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A ROLE FOR ADIPONECTIN IN TROPHOBLAST FUNCTION

BY

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Submitted to the graduate degree program in Molecular and Integrative Physiology
and the Graduate Faculty of the University of Kansas Medical Center
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy.

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II. Acknowledgements

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and shared in my joys and frustrations. A special thanks to Beth Dille and Lacey Luense, who am I lucky to count as great friends.

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IV. Abstract

As the ultimate mediator of fetal growth and well-being, the placenta is inundated with a number of molecular cues from both the maternal and fetal systems. Successful integration of these signals is critical to the ongoing health of the pregnancy, and may have important implications for the future health of both mother and child. Maternal adipose tissue releases a variety of adipokines, whose primary aim is to regulate energy metabolism. Given this, and the importance of this process in the placenta, it follows that adipokines may have profound effects on the trophoblast cells of the placenta. However, the influence of most of these adipokines on the placenta remains poorly understood. We examined the role the adipokine, adiponectin, has on the human placenta using isolated trophoblasts collected from healthy term pregnancies. Treatment of these cells with adiponectin in culture inhibited the production of human chorionic gonadotropin (hCG), placental lactogen and progesterone, all hormones produced by the placenta that play a key role in the continued health and viability of the pregnancy. In addition, adiponectin induced a pro-inflammatory environment in trophoblast cells, with increases in production of both interleukin (IL)-1β and IL-8. We have gone on to show that adiponectin can mediate its actions on trophoblast cells through the adaptor protein APPL1, the signaling molecules mitogen-activated protein kinases (MAPKs), and the epidermal growth factor (EGF) receptor. Finally, we examined the effects of adiponectin on gene expression by term trophoblasts. These data have highlighted a number of additional processes influenced by adiponectin, including insulin-like growth factor bioavailability and cortisol metabolism. The implications for these effects at the maternal-fetal interface are great. From these data, we hypothesize that adiponectin

works to maintain the balance between the energy demands of the fetus and the needs of the mother, with specific functions at the placenta that appear to favor the mother. Such a role is increasingly critical as gestation progresses and fetal demands continue to increase. With the continued prevalence of metabolic health diseases, such as obesity, it is especially important to understand the influences hormones such as adiponectin have on the placenta.

V. Chapter One:

Introduction

1. The Human Placenta

Classification

There are many different characteristics that have been used to classify placentas of different species. Based on the shape and region of maternal-fetal exchange, the human placenta is classified as a discoid placenta, meaning it contains a single zone in which intimate maternal-fetal contact is possible (Boyd and Hamilton, 1970). The human shares this type of placenta with other primates and rodents.

Based on the Grosser classification (reviewed in (Boyd and Hamilton, 1970)), the human placenta is of the hemochorial type, meaning it is composed of three layers, with trophoblast cells of fetal origin in direct contact with maternal blood. Such classification has been criticized and further refined such that during the first trimester of gestation, the human placenta can be further classified as hemodichorial, meaning there are two distinct layers, one of mononuclear cytotrophoblasts and a single layer of multi-nucleated syncytiotrophoblast cells (Enders, 1965a). By the end of gestation however, the human placenta has become hemomonochorial, with significant fusion of the cytotrophoblast layer into the syncytiotrophoblast layer.

Pre-lacunar Stage of Development

The first distinct cell lineage to differentiate during the blastocyst stage of embryonic development are mononuclear trophoblast cells. These cells surround the inner cell mass (ICM) and the blastocoel, and will eventually differentiate into a fully functional placenta. Placental development is considered to begin at the initiation of

blastocyst implantation into the uterine epithelium (6-7dpc) (Huppertz, 2008). At this point the trophoblast cells apposed to the inner cell mass (i.e. the polar trophoblast cells) gain contact with the uterine lining (Boyd and Hamilton, 1970). After attachment of the blastocyst, the polar trophoblast cells proliferate to form two layers. The layer most distal to the ICM then differentiates to a multi-nucleated syncytiotrophoblast. During this period the syncytiotrophoblast is invasive, allowing the blastocyst to penetrate into the uterine epithelium.

