Exposure and Fetal Growth-Associated miRNA Alterations in the Human Placenta

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Curriculum Vitae

Matthew Alan Maccani was born in Vineland, NJ, USA on January 8th 1985. He graduated from Washington Township High School in Sewell, NJ in June 2003 with high honors as valedictorian and went on to achieve his B.A. at Dartmouth College in Hanover, NH, USA, double-majoring in "Cell and Developmental Biology and Genetics" and "German Studies". While still a student at Dartmouth, Matthew won an internship at the Maximilian Planck Institute for Chemical Ecology in Jena, Germany, and worked for a semester in the lab of Dr. Ian Baldwin on characterizing the biological and biochemical properties of genetically-modified tobacco. Back at Dartmouth, Matthew worked in the lab of Dr. Mardi Crane-Godreau and conducted research on the effects of cigarette smoke exposure on antimicrobial peptide production and function in human airway epithelial cells; Matthew had the great opportunity to present his research at the New England Immunology Conference in Woods Hole, MA in October 2006 and at the 94th Annual Meeting of the American Association of Immunologists in Miami Beach, FL in May 2007. After graduating from Dartmouth in June 2007, Matthew began his PhD work in the Pathobiology Graduate Program at Brown University in July 2007 with a summer rotation in the lab of Dr. Carmen Marsit. Matthew did his second rotation in the lab of Dr. Joseph Bliss in which he worked on a project characterizing phagocytosis of *Candida parapsilosis* by human neutrophils. In January 2008, Matthew officially joined the Marsit lab to continue his doctoral training, working to characterize epigenetic alterations in the human placenta associated with *in utero* exposures and fetal growth. After passing his preliminary exam in May 2009, Matthew became a doctoral candidate and continued his research in the Marsit lab. Matthew has presented his work at a number of scientific

conferences, including the 57th Annual Meeting of the Society for Gynecologic Investigation in Orlando, FL in March 2010, the 36th Annual New England Conference on Perinatal Research in Chatham, MA in October 2010, the Clinical Epigenetics International Meeting (CLEPSO) in Homburg/Saar, Germany, in March 2011, and the Epigenetics and Developmental Programming Conference in Newcastle upon Tyne, UK in March 2011. While a PhD student, Matthew won a number of awards, including a Pre-Doctoral Training Fellowship in Environmental Pathology from the National Institute of Environmental Health Sciences, the Mander Research Pre-Doctoral Fellowship for FY 2010 for excellence in genomics and proteomics research at Brown University, the Wellcome Trust Young Investigator Bursary Award from the Epigenetics and Developmental Programming Conference in March 2011, and a Sheridan Teaching Certificate I from Brown University. Matthew has been an invited speaker at a number of conferences and research colloquia, mentored a number of undergraduates in the lab, and authored several peer-reviewed original manuscripts and reviews.

Preface and Acknowledgments

The work presented in this PhD dissertation, including the experiments, analysis, and discussion, have been executed by me. Critical collaborations have been acknowledged appropriately throughout.

This thesis describes four years of drive, desire, and dedication geared at better understanding alterations of miRNA expression and function in the placenta associated with *in utero* exposures and fetal growth. I believe this work represents an important addition to the understanding of how miRNA may be playing important roles in placental function and ultimately, how aberrant miRNA expression in the placenta may have important downstream effects on the fetal programming of the infant.

It is a humbling honor to be achieving a doctorate degree. There are a number of individuals whose guidance, mentoring, and support has made such an awesome endeavor not only possible but fruitful along the journey. My training at Brown has provided me with opportunities to grow and develop, both inside and outside of the laboratory. My advisor and mentor, Dr. Carmen Marsit, has been an exceptional mentor, challenging me to strive for excellence while at the same time keeping my best interests in mind along the way. From the early days of my summer rotation in 2007, I knew that if blessed with the opportunity to work on my thesis in the Marsit lab, it would be an amazing experience. The opportunities that Carmen has given me have broadened my horizons and have continued to cultivate my scientific and creative mind. It has been an honor to work under the guidance of such a driven, intelligent, and accomplished individual. My thesis committee, chaired by Dr. Joseph Bliss, and including Dr. James Padbury and Dr. Jared Robins, has offered guidance, helpful suggestions, and

constructive criticism which have proved very beneficial throughout the process. Members of my committee have also invited me to engage in opportunities to enrich both my public speaking skills as well as to engage in scientific dialogue with colleagues and collaborators – such as presenting my work on a number of occasions at the Pediatric Research Colloquium at Women & Infants' Hospital in Providence – for which I am very grateful. My outside reader, Dr. Richard Miller, has also contributed his expertise in the areas of placental toxicology and placental biology, and I am very thankful for his contributions. I would also like to thank the members of the Marsit lab, past and present, for without their assistance – whether working out the nuances of a new protocol or aiding in the collection of samples and questionnaire data compilation – this work would be nearly impossible.

My journey as a scientist began very early in my life. My love of nature was instilled in me by my grandparents and great-grandmother, and I enjoyed exploring the wilds of the natural world surrounding our home in rural southern New Jersey. As I continued through young adulthood, my parents and family nurtured this love and supported me as I strove to make my dreams and aspirations a reality. My family also instilled in me the value of hard work; for this and for their continued love, guidance, and support, I am forever grateful. Following high school, I attended Dartmouth where I trained in the lab of Dr. Mardi Crane-Godreau. Her creativity both inside and outside of the laboratory has been an inspiration for me; Mardi's mentoring while I was a student in her lab and beyond, as well as her friendship, has been truly wonderful and has enriched my life greatly.

Last but definitely not least, I would like to thank my girlfriend, Jennifer Z. Joukhadar, for her friendship, love, and support. She is truly a partner for the journey – and has made my life all the sweeter. Many thanks to my friends and family all over the globe, most especially Kiran Parkhe, Vedant Mehra, Lizzie Newton, Kristin Krogh, Iden Sinai, Andrew Robson, Weizhi Zhang, Allison Citro, Nathan Chung, and many more. Your friendship has meant the world to me.

My PhD dissertation experience was, for me, yet another example which once more underscores that timeless adage: “Life is a journey, not a destination.”

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Abstract of Exposure and Fetal Growth-Associated miRNA Alterations in the Human Placenta, by Matthew Alan Maccani, Ph.D., Brown University, May 2011

Exposure of the developing fetus to harmful chemicals, such as xenoestrogens, heavy metals, alcohol, and cigarette smoke, is associated with poor fetal and developmental outcomes. The overarching hypothesis of this work is that environment-associated differential expression of placental miRNA has consequences for the growth and development of the placenta and fetus and plays a role in fetal programming. In 25 human placentas, qRT-PCR revealed that *miR-16, miR-21,* and *miR-146a* were significantly downregulated in cigarette smoke-exposed placentas (n=17) compared to controls (n=8), and TCL-1 cells exposed to both nicotine and benzo(a)pyrene exhibited significant, downregulation of *miR-146a*. In an independent, population-based birth cohort, 107 human placenta samples were examined for the expression of 6 miRNA expressed in the placenta and previously shown to regulate cell growth and development pathways – *miR-16, miR-21, miR-93, miR-135b, miR-146a,* and *miR-182* – and were investigated for associations of fetal growth and miRNA expression. The expression of *miR-16* and *miR-21* was markedly reduced in infants with the lowest birthweights ($p<0.05$). Logistic regression models suggested that low expression of *miR-16* in the placenta predicts an over 4-fold increased odds of small for gestational age (SGA) status ($p=0.009$). Moreover, having both low *miR-16* and low *miR-21* expression in the placenta predicts a greater increase in odds for SGA than having just low *miR-16* or *miR-21* expression ($p<0.02$), suggesting an additive effect of both of these miRNA. Placental cell lines transfected to overexpress miRNA of interest were utilized to analyze changes in cell viability, growth, proliferation, migration, and invasion, as well as to empirically

validate miRNA targets predicted using in *silico* bioninformatic approaches. Overexpression of *miR-21* in 3A cells resulted in a trend to increased cell migration. TCL-1 cells overexpressing *miR-21* exhibited a 50% reduction in protein levels of PTEN ($p<0.05$), and 3A cells overexpressing *miR-146a* exhibited a 25% reduction in STAT-1 protein levels ($p<0.05$). Taken collectively, this work further elucidates the association of placental miRNA expression with *in utero* exposures and fetal growth and seeks to better understand how an adverse intrauterine environment acts through epigenetic mechanisms to alter infant and child health.

Chapter 1

Thesis Overview and Introduction

Thesis Overview

The study of epigenetics is focused on understanding the control of gene expression beyond what is encoded in the DNA sequence. Central to growing interest in the field is the hope that more can be learned about the epigenetic regulatory mechanisms underlying processes of human development and disease. Researchers have begun to examine epigenetic alterations to learn more about epigenetic regulation in the placenta. A number of studies are now making important links between alterations to appropriate epigenetic regulation and diseases of gestation and early life; promising links have been examined between environmental exposures and altered expression of microRNA (miRNA), small RNAs involved in epigenetic mechanisms of gene regulation. Examining changes in miRNA expression associated with environmental exposures and fetal growth will prove especially important in providing critical insights into the biology of development and response to *in utero* exposure. Furthermore, miRNA expression profiles in the placenta may aid in determination of *in utero* exposures, as well as future disease risk assessment. This thesis aims to analyze changes in miRNA expression in the human placenta associated with *in utero* exposure and fetal growth and seeks to determine the cellular consequences of such changes in miRNA expression to ultimately understand potential downstream effects on infant growth and development.

This thesis begins with a broad introduction to the placenta and to the endocrine and chemical processes it controls and regulates. The introduction to the thesis continues with a definition of epigenetics, a description of the field, the main modes of epigenetic gene regulation, and the strides that are being made in the study of placental epigenetics. The introduction continues with a discussion of miRNA and work that has been done to

better characterize placental miRNA and their crucial involvement in ensuring the proper growth and development of the fetus. Chapter two details the results of a study which sought to characterize the modulation of placental miRNA by maternal cigarette smoking during pregnancy by analyzing the expression of four candidate miRNA for associations with maternal cigarette smoking during pregnancy. Chapter three describes the results of a study which investigated associations of the expression of six candidate miRNA in the placenta with fetal growth and attempted to elucidate some of the implications of aberrant expression on the downstream growth and development of the fetus. Chapter four shows data from work aimed at better understanding the effects of aberrant miRNA expression on the placental cell level, further suggesting that miRNA play important roles in regulating cell processes crucial for the proper function of placental cells during various stages of pregnancy. Finally, Chapter five summarizes the conclusions of the previous chapters, highlights the relevance of these findings, and discusses future directions for this work.

Fetal Programming

Our understanding of the interplay between genes and the environment is being greatly enhanced in the post-genome era. There are only a few settings where the importance of this gene-environment interface is more profound than during intrauterine development, where the "critical windows" are narrower and where disruption or modification can influence fetal development as well as lead to programming of health throughout the life course. Over the years, many have speculated that adverse

intrauterine conditions might play a role in determining not only the growth and development of the fetus but also the health of the child and adult.

A number of epidemiological studies exploring links between adverse intrauterine conditions and diseases or conditions later in life have used as their cohort of interest the Dutch Famine Birth Cohort, which consists of men and women born as term singletons in Amsterdam, The Netherlands (1, 2). The formation of the Dutch Famine Birth Cohort resulting from the Dutch Famine of 1944-45 provided researchers with an opportunity to study starvation during pregnancy and a number of health and developmental outcomes potentially associated with this intrauterine exposure. As described by Stein, et al.(2), the Nazi food blockade and embargo of part of the Netherlands leading to the Dutch Famine was quite remarkable in a number of respects. Extensive data existed on a number of conditions of the social environment at the time of the famine, the definitive demarcation of the famine in both time and place, and the degree of nutritional deprivation was known with a great degree of precision (2). Throughout the 6 months of famine, the population's daily rations were 400-800 calories and at the height of the famine, the official food rations of the famine area were 450 calories per day, considered only 25% of the minimum standard while cities and towns outside of the famine area had daily caloric intake of approximately 1300 calories (1, 2). In developing the Dutch Famine Birth Cohort, these nutrition levels as well as a number of other key confounders were assessed and included in the data. Associations between adverse intrauterine environment as represented by famine and a number of diseases and conditions, including but not limited to type 2 diabetes mellitus, cardiovascular disease, other metabolic disorders, and decreased cognitive function later in life (1, 3, 4) have been reported in the years since

the creation of the Dutch Famine Birth Cohort. In addition, researchers found that maternal weight loss or moderate to low weight gain was significantly associated with infant birthweight, length, and ponderal index, as well as with trimester of exposure to famine, controlled for confounders (5). Furthermore, it was noted that among women whose weight changed more than 0.5 kg/week the "association between third-trimester weight change and birthweight among mother-daughter pairs exposed to famine in early or mid-pregnancy was stronger than the association observed among the unexposed cohort or among those exposed only late in pregnancy" (5). Additional observations of increased risk of affective disorders among males exposed to famine during their second trimester of gestation have been characterized (6), further underscoring the notion that timing of prenatal exposure to famine as well as potential confounding elements, such as gender of the infant, may be playing important roles in modulating the programming of the fetus. Birth cohorts such as the Dutch Famine Birth Cohort have provided researchers with many of the tools necessary to investigate epidemiological associations between adverse intrauterine conditions and postnatal health and disease.

Observations and associations reported in studies on the Dutch Famine Birth Cohort led many to further hypothesize that adverse intrauterine conditions could play a part in programming the fetus. The phenomenon now known as "fetal programming" is a model of gene-environment interaction and can inform the mechanistic basis of the synergistic effect(s) of the environment and the molecular character of development (4, 7). One of the forefathers of the theory of fetal programming is David Barker who, starting in the 1980s and 1990s, proposed and tested the hypothesis that an adverse fetal environment but plentiful food in adulthood might be a major factor associated with a

number of adult diseases (8). This "Barker Hypothesis" as it became known further hypothesized that adverse intrauterine conditions may result in a negative or poor maternal forecast, commonly manifested in small for gestational age (SGA) status or reduced infant birthweight. This poor maternal forecast predicts that the child will be born into a postnatal environment in which resources are scarce, and thus, the child has been forecasted or programmed to thrive in such a poor environment. Poor maternal forecasts can prove incorrect if a child is born into what is or soon becomes a nutrient-rich environment.

Several studies suggest that incorrect maternal forecasts may be linked to a number of diseases later in life, including type 2 diabetes mellitus, cardiovascular disease, other metabolic disorders, and decreased cognitive function later in life (1, 3, 4). A growing body of evidence suggests the importance of prenatal growth and development on later morbidity, further suggesting a link between developmental responses to *in utero* environments and adult biological outcomes (9). Current and future work in the developmental origins of health and disease is continuing to investigate the molecular mechanisms of fetal programming, both in epidemiological studies and model systems, often focusing on one of the most important tissues responsible for regulating *in utero* development – the placenta.

The Placenta

Throughout the *in utero* development of the fetus, the placenta is of utmost importance to ensure proper growth and development. The placenta begins development at the time of fertilization (10). Four days after fertilization, the morula enters the uterus.

As uterine fluid penetrates its outer layer, the zona pellucida, a blastocyst with a cavity is formed; further penetration results in the hatching of the blastocyst into two parts, the inner cell mass and the trophoblast. The blastocyst is then bathed in uterine secretions that provide the embryo with oxygen and key metabolites; soon this becomes inadequate for further embryonic development and the embryo must implant in the uterine wall (10). Invasion of the uterine wall begins at this stage (11). Proper invasion is crucial to further placental development in a normal pregnancy.

Following implantation of the blastocyst, trophoblast cells begin to differentiate, migrate, and invade into the uterine stroma. The cytotrophoblast stem cells either fuse to form the syncytiotrophoblast or aggregate to form anchoring villous trophoblast cells. Anchoring villous trophoblast cells give rise to a sub-population known as extravillous trophoblast (EVT) cells which invade the uterine wall and its blood vessels, particularly the spiral arteries. Both interstitial invasion of the stroma and endovascular trophoblast invasion of the spiral arteries occur (11). EVT cells are generated from cell columns at the tips of anchoring villi with resulting cells migrating deeper into maternal tissues (11). The EVT are responsible for invasion, thereby anchoring the placenta to the decidua and myometrium. Additionally, the EVT cells remodel the maternal spiral arteries in a process that spans the first few weeks in pregnancy; while the specifics of this process remain controversial, spiral artery remodeling seems to follow a mechanism displacing smooth muscle and endothelial cells, in order to produce a blood vessel capable of allowing increased blood flow and reduced resistance (12). This is an essential step in establishing and maintaining a normal pregnancy and is necessary for the higher blood requirement of the fetus later in pregnancy (12). Shallow or incomplete trophoblast

invasion and lack of or limited spiral artery modification have been implicated in complications of pregnancy such as preeclampsia and intrauterine growth restriction (12, 13).

Formed from fused cytotrophoblast stem cells, the syncytiotrophoblast is a multinucleated but continuous cell layer which ultimately covers the surface of the placenta and is a site of metabolic activity resulting in one function of the placenta as a barrier (though not completely impervious to insult) (10). The syncytiotrophoblast is a specialized epithelium covering the villous tree and has several functions, such as transport of gases, nutrients, and waste products and synthesis of peptide and steroid hormones that regulate placental, fetal, and maternal systems. Alterations in villous trophoblast differentiation are seen in various pathophysiological situations and may underlie several pregnancy disorders (10).

Fetal circulation begins around 6 weeks (14) and maternal flow starts as intravascular plugs are dissolved and endovascular trophoblast migration into spiral arteries is completed (15). It is at this stage that most consider the placenta as fully functional with both fetal and maternal circulations established and functioning (11). During the stages of further placental growth and development, intermediate and terminal villi branch off from the main stem villi, forming what is considered to be a system of villous trees (11). Additionally, the placenta subdivides into functional units called placental lobules (11). As an overall summary, basic development of the human embryonic and extraembryonic tissues is described schematically by **Figure 1** (16).

Called by some a hallmark of mammalian development (17), the functional placenta provides the fetus with nutrients, allows for waste to be transferred and

ultimately excreted by the mother, and protects the fetus from what would otherwise be a type of immune detection which would result in an attack of the placenta by the maternal immune system. In addition, the placenta has a degree of metabolic and endocrine activity, is involved in secreting hormones responsible for maintaining and regulating various stages of pregnancy, and performs biochemical reactions to protect, whenever possible, the fetus from exposure to toxicants or other harmful chemicals (18). All of these functions of the placenta as well as placental gene expression thus respond to and are marked by environmental insults (18, 19), and in many ways, the placenta can serve as a record of *in utero* exposure and pathology (20). Various compounds and drugs, including but not limited to alcohol (21), nicotine (22), cocaine (23) (24), lead (25), and phthalates (26), have been shown to cross the placenta and alter placental gene expression; some even accumulate in the placental tissue. Depending on the timing and type of environmental insult, such insults may affect various aspects of placental development and function and can have a variety of ramifications on the developing fetus.

One of the most common environmental exposures during pregnancy is maternal cigarette smoking during pregnancy. Maternal cigarette smoking has been reported to be associated with increased risk for spontaneous abortion (27) and preterm delivery (27-29). Previous studies have shown that there are placental complications linked to cigarette smoke exposure during pregnancy, including alterations to the development and function of the placenta (30). There are more than 4,000 chemicals in a cigarette, including nicotine, benzo(a)pyrene, and carbon monoxide; more than 43 of these chemicals are known carcinogens (31). Nicotine readily crosses the placenta and can

result in fetal concentrations that are 15% higher than maternal concentrations (32). While a number of studies have shown a decrease in overall prevalence of smoking in women in the past 20 years, the prevalence of smoking in young pregnant women has increased (33, 34). Additional studies have reported that 12-15% of all women smoke during their pregnancies (35, 36). Taken collectively, these observations suggest that maternal smoking during pregnancy remains an important common exposure that can have major ramifications on not only the normal growth and development of the fetus but also on fetal programming.

Studies continue to reveal how developmental plasticity and the developmental origins of health and disease may be mechanistically linked to the placenta (37). Also, data have linked the placenta to fetal cardiovascular adaptations(38), fetal body composition(39, 40), and fetal endocrinology and metabolism(40). Investigations are underway to determine changes in the genetics and epigenetics of the placenta which are characteristic of such exposures and pathological responses resulting from these exposures. Many of these investigations are revealing the epigenetic mechanisms underlying molecular fetal programming.

Epigenetics

Epigenetics is broadly defined as the field of research which studies changes in gene expression that are not caused by changes in the sequence of DNA (41), and the field has seen relatively rapid growth over the past few decades, accelerated by advancements in molecular biology, biotechnology, and genomics. The emergence of a new field – namely, "environmental epigenetics" (42) – combines the traditional ways of

studying epigenetics with the understanding that environmental exposures affect such epigenetic mechanisms as well. Research in fetal programming and many other disciplines is now focusing on the paradigm that gene regulation occurs beyond the DNA sequence. Most of the acquired adverse effects related to intrauterine environment cannot be due to genetic alterations. This critical role of *epigenetic* regulation, the mitotically and meiotically heritable control of gene expression not related to DNA sequence, during development is becoming increasingly appreciated. Thus, an understanding of changes to the cellular epigenome is at the interface of the interaction between genes and environment, and can provide a mechanistic basis for the synergistic effects. Research in model systems and now expanding to human studies has suggested that the causes and consequences of a variety of pathologies are related to environmental influence on epigenetic regulation. Examination of the specific molecular character of these epigenetic alterations in perinatal development has been less comprehensive. As the field of epigenetics continues to grow and be defined, there remains a central focus on examination of 4 main modes of epigenetic regulation: DNA methylation, imprinting, histone modification and small RNA-mediated control, specifically miRNAs.

DNA Methylation

DNA methylation has become the most heavily studied mode of epigenetic regulation (41). In brief, DNA methylation is carried out by one of a variety of DNA methyltransferases responsible for adding a methyl group to cytosine residues in cytosine/guanine-rich regions of DNA (called “CpG islands”). A general rule (one that is usually, but not always, true) is that when a given stretch of cytosines in a CpG island or

islands located in the promoter region of a gene is methylated, that gene will be effectively silenced by methylation; such a CpG island would be termed "hypermethylated". Conversely, when a given stretch of cytosines in a CpG island or islands located in the promoter region of a gene is not methylated, that gene will not be silenced by methylation; the CpG island in this case would be said to be "hypomethylated". It should also be noted that it is not the methylation of DNA itself which contributes to transcriptional repression but rather the binding of various elements (proteins that act as transcriptional repressors, proteins that block the movement of RNA polymerase, etc.) to methylated stretches of DNA that most greatly contribute to the transcriptional repression characteristic of genes with methylated CpG islands in their promoters. Researchers continue to work on attempting to decipher a type of chromatin code – one that may give scientists clues as to what degree of promoter methylation as well as interaction with histone post-translation modifications may be necessary to silence a particular gene.

