

**THE ROLE OF GENDER AND GENETIC POLYMORPHISMS IN
DE NOVO CHOLINE SYNTHESIS**

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ABSTRACT

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The Role of Gender and Genetic Polymorphisms in *De Novo* Choline Synthesis (Under the direction of Dr. Steven H. Zeisel)

Choline is an essential nutrient for humans, though some of the requirement can be met by endogenous synthesis catalyzed by phosphatidylethanolamine *N*-methyltransferase (*PEMT*). The human *PEMT* gene encodes three unique transcripts, A (NM_148172), B (NM_007169), and C (NM_148173) which encode two different protein isoforms. *PEMT* I, encoded by transcript A, localizes to the endoplasmic reticulum whereas *PEMT* II, synthesized from transcripts B and C, is found primarily in the mitochondrial associated membrane and is functionally distinct from *PEMT* I. Studies in mammals indicate a connection between estrogen and protection against choline deficiency syndrome (CDS) but the nature of this interaction is not understood. Examining the entire *PEMT* locus by chromatin immunoprecipitation coupled to microarray (ChIP-chip), we identified several estrogen receptor-alpha (ER- α) enriched regions in the *PEMT* locus specifically implicating a critical regulatory region located in intron 1 of the A transcription start site, 7500 nucleotides (nt) upstream of the transcriptional start site of transcript B. We found that *PEMT* transcription was increased in a dose-dependent manner when primary mouse and human hepatocytes were treated with 17- β -estradiol for 24 hours and this increased message was associated with an increase in protein expression and enzyme activity. ER- α regulation of the *PEMT* gene is transcript specific,

whereby estrogen binding results in an increase in transcripts B and C but not transcript A. We suggest that differences in dietary choline requirements occur between men and women because estrogen induces expression of the *PEMT* gene, allowing premenopausal women to make more choline endogenously.

In humans, young women harboring a *PEMT* promoter SNP are 25X as likely to develop CDS as are non-carriers of this SNP. Here we demonstrate, in human hepatocytes, that a haploblock of SNPs within a key estrogen regulatory region in the *PEMT* gene disrupt ER- α DNA binding. Hepatocytes homozygous for the risk allele were not estrogen responsive. For the first time, we report a putative mechanism underlying the association of *PEMT* genetic variation and susceptibility to choline deficiency syndrome in women.

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CHAPTER I

GENERAL INTRODUCTION

CHOLINE IS AN ESSENTIAL NUTRIENT

Choline is a dietary component essential for normal function of all cells (Zeisel and Blusztajn, 1994). It, or its metabolites, assures the structural integrity and signaling functions of cell membranes; it is the major source of methyl-groups in the diet (following its oxidation to betaine, it participates in the methylation of homocysteine to form methionine); and it is the precursor for the neurotransmitter acetylcholine; and it is involved in lipid transport from liver (Zeisel and Blusztajn, 1994). Choline is particularly important during pregnancy because it has been implicated in fetal brain development. When rat dams received choline supplements (during days 12-17 of gestation), their offspring had significant and lifelong enhancement of spatial memory and attention with changes in hippocampal anatomy, chemistry, electrophysiology, neuronal stem cell proliferation and apoptosis (Albright et al., 2001; Albright et al., 1999; Craciunescu et al., 2003; Loy et al., 1991; Meck and Williams, 1997a, b, c; Meck et al., 1988, 1989; Meck and Williams, 1999; Meck and Williams, 2003; Mellott et al., 2004; Montoya et al., 2000; Pyapali et al., 1998). Choline is widely distributed in all cells, mainly in the form of the phospholipids, phosphatidylcholine (PtdCho), lysophosphatidylcholine (lysoPtdCho), choline plasmalogen, platelet-activating factor, and sphingomyelin (SM), essential components of all eukaryotic cell membranes. Choline can also be acetylated, phosphorylated, oxidized or hydrolyzed, and forms water-soluble choline metabolites, acetylcholine, phosphocholine (PCho), betaine, or glycerophosphocholine (GPCho).

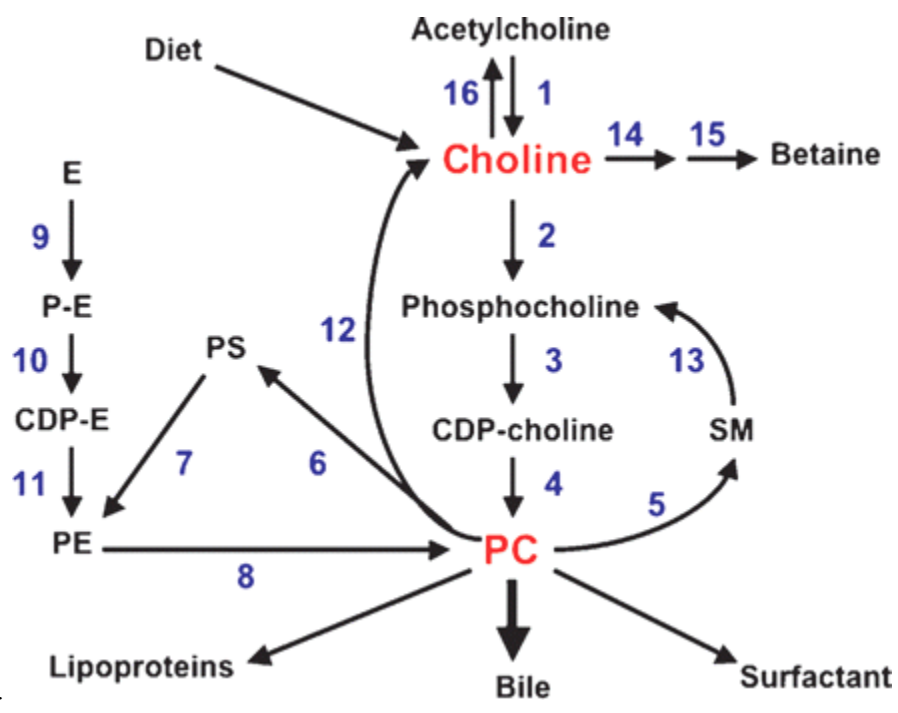
There are multiple functional consequences of dietary choline deficiency in humans, including the development of fatty liver (hepatosteatosis) (Buchman et al., 1995; Zeisel et al., 1991) because a lack of phosphatidylcholine (PtdCho) limits the export of

excess triglyceride from liver in lipoproteins (Yao and Vance, 1988, 1989). Also, choline deficiency is associated with liver damage (elevated serum aminotransferases) (Albright et al., 2005; Albright et al., 1996; Albright and Zeisel, 1997; Zeisel et al., 1991) and liver dysfunction due to apoptosis of liver cells (Zeisel 1996) and massive accumulation of triacylglycerol (TG) within the hepatocyte in mammalian species (Zeisel 1991). The phenotypic clinical manifestations associated with restricted dietary choline will subsequently be referred to as choline deficiency syndrome (CDS).

PtdCho is made in all nucleated cells via the choline pathway. The majority of PtdCho derives from pre-formed choline, with CTP: phosphocholine cytidylyltransferase (CCT) catalyzing the rate-limiting step in the CDP-choline pathway (Sundler 1972; Kent 1997). **(Figure 1-1)**

Figure 1-1. Pathways of Choline Metabolism

Pathways involved in choline and phosphatidylcholine (PC) homeostasis: E, ethanolamine; P-E, phosphoethanolamine; CDP-E, CDP-ethanolamine; PE, phosphatidylethanolamine; SM, sphingomyelin. Enzyme names are indicated by numbers: 1, choline acetyltransferase; 2, choline kinase; 3, CTP:phosphocholine cytidylyltransferase; 4, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; 5, sphingomyelin synthase; 6, phosphatidylserine synthase 1; 7, phosphatidylserine decarboxylase; 8, phosphatidylethanolamine *N*-methyltransferase; 9, ethanolamine kinase; 10, CTP:phosphoethanolamine cytidylyltransferase; 11, CDP-ethanolamine:1,2 diacylglycerol ethanolaminephosphotransferase; 12, various phospholipase and lysophospholipase activities; 13, sphingomyelinase; 14, choline oxidase; 15, betaine aldehyde dehydrogenase.



CHOLINE BIOSYNTHESIS

The only source of choline other than diet is from the *de novo* biosynthesis of PtdCho catalyzed by phosphatidylethanolamine-*N*-methyltransferase (PEMT; EC 2.1.1.17). PEMT, ~20 kDa protein, is primarily found in the liver (Vance and Ridgway, 1988). PEMT spans the membrane with four transmembrane sequences (Shields et al., 2003b). The methyl donor for the methylation reactions is *S*-adenosylmethionine (AdoMet), which binds to several residues of PEMT exposed on the cytosolic surface of the endoplasmic reticulum (Shields et al., 2003a).

The human *PEMT* gene, which is located on chromosome 17p11.2, has 9 exons and 8 introns spanning its 86 kb length, and it encodes three unique transcripts annotated by three Refseq accessions: A (NM_148172), B (NM_007169), and C (NM_148173) which make two different protein isoforms. Each of the three transcript variants has only 7 exons and 6 introns due to splicing of the leading exons, which are alternatively spliced to a common exon 2, where translation begins (Shields et al., 2001). Each of the three leading exons is unique for transcript A, B, and C and thus will be referred to as exons 1A, 1B, and 1C throughout the manuscript. PEMTI, encoded by transcript A, localizes to the endoplasmic reticulum and transcripts B and C encode an isoform, PEMTII, which is localized to the mitochondrial associated membrane (a subfraction of the endoplasmic reticulum) (Cui et al., 1993).

The significance of phosphatidylethanolamine methylation is only now being understood. The CDP-Choline and PEMT pathways have distinct roles and are not interchangeable (DeLong et al., 1999). Bile secretion or lipoprotein secretion may specifically require PEMT-derived PtdCho (Agellon et al., 1999; Li et al., 2005; Sehayek

et al., 2003). PEMT is required for the secretion of apoB100-containing VLDLs (Noga et al., 2002). The PEMT pathway generates PtdCho species that are much more diverse and enriched with long chain, and very long chain, polyunsaturated (e.g. 18:0/20:4) fatty acids. In contrast, PtdCho derived from the CDP-choline pathway is mainly comprised of long chain saturated (e.g. 16:0/18:0) species (Magret et al., 1996).

To explore the physiological importance of the PEMT pathway, mice that lack *Pemt* (*Pemt*^{-/-}) were created by targeted deletion of exon 2 which contains the transcription start site (Walkey 1997). When fed a choline-deficient (CD) diet, the mice rapidly developed steatosis, steatohepatitis, and died from liver failure after 3 days (Walkey et al., 1998). The liver failure was associated with a 50% decrease in hepatic PtdCho content; thus, it was suggested that this was the reason for the steatohepatitis and liver failure. Zhu et al found that *Pemt*^{-/-} mice developed significant lipid accumulation and cell damage in liver despite having extra choline provided in their diet, and that condition was exacerbated during pregnancy (Zhu et al., 2003). *Pemt*^{-/-} mice have lower choline pools in liver despite being fed sufficient or supplemental amounts of dietary choline (Zhu et al., 2003). Lipid analyses of plasma and liver indicated that male *Pemt*^{-/-} mice accumulated triacylglycerols (TG) in their livers and were unable to secrete the same amount of TG from the liver as did *Pemt*^{+/+} mice (Noga and Vance, 2003). Another study found that cultured hepatocytes from *Pemt*^{-/-} mice incubated with oleate secreted ~50% less TG and ~70% less apoB100 than do hepatocytes from *Pemt*^{+/+} mice and although the total amount of PtdCho secreted was not altered in *Pemt*^{-/-} hepatocytes, the PtdCho recovered in the VLDL fraction was decreased. Thus, PEMT has a special function that is required for normal secretion of TG and apoB100 particles from

hepatocytes (Noga et al., 2002). These results underscore the importance of the PEMT pathway as a significant and indispensable source of choline.

ESTROGEN STATUS MAY MODULATE CHOLINE REQUIREMENT

Gender is an important modifier of susceptibility to developing organ dysfunction when fed a low choline diet. Premenopausal women, relative to males and postmenopausal women are resistant to developing organ dysfunction when fed a low choline diet (Fischer et al., 2007). Interestingly, within each gender category, only a subset of subjects were found to be susceptible to developing organ dysfunction, suggesting that other factors, such as genetic polymorphisms, may contribute to susceptibility to developing organ dysfunction when fed a low choline diet. It is reasonable to suggest that this protective effect observed in young women is due to an enhanced capacity for *de novo* biosynthesis of the choline moiety. Studies in animal models support this hypothesis, as female rats are less prone to becoming symptomatic on a low choline diet due their ability to generate more PtdCho via the PEMT pathway as compared to male mice (Tessitore et al., 1995) Female rats have greater PEMT enzymatic activity relative to male rats (Bjornstad and Bremer, 1966; Lindblad and Schersten, 1976; Lyman et al., 1971) and male castrated rats treated with estradiol have increased hepatic PEMT activity (Young, 1971). Thus, estrogen could be the mediator of increased PEMT activity in humans.

Pregnancy and lactation are times when demand for choline is especially high, thus it makes sense evolutionarily that women have adequate stores of choline prior to becoming pregnant (Sweiry et al., 1986; Sweiry and Yudilevich, 1985). During pregnancy, estradiol concentration rises from approximately 1 nM during the first

trimester to 60 nM at term, allowing for maximum endogenous synthesis of choline during the period when females need to support fetal development (Adeyemo and Jeyakumar, 1993). Transport of choline from mother to fetus depletes maternal plasma choline in humans (McMahon and Farrell, 1985). Because milk contains a large quantity of choline, lactation further increases maternal demand for choline resulting in depleted tissue stores (Holmes-McNary et al., 1996; Zeisel et al., 1995). Thus, choline availability during pregnancy and lactation is marginal despite enhanced capacity for endogenous biosynthesis of choline. *PEMT*^{-/-} mice abort pregnancies around day 9-10 of gestation unless fed supplemental choline (Zhu et al., 2004). The same may be true for pregnant women eating too little choline. Indeed, a study conducted by the March of Dimes reports that high periconceptional intake of choline and betaine reduced the risk of neural tube defects and cleft palate (Shaw et al., 2006; Shaw et al., 2004). This group also reported that women in the United States consume <300 mg/d to >500 mg/d dietary choline (Shaw et al., 2004).

GENETIC POLYMORPHISMS AND CHOLINE REQUIREMENT

Genetic polymorphisms are found throughout the genome. Polymorphisms that affect the regulation of gene expression are critical to understanding human genetic differences in susceptibility to diseases and sensitivity to environmental exposures (Olivier, 2004; Pastinen et al., 2004). Single nucleotide polymorphisms (SNPs), changes of a single DNA base, account for approximately 90% of human sequence variations and have been estimated to occur every 500–1000 base-pairs throughout the human genome (Collins et al., 1998; Sherry et al., 1999). Apart from SNPs, other common polymorphisms include tandem repeated segments (minisatellite and microsatellites), and copy number variants (Ponomarenko et al., 2002). SNPs that occur in coding regions (cSNPs) may alter the amino-acid sequence, resulting in changes in the structure and function of the encoded protein; whereas SNPs that affect gene expression levels in an allele-specific manner are generally found in gene regulatory regions.

Polymorphisms in choline metabolism genes that affect enzymatic activity and transcriptional regulation could influence human requirements for the nutrient choline. SNPs in the *PEMT* gene could modify *de novo* PtdCho synthesis, thereby altering susceptibility to developing organ dysfunction when fed a low choline diet. Ninety-eight SNPs were identified in *PEMT* from 48 Japanese individuals by direct sequencing of their entire genomic regions except for repetitive elements (Saito et al., 2001), but only one cSNP was found to be functionally significant (Song et al., 2005). We recently reported that a *PEMT* B promoter SNP, rs12325817, was associated with increased susceptibility to CDS in women (da Costa et al., 2006). Presumably rs12325817 affects transcriptional regulation of the gene or is in linkage disequilibrium (LD) with a SNP that

affects *PEMT* function. The SNP was associated with CDS in women but not in men, suggesting that an estrogen regulatory mechanism is involved. Premenopausal women homozygous for the SNP are sensitive, perhaps premenopausal women heterozygous for the rs12325817 risk allele have sufficient estrogen to overcome the effects on estrogen of the single allele, whereas postmenopausal women with lower estrogen levels are sensitive to the SNP and men, with no estrogen, are not sensitive to the SNP at all. This promoter SNP does not fall within a mapped transcription factor binding site nor is it a HapMap (a catalog of common genetic variants that occur in human beings) SNP, which would permit, by imputation, identification of potential regulatory SNPs in linkage disequilibrium with rs12325817 (Frazer et al., 2007). We propose studies to uncover the mechanism whereby rs12325817 or an associated SNP confers risk to CDS. A cSNP that alters the tertiary structure of the active site in *PEMT* enzyme could potentially decrease the enzyme activity, and people with such a polymorphism would need extra choline for normal liver function. Analogously, a regulatory SNP that affects *PEMT* gene expression could result in less *PEMT* message and subsequently less choline availability *de novo*. As discussed previously, SNPs that affect choline bioavailability during pregnancy could markedly increase a woman's risk of having a baby with a birth defect.

ESTROGEN AND LIVER

Estrogens have important roles in both males and females. However, it has been reported that in postmenopausal women and in men, 17- β -estradiol (E2) is not a circulating hormone; rather, it is synthesized in a number of sites other than the ovaries such as breast, brain, and bone (Glass, 2006). Thus, in postmenopausal women and in men estrogen biosynthesis in these sites depends on a circulating source of androgenic precursors such as testosterone. Plasma steroid E2 levels are approximately 0.04 nmol/l and 0.10 nmol/l in post-menopausal women and men, respectively whereas in pre-menopausal women levels range from 1.0 to 60nmol/l. (Adeyemo and Jeyakumar, 1993; Labrie et al., 2003)

Estrogen biosynthesis is catalyzed by aromatase cytochrome P450, a microsomal member of the cytochrome P450 superfamily (Harada et al., 1990; Means et al., 1989). Estrogens induce cellular changes through several different mechanisms. In the “classical” mechanism of estrogen action, estrogens diffuse into the cell and bind to the estrogen receptor (ER), which is located in the nucleus. This nuclear estrogen-ER complex binds to estrogen response element sequences directly –via a 13-bp palindromic repeat– or indirectly through protein-protein interactions with activator protein 1 (AP1) or Sp1 transcription factor (SP1) sites in the promoter region of estrogen-responsive genes. Coregulatory proteins (coactivators or corepressors) are recruited to the promoter, which results in either an increase or decrease in gene expression. Genomic mechanisms, which alter gene expression, typically occur over the course of hours. In contrast, estrogen can act more quickly (within seconds or minutes) via “nongenomic” mechanisms, either through the ER located in or adjacent to the plasma membrane, or

through other non-ER plasma membrane-associated estrogen-binding proteins resulting in cellular responses such as increased levels of Ca^{2+} or NO, and activation of kinases (Deroo and Korach, 2006). In addition, recent reports suggest “nontranscriptional” action mediated by ER situated in the plasma membrane through the p85/Akt pathway (Bjornstrom and Sjoberg, 2005). The ER exists in 2 main forms, ER- α and ER- β , which have distinct tissue expression patterns in both humans and rodents (Mueller and Korach, 2001).

Significant progress has been made in defining the interactions between chromatin and a complex array of transcription factors involved in ER-mediated gene expression (Halachmi et al., 1994; Metivier et al., 2003; Shang et al., 2000). Studies have revealed the cyclical, combinatorial association of ER, p160 coactivators, histone acetyl transferases (HAT), and chromatin modifying molecules, such as p300/CBP and p/CAF, with gene promoters (Metivier et al., 2003; Shang et al., 2000). Many gene expression profiling studies have been conducted in breast cancer cell lines and have identified potential ER target genes such as *TFF-1* (*pS2*), *EBAG9*, and *Cathepsin D*. (Augereau et al., 1994; Berry et al., 1989; Ikeda et al., 2000). Recently, chromatin immunoprecipitation coupled to microarrays (ChIP-chip) have been explored to pursue the identification of ER binding in the human genome in breast cancer cells (Carroll et al., 2005; Carroll et al., 2006; Kwon et al., 2007; Lin et al., 2007). The results of these studies, which found that gene proximal-promoter regions are not the major sites of ER binding, dispute the classical mechanism of ER mediated regulation. For example, one study found that only 5% of the mapped ER- α binding sites are located within 5 kb upstream of the transcriptional start sites of adjacent genes and the vast majority of the

ER- α sites were within intronic or distal locations (Lin et al., 2007). This result suggests ER- α mediates transcriptional regulatory mechanisms over significant physical distances from the site of gene transcription. Carroll et al. identified six ER- α binding sites within 200kbp of the *PEMT* A promoter (Carroll et al., 2006). Another genome-wide promoter ChIP-chip study performed in breast cancer cells, identified enrichment at *PEMT* promoter A (NM_148172) (Kwon et al., 2007).

