IDENTIFICATION OF SIGNIFICANTLY REGULATED GENES IN THE ESTROGEN INDUCED *GALLUS GALLUS* LIVER OVER A 24-HOUR TIME COURSE

A Thesis

by

ERICA RENEE TROJACEK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Nutrition

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Approved by:

Chair of Committee,	Rosemary Walzem
Committee Members,	Alice Villalobos
	Huaijun Zhou
Intercollegiate Faculty Chair,	Steven Smith

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ABSTRACT

Identification of Significantly Regulated Genes in the Estrogen Induced *Gallus gallus* Liver Over a 24-Hour Time Course. (December 2011)

Erica Renee Trojacek, B.S.; B.S., Texas A&M University

Chair of Advisory Committee: Dr. Rosemary Walzem

In birds, estrogen is a strong stimulator of gene programs that regulate the formation of very low density lipoproteins (VLDL). Apolipoprotein-B (ApoB) is an integral part of very low density lipoproteins. In mammals, the rate of ApoB synthesis is controlled by post-translational means. In contrast, estrogen treated birds show changes in ApoB transcript level; in a natural setting, the bird's metabolism and transcription are in great flux due to yolk formation. Besides the ApoB gene, the entire complement of genes that is necessary to form a VLDL is not known. To determine the genes that play a role in the formation of VLDL 7-10d old chicks were injected with estrogen at several time points over a 24hr period. Following exsanguinations by cardiac puncture, livers were removed and RNA was extracted. The RNA was quantified and hybridized to microarrays using a dual-dye system. Slides were scanned and analyzed, and features were extracted. To qualify microarray results, quantitative real time PCR (q-RTPCR) was done on a selection of genes.

Previous studies had shown that approximately 200 genes are upregulated by the treatment of hormone naïve chickens with estrogen. As a result of our liver transcriptional profiling, we identified 1,528 genes at 1.5hrs, 1,931 genes at 3hrs, 2,398 genes at 6hrs, 2,356 at 12hrs, and 1,713 genes at 24hrs following estrogen exposure. We determined that these regulated genes include those responsible for the transcription of RNA used to create the gene products that serve as components of VLDL itself or that act in VLDL assembly. These include genes encoding structural proteins, like ApoB, and genes encoding assembly-related proteins. Of the differentially expressed genes as compared to time 0, there were approximately 30% which were unannotated with regards to function limiting conclusions. We hope to determine the function of these genes and to annotate them based on this information.

DEDICATION

This thesis is dedicated to my family and Ryan for their constant love, support, and encouragement.

ACKNOWLEDGEMENTS

First, I'd like to thank my family for their constant love and support. Thank you to Ryan for your encouragement, love, and belief in me.

Thanks go to Dr. Walzem for her patience, guidance, and knowledge along this journey. Thank you to my committee, Dr. Alice Villalobos and Dr. Huaijun Zhou, for their expertise and support.

I'd like to thank the TAMU Poultry Science Department for all of the help I received with raising birds, using equipment in the lab and at the farm, and doing experiments. This was greatly appreciated.

NOMENCLATURE

ApoB	Apolipoprotein B
$ApoB_{100}$	Apolipoprotein B_{100}
FDR	False discovery rate
MTP	Microsomal triacylglyceride transfer protein
nt	Nucleotide
q-value	Adjusted p-values found using an optimized FDR approach
RER	Rough endoplasmic reticulum
SER	Smooth endoplasmic reticulum
TAG	Triacylglycerol
VLDL	Very low density lipoprotein
VLDLy	Yolk-targeted very low density lipoprotein

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Lipoprotein Particle

Lipoproteins are macromolecular structures that are composed of phospholipid, cholesterol, cholesteryl esters (CE), triacylglycerol (TAG), and apolipoprotein; of these components, cholesterol, apolipoproteins, and phospholipids are on the surface and TAGs and CE are located in the core. Different lipoprotein classes contain unique apolipoproteins on the surface which serve as receptor ligands, enzyme cofactors, and lipid transfer carriers. In addition, the classes differ in diameter, density, and lipid fractions.

Specifically, very low density lipoproteins (VLDLs) are a class of lipoproteins that contain triacylglyceride (TAG), phospoholipid, cholesterol, CE and the ApoB protein [1]. The source of the phospholipids, CE and cholesterol that make up a VLDL is variable, and can either be from *de novo* synthesis or preexisting pools [2]. Immediate precursors to TAGs destined for VLDL inclusion are hepatic fatty acids derived from *de novo* synthesis from acetyl CoA or from uptake of free fatty acids from the circulation [3]. The mass distribution of this macromolecular structure is 43.7-60.1% TAG, 11-14.2% cholesteryl esters, 5.1-8.4% free cholesterol, 19.7-22.6% phospholipids, and 4.1-11.1% protein [4].

This thesis follows the style of *BMC Genomics*.

The apolipoproteins associated with lipoproteins contribute to the lipoprotein's structure and stability. There is a difference in molecular weight and the theoretical isoelectric point (pI) between the apolipoproteins (Table 1). The primary apolipoprotein of TAG rich lipoproteins (VLDL, intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and portomicrons in the chicken) is ApoB. Each VLDL contains one $ApoB_{100}$ molecule [5] and is 250-800 Å in diameter [6]. In addition to $ApoB_{100}$, VLDLy in chickens contain one other apolipoprotein, very low density lipoprotein II (ApoVLDL-II). In humans, VLDL can contain $ApoB_{100}$, ApoE, and isoforms of apoC [7].

Apolipoproteins have two roles in the body: structure and signaling. Through these two functions, apolipoproteins provide the means for the regulated transport and metabolism of nutrient cargo that they convey. Very low density lipoproteins, IDLs, LDLs, high density lipoproteins (HDL), and portomicrons in chickens are essential for normal growth and development by acting as the structures that allow dietary and *de novo* synthesized lipids to be shuttled through the blood to tissues for use as fuel or for storage [8-10]. In addition, these macromolecules transport essential fatty acids, fat soluble vitamins and hormones, pigments, phytosterols, hydrophobic bile acids, signaling lipids, and other elements. The other necessary function of apolipoproteins is signaling; the signaling lipids transported by lipoproteins include sphingomyelin, ceramides, lysophosphatidylcholine, and diacylglycerides.

Overall, lipoproteins are remarkable macromolecular structures that have unique characteristics for each of the density classes. They perform vital roles in the body and

Table 1: Apolipoprotein characteristics. Approximate molecular weight (MW) and theoretical isoelectric points (pI) of various apolipoproteins found in avian species. Calculations of theoretical pI were done with ExPASy. [11]

Apolipoprotein	Approximate MW (kDa)	Theoretical pI
Apo B	550	6.59
Apo A-I	28	5.26
Apo C-I	6.6	7.93
ApoVLDL II	9.3	9.21

contain unique proteins to help carry out these roles. The focus of this study is the identification of genes involved in the hepatic assembly of VLDL, which is the precursor particle to LDL, an atherogenic lipoprotein.

Lipoprotein Particle Formation

Lipoproteins can form intracellularly or extracellularly. Hepatic VLDL synthesis constitutes a highly specialized form of post-translational protein modification of $ApoB_{100}$ referred to as ApoB-LP assembly. This assembly process occurs intracellularly and engages several subcellular compartments. The complete assembly process and intracellular locations of ApoB-LP assembly are unknown and continue to be studied. However, a general outline of events broken into broad subprocesses with many individual steps creating two intermediate particles, is relatively well agreed upon (Figure 1). The first of the two intermediate particles is the small, $ApoB_{100}$ containing particle found in the rough endoplasmic reticulum (RER), and the second intermediate particle is the ApoB-less, large lipid filled particle formed within the smooth endoplasmic reticulum (SER). In step 1, translation and simultaneous folding to form the tertiary structure of ApoB occurs. This folding creates a lipid binding cavity that MTP, in step two, begins to deposit small amounts of TAG into. Steps 3 and 4 go into more detail of the vital role MTP plays in transferring lipid to the first step particle. Step 5 depicts the completion of the first step particle and its liberation from the ribosome down the secretory pathway towards the SER. The large, protein free second step particle then meets the first step particle in step 6. The events leading to the formation of the large, ApoB-less particle are largely unknown. The sequence of events leading to

assembly of TAG-rich ApoB-LP has been defined using a variety of techniques including *in situ* antibody mapping to track the subcellular movement of ApoB, pulse-chase experiments with radioactive precursors of both lipids and proteins, and measurement of pertinent enzymatic activities in subcellular fractions [12].

Alexander, Hamilton, and Havel in the late 1970's used electron microscope imaging of immuno gold particles to locate significant sub-cellular sites involved in ApoB-LP assembly; two broad steps were seen to encompass the assembly of an ApoB-LP. The trio found that ApoB is synthesized within the RER [13] as are CE [14]. The TAG-rich ApoB-less particle originates in the SER where TAGs and cholesterol are synthesized [6]. The ER is a true membrane, and organized as a lipid bilayer. Orientation of the hydrophobic elements (fatty acyl chains of phospholipids and rings and side chains of cholesterol) of the lipid bilayer perpendicular to the surface of the membrane creates a hydrophobic region suitable for TAG and CE accumulation [12]. The ability of membranes to bud off enables the vesicular mode of protein transport through the ER [15]. The actual initiator or nucleation event leading to VLDL assembly is unknown. It is a point of contention whether VLDL assembly is most closely linked to the synthesis of TAG or CE [16-18]. Most likely, TAGs are at least equal to CE in their ability to initiate VLDL assembly in the laying hen [12].

The first step ApoB-lipid complex converges with the ApoB-free TAG-rich core second step particle formed within the SER [12]. Chaperone proteins are likely involved in this convergence. Studies in cultured cells found the chaperone proteins glucose

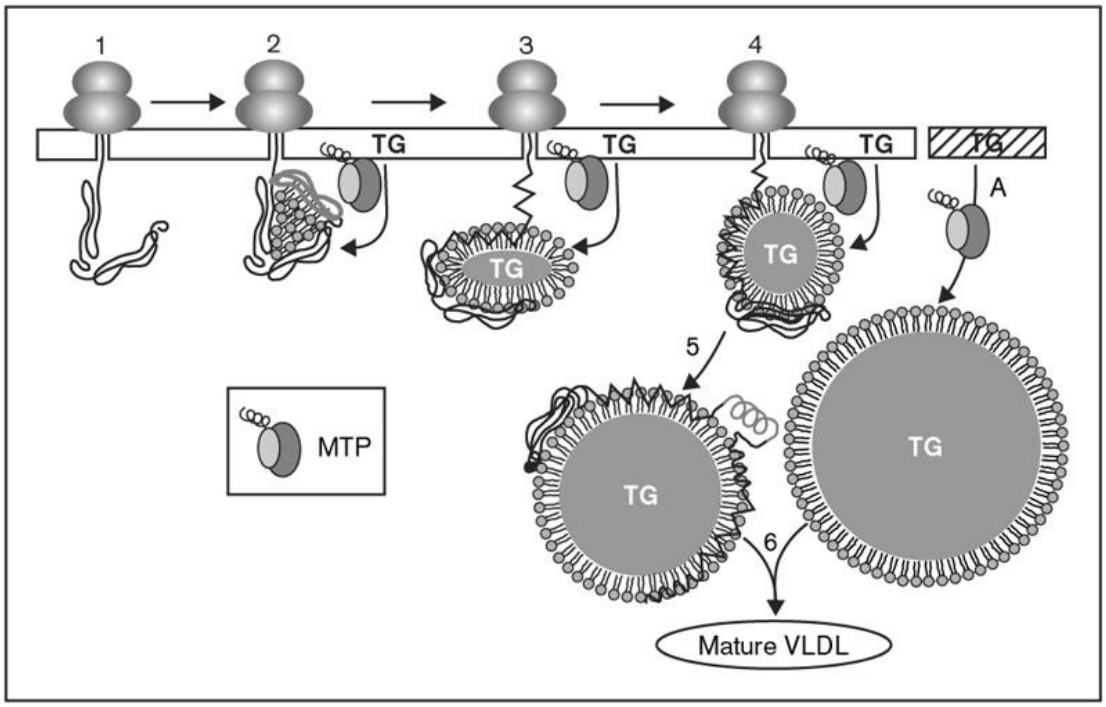

Figure 1. General outline of the two step model of VLDL assembly. Lipid is added to ApoB co-translationally, mediated by MTP. After release from the ribosome, the first step particle travels to the junction of the SER. It can then fuse with the large TG containing second step particle forming mature VLDL. [19]

regulated protein 94 kDa, glucose regulated protein 78 kDa, calreticulin, and cyclophilin B active in ApoB folding [20]. In addition to chaperone proteins, intracellular lipid transporters and nuclear factors are expected to be involved in ApoB-LP assembly, such as hepatic nuclear factor-α (HNFα) and sterol regulatory element-binding protein cleavage-activating protein (SCAP) [21, 22]. $ApoB_{100}$ translocation across the ER membrane creates a steady-state pool of membrane-associated ApoB [1]. A major determinant of the $ApoB_{100}$ translocation is the microsomal TAG transfer protein (MTP), an ER luminal protein with lipid transfer activity [1]. It is thought that the MTP protein is located at the site of ApoB translocation and facilitates regulated transfer of lipids and folding of ApoB as it exits the ribosome and enters the ER lumen [23]. It is expected that MTP gene expression will increase following estrogen treatment: this theory is directly testable.

Specialized tubules transport the particle, now a nascent lipoprotein, to the Golgi apparatus where concentration occurs in secretory vesicles [6]. These secretory vesicles transfer to the sinusoidal surface where the particles are secreted into the space of Disse by fusion of the vesicular membrane with the plasma membrane of the hepatocytes [6]. In both male and immature female birds, dietary and liver-synthesized fats leave the liver as TAG-rich VLDL for metabolism by extrahepatic tissues [12].

Clearly the assembly of a VLDL is a complex, multi-variable process. The exact details of this process are not wholly known. A candidate gene method was utilized as our strategy to identify candidates in a first-pass approach to assess gene involvement in assembly. Candidate genes were selected according to reports in the

peer-reviewed literature that, when the gene in question was absent or altered VLDL assembly failed or was otherwise compromised, the gene was shown or speculated to be involved in assembly of an apoB-LP or to be a structural part of an apoB-LP, the gene was shown or speculated to be estrogen responsive, or the gene was responsible for a protein involved in making lipids or transporting lipids. This list of candidate genes is shown in Table 2.

VLDLy

VLDLy is a remarkable lipoprotein species that is unique to oviparous species and has a complex assembly process. VLDLy depend on estrogen exposure for specific assembly. VLDLy contain two lipoproteins, $ApoB_{100}$, which is regulated transcriptionally in birds: a unique trait as compared to mammals, and ApoVLDL-II [1].

In laying hens, VLDLy are synthesized to deliver all TAG contained in the yolk-follicles during egg production [12]. Enrichment in TAG and smaller diameter (30nm vs 60nm) than VLDL allows for this specialized yolk deposition [24]. It has been proposed that the small diameter of VLDLy avoided the probable size exclusion properties of the granulosa basal lamina of the ovarian follicle [25]. In actively laying hens, the uniform diameter of 30 nm diameter is indicative of VLDLy synthesis and secretion [12].
In a natural egg-laying cycle, estrogen and progesterone produced by the ovarian follicles initiate and control VLDLy synthesis and egg production [12].

Table 2: Candidate genes grouped by differential expression as compared to time 0.

Genes With Significant Change	Genes Without Significant Change
Fatty Acid Synthase (FAS)	Sterol Regulatory Element Binding Protein Cleavage-Activating Protein (SCAP)
Diacylglycerol O-Acyltransferase 2 (DGAT2)	Sterol Regulatory Element Binding Protein (SREBP)
Glycerol-3-phosphate acyltransferase-2 (AGPAT-2)	ATP citrate lyase (ACLY)
Glycerol-3-phosphate acyltransferase-3 (AGPAT-3)	Acetyl-CoA Carboxylase (ACC)
Thyroid hormone responsive spot 14 (THRSP)	Stearoyl CoA desaturase (D9D)
Ferritin repressor protein (aconitase 1)	Fatty acid elongase (ELOVL)
Cytochrome P450, family 51 (CYP51)	6-phosphogluconate dehydrogenase (PGD)
Squalene synthase (FDFT1)	Protein Kinase, AMP-activated, beta 2 (PRKAB2)
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (HMGCS1)	Hepatic Nuclear Factor alpha (HNFα)
Microsomal Triglyceride Transfer Protein (MTP)	Vitamin D3 receptor (VDR)
Heat Shock Protein 70 (HSP-70)	Calreticulin (CALR)
Heat Shock Protein 90 kDa beta (GRP94)	Acetyl-CoA Acetyltransferase 1 (ACAT1)
Heat Shock 70kDa Protein 5 (HSPA5)	Acetyl-CoA Acetyltransferase 2 (ACAT2)
Heat Shock Protein Cognate Beta (HSPCB)	Carboxylesterase B (CES2)
Apolipoprotein B (ApoB)	ADP-ribosylation factor 1 binding protein (ARF1BP)
Riboflavin Binding Protein (RBP)	3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)
Dolichyl Pyrophosphate Phosphatase-1 (DOLPP1)	Farnesyl Diphosphate Synthase (FDPS)
Phosphatidylethanolamine N-methyltransferase (PEMT)	Lipin 1 (LPIN1)
Choline Kinase (CHK)	Peroxisomal 2,4-dienoyl-CoA Reductase (DECR2)
Very low density lipoprotein II (ApoVLDL-II)	acyl-CoA synthetase long-chain family member 6 (ACSL6)

Table 2 Continued

Genes With Significant Change	Genes Without Significant Change
Vitellogenin I (VTGI)	Aldo-keto reductase family 1, member B1 (AKR1B1)
Liver Fatty Acid Binding Protein (FABP)	Phosphatase and actin regulator 3 (PHACTR3)
Lipoprotein Lipase (LPL)	SAM domain, SH3 domain and nuclear localisation signals protein 1 (SAMSN1)
VLDL receptor (VLDLR)	Ribosomal Protein S9 (RPS9)
Cell death-inducing DFFA-like effector a (CIDEA)	RNA polymerase III (DNA directed) polypeptide F, 39 kDa (POLR3F)
Diazepam Binding Inhibitor (DBI)	peptidylprolyl isomerase B (cyclophilin B) (PPIB)
Malic enzyme 3 (ME3)	Mitogen Activated Protein Kinase 12 (MAPK12)
Carboxy-terminal domain, RNA polymerase II, polypeptide A phosphatase, subunit 1 (CTDP1)	Apolipoprotein E receptor 2 (LRP8)
Forkhead Box A2 (FOX A2)	Delta-6 fatty acyl desaturase (D6D)
Insulin Induced Gene 1 (INSIG 1)	Delta-5 fatty acyl desaturase (D5D)
Fas associated factor family member 2 (Ubxd8)	

Estrogen changes the assembly of these unique VLDLy particles and also influences the transcription and stabilization of mRNA of specific gene transcripts to achieve desired effects. The net consequence of estrogen induced changes in hepatic lipid and protein metabolism in birds is the production of small VLDL with distinctive changes in chemical composition.

