**SIGNIFICANCE**

**Clinical Significance of Spontaneous Preterm Birth:** Preterm birth (PTB) is the leading cause of newborn death worldwide, is responsible for the majority of newborn morbidity[1], and represents a significant financial and social burden to caregivers and the healthcare system. Approximately 40% of PTB results is spontaneous (sPTB); labor exclusive of premature membrane rupture or medically indication i.e. preeclampsia[2] (***Fig. 1***). Despite significant research efforts, the underlying mechanisms of sPTB remain unclear. The majority of data driven research has focused on primarily on medically indicated preterm birth, and only 18% of transcriptomics analyses capture sPTB [3]. We will use systems biology to address this *critical research gap* by examining changes in transcriptional regulation related to both maternal plasma CRH and gestational age in otherwise asymptomatic pregnancies.

**Molecular Mechanisms implicated in premature birth:** PTB is the end result of a multifactorial process involving maternal and environmental components.In cases of asymptomatic sPTL, pathological changes have been observed involving placental insufficiency, subclinical infections, disruptions in maternal tolerance to pregnancy, and decidual senescence [4]. Glucocorticoid signaling regulates inflammation and immune response, and thus is intimately linked in many of these processes. Glucocorticoid signaling is carefully synchronized by the placenta, which is the master regulator of the *in-utero* environment. Maternal glucocorticoid response is modified by maternal stress, which inhibits the placentas ability to sequester excessive glucocorticoids and is associated with negative pregnancy outcomes[5]. Psychosocial stress during pregnancy is a well established risk factor for preterm birth[6].

**Placental Corticotrophin releasing hormone production and function:** CRH is a neuropeptide hormone produced by the periventricular nucleus within the hypothalamus in response to stress and by placental syncitiotrophoblasts during pregnancy. Placental CRH is secreted into both fetal and maternal compartments, but preferentially the maternal compartment as CRH levels are 10-20 times higher in maternal plasma than in fetal umbilical blood [7], and rise exponentially, reaching levels up to 1000 times higher than before pregnancy. Placental *CRH* expression occurs only in higher primates and is dependent upon species specific nuclear transcription factors[8]. Placental CRH plays a functional role in fetal development by influencing placental blood flow and metabolism. CRH acts as a vasodilator within the placental bed and regulates placental blood circulation, regulating nutrient transport and endocrine signaling [9]. Placental CRH also modulates glucose transport to the fetus by modulating GLUT expression[10].CRH is involved in steroid production in the placenta and modulates the levels of key hormones involved in pregnancy including estrogen and progesterone[11]. Placental CRH crosses the placental barrier and stimulates the fetal pituitary-adrenal axis, resulting in increased production of fetal adrenal steroids, which aid in fetal lung development[12]. Hypothesis driven analyses have uncovered multifaceted roles of placental CRH in placental function and fetal development, but there is a *critical research gap* in the understanding of the underlying transcriptional mechanisms by which this occurs.

**Multifaceted regulation of CRH during pregnancy:** The massive influx of CRH into maternal serum results indisrupted glucocorticoid signaling throughout pregnancy and during the postpartum period [12]. Although placental CRH is identical to hypothalamic CRH in terms of structure and bioreactivity, it exhibits differential transcriptional response to glucocorticoids. In the hypothalamus, glucocorticoids act as negative regulators of *CRH* by inhibiting transcription and decreasing mRNA stability[13]. In the placenta, glucocorticoids stimulate CRH activity through interactions with its promoter. These differences are related to placenta specific transcriptional regulators including cAMP regulatory elements, estrogen regulatory elements, and ecdysone regulatory elements[14]. Hypothesis driven studies have revealed cell type specific differences in transcriptional regulatory elements, but comprehensive characterization of transcriptional regulatory network differences in response to CRH remains a *critical research gap.*

**CRH as the “Placental Clock”:** CRH is a fundamental player in the complex mechanisms which orchestrate parturition. Plasma levels of CRH increase throughout pregnancy and are associated with a decrease in CRH binding protein during late gestation which results in high circulating, bioavailable CRH at the onset of partition [15]. The rate at which CRH increases is *accelerated* in women who deliver prematurely[15][16], indicating these changes in CRH signaling occur early in gestation (***Fig. 2***). CRH signaling is involved in labor initiation through a number of mechanisms. The steroid hormone progesterone binds to CRH and maintains pregnancy by promoting myometrial quiescence and maintaining maternal immune tolerance[4].In late gestation, cortisol outcompetes progesterone in this binding, which may be involved in the initiation of labor [17]. CRH also stimulates the release of prostaglandins on myometrial tissue[18], which stimulate contractions and initiate cervical ripening. Thus, there are converging epidemiological associations and functional roles that support the fundamental role of CRH on birth timing, but these exact mechanisms *remain unclear*.