The liver is an estrogen-sensitive organ which primarily expresses ER- α . Human liver from both males and females expresses ER- α (Rossini et al., 1989). To date, only one study has been conducted to evaluate ER- α binding patterns in liver (Gao et al., 2008). In contrast to the studies conducted in human breast cancer cells, this group determined in mouse liver that almost 50% of ER- α binding regions overlap with genes including 8% overlap with exons (Gao et al., 2008). Carroll and colleagues demonstrated that ER- α binding requires a Forkhead protein, FOXA1, at these ER- α sites, to mediate transcriptional regulation (Carroll et al., 2005). FOXA transcription factors are integral components of transcriptional regulatory networks in mammalian liver (Odom et al., 2004).

Nonalcoholic fatty liver disease (NAFLD) encompasses a disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis (Neuschwander-Tetri and Caldwell, 2003). A “two-hit” model has been proposed to explain the progression of NAFLD to non-alcoholic steatohepatitis (NASH). The “first hit” constitutes the deposition of triglycerides in the cytoplasm of the hepatocyte. Additional cellular events must occur (the “second hit”) such as inflammation, apoptosis

and fibrosis for the disease to progress to NASH. NASH has become the second or third most common liver disease in outpatient hepatology practice in North America (James and Day, 1999). The underlying molecular mechanism involving the progression of hepatic steatosis to NASH remains to be elucidated in humans (Browning and Horton, 2004).

It has been reported that in nonobese nondiabetic breast cancer patients treated with tamoxifen induces NASH and liver cirrhosis (Oien et al., 1999; Pratt et al., 1995). Tamoxifen is a potent antagonist of estrogen in liver and breast tissue, and estrogen is believed to be profoundly involved in hepatic lipid metabolism (Djouadi et al., 1998). Previous studies suggest that tamoxifen increased hepatic fat content by blocking estrogenic regulation of hepatic lipid homeostasis (Osman et al., 2007). Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout (ArKO) mouse (Hewitt et al., 2004). Collectively, these findings suggest the involvement of estrogen deficiency in the development of liver steatohepatitis.

SUMMARY

Choline is an essential nutrient required for maintaining normal liver function and sustaining pregnancy and lactation. *PEMT* is the only source of endogenous choline biosynthesis. Genetic polymorphisms that affect *PEMT* gene function may contribute to susceptibility to nonalcoholic steatohepatitis and other liver related metabolic disorders especially within dietary choline deficient populations. A subset of premenopausal women is resistant to choline-deficiency syndrome when fed a choline deficient diet. This protective mechanism observed in young women is critical during pregnancy and fetal development when choline stores are limiting. We hypothesize that *PEMT*, the gene responsible for endogenous biosynthesis of choline, is regulated by estrogen and suggest that a plausible explanation for the mechanism in which young women are resistant to choline deficiency syndrome could involve estrogen-mediated induction of *PEMT*. We propose studies to determine if the *PEMT* gene is regulated by estrogen in human liver and elucidate the mechanism whereby regulatory SNPs identified in our clinical studies affect estrogenic regulation of gene expression. Women with SNPs which affect estrogen regulation of *PEMT* may be at risk for hepatosteatosis as well as aberrant pregnancy outcomes.

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CHAPTER II

PHOSPHATIDYLETHANOLAMINE N-METHYLTRANSFERASE (*PEMT*) GENE EXPRESSION IS INDUCED BY ESTROGEN IN HUMAN AND MOUSE PRIMARY HEPATOCYTES

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ABSTRACT

Choline is an essential nutrient for humans, though some of the requirement can be met by endogenous synthesis catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT). Pre-menopausal women are relatively resistant to choline deficiency compared to post-menopausal women and men. Studies in animals suggest that estrogen treatment can increase PEMT activity. In this study we investigated whether the *PEMT* gene is regulated by estrogen. *PEMT* transcription was increased in a dose-dependent manner when primary mouse and human hepatocytes were treated with 17- β -estradiol for 24 hours. This increased message was associated with an increase in protein expression and enzyme activity. In addition, we report a region that contains a perfect estrogen response element (ERE) approximately 7.5 kb from the transcription start site corresponding to transcript variants NM_007169 and NM_148173 of the human and murine *PEMT* genes respectively, three imperfect EREs in evolutionarily conserved regions and multiple imperfect EREs in non-conserved regions in the putative promoter regions. We predict that both the mouse and human *PEMT* genes have three unique transcription start sites which are indicative of either multiple promoters and/or alternative splicing. This study is the first to explore the underlying mechanism of why dietary requirements for choline vary with estrogen status in humans.

INTRODUCTION

Choline is an essential nutrient (Institute of Medicine and National Academy of Sciences USA, 1998); it is used to form cell membranes, it is the major source of methyl-groups in the diet, and it is a precursor for biosynthesis of the neurotransmitter acetylcholine (Zeisel, 2006). Choline is critical during fetal development, when it influences stem cell proliferation and apoptosis, thereby altering brain structure and function (Albright et al., 1999b; Craciunescu et al., 2003; Loy et al., 1991; Meck and Williams, 2003a; Mellott et al., 2004). Similarly it influences neural tube development (Fisher et al., 2001; Shaw et al., 2004). In later life, choline deficiency causes fatty liver, liver damage and muscle damage (da Costa et al., 2004; da Costa et al., 2005) and reduces the capacity to handle a methionine load, resulting in elevated homocysteine (da Costa et al., 2005), a risk factor for cardiovascular disease (Guba et al., 1996). Though many foods contain choline (Zeisel et al., 2003a; Zeisel et al., 2003b), there is at least 2-fold variation in dietary intake in humans (Fischer et al., 2005; Shaw et al., 2004).

Choline can also be derived from *de novo* biosynthesis of phosphatidylcholine catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT, EC 2.1.1.17) (Bremer and Greenberg, 1961; Vance et al., 1998). Most of this enzyme's activity is in the liver (Vance et al., 1997).

When fed a diet low in choline, premenopausal women were much less likely to develop choline-deficiency associated organ dysfunction compared to men or postmenopausal women (Fischer et al., 2007). This suggests that premenopausal women might have an enhanced capacity for *de novo* biosynthesis of choline. Such a finding would be important during pregnancy and lactation, when the demand for choline is

especially high because the transport of choline from mother to infant, via placenta or mammary gland, constitutes an extraordinary drain on maternal choline stores (Zeisel et al., 1995).

Studies in animal models support this hypothesis. Female rats are less sensitive to choline deficiency than are male rats (Tessitore et al., 1995) and female mice produce more phosphatidylcholine via the PEMT pathway than do male mice (Noga and Vance, 2003). Estrogen status may be important for this increased PEMT activity; estradiol treatment increased PEMT activity in pituitary of rats (Drouva et al., 1986) as well as in liver of castrated-rats (Young, 1971) and PEMT activity in liver was increased in diethylstilbestrol treated roosters (Vigo and Vance, 1981b). Thus, estrogen-mediated increases in PEMT activity in humans could be an explanation for the lower dietary choline requirements of premenopausal women.

The mechanism whereby estrogen increases PEMT activity is not known. In the present study, we report that estrogen treatment up-regulates the expression of this gene in both mouse and human primary hepatocytes, with resulting increases in PEMT enzyme activity and that there are motifs for estrogen response elements (EREs) in the promoter region(s) of the *PEMT* gene.

METHODS AND METHODS

Materials. All reagents were obtained from Fisher Scientific (Fair Lawn, NJ), unless otherwise noted.

Animals. All animal procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Male C57BL/6J mice (5-7 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME) were housed individually in

cages in a climate-controlled room (24°C) exposed to a 12-hr light cycle, fed AIN76A semipurified diet (Dyets, Bethlehem, PA) and water *ad libitum*.

Primary mouse hepatocyte isolation. Mice were anesthetized (200 mg ketamine/kg and 16 mg xylazine/kg) by subcutaneous injection, and the livers harvested. Hepatocytes were isolated from the livers by a modification of the method described previously (Morita et al., 1995). Briefly, the liver was perfused, through a needle aligned along the inferior vena cava, with buffer (pH 7.5) containing 137 mmol/L NaCl, 5.4 mmol/L KCl, 0.5 mmol/L NaH₂PO₄, 0.42 mmol/L Na₂HPO₄, 10 mmol/L HEPES, 0.5 mmol/L EGTA, 4.2 mmol/L NaHCO₃, and 5 mmol/L glucose; collagenase buffer, pH 7.5, contained 137 mmol/L NaCl, 5.4 mmol/L KCl, 5 mmol/L CaCl₂, 0.5 mmol/L NaH₂PO₄, 0.42 mmol/L Na₂HPO₄, 10 mmol/L HEPES, 0.15 g/L collagenase B (Boehringer Mannheim Corp, Indianapolis, IN), 0.05 g/L trypsin inhibitor, 4.2 mmol/L NaHCO₃, and 0.016 mmol/L phenol red. The collagenase-perfused liver was then dissected, suspended in Hanks' solution (30 mL), and filtered through cheesecloth and a 100- μ m nylon membrane to remove connective tissue debris and cell clumps. Hepatocytes were subjected to centrifugation (42 x g, 2 min at 4° C) and resuspended in Hanks solution, this was repeated 4x. Then hepatocytes were purified using density gradient centrifugation (45% Percoll solution, 42 x g for 10 min at 4° C). Cell viability, measured by trypan blue exclusion, was more than 90%.

Preparation of estradiol-free serum for hepatocyte culture. Charcoal (Norit A, acid washed, Sigma-Aldrich, St. Louis, MO) was washed twice with cold sterile water immediately before use. A 5% charcoal-0.5% dextran T70 (Pharmacia-LKB, Uppsala, Sweden) suspension was prepared, and aliquots were centrifuged at 1600 x g for 10 min.

Supernatants were aspirated, and fetal bovine serum (Biomed, Foster City, CA) was mixed with the charcoal pellets. This charcoal-serum mixture was kept in suspension by rolling at 4 cycles/min at 37°C for 1 hour. After centrifugation at 1600 x g for 20 min, the supernatant was passed through a 0.45µm filter (Nalgene, Rochester, NY). The charcoal stripped serum was then stored at -20°C until needed. More than 99% of serum sex steroids are reported to be removed by this treatment (Soto and Sonnenschein, 1985).

Murine hepatocyte culture. Primary mouse hepatocytes were maintained in DMEM/F12 medium (GIBCO- BRL, Carlsbad, CA), supplemented with 10% fetal bovine serum (Biomed, Foster City, CA) and antibiotics (100 U/ml penicillin and 100 µg/ml of streptomycin; Sigma-Aldrich, St. Louis, MO), at 37 °C in humidified air containing 5% CO₂. Briefly, 1.5 X 10⁶ cells were seeded onto 60 mm culture dishes (BDBiosciences, Franklin Lakes, NJ), incubated in fresh medium for 24 h and then washed with 1 × phosphate buffered saline (PBS) twice before further incubation in medium which contained 90% phenol red-free DMEM/F12 supplemented with 10% charcoal stripped E2-free serum for 48 h. This medium was used to effect growth factor deprivation (Berthois et al., 1986). Cells then were treated with 0-100 nmol/L 17-β-estradiol (prepared and stored in phenol red-free DMEM/F12 at 73 µmol/L per manufacturer's instructions; Sigma-Aldrich, St-Louis, MO). After 24 or 48 h incubation, cells were harvested for total RNA and separately for the preparation of the microsomal protein fraction for PEMT activity assay, respectively.

Murine Pemt Relative real-time mRNA quantification. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Primers and probes for murine *Pemt* and *β-actin* were designed using

JaMBW software (<http://www.bioinformatics.org/JaMBW>; **Table 2-1**). The *Pemt* probe was labeled with a reporter dye (FAM, 6-carboxyfluorescein) at the 5' end and a quencher dye (TAMRA, 6-carboxytetramethylrhodamine) at the 3' end. The β -actin probe was labeled with a reporter dye (TET, tetramethylrhodamine) at the 5' end. Quantitation of *Pemt* and β -actin mRNA levels was performed by a real-time RT-PCR assay using an ABI prism 7700 sequence detection system (ABI, Foster City, CA). A 30 μ L reaction mixture contained 500 ng of total RNA and 0.5 μ mol/L of each primer. The reaction conditions were designed as follows: reverse transcription (RT) at 48°C for 30 min and initial denaturation at 95°C for 10 min followed by 40 cycles with 15s at 95°C for denaturing and 1 min at 60°C for annealing and extension. Relative quantification of *Pemt* mRNA expression was calculated by the comparative threshold (Ct) method described elsewhere (Livak and Schmittgen, 2001). The relative quantification value of the target gene, normalized to an endogenous control gene and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (Power/folds), where $\Delta Ct = Ct$ of target gene (*Pemt*) – Ct of endogenous control gene (β -actin), and $\Delta\Delta Ct = \Delta Ct$ of samples for target gene (estrogen treatment) - ΔCt of the calibrator (without estrogen treatment, negative control) for the *Pemt* gene. Final results are expressed as mean ratio of *Pemt* expression for each estrogen treatment normalized to *Pemt* gene expression without estrogen.

Murine Pemt Immunoblot. 50 μ g of protein was separated by SDS-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Sigma-Aldrich, St-Louis, MO), which was probed with anti-*Pemt* antibody (a kind gift from Dr. Dennis E. Vance), washed extensively, and then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL).

Table 2-1. Real-Time PCR primers and probes used for gene expression analysis.

Primers and probes for murine *Pemt* and β -*actin* were designed using JaMBW software (<http://www.bioinformatics.org/JaMBW>). The *Pemt* probe was labeled with a reporter dye (FAM, 6-carboxyfluorescein) at the 5' end and a quencher dye (TAMRA, 6-carboxytetramethylrhodamine) at the 3' end. The β -actin probe was labeled with a reporter dye (TET, tetramethylrhodamine) at the 5' end. PCR primers for human *PEMT*, Proteinase Inhibitor-9 (*PI-9*; positive control) and Tata-box Binding Protein (*TBP*; housekeeping gene) were designed using online Gene Fisher interactive primer design software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher/>). ^aPrimer binds all three *Pemt* transcript variants.

Gene	GenBank Accession #	Forward Primer	Reverse Primer	bp	Murine Gene Expression Probe
<i>Pemt</i> ¹	NM_008819	ACTCATGCATG CTAGTCCA	AGCAGTGAAGGG CTCTTCAT	178	5'-FAM-CGAGACAATTG CCACCAGCACGT-3'
<i>β-actin</i>	NM_007393	CTGCCTGACG GCCAAGTC	CAAGAAGGAAGG CTGGAAAAA	220	5'-TET-CACTATTGGCA ACGAGCGGTTCCG-3'
<i>PEMT</i> ¹	NM_148172	AAGACCCGCAA GCTGAGA	AGTACATGGGGT TGTCAGCA	298	Not applicable
	NM_007169				
	NM_148172				
<i>TBP</i>	NM_003194	TTCGGAGAGTT CTGGGAG	TGGAAGTTCTT CACTCTTGC	227	Not applicable
<i>PI-9</i>	L40378	GTGGCAGGCC CTGCATCA	CACCCTTTATGG CGATGA	271	Not applicable

Pemt protein was visualized by a reaction with Supersignal chemiluminescent substrate (Pierce) and exposed to X-ray film (Denville Scientific, Metuchen, NJ).

Human hepaocyte isolation. Primary human liver cells were provided as a gift by Admet Technologies (Durham, NC). They used donated livers not suitable for orthotopic liver transplantation obtained from federally designated organ procurement organizations. Informed consent was obtained from next of kin for use of the livers for research purposes. Isolation of hepatocytes was performed by members of the Admet Technologies research team as described previously (LeCluyse et al., 2005). These hepatocytes were transferred to us and used within 96 h of isolation.

Human hepatocyte culture. Cells were plated at a density of approximately 1.8×10^6 cells per well on collagen-coated 6-well culture plates (BDBiosciences, Franklin Lakes, NJ) and incubated for 6 h at 37°C in humidified air containing 5% CO₂ in William's complete medium E (WCME; GIBCO-BRL, Carlsbad, CA) containing 10% Dextran-treated charcoal stripped fetal bovine serum (Biomeda, Foster City, CA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Sigma-Aldrich, St-Louis, MO). Medium was replaced at 6 h with serum-free WCME. Following a 48 h “recovery period” in serum-free WCME, human primary cells were incubated for 24 h in WCME with 0-1000 nmol/L 17-β-estradiol (prepared and stored per manufacturer’s instructions). After 24 or 48 h incubation, cells were harvested for cytoplasmic RNA and the total cellular protein fraction for the PEMT activity assay, respectively.

Human PEMT mRNA quantification. Cytoplasmic RNA from liver cells was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). PCR primers for human *PEMT*, Proteinase Inhibitor-9 (*PI-9*; positive control) and Tata-box Binding Protein (*TBP*;

housekeeping gene) were designed using online Gene Fisher interactive primer design software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher/>; Table 1) and purchased from Operon (Germantown, MD). Real-time PCR reactions were performed using 100 ng RNA, 0.5 μ mol of each primer, and the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) in a 40 μ L reaction. The reaction conditions were designed as follows: RT at 50°C for 30 min and PCR initial activation step at 95°C for 15 min. Initial denaturation at 94°C for 15 sec was followed by 40 cycles of annealing (58°C for 30 sec) and extension (72°C for 30 sec). Changes in PEMT mRNA levels were detected using an iCycler iQ Real-Time PCR Detection System (BioRad, Hercules, CA). Relative quantification of PEMT mRNA expression was calculated by the Ct method as described earlier and final results expressed as ratio change relative to untreated and normalized for Tata-Box Binding Protein (TBP). We used expression of the protease inhibitor 9 (PI-9) gene, known to be estrogen responsive (Kanamori et al., 2000), as a positive control (data not shown).

PEMT protein expression and activity assay. Mouse and human hepatocytes were harvested 48 h after 17- β -estradiol treatment with two washes of 1X PBS, scraped into Medium 1 (250 mmol/L sucrose, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, and 1 mmol/L dithiothreitol) and homogenized with 10 complete strokes in a Teflon-glass homogenizing vessel. For the murine cellular homogenate, an aliquot of total cellular homogenate was then centrifuged at 100,000 x g for 1 h to obtain total particulate (membrane) fraction. 50 μ g of total murine particulate protein was used for PEMT activity assays as described previously using phosphatidylmonomethylethanolamine (PDME; Avanti Polar Lipids, Alabaster, AL) as the methyl acceptor and S-adenosyl-L-

[*methyl*-³H] methionine (American Radiolabeled Chemicals Inc, St. Louis, MO) as the methyl group donor (Audubert and Vance, 1983).

Comparative bioinformatics promoter analysis:

CAGE analysis. CAGE analysis (Cap Analysis Gene Expression) data was retrieved from the FANTOM3 website (<http://fantom.gsc.riken.go.jp/>). CAGE analysis (Shiraki et al., 2003) was used to identify the major transcription start sites (TSS) for the mouse and human PEMT gene.

EST abundance of various PEMT transcripts. The positions of expressed sequence tag (EST) sequences from the NCBI dbEST (<http://www.ncbi.nlm.nih.gov/dbEST/>; last accessed on 01/03/07) were displayed relative to the mouse and human PEMT gene of the Mouse and Human genome (May 2004 release) using the GenomBench tool of Vector NTI 10.1 (Invitrogen, Carlsbad, CA). All potentially full length ESTs were selected and found to group into three clusters, differing in the 5' start of the ESTs for both human and mouse.

Identification of Evolutionarily Conserved Regions (ECRs) and conserved transcription factor binding sites. The analysis of human and mouse syntenic relationships and conservation profiles was done through the annotation of ECRs in the alignments of genomes. We employed the BLASTZ-based genome alignments generated by the ECR Browser (<http://ecrbrowser.dcode.org>; last accessed 01/03/07) (Ovcharenko et al., 2004). A genomic interval was annotated as an ECR if it was >100 bp and >70% identity as defined by the number of nucleotide matches in a sliding window (default settings). Prediction of potential conserved transcriptional binding sites was then done using the

rVista 2.0 search tool within the ECR Browser. The Transfac V10.2 database was used to scan these ECRs using default settings.