Throughout the development of the embryo, important resetting of methylation patterns of germline and somatic lineages occur. Methylation throughout the genome of the zygote is almost completely removed during the cleavage phase of development; in between the implantation and gastrulation phases of development, *de novo* methylation reestablishes the developing organism's methylation patterns which, under normal conditions, are maintained throughout the rest of the organism's life (17, 43-45). This patterning is not limited to the embryo, but also occurs in a specific fashion in the extraembryonic lineages, although the overall levels of methylation in extraembryonic cell lineages are significantly lower than that in the somatic lineage (17). Crucial for the

health and survival of the organism is the need for the appropriate removal and resetting of methylation patterns during development thereby making this period a critical window during which the environment can have profound effects on the epigenetic pattern of the offspring.

Imprinting

Epigenetic regulation is central to the phenomenon of genomic imprinting, the parent-of-origin, allele-specific expression of genes. Genes controlled through imprinting are often located and regulated coordinately in clusters. Imprinted genes are theorized to be controlled at differentially methylated regions (DMRs) by DNA methylation(46). One type of DMR is one that is differentially methylated in all tissues throughout development and is commonly called an imprinting control region (ICR) because such ICRs are hypothesized to be key regulators of imprinting in their particular chromosomal domains (47). The other type of DMR is one that has differential patterns of tissue-specific methylation during stages of somatic development (47).

Both non-coding RNAs and changes in DNA methylation at sites in DMRs are responsible for the regulation of the imprint. Although DNA methylation is involved, it does not function in a manner similar to that seen in promoter regions, but instead functions to alter the binding of specific transcription factor and/or enhancer elements which control the allele-specific expression of the region (48, 49). The marks of imprinting are erased in germline cells, and re-established dependent on the sex of the individual (i.e. in sperm, paternal imprints become established and in oocytes, maternal imprints) (50).

Imprinting has been theorized to be one of the mechanisms involved in the so-called "parent conflict" theory (51). The "parent conflict theory" suggests that paternally expressed genes strongly favor using maternal resources to benefit offspring while maternally expressed genes attempt to preserve such maternal resources and thus, are in direct conflict with one another (51). In such a way, one could argue that paternally expressed (and maternally imprinted) genes would work to foster the growth of offspring while maternally expressed (and paternally imprinted) genes would function to better ensure that each offspring has approximately the same access to maternal resources as its siblings (52).

Imprinted genes are thought to function in the control of embryonic development, including placental development (53), as well as in functions later in life such as behaviors and metabolism (54). Alterations to normal imprinting patterns lead to well characterized syndromes, including Praeder-Willi and Angelman Syndromes related to inappropriate imprinting at chromosome 15q11. Developmental environment has also been suggested to affect the appropriate establishment of imprinted genes, particularly at chromosome 11p15 leading to Beckwith-Wiedemann Syndrome, which has been linked to the use of assisted reproductive technologies (55-57). Although methylation patterns are critical in the regulation of genomic imprinting, their utility to examine imprinted genes is limited by the ability to determine allele-specific methylation patterns. Most often, imprinting status is determined using allele specific PCR reactions to examine allele specific expression (58), although new methods based on genome-wide SNP arrays are allowing for the examination of allelic-specific expression and alterations to normal imprinting status on the genomic scale (59, 60), and may be useful for the examination of

imprinting patterns in the placenta and their association with normal fetal growth and development.

Histone modification

Modifications of the chromatin environment play key roles in epigenetic regulation of gene expression as well. One of these epigenetic regulatory mechanisms involves the acetylation, methylation, phosphorylation, and ubiquitinylation of histones, leading to regulation of gene expression (61, 62). The combined effects of the modification of the amino-terminal tails of core histones by acetylation, phosphorylation, and methylation play a major role in determining gene activity (63-65). A number of classes of histone methyltransferases – key enzymes involved in the transfer of methyl groups to histones – have been discovered (64), including the H3-K4 methyltransferase (66, 67) and five H3-K9 methyltransferases (68-71). Additionally, researchers have identified a number of transcription co-activators that have characteristic histone acetyltransferase (HAT) activity and histone deacetylases (HDACs), both of which play important roles in histone modification (72).

Histone modifications can be established in particular reactions or in a sequential order (63, 65); recent studies have indicated that the sequential order of modifications may be gene specific (73, 74). Other research has suggested that site-specific combinations of covalent histone modifications may comprise a type of histone code that can not only affect the structure of chromatin but can also affect targeting of transcriptional complexes (75-77). Such histone modifications can lead to gene activation or gene silencing, depending on the effects on transcriptional complexes.

Alterations to patterns of histone modification can have a number of negative consequences, such as developmental dysregulation, X-chromosome inactivation, or might lead to a number of diseases (72). Research is continuing to better define how the patterns of histone modifications are utilized by the cell to control gene expression, as well as how these marks are involved in regulating additional epigenetic processes (78).

Small regulatory RNAs and microRNAs (miRNA)

In the early 1990s, researchers first published observations characterizing two small regulatory RNAs, known as *lin-4* and *let-7*, which were shown to control the timing of larval development in *C. elegans* (79, 80). These RNAs, initially termed "*lin-4* and *let-7* RNAs", were initially suggested to represent a class of endogenous RNAs found in worms, flies, and mammals and since have been renamed "microRNAs (miRNAs)" (81-83). Subsequent work suggested that these small regulatory RNAs could be found in plants, mammals, green algae, and viruses (84). Other classes of small RNAs have been found in plants, animals and fungi; small interfering RNAs (siRNAs) (85, 86) and Piwi-interacting RNAs (piRNAs) (87) are two examples. miRNAs are different from these other classes of small RNAs in that they are formed from transcripts that have been shown to fold back on themselves, generating characteristic hairpin structures (88); other small RNA classes are formed from longer hairpins (siRNAs) or from precursor forms lacking a double-stranded nature (piRNAs) (88). Generally, as these small regulatory RNA molecules can alter gene and protein expression without altering the underlying genetic code, they too are considered critical mechanisms in epigenetic regulation.

miRNA are transcribed by RNA Polymerase II as part of transcripts called primary miRNAs (pri-miRNAs) and include 5' caps and 3' poly(A) tails (89-91). The miRNA portion of the pri-miRNA then forms a hairpin (89). The pri-miRNA is then digested by the dsRNA-RNA-specific ribonuclease Drosha, and the hairpin that is ultimately released is called precursor miRNA (pre-miRNA) (92). pre-miRNA has been characterized to be 70-75 nucleotides of RNA in length with 1-4 nucleotide 3' overhangs, 25-30 base pair stems, and small loops (92, 93). Data have also suggested that Drosha processes either the 5' or 3' terminus of the mature miRNA, depending on which strand of the pre-miRNA associates with the RNA-induced silencing complex (RISC) (92, 93). The pre-miRNA is then exported from the nucleus to the cytoplasm by a complex containing Exportin-5 (Exp5) (93, 94). After arrival in the cytoplasm, the pre-miRNA is cleaved by Dicer, an RNase III superfamily member (92, 93). After cleavage by Dicer, the resulting double-stranded RNA has short 3' overhangs at either end (94). It should be noted that only one of the two strands in the post-Dicer-processed dsRNA is the true mature miRNA; some mature miRNAs are formed from the leading strand of the miRNA strand while others are formed from the lagging strand, in a mature miRNA-specific fashion (94). In order to effectively control the translation of target mRNAs, the dsRNA that Dicer has processed must be separated into two strands, and the single-stranded mature miRNA has to associate with the RISC in order to be trafficked to its mRNA target (95). Researchers have shown that determination of the active strand in the ds-RNA has a direct relationship with the stability of the ends of the dsRNA (96, 97); in brief, their work revealed that the strand with less stable base pairing of the 2-4

nucleotides at the 5' end of the duplex associates with RISC and ultimately takes on the role as the active miRNA strand (96).

Data have suggested that miRNA regulate gene expression by base-pairing to a target mRNA transcript; the exact mechanism for this post-transcriptional gene regulation varies depending on a number of factors, the most noteworthy of which seems to be the degree of complementarity of the miRNA to its target mRNA sequence (81). The active strand of the mature miRNA associates more specifically with the Argonaute protein of the RISC and upon trafficking to the target mRNA, participates in post-transcriptional repression (95, 98). As a general rule, a miRNA with perfect complementarity to its target mRNA will cause the degradation of the mRNA transcript through Argonaute-catalyzed mRNA cleavage (95, 99, 100) while miRNA with imperfect complementarity to a target mRNA will cause translational repression by blocking or altering the normal function of machinery that would otherwise aid in translation of mRNA into protein (81). Mechanisms by which translational repression is carried out include inhibition of translation initiation and poly(A) shortening (101). Some groups have also shown data which suggest that miRNA can use a combination of both mRNA degradation and disruption of translation to carry out post-transcriptional repression (102).

miRNA have been shown to carry out important roles in a number of responses to stress and disease. Work by van Rooij and colleagues demonstrated that a cardiac-specific miRNA, miR-208, was crucial for hypertrophy of cardiomyocytes and fibrosis in response to stress and hypothyroidism (103). Findings such as this have led several to hypothesize that there may be important miRNA-based therapies for heart disease that have yet to be developed (104). Several groups have suggested roles for miRNA in

preventing or even contributing to the development and progression of cancer (105). miR-21, shown to be upregulated in human brain tumor glioblastoma, has also been shown to have anti-apoptotic properties in human glioblastoma cells (106). Several other miRNAs have been described as having oncogenic or even tumor-suppressive characteristics, and many have been selected for use in developing cancer-specific therapies (105).

There exist a number of technologies and methods that have given researchers the power to measure even the smallest of changes in miRNA expression. Reverse transcription quantitative real-time PCR has been used to determine changes in the expression of particular miRNA associated with particular exposures or diseases. High-throughput assays, including miRNA microarrays, have been used to interrogate the expression of thousands of reported and predicted miRNA sequences in tissues that have a particular exposure or disease. Downstream effects of miRNA on post-transcriptional gene regulation remain more challenging and require use of bioinformatics approaches to first predict mRNA targets of specific miRNA and then confirm the effects of over- or underexpression of miRNA on that particular target. Due to the miRNAs' ultimate role in controlling protein translation, true confirmation of targets requires examination of the proteins of interest using specific antibodies, or through in-vitro approaches coupling targeted miRNA binding regions to reporter constructs. The understanding of miRNAs' role in the human placenta is in its infancy but due to the known critical role of miRNA in human development, it is certainly an attractive and exciting field of study.

Placental miRNA

Several groups have published data demonstrating that miRNA expression is tissue-specific and that several miRNAs are expressed in the human placenta (102, 107). Since such discoveries, much interest has been generated for investigating the involvement of miRNA in placental gene regulation and the possible utility of discovering placental miRNA which can serve as clinical biomarkers of exposure or disease.

Pineles and colleagues measured miRNA levels in placentas to investigate whether placental miRNA expression patterns are associated with preeclampsia or small for gestational age diagnoses (108). This work revealed differential expression of *miR-210* and *miR-182* in preeclampsia versus control patients and showed key associations in miRNA expression patterns and preeclampsia, furthering efforts to find a miRNA biomarker of preeclampsia pathology (108). Zhu and coworkers found that thirty four miRNAs were differentially expressed in preeclamptic placentas compared to normal placentas further suggesting a role for miRNA in the pathogenesis of preeclampsia (109). Additional studies have been conducted linking differential miRNA expression in placental tissue with preeclampsia (110). Such biomarkers would prove especially useful in giving clinicians and pathologists sensitive molecular tools to better diagnose preeclampsia, a disorder that has great impacts on both pregnant women and their newborns.

It has long been speculated that varying oxygen levels have effects on the genetic and epigenetic control mechanisms involved in placental growth and cell survival – and ultimately, on the health and survival of the developing fetus. To investigate this further,

Donker and coworkers analyzed the relationships between expression of Argonaute 2, an important RNAi enzyme, and other miRNA in trophoblasts and in environments with varying oxygen level (111). Donker and colleagues' data showed that not only is the miRNA processing machinery present and functional in human trophoblasts but that varying expression of *miR-93* and *miR-424* is associated with different levels of oxygen (111). Such data may prove especially helpful in determining whether particular placental abnormalities – and ultimately, fetal abnormalities – may be associated with aberrant levels of oxygen at particular critical windows of development.

Some groups have observed and detected placental miRNA in maternal plasma and have conducted comparative studies on circulating miRNA and other circulating nucleic acids (CAN) in the maternal sera of pregnant and non-pregnant women (112, 113). Chim and colleagues showed that placental miRNAs (*miR-141*, *miR-149*, *miR-299-5p*, and *miR-135b*) were highly expressed in maternal plasma in pregnant mothers and suggested that such expression patterns could serve as important biomarkers for monitoring pregnancy (112). Gilad and coworkers demonstrated that placental miRNA levels in sera from pregnant women were higher than those in sera from non-pregnant women; moreover, levels of miRNA in sera of pregnant women correlated with pregnancy stage (113). Thus, placental miRNA levels detectable in maternal serum may serve as important clinical biomarkers of pregnancy, pregnancy stage, and other pregnancy-related outcomes.

The Placenta as a Functional Model of Epigenetics in Fetal Programming

Data have suggested that environmental exposures can alter miRNA expression in in-vitro systems (114) and various tissues (42) and that altered levels of miRNA expression can be associated with particular diseases or risk factor for disease (115). As reviewed above, work has also been done to investigate biomarkers of diseases, such as cancer, growth retardation, and other records of *in utero* environment in placental patterns of DNA methylation, imprinting, histone modification, and miRNA expression. Studies aimed at further revealing the importance of miRNA in responding to environmental exposure and to disease may prove very useful in better understanding how such exposures and diseases affect the body. Current and past research into diseases of pregnancy – such as finding miRNA associated with preeclampsia (108, 109) or gene-specific placental methylation patterns associated with gestational trophoblastic disease (GTD) (116) – might be expanded to include work to better understand associations between epigenetic factors in the placenta and diseases such as choriocarcinoma, childhood cancers, and other diseases of childhood and adolescence, as well as alterations to normal placental immunology and function.

Using the placenta as a reference tissue allows researchers to utilize an important residual tissue whose respective miRNA expression and DNA methylation patterns may prove to be powerful biomarkers possessing predictive capability for a number of diseases or disease progression. Modern advances in bioinformatics – such as pathway analysis tools and target gene prediction software – as well as advances in technology, such as microarray technology, have given researchers and clinicians tools to better detect patterns in DNA methylation, imprinting, histone modification and miRNA

expression that are associated with particular exposures and diseases. Aberrant patterns of miRNA expression or DNA methylation may ultimately serve as biomarkers for exposure, disease burden, or even as "early indicator" diagnostics of increased risk for developing future disease or disorders.

Important advances in placental epigenetics continue to elucidate a better understanding of the epigenetic regulatory mechanisms of in the placenta. Knowledge of such epigenetic mechanisms may be useful in identifying novel biomarkers for exposure, burden, or risk for disease. Such biomarkers may prove essential for developing new diagnostics for early diagnosis of risk factor and levels of exposure. Additionally, these aberrant patterns of miRNA expression, imprinting, DNA methylation, or histone modification may identify previously unknown pathways targeted for alteration, which, in turn, may serve as targets for novel drug treatment or prevention strategies. These epigenetic biomarkers can be brought from the benchtop to the bedside and will be useful in helping clinicians better diagnose and prevent the onset of disease.

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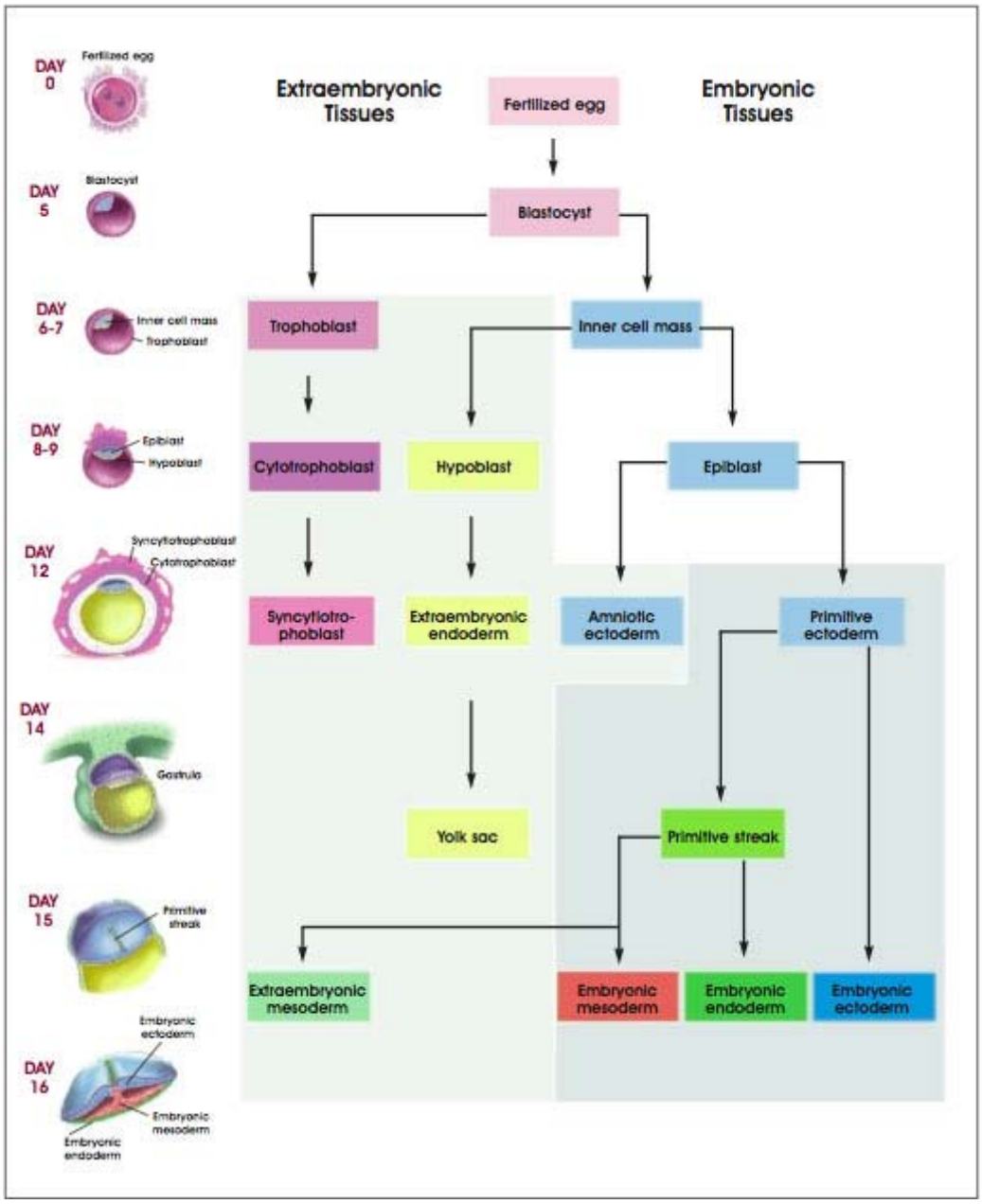

Figure 1. Basic development of the human embryonic and extraembryonic tissues. Figure adapted from (16).

Chapter Two

Maternal Cigarette Smoking During Pregnancy is Associated with Downregulation of *miR-16, miR-21,* and *miR-146a* in the Placenta

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Maternal Cigarette Smoking During Pregnancy is Associated with Downregulation of *miR-16, miR-21,* and *miR-146a* in the Placenta

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ABSTRACT

Maternal cigarette smoking during pregnancy is associated with poor fetal outcome and aberrant miRNA expression is associated with adverse pregnancy outcomes. In 25 human placentas, we analyzed the expression of four candidate miRNA previously implicated in growth and developmental processes: *miR-16*, *miR-21*, *miR-146a*, and *miR-182*, and used three immortalized placental cell lines to identify if specific components of cigarette smoke were responsible for alterations to miRNA expression. *miR-16*, *miR-21*, and *miR-146a* were significantly downregulated in cigarette smoke-exposed placentas compared to controls. TCL-1 cells exposed to both nicotine and benzo(a)pyrene exhibited significant, dose-dependent downregulation of *miR-146a*. These results suggest that *miR-146a* is particularly responsive to exposures, and that smoking may elicit some of its downstream effects through alteration of miRNA expression.

KEY WORDS: miRNA, placenta, cigarette smoking, nicotine, benzo(a)pyrene, epigenetics

RUNNING TITLE: Cigarette smoking downregulates miRNA in placenta

INTRODUCTION

Gene-environment interactions occur throughout the lifetime but there are few times when such interactions are more important than during intrauterine development when perturbations can affect fetal growth and development. Further, there can be lasting effects due to altered fetal programming. During *in utero* development, the placenta plays a critical role supporting normal growth and development, providing the fetus with nutrients, assisting in waste removal, and protecting the fetus from both maternal immune rejection and from other environmental insults. Additionally, the placenta is a metabolic and endocrine center, producing and secreting hormones which support each stage of pregnancy. The placenta also expresses metabolic compounds responsible for the reactions which ultimately protect the fetus from exposure to toxicants. A number of drugs and toxicants, including nicotine,[1] alcohol,[2] and benzo(a)pyrene,[3] have been found to accumulate in placenta tissue and affect placental gene expression. Maternal cigarette smoking has been reported to be associated with increased risk for spontaneous abortion[4] and preterm delivery.[4-6] Previous studies have shown that there are placental complications linked to cigarette smoke exposure during pregnancy, including alterations to the development and function of the placenta.[7] There are more than 4,000 chemicals in a cigarette, including nicotine, benzo(a)pyrene, and carbon monoxide; more than 43 of these chemicals are known carcinogens.[8] Nicotine readily crosses the placenta and can result in fetal concentrations that are 15% higher than maternal concentrations.[1] While a number of studies have shown a decrease in overall prevalence of smoking in women in the past 20 years, the prevalence of smoking in young pregnant women has increased.[9, 10] Additional studies have reported that 12-15%

of all women smoke during their pregnancies.[11, 12] Taken collectively, these observations suggest that maternal smoking during pregnancy remains an important common exposure that can have major ramifications on not only the normal growth and development of the fetus but also on fetal programming.

