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Elyse Katz

Chaperone, Co-chaperone, and Transcription Factor Proteins of the Glucocorticoid Receptor and Depression in Pregnancy

By

Elyse Katz
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Neuroscience

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Elyse Katz
B.S., Emory University, 2001

Adviser: Joseph Cubells, MD, Ph.D.
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An Abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
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ABSTRACT

Depression during pregnancy is a major women's health concern. Similar to endocrine alterations observed in certain subtypes of depression, pregnancy induces dramatic changes in the function of the hypothalamus-pituitary adrenal (HPA)-axis, leading to elevated maternal levels of cortisol throughout gestation and increased glucocorticoid receptor (GR)-resistance. This dissertation investigates the hypothesis that sex-steroid-induced changes in the gene expression levels of proteins that regulate GR-mediated signaling adaptively alter GR-sensitivity in pregnancy. Dysfunction of these putative physiologic regulatory mechanisms may increase some women's risk for depression during by altering GR-sensitivity in a suboptimal manner.

Changes in peripheral-blood mRNA levels of 17 GR-associated proteins, serum levels of estrogen, progesterone, and cortisol over pregnancy, were compared in 202 women with and without depressive symptoms. In a subset of women, we characterized GR-sensitivity with an *ex-vivo* bioassay and observed an increase in GR-resistance with progressing gestation that was paralleled by a significant up-regulation of 8 of the investigated genes in non-medicated, non-depressed pregnant women. mRNA levels of 4 genes were significantly different between the depressed and non-depressed groups. mRNA levels of heat shock protein 70 and P23 significantly correlated with GR-sensitivity, possibly providing a mechanism for the observed changes in GR-sensitivity over pregnancy and with depressive symptoms.

Alterations in GR-sensitivity in depression during pregnancy may also be a molecular mechanism for the previously reported negative impact of maternal stress

and depression in pregnancy on the health and well-being of offspring. Here, we show that maternal GR-sensitivity in pregnancy, genetic polymorphisms in FK506 binding protein 5 (FKBP5) impacting GR function, and gene expression of BAG1 and PPP5C associate with gestational duration. In addition, genetic polymorphisms in FKBP5 are associated with birth weight.

In summary, the results reported in this dissertation provide possible novel molecular mechanisms for the regulation of GR-sensitivity in pregnancy and show that depression during pregnancy associates with a dysfunction of pregnancy-related GR-chaperone up-regulation and steroid receptor sensitivity. Such dysfunction might have a direct impact on offspring and long-term consequences as prematurity and low birth weight have been linked to behavioral and metabolic disorders in childhood that can persist into adulthood.

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CHAPTER 1:

INTRODUCTION

1 Overview

Symptoms of depression can occur in a number of psychiatric diagnoses, including Major Depressive Disorder (MDD), Anxiety Disorders, and Bipolar Disorder (BPD). These depressive symptoms include dysphoric mood, anhedonia, changes in affect, such as irritability or social withdrawal, "neurovegetative" signs including disturbed sleep, appetite, or libido and disturbances of thought, such as excessive guilt or preoccupation with death or suicide. The majority of research exploring the impact of maternal psychiatric disorders on the mother and the infant's well being has used a measure of symptoms of stress and/or depression, rather than clinical diagnosis to elucidate their findings. Therefore, the studies performed in this dissertation also focused on depressive symptomatology as a measure of psychiatric illness.

Biological mechanisms of depressive symptoms, in general, are poorly understood, however research from the last 20-30 years has pointed to the involvement of the hypothalamic-pituitary-adrenal (HPA)-axis. Our knowledge of the biological underpinnings of depressive symptoms during pregnancy, a time when HPA-axis function is normally altered, is accompanied by a further paucity of information, despite the high prevalence of occurrence. In addition to being harmful to the mother, depressive symptoms during pregnancy have adverse effects on the fetus, possibly via intrauterine programming of the HPA-axis, which can be detected at birth and can increase the offspring's risk for chronic disorders in adulthood.

The three main purposes of this work were to: 1) Describe at the level of gene expression and receptor function the regulation of hypothalamic-pituitary-axis (HPA-axis) function during pregnancy in women at high risk for depression. 2) To investigate potential differences in HPA-axis regulation between women with and without depressive symptoms during pregnancy and 3) to explore how maternal HPA-axis dysfunction impacts neonatal outcomes.

2 Depressive Symptoms during Pregnancy

2.1 Assessment and Symptomatology

Within a woman's lifetime, the childbearing years represent a time in which she is most susceptible to depression (Burke et al., 1991). It is becoming increasingly apparent that depressive symptoms surrounding childbirth often begin during pregnancy(Evans et al., 2001). In fact, depressive symptoms during pregnancy are the number one predictor of postpartum depression (Beck, 1996). As symptoms start in pregnancy and continue into the postpartum time period, it may be more helpful to examine causes of depressive symptoms during pregnancy, rather than following delivery. Therefore, for the purposes of this dissertation, we will focus on the presence or absence of clinically relevant depressive symptoms during pregnancy.

Depressive symptoms can be grouped into two categories, vegetative and cognitive symptoms (Overall and Rhoades, 1982). Vegetative symptoms are those that

affect appetite and weight gain/loss, sleep disturbances, and sexual function while cognitive symptoms are related to the core mood symptoms of negative affect as well as concentration and decision making. As changes in a series of vegetative symptoms are inherent to normal pregnancy, standard instruments used to establish the diagnosis of major depression (e.g. the Structured Clinical Interview (SCID) for the DSM-IV (First et al., 1995), or to measure symptom severity (e.g. Beck Depression Inventory (BDI) (Beck et al., 1961) and the Hamilton Rating Scale for Depression (HAM-D) (Hamilton, 1960)) that contain items specific to vegetative changes are confounded for use during pregnancy. Cut-off values for determining depressed and non-depressed patients during pregnancy for these scales should be determined taking into account the likelihood that scores related to vegetative symptoms will be elevated even in normal pregnancy. At least two tools have been developed specifically for assessing depressive symptoms during pregnancy or after childbirth. The more frequently used Edinburgh Postnatal Depression Scale (EPDS) is a self-reported scale that consists of 10 questions that address anhedonia, guilt, anxiousness, sleep disturbances, feelings of being overwhelmed or panicked, depressed mood, crying, and suicide (Cox et al., 1987). Each item can be answered on a scale from 0-3, where 0 represents the absence of the symptom endorsement addressed by that item, and 1 through 3 indicates increasing strength and frequency of endorsement of the item. The Postpartum Depression Screening Scale (PDSS) is a 35-question self-report scale consisting of 7 dimensions: Sleeping/Eating Disturbances, Anxiety/Insecurity, Emotional Lability, Cognitive Impairment, Loss of Self, Guilt/Shame, and Contemplating Harming Oneself. Each

dimension consists of 5 questions rated on a 35 to 175 point scale (Beck and Gable, 2000).

Few studies have directly compared general and specific screening tools for depressive symptoms during the peripartum period. Beck and Gable (2001) showed that the PDSS had the highest sensitivity and specificity to identify cases of postpartum depression against the SCID, compared to the EPDS and the BDI. However, Hanusa et al (2008) reported the EPDS to be more accurate in recognizing postpartum depression cases compared to the PDSS and the Patient Health Questionare (PHQ). Finally, a Taiwanese group found the EPDS to be more sensitive and specific against the Mini-International Neuropsychiatric Interview (MINI) compared to the BDI in picking up depressive symptoms during pregnancy in a Taiwanese population (Su et al., 2007).

For this study we used the BDI as this scale provided the most complete dataset. The BDI had been previously validated using the Clinical Global Impressions (CGI) as the reference scale in a similar sample of subjects from the same clinic. The specificity and sensitivity of the BDI was .829 and .811 using a CGI cut-off of 3, which corresponded to a cut-off of 14 on the BDI. This validation study showed a high correlation between BDI scores and the HAM-D-17 (R=.807) and the EPDS (R=.866) (Berg et al., 2006). In the sample that was used for this dissertation, the correlation between the BDI and the HAM-D-17 was also high (R=.801).

Future studies could benefit from using a specific tool (i.e. EPDS or PDSS) for identifying depressive symptoms in pregnancy and the postpartum period, as these

scales are less confounded with items related to vegetative symptoms that are common in pregnancy regardless of depressive symptoms (i.e. changes in eating and sleeping).

A number of studies have compared depressive symptoms during the postpartum period to those reported in episodes unrelated the postpartum period (Cooper et al., 1988; Wisner et al., 1994). These studies find no evidence to support the hypothesis that depressive symptoms occurring in the postpartum period are phenomenologically unique. However, we are aware of no studies that have examined this issue during pregnancy. In a preliminary study by Dr. Newport in our group, responses to 5 items from the BDI were identified as being highly correlated with the total BDI score in a sample similar to that used in this dissertation work. Interestingly, none of these 5 items (sadness, pessimism, loss of pleasure, self-dislike, and crying) are identified as being associated with vegetative symptoms, further suggesting the validity of the BDI as a tool to assess depressive symptoms during pregnancy. Furthermore, these are common symptoms that are endorsed in non-pregnant cases of depression, supporting the conclusion that depression during pregnancy is not associated with atypical symptomatology. These 5 items comprise what has been termed the "Baby Beck," and may be useful for obstetricians, pediatricians, and general practitioners to quickly identify women in the peripartum period who may be depressed and could benefit from psychiatric treatment (Berg et al., 2006).

2.2 Prevalence and Implications

The point prevalences of postpartum depression and depression during pregnancy are similar ranging from 8-13% (Cox et al., 1993; Evans et al., 2001; Gavin et al., 2005; O'Hara et al., 1990). However, the number of studies devoted to identifying the point and lifetime prevalence of depressive symptoms specifically during pregnancy is much smaller, compared to the number of studies in the postpartum literature. Since depressive symptoms during pregnancy have been associated with adverse outcomes for the mother and her fetus that could be long-lasting (Clark, 1998; Nomura et al., 2007), it will be important to perform future studies to confirm the prevalence rates for depressive symptoms during pregnancy.

Previous research in neonates born to depressed mothers suggests that prenatal stress and depression may represent a child's first adverse life event, which can be detected at birth and can have life-long effects (Newport et al., 2002a; Newport et al., 2002b). Shortly after birth, neonates born to depressed mothers show abnormal muscle tone, more irritability, lower scores on orienting to stimuli, and less robustness and endurance (Abrams et al., 1995) as measured by the Brazelton neonatal behavioral assessment scale (Brazelton and Nugent, 1995), compared to neonates born to non-depressed mothers. In addition, increased right frontal activation, as measured by electroencephalogram (EEG), in neonates of depressed mothers compared to non-depressed mothers (Diego et al., 2006), suggests an increased predisposition for negative affect (Davidson, 2000). These newborns are also at increased risk for low birth

weight (Hoffman and Hatch, 2000) and prematurity (Field et al., 2004) which have been implicated in glucose intolerance, obesity, and hypertension in adulthood (Clark, 1998).

Behavioral abnormalities have been described in children up to 4 ½ years old, born to mothers who were depressed in pregnancy, the postpartum period, or both (Downey and Coyne, 1990; Luoma et al., 2001; Luoma et al., 2004; Lyons-Ruth et al., 2000; O'Connor et al., 2002a; O'Connor et al., 2002b; Philipps and O'Hara, 1991). It is hypothesized that an additive model is at work, where depressive symptoms in pregnancy along with continuing depressive symptoms in the postpartum period doubles the risk for behavioral issues in these children (O'Connor et al., 2002a).

2.3 Intrauterine Progamming and the HPA-axis

Intrauterine programming can broadly be defined as prenatal conditions that have long-lasting effects on the structure and/or function of physiological processes in the offspring. For example, exogenous glucocorticoids are commonly administered to pregnant women in order to accelerate fetal lung development of when there is a high risk for premature delivery. This administration of glucocorticoids is an alteration of the fetal environment that, while having the short-term benefit of increased lung development, has been associated with intrauterine growth restriction and low birth weight, both of which associate with increased risk for chronic disorders in adulthood. The clear impact of exogenously administered glucocorticoids on neonatal outcome suggests that endogenous HPA-axis hormones such as corticotrophin releasing hormone (CRH), adrenocorticotropin (ACTH), and cortisol may play important roles in intrauterine programming. Recent studies have shown that maternal symptoms of depression,

anxiety, and trauma are associated with alterations in the levels of cortisol in the infants at 6 months of age, the reactivity of cortisol in response to a stressor at 6 months of age, and cortisol levels at 1 year old, respectively(Brennan et al., 2008; Yehuda et al., 2005). Furthermore, adult offspring of Holocaust survivors who had Post Traumatic Stress Disorder (PTSD) had lower mean cortisol levels than offspring of Holocaust survivors without parental PTSD or offspring from unexposed parents (Yehuda et al., 2007). These studies show that psychiatric symptoms in the mother can have short-term and long-term consequences on HPA-axis output and function. We hypothesize that depression-related differences in HPA-axis function may mediate some or all of the relationship between maternal depression and child outcome.

Fetal development is a multi-faceted process that involves a large number of systems and molecules. CRH, in particular, has been identified as being associated with timing of onset of labor, birth weight, as well as fetal neurodevelopment and learning (Sandman et al., 1999; Sandman et al., 2003; Smith and Nicholson, 2007(Wadhwa, 2004 #735(Sandman, 1997 #764; Wadhwa et al., 2004)). Women with increased levels of CRH during pregnancy, thus, are at risk for preterm labor and their infants are at increased risk for being born small for their gestational age, in addition to having abnormal neurodevelopment. It has been found that women with depressive symptoms during pregnancy are more likely to deliver prematurely and have low birth weight babies (Wadhwa et al., 1993), but the direct link between depressive symptoms, high levels of CRH, and baby outcome has not yet been investigated.

Recent unpublished studies in our laboratory suggest significant associations between the levels of maternal HPA-axis hormones and infant HPA-axis hormones. These studies comparing plasma levels of CRH, ACTH, cortisol, and cortisol binding globuling (CBG) in maternal and umbilical cord blood at parturition showed that maternal cortisol and ACTH were highly

predictive of neonatal cortisol and ACTH at delivery (Smith et al., in preperation). Since cortisol is passed through the placenta, while ACTH is not, these results suggest both direct and indirect transfer of hormonal information from mother to fetus during pregnancy.

In summary, these data suggest that maternal psychiatric symptoms can alter infant HPA-axis hormone level and reactivity. In addition, the maternal and fetal HPA-axis communicate with each other directly and indirectly through the placenta, so that maternal HPA-axis hormones, such as CRH, can impact neonatal outcomes. However, it is not known whether these phenomena are related. To date, the impact of maternal psychiatric symptoms on maternal HPA-axis function, infant HPA-axis function, and infant outcome has not been studied in the same prospectively followed cohort of women (and their infants) with or without a history of psychiatric disorders throughout pregnancy and the postpartum period. While this issue will not be addressed in its entirety in this dissertation, parts of it will be examined. Furthermore, due to adequate access to subjects on a prospective basis, on-going studies to understand these relationships are being performed within the Women's Mental Health Clinic at Emory University.

2.4 Risk Factors for Depression during the Peripartum Period

As with other complex medical conditions, a combination of risk factors can increase the likelihood of depressive symptoms during pregnancy and the post-partum period. While a number of environmental risk factors have been consistently found to be associated with increased risk for depressive symptoms in pregnancy and during the postpartum period, biological risk factors have been more elusive.

2.4.1 Environmental Risk Factors

Stressful live events have been associated with risk for Major Depressive Disorder (MDD) (Korte et al., 2005; McEwen, 2000; McEwen, 2003), including depression during pregnancy and the postpartum period. In particular, *psychosocial* stressors have been associated with an increased risk for depressive symptoms during this time period (Brett and Barfield, 2008; Gotlib et al., 1991; O'Hara et al., 1983; O'Hara et al., 1984; Paykel et al., 1980). Similar to studies examining prevalence rates of depressive symptoms during the peripartum period, the majority of research identifying environmental risk factors for depressive symptoms in the time surrounding childbirth has focused on the postpartum period, rather than pregnancy. While more research is needed in this area, previous studies have shown a similarity in risk factors for the development of depressive symptoms both during pregnancy and the postpartum period. These studies are reviewed below.

A recent report by the CDC, that surveyed nearly 52,000 women in 17 states in the United States between the years 2004 and 2005, found that tobacco use in the last 3 months of pregnancy, physical abuse in the 12 months before or during pregnancy, partner-related stress during pregnancy, traumatic stress during pregnancy, and financial stress during pregnancy were significant risk factors for the development of depressive symptoms in the postpartum period (Brett and Barfield, 2008). Other studies have found similar results with respect to marital conflict specifically, and social support more generally during pregnancy (Gotlib et al., 1991; O'Hara et al., 1984; Westdahl et al., 2007) or following delivery (Gotlib et al., 1991; Paykel et al., 1980), with higher levels

of perceived or actual stress during pregnancy or the postpartum period (Gotlib et al., 1991) and financial poverty (Segre et al., 2007) predicting depressive symptoms. Consistent with other studies (Garvey et al., 1983; Paykel et al., 1980), the CDC report also found that women who were younger or less well educated were more likely to report depressive symptoms in the postpartum period (Brett and Barfield, 2008). Finally, studies have identified illness in a neonate (Gotlib et al., 1991; O'Hara et al., 1984), a larger number of children (Faisal-Cury et al., 2004), and absence of a partner (Gotlib et al., 1991; O'Hara et al., 1984) as factors associated with an increased risk for development of postpartum depression.

2.4.2 Biological Risk Factors

Pregnancy and the postpartum period represent a time where there are large fluctuations in gonadal hormone levels. Some researchers have hypothesized that a subset of women are more susceptible to these changes that, on a smaller scale, can also occur monthly as a result of the menstrual cycle and during the perimenopausal period. A number of studies have supported this idea of hormonal risk factors. Women who developed depression in the postpartum period had an increased prevalence of Premenstrual Dysphoric Disorder (PMDD) (Bloch et al., 2006), mood symptoms in the first 2-4 days postpartum and mood symptoms secondary to oral contraceptive use (Bloch et al., 2005). Also, women who have had an episode of postpartum depression are up to 75% more likely to have a subsequent postpartum depressive episode (Cooper

et al., 1988; Cooper and Murray, 1995; Cox et al., 1993; Garvey et al., 1983).

A significant personal or family history of postpartum depression (Forty et al., 2006; Paykel et al., 1980) or MDD outside of the peripartum period (Bloch et al., 2006; Kumar and Robson, 1984; O'Hara et al., 1984; Watson et al., 1984) has also been associated with an increased risk for peripartum depression, suggesting a genetic link. In a meta-analysis, O'Hara et al.(1996) found that a personal history of depression, but not a family history of depression was a predictor of postpartum depression. Steiner (2002) found that out of 254 women diagnosed with postpartum depression, 54.1% had a personal history of MDD and 63.9% had a family history of MDD. A recent study of sibling pairs from families with multiple affected relatives ascertained through probands with early-onset recurrent depression showed significant elevations in risk for perinatal depression (PND; odds ratio, OR, = 2.87) and postpartum depression (OR = 3.96) in sisters of women with histories of PND or postpartum depression, respectively (Murphy-Eberenz et al., 2006). This study provides evidence for familiality of postpartum depression. To our knowledge, only one twin study directly addresses whether risk for postpartum depression is genetic (Treloar et al., 1999). That study estimated a heritability of .38 for postpartum depressive symptoms, and .25 for DSM-IV major depression occurring postnatally. Although this study had several methodological limitations, including diagnosis of depression based on retrospective interviews, the results support the conclusion that genes contribute modestly, but significantly, to risk for postpartum depression.

While little is known about biological risk factors for postpartum depression, even

less is known about biological risk factors for depression during pregnancy. As the HPA-axis has been implicated in the pathophysiology of depression outside of pregnancy, the HPA-axis is altered in pregnancy, and HPA-axis hormones have been implicated in intrauterine programming and neonatal outcomes, one of the main objectives of this work was to examine HPA-axis function during pregnancy in a population of women at high risk for developing depressive symptoms during pregnancy. We aimed to identify differences in HPA-axis function in women with depressive symptoms compared to those without such symptoms.

3 The Endocrine System and Depression:

The endocrine system is a multifaceted system that works in conjunction with the nervous system to regulate homeostasis and growth. It consists of a number of signaling cascades, or axes, that send releasing factors from the hypothalamus to the pituitary gland, which in turn releases hormones into the peripheral circulation, where they act on target endocrine organs via membrane-bound and/or cytoplasmic receptors, which in turn release hormones that act on target tissues. Hyperactivity and hyposensitivity to negative feedback of the HPA-axis are two of the most consistent findings in the depression literature (Holsboer, 2000). Normally, corticotrophin releasing hormone (CRH; also known as corticotropin releasing factor, CRF) is released from the paraventricular nucleus of the hypothalamus into the anterior pituitary, where it stimulates the release of adrenocorticotrophic hormone (ACTH) into the circulation.

ACTH, in turn, binds to its receptors in the adrenal cortex where it stimulates the conversion of cholesterol to cortisol, which is subsequently released into the circulation. This chain of events is regulated by glucocorticoid receptor (GR)-mediated negative feedback at the level of the hippocampus, hypothalamus, and the pituitary gland (see Figure 1.1 – Nonpregnant state).

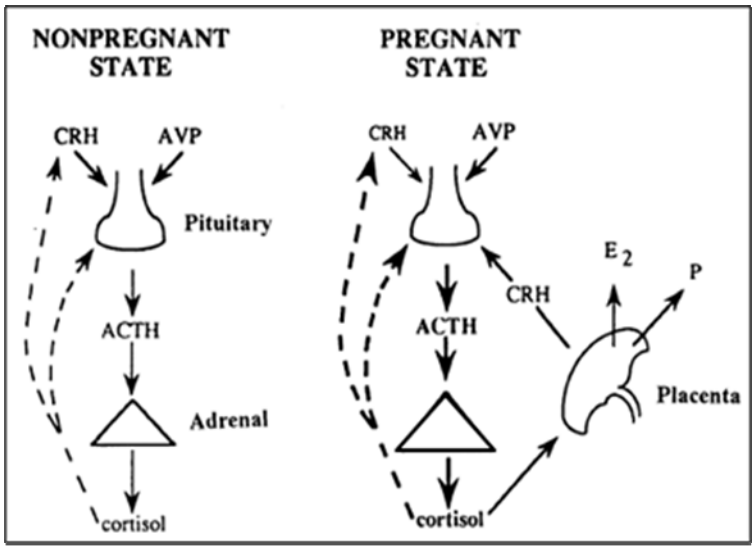

Figure 1.1. HPA-axis in nonpregnant and pregnant states. Dashed lines represent negative cortisol feedback. Solid lines represent positive cortisol feedback. Adapted from Mastorakos and Illias 2000.