Estrogen Response Element identification. EREs were identified with detection parameters set on the basis of optimized settings for the Dragon ERE Finder (<http://sdmc.lit.org.sg/ERE-V2/index>; last accessed 01/03/07) using the default 83% sensitivity (Bajic et al., 2003). Conserved EREs were identified as elements present in both the human and mouse PEMT gene at distances comprised between -10 to +15 kb from their respective transcription start sites (TSS A, TSS B, TSS C)

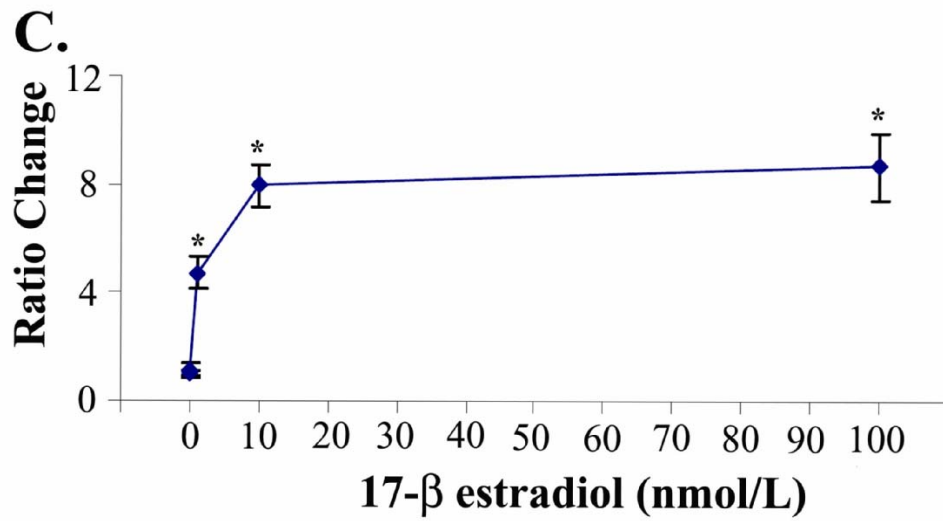
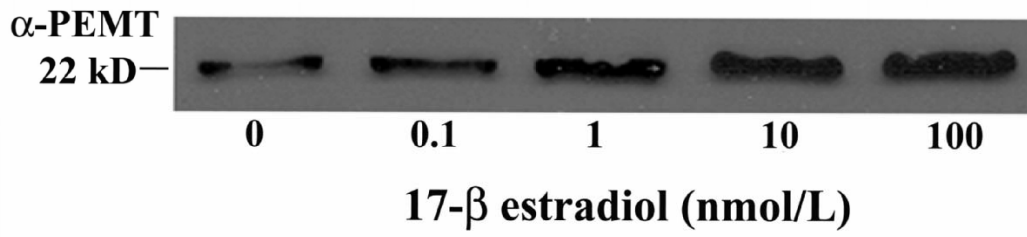
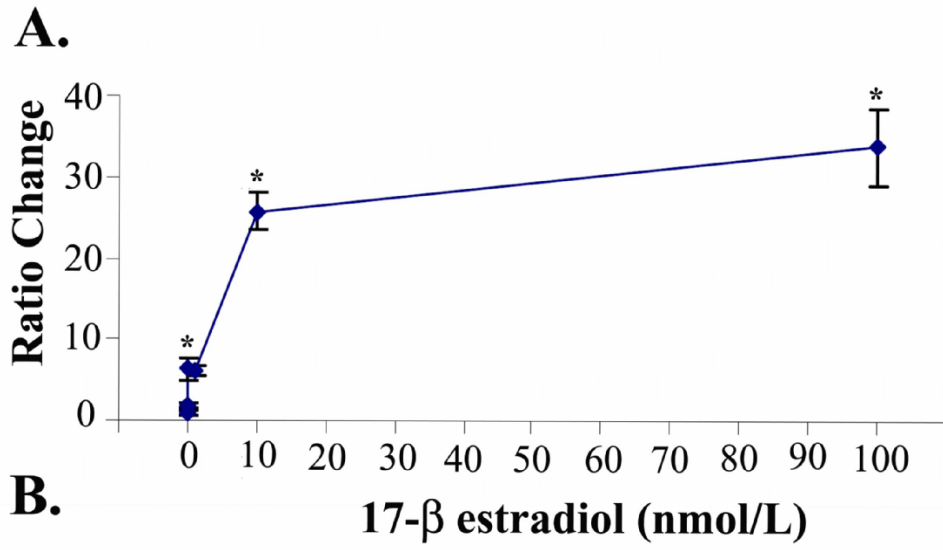
Data Analysis: Prior to analysis, all data was normalized to the 0 nmol/L estrogen treatment for each individual (mouse and human). Data were expressed as mean ratio change of each estrogen treatment versus no treatment \pm SE. Ratios were transformed to a log(2) scale to ensure normal distribution of the data, required by the parametric testing assumptions. Protein expression and gene expression significance of change statistical differences were assessed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer ($P < 0.05$) multiple comparison test (JMP Version 3.2, SAS Institute Inc, Cary, NC). Logistic regression analysis was performed to assess the trend significance of the dose response to estrogen (JMP Version 3.2), using ratio values. Linear Fit was used to determine the correlation between PEMT gene expression and activity, using only the points that have both gene expression and activity data.

RESULTS

Estrogen up-regulates murine Pemt gene expression, protein levels and enzyme activity.

In mouse hepatocytes gene expression, protein expression and activity all were significantly increased by 17- β -estradiol treatment (**Figure 2-1**) and there was a strong

Figure 2-1 Estrogen induces Pemt gene and protein expression and increases enzyme activity in primary cultured mouse hepatocytes. Primary mouse hepatocytes were incubated with 17- β -estradiol (0-100 nmol/L) for 24 h before harvesting. **A**, Detection of Pemt and β -actin mRNA levels was performed by a real-time RT-PCR assay. Relative quantification of Pemt mRNA expression was calculated by the comparative threshold cycle (Ct) method as described in "*Experimental Procedures*". Results are expressed as mean ratio change in gene expression \pm SEM. (n=3/point). *= P <0.05 different from no treatment. **B**, Total particulate protein was isolated and analyzed for PEMT protein expression by Western blotting as described in "*Experimental Procedures*". Equal protein loading of lanes was assessed by Coomassie Blue gel staining (data not shown). **C**, Pemt enzyme activity was assayed by a radio-enzymatic assay as described in "*Experimental Procedures*". Results are expressed relative to no treatment as mean ratio change \pm SEM. (n= 3/point). *= P <0.05 different from no treatment.



dose-response relationship for both activity (EC50= 1nmol/L; p<0.0001) and gene expression (EC50= 5 nmol/L; p<0.0001). Additionally, there was a strong correlation between gene expression and activity (correlation coefficient = 0.91).

Estrogen up-regulates human PEMT gene expression and enzyme activity. In human hepatocytes gene expression and activity were significantly increased by 17- β -estradiol treatment (**Figure 2-2**) and there was a strong dose-response relationship for both activity (EC50= 1nmol/L; p<0.0006) and gene expression (EC50= 10 nmol/L; p<0.0001) using logistic regression analysis. Gene expression and activity further increased at 1000 nmol/L estrogen treatment (data not shown). However, a linear fit model suggests that there is only a weak correlation between PEMT gene expression and activity (0.34).

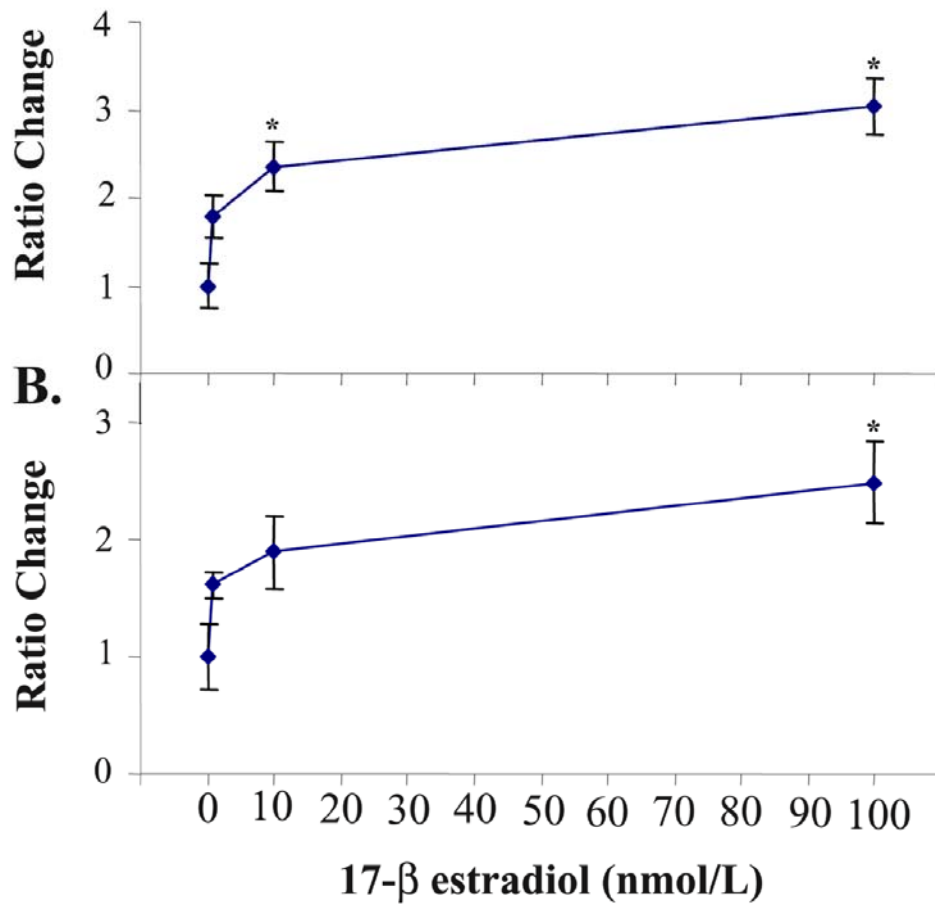
Characterization of the Major Human and Mouse PEMT transcripts. The human *PEMT* gene, which is located on chromosome 17p11.2, has 9 exons and 8 introns spanning its 86 kb length, and it encodes three variant transcripts

(http://www.ensembl.org/Homo_sapiens/geneview?gene=OTTHUMG00000059290;db=vega, date last accessed 1/02/07). Each of the three transcript variants has only 7 exons and 6 introns due to splicing of the leading exons 1-3, which are alternatively spliced to a common exon 4, where translation begins. (Shields et al., 2001). Three RefSeq accessions have been annotated for the human *PEMT* gene by NCBI, NM_148172, NM_007169, and NM_148173. Murine *Pemt*, located on chromosome 11, has been reported to have 7 exons and 6 introns spanning approximately 75 kb and encoding one major transcript, annotated by NCBI RefSeq accession NM_008819.

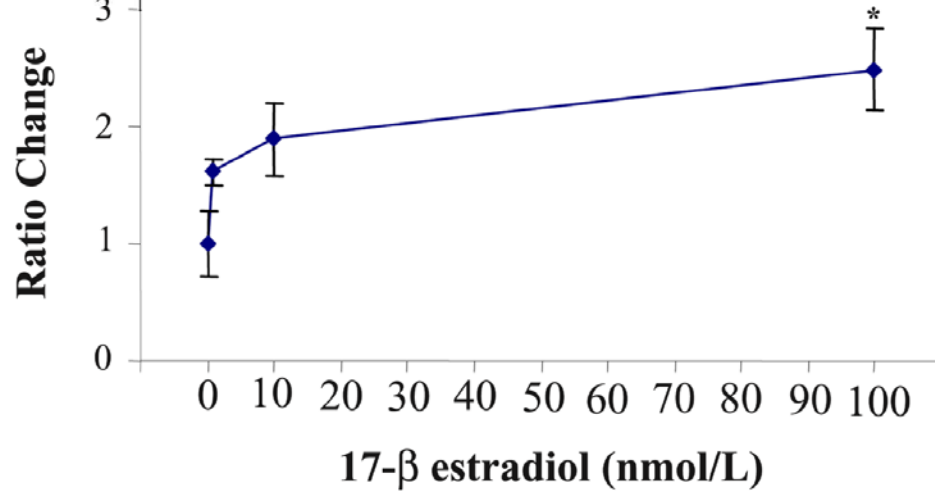
(http://www.ensembl.org/Mus_musculus/geneview?gene=OTTMUSG00000005808;db=vega, date last accessed 1/02/07) (Walkey et al., 1996). Primer extension analysis of the

Figure. 2-2 Estrogen induces *PEMT* gene expression and increases enzyme activity in primary cultured human hepatocytes. Primary human hepatocytes from various donors were incubated with 17- β estradiol (0-100 nmol/L) for 24 h before harvesting. **A**, Detection of *PEMT* and Tata-Box Binding Protein (TBP) mRNA levels was performed by a real-time RT-PCR assay. Relative quantification of *PEMT* mRNA expression was calculated by the comparative threshold cycle (Ct) method described in “*Experimental Procedures*”. Results are expressed as ratio fold change in gene expression relative to untreated samples \pm SEM. (n= 4-5/point). **B**, *PEMT* enzyme activity was assayed by a radio-enzymatic assay as described in “*Experimental Procedures*”. Results are expressed as ratio change in enzyme activity (pmol/mg protein/min) relative to untreated samples \pm SEM. (n= 4-7/point). *= $P < 0.05$ different from no treatment.

A.



B.



murine *Pemt* gene demonstrated experimentally that the major *Pemt* TSS is located 139 bp upstream of the initiator methionine codon (Walkey et al., 1996). Human and murine PEMT proteins are 80% homologous, but unlike its human counterpart, murine Pemt protein translation initiation begins in exon 2. It was previously reported that the transcript that begins with exon 2 [NM_007169] is the most abundant in human liver and that only one transcript [NM_008819] exists for the mouse *Pemt* gene (Shields et al., 2001; Walkey et al., 1996).

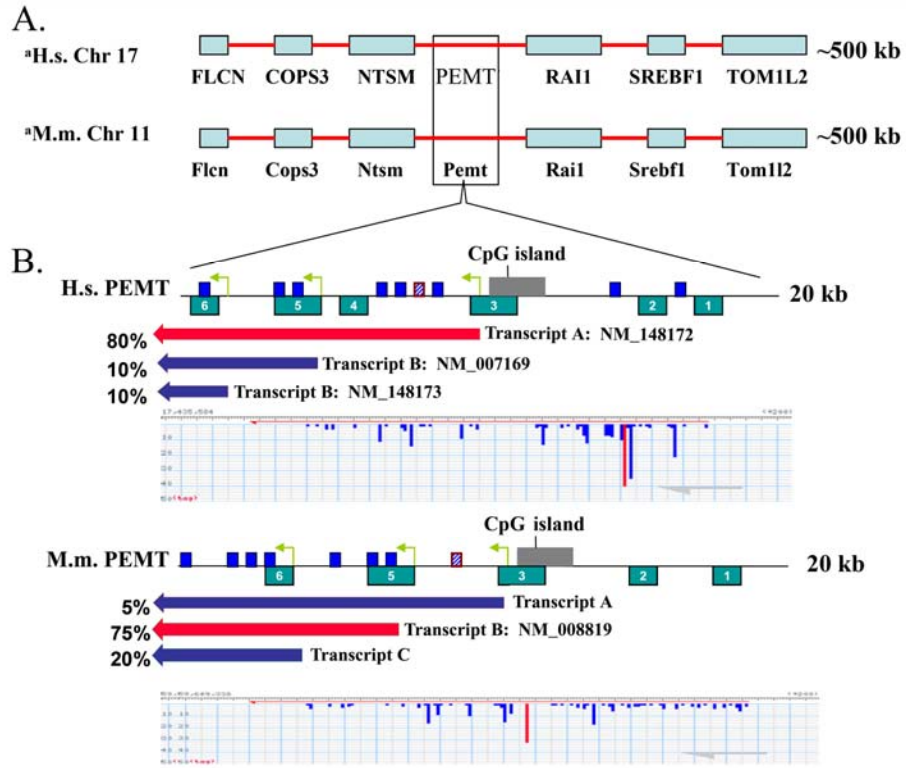
To more fully characterize the various alternative TSS in the human and mouse PEMT orthologs, we estimated the relative abundance of the major PEMT transcripts in human and mouse liver based on the ESTs in dbEST and used FANTOM3 CAGE analysis viewer to ascertain the location of the various TSS (**Figure 2-3**). Using publicly available data from dbEST we found that the vast majority of the 5' ends of the ESTs cluster around the start point of NM_148172 (bp 17,435,719 of ch. 17) (denoted as human TSS A), suggesting that this is the most abundant transcript in the liver for the human *PEMT* gene. This TSS is independently confirmed by the results of the FANTOM CAGE analysis where the representative CAGE TSS for the *PEMT* gene is at bp 17,435,731, 12 bp upstream of the RefSeq defined start. For NM_007169 (start defined at bp 17,426,470) (denoted as human TSS B), a representative CAGE TSS is found 40 bp upstream at bp 17,426,510. For NM_148173 (start defined at bp 17,421,504) (denoted as human TSS C), a representative CAGE start is found at bp 17,421,107 while the start based on the average of ESTs is 17,421,043; both are several hundred basepairs downstream of the RefSeq defined start.

Figure 2-3. Comparative analysis of murine and human *PEMT* gene promoters.

A. 500 kbp alignment of conserved syntenic blocks of human chromosome 17 and mouse chromosome 11, with blue boxes showing the relationships between orthologous genes.

B. A 20kbp enlargement of the human *PEMT* gene encoded on the minus strand, the promoter region and the mouse orthologous region. The *PEMT* gene promoter region of the human and mouse contains six distinct evolutionarily conserved regions (ECR 1-ECR 6). ECR 4 in murine *Pemt* has been translocated to a different chromosome (data not shown). There are three conserved estrogen response elements within ECR 5 and 6, shown as dark blue boxes, and a perfect consensus ERE in a distal promoter/enhancer region approximately 7.5kb from transcription start site (TSS) B, shown as hatched blue boxes with red outline. Block arrow bars displayed beneath *PEMT* gene promoter regions represent the major transcripts relative to TSS A, B, C, (indicated by yellow-green arrows) with the percentages reflecting the EST abundance of each transcript in liver (purple arrows indicate minor transcripts; red indicates the major transcript).

Beneath the transcript arrow bars is shown a representative CAGE analysis for the major human and mouse TSS. The highest peak, shown in red, represents the predominant TSS for the *PEMT* gene. Lower peaks around the major TSS (s) would represent some variation in the actual *in vivo* TSS. ^aMotif Abbreviations for gene names are from LocusLink (www.ncbi.nlm.nih.gov/LocustLink/list.cgi; last accessed 01/02/07



We also suggest that the mouse *Pemt* gene has two previously unreported transcripts (denoted murine transcripts A and C) in addition to the previously described transcript, NM_008819 (denoted murine transcript B), which is also the most abundant transcript based on the abundance of ESTs in dbEST. The majority of the 5' ends of the available ESTs (the EST average start; 59,649,465) cluster around the start point of NM_008819 (bp 59,649,379 of ch. 11) (denoted murine TSS B), while CAGE analysis predicts the representative TSS at bp 59,649,447, which is 68 bp upstream of the RefSeq defined start for NM_008819. Available EST data suggest two additional mouse transcripts, transcript A, initiating approximately 9 kp upstream of NM_008819 and transcript C, initiating approximately 5 kb downstream. A representative CAGE TSS for transcript A is at 59,659,083, denoted murine TSS A, while the EST average start suggests a start at 59,658,975, within 108 bp of the CAGE start. For transcript C a representative CAGE TSS is at 59,644,334, denoted murine TSS C, and with the EST average start position is 59,644,300. Thus, based on both EST sequences from dbEST and from CAGE analysis from the FANTOM3 database we conclude that both the mouse and human *PEMT* genes have three unique transcription start sites which are indicative of multiple promoters. While the identification of two additional transcripts in the mouse gene suggests conserved transcriptional regulation between species, it is interesting to speculate why transcript variant B (NM_008819) is the more abundant transcript in mice whereas transcript variant A (NM_148173) is more abundant in humans.

Initial analysis of the human *PEMT* putative promoter B sequence for the exon-2 containing transcript, NM_007169, suggested that it contained transcription factor binding sites critical for SREBP and C/EBP; however, promoter B lacks core promoter

elements such as a TATA or CCAAT boxes (Shields et al., 2001). We report that the region upstream of putative promoter A, exon-1 containing and the most abundant transcript variant NM_148172, contains a CpG island as well as core promoter elements including an experimentally validated 600 bp RNA polymerase II binding site (Pol 9419, $p < 0.00001$) (**Figure 2-3, Table 2-2**)(Carroll et al., 2006).

Table 2-2. Human and Mouse Conserved Promoter Motifs. For both human and mouse *PEMT* genes motif chromosomal position is displayed relative to transcription start site B (TSS B) (+1) based on the literature defined major TSS for each (Shields et al., 2001; Walkey et al., 1996); +/- indicates upstream or downstream orientation, respectively. For the human +1 site, this corresponds to bp 17,426,514 of ch17. For mouse this corresponds to bp 59,649,481 on ch11 (UCSC May 2004 Release of the *Mus musculus* genome, mm5) which corresponds to bp 59,853,136 in the current release of the *M. musculus* genome (mm8). The mouse and human *PEMT* gene promoters are highly conserved in six distinct evolutionarily conserved regions (ECR 1-ECR 6). ECR 4 in mouse *Pemt* has been translocated to a different chromosome (data not shown). There are conserved estrogen response elements and transcription factor binding sites within ECR 5 and 6 in mouse and human proximal promoter regions. The human and murine *PEMT* gene contains additional EREs including a consensus ERE in a distal promoter/enhancer region approximately 7.5kb from TSS-B. The human promoter A region contains a CpG island and an experimentally validated 600 bp RNA polymerase II binding site (Pol 9419, p<0.00001) (**Figure 2-3, Table 2-2**) (Carroll et al., 2006). ECR browser, rVista TFBS search engine, Dragon ERE Finder, and TRANSFAC were used to identify ECRs, EREs, and TFBS respectively. Each evolutionarily conserved region is outlined and double-underlined, italicized sequences correspond to nucleotides that differ from the estrogen response element consensus sequence. Underlined, italicized letters indicate transcription start sites for each proximal promoter region denoted A, B, C.