Estrogen appears to selectively alter gene expression to support massive increases in ApoB-LP assembly and secretion necessary to sustain yolk formation. Some genes necessary in yolk formation process are termed "estrogen dependent" while others are modulated by, but not dependent on, the hormone [26]. It has been shown in several studies that estrogen induces the synthesis of the egg specific proteins ApoVLDL-II and vitellogenin (VTG) [26-30]. Specific induction profiles for increases in protein synthesis following estrogen treatment differ for ApoB, ApoVLDL-II and the VTGs [26, 28, 31-33]. A study by Kirchgessner in 1987 illustrated that administration of estrogen produced a six fold increase in ApoB mRNA in the liver [34]. In a study by Capony and Williams, in response to estrogen treatment, ApoB synthesis increased sharply after a lag period of 1.5hrs, reached a maximum at 15-24hrs, and then returned to the control level [35].

Janero and Lane showed in a pulse-chase study that VLDL particle assembly requires about 30 minutes [36]. Using estrogen induced avian parenchymal liver cells, they determined that phospholipids were secreted in a biphasic pattern, the first secretion wave occurred 5-15 minutes into the chase, and the second wave after 30 minutes.

Appearance of radio-labeled TAGs occurred 20-25 minutes into the chase, and apolipoproteins were secreted after 30-35 of chase time [36].

The yolk-specific proteins whose synthesis is induced by estrogen include VTG, ApoVLDL-II, and riboflavin binding protein (RBP); ApoVLDL-II is associated with VLDLy assembly, and all three are part of yolk assembly. The gene expression of these three genes can be used to judge the efficacy of exogenous estrogen treatment. The genes encoding ApoVLDL-II and VTGII exhibit estrogen-dependent nuclease hypersensitive sites, and an estrogen receptor-binding region has been identified on VTGII [34].

Following induction of the synthesis of yolk-specific proteins, hepatic VLDLy apoprotein composition changes [37-39]. Instead of containing the usual ApoB plus about five other apoproteins, VLDLy contains predominantly ApoB plus one other apoprotein, ApoVLDL-II [37-39]. Competitive exclusion of other apoproteins from the surface of VLDLy by ApoVLDL-II is believed to be responsible for the simplicity of VLDLy's apoprotein content [38]. ApoVLDL-II synthesis accounts for 11% of soluble protein synthesized in the liver following a single estrogen injection [40]. Estrogen acts to stabilize the ApoVLDL-II mRNA, while estrogen removal selectively destabilizes the transcript [26, 33]. Estrogen's presence specifically stabilizes the mRNA by inducing the formation of a poly-adenosine tail, thus increasing the mRNA's half life and leading to an accumulation of ApoVLDL-II mRNA [41]. This is why VLDLy, through the action of ApoVLDL-II, continues to be produced approximately 24hrs after a single dose of estrogen [42]. Estrogen also appears to stimulate the rate of transcription of the

ApoVLDL-II gene prior to stimulation of lipid synthesis and ApoVLDL-II mRNA increases 100-fold within 3 hours of treatment [39].

Estrogen induces ApoVLDL-II synthesis by directly inducing transcription of ApoVLDL-II mRNA [26]. ApoVLDL-II mRNA was shown to increase 12 fold after approximately fifty hours of estrogen exposure in rooster liver in a study done by Chan et al. [42]; VTG mRNA was shown to increase several thousand fold after 24hrs in a study done by Deeley et al. [43].

ApoVLDL-II contains two 82-amino acid residues linked by a single disulfide bond at residue 76 [44]. ApoVLDL-II is unique to avian species. ApoVLDL-II is found in the chicken as a homodimer, with each subunit being 9 kDa [45]. Conversely, in other avian species like the quail, ApoVLDL-II is found as a monomer. Both monomeric and dimeric forms inhibit LPL [46]. The typical metabolic course of TAG-rich VLDL in non-egg laying states ends in TAG hydrolysis by the enzyme LPL [47]. Hepatic VLDL assembly processes are retooled to form VLDLy in response to an estrogen dependent transcription program. These VLDLy are unique among TAG rich lipoproteins as they resist hydrolysis by LPL [12]. The presence of ApoVLDL-II in VLDLy is believed to mediate the reduction in this lipoprotein's diameter [12], possibly by competitively excluding other exchangeable apoproteins, such as ApoC-II, from the surface of VLDLy causing LPL resistance [38].

VTGs are ancient, very high density lipoproteins, responsible for lipid transport and protein storage that are not physically associated with VLDLy, but play a role in yolk formation [48]. Deeley showed that VTG is the only phosphoprotein present in the

plasma of the estrogen treated rooster [27]. There are three VTGs, one major (VTGII) and two minor (VTGI and VTGIII) protein isoforms. Avian VTG is composed of two polypeptides, each having a molecular weight of 240,000, and is actually a cryptic peptide that becomes the egg yolk phosphoproteins lipovitellin and two phosvitins, one with a molecular weight of 28,000 and the other 34,000 [27]. In the yolk, the products form granules, which also contain lipids and metals, including zinc, which were carried into the yolk with the VTG [49].

For oviparous species, vitamin delivery to the oocyte is a special challenge which must occur prior to egg fertilization and laying [50]. Avian RBP is a 29 kDa phosphoglycoprotein, which is a necessary component of chicken eggs; it supplies the oocyte and egg white with the riboflavin necessary to sustain embryonic development [50]. A study by Mac Lachlan showed that RBP associates with VTG in serum, "piggybacking" with VTG into the growing oocyte through the interaction of VTG with the 95 kDa VLDL/VTG receptor [50].

The vital apolipoprotein in VLDLy particles is $ApoB_{100}$, which is an amphipathic protein that is synthesized in the liver as a 4536-amino acid polypeptide [5]. It has five secondary structural domains and contains an oddly large number of cysteine residues in disulfide linkages which are believed to be essential for VLDL assembly [5]. $ApoB_{100}$ serves as the ligand for the ApoB receptor. The receptor binding domain of $ApoB_{100}$ includes a cluster of positively-charged residues at amino acids 3359-3367 [5].

$ApoB_{100}$ is a positive risk factor in the development of atherosclerosis in humans and it is present in the arterial wall in atherosclerotic lesions [2]. A study by Williams

showed that ApoB correlated significantly ($p<0.05$) inversely with HDL cholesterol, LDL size, and insulin sensitivity, and positively with body mass index, waist circumference, TG, DBP, systolic blood pressure, 2-hour and fasting glucose and insulin levels, CRP, fibrinogen, PAI-1, and both common and internal carotid artery intima-media thicknesses (IMTs); this makes ApoB concentration an important risk factor for developing heart disease [51].

The $ApoB_{100}$ protein remains on the ApoB-LP molecule from synthesis to degradation because $ApoB_{100}$ does not undergo exchange reactions [2]. It is insoluble, susceptible to degradation, oxidative cleavage, and aggregation [2]. The quality and amounts of VLDLy that are assembled and secreted are controlled at several points within the apoB assembly process [13, 52]. The first hypothetical sorting site is during translation and translocation of a growing ApoB peptide chain [12]. $ApoB_{100}$ that is not translocated to the lumen of the RER is then included into the outer leaflet of the ER where it is eventually degraded by intracellular proteases [12]. Sorting of $ApoB_{100}$ at this step controls the amount of $ApoB_{100}$ available for VLDL assembly [12]. The second sorting step follows translocation of nascent $ApoB_{100}$ and depends on neutral lipid availability for lipoprotein core formation [12]. The $ApoB_{100}$, at this second sorting site, must achieve sufficient TAG and cholesterol ester to gain a buoyant density of 1.0063 g/mL [53]. If this does not occur, then the particle is termed "misfolded" with regard to ApoB conformation; thus, it is sorted for degradation [52]. A final sorting step occurs immediately post secretion at the surface of the hepatocyte, where overly small VLDL are endocytosed by the LDL receptor [54].

A lipoprotein unique to egg-laying species, VLDLy has been shown to have a complex assembly process that produces a specialized particle that is vital to the growing bird. It is useful to experimentation that VLDLy depend on estrogen exposure for specific assembly. This fact allows for induced VLDLy assembly to occur within an experimental environment with application of exogenous estrogen.

Animal Model Selection

Of particular interest is the physiologic difference between egg laying organisms and mammals, whose offspring are of placental origin. It is possible to take advantage of the developmental strategy of birds to study very low density lipoproteins (VLDL). In humans, ApoB-LP can contain one of two isoforms of ApoB: $ApoB_{100}$ and $ApoB_{48}$; this is believed to be the same in rats and rabbits [55, 56]. Mouse and hamster models also contain both $ApoB_{100}$ and $ApoB_{48}$ [57]. $ApoB_{48}$ is a product of ApoB mRNA editing by the enzyme APOBEC-1, expressed in the human small intestine [55]. This editing changes a glutamine codon to a stop codon, thus creating a shorter transcript that encodes a protein that has a molecular weight approximately 48% of an apolipoprotein. Human liver does not edit ApoB because it does not express APOBEC-1 [55].

Avian species do not edit $ApoB_{100}$ mRNA as do mammals; their intestines, as well as livers, produce full-length $ApoB_{100}$ [1]. In contrast, isolated rat liver and rat hepatocytes in culture produce both $ApoB_{48}$ and $ApoB_{100}$, making them less useful for an $ApoB_{100}$ centered study [58]. Also, assembly processes may not be equivalent between rodent and avian species. The chicken, *Gallus gallus*, produces $ApoB_{100}$ in the kidney, intestine, and liver, as shown through in a study by Blue, where in vitro pulse labeled

tissues were measured in a double antibody procedure [59]. Only $ApoB_{100}$ synthesis in liver is increased by elevation of plasma estrogen, making the chicken a useful model organism for the selective study of $ApoB_{100}$.

Estrogen treatment of male birds mimics the effects of female sexual maturation and increases ApoB-LP assembly in male birds fourfold 16hrs after injection [60]. In one study, 60 mg of 17-β estradiol dissolved in 1.5 ml sodium benzoate was needed for maximal effectiveness in inducing hepatic synthesis of VLDL in 2.0-4.5 kg White Leghorn roosters [60]. Estrogen induction of male chicks is one of the techniques applied in this thesis.

In mammals, a constant supply of $ApoB_{100}$ supports VLDL assembly due to the constitutive expression of the gene [61, 62]. The fixed rate of gene expression in mammals demanded that non-transcriptional mechanisms be used to explain changes in $ApoB_{100}$ secretion rates with various treatments [12].

Genes Thought to be Involved in ApoB-LP Assembly

Genes shown to be critical for VLDLy formation in various knockout models are ApoB [63], MTP [20], and DGAT-2 [64], among others. ApoB is a gene that is responsible for the $ApoB_{100}$ protein; $ApoB_{100}$ is a necessary structural and functional part of VLDLy [1]. MTP is an ER luminal protein with lipid transfer activity [1]. It is thought that MTP facilitates regulated transfer of lipids and folding of ApoB as it exits the ribosome and enters the ER lumen [23]. Diacylgylcerol phosphate acyl transferase 2 (DGAT-2, E.C. 2.3.1) is an enzyme that catalyzes the acylation of diacylglycerol, which is necessary for the formation of TAG [65].

Two other genes expected to supply the fatty acids required for the massive demand for acylglycerol lipid components following estrogen mediated induction of VLDLy assembly and secretion were fatty acid synthase (FAS, E.C. 2.3.1.85) and acetyl Co-A carboxylase (ACC, E.C. 6.4.1.2). Fatty acid synthase was selected as it is a key enzyme in fatty acid synthesis, catalyzing the synthesis of long chain fatty acids through the condensation of acetyl-CoA and malonyl-CoA in a complex seven-step reaction [66]. Acetyl Co-A carboxylase is the rate limiting enzyme in fatty acid synthesis; it catalyses the carboxylation of acetyl-CoA to malonyl-CoA. Malonyl-CoA synthesized by ACC is a critical regulator of lipid metabolism, and plays roles in the regulation of oxidation and synthesis of fatty acids [67].

When cells are exposed to stressful conditions, including heat shock, a small number of highly conserved proteins, known as heat shock proteins (HSP), must be quickly expressed as part of an important survival response. Heat shock protein, particularly HSP-70, can repair proteins that unfold, misfold or aggregate upon stress, assisting in the recovery of the cell [68], and is believed to be a key chaperone in ApoB100 folding [69]. HSP-70 may play an important role in cell survival by interfering with apoptotic programs [70]. Overexpression of HSP-70 during heat shock may protect cells from stress-induced apoptosis preventing the activation of pro-caspases 9 and 3 [70]. VLDL assembly requires several cell process that work in a synchronized fashion. This puts stress on the cell and calls HSPs into play to elicit a survival response.

Malic enzyme (E.C. 1.1.1.40) catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2, simultaneously generating NADPH from NADP+ [71]. In the avian liver, most of the NADPH used by FAS to catalyze the synthesis of the fatty acid palmitate (16:0) is generated by malic enzyme [72].

Circadian Rhythms

Circadian rhythms are known patterns of biochemical, physiological, or behavioral processes that occur in a cyclic pattern. Circadian rhythms are endogenous to an organism and can be adjusted by external cues such as the environment. The primary environmental cue seen in nature is sunlight. It is known that genes related to lipid synthesis will vary in a circadian manner [73, 74]. Due to this fact, all animals used in this study were sacrificed at the same time of day (approximately 17:00) and at the same nutritional status to minimize circadian variations between the animals.

Much of circadian science is based on the concept of zeitgeber, meaning "time giver" in German. Zeitgeber is any exogenous cue that synchronizes an organism's endogenous clock to earth's light/dark cycles. Light is the strongest zeitgeber for both plants and animals.

There are two locations of organs and tissues, central and peripheral, which control circadian rhythms; these tissues are termed oscillators. In avian species, the central part of the circadian system is made up of the suprachiasmatic nucleus, retina, and the pineal gland [75]. Peripheral oscillators are present in tissues including the heart and liver [76]. These central and peripheral oscillators interconnect to synchronize phase relationships inside the organism to the external environment [77].

Transcriptional Profiling

Transcriptional profiling is a method to measure gene expression within a controlled setting, such as disease vs. non-disease state; it can be done by using various techniques that measure the levels of transcription in a tissue of interest. The most common method of transcriptional profiling is through microarray. Transcriptional profiling is useful because it can give an overall "snapshot" of what is occurring at the cell at one point in time. A limitation is that annotation of the profiled genes must be adequate for results and conclusions from transcriptional profiling to be made.

Prior to gene transcriptional profiling, messenger RNA (mRNA) that are gene transcripts must be extracted from tissue and its quality assessed. This extract will contain transcripts of both "genes of interest" that may have been identified by some prior knowledge, as well transcripts for which the experimentor has no prior expectations. Microarray studies are a powerful approach to discover new genes of interest. An estimate of the quality of the initial transcript collection extracted from the tissue typically involves both spectrophotometric and electrophoretic analyses.

Spectrophotometrically, mRNA's absorbance should be measured at 230 nm, 260 nm, and 280 nm. High quality mRNA extracts possess specific (optimal) ratios of optical absorbance at certain wavelengths, namely 260/280 and 260/230. Both ratios give information on the quality of the mRNA. The 260/280 value estimates the level of protein contamination in the mRNA sample; the ideal range of this value is 1.7-2.0. The 260/230 value estimates the level of organic contamination of the mRNA, and the desired range of this ratio is 2.0-2.3. Solutions of extracted mRNA that have optimal

values for these two ratios are assumed to be relatively free of protein and other organic contaminants and suitable for use in transcriptional profiling studies.

An additional method to judge the quality of the mRNA is the use of agarose gel electrophoresis. This is a method to separate nucleic acids by length. An electric field is applied to the gel to move the negatively charged nucleic acids through the gel. Shorter nucleic acids migrate through the agarose faster than longer nucleic acids due to the increased ease of transport of smaller particles through the pores of the gel. Intercalation of ethidium bromide between RNA bases provides a means of band identification. High quality RNA should have one bright, distinct band at 18S and another at 28S. Any samples in which bands were smeared or that did not have bright bands must be purified or re-extracted.

To validate outcomes of transcriptional profiling through microarray, a second, independent method to assess changes in gene expression should be applied to sample mRNA. Quantitative real time polymerase chain reaction (q-RTPCR) is one such independent method. In q-RTPCR, a so-called "housekeeping gene", that is, a gene that is expressed at the same level at all times in each sample are measured along with genes of interest. The genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12) are often used as housekeeping genes to normalize each gene of interest. Comparing the level of expression of the housekeeping gene to the level of expression of the gene of interest gives the level of expression of the gene of interest relative to a constant value. In this way, changes in gene expression can be estimated.

The recent development of microarray technology, along with specific arrays for various species including the chicken, has allowed for extensive analysis of genetic markers and pathways. A large collection of EST's enabled the creation of high density microarrays [78]. Tissue specific, as well as global, microarrays have been created for the chicken. These arrays include a 3,011 lymphocyte array [79], a 3,072 intestinal array [80], an 11K heart specific array [81], a 14,718 macrophage specific array [82], a 13K cDNA chicken genome array [83], a 5K immune related array [83, 84], a 20K long oligo chicken genome array [85], and a 33K Affymetrix chicken genome array [86].

There are several classes of microarray technology. Affymetrix was the pioneer in this field with the GeneChip platform. GeneChip technology is based on short, single-stranded DNA segments, or oligonucleotides, that are built to order by chemical synthesis [87]. High density arrays are constructed using light directed DNA synthesis, namely photolithography and solid-phase DNA synthesis [87]. The photolithographic technique uses a silicon substrate onto which photochemically removable protecting groups are attached [88]. Light is directed through a photolithographic mask to produce specific photodeprotection on the chip surface [88]. Specific deoxynucleosides are then incubated with the surface and chemical coupling to the surface occurs; using new masks the process is repeated until the chip of 25-mer probes is complete [88]. Advantages to the GeneChip are *in silico* design and probe redundancy, present because multiple independent oligonucleotides are designed to hybridize to different regions of the same RNA [88].