Lacunar Stage

On day 8 following conception (8 dpc) fluid-filled spaces begin to form within the syncytiotrophoblast and merge to create lacunae (Boyd and Hamilton, 1970). The lacunae as well as the syncytiotrophoblast layers that separate them, known as the trabeculae, will eventually form the villous tree structures of the mature placenta. By 12 dpc the blastocyst has invaded so deeply that the uterine epithelium closes over the site of implantation, allowing differentiation of a multinucleated syncytium completely surrounding the blastocyst (Benirschke and Kaufmann, 1990). Although this syncytium and the underlying cytotrophoblast cells are proliferative, they will subsequently regress, creating the smooth membranes of the fetal compartment. The three "zones" of the placenta can now be observed: the chorionic plate, a region of mononuclear cytotrophoblasts facing the embryo; the primitive villous region containing lacunae and trabeculae; and the trophoblastic shell, the attachment site of the developing placenta in contact with the maternal endometrium.

Beginning on 12 dpc, cytotrophoblast cells invade the syncytiotrophoblast trabeculae, reaching maternal tissue by 15 dpc (Huppertz, 2008). From this point

onward, the cytotrophoblasts, now termed extravillous trophoblasts, are responsible for all of the invasive characteristics of the placenta. Furthermore, a subset of these invasive cells, the endovascular trophoblasts, will reach maternal blood vessels and replace the endothelial cells of these vessels to transform the uterine spiral arteries.

Villous Stage

Beginning around 13 dpc, the trabeculae begin to branch out, forming primary villi that reach into the lacunae, henceforth referred to as intervillous space. Extraembryonic mesodermal cells then begin to invade as well, following the path of the cytotrophoblast cells through the trabeculae. The mesodermal cells do not invade all the way into the maternal space as the cytotrophoblasts do, and therefore fill the trabeculae and primary villi, leaving cytotrophoblast cells alone to form columns in the area of the trabeculae closest to the maternal endometrium. Invasion of mesodermal cells into the primary villi establishes a mesodermal core. Once a mesodermal core is established in a villous it is classified as a secondary villous. Mesodermal cells of the secondary villi begin to develop a vascular system that is independent from the vascular system of the embryo proper. With the development of vasculature, the villi are then classified as tertiary villi. Those villi that maintain their contact with the trophoblastic shell are referred to as anchoring villi, with the remaining classified as floating villi, “floating” in the intervillous space.

Villous types

The body of the placenta is made up of the villous trees. A mature placenta contains an average of 60-70 villous trees (Huppertz, 2008). The villi are widely classified into one of five categories (Kaufmann et al., 1979):

1. The stem villi contain a highly fibrous stroma and large arteries and veins. They have the largest diameter of all of the villi types, and provide the mechanical strength of the placenta.
2. The mature intermediate villi are derived from the stem villi. They are long and slender and are characterized by a loose stromal compartment with small blood vessels.
3. The terminal villi are the last of the villous types to develop, and are commonly thought of as the most physiologically important class of villi within the placenta. They are the final branches on the villous tree and contain a large amount of capillaries. These capillaries often dilate to the point of being in direct juxtaposition with a thin layer of syncytiotrophoblast, making the distance between maternal and fetal blood spaces the smallest of all villi.
4. The immature intermediate villi arise from the developing mesenchymal villi early on in gestation. They are large and bulbous with a large amount of stroma and relatively few arterioles and venules. The stem villi will arise from these villi, with only a few immature intermediate villi remaining at term.
5. The mesenchymal villi are the earliest villi to form in the developing placenta, and are therefore most prominent during the early stages of

gestation. They contain a weakly organized stromal compartment with rudimentary vessels.

Trophoblast types

The villous cytotrophoblast cells (also known as Langhan's cells; (Boyd and Hamilton, 1970)) are in direct contact with the basement membrane next to the fetal compartment, with their apical membrane in contact with the syncytiotrophoblast layer (Figure V-1). During the first trimester of pregnancy, the villous cytotrophoblasts form a complete layer directly below the syncytiotrophoblast. By term however, this continuity is lost, with cytotrophoblasts sporadically located below the syncytiotrophoblast. Nonetheless, the absolute number of the proliferative cytotrophoblasts is greater at term compared to the first trimester. By term, the amount of syncytium has grown to the extent that the cytotrophoblasts no longer make a complete monolayer beneath it. Cytotrophoblasts serve as progenitor cells for all other trophoblast subtypes within the placenta, and allow for regeneration of the various subtypes throughout gestation.