**CRH as a biomarker of pregnancy complications:** CRH is detectable in maternal plasma and is well established to be positively associated with gestational age and preterm birth[19]. Elevated Plasma CRH measurements measured at 33 weeks was associated with 3.6 fold increased risk of preterm birth, after adjustment for confounding variables. CRH is an attractive biomarker for preterm birth, but any one single measure of CRH has limited clinical utility due to low sensitivity (<50%) [20]. This is likely due to the complex nature of CRH signaling, as well as the other confounding factors which are associated with both CRH and risk of preterm birth, such as ethnicity[21]. Elevated plasma CRH is observed in other pregnancy pathologies, including preeclampsia[22], In utero growth restriction[16], and chorioamniotis [23]. Beyond its role in partition, altered glucocorticoid signaling and prenatal stress are implicated in increased risk of later life cardiovascular metabolic, neuroendocrine and psychological disorders through the “prenatal programming of adult disease” paradigm[24]. Epidemiological studies have revealed that changes in CRH are highly indicative of adverse pregnancy outcomes and later life health.

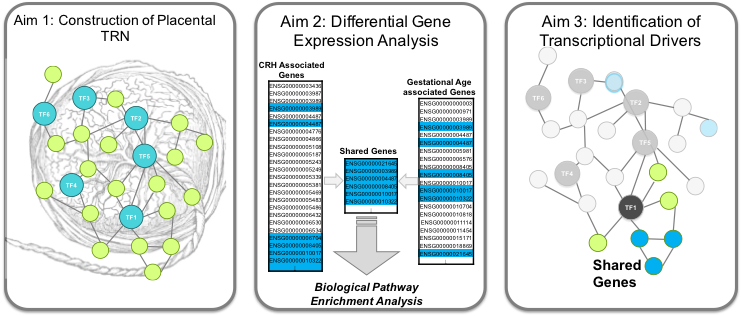
***IN SUM***, CRH is a crucial glucocorticoid response hormone which is heavily involved in fetal development, placental function, and parturition. CRH is strongly linked to gestational length and is implicated in preterm birth pathology. I have highlighted a number of critical *research gaps,* includinglimited transcriptomics analyses of asymptomatic preterm labor, lack of understanding of the role of CRH in placental metabolism, and transcriptional regulatory network function. Overall, the analyses described in this proposal will seek to fill these gaps using highly sophisticated, multimodal approaches.

**INNOVATION**

This proposal is designed to address knowledge gaps about CRH signaling and gestational age timing using a systems biology based, multidisciplinary approach. This proposal is innovative specifically because:

1. We generate the first placenta specific transcriptional regulatory network using the large transcriptomics data provided by ECHO-PATHWAYS. This TRN will be publicly available and can be used by other members of the placental research community interested in relationships between specific transcription factors and their target genes.
2. We will apply this model to learn how CRH signaling quantified in mid-late pregnancy influences transcription, and validate these findings in placental derived cell lines. This will help us understand crucial knowledge gaps involving CRH signaling during pregnancy.
3. We will identify both biological pathways and transcriptional regulatory networks that are altered in relation to both CRH signaling and gestational age, providing insight on convergence in molecular mechanisms shared by these 2 phenotypes.
4. I have assembled a *multidisciplinary team* with expertise in computational biology, epidemiology, CRH signaling, and obstetrics and gynecology. This team will ensure that the results we produce are technically correct, biologically relevant, and impactful on preterm birth outcomes.