The majority of patients with depression have increased levels of circulating free cortisol and glucocorticoid resistance resulting in a lack of inhibition of cortisol release by cortisol or artificial glucocorticoids, such as dexamethasone (Holsboer, 2000; Pariante and Miller, 2001). HPA-axis dysregulation is not thought to cause depressive symptoms, but to be a peripheral measure of central dysregulation of the GR and CRH systems in regions of the limbic system that are thought to regulate mood. The HPA-axis profile in depression seems to be a state marker in that it normalizes with treatment response (De Bellis et al., 1993).

During pregnancy, the level of circulating immunoreactive CRH in the mother increases up to one-thousand fold starting in the eighth week of gestation (Goland et

al., 1986). This CRH is synthesized and released by syncytiotrophoblasts in the placenta and appears to participate in regulating the length of gestation and the timing of the onset of labor (Challis, 1995; McLean et al., 1995). CRH mRNA and protein levels are under direct genetic control of cortisol, estrogen, and progesterone. Opposite to their roles in regulating hypothalamic CRH, cortisol acts as a positive regulator of placental CRH production (Robinson et al., 1988), while estrogen and progesterone act as negative regulators (Karalis et al., 1996; Mastorakos and Ilias, 2000; Ni et al., 2002). Maternal levels of ACTH rise in response to the increasing levels of placenta-derived CRH, but remain within the normal range. Cortisol levels, in the last trimester of pregnancy, rise to twice the levels of the first trimester (see Figure 1.1 – Pregnant state) (Stalla et al., 1989).

Following delivery of the placenta after childbirth, maternal levels of circulating CRH, ACTH, and cortisol rapidly decline. Interestingly, it takes several days for HPA-axis function to return to baseline, with 80-90% of euthymic women at 3-5 days postpartum showing a glucocorticoid resistance similar to that seen in depressive episodes not related to pregnancy (Greenwood and Parker, 1984). Pregnancy-associated glucocorticoid resistance, as measured by the dexamethasone suppression test, begins in the second trimester and continues until at least 5 days postpartum (Allolio et al., 1990; Greenwood and Parker, 1984; O'Hara et al., 1991b; Smith et al., 1987). The full duration of glucocorticoid resistance in the postpartum period is not known.

Recovery of pituitary sensitivity to CRH appears to take several weeks to normalize. In a small study, Magiakou et al (1996) showed that postpartum women given

exogenous ovine CRH, exhibit a blunted ACTH response for up to 12 weeks following delivery. The blunted ACTH response was more severe and lasted significantly longer in women with depressive symptoms. Another study found no association of depressive symptoms with total cortisol, urinary free cortisol, or dexamethasone suppression with measures of postpartum mood (O'Hara et al., 1991a).

4 Compensation for Endocrine Changes during Pregnancy and the Postpartum Period

4.1 Overview

Several regulatory mechanisms appear to limit the activity of cortisol during pregnancy. One mechanism for doing so is the induction by estrogen of hepatic synthesis of corticosteroid binding globulin (CBG). CBG concentration increases dramatically during pregnancy, limiting the amount of circulating free cortisol (Bloch et al., 2003). Nonetheless, Carr et al (1981) have described a rise in free, as well as total cortisol during pregnancy, suggesting that additional modulatory mechanisms may be important. For example, levels of progesterone, which also dramatically increase during pregnancy, can antagonize the effects of the GR-signaling in vitro and in vivo (Duncan and Duncan, 1979; Keller-Wood et al., 1988; Rousseau et al., 1972). In addition, the sensitivity of the GR appears to change during pregnancy, as several groups report dexamethasone non-suppression in about 80% of pregnant and postpartum women

(Greenwood and Parker, 1984; O'Hara et al., 1991b). Thus, we propose that one possible mechanism for protecting the central nervous system (CNS) from pregnancy-induced hypercortisolemia is down-regulation of GR sensitivity, via altered expression of molecules forming the large GR-chaperone complex.

4.2 Chaperone and Co-chaperone Proteins of the GR

Cortisol that is not bound to its plasma binding protein, CBG, can diffuse freely across the cell membrane. Once in the cytosol, cortisol binds to the GR, which then forms either a homodimer or heterodimer with other steroid receptor complexes. This complex then translocates to the nucleus where it acts as a transcription factor that affects transcription of target genes. The GR is then recycled or degraded by the cell's ubiquitin-proteosome system (Grad and Picard, 2007).

There are a number of chaperone and co-chaperone proteins that regulate each step of GR processing. These steps can broadly be grouped as GR folding, maturation, trafficking to the nucleus, DNA binding/RNA transcription, and degradation (Grad and Picard, 2007). At each level of processing chaperone and co-chaperone proteins play an essential role in either increasing or decreasing the efficiency of cortisol's influence on downstream gene expression in target cells.

Proper folding of the GR is initiated when HSP70 (Heat Shock Protein 70) binds to newly synthesized hydrophobic peptides. This is an ATP-dependent process whereby HSP70 is tightly bound to the GR in its ADP-bound state and dissociates from the GR in

its ATP-bound state. HSP70 cycles between these two forms facilitating folding of the GR (Smith and Toft, 1993). Next, STIP1 (Stress-Induced-phosphoprotein 1; also known as Hop; HSP70/HSP90 Organizing Protein) transfers the newly folded GR from HSP70 to Heat Shock Protein 90 (HSP90). While HSP70, HSP90, and STIP1 are essential for folding of the GR, other co-chaperone proteins such as ST13 (suppression of tumorigenicity 13; also known as Hip; HSP70 interacting protein), which by binding to the ATPase domain of HSP70, prevents dissociation of ADP, contributes to the stability of HSP70 and the GR polypeptide chain (Hohfeld et al., 1995). BAG1 (BCL2-associated athanogene) can also bind to the ATPase domain of HSP70, however, this protein inhibits the release of the GR from HSP70 and can prevent further processing of the receptor (Bimston et al., 1998).

Maturation of the GR from the properly folded state to the conformation where it has a high binding affinity for cortisol is mediated primarily by HSP90. In an ATP-dependent manner, HSP90 binding to the GR facilitates the opening of the ligand-binding cleft (Grenert et al., 1999). Other co-chaperone proteins, such as CDC37L1 (Cell Division Cycle 37 Homologue-like 1; also known as HARC) have been shown to bind to the heat shock proteins and other co-chaperones and may mediate the maturation of the receptor (Cartledge et al., 2007; Scholz et al., 2001). The co-chaperone protien, P23, is thought to stabilize the HSP90-GR complex (Wochnik et al., 2004). Interestingly, disruption of the P23 gene in a mouse model mimics the developmental phenotype of GR and CRH knockout animals, suggesting the essential role of P23 in GR function (Grad et al., 2006). Immunophilins, such as FKBP4 (FK506 binding protein 4), FKBP5 (FK506

binding protein 5), PPID (peptidylprolyl isomerase D), PPIA (peptidylprolyl isomerase A), and PPP5C (protein phophatase 5) are thought to compete with STIP1 for access to HSP90 and by doing so, alter hormone binding affinity and subsequent processing and trafficking of the receptor complex (Ratajczak et al., 2003). These proteins may also facilitate proper folding of the GR via their peptidyl-prolyl-cis-trans isomerase (PPIase) activity (Yao et al., 2005).

Dynein is a molecular motor responsible for moving organelles and proteins throughout the cell by way of the microtubule cytoskeleton. FKBP4 contains a dynein binding domain and has been shown to facilitate the translocation of the GR-complex from the cytosol to the nucleus where it can function as a transcription factor (Czar et al., 1994; Harrell et al., 2004). Conversely, FKBP5 can prevent this action from occurring (Davies and Sanchez, 2005). Lymphocytes from New World monkeys have the same hormone binding affinity for the GR as humans, but because of a 13-fold increase in expression of FKBP5, these cells are relatively resistant to glucocorticoids, needing higher levels of cortisol to induce activation of the GR (Denny et al., 2000; Reynolds et al., 1999; Scammell et al., 2001). Alternate mechanisms of GR transport into the nucleus that are not HSP90 or FKBP4 dependent have also been proposed (Freedman and Yamamoto, 2004; Tanaka et al., 2003).

Previous studies have shown that cortisol, estradiol, and progesterone exposure alter the expression of many co-chaperone proteins *in vitro* (Hubler and Scammell, 2004; Kumar et al., 2001; Tang et al., 1995). In FKBP5, for example, hormone response elements (HREs) responsive to cortisol and progesterone are located in introns 2 and 5

of the gene (Hubler and Scammell, 2004; U et al., 2004), thus suggesting a molecular mechanism for hormonal regulation of this co-chaperone gene.

Following translocation to the nucleus, the GR-HSP90 complex binds to the promoter region of genes that contain Glucocorticoid Response Elements (GREs) (Beato and Klug, 2000). There is some debate as to the exact mechanisms involved in this process. However, evidence points to the involvement of HSP90, P23, and BAG1 (Schmidt et al., 2003; Stavreva et al., 2004). HSP90 has been found to increase nuclear mobility of both ligand bound and non-ligand bound GR (Elbi et al., 2004) in addition to stabilizing the DNA bound GR-HSP90 complex, promoting increased transcription (Stavreva et al., 2004). However, this is contradicted by other studies that suggest HSP90 may inhibit GR-mediated transcription (Kang et al., 1999). P23 and BAG1 have been found to disrupt the binding of the activated GR-complex to other coactivators (Freeman et al., 2000) and to DNA (Schmidt et al., 2003), respectively. These properties likely cause reduced transcription of GR target genes.

The primary co-chaperone protein responsible for recycling and degradation of the GR is STUB1 (STIP1 homology and U-box containing protein 1; also known as CHIP) (Hohfeld et al., 2001). STUB1 has two main mechanisms for mediating these effects. First, the STUB1 protein contains three tandem tetratricopeptide (TPR) domains, which together with a nearby α-helix forms an adaptor that can bind to both HSP70 and HSP90 (Ballinger et al., 1999; Connell et al., 2001). When bound to HSP70, STUB1 inhibits the hydrolysis of ATP to ADP, preventing HSP70 from facilitating proper folding of the GR peptide (Ballinger et al., 1999), whereas STUB1 binding to HSP90 prevents association of

the chaperone with its co-chaperones and halts further processing of the GR (Connell et al., 2001). Second, STUB1 has been identified as an E3 ubiquitin ligase which provides the GR an entrance point into the proteosome-mediated degradation process (Demand et al., 2001; Hatakeyama et al., 2001; Jiang et al., 2001). Interestingly, the BAG1 protein contains an ubiquitin-like domain (Jentsch and Pyrowolakis, 2000) and may provide redundancy in linking the GR-HSP70 complex with the proteosome (Luders et al., 2000).

Table 1.1 lists the genes examined in this dissertation; they were chosen based on evidence, described above, that their encoded proteins play important roles in regulating GR-signaling by interacting with the GR or its associated proteins.

Table 1.1. Chaprerone, Co-chaperone, and Transcription Factor Genes of Interest

Gene ID and common synonyms	Gene name	Involvement in GR processing
NR3C1	glucocorticoid receptor	receptor protein
HSP70	heat shock 70kDa protein 1	folding
ST13 (Hip)	suppression of tumorigenicity 13/HSP70 interacting protein	folding
STIP1 (Hop, p60)	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	folding
HSP90	heat shock 90kDa protein 1, alpha	maturation
P23 (TEBP)	unactive progesterone receptor, 23 kD	maturation, DNA binding
FKBP5 (FKBP51)	FK506 binding protein 5	maturation
PPP5C (PP5)	protein phosphatase 5, catalytic subunit	maturation
CDC37L1 (HARC)	cell division cycle 37 homolog-like 1/Hsp90-associating relative of Cdc37	maturation
PPIA (CypA)	peptidylprolyl isomerase A (cyclophilin A)	maturation
PPID (CypD)	peptidylprolyl isomerase D (cyclophilin D)	maturation
FKBP4 (FKBP52)	FK506 binding protein 4, 59kDa	trafficking
BAG1 (RAP 46)	BCL2-associated athanogene	folding, DNA binding, degradation
STUB1 (CHIP)	STIP1 homology and U-Box containing protein 1	degradation
NCOR1	nuclear receptor corepressor 1	DNA binding
NCOA1 (SRC-1)	nuclear receptor coactivator 1	DNA binding

4.3 Transcription factors:

DNA binding of the GR complex in the nucleus recruits co-activators and co-

repressors of transcription that, respectively, stabilize and destabilize the binding of the complex to the DNA. Upon stabilization, a myriad of nuclear transcription factors bind to the DNA to facilitate transcription of downstream genes. The nuclear receptor co-activator 1 (NCOA-1 or SRC-1) and the nuclear receptor co-repressor (NCOR1) have been shown to have direct roles in regulating the downstream effects of steroid hormone receptor actions in the nucleus (Hu and Lazar, 1999; Iannacone et al., 2002; Kurihara et al., 2000; Meijer et al., 2005). Similar to some co-chaperone proteins, NCOA1 is part of an ultra-short feedback loop in which cortisol downregulates the expression of the gene (Kurihara et al., 2002). As with the actions of the chaperone and co-chaperone proteins, a hypothesis of this dissertation is that differential regulation of NCOA1 and/or NCOR1, plays an active role in regulating downstream effects of endocrine changes in pregnancy and the postpartum period.

5 Chaperones, Co-chaperones, and Transcription Factor Proteins in Psychiatry

The majority of the seminal research in the field of steroid hormone signaling, specifically as it relates to chaperone and co-chaperone machinery, has taken place in the last decade. This field is thus in its infancy and implications of this research have yet to be realized. Nonetheless, there are a number of studies that have identified aberrations in this system with regard to psychiatric phenotypes. The most consistent

replicated findings have been with FKBP5 and HSP70, while some reports implicate HSP90, BAG1, and HDAC proteins in the pathophysiology of, or treatment response in, psychiatric disorders.

Single nucleotide polymorphisms (SNPs) in FKBP5 have been found to associate with rapid response to antidepressants and lifetime number of recurrent major depressive episodes in two independent samples of patients with MDD (Binder et al., 2004). These SNPs were also associated with FKBP5 protein levels and HPA-axis reactivity (Binder et al., 2004). Similarly, SNPs within this gene have been associated with bipolar disorder in a family-based association study as well as attempted suicide and recurrence of depressive episodes in bipolar patients in a covariate-based association study (Willour et al., 2008). Finally, variations in the FKBP5 gene have been associated with dissociation in children with traumatic medical emergencies (Koenen et al., 2005) and, more recently, in adults with PTSD symptoms and a history of child abuse (Binder et al., 2008).

Three studies have examined the role that HSP70 may play in psychiatric disease. Arion et al. (2007) used microarray analysis in brain tissue from people diagnosed wtih schizophrenia and found a significant upregulation of many co-chaperone proteins, including HSP70 in the dorsolateral prefrontal cortex in the patient tissue compared to the controls. In another study, antibody levels to HSP70 and HSP90 were found to be higher in patients with schizophrenia, highly correlated with each other, and HSP70 antibodies were associated with symptom severity and response to antipsychotics (Kim et al., 2001). SNPs in the HSP70 gene were found to be associated with poorer antidepressant efficacy in a hospitalized population of patients with MDD (Pae et al.,

2007).

Using a microarray approach in tissue from normal rat hippocampi, BAG1 was identified as a novel target for mood stabilizers, such as valproate and lithium. Chronic administration of each of these mechanistically different mood stabilizers results in upregulation of BAG1 gene expression (Zhou et al., 2005).

6 Summary

Depression during pregnancy is highly prevalent and understudied. A number of psychosocial risk factors, such as lack of social support, have been identified for this disorder. While postpartum depression appears to have a biological and genetic component, little is known about biological risk factors for depression during pregnancy. Furthermore, specific risk factors of these types have yet to be identified for either postpartum depression or depression during pregnancy.

Women are at increased risk for depressive symptoms at times when sex hormone levels are changing, such as during the luteal phase of the menstrual cycle, pregnancy, the postpartum period, and perimenopause. No absolute changes in levels of sex hormones have been associated with the development of peripartum depressive symptoms, although a sensitivity to changing levels of these hormones has been demonstrated (Bloch et al., 2000).

A decrease in glucocorticoid receptor sensitivity has been consistently implicated in depression, however mechanisms that account for changes in GR sensitivity are still

largely unknown. The hypothesis addressed in this dissertation is that, during pregnancy, regulation of chaperone, co-chaperone, and transcription factor proteins are part of a maternal compensatory mechanism, the net result of which is a reduction in GR-mediated signaling in response to elevated levels of cortisol associated with pregnancy. Furthermore, we hypothesize that depressive symptoms during pregnancy result, in part, from dysregulation of that compensatory mechanism, reflected in altered expression of transcripts encoding chaperone, co-chaperone, and transcription proteins associated with the GR. Lastly, due to the role of the HPA-axis in intrauterine processing, maternal depression during pregnancy, has adverse effects on neonatal outcomes that results in increased risk for chronic disorders later in life.

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CHAPTER 2:

EXPRESSION OF MRNA ENCODING CHAPERONE, CO-CHAPERONE, AND TRANSCRIPTION FACTOR PROTEINS OF THE GLUCOCORTICOID RECEPTOR DURING PREGNANCY IN A PSYCHIATRIC POPULATION

Introduction

In 2000, depression was the leading cause of the non-fatal disease burden in the world (Ustun et al., 2004) and is projected to remain among the top three causes of burden of disease in the coming decades (Mathers and Loncar, 2006). The lifetime risk of major depression is nearly two times higher for women than for men (Kessler et al., 1994) and within a woman's lifetime, the childbearing years represent a time in which she is most susceptible to this disorder (Burke et al., 1991). With an estimated point prevalence of between 8 and 13% (Cox et al., 1993; O'Hara et al., 1990), depression during pregnancy is a significant health problem that adversely affects both mothers and their offspring.

Previous research in neonates born to depressed mothers suggests that prenatal stress may represent a child's first adverse life event, which can be detected at birth and can have life-long effects (Newport et al., 2002a; Newport et al., 2002b). Shortly after birth, neonates born to depressed mothers show abnormal muscle tone, more irritability, lower scores on orienting to stimuli, and less robustness and endurance (Abrams et al., 1995) compared to neonates born to non-depressed mothers. In addition, depression during pregnancy (but not the postpartum period) is a predictor for an abnormal stress response (Huot et al., 2004), increased right frontal activation (Diego et al., 2006) and an increased predisposition for negative affect (Davidson, 2000) in these infants. Offspring of women who were depressed during pregnancy are also at increased risk for low birth weight (Hoffman and Hatch, 2000) and prematurity (Field et

al., 2004), which in turn predict glucose intolerance, obesity, and hypertension in adulthood (Clark, 1998). The pressing need for effective prevention and treatment of depression during pregnancy is in stark contrast with the relative lack of knowledge about the specific pathophysiology of this disorder.

Psychosocial stressors, as well as personal and family histories of major depression, associate with increased risk for depression during the peripartum period (Gotlib et al., 1991; Murphy-Eberenz et al., 2006; O'Hara et al., 1983; O'Hara et al., 1984; OHara and Swain, 1996; Paykel et al., 1980). However, relatively few data are available that address the degree to which genetic factors contribute to these depressive symptoms.

Several groups have tested the hypothesis that differences in the levels of sex hormones over pregnancy and the postpartum period may be associated with differences in mood symptoms, but most of these data do not support this idea (Harris et al., 1989; Harris, 1994; Nott et al., 1976; O'Hara et al., 1991a; Wisner and Stowe, 1997). In contrast, a study by Bloch et al. (2000) suggests that a difference in the *sensitivity* to changing levels of hormones, rather than levels of hormones themselves, appear to associate with elevated depressive symptoms.

During pregnancy, circulating levels of glucocorticoids (as well as sex-steroids) increase dramatically. Starting in the eighth week of pregnancy, the level of circulating immunoreactive corticotropin releasing hormone (CRH) in the mother increases approximately one-thousand fold over levels found in nonpregnant women (Goland et al., 1986). This peripherally circulating CRH, mostly synthesized and released by syncytiotrophoblasts in the placenta, is thought to regulate the length of gestation and

the timing of the onset of labor (Challis, 1995; McLean et al., 1995). Contrary to the negative feedback of cortisol on CRH release in the hypothalamus, cortisol acts as a positive regulator of CRH production in the placenta. Rising cortisol levels in the mother appear to be a by-product of increasing placental CRH production (Robinson et al., 1988).

Several mechanisms buffer the effects of increased maternal cortisol levels during pregnancy. Estrogen induces the expression of corticosteroid binding globulin (CBG), which sequesters biologically active cortisol (Bloch et al., 2003). Despite this rise in CBG, however, levels of circulating free cortisol also rise during pregnancy (Carr et al., 1981). The effects of free cortisol are at least partially attenuated by elevated progesterone, which can antagonize the effects of glucocorticoid receptor (GR) signaling (Duncan and Duncan, 1979; Keller-Wood et al., 1988; Rousseau et al., 1972). In addition, the sensitivity of the glucocorticoid receptor (GR), as measured by the dexamethasone suppression test, decreases beginning in the second trimester of pregnancy and continuing until at least five days postpartum (Allolio et al., 1990; Greenwood and Parker, 1984; O'Hara et al., 1991b; Smith et al., 1987).

Cortisol signaling through the GR involves a complex series of events that are orchestrated by a myriad of chaperone, co-chaperone, and transcription factor proteins. Specifically, these molecules regulate the folding and maturation of the GR, its affinity for and activation by cortisol, intracellular transport of the GR complex from the cytosol to the nucleus, the binding of this complex to DNA elements in target genes, and the recycling of GR (for review see Grad and Picard, 2007). The function of each protein is

critical for proper signaling, and the balance of function among these proteins can either increase or decrease the effect that cortisol will have on downstream gene expression in target cells. Interestingly, the expression of some these GR-regulating molecules can be induced by sex-steroids (Hubler et al., 2003; Hubler and Scammell, 2004; Kumar et al., 2001; U et al., 2004) Chaperones, co-chaperones, and transcription factor proteins of the GR are thus prime candidates as mediators of pregnancy-related changes in GR-sensitivity.