The syncytiotrophoblast is the multinucleated continuous layer that covers the surface of the villi, and is the layer responsible for nutrient and gas exchange between the fetal and maternal compartments (Figure V-1). The apical surface of the syncytiotrophoblast is also covered in microvilli, increasing the surface area exposed to the intervillous space by about 7-fold (Huppertz, 2008). The syncytiotrophoblast layer does not proliferate, and thus relies entirely on the underlying cytotrophoblasts for regeneration throughout gestation (Enders, 1965b). The syncytium is regenerated through an apoptotic mechanism (Huppertz et al., 1999). Late-stage apoptotic nuclei

in the syncytium are condensed into syncytial knots, which are then extruded into the intervillous space (Gauster et al., 2009).

Extravillous trophoblasts arise from villous cytotrophoblasts as soon as the cytotrophoblast loses contact with the basement membrane (Huppertz, 2008). Once the initial invasion process has occurred during implantation, the extravillous trophoblast cells are the only cells that display invasive characteristics. These cells are responsible for the remodeling of spiral arteries and cross-talk with maternal immune cells.

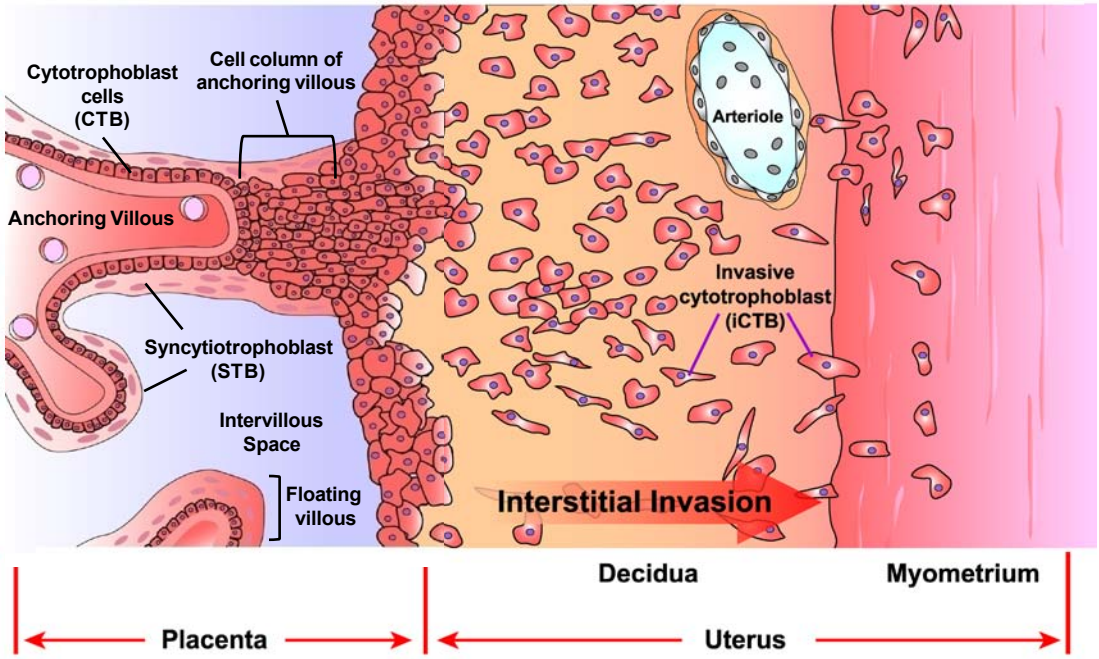
Placental Anatomy

At term, the mature placenta is composed of the basal plate, the villous lobes, and the chorionic plate. The basal plate is the section of decidua that is delivered along with the placenta. This region contains a variety of different cell types, including extravillous trophoblast cells, maternal immune cells, and stroma. Along the edge of the placenta the basal plate becomes continuous with the chorionic plate from the fetal side of the placenta to form the smooth membranes that surround the fetus during gestation (Huppertz, 2008). These are composed of the amnion, the chorion, and the decidua capularis (Benirschke and Kaufmann, 1990). The amnion contains a single layer of amniotic epithelial cells and the amniotic mesenchyme, while the chorion is composed of chorionic mesenchyme and cytotrophoblast cells.