**APPROACH**

**A) Overview:** The overall goal of this proposal is to characterize CRH related changes in the placental transcriptome, and to determine how these changes influence gestational length through transcriptional regulatory network analysis (TRENA), as shown in **Fig 3.** In the mentored phase of this proposal, we will construct a placental transcriptional regulatory network using ECHO-PATHWAYS transcriptomics data. This model provides directional, causal models of gene expression at the genome scale, which will be experimentally validated using knock out experiments of key transcription factors. In the independent phase, we will identify genes that are associated with both CRH expression in mid-late gestation as well as genes associated with gestational age, and identify overlaps in gene expression between these conditions. We use the placental TRN to identify transcription factor modules (networks of genes and transcription factors) which regulate the differentially expressed genes we identify, and experimentally validate our findings with CRH by treating cells with CRH and examining expression of transcription factors. These independent but complementary aims will provide crucial insight into the molecular mechanisms of CRH as a “placental clock”.

**B) Experimental Methods and Design:**

*ECHO Cohort:* University of Washington ECHO PATHWAYS is a multi-year NIH center grant focused on examining prenatal environmental exposures, placental transcriptome, and childhood health outcomes. The primary objective of PATHWAYS is to examine the influence of these exposures on the placental transcriptome by generating a large set of RNA sequencing data from 1568 placentae across 3 separate cohorts with data available, which is set to be completed by mid 2018. We will utilize a subset of these previously generated transcriptomes from the CANDLE cohort (generated as a part of ECHO-PATHWAYS), which have appropriate covariate information including gestational age and CRH measurements taken at 2 time points (N=1200). The age range of these samples is 22.4-42 weeks, mean age 38.8 and 10.22% of infants born prematurely (<37W). These studies were not designed to specifically investigate preterm birth, and as CRH has been specifically related to timing of delivery in previous studies, we use gestational age in our analyses. We will exclude transcriptomes generated from infants with evidence of severe pregnancy complications. The ECHO PATHWAYS group will be directly involved with the analysis and publication of this data.

*CRH quantification:*In this analysis we will utilize CRH measurements measured in maternal plasma in mid-late gestation. CRH produced by the hypothalamus is not detectable within the peripheral circulation, but during pregnancy the high levels of CRH produced by the placenta are reliably detectable in maternal plasma through the second half of gestation[25]. Unlike other hormones, CRH is not diurnal, so the the time of collection will not influence measurements. Factors that may influence CRH bioassay results include storage conditions, sample processing, temperature and time to processing, and sample additives. As changes in the rate of CRH have been previously associated with adverse pregnancy outcomes it is imperative to measure differences in CRH between trimesters, which requires CRH measurements at 2 timepoints.CRH is quantified in X lab, and Y procedures are in place to ensure the highest quality. CRH will be normalized and absolute quantification is provided (?) . CRH will be measured in comparison to baseline….

*Transcriptional Regulatory Network Analysis (TReNA):*A transcription factor network is a quantitative mapping of the interactions between sequence specific DNA binding proteins (i.e. transcription factors or TFs) and the genes to which they bind and regulate, known as target genes, and provides valuable insight into the underlying causes of expression differences between samples [26]**.** *TRNs provide directional, causal models of gene expression at a genome scale and are mechanistically informed.* The Price lab has developed a novel approach to this called Transcriptional Regulatory Network Analysis (TRENA) which expand this approach by generateing tissue-specific transcriptional regulatory network (TRN) models (Figure 4). Binding of all transcription factors in a specific tissue can be learned from DNase Hypersensitivity (DHS) data such as is generated by the ENCODE project. To this end, we have generated a pipeline that identifies open-chromatin regions and characterizes these by applying several different foot-printing algorithms to identify locations where a TF is likely protecting the DNA from DNase I cleavage. These footprints are checked against a catalog containing all known TF-binding motifs (as identified through position weight matrices) to make a genome-wide map of putative TF binding sites that can be used to test the relationship between binding sites and target genes [27] (Fig 4). We will utilize the high quality data available through ECHO to enhance our placental TRN and use it to investigate specific phenotypes related to maternal CRH in mid-late gestation and gestational age.



**C) Preliminary studies:** We performed preliminary analyses using publicly available transcriptomics data from microarrays and RNA sequencing, which did not provide maternal CRH plasma expression or appropriate covariate information. Here, we utilize placental *CRH* gene expression as a proxy for maternal CRH serum measurements in mid-late gestation. Prior studies of CRH and pregnancy outcomes have largely utilized maternal serum, as changes CRH placental expression represent the end of the gestational process and we acknowledge are not the most appropriate time point. These preliminary analyses provide proof of concept, which would need to be analyzed with appropriate CRH measurements.