Interestingly, the profile of increased cortisol release and relative GR resistance during pregnancy resembles the pattern often observed in non-gravid major depression (Holsboer, 2000; Pariante and Miller, 2001). In this study we test the hypothesis that depressive symptoms during pregnancy is associated with changes in GR sensitivity above and beyond those observed in pregnancy, and whether these mood related differences in GR sensitivity are related to changes in chaperone, co-chaperone and transcription factor gene expression. We examined peripheral blood cell expression of mRNA encoding chaperones, co-chaperones, and transcription factor proteins of the GR, and characterized GR sensitivity using an ex-vivo bioassay over the course of pregnancy in a sample of women at high risk for PPD due to a previous history of psychiatric illness.

Methods

Subject Ascertainment and Assessment

Six hundred sixty women were recruited within the Specialized Center for Research on Sex and Gender Factors Affecting Women's Health, a federally funded research program that focuses on evaluation and treatment of women at high risk for mood disorders who are either pregnant or planning to become pregnant. Subjects were recruited through self-referral, referral from community obstetrical practices, or because they had received care during previous pregnancies at the Emory Women's Mental Health Clinic. Eligibility criteria were: (1) a positive history of an Axis I mood or anxiety disorder as defined by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (*DSM-IV) (First et al., 1995) (2) either taking no psychotropic medication for at least 2 weeks, or currently taking a selective serotonin reuptake inhibitor (SSRI) for at least 2 weeks; (3) pregnant or trying to become pregnant; (4) willing and able to give written informed consent for research participation as approved by the Emory University Institutional Review Board. Women were excluded from the study if they (1) were actively suicidal; exhibited current psychotic symptoms; (3) were severely anemic; (4) had a positive urine drug screen; (5) had an abnormal TSH; or (6) were actively abusing alcohol or drugs in the past 12 months. Two-hundred and two subjects (a total of 207 pregnancies) met criteria for entry into this study.

Blood was collected by standard venipuncture up to 8 times per patient from March 2005 through June 2007. Samples for estimating relative levels of peripheral blood mRNA, or for measurement of serum hormone levels, were frozen until RNA extraction or assay. Blood from a subset of 53 women was processed immediately after collection for use in the ex-vivo bioassay of GR sensitivity, as described below.

A DSM-IV primary diagnosis was established at enrollment using the Structured Clinical Interview (SCID) (First et al., 1995). Comorbid diagnoses were also noted at this time. The severity of mood symptoms was assessed during the course of the study using the Beck Depression Inventory (BDI) (Beck et al., 1961).

RNA extraction and quantification by real-time PCR

In this study, we examined levels of RNA extracted from peripheral blood monocytes from 522 samples from 202 subjects. Blood for RNA extraction was collected directly into either PAXgene blood tubes (PreAnalytix, Hombrechtikon, Switzerland) or Tempus blood RNA tubes (Applied Biosystems, Inc., Foster City, CA). RNA extraction for both Paxgene and Tempus tubes was performed using a modified protocol (see Appendix B) from the Versagene RNA Purification Kit for Cell Culture in a 96-well format (Gentra Systems, Inc., Minneapolis, MI).

Total RNA was reverse transcribed according to the manufacturer's protocol using the cDNA Archive kit (Applied Biosystems, Inc., Foster City, CA) and the resulting cDNA was quantified using the Quant-it Picogreen Kit (Invitrogen, Carlsbad, CA). Each

sample was diluted to 0.5ng/ul and plated in duplicate into 384-well plates, for assay by Real-Time PCR (RT-PCR) using Taqman human gene expression assays (Applied Biosystems, Inc., Foster City, CA) for transcripts listed in Chapter 1, Table 1.1 (pg 22). Assays were performed in a 10ul volume according to the manufacturer protocol on a 7900HT ABI 7900 Real Time PCR System (Applied Biosystems, Inc., Foster City, CA). For quality control, concordance of duplicates was assessed and samples with a crossing threshold (CT) greater than 35 and a standard deviation more than .25 CTs apart in each duplicate pair were excluded (18%) from further analyses.

Raw fluorescence data were used to estimate the PCR efficiency for each reaction using the LinRegPCR program (Ramakers et al., 2003). Mean assay-specific efficiencies were then used in conjunction with the CT values to calculate relative ratios of the target mRNA to that of the endogenous control gene, RPLP0 (Karlen et al., 2007; Ramakers et al., 2003). CT values were calculated using default settings within the RQ Manager version 1.2 software (Applied Biosystems, Inc., Foster City, CA). We observed a systematic difference in estimated mRNA levels between samples collected in PAXgene versus Tempus RNA tubes. To address this bias, we performed a Z-score transformation (see Appendix A) on the raw data for each type of tube. This type of correction is commonly used in studies with gene expression data derived from different batches of extracted RNA or different assay platforms (Cheadle et al., 2003). A rank order analysis was performed on the transformed values to compare the magnitude of change in gene expression over pregnancy for each candidate transcript.

Selection of endogenous and positive control genes

In order to identify an appropriate endogenous control gene for the RT-PCR assays, we pooled 4 samples each from women who were preconception, 12 weeks, 21 weeks, 36 weeks, and between 0 and 8 weeks postpartum. These samples were run on the Taqman Human Endogenous Control Array (Applied Biosystems, Inc., Foster City, CA). The array contained the genes: ACTB, AVPR1B, B2M, CANX, CCT7, CPB1, GAPD, GUSB, HPRT1, HSPCB, 4342379-18S, IFITM2, JUNB, KCNMB1, LDHB, PFDN5, PGK1, PLN, PPIA, RPLP0, TAGLN2, TBP, TFRC, and TGFBI. The CT values for RPLP0 were the least variable between the pregnancy time points, equal to or less than 0.15 CT difference, therefore this transcript was chosen as the endogenous control gene for this study. As expected based on prior studies (Akesson et al., 1998; Choi et al., 2000), TFRC, which encodes the transferrin receptor, was highly regulated during pregnancy and was thus chosen as a positive-control transcript for pregnancy-dependent regulation.

Hormone Measures

Serum levels of cortisol, estradiol, and progesterone were measured using direct radioimmunoassay kits from Diagnostic Systems Laboratories (Webster, TX). The sensitivity of these kits were 0.11 µg/dL, 4.7 pg/mL and 0.12 ng/mL and the inter-assay correlation of variation was 11.13%, 6.89%, and 4.5% for cortisol, estradiol, and progesterone, respectively.

GR Sensitivity

To assess GR function we used an *ex vivo* assay that estimates the degree of suppression of IL-6 secretion by the synthetic glucocorticoid, dexamethasone, in peripheral leukocytes, as described by DeRijk (1996) and modified by Miller et al (2005). Eight milliliters (mls) of blood were drawn into lithium-heparin anti-coagulating vacutainers (Becton-Dickinson, Franklin Lakes, NJ) and diluted 10:1 with .9% saline solution within 60 minutes of collection. Eight hundred microliters of the diluted blood was added to 100 µl of lipopolysaccharide (LPS; Sigma Chemical, Saint Louis, MO) and 100µl of dexamethasone (Sigma Chemical, Saint Louis, MO) in each of 6 wells in a 24-well flat bottom plate. The final concentration of LPS was 100 ng/ml, while the final concentration of dexamethasone was 0, 1, 10, 100, 1000, or 10000 nM. Following a 6 hour incubation at 37°C in an atmosphere containing 5% CO_2, the plate was centrifuged for 10 minutes at 1000*g.* The plasma was then aspirated and stored at -20°C until assay of IL-6 using a commercial ELISA kit (Biosource Diagnosics, Belgium). The sensitivity of the ELISA kit was <2 pg/mL and the intra-assay coefficient of variation was 2.9%.

Using Sigmaplot 10.0 (San Jose, CA), a dose response curve was determined for each sample from the ELISA results and the concentration of dexamethasone necessary to suppress 50% of the IL-6 expression (IC_{50}) was calculated.

Data Analysis

Differences in hormone levels and relative levels of transcripts across pregnancy, with and without respect to mood symptoms, were assessed using a mixed model analysis accounting for repeated measures within subjects using SAS version 9.1 (Cary, NC). The dependent variable was mRNA ratios expressed as Z-scores (see Appendix A). The predictors were depression status and trimester of pregnancy. Each analysis included a random intercept to control for individual level variability in the mRNA levels. A dichotomous variable for depression status was created by defining "depressed" as a total BDI score > 15, and "non-depressed" as < 15 at the time of blood draw. This relatively high cut-off value for clinically significant depression reflects the fact that certain items on this scale (e.g., changes in sleep, libido, etc) are endorsed more frequently during pregnancy, and so do not always reflect mood-related symptoms. Data were grouped by time relative to gestation, i.e. preconception and pregnancy trimesters in 12 week intervals. Results for each subject were averaged within a trimester, if more than one sample was collected during this time frame.

In the analyses where samples collected from women who were taking SSRIs were included, main effects between medication status (i.e. on SSRIs vs. no psychotropic medication) on the outcome variable were tested. Medication status was not significantly associated with any of our outcome variables and thus was not included in our final models.

Analyses of GR sensitivity and hormone levels were performed using SPSS version 15 (Chicago, IL). GR sensitivity measures across pregnancy, with and without

respect to mood, were analyzed using a linear regression model in a subset of patients (42%). The association between hormone levels and mRNA levels of our genes of interest were explored using correlation analysis. The significance level was set to 0.05. Age, years of education, marital status, race, number of children, number of pregnancies, and SCID diagnosis were examined for associations with outcome variables. SCID diagnosis was the only of these variables that was significantly associated with mRNA levels with a number of our genes of interest (data not shown). In analyses using mRNA levels of these transcripts (CDC37L1, HSP70, HSP90, NCOR1, P23, STIP1, STUB1, and ST13), associations with predictor variables were verified with and without SCID diagnosis entered into the model as a covariate. Only those results where the results were consistent with and without SCID diagnosis in the model are reported.

Results

Patient Demographics and Clinical Characteristics

Table 2.1 shows the demographic and clinical characteristics of the participants in this study. The mean age of the women was 32.4 years (SD=4.9). One hundred and twenty six (60.9%) women were seen at least twice during pregnancy. Using the cut-off BDI score of 15, 71.7% of samples were taken at time points at which women were significantly depressed. The mean (SD) BDI score for samples from women who were categorized as non-depressed and depressed, respectively, was 5.3 (4.1) and 23.9 (9.7)

Table 2.1. Patient demographics and clinical characteristics. Age, Education, Race, Marital status, Gravid, and Para statistics are given per subject (N=202). Medication status, psychiatric diagnosis, and number of visits are given per pregnancy (N=207).

Measure	Mean ± SD
Age, years	32.42± 4.9
Education	N (%)
High School/Some College	50 (24.9)
College	85 (41.8)
Graduate School	67 (33.3)
Race	N (%)
White	173 (85.6)
African American	20 (9.9)
Asian	6 (3.0)
Other	3 (1.5)
Marital Status	N (%)
Married	83 (78.3)
Single	22 (21.7)
Gravida	N (%)
0	17 (8.5)
1	43 (21.4)
2	60 (29.9)
3	45 (22.4)
4	19 (9.5)
5 or greater	17 (8.5)
Para	N (%)
0	83 (41.3)
1	80 (39.8)
2	28 (13.9)
3 or greater	10 (5.0)
Medication Status	N (%)
No medication	85 (41.1)
Only SSRI	98 (47.3)
Switched during Pregnancy	24 (11.6)
Psychiatric Diagnosis	N (%)
Major Depressive Disorder	120 (58.0)
Anxiety Disorder	61 (29.4)
Bipolar Disorder	20 (9.7)
Other	6 (2.9)
Number of Visits During Study	N (%)
1	81 (39.1)
2	40 (19.3)
3	31 (15.0
4	23 (11.1)
5	24 (11.6)
6 or greater	8 (3.9)

for preconception, 6.2 (4.0) and 26.9 (6.9) for 1st trimester, 6.1 (4.2) and 23.1 (6.6) for 2nd trimester, and 6.8 (3.9) and 23.4 (7.2) for 3rd trimester.

Plasma levels of Cortisol, Estradiol, and Progesterone during Pregnancy in Depressed and Non-Depressed Women

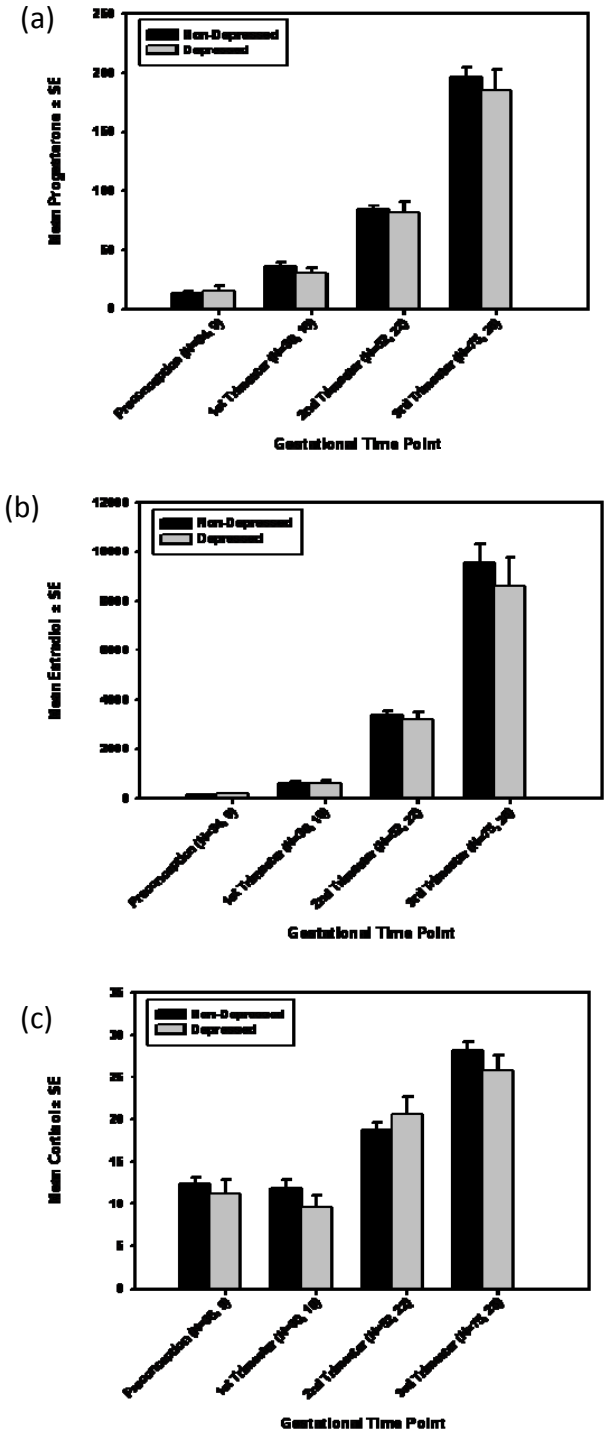
Using a mixed model analysis with pregnancy trimester and depression as predictor variables we found a significant effect of trimester on estrogen, progesterone and cortisol levels ($p<.0001$ in each case) but no effect of depressive symptoms. Third trimester levels of cortisol were 27-fold higher, progesterone 195-fold higher, and estrogen 9241-fold higher, compared to preconception levels in this mixed longitudinal and cross sectional sample (Figure 2.1a-c).

GR Sensitivity during Pregnancy

We first examined GR sensitivity using the *ex vivo* assay only in pregnant women who were euthymic at the time of blood collection (N=24; BDI mean(SD)=5.8(4.5)). Examination of IC_{50} levels by trimester showed a relationship ($p=.009$) between these two variables indicating increased GR resistance with increased gestation time (Figure 2.2).

To assess the association of mood with GR sensitivity, we compared mean IC_{50} values of pregnant non-depressed women (N = 24) to pregnant depressed women (N =

Figure 2.1a-c. (a) Progresterone, (b) estrogen, and (c) cortisol levels in depressed (N=69) and non-depressed (N=191) women at preconception and during pregnancy.



6; BDI mean(SD)=19.7(5.5)). We found a significant effect of depression status on GR-resistance (p=.0001) with higher GR resistance in depressed women (Figure 2.3a).

To reduce biases due to the small number of depressed patients with the binary BDI cut-off, we re-investigated this question using a correlation between the continuous variable of BDI score and IC_{50} in all pregnant women. After accounting for the effect of trimester, we found a significant positive correlation between the two variables (Pearson's R=.427, p=.019, N=30), confirming the relationship between mood status and GR resistance observed in the primary analysis (Figure 2.3b). Interestingly, medication status did not show an association with depressive symptoms using either the categorical definition or the continuous BDI measure, nor did medication show a main effect with IC_{50} values. Therefore, medication status was not included in the statistical models.

Figure 2.2. GR sensitivity in non-depressed women during pregnancy

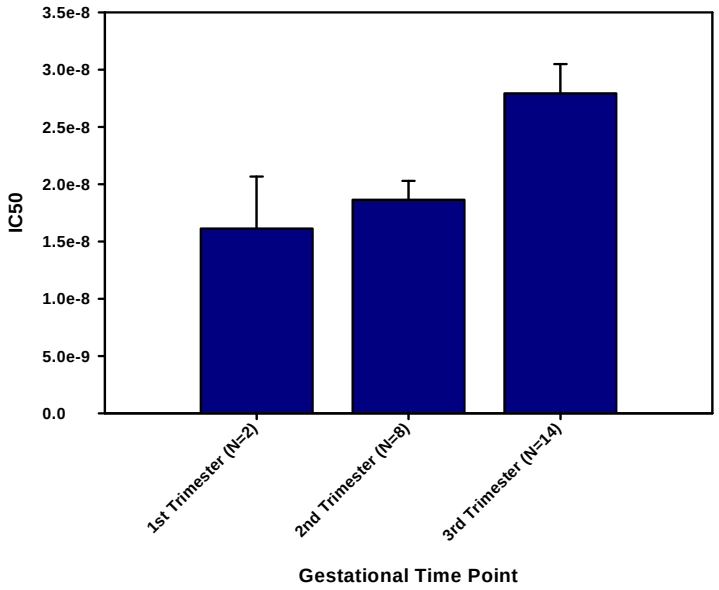
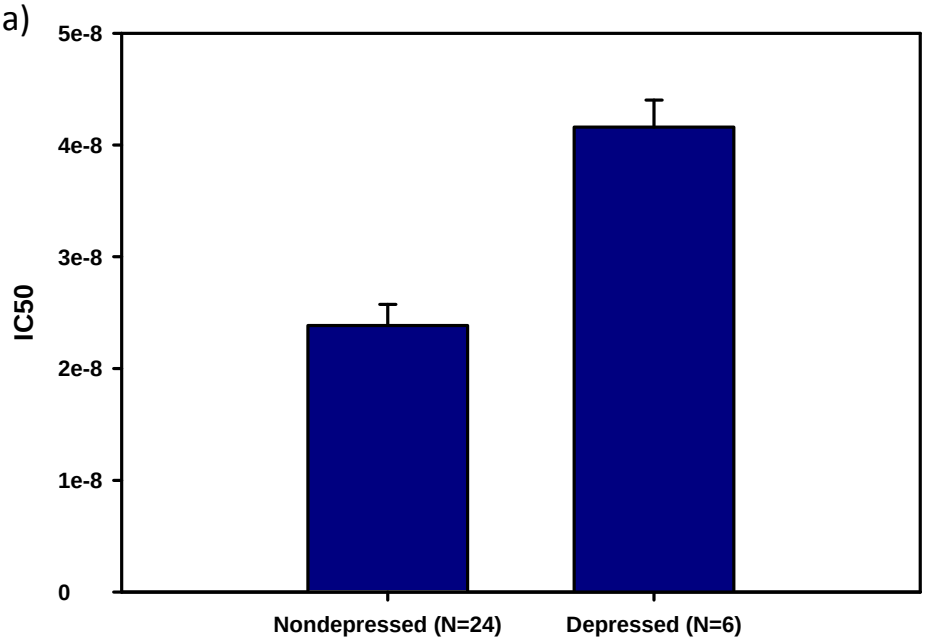
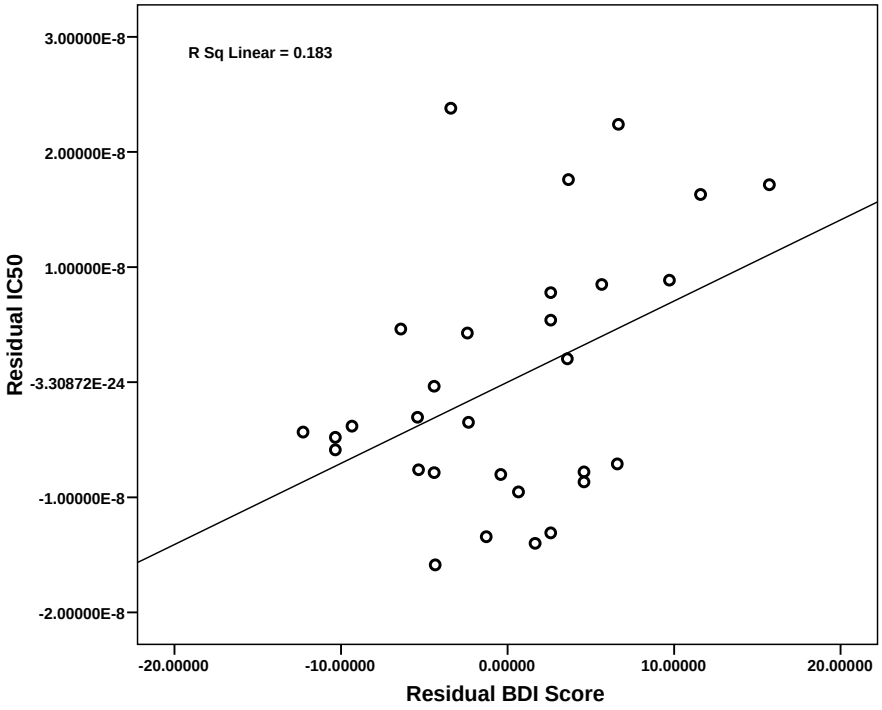


Figure 2.3a,b. GR sensitivity in depressed and non-depressed women during pregnancy. Non-depressed women in pregnancy are less GR resistant in pregnancy than depressed women in pregnancy where depression is either a categorical variable (a) or a continuous variable (b). (b) is partial correlation plot adjusting for affects of trimester