The mechanisms by which exposures, such as cigarette smoke, affect the complex regulatory mechanisms of the placenta are still being characterized. One such mode of toxicity may be through the altered expression of microRNA (miRNA), small ~22 nucleotide-long noncoding RNA molecules, which are highly ubiquitous and possess conservation across many species.[13] MicroRNA posttranscriptionally regulate gene expression by base-pairing to the 3'-untranslated region of a target mRNA resulting in either translational repression or direct degradation of the mRNA, the exact mechanism of which depends largely on the degree of complementarity of the miRNA to its mRNA target. Because partial complementarity of a miRNA to an mRNA target can still lead to translational repression, a single miRNA has the capability of regulating a large number of genes.[14] By negatively regulating their mRNA targets, miRNA have been implicated in regulating cell proliferation, growth and differentiation, and apoptosis.[15] Highly conserved clusters of primate-specific miRNA are expressed in the placenta and other tissues.[16-19] Additionally, multiple groups have further characterized a number of placental miRNA whose aberrant expression is associated with derangements in the intrauterine environment or maternal condition.[20-22] These discoveries have generated much interest in the involvement of miRNA in placental gene regulation and the possible utility of discovering placental miRNA which can serve as clinical biomarkers of exposure or disease.

As these miRNA play critical roles in development both of the fetus and placenta, and because toxicant exposures and stress can alter the expression of miRNA,[23, 24] we sought to characterize the modulation of placental miRNA by maternal cigarette smoke, by analyzing the expression of four candidate miRNA for associations with maternal cigarette smoking during pregnancy.

RESULTS

Twenty-five placentas, 8 with a history of maternal cigarette smoking during pregnancy and 17 non-smoking controls, were analyzed for expression of candidate miRNA. Table 1 illustrates the demographics of the sample population. There were no significant differences in the gender, gestational age, birth weight, or maternal age between exposed and unexposed infants. Quantitative RT-PCR analysis revealed downregulation of *miR-16* ($p<0.0001$), *miR-21* ($p<0.0001$), and *miR-146a* ($p<0.01$) associated with maternal cigarette smoking during pregnancy (Figure 1). *miR-182* expression showed no differential expression by maternal cigarette smoking during pregnancy. To control for potential confounding effects of clinical variables in these results, we utilized multivariable linear regression to examine the association between *miR-16, miR-21,* and *miR-146a* expression with maternal smoking during pregnancy, controlled for gestational age, birth weight, gender, and maternal age. The respective regression coefficients and p-values are provided in Table 2. These models suggest that maternal smoking leads to a statistically significant 3.9% decrease in relative expression of *miR-146a*, a 5.8% decrease in the relative expression of *miR-16*, and a 9.4% decrease in the relative expression of *miR-21*, each independent of infant gender, gestational age, maternal age, and infant birth weight.

Three immortalized placental cell lines representing three different stages and aspects of placental development were chosen to examine the effects of nicotine and benzo(a)pyrene specifically on the expression of the three miRNA found to be significantly altered in the primary tissues in cells from these different stages and aspects of placental development. We sought to investigate the effects of nicotine and

benzo(a)pyrene exposure on the expression of these three miRNA in the first trimester villous 3A cells, the first trimester extravillous HTR8 cells, and the third trimester extravillous TCL-1 cells. As shown in Figure 2A, qRT-PCR analysis revealed that *miR-146a* was significantly altered across nicotine exposures in TCL-1 (ANOVA $p<0.03$), with a specific significant downregulation of approximately 2.5 fold in TCL-1 exposed to 1μM nicotine compared to mock control ($p<0.02$). No differential expression of *miR-16* or *miR-21* occurred in TCL-1, 3A, and HTR8 cells exposed to nicotine (data not shown). Similar to nicotine exposure, qRT-PCR analysis revealed that *miR-146a* was downregulated in TCL-1 cells across all benzo(a)pyrene exposure levels (Fig 2B, ANOVA $p<0.006$), with 1 μM exposures leading to a greater than 50% downregulation, and 10 μM an almost 75% downregulation of the miRNA. Again, no differential expression of *miR-16* or *miR-21* occurred in TCL-1, 3A, and HTR8 cells exposed to benzo(a)pyrene (data not shown).

Table 3 shows the targets as predicted by using the three-algorithm miRNA target prediction approach described, and confirmed targets with respective sources[6, 25-29] for the three candidate miRNA with significant differential expression in primary placenta samples exposed to cigarette smoking *in-utero*. Predicted targets were as follows: for *miR-16*, BCL2L2 and EDA; for *miR-21*, PLAG1 and SATB1; and for *miR-146a*, TRAF6. Minimum free energies (MFE) are listed for each respective miRNA and target mRNA duplex and were determined using *RNA-hybrid*.

DISCUSSION

Taken as a whole, our work identifies the association of cigarette smoking during pregnancy with the aberrant expression of several miRNA involved in critical cell processes. A number of groups have previously demonstrated that nicotine[1] and benzo(a)pyrene[3] interact with the placenta and may affect placental growth and development. Previous work demonstrated the effects of toxicants and agents of cell stress on expression of *miR-146a*,[23, 24, 30, 31] as well as on *miR-16*[32] and *miR-21*.[33, 34] While our observations are limited by incomplete information regarding the duration of cigarette smoking during pregnancy, cigarette per day usage, or more extensive environmental exposure information (such as alcohol usage, environmental pollutant exposure, or secondhand cigarette smoke exposure), our data comprise an important first step in determining associations between maternal cigarette smoking during pregnancy and aberrant miRNA expression in the placenta.

To further investigate the effects of cigarette smoke, we utilized three placental cell lines from different stages of placental development to investigate mechanisms of aberrant miRNA expression associated with cigarette smoke exposure. Downregulation of *miR-146a* in TCL-1 cells treated with nicotine and benzo(a)pyrene suggests that *miR-146a* may be especially sensitive to agents of cellular stress. Moreover, this result suggests that two components of cigarette smoke which affect the expression of *miR-146a* in term placentas may be nicotine and benzo(a)pyrene. The lack of differential expression of *miR-16* and *miR-21* in cells treated with nicotine or benzo(a)pyrene does not necessarily rule out that their expression is not modulated by cigarette smoke. Other components of cigarette smoke may modulate the expression of these miRNA.

Furthermore, the effect of the mixed exposures encountered with the myriad chemicals in cigarette smoke may be truly responsible for the modulation of expression of these miRNA. Experiments conducted with different components of cigarette smoke and complex mixtures of these components may provide more extensive information on the mechanistic effects of cigarette smoke on miRNA expression.

Previous studies on benzo(a)pyrene-exposed human choriocarcinoma cell lines BeWo and JEG-3 showed that placental cell line proliferation was largely unchanged following exposure to 10 and 50 μM doses of benzo(a)pyrene in serum-containing medium.[35] Genbacev and colleagues used nicotine-exposed placental explants to conclude that nicotine is an important molecule that can drastically placental growth and development.[36] In our cell culture experiments, we saw no detectable change in cell growth or morphology in any of our cell lines treated with any of the doses of benzo(a)pyrene or nicotine compared to respective mock treated-cells. Doses of benzo(a)pyrene and nicotine higher than the ones we used may have led to increased cell toxicity and alterations in cell growth due to increased cell stress. Future investigations using higher doses may discover miRNA whose expression and function is altered by higher levels of benzo(a)pyrene, nicotine, and other agents of environmental stress.

One of the more difficult challenges is miRNA target prediction; complementarity between miRNAs and their target mRNAs is generally far from perfect, and is not required for miRNA to functionally silence the expression of a protein product. As a result of this complexity, a simple search for sequence complementarity between the miRNA and its mRNA target, the basis of many target prediction algorithms, can be expected to produce many false-positive hits. Moreover, most miRNAs are thought to

have potentially hundreds of targets, some of which will be targeted more strongly than others, and in cell-type specific contexts. Thus, it is essential to devise a strategy which predicts targets based not solely on sequence homology, but which also incorporates additional characterizations when choosing which targets should be examined with further testing.

Our target prediction strategy suggested that PLAG1 and SATB1 were targets of *miR-21*. Both of these genes are transcription factors implicated in tissue-specific control of the cell cycle and cellular proliferation, and PLAG1 has been previously demonstrated to be a target of *miR-21*.[26] Downregulation of *miR-21* due to cigarette smoke exposure might lead to overproduction of PLAG1 and SATB1 protein, resulting in the overexpression of these key genes involved in cell cycling and proliferation.

Our target prediction strategy predicted BCL2L2 and EDA as targets for *miR-16*. BCL2L2, or B-cell CLL/lymphoma 2 like 2 protein, is a pro-survival molecule previously reported to be targeted by *miR-133b*.[37] Like other anti-apoptotic proteins in the BCL-2 family, BCL2L2 has been shown to be regulated both pre-transcriptionally and post-transcriptionally.[38] The prediction that BCL2L2 is a target of *miR-16* supports hypotheses that by downregulating *miR-16*, cigarette smoke upregulates BCL2L2 and may enhance signaling through anti-apoptotic pathways. The EDA gene is mutated in anhidrotic ectodermal dysplasia and under normal circumstances, produces the protein ectodysplasin, a member of the TNF superfamily that is involved in activation of the NF-κβ signaling pathway.[39] Again, *miR-16* downregulation by cigarette smoke exposure, could lead to an upregulation of EDA protein thereby enhancing NF-κβ signaling and increased tendency toward survival.

Our *in-silico* analysis predicted TRAF6 as a target for *miR-146a*, suggesting that downregulation of *miR-146a* could result in an upregulation of TRAF6, a protein also important for signaling through both the NFκβ signaling pathway[40] as well as a mediator of inflammation in the toll-like receptor 4 (TLR4) pathway.[41, 42] Previous reports using different target prediction strategies have hypothesized TRAF6 as a target of *miR-146a*[28] and have validated TRAF6 as a target in murine macrophages.[29] TLR4 mediates the inflammatory response, and aberrant TLR-4 signaling is associated with inflammation-induced preterm delivery.[43] Such a consequence could be the result of dysregulated TRAF6 production as a result of downregulation of *miR-146a*. TRAF6 overexpression due to downregulation of *miR-146a* could result in overactive TLR signaling which could have a number of downstream consequences for both placenta and fetus. Further investigation into such relationships would prove vital into better understanding both the role of TRAF6 in TLR4 signaling, as well as the association of aberrant expression of *miR-146a* with adverse pregnancy outcomes.

As with *miR-16*, downregulation of *miR-146a* can lead to enhanced signaling through the anti-apoptotic and pro-survival NFκβ pathway, resulting in placental cells that avoid apoptosis and prolong their survival. Components of cigarette smoke have been suggested to function through the NFκβ pathway,[44] and in light of our results with both placental tissues as well as cell culture experiments, both benzo(a)pyrene and nicotine may be acting through the NFκβ signaling pathway through downregulation of *miR-146a*. Studies by other groups have demonstrated that an NFκβ-modulated pathway upregulates *miR-146a* when *miR-146a* expression is induced with IL-1β.[45] While this may be true under normal circumstances under which conditions of cellular stress are

held to a minimum, our data suggest that agents of cell stress, such as nicotine and benzo(a)pyrene, may downregulate *miR-146a* expression which, by consequently upregulating TRAF6 expression, enhances signaling through NFκβ and promotes cell survival. The complexities of this potential duality of effect, namely the notion that *miR-146a* both negatively regulates and can be regulated by the NFκβ pathway, remain to be further elucidated and future experiments involving knock-downs of various components of the pathway, including the miRNA acting as negative regulators of the pathway, may clarify the mechanisms at play. Our results suggest then that TRAF6, and therefore the NFκβ and TLR4 signaling pathways, may be important mediators of the effects of cigarette smoke exposure on the placenta.

Our results indicate a potential cascade of molecular changes that occur in the placenta upon exposure to cigarette smoke. We suggest that cigarette smoke exposure during pregnancy is associated with downregulation of *miR-16, miR-21,* and *miR-146a* in the placenta. Collectively, aberrant repression of these miRNA upregulates the targets of these miRNA and may affect cell cycle regulation, growth, immunomodulation, and development in the placenta. These changes in target gene expression may have further effects downstream for both placenta and fetus, ultimately resulting in altered fetal programming. Future studies are ongoing to further characterize the effects of environmental exposures on placental miRNA, on understanding the downstream phenotypes of aberrant miRNA expression, and in investigating the placenta as a record of the intrauterine environment.

MATERIALS AND METHODS.

Placenta Samples. Placenta samples were collected within two hours of delivery at Women and Infants' Hospital in Providence, RI. An approximately 1 g biopsy of placenta was excised, free of maternal decidua, from the maternal side of the placenta 2 cm from the umbilical cord insertion site, and the sample was placed immediately in RNAlater and stored at 4ºC. At least 72 hours later, placenta samples were removed from the RNAlater, blotted dry, aliquoted, and stored in sample tubes at -80ºC until needed for examination. Medical information, including cigarette smoking during pregnancy, available on patient charts was also collected. All samples were collected in under appropriate IRB protocols for Women and Infants' Hospital, and Brown University.

RNA extraction. RNA was extracted from placenta samples and cultured cells using the miRvana miRNA Isolation Kit (Ambion) following the manufacturer protocols. For tissue samples, a 200 mg piece was cut and placed in ice cold, sterile PBS and homogenized using a PowerGen 125 Tissue Homogenizer (Fisher Scientific). The homogenized sample was removed from PBS, and RNA was extracted using manufacturer protocols. Extracted RNA was quantified using a Nanodrop spectrophotometer and then aliquoted into single-use aliquots and stored at -80ºC.

Quantitative RT-PCR (qRT-PCR) for mature miRNA. Expression of mature miRNAs was measured using commercially available TaqMan MicroRNA Assays or TaqMan Gene Expression Assays (Applied Biosystems, Valencia, CA) on an Applied Biosystems 7500 Real Time PCR system, and analyzed with 7500 System Software All reactions were run in triplicate, with RNU44 serving as the referent for miRNA expression. In addition, a no-RT control was run with each plate.

Cell culture. 3A and TCL-1 placental cell lines were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS and 1% Pen-Strep. HTR8 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 5% FBS. These three placental cell lines represent three different stages and aspects of placental development and were selected to examine the effects of components of cigarette smoke in models of different time-points during pregnancy. 3A cells are first-trimester villous cells, HTR-8s are first trimester extravillous cells and TCL-1s are third trimester extravillous cells. Placental cell lines were exposed to 0 μM, 1 μM, 5 μM, and 10 μM doses of nicotine (Sigma-Aldrich) in medium for 6 days, with 1 μM being closest to a physiologically relevant dose and higher doses used to investigate the effects of more concentrated doses of nicotine on placental cells.[46, 47] Placental cell lines were exposed to 0.1 μM, 1 μM, and 10 μM doses of benzo(a)pyrene (Sigma-Aldrich) or DMSO (< 0.1%) alone in medium for 6 days based on previously published methods and physiologically-relevant doses.[23, 48] None of the doses of nicotine or benzo(a)pyrene led to increased cell toxicity compared to controls. Cells were cultured for 6 days, with exposure medium refreshed on days 2 and 4. The 6-day period of exposure was chosen to mimic a long-term chronic exposure, but to avoid sub-culturing of the cells, and has been previously described.[23] All experimental and control conditions were performed in triplicate. Following exposure, cells were harvested and RNA was extracted for analysis by qRT-PCR as described above.

miRNA target prediction. 3 target prediction algorithms were used to predict targets for miRNA of interest, and a fourth algorithm was used to evaluate predicted targets for base-pairing and minimum free energy. The three algorithms used for target prediction were *miRanda* (September 2008 release, available online at

http://www.microrna.org/microrna/home.do), *PicTar* (as cited by [49] and available online at http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi), and *TargetScan 5.1* (available online at http://www.targetscan.org/). In order to be considered a predicted target for further investigation, the target must have appeared in the top 100 targets in all three prediction algorithms. The *RNA-hybrid* algorithm was used to evaluate predicted targets for information on secondary structure and thermodynamic stability of the miRNA-mRNA duplex.[50] *RNA-hybrid* is available online at http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html.

Statistical analysis. Two-tailed *t* tests were used to compare miRNA expression levels determined by real-time PCR in human tissue samples. In order to examine the association between exposure to cigarette smoke and miRNA expression, controlling for potential confounders, we employed multivariable linear regression modeling, in SAS 9.1 (SAS Institute, Cary, NC). ANOVA was used to determine differential expression of miRNA across treatments of nicotine and benzo(a)pyrene on cells in cell culture experiments.

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Table 1. Demographics of the study population.			
	Total	Maternal Smoking During Pregnancy	No Maternal Smoking During Pregnancy
Total, n (%)	25 (100)	8 (32)	17 (68)
Gender, n (%)			
Female	13 (52)	3 (37.5)	10 (59)
Male	12 (48)	5 (62.5)	7 (41)
Gestational Age in weeks, mean (SD)	38.7 (0.88)	38.4 (1.20)	38.9 (0.66)
Birthweight in g, mean (SD)	3080 (540)	3091 (682)	3071 (484)
Maternal Age in years, mean (SD)	27.3 (6.66)	24.3 (7.94)	28.8 (5.68)

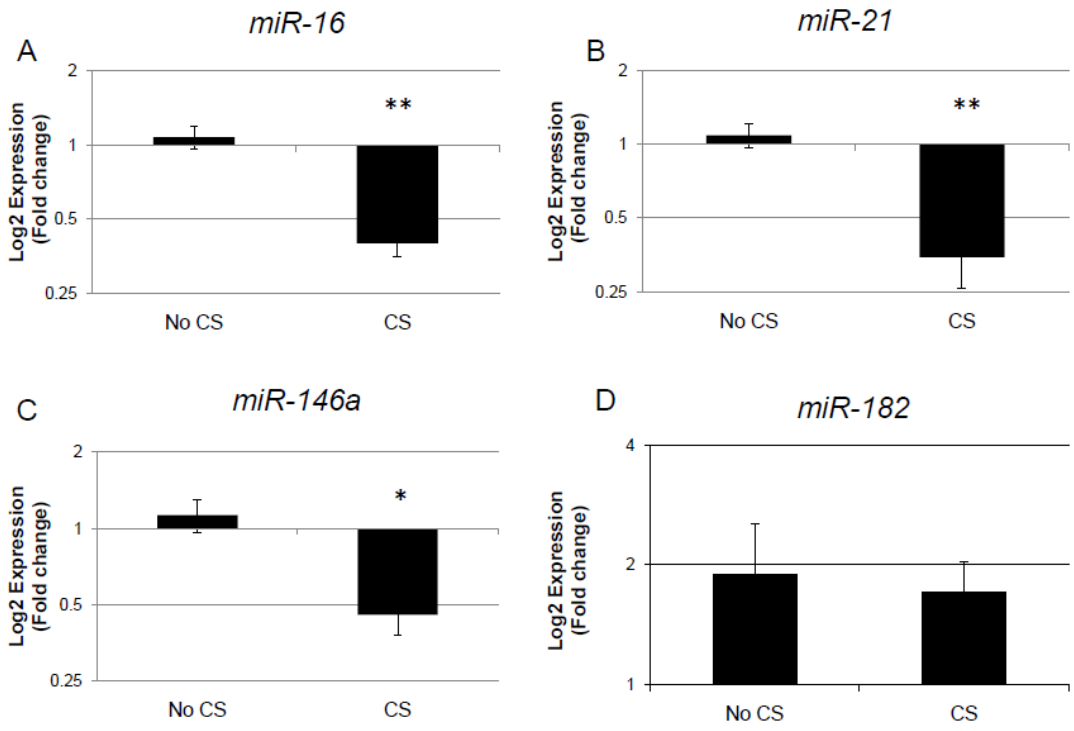

Figure 1. Maternal cigarette smoking during pregnancy is associated with downregulation of *miR-16* , *miR-21,* and *miR-146a*. Quantitative RT-PCR analysis was used to examine the expression of the mature forms of *miR-16* (A), *miR-21* (B), *miR-146a* (C), and *miR-182* (D), in primary placenta tissue samples from infants whose mothers smoked cigarettes (CS) during pregnancy (n=8) and infants whose mothers did not smoke during pregnancy (No CS, n=17). * indicates $p<0.01$ and ** indicates $p<0.0001$ as determined by t-test.

Table 2. Multivariable linear regression model of individual miRNA expression.						
	miR-16		***miR-21***		***miR-146a***	
	Reg. coeff.	**p**	**Reg. coeff.**	**p**	**Reg. coeff.**	**P**
Maternal Cigarette Smoking During Pregnancy						
No	Reference		Reference		Reference	
Yes	-0.058	<0.0001	-0.094	<0.0001	-0.039	0.003
Gender						
Female	Reference		Reference		Reference	
Male	-0.008	0.4	-0.003	0.9	0.001	0.9
Gestational Age, per week	0.004	0.5	0.007	0.5	0.005	0.5
Birthweight, per gram	-8.85×10^{-06}	0.4	-1.26×10^{-06}	0.9	-7.23×10^{-06}	0.5
Maternal Age, per year	-0.0003	0.7	-0.002	0.1	-0.001	0.2
Note: Each miRNA is modeled individually, and the models are controlled for all co-variates in the table.						

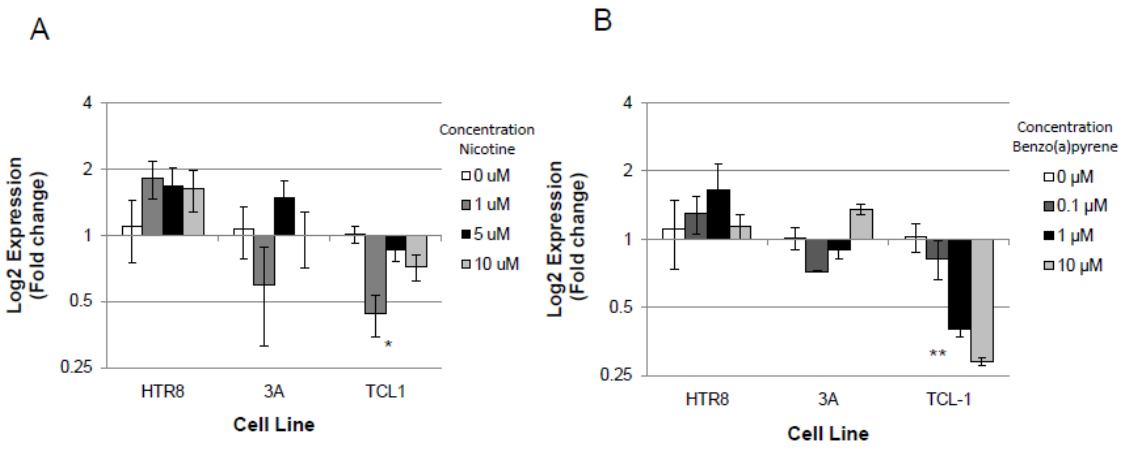

Figure 2. *miR-146a* is downregulated in TCL-1 cells exposed to nicotine and benzo(a)pyrene. Three placental cell lines (HTR8, 3A, and TCL1) were exposed to increasing doses of nicotine (A) and benzo(A)pyrene (B) for 6 days, and the expression of *miR-146A* was determined through qRT-PCR. Error bars indicated standard error of the mean, * indicates a significant downregulation of *miR-146a* across doses of nicotine (ANOVA , $p<0.03$). ** indicates a significant downregulation of *miR-146a* across doses of benzo(a)pyrene (ANOVA, $p<0.006$).