**Figure 5: Schematic for reconstruction and validation of a transcriptional regulatory network (TRN) in the placenta. TF binding sites from JASPR is combined with placental specific cis motifs from ENCODE, to generate predicted TFBS. LASSO regression is used to identify active edges (i.e. gene-TF expression relationships). Performance accuracy of the model is evaluated by using a subset of data to train the model and evaluate in an independent test set. Adapted from Ament *et. al*, 2017 in prep**

*Generation of Draft Transcriptional Regulatory Network:* We have completed a draft transcriptional regulatory network using X healthy term placentas from publicly available RNA sequencing data generated in 200 term placentae[28]. We intend to greatly expand this TRN using high quality RNA transcriptomes in the process of being generated, and to make data publicly available. To build this network, we used ENCODE data to restrict our analysis to regions of open chromatin, and identified transcription factors uing X. We used X(Random Forest) to build a series of models linking expression of transcription factors to their target genes. We identified X nodes and Edges and got X accuracy on the model (?) U

*Identification of shared and distinct genes and pathways associated with gestational age CRH expression:* There is a paucity of placental transcriptomics data involving pregnancies complicated by spontaneous preterm birth independently of other pregnancy complications (i.e. preeclampsia). We overcame this research gap by generating an aggregated dataset of all publicly available microarray data on these infants. We identified publicly available microarray transcriptome studies that included term and preterm placental villous samples within the Gene Expression omnibus (GEO)[29] and removed samples with pregnancy complications, resulting in 55 preterm and 78 term samples from 6 datasets (one unpublished)[30][31][32][33][34]. Microarray datasets were preprocessed as described in [35], with cross hybridizing probes removed and gene level expression values calculated by the mean of all probes which mapped to the gene. Data was aggregated using genes in common among all arrays (N=14,251 genes), and batch effects were mitigated using empirical Bayesian adjustments implemented within combat [36]. In this dataset, we identified significant positive correlations between placental CRH expression at delivery and gestational age (*P*=0.003, R2=0.06). We performed preliminary analysis of genes and pathways which were associated with both gestational age and placental *CRH* expression as a proof of concept for *aim 2,* using linear models generated within LIMMA[37]. We identified 2631 genes which were associated with placental CRH expression, and 618 genes associated with gestational age (significance defined as FDR adjusted Q<0.05) (**Fig 5A**). We identified 269 genes which overlapped between these 2 pathways, which were enriched for 21 biological pathways (calculated using hypergeometric tests, with significance defined as P<0.05, pathways defined as gene ontology biological processes). Pathways were visualized and clustered using ClueGO, where we identified 9 distinct clusters of these pathways, with most of the genes involved in beta cell proliferation and differentiation(Fig 6B)[38]. This is complementary to what is known about both CRHs role in stress response and lymphocyte production[39] as well as clinical observations involving changes to lymphocytes during preterm labor[40]. Based on this preliminary data, *we hypothesize* that CRH production in mid and late gestation may influence expression of genes involved several key metabolic pathways which leads to their dysregulation during preterm labor.

*Shared and Distinct Transcriptional drivers of CRH and gestational age: WAITING ON PLACENTAL TRN*

How many TF modules are associated with the 2631 genes associated with CRH expression and how many TF modules are associated with the 618 genes associated with GA? What insight can we glean from these TF Modules? Enrichments in specific pathways?

**D) Specific Aim 1(Mentored):** *Construction of Placental TRN using ECHO PATHWAYs data*

*Strategy*: The goal of this aim is to generate a genome scale model which maps transcription factors to the genes which they regulate (target genes), which can be used to understand placental gene regulation. We will accomplish this goal using established software tools and pipelines developed in the Price lab, executed through the R package “TRENA”. The Transcriptional Regulatory Network Analysis approach (TRENA) uses linear solvers to capture the relationships between a transcription factor (TF) and target gene in a tissue specific manner [27]. These models are restricted to regions of open chromatin for each tissue available through DNASe hypersensitivity data generated through ENCODE[41], increasing the specificity of the model. This model allows us to establish directional, causal models between transcription factors and target genes in the placental transcriptome.