^aMotif Abbreviations for gene names are from LocusLink
(www.ncbi.nlm.nih.gov/LocusLink/list.cgi; last accessed 01/02/07)

Evolutionarily Conserved Region	^a Motifs	Position	Sequence	
<i>Human</i>				
ECR 1 [-13399-13206]	PPARA	-13233	TGATCT	
	SP1	-13118	CCACAGCCCC	
ECR 2 [-10919-10748]	ERE	-11388	TT-GGTCA-GGC-TGGTC-TT	
	MYB	-10839	GGCCAGTTC	
ECR 3 [-9246-9038]	ERE	-10266	TT- <u>AG</u> TCA-CGC-TGGTC-TC	
	Pol II binding site	-9038	(bp 17,435,552-17,436,153)	
	CpG island	-8774	CpG Repeat	
	Exon 1	-9151		
Transcript A: NM_148172	TSS A	-9205	TTCCGGGGG (bp 17,435,719)	
	ERE	-7647	GG-GGTCA-TGA-T <u>C</u> ACC-TC	
Consensus	ERE	-7401	CG-GGTCA-GGG-TGACC-CT	
Transcript B: NM_007169	ERE	-6707	GA-G <u>AC</u> CA-GCC-TGACC-AA	
	ERE	-5879	CG-GGGTA-TCT-TGACT <u>G</u> C	
	ECR 4 [-3324-2994]	FOXA1	-3309	AAGTTGTTCCATT
		MYB	-3264	AAACTGCCA
		SOX6	-3190	TGCATTGTTATCA
		GATA	-3186	TGTTATCATT
	SP1	-862	GTGGCGTGAT	
	AP1	-386	CTGACTCCT	
	AP1	-206	CCCGAGTCAGC	
	ECR 5 [-35-1073]	TSS+1	+1	TTGTCCATG (bp 17,426,514)
TSS B		+40	GACCACAA (bp 17,426,470)	
Exon 2		+50		
C/EBP		+175	AAATTACCA	
AP1		+631	CATTAGTCATT	
ERE		+725	TG- <u>AG</u> ACA-GGC-TGACC-TG	
Transcript C: NM_148173	ERE	+741	GA-GG <u>C</u> CA-TTG- <u>GG</u> ACC-TG	
	ECR 6 [+5192-5579]	TSS C	+5010	TGTGGGCGA (bp 17,421,504)
		GATA	+5284	TTTTATCTTC
ERE	+5476	TG-GG <u>C</u> TA-CGT- <u>GG</u> ACC-CC		
<i>Mouse</i>				
Transcript A	ECR 1 [-12997-12808]	Ppar- α	-12,980	AGATCA
		Sp1	-12,884	CCCACC
Transcript A	ECR 2 [-11,874-11,709]	Ppar- α	-11,788	AGGTCA
		TSS A	-9602	CATCAGATA (bp 59,659,083)

	Evolutionarily Conserved Region	^a Motifs	Position	Sequence
Transcript B: NM_008819	ECR 3 [9592-9382]	CpG island	⁻ 9622- ⁻ 9169	CpG repeats
	Consensus	ERE	⁻ 7919	TA-GGTC A-GGA-TGACC-TT
	ECR 5 (⁻ 190- ⁺⁸⁸⁴)	TSS B (⁺¹)	⁺¹	CCCAGTGTG (bp 59,649,481)
		TSS B	⁺¹¹⁰	TTCC TTCTG (bp 59,649,379)
		Ap1	⁺⁴²⁹	CGTTAGTCACT
		ERE	⁺⁷⁹⁰	TG- <u>AGG</u> CA-GGC-TGACC-AG
		ERE	⁺⁸¹³	CA-GGG <u>CA</u> -CGG- <u>GG</u> ACC-TG
		ERE	⁺⁵¹⁵⁰	TT- <u>AGT</u> CA-TGT-TGG <u>CT</u> -GC
Transcript C	ECR 6 (⁺⁴⁸⁴⁸ - ⁺⁵²⁴³)	Gata	⁺⁵⁴⁴¹	CTTTTATCTTC
		TSS C	⁺⁵¹⁴⁷	GCTGATCTC (bp 59,644,334)
		ERE	⁺⁵¹⁹²	TG-GGT <u>TA</u> -CAT- <u>GG</u> ACC-CC
		ERE	⁺⁵⁷⁸⁰	GG- <u>TGT</u> CA-AGG-TGACC-TA
		ERE	⁺⁵⁸⁶⁰	AA-G <u>ACCA</u> -CTG-TGACC-TC
		ERE	⁺⁷⁹⁶¹	CA-GGT <u>GG</u> -GCC-TGACC-CT

Identification of an evolutionarily shared promoter framework in the PEMT gene. In orthologous promoters, elements important for transcriptional regulation may be expected to be conserved during evolution (Elnitski et al., 2003). Evolutionarily conserved regions (ECRs) were identified from two species (*Homo sapiens* and *Mus musculus*) in the PEMT promoter(s) by using the comparative genomics tool ECR Browser (as described in “*Experimental Procedures*”). We determined that the *PEMT* gene promoters contain six evolutionarily conserved regions within the region encompassing the three TSS predicted for the PEMT gene in each species (**Figure 2-3, Table 2-2**). The region containing this cluster of ECRs is approximately 18 kb for each organism with ECR 6 overlapping the TSS of the shortest predicted transcript for each and ECR 1 being 4 kb upstream of NM_148172 (human) and 9.7 kb of transcript A (mouse). No other ECRs were found in the 15 kb upstream of ECR 1 in the mouse or within 29 kb of the human ECR 1. Within these conserved regions predicted transcription factor binding sites are likely to be conserved (**Table 2-2**).

Approximately 1% of previously identified EREs in human genes have counterparts at similar positions in their mouse orthologs. (Bourdeau et al, Mol Endocrinol, June 2004, 18(6):1411–1427). Since conservation of EREs may indicate a functional role of these elements, we searched for the presence of conserved EREs with less than 9 kb difference in distance from their respective transcriptional start sites. For estrogen binding-site predictions we used the previously described ERE model (Bajic et al., 2003) and Ap1 and Sp1 binding-site position weight matrices from the TRANSFAC database (Heinemeyer et al., 1999). We identified one perfect consensus estrogen response element approximately 7.5 kb from transcription start site B, TSS (+1), in both

the human and murine *PEMT* promoters. In the murine *Pemt* gene promoter region we identified seven imperfect EREs differing from the consensus by 1-3 nucleotides, three of which are located in ECRs in close proximity to TSS B and TSS C (**Figure 2-3, Table 2-2**). The human *PEMT* promoter region contains eight imperfect estrogen response motifs, three of which occur in an evolutionarily conserved region in close proximity to Promoter B (TSS B) and Promoter C (TSS C) (**Figure 2-3, Table 2-2**). We also found several AP-1, SP-1 and FOXA1 sites in these evolutionarily conserved regions.

DISCUSSION

Choline is derived not only from the diet, but as well from *de novo* synthesis of phosphatidylcholine catalyzed by PEMT (Zeisel, 2006). We have previously reported that when deprived of dietary choline, men and postmenopausal women are more likely to develop fatty liver or muscle damage compared to pre-menopausal women (Fischer et al., 2007). In the present study, we observed that estrogen, at doses bracketing physiological concentrations in humans (0-100 nmol/L), caused a marked up-regulation in *PEMT* mRNA expression and enzyme activity, and that the *PEMT* gene has motifs (Walter et al., 1985) that may act as EREs in its promoter regions. This observation may help to explain why premenopausal women usually do not develop organ dysfunction when fed a diet low in choline (Fischer et al., 2007); they have estrogen-induced increased capacity for endogenous biosynthesis of the choline moiety.

Pregnancy and lactation are times when demand for choline is especially high. Indeed, transport of choline from mother to fetus (Sweiry et al., 1986; Sweiry and Yudilevich, 1985) depletes maternal plasma choline in humans (McMahon and Farrell, 1985). Thus, despite an apparent enhanced capacity to synthesize choline, the demand

for this nutrient is so high that stores are depleted (Zeisel et al., 1995). Because milk contains a great deal of choline, lactation further increases maternal demand for choline resulting in further depletion of tissue stores (Holmes-McNary et al., 1996; Zeisel et al., 1995). *Pemt*^{-/-} mice abort pregnancies around 9-10 days gestation unless fed supplemental choline (personal observation). Women in the USA vary enough in dietary choline intake (from <300mg/d to >500 mg/d) to influence the risk that they will have a baby with a birth defect; low dietary intake of choline during pregnancy was associated with a 4-fold increased risk of giving birth to an infant with a NTD (Shaw et al., 2004) and 1.5-fold increased risk for orofacial clefts (Shaw et al., 2006). Choline nutrition during pregnancy is especially important because it influences brain development in the fetus (Albright et al., 1999a; Albright et al., 2005; Albright et al., 2001; Albright et al., 2003; Albright et al., 1999b; Albright et al., 1998; Craciunescu et al., 2003; Meck and Williams, 1997; Meck et al., 1988; Meck and Williams, 2003b; Mellott et al., 2004; Niculescu et al., 2006; Pyapali et al., 1998). These observations suggest that women depend on high rates of endogenous biosynthesis of choline induced by estrogen, as well as on dietary intake of choline to sustain normal pregnancy. It is biologically plausible that, during evolution, appropriate mechanisms were developed to assure that young women are less susceptible to dietary choline deficiency and have adequate stores of choline prior to, and during pregnancy.

Our observation that *PEMT* gene expression in mice and humans is induced by physiological concentrations of estrogen in humans (0-100 nmol/L) (Peck et al., 2002) correlates with the previous finding that estrogen increases PEMT activity in liver of the bird (Vigo et al., 1981; Vigo and Vance, 1981a, b), the rat liver (Young, 1971) and the rat

pituitary (Drouva et al., 1986; Drouva et al., 1987). The classic actions of estrogen occur through its receptors ER α and ER β which bind as homodimers or heterodimers to EREs in the promoters of many estrogen-responsive genes (Walter et al., 1985). The consensus ERE (PuGGTCAnnnTGACCPy) is an inverted palindromic sequence separated by three intervening nucleotides (Walter et al., 1985). This motif is usually surrounded by 50-nucleotide flanking regions that contain other transcription factor binding sites (Tang et al., 2004). Imperfect ERE half site motifs (ERE1/2) also bind with ER α and ER β (Agarwal et al., 2002; Lopez et al., 2002; Xie et al., 1999). It is noteworthy that the majority of known estrogen responsive genes contain imperfect EREs that differ from the consensus sequence (Driscoll et al., 1998). EREs are usually found within -10 to +5 kb of transcriptional start sites and approximately 1% of elements appear to be conserved in the flanking regions of orthologous human and mouse genes (Bourdeau et al., 2004). Functional evolutionarily conserved EREs are most abundant in the 0 to +1 kb region around transcriptional start sites (Bourdeau et al., 2004). Estrogen also can mediate its effect on proximal gene promoters from distances up to 10 kb by acting as an enhancer element (Bourdeau et al., 2004). This seems to be especially true when estrogen response motifs are located in the proximal gene region. EREs that are located far away from the promoter of a single gene can interact synergistically with EREs in the proximal promoter by DNA looping (Sathya et al., 1997).

In mice, we found three conserved EREs at position +799 bp and +822 bp downstream of the TSS B and +66 bp upstream of TSS C. In humans, we found three conserved EREs at positions +725 bp and +741 bp downstream of TSS B and +466 bp of TSS C. One of the ERE1/2 we found was embedded in an Alu repeat sequence. Alu

elements, the most abundant interspersed repeats in the human genome, have repeatedly been found to be involved in gene rearrangements in humans (Norris et al., 1995).

Ubiquitous presence of Alu repeats and their specific properties suggest a number of functions for the Alu elements, one of which is the introduction of functional estrogen response elements into gene promoters (Norris et al., 1995). Finally, not all genes that are regulated by estrogen contain an ERE. Estrogen may regulate these target genes through interactions with other transcription factors such as activator protein 1, AP-1 (Vigo et al., 1981; Vigo and Vance, 1981a, b), NF- κ B (Drouva et al., 1987), or specific factor, Sp-1 (Walter et al., 1985), and FoxA1 (Carroll et al., 2005; Laganieri et al., 2005). We found several AP1, FOXA1, and SP1 sites in mouse and in human conserved promoter regions as well as in non-conserved regions.

It was previously reported that the murine *Pemt* gene encodes one major transcript and that transcription was initiated from a single promoter (Walkey et al., 1996). Both EST and CAGE analysis suggests the existence of three transcripts for the murine *Pemt* gene and for its human ortholog. There is a cluster of ECRs within the putative promoter region containing the multiple TSS for each species and many conserved predicted binding sites for transcription factors within these ECRs and throughout the sequence analyzed above. Based on multiple similarities in gene and protein structure, it is reasonable to predict that the murine and human *PEMT* genes are regulated in a similar manner.

This report is the first to identify a possible mechanism through which estrogen induces *PEMT* expression and may explain the observation that premenopausal women are relatively resistant to choline deficiency. Future studies may include measuring the

abundance of the three *PEMT* transcripts in human and murine hepatocytes cultured in the presence and absence of 17- β -estradiol in order to determine whether estrogen treatment causes alternative promoter use and/or splicing. We are currently determining whether recently identified single nucleotide polymorphisms that increase women's dietary requirement for choline (da Costa et al., 2006) work by altering estrogen-mediated induction of the *PEMT* gene.

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CHAPTER III

***PEMT* POLYMORPHISMS AFFECT ESTROGEN REGULATION OF CHOLINE BIOSYNTHESIS**

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ABSTRACT

Studies in mammals indicate a connection between estrogen status and resistance to choline deficiency syndrome (CDS), but the nature of this interaction is not understood. Synthesis by hepatically expressed phosphadylethanolamine N-methyltransferase (*PEMT*) constitutes the only *de novo* source of choline and is regulated by estrogen. In humans, pre-menopausal women are relatively resistant to CDS suggesting young women have an enhanced capacity to generate choline *de novo*. We identified a polymorphism highly associated ($p < 0.0001$) with risk of CDS in a subset of premenopausal women which falls within 1kb of a key estrogen receptor (ER) regulatory region in the *PEMT* gene. Hepatocytes homozygous for the risk allele are not estrogen responsive and exhibit decreased ER-DNA enrichment in the *PEMT* locus. We propose a model in which an allele-specific ablation of ER-DNA interactions in a key regulatory region in the *PEMT* locus leads to a decrease in *PEMT* gene expression, thereby conferring risk of CDS in women.

INTRODUCTION

Humans fed a choline deficient diet develop liver and muscle damage characterized by hepatosteotosis, liver ALT/AST enzyme leakage, elevated CPK levels, muscle cell apoptosis and lymphocyte cell death (subsequently referred to as choline deficiency-induced syndrome, CDS) (da Costa et al., 2006b; Li et al., 2005; Zeisel, 2006). Although choline deficiency syndrome is consistently observed in men and post-menopausal women, more than half of young women seem to be resistant to symptoms associated with low dietary choline. However, approximately 44% of young women do develop choline deficiency (Fischer et al., 2007; Zeisel, 2008). Synthesis of choline by liver, catalyzed by phosphadylethanolamine N-methyltransferase (PEMT) constitutes the only *de novo* source of the choline moiety. *PEMT* message is synthesized from three promoters which direct expression of two isoforms (Shields et al., 2001). Since endogenous production of choline may protect from CDS, we had previously examined the regulation of *PEMT* and demonstrated that *PEMT* is regulated by estrogen in human and mouse hepatocytes (Resseguie et al., 2007). Based on this finding, we hypothesized that premenopausal women may be protected from CDS by estrogenic regulation of *PEMT*. We examined the association of single nucleotide polymorphisms (SNPs) in the *PEMT* locus and found that premenopausal women harboring a SNP in the *PEMT* promoter are 25 times as likely to develop CDS as non-carriers (da Costa et al., 2006a).

Although we had demonstrated that estrogen can regulate *PEMT* in hepatocytes, the specific mechanism for this regulation remains unknown. Estrogen receptor mediates gene regulation by estrogenic hormones by directly interacting with DNA. In order to comprehensively identify these sites of estrogen receptor/DNA contact, chromatin

immunoprecipitated (ChIP) with anti-estrogen receptor antibody has been analyzed by DNA microarrays (chip) (ChIP-on-chip) (Carroll et al., 2006; Gao et al., 2008; Kwon et al., 2007; Lin et al., 2007). Examination of these data sets failed to reveal sites of estrogen receptor interaction near the *PEMT* locus. However, these studies were performed in breast cancer cells and mouse liver cells.

In order to determine the mechanism whereby the *PEMT* gene is regulated by estrogen in humans, we identified sites of ER- α binding to chromatin throughout the *PEMT* locus and examined estrogen regulation of the various *PEMT* transcripts. Using an expanded panel of SNPs we narrowed the CDS susceptibility locus to eleven strongly linked SNPs spanning a region of 70.5 kilobases (kb) in the promoter regions and first and second intron of the *PEMT* gene. We identified ER- α binding in a region of the *PEMT* gene located in the first intron of the *PEMT* A transcript and -40 bp 5' of the transcriptional start site for *PEMT* B. The intronic site is within 1 kb of the SNP most highly associated with CDS in women (rs4646343 (C/T); p=0.0001). Using allele-specific quantitative PCR (qPCR), we found decreased estrogenic activation of the *PEMT* gene from the risk allele. To identify the functional polymorphism, we examined ER-DNA interactions for the SNPs within the ER- α binding site that are in linkage disequilibrium (LD) with the most strongly susceptibility-associated SNP, rs4646343, in human hepatocytes and found reduced enrichment across this region in subjects homozygous for the risk allele relative to protective allele carriers.

Taken together, our results suggest that SNPs within a critical estrogen regulatory region of the *PEMT* locus abrogate estrogenic regulation of *PEMT*, explaining how genetic variation can influence gender-specific dietary choline requirements.

MATERIALS AND METHODS

ChIP-chip and ChIP-qPCR. Chromatin prepared from formaldehyde-fixed primary hepatocytes was immunoprecipitated with a mixture of two anti-ER- α antibodies (Ab-10, Neomarkers; HC-20, Santa Cruz) as previously described (Carroll et al., 2006; Lee et al., 2006). Chromatin was analyzed by qPCR or by microarray hybridization as described previously (O'Geen et al., 2006). Immunoprecipitated chromatin, as well as unfractionated input control chromatin, were fluorescently labeled and hybridized to a custom designed array (Nimblegen) which tiled 400 kb of the *PEMT* locus as well as several ER- α binding sites identified in other studies, (Carroll et al., 2005; Carroll et al., 2006; Kwon et al., 2007; Lin et al., 2007) with 60-nt oligonucleotide overlapping isothermal probes after masking for repetitive regions. We identified regions of enrichment by the intersection of two independent methods. Using SignalMap (Nimblegen), we filtered for peaks with peak height enrichment ratio ≥ 2.0 ; peak width ≥ 200 bp. Chromatin was isolated from human hepatocytes derived from two different organ donors. Statistical analyses were performed using Student's t test comparison for unpaired data.