(a)



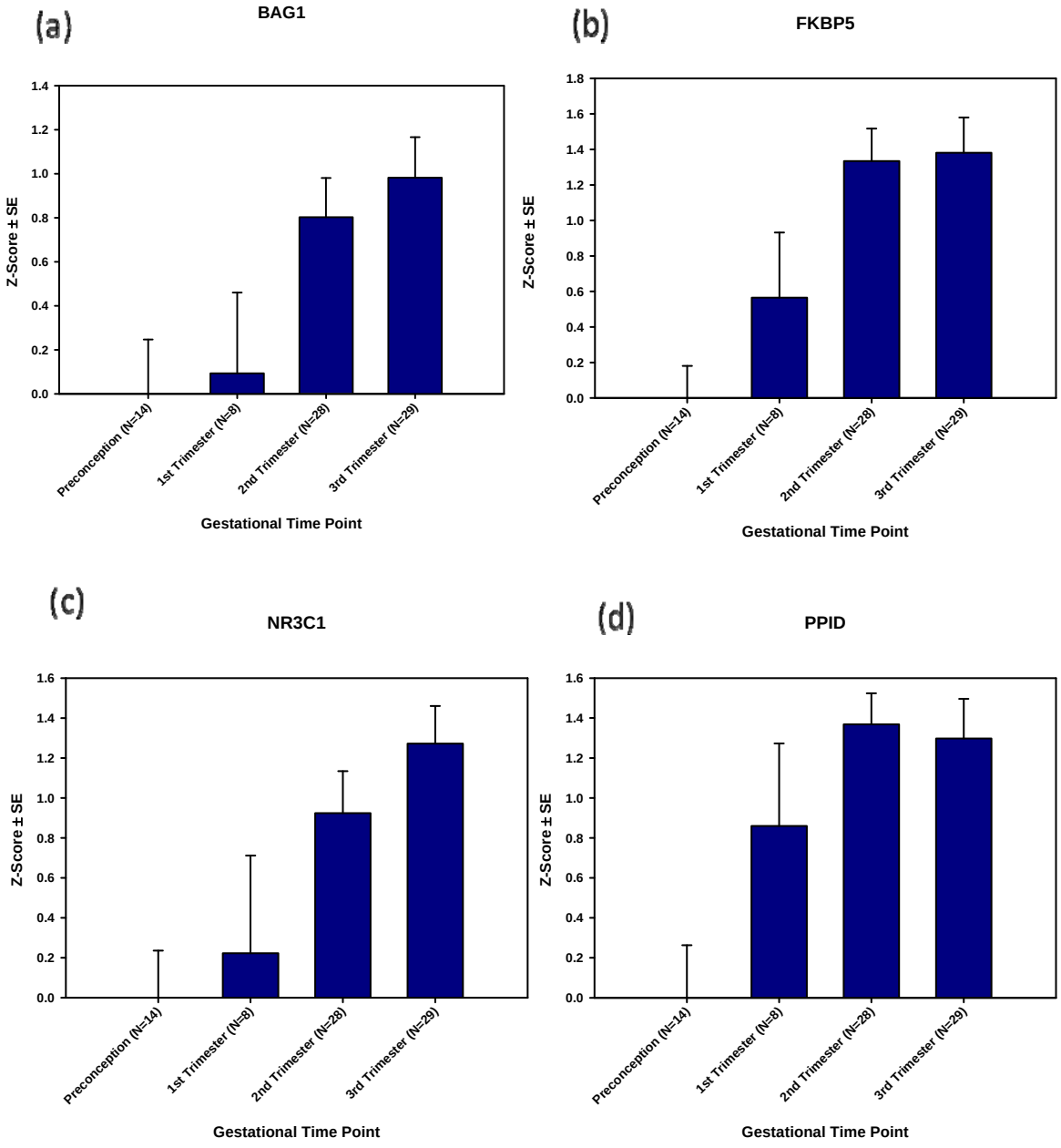
(b)



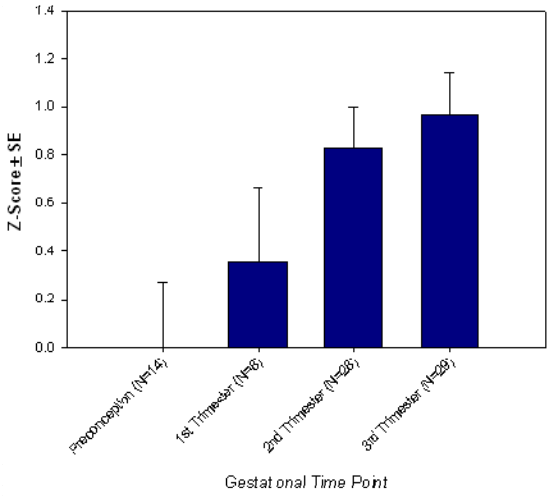
Gene Expression Changes During Pregnancy in Non-medicated, Non-depressed Women

Using only data from non-depressed women (BDI mean(SD)=7.3(4.2)) who were not taking any psychotropic medication at the time of blood draw, we compared levels of mRNA encoding proteins known to regulate GR signaling across trimesters using a mixed model analysis, using trimester as the predictor variable (N=79). Significant effects of trimester were observed for the following genes: BAG1 (p=.01), FKBP5 (p=.004), HSP70 (p=.036), NR3C1 (p=.016), PPID (p=.004), STIP1 (p=.023), ST13 (p=.032). The positive control gene TFRC was also significantly up-regulated over pregnancy (p=.002). Figures 2.4a-h show the mean and standard error of the mean for the standardized gene expression levels for the genes that were significantly regulated in pregnancy for each trimester. All values were normalized first to the endogenous control gene, RPLP0, and subsequently normalized to preconception values. In a rank order analysis comparing the combined 2nd and 3rd trimester gene expression levels to preconception levels, we found that the magnitude of the gene expression change from highest to lowest was in the following order: FKBP5, PPID, TFRC, NR3C1, STIP1, ST13, NCOR1, HSP70, BAG1, NCOA1, P23, CDC37L1, HSP90, PPIA, FKBP4, PPP5C, and STUB1 (data not shown).

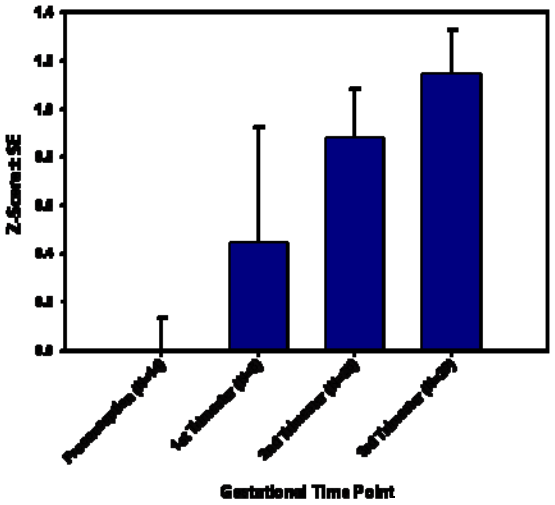
Figure 2.4a-h. Significant gene expression changes during pregnancy (N=79) in non-medicated, non-depressed women. (a) BAG1, (b) FKBP5, (c) NR3C1, (d) PPID, (e) HSP70, (f) STIP1, (g) ST13, (h) TFRC.



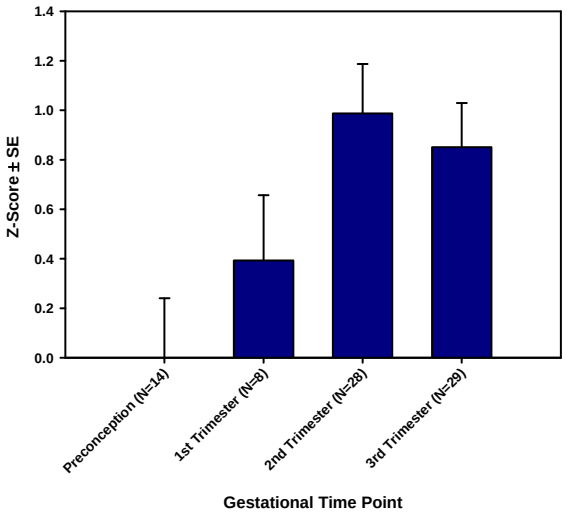
(c)


HSP70
Z-Score ± SE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Preconception (N=14)
1st Trimester (N=8)
2nd Trimester (N=28)
3rd Trimester (N=29)
Gestat onal Time Point

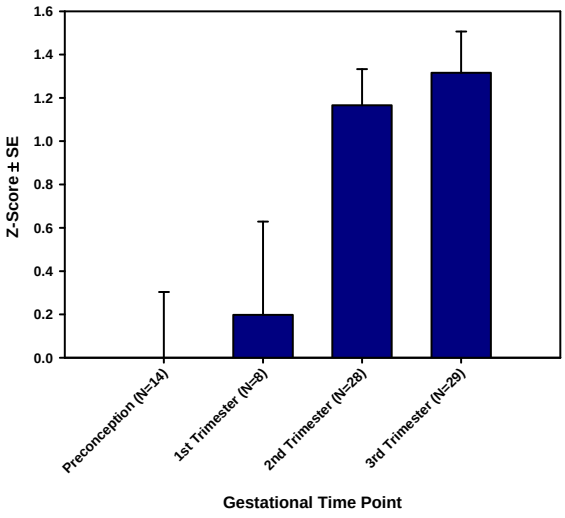
(f)


STIP1
Gestational Time Point

(g)


Z-Score ± SE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Preconception (N=14)
1st Trimester (N=8)
2nd Trimester (N=28)
3rd Trimester (N=29)
Gestational Time Point

(h)


TFRC
Z-Score ± SE
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Preconception (N=14)
1st Trimester (N=8)
2nd Trimester (N=28)
3rd Trimester (N=29)
Gestational Time Point

Differential Regulation of Co-chaperone Genes in Non-medicated, Depressed and Non-depressed Women During Pregnancy

Extending the analysis to include women with significant depressive symptoms (N=48; total N=127), but excluding all samples with SSRI intake in the 2 weeks prior to blood collection we used a similar mixed model with trimester and depressive symptom status as independent predictor variables. There were no significant interactions between trimester and depression status for any of the genes, so we were able to interpret the marginal main effects. We observed a significant main effect of depression status on gene expression for BAG1 (p=.04), FKBP5 (p=.04), NCOA1 (p=.05), and PPID (p=.02). A significant trimester effect was observed for all 4 genes.Figure 2.5a-d shows the mean and standard error of the mean for standardized gene expression levels in preconception and each trimester in pregnancy in depressed and non-depressed women.

GR Sensitivity and Co-Chaperone Gene Expression

In order to explore which molecules may be important in mediating GR signaling, we examined associations between levels of candidate transcripts and GR sensitivity as measured by the ex vivo bioassay. Results, shown in figures 2.6a-b, illustrate that the expression of transcripts encoding HSP70 and P23 significantly correlated with GR sensitivity in women during the peripartum period (N=30, p=.031 and p=.047, respectively).

Figure 2.5a-d. Gene expression changes in non-medicated, depressed and non-depressed women during pregnancy. (a) BAG1, (b) FKBP5, (c) NCOA1, (d) PPID).

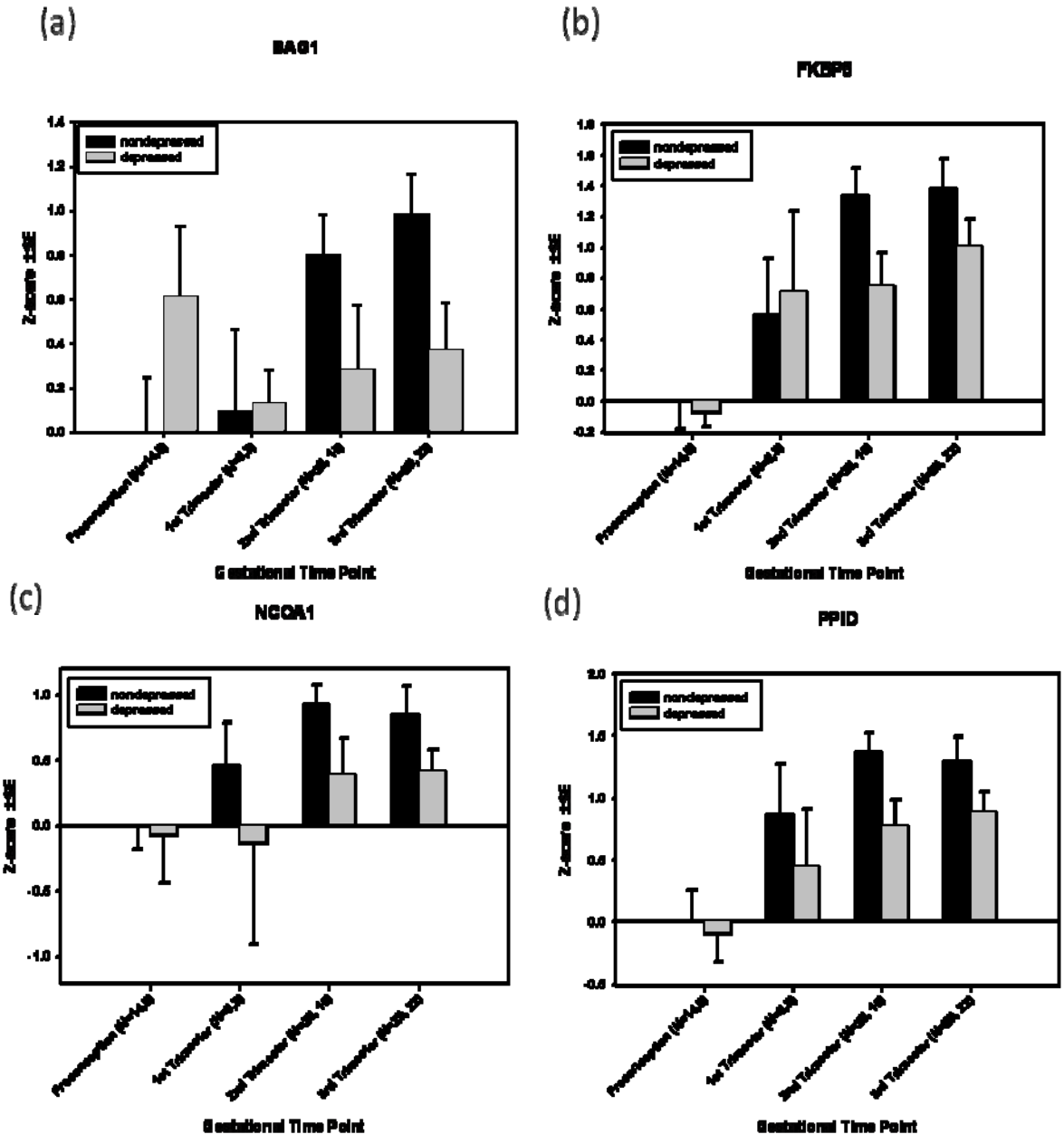
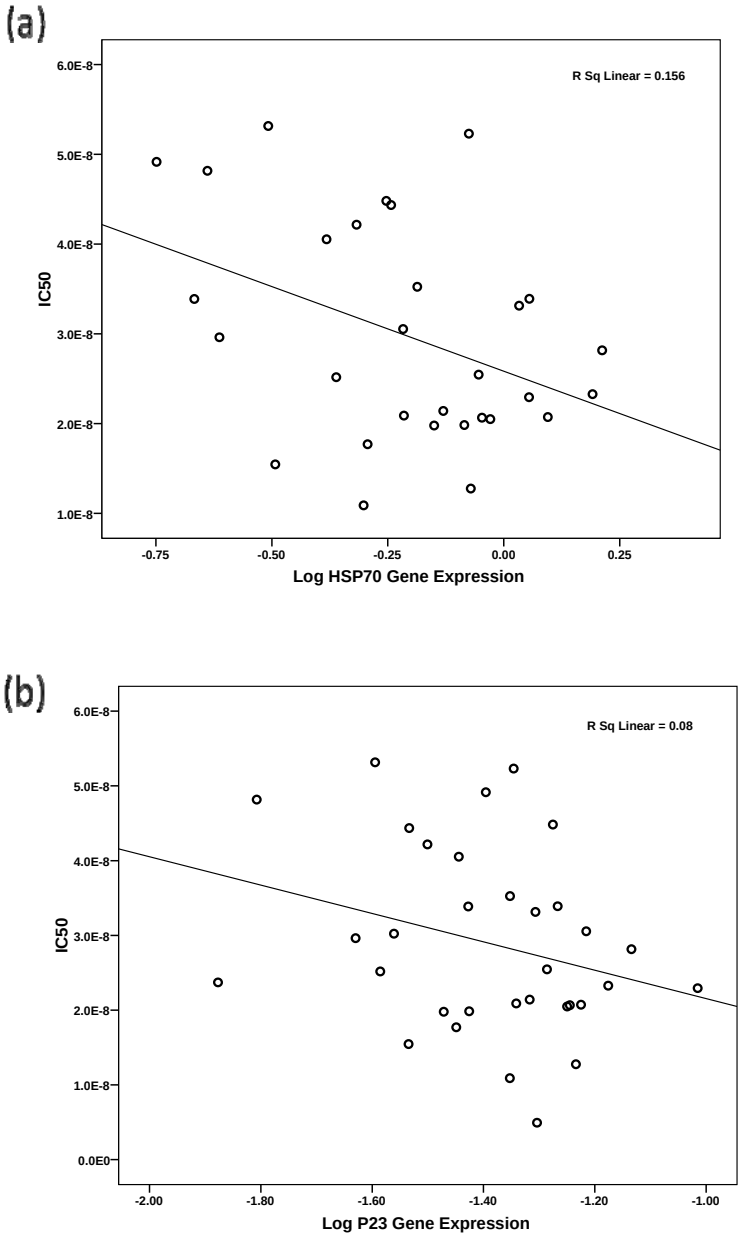


Figure 2.6a,b. Correlation between (a) HSP70 (N=30, p=.031) and (b) P23 (N=30, p=.047) with IC_{50} values from the GR sensitivity assay.



Cortisol, Estradiol and Progesterone Levels and Gene Expression in Pregnancy

Building on evidence from *in vitro* experiments that changes in levels of steroid hormones can affect expression of chaperone, co-chaperone, and transcriptions factor proteins via HREs (Beato and Klug, 2000; Hubler and Scammell, 2004), we explored whether we could detect a similar effect in our sample. Specifically, we examined whether there was a significant correlation of estrogen, progesterone and cortisol levels of our transcripts of interest. Table 2.2 shows the correlation coefficients and p-values for our genes of interest and the hormone levels. Our analyses revealed that while the expression of some genes (CDC37L1, FKBP4, FKBP5, HSP70, NCOR1, NR3C1, PPID, and TFRC) is highly associated with cortisol, estrogen and progesterone levels, others only correlate with one or two hormones or none of them. NCOA1, PPIA, P23, ST13, STIP1, and STUB1 are associated with sex hormone levels only. BAG1 gene expression is only associated with estradiol levels and P23 gene expression only with progesterone levels.

Discussion

In this manuscript, we present data that suggest a novel mechanism for pregnancy-related changes in steroid hormone receptor sensitivity. We observe that levels of mRNA encoding chaperone, co-chaperone, and transcription factor proteins critical for steroid hormone receptor function are up-regulated in pregnancy. Our data further suggest that for some transcripts, regulation of expression during pregnancy

differs in women with depressive symptoms as compared to those without these symptoms. Examination of GR sensitivity using an ex vivo bioassay supports previous studies suggesting that differences in steroid receptor sensitivity, rather than differences in overall hormone levels during pregnancy might be associated with peripartum depressive symptoms. Finally, although absolute levels of steroid hormones are not different in depressed and non-depressed women, levels of these hormones associate with mRNA levels of molecules that modulate steroid hormone signaling.

Table 2.2. Results from correlation analysis of gene expression with hormone levels

Log transformed hormone levels with log transformed standardized gene expression			
	Cortisol	Estradiol	Progesterone
BAG1 (N=408)	0.122 (0.076)	0.035* (0.104)	0.144 (0.072)
CDC37L1 (N=391)	0.043* (0.102)	0.001** (0.166)	0.001** (0.174)
FKBP4 (N=375)	0.02* (0.119)	6.2E-05** (0.205)	2.4E-05** (0.216)
FKBP5 (N=418)	2.9E-06** (0.226)	8.0E-10** (0.294)	4.5E-08** (0.263)
HSP70 (N=414)	0.041* (0.1)	2.0E-07** (0.252)	9.0E-07** (0.238)
HSP90 (N=374)	0.36 (-0.047)	0.622 (0.026)	0.789 (0.014)
NCOA1 (N=433)	0.082 (0.083)	0.006** (0.133)	0.015* (0.117)
NCOR1 (N=359)	0.023* (0.12)	5.5E-07** (0.26)	1.4E-05** (0.226)
NR3C1 (N=375)	0.041* (0.105)	4.8E-05** (0.208)	1.5E-4** (0.194)
PPIA (N=438)	0.755 (0.015)	0.022* (0.109)	0.029* (0.104)
PPID (N=427)	4.9E-05** (0.195)	6.3E-11** (0.309)	9.6E-10** (0.29)
PPP5C (N=313)	0.981 (-0.001)	0.625 (0.028)	0.946 (-0.004)
P23 (N=385)	0.518 (-0.033)	0.066 (0.093)	0.013* (0.126)
ST13 (N=404)	0.059 (0.094)	1.0E-4** (0.191)	1.9E-4** (0.184)
STIP1 (N=312)	0.074 (0.101)	0.008** (0.149)	0.004** (0.162)
STUB1 (N=406)	0.797 (-0.013)	0.01* (0.127)	0.003** (0.146)
TFRC (N=431)	3.8E-06** (0.22)	2.1E-14** (0.356)	1.0E-13** (0.319)
	Values represent p-values (Pearson's R)		
	* p<.05		
	** p<.01		

Epigenetic changes affect chromatin structure of DNA and subsequent transcription of genes via methlyation, acetylation, phosphorylation or ubiquitylation of histone tails (Nemeth and Langst, 2004; Rice and Allis, 2001; Strahl and Allis, 2000). The more tightly bound the chromatin is to histone proteins, the less available the regulatory regions of DNA are to transcription factor binding, leading to decreased transcription rates of the genes associated with those promoters. Steroid hormone receptors, through their recruitment of co-activators and co-repressors can cause epigenetic changes (Misiti et al., 1998; Thomassin et al., 2001) that impact mRNA levels of downstream molecules, such as the co-chaperone proteins. Thus, epigenetic changes may play an intermediate role between steroid hormones and mRNA levels of molecules that in turn effect steroid hormone signaling.