*Methodology*

(Aim 1A): Construction of TRN: First, we will perform digital genomic foot printing to identify binding sites for transcription factors in the human placenta, using DNASE hypersensitivity data from 9 placental samples from the NIH Roadmap Epigenomics Consortium, which are available on ENCODE[42]. DNase I cleavage patterns predict occupied binding sites for TFs and other DNA binding proteins[43][44]. We will intersect the DNAse-1 footprints with DNA sequence motifs from JASPAR[45], UniProbe[46], and SwissRegulon[47] to predict binding sites for specific TFs. These TF binding sites (TFBSs) are used as constraints, and only those TFs whose TFBSs were enriched +/-10 kb from a gene’s transcription start site were considered as candidate regulators, in this way making the model tissue specific. Next, we will construct our placental TRN using the transcriptomics data from individuals in the ECHO cohort who met inclusion criteria (N=1200), which will be split into a training and hold out data set. In our training dataset, we will build a series of models which predict relationships between transcription factors and target genes using linear “solvers” (i.e. LASSO regression, L2 penalized logistic regression, random forest), and model accuracy will be evaluated by testing ability of TFs to predict expression of target genes within the hold-out dataset. This will result in a large final model consisting of lists of TFs and their target genes, which we will make publicly available as a search tool on the Institute for Systems Biology website, as part of a grant through the Human Placenta Project.

(Aim 1B) Experimental Validation of TRN: We will validate the accuracy of our placental TRN through a series of *in-vitro* experiments conducted in JEG3 Placental Choriocarcinoma cells. We will select a series of 2-5 transcription factors which have the strongest relationships with target genes based on relationships captured in linear solvers, and silence these transcription factors using lentiviral delivery of CRISPR/Cas9 sgRNA. We will identify 3 guide sites using MITs online CRISPR design tool (http://crispr.mit.edu:8079/), which generates primers based on detailed, well established protocols[48], which have been previously used in the Price lab (citation). We will quantify expression of target mRNAs using nanostring N-Counter technology, and compare the strength and directionality of changes in gene expression to what is predicted in the model. We will quantify expression of genes which are considered to be directly influenced by these transcription factors determined by a literature review (positive controls) and negative controls which are not predicted targets in our model. All experiments will be completed in triplicate to ensure reproducibility.

*Anticipated Outcomes:* In this aim, we expect to build a robust, genome scale model which accurately predicts the relationship between transcription factors and their target genes. This model will only capture relationships of genes and transcription factors which are expressed in the placenta, reducing the complexity and chances of incorrectly predicting expression. We will validate this model *in vitro* by knocking out key TFs identified in our network, and we expect that the downstream genes will have altered expression. For example, HAND1 is a key placental transcription factor required for placental development [49], which induces expression of genes such as TCF3, and thus we expect that expression of these downstream genes would be decreased in response to knocking out HAND1. As the completed TRN will become a publicly available resource as part of the Human Placenta Project, we anticipate this will help other placental biology researchers.

*Potential Pitfalls and Alternative Approaches:*We anticipate some confounding in our transcriptional regulatory network as our network is built to be tissue specific instead of n cell type specific, and the placenta is a heterogeneous mix of cells. TRNS are based on epigenetic regulation which is intrinsically cell and not tissue type specific. Our results will reflect a composite of different cell types. This can create false positive relationships and dilute signal strengths between known and target genes. Furthermore, there TRNs do not take into account all levels of epigenetic regulation, and do not include post translational modifications or other mechanisms which may influence TF-target gene relationships. This problem is not unique, and as the Price labs ability to generate and analyze TRNs in other tissues becomes more developed, we can use other publicly available data to incorporate other modes of development or leverage more evidence to reduce the model further. In the validation stage, we will need to be selective of which transcription factors we knock out, as some TFs may be crucial for cellular survival, and a thorough literature search will be performed to ensure reasonable TFs are selected. JEG3 cells are derived from a choriocarcinoma, and may not accurately reflect placental gene expression, so we may use other placental cell lines (BeWO, Jar), or villous explants from placentae derived at birth. These cell lines have been established as popular in vitro models to study placental function in relation to disease[50], and in-vitro models are advantageous to animal models as placental gene expression is highly species specific.