Table 3. mRNA targets predicted and/or experimentally validated for miR-16, miR-21, and miR-146a.

miRNA	Predicted target mRNA	Minimum free energy of duplex (kcal / mol)	Experimentally confirmed target mRNA	Source of target confirmation
miR-16	BCL2L2	-23.1	Caprin-1, HGMA-1	Kaddar *et al*. 2009
	EDA	-25.3		
miR-21	PLAG1	-22.7	PTEN	Meng *et al*. 2007
	SATB1	-23.8	PLAG1	Tran *et al.* 2007
miR-146a	TRAF6	-26.1	CCL8/MCP-2	Rom et al. 2010
			TRAF6	Taganov, *et al*. 2006; Hou *et al*. 2009

Note: In order to be considered a "predicted target", the target must appear in the "top 100" targets for the respective miRNA using all of the following target prediction tools: miRanda (September 2008 Release), PicTar (based on Lall, et al., 2006), and TargetScan 5.1. Minimum free energies of the miRNA-mRNA duplexes were determined using *RNA-hybrid*.

Chapter Three

miR-16 and *miR-21* Expression in the Placenta is Associated with Fetal Growth

Matthew A. Maccani, James F. Padbury, Carmen J. Marsit

Under Review

miR-16 and *miR-21* Expression in the Placenta is Associated with Fetal Growth

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The authors have declared that no conflict of interest exists.

ABSTRACT

Novel research has suggested that altered miRNA expression in the placenta is associated with adverse pregnancy outcomes and with potentially harmful xenobiotic exposures. We hypothesized that aberrant expression of miRNA in the placenta is associated with fetal growth, a measurable phenotype resulting from a number of intrauterine factors, and one which is significantly predictive of later life outcomes. We analyzed 107 primary, term, human placentas for expression of 6 miRNA reported to be expressed in the placenta and to regulate cell growth and development pathways: *miR-16*, *miR-21*, *miR-93*, *miR-135b*, *miR-146a*, and *miR-182*. The expression of *miR-16* and *miR-21* was markedly reduced in infants with the lowest birthweights ($p<0.05$). Logistic regression models suggested that low expression of *miR-16* in the placenta predicts an over 4-fold increased odds of small for gestational age (SGA) status ($p=0.009$). Moreover, having both low *miR-16* and low *miR-21* expression in the placenta predicts a greater increase in odds for SGA than having just low *miR-16* or *miR-21* expression ($p<0.02$), suggesting an additive effect of both of these miRNA. Future research on miRNA whose expression is associated with *in utero* exposures and markers of fetal growth is essential for better understanding the epigenetic mechanisms underlying the developmental origins of health and disease.

INTRODUCTION

Fetal development represents a critical period during which perturbations to the intrauterine environment through various factors in the extrauterine environment can have major ramifications on not only the proper growth and development of the fetus but also on risk for disease later in life(1) . Barker and Hales(1) hypothesized that fetuses receive a poor or rich maternal forecast depending, in part, on the intrauterine and extrauterine conditions during pregnancy, but that this maternal forecast may not always accurately predict the post-birth environment and that such mismatches give rise to disease risk later in life. This has played out in a number of epidemiologic studies linking low birthweight with morbidity and mortality in early infancy(2) as well as with an increased risk for certain diseases later in life, particularly coronary heart disease, diabetes mellitus type 2, and hypercholesterolemia(2).

The placenta is of critical importance to ensure the proper growth and development of the fetus while *in utero*. It is involved in providing the fetus with nutrients and is involved in waste and gas exchange. The placenta's metabolic activity is crucial for protecting the fetus from potentially harmful maternal factors and xenobiotic toxicants that may alter fetal growth and development. The environment during pregnancy is thought to impact the appropriate function of the placenta during development, thus identification of alterations to the placenta and to placental gene expression may serve as a record of *in utero* exposures and of the intrauterine and extrauterine environments during pregnancy(3).

The mechanisms by which in utero exposures may dysregulate the regulatory mechanisms of the placenta continue to be studied. One mode of alteration may be

through the aberrant expression of microRNA (miRNA), 21-25 nucloeotide long non-coding RNA involved in post-transcriptional gene regulation (4, 5). miRNA base-pair to the 3'-untranslated region of target mRNA and effectively silence gene expression by a mechanism of either translational repression or direct mRNA degradation. The particular mechanism of this post-transcriptional regulation depends greatly on the degree of complementarity of the miRNA to its mRNA target. Previous work has shown that partial complementarity of a miRNA to an mRNA target may result in effective repression of translation; therefore, a single miRNA can regulate a vast number of genes(5). Through this mechanism of post-transcriptional gene regulation, miRNA have been shown to regulate a number of key cellular functions including migration, invasion, growth, and death(6). miRNA exhibit tissue-specific expression and function and have been shown to be expressed in the placenta in addition to a variety of other tissues(7). Alterations to placental miRNA expression have been associated with *in utero* exposures(8, 9) and adverse pregnancy outcomes (10-13).

Since miRNA have been described as playing important roles in development and are susceptible to the environment, we sought to further characterize the expression of six candidate miRNA previously shown to be expressed in the placenta and involved in regulating key cell processes – *miR-16*(14), *miR-21*(15), *miR-93*(12, 13), *miR-135b*(11), *miR-146a*(16), and *miR-182*(17) – in a large series of human placentas for associations with fetal growth.

RESULTS

One hundred seven human placenta samples were analyzed for the expression of candidate miRNA previously shown to be expressed in the placenta and involved in regulating cell growth and developmental processes, specifically, *miR*-16*, miR-21, miR-93, miR-135b, miR-146a,* and *miR-182*. The demographics of the sample population are given in **Table 1.** Of note, the study population was oversampled for small for gestational age (SGA) infants, defined as infants whose birth weight was $<10^{th}$ percentile for their gestational age, calculated as in Fenton 2003(18). Placentas from SGA neonates comprised approximately 30% of the study population. **Table 2** describes the expression of the 6 candidate miRNA in all of the 107 samples, based on qRT-PCR and absolute quantification from a standard curve. The range of expression values differed for the miRNAs, with *miR-146a* and *miR-182* exhibiting expression in the 0.001-3.95 amol range, while *miR-16* and miR-*21* expression was 2 orders of magnitude greater with expression ranging from 3.53-434.74 amol range. Within each miRNA, the majority of the samples exhibited relatively homogenous low levels of expression, but the distributions of expression exhibited a right skew, suggesting there were still a number of samples with relatively moderate to high expression. As these data were not normally distributed, and as we hypothesized that aberrant high or low expression may be associated with exposures or outcomes, the expression profiles were split into quartiles, and birthweight percentile was plotted by quartile of miRNA expression **(Figure 1)**. Kruskal-Wallis tests were used to assess differences in birthweight percentile across quartiles of miRNA expression **(Figure 1).** Analysis revealed that birthweight percentile

significantly differed across quartiles of *miR-16* and *miR-21* expression, p=0.04 and p=0.02, respectively.

Observing that expression in the lowest quartiles of *miR-16* and *miR-21* was associated with reduced birthweight percentile, we more specifically examined the association between low expression (≤median vs. >median) of the miRNA and infants considered small for gestational age (SGA), using logistic regression to control for potential confounders. These models (**Table 3)** demonstrate that low *miR-16* expression in the placenta predicts an odds of 4.13 for SGA (95% CI=1.42, 12.05) compared to infants with high placenta *miR-16* expression, controlled for confounders. Additionally, low *miR-21* expression in the placenta predicts a marginally-significant, 2.43-fold increased odds for SGA (95% CI=0.93, 6.37) compared to infants with high placenta *miR-21* expression controlled for confounders. Maternal cigarette smoking during pregnancy, as expected, also demonstrated greatly increased risks for growth restriction in both models.

As both miRNA demonstrated independent correlations with SGA status, we further examined if there was an interaction between expression of these 2 miRNA in their association with infant growth outcome. This model **(Table 4)** demonstrated that compared to infants having high expression (>median) of both miRNA, infants with low *miR-21* only or low *miR-16* only had non-significant elevation in SGA risk, but infants exhibiting reduced expression of both *miR-16* and *miR-21* were significantly more likely to be classified as SGA (OR 5.38, 95% CI 1.52, 19.01). Although a likelihood ratio test suggested no significant multiplicative interaction between *miR-16* and *miR-21* (p>0.05), there was a significant trend for increased risk of being classified as SGA from having

only *miR-21* reduced or only *miR-16* reduced in expression to having both reduced in expression ($p<0.02$).

DISCUSSION

We have demonstrated the expression of key candidate miRNA in a large, population-based series of primary human placenta samples and their association with poor fetal growth, specifically identifying that reduced expression of *miR-16* and *miR-21* are strongly associated with growth restriction. A number of groups have previously described placental miRNA expression associations with maternal conditions such as preeclampsia(17), with maternal cigarette smoking during pregnancy(8), and as markers of pregnancy itself(11). Furthermore, more is being uncovered about the role of *miR-16* and *miR-21* in regulating key cellular processes, especially the involvement of *miR-16* in regulating cell cycle progression(14) and *miR-21*'s capability of regulating cell cycling and cell proliferation(15).

miR-21 has been described as an oncogene, plays a role in enhancing tumor phenotypes including proliferation and migration, and has been shown to target a number of key regulators of these processes, including but not limited to PLAG1(22) and PTEN(19, 20). Meng and coworkers showed that *miR-21* regulates PTEN in human hepatocellular cancer(20), and Lou and colleagues demonstrated that in ovarian epithelial carcinomas, *miR-21* promotes proliferation, invasion and migration abilities by inhibiting PTEN(19). As the function of the placenta, though, is to promote fetal growth through its own proliferation and invasion into the maternal decidua, downregulation of *miR-21* in the placenta could, through dysregulation of PTEN, result in decreased invasion of the maternal decidua, decreased migration, and decreased growth – the opposite of what has been observed to occur in the case of upregulated *miR-21*(20).

As with many miRNA, *miR-16* exhibits tissue-specific function and expression. In a number of cancer cell lines, *miR-16* has been shown to be involved in the induction of apoptosis by targeting *BCL-2(23)* and in cell cycle regulation by targeting CDK6(24), CDC27(25), and CARD10(26). In other cell types, *miR-16* has different functions, such as targeting HMGA1 and Caprin-1(21), further suggesting that *miR-16* may have cell-type function and expression(21, 23). Dysregulation of *miR-16* in the placenta may lead to aberrant expression of its targets and may lead to functional and developmental abnormalities in the placenta that might result in reduced infant birthweight. Mechanistic research using model systems is needed to further elucidate the pathways regulated by *miR-16* and *miR-21* and to better determine the functional consequence of downregulation of *miR-16* and *miR-21* in the placenta. Additionally, more work to determine the functional effects of miRNA crosstalk – that is, miRNAs whose differential expression may have additive associations with risk for phenotype or disease – will be necessary to better understand the complex regulatory networks involved.

Poor fetal growth associated with adverse intrauterine conditions continues to be characterized. Fetal malnutrition linked to growth restriction has been shown occur in a variety of conditions, including but not limited to poverty, pregnancy in women with eating disorders, and pregnancy in high altitude(27). Maternal cigarette smoking during pregnancy is associated with an increased risk of fetal growth restriction(28, 29), and exposure to environmental toxicants *in utero*, such as those found in cigarette smoke, is associated with increased placental aberrations(30) and decreased placental function (31). A number of animal models have been generated to further study low birthweight in a controlled, experimental system (27, 32), and it will be important to consider the role of

miRNA in these animal models. Because poor maternal forecasts leading to low birthweight may not accurately predict the post-birth environment and because low birthweight increases one's risk for a number of diseases later in life(2), more research is necessary to more fully understand the pathways whose dysregulation may ultimately affect birthweight.

In summary, our data suggesting that low expression of *miR-16* and *miR-21* in the placenta is associated with poor fetal growth may have many important implications. Future work to determine the roles of miRNA in specific pathways leading to altered fetal growth will be key to better understanding fetal growth as both a marker of the intrauterine environment as well as a developmental outcome and in better comprehending the developmental origins of health and disease.

MATERIALS AND METHODS.

Placenta samples. All placenta samples used were collected as part of the Rhode Island Child Health Study (RICHS), an ongoing, population-based birth cohort at Women and Infants' Hospital in Providence, RI. In brief, for each sample, 12 biopsies of placenta tissue, 3 from each of 4 quadrants (totaling approximately 1 g of tissue) were excised, from the maternal side of the placenta 2 cm from the umbilical cord insertion site, free of maternal decidua. The samples were placed immediately in RNAlater and stored at 4°C. At least 72 hours later, placenta samples were removed from RNAlater, blotted dry, snap-frozen in liquid nitrogen, homogenized using a mortar and pestle, and stored in sample tubes at -80°C until needed for examination. A structured chart review was used to collect information from the maternal inpatient medical record from delivery, and mothers were subjected to an interviewer-administered structured questionnaire to obtain information on the lifestyle, demographics, and exposure histories of the participants. All samples and information were collected under appropriate protocols approved by the Institutional Review Boards for Women and Infants' Hospital and Brown University.

RNA extraction. RNA was extracted from placenta samples and cultured cells using the miRvana miRNA Isolation Kit (Ambion) and manufacturer protocols as described previously(8). For tissue samples, 200 mg of homogenized tissue was used for extraction. Extracted RNA was quantified using a Nanodrop spectrophotometer and then aliquoted into single-use aliquots and stored at -80°C.

Quantitative RT-PCR (qRT-PCR) for mature miRNA. Expression of mature miRNAs was measured using commercially available TaqMan microRNA Assays or TaqMan Gene Expression Assays (Applied Biosystems, Valencia, CA) on an Applied Biosystems

7900HT Real-Time PCR system and analyzed with 7900HT System Software. Absolute quantitation of miRNA was calculated using a standard curve generated from serial dilutions of miRNA-specific pre-miR oligonucleotides (Ambion) run on each plate for each miRNA of interest, as previously described for array analyses by Bissels and colleagues (33). All reactions were run in triplicate on 384-well plates, and RNU-44 was used as an internal control for each sample to assess sample performance. No-RT controls were also run for each sample on each plate to assure samples were free of genomic DNA.

Statistical analysis. Birthweight percentile was calculated using the Fenton growth chart(18). SGA was defined as the lowest 10% of birthweight percentile. Kruskal-Wallis tests were used to determine if birthweight percentile significantly differed across quartiles of miRNA expression. Logistic regression models were used to analyze if differential expression of miRNA predicted increased risk for SGA while also considering potential confounders. All analyses were conducted in SAS 9.2 (SAS Institute, Cary, NC).

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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Table 1. Demographics of the study population (n=107)

Birthweight, grams, mean (sd)	3308 (742)
Gestational Age, weeks, mean (sd)	38.9 (1.2)
Small for Gestational Age (SGA) Status, n (%)	
SGA	32 (29.9%)
Non-SGA	75 (70.1%)
Infant Gender, n (%)	
Female	60 (56.1%)
Male	47 (43.9%)
Mode of delivery, n (%)	
Caesarian section	39 (36.4%)
Vaginal delivery	67 (62.6%)
Maternal Age, years, mean (sd)	29.4 (6.0)
Maternal Ethnicity, n (%)	
Non-white	24 (22.4%)
White	80 (74.8%)
Unknown	3 (2.8%)

* One sample was missing mode of delivery information.

Table 2. Expression of *miR-16, miR-21, miR-93, miR-135b, miR-146a,* and *miR-182* determined through qRT-PCR in 107 primary human term placenta samples.

	Median (amol)	Range (amol)
miR-16	18.64	3.53-399.50
miR-21	54.86	5.25-434.74
miR-93	4.26	0.14-118.39
miR-135b	4.72	0.13-216.92
miR-146a	0.1	0.002-3.95
miR-182	0.23	0.001-3.63

Figure 1.

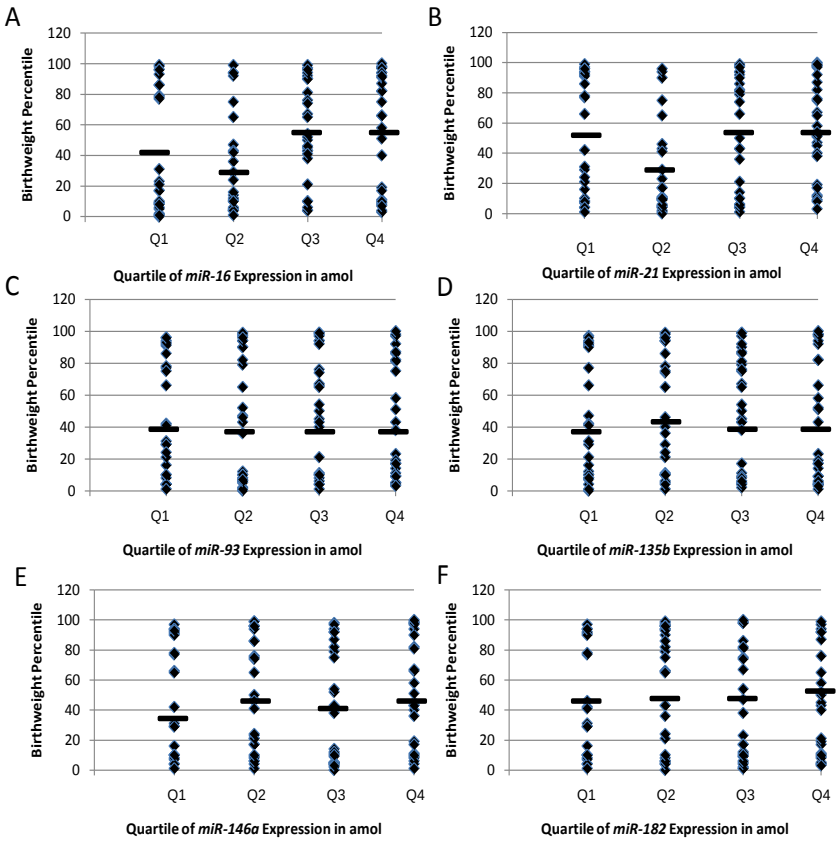

Figure 1. Distribution of infant birthweights (y-axis) by primary term human placenta miRNA expression quartiles (x-axis) determined through qRT-PCR for (A) *miR-16* (p=0.04), (B) *miR-21* (p=0.02), (C) *miR-93* (p=0.88), (D) *miR-135b* (p=0.84), (E) *miR-146a* (p=0.46), and (F) *miR-182* (p=0.55). Black bars indicate median of birthweight percentile within each quartile. Kruskal-Wallis tests revealed that birthweight percentile significantly differed across quartiles of *miR-16* and *miR-21* expression ($p<0.05$).

Table 3. Logistic regression for the association between individual miRNA expression and SGA status.

Effect	Odds Ratio	95% Wald Confidence Limits	p
***miR-16* Expression in Placenta, n (%)**			
High n=52 (51%)	**Reference**		
Low n=50 (49%)	**4.13**	**1.42-12.05**	**0.009**
Maternal Smoking During Pregnancy, n (%)			
No n=97 (95%)	**Reference**		
Yes n=5 (5%)	**22.18**	**1.72-286.86**	**0.018**

Effect	Odds Ratio	95% Wald Confidence Limits	p
***miR-21* Expression in Placenta, n (%)**			
High n=52 (51%)	**Reference**		
Low n=50 (49%)	**2.43**	**0.93-6.37**	**0.069**
Maternal Smoking During Pregnancy, n (%)			
No n=97 (95%)	**Reference**		
Yes n=5 (5%)	**10.82**	**0.82-143.58**	**0.071**

*Also included in models: Relative Weight Gained During Pregnancy, Maternal Ethnicity, Maternal Age, Delivery Method, Insurance, and Infant Gender

Table 4. Logistic regression to examine the interaction of low *miR-16* and low *miR-21* expression on the association with SGA status

Effect	**Odds Ratio**	**95% Wald Confidence Limits**
***miR-21* and *miR-16*, n (%)**		
Both High, n=36 (35%)	Reference	
Low *miR-21* only, n=16 (16%)	1.54	0.29-8.21
Low *miR-16* only, n=16 (16%)	3.35	0.69-16.32
Both Low, n=34 (33%)	5.38	1.52-19.01

*Also included in model: Relative Weight Gained During Pregnancy, Maternal Ethnicity, Maternal Age, Delivery Method, Insurance, and Infant Gender

**p for trend ($p<0.02$).

Chapter Four

Functional Consequences of Overexpression of miRNA in Placental Cell Lines

Matthew A. Maccani and Carmen J. Marsit

ABSTRACT

Key cell processes have been shown to be regulated, in part, by miRNA. Previous work in our lab has suggested that *in utero* exposures and poor fetal growth are associated with the differential expression of several miRNA in the placenta. Using HTR8, 3A, and TCL-1 placental cell lines, representing early and later stages of placental development, we sought to characterize effects of dysregulation of *miR-16, miR-21,* and *miR-146a* – three miRNA previously shown by our lab and others to be expressed in the placenta and to be involved in the regulation of key cell processes – on placental cell viability, growth, proliferation, invasion, and migration. Additionally, we investigated effects of dysregulated miRNA expression on experimentally confirmed targets, specifically PTEN for *miR-21* and TRAF6 and STAT-1 for *miR-146a*. Our data suggested that 3A cells overexpressing *miR-21* exhibited a trend to increased migration compared to negative control cells ($p<0.08$). TCL-1 cells overexpressing *miR-21* had 50% less PTEN than cells transfected with negative control ($p<0.05$). 3A cells overexpressing *miR-146a* had 26% less STAT-1 than cells transfected with negative control ($p<0.05$). Taken collectively our data suggest that miRNA play important roles in regulating cell processes crucial for the proper function of placental cells during various stages of pregnancy.

INTRODUCTION

The placenta is of critical importance to ensure the proper growth and development of the fetus while *in utero*. It is involved in providing the fetus with nutrients and is involved in waste and gas exchange. The placenta's metabolic activity is crucial for protecting the fetus from potentially harmful toxicants that may alter fetal growth and development. The placenta expresses compounds capable of metabolizing toxicants that would otherwise harm the fetus or lead to fetal demise. In order to perform this multitude of important functions, the placenta needs to differentiate, invade maternal decidua, illicit vascularization and immune changes, while still growing and performing the task of maintaining proper fetal growth and development. Alterations to the placenta and to placental gene expression may alter these important tasks required for appropriate function, and may therefore illicit downstream effects on the fetus (1).