Figure V-1. **Schematic representation of the first trimester human placenta.**

The human placenta is composed of villous cytotrophoblast cells (CTB), from which both syncytiotrophoblast and extravillous, invasive cytotrophoblast cells arise. Villous cytotrophoblasts lie directly under the syncytial layer, and thereby regenerate it by fusing with its basal membrane. Other villous cytotrophoblasts stream down the anchoring villi, becoming invasive cytotrophoblasts. These cells will subsequently invade deep into the maternal tissue and remodel uterine spiral arteries.

Figure V-1.


Cytotrophoblast cells (CTB)
Cell column of anchoring villous
Anchoring Villous
Syncytiotrophoblast (STB)
Intervillous Space
Floating villous
Arteriole
Invasive cytotrophoblast (iCTB)
Interstitial Invasion
Decidua
Myometrium
Placenta
Uterus

Endocrine Function

Human chorionic gonadotropin (hCG) is one of the predominant hormones produced by the syncytiotrophoblast, and allows for the maternal recognition of pregnancy in the human. It is widely used as a marker of pregnancy, as it can be detected in the urine of pregnant women very early on in gestation. The hormone is a member of the glycoprotein family of hormones, and is composed of the common α-subunit and a β-subunit unique to hCG. It is very similar in sequence and function to luteinizing hormone (LH), and the two hormones share the same receptor. Its primary function during pregnancy is to prevent lysis of the corpus luteum. Production of hCG peaks around 8-10 weeks of pregnancy, after which the placenta is producing large amounts of progesterone, and thus the corpus luteum is no longer needed. The production of hCG declines to a relative low level for the rest of gestation.

Progesterone production by the placenta begins almost immediately after implantation, however until about the 10^{th} week of gestation progesterone production by the corpus luteum of the ovary is the primary source of the hormone. Progesterone levels produced by the placenta continue to rise throughout gestation. Progesterone serves a number of functions throughout gestation, including priming the uterus for implantation and decidualization of the endometrium (Ramathal et al., 2010). Progesterone secretion also maintains uterine quiescence throughout gestation by inhibiting myometrial contractions. In addition, the high levels of progesterone produced by the placenta causes the production of gelatinous secretions from the cervix, which may help deter pathogens from entering the uterus (Hedge et al., 1987).

Progesterone of placental origin is also important in priming the mammary glands for lactation following parturition.

Similar to progesterone, estrogens are produced in very high amounts during gestation, and continue to rise as gestation progresses. Unlike during the menstrual cycle, the primary estrogen produced by the syncytiotrophoblast is estriol. Estriol is a relatively weak estrogen, but nevertheless is instrumental in mammary gland development throughout gestation (Hedge et al., 1987). The placenta lacks the steroidogenic enzyme 17α-hydroxylase/C_{17-20} lyase, making it incapable of producing DHEA from pregnenolone, a crucial step in estrogen biosynthesis (Villee, 1969). The fetal adrenal is thus responsible for conversion of pregnenolone to DHEA, which is sulfated and hydroxylated by the fetal liver (Villee, 1969). The resulting 16α-OH-DHEA is then transported to the placenta, which can convert it to estriol through the actions of 3β-hydroxysteroid dehydrogenase (3βHSD), 17βHSD, and aromatase. In this manner the production of estrogens by the placenta is intimately associated with the development of the fetus as well.

The placenta also produces the protein hormone placental lactogen (hPL), which is similar in structure to growth hormone. Placental lactogen production increases steadily throughout gestation, until reaching a plateau around the 35th week (Hedge et al., 1987). In a manner similar to growth hormone, hPL has growth promoting and lactogenic activities, although its potency is significantly less than that of growth hormone. It is thought that hPL may play an important role in mediating glucose utilization by the mother, as the placenta and fetus continue to place increasing demands for energy on the mother as gestation progresses. Indeed, it has

been suggested that hPL is the primary hormone responsible for mediating the insulin resistance that occurs during normal late gestation, in order to meet the high demands of the fetus (Handwerger and Freemark, 2000).

Immune Evasion

The placenta serves two very important, if somewhat discordant, functions with regards to immunological protection of the fetus; 1) protection of the allogeneic fetus from the immune system of the mother, and 2) passive immunity through the transfer of select maternal immunoglobulins. The avoidance of maternal immune activation during implantation is achieved in part through expression of specific human leukocyte antigens (HLAs) on the part of the trophoblast cells.