**E) Specific Aim 2(Independent):**

*Strategy*: Based on CRHs role as the “placental clock” governing the length of gestation and our preliminary analysis, we hypothesize that maternal plasma CRH and gestational length are both associated with changes in the same genes and biological pathways. We aim to identify shared and distinct genes and pathways related to maternal plasma CRH quantified in both mid and late gestation, as well as gestational age. We will build a series of linear regression models for both phenotypes using transcriptomics data from ECHO-PATHWAYS (N=1200), and perform enrichment analysis of the intersecting genes using Gene Ontology terms related to biological processes to identify overlapping pathways, which will provide additional insight into the underlying molecular mechanisms related to both of these processes.

*Methodology*

(Aim 2A) Identification of genes associated with gestational age: We will generate a series of linear models relating gestational age (dependent variable) to gene expression (independent variables) captured through RNA sequencing using EdgeR [51]. Models will be adjusted for confounding variables including fetal sex, maternal age, and maternal ethnicity, and we will correct for multiple comparisons using the Benjamini Hotchberg approach[52].

(Aim 2B) Identification of genes associated with genes associated with CRH in mid-late gestation: We will generate a series of linear models relating to maternal plasma CRH (dependent variable) to gene expression (independent variables) captured through RNA sequencing using EdgeR [51]. Models will be adjusted for confounding variables including fetal sex, maternal age, and maternal ethnicity, and we will correct for multiple comparisons using the Benjamini Hotchberg approach[52]. Here, we will perform 3 separate analyses, quantifying genes associated with CRH at mid gestation (time-point 1), genes associated with CRH at late gestation (time-point 2), and genes associated with the difference in CRH quantified at these time points.

(Aim 2C) Identification of shared and distinct genes and gene pathways: We will intersect the gene lists generated in aim 2A and 2B to generate a list of genes associated with both phenotypes. We will perform pathway enrichment analysis of using hypergeometric tests to identify pathways enriched for these the genes which are shared and unique related to gestational age and plasma CRH. These hypergeometric tests will be performed using gene ontology terms[53], and visualized using ClueGO[38], which allows for easier interpretation and comparison of these gene ontology terms.

*Anticipated Outcomes:* This aim will deliver a list of shared and distinct genes and gene ontology terms related to maternal plasma CRH and gestational age. We expect to identify a number of differentially expressed genes associated with both CRH and gestational age based on our high power. With a sample size of 1200, we will be able to reliably identify associations between our gene expression data and outcomes of interest with estimates as small as 0.158 with a power of 0.8 and a bonferonni adjusted false discovery rate of Q<0.05. We expect to see enrichments in similar GO biological pathways which we identified in the preliminary analysis, with overlapping genes involved pathways related to immune regulation and stress response pathways. This will provide crucial insight into shared and distinct molecular mechanisms related to maternal Plasma CRH and gestational age.

*Potential Pitfalls and Alternative Approaches:* In our preliminary analysis using aggregated publicly available data, we identified 528 genes which were significantly associated with gestational age and 371 genes associated with placental CRH expression with an estimate at or above this threshold of 0.158. With a stronger data set that is not confounded by study design and with more appropriate measurements, we will likely detect even more genes. We may not find overlaps between these genes, or the gene lists we identify may be too large or not biologically meaningful. In this case, we can further refine these lists using a more sophisticated approach such as WGCNA[54]. We may run into challenges with using gene ontology pathways (i.e no pathways significant, irrelevant pathways significant), and may use other pathway annotation tools such as reactome[55] or KEGG[56]. This study is not designed to examine causality between maternal plasma CRH and gestational age, and we will not infer directionality based on our overlapping gene lists. A more sophisticated mediation analysis would be needed to infer this, which is beyond the scope of this study and is not commonly performed in systems biology analyses.

**F) Specific Aim 3(Independent):**

*Strategy* We hypothesize that there are shared and distinct transcriptional drivers which regulate the expression of the differentially expressed genes related to both CRH and gestational length. We will investigate the transcriptional drivers of these differentially expressed gene using the TRN we constructed in aim 1. We will validate our CRH related findings by treating JEG3 cells with CRH to establish causality of the CRH related changes in gene expression.