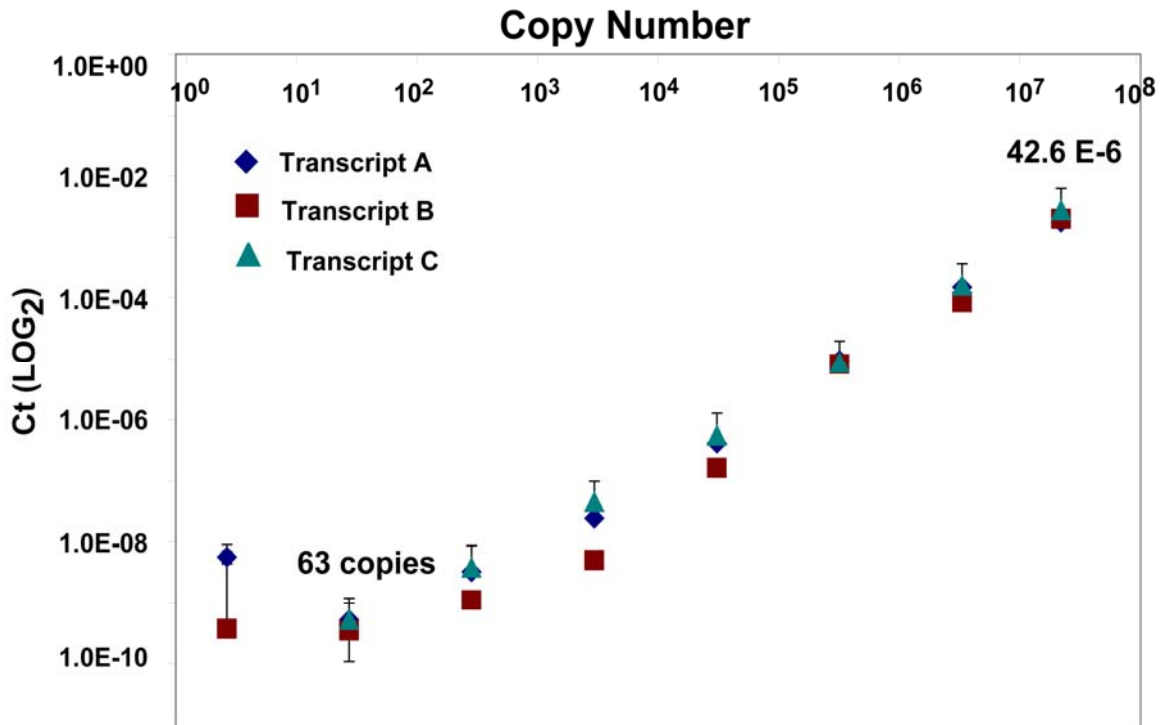
Construction of plasmids and luciferase reporter assay. ER- α ChIP binding sites were amplified from human liver genomic DNA by PCR and cloned into the pGL4-SV40 vector (an SV40 promoter cloned upstream of pGL4-Basic (Promega)). Sites were sequenced using a capillary sequencing machine (model 3100, Applied Biosystems) at the University of North Carolina at Chapel Hill Genome Analysis Facility. Human primary hepatocytes, grown in hormone-depleted medium for at least 2 days, were co-transfected with the ChIP- ER- α constructs and HSV-TK renilla with JETPEI hepatocyte

transfection reagent (Genesee Scientific). 24 hours after transfection, cells were treated with 100 nM Moxestrol (MOX) for 24 hours, and cell lysates were harvested and assessed for firefly and renilla luciferase activity using the Dual Luciferase Reporter Assay system (Promega). The data represent the average of four individual experiments.

Transcript-Specific Gene Expression. Primary human hepatocytes were isolated and cultured as described previously (Resseguie et al., 2007). Briefly, hepatocytes were cultured for 24 hours in hormone-free media in 6-well collagen coated plates (BD biosciences) prior to treatment. Cells were treated with 100nM MOX (Steraloids) for 4-hour, 8-hour, and 24-hour time points. RNA was isolated at each time point using RNeasy kit (Qiagen). RNA was analyzed by quantitative PCR (Taqman, Applied Biosystems) with probes specific for each PEMT transcript (Transcript A: NM_148172: assay ID *Hs01002998_m1*; Transcript B: NM_007169: assay ID *Hs00200354_m1*; Transcript C NM_148173: custom assay ID 4331348). To determine the absolute copy number of the target transcripts, we generated cDNA standards from human liver RNA, SuperScript III First (SSIII) Strand synthesis kit (Invitrogen), for each transcript. 429-bp cDNA fragments unique to each PEMT transcript were cloned into a TOPOII TA (Invitrogen), linearized with KpnI, gel purified and quantitated. A standard curve for each transcript was generated (**Figure 3-1**). The copy number of unknown samples (RNA extracted at various time points from human hepatocytes) was determined by linear regression analysis utilizing transcript specific standard curves. To correct for differences in both RNA quality and quantity between samples, the expression levels of transcripts of interest were normalized to the housekeeping gene, GAPDH, and expressed a gene expression ratio change.

Figure 3-1. Validation of Transcript Specific Gene Expression Using Real-Time Quantitative PCR.

(A) Real-time PCR was performed with Taqman assays (Applied Biosystems) specific for each PEMT transcript: Transcript A: NM_148172: assay ID *Hs01002998_m1*; Transcript B: NM_007169: assay ID *Hs00200354_m1*; Transcript C: NM_148173: custom assay ID 4331348. To determine the absolute copy number of the target transcripts, we generated cDNA standards for each transcript. (B) A standard curve for each transcript was generated using serial dilutions of cloned products ranging from 1 or 10 to 10^9 molecules/ μ l. The copy number of unknown samples (RNA extracted at various time points from human hepatocytes) was determined by linear regression analysis utilizing the transcript-specific standard curve. The limit of detection was ~1300 copies per 150 ng cDNA for each transcript-specific standard ($R^2=0.999$).



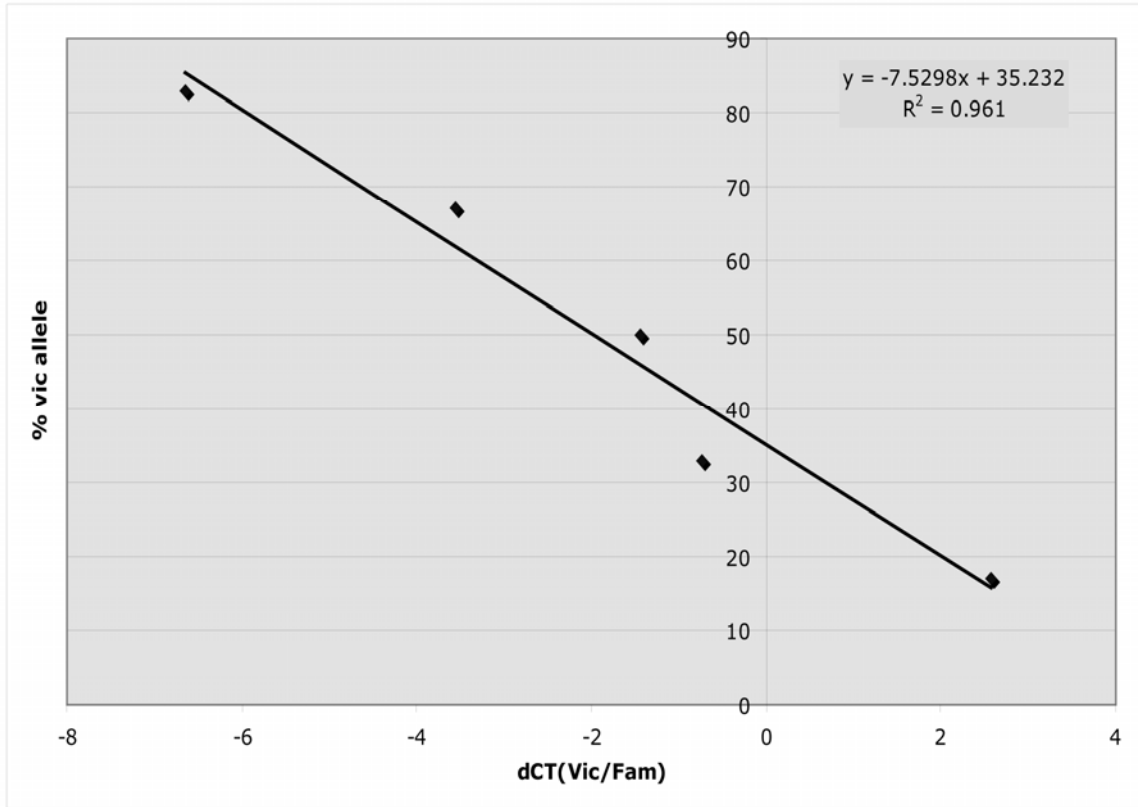
Mapping Choline-Deficiency Syndrome Risk Haplotype in PEMT locus. Humans eating diets deficient in the essential nutrient choline can develop organ dysfunction. We hypothesized that common SNPs in genes involved in choline metabolism influence the dietary requirement of this nutrient. Fifty-seven humans were fed a low choline diet until they developed organ dysfunction or for up to 42 days. We tested DNA SNPs for allelic association with susceptibility to developing organ dysfunction upon subjection to a choline-deficient diet. We identified a SNP in the promoter region of the phosphatidylethanolamine N-methyltransferase gene (*PEMT*; -744 G-->C; rs12325817) for which 18 of 23 carriers of the C allele (78%) developed organ dysfunction when fed a low choline diet (odds ratio 25, P=0.002) (da Costa et al., 2006a). DNA was isolated from women (n=31) and men (n=26) who participated in the human choline study. DNA was genotyped for a selection of SNPs in the *PEMT* locus using a custom bead array (Goldengate, Illumina). SNPs selection was based on the TAMAL algorithm which selects SNPs in genes that likely affect function of the gene (e.g. predicted transcriptionally active regions, exonic (non-synonymous), transcription factor binding sites) (Hemminger et al., 2006). Fisher's exact test was used to determine statistical significance of SNP association with organ dysfunction.

Allele Specific Gene Expression. For allele imbalance experiments, we genotyped human liver donors for rs897453 (G/A; located in exon 3 of the *PEMT* gene common to all three transcripts) and rs4646343 (C/A; risk allele (A) highly associated with susceptibility ($p=1 \times 10^{-9}$) to CDS in women (as described above) and within 1kb of the *PEMT* intron 1A ER α binding cluster). SNP rs897453 is highly associated with SNP rs4646343 ($p < 1 \times 10^{-5}$) using qPCR (Assays-on-Demand SNP Genotyping assay, Applied

Biosystems) and the following Taqman primer-probe assays: SNP rs897453 (assay ID: *C___7443062_1_*); SNP rs4646343 (assay ID: *C___9246129_10*). To quantify differences in allelic expression, we then generated a standard curve by mixing genomic DNA from homozygous risk individuals (rs4646343 (A/A)) and homozygous protective individuals (rs4646343(C/C)) at five different ratios in a TaqMan assay for exonic SNP rs897453 (**Figure 3-2**). RNA was isolated from hepatocytes with RNeasy kit (Qiagen) and cDNA was generated using SSIII First Strand synthesis kit (Invitrogen). Using real-time qPCR and these standard curves, we were able to deduce the ratios of gene expression between the two alleles by measuring the fluorescent intensity of the two alleles in cDNA samples as described previously (Lo et al., 2003). RNA expression was normalized by comparing to the allele-specific (FAM) 6-carboxyfluorescein intensity/VIC (a proprietary dye) intensity) gDNA quantitation from heterozygous subjects. The average gDNA ratios were equilibrated to 50% for each allele and the same factor was applied to cDNA ratios. Results are expressed as a percent of relative risk allele expression. For determination of hormone mediated allele-specific expression, hepatocytes were treated with 100 nM MOX for 24h as described above. The levels of risk allele expression from nine hepatocytes donors were calculated.

Figure 3-2. Validation of Standard for Detection of Allele-Specific Gene Expression.

SNP rs897453 is within a transcribed region of the *PEMT* gene (exon 3) that is common to all transcripts and is associated with SNP rs4646343 (a SNP associated with risk of developing choline deficiency syndrome in women deprived of choline ($p=1 \times 10^{-9}$)). To estimate allele-specific differences in *PEMT* gene expression, we created a standard curve by combining genomic DNA from two individuals at various ratios that were homozygous for the risk allele and homozygous for the protective allele. A Taqman SNP Genotyping Assay for rs897453, in which the FAM probe is specific for the protective allele and the VIC probe is specific for the risk allele, was used to estimate the percent abundance of each allele at each ratio as described previously (Lo et al., 2003). Using trend analysis, we determined that we could detect quantifiable differences between the VIC and FAM alleles ($R^2=0.961$).



Transcript-specific allele imbalance experiments were performed with primer-probe sets unique for each transcript (as described above) in hepatocytes that were homozygous for the risk allele (n=5), homozygous for the protective allele (n=6), and heterozygous for the risk/protective allele (n=5).

Allele Specific ChIP. Primers were designed (Primer 3, (Rozen and Skaletsky, 2000) to amplify regions of approximately 100 bp in close proximity to SNPs within ER binding regions and a negative control region (region of the genome not suspected to bind any of the transcription factors of interest). ChIP qRT-PCR experiments were performed as described above except 30 µg of chromatin was used for the chromatin IP experiments from subjects homozygous for the risk (n=5) and protective allele (n=6) rs4646343 as well as for heterozygous subjects (n=4). ChIP-chip experiments were performed in a subject homozygous for the risk allele and in a subject homozygous for the protective allele. To validate SNP status and to identify potentially new SNPs, the 2.2 kb intron 1A region was sequenced in human hepatocytes and in subjects from the human choline study who were homozygous for the rs4646343 risk (A/A) and protective allele (C/C).

RESULTS

*Identification of ER- α Binding Sites in *PEMT* Locus in Human Hepatocytes*

Chromatin isolated from 17-beta estradiol (E2)-treated primary human hepatocytes was immunoprecipitated with an ER- α specific antibody as described previously. Enriched chromatin and unfractionated input control DNA chromatin were hybridized to a custom Nimblegen-Roche microarray that comprehensively tiled virtually the entire *PEMT* gene with 60-bp overlapping, isothermal probes. Previous genome-wide ER- α ChIP-chip studies reported that only 4% enrichment occurred at proximal promoter regions for all genes and had identified six ER- α binding sites within 200 kbp of the *PEMT* promoter at the following chromosomal coordinates (bp) relative to transcription start site (TSS) A (Carroll et al., 2006):

Peak 1: {bp 17,599,362-17,599,962; $+1.6 \times 10^5$ bp TSSA }

Peak 2: {bp 17,574,823-17,575,536; $+1.39 \times 10^5$ bp TSSA }

Peak 3: {bp 17,572,850-17,573,450; $+1.37 \times 10^5$ bp TSSA }

Peak 4: {bp 17,541,224-17,541,831; $+1.05 \times 10^5$ bp }

Peak 5: {bp 17,535,860-17,536,461; $+1.0 \times 10^5$ bp TSSA }

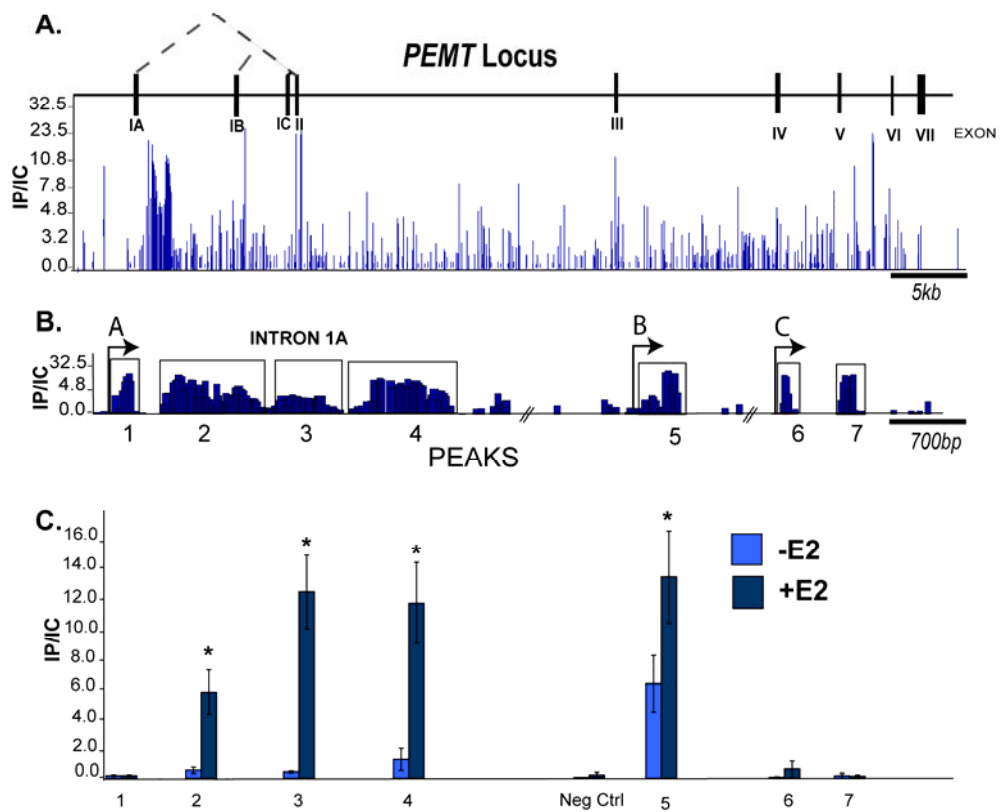
Peak 6: {bp 17,448,820-17,449,421; $+1.3 \times 10^4$ bp TSSA }

In light of this, we designed our arrays to tile 340 kb flanking the genes. Another genome-wide promoter ChIP-chip study, also performed in breast cancer cells, identified enrichment at *PEMT* promoter A (NM_148172) (Kwon et al., 2007). In contrast, a genome-wide identification of ER- α sites in mouse liver reported that almost 50% of ER- α -binding regions overlap with genes, including 8% that overlap with exons (Gao et al., 2008). In order to identify putative sites of estrogen receptor interaction, we designed a

finely tiled array covering virtually the entire *PEMT* locus. Using SignalMap (Nimblegen), we filtered for peaks with peak height enrichment ratio ≥ 2.0 ; peak width ≥ 200 bp. A total of 7 binding sites were identified and were all located within the *PEMT* gene. The data from the two human hepatocyte donors highly correlate with approximately 86% (6/7 peaks identical) overlap of binding sites (**Figure 3-3, A and B**). As positive controls, we also tiled several regions found to bind ER- α in MCF-7 cells. We found that approximately 60% of the positive control gene exhibited ER- α enrichment.

Figure 3-3. ER- α Enrichment Regions in the Human *PEMT* Gene

A) We tiled 340 kbp of the human *PEMT* gene and identified seven regions that were significantly enriched with ER- α by ChIP-chip. **B)** A 20 kb wide view of the probe signals is illustrated. The majority of the ER- α regions identified on the array were within the *PEMT* gene, including a region (Peak 4), which contains a consensus ERE/FOXA site. **C)** We validated the regions identified on the array using ChIP coupled to real-time PCR and found that the peaks within intron 1A and the site at promoter B were significantly enriched with ER- α complex in the presence of E2 (n=3, *p<0.05; two-tailed; paired-Student's test).



regions also exhibited ER- α enrichment in our experiments (data not shown).

To validate the sites identified on the array, we performed three independent ChIP assays in primary human hepatocytes in presence or absence of E2 followed by real-time PCR (**Figure 3-3, C**). The results of the ChIP-qPCR suggest the major sites of enrichment, which include a cluster of contiguous binding events within intron 1A and within promoter B. The cluster of peaks within intron 1A, which spans ~2.2 kb, was divided into four regions. Each site was validated by RT-PCR. The segmented regions exhibited between 4 to 11-fold enrichment ($p < 0.05$) in the presence of E2, with the exception of the peak that overlapped exon 1a (Peak 1). These sites were located approximately 7.8kb 5' from the transcription start site for *PEMT* B. Although we densely tiled the region spanning the *PEMT* gene, the limiting resolution of this assay (approximately 250bp) makes it difficult to precisely identify the binding site. The region annotated as Peak 4 within this cluster contained an evolutionarily-conserved consensus ERE (GGTCA-nnn-TGACC) and a proximal FOXA1 site. (See **Table 3-1** for chromosomal positions of validated ER- α regions relative to TSS B.)

Table 3-1. Genomic Coordinates of ER Binding Regions, SNPs, and Transcription Factor Binding Sites

Using ChIP-chip we identified 7 regions in the *PEMT* locus enriched with ER- α annotated as Peaks 1-7. Using ChIP-qPCR and gene-reporter assays, we narrowed down the critical ER regulatory region to the intron1a cluster (Peaks 2-4). This region was sequenced. Transcription factor binding sites were identified with TRANSFAC motif finder. The minor allele for each SNP is in bold, underlined, and italicized. All SNP sequences are given for (+) strand orientation; however in text the sequences for each SNP are given for (-) strand due to anti-sense orientation of the *PEMT* gene. SNPs with linkage disequilibrium (r^2) data relative to rs4646343 (Hapmap.org) are presented in the last column. The majority of the hepatocyte organ donors from which data were obtained were of European descent, therefore the r^2 values are presented for Utah residents with ancestry from northern and western Europe (CEU). SNPs rs4646343 and rs12325817 (shown in red) are associated with CDS in women ($p < 0.0001$).