Extravillous trophoblasts only express HLA-C, -G, and -E, thus avoiding the highly polymorphic (and consequently highly prone to immune activation) HLA-A and -B. In addition, the first trimester decidua contains relatively few cytotoxic T cells. Instead, the predominant immune cell type in the early decidua is uterine natural killer (uNK) cells. These specific uNK cells express killer inhibitory receptors (KIRs), which can recognize the specific HLAs expressed by the trophoblast (Moffett-King, 2002). Binding of HLA-C to a specific haplotype of KIR on uNK cells will activate the uNK cell to increase cytokine production, rather than behave as a killer cell (Moffett-King, 2002). Studies have also shown that HLA-G is expressed by extravillous trophoblast cells, and can also act as a pro-gestation signal by activating uNK cells to increase cytokine production.

In contrast to extravillous trophoblasts, the syncytiotrophoblast do not express HLA-C, in order to avoid maternal immune recognition (Clark et al., 2010). In this

manner, it is thought that the initial invasion by syncytiotrophoblasts early in implantation may allow for a certain level of protection of the embryo.

The placenta also allows for the selective transport of maternal IgG, equipping the fetus with a low level of passive immunity. The placenta forms a barrier to transfer of all other immunoglobulins however. The human placenta transfers $IgG_{1, 3,}$ and $_4$ subclasses to such an extent that the levels of these in fetal circulation can reach or surpass levels in maternal serum (Lanman, 1975). All of these are weakly cytotoxic, allowing for low levels of immunity. Although the mother will also pass immunoglobulins to the infant through the delivery of colostrum in the early stages of lactation, the placenta aids in this passive immunity through the transfer of IgG across its surface and into the fetal compartment.

Nutritional Support

The placenta serves as the sole pipeline for nutritional support and gas exchange of the developing fetus. As such it is critically important to the maintenance of a healthy pregnancy. This process can be complicated by suboptimal nutritional conditions of the mother. Indeed, there is increasing interest in the effect of maternal energy irregularities (e.g. maternal obesity) on long-term fetal health, extending past gestation and affecting the offspring's health throughout its lifetime. Regulation of the placenta's response to such stresses may have a profound effect on the subsequent programming of the fetus. The placenta is responsible for moving a large number of different molecules across its surface, and consequently has developed a variety of different mechanisms and specific transporters to deal with each of these. Discussed

below are some of the major molecules that need to be made available to the fetus as it continues to grow and develop.

With respect to transfer of nutrients across the placenta, the human appears to be relatively efficient in that the terminal villi, where most of the nutrient transfer occurs, is composed of only a thin layer of syncytiotrophoblast separating the maternal and fetal blood flows (Kaufmann et al., 1979). As such, the exchange rate of electrolytes such as sodium, which largely utilize the principle of diffusion based on concentration gradients, increases steadily as gestation progresses and the syncytiotrophoblast surface area increases (Dancis and Schneider, 1975).

In the case of iron, the placenta must utilize an active transport mechanism to direct the fetus' high requirements for iron against a concentration gradient. By the final trimester of gestation, the fetus requires a large amount of iron, such that over 90% of the iron in maternal circulation will be transferred through the placenta to the fetus, with no transfer in the opposite direction (Dancis and Schneider, 1975).

Glucose is the primary energy source for the developing fetus. Transfer of glucose across the syncytiotrophoblast occurs through the actions of a specific isoform of the glucose transporters, GLUT1. GLUT1 is expressed on both the apical microvilli and on the basal surface of the syncytiotrophoblast, with a higher level of expression on the microvilli side (Jansson et al., 1993, Takata et al., 1992), suggesting this isoform may play a predominant role in uptake of glucose from the maternal circulation. In addition, the fetal arteriole endothelium expresses the GLUT3 isoform, suggesting that it functions to transfer glucose to the fetal circulation, and thus further increase the fetal:maternal glucose ratio (Hauguel-de Mouzon et al., 1997). There has

been one report of expression of the insulin-sensitive isoform, GLUT4, in the cytosol of the first trimester syncytiotrophoblast (Ericsson et al., 2005), although the physiological relevance of this is debated (Jansson et al., 1993). The increased density of GLUT1 on the microvillous side of the syncytiotrophoblast serves to move a large amount of glucose into the syncytium in order to serve the placenta as well as the fetus. Neither GLUT1 nor GLUT3 are responsive to insulin, which promotes a steadier influx of glucose into the placenta (Illsley, 2000). As such, glucose levels in the fetus at any given time can be directly correlated with the levels of glucose in maternal circulation (Illsley, 2000).