Agilent (Palo Alto, CA, US) uses inkjet printing through phosphoramidite ($(RO)_2PNR_2$) chemistry to carry out *in situ* synthesis of 60-mer oligonucleotide probes on or near the surface of the microarray slide [89]. In a study by Hughes, it was found that 60-mer arrays can reliably detect transcript ratios at one copy per cell in complex biological samples, and that these arrays are compatible with different sample amplification and labeling techniques [89]. An advantage to Agilent technology is that Agilent probes are 60-mer oligonucleotides, while GeneChip probes are only 25, making the Agilent probes more site-specific. In addition, the Agilent probes are able to reliably detect transcript ratios of one copy per cell in complex samples.

The Agilent 60-mer oligonucleotide 44K chicken array was developed in 2008 by a group at Texas A&M University [90]. The 44K array consists of 42,034 features, or individual spots on the array which hybridize to a specific oligonucleotide sequence, based on the whole chicken genome sequence plus 1264 positive control features and 153 negative control features [90].

The development of microarray technology has opened countless doors allowing whole-genome research. These highly specific slides also allow the researcher to create a specialized array, focus on a set of particular genes or encompassing an organism's genetic makeup. With this technology, transcriptional profiling is quickly and easily accomplished. The custom 44K Agilent array was utilized for the current study, as it is based upon the chicken's whole genome sequence.

Bioinformatic Analysis of Transcription Data

A series of basic steps are followed to process microarray derived data for transcriptional profiling: 1. Normalize the raw data outputs using technique-specific software → 2. Remove any background technique generated noise (a form of systematic error) → 3. Normalize the data to determine significance of individual data points → 4. Carry out specialized statistical methods to answer specific questions → 5. Use literature and online databases to determine the identity or homology of specific gene sequences if annotation is unavailable. Annotation is the process by which important pieces of information about a particular gene's raw gene sequence or gene product function are added to a database. This includes describing different sections of the raw gene sequence as protein-coding, nonfunctional, or regulatory, as well as providing links to publications that describe gene product function, regulation or similarity to other coding regions.

To determine if results are statistically meaningful, and the gene of interest is present, significance can be assessed by using the statistical formula and significance boundaries of the researcher's choice. In this particular study, transcription data was analyzed using Lowess normalization and a significance level of $p<0.05$ was chosen. Lowess normalization allows the researcher to correct for background signal, and determine that a gene is expressed at a level above or below that background signal. After statistically meaningful results are determined, the researcher is able to use the genes expressed differently from background signal to cluster genes and do further gene identification, annotation, analysis.

There are countless online databases that are useful tools for analyzing nucleotide sequence for similarity and identity, finding gene localization, and determining gene pathways, among others. NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) can identify similarities in gene sequences with great speed [91]. NCBI also has a feature termed Homologene which is an automated system for detecting homologs among the annotated genes of several completely sequenced eukaryotic genomes, including those of chickens, mice, and humans [91].

The Database for Annotation, Visualization and Integrated Discovery (DAVID) is a database used for large scale functional annotation of identified genes [92, 93]. It utilizes a gene list and gene background, both entered by the user. The program then uses available online resources, including the Gene Ontology project, to compare the "interesting" gene list to the background. According to DAVID, a "good" gene list contains many important markers, including containing a reasonable number of genes (100-2,000), and passing the statistical threshold for selection in the particular experiment being done [92, 93]. For example, in the estrogen induction experiment, the statistical threshold for inclusion of a particular gene's outcome in a DAVID analysis was a signal to noise ratio (SNR) greater than three at a minimum of one sampling point, and a significance of $p<0.05$ after Lowess normalization [94]. Importantly, in experiments where two fluorescent dyes (red and green) are used, intensity-dependent variation in dye bias may introduce spurious variations in the collected data. Lowess normalization merges two-color data, applying a smoothing adjustment that removes such variation [95]. This correction is "Step2" in transcriptional profiling data analysis

for this specific technique. After this process the transcripts for which sufficient statistical confidence exists to declare the gene "present" in the sample will have been identified. This dataset can be subjected to further statistical analysis to determine whether experimental treatments alter the relative amounts of each transcript that is present.

Gene Ontology (GO) is a collaborative effort to address the need for consistent descriptions of gene products in different databases [96]. Within GO, three structured, controlled "vocabularies" (ontologies) describe gene products in a species-independent manner [96]. The three ontologies are molecular function, cellular component, and biological process. Cellular component describes a component of the cell that is part of some larger object. Molecular function represents activities rather than the entities (molecules or complexes) that perform the actions. They do not specify where or when, or in what context, the action takes place. Biological process describes a series of events accomplished by one or more ordered assemblies of molecular functions [96]. These events must have more than one distinct step. Determining biological process data, including signal transduction and specific gene/protein interactions is a long term objective of this work.

GeneCards is an easy to use, searchable online database that integrates information from several online databases onto one page for each gene. The data presented on the GeneCards site is extracted or linked to over 80 external databases and digital sources [97]. The data for each available gene includes function, sequences, orthologs, variants, aliases, and pathways, among many other pieces of information.

In all, transcriptional analysis can give a rich dataset of significant values. Further statistical analysis can give interesting clusters and patterns of data, and are invaluable to a whole-genome array experiment. Online databases sponsored by the government, institutes of higher education, and non-profit groups lend insight to genes whose function or homology is unknown, further enriching the experiment outcomes.

Conclusion

In conclusion, the assembly of VLDL (and VLDLy) is a complex process that is incompletely understood. The chicken has frequently been used for studies of VLDL assembly since massive increases in plasma VLDL occur in response to exogenous estrogens in the rooster or with the onset of egg laying in the hen [60]. In avian species, the liver is the main site of *de novo* fatty acid synthesis, accounting for 95% in young chicks [98-100].

The current study hypothesized that estrogen induction will selectively alter hepatic gene expression to facilitate VLDLy assembly and secretion. An underlying assumption in this study was that the same processes used to assemble VLDL operate during VLDLy assembly, albeit perhaps modified to accommodate the inclusion of ApoVLDL-II into VLDLy structure. We further hypothesized that by measuring changes in hepatic gene expression in relation to time following estrogen exposure requisite changes in cellular processes to "retool" VLDL assembly for VLDLy assembly, including a general up regulation of all gene products required for ApoB-LP assembly, that all components of that assembly process might be identified.

Objectives of this study included: using the estrogen induced chick model to identify which genes are expressed before and at each of five time points following a maximally stimulating estrogen dose, characterizing identified genes using publically available databases, correlating changes in gene expression with phenotypic changes in plasma lipoproteins and selected tissue weights, determining whether anticipated changes in ApoB gene expression could be used to identify other genes associated with VLDLy assembly processes.

It is hoped that this project will lead to a better understanding of the assembly of VLDLy particles. This study is a first-pass analysis of the feasibility of using gene transcription assays to characterize the assembly of a VLDLy. Information gained on gene expression patterns and experimental techniques from this project will benefit future gene-centric projects focusing on particle assembly.

CHAPTER II

MATERIALS AND METHODS

Animal Subjects: Experiment 1

A total of twenty-four, 7-10 day old chicks were studied in the microarray survey. The chicks were maintained at the Texas A&M University Poultry Research and Teaching Facility in accordance with AUP #2009-245. The chicks were fed a nutritionally adequate non-purified grower ration (Table 3) in routine use at the TAMU Poultry Research and Teaching Facility.

Animal Subjects: Experiment 2

To compare a natural estrogen cycle to the artificially induced model employed in Experiment 1, twelve female Lohmann birds were studied during the pullet/layer transition. Four pre-production pullets (15wk 4d), four immediate pre-production hens (16wk 3d), and four egg-laying hens (26wk 1d) were used. The hens were maintained at the Texas A&M University Poultry Research and Teaching Facility. Pullets were provided the same non-purified grower ration (Table 3) until transfer to a nutritionally adequate layer ration at 16 weeks of age (Table 4). This feed is in routine use at the TAMU Poultry Research and Teaching Facility and is provided for ad-libitum consumption according to AUP#2009-245. The hens were not fasted prior to tissue sampling.

Table 3: Chick grower ration.	
Ingredient	
Corn (%)	64.21
Soybean Meal (%)	23.60
Limestone (%)	10.09
Biofos (%)	1.17
Salt (%)	0.46
Vitamins Mix (%)	0.25
DL-MET98 (%)	0.17
Trace Minerals Mix (%)	0.05
Calculated composition	
Protein (%)	2.319
Calcium (%)	-0.027
AV Phosphate (%)	0.829
TSAA (%)	2.544
Sodium (%)	-0.052
ME (kcal/kg)	2733.21

Table 4. Composition of the basal diet - pre lay.	
Ingredient	
Corn (%)	70.3
Soybean Meal (%)	22.7
Limestone (%)	5.29
Biofos (%)	1.02
Salt (%)	0.38
Vitamins Mix (%)	0.25
DL-MET98 (%)	0.05
Trace Minerals Mix (%)	
Calculated composition	2.974
Protein (%)	-0.037
Calcium (%)	0.818
AV Phosphate (%)	-0.0618
TSAA (%)	2909.22

Treatment and Sampling Protocol: Experiment 1

The experiment was conducted in a way that produced different lengths of exposure to exogenous estrogen, but tissue was collected at the same "clock hour" for all birds (Table 5). Four chicks were injected with 10 mg/kg diethylstilbesterol in extra light olive oil (Star, Borges USA, Fresno, CA) at the start of experimentation and at four time points following [101].

Injections occurred 12 hours, 18 hours, 21 hours, and 22.5 hours before liver harvest (Figure 2). Four chicks served as vehicle-injected controls and were killed at time zero. This scheme generated four chicks that were exposed to estrogen for each of the following lengths of time: 0, 1.5, 3, 6, 12, 24 hours. Dosing was timed so that all birds were killed at constant clock hour, specifically 17:30h.

This component of the experimental design aimed to remove any changes in gene expression due to circadian rhythms. Birds were anesthetized in a chamber filled with 4% isoflurane (IsoFlo, Abbott Laboratories, Abbott Park, Illinois) and 96% oxygen (Praxair, College Station, TX). Birds were checked to ensure full anesthetization had occurred by observing animal reaction to an abdominal skin pinch. After full anesthetization occurred, the bird was removed from the chamber and transferred to a prepared surgical table; a face mask was attached to the bird to provide continued sedation. Birds were exsanguinated by cardiac puncture; their livers were removed rapidly and immediately frozen on dry ice in between two pieces of foil. After freezing the livers were sealed between the foil pieces and placed into a -80°C freezer. Precaution was taken with the foil to avoid any contamination of the foil prior to use and

Table 5. Estrogen induction and sacrifice time course.

Length of Estrogen Exposure (hrs)	Time of Estrogen Injection	Time of Sacrifice
24.0	Day 1 17:30	Day 2 17:30
12.0	Day 2 5:30	Day 2 17:30
6.0	Day 2 11:30	Day 2 17:30
3.0	Day 2 14:30	Day 2 17:30
1.5	Day 2 16:00	Day 2 17:30
0.0	Day 2 17:30	Day 2 17:30

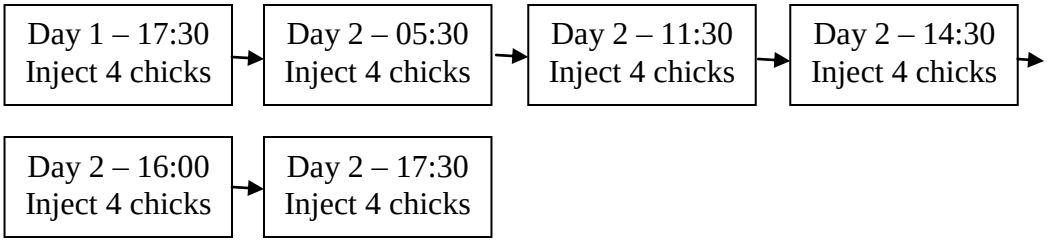

Figure 2. Estrogen induction flowchart.

to avoid the foil becoming frozen in folds of the livers during freezing.

Treatment and Sampling Protocol: Experiment 2

Hens received an electric current to the temple using a SKVS electric stunning knife (Pickwick-Knase, Eden Prairie, MN), and their throats were punctured. Blood was collected from the neck and stored on ice in 10 mL Vacutainer (Beckton Dickinson, Franklin Lakes, NJ) collection tubes. Livers were removed, weighed, and immediately frozen on dry ice as described for Experiment 1. The hen's body weight, liver weight, pelvic width at midpoint of the ischium, ovary weight, oviduct weight, ovarian status, and follicle presence and weight were recorded

Biochemical Measurements: Experiment 2

A portion of the processed plasma was sent to the Texas Veterinary Medical Diagnostic Laboratory on the campus of Texas A&M University for 17β-Estradiol quantification. Estrogens were extracted from plasma using 1.0 mL of dichloromethane and 0.5 mL plasma sample. After extraction, the sample was dried under a stream of nitrogen and rehydrated with phosphate buffered saline; 200 µl was used in duplicate for radioimmunoassay. The Siemens Estradiol Double Antibody Kit #KE2D1 (Siemens, Munich, Germany) was used for analysis. The sample was incubated with antibody supplied with the kit for 2hrs, and then incubated for 1hr with the tracer supplied with the kit. Finally, the antibody bound fraction was precipitated and counted using a Genesys 5000 gamma counter Model LTI505 (Laboratory Technologies, Inc., Maple Park, IL).

Lipoprotein Diameter Measurements: Experiment 2

Reduction in VLDL particle diameter is characteristic of VLDLy formation. In order to determine whether patterns of gene expression coincided with this physiological marker, VLDL diameters were determined in intact female chickens undergoing the pullet/layer transition. Venous blood samples were placed in a pre-chilled Sorvall RT-6000B (Thermo Fisher Scientific Inc., Asheville, NC) tabletop refrigerated centrifuge and spun for 30 minutes. The plasma was removed from the tubes and transferred to collection tubes containing EDTA. One milliliter of plasma was put into each UltraClear centrifuge tube catalog # 344624 (Beckman Coulter, Inc., Brea, CA). Five milliliters of d=1.0255 solution was added to total 6 mL per tube with a final density of 1.022 g/mL. The tubes were placed in a Beckman TFT 44.5 rotor (Beckman Coulter, Inc., Brea, CA) and subjected to ultracentrifugation at 14˚ C at a speed of 40,000 RPM for 18 hours with a maximum temperature of 25˚ C using a Beckman L8-70M Ultracentrifuge (Beckman Coulter, Inc., Brea, CA).

After centrifugation was complete, the diameters of lipoproteins recovered from the floating lipid layer were measured using a Nanotrac NPA 250 (Microtrac software version 10.5, Clearwater, FL). Each sample was analyzed in triplicate with a measurement time of 60 seconds for individual measurements, with the following parameters: particle refractive index=1.46, transparent/irregular particle, density=0.98, fluid refractive index=1.34, high temperature=26°C, viscosity=0.889, low temperature=20°C, fluid viscosity=1.002, analysis option=high sensitivity, perspective=number, progression=geometric 8 root.

Molecular Measurements: Experiment 1

RNA was extracted from all livers using the Ribopure kit using approximately 10-15 mg of tissue and according to standard kit protocol (Ambion, Inc., Foster City, CA). Tissues were homogenized using a VWR VDI 12 SI homogenizer (VWR International, Radnor, PA). The concentration (ng/uL), level of organic contamination (260/230 ratio) and level of protein contamination (260/280 ratio) of each sample was determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Samples with ratios outside of the desired range (1.7-2.0 for 260/280 and 2.0-2.3 for 260/230) were concentrated using Ambion's (Ambion, Inc., Foster City, CA) sodium acetate precipitation of small nucleic acids protocol by combining the total RNA with 0.1 vol 3.0 M sodium acetate (Ambion, Inc., Foster City, CA), 3 vol 100% ethanol (KOPTEK, King of Prussia, PA), and 3 vol of 70 µg glycogen, Lot #411291 (Illumina, Inc., San Diego, CA). Mixtures were then placed in a -80°C freezer for fifteen hours. The samples were centrifuged at 14,000 rpm at 4˚C for fifteen minutes. The supernatant was removed and 200 µL of 75% ethanol was added to the pellet. The pellet and 75% ethanol were centrifuged for five minutes at 8,000 rpm at 4˚C. The supernatant was carefully removed from the tube, and the pellet was allowed to air dry for five minutes in the same tube, on ice, with the tube opening parallel to the surface of the ice. The samples were then diluted to 800 ng RNA/µL in RNAse free water to a total volume of 40 µL. To do this, the sample concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). The formula (800 ng/µL)*(40µL)/(concentration in ng/µL) was used to determine the volume of RNA to

add to make 40 µL at a final concentration of 800 ng/µL. The amount of RNAse free water to add was determined by subtracting the volume of RNA stock solution added, using the above calculation, from 40 µL. The resulting samples were stored at -80˚C until used.

Additional confidence in the integrity of the RNA was sought through the use of denaturing agarose gel electrophoresis. 100 mL of 1% denaturing agarose gel was made by combining 1.0 g agarose (Bio-Rad, Hercules, CA) with 90 mL of RNase free water in an Erlenmeyer flask. The flask was heated in the microwave until the agarose was melted, and then placed in a 60°C water bath until flask temperature was equilibrated. 10 mL of NorthernMAX 10X running buffer (Ambion, Inc., Foster City, CA), and 1.5 µL of 10 mg/mL ethidium bromide (Promega, Madison, WI) were added to the flask and swirled to mix. The agarose solution was poured into an RNase free BioRad Sub-Cell GT System Horizontal Gel Electrophoresis System with a 15x15 cm gel tray size (Bio-Rad, Hercules, CA). After the gel hardened, 1X MOPS running buffer (Lonza, Basel, Switzerland) was added to cover the gel. A 1.0 µg aliquot of each sample was combined with 2.5X the sample volume of NorthernMAX formaldehyde load dye (Ambion, Inc., Foster City, CA) in a 0.2 µL tube. Prepared sample tubes were thoroughly mixed with a VortexGenie (Scientific Industries, Bohemia, New York) and incubated for 10 minutes at 65°C. Tubes were removed from the incubation and placed on ice for 5 minutes. Samples were loaded into each well and run at 140 volts for 25 minutes. Bands were visualized using a Kodak EDAS 290 Electrophoresis Documentation and Analysis System (Kodak, Rochester, NY). High quality RNA should have one bright, distinct

band at 18S and another at 28S. Intercalation of ethidium bromide between RNA bases provides a means of band identification. Any samples in which bands were smeared or that did not have bright bands were purified or re-extracted. All previously used techniques to determine concentration and purity were applied to these subsequent samples.