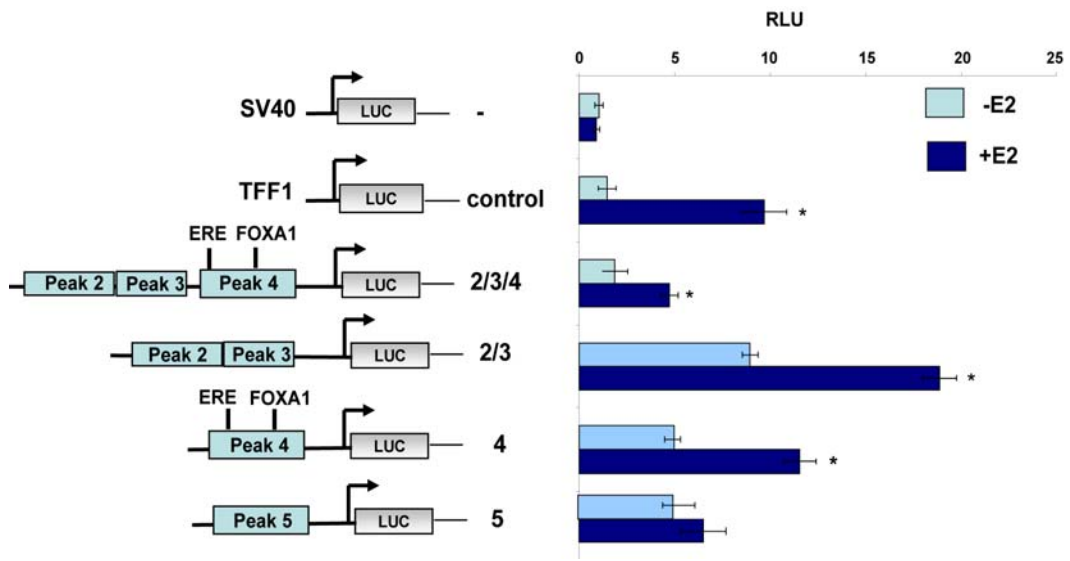
ER Binding Site	5'	3'	Size (bp)	Sequence	r ² {CEU} (rs464343)
TSS A	17435719				
Peak 1	17435563	17435779	216		
Peak 2	17434730	17435315	585		
un-annotated SNP	17434794			G/ <u>A</u>	
rs4646340	17434740			G/ <u>A</u>	0.659
Peak 3	17434183	17434679	496		
rs4646341	17434535			<u>C</u> /T	0.671
Peak 4	17433337	17434067	730		
un-annotated SNP	17434032			C/ <u>G</u>	
rs4646342	17433997			C/ <u>T</u>	0.858
ERE	17433899			GGTCA-GGG-TGACC	
rs46464459	17433811			A/ <u>G</u>	0.834
FOXA1	17433694	17433717		TCCCT-GGCCTG-GGAGAGT-TGCAAG	
rs4646343	17432802			C/ <u>A</u>	1.0
rs12325817	17427244			C/ <u>G</u>	
TSS B	17426470				
Peak 5	17426283	17426611	328		
TSS C	17421504				
Peak 6	17421386	17421571	185		
Peak 7	17420889	17421092	203		

ER- α Sites in Intron1A Mediate Transcriptional Activation

To examine whether ChIP ER- α binding sites harbor transcriptional enhancer activities, we cloned the ER- α enrichment cluster in intron 1A containing Peaks 2-4 and the region within Promoter B, Peak 5, isolated from human liver genomic DNA upstream of the pGL4-SV40 luciferase reporter. Hormone-depleted primary human hepatocytes were transiently transfected with these constructs were treated with MOX for 24 hours and then assayed for luciferase activity. Since the prototypical estrogen, E2, is rapidly metabolized in p450-containing liver cells (Barkhem et al., 1997; Mattick et al., 1997), we stimulated cells with the synthetic estrogen MOX (Barkhem et al., 1997; Kanamori et al., 2000; Mattick et al., 1997; Petz et al., 2002). The region containing peaks 2, 3 and 4 can mediate estrogenic activation (**Figure 3-4**). Peak 5 stimulated basal transcription from the SV40 promoter, but was incapable of mediating estrogen activation. Peaks 2 and 3, as well as the ERE region containing Peak 4, demonstrated estrogen-mediated induction. This suggests that multiple regions located in intron 1 can mediate estrogen induction, even in the absence of a consensus ERE.

Figure 3-4. Intron 1A ER- α CHIP Binding Sites are Functional Transcriptional Enhancers

ER binding sites were cloned into the pGL4-SV40 luciferase reporter construct and transfected into primary hepatocytes that were grown in hormone-depleted medium for at least two days. The cells were then either left untreated or treated with 100 nM MOX for 24 hours before harvesting for luciferase activity. pGL4-SV40 and pGL4-TFF1 (TFF1 promoter cloned into a pGL4-luc construct) were used as negative and positive controls, respectively. The cells were also cotransfected with the -TK renilla vector as an internal control for transfection efficiency. The data represent the average of four individual experiments (*= $p < 0.05$).

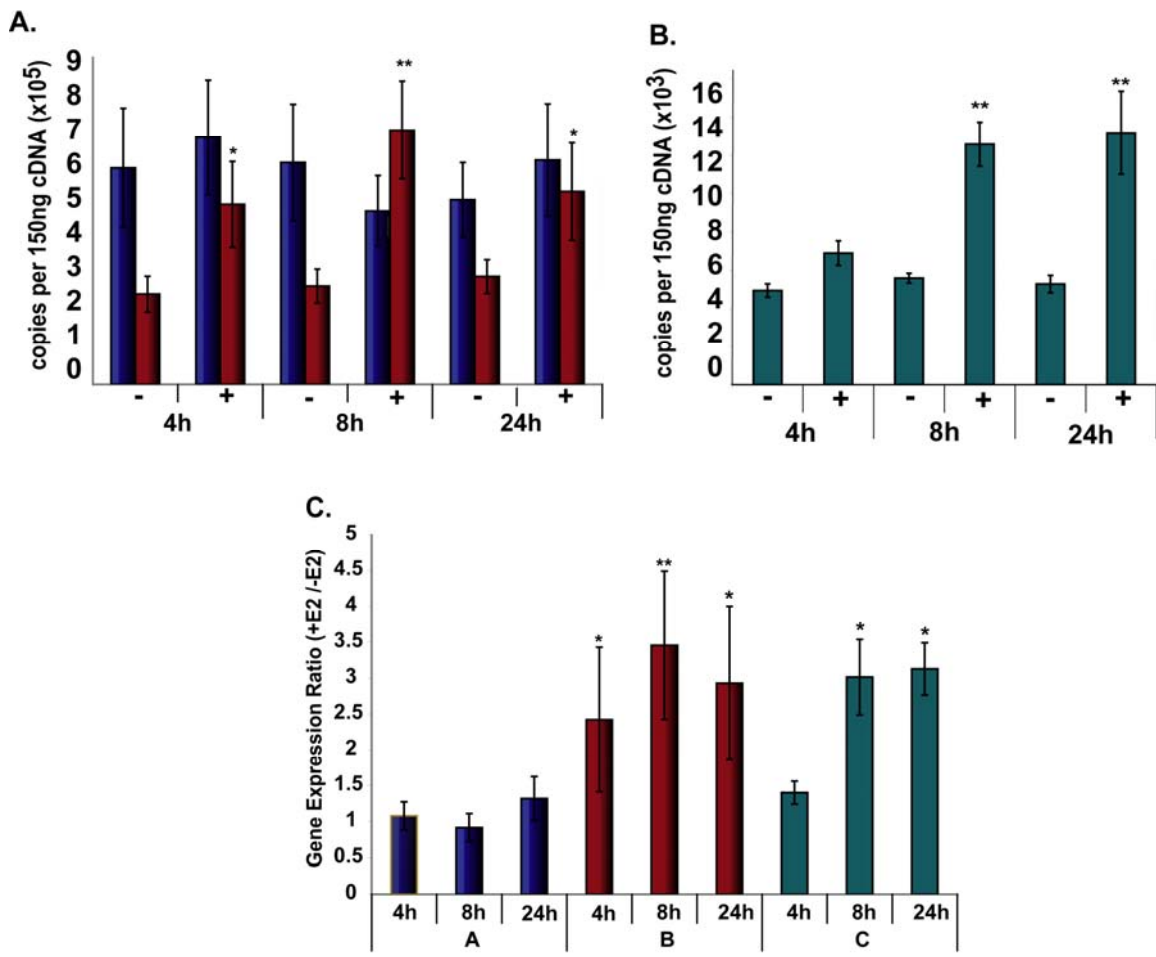


Estrogen Regulation of PEMT Gene is Transcript-Specific

Previous studies of *PEMT* activation by estrogen were unable to examine which promoters were induced and which isoforms were made. To further characterize estrogenic regulation of the gene, we utilized transcript-specific primer-probe sets. To accurately quantify expression level (gene copy number) differences between transcripts, we also generated cDNA standards for each transcript. To assess the kinetics of *PEMT* induction for each transcript, we harvested mRNA at various time points. Transcriptional changes at 4 hours represent immediate transcriptional activation whereas activation at 8 and 24 hour time points suggest delayed activation. Transcript A was the most abundant of the three transcripts in un-stimulated cells with approximately 750,000 copies per 150 ng cDNA (**Figure 3-5, A**), however it was not responsive to MOX treatment any time point tested (**Figure 3-5, A and C**). In contrast, transcript B was induced at all time points. Hepatocytes contained approximately 300,000 copies per 150 ng cDNA in untreated cells, but this level increases to ~500,000 and ~750,000 at 4 and 8 hours, respectively, of MOX treatment (**Figure 3-5, A**). Transcript B became the predominant transcript in cells 4 hours after MOX treatment and was almost equivalent to expression of transcript A by 24 hours. Transcript C was the least abundant transcript. Nonetheless, treatment with MOX markedly induced gene expression by 8 hours (~15,000 copies per 150 ng cDNA) and was maximally induced by 24 hours (~18,000 copies per 150 ng cDNA) (**Figure 3-5, B**). These results suggest that transcript B is an early estrogen target, whereas transcript C is likely a late target, and that transcript A is not significantly regulated by estrogenic mechanisms.

Figure 3-5. Estrogen Regulation of *PEMT* Gene Expression is Transcript-Specific

Using RT-PCR, we measured transcript-specific gene expression of *PEMT* transcripts A, B, and C. **A)** Transcript A is the most abundant transcript ($\sim 6.5 \times 10^5$ copies per 150 ng cDNA) but is not responsive to hormone treatment. Transcript B is significantly induced in response to hormone treatment at both early and late time points ($\sim 3.0 \times 10^5$ copies per 150 ng cDNA in untreated cells vs. $\sim 7.0 \times 10^5$ copies per 150 ng cDNA in treated cells). **B)** Transcript C was the least abundant transcript but was significantly induced in response to hormone treatment by 8 and 24 hours ($\sim 5.0 \times 10^3$ copies per 150 ng cDNA). **C)** Transcript B was induced by approximately 2.5-fold by 4 hours and maximally by 8 hours whereas transcript C was induced 3-fold by 8 and 24 hours. Results are expressed as ratio-fold change in gene expression relative to untreated samples, normalized to GAPDH. (n= 4/point; * = $p < 0.05$ by paired, two-tailed Students t-test).

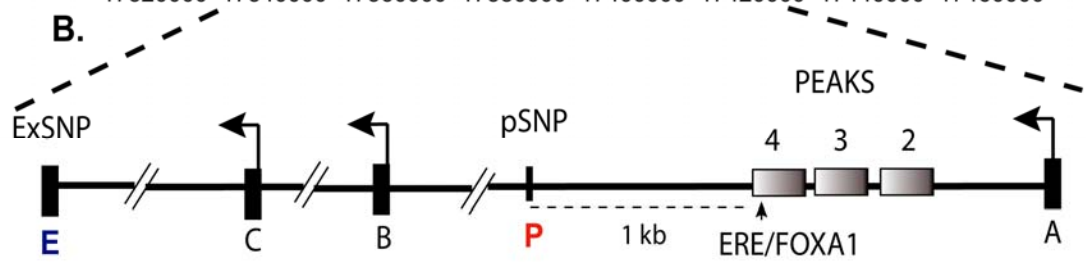
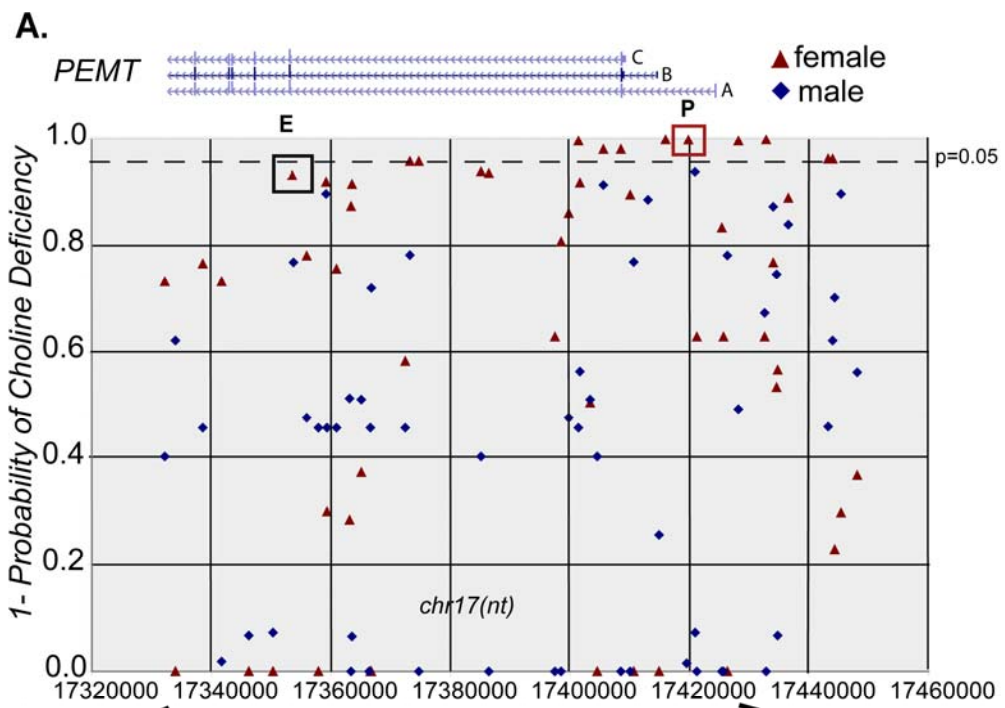


The ER-Binding Region is Proximal to an Organ Dysfunction-Associated SNP

Our previous study of choline deprivation in human subjects identified a specific SNP that was highly associated with CDS in women (SNP rs12325817; $p=1 \times 10^{-9}$) (da Costa et al., 2006a). To further define the region of the *PEMT* locus associated with risk for CDS in women, we genotyped subjects for an extended panel of SNPs that span the *PEMT* locus using a custom Goldengate Illumina Bead array. Eleven SNPs were found to be significantly associated with susceptibility to CDS in women but not men (**Figure 3-6, A**). The minor allele of SNP rs4646343 (C/A) was most strongly associated with the CDS phenotype ($p=1 \times 10^{-7}$). SNP rs464343 is located approximately 1 kb from the ER- α enriched region in intron 1A (**Figure 3-6, B**). Other SNPs within this binding region are in high LD with rs464343. In addition, a SNP located in exon 3, rs897453, was also found to be statistically significantly associated with rs464343 ($p=1 \times 10^{-6}$) and rs12325817 ($p=1 \times 10^{-4}$). Exonic SNP rs897453, a missense polymorphism (Val-Ile) was not found to be significantly associated with CDS in women relative to men ($p=0.0812$). The identification of a SNP in a common coding exon permits detection of allele-specific expression.

Figure 3-6. SNP Highly Associated with CDS in LD with *PEMT*-ER Region

A) A study conducted in a cohort of women (n=31) and men (n=26) identified SNP rs12325817 ($p=1 \times 10^{-9}$) as being strongly associated with CDS in women (da Costa et al., 2006a). To determine whether this SNP acts a regulatory SNP or is just in LD with a regulatory SNP, we genotyped these subjects for a panel of SNPs that spanned the *PEMT* locus (Custom Goldengate Illumina Bead array) to identify regions associated with organ dysfunction. Eleven SNPs were found to be significantly associated with susceptibility to CDS in women but not men. A map of these SNPs is displayed graphically as a function of chromosomal position (x-axis) relative to association with CDS (1-probability; y-axis). SNP rs4646343 was most strongly associated with the phenotype ($p=1 \times 10^{-7}$) and an exonic SNP rs897453 was found to significantly associated with rs4644343 ($p=1 \times 10^{-6}$) and rs12325817 ($p=1 \times 10^{-4}$). **B)** SNP rs464343 is located approximately 1 kb from the intron 1A cluster of ER- α enrichment and is in high LD with several SNPs within this binding region (statistical cut-off: $p < 0.05$; Fishers exact test).



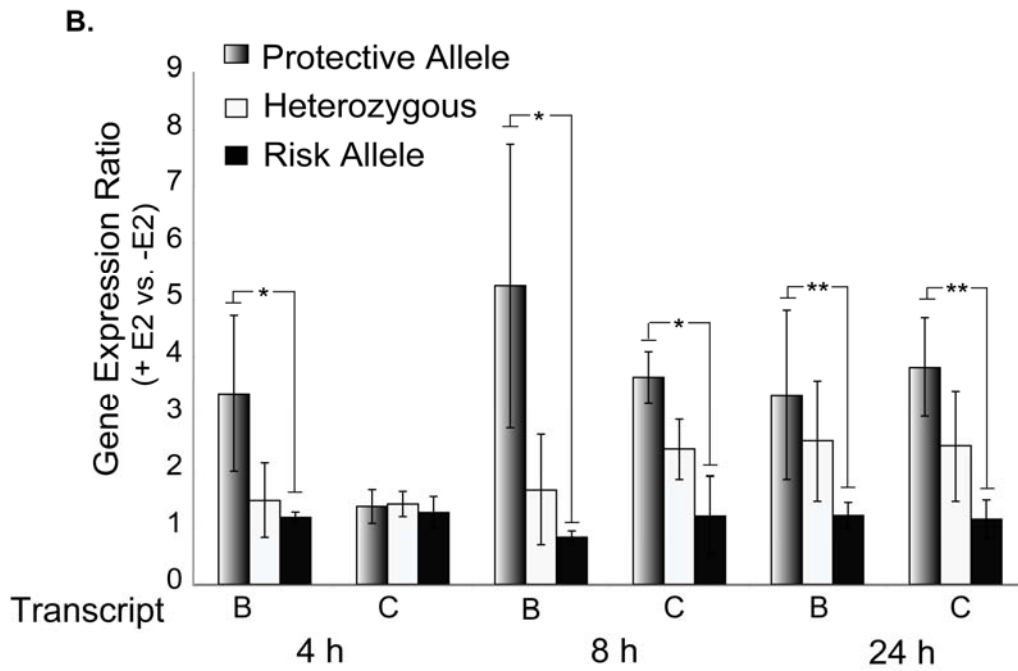
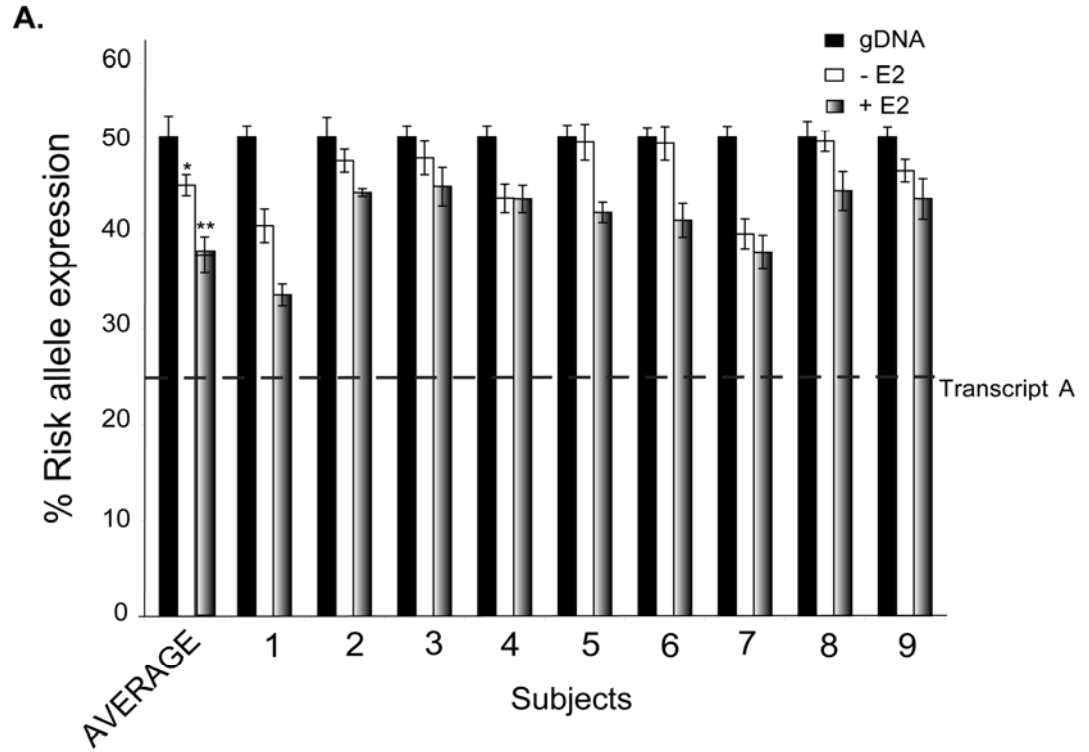
Risk Allele is Not Estrogen Responsive

The minor allele (A) of SNP rs897453 was highly associated with the risk allele (A) of SNP rs4646343 but not with risk of CDS in women relative to men in the human choline study cohort. To determine if the risk allele was associated with aberrant *PEMT* gene expression, we genotyped human liver organ donors to determine their SNP status at rs4646343 and rs897453 (based on the association that minor allele A is highly associated with risk allele A). We then generated a standard curve by mixing gDNA from homozygous risk allele subjects with homozygous protective subjects at the several ratios to validate the reliable detection of differences between the risk and protective allele ($r^2=0.961$, **Figure 3-2**). Quantitative PCR of cDNA from estrogen treated and non-treated heterozygous subjects followed by linear regression analysis permitted us to estimate the relative expression of the risk allele versus protective allele in nine subjects. We found that, for all transcripts, in the absence of estrogen treatment, the risk allele was under-expressed by ~4% relative to the protective allele ($p=0.01$) and under-expressed by ~8% ($p=0.001$) after treatment with MOX (**Figure 3-7, A**).