Amino acids are transported across the placenta through active transport mechanisms. Although some of the transporters utilize ATP or sodium, many of them exchange one amino acid for another across the membrane. It is thought that both the transporters that accumulate and those that exchange amino acids work together to attain the appropriate amount and specificity of amino acids (Cleal and Lewis, 2008). Although amino acid transport across the microvilli and into the syncytiotrophoblast is understood to occur largely through the work of amino acid accumulator and exchange transporters, the mechanism(s) by which amino acids leave the syncytiotrophoblast through the basal membrane are poorly understood. There is evidence to suggest that another family of amino acid transporters that function as facilitated transporters are at work on the basal membrane, to push the amino acids to the fetal compartment against a steep concentration gradient (Cleal and Lewis, 2008).

In contrast to the more complex, regulated mechanisms employed in the transport of glucose and amino acids, the placenta is thought to exchange gas through

simple diffusion. Although the gases, primarily oxygen and carbon dioxide, will favor diffusion to the compartment with the lowest concentration, there are some complicating factors that favor the movement of oxygen to the fetal compartment. Fetal hemoglobin has a higher affinity for oxygen than does adult hemoglobin, and therefore the levels of dissolved oxygen in the maternal blood may be higher than in fetal blood. In addition, movement of oxygen to the fetal compartment is favored by the simultaneous transfer of carbon dioxide from the fetal to the maternal compartment, through the Bohr Effect phenomenon, which will decrease maternal hemoglobin's affinity for oxygen as more carbon dioxide enters the maternal circulation (Dancis and Schneider, 1975). The remodeling of the spiral arteries also allows for low resistance blood flow in the placental compartment, further enhancing the ability of the syncytiotrophoblast to efficiently exchange the necessary nutrients, gases, etc.

2. Diseases of Pregnancy

Due to the intimate association between a mother and her developing fetus, damage to her health can also be detrimental to the fetus. Therefore, a discussion of the diseases that affect pregnancy could by quite extensive. In this section however, I have chosen to focus on three major diseases that are uniquely related to pregnancy, i.e. they do not manifest until pregnancy is attained.

Gestational Diabetes

The incredible energy demands placed on the mother as gestation advances usually result in some degree of maternal insulin resistance (reviewed in (Lain and

Catalano, 2007)). In approximately 7% of pregnant women however the degree of insulin resistance is exacerbated, resulting in gestational diabetes (Reece, 2008). Gestational diabetes mellitus (GDM) is largely a disease of late gestation, as the mother's metabolism early in gestation is primarily anabolic, that is she stores energy to accommodate the forthcoming demands of the fetus. This switches to a metabolism that is profoundly catabolic during the latter part of gestation, as the fetus grows dramatically and places increasing energy demands on the mother (Lain and Catalano, 2007).

The normal amount of insulin resistance that occurs in a late-gestation pregnant woman is exacerbated in women with GDM, not only by down-regulation of the insulin signaling protein, insulin receptor substrate (IRS)-1, but also a decreased ability of the β component of the insulin receptor to undergo tyrosine phosphorylation and perpetuate the insulin signal intracellularly (Lain and Catalano, 2007). Women with GDM are also thought to have decreased pancreatic β-cell function relative to women that maintain normal glucose tolerance throughout pregnancy, and thus, while able to respond to the increased need for insulin, are unable to do so to an adequate extent (Lain and Catalano, 2007). Development of GDM also puts the mother at a significantly higher risk of becoming diabetic later in life, and her risk of developing a number of complications including heart disease, stroke, and hypertension increases substantially (Metzger, 2007).

A number of maternal hormones and cytokines are known to be altered in GDM, including the metabolic hormone adiponectin (Altinova et al., 2007, Ranheim et al., 2004, Retnakaran et al., 2007, Tsai et al., 2005, Williams et al., 2004, Cseh et

al., 2004, Georgiou et al., 2008, Gao et al., 2008, Lain et al., 2008, Cortelazzi et al., 2007). Other hormones and cytokines such as leptin, tumor necrosis factor-α (TNF-α) and adipocyte fatty acid binding protein (AFABP) have been shown to be increased in GDM, even before the levels of maternal glucose are significantly higher than normal controls (Gao et al., 2008, Kralisch et al., 2009). The hormone placental lactogen has been suggested to be involved in mediating insulin resistance during pregnancy due to it similarities to growth hormone, and has also been found to be elevated in the serum of women with GDM (Ursell et al., 1973). Additionally, human placental growth hormone, a hormone in the same family as hPL, has been shown to induce insulin resistance in non-pregnant mice (Barbour et al., 2002).