Highly concentrated RNA samples with clear bands on a denaturing gel and acceptable 260/280 and 260/230 ratios were labeled and hybridized to the 44K Agilent chicken microarray (Agilent Technologies, Inc., Santa Clara, CA). Sample labeling was accomplished using a dual-dye system, shown in Table 6.

The Agilent 44K chicken array is a two-color microarray where two different cDNA samples, one labeled with one color dye, and one labeled with another color, are hybridized to a single probe. Dual dye systems function to eliminate dye bias, as half the control samples on each slide were labeled with cyanine 3 (Cy3) and the other half of the control samples labeled with cyanine 5 (Cy5). The concentration and purity of the labeled samples was again measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) prior to hybridization to a *Gallus gallus* 44K Agilent array containing 43,803 probes [90]. RNA isolated from samples taken at time points 24, 12, 6, 3, and 1.5 was hybridized with one zero time point control sample. Under this scheme, four replicates of the entire time course were generated.

Table 6: Microarray dual dye scheme.

Slide #	Array #	Cy5 Labeled Sample #	Time Point (hr)	Cy3 Labeled Sample #	Time Point (hr)
1	1	6	1.5 h	2	0
	2	7	0	18	1.5 h
	3	34800	1.5 h	9	0
	4	34776	0	34783	1.5 h
2	1	11	3.0 h	2	0
	2	7	0	13	3.0 h
	3	34788	3.0 h	9	0
	4	34776	0	34789	3.0 h
3	1	5	6.0 h	2	0
	2	7	0	10	6.0 h
	3	34780	6.0 h	9	0
	4	34776	0	34790	6.0 h
4	1	3	12.0 h	2	0
	2	7	0	4	12.0 h
	3	34794	12.0 h	9	0
	4	34776	0	34796	12.0 h
5	1	8	24.0 h	2	0
	2	7	0	12	24.0 h
	3	34787	24.0 h	9	0
	4	34776	0	34778	24.0 h

The slides were incubated at 65°C for seventeen hours in Agilent's microarray hybridization chambers. After hybridization, arrays were washed according to the Agilent Two-Color Microarray-Based Gene Expression Analysis Protocol Version 5.7, Manual # G4140-90051 (Agilent Technologies, Inc., Santa Clara, CA). Labeling, hybridization, and washing procedures were all done following Agilent's Two-Color Microarray-Based Gene Expression Analysis Protocol Version 5.7, Manual # G4140-90051 (Agilent Technologies, Inc., Santa Clara, CA).

A 1.0 ug aliquot of total RNA was used to synthesize first-strand cDNA using the Thermoscript™ RT-PCR system (Invitrogen, Carlsbad, CA), and utilizing the random hexamers provided in the kit in a reaction volume of 20 μL. This reaction was done in a MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) with the following program: 94°C for 15 min, 30 cycles of 64°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec, and 64°C for 15 min. The resulting cDNA was used to amplify GAPDH primers (Sense-5'-GTGAGTCCACTGGTGTCTTCAC-3', Antisense-5'-CTTAGCACCACCCTTCAGATG-3'), using the program: 94°C for 15 min, 30 cycles of 64°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec, and 64°C for 15 min.

Agarose gel electrophoresis of the amplification products using a 2% agarose gel was done to check cDNA integrity. To make the gel, 150 mL of 1X tris/borate/EDTA buffer (TBE) was added to 3 g agarose (Bio-Rad, Hercules, CA). The flask was microwaved until the agarose was melted (approximately one minute on high power). Three microliters of 10 mg/mL ethidium bromide (Promega, Madison, WI) was added to the agarose solution after approximately one minute, and swirled to mix. The gel was

poured into a BioRad Sub-Cell GT System Horizontal Gel Electrophoresis System with a 15x15 cm gel tray size (Bio-Rad, Hercules, CA) and cooled until solid. The solidified gel was covered with 1X TBE. A 5 µL aliquot of amplification product and 1 µL of 6X blue gel loading dye (New England BioLabs, Ipswich, MA) were used in each well. The amplification products were separated following electrophoresis at 140 volts for 50 minutes. Bands were visualized using a Kodak EDAS 290 Electrophoresis Documentation and Analysis System (Kodak, Rochester, NY).

Seventeen genes that displayed changes in gene expression by microarray analysis were chosen for q-RTPCR, with gene choice based upon genes shown to be necessary for VLDL assembly found using the literature. Included in this list were both induced and repressed genes that either showed significant change from baseline expression levels or did not show a significant change in expression level . Genes whose expression level did not change significantly as judged by microarray analysis were amplified using two different time point samples in the q-RTPCR analysis. Quantitative real time PCR was done to determine if microarray outcomes were trustworthy and repeatable. A list of gene symbols, gene names, and primers employed for q-RTPCR are shown in Table 7.

Primers were designed using the Roche Universal Probe Library (Roche Applied Science, Indianapolis, IN). First, the gene's nucleotide sequence was found using the Ensembl online software (http://uswest.ensembl.org/index.html).

Table 7: Primer names and sequences used in q-RTPCR analysis.

Gene Name	GenBank Accession	Primers
HSP-70	NM_001006685	F: TCTGGGCACCACGTATTCTT
		R: GGCAATGATCTCCACTTTGC
Glycerol-3-phosphate acyltransferase	CR385377	F: AGTAAACTGTCGCCTTGCCTA
		R: CACCATTCCAGCAGCATTAC
THRSP	AY568628	F: AAGCTCTCACCCGGAGGTA
		R: CTCTGACCTTCACCGAGGTT
LPL	BU397801	F: AGAGTGAGAACATTCCTTTCACG
		R: GGAAGGAGAAGGTCTTGTTGG
Apolipoprotein B (early sequence)	M18421	F: TTCAGTCCAATCAGAGATTATGTCA
		R: CAGAAGGGTAGATAGTGGGATGTT
Apolipoprotein B (later sequence)	M18421	F: GCAGCTCAACGTTACAATTCC
		R: GCTGCCTACGGTCTTCTTTACA
Vitellogenin II	nm_001031276	F: CAGTAACTGTTGGCTTCCACTG
		R: TCTGTTTGTCAGTCAGGCTGTT
D9D	X60465	F: CCTGCGGATCTTCTTGACTATT
		R: GGGCCCACTCATAGATGTCA
ApoVLDL-II	M25774	F: ATGGTGCAATACAGGGCATT
		R: GTCAATGATGGACTTTGAGTGC
Cyp 7A	AY700578	F: TGGTTTCTTTTTGGGAGGAG
		R: TGGAAGGAACCCATTTTCAA
Ppar-α	AF163809	F: AATGGTCCAGGATCTGATGG
		R: GGCAAAATTAATGGATGAAGGA
Fatty Acid Synthase	J03860	F: TGAACTGTCCTCCAAGACTGG
		R: CTGGGCCTGTCTTCAACACT
MTP	BX934807	F: CCGAATGCAAGAAGTGTCCT
		R: AGGATGCATGTGTTCTTTGATG
Riboflavin Binding Protein	X74247	F: GAAGGGGACACCCACAAA
		R: TGTGAAGTTTGCATAGCAACAG
LXR	AJ851708	F: GAGCTACAACTCAATGATGCTGA
		R: GACTGGTCCTGCACATTCG
Phosphatidylethanolamine N-methyltransferase	AJ720523	F: CACAATTGCAGTGCTGTACGA
		R: GCTCCCTTCTGTTTCTGACG
Hepatic Nuclear Factor 4-α	AY700581	F: CAATGAATACGCCTGCTTGA
		R: GGATCGCTCAGTCCTTTGG

The sequence was input to the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) beginning with a > symbol and with introns denoted using []. The choice "Automatically Select an Intron Spanning Assay" was clicked. The first primer option was chosen. Primers were ordered from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, Iowa), with a scale of 25 nanomole DNA and with the standard desalting purification option chosen. The GAPDH primer pair sequences were obtained from the Dr. Michael J. Bailey lab, and were ordered in the same manner as listed above. All seventeen primers were included in analysis, as no primer dimers were visualized on the melting curve during q-RTPCR. Primer dimers appear on the q-RTPCR graph with two peaks instead of a single peak.

The primers were first amplified using cDNA. As described in the previous paragraph, the integrity of the products of the PCR amplification of the primers was determined by separation using electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining using a Kodak EDAS 290 Electrophoresis Documentation and Analysis System (Kodak, Rochester, NY). A 5 µL aliquot of amplification product and 1 µL of 6X blue gel loading dye (New England BioLabs, Ipswich, MA) were used in each well. The PCR reactions including both forward and reverse primers were performed in a 10 ul reaction volume using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and sample cDNA, on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). There was one "hot start" incubation stage (95°C for 10 min), one cycling stage (40 cycles of 95°C for 15 sec,

59°C for 30 sec, and 72°C for 30 sec), and one dissociation curve stage (95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec with a 2% ramp rate). A dissociation curve was utilized to distinguish between specific and non-specific amplicons. GAPDH was used as the internal control, or "housekeeping" gene. GAPDH is a commonly used reference gene whose product is necessary to a cell's maintenance and survival; it is a gene that is expressed in cells at a constant level, therefore it is used as a standard for molecular assays [102]. All samples were measured in duplicate qRTPCR determinations. Duplicates were averaged, and the single value used for further calculations and statistical analysis. The comparative Ct method was used to calculate the relative gene expression level across the tissues using the ABI PRISM 7700 Sequence Detection System, User Bulletin #2, part # 4303859 (The Perkin-Elmer Corporation, Waltham, MA).

The relative expression level of each gene in one tissue (ΔCt) was calculated by Ct target gene-Ct GAPDH; relative expression of each gene in two different tissues (ΔΔCt) was calculated by ΔCt A -ΔCt B (The Perkin-Elmer Corporation, Waltham, MA). The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by: $2^{-\Delta\Delta CT}$ (The Perkin-Elmer Corporation, Waltham, MA).

Molecular Measurements: Experiment 2

RNA was extracted from all livers using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The concentration, level of organic contamination (260/230 ratio) and level of protein contamination (260/280 ratio) of each sample was determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Samples with ratios

outside of the desired range (1.7-2.0 for 260/280 and 2.0-2.3 for 260/230) were re-purified by combining with 0.1 vol 3M sodium acetate, 3 vol 100% ethanol, and 3 vol glycogen and placed in a -80°C freezer for fifteen hours. The samples were centrifuged, supernatant removed, and the resulting pellets washed with 75% ethanol. Samples were then diluted to 800 µg RNA/µL in RNAse free water and replicate aliquots were stored frozen at -80°C until used.

A 1.0 ug aliquot of total RNA was used to synthesize first-strand cDNA using random hexamers and the Thermoscript™ RT-PCR system (Invitrogen, Carlsbad, CA) in a reaction volume of 20 µL. This reaction was done in a MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) with the following program: 94°C for 15 min, 30 cycles of 64°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec, and 64°C for 15 min. The resulting cDNA was used to amplify GAPDH primers (Sense-5'-GTGAGTCCACTGGTGTCTTCAC-3', Antisense-5'-CTTAGCACCACCCTTCAGATG-3') using the same time/temperature program.

Agarose gel electrophoresis of the amplification products using a 2% agarose gel was conducted as described previously to verify cDNA integrity. To make the gel, 150 mL of 1X tris/borate/EDTA buffer (TBE) was added to 3 g agarose (Bio-Rad, Hercules, CA). The flask was microwaved until the agarose was melted. Three microliters of 10 mg/mL ethidium bromide (Promega, Madison, WI) was added to the agarose solution after approximately 1 minute, and swirled to mix. The gel was poured into a BioRad Sub-Cell GT System Horizontal Gel Electrophoresis System with a 15x15 cm gel tray size (Bio-Rad, Hercules, CA) and allowed to solidify at room temperature. The gel was

covered with 1X TBE. A 5 µL aliquot of amplification product was combined with 1 µL of 6X blue gel loading dye (New England BioLabs, Ipswich, MA), resulting in a 1X concentration of blue gel loading dye in each well. The gel was run at 140 volts for 50 minutes. Ethidium bromide stained bands arising from PCR were visualized using a Kodak EDAS 290 Electrophoresis Documentation and Analysis System (Kodak, Rochester, NY).

The genes listed in Table 7 used to evaluate the microarray outcomes from the estrogen induction study were used for quantification of hen mRNA using q-RTPCR. The PCR reactions were performed in a 10 ul reaction volume, combining forward and reverse primers with sample cDNA, and using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Analysis was done on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). There was one "hot start" incubation stage (95°C for 10 min), one cycling stage (40 cycles of 95°C for 15 sec, 59°C for 30 sec, and 72°C for 30 sec), and one dissociation curve stage (95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec with a 2% ramp rate). GAPDH was used as the comparative control in order to control for experimental variations in the amount of RNA used for each q-RTPCR reaction as well as batch to batch variation in PCR reagents [102]. All of the samples were measured in triplicate.

The three measurements for each tissue sample were averaged prior to further calculation or statistical analysis. The comparative Ct method was used to calculate the relative gene expression level across the tissues. Relative expression level of each gene in one tissue (ΔCt) was calculated by: Ct target gene-Ct GAPDH; relative expression of

each gene in two different tissues (ΔΔCt) was calculated by: ΔCt A -ΔCt B. During analysis, relative fold change was determined between the pre-production hens and the immediate pre-production hens, the pre-production hens and the laying hens, and the immediate pre-production hens and the laying hens.

Bioinformatic Analysis: Experiment 1

Imaging of the microarray is a first critical step to data analysis for this technique. GenePix Pro 5.0 Personal 4100A array scanner and software (Molecular Devices, Sunnyvale, CA), was used to scan the microarrays at the level of 5 µm resolution; images from this analysis were saved in TIFF format. Photomultiplier tube (PMT) gains were adjusted individually for each array to achieve a ratio of the overall intensities of the two channels (Cy3 and Cy5) of 0.95 to 1.05 [90]. Photomultiplier tubes are optical pieces of hardware that convert incident photons into electrons due to the photoelectric effect [103]. When an incident photon encroaches on the active surface of the photocathode, a photoelectron is generated [103]. The PMTs should be balanced so that the fluorescent intensities from both channels (635 nm and 532 nm) are similar [104]. The PMTs were adjusted so that the normalization factor, calculated by the GenePix Pro software, was close to one. This optimizes dynamic range of the two channels and achieves the best visual effect of an image [104]. Second, each microarray feature (spot on the array) was manually identified and extracted by moving and changing the size of its feature identifier on the array grid using a GenePix Array List (GAL) file. This was repeated for each of the 43,804 features on each of the twenty

arrays using the GenePix Pro 6.0 Software. This is a total of 876,080 individual adjustments.

The SNR is used to define the detection limit and the optimal PMT gain setting of the microarray scanner. This value is calculated for each array element using the difference of the median intensity, minus the median background, divided by the standard deviation of the background [105]. The data from a gene feature was withdrawn from further analysis if it did not have at least one feature sampling spot on one array with an SNR greater than three. This means that if a gene had SNRs of less than three at every sampling spot, the gene was deemed absent under the defined experimental conditions.

Data were normalized using R [106]. Next, Lowess normalization, or locally weighted least squares regression, using a mixed linear model was performed with SAS (SAS, Cary, NC) to fit a smoothing curve onto the microarray dataset [107]. Lowess normalization is used to merge two-color data, such as in microarrays with two dyes used, and all samples in the dataset are corrected independently [107]. Genes with a significant SNR (SNR>3 at a minimum of one sampling point) and a p-value of less than 0.05 were termed "significant" and included in further mathematical and statistical analysis. In this context the term "significant" implies that the gene is expressed at some level within the tissue being analyzed.

JMP (SAS, Cary, NC) was then used as a comparative tool to determine uniquely shared genes between every possible time point combination, namely 1.5hr-3hr, 1.5hr-6hr, 1.5hr-12hr, 1.5hr-24hr, 3hr-6hr, 3hr-12hr, 3hr-24hr, 6hr-12hr, 6hr-24hr, 12hr-24hr,

1.5hr-3hr-6hr, 1.5hr-3hr-6hr-12hr, 1.5hr-3hr-6hr-12hr-24hr, 3hr-6hr-12hr, 3hr-6hr-12hr-24hr, and 6hr-12hr-24hr. To use JMP, the following steps were followed:

- The data from each time point was placed into a single excel file, with data from each time point located on individual worksheets within the Excel workbook.
- JMP was opened.
- Under the data file, the workbook name containing the individual worksheets with the data was chosen.
- Click the first worksheet to be used in the comparison to activate.
- Choose "Tables", and then "Join".
- Next, choose the second worksheet to be used in the comparison.
- The option "Gene Index" under each worksheet was highlighted as the point of comparison between the worksheets.
- "Match" was clicked.
- The option "Include non-matches" was clicked to include genes which are not shared by the two worksheets in the output file.
- Choose "OK".
- A new JMP output file is created that can be saved with a unique file name.

For genes that were included in JMP outcome files, and were on the array listed as "no hits", "unknown", or without a description, the GenBank ID was searched within the NCBI chicken specific map viewer (www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9031). To do this, the GenBank ID was entered in the "Search for" box and "Find" was clicked. The search

results page produces a nucleotide sequence for the GenBank ID of interest. This sequence was entered into the NCBI BLAST [108] program to determine homology to genes in other organisms, and to provide further information about the gene in the pursuit of gene identification.

The DAVID web-based software was next used for large scale functional annotation of identified genes [92, 93]. The entire list of probes on the array, received from the microarray developers, was pasted into the DAVID upload box as a background for analysis. The genes of interest (example: all 1,528 genes, with significantly different levels of expression at 1.5 hours when compared to 0.0 hrs) were pasted into the upload box as a gene list. The "Identifier" selected in Step 2 is GENBANK_ACCESSION. "Start analysis" was clicked and then "Functional Annotation Tool" was clicked. Following analysis, the Uploaded List can be removed from the "List Tab" and the next set of genes can be input. The background genes remain in the program for these additional analyses.