The mechanisms by which *in utero* exposures may dysregulate the regulatory mechanisms of the placenta continue to be studied. miRNA base-pair to the 3'-untranslated region of target mRNA and effectively silence gene expression by a mechanism of either translational repression or direct mRNA degradation. The particular mechanism of this post-transcriptional regulation depends greatly on the degree of complementarity of the miRNA to its mRNA target. Previous work has shown that partial complementarity of a miRNA to an mRNA target may result in effective repression of translation; therefore, a single miRNA can regulate a vast number of genes(2). Through their mechanism of post-transcriptional gene regulation, miRNA have been shown to regulate a number of key cell pathways including migration, invasion, growth, and proliferation(3). miRNA exhibit tissue-specific expression and function and

have been shown to be expressed in the placenta in addition to a variety of other tissues(4).

Work in our lab and elsewhere has investigated the association of aberrant placental miRNA expression with *in utero* exposures(5, 6) and adverse intrauterine and extrauterine environment(7-9). Work in our lab has demonstrated that downregulation of *miR-16, miR-21,* and *miR-146a* in the placenta is associated with maternal cigarette smoking during pregnancy(5), that low *miR-16* and *miR-21* expression is associated with fetal growth (Maccani, et al., *submitted*), and that bisphenol A (BPA) exposure dysregulates miRNA expression in placental cell lines(6). Pineles and colleagues investigated miRNA expression associated with preeclampsia and suggested that differential expression of *miR-210* and *miR-182* was associated with preeclampsia versus control patients(7). Others have shown dysregulated miRNA expression in trophoblasts exposed to varying concentrations of oxygen(9-11). This research suggests the importance of miRNA in regulating processes crucial for proper function of the placenta and of placental cells, thereby ensuring the proper health and development of the fetus *in utero*.

As miRNA can regulate key cell functions and as these functions are critical to the proper growth, development and function of the placenta, we hypothesized that overexpression of *miR-16, miR-21*, and *miR-146a* in placental cell lines may alter the growth, viability, proliferation, migration, and invasion of these cells. Western blots were used to detect the differential expression of previously predicted and empirically confirmed targets for miRNA, specifically PTEN(12, 13) for *miR-21* and STAT-1(14) and TRAF6(15) for *miR-146a*. Taken collectively, our work attempts to further elucidate

some of the pathways that are regulated by miRNA and whose perturbation in the placenta could have major ramifications for both placental function and proper health and development of the fetus.

RESULTS

Overexpression of miRNA and assessment of transfection efficiency

Effects of overexpression of *miR-16, miR-21,* and *miR-146a* on placental cell viability, growth, proliferation, invasion, and migration were investigated using a variety of *in vitro* strategies. HTR8, 3A, and TCL-1 placental cell lines were cultured as described previously(5). These three placental cell lines represent three different stages and aspects of placental development and were selected to examine the effects of dysregulated miRNA expression on models of different timepoints during pregnancy. In brief, 3A cells are first-trimester villous cells, HTR8 are first trimester extravillous cells, and TCL-1s are third trimester extravillous cells. Placental cells were transfected using the pre-miR miRNA precursors (Ambion) system and siPORT NeoFX transfection agent (Ambion) following manufacturer's protocols. The *Silencer* siRNA Transfection Kit (Ambion) and KDalert GAPDH Assay (Ambion) were used to determine transfection efficiency. **Figure 1** shows that an approximately 45% knockdown of GAPDH enzyme activity was observed in HTR8, 3A, and TCL-1 placental cells transfected with an siRNA to GAPDH, suggesting that a transfection efficiency of approximately 45% exists for these three cell lines using this system of transfection.

Growth

To determine the effects of overexpression of miRNA on cell growth, cell growth was characterized using cell counts. Cells were enumerated at time 0, 24, and 48 hours post-transfection using a Coulter counter. Differential growth of cells overexpressing *miR-16, miR-21,* or *miR-146a* was determined by comparing cell counts to those of negative control cells. **Figure 2** presents results from cell growth analyses using

transfected cells and cell counts to investigate whether overexpression of miRNA results in differential cell growth. The data suggest that overexpression of *miR-16, miR-21,* and *miR-146a* in HTR8, 3A, and TCL-1 does not alter cell growth compared to negative control.

Viability

To ensure that cell death was not being increased as a result of overexpression of *miR-16, miR-21*, or *miR-146a*, cell viability tests were conducted. At 0 hours, 24 hours, and 48 hours post-transfection, cell viability was assessed using the trypan blue exclusion test of cell viability. Cells were deemed viable if they successfully excluded trypan blue. **Figure 3** displays results from cell viability assays. The results suggest that overexpression of *miR-16, miR-21*, and *miR-146a* in HTR8, 3A, and TCL-1 does not alter cell viability compared to negative control.

Proliferation

To confirm the cell growth data by quantifying metabolically active cells, cell proliferation was assessed using the MTS assay at 0 hours, 24 hours, and 48 hours post-transfection. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of metabolically active cells in culture. Differential proliferation of cells was determined by comparing number of proliferating cells overexpressing *miR-16, miR-21,* or *miR-146a* versus number of proliferating cells transfected with negative control. **Figure 4** shows results from proliferation assays

conducted on HTR8, 3A, and TCL-1 overexpressing *miR-16*, *miR-21*, and *miR-146a* or negative control cells. The data suggest that overexpression of *miR-16, miR-21,* and *miR-146a* does not alter the proliferation of HTR8, 3A, and TCL-1 cells.

Migration

Placental cell migration is a key process ensuring proper placental development and function. Migration assays using a modified protocol from Shaw, et al, 1996(16) were conducted to determine whether overexpression of *miR-16*, *miR-21*, and *miR-146a* in placental cells leads to aberrant cell migration. Migration was assessed using an MTS assay as a more conservative, first-pass assay and the technique of staining with hematoxylin and counting described previously(17) was used as a confirming assay that is more sensitive to smaller changes in migration. **Figure 5** shows data from migration assays conducted with 3A, HTR8, and TCL-1 cells overexpressing *miR-16*, *miR-21*, or *miR-146a* or negative control cells. 3A cells overexpressing *miR-21* exhibited a trend of increased invasion, 250% versus control ($p<0.08$). Overexpression of the other miRNA in other cell types showed no differences in migration compared to controls.

Supplemental Figure 1 shows data from migration assays using the technique of staining with hematoxylin and cell counting described previously(17) to confirm results trending significance ($p<0.2$) as determined using MTS to quantify migrated cells.

Supplemental Figure 1 shows that 3A cells overexpressing *miR-21* exhibited a trend of increased invasion, 150% versus control ($p<0.08$), confirming previous results shown in **Figure 5** using MTS to quantify migrated cells.

Invasion

Placental cell invasion of the maternal decidua is a crucial process in not only anchoring the placenta but also ensuring the proper growth and development of the placenta. Invasion assays using a modified protocol from Shaw, et al, 1996(16) were utilized to investigate if overexpression of *miR-16, miR-21,* or *miR-146a* leads to altered placental cell invasion. Invasion was assessed using an MTS assay as a more conservative, first-pass assay and the technique of staining with hematoxylin and counting described previously(17) was used as a confirming assay that is more sensitive to smaller changes in invasion. **Figure 6** shows data from invasion assays conducted with 3A, HTR8, and TCL-1 cells overexpressing *miR-16, miR-21,* or *miR-146a* or negative control cells. Analysis revealed there was no significant difference in invasion in cells overexpressing any of the three miRNA versus negative control cells.

Empirical validation of miRNA gene targets

Table 1 displays an informative, though not exhaustive, list of predicted and empirically validated gene targets of *miR-16, miR-21,* and *miR-146a* reported in the literature. Predicted targets were predicted using the target prediction strategy reported previously(5). Targets on this list were considered for empirical validation in placental cells overexpressing *miR-21* and *miR-146a*. PTEN was selected as a target for validation due to its important role as a regulator of the PI3K pathway, a pathway important for cell survival, migration, and Akt signaling. STAT-1 was selected as a target for validation due to its role as a transcription factor and its involvement in the JAK/STAT signaling pathway. TRAF6 was selected for further validation in placental cells because it was predicted as a target using a previously-reported target strategy(5) and has been

previously identified as an important molecule responsible for signaling through the TLR4 pathway. All of the selected targets have been previously empirically validated as targets in non-placental cell types **(Table 1)**, and as suggested, are involved in regulating potentially crucial cell processes in the placenta.

Protein from HTR8, 3A, and TCL-1 placental cells overexpressing *miR-21* or *miR-146a*, or transfected with negative control, was harvested and Western blots were used to investigate whether bioinformatically-predicted and/or previously empirically-validated targets of *miR-21* and *miR-146a* are in fact targeted in placental cells. **Figure 7A** shows that TCL-1 cells overexpressing *miR-21* express approximately 50% less PTEN protein than TCL-1 cells transfected with negative control ($p<0.05$). **Figure 7B** shows that 3A cells overexpressing *miR-146a* express approximately 26% less STAT-1 protein than 3A cells transfected with negative control ($p<0.05$). Overexpression of *miR-146a* in HTR8, 3A, and TCL-1 did not significantly alter levels of TRAF6 compared to cells transfected with negative control **(Figure 7C)**.

DISCUSSION

Our work sought to investigate the effects of miRNA overexpression on a number of key placental cell processes, specifically cell viability, growth, proliferation, invasion, and migration, as well as further confirm previously empirically validated targets of miRNA in placental cells. Previous findings have suggested that these miRNA are significantly altered in human placentas associated with a number of conditions and exposures(5). Taken collectively, our work contributes to the better understanding of how dysregulated *miR-16*, *miR-21*, and *miR-146a* may alter key cellular processes and expression of target genes.

As previous studies have suggested, one of the more challenging aspects of miRNA research is target prediction and validation(5). By using *in silico* bioinformatic approaches combined with empirical techniques, targets of specific miRNA can be predicted and validated. A number of target prediction strategies utilizing one or more of a number of published and publicly-available target prediction algorithms have predicted targets for *miR-16*(5, 18), *miR-21*(5, 12, 19), and *miR-146a*(5). Several of these predicted targets have also been empirically confirmed through experimental techniques demonstrating that the specific miRNA is capable of downregulating or, in some cases, silencing, its target (18, 19).

As with many miRNA, *miR-16* exhibits tissue-specific function and expression. In a number of cancer cell lines, *miR-16* has been shown to be involved in the induction of apoptosis by targeting *BCL-2*(20) and in cell cycle regulation by targeting CDK6(21), CDC27(22), and CARD10(23). In other cell types, *miR-16* has different functions, such as targeting HMGA1 and Caprin-1(18), further suggesting that *miR-16* may have cell-

type-specific function and expression (18, 20). Dysregulation of *miR-16* in the placenta may lead to aberrant expression of its targets and may lead to dysregulated placental cell processes. While our data suggest that overexpression of *miR-16* does not lead to significant differences in cell viability, growth, proliferation, invasion, or migration, future studies addressing potential effects on apoptosis and target gene expression may prove to be especially useful in better determining the biological ramifications of dysregulated *mir-16* expression in the placenta, which may ultimately lead to abnormalities in the placenta that might result in reduced infant birthweight.

miR-21 has also been shown to target a number of key genes, including but not limited to and PTEN(12, 13). Meng and coworkers showed that *miR-21* regulates PTEN in human hepatocellular cancer(12), and Lou and colleagues demonstrated that in ovarian epithelial carcinomas, *miR-21* promotes proliferation, invasion and migration abilities by inhibiting PTEN(13). Our results that *miR-21* targets PTEN in the TCL-1 placental cell line further suggest that *miR-21* may be one mechanism regulating PTEN expression in the third trimester placenta. Lack of targeting of PTEN by *miR-21* in 3A and HTR8 cells suggests that *miR-21* may not be the key or major regulator of PTEN in first trimester villous and extravillous placental cells, but more work is necessary to further investigate the epigenetic regulation of PTEN in these cell types. Downregulation of *miR-21* in the placenta could thus lead to a dysregulation of PTEN in the placenta and could result in decreased invasion of the maternal decidua, decreased migration, and decreased growth – the opposite of what has been observed to occur in the case of upregulated *miR-21*(12). Our results suggesting that overexpression of *miR-21* in 3A cells leads to a trend towards increased migration compared to cells transfected with negative control may further

underscore the involvement of *miR-21* in regulating placental cell migration in first trimester villous cells. Lack of differential migration in HTR8 and TCL-1 cells overexpressing *miR-21* may suggest that *miR-21* may not be a major modulator of migration in these cell types. Future investigations are necessary to better determine mechanisms controlling migration throughout the various stages of pregnancy and are essential to generating a more complete understanding of *miR-21*'s regulation of placental cell processes.

Our *in-silico* analysis predicted TRAF6 as a target for *miR-146a*, suggesting that downregulation of *miR-146a* could result in an upregulation of TRAF6, a protein also important for signaling through both the NFκβ signaling pathway(24) as well as a mediator of inflammation in the toll-like receptor 4 (TLR4) pathway (25, 26). Previous reports using different target prediction strategies have hypothesized TRAF6 as a target of *miR-146a*(5, 27) and have validated TRAF6 as a target in murine macrophages(15). TLR4 mediates the inflammatory response, and aberrant TLR-4 signaling is associated with inflammation-induced preterm delivery(28). Such a consequence could be the result of dysregulated TRAF6 production as a result of downregulation of *miR-146a*. TRAF6 overexpression due to downregulation of *miR-146a* could result in overactive TLR signaling which could have a number of downstream consequences for both placenta and fetus. Unfortunately, our Western blot analysis suggested that TRAF6 protein levels were not significantly different in placental cells overexpressing *miR-146a* versus cells transfected with negative control. This may be due, in part, to a more complex regulation of TRAF6 in these placental cell lines than is currently understood. While *miR-146a* may be capable of regulating TRAF6, it does not appear to be the major or most important

regulator of TRAF6 in these placental cell lines. Further investigation into such relationships and regulatory networks would prove vital into better understanding both the role of TRAF6 in TLR4 signaling, as well as the association of aberrant expression of *miR-146a* with adverse pregnancy outcomes.

Previous work has demonstrated that STAT-1 is targeted by *miR-146a*(14). Our Western blot analysis revealed that 3A cells overexpressing *miR-146a* had 26% less STAT-1 protein than did cells transfected with negative control, a potentially important result since STAT-1 plays a role as a key transcription factor. STAT-1 is activated upon interferon (IFN) binding to its receptor. Once phosphorylated by JAKs, STAT-1 forms a homodimer and translocates to the nucleus where it binds the Interferon-Gamma activation site (GAS) in the promoter of interferon stimulated genes (ISG)(29). Our data suggest that STAT-1 may be regulated, in part, by *miR-146a* in 3A placental cells. 3A cells are first trimester villous cells, and lack of STAT-1 regulation by *miR-146a* in HTR8 or TCL-1 cells may suggest that time in pregnancy (i.e. first trimester vs. third trimester) as well as cell type (i.e. villous vs. extravillous) may play a role in determining targeting by *miR-146a*. More work is necessary to better understand the factors underlying this differential targeting of STAT-1 by *miR-146a*. Dysregulation of *miR-146a* may thereby lead to aberrant production of STAT-1 and dysregulated transcription of ISG, potentially leading to a cascade of events which may negatively alter placental function and ultimately lead to altered fetal programming.

While *in vitro* studies are important for proof-of-principle aims, such as targeting of particular genes by miRNA or analyzing the potential effects of dysregulated miRNA expression on one particular cell type, much of the complexity of an *in vivo* system is lost

when using an *in vitro* cell culture model alone. The use of an *ex vivo* model, such as harvesting primary, term, human placental tissue for experiments where one overexpresses a miRNA of interest and then characterizes effects on cell growth, viability, or migration, may prove worthwhile in better demonstrating the effects of dysregulated miRNA expression on primary tissue. Some challenges in such a system could be the difficulty in transfecting cells harvested from primary, term, human placental tissue, as well as the challenge of maintaining a healthy culture for the time period needed to perform the suite of assays aimed at characterizing effects of dysregulated miRNA expression on cell function. Additionally, the possibility of characterizing the effects of aberrant miRNA expression on cells from pre-term placental tissue, especially cells derived from human placental tissue from the first trimester, would be nearly impossible due to the practical difficulty of obtaining placental tissue from first trimester human placenta.

Model systems may prove useful in determining the effects of dysregulated miRNA on both the placenta as well as the developing fetus. Animal models have been generated to further study low birthweight (30, 31), and it will be important to consider the role of dysregulated miRNA expression in these animal models. Mouse lines generated to have tissue-specific (such as placenta-specific) dysregulation of miRNA may be especially useful in determining the effects of differential miRNA expression on both the placenta as well as the fetus at various stages of pregnancy. Placenta-specific knockouts of miRNA might be generated using a combined approach of the Cre/LoxP system and integrase-defective lentiviral (IDLV) vectors to help reduce the otherwise observed toxic effects of Cre expression in miRNA-specific floxed mice; while this

system has not been applied to miRNA to date, it has been described previously (32). Of course, such a system presents a number of challenges, including difficulties in developing a miRNA-specific floxed mouse line not to mention the possibility that a total knock-out of a miRNA in the placenta might result in lethality. Nonetheless, future work in such systems will be necessary to further demonstrate and understand the effects of aberrant miRNA expression on placental function and fetal programming.

Collectively, dysregulation of these miRNA can lead to aberrant expression of their targets and may affect a number of key placental cell processes, ultimately leading to aberrant cell cycle regulation, growth, immunomodulation, and development in the placenta. These changes in target gene expression may have further effects downstream for both placenta and fetus, ultimately resulting in altered fetal programming. Future studies are ongoing to further characterize the effects of environmental exposures on placental miRNA, on understanding the downstream phenotypes of aberrant miRNA expression, and in investigating the placenta as a record of the intrauterine environment.

MATERIALS AND METHODS

Cell culture and transfection. HTR8, 3A, and TCL-1 placental cell lines were cultured as described previously(5). Placental cells were transfected using the pre-miR miRNA precursors (Ambion) system and siPORT NeoFX transfection agenet (Ambion) following manufacturer's protocols. The *Silencer* siRNA Transfection Kit (Ambion) and KDalert GAPDH Assay (Ambion) were used to determine transfection efficiency.

Growth assays. Effects on cell growth were characterized using cell counts enumerated by a Coulter Counter. Briefly, cells were transfected as above and plated in triplicate, approximately 250,000 cells per well in 6-well dishes. Cells were enumerated at time 0, 24, and 48 hours post-transfection using a Coulter counter. Differential growth of cells overexpressing *miR-16, miR-21,* or *miR-146a* was determined by comparing cell counts to those of negative control cells.

Viability assays. Cells were transfected as above. At the noted timepoints, cells were trypsinized, spun down, and washed 2x with PBS before being resuspended in PBS. 15 µL of cells resuspended in PBS were mixed with 15 µL of Trypan blue (Sigma-Aldrich) and incubated at room temperature for 3 minutes prior to counting using a hemacytometer (Hausser). Cells were deemed viable if they successfully excluded Trypan blue (hence, the Trypan Blue Exclusion Test of Cell Viability).

Proliferation assays. Cells were transfected as above and seeded into 96 well plates in triplicate. Twenty-four hours following transfection, transfection medium was removed and replaced with 100 µL fresh medium and 20 µL MTS reagent (Promega). Following incubation at 37 degrees C for 3 hours, absorbance at 490 nm was observed using a

spectrophotometer and differential proliferation of cells was determined by comparing number of proliferating cells overexpressing *miR-16*, *miR-21*, or *miR-146a* versus number of proliferating cells transfected with negative control.

Migration assays. Migration assays were conducted using a modified protocol based on Shaw et al, 1996(16). In brief, placental cells were transfected and serum-starved as above. Following serum-starvation, 200,000 cells in 100 µL serum-free medium were seeded on filter-inserts in Boyden chambers (Corning). As above, complete medium with serum was placed in the lower chamber below the filter-insert and was used as a chemoattractant. After incubation at 37 degrees C for 20 hours, cells that had migrated to the lower side of the filter-insert were quantified using MTS assay (Promega) and manufacturer protocols. MTS assay was utilized as a more conservative, first-pass assay for quantifying cells that had migrated and the technique of staining with hematoxylin and cell counting described previously(17) that is more sensitive to smaller changes in migration was used to confirm results trending significance ($p<0.2$) as determined by MTS.

Invasion assays. Placental cells were transfected as above. Invasion assays were conducted using a modified protocol by Shaw, et al, 1996(16). Twenty-four hours after transfection with either miRNA-specific pre-miR precursor or negative control, media containing transfection reagents and molecules was removed, cells were washed once with PBS, and serum-free medium was added to serum-starve cells for 24 hours. Following serum-starvation, 200,000 cells in 100 µL serum-free medium were seeded on solidified growth-factor reduced Matrigel (BD Biosciences) poured onto Costar

Transwell filter-inserts in Boyden chambers (Corning). Complete medium with serum was added to the lower chamber below the inserts and used as a chemoattractant. After incubation at 37 degrees C for 20 hours, cells that had invaded through the Matrigel and to the lower side of the filter-insert were quantified using MTS assay (Promega) and manufacturer protocols. MTS assay was utilized as a more conservative, first-pass assay for quantifying cells that had invaded and the technique of staining with hematoxylin and cell counting described previously(17) that is more sensitive to smaller changes in invasion was used to confirm results trending significance ($p<0.2$) determined by MTS assay. However, since no data trending significance ($p<0.2$) were determined using MTS, no follow-up invasion assays with hematoxylin staining and counting were performed.

Western blot. Western blots were used to confirm that overexpression of miRNA resulted in decreased protein levels of previously confirmed targets of miRNA. Following cell harvest and lysate collection, total protein was quantified using the BCA assay (Thermo Scientific) and manufacturer protocols. Cell lysates were then separated electrophoretically using 10% Tris-HCl gels (Bio-Rad). Proteins were transferred to Immuno-Blot PVDF membranes (Bio-Rad) through overnight night transfer at 4 degrees C and following transfer, membranes were washed with TBSt (Boston Bioproducts) and blocked with 5% milk in TBSt. For Western blot analysis, antibodies against PTEN (Invitrogen), STAT-1 (Cell Signaling), TRAF6 (Santa Cruz), and gamma-Tubulin (Cell Signaling) were used to visualize corresponding proteins following established protocols for staining and washing. Proteins were visualized using the Amersham ECL Western Blotting Analysis System (GE Healthcare) following the manusfacturer's protocol.

Resulting images were scanned and protein quantitation was performed using Image J software.