To explore whether over-expression of the protective allele may compensate for the lower expression of the risk allele, we compared total gene expression levels in human hepatocytes from three genetic backgrounds (i.e. homozygous for protective (C/C), risk (A/A) and heterozygous (C/A) alleles) before and after treatment with estrogen. We found that in hepatocytes homozygous for the risk allele, transcripts B and C were not hormone-inducible at any time point tested ($p \leq 0.02$) (**Figure 3-7, B**).

Figure 3-7. Risk Allele is Not Estrogen Responsive

A) To assess differences in allelic expression, we utilized RT-PCR and isolated hepatic cDNA from MOX-treated and non-treated heterozygous (risk SNP rs4646343; exonic SNP rs897453) subjects (n=9) and used linear regression analysis to estimate the relative % expression of the risk allele versus protective allele. In the absence of MOX treatment, the risk allele was under-expressed by ~4% relative to the protective allele (p=0.01) and under-expressed by ~8% (p=0.001) after treatment with MOX. Results are presented as an average +/- SD of the nine subjects and individually. **B)** Total gene expression levels were determined by quantitative RT-PCR in human hepatocytes treated with estrogen representative of all three genetic backgrounds (homozygous for protective (C/C), risk (A/A) and heterozygous (C/A) alleles). Transcripts B and C in hepatocytes that were homozygous for the risk allele were not estrogen inducible at any time point tested (p<0.02). There was a trend of reduced estrogen-mediated induction in heterozygous hepatocytes that approached statistical significance (n= 6 protective homozygous subjects; 5 heterozygous subjects; 5 risk homozygous subjects; *p=paired student's t-test; **p= 0.002).

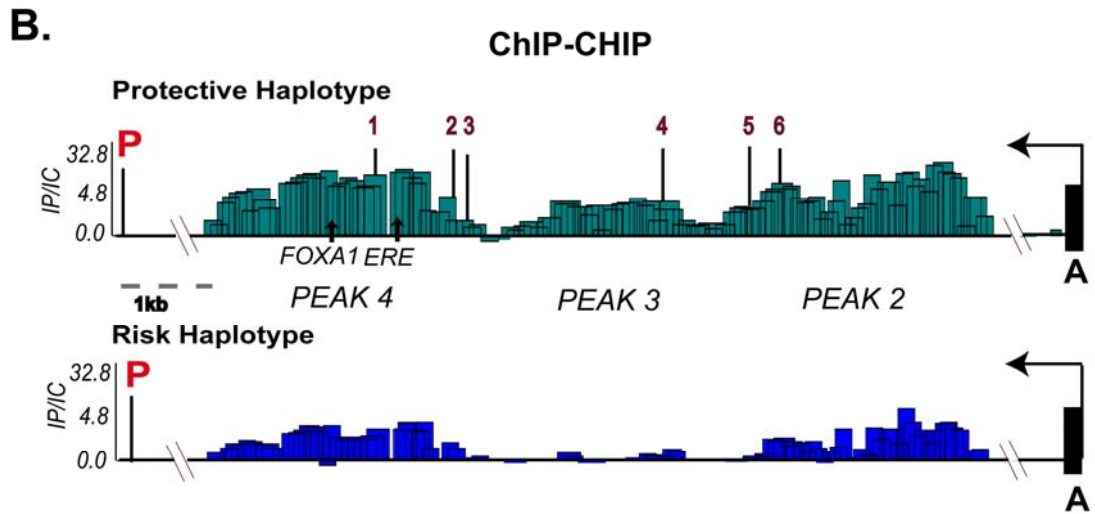
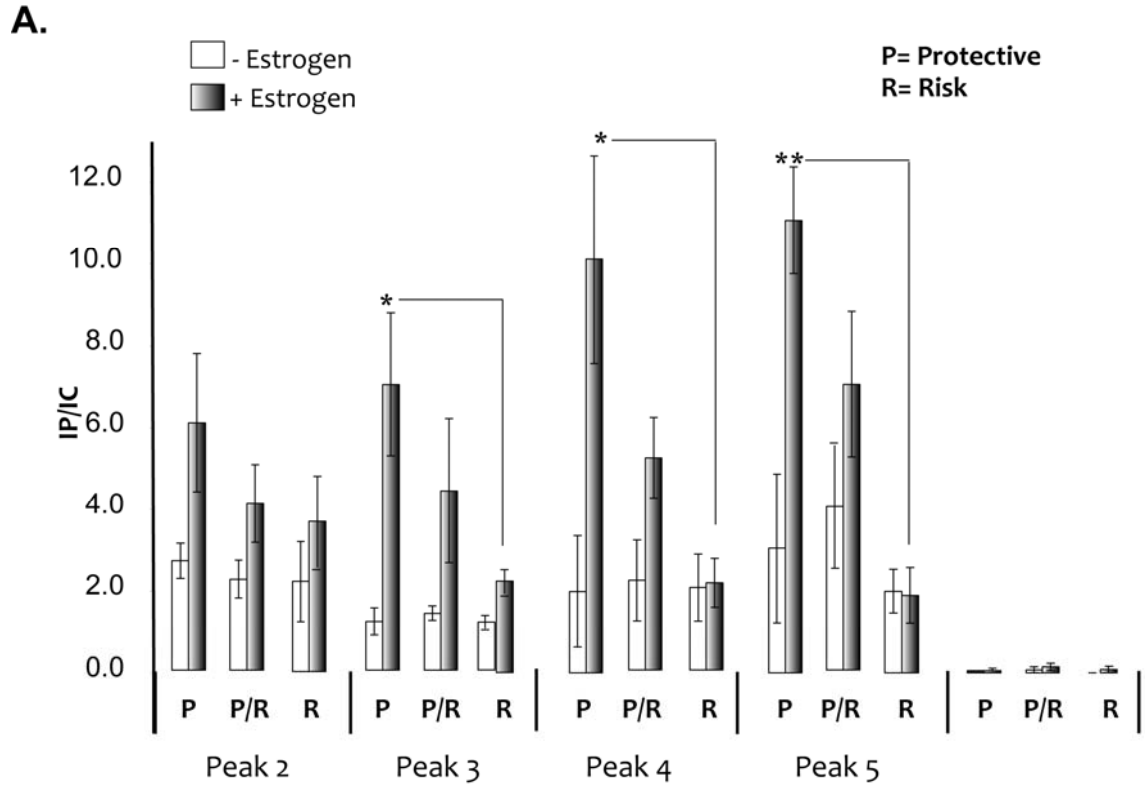


Allele-Specific ER- α Enrichment within Intron 1A Binding Region.

As previously noted, the SNP most highly associated with CDS in women (rs4646343 (C/A); p=0.0001) is within 1kb of an ER- α binding region. This 2.2 kb region contains three sites (annotated as Peak 2, 3 and 4) that can act as a transcriptional enhancer. Peak 4 contains a consensus ERE and FOXA1 site. To determine if SNP status affects ER- α DNA binding at any of these sites, we performed quantitative ChIP in primary hepatocytes from subjects from the three genetic backgrounds (homozygous for risk allele, homozygous for the nonrisk allele, and heterozygous). We found decreased enrichment at all sites in chromatin from subjects homozygous for the risk allele compared to homozygous protective and heterozygous subjects. In contrast, ER- α binding at Peaks 4 and 5 was identical in treated vs. non-treated hepatocytes homozygous for the risk allele (**Figure 3-8, A**). In order to evaluate for additional SNPs in this region that may not have been represented in the Illumina bead array, we sequenced the 2.2 kb region from several subjects. We identified six SNPs that differ between the homozygous risk and protective subjects.

Figure 3-8 Allele Specific ER- α Enrichment within Intron 1A Binding Region

A) SNP rs4646343 ((C/A); p=0.0001) is within 1kb of a region enriched with ER- α binding in the *PEMT* gene. This 2.2 kb region contains three ER- α binding sites (annotated as Peak 2, 3 and 4). To determine if SNP status affects ER-DNA binding at any of these sites, we performed ChIP-qPCR in hepatocytes representative of all three genetic backgrounds. We found decreased enrichment at all sites tested in subjects that were homozygous for the risk allele relative to homozygous protective and heterozygous subjects; however there was no difference in enrichment between treated and non-treated hepatocytes that were homozygous for the risk allele at Peaks 4 and 5. **B)** By sequencing, we identified six SNPs that differ between the homozygous risk and protective subjects. These include SNPs (rs4244599 (A/G), rs4646342 (C/T), rs4646340 (A/G), rs4646341 (C/T) and two previously unidentified SNPs at Chr17 nt position 17434032 (C/G)) and 17434794 (G/A). Comparison of ChIP-chip results between subject #1 (homozygous for the risk allele) and subject #2 (homozygous for the protective allele) reveals less ER- α enrichment in subjects homozygous for the risk allele across the intron1A region, however Peak 3 is absent in subject #1. ChIP-qPCR in hepatocytes homozygous for the risk allele did exhibit enrichment at the Peak 3 site, albeit less enrichment than in homozygous protective hepatocytes and in heterozygous subjects.



This includes four previously annotated SNPs, rs4244599 (C/G), rs4646342 (A/T), rs4646341 (G/C), rs4646340 (G/A), and two previously unidentified SNPs at Chr17 nt positions 17434032 (C/G) and 17464794 (G/A). There was a perfect concordance between the SNPs among homozygous risk subjects and homozygous protective subjects (**Figure 3-8, B**). In Figure 3-8 we present ChIP-chip data from the subject that is homozygous for the risk allele (subject #1) versus a subject that is homozygous for the protective allele (subject #2). There is less enrichment in subjects homozygous for the risk allele across the intronic region ER region however Peak 3 is absent in subject #1. ChIP-qPCR in hepatocytes that were homozygous for the risk allele did exhibit enrichment at the Peak 3 site, albeit less than in homozygous protective hepatocytes and in heterozygous subjects.

DISCUSSION

Choline is an essential nutrient in humans. Insufficient dietary choline results in a syndrome that includes muscle and liver dysfunction characterized by hepatosteotosis, liver ALT/AST enzyme leakage, elevated CPK levels, muscle cell apoptosis, inflammation, and hepatocyte cell death. We previously identified a subset of young women who are protected against CDS relative to men and post-menopausal women. Since PEMT functions as the only source of *de novo* choline, we looked for an association between SNPs in the *PEMT* locus and CDS. We found that premenopausal women harboring a *PEMT* promoter SNP are twenty five times as likely to develop CDS as non-carriers of this SNP.(da Costa et al., 2006a)

The *PEMT* gene is estrogen responsive in a dose-dependent manner.(Resseguie et al., 2007) However, the mechanism whereby estrogen regulates the gene remains

underdetermined. In this study we sought to identify regions in the *PEMT* locus that could be estrogen regulatory and to determine if SNPs, previously identified as conferring an increased risk to CDS in women, affect estrogenic regulation of *PEMT* gene expression.

Examining the entire *PEMT* locus by ChIP-chip we identified several ER- α enriched regions in the *PEMT* gene, specifically implicating a critical regulatory region located in intron 1 of the A transcription start site, 7500 nt upstream of the transcriptional start site of transcript B. We were able to resolve this region into three potential binding sites—annotated as Peak 2-4. Prototypically estrogen receptor binds a 13 bp palindromic repeat and its binding seems to be facilitated by proximal binding of FOXA1, a transcription factor shown to be essential for ER gene regulation at distal sites. (Carroll et al., 2005) We found that peak 4 contains a canonical ERE as well as a FOXA1 site. These sites were not identified in a previous genome-wide examination for ER- α binding sites in the estrogen responsive breast tumor cell line MCF-7, suggestive of potential tissue specificity. (Carroll et al., 2006) Peak2/3 as well as Peak 4 are independently able to act as enhancers. A site in the proximal promoter for transcript B was also found to be bound by ER, by ChIP-chip and ChIP-qPCR. However, this site lacked the ability to act as an enhancer in transfection-based reporter assays in primary hepatocytes. This suggests that this region does not have the ability to act alone to initiate transcription and may physically interact with the more distal enhancer region. Similarly, there was a peak in promoter C that may play the same role. This hypothesis is corroborated by the finding that transcripts B and C are estrogen inducible transcripts in contrast to transcript A. Although transcript A is the most abundant transcript in untreated hepatocytes, in the

presence of estrogen transcript B is strongly induced and becomes the majority *PEMT* transcript. Transcript C is also strongly induced, but was expressed at much lower levels relative to A and B, and thus is unlikely to contribute significantly to an estrogen-mediated increase in *PEMT* activity. Transcripts B and C encode the same ER-directed isoform, *PEMT* II which is functionally distinct from *PEMT* I. Previously it was shown that *PEMT* II is highly expressed in female mouse liver, but not in male liver suggesting a gender specific role. (Noga and Vance, 2003)

We genotyped the cohort of men and women from a clinical study on human dietary choline requirements (da Costa et al., 2006a) for a subset of SNPs in the *PEMT* locus to identify potential regions associated with susceptibility to CDS. A subset of SNPs was strongly associated with susceptibility to CDS in women but not in men, suggesting that these regions may be critical to *PEMT* expression in women. We compared the risk associated SNPs with the regions of ER binding identified by ChIP-chip and found that the SNP most highly associated with susceptibility to CDS in women (rs4646343 (C/A); p=0.0001) was within 1kb of peak 4. A SNP located in LD with rs4646343 provided us the opportunity to examine allele-specific expression in heterozygous hepatocytes. We found that the risk allele was significantly under-expressed both in the presence and absence of hormone treatment. In hepatocytes homozygous for the risk allele, *PEMT* was not estrogen-responsive. We examined the influence of these SNPs on ER binding to the transcriptionally active regions identified in transcript A, exon 1. We found that Peak 4, which contains an ERE and FOXA1 site, was not enriched with ER- α in hepatocytes homozygous for the risk allele. Peak 4 contains three SNPs that are in high linkage disequilibrium with the most strongly risk-

associated SNP---rs4646343—in human hepatocytes. Peak 5 was also diminished in the context of the rs4646343 risk SNP, supporting the hypothesis that the critical ER- α binding site in peak 4 interacts with proximal promoter B and both sites of interaction are disrupted by the SNP. We suggest that potential regulatory SNP(s) reside within Peak 4, and these alter binding affinity for transcription factor ER- α , prevent looping to Promoter B and result in decreased estrogen-mediated induction of *PEMT* transcripts B and C.

The results presented here provide a potential mechanism which explains how SNPs within a critical estrogen regulatory region of the *PEMT* locus abrogate estrogenic regulation of *PEMT*. This study is the first to propose a model which explains how genetic variation influences gender specific requirements for choline.

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CHAPTER VI
OVERALL DISCUSSION

IMPORTANCE OF CHOLINE

Choline is essential for normal function of all cells. (Zeisel and Blusztajn, 1994) It is the major source of methyl-groups in the diet. Following its oxidation to betaine, choline participates in the methylation of homocysteine to form methionine. (da Costa et al., 2005). In addition, choline is used for the biosynthesis of cell membranes, bioactive phospholipids, and is the precursor molecule of the neurotransmitter acetylcholine. (Zeisel and Blusztajn, 1994) Insufficient dietary choline results in a syndrome characterized by hepatosteotosis (due to lack of phosphatidylcholine which limits the export of excess triglyceride from liver in lipoproteins)), liver ALT/AST enzyme leakage, elevated CPK levels, muscle cell apoptosis, inflammation, and hepatocyte cell death. (Albright et al., 2005; Albright et al., 1996; Albright and Zeisel, 1997; Buchman et al., 1995; da Costa et al., 2004; Fischer et al., 2007; James et al., 1997; Zeisel, 2000) Shaw and colleagues, studying pregnant women in California observed intakes of choline in 25% of the population that were less than needed to prevent birth defects in their fetuses. (Shaw et al., 2006; Shaw et al., 2004)

The only source of choline other than diet is from the *de novo* biosynthesis of phosphatidylcholine catalyzed by phosphatidylethanolamine-*N*-methyltransferase (PEMT) in liver. When fed a diet deficient in choline, *Pemt* $-/-$ mice developed fatty liver, severe liver damage and died; a choline supplemented diet prevented this (Walkey et al., 1998) and reversed hepatic damage if begun early enough. (Waite et al., 2002) It is now accepted that the PEMT pathway is not just a minor pathway that backs up the CDP-choline pathway for phosphatidylcholine biosynthesis. *Pemt* $-/-$ mice have lower choline pools in liver despite being fed sufficient or supplemental amounts of dietary choline,

suggesting that choline production by PEMT is a significant source of choline relative to dietary intake. (Zhu et al., 2003)

ESTROGEN AND THE REQUIREMENT FOR CHOLINE

A subset of premenopausal women, relative to males and postmenopausal women, are resistant to developing choline deficiency syndrome. (Fischer et al., 2007) Treatment with estradiol at doses bracketing physiological concentrations in humans (0 – 100 nmol/l), causes a marked up-regulation in *PEMT* mRNA expression and enzyme activity in human hepatocytes. (Resseguie et al., 2007) Thus, premenopausal women have an enhanced capacity for *de novo* biosynthesis of choline moiety.

Although the *PEMT* gene is estrogen responsive in a dose-dependent manner; the mechanism whereby estrogen regulates the gene remains undetermined. The human *PEMT* gene encodes three unique transcripts, A (NM_148172), B (NM_007169), and C (NM_148173) which encode two different protein isoforms. PEMT I, encoded by transcript A, localizes to the endoplasmic reticulum whereas PEMT II, synthesized from transcripts B and C, are found primarily in the mitochondrial associated membrane, a subfraction of the endoplasmic reticulum.

Recently, chromatin immunoprecipitation (ChIP) has been used coupled with DNA microarrays (chip) (ChIP-on-chip) to pursue whole-genome identification of ER-binding sites in intact chromatin of cultured breast cancer cell lines. (Carroll et al., 2005; Carroll et al., 2006) (Kwon et al., 2007; Lin et al., 2007) A genome-wide ChIP-on-chip study identified DNA regions that bind ER- α in intact chromatin from mouse liver, and reported that approximately 1.2% of the ER- α binding regions overlapped with human studies. (Gao et al., 2008)

Examining the entire *PEMT* locus by CHIP-chip we identified several ER- α enriched regions in the *PEMT* locus, specifically identifying a critical regulatory region located in intron 1 of the A transcription start site, 7500 nt upstream of the transcriptional start site of transcript B. We were able to resolve this region into three potential binding sites—annotated as Peak 2-4. We found that peak 4 contained a canonical ERE as well as a FOXA1 site, which are essential to ER gene regulation. A site in the proximal promoter for transcript B, was found to be bound by ER, by CHIP-chip and CHIP-qPCR. However, this site lacked the ability to act as an enhancer in transfection-based reporter assays in primary hepatocytes. This suggests that this region does not have the ability to act alone to initiate transcription and may physically interact with the more distal enhancer region. This hypothesis is corroborated by the finding that transcripts B and C are estrogen inducible transcripts in contrast to transcript A. Although transcript A is the most abundant transcript in untreated hepatocytes, in the presence of estrogen, transcript B is strongly induced and becomes the majority *PEMT* transcript. Transcript C is also strongly induced, but was expressed at a much lower level relative to A and B and, thus, is unlikely to contribute significantly to an estrogen-mediated increase in *PEMT* levels. Transcripts B and C encode the same endoplasmic reticulum-directed isoform, it is possible that the role of *PEMT* II is functionally distinct from *PEMT* I. This isoform specificity has been suggested by previous findings that *PEMT* II which is highly expressed in female mouse liver but in not male liver suggesting a gender specific role for this isoform.(Noga and Vance, 2003)

GENETIC VARIATION IN CHOLINE REQUIREMENTS

Though some humans develop fatty liver, as well as liver and muscle damage when fed a low choline diet, others do not. Even among premenopausal women who should be resistant to choline deficiency, a subset develops organ dysfunction when deprived of choline. (Fischer et al., 2007) Genetic variation likely underlies these differences in dietary requirements. Several pathways influence how much choline is required from diet and functional SNPs in genes for these pathways might be of importance. Specifically, polymorphisms in the *PEMT* gene might alter endogenous synthesis of choline thereby influencing choline dietary requirements.