Specific Gene Focus: Experiment 1

The first gene focused on was TAG hydrolase, an intracellular lipase that is believed to catalyze hepatic TAG store mobilization for VLDL assembly. The term "triacylglycerol hydrolase" was searched in the Genecards (www.genecards.org) database[97]. Carboxylesterase 1 (CES1) was chosen, as it is an also known as term for triacylglycerol hydrolase. In the section labeled "Gene Function for CES1 gene" the Enzyme Number (IUBMB) was noted to be 3.1.1.1. Next in Uniprot (www.uniprot.org) this IUBMB number was searched for further information.

The death domain genes and cell-death-inducing DFF45-like effector (CIDE) genes were investigated next. CIDE genes are a family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor that play an important role in lipid metabolism[109]. CIDE-B has specifically been shown to mediate VLDL lipidation and maturation by interacting with apolipoprotein B [110].

CHAPTER III

ESTROGEN INDUCTION OF MALE CHICKS

Introduction

The chicken egg is a product of an intense, hormone driven process that creates an environment fit for the incubation of a chick embryo. Important to the chick's survival is an adequate supply of energy which is shuttled to the egg in the form of VLDLy, a TAG rich lipoprotein. TAG is the most concentrated form of stored energy, and the 6.0 g of TAG in an egg yolk is enough for the chick's survival prior to hatch [1]. It has been observed that estrogen treatment of male birds mimics the effects of female sexual maturation on circulating lipoprotein profiles [60]; in that same study by Luskey, ApoB-LP assembly in male birds was seen to increase fourfold 16 hours after estrogen treatment [60]. We hypothesized that inducing seven to ten day old male chicks with estrogen would produce effects on hepatic ApoB-LP assembly similar to that of a hen reaching sexual maturity. Expanding on earlier estrogen induction studies, the Agilent 44K Chicken Array was used to measure levels of individual mRNA transcripts in the livers of chicks.

As all aspects of the VLDL assembly process are not known, microarray technology was used to compare the temporal expression patterns of genes in a tissue (liver) known to be involved in VLDL assembly under conditions where this process is known to be undergoing rapid and massive increases in capacity. One of our aims was to evaluate a strategy to cluster genes with similar kinetic schemes in order to determine

whether additional biological inference about their contribution to higher order biological processes. Specifically, we wondered whether previously unrelated genes could be specifically associated with VLDL assembly by having a kinetic pattern similar to that of $ApoB_{100}$. In a second approach a list of candidate genes shown to be essential to the VLDL assembly process was identified using peer-reviewed literature. The stipulations for inclusion in the candidate gene list are listed in Chapter I.

Materials and Methods

Based on a scheme described in Chapter II, and also described in Table 5 and Figure 2, four chicks were exposed to estrogen for each of the following lengths of time: 0, 1.5, 3, 6, 12, 24 hours post-estrogen exposure. Birds were killed at a constant clock hour by exsanguination via cardiac puncture. Following full sedation, livers were quickly removed and frozen on dry ice.

RNA was extracted from all livers with the Ribopure kit using approximately 10-15 mg of tissue and according to standard kit protocol (Ambion, Foster City, CA). Following concentration determination, select samples were concentrated using a sodium acetate precipitation, thoroughly described in Chapter II. Denaturing agarose gel electrophoresis was done to measure RNA integrity.

Highly concentrated RNA samples with clear bands on a denaturing gel and acceptable 260/280 and 260/230 ratios were labeled and hybridized to an Agilent microarray using a dual dye system (Santa Clara, CA). RNA isolated from samples taken at time points 24, 12, 6, 3, and 1.5 was hybridized with one zero time point control sample, creating four replicates of the time course.

Features were extracted and optimized as listed in Chapter II. The SNR was calculated for each array element using the difference of the median intensity, minus the median background, divided by the standard deviation of the background [105].

Data were normalized using R [106]. Lowess normalization using a mixed linear model was performed with SAS (SAS, Cary, NC) [107]. Genes with a p-value of less than 0.05 were termed significant and included in analysis. The program JMP (SAS, Cary, NC) was then used as a comparative tool to determine uniquely shared genes between every possible time point combination described in Chapter II.

Unknown sequences of interest were found using the NCBI map viewer (www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9031). Sequence was entered into the NCBI BLAST [108] program to determine homology to genes in other organisms, and to identify the gene. The web-based DAVID software was next used for large scale functional annotation of identified genes [92, 93].

Total RNA was used to synthesize first-strand cDNA as described in Chapter II. cDNA integrity was tested by amplifying GAPDH primers and running the amplification product on an agarose gel. Next, q-RTPCR was used for quantification of seventeen genes of interest to qualify microarray results. All samples were measured in duplicate, and values averaged prior to further computational analysis. Following measurement, the comparative Ct method was used to calculate the relative gene expression level.

A list of candidate genes was created apart from any microarray data (Table 2). The stipulations for inclusion in the candidate gene list are listed in Chapter I. Briefly, when the gene in question was absent or altered VLDL assembly failed or was otherwise

compromised, the gene was shown or speculated to be involved in assembly of an apoB-LP or to be a structural part of an apoB-LP, the gene was shown or speculated to be estrogen responsive, or the gene was responsible for a protein involved in making lipids or transporting lipids. These genes were compared to the results seen in the chick estrogen induction model. It was noted whether the genes on the candidate gene list showed significant changes in transcript levels as estimated by binding to microarray probes in the chick estrogen induction model. Next, the genes in the candidate gene list were placed into groups with candidate genes that have similar functions or are structurally.

Results

Seventeen genes were tested using q-RTPCR to qualify the microarray results. The coefficient of variation (CV) between the replicate q-RTPCR reactions ranged from 1%-5%, with a mean CV of 4.1% ± 1.0%. The outcomes of q-RTPCR are listed in Table 8. For testing purposes, several genes that were not differentially expressed in relation to time 0 in the microarray experiment were chosen for q-RTPCR inclusion. For these genes, samples from two different time points were tested using q-RTPCR. These gene names are denoted with a '*' in Table 8. Statistical significance values are presented in Table 9. Standard deviation and standard error of the mean compare the raw Ct values from q-RTPCR at one time point. The student's t-test compares the Ct values of a gene at the noted time point to the Ct values of the same gene at time 0.

Table 8. Fold differences in expression between an internal reference gene (GAPDH) and target genes using q-RTPCR. Those gene names with a * are genes tested against samples at two time points for additional confidence.

Gene Name	1.5hrs	3hrs	6hrs	12hrs	24hrs
Heat Shock Protein 70kDa*	-2.08	-	-	-	-1.45
Glycerol-3-phosphate acyltransferase-3*	-	1.20	-	-	-2.56
Thyroid hormone responsive spot 14	-	-	-3.33	-	-
Lipoprotein Lipase	-	-	-	-	-7.69
Apolipoprotein B (taken from early part of ApoB sequence)	-1.39	-	-	-	-
Apolipoprotein B (taken from late part of ApoB sequence)	-	-	-	10.36	-
Vitellogenin 1I	-	849.70	-	-	-
Delta 9 Desaturase*	1.62	-	-	2.17	-
Very low density lipoprotein II	-	-	6618.62	-	-
Cytochrome P450, family 7, subfamily A *	-	1.38	-	2.95	-
Peroxisome Proliferator – Activated Receptor α	-	-	-	-1.43	-
Fatty Acid Synthase	-	-	-1.67	-	-
Microsomal Triglyceride Transfer Protein	-	-	-	-	-4.55
Riboflavin Binding Protein	-	-	33.71	-	-
Liver X Receptor*	1.08	1.53	-	-	-
Phosphatidylethanolamine N-methyl transferase	-	-	-	1.87	-
Hepatocyte Nuclear Factor 4 alpha*	-	-	-	1.16	0.76

Table 9. Statistical outcomes of chick q-RTPCR results.

Gene Name	Time Point	Standard Deviation	Standard Error of the Mean	Student's T-Test p value
Heat Shock Protein 70kDa	1.5hrs	1.31	0.46	0.06
Heat Shock Protein 70kDa	24hrs	1.38	0.49	0.39
Glycerol-3-phosphate acyltransferase-3	3hrs	0.94	0.33	0.65
Glycerol-3-phosphate acyltransferase-3	24hrs	2.00	0.76	0.06
Thyroid hormone responsive spot 14	6hrs	0.88	0.31	0.06
Lipoprotein Lipase	24hrs	2.28	0.86	0.01
Apolipoprotein B (early)	1.5hrs	1.06	0.37	0.48
Apolipoprotein B (late)	12hrs	0.30	0.14	2.12E-5
Vitellogenin 1I	3hrs	1.45	0.51	9.39E-8
Delta 9 Desaturase	1.5hrs	1.41	0.50	0.76
Delta 9 Desaturase	12hrs	0.92	0.35	0.053
Very low density lipoprotein II	6hrs	0.59	0.24	7.94E-10
Cytochrome P450, family 7, subfamily A	3hrs	1.73	0.65	0.81
Cytochrome P450, family 7, subfamily A	12hrs	1.62	0.57	0.0079
Peroxisome Proliferator –Activated Receptor α	12hrs	0.27	0.10	0.48
Fatty Acid Synthase	6hrs	0.56	0.21	0.63
Microsomal Triglyceride Transfer Protein	24hrs	3.06	1.08	0.095
Riboflavin Binding Protein	6hrs	0.95	0.34	2.40E-10
Liver X Receptor	1.5hrs	0.89	0.31	0.91
Liver X Receptor	3hrs	0.94	0.33	0.68
Phosphatidylethanolamine N-methyl transferase	12hrs	0.47	0.16	0.003
Hepatocyte Nuclear Factor 4 alpha	12hrs	1.24	0.44	0.09
Hepatocyte Nuclear Factor 4 alpha	24hrs	1.71	0.61	0.68

After JMP analysis comparing all possible combinations of time points, it was determined that an interesting JMP comparison was the comparison of all possible time points (1.5-3-6-12-24). This comparison determined that 119 genes were differentially expressed as compared to time 0 at every time point.

When the NCBI map viewer was utilized to identify the 119 genes expressed at every time point, several interesting points were determined. Six of the 119 probes (5%) showed no identity to annotated amino acid sequences. Thirty-four of the 119 probes (29%) showed 98%, 99%, or 100% of identity to annotated amino acid sequences. Fifteen of the 119 probes (13%) showed identity to a predicted, hypothetical, or unknown annotated amino acid sequence. The remaining 53% of genes were identified on the Agilent 44K Chicken Array.

ApoB had five probes on the microarray, each assessing a different region of its 4536 bases. The expression patterns of each probe differed (Figure 3). BX258989 (nt 2,517 to nt 2,576) had the highest individual fold change of the probes, peaking at a fold change of 7.6 at 6 hrs (Figure 3). BX258989 also had the highest fold change at every other time point except for 24 hrs. At 24 hrs, BU125204 (nt 33,310 to nt 33,369) had the highest fold change. BX935480 (nt 10,608 to nt 10,667) had the lowest fold change at 1.5, 3, and 6 hrs. CR407271 (nt 27,799 to nt 27,858) had the lowest fold change at 12 and 24 hrs. CR407271 is located late in the ApoB sequence.

Candidate genes were chosen, based upon the stipulations listed in Chapter I. Briefly, genes that had been shown to significantly impact VLDL assembly or secretion in a natural or induced gene absence were included.

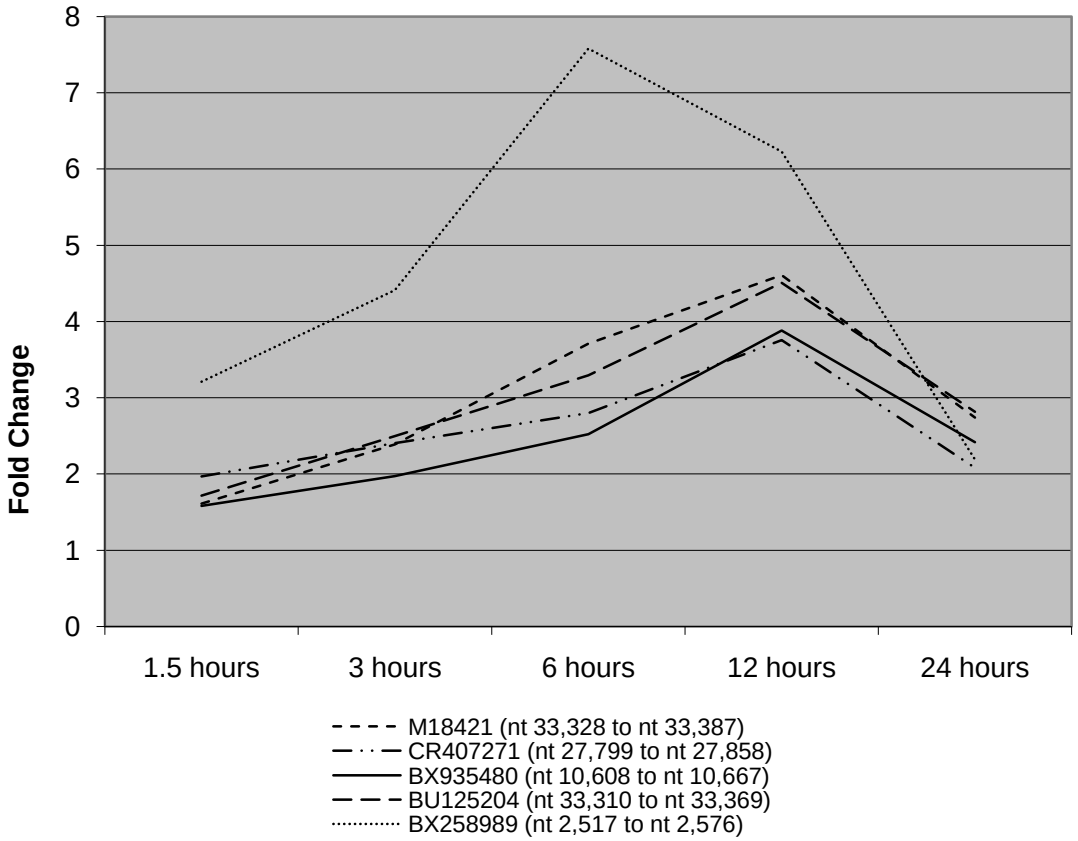

Figure 3. Expression patterns of five apoB probes analyzed by microarray.

When these report findings were compared to the chick estrogen induction data, it was seen that expression levels of 31 of the 61 candidate genes found in literature (51%) were not significantly different at any time point post estrogen exposure. Fold changes for candidate genes are seen in Table 10. Of the candidate genes, seven unique genes had differential expression in relation to time 0 at all five time points. Several of these seven genes had two probes on the microarray. The seven genes included AGPAT-2, HSP70, ApoB, RBP, VLDLII, VTGI, LPL. Of the thirty genes with differential expression, ten had negative fold changes.

The candidate genes were next organized into groups involved in similar processes. The first group of genes was the nuclear transcription factors associated with either assembly specifically or with genes responsible for particle component synthesis. These genes included forkhead box A2 (FOX A2), HNFα, vitamin D3 receptor, and calreticulin. The first, FOX A2, was differentially expressed in relation to time 0 and the last three were not. FOX A2 regulates gene expression in differentiated tissues; it is a transcriptional activator for liver-specific genes like albumin and transthyretin.

Another group included genes known to be involved in VLDL assembly, namely ApoB, MTP, choline kinase (CHK, E.C. 2.7.1.32), diacylglycerol O-acyltransferase 2 (DGAT2, E.C. 2.3.1.20), 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT-3, E.C. 2.3.1.51), phosphatidylethanolamine N-methyltransferase (PEMT, E.C. 2.1.1.17), which were all regulated at one or more time points. These genes are directly involved in particle component synthesis or putting together the interdependent parts of a VLDL.

Table 10. **Fold changes of candidate genes whose level of transcription differed significantly from 0 hour post estrogen exposure**. Gene names listed twice represent a gene with two probes on the microarray.

Gene Name	Accession	1.5hr	3hr	6hr	12hr	24hr
Fatty Acid Synthase	BU301374	-	-	-	-	-1.59
Diacylglycerol O-Acyltransferase 2	BX275561	-	-1.43	-1.54	-	-
Glycerol-3-phosphate acyltransferase-2	BU295914	1.77	2.89	3.57	4.56	1.87
Glycerol-3-phosphate acyltransferase-3	CR385377	-	-	-	-	-1.67
Thyroid Hormone Responsive Spot 14	AY568628	-	-1.75	-2.44	-	-
Ferritin repressor protein	D16150	1.46	1.78	1.81	1.84	-
Cytochrome P450, Family 51	BU467769	-	-	-	-	2.05
Cytochrome P450, Family 51	BU221671	-	-	-	-	1.78
Squalene synthase	AJ719973	-	-	-3.13	-	-
HMG CoA Synthase-1	M60657	2.51	2.38	-	-	-
Microsomal Triglyceride Transfer Protein	BX934807	-1.67	-1.89	-2.17	-2.04	-
Heat Shock Protein 70	BU308587	-1.75	-2.17	-2.04	-1.72	-1.75
Heat Shock Protein 90 kDa beta	M14772	-2.70	-	-	-	-
Heat Shock 70kDa Protein 5	M27260	-3.13	-	-	-	-2.44
Heat Shock Protein Cognate Beta	X70101	-1.64	-1.79	-1.59	-	-
Apolipoprotein B	M18421	1.61	2.39	3.71	4.61	2.74
Apolipoprotein B	BU125204	1.72	2.50	3.30	4.51	2.82
Riboflavin Binding Protein	X74247	6.27	11.24	20.62	45.22	56.24

Table 10 Continued

Gene Name	Accession	1.5hr	3hr	6hr	12hr	24hr
Riboflavin Binding Protein	J03922	4.06	10.47	17.80	59.44	45.53
Dolichyl Pyrophosphate Phosphatase-1	BX932586	-	-	-	-	-1.35
Phosphatidyletha-nolamine N-methyltransferase	AJ720523	-	-	-	1.93	-
Choline Kinase	CF257275	4.51	5.19	5.10	5.10	-
Very low density lipoprotein II	M25774	142.32	151.74	177.01	94.06	90.50
Very low density lipoprotein II	V00449	113.19	102.14	127.13	80.67	75.97
Vitellogenin I	D89547	-	8.13	57.33	111.76	56.87
Vitellogenin I	BU125536	41.56	163.19	226.93	51.32	65.57
Liver Fatty Acid Binding Protein	AF380999	-	-	2.37	1.62	1.85
Lipoprotein Lipase	BU397801	-4.00	-7.69	-6.67	-6.25	-2.22
VLDL receptor	X80207	-	1.74	-	-	-
Cell death-inducing DFFA-like effector a	BU456248	-	-	-	-	2.39
Diazepam Binding Inhibitor	BU476960	-	-	1.42	1.78	1.74
Malic enzyme 3	BQ038051.2	-	-	2.64	-	-
Carboxy-terminal domain, RNA polymerase II, polypeptide A phosphatase, subunit 1	CR387746	-	-1.43	-1.49	-1.37	-1.41
Forkhead Box A2	AF150749	-	-1.45	-	-	-
Insulin Induced Gene 1	AJ719295	2.33	2.33	-	-	-
Fas associated factor family member 2	CR387756	-	1.44	-	-	-

Several candidate genes involved in VLDL assembly which were expected to be differentially expressed when compared to time 0, but were not, included ACC, lipin 1 (LPIN1), and fatty acid elongase (ELOVL, E.C. 2.3.1). A reproduction specific gene group was comprised of RBP, VTGI, and ApoVLDL-II. Transcription of these genes was shown by other studies to be estrogen dependent [26-30] and their transcription is increased in the presence of estrogen (Figure 4). ApoVLDL-II's transcript is stabilized by estrogen [26]. As estrogen declines, ApoVLDL-II is no longer stable and the protein is not present. These genes were all seen to be differentially expressed compared to time 0 in the estrogen induced chick model (Figure 4).

A candidate gene group involved in glycerol lipid synthesis included the regulated FAS, acyl-CoA:diacylglycerol acyltransferase, 1-AGPAT-2, 1-AGPAT-3, thyroid hormone responsive spot 14 (THRSP), and aconitase (E.C. 4.2.1.3). The genes with unchanging expression levels in this group included ATP citrate lyase (ACLY, E.C. 2.3.3.8), ACC, D9D, ELOVL, 6-phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), and 5' adenosine monophosphate-activated protein kinase (AMP-K, E.C. 2.7.11).

Conclusions

The results observed from the chick estrogen induction trial aligned with the individual steps required for VLDL assembly. 31 out of 61 candidate genes (51%) were seen to significantly change at one or more time points. Vitellogenin I, lipoprotein lipase (LPL), and ApoVLDL-II were three genes included on the candidate gene list whose expression level was significantly different from 0 time at one or more time points.

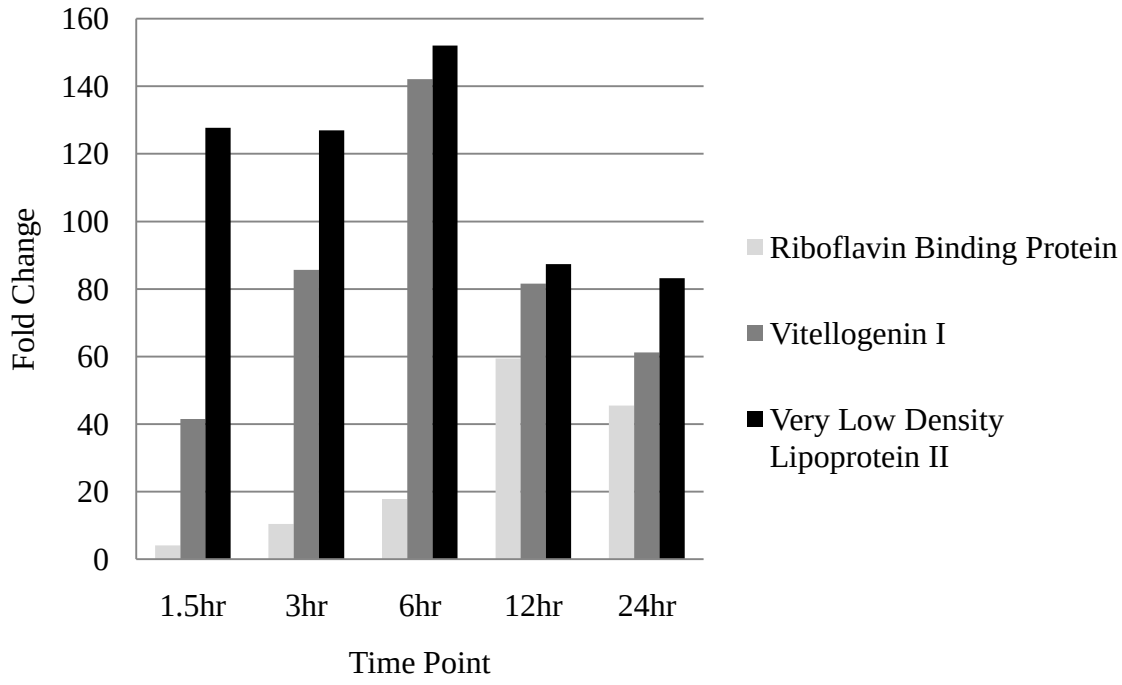

Figure 4. Changes in the expression of estrogen responsive genes following a single maximally stimulating estrogen dose. Note: Vitellogenin I and Very Low Density Lipoprotein II data are an average of two probe fold changes.

Vitellogenin I, an estrogen responsive gene, peaked at a fold change of 166 at 3hrs. Lipoprotein lipase was a gene that was suppressed 8 fold at 3hrs. ApoVLDL-II, another estrogen responsive gene, peaked at a fold change of 177 at 6hrs.

Not all of the genes expected to be necessary for VLDL assembly were seen to be differentially expressed as compared to 0 time across the time course. This may be due to variations in the chicken's physiology as compared to a human or mouse model, or other variations in the experiment, including factors like feeding state, time of day, or physiological stage of the animal. Alternatively, the expression levels of these genes may not have been limiting to increases in VLDL assembly and so alteration in their expression was not needed.

When observing the 24-hour time course, it was clear that gene programs were predominantly working to prepare the cell to produce VLDL for three to six hours post estrogen exposure. These preparatory processes included increases in mRNA responsible for ribosome synthesis, specific structural proteins like ApoB and ApoVLDL-II, lipids, and expression of proteins that serve to reduce cell stress. Later regulated processes (6-24 hours) were predominately related to lipid filling and lipid transport, most likely correlated to the assembly of a VLDL. This information formed the rationale of what we expected the gene expression patterns of the laying hen survey to be; the pre-lay transitioning to immediate pre-lay hens were expected to have more genes in common with the 0-6 hour chick data, and the laying hen gene expression was expected to be more like the 6-24 hour chick data.

CHAPTER IV

NATURAL ESTROGEN EFFECTS ON PULLETS

Introduction

We hypothesized that patterns of change in mRNA levels of estrogen induced male chicks would be similar to those of hens naturally approaching and achieving sexual maturity. To test this idea, changes in gene expression of female chickens undergoing the pullet to layer transition were studied. Towards this end, four pullets, 15wk 4d old, (~12wk prior to lay), four pullets, 16wk 3d old, (~11wk prior to lay), and four hens, 24wk 1d old, (actively laying), were used in this experiment. The data from these twelve birds was compared to the data from the twenty-four estrogen induced chicks to identify similarities and differences in expression of seventeen selected genes.

We expected the same genes whose expression increased massively in the estrogen induced chicks at three and six hours to be similarly elevated in laying hens in comparison to 15 wk old pullets. Three hours and six hours were judged "maximal" for gene expression. The pre-production and immediate pre-production hens were expected to have similar gene profiles to the chicks exposed to estrogen for 1.5 to 6 hours, respectively. This hypothesis was based on the fact that estrogen is responsible for the physiologic and gene expression changes that occur in a hen allowing it to produce an egg, and the idea that estrogen levels in a hen are in flux as it reaches sexual maturity.

Materials and Methods

Twelve female chickens at different stages of reproductive maturity were studied. Four pre-production pullets, four immediate pre-production pullets, and four egg-laying hens were used. Hens were killed and blood and livers collected according to section 2. Each hen's body weight, liver weight, pelvic width at the midpoint of the ischium, ovary weight, oviduct weight, ovarian status, and follicle presence and weight were noted.

After blood processing, the diameters of lipoproteins recovered from the floating lipid layer were measured using a Nanotrac UPA 250 (Microtrac software version 10.5, Clearwater, FL). A complete description is found in section two. A portion of the processed plasma was sent to the Texas Veterinary Medical Diagnostic Laboratory on the campus of Texas A&M University for 17β-Estradiol quantification to assess estrogen levels in the pre-production, immediate pre-production, and laying hens. Briefly, 1.0 mL of dichloromethane was added to 0.5 mL plasma sample to extract estrogens. Solvent was evaporated from plasma extracts, and rehydrated with phosphate buffered saline. A 200 µl aliquot of the solubilized extract was used in each duplicate for radioimmunoassay using the Siemens Estradiol Double Antibody Kit #KE2D1 (Siemens, Munich, Germany). The sample was incubated with antibody supplied with the kit for 2hrs, and then incubated for 1hr with the tracer supplied with the kit. Finally, the antibody bound fraction was precipitated and counted using a Genesys 5000 gamma counter Model LTI505 (Laboratory Technologies, Inc., Maple Park, IL).

RNA was extracted from all livers using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using random hexamers and the Thermoscript™

RT-PCR system (Invitrogen, Carlsbad, CA). Second, the cDNA was used to quantify the transcript levels of the same seventeen genes used in section three using q-RTPCR. See Chapter II for a complete protocol.

Results

The pre-production (15wk 4d), immediate pre-production (16wk 3d), and laying hens (24wk 1d) had an average body weight of 1033.3 g ± 30.4 g, 1183.8 g ± 50.7 g, and 1448.5g ± 64.4 g, respectively. Average liver weight as percent of body weight was 2.30% ± 0.27% for pre-production, 2.49% ± 0.078% for immediate pre-production, and 2.48% ± 0.15% for laying hens. Average pelvic width was 2.71 cm ± 0.085 cm, 5.75 cm ± 0.32 cm, and 6.38 cm ± 0.24 cm for pre-production, immediate pre-production, and laying hens, respectively. Average ovary weight drastically increased: 0.42 g ±0.073 g for pre-production, 0.84 g ± 0.061 g for immediate pre-production, and 57.22 g ± 7.68 g for laying hens. Average oviduct weight increased from 0.56 g ± 0.094 g in pre-production hens to 15.32 g ± 0.82 g in immediate pre-production hens to 51.69 g ± 5.07 g in laying hens. There were no follicles present in the pre-production hens. There were follicles present in one of the immediate pre-production hens; this hen had two follicles, F1 of 0.40 g and an F2 of 0.34 g. The laying hens had multiple follicles. The average weights of follicles F1 through F6 of the laying hens were 12.99 g ± 0.95 g, 12.4 g ± 1.28 g, 9.67 g ± 1.72 g, 6.68 g ± 1.55 g, 4.52 g ± 1.50 g, and 4.46 g ± 0.30 g, respectively. The average VLDL diameter was 72.40 nm ± 10.18 nm in the pre-production hens, 21.95 nm ± 0.71 nm in the immediate pre-production hens, and 26.97 nm ± 0.96 nm in the laying hens. This physiologic data is summarized in Table 11.

Table 11. Hen physiological data. Each data point is an average of four birds. Note: Follicles are not present in immature birds, thus follicle weights are not available and statistics are not possible.

Feature Measured	Pre-Production Hens	Immediate Pre Production Hens	Laying Hens
Sacrifice Time	14:52	14:43	15:33
Body Wt	1033.25 g	1183.75 g	1448.50 g
Liver Wt	23.72 g	29.44 g	35.89 g
Liver Wt as a % of Body Wt	2.30%	2.49%	2.48%
Pelvic Width	2.71 cm	5.75 cm	6.38 cm
Ovary Wt	0.42 g	0.84 g	57.22 g
Oviduct Wt	0.56 g	15.32 g	51.69 g
F1 Wt	n/a	0.09 g	12.99 g
F2 Wt	n/a	0.10 g	12.40 g
F3 Wt	n/a	n/a	9.67 g
F4 Wt	n/a	n/a	6.68 g
F5 Wt	n/a	n/a	4.52 g
F6 Wt	n/a	n/a	4.46 g
VLDL Diameter	72.40 nm	21.95 nm	26.97 nm
Plasma Estrogen Levels	109.00 pg/uL	177.95 pg/uL	37.15 pg/uL

Average pelvic width was positively correlated with the VLDL diameter (nm) when average (Figure 5). The trend line of average plasma estrogen concentration was also negatively correlated with pelvic width, oviduct weight, and liver weight.

17β-Estradiol analysis showed that the immediate pre-production hens had the highest average of plasma estrogen, with 109.0 ± 74 pg/mL. The pre-production hens had an average plasma estrogen of 178.0 ± 84 pg/mL. The laying hens had the lowest concentration of plasma estrogen, with 40.9 ± 5.5 pg/mL. This general pattern aligns with a study by McKeegan which showed that a laying hen's plasma estrogen level peaks at approximately 200 pg/mL around 15-17 weeks, and drops to approximately half of that by 24 weeks [111].

Seventeen genes were analyzed using q-RTPCR. The pre-production hens were compared with the immediate pre-production and the laying hens to determine fold changes of gene expression. In addition, the immediate pre-production hens were compared with the laying hens to determine fold changes in gene expression. This data is summarized in Table 12. The average standard deviation for each set of replicates of a gene was 1.41. Six genes showed negative gene expression patterns in relation to another maturity group of birds. Cyp 7A and HNFα were the genes whose expression was observed to be both up and down in comparison to one of the other bird age groups. VTGII had the highest expression level (1653.2) as compared to time 0 of all comparisons, and riboflavin binding protein had the second highest fold change (851.8).

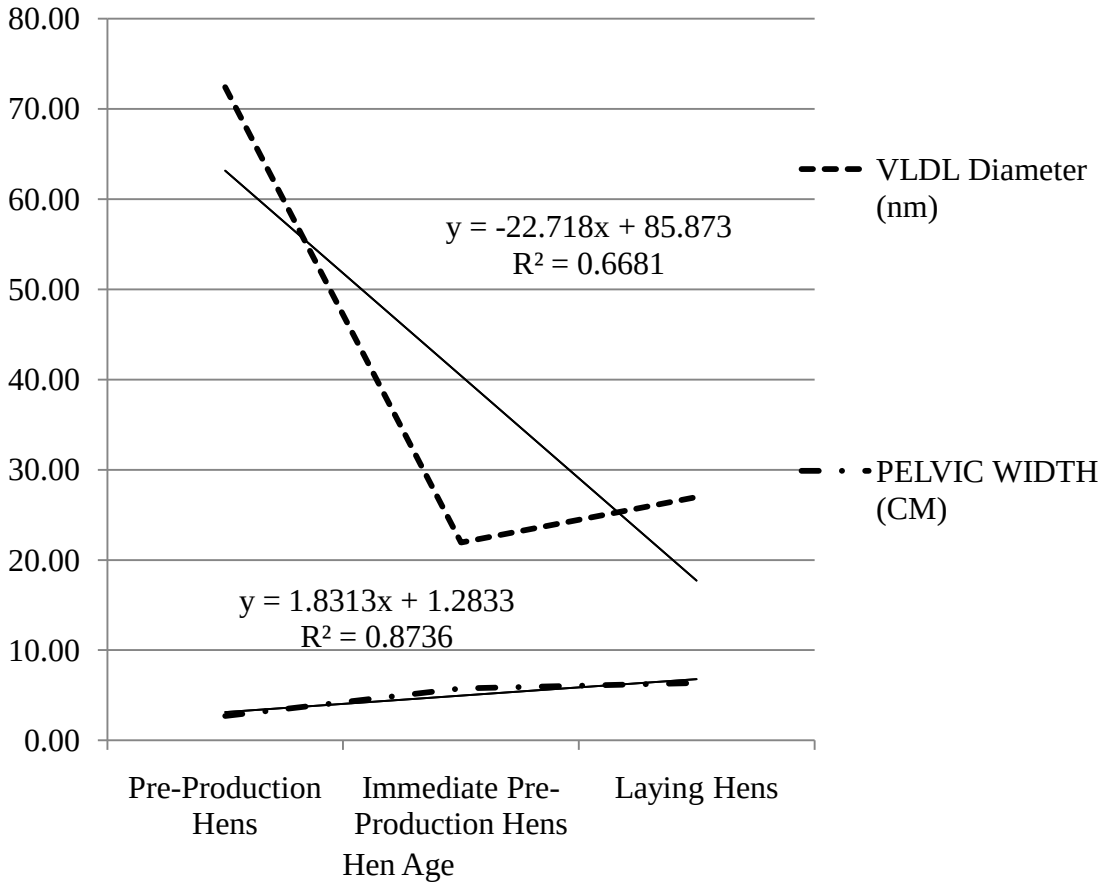

Figure 5. **Comparison of VLDL diameter trend line to average pelvic width trend line.**

Table 12. Hen q-RTPCR results.

Gene Name	Pre-production and immediate pre-production	Immediate pre-production and lay	Pre-production and lay
HSP-70	1.03	3.79	3.92
1-AGPAT 3	1.39	1.67	2.31
THRSP	-1.06	-2.27	-2.44
LPL	-6.25	-2.04	-12.50
ApoB (early)	7.06	1.55	10.93
ApoB (late)	4.58	1.73	7.90
VTGII	638.36	2.59	1653.20
D9D	-2.44	-1.30	-3.23
ApoVLDL-II	63.98	3.05	194.87
Cyp-7A	1.39	-1.89	-1.35
Ppar-alpha	1.56	1.11	1.73
Microsomal Triacylglyceride transfer protein (MTP)	-2.04	-1.43	-2.94
Riboflavin binding protein	161.50	5.27	851.80
LXR	1.32	2.00	2.65
PEMT	1.08	2.20	2.37
Hepatic Nuclear Factor-alpha	-1.64	10.34	6.34

Conclusions

Physical examination of birds at necropsy showed that pre-production hens' ovarian status was extremely undeveloped, the immediate pre-production hens' ovarian status was clearly developing, and the laying hens' ovarian status was fully developed. This anatomical data combined with physiologic data confirmed that the terms pre-production, immediate pre-production, and laying hens were correct for the stage of sexual development assigned to each group. Based upon the increase in oviduct and ovary weight, it was apparent that the hens physically progressed into sexually maturity very quickly.

One interesting find was the concentration of estrogen in the different aged hens. We initially expected the laying hens to have the highest level of plasma estrogen of the three sets of birds, as they were actively undergoing lay and producing approximately one egg per day. What was seen, though, was that the laying hens had the lowest concentration of estrogen in the plasma among the three groups. The birds with the highest level of estrogen in their plasma were the immediate pre-production pullets, which were ~eleven weeks from lay. It can be ruled out that estrogen levels differed due to the time of death, because the immediate pre-production pullets were killed from 14:40 hrs to 14:48 hrs, which was very similar to the time of death of the pre-production hens (14:51 hrs to 14:54 hrs). The laying hens had the latest time of death, from 14:58 hrs to 15:59 hrs. It could be hypothesized that these hens were undergoing drastic physiological changes preparing their bodies for egg production.

One clue to support this idea is the rapid 27-fold increase in oviduct weight in the one week period between the harvest of the pre-production and immediate pre-production hens. The average oviduct weight increased from 0.56 g to 15.32 g, as the average plasma estrogen concentration increased from 109.00 pg/mL to 177.95 pg/mL. The general pattern of change in estrogen concentration observed in our study aligns with a study by McKeegan which showed that a laying hen's plasma estrogen level peaks at approximately 200 pg/mL around 15-17 weeks, and drops to approximately half of that by 24 weeks [111].

The q-RTPCR analysis showed that VTGII, ApoVLDL-II, and RBP had massive upregulation, as expected. Another interesting thing noticed from q-RTPCR analysis was the difference in expression between the two ApoB primers. The primer which is early in the ApoB sequence had a fold change of 10.93 when the pre-production and laying hens were compared. Using this same comparison, the primer localized later in the ApoB sequence had a fold change of 7.90. This differential degree of expression was also observed in the microarray results from the estrogen induced chicks (Figure 3). It is thought that the placement of the sequence to be amplified plays a role in efficiency of the amplification and affects the actual amplification results. This may be due to the aspects of the particular amplification procedure used or simply the length of the mRNA sequence.

The q-RTPCR outcomes from both estrogenized male chicks and female chickens at the pullet-layer transition were used in general comparisons to one as a way to evaluate the similarity in patterns of gene expression and perhaps provide a

physiological frame of reference for the estrogen induction protocol. Comparisons between pre-lay and immediate pre-lay pullet patterns of gene expression were similar in magnitude to the changes observed in comparisons of estrogen naïve male chicks to those exposed to estrogen for 1.5hr to 6hrs. Specifically, VTGII had its greatest fold change in male chicks during this time frame, and similarly the greatest fold of 166 at 3hrs in the estrogen induced chick microarray. In the hen q-RTPCR data, the largest fold change was 638, seen when the same pre-lay to immediate pre-lay comparison was made. In just seven days, the hens exhibited massive changes that caused VTGII expression to increase. In the estrogen induced chicks, LPL was suppressed 8 fold at 3hrs. In the hens, LPL was maximally suppressed 6-fold when pre-lay and immediate pre-lay birds were compared. ApoVLDL-II, peaked at 6 hrs at a fold change of 177. When the pre-lay and immediate pre-lay birds were compared, ApoVLDL-II had its maximal fold change of 64. The estrogen induced chick model clearly provides information on an extremely compressed time-scale that seems to be comparable to an approximately one week long transition of a pullet to a layer bird.

CHAPTER V

STATISTICAL ANALYSIS OF EXPRESSION KINETICS

Introduction

From the start of the study, it was a goal to kinetically group genes. It was hoped that kinetic clustering would group genes known to be affected by the presence of estrogen and genes involved in VLDL assembly with genes previously unknown to act similarly. The candidate gene approach was helpful for analysis focusing on independent time points, but it was not able to capture patterns of change in gene expression over time. Thus, we sought help from statistics experts to achieve this goal.

The hypothesis of the study was explained to the statistical experts, Dr. Scott Schwartz and Dr. Jiawei Wei, members of the Training program in Bioinformatics in the Department of Statistics at Texas A&M University. Simply described, we gave estrogen to male chicks, knowing that this causes ApoB gene expression to increase. In response, massive amounts of VLDLy are made. We believe that if genes follow the same expression pattern as $ApoB_{100}$, there is an enhanced chance that those genes may be specifically involved in that assembly process as well.

By working with Dr. Scott Schwartz and Dr. Jiawei Wei, this aim was achieved with the creation of a novel Bayesian model based classification of temporal patterns. This modeling system grouped genes based on their simple kinetic patterns, and allowed genes some flexibility with regards to which model they fell into. There were 243 possible models for each gene to fall into; each of the five time points were classified as

up (+1), down (-1) or no change (0). 395 genes with significant q-values were included in this portion of analysis. The group of 395 genes was the most highly significant group of genes after false discovery rate (FDR) adjustment was made. This includes removing a percentage of genes with significant p-values believed to be false positives.

Methodology (taken from Schwartz 2011 Manuscript)

An apparent strategy to address this question was to cluster j according to the vector $\{\bar{}ytj : t = 1, \cdots, T\}$, e.g., using a K-means approach [112] or a model based approach [113]. We initially employed both options, however, we found that clustering did not well discern the kinetic pattern of the elements of $\{\bar{}ytj : t = 1, \cdots, T\}$. Instead, clustering using these methodologies constructed classifications on the basis of the overall magnitudes of the vectors. Thus, for our setting, it appeared that clustering based on euclidean metrics of the T dimensional variable $\{\bar{}ytj : t = 1, \cdots, T\}$ was not conducive to distinguishing kinetic patterns in $\bar{}ytj$. Certainly, there may be some potential for addressing pattern identification in our setting via clustering, e.g., using some form of standardized data $\{ztj : t = 1, \cdots, T\}$, but we do not pursue this further here.

Instead, we provide a model framework that characterizes outcomes according to their kinetic patterns in $\{yitj : i = 1, \cdots, n; t = 1, \cdots, T\}$. Our methodology has three key components: 1. a cumulative mean changing from one time point to the next, 2. a variable selection prior that characterizes kinetic patterns, and 3. a hierarchical component that for correlated/longitudinal data. Our specification is

$$
\begin{aligned}
y_{itj} &= B_{ij} + \sum_{k=2}^{T} I(t \geq k)\beta_{kj} + \epsilon_{itj}, \\
B_{ij} &\sim \text{Normal}(\beta_{1j}, s_j^2), \\
s_j^2 &\sim \text{InverseGamma}(a_{1j}, b_{1j}), \\
f(\beta_{tj}) &= (1 - d_{tj})I(\beta_{tj} = 0) + d_{tj}\text{Normal}(\mu_{tj}, \sigma_{tj}^2), \\
d_{tj} &\sim \text{Binomial}(1, 1 - p), \\
\epsilon_{itj} &\sim \text{Normal}(0, \sigma_{ej}^2), \\
\sigma_{ej}^2 &\sim \text{InverseGamma}(a_j, b_j).
\end{aligned}
$$

The j subscripts are retained to emphasize that the univariate data {yitj : i = 1, · · · , n; t = 1, · · · , T} is considered independently. Our specification results in a cumulative expected value for yitj that is equal to the expected value of the previous time point E(yi(t−1)j) = Bij + Pt−1 k=1 βkj adjusted by some βtj . That is, E(yitj) = E(yi(t−1)j) + βkj . Thus, through this parameterization, the kinetic changes from one time point to the next are directly captured. The inclusion of the point mass mixture prior specification f(βtj) of Geweke [114] allows for each modeled βtj to either increase, decrease, or not affect the expected value accumulated up to the previous time point. Through this mechanism, gene j's relationship to estrogen exposure over time is characterized according to kinetic pattern comprised of up/down/steady transitions in mRNA response over time. Finally, we allow each {yitj : t = 1, · · · , T} to have a unique offset Bij = β1j + (Bij − β1j). This allows the methodology to be applied to longitudinal settings or other settings (such as the one we describe in Section 3) where there is correlation between {yitj : t = 1, · · · , T}. By specifying Bij as the first level of a hierarchy centered around β1j , inference on the correlation is provided by (Bij − β1j), and full kinetic inference on the overall intercept β1j is still available via the point mass mixture prior f(β1j). If there

is no reason to expect {yitj : t = 1, · · · , T} are correlated, as in true cross-sectional data settings, Bij and s2j may be simply dropped from the modeling. We explore sensitivity of the estrogen data results to this non-correlated specification in Section 3. As always, the model will be sensitive to dominating specifications of the prior parameters. Of particular interest in this regard is the choice of p, the prior probability of each βtj being 0. In our experience, p behaves as an analysis tuning parameter. As such, a sensitivity analysis over plausible values for p must be implemented to assess the influence of the prior specification of p on the results. For example, in our actual analysis we use a subset of genes expected to show temporal patterns and so we examine results for p = .5, p = .2, and p = .05. This corresponds to a prior belief that 50%, 20%, and 5% of the βtj are 0, respectively. In addition to examining sensitivity to p, because the behavior of certain genes in response to estrogen exposure is approximately known we may also criticize our selections.On a final note, we could incorporate a hierarchical information sharing structure by substituting ptj ∼ Beta(aθ, bθ) in place of the single parameter p. However, we feel in the case of T = 5 coefficients, the status dtj of a single βtj would provide questionable information regarding the status dt′j of another βt′j , and so we do not pursue this extension.

As noted, our approach for characterizing mRNA estrogen response is to classify each gene j by its kinetic pattern:{sign(βtj)dtj : t = 1, · · · , T}. That is, each gene j is enumerated according to a model of the form Mj = {m1j , · · · ,mtj}, where mtj ∈ {−1, 0, 1} and t = 1, · · · , T. For example, the model, or kinetic pattern for the mRNA expression data displayed in Figure 1 (see appendix) appears to be MFig1 = {1, 1, 1,

1,−1} or up-up-up-up-down, while a gene that is not affected by estrogen should be M0 = {0, 0, 0, 0, 0} or steady-steady-steady-steady-steady. Note that m1j represents the change relative to the baseline, whereas each subsequent mkj for k = 2, · · · , T represents the change from the previous time point. The Mj are meant to provide high level classification capabilities for distinguishing between various combination of increasing, decreasing, and steady kinetic patterns across ordinal treatment regimes t. Full inference on {βtj : t = 1, · · · , T} is still available for finer levels of inference, should they be required. Once inference on each Mj is complete, classification is complete, and inference on specific Mj′ may be compared to that of any other Mk, k 6= j for relative classification purposes. In many model selection settings, finding high posterior probability models is challenging. It is not a problem here however. First, for a small number of time points, there are not very many models, e.g., 35 = 243 when T = 5, and so in principal all may be examined. Second, models with high posterior probability are in fact easily found under this specification since each βtj is essentially estimating a mean. Thus, advanced stochastic search variable selection (SSVS) procedures (see, e.g., Hans *et al.* [115]) are not necessary.

Results

By design, there were 3^5 (243) possible models over five time points; each of the 395 genes with significant q-values fell into models most closely matching the gene's individual expression pattern. Biologically, the most interesting patterns were those which included genes known to be related to the ApoB-LP assembly process. These interesting genes include apolipoprotein B (ApoB), ApoVLDL-II, MTP, and AGPAT3,

among others. Along with these noteworthy genes, it was anticipated that additional genes related to transport, binding, enzyme activity, and secretion might be identified by possession of an expression pattern that corresponded to that of ApoB.

Analysis based upon a time course gives the ability to tease out necessary and possibly unknown components that enable process flow. An early time course study by Janero and Lane determined that VLDL particle assembly required about 30 minutes, focusing on apolipoprotein, TAG, and phospholipid secretion rates [36]. Their pulse-chase study of estrogen induced parenchymal liver cells determined that phospholipids were secreted 5-15 minutes into the chase and again after 30 minutes, TAGs were secreted 20-25 minutes into the chase, and apolipoproteins were secreted after 30-35 of chase time [36]. This secretion pattern gives a basic framework upon which to place individual genes involved in production of individual particles.

The 395 genes with significant q-values were first analyzed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) [92, 93]. DAVID recognized 101 unique genes (25.57%) and placed them into fourteen functional annotation clusters. Thirteen of the fourteen functional clusters (92.86%) contained at least one gene ontology (GO) term containing the key word “fat”. The cluster with the highest enrichment score was involved in lipid transport and lipid localization.

The correlated models containing ApoB contained a total of 285 genes. The uncorrelated models containing ApoB contained 233 genes. When the data were not correlated, ApoB statistically fell into four of the possible 243 patterns when $p=0.5$, one pattern when $p=0.2$ and three patterns when $p=0.05$. When the data were correlated,

ApoB statistically fell into three patterns when p=0.5, six patterns when p=0.2 and three patterns when p=0.05. At p=0.05, the expression patterns that ApoB matched were the same when using the correlated and non-correlated algorithms. It was interesting that out of the 243 patterns, ApoB falls into a limited number of patterns; only ten patterns contained ApoB (4.1%). It should be noted that apolipoprotein-B is a large protein (4536 amino acids long) and there are five oligonucleotide probes for ApoB on the 44K Agilent Chicken Array. Only two of the five ApoB probes, M18421 and BU125204, had significant q-values and appeared in the analysis. Interestingly, of the twenty models ApoB appeared in across the multiple model analysis, only five of these models were representative of both M18421 and BU125204. These two ApoB probes had similar expression patterns, and together guide further comments related to ApoB.

Several genes appeared to share the kinetic profile of ApoB, appearing in each of the three non-correlated groups. One of these genes is putative porin precursor, which functions in ion and general cell transport. This supports the necessity of transport within the cell during a massive upregulation of VLDLy production. Another gene that appeared with ApoB in each of the non-correlated p groups was 1-AGPAT 2. This gene is expected to be upregulated in course with ApoB, as it provides ample phospholipids to supply developing VLDLys. Molybdenum cofactor sulfurase (MOCOS, E.C. 4.4.-) was also seen across the three non-correlated groups, probably assisting the process by acting as an antioxidant, as VLDL secretion is known to be impaired with oxidative stress in the RER [116]. Another gene that was in each of these groups was liver fatty acid

binding protein (FABP). The FABPs act as carriers for fatty acids and other lipophilic substances between intra- and extracellular membranes.

Like the non-correlated groups, the putative porin precursor appeared in all three correlated groups, showing that the expression patterns of this transporter protein and ApoB are extremely similar. Alanine aminotransferase 2 (GPT2, E.C. 2.6.1.2) was also seen in each of the three correlated groups. GPT2 is an enzyme which acts to catalyze the reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate, processes important in gluconeogenesis and amino acid metabolism. Fatty acid binding protein participates in the uptake, intracellular metabolism and transport of long-chain fatty acids. This gene was seen to follow all three groups. An interesting gene that was seen in this grouping was pescadillo homolog 1 (PES1). This gene is part of the PeBoW complex, which is required for maturation of 28S and 5.8S ribosomal RNAs and the formation of the 60S ribosome. PES 1 was unseen in any ApoB containing non-correlated groups, which is likely due to the stricter statistical requirements of the non-correlated groups as compared to the correlated groups.

A gene expected to more closely follow ApoB was VLDL-II. VLDL-II is an inhibitor of LPL, protecting the VLDLy from enzymatic breakdown and allowing the deposition of lipid into the developing oocyte [117]. VLDL-II may be a protein present in excess or the timing of its expression may be slightly different from that of ApoB. Another gene with interesting grouping was VTG. VTG appeared in two correlated groups and in two non-correlated groups. VTG is a phosphoprotein exclusively expressed in the livers of estrogenized birds that is cleaved to form the egg yolk

phosphoproteins, lipovitellin, and phosvitin [27]. It was seen to increase expression by 200-fold in this microarray study.

Of the 31 candidate genes that were significantly expressed at one or more time points, only eight (26%) fell into at least one Bayesian model. This may be because not all of these genes had a q-value of <0.05, and were not included Bayesian modeling. Several of these significantly expressed genes had two representative probes on the microarray. The candidate genes included in Bayesian modeling were ApoB, AGPAT-2, ApoVLDL-II, VTGI, FABP, RBP, CHK, and LPL. All eight of these genes fit into at least one model in each of the correlated and uncorrelated p=0.05, p=0.20, and p=0.50 significance groups. Three genes fell into six models. These were AGPAT-2 at the correlated p=0.20 level, one ApoVLDL-II probe at the correlated p=0.05 level, and FABP at the correlated p=0.05 level. The only similar model between any of these three genes was model 105, which fit ApoVLDL-II and FABP.

Of the 119 genes that were differentially expressed in relation to time 0 across all time points, 37 of the 119 genes (31%) were not included in a correlated Bayesian model of any significance level. Forty of the 119 genes (34%) were included in only one Bayesian model in the correlated, p=0.50 significance group. Twenty-six of the 119 genes (22%) were included in only one Bayesian model in the correlated, p=0.20 significance group. Six of the 119 genes (5%) were included in only one Bayesian model in the correlated, p=0.05 significance group. Of these six groups, model 81 appeared three times in three different genes (glypican-1 protein, claudin domain containing-1 protein, and unknown). Glypican-1 is a cell surface proteoglycan that bears

heparin sulfate, and may regulate cell response to growth factors, cell adhesion molecules, and extracellular matrix components [118].

Conclusions

From this interaction, I learned how to convey biological messages to non-biology scholars. It was important that the statistics experts involved in the project understood the basic biology behind the project and knew our precise goals in proceeding with the interaction. From this information and understanding, they would be able to apply appropriate statistical methodologies to the data set.

The Bayesian modeling allowed me to classify genes based upon kinetic patterns, a feat otherwise not possible. The modeling identified several genes following ApoB's kinetic pattern which were novel to our study, which were PES1, the PeBoW complex, and MOCOS. It also classified several genes in groups not containing ApoB. These genes were expected to be classified along with ApoB.

It is not possible to determine the nature of the clusters at this point, but as annotation knowledge is furthered, determination of the functionality of the clusters may be possible. This interaction was also an important stepping stone to future interactions with statisticians using data from future projects in the lab creating large datasets, similar to the dataset used in this thesis.

CHAPTER VI

INTEGRATED CONCLUSIONS

This study has shown that estrogen exposure using male chicks followed by whole genome microarray analysis produces similar results to previously done estrogen induction surveys as well as the events accompanying the natural onset of yolk formation. As technology advances and gene annotation is added, the microarray will produce richer, and more extensive data. Even with the available annotation, this chicken microarray project has proven to be a valuable addition to available work on VLDL assembly.

As acquired, the Agilent 44K Chicken array was well annotated for immunological and cytokine genes. In the course of this study, extensive translation of feature IDs to current GenBank ID's was done. In addition, annotation of unknown feature IDs was added to the array list. One gene of interest, THRSP, was extensively searched using the map viewer and BLAST. It was determined that THRSP has the GenBank accession AJ719312 and its alias, LXR, was listed on the 44K array probe list.

A candidate gene list was created in this microarray study as a starting point to assess the alignment of array results with pertinent findings in published literature. This turned out to be a useful tool, as it showed us that estrogen induction in the male chick produces similar, but not exactly identical results to those shown in the murine "knock-out" literature.

Indeed, several genes reported in the literature to be necessary to produce functional VLDL were not seen to be differentially expressed in relation to time 0 in the estrogen induced chick model. There are several hypotheses on this seeming disagreement. First, the chicken is a primordial organism that is functionally different from the models most used in the studies included in the candidate gene list, i.e. human cell cultures, murine, and rat models. This evolutionary difference may be one reason for the difference in VLDL assembly between chickens and other models. Second, there may have been experimental factors that caused the gene expression patterns in the estrogen induced chicks to behave differently than the literature proposes. These factors include feeding and fasting states, age of the chicks, sex of the chicks, and time of day of harvest. Specifically, the number of estrogen receptors in a chick's liver increases from 1000 receptors per cell at 1 week after hatch, to 3,500 receptors per cell 6 weeks after hatch [119]. In addition, gene knock out studies are "all or nothing" examples of gene expression. In a natural setting such as the one presented, genes may be permissive, expressed at low levels at all times, or controlling, with expression playing a regulatory role.

By comparing the microarray data from the estrogen induced male chicks and q-RTPCR data from female chickens during the pullet/layer transition, it was observed that the massively upregulated genes in the estrogen induced chick model behaved similarly in both models. It is important to note that q-RTPCR is a significantly more sensitive tool than microarrays, and there was a "cut-off" limit for highly upregulated genes in the microarray study. This means that q-RTPCR fold changes had the potential to be much

greater than microarray data for the same gene. VTGII was one such massively upregulated gene; it was included in the 17 genes tested using q-RTPCR. In the estrogen induced chick model using microarray, VTGII had a fold change of 227 at its peak at 6 hours; using q-RTPCR in the transition pullets, VTGII had a fold change of 1653 when the pre-production and laying hens were compared. At its peak of 6 hours, ApoVLDL-II had a fold change of 177 using microarray. ApoVLDL-II had a fold change of 195 when pre-production and laying hens were compared using q-RTPCR. RBP had a peak fold change of 59 at 12 hours using microarray; using q-RTPCR, RBP had a fold change of 852 when pre-production and laying hens were compared.

Across all microarray and q-RTPCR surveys, $ApoB_{100}$, interestingly, had different expression patterns related to the location of the probe within the nucleotide sequence. This may be due to efficiency of the methods used, chaperone availability, or factors specific to the $ApoB_{100}$ sequence, but it is beyond the scope of this thesis to decipher this variance.

When the q-RTPCR data from the estrogen induced chicks and data from the laying hens was compared, it was observed that the same genes with huge fold changes in the chicks had similar results in the hens. When comparing q-RTPCR data, the estrogen induced chick model at 3 hrs showed VTGII had a fold change of 850; VTGII had a fold change of 1653 when the pre-production and laying hens were compared. At its peak of 6 hrs, ApoVLDL-II had a fold change of 7430 using q-RTPCR. ApoVLDL-II had a fold change of 195 when pre-production and laying hens were compared using q-RTPCR. RBP had a peak fold change of 34 at 6 hours using q-RTPCR; using the

same technology, RBP had a fold change of 852 when pre-production and laying hens were compared.

In addition, we were able to show that estrogen induced male chicks exhibit similar expression patterns to hens in a pullet to layer transition. Gene expression from 1.5 to 6 hrs after estrogen introduction closely mirrored the gene expression patterns seen in a hen transitioning from a pullet to a layer. In a very compressed time-scale, the estrogen induced chick model was able to mirror the genetic regulation of the approximately one week long transition of a pullet to a layer hen.

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APPENDIX A

GLOSSARY OF GENES DISCUSSED

ApoB - This gene product is the main apolipoprotein of chylomicrons and low density lipoproteins. It occurs in plasma as two main isoforms, apoB-48 and apoB-100: the former is synthesized exclusively in the gut and the latter in the liver. The intestinal and the hepatic forms of apoB are encoded by a single gene from a single, very long mRNA. The two isoforms share a common N-terminal sequence. The shorter apoB-48 protein is produced after RNA editing of the apoB-100 transcript at residue 2180 (CAA->UAA), resulting in the creation of a stop codon, and early translation termination. Mutations in this gene or its regulatory region cause hypobetalipoproteinemia, normotriglyceridemic hypobetalipoproteinemia, and hypercholesterolemia due to ligand-defective apoB, diseases affecting plasma cholesterol and apoB levels. [provided by RefSeq].

Vitellogenin – estrogen responsive gene in oviparous species that binds PL and zinc, is deposited in egg yolk.

Riboflavin Binding Protein – an estrogen responsive gene in oviparous species. The protein encoded by this gene acts to carry riboflavin into the yolk. Associates with HDL and VTG for transport into the follicle.

ApoVLDL-II – an estrogen responsive gene in oviparous species. Encodes a protein that becomes incorporated in the surface of VLDL particles and inhibits lipase. Is also thought to mediate a decrease in VLDL particle diameter.

MTP - MTP encodes the large subunit of the heterodimeric microsomal triglyceride transfer protein. Protein disulfide isomerase (PDI) completes the heterodimeric microsomal triglyceride transfer protein, which has been shown to play a central role in lipoprotein assembly. Mutations in MTP can cause abetalipoproteinemia. [provided by RefSeq].

Clock - This gene encodes a protein that belongs to the basic helix-loop-helix (bHLH) family of transcription factors. Polymorphisms within the encoded protein have been associated with circadian rhythm sleep disorders. A similar protein in mice is a circadian regulator that acts as a transcription factor and forms a heterodimer with aryl hydrocarbon receptor nuclear translocator-like to activate transcription of mouse period 1. [provided by RefSeq].

PER2 - This gene is a member of the Period family of genes and is expressed in a circadian pattern in the suprachiasmatic nucleus, the primary circadian pacemaker in the mammalian brain. Genes in this family encode components of the circadian rhythms of locomotor activity, metabolism, and behavior. Circadian expression in the suprachiasmatic nucleus continues in constant darkness, and a shift in the light/dark

cycle evokes a proportional shift of gene expression in the suprachiasmatic nucleus. The specific function of this gene is not yet known. [provided by RefSeq].

Bmal-1 - The protein encoded by this gene is a basic helix-loop-helix protein that forms a heterodimer with CLOCK. This complex binds an E-box upstream of the PER1 gene, activating this gene and possibly other circadian rhythym-associated genes. Three transcript variants encoding two different isoforms have been found for this gene. [provided by RefSeq].

Cry1 – part of clock gene complex that controls circadian rhythms.

Cry2 – cryptochrome 2 (photolyase-like); part of clock gene complex that controls circadian rhythms.

SCAP - This gene encodes a protein with a sterol sensing domain (SSD) and seven WD domains. In the presence of cholesterol, this protein binds to sterol regulatory element binding proteins (SREBPs) and mediates their transport from the ER to the Golgi. The SREBPs are then proteolytically cleaved and regulate sterol biosynthesis. [provided by RefSeq].

INSIG2 - The protein encoded by this gene is highly similar to the protein product encoded by gene INSIG1. Both INSIG1 protein and this protein are endoplasmic

reticulum proteins that block the processing of sterol regulatory element binding proteins (SREBPs) by binding to SREBP cleavage-activating protein (SCAP), and thus prevent SCAP from escorting SREBPs to the Golgi. [provided by RefSeq].

SREBP-1 - This gene encodes a transcription factor that binds to the sterol regulatory element-1 (SRE1), which is a decamer flanking the low density lipoprotein receptor gene and some genes involved in sterol biosynthesis. The protein is synthesized as a precursor that is attached to the nuclear membrane and endoplasmic reticulum. Following cleavage, the mature protein translocates to the nucleus and activates transcription by binding to the SRE1. Sterols inhibit the cleavage of the precursor, and the mature nuclear form is rapidly catabolized, thereby reducing transcription. The protein is a member of the basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor family. This gene is located within the Smith-Magenis syndrome region on chromosome 17. Two transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq].

SREBP-2 - This gene encodes a ubiquitously expressed transcription factor that controls cholesterol homeostasis by stimulating transcription of sterol-regulated genes. The encoded protein contains a basic helix-loop-helix-leucine zipper (bHLH-Zip) domain. [provided by RefSeq].

UBDX8 – official gene symbol replaced by FAF2; The protein encoded by this gene is highly expressed in peripheral blood of patients with atopic dermatitis (AD), compared to normal individuals. It may play a role in regulating the resistance to apoptosis that is observed in T cells and eosinophils of AD patients. [provided by RefSeq] From PNAS 108(31):2010 Ubxd8 inhibits TG synthesis by blocking conversion of diacylglycerols (DAGs) to TGs. Excess unsaturated but not saturated FAs relieve this inhibition. As a result, unsaturated FAs are incorporated into TGs, whereas saturated FAs are incorporated into DAGs. In vitro, unsaturated but not saturated FAs alter the structure of purified recombinant Ubxd8 as monitored by changes in its thermal stability, trypsin cleavage pattern, and oligomerization. These results suggest that Ubxd8 acts as a brake that limits TG synthesis, and this brake is released when its structure is altered by exposure to unsaturated FAs.

Choline Kinase - Choline kinase (CK) and ethanolamine kinase (EK) catalyze the phosphorylation of choline/ethanolamine to phosphocholine/phosphoethanolamine. This is the first enzyme in the biosynthesis of phosphatidylcholine/phosphatidylethanolamine in all animal cells. The highly purified CKs from mammalian sources and their recombinant gene products have been shown to have EK activity also, indicating that both activities reside on the same protein. The choline kinase-like protein encoded by CHKL belongs to the choline/ethanolamine kinase family; however, its exact function is not known. Read-through transcripts are expressed from this locus that include exons from the downstream CPT1B locus. [provided by RefSeq].

DGAT2 - Acyl-CoA:diacylglycerol acyltransferase, or DGAT (EC 2.3.1.20), is responsible for the synthesis of triglycerides. It catalyzes a reaction in which diacylglycerol is covalently joined to long chain fatty acyl-CoAs [supplied by OMIM].

AGPAT-3 - 1-acylglycerol-3-phosphate O-acyltransferase 3; The protein encoded by this gene is an acyltransferase that converts lysophosphatidic acid into phosphatidic acid, which is the second step in the de novo phospholipid biosynthetic pathway. The encoded protein may be an integral membrane protein. Two transcript variants encoding the same protein have been found for this gene. [provided by RefSeq].

AGPAT-2 - 1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta) This gene encodes a member of the 1-acylglycerol-3-phosphate O-acyltransferase family. The protein is located within the endoplasmic reticulum membrane and converts lysophosphatidic acid to phosphatidic acid, the second step in de novo phospholipid biosynthesis. Mutations in this gene have been associated with congenital generalized lipodystrophy (CGL), or Berardinelli-Seip syndrome, a disease characterized by a near absence of adipose tissue and severe insulin resistance. Alternate transcriptional splice variants, encoding different isoforms, have been characterized. [provided by RefSeq].

PEMT - phosphatidylethanolamine N-methyltransferase; This gene encodes an enzyme which converts phosphatidylethanolamine to phosphatidylcholine by sequential

methylation in the liver. The protein localizes to the endoplasmic reticulum and mitochondria-associated membranes. The gene is within the Smith-Magenis syndrome region on chromosome 17. Alternate splicing of this gene results in three transcript variants encoding two different isoforms. [provided by RefSeq].

Alpha Tocopherol Associated Protein - SEC14-like 2 (S. cerevisiae). This gene encodes a cytosolic protein which belongs to a family of lipid-binding proteins including Sec14p, alpha-tocopherol transfer protein, and cellular retinol-binding protein. The encoded protein stimulates squalene monooxygenase which is a downstream enzyme in the cholesterol biosynthetic pathway. Alternatively spliced transcript variants encoding different isoforms have been identified for this gene. [provided by RefSeq].

FABP - The intracellular fatty acid-binding proteins (FABPs) belongs to a multigene family. FABPs are divided into at least three distinct types, namely the hepatic-, intestinal- and cardiac-type. They form 14-15 kDa proteins and are thought to participate in the uptake, intracellular metabolism and/or transport of long-chain fatty acids. They may also be responsible in the modulation of cell growth and proliferation. Fatty acid-binding protein 3 gene contains four exons and its function is to arrest growth of mammary epithelial cells. This gene is a candidate tumor suppressor gene for human breast cancer. [provided by RefSeq].

Fatty Acid Synthase - The enzyme encoded by this gene is a multifunctional protein. Its main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated fatty acids. In some cancer cell lines, this protein has been found to be fused with estrogen receptor-alpha (ER-alpha), in which the N-terminus of FAS is fused in-frame with the C-terminus of ER-alpha. [provided by RefSeq].

ATP Citrate Lyase - ATP citrate lyase is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA in many tissues. The enzyme is a tetramer (relative molecular weight approximately 440,000) of apparently identical subunits. It catalyzes the formation of acetyl-CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP to ADP and phosphate. The product, acetyl-CoA, serves several important biosynthetic pathways, including lipogenesis and cholesterogenesis. In nervous tissue, ATP citrate-lyase may be involved in the biosynthesis of acetylcholine. Two transcript variants encoding distinct isoforms have been identified for this gene. [provided by RefSeq].

Stearoyl CoA Desaturase - Stearoyl-CoA desaturase (SCD; EC 1.14.99.5) is an iron-containing enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids. The principal product of SCD is oleic acid, which is formed by desaturation of stearic acid. The ratio of stearic acid to oleic acid has been implicated in the regulation

of cell growth and differentiation through effects on cell membrane fluidity and signal transduction.

Fatty Acid Elongase - ELOVL5 plays a role in elongation of long-chain polyunsaturated fatty acids (Leonard et al., 2000 [PubMed 10970790]).[supplied by OMIM].

PGD - 6-phosphogluconate Dehydrogenase - 6-phosphogluconate dehydrogenase is the second dehydrogenase in the pentose phosphate shunt. Deficiency of this enzyme is generally asymptomatic, and the inheritance of this disorder is autosomal dominant. Hemolysis results from combined deficiency of 6-phosphogluconate dehydrogenase and 6-phosphogluconolactonase suggesting a synergism of the two enzymopathies. [provided by RefSeq].

AMP-K - protein kinase that phosphorylates and inhibits acetyl-CoA carboxylase (ACC); rate-limiting enzyme in malonyl-CoA synthesis [RGD].

FADS2 - The protein encoded by this gene is a member of the fatty acid desaturase (FADS) gene family. Desaturase enzymes regulate unsaturation of fatty acids through the introduction of double bonds between defined carbons of the fatty acyl chain. FADS family members are considered fusion products composed of an N-terminal cytochrome b5-like domain and a C-terminal multiple membrane-spanning desaturase portion, both

of which are characterized by conserved histidine motifs. This gene is clustered with family members FADS1 and FADS2 at 11q12-q13.1; this cluster is thought to have arisen evolutionarily from gene duplication based on its similar exon/intron organization. [provided by RefSeq].

FADS1 - The protein encoded by this gene is a member of the fatty acid desaturase (FADS) gene family. Desaturase enzymes regulate unsaturation of fatty acids through the introduction of double bonds between defined carbons of the fatty acyl chain. FADS family members are considered fusion products composed of an N-terminal cytochrome b5-like domain and a C-terminal multiple membrane-spanning desaturase portion, both of which are characterized by conserved histidine motifs. This gene is clustered with family members FADS1 and FADS2 at 11q12-q13.1; this cluster is thought to have arisen evolutionarily from gene duplication based on its similar exon/intron organization. [provided by RefSeq].

ACAT - Acyl-coenzyme A:cholesterol acyltransferase (ACACT; EC 2.3.1.26) is an intracellular protein located in the endoplasmic reticulum that forms cholesterol esters from cholesterol. Accumulation of cholesterol esters as cytoplasmic lipid droplets within macrophages and smooth muscle cells is a characteristic feature of the early stages of atherosclerotic plaques (Cadigan et al., 1988 [PubMed 3335499]).[supplied by OMIM].

ACAT 2 - This gene is a member of a small family of acyl coenzyme A:cholesterol acyltransferases. The gene encodes a membrane-bound enzyme localized in the endoplasmic reticulum that produces intracellular cholesterol esters from long-chain fatty acyl CoA and cholesterol. The cholesterol esters are then stored as cytoplasmic lipid droplets inside the cell. The enzyme is implicated in cholesterol absorption in the intestine and in the assembly and secretion of apolipoprotein B-containing lipoproteins such as very low density lipoprotein (VLDL). Several alternatively spliced transcript variants of this gene have been described, but their full-length nature is not known. [provided by RefSeq].

Lipin - This gene represents a candidate gene for human lipodystrophy, characterized by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance. Mouse studies suggest that this gene functions during normal adipose tissue development and may also play a role in human triglyceride metabolism. [provided by RefSeq].

PeBoW Complex - A protein complex that is involved in coordinating ribosome biogenesis with cell cycle progression. In human, it is composed of Pes1, Bop1, and WDR12; in Saccharomyces the proteins are known as Nop7p, Erb1 and Ytm1 respectively.

PES1 - This gene encodes a nuclear protein that contains a breast cancer associated gene 1 (BRCA1) C-terminal interaction domain. The encoded protein interacts with BOP1

and WDR12 to form the PeBoW complex, which plays a critical role in cell proliferation via pre-rRNA processing and 60S ribosomal subunit maturation. Expression of this gene may play an important role in breast cancer proliferation and tumorigenicity. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. Pseudogenes of this gene are located on the long arm of chromosome 4 and the short arm of chromosome 9. [provided by RefSeq].

MOCOW - This gene encodes a nuclear protein that contains a breast cancer associated gene 1 (BRCA1) C-terminal interaction domain. The encoded protein interacts with BOP1 and WDR12 to form the PeBoW complex, which plays a critical role in cell proliferation via pre-rRNA processing and 60S ribosomal subunit maturation. Expression of this gene may play an important role in breast cancer proliferation and tumorigenicity. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. Pseudogenes of this gene are located on the long arm of chromosome 4 and the short arm of chromosome 9. [provided by RefSeq].

WDR12 - This gene encodes a member of the WD repeat protein family. WD repeats are minimally conserved regions of approximately 40 amino acids typically bracketed by gly-his and trp-asp (GH-WD), which may facilitate formation of heterotrimeric or multiprotein complexes. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. This protein is highly similar to the mouse WD repeat domain 12 protein at

the amino acid level. The protein encoded by this gene is a component of a nucleolar protein complex that affects maturation of the large ribosomal subunit.

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