GR-function during pregnancy:

Using an *ex vivo* bioassay for GR sensitivity, our data confirm results from studies performed over two decades ago using the dexamethasone suppression test (Greenwood and Parker, 1984; Smith et al., 1987), showing that pregnant women develop increasing GR resistance with gestational time. It has been hypothesized that this increased GR resistance during pregnancy is a mechanism to protect the mother from increasing plasma cortisol levels related to the placental release of CRH, which is important for timing of delivery (McLean et al., 1995; Smith and Nicholson, 2007). In addition to confirming that GR resistance increases during pregnancy, our data suggest that this change in GR sensitivity is associated with up-regulation of several chaperone

and co-chaperone genes regulating GR function over pregnancy. In fact, gene expression levels of HSP70 and P23 were significantly associated with GR sensitivity in our sample. *In vitro* studies show that these two molecules are integral to GR function. HSP70 recognizes hydrophobic nascent strands of GR protein and ensures proper folding (Palleros et al., 1991), while P23 stabilizes the HSP90-GR complex allowing greater hormone binding (Wochnik et al., 2004). Interestingly, disruption of the P23 gene in a mouse model mimics the developmental phenotype of GR and CRH knockout animals suggesting that P23 plays an essential role in HPA-axis function (Grad et al., 2006).

We also observed correlations between levels of transcripts regulated during pregnancy and rising steroid hormone levels. These hormones might induce chaperone gene expression through regulation of gene transcription via the interaction of glucocorticoid, estrogen, and/or progesterone receptors with hormone response elements (HREs). These consist of DNA binding sites in the target genes for these receptors and allow them to act as transcriptional regulators, the major mode of action of steroid receptors (Beato and Klug, 2000). Previous studies have shown that cortisol, estradiol, and progesterone exposure alter the expression of many of our genes of interest using *in vitro* systems (Hubler and Scammell, 2004; Kumar et al., 2001; Tang et al., 1995). In FKBP5, for example, HREs responsive to cortisol and progesterone are located in introns 2 and 5 of the gene (Hubler and Scammell, 2004; U et al., 2004), thus suggesting a molecular mechanism for hormonal regulation of this co-chaperone gene.

Interestingly, we also observed up-regulation of NR3C1 mRNA, encoding the GR itself during pregnancy. Because we did not observe differences in the maximal binding

capacity (Vmax) in the ex-vivo GR sensitivity assay at different gestational time points, it is likely that this increase in mRNA expression is due to increased GR turn-over and does not result in a net increase in number of active receptors. Consistent with this hypothesis, BAG1, the expression of which also increases over pregnancy, interacts with STUB1 to increase proteasomal degradation of the GR (Demand et al., 2001; Luders et al., 2000).

While our study focused on the GR due to its relevance to mood symptoms, it is likely that these changes in chaperone and co-chaperone expression also influence the sensitivity of the estrogen and progesterone receptors, as these molecules are to some degree promiscuous in their association with target proteins (Pratt and Toft, 2003).

One of the limitations of this work is the lack of a healthy, control cohort to test whether the observed changes are related to a history of a mood or anxiety disorder. It will thus be important to replicate these data in pregnant women without a psychiatric history in order to assess the generalizability of these findings.

GR function in peripartum depression:

Regulation of several chaperone, co-chaperone, and transcription factor transcripts during pregnancy appear to be altered in women with depressive symptoms. Of the 4 transcripts that were differentially regulated in depressed and non-depressed women, two have a proposed negative role in GR signaling (BAG1 and FKBP5) and two a proposed positive role in GR signaling (NCOA1 and PPID) (Bimston et al., 1998; Davies et al., 2002; Kimmins and MacRae, 2000; Kullmann et al., 1998; Kurihara et al., 2000;

Meijer et al., 2005; Morishima et al., 2000; Odunuga et al., 2004; Ratajczak et al., 2003; Schneikert et al., 1999). Women with depressive symptoms may up-regulate BAG1, FKBP5, NCOA1, and PPID less over pregnancy than women without depressive symptoms. In our data is also accompanied by an increase in GR-resistance, which would have been difficult to predict from the known effects of the molecules on GR function. This may be due to several factors. First, with the exception of FKBP5, most functional predictions for the net effect of chaperone action on GR function results from *in vitro* manipulations and may not be extrapolated to the *in vivo* system (Grad and Picard, 2007). Second, levels of mRNA might not correlate with protein function, as increased mRNA levels may be the result of increased protein turnover or altered mRNA degradation, for example. Finally, chaperone and co-chaperone genes act in concert and their activity is dependent on the presence of other chaperone genes. Building ratios or matrices of expression levels may assist in a better prediction of how multiple molecules affect GR function. Statistical analyses, such as principle component analyses, that would aid in further determining the combination of proteins having the most impact on GR sensitivity require large datasets that were not available to us at the time this paper was drafted. Future studies should employ these techniques to gain further insight into the relationship between chaperone protein gene expression and GR sensitivity.

Data from the ex-vivo GR function assay show that increased severity of depressive symptoms is associated with increased GR resistance above and beyond what is expected in normal pregnancy. These data parallel what is seen in non-gravid depression in which increases in GR resistance are observed in a subset of patients with

major depression (Holsboer, 2000; Pariante and Miller, 2001). As all our results come from the study of peripheral blood monocytes, we cannot directly extrapolate these data to the function of the GR in other tissues, such as the brain. Nonetheless, a series of studies support the idea that glucocorticoid receptor signaling in these cells can reflect overall GR function. Glucocorticoid receptor resistance as measured by endocrine stimulation tests and its resolution with antidepressant treatment is one of the most consistent biological findings in major depression (Holsboer and Barden, 1996; Pariante, 2004). This overall steroid resistance has also been reported for the activation of T-cells and monocytes in major depression and bipolar disorder (Pariante and Miller, 2001) suggesting comparable GR impairment in immune cells and the central nervous system (Lowy et al., 1984; Lowy et al., 1988). GR function in PBMCs might thus in fact be a peripheral marker of general GR signaling dysfunction.

Potential mechanisms for depressive symptom-related differences in chaperone and GR function:

As mentioned above, chaperone and co-chaperone gene expression is to some extent modulated by steroid receptor activation. While our data show associations between total blood levels of cortisol, estradiol, and progesterone and mRNA levels of our genes of interest, differences in overall levels of steroid hormones in depressed compared to non-depressed women do not appear to account for the differences in depressive symptoms during pregnancy. The fact that there are no significant

differences in hormone levels between depressed and non-depressed women, but that there are symptom-related differences in gene expression levels in BAG1, FKBP5, NCOA1, and PPID suggests differences in the transcriptional regulation of these genes by steroid hormones, due to altered receptor sensitivity, downstream signal transduction or epigenetic changes of the target genes, for example.

Conclusions:

The results of the current study suggest that depressive symptoms during pregnancy associate with a decreased up-regulation of chaperone and co-chaperone gene expression over pregnancy. Such differences could lead, in turn, to depression-related alterations in steroid receptor sensitivity during pregnancy, as suggested by the positive correlation between GR resistance and severity depressive symptoms.

Absolute glucocorticoid levels have been associated with a number of adverse fetal outcomes. Specifically, cortisol levels may be involved in preterm birth by increasing placental CRH release[34] or by acting as an inhibitor of progesterone on prostaglandin-inactivating enzymes (Karalis et al., 1996; Patel and Challis, 2002; Robinson et al., 1988). Increased maternal cortisol has also been implicated in intrauterine growth retardation (Goland et al., 1993). Likewise, the programming of the fetal HPA-axis, in part by maternal hormone levels, has been described and implicated in the development of diseases such as hypertension and obesity in adulthood (Clark, 1998; Meaney et al., 2007). Mechanistically, it is possible that the efficiency of GR signaling and not absolute levels of glucocorticoids may be responsible for these

adverse outcomes. The work presented here supports the reexamination of these birth outcomes in the context of GR signaling.

Monitoring chaperone gene expression over pregnancy, as well as GR signaling, may thus serve as biomarkers for women at risk for depressive symptoms and may allow for earlier intervention which could be beneficial to both mother and baby.

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CHAPTER 3:

MODULATORS OF THE GLUCOCORTICOID RECEPTOR, GLUCOCORTICOID RECEPTOR FUNCTION, AND NEONATAL OUTCOMES

Introduction

Offspring of women who have high levels of stress or depression during pregnancy are at increased risk for motor, behavioral, and metabolic abnormalities that are present at birth (Abrams et al., 1995; Davidson, 2000; Diego et al., 2006; Newport et al., 2002a; Newport et al., 2002b), persist through childhood (Downey and Coyne, 1990; Luoma et al., 2001; Luoma et al., 2004; Lyons-Ruth et al., 2000; O'Connor et al., 2002a; O'Connor et al., 2002b; Philipps and O'Hara, 1991), and may have long-term consequences extending into adulthood (Clark, 1998; Nomura et al., 2007). While specific mechanisms that account for these adverse outcomes are still being investigated, a number of studies have suggested a role of the maternal hypothalamic-pituitary-adrenal (HPA)-axis. Prematurity and low birth weight is of special concern, as these outcomes, specifically, have been associated with the development of diseases such as heart disease, hypertension, glucose intolerance, and obesity in adulthood (Field et al., 2004; Hoffman and Hatch, 2000; Nomura et al., 2007).

Timing of the onset of labor is thought to be dependent, in large part, on the balance between endocrine and immune mechanisms. High plasma levels of corticotropin releasing hormone (CRH), which drives the HPA-axis, and of pro-inflammatory cytokines, such as interleukin-1 (IL-1β), and interleukin-6 (IL-6) have been associated with preterm labor and delivery. The anti-inflammatory molecule interleukin-10 (IL-10) is thought to play a modulatory role in this process (Greer et al., 1994; Schmeelk et al., 1999).

There is a significant bidirectional regulation that exists between the endocrine and immune systems. For example, IL-1 activates the hypothalamic-pituitary-adrenal (HPA) axis by directly stimulating the production of CRH in the hypothalamus (Sapolsky et al., 1987). In turn, cortisol, the final output of the HPA-axis, counters IL-1 effects through its anti-inflammatory properties. CRH has been shown to increase expression of the IL-1 receptor antagonist (IL-1ra), providing another mechanism for dampening the immune response (Schmeelk et al., 1999). Interestingly, IL-6 production is under the direct control of both CRH and cortisol, although CRH causes increases in IL-6 expression (Angioni et al., 1993) while cortisol signaling can decrease the expression of this molecule (Miller et al., 2005). Maternal stress and depression during pregnancy is associated with increased levels of pro-inflammatory cytokines (Coussons-Read et al., 2007).

Independent of its role in timing of labor and delivery, CRH has been shown to have significant effects on intrauterine growth. Wadhwa et al (2004) found that CRH levels at 33 weeks gestation were highest in the group of women who delivered preterm infants who were also small for gestational age (SGA). The group with the next highest CRH levels delivered preterm non-SGA neonates, while the term non-SGA neonates were associated with the lowest levels of CRH.

Cortisol is a negative regulator of CRH expression and release in the hypothalamus, while it is a positive regulator of this peptide in placental tissues (Robinson et al., 1988). Cortisol signaling through the glucocorticoid receptor (GR) involves a complex series of events that are mediated by a large number of chaperone,

co-chaperone, and transcription factor proteins (Grad and Picard, 2007). In fact, the tissue-specific effects of cortisol on CRH production are thought to be obtained through differential association of the GR with various chaperone, co-chaperone and nuclear proteins (Nicholson et al., 2004). In the following analyses we test the hypothesis that GR function and the molecules that modulate GR function also affect timing of onset of labor and intrauterine growth, possibly by altering CRH expression and immune-regulation. Furthermore, we examine the relationship between single nucleotide polymorphisms (SNPs) in FKBP5 that have been shown to alter GR sensitivity (Binder et al., 2004; Binder et al., 2008) and neonatal outcomes. Although we hypothesize that alleles at FKBP5 that have been associated with increased GR sensitivity will also associate with differences in birth weight and timing of delivery, we leave the hypothesis two-tailed, because the complexity of GR regulation precludes straightforward prediction of the direction of the hypothesized associations.

Methods

Subject Ascertainment and Assessment

A subset of women from a large clinic-based study of mood disorders in pregnancy was selected for the current analyses based on the availability of delivery-outcome information (i.e. estimated gestational age (EGA) at delivery and birth weight), and of blood samples allowing analysis of gene expression and SNP genotypes. One hundred and fifty-four women (76% of the total sample) with delivery outcome data

had gene expression data during the 2nd and/or 3rd trimester of pregnancy and 177 (87%) women of the original sample had at least partial SNP genotype data.

Genotyping

Four single nucleotide polymorphisms (SNPs) in the gene encoding FK506 binding protein 5 (FKBP5) were selected based on data showing associations between these SNPs (rs3800373, rs9470080, rs1360780, and rs9296158) and functional measures of GR signaling, namely the dexamethasone suppression test (Binder et al., 2008) and the combined dexamethasone-suppression/CRH stimulation test (Binder et al., 2004). SNPs were genotyped using Taqman SNP genotyping assays (Applied Biosystems, inc., Foster City, CA). All genotype calling was done automatically using SDS v2.3 (Applied Biosystems, inc., Foster City, CA). Only genotypes in Hardy-Weinberg Equilibrium (HWE) were used for analysis (see Table 3.1).

Table 3.1. List of FKBP5 SNPs tested, their positions on Human Chromosome 6 according to University of California Santa Cruz Genome Browser Version hg17, Hardy-Weinberg Equilibrium Test p-value, and minor allele frequency

dbSNP ID	Position	Minor Allele Frequency (MAF)	Hardy-Weinberg Equilibrium Test P Value
rs3800373	35650460	0.37	0.76
rs9470080	35754410	0.38	0.92
rs1360780	35715550	0.37	0.81
rs9296158	35675060	0.36	0.72

RNA extraction and quantification by real-time PCR

See Chapter 2 for these methods.

Selection of endogenous and positive control genes

See Chapter 2 for these methods.

GR Sensitivity

See Chapter 2 for these methods.

Neonatal Delivery Measures

Neonatal gestational age at delivery (in days) was estimated using the last menstrual period (LMP) as a reference. Birth weight was determined in kilograms shortly following delivery.

Data Analysis

All analyses were performed in SPSS version 15 (Chicago, IL). Gene expression was examined by estimating levels of mRNA encoding chaperone, co-chaperone, and transcription factor proteins in peripheral blood gathered during the 2^{nd} and 3^{rd} trimesters. If a woman contributed more than one sample during each trimester, the

mRNA levels were averaged within trimester. Data from samples collected into PAXgene (N=80) and Tempus tubes (N=77) were analyzed together after performing a Z-score transformation of the raw data for each tube separately (see Appendix A).

Associations between mRNA levels of our genes of interest and the neonatal birth weight were tested using linear regression, adjusting for EGA at delivery. The association between the continuous measure of EGA at delivery and gene expression was tested using a Pearson's correlation, while the relationships between the categorical variables of preterm (<36 weeks), term (36 to 40 weeks), and post-term (>40 weeks) delivery and gene expression values were explored using analysis of variance (ANOVA). Main effects of depression status, medication status, and SCID diagnosis were tested as possible confounding variables for birth weight and EGA at delivery, but none of these factors were significantly associated with either outcome (data not shown).

Pearson's correlation was used to assess the relationship between GR function (IC_{50}) and estimated gestational age at delivery. Main effects for parity and maternal age with estimated gestational age at delivery were examined, but no significant associations were found.

All SNP genotyping analyses were performed in SPSS version 15 (Chicago, IL). Additive genotypic effects of each SNP on EGA at delivery were analyzed using linear regression analysis with EGA at delivery and each genotype entered separately in the model as a main effect. Additive allelic effects on birth weight were assessed using a linear regression model between each genotype and birth weight after adjusting for

EGA at delivery. These analyses were exploratory and thus, significance levels were not corrected for multiple testing.

Results

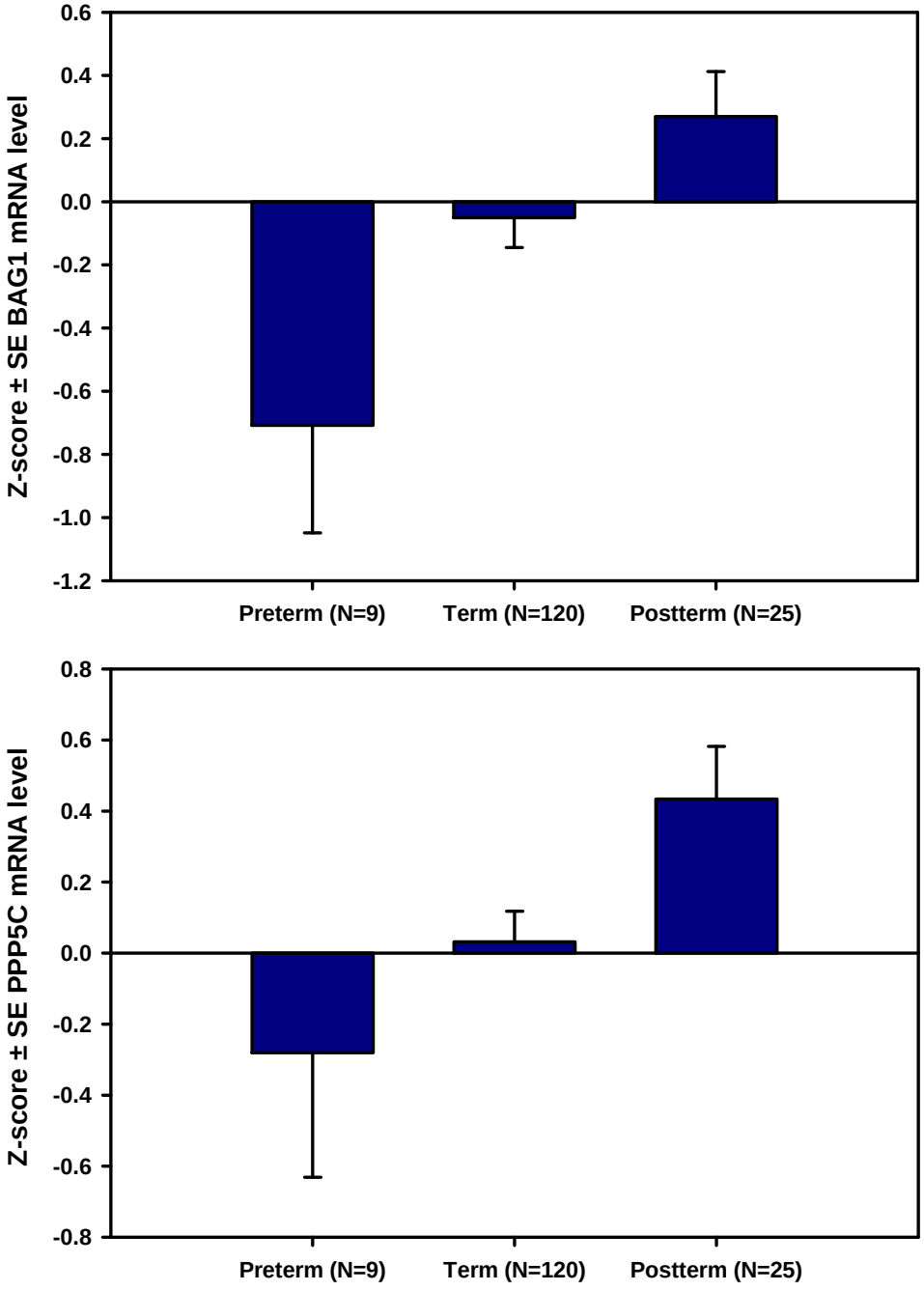
Gene Expression and EGA at Delivery

There were no significant associations between the continuous measure of EGA at delivery and standardized gene expression levels. However, third trimester levels of the mRNAs encoding the co-chaperones PPP5C and BAG1, showed significant (p=.032 and p=.019, respectively) associations with timing of delivery, in that mRNA levels of each transcript were significantly different between women who experienced pre-term birth (<36 weeks, N=9), term birth (36 to 40 weeks, N=120), or post-term birth (>40 week, N=25) (Figure 3.1a-b). Women who delivered before 36 weeks had the lowest mRNA levels of both PPP5C and BAG1, while the women who delivered after 40 weeks had the highest mRNA levels of these transcripts.

Gene Expression and Birth Weight

There were no significant associations between levels of transcripts for our genes of interest and baby weight, after adjusting for EGA at delivery.