*Methodology*

(Aim 3A) Identification of TF modules: Using our TRN, we will identify what transcription factors regulate or are regulated by the differentially expressed genes identified in Aims 2A and 2B, generating a series of TF modules. We will find and categorize these TF modules, and find overlapping master transcriptional regulators related to both CRH expression in mid-late gestation and gestational age.

(Aim 3B) Validation of TF modules related to CRH: We will validate the relationship between CRH and transcriptional regulation we calculated *in silico* using JEG3 choriocarcinoma cells. We will treat cells with a range of 10-100 nM of CRH or vehicle, harvest cells between-24, and perform genome scale analysis of changes in transcription through RNA sequencing. These concentrations and times were selected based on prior work revealing biological effects but no changes in cell viability or apoptosis[57][58][59]. We will then see if the changes to transcription factors and target genes we predicted using TRENA also occur after CRH treatment using a series of linear regression models implemented within EdgeR on the shortened list of target genes and transcription factors identified in *Aim 3A*. As in *Aim 2A*, the models would be designed so that *CRH* treatment is the dependent variable and gene expression of the shortened list of target genes is the independent variable.

*Anticipated Outcomes:* This aim will deliver list of transcriptional drivers related to altered CRH signaling *in utero,* improving mechanistic insight and providing potential biomarkers, as well as a list of TFs which are associated with changes related to gestational age. By finding overlapping modules between between these 2 pathologies, we will gain insight into how transcriptional regulation by CRH may influence gestational age. We will perform in-vitro analysis of the CRH related changes, providing further credence to our model and providing enhanced mechanistic understanding of how CRH may influence placental transcription and function.

*Potential Pitfalls and Alternative Approaches:*We anticipate finding a high number of differentially expressed genes related to either maternal plasma CR or gestational age based on our preliminary data, and we expect that at least some of these genes will be transcription factors or will be transcriptionally regulated. We may pick up an overwhelming number of TF modules, and in this case would need to further refine our gene lists based on fold changes or something similar in order to have a manageable number of modules and lists of TFs. We intend to treat our cells using a range of 10-100 nM of exogenous CRH, as this range has been shown to not induce apoptosis or cell death in placental derived cell lines[57]. However, this does not accurately capture the *in utero* environment, where the placenta itself is producing massive amounts of CRH, and we may face challenges overcoming the internal transcriptional machinery regulating CRH in these placental cells. We then may need to perform knock out experiments of CRH (similar to those described in aim 1) in order to determine changes that occur in relation to changes in CRH hormone levels specifically in placental cells.

**G)TIMETABLE**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **K99** | | **R00** | | |
|  | Y1 | Y2 | Y3 | Y4 | Y5 |
| **Aim 1** |  |  |  |  |  |
| 1A: Completion of Placental TRN |  |  |  |  |  |
| 1B. *In Vitro* Validation |  |  |  |  |  |
| **Aim 2** |  |  |  |  |  |
| 2A. &2B. Identification of DEGs |  |  |  |  |  |
| 2B. Pathway Enrichment Analysis |  |  |  |  |  |
| **Aim 3** |  |  |  |  |  |
| 3A. Identification of TF modules |  |  |  |  |  |
| 3B. *In-vitro* CRH validation |  |  |  |  |  |
| Apply for future grants |  |  |  |  |  |

**FUTURE DIRECTIONS**

This analysis will provide functional insight into the mechanism by which CRH acts as the “placental clock” and mediates the length of gestation. This proposal contains hypothesis generating results which will be tested through in vivo experiments, and may be further validated or extending using transcriptomics data from existing populations as well as through additional in vivo analysis which is beyond the scope of this grant. Through the analysis plan outlined in this proposal, I will build a pipeline to specifically investigate transcriptional drivers related to both maternal plasma CRH and gestational age. intend to use this same approach to investigate perinatal exposures concurrently being measured within the ECHO cohort i.e. phthalate and particulate matter exposure, as well as other outcomes of interest, including neurobehavioral outcomes, and airway health. This pipeline and our placenta specific TRN could also be utilized to probe functional consequences related to a number of perinatal outcomes, and I look forward to working with collaborators to explore other hypothesis of interest. I anticipate submitting these emerging research interests as independent proposals, allowing me to gain independence and developed a research focus that is independent but complementary to my mentors.