Statistical analysis. ANOVA for repeated measures was used to determine if overexpression of candidate miRNA resulted in differential growth, viability, and proliferation of cells compared to negative control. Student's T-tests were used to determine if overexpression of candidate miRNA resulted in differential invasion and migration of cells compared to negative control. Student's T-tests were used to determine if overexpression of candidate miRNA resulted in differential protein levels as determined by densitometry of PTEN and STAT-1.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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Table 1. Predicted and empirically-confirmed gene targets of *miR-16, miR-21,* and *miR-146a*. Predicted targets were predicted using the target prediction method described by Maccani, et al., 2010(5).

miRNA	**Predicted Targets**	**Confirmed Targets**
miR-16	BCL2L2 EDA	Caprin-1(18) HGMA-1 (18) SERT (33)
miR-21	PLAG-1 SATB1	PTEN (12) PDCD4 (34) TM1 (34)
miR-146a	TRAF6	TRAF6 (15) STAT-1 (14) TBP (35)

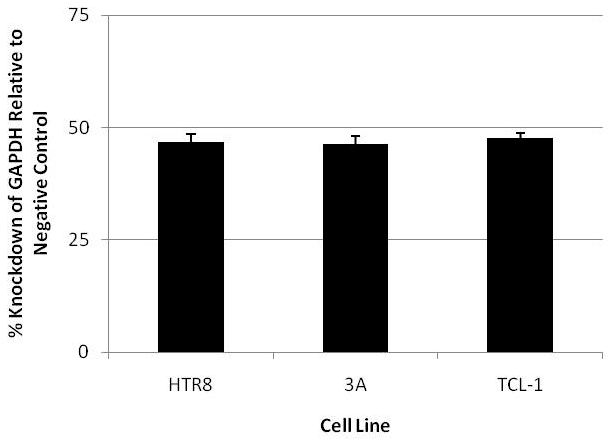


Figure 1. HTR8, 3A, and TCL-1 cells transfected with siRNA targeting GAPDH exhibit a 45% knockdown of GAPDH enzyme activity relative to cells transfected with negative control. Error bars show standard error from five replicates.

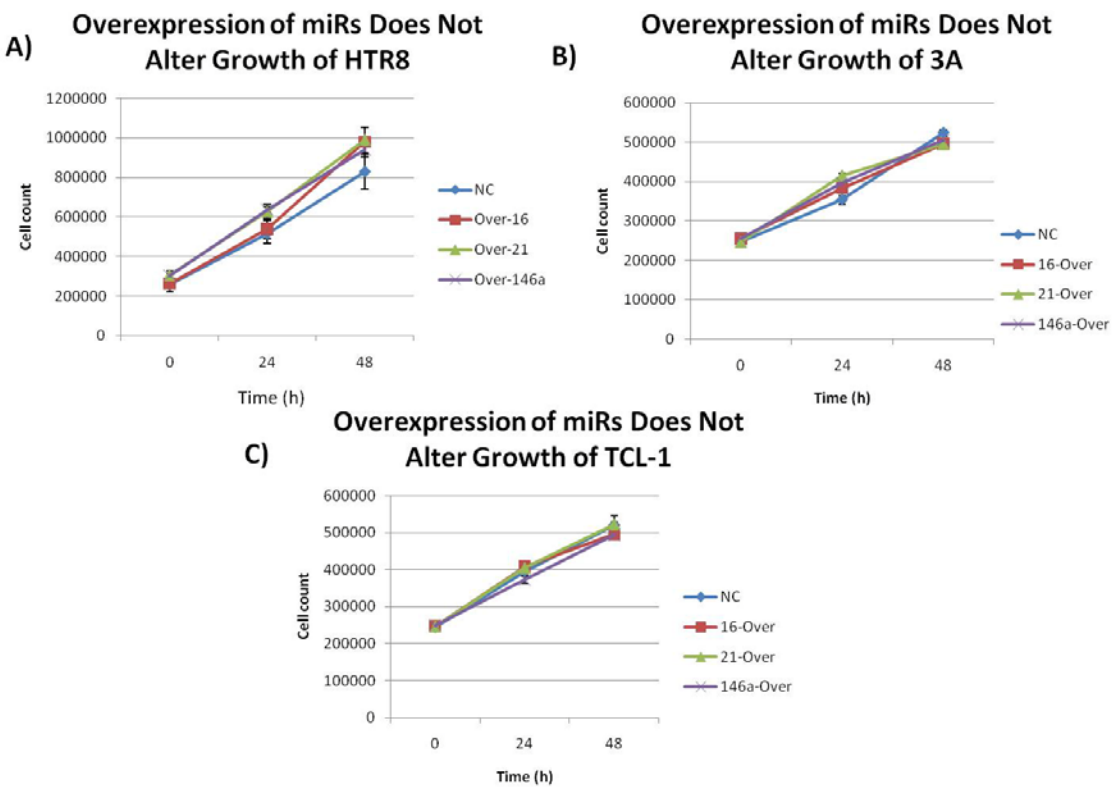


Figure 2. Overexpression of *miR-16*, *miR-21*, or *miR-146a* in HTR8 (A), 3A (B), and TCL-1 (C) cells does not lead to significantly different cell growth compared to negative control cells.

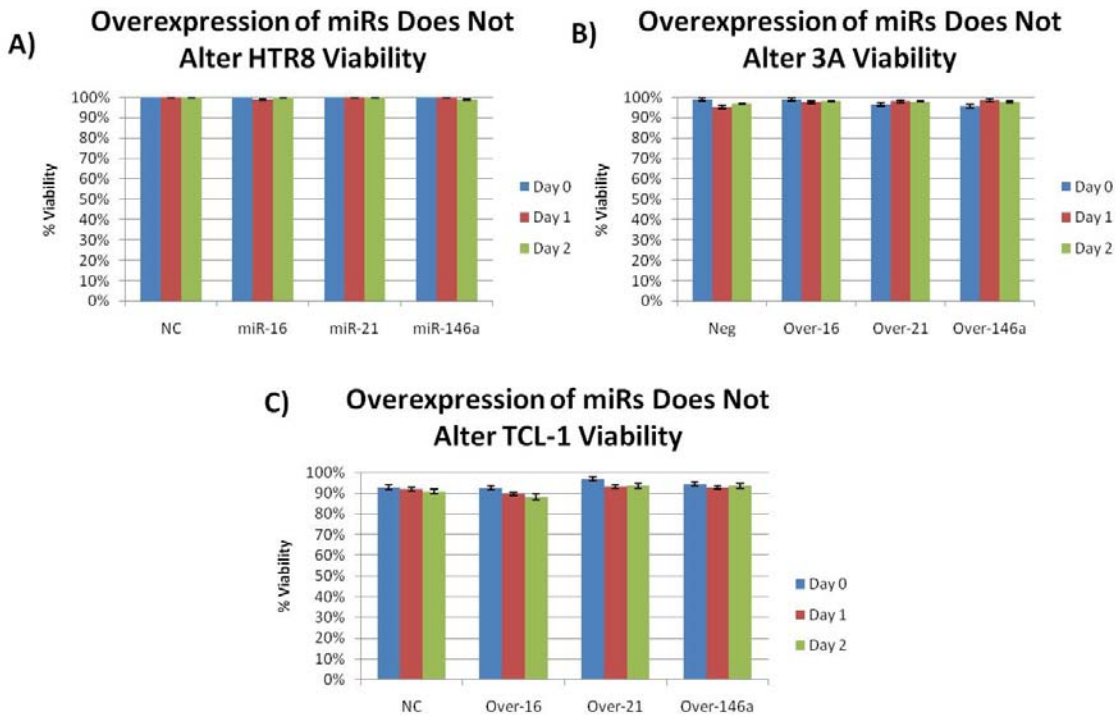


Figure 3. Overexpression of *miR-16, miR-21,* or *miR-146a* in HTR8 (A), 3A (B), and TCL-1 (C) cells does not lead to significantly different cell viability compared to negative control cells.

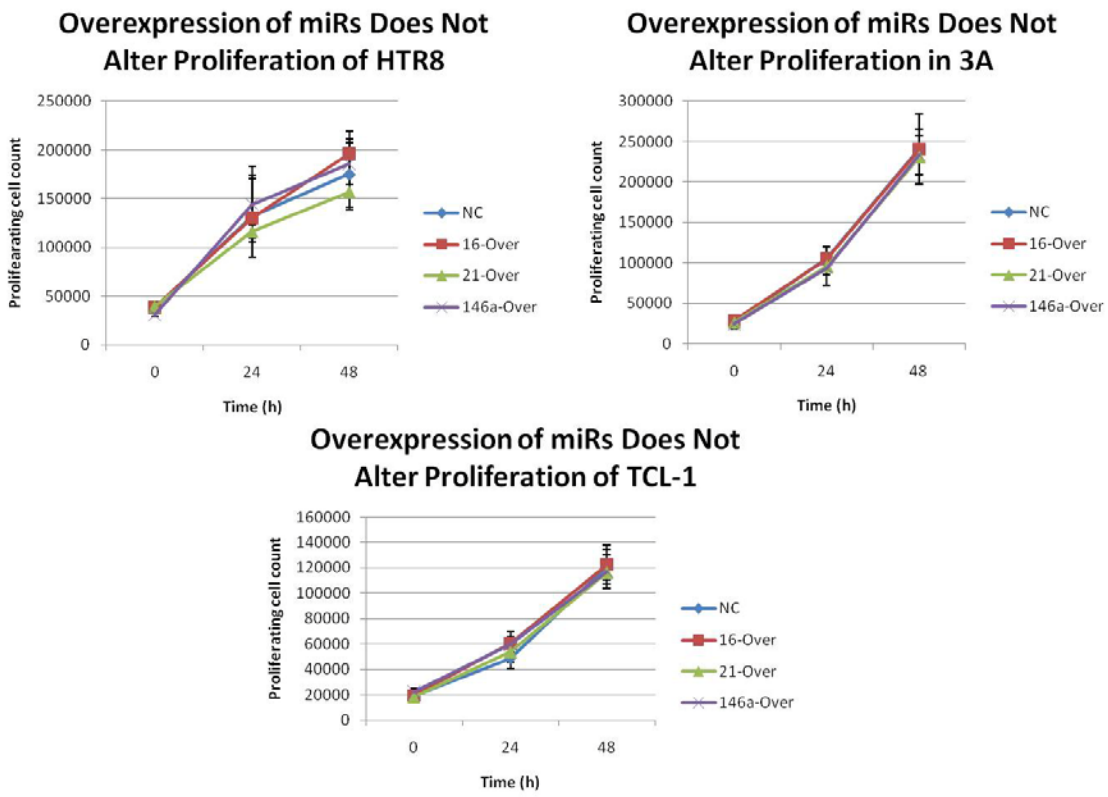


Figure 4. Overexpression of *miR-16*, *miR-21*, or *miR-146a* in HTR8 (A), 3A (B), and TCL-1 (C) cells does not lead to significantly different cell proliferation compared to negative control cells.

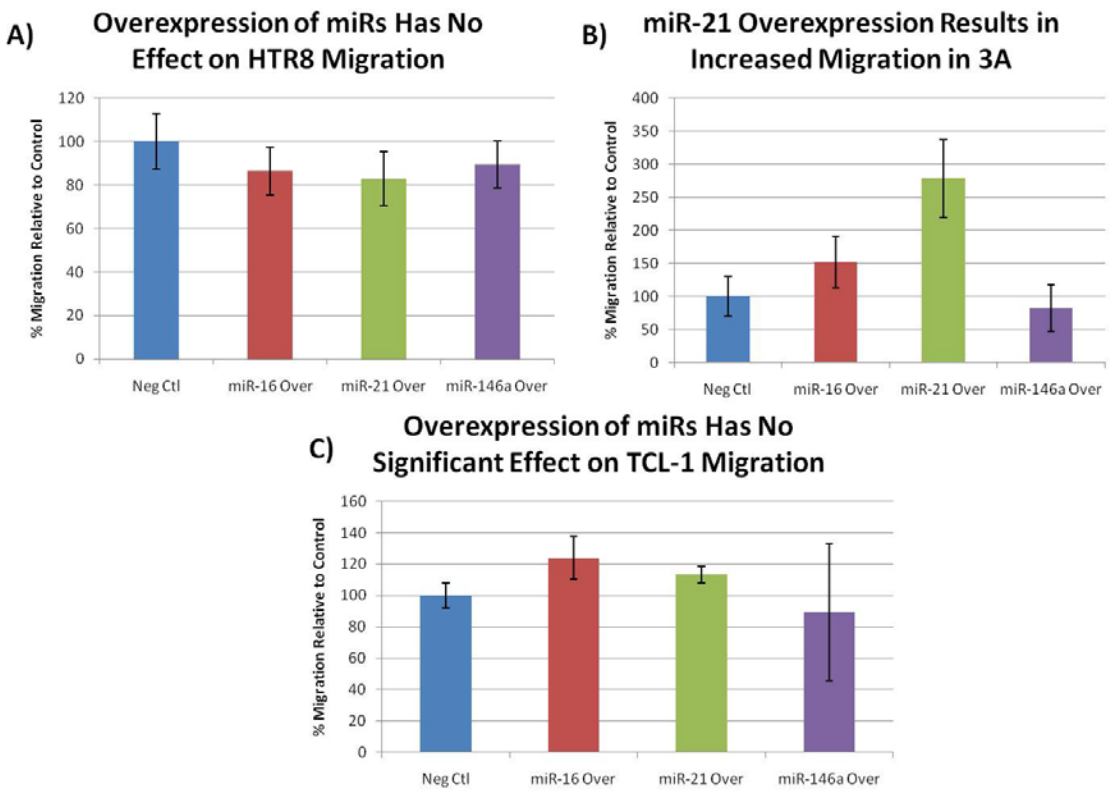
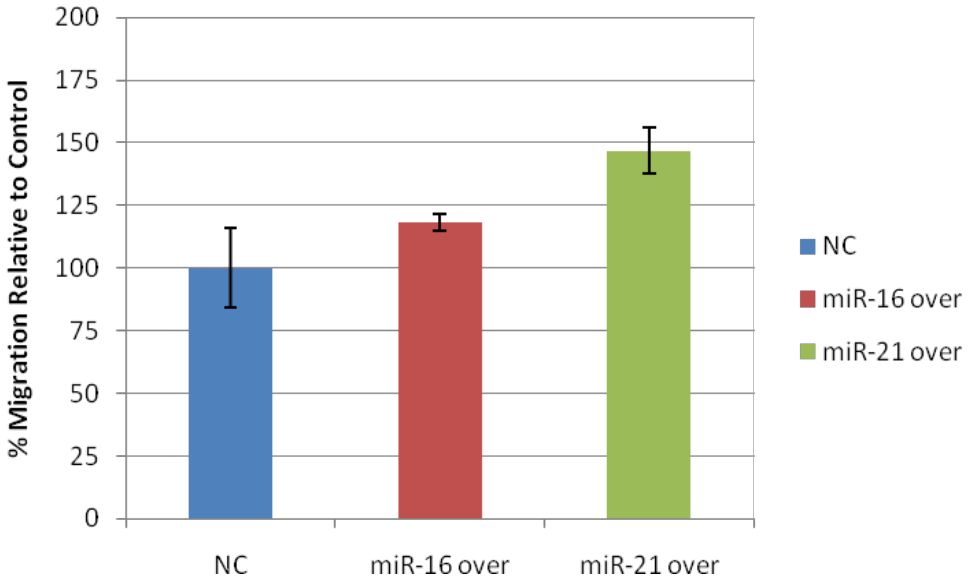


Figure 5. Overexpression of *miR-16*, *miR-21*, or *miR-146a* in HTR8 (A), 3A (B), and TCL-1 (C) and investigation of differential cell migration compared to negative control cells. Overexpression of *miR-21* in 3A cells results in a trend to increased migration ($p<0.08$).



Supplemental Figure 1. Migration assays using the technique of staining with hematoxylin and cell counting described previously(17) to confirm results trending significance ($p<0.2$) as determined using MTS to quantify migrated cells (see **Figure 5**). 3A cells overexpressing *miR-21* exhibited a trend of increased invasion, 150% versus control ($p<0.08$), confirming previous results using MTS to quantify migrated cells.

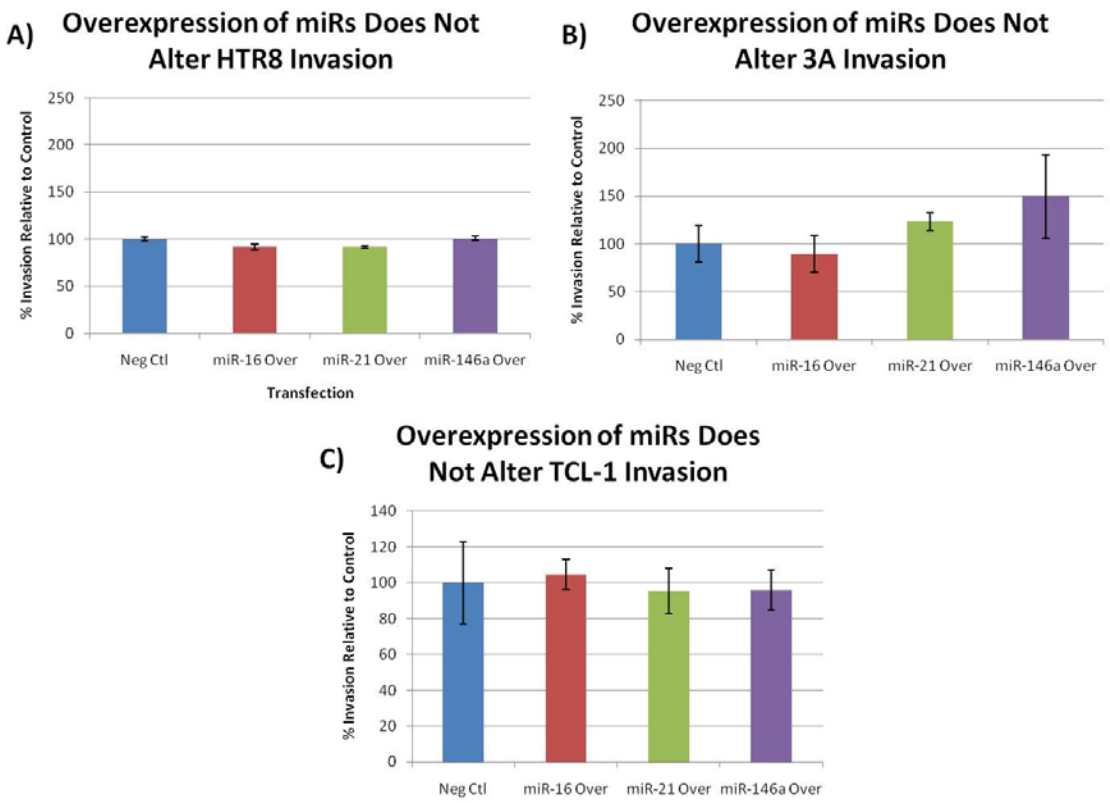


Figure 6. Overexpression of *miR-16, miR-21,* or *miR-146a* in HTR8 (A), 3A (B), and TCL-1 (C) cells does not lead to significantly different cell invasion compared to negative control cells.

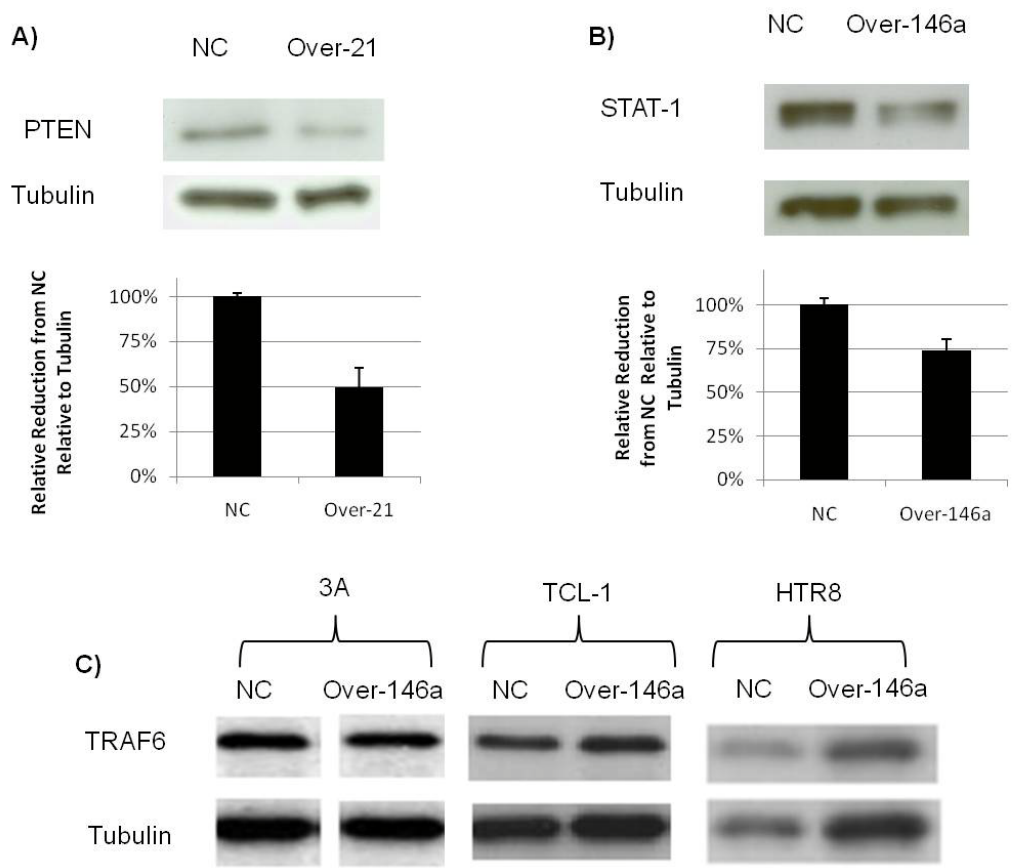


Figure 7. Western blots for PTEN and STAT-1 demonstrate targeting by *miR-21* and *miR-146a*, respectively. **A)** TCL-1 cells overexpressing *miR-21* express approximately 50% less PTEN protein than TCL-1 cells transfected with negative control ($p<0.05$). **B)** 3A cells overexpressing *miR-146a* express approximately 26% less STAT-1 protein than 3A cells transfected with negative control ($p<0.05$). **C)** 3A, TCL-1, and HTR8 cells overexpressing *miR-146a* have no differential levels of TRAF6 compared to cells transfected with negative control.

Chapter Five:

Discussion

This thesis aimed to analyze changes in miRNA expression in the human placenta associated with *in utero* exposure and fetal growth and sought to determine the cellular consequences of such changes in miRNA expression to ultimately understand potential downstream effects on infant growth and development. In this concluding chapter, I summarize my key findings from the previous chapters, attempt to put them into the context of previous and current research, and provide the reader with examples of future experiments important for better understanding links between exposures and fetal growth and miRNA expression in the placenta.

Central to growing interest in the field of epigenetics is the hope that more can be learned about the epigenetic regulatory mechanisms underlying processes of human development and disease. Important links have been examined between environmental exposures and altered expression of miRNA. miRNA expression profiles in the placenta may aid in determination of *in utero* exposures, as well as future disease risk assessment. Many of these important advances in placental epigenetics were discussed in Chapter one of the thesis.