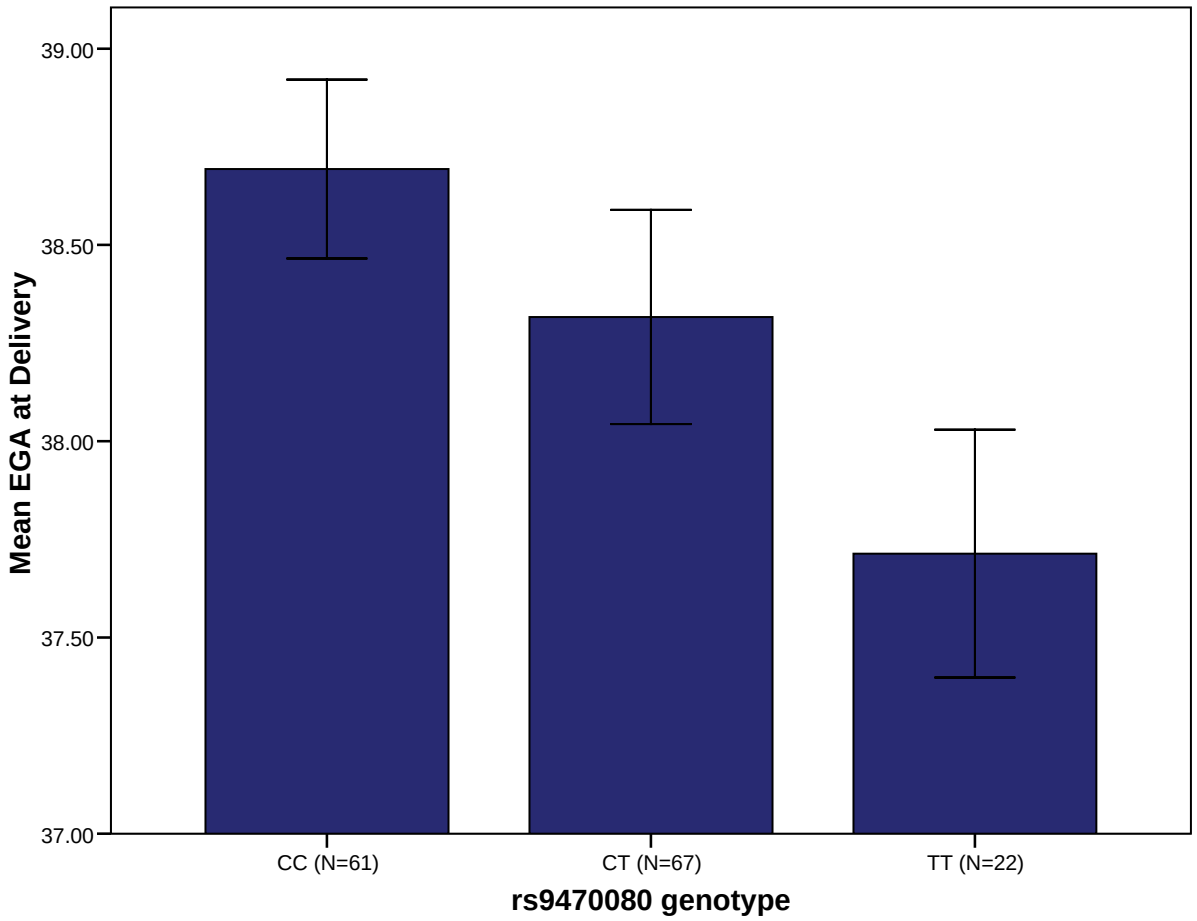
Figure 3.1a,b. Third trimester PPP5C and BAG1 gene expression levels associate with preterm, term, and post-term labor and delivery (p=.032 and p=.019, respectively) N=154). Y-axis values represent gene expression values standardized with a Z-score transformation as described in the text.



SNPS and EGA at Delivery

One SNP, rs9470080, in FKBP5 was significantly associated with EGA at delivery (p=.045) (Figure 3.2). Individuals with a TT genotype at this locus delivered at an average (SD) of 37.7 weeks (1.5 weeks) compared to those women with a CC genotype at this locus who delivered at 38.7 weeks (1.8 weeks). EGA at delivery for heterozygotes was intermediate between the homozygotes at 38.3 weeks (2.2 weeks).

Figure 3.2. FKBP5 SNP rs9470080 significantly associates with EGA at delivery (p=.045, N=150).



SNPS and Birth Weight

All four SNPs in FKBP5 that were tested (rs3800373, rs9470080, rs1360780, and rs9296158) were significantly associated with birth weight of neonates, after adjusting for EGA at delivery (Figure 3.3a). The risk alleles at these loci were associated with increased birth weight. There is a high level of linkage disequilibrium across this region of the FKBP5 with pair-wise r^2 values ranging from .74 to .97 (Figure 3.3b), thus these SNPs represent similar information regarding the relationship between genetic variation and timing of delivery.

GR Sensitivity and EGA at delivery

GR sensitivity, as measured by IC_{50} in the *ex vivo* bioassay, was significantly associated with the timing of delivery (p=.012) in 22 individuals (Figure 3.4). The GR sensitivity measures were restricted to pregnancy time points. Two women were in the 1^{st} trimester of pregnancy, 7 women were in the 2^{nd} trimester of pregnancy, and 13 were in women were 3^{rd} trimester of pregnancy when the samples was taken. This association remained significant (p=.013) after adjusting for the time point in pregnancy at which the sample was taken. Due to a lack of obstetrical information, method of delivery (vaginal versus C-section), presence or absence of induction of labor, or presence or absence of spontaneous labor was not taken into account for this analysis. However, when this analysis was repeated with only those samples from women who had vaginal deliveries without induction of labor, a similar association was found.

Figure 3.3a,b. Linkage disequilibrium (LD) relationship of FKBP5 SNPS rs3800373, rs9470080, rs1360780, rs929658, position on chromosome 6, and association of these SNPs with birth weight after adjusting for estimated gestation age (EGA) at delivery. (a) SNPs in the FKBP5 region are associated with birth weight after adjusting for EGA at delivery (p=.01 – p=.037, N=112-114, MAF=.36-.38). The y-axis shows the –log P values of the association, while the x-axis shows the positions of the SNPs according to the University of California-Santa Cruz genome browser (March 2006 assembly) (b) Representation of the LD relationship between rs3800373, rs9470080, rs1360780, and rs929658 using r^2 and relative position of these SNPs within the FKBP5 gene on chromosome 6. Vertical lines on chromosome 6 diagram represent exons in FKBP5. Numbers in the boxes in the LD plot represent r^2 values.

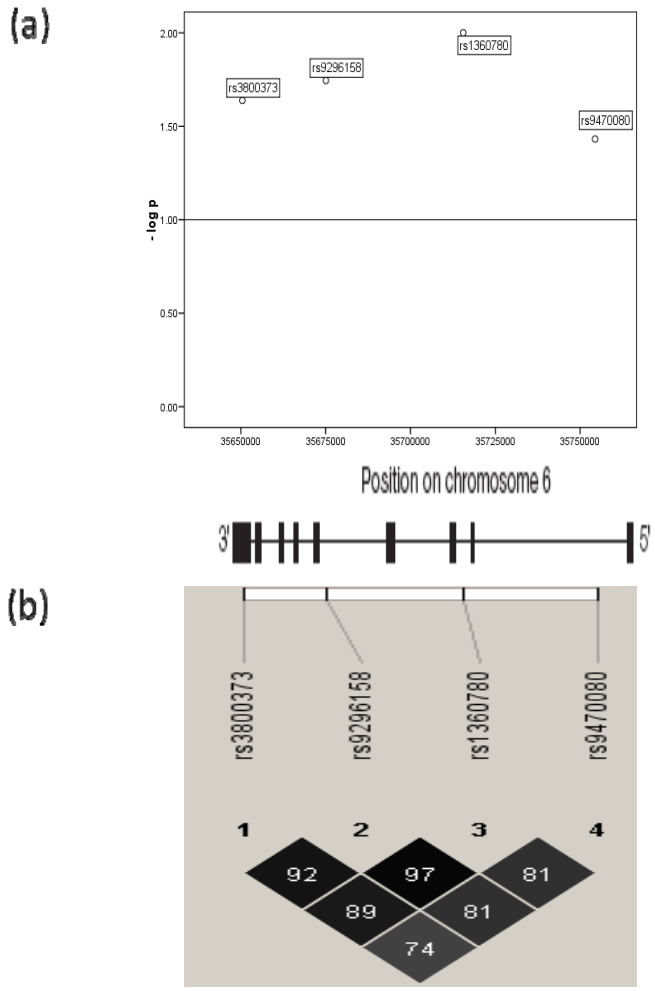
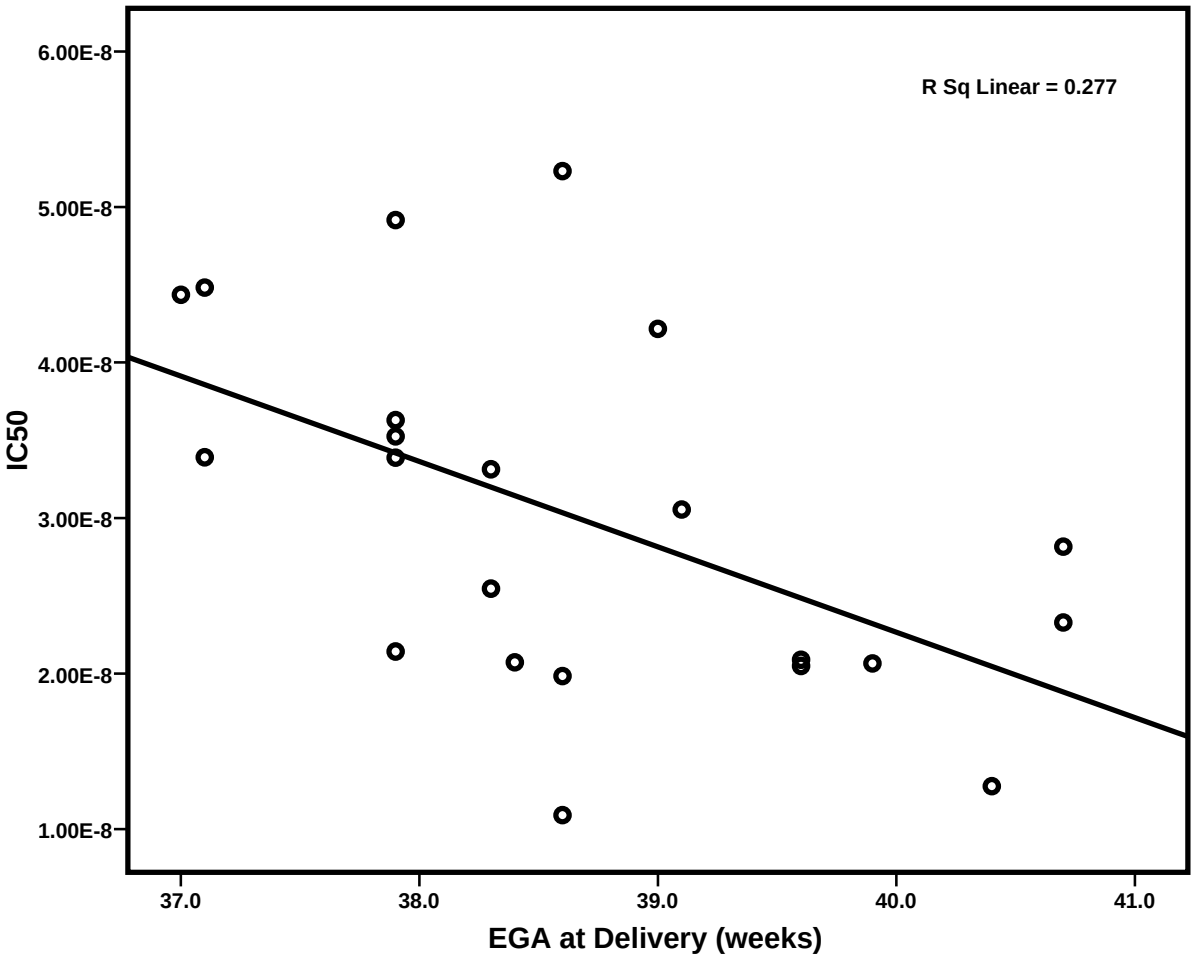


Figure 3.4. GR function is associated with timing of onset of labor.



Depressive Symptoms and Birth Outcomes

Depressive symptoms during pregnancy, in the form of either average or maximum BDI scores, are not associated with either EGA at delivery (p=.84 and p=.52, respectively; N=210) or birth weight, after adjusting for EGA at delivery (p=.41 and p=.7, respectively; N=199).

Discussion

Birth weight and timing of delivery are complex traits reflecting many factors. Maternal stress and depression during pregnancy have been shown to be significant mediators of these neonatal outcomes (Wadhwa, 2005). Many studies have suggested that a balance between the HPA-axis and the immune system is important for maintaining fetal growth *in utero,* in addition to ensuring the length of gestation is adequate for proper fetal development. Cortisol, the final output hormone of the HPA-axis and an important molecule in stress and depression, has downstream effects on the expression of molecules such as CRH, via signaling of the GR, and has been implicated as playing a major role in neonatal growth and development (Field et al., 2006).

Here, we show that GR function, as measured by a bio-assay in whole blood, is significantly associated with timing of delivery. Furthermore, functional SNPs in FKBP5, a known modulator of GR function, are also associated with length of gestation. Alleles that are associated with increased GR sensitivity in patients with MDD and PTSD are associated with earlier delivery in this sample. Finally, there is a "dose-dependent" effect of mRNA levels of two other chaperones proteins, BAG1 and PPP5C, on gestational length, with lower levels of both proteins associated with earlier onset of labor and delivery. We have also found that transcript levels of BAG1 and FKBP5 are significantly different between non-medicated, non-depressed women and non-medicated, depressed women in pregnancy, with the majority of the effect in the 2nd and 3rd trimesters (Chapter 2, pg 55). Here, lower levels of the transcripts of BAG1 and

and FKBP5 are associated with the presence of depressive symptoms. Measures of BAG1, PPP5C, FKBP5, and GR sensitivity may therefore be important in investigating the role of the HPA-axis in timing of delivery, as well as serving as biomarkers to predict and prevent preterm labor.

Interestingly, the T allele at rs9470080 in *FKBP5* has previously been associated with PTSD and depressive symptoms in subjects with child abuse and MDD, respectively. In this study, this allele associated with increased birth weight, after adjusting for EGA at delivery, as well as earlier timing of delivery. The same T risk allele has also associated with differences in GR sensitivity (Binder et al., 2004; Binder et al., 2008), dependent on the presence or absence of psychiatric symptoms (Binder et al., 2008; Ising, unpublished). Binder et al. (2008) showed that carriers of the T allele at rs9470080 that did not have PTSD symptoms showed more GR resistance in the dexamethasone suppression test (DST) than carriers of the T allele with PTSD symptoms compared to asymptomatic and symptomatic subjects with a CC genotype. While it is difficult to directly extrapolate from the PTSD data to depression during pregnancy due to differences in HPA-axis abnormalities in the two disorders (Holsboer, 2000; Nemeroff, 1996; Yehuda et al., 1991; Yehuda, 2001; Yehuda et al., 2004a; Yehuda et al., 2004b), it is likely that the interaction between maternal genotype and mood state during pregnancy is important for predicting GR sensitivity in this population. Currently, specific mechanisms related to how glucocorticoids and glucocorticoid signaling may affect birth weight and timing of delivery are unknown, however, the positive feedback

of cortisol on placental CRH is one mechanism that has been proposed(Wadhwa et al., 2004; Wadhwa, 2005).

We did not observe a significant association between mRNA levels of any of the genes tested and birth weight. However, our power to detect such associations was limited by the small number of patients available at the time of this study with both delivery outcome data and mRNA levels. Standardizing the data using a Z-score transformation further reduced our ability to detect differences in magnitude between groups (see Appendix A). Lastly, because treatment with selective serotonin reuptake inhibitors (SSRIs) has a significant effect on birth weight in this sample (data not shown), we were required to analyze medicated and non-medicated patients separately, which reduced our statistical power.

A major limitation in this study is the small sample size, which limited statistical power. However, the preliminary results in this sample suggest that GR function, gene expression, and genetic variants of molecules that participate in the regulation of GR function may be valuable for predicting preterm delivery. Identification of women at risk for preterm labor could result in early intervention and better outcomes for the neonate.

While the samples sizes for the SNP analyses were also limited, high levels of linkage disequilibrium (LD), such as what is found between the SNPs in FKBP5 studied here, decreases the need for correcting significance levels for multiple testing. The genotype at one locus highly predicts the genotypes at the other loci (Figure 3.3b). Future studies with larger samples sizes should aim to replicate these findings and

examine the association of haplotypes in this region of the genome with neonatal outcomes.

Another limitation of this study is that GR function in addition to gene expression of GR-associated molecules was measured in peripheral lymphocytes rather than placental tissue. As cortisol has regulatory effects on CRH expression that are tissue-specific (Nicholson et al., 2004), it is possible the GR function assessed in lymphocytes does not directly mimic the function of the GR in the placenta or at the level of the fetus. A protocol for obtaining tissue from human placenta during elected C-sections is currently being implemented. This may provide an opportunity to replicate these findings in the most relevant tissue type.

A number of models can be formulated to explain apparent associations among maternal stress and depression, HPA-axis function (GR function and CRH expression), immune reactivity and timing of onset of labor. For example, data from chapter 2 of this dissertation show that women with high levels of depression in pregnancy exhibit decreased GR function. Decreased GR signaling could lead to decreases in CRH expression, which in turn would decrease IL-1ra expression, resulting in increased IL-1 signaling and an increased risk for preterm labor (Schmeelk et al., 1999). Alternatively, as GR signaling is anti-inflammatory in general, maternal stress leading to decreases in GR function could increase levels of the pro-inflammatory cytokine, IL-6, predisposing women to preterm labor and delivery (Miller et al., 2005).

Based on in vitro studies, BAG1 and FKBP5 are known to function as negative regulators of GR function (Kullmann et al., 1998; Schneikert et al., 1999). Levels of

mRNA encoding these proteins are lower in depressed patients and lower in women who deliver preterm neonates. These observations support the hypothesis that altered regulation of the molecules that regulate GR function, and GR function itself, are associated with maternal stress and depression. Those maternal outcomes in turn can have negative consequences on neonatal outcomes, such as preterm delivery and low birth weight. The prediction or prevention of preterm labor using these measures could be extremely beneficial on the level of the individual, but may also provide insight into the larger public health concern of maternal-child health.

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CHAPTER 4:

DISCUSSION

Summary of Findings and Discussion

The overarching hypothesis framing the studies in this dissertation was that altered regulation of chaperone, co-chaperone, and transcription factor proteins that modulate the function of the GR in pregnancy may contribute to risk for depressive symptoms in pregnancy. Furthermore, these proteins and their effects on GR function may also mediate the previously reported negative effects of maternal HPA-axis dysregulation on neonatal outcomes that have been associated with long-term behavioral and metabolic syndromes.

This set of hypotheses was based on previous findings that absolute hormone measures do not distinguish between women with and without mood symptoms in the peripartum period, but that differences in sensitivity to changing levels of hormones appear to differ between symptomatic and asymptomatic women (Bloch et al., 2000). This dissertation focused on the regulation of the GR, as GR dysfunction is one of the most consistent findings in patients with depressive symptoms outside of pregnancy (Holsboer, 2000; Nemeroff, 1996) and there are marked differences in GR function within normal pregnancy (Greenwood and Parker, 1984; Smith and Thomson, 1991).

We first show that in our sample there are no absolute differences in serum levels of total cortisol, estrogen, or progesterone in depressed as compared to non-depressed women in pregnancy. This experiment was important, first, because it was performed in the largest population of women at risk for the development of depression during pregnancy to date and second, it set up the premise for looking downstream of

absolute levels of hormones to intracellular steroid hormone signaling as a mechanism that may be involved in the development of mood symptoms during pregnancy.

Next, we extended findings based on the dexamethasone suppression test from two decades ago suggesting that GR resistance increases in non-depressed women throughout gestation. We confirmed that hypothesis in our study by testing GR function directly, using an ex-vivo assay in whole blood in depressed and non-depressed pregnant women. Our results confirm that GR resistance (in leukocytes) increases over pregnancy. We postulate the phenomenon is likely a protective mechanism for the mother in response to increasing levels of cortisol. Second, we showed that there was a highly significant difference in GR function between the depressed and non-depressed pregnant women, such that women that are depressed have higher levels of GR-resistance. Our study is the first to examine GR sensitivity in depressed pregnant women. With altered GR sensitivity over pregnancy and the positive feedback of cortisol on placental CRH release, it was not clear that we would observe a change in the same direction as the one observed in depressed non-pregnant patients.

As chaperone, co-chaperone, and transcription factor proteins are integral in mediating GR function, the next set of studies examined the levels of mRNA encoding these proteins during pregnancy, in depression during pregnancy, and in relation to GR function in peripheral blood monocytes (PBMCs). The data from these experiments show that a subset of our genes of interest (BAG1, FKBP5, PPID, HSP70, STIP1, and ST13) is upregulated during pregnancy. In addition, an overlapping subset of genes was differentially regulated in the depressed women compared to the non-depressed

women in pregnancy (BAG1, FKBP5, NCOA1, and PPID), with a lack of up-regulation of these genes observed primarily in the 2nd and 3rd trimester in the depressed women.

The majority of the information known about the effects of the chaperone, co-chaperone, and transcription factor proteins of the GR has been gathered from experiments performed studying one or a few proteins at one time. In general, these studies rely on molecular techniques that overexpressed, knocked-down, or mutated these proteins in cell culture or animal models in order to identify their roles in GR signaling. Based on these experiments, two of our transcripts of interest, FKBP5 and BAG1, have been shown to have negative effects on GR-signaling. We observed that pregnancy is associated with upregulation of BAG1 and FKBP5 that in turn paralleled an increase in GR-resistance, consistent with our hypothesis that changes in the mRNA levels of genes that modulate GR-function would be reflected in altered GR sensitivity. However, the similar upregulation of transcripts, such as PPID, which has a putative positive effect on GR-signaling, is not consistent with this hypothesis. Furthermore, the group of depressed women showed a further increase in GR-resistance that corresponded to lower levels of FKBP5 and BAG1 compared to the non-depressed group. These points highlight the fact that mRNA levels may not reflect protein levels and function. In addition, cell culture models may not accurately represent the complex interactions of this large number of molecules. More data, from a larger number of subjects, along with statistical modeling using data reduction techniques together with studies in animal models, may help to identify more precisely the most important

components of this complex system that differ between symptomatic and asymptomatic women.

Despite the complexity of the interactions amongst chaperones and co-chaperones that determine the final output of GR signaling, we found evidence for the association of HSP70 and P23 expression with ex-vivo GR sensitivity in two independent samples. The first sample is the pregnancy sample that is the focus of this dissertation. Here, gene expression levels of HSP70 and P23 directly correlated with GR sensitivity measurements (IC_{50}) in 22 pregnant women. In a small sample (N=10) of college age males without a psychiatric history, we found these molecules were also main predictors of GR function using the same methods (Appendix C – Figure 1). Both HSP70 and P23 are molecules that are critical for GR function. In fact, knock-out mice lacking P23 have a similar phenotype to GR knock-outs (Grad et al., 2006). The fact that we observe a significant association of mRNA expression of these two molecules with GR sensitivity in 2 independent samples is an indication that chaperone mRNA changes may indeed be related to changes in GR function. To investigate more specific questions, as to which chaperone expression changes have the largest impact on GR sensitivity with increasing pregnancy or depression, we will need additional samples in each of the relevant groups, as our current collection has insufficient power to investigate subsamples.