The occurrence of GDM has been associated with induced placental expression of a number of genes, predominantly ones involved in stress and inflammatory responses, although other genes involved in metabolism and endothelial differentiation are also altered in GDM placentas (Radaelli et al., 2003). These data point to the induction of an inflammatory response and remodeling of the placental vasculature during GDM, both of which could have profound effects on the development of the fetus.

Gestational diabetes can have deleterious effects on both the mother and fetus, frequently resulting in a macrosomic fetus and delivery complications (Gilbert, 1949, The Hapo Study Cooperative Research Group, 2008). Pregnancies complicated by GDM frequently result in a fetus that is hyperinsulinemic (Desoye and Hauguel-de Mouzon, 2007). This is thought to be a result of high levels of glucose in maternal circulation, and not a response to increases in placental transporter expression.

Although the syncytiotrophoblast expresses high levels of insulin receptor, this is predominantly early in gestation, and the receptor density pattern shifts from the microvillous membrane of the syncytiotrophoblast during the first trimester, to the villous endothelial cells by term (Desoye et al., 1997). Thus, insulin's control over the placenta appears to be primarily during the first trimester (Hiden et al., 2006).

The transition of insulin receptor expression to the endothelium toward the end of pregnancy may have profound implications in gestational diabetes in particular, allowing the abnormally high levels of insulin being produced by the fetus to modify placental behavior as well. Increasing amounts of fetal insulin will also increase fetal demand for oxygen, as aerobic glucose metabolism is stimulated (Desoye and Hauguel-de Mouzon, 2007). This in turn will stimulate growth of the placental endothelium and the syncytiotrophoblast surface area (Desoye and Hauguel-de Mouzon, 2007). Indeed, an enlarged placental weight is a characteristic of GDM, and this may be one mechanism by which a GDM fetus becomes macrosomic.

Although GLUT1 expression on the microvillous membrane of the syncytiotrophoblast does not change dramatically during GDM, GLUT1 expression on the basal membrane of the syncytiotrophoblast increases during cases of GDM, possibly serving as another mechanism by which the fetus receives increased levels of glucose during GDM (Gaither et al., 1999). In addition, increases in the insulin-like growth factors (IGF)-1 and -2 are also observed in cases of GDM (Hiden et al., 2009). Increased IGF-1 and -2 can lead to increases in expression of amino acid transporters on the placenta, which in turn may explain the increased levels of amino acids in fetus' from GDM pregnancies (Cetin et al., 2005).

The obesity epidemic in the developed world serves to further increase the number of women who develop this serious and relatively common disorder during pregnancy. Indeed, the risk of gestational diabetes mellitus (GDM) rises 5.2 fold in obese women versus women of normal body mass index (BMI; (Baeten et al., 2001)).

A growing body of evidence suggests that GDM may put the fetus at an increased risk of metabolic disorders such as diabetes and obesity later in life (Reece, 2008). Although GDM poses a grave threat to both mother and fetus, we have only a rudimentary understanding of the molecular events that are altered during GDM. It is especially important that the effect of GDM on the placenta be evaluated in more depth, as the placenta is the sole mediator of interaction between mother and fetus.

Intrauterine Growth Restriction

A fetus that is pathologically small, i.e. not simply small for gestational age (SGA), but is malformed or malnourished, will often be diagnosed with intrauterine growth restriction (IUGR). IUGR is second only to prematurity as the leading cause for perinatal mortality (Baschat et al., 2007). The most widely used definition for IUGR is a fetus with an estimated weight below the 10^{th} percentile for its gestational age with an abdominal circumference below the 2.5^{th} percentile. IUGR can be classified as either symmetric or asymmetric based off of the pattern of growth. Asymmetric growth is diagnosed when the fetus directs most of its available nutrients to the growth of vital organs, and thus may have a normal size head, but small abdominal girth and small limbs. Asymmetric growth may be a consequence of insufficient transfer of nutrients