Chapter two detailed the findings from a study which sought to characterize the modulation of 4 candidate placental miRNA by maternal cigarette smoking during pregnancy. Taken as a whole, the work identified the association of maternal cigarette smoking during pregnancy with the downregulation of *miR-16, miR-21,* and *miR-146a*, miRNA reported to be involved in critical cell processes. Previous work had demonstrated that nicotine (1) and benzo(a)pyrene (2) interact with the placenta and may affect placental growth and development and the effects of toxicants and agents of cell stress on expression of *miR-146a,* (3-6) as well as on *miR-16* (7) and *miR-21*. (8, 9)

As noted in the discussion section of Chapter two, our observations were limited by incomplete information regarding the duration of cigarette smoking during pregnancy, cigarette per day usage, or more extensive environmental exposure information (such as alcohol usage, environmental pollutant exposure, or secondhand cigarette smoke exposure). Despite these limitations, our data comprised an important first step in determining associations between maternal cigarette smoking during pregnancy and aberrant miRNA expression in the placenta.

Three placental cell lines from different stages of placental development were then utilized to further investigate the effects of components of cigarette smoke, namely nicotine and benzo(a)pyrene. Downregulation of *miR-146a* in TCL-1 cells treated with nicotine and benzo(a)pyrene suggested that *miR-146a* may be especially sensitive to agents of cellular stress. Moreover, this result suggests that two components of cigarette smoke which affect the expression of *miR-146a* in term placentas may be nicotine and benzo(a)pyrene. The lack of differential expression of *miR-16* and *miR-21* in cells treated with nicotine or benzo(a)pyrene does not necessarily rule out that their expression is not modulated by cigarette smoke. Other components of cigarette smoke may modulate the expression of these miRNA. Furthermore, the effect of the mixed exposures encountered with the myriad chemicals in cigarette smoke may be truly responsible for the modulation of expression of these miRNA. Experiments conducted with different components of cigarette smoke and complex mixtures of these components may provide more extensive information on the mechanistic effects of cigarette smoke on miRNA expression.

A final, but nonetheless important, contribution of Chapter two was the design and implementation of a miRNA target prediction strategy. As has been widely

reported, one of the more difficult challenges is miRNA target prediction; complementarity between miRNAs and their target mRNAs is generally far from perfect, and is not required for miRNA to functionally silence the expression of a protein product. As a result of this complexity, a simple search for sequence complementarity between the miRNA and its mRNA target, the basis of many target prediction algorithms, can be expected to produce many false-positive hits. Additionally, most miRNAs are thought to have potentially hundreds of targets, some of which will be targeted more strongly than others, and in cell-type specific contexts. Thus, it is essential to devise a strategy which predicts targets based not solely on sequence homology, but which also incorporates additional characterizations when choosing which targets should be examined with further testing.

Our target prediction strategy was as follows. Three target prediction algorithms were used to predict targets for miRNA of interest, and a fourth algorithm was used to evaluate predicted targets for base-pairing and minimum free energy. The three algorithms used for target prediction were *miRanda* (September 2008 release, available online at http://www.microrna.org/microrna/home.do), *PicTar* (as cited by (10) and available online at http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi), and *TargetScan 5.1* (available online at http://www.targetscan.org/). In order to be considered a predicted target for further investigation, the target must have appeared in the top 100 targets in all three prediction algorithms. The *RNA-hybrid* algorithm was used to evaluate predicted targets for information on secondary structure and thermodynamic stability of the miRNA-mRNA duplex (11). *RNA-hybrid* is available online at http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html. The design and

implementation of target prediction algorithms and strategies are important for determining which gene targets to empirically validate using proteomic approaches as targets of miRNA.

A number of future experiments aimed at further characterizing the modulation of miRNA by cigarette smoke are needed. Future experiments conducted using cigarette smoke condensate as reported by Crane-Godreau and colleagues (12) may prove especially useful in characterizing the effects of complex mixtures of components of cigarette smoke on placental cells. Of course, such experiments also have the caveat of presenting challenges in determining the specific composition of the complex mixture as well as physiological relevance of exposure to such a mixture on placental cells. Other more extensive experiments aimed at better understanding the more complex effects of organism-wide cigarette smoke exposure might be designed using a mouse model of chronic environmental tobacco smoke exposure as described previously (13). In such an experimental design, pregnant mice would be exposed to environmental tobacco smoke throughout their entire gestation and euthanized at key timepoints in gestation, with placentas and other key organs harvested for measurement of miRNA expression. Findings would elaborate on work done to characterize the association of maternal cigarette smoking during pregnancy with the dysregulation of placental miRNA. Additionally, such findings using an *in vivo* model system may provide key mechanistic data that may further strengthen current epidemiologic association studies with findings from a model system experimental paradigm.

In Chapter three, I showed data from an investigation of the expression of key candidate miRNA in a large, population-based series of primary human placenta samples

and their association with poor fetal growth, specifically identifying that reduced expression of *miR-16* and *miR-21* are strongly associated with growth restriction. While I controlled for a number of confounders in my analysis and while my data suggest that reduced *miR-16* and *miR-21* expression in the placenta may be associated with SGA status, there remains a somewhat difficult challenge of data interpretation. Because this study was performed using human tissues, it is a retrospective association study – one that is limited by the fact that it can reveal associations between expression of miRNA and fetal growth but it cannot reveal causality. Furthermore, the study cannot adequately answer the question of whether dysregulated miRNA expression in the placenta leads to growth restriction (i.e. SGA status) or growth restriction leads to dysregulated miRNA expression in the placenta. Future animal models designed to study growth restriction (14, 15) will be essential in answering this "chicken and egg" causality dilemma.

A number of groups had previously described placental miRNA expression associations with maternal conditions such as preeclampsia (16), with maternal cigarette smoking during pregnancy (17), and as markers of pregnancy itself (18). Furthermore, more is being uncovered about the role of *miR-16* and *miR-21* in regulating key cellular processes, especially the involvement of *miR-16* in regulating cell cycle progression (19) and *miR-21*'s capability of regulating cell cycling and cell proliferation (9).

miR-21 had been shown to target a number of key regulators of cell proliferation and migration, including but not limited to PLAG1 (20) and PTEN (21, 22). Meng and coworkers showed that *miR-21* regulates PTEN in human hepatocellular cancer (21), and Lou and colleagues demonstrated that in ovarian epithelial carcinomas, *miR-21* promotes proliferation, invasion and migration abilities by inhibiting PTEN (22). Like *miR-21* and

many miRNA, *miR-16* exhibits tissue-specific function and expression. In a variety of cancer cell lines, previous work has shown that *miR-16* is involved in the induction of apoptosis by targeting *BCL-2* (23) and in cell cycle regulation by targeting CDK6 (24), CDC27 (25), and CARD10 (26). In other cell types, *miR-16* has different functions, such as targeting HMGA1 and Caprin-1 (27), further suggesting that *miR-16* may have cell-type-specific function and expression (23, 27).

Mechanistic research using model systems is needed to further elucidate the pathways regulated by *miR-16* and *miR-21* and to better determine the functional consequence of downregulation of *miR-16* and *miR-21* in the placenta. A number of animal models have been generated to further study low birthweight in a controlled, experimental system (14, 15), and it will be important to consider the role of miRNA in these animal models. Recently, Pernaute and colleagues have made important steps in characterizing the roles of miRNA in controlling cell cycle processes in the developing mouse embryo as well as extra-embryonic tissues (28). Future work may include studying the effects of viral or bacterial infections at different stages during pregnancy on placental miRNA expression in the mouse. Such experiments could be accomplished by exposing mice to sub-lethal doses of Influenza A as described by Mackenzie and colleagues (29) or LPS as described by Clark (30) and investigating subsequent effects on miRNA expression in placental tissues. One might hypothesize that miRNA particularly sensitive to infections and miRNA reported to be involved in regulating inflammatory pathways, such as *miR-146a*, might be especially sensitive to such infections. Additionally, the effects of Influenza A-induced or LPS-induced dysregulation of miRNA on rates of resorption, pre-term birth, and birthweight might be investigated to

further understand the roles of miRNA in mediating or responding to infection during pregnancy.

In summary, data from Chapter three suggesting that low expression of *miR-16* and *miR-21* in the placenta is associated with poor fetal growth may have many important implications. Future work to determine the roles of miRNA in specific pathways leading to altered fetal growth will be crucial to better understanding fetal growth as both a marker of the intrauterine environment as well as a developmental outcome and in better comprehending the developmental origins of health and disease.

Chapter four reported findings from important follow-up experiments in placental cell lines to determine effects of aberrant miRNA expression on important cell processes and function, as well as to determine the effects of dysregulated miRNA expression on protein levels of target genes. More specifically, our work sought to investigate the effects of miRNA overexpression on a number of key placental cell processes, specifically cell viability, growth, proliferation, invasion, and migration, as well as further confirm previously empirically validated targets of miRNA in placental cells. Taken collectively, our work contributes to the better understanding of how dysregulated *miR-16*, *miR-21*, and *miR-146a* may alter key cellular processes and expression of target genes and further explore how miRNA may control cell processes in placental cells from various stages of pregnancy.

As with many miRNA, *miR-16* exhibits tissue-specific function and expression. We hypothesized that dysregulation of *miR-16* in the placenta may lead to aberrant expression of its targets and may lead to dysregulated placental cell processes. While our data suggest that overexpression of *miR-16* does not lead to significant differences in cell

viability, growth, proliferation, invasion, or migration, future studies addressing potential effects on apoptosis and target gene expression may prove to be especially useful in better determining the biological ramifications of dysregulated *mir-16* expression in the placenta, which may ultimately lead to abnormalities in the placenta that might result in reduced infant birthweight. Future work using both cell lines as well as animal models will be important in better characterizing the effects of dysregulated *miR-16* on the placenta.

miR-21 has also been shown to target a number of key genes, including but not limited to PTEN (21, 22). Our results that *miR-21* targets PTEN in the TCL-1 placental cell line further suggest that *miR-21* may be one mechanism regulating PTEN expression in the placenta. Downregulation of *miR-21* in the placenta could thus lead to a dysregulation of PTEN and could result in decreased invasion of the maternal decidua, decreased migration, and decreased growth – the opposite of what has been observed to occur in the case of upregulated *miR-21* (21). Our results suggesting that overexpression of *miR-21* in 3A cells leads to a trend towards increased migration compared to cells transfected with negative control may further underscore the involvement of *miR-21* in regulating placental cell migration, especially in first trimester placental cells. Future work using *in vivo* animal models of dysregulated placental miRNA expression will be crucial in determining the causal effects aberrant placental *miR-21* expression on placental cell migration.

Our *in-silico* analysis predicted TRAF6 as a target for *miR-146a*, suggesting that downregulation of *miR-146a* could result in an upregulation of TRAF6, a protein also important for signaling through the NFκβ signaling pathway (31) as well as a mediator of

inflammation in the toll-like receptor 4 (TLR4) pathway (32, 33). Previous reports using different target prediction strategies have hypothesized TRAF6 as a target of *miR-146a* (17, 34) and have validated TRAF6 as a target in murine macrophages (35). TLR4 mediates the inflammatory response, and aberrant TLR-4 signaling is associated with inflammation-induced preterm delivery (36). Such a consequence could be the result of dysregulated TRAF6 production as a result of downregulation of *miR-146a*. TRAF6 overexpression due to downregulation of *miR-146a* could result in overactive TLR signaling which could have a number of downstream consequences for both placenta and fetus. Unfortunately, our Western blot analysis suggested that TRAF6 protein levels were not significantly different in placental cells overexpressing *miR-146a* versus cells transfected with negative control. This may be due, in part, to a more complex regulation of TRAF6 in these placental cell lines than is currently understood. While *miR-146a* may be capable of regulating TRAF6, it does not appear to be the major or most important regulator of TRAF6 in these placental cell lines. Further investigation into such relationships and regulatory networks using *in vivo* animal models would prove vital into better understanding both the role of TRAF6 in TLR4 signaling, as well as the association of aberrant expression of *miR-146a* with adverse pregnancy outcomes.

Previous work has demonstrated that STAT-1 is targeted by *miR-146a* (37). Our Western blot analysis revealed that 3A cells overexpressing *miR-146a* had 25% less STAT-1 protein than did cells transfected with negative control, a potentially important result since STAT-1 plays a role as a key transcription factor. STAT-1 is activated upon interferon (IFN) binding to its receptor. Once phosphorylated by JAKs, STAT-1 forms a homodimer and translocates to the nucleus where it binds the Interferon-Gamma

activation site (GAS) in the promoter of interferon stimulated genes (ISG) (38). Our data suggest that STAT-1 may be regulated, in part, by *miR-146a* in 3A placental cells. Dysregulation of *miR-146a* may thereby lead to aberrant production of STAT-1 and dysregulated transcription of ISG, potentially leading to a cascade of events which may negatively alter placental function and ultimately lead to altered fetal programming. Once again, future work using animal models of aberrant placental miRNA expression will be important for further determining the role of dysregulated *miR-146a* expression in the placenta.

Whenever one is working with placental cell lines, one must be aware of the potential limitations involved. Cell lines have important utility as tools for proof-of-concept experiments but cannot serve as adequate substitutes for animal model systems used to answer questions of causality. One recent study pointed out several limitations regarding human placental cell lines, including but not limited to unexpected effects of transformation, unanticipated cell contamination resulting from site of initial derivation, and varying immunological characteristics of different human placental cell lines (39). In addition, varying degrees of transfection efficiency can impact the likelihood of observing hypothesized changes in cell culture. For example, transfection efficiency for HTR8, 3A, and TCL-1 cells using our transfection protocol was estimated at approximately 45%; this equates to only 45% of the cells in culture actually being transfected with the pre-miR mimic or negative control of interest. Higher transfection efficiencies may have led to more cells actually being transfected with the pre-miR mimic of interest and may have resulted in increasing the likelihood of observing phenotypic changes to placental cells and cell function in culture. Collectively,

considering these limitations is important when determining the scope of conclusions from data using human placental cell lines; nonetheless, human placental cell lines can serve as important tools for proof-of-concept experiments, providing researchers basis for following up such experiments in primary cell culture or in *in vivo* animal models.

Collectively, dysregulation of these miRNA can lead to aberrant expression of their targets and may affect a number of key placental cell processes, ultimately leading to aberrant cell cycle regulation, growth, immunomodulation, and development in the placenta. These changes in target gene expression may have further effects downstream for both placenta and fetus, ultimately resulting in altered fetal programming. Additionally, more work to determine the functional effects of miRNA crosstalk – that is, miRNAs whose differential expression may have additive associations with risk for phenotype or disease – will be necessary to better understand the complex regulatory networks involved. Also, future studies comparing the severity of effects resulting from the downregulation of a particular miRNA versus the upregulation of that miRNA will be important in further understanding the boundaries of what might be considered the range of specific miRNA expression that allows for biological equilibrium; this range of miRNA expression may be miRNA-specific as well as cell type-specific. Future studies are ongoing to further characterize the effects of environmental exposures on placental miRNA, on understanding the downstream phenotypes of aberrant miRNA expression and aberrant miRNA target gene expression, and in investigating the placenta as a record of the intrauterine environment. In such a way, the environment can act through the alteration of miRNA which may lead to aberrant mediation of placental function, potentially leading to downstream effects on infant growth and development.

Understanding how the variety of stimuli that comprise the intrauterine environment elicit their effects on the placental epigenome and how these epigenetic effects mechanistically alter placental function will be important in better characterizing the downstream effects of the intrauterine environment on fetal programming.

In conclusion, this work further elucidates the association of placental miRNA expression with *in utero* exposures and fetal growth and seeks to better understand how an adverse intrauterine environment acts through epigenetic mechanisms to alter infant and child health. Data have suggested that environmental exposures can alter miRNA expression in in-vitro systems (3) and various tissues (40) and that altered levels of miRNA expression can be associated with particular diseases or risk factor for disease (41). Research on diseases of pregnancy – such as finding miRNA whose expression may be associated with preeclampsia (16, 42) or gestational diabetes – might be expanded to include work to better understand associations between epigenetic factors in the placenta and diseases of childhood and adolescence, as well as alterations to normal placental immunology and function.

As has been reported previously (43), using the placenta as a reference tissue allows researchers to utilize an important residual tissue whose respective miRNA expression may prove to be powerful biomarkers possessing predictive capability for a number of diseases or disease progression. Modern advances in bioinformatics – such as pathway analysis tools and target gene prediction software – as well as advances in technology, such as microarray technology, have given researchers and clinicians tools to better detect patterns in a number of epigenetic mechanisms, including DNA methylation, imprinting, histone modification and miRNA expression, that are associated with

particular exposures and diseases. Aberrant patterns of miRNA expression or DNA methylation may ultimately serve as biomarkers for exposure, disease burden, or even as "early indicator" diagnostics of increased risk for developing future disease or disorders.

Research continues to elucidate a better understanding of the epigenetic regulatory mechanisms of in the placenta. Panels of miRNA determined to be associated with particular exposures, such as heavy metal exposure or other xenobiotic toxicants, may prove to be useful biomarkers in assessing a newborn's degree of *in utero* exposure to such toxicants; in this way, an aberrant miRNA expression profile previously associated with a heavy metal or toxicant exposure might give clinicians an important indication of exposure level in their patients. Such miRNA biomarkers may be especially important in circumstances where exposure assessments have not yet been conducted. miRNA biomarkers may prove essential for developing new diagnostics for early diagnosis of risk factor and levels of exposure; in this way, panels of miRNA whose altered expression is shown to be associated with a particular disease or risk for disease might be investigated to inform clinicians of a patient's particular disease state or risk for disease. Additionally, these aberrant patterns of miRNA expression may identify previously unknown pathways targeted for alteration, which, in turn, may serve as targets for novel drug treatment or prevention strategies. These epigenetic biomarkers can be brought from the benchtop to the bedside and will be useful in helping clinicians better diagnose and possibly prevent the onset of disease.

Because poor maternal forecasts leading to low birthweight may not accurately predict the post-birth environment and because low birthweight increases one's risk for a number of diseases later in life (44), more research is necessary to more fully understand

the pathways whose dysregulation may ultimately affect birthweight. In addition, it will be important to gain a better understanding of how *in utero* exposures and adverse intrauterine conditions mechanistically lead to changes which alter the placental epigenome and may thereby alter placental function, ultimately leading to impacts on fetal programming and future health. Future work to determine the roles of miRNA in pathways responsive to *in utero* exposures and in pathways leading to altered fetal growth will be key to better understanding fetal growth as both a marker of the intrauterine environment as well as a developmental outcome and in better comprehending the developmental origins of health and disease.

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Appendix:

Birthweight is associated with DNA promoter methylation of the glucocorticoid receptor in human placenta

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Birthweight is associated with DNA promoter methylation of the glucocorticoid receptor in human placenta

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ABSTRACT

Birthweight has been associated with a number of health outcomes throughout life. Crucial to proper infant growth and development is the placenta, and alterations to placental gene function may reflect differences in the intrauterine environment which functionally contribute to infant growth and may ultimately affect the child's health. To examine if epigenetic alteration to the glucocorticoid receptor (GR) gene was linked to infant growth, we analyzed 480 human placentas for differential methylation of the GR gene, exon 1F, and examined how this variation in methylation extent was associated with fetal growth. Multivariable linear regression revealed a significant association ($p<0.0001$) between differential methylation of the GR gene and large for gestational age (LGA) status. Our work is one of the first to link infant growth as a measure of the intrauterine environment and epigenetic alterations to the GR and suggests that DNA methylation may be a critical determinant of placental function.

KEYWORDS: DNA methylation, placenta, fetal development, birthweight, epigenetics

RUNNING TITLE: Birthweight is associated with placental GR methylation

INTRODUCTION

The period of intrauterine development represents a sensitive period where disruption or modification of the intrauterine environment can influence fetal development as well as lead to programming of health throughout the life course.[1, 2] In particular, environmental exposures during intrauterine development have been shown to be associated with several chronic diseases in adulthood[3] and adverse intrauterine conditions have been shown to associate with fetal growth outcomes.[2, 4-6] There is growing interest in determining the functional molecular basis of this fetal programming.

The metabolic and endocrine activity and ability to transport nutrients, water, gas and waste products marks the placenta as a vital organ for the growing fetus.[7] Dysregulated placental gene expression may result from *in utero* exposures and thus represent a record of the intrauterine environment during pregnancy. These alterations may also play a critical role in establishing a fetal program which can influence health outcomes throughout life.[7] Thus, as a functional marker of intrauterine environment,[8] investigation of epigenetic alterations in the placenta may further the understanding of the molecular mechanisms behind many developmental outcomes which may be influenced by adverse intrauterine conditions.

Low birthweight has been associated with increased infant morbidity and mortality and with an increased risk for certain diseases later in life, particularly those comprising the metabolic syndrome.[9] High birthweight, as well, has been shown to have serious adverse health outcomes in both developing children and later in life.[10] Previous studies have shown high birthweight as a risk factor for insulin resistance,[11] obesity,[12] and cancers[13] such as leukemia and breast, prostate and colon cancers. Studies to

determine the potential risk factors for mothers of large for gestational age (LGA) babies are ongoing,[10] and more research is needed to identify potential risk factors behind this developmental outcome.

The glucocorticoid receptor (GR), a known mediator of glucocorticoid signaling, has been shown to be regulated, in part, by epigenetic mechanisms, specifically DNA methylation.[14-19] Both rat and human studies have shown an association between methylation of the hippocampal GR gene and early postnatal outcomes.[16] Early maternal care of rat pups leads to altered methylation status of the NGFI-A consensus binding site within the promoter of the GR gene in the hippocampus, and these changes have been linked to altered stress responses later in life, as well as to growth restriction in-utero.[14, 15, 20-22] Oberlander and colleagues suggested a potential association between methylation status of the analogous human NGFI-A binding site in promoter region 1F of the GR gene in infant cord blood and maternal mood.[18, 20]

As birthweight is known to be associated with a variety of later life health outcomes and aberrant methylation of the GR gene has been linked with adverse developmental outcomes in animal models, we sought to investigate the associations between birthweight and GR promoter methylation in human placenta.

RESULTS

We examined the variation in the extent of methylation of the GR gene promoter 1F region in a large series of human placenta samples obtained from term infants of various birthweights. **Table 1** shows the demographics of the study population in total (n=480) and by birthweight grouping: small for gestational age (SGA, $<10^{th}$ percentile of birthweight for gestational age, n=102), appropriate for gestational age (AGA, 10-90^{th} percentile of birthweight for gestational age, n=343), and large for gestational age (LGA, $>90^{th}$ percentile of weight for gestational age, n=35). Distributions of gestational age, infant gender, maternal race, prenatal vitamin use, and maternal age were not significantly different across birthweight groupings. As expected, maternal tobacco use during pregnancy was more common amongst SGA infants, and LGA infants were more often delivered through Caesarean section. There was a low prevalence of recreational drug use, alcohol use, and maternal gestational diabetes across all groups.