Our next set of research questions examined the relationship between maternal molecular events during pregnancy and neonatal outcomes, such as timing of delivery and birth weight. We found preliminary evidence supporting associations between

maternal GR sensitivity and genetic polymorphisms in the GR modulator, FK506 binding protein 5 (FKBP5), with length of gestation. These observations support the hypothesis that the HPA-axis is essential in the study of timing of delivery, and further suggest that polymorphic variation at *FKBP5* alters HPA-axis function relevant to timing of delivery. These measures, along with mRNA levels of BAG1 and PPP5C, may be important for predicting and preventing preterm delivery. In addition, the finding that genetic polymorphisms in *FKBP5* are associated with birth weight provides an additional avenue of exploration in understanding the role of the HPA-axis in intrauterine growth.

Limitations of the Study Sample

The sample studied in this dissertation was comprised mainly of women with a personal or family history of a major psychiatric illness, who were wither referred by their obstetricians or self-referred to a clinic specializing in the care of pregnant women with psychiatric disorders. These women were pregnant or trying to become pregnant and sought out professional medical help for managing symptoms and/or medication related to psychiatric illness during this time. Studying these at-risk patients provides a unique opportunity to identify pathophysiology of disease states. With an increased number of affected individuals comes increased power to detect differences between groups. However, utilizing a clinical population, such as this one, limits the generalizability of the results to non-psychiatric populations. This clinic-based sample also presents a challenge in obtaining true longitudinal data. Subjects were scheduled

to participate in this study every 4 weeks during pregnancy, however, repeated samples were not available for a substantial proportion (40%) of the subjects. The sample, therefore, was largely cross-sectional in nature. The mixed model analysis containing the random intercept parameter allowed us to capture both longitudinal and cross-sectional aspects of the data. This complex modeling, however, limits our ability to directly compare effects of depression status across time or time points to each other due to different subjects comprising each depression status group at various trimesters. In addition, while medication use did not statistically interact with gene expression outcomes, past or present medication use may affect the biological phenomena we are interested in. Finally, SCID-based diagnosis did have a significant association with a small number of transcripts studied in this dissertation, however, based on a number of studies (for review see Wadhwa, 2005), it is likely that maternal stress and depressive symptoms during pregnancy, rather than a psychiatric diagnosis is the more relevant characteristic, especially with regard to neonatal outcomes.

Peripheral Blood Monocytes (PBMCs) and Brain Function

A number of studies have examined whether GR function in PBMCs might be a peripheral marker of central GR signaling function and dysfunction. Glucocorticoid receptor resistance as measured by endocrine stimulation tests and its resolution with antidepressant treatment are among the most consistent biological findings in major depression (Holsboer and Barden, 1996; Pariante, 2004). This overall steroid resistance

has also been reported for the activation of T-cells and monocytes in major depression and bipolar disorder (Pariante and Miller, 2001) suggesting comparable GR impairment in immune cells and in the central nervous system (Lowy et al., 1984; Lowy et al., 1988).

In addition, levels of a downstream target of GR signaling, NF-κB, have been shown to increase in response to social stress in PBMCs in humans (Bierhaus et al., 2003), as well as in response to immobilization stress in the rat cortex (Madrigal et al., 2002). These studies suggest parallel effects of GR signaling in the periphery and areas of the brain relevant to mood regulation.

In order to further investigate the relationship between peripheral and central markers of modulators of GR function, we conducted a preliminary study measuring mRNA levels of FKBP5 in the blood (PBMCs), hippocampus, amygdala, prefrontal cortex, and the hypothalamus in normal rats at 4 time points across pregnancy and one postpartum time point (E5, E10, E15, E20, and P21). We found that FKBP5 gene expression was significantly correlated between blood and the amygdala at the late pregnancy (E20) time point ($p=.006$, $N=4$, $R^2=.99$, Figure 2 – Appendix C). While this and other animal models that we investigated (ovine and baboon) did not prove to be useful tools for examining central effects of pregnancy on steroid signaling, the parallel between peripheral and central gene expression in pregnancy is an important preliminary finding. Animal models of HPA-axis function changes in pregnancy are difficult due to uniquely human characteristics of pregnancy, including placental release of CRH. Placental release of CRH may drive the maternal HPA-axis during pregnancy and while placental CRH has been found in some primate species, such as baboon, it is

debated whether it is present in sheep (Jones et al., 1989; Thorburn et al., 1991) and has been found not to exist in rodent species (Robinson et al., 1989). Although, the baboon placenta does express CRH, it's regulation varies from humans in that levels peak in mid-gestation rather than late-gestation (Power and Schulkin, 2006). This likely leads to divergent alterations in the HPA-axis in this primate species, compared to humans and thus baboons may be a poor model for understanding pregnancy-related changes in HPA-axis function.

Future Directions

Molecular Predictors of Relapse of Depressive Symptoms

Pregnancy is often thought of as a time when women are protected from mood disorders. Epidemiological data, however, show that antepartum depression is highly prevalent with estimates of 10-20% (Brett and Barfield, 2008; Campbell and Cohn, 1991; Robinson and Stewart, 1986). Furthermore, approximately two-thirds of women with a history of major depression who choose to discontinue antidepressant use during pregnancy will experience a relapse (Cohen et al., 2006) during that time. The decision to discontinue medication use should be weighed against the known adverse outcomes to both the mother and the fetus with respect to maternal depression (Nonacs and Cohen, 2003).

Cohen et al (2006) examined rates of relapse of depressive symptoms during pregnancy in women who maintained, increased, decreased, or discontinued antidepressant treatment during pregnancy. All of the women (N=201) were euthymic and receiving antidepressant therapy within three months of their last menstrual period (LMP). This study found that 26% of women who continued antidepressant use during pregnancy experienced a relapse of depressive symptoms, whereas 68% of those discontinuing antidepressant medications relapsed.

Outside of pregnancy, GR function measures are thought to be state markers for depression, with GR function normalizing with effective treatment (Linkowski et al., 1987). It would be important to examine whether this is also the case for depression in pregnancy. Furthermore, changes in gene expression of the molecules that mediate GR function may also differ in pregnant women who are effectively being treated for depression compared to the women who, while medicated, remain or become depressed. Thus, measuring gene expression over time might be a useful tool as early predictor of non-response.

Preliminary experiments from our lab measured gene expression levels of the GR-associated chaperone, co-chaperone, and transcription factor proteins in women who were taking antidepressants for more than 2 weeks and were not depressed (BDI score <15) compared women who were taking antidepressants and were depressed (BDI score 15 or greater). The data suggest that different genes are associated with relapse of depressive symptoms in medicated women at different reproductive time points. For example, at preconception, PPP5C gene expression levels are significantly different

(p=.02) between medicated depressed (N=21) and non-depressed women (N=4) (Appendix C -Figure 3a), while 1st trimester relapse is associated with differential CDC37L1 (p=.032, N=18, 6) and FKBP4 gene expression (p=.017, N=18, 5) (Appendix C - Figure 3b,c), and 3rd trimester relapse shows a trend association with differential expression of HSP70 gene expression (p=.082, N=55, 13) (Appendix C - Figure 3d). Our sample lacked sufficient power to investigate these predictors with confidence, but our preliminary findings suggest that further study of these factors may yield useful predictors of maternal outcomes. Understanding underlying molecular mechanisms of relapse in any patient with depressive symptoms, including pregnant women may be helpful in predicting which patients will be responders and non-responders to treatment before clinical outcomes can be established.

Steroid Hormones, Brain Structure, and Function

Another topic of interest is whether the regulation of levels of gene expression, protein expression, and protein function of the molecules that mediate steroid hormone signaling, which we studied in peripheral blood, translates into changes in brain structure and function. Secondary to that, is the question, can differences in these processes contribute to psychiatric disorders?

The age of onset for many psychiatric disorders is during late adolescence and early adulthood. This is a time when there is significant reorganizing of limbic and frontal lobe areas of the brain due to increases in steroid hormones (Sisk and Zehr,

2005). These changes have been shown to occur in a sex-specific manner perhaps explaining, in part, gender differences in the prevalence of some psychiatric disorders.

Researchers are just starting to uncover how exposure to high levels of steroid hormones or other peptides, such as oxytocin and prolactin, during pregnancy cause a similar reorganization of the maternal brain. Structural and functional brain imaging performed prior to conception and throughout the postpartum period in combination with some of the molecular methods presented in this dissertation may contribute significantly to the literature related to mechanisms of steroid hormone action in normal pregnancy, as well as in pregnancy related depression.

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APPENDIX A:

STANDARDIZATION OF GENE EXPRESSION MEASURES IN PAXGENE AND TEMPUS RNA TUBES

Background

PAXgene blood tubes (PreAnalytix, Hombrechtikon, Switzerland) and Tempus blood RNA tubes (Applied Biosystems, Inc., Foster City, CA) are specialized 10ml vacutainer tubes that contain proprietary buffers that immediately stabilize intracellular RNA upon collection. RNA from blood collected directly into these tubes is stable at room temperature for 2-3 days, at +4˚C for up to 1 week, and at either -20˚C or -80˚C for at least 2 years. Batched purification of samples collected into either type of tube can be carried out through either membrane or magnetic bead based nucleic acid purification. Purified RNA from these sources is ideal for downstream applications, such as real-time PCR (RT-PCR) and microarray analysis.

In the following report, we present data that describes how these tubes compare at various levels of processing prior to and including purification (yield, quality, cost, ease of purification). We also compare RT-PCR results (PCR efficiency and relative levels of transcript) for these two types of tubes in our study of transcripts that encode glucocorticoid receptor associated proteins during pregnancy in humans.

Methods

While PAXgene tubes had been introduced in 2001, Tempus tubes were not introduced until 2004. Therefore, PAXgene tubes were used to collect the first 377 samples in our study and Tempus tubes were used to collect 314 subsequent samples.

All tubes were stored at +4˚C for 1-3 days before being frozen at -20˚C until batch purification was performed using a modified protocol (see Appendix B) from the

Versagene RNA Purification Kit for Cell Culture in a 96-well format (Gentra Systems, Inc., Minneapolis, MI).

Total RNA was reverse transcribed according to the manufacturer's protocol using the cDNA Archive kit (Applied Biosystems, Inc., Foster City, CA) and the resulting cDNA was quantified using the Quant-it Picogreen Kit (Invitrogen, Carlsbad, CA). Each sample was diluted to 0.5ng/ul and plated in duplicate into 384-well plates, for assay by Real-Time PCR (RT-PCR) using Taqman human gene expression assays (Applied Biosystems, Inc., Foster City, CA) for transcripts listed in Chapter 1, Table 1.1 (pg 22). Assays were performed in a 10ul volume according to the manufacturer protocol on a 7900HT ABI 7900 Real Time PCR System (Applied Biosystems, Inc., Foster City, CA). For quality control, concordance of duplicates was assessed and samples with a crossing threshold (CT) greater than 35 and a standard deviation more than .25 CTs apart in each duplicate pair were excluded (18%) from further analyses.

Raw fluorescence data were used to estimate the PCR efficiency for each reaction using the LinRegPCR program (Ramakers et al., 2003). Mean assay-specific efficiencies were then used in conjunction with the CT values to calculate relative ratios of the target mRNA to that of the endogenous control gene, RPLP0 (Karlen et al., 2007; Ramakers et al., 2003). CT values were calculated using default settings within the RQ Manager version 1.2 software (Applied Biosystems, Inc., Foster City, CA). Differences in mean PCR efficiency in the PAXgene and Tempus tubes were established using a Student's T-test.

Quality of RNA was assessed in a subset of samples using the Agilent Bioanalyzer 2100 (Santa Clara, CA).

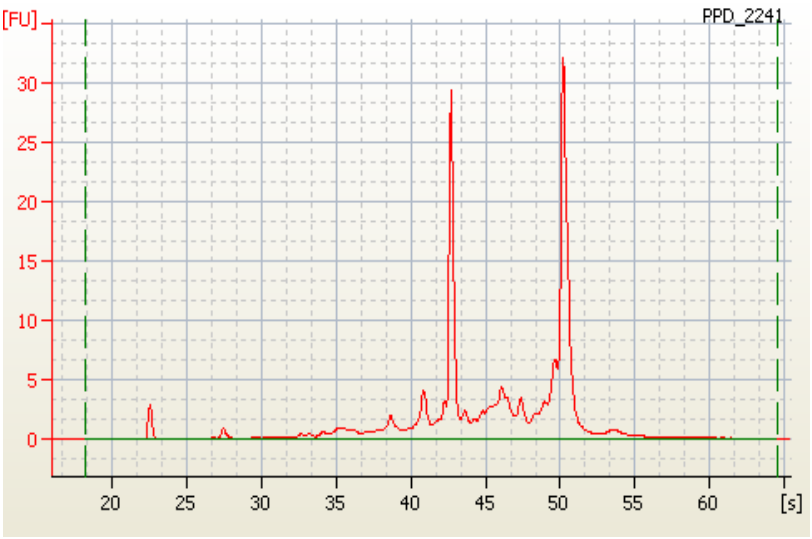
Standardization of RT-PCR results across PAXgene and Tempus tubes was achieved using a Z-score transformation on the raw data for each type of tube. The Z-score transformation (Equation 1) is a linear transformation that redistributes the data such that the mean is 0 and the standard deviation is 1. This is a common way to combine gene expression data across experiments across subjects, experiments, and platforms (Cheadle et al., 2003). After performing the Z-score transformation independently for each tube type and each gene, this data was combined and used for all further analyses.

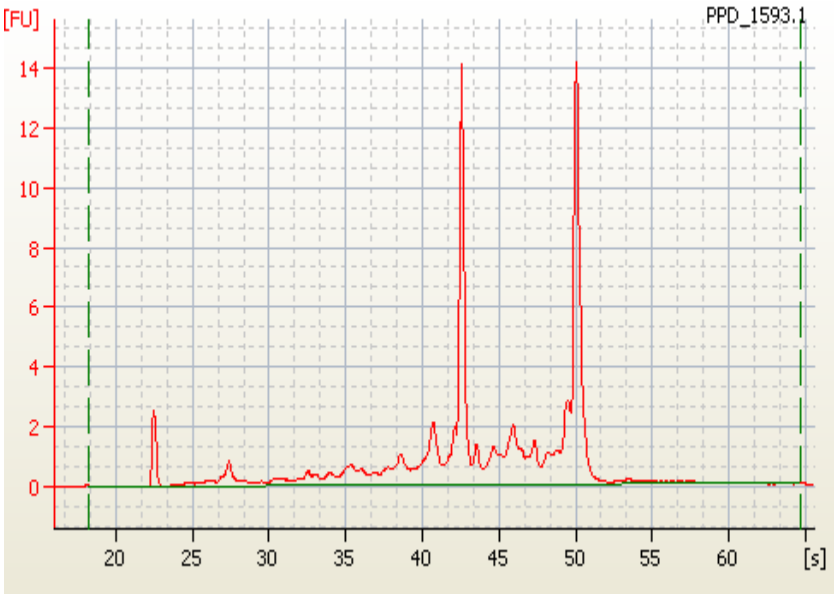
$$z = \frac{(x - \mu)}{\sigma} \quad \text{or} \quad z = \frac{(\text{data point} - \text{mean})}{\text{standard deviation}} \qquad (1)$$

Results

We found properties of the Tempus tubes to be superior to those of the PAXgene tubes in a number of areas. The average yield of cDNA was approximately 4.2 times greater (1.25 ± .94µg vs. 5.26 ± 2.84µg), the cost of the tubes was 36% less ($7.00 vs. $4.50 per tube), the cost of the RNA purification per sample was 66% less ($7.15 vs. $2.17), the extraction procedure took 1 person instead of 2 people due to an extremely time sensitive step found only in the PAXgene protocol, and the extraction method was more straight forward and took 50% less time.

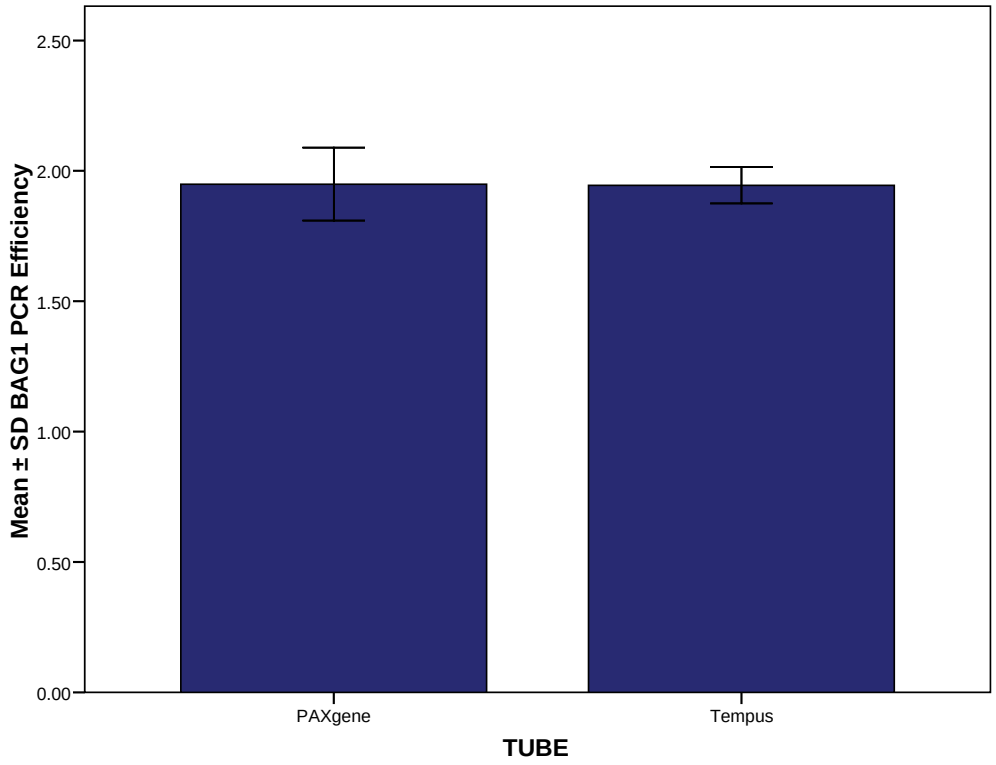
Figure 1. (a) Bioanalyzer plot for Tempus tube sample 2241 and (b) PAXgene sample 1593.1.





The quality of the RNA between these 2 tubes was comparable, however the Tempus tube quality exceeded that of the PAXgene tubes more often than not. For example, Figure 1 shows results from the Agilent Bioanalyzer 2100 (Santa Clara, CA) for a (a) Tempus tube sample (28S/18S ratio=1.87) and a (b) PAXgene tube (28S/18S ratio=1.42). The 28S/18S ratio is a well established measure of RNA integrity and is optimal for RNA between 1.8 and 2.

Figure 2.Mean and standard deviation of PCR efficiency for BAG1 in PAXgene and Tempus tubes.



For the purposes of comparison, we present data for one of our transcripts of interest, BCL2-associated athanogene (BAG1). PCR efficiency was comparable between the PAXgene and Tempus Tubes (Figure 2, p=.576). After analyzing relative ratio data from the PAXGene and Tempus tubes, however, it was clear that while the overall pattern of gene expression was similar, the scale of the gene expression changes was different. Figure 3 shows the raw ratio data for both PAXgene and Tempus tubes for BAG1, while figure 4 shows the same PAXgene data on an appropriate scale.

Figure 3. Gene expression levels for BAG1 before standardization. The top graph represents that PAXgene data. The bottom graph represents the Tempus tube data.

