B. Hagenbuch, Drug Uptake Systems in Liver and Kidney: A Historic Perspective,

2009.

[32] J. K. F. M.-W. V. G. C. V. D. L.-C. B. K. S. V. D. L.-C. B. K. S. B. a. H. K. Ulrich Karbach, "Localization of organic cation transporters OCT1 and OCT2 in rat kidney," *Am J Physiol Renal Physiol,* pp. 679-687, 2000.

[33] M. O. H. S. K.-i. I. Yumiko Urakami, "Hormonal regulation of organic cation transporter OCT2 expression in rat kidney," *FEBS Letters,* vol. 473, no. 2, pp. 173-176, 2000.

[34] J. C. L. a. B. T. H. Carmen M. Rodriguez, "Organic Cation/Carnitine Transporter, OCTN2, Is Differentially Expressed in the Adult Rat Epididymis," *Biology of Reproduction,* pp. 314-319, 2002.

[35] M. O. H. S. K.-i. I. Yumiko Urakami, "Hormonal regulation of organic cation transporter OCT2 expression in rat kidney," *FEBS Letters,* vol. 473, no. 2, pp. 173-176, 2000.

[36] R. L. J. &. M. K. Skinner, "Environmental epigenomics and disease susceptibility," *Nature Reviews Genetics ,* pp. 253-262, 2007.

[37] M. M. C. G.-B. Michael K. Skinner, "Epigenetic transgenerational actions of environmental factors in disease etiology," *Trends Endocrinol Metab,* pp. 214-222, 2010.

[38] J. R. Waterland RA, "Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases.," *Nutrition,* pp. 63-68, 2004.

[39] M. A. H. C. C. a. K. L. T. Peter D. Gluckman, "Review: Effect of In Utero and Early-Life Conditions on Adult Health and Disease," *N Engl J Med,* pp. 61-73, 2008.

[40] E. Li, "Chromatin modification and epigenetic reprogramming in mammalian development," *Nat Rev Genet,* pp. 662-673, 2002.

[41] K. R. M. S. C. C. Wolf GL, "Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice," *FASEB J,* p. 949–957, 1998.

[42] A. V. M.-S. G. C. B. J.-P. R. J. F.-N. J.-P. J. C. J. Catherine Gallou-Kabani, "Resistance to high-fat diet in the female progeny of obese mice fed a control diet during the periconceptual, gestation, and lactation periods," *AJP - Endo,* pp. 1095-1100, 2007.

[43] D. M. S. T. K. M. H. V. G. J. Howie, "Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet," *The Journal of Physiology, ,* pp. 905-915, 2009.

[44] C. A. U. M. S. M. Anway MD, "Epigenetic transgenerational actions of endocrine disruptors and male fertility," *Science,* pp. 1466-1469, 2005.

[45] Q. L. S. J. J. S. L. K. S. M. S. J. X. Y. L. W. C. W. C. G. G. G. M. C. K. H. A. R. A. M. R. H. L. X. Ke, "Uteroplacental insufficiency affects epigenetic determinants of chromatin structure in brains of neonatal and juvenile IUGR rats," *Physiol. Genomics ,* pp. 16-28, 2006.

[46] W. B. S.-D. A. H. A. L.-C. B. H. G. Xiao Y, "Age and gender affect DNMT3a and DNMT3b expression in human liver.," *Cell Biol Toxicol.,* pp. 265-272, 2008.

[47] X. Y. e. al., "Tissue-specific expression and regulation of sexually dimorphic genes

in mice," *Genome Res.,* pp. 995-1004, 2006.

[48] D. J. W. Valerie Wauthier, "Sex-Specific Early Growth Hormone Response Genes in Rat Liver," *Molecular Endocrinology,* pp. 1962-1974, 2008.

[49] e. a. Z. Hochberg, "Child Health, Developmental Plasticity, and Epigenetic Programming," *Endocrine Reviews ,* pp. 159-224, 2011.

[50] R. S. B. Clark, H. Bayir and L. W. Jenkins, "Posttranslational protein modifications," *Crit Care Med,* vol. 33, no. 12, 2005.

[51] B. A. Jaenisch R, " Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals," *Nature Genetics ,* p. 245–54., 2003.

[52] N. a. E. H. Anzai, " Renal drug transporters and nephrotoxicity," pp. 447-452, 2007 .

[53] N. e. a. Kimura, "Metformin is a Superior Substrate for Renal Organic Cation Transporter OCT2 rather than Hepatic OCT1," 2005.

[54] L. T. L. D. P. H. K. H. P. H. H. J. J. U. Z. J. a. S. E. Ciarimboli G, "Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2.," *Am J Pathol ,* vol. 167, p. 1477–1484, 2005.

[55] O. M. M. S. A. M. S. H. a. I. K. Urakami Y, "Distinct characteristics of organic cation transporters, OCT1 and OCT2, in the basolateral membrane of renal tubules," *Pharm Res ,* p. 1528–1534, 2001..

[56] W. S. C. V. B. O. Dantzler WH, " Basolateral tetraethylammonium transport in intact tubules: specificity and trans-stimulation," *Am J Physiol,* p. F386–F392, 1991.

[57] J. R. W. R. L. J. Dana C. Dolinoy, "Epigenetic gene regulation: Linking early developmental environment to adult disease," *Reproductive Toxicology,* vol. 23, no. 3, pp. 297-307, 2007.

[58] R. R. P.-B. E. J. W. N. a. H. J. J. Newbold, "Effects of endocrine disruptors on obesity," *International Journal of Andrology,* p. 201–208, 2008.