Figure 1 depicts the difference in the extent of methylation of the 13 CpG sites examined in the GR exon 1F region in SGA, AGA, and LGA placentas. Differential methylation was noted across groups at all CpG sites in the GR exon 1F but only CpG site 7 and the mean across all CpG sites showed a significantly different extent of methylation across the groups following a Bonferroni correction for multiple comparisons ($p<0.0036$).

As these sites are in close proximity, and as methylation likely occurs throughout the region in a coordinated fashion, we examined the correlation between the extent of methylation at the individual CpG sites, using pairwise correlations for methylation extent between each of the 13 CpG sites (**Figure 2**). Of note, there is a moderate to

strong correlation in methylation between many of the 13 CpG sites. Moreover, the correlation in methylation between CpG sites appears to be a function of the distance between sites, consistent with the findings of Nautiyal and colleagues.[23] This relatively high degree of correlation suggests that use of the mean may be a reliable marker of the extent of methylation across this region.

Examination of the correlation of each of the covariates listed in **Table 1** with log mean GR methylation extent was conducted (**Supplemental Table 1**). This examination revealed that birthweight was significantly correlated with mean GR methylation extent ($r=0.16$, $P=0.0004$). None of the other demographic or clinical characteristics of the subjects was significantly correlated with GR methylation. To further investigate the association between birthweight category and mean GR methylation, controlling for potential confounders, we performed multivariable linear regression **(Table 2)**. Analysis revealed that in LGA infants, the log mean percent methylation of the GR increased significantly by 0.40 ($p<0.0001$), controlled for gestational age, maternal age, maternal ethnicity, tobacco use during pregnancy, alcohol use during pregnancy, recreational drug use during pregnancy, prenatal vitamin use, delivery method, maternal gestational diabetes, and infant gender. Being SGA was not a significant predictor of GR methylation extent, nor were any of the covariates included as confounders in the model, consistent with the univariate findings.

To demonstrate the functional role of promoter methylation on GR gene expression, the placenta choriocarcinoma cell line JEG3 was treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AzaC). Pyrosequencing of bisulfite modified DNA from JEG-3 cells treated with 5-azaC showed decreased

methylation with increasing doses of 5-azaC across all 13 CpG sites in the GR **(Fig. 3a).** The mean methylation across this region demonstrated an almost 20% decrease in cells treated with 1.25μM 5-azaC, and a 50% decrease in methylation in cells treated with 2.5 μM 5-azaC, compared to cells treated with DMSO alone. GR gene expression, measured by qRT-PCR, also showed a significant ($p<0.004$) dose-dependent increase with increasing exposure to 5-azaC (**Fig. 3b).** Our cell culture data suggests that JEG-3 cells do not express GR unless treated with 5-AzaC, and increasing doses of 5-AzaC resulted in a dose-dependent increase in GR gene expression.

DISCUSSION

Perturbations to placental gene expression may result in the altered metabolic and endocrine function of the placenta and may also affect the placenta's ability to transport water, gas, nutrients, and waste products crucial for the proper growth and survival of the fetus.[7] Previous findings have reported that DNA methylation marks are stable and accessible for measurement in placenta,[24] and that the GR gene is expressed in placenta.[25] Taken collectively, our data combined with this previous work suggests that DNA methylation of the GR gene promoter may be important in regulating GR gene expression, and this epigenetic alteration is linked to infant birthweight. Specifically, we have demonstrated that infants considered LGA have significantly higher GR methylation and that birthweight classification as LGA explains a great deal of the variability in methylation of this promoter region. While there is a difference in the percent methylation observed in the placental choriocarcinoma cell lines versus that in tissue, the levels of GR methylation that we observed in tissue are consistent with previous findings in other primary human tissues.[18] As birthweight is a multifactorial outcome as well as a predictor of later life health and disease, this finding begins to provide a mechanistic link between the role of the intrauterine environment on health throughout life.

Previous work has shown that altered methylation status of the NGF1-A consensus binding site within the rat exon 1-7 and analogous human exon 1F of the GR gene can lead to reduced NGF1-A binding, and reduced expression.[14, 15, 19, 26] Our work is an extension of the previous work focused on the 13 CpG sites in exon 1F[15, 18], driven by the hypothesis that the environment may be playing a critical role in determining the DNA methylation status of this gene regulatory region. The NGFI-A binding site occurs

at CpG sites 3 and 4. Our results suggest elevated methylation at these sites in LGA compared to SGA and AGA infants, although the differences were not significant. Methylation at these sites in infant cord blood was previously associated with maternal mood and stress.[18] Although we could not examine these associations in this population, our results suggest that the environment represented by birthweight may have greater impacts at other CpG sites in the 1F promoter than at the NGF1A sites. This difference may be related to differences in the environment influencing methylation, or may be related to differences in susceptibility of the tissues examined. Specific examinations of potential environmental influences on methylation of this region in placenta and other tissues are warranted to better explain this difference. There are also several alternative first exons of the GR involved in gene expression regulation in a tissue specific manner. Future studies aimed at exploring and more completely characterizing the complex regulatory mechanisms controlling GR gene expression are needed in the placenta and in other primary human tissues.

Our *in-vitro* work in human placenta choriocarcinoma cells suggests that methylation of this region of the gene is also associated with reduced gene expression in this cell type, thereby suggesting a relationship between the methylation of this region in human placentas. Our cell culture data suggests that JEG-3 cells do not express GR unless treated with 5-AzaC, and increasing doses of 5-AzaC resulted in a dose-dependent increase in GR gene expression. Because 5-AzaC acts as a non-specific DNA methyltransferase inhibitor, we cannot exclude the possibility that its effects were not directly related to changes in exon 1F, but these results are consistent with previous work performed in other cell types.[26] Future follow-up studies can be performed to more

completely characterize the molecular mechanisms underlying control of GR gene expression.

Rich maternal forecasts represented by LGA status can prove to be incorrect if the child is born into a nutrient poor environment. Moreover, high birthweight, especially birthweights that would categorize babies as LGA, is associated with an increased risk for disease in both children and adults. Previous work has shown high birthweight as a risk factor for insulin resistance[11] and obesity,[12] as well as for a number of cancers,[13] and it is clear that glucocorticoid signaling can play a role in these pathologic processes. While normal physiological levels of glucocorticoids and GR are essential for metabolic control, altered glucocorticoid action has also been shown to be associated with a variety of metabolic diseases such as obesity and type 2 diabetes.[27] Altered methylation of the GR in the placenta and subsequent dysregulated expression of the GR and thereby altered glucocorticoid signaling may lead to an adult phenotypes, such as glucocorticoid resistance, as well as to increased risks of metabolic disorders, such as Type 2 diabetes and obesity.[28-30] To date, associations between placental GR methylation and adult metabolic outcomes have yet to be elucidated. Ke and colleagues demonstrated an increase in expression of some forms of GR in the hippocampus of IUGR rats.[17] Our data suggest an increase in DNA methylation of the exon 1F of the GR associated with LGA status, which would be consistent with the findings of Ke and colleagues. More research is needed to identify risk factors that may lead to the developmental outcome of high birthweight, as well as further elucidate the role of the glucocorticoid pathways as well as other critical molecular mediators leading to this phenotype and the associated later onset disease risk including metabolic disorders.

In summary, our study is one of the first to show an association between the methylation status of the promoter of the GR gene in human placenta and LGA status. This may serve as an early epigenetic marker of maternal exposures and may be involved in the pathway leading to the adverse health outcomes associated with increased birthweight. More work is necessary to further elucidate the mechanisms involved in this complex and multifactorial pathway and is crucial for better understanding the developmental origins of health and disease.

MATERIALS AND METHODS

Placenta samples. 480 placenta samples were collected at Women and Infants Hospital in Providence, Rhode Island, in accordance with protocols approved by the Institutional Review Boards of both Women and Infants Hospital and Brown University. Samples were collected from women in good physical health between the ages of 18-42, whose infants were at term and viable with no known genetic disorders. Approximately a 1g biopsy of placenta was excised, free of maternal decidua, from the maternal side of the placenta 2 cm from the umbilical cord insertion site within 4 hours of delivery, using a protocol designed to reduce potential sample degradation. The sample was immediately placed in RNAlater (Applied Biosystems, Inc.) and stored at 4ºC. At least 72 hours later, placenta samples were removed from the RNAlater, blotted dry and stored at -80ºC until further analysis. Medical information, such as infant's birth weight, length, gender, mode of delivery, gestational age, and maternal demographics was recorded from patient medical charts using a structured chart review. Birthweight percentiles were calculated using the method of Fenton (2003).[31]

DNA extraction and modification. DNA was extracted from the placenta samples using the QIAmp DNA Mini Kit (Qiagen, Inc.) following manufacturer's protocols. Purified DNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), and DNA samples (1μg) were bisulfite-modified using the EZ DNA Methylation Kit (Zymo Research, CA, USA.) and stored at -20ºC .

Bisulfite pyrosequencing DNA methylation analysis. Pyrosequencing was performed on PCR product amplified from bisulfite-modified DNA as described previously.[18] Bisulfite conversion was performed using individual columns using the EZ DNA Methylation Kit

(Zymo Research) and manufacturer's protocol. In order for a sample's methylation extent to be called, it must exhibit at least a 93% bisulfite conversion rate, as assessed by pyrosequencing, and all samples examined exhibited a rate >95%. To prevent batch effects from bisulfite treatments interfering with the analysis, samples were randomized across batches. 10% of the samples were repeated independently and the R^2 for repeats is 0.98. The region of interest, exon 1F of the human GR (*NR3C1*) gene, has 13 CpG sites, and is considered the human homologue of the rat *NR3C1* gene, exon 1-7 previously shown to have differential methylation in response to maternal exposures.[19, 32]

In brief, HotStar Taq DNA Polymerase (Qiagen) and the following forward and biotinylated reverse primers were used: PMHumGCCRF, 5'-TTTTTTTTTTGAAGTTTTTTTA-3' and PMHumGCCRR, 5'-CCCCCAACTCCCCAAAAA-3'(IDT Inc., Coralville, IA.) Cycling conditions were 94ºC for 15 minutes followed by 50 cycles of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute with a final extension of 10 minutes at 72ºC. PCR products were sequenced using a PyroMark MD system and the two following sequencing primers (IDT): PMHumGCCRS2, 5'GAGTGGGTTTGGAGT-3' and PMHumGCCRS3, 5'-AGAAAAGAATTGGAGAAATT-3'. The first sequencing primer was designed to sequence the first five CpG sites, and the second sequencing primer was designed to sequence the following eight CpG sites for a total of thirteen sites sequenced. The dispensation orders for the two assays were GTCTGTCGAGTAGTCGGTCGAGAGCTATGTCGAG for the first assay and ATCGTGTTGATCTGTCGCTTAGAGAGACTATGTCAGTTCTGTCGTAGTCTGTCGTA for the second. The percent methylation at each CpG site was quantified using the

Pyro Q-CpG software, version 1.0.9. (Qiagen). A >99% success rate of the pyrosequencing reaction was observed for the samples examined.

RNA extraction. RNA was isolated from cultured cells using the miRvana miRNA Isolation Kit (Ambion, Inc., Austin, TX) following the manufacturer protocol. Extracted RNA was quantified using a ND-1000 spectrophotometer and stored at -80ºC.

Quantitative RT-PCR (qRT-PCR). Gene expression was measured using commercially available TaqMan Gene Expression Assays (Applied Biosystems, Valencia, CA) on an Applied Biosystems 7500 Real Time PCR system and analyzed with 7500 System Software. All reactions were run in triplicate, with GAPDH serving as a referent. In addition, a no-RT control was run with each plate.

Cell Culture. The human choriocarcinoma cell line JEG-3 was cultured in Eagle's minimal essential medium (MEM) (ATTC, Manassas, VA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. JEG-3 cells were treated with the inhibitor of DNA methylation, 5-aza-2'deoxycytidine (Sigma-Aldrich), using doses of 1.25μM and 2.5μM in DMSO for 7 days, based on previously published studies.[33] Media containing DMSO vehicle only was used as the mock treatment. All experimental and mock exposures were done in biological triplicate.

Statistical Analysis. Differences in covariates between birthweight groups (SGA, AGA, and LGA) were assessed by ANOVA for continuous variables and chi-square test for categorical variables. Pearson correlation coefficients and corresponding p-values were used to examine the correlation of each of the covariates with log GR mean methylation extent. Univariate associations between birthweight status and methylation extent were assessed using the nonparametric Kruskal-Wallis test, with Bonferroni correction to

control for multiple comparisons. Controlling for potential confounders, multivariable linear regression modeling was used to examine the association between DNA methylation (log-transformed to fit the normality assumptions of the model) and birthweight. Covariates were added to the model because of their potential as confounders of birthweight. Two-tailed t-tests were used to analyze gene expression of 5-aza-2'deoxycytidine exposed cells compared to control cells. Data were analyzed by SAS 9.1 and R.

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Table 1. Demographics of the study population

	Total (n=480)	**SGA** (n=102)	**AGA** (n=343)	**LGA** (n=35)	***P****
Birthweight in g, mean (sd)	3035.6 (671.5)	2369.8 (396.1)	3118.7(553.5)	4161.1 (320.2)	<0.0001
Gestational age in wks, mean (sd)	38.3 (2.1)	38.0 (2.2)	38.3 (2.1)	38.7 (1.2)	0.185
Maternal age in yrs, mean (sd)	28.2 (5.8)	27.6 (6.0)	28.3 (5.8)	28.7 (4.9)	0.488
Infant gender, n (%)					0.102
Male	236 (49%)	43 (42%)	179 (52%)	14 (40%)	
Female	243 (51%)	59 (58%)	163 (48%)	21 (60%)	
Maternal race, n (%)					0.215
White	280 (58%)	49 (48%)	209 (61%)	22 (63%)	
Black	50 (10%)	14 (14%)	33 (10%)	3 (9%)	
Hispanic	105 (22%)	27 (26%)	69 (20%)	9 (26%)	
Asian	27 (6%)	8 (8%)	19 (6%)	0 (0%)	
Other/Unknown	18 (4%)	4 (4%)	13 (3%)	1 (2%)	
Alcohol during pregnancy, n (%)					0.153
No	477 (99%)	100 (98%)	342 (100%)	35 (100%)	
Yes	3 (1%)	2 (2%)	1 (0%)	0 (0%)	
Tobacco during pregnancy, n (%)					0.007
No	433 (90%)	84 (82%)	315 (92%)	34 (97%)	
Yes	47 (10%)	18 (18%)	28 (8%)	1 (3%)	

	Total	**SGA**	**AGA**	**LGA**	***P****
	(n=480)	(n=102)	(n=343)	(n=35)	
Recreational drugs during pregnancy, n (%)					0.511
No	471 (98%)	99 (97%)	337 (98%)	35 (100%)	
Yes	9 (2%)	3 (3%)	6 (2%)	0 (0%)	
Prenatal vitamin use, n (%)					0.249
No	95 (20%)	18 (18%)	74 (22%)	3 (9%)	
Yes	385 (80%)	84 (82%)	269 (78%)	32 (91%)	
Delivery method, n (%)					0.0004
C-section	165 (34%)	34 (33%)	108 (31%)	23 (66%)	
Vaginal	315 (66%)	68 (67%)	235 (69%)	12 (34%)	
Maternal gestational diabetes, n (%)					0.202
No	460 (96%)	96 (94%)	332 (97%)	32 (91%)	
Yes	20 (4%)	6 (6%)	11 (3%)	3 (9%)	

Supplemental Table 1. Pearson correlation coefficients and corresponding P-values examining the correlation of each of the covariates with log GR mean methylation extent.

	Correlation coefficient	p-value
Birthweight in g	0.161	0.0004
Gestational age in wks	0.075	0.099
Maternal age in yrs	-0.064	0.158
Infant gender	-0.010	0.833
Maternal race	0.013	0.768
Alcohol during pregnancy	-0.058	0.204
Tobacco during pregnancy	-0.019	0.687
Recreational drugs during pregnancy	-0.024	0.606
Prenatal vitamin use	0.017	0.705
Delivery method	0.089	0.052
Maternal gestational diabetes	0.033	0.468

Figure 1.

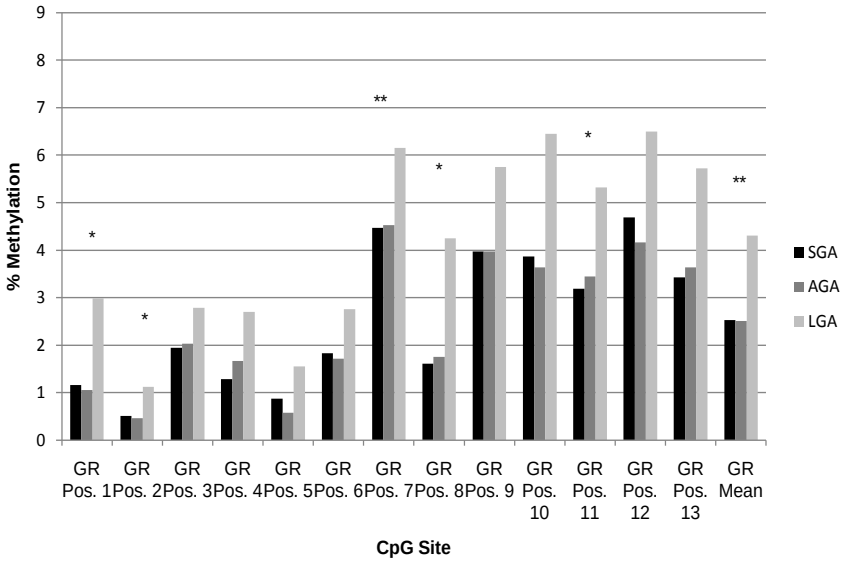


Figure 1. Comparison of the mean extent of methylation of the 13 CpG sites in the GR exon 1F promoter region in SGA (n=102), AGA (n=343), and LGA (n=35) placentas determined by bisulfite pyrosequencing. Error bars represent standard error. * indicates $p<0.05$ and ** indicates significant differential methylation following Bonferroni correction ($p<0.0036$).

Figure 2.

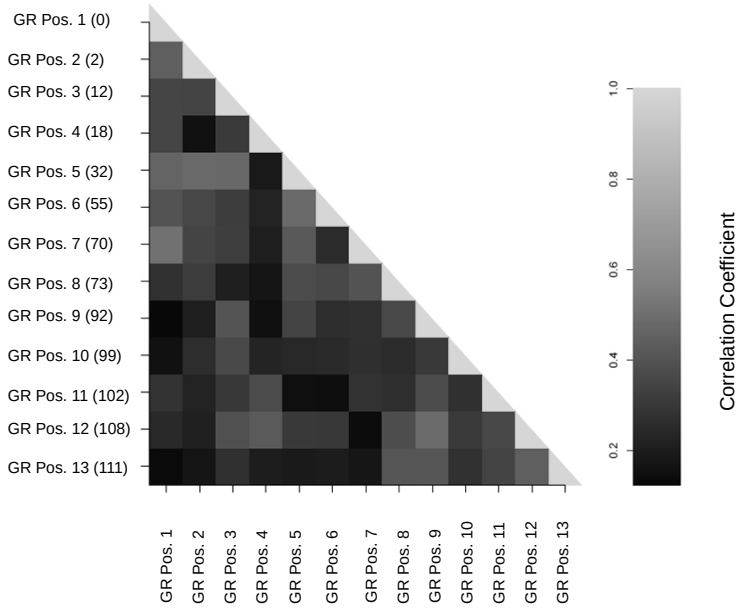


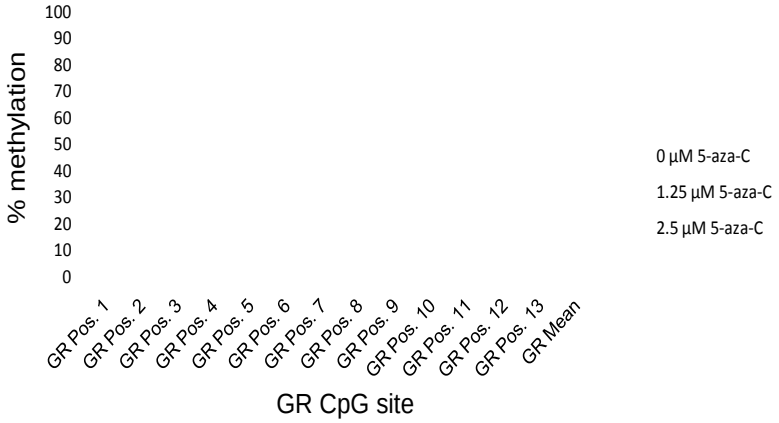
Figure 2. Correlation matrix describing pairwise Pearson correlations of methylation status among the 13 CpG sites within the exon 1F promoter region of the GR in all placenta samples (n=480). Numbers in () indicate bp distance of specific GR Position from GR Position 1.

Table 2. Multivariable linear regression model of log-transformed mean GR methylation extent

Covariate	N	Estimate	*P*
AGA Status	327	referent	
LGA status	35	0.40	<0.0001
SGA status	101	-0.05	0.3504

Model is controlled for covariates in table as well as gestational age, maternal age, maternal ethnicity, tobacco, alcohol, and recreational drug use during pregnancy, prenatal vitamin use, method of delivery, infant gender, and diagnosed gestational diabetes.

3A



3B

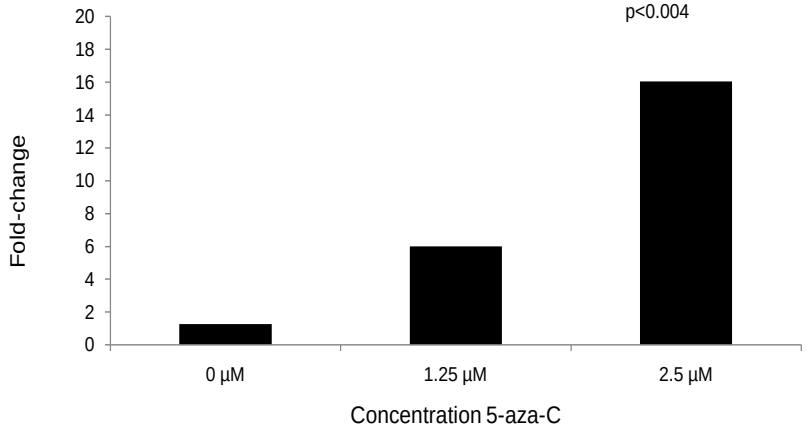


Figure 3. Examination of the effects of 5-aza-2'deoxycytidine (5-azaC) on placenta choriocarcinoma cell line JEG-3. A) Extent of methylation at CpG sites in GR exon 1F region examined by bisulfite pyrosequencing with increasing doses of 5-azaC. B) Expression of GR mRNA examined by qRT-PCR with increasing doses of 5-azaC.