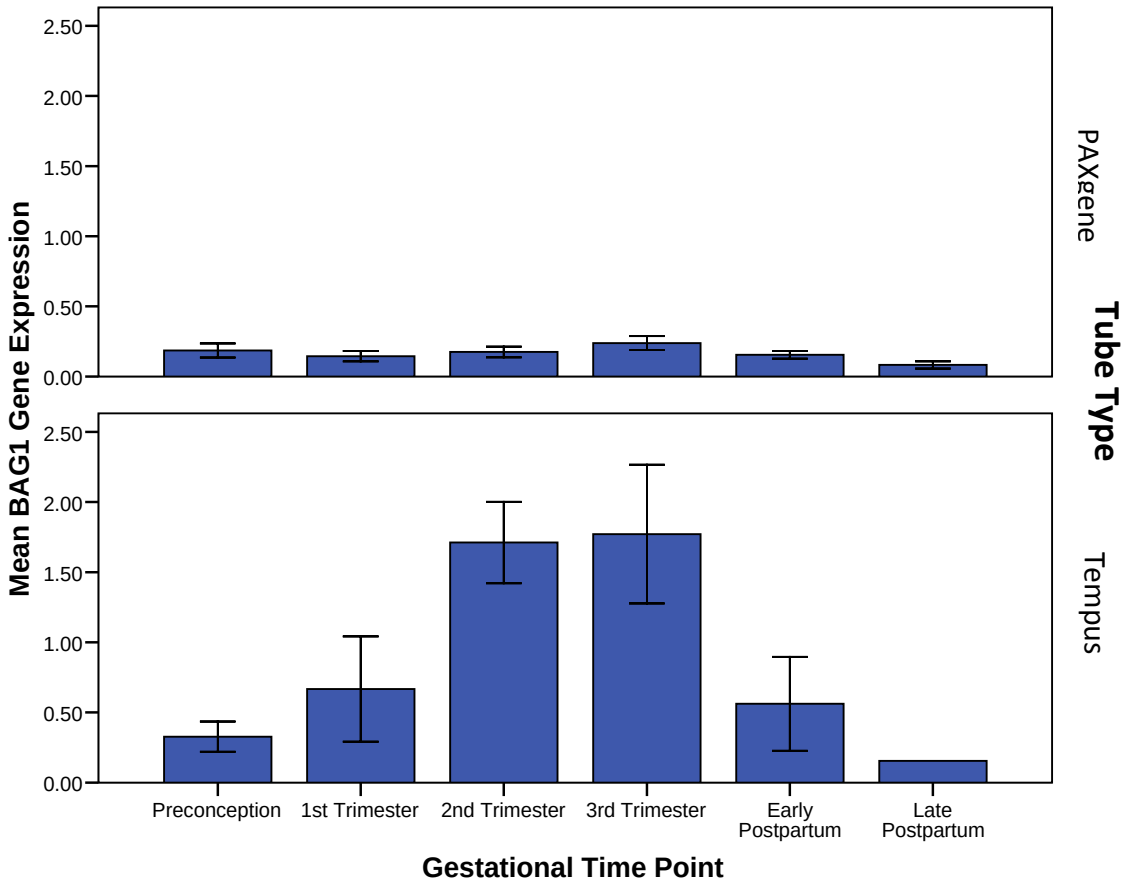
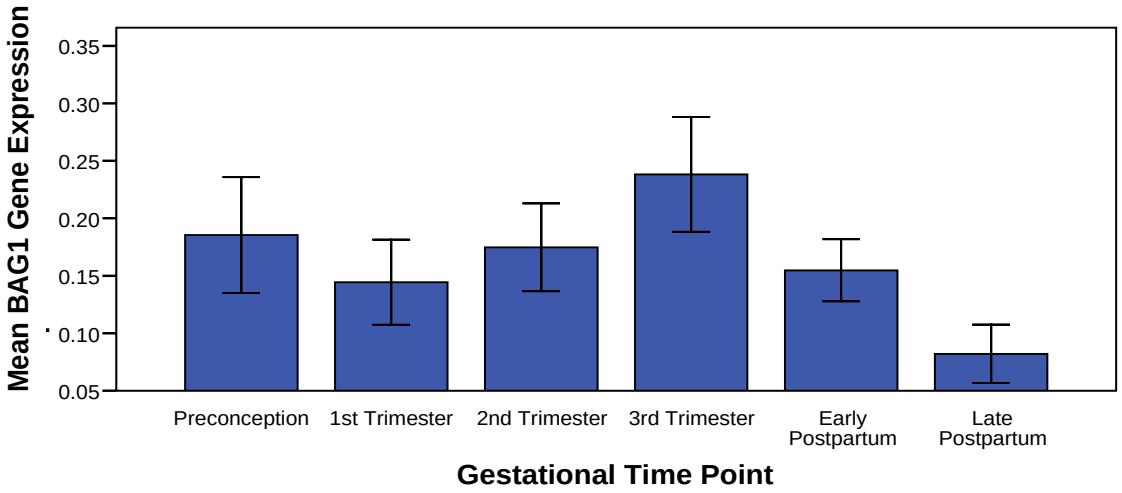
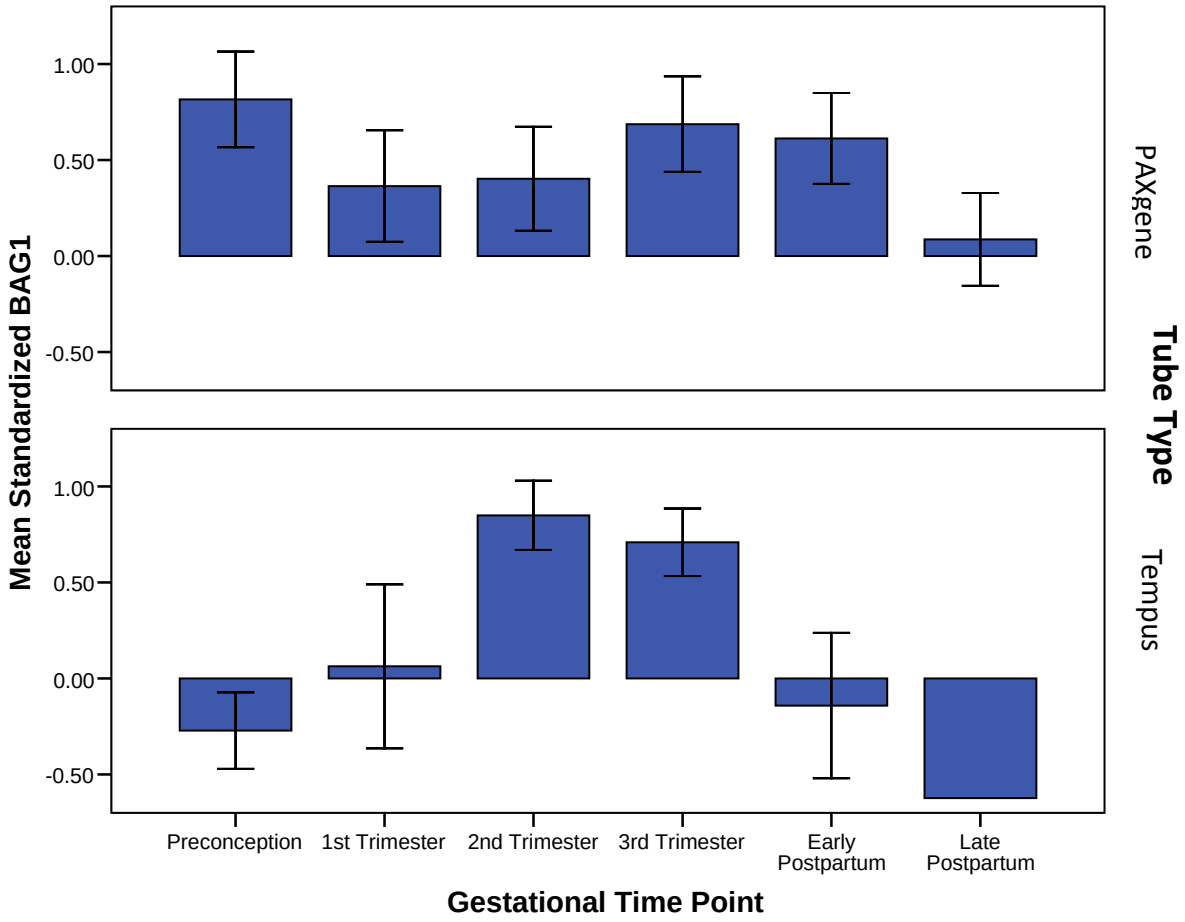


Figure 4. Rescaled PAXGene tube data from Figure 2.



In order to avoid decreasing the sample size if data from each tube type was analyzed separately, we chose to perform a Z-score transformation on the data from each tube type prior to combining this data for further analysis. Figure 5 shows the BAG1 data from Figure 3 following the Z score transformation. This figure shows that the scale is appropriate for each tube type and the overall pattern of the data is preserved with an up-regulation of the BAG1 during pregnancy and a down regulation in the postpartum period.

Figure 5. Data from Figure 2 after a Z-score transformation



Conclusion

Tempus tubes provide researchers with easy way to produce high quantities of RNA with easy processing, at a low cost. Datasets that contain data from Tempus and PAXgene tubes can be combined, but there is a tradeoff between resolution of the magnitude of gene expression change in the case of performing a Z-score transformation and ability to detect statistically significant differences between groups in the case of choosing to use data from either PAXgene or Tempus tubes.

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APPENDIX B:

MODIFIED PROTOCOL FOR MRNA EXTRACTION FROM PAXGENE AND TEMPUS RNA TUBES

PAXgene blood tubes (PreAnalytix, Hombrechtikon, Switzerland) and Tempus blood RNA tubes (Applied Biosystems, Inc., Foster City, CA) are specialized 10ml vacutainer tubes that contain RNA stabilization buffer. RNA from blood collected directly into these tubes is stable at room temperature for 2-3 days, at +4˚C for up to 1 week, and at either -20˚C or -80˚C for at least 2 years.

For the studies described in this dissertation, samples were collected in either the PAXgene or Tempus tube, stored immediately at +4˚C for up to 3 days before being frozen at -20˚C until the day before extraction. At this time tubes were stored at +4˚C overnight to thaw and the protocol below was followed.

This protocol is modified from two separate kits that were marketed by Gentra Systems (Minneapolis, MI). The first kit was a single tube kit for RNA extraction from Tempus Tubes, while the second kit was a 96-well kit for RNA extraction from cell culture. Other kits that were tested were from PreAnalytix (Hombrechtikon, Switzerland) and Agencourt (Beverly, MA). These other kits were more expensive, more time consuming and had lower yields of RNA. For example, the PreAnalytix kit costs $7.15 per sample, it takes 17 minutes per sample to extract, and yields 1-5ug of RNA. In comparison, the Gentra Systems kit costs $2.17 per sample, it takes 2.5 minutes per sample to extract and yields 15-25ug of RNA. These factors along with ease of use were considered when choosing and implementing the RNA extraction kits. To date, we have collected 3990 RNA tubes, of which 1619 tubes have been processed with the protocol below.

Protocol for Tempus Tube Extraction with 96-Well Plate

DNase Solution – See Appendix C below for reconstitution instructions

1. If completing the entire protocol in 1 day, prepare DNase Solution and put on ice
2. If doing sample homogenization on day 1 and RNA purification on day 2, prepare DNase solution on day 2

Sample Homogenization for PAXgene Tubes

1. Centrifuge samples for 10 minutes at 3000 x g
2. Pour off the supernatant and blot on clean absorbent paper
3. Add 5ml RNase free water, cap tube, vortex for 20 sec
4. Centrifuge for 10 minutes at 3000 x g
5. Pour off the supernatant and blot on clean absorbent paper
6. Add 5ml RNase free water, cap tube, vortex for 20 sec
7. Centrifuge for 10 minutes at 3000 x g
8. Pour off the supernatant and blot on clean absorbent paper
9. Add 150ul Resuspension Buffer
10. Vortex 20 seconds
11. Immediately add 310ul of Lysis Solution/Proteinase K
12. Vortex 20 seconds
13. Incubate on ice for 15 minutes, vortex when done with all samples for 30 seconds
14. Vortex iced samples again for 30 seconds

Sample Homogenization for Tempus Tubes

1. Mix the sample in the Tempus tube by inverting 2-3 times and pour blood into a clean 50 ml conical tube.
2. Add 3 ml of 95% reagent grade ethanol or better and vortex the sample on high for 2 minutes. (Ensure that the sample is adequately vortexed in order to completely thin the lysate.)
3. Centrifuge the sample at 3000 x g for 30 minutes at 0ºC.
   NOTE: Increasing centrifugation to up to 5,800 x g and 60 minutes can yield up to 30% more RNA per tube.
4. Pour off the supernatant and blot on clean absorbent paper. Keep tubes inverted for 2 minutes on absorbent paper.
   NOTE: No pellet will be visible and residual foaming is normal and does not interfere with the purification.
5. Add 300 µl of Lysis Solution to the tube and vortex for 60 seconds on high speed to thoroughly dissolve the RNA pellet.
6. Place 50ml tubes in freezer at -20 ºC until ready to load them onto the 96-well plate

a. According to Gentra Systems, RNA is stable in lysis buffer for 1 hr at room temperature, a few hours on ice, 1 week at -20ºC, and 3 weeks at -80ºC

RNA extraction

1. Use a Nunc Deep Well 1ml plate for waste plate instead of the waste plate that comes with the kits. The waste plate sent with the kit cracks in the centrifuge and makes a mess.
3. Pipette lysate onto plate, seal w/ adhesive seals
a. You should be able to fit all of the lysate (~350ul) into one well
4. Centrifuge to pull lysate through the columns at 3,220 x g for 2 minutes
5. Add 100ul WASH 1, seal plate, centrifuge at 3,220 x g for 2 minutes
6. Add 100ul WASH 2, seal plate, centrifuge at 3,220 x g for 2 minutes
7. Add 50ul DNase Solution to center of each well with repeat pipetter w/ 2.5 ml tip
8. Incubate at room temperature for 20 minutes
9. Add 100ul WASH 1 to each well seal plate, centrifuge at 3,220 x g for 2 minutes
10. Add 100ul WASH 2, seal plate, centrifuge at 3,220 x g for 2 minutes
11. Add 100ul WASH 2, seal plate, centrifuge at 3,220 x g for 5 minutes
12. Carefully place purification plate on Elution plate, discard waste plate
13. Add 50ul ELUTION SOLUTION to center of each well
14. Seal plate, centrifuge at 3,220 x g for 2 minutes, discard purification plate
15. Cover the Elution Plate containing purified RNA with the pierceable seal.
16. Store purified RNA at -70º to -80ºC or immediately convert RNA to cDNA
    a. We convert 25ul of the 50ul elution to cDNA with the ABI archive kit
    b. We store the rest of the RNA in 500ul screw top tubes from USA scientific

Appendix C: DNase Reconstitution Protocol

This protocol is to be used for reconstituting the DNase enzyme for use in the 96-well RNA purification protocol.

NOTE: Each vial of lyophilized DNase I enzyme must be resuspended in the entire 11 ml of DNase Buffer before starting the purification procedure.

Prior to using DNase:

1. Add 1 ml DNase Buffer to the lyophilized DNase I, recap the bottle and invert gently to mix.
2. Add the 1 ml of reconstituted enzyme back into the DNase Buffer vial, recap the bottle and invert to mix. The enzyme needs to be reconstituted in the entire 11 ml of DNase Buffer before starting the purification.
3. Store enzyme on ice until use.

The reconstituted DNase enzyme will treat two plates.

Store the unused DNase at -20°C. This enzyme should be subjected to only one freeze/thaw cycle.

It is recommended that a repeat pipettor be used to dispense the DNase enzyme into the center of each well during purification.

DO NOT POUR THE DNase ENZYME INTO A REAGENT RESERVOIR.
DO NOT USE A MULTI-CHANNEL PIPETTOR.

Appendix C:

FIGURES FROM DISCUSSION (CHAPTER 4)

Figure 1. HSP70 gene expression and IC_{50} from GR functional bio-assay in college age males without a history of psychiatric disorders (N=10).

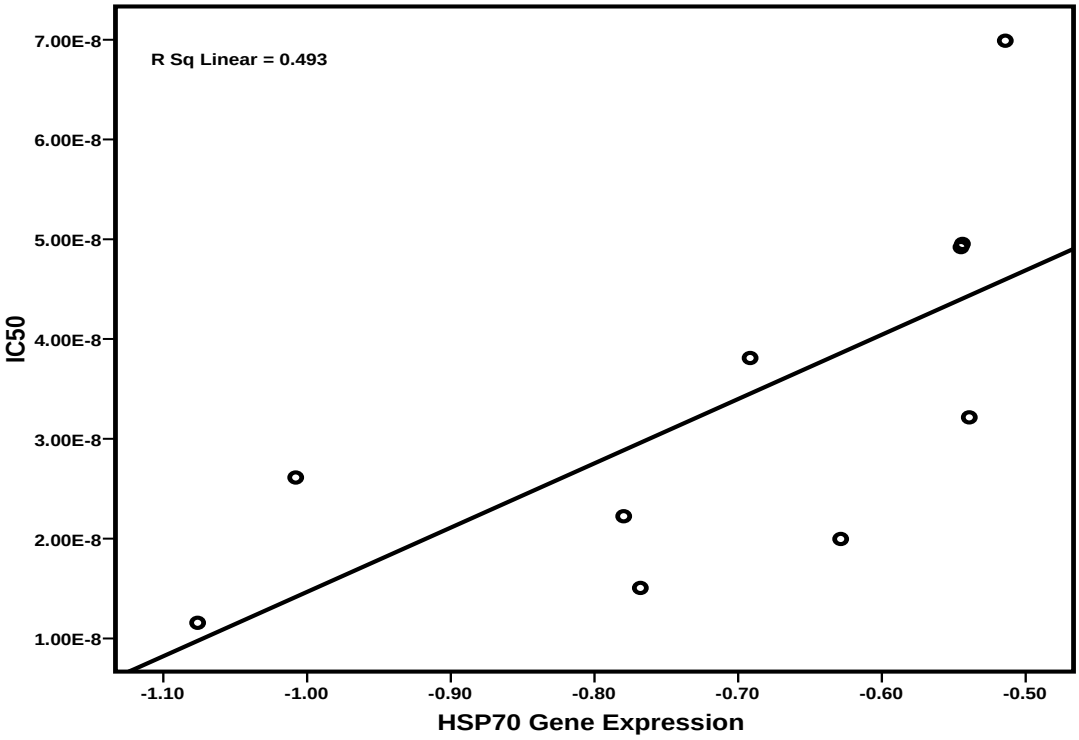
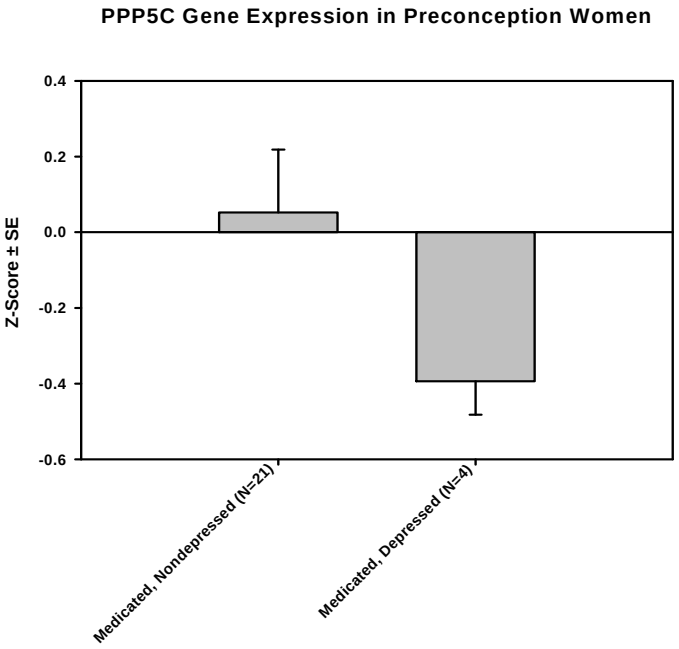
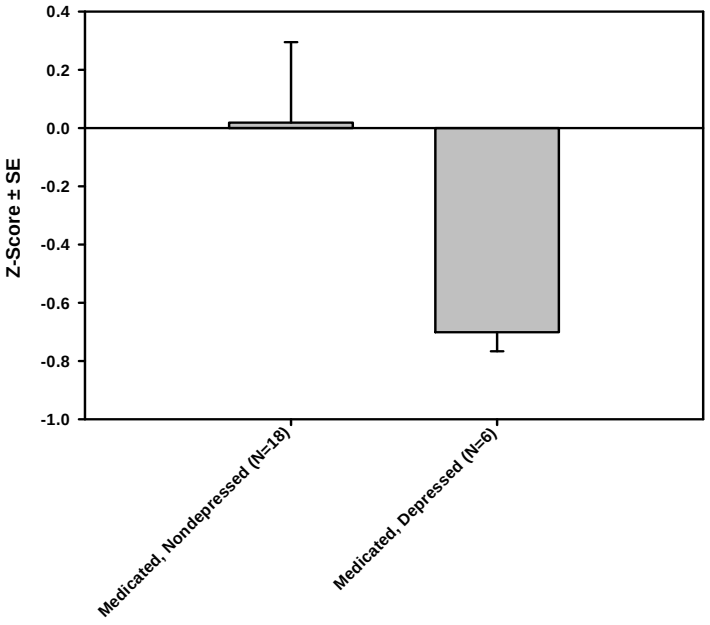


Figure 2a-d. Gene expression of (a) PPP5C in preconception (b) CDC37L1 and (c) FKBP4 in 1st trimester, and (d) HSP70 in 3rd trimester women taking antidepressants who are depressed or not depressed

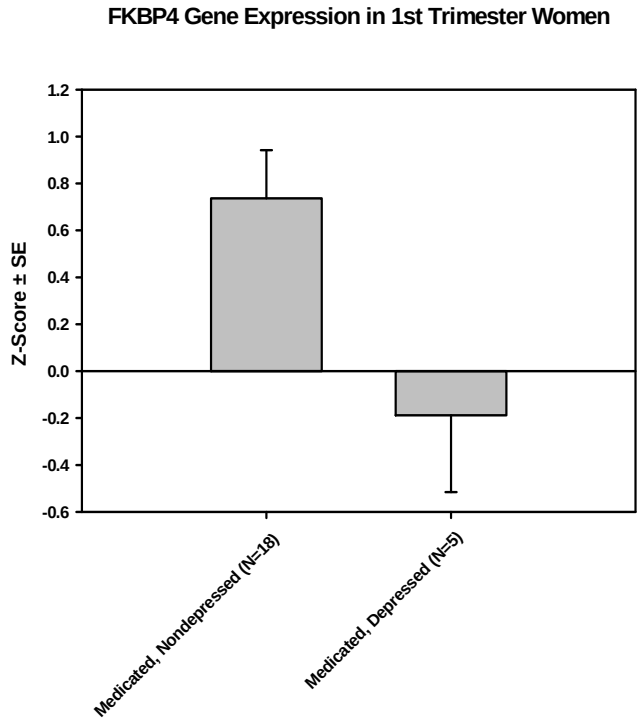
(a)



(b)



(c)


FKBP4 Gene Expression in 1st Trimester Women
Z-Score ± SE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
Medicated, Nondepressed (N=18)
Medicated, Depressed (N=5)

(d)

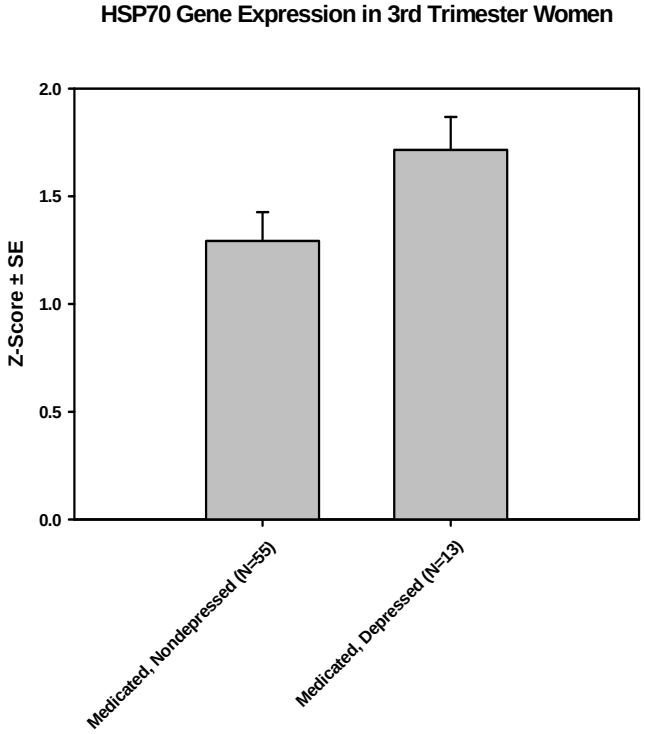

HSP70 Gene Expression in 3rd Trimester Women
Z-Score ± SE
2.0
1.5
1.0
0.5
0.0
Medicated, Nondepressed (N=55)
Medicated, Depressed (N=13)

Figure 3. Gene expression of FKBP5 in the rat amygdala correlates with gene expression of FKBP5 in rat PBMCs (p=.006, R2=.99, N=4).