The human *PEMT* gene is highly polymorphic, and one of the primary goals of this dissertation is the identification of SNPs that affect choline biosynthesis. We believe that people with such SNPs will be more vulnerable to developing organ dysfunctions when dietary choline intake is marginal. We found that premenopausal women harboring a *PEMT* promoter SNP are twenty five times as likely to develop CDS as are non-carriers of this SNP.(da Costa et al., 2006) Given the sexual dimorphism in the effect of *PEMT* rs12325817, it is possible that this SNP or an associated SNP alters the estrogen responsiveness of the promoter. The frequency of this variant allele was 0.74.

We genotyped the cohort of men and women from the human choline study (da Costa et al., 2006) for a subset of SNPs in the *PEMT* locus to identify potential regions in the *PEMT* locus associated with susceptibility to CDS. A subset of SNPs was strongly associated with risk in women but not in men suggesting these regions may be critical to *PEMT* expression in women. We compared the risk-associated SNPs to with the regions of ER- α binding identified by ChIP-chip and found that the SNP most highly associated

with CDS in women (rs4646343 (C/A); $p=0.0001$) is within 1kb of peak 4. A SNP located in linkage disequilibrium with rs4646343 provided us the opportunity to examine allele-specific expression in heterozygous hepatocytes.

A recent genome-wide association (GWA) study profiled more than 39,000 transcripts and genotyped approximately 800,000 unique SNPs in more than 400 human liver samples to determine the association between allelic imbalance in gene expression in the human liver and the association to various diseases (Schadt et al., 2008). In order to map cis-acting regulatory SNPs, this group identified genes which exhibit allelic imbalance in gene expression by collating transcript expression levels in the human liver samples with each of the SNPs in the analysis. Results from this study did not support the hypothesis that *PEMT* gene expression is under cis regulatory genetic control in human liver. We found that the risk allele was significantly under-expressed both in the presence and absence of hormone treatment. Since we determined that *PEMT* A is the most abundant transcript in human hepatocytes but is not induced by estrogen, we hypothesize that the allelic imbalance we observed between risk and protective allele primarily affects *PEMT* transcripts B and C and that transcript A remains unchanged. A potentially greater reduction in transcript B and C expression levels would be not detected by the allele imbalance detection assay utilized by our experiments nor would this difference have been detected in the GWA study conducted by Schadt and colleagues, due to an inability to discriminate between transcripts. To further support our hypothesis that risk allele-associated under-expression is transcript-specific, we looked at total expression levels and found that neither *PEMT* transcript B or C were estrogen-responsive in hepatocytes homozygous for the risk allele.

We examined the influence of these SNPS on ER binding to the transcriptionally active regions identified in transcript A, exon 1. We found that Peak 4, which contains an ERE and FOXA1 site, was not enriched with ER- α in hepatocytes homozygous for the risk allele. Peak 4 contains three SNPs that are in high LD with the most strongly risk-associated SNP---rs4646343—in human hepatocytes. Peak 5 was also diminished in the context of the rs4646343 risk SNP, supporting the hypothesis that the critical ER- α binding site in peak 4 interacts with proximal promoter B and both sites of interaction are disrupted by the SNP. We suggest that potential regulatory SNP(s) reside within Peak 4, and these alter binding affinity for transcription factor ER- α , prevent looping to Promoter B and result in decreased estrogen-mediated induction of *PEMT* transcripts B and C.

The results presented here provide a potential mechanism which explains how SNPs within a critical estrogen regulatory region of the *PEMT* locus abrogate estrogenic-regulation of *PEMT*. Characterizing estrogenic regulation of choline biosynthesis provides an explanation for studies that have reported that, in animal models, females are less sensitive to choline deficiency than are males because they have higher capacity to form choline *de novo* via the PEMT pathway. (Noga and Vance, 2003; Tessitore et al., 2000)

During pregnancy, choline stores become depleted and lactation further increases the demand for choline.(Zeisel et al., 1995) Pregnant *Pemt* knockout females had the most severe triglyceride accumulation in liver as compared to males and non-pregnant females maintained on the same diet.(Zhu et al., 2003) Studies conducted in collaboration with the March of Dimes, found that women in the lowest quartile of dietary choline intake, were 4 times as likely to have a baby with a neural tube defect.

(Shaw et al., 2006) We propose that women with functional SNPs that affect estrogen regulation will be susceptible to CDS and will have an increased risk of having a baby with a neural tube defect. This study is the first to propose a model which explains how genetic variation influences gender specific requirements for choline

SUMMARY

Studies aimed at defining the human dietary choline requirement underscore the importance of research exploring genetic and variation. Choline is a major dietary source of methyl-groups (one of choline's metabolites, betaine, participates in the methylation of homocysteine to form methionine); also choline is needed for the biosynthesis of cell membranes, bioactive phospholipids and the neurotransmitter acetylcholine. A recommended dietary intake for choline in humans was set in 1998, and a portion of the choline requirement can be met via endogenous *de novo* synthesis of phosphatidylcholine catalyzed by phosphatidylethanolamine *N*-methyltransferase (*PEMT*) in the liver. Though many foods contain choline, many humans do not get enough in their diets. When deprived of dietary choline, most adult men and postmenopausal women developed signs of organ dysfunction (fatty liver, liver or muscle cell damage, and reduces the capacity to handle a methionine load, resulting in elevated homocysteine). However, only a portion of premenopausal women developed such problems. For the first time, we present evidence that choline biosynthesis is induced by estrogen and suggest that differences in dietary choline requirements occur because estrogen induces expression of the *PEMT* gene and allows premenopausal women to make more of their needed choline endogenously. Further, we identified SNPs in an ER regulatory region that may affect estrogen-mediated induction of the *PEMT* gene. We postulate that these SNPs are

responsible for the significant variation in the dietary requirement for choline observed in women. Preliminary studies conducted by members of our clinical research team, corroborate this hypothesis. That is, we find that the administration of Premarin, an estrogen derivative, does not prevent CDS in post-menopausal homozygous for the risk allele. (manuscript in preparation).

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CHAPTER V
FUTURE RESEARCH

The preceding studies demonstrated that estrogen regulates the choline biosynthetic gene, *PEMT* and determined that regulation is conferred by a 2.2 kb region in intron 1A. A SNP (rs4646343) which conferred risk to choline deficiency syndrome in women was associated with under-expression of the *PEMT* gene in response to estrogen treatment in human hepatocytes. SNPs in linkage disequilibrium with rs4646343 that map to ER regulatory regions affect ER-DNA interactions. Based on this work, the following areas are proposed for further investigation:

I. Characterize SNP-ER Impact on Choline Biosynthesis in Human Hepatocytes.

We observed that the *PEMT* gene is responsive to estrogen treatment and examined the entire locus by ChIP-chip. We identified several ER- α enriched regions in the *PEMT* locus specifically implicating a critical regulatory region located in intron 1 of the A transcription start site, 7500 nt upstream of the transcriptional start site of transcript B. We were able to resolve this region into three potential binding sites—annotated as Peak 2-4. We found that peak 4 contains a canonical ERE as well as a FOXA1 site and suggest that the effect may be conferred by the ERE-FOXA1 site within the ER- α binding site. Promoter B was also enriched with ER- α binding although gene reporter assays indicated that this region does not harbor independent transcriptional activity. Hepatocytes homozygous for the risk allele rs4646343 were not estrogen responsive. ER-DNA binding at Peaks 2-4 and at Promoter B was diminished in hepatocytes homozygous for the rs4646343 risk allele. We sequenced this region and identified 6 SNPs, 4 of which are in high LD ($r^2 > 0.6$) with rs4646343. We hypothesize that a regulatory SNP(s) within this region acts to prevent ER-DNA binding and potentially prohibits inter-chromosomal looping of the intronic binding site to Promoter B which

resulting in under-expression of the estrogen inducible transcripts B and C. To examine the effects of genetic variation on the ER transactivational capabilities of the risk haplotype vs. the protective haplotype, we will clone the region spanning Promoter B through intron 1A from human hepatocytes homozygous for the risk allele and homozygous for the non-risk allele into luciferase report constructs. Site-directed mutagenesis will be used to examine the effects of individual SNPs within the ER- α binding region. Following transient transfection into primary human hepatocytes and induction with estrogen, we can measure luciferase activity to evaluate the individual contributions of each SNP and as well as the combined effect impact on promoter activities. We predict that the risk haplotype will result in decreased estrogen mediated promoter B induction.

Chromosome conformation capture assays (3C) have been utilized to explore long range estrogen-induced control of gene expression which is mediated through direct physical interactions between gene promoters and estrogen regulatory elements. (Perillo et al., 2008) This inter-chromosomal looping interaction can be detected using the chromosome conformation capture (3C) methodology. (Gondor et al., 2008) Studies will be conducted too determine whether the causative SNP(s) prevent or decrease inter-chromosomal interactions between the intron1A sites and Promoter B. We predict that intron 1A interactions with Promoter B would be absent in hepatocytes homozygous for the risk allele in the presence of estrogen. Analogously, our allele specific ChIP findings demonstrated that hepatocytes homozygous for the risk allele were not enriched with ER- α at Promoter B in the presence of estrogen treatment.

II. The Influence of *PEMT* Copy Number Variation on Choline Deficiency Syndrome in Women.

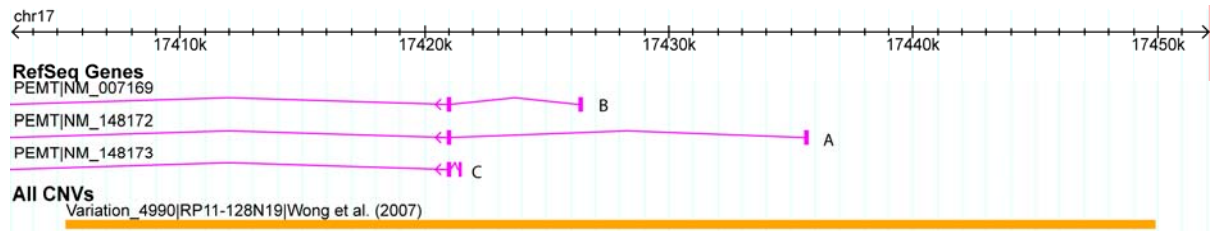
The human genome shows extensive copy-number variation (CNV), the presence of variable numbers of copies of large, multi-kilobase genomic regions in the genomes of different individuals. (Young et al., 2008) It is reasonable to speculate that copy number variants which overlap genes have significant potential to influence gene expression at the transcriptional and translational levels. The association of specific CNVs with clinical phenotypes makes it reasonable to hypothesize that CNVs in the *PEMT* locus could account for some of the variation we see in susceptibility to CDS in women. (Perry et al., 2007; Wong et al., 2007)

A study using a whole-genome comparative genomic hybridization (CGH) array, which measured large scale (>40 kb) segmental gains and losses in >100 individuals identified a CNV spanning *PEMT* locus. Of the 95 individuals analyzed, 3% of the subjects exhibited this genomic variant. (**Figure 5-1**) To determine if a CNV confers susceptibility to CDS, a robust method that allows for accurate quantitation of DNA copy number should be employed.

CGH arrays and fluorescence in situ hybridization (FISH) are techniques that have commonly been used to detect CNVs in mammalian genomes. (Babovic-Vuksanovic et al., 2004; Ishkanian et al., 2004) The resolution of CGH arrays is too low to detect microdeletions and microduplications (Mantripragada et al., 2004a; Mantripragada et al., 2004b) and would be too costly to screen large numbers of subjects. FISH is useful for regions that have previously been identified and is costly and time-consuming. Competitive PCR, a highly sensitive technique for detecting CNVs, accurately distinguishes normal copy number (2 copies) from a single copy deletion (1

copy) and a single copy gain (3 copies). (Williams et al., 2008) High-density SNP genotyping platforms, in which a signal intensity measure is summarized for each allele of a given SNP, are used to identify regions with multiple SNPs that fall within deletions or duplications. (Cooper et al., 2008) Due to the high number of polymorphisms in LD within the *PEMT* locus, high-density SNP genotyping arrays may be a good method for copy number detection and analysis. (Locke et al., 2006)

Figure 5-1. CNV identified in the *PEMT* locus. A whole-genome tiling BAC array CGH approach identified a segmental loss in in 3% of the study population in the *PEMT* gene spanning 44.5kb (Chr 17; bp 17,405,360—17,449,863).



Using quantitative PCR, an established method for detection of CNVs, (Weksberg et al., 2005), we are currently developing an assay for detection of the *PEMT* CNV previously reported by Wong and colleagues. This technique provides a quantitative measurement of DNA copy number and has several advantages, such as low technical difficulty, cost effectiveness and requires a fraction of the time required by FISH or array hybridizations.

III. The Role of Estrogens on *Pemt* Expression in Fetal Mouse Brain

ER α and ER β are differentially expressed throughout the rodent brain. (Mitra et al., 2003; Shughrue et al., 1997) Estrogen enhances learning in a range of rodent memory tasks, including the Morris water maze and inhibitory avoidance tasks and the underlying mechanisms have been elucidated. (Frye and Rhodes, 2002; Li et al., 2004)(Adams et al., 2004) Maternal dietary choline supplementation in rats results in lifelong cognitive and memory enhancement in the offspring. (Craciunescu et al., 2003) Choline availability during fetal development is critical for the developing fetal brain (Albright et al., 1999a; Albright et al., 2001; Albright et al., 1999b); however the contribution of fetal *Pemt* derived PtdCho to the choline pool is unclear. (Blusztajn et al., 1985) Early studies in rat brain found that *Pemt* enzymatic activity was approximately 4-fold higher in neonatal than in adult brains. (Blusztajn et al., 1985). Brain development is perturbed in the *Pemt*^{-/-} mouse, with increased cell proliferation in the fetal hippocampus and decreased expression of calretinin (a marker of GABAergic cell differentiation). (Zhu et al., 2004) Although the knockout mice had diminished choline and metabolite concentrations in fetal brain, unlike animals fed a choline-deficient diet, the *Pemt*^{-/-} mice had increased

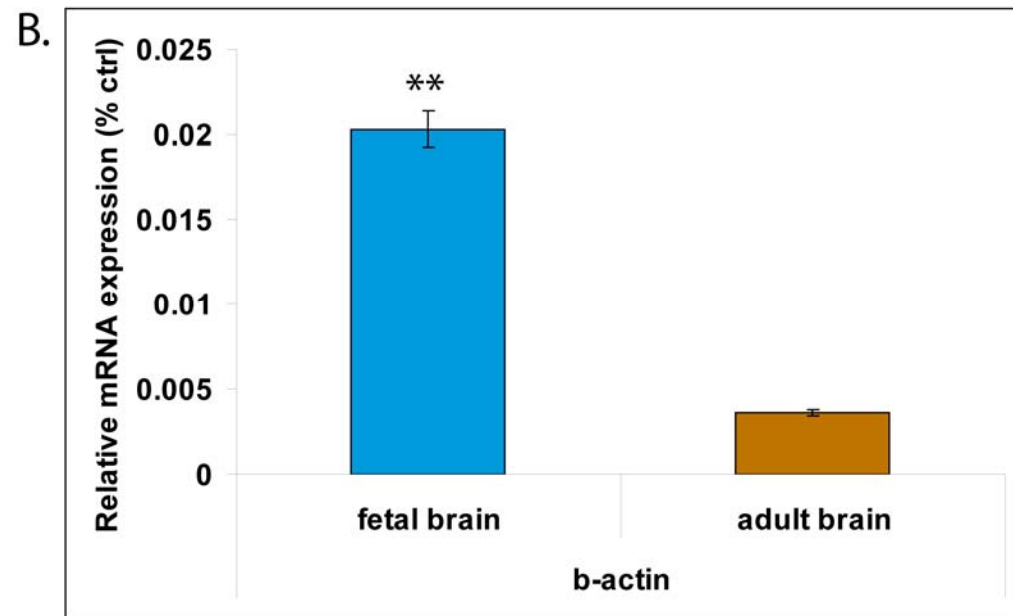
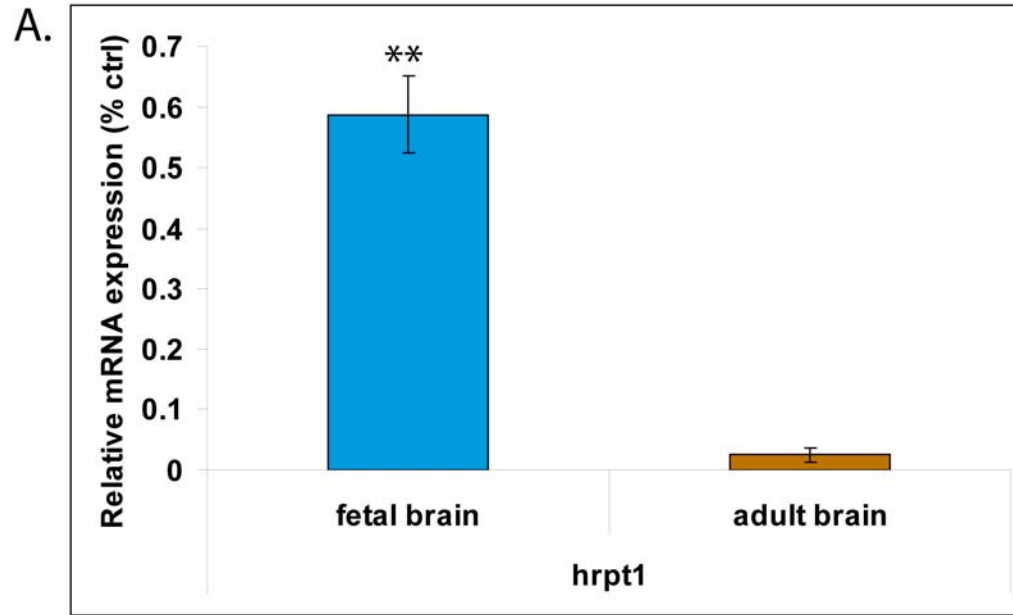
concentrations of S-adenosylmethionine in brain, with hypermethylated DNA and proteins. (Zhu et al., 2004)

The effect of maternal estrogen on fetal brain development is not well established. Pregnancy provides a high progesterone and estrogen environment for both fetus and mother. Understanding the impact of the hormones produced by the materno-feto-placental unit on brain development is an area of active investigation. Penn and colleagues at Stanford School of Medicine report that many neurons in “non-sexually dimorphic” regions are responsive to these hormones, including cerebellar and hippocampal neurons. We would like to extend our hepatic *Pemt* studies to the developing fetal brain. If estrogen regulation of *Pemt* is important during development, then SNPs that have functional significance could perturb brain development.

Preliminary studies in which we compared the relative *Pemt* expression levels in fetal vs. adult brain revealed that *Pemt* gene expression is significantly higher in fetal than in adult brain. **(Figure 5-2)** Briefly, whole brains were isolated from fetal and adult male C57Bl6 mice. Brains were homogenized in lysis buffer and total RNA isolated (Qiagen’s RNasy kit) per manufacturer’s instructions. RNA was reverse transcribed using Superscript III First Strand synthesis kit (Invitrogen) and amplified by PCR using ABI Prism 7900 sequence-detection system (Roche-Applied Biosystems, Foster City, CA). Since stable gene expression of housekeeping genes varies between tissues and throughout development, we choose to normalize *Pemt* gene expression relative two house-keeping genes, namely b-actin (*b-act*) and hypoxanthine guanine phosphoribosyl transferase I (*Hprt1*). *Hprt1* is one of the most stably expressed genes in hippocampal neurons. (Santos and Duarte, 2008) The following gene-specific Taqman assays were

used for real-time PCR: *Pemt*: Mm00839436_m1; *b-act*: Mm01205647_g1; and *hrpt1*:
Mm01305687_g1.

Figure 5-2. *Pemt* gene expression in fetal brain is markedly higher than in adult brain. To determine relative *Pemt* gene expression levels in fetal and adult brain. We performed Real time quantification of *Pemt* in mRNA isolated from fetal (E17) and adult whole brain. We normalized *Pemt* gene expression relative to two house-keeping genes (A) hypoxanthine guanine phosphoribosyl transferase I (*Hprt1*) and (B). b-actin (*b-act*). Result are expressed as mRNA expression as a percent of control (Ctrl). Results represent an average of three biological replicates. (**p<0.001; as determined by Student's *t*-test performed on log-transformed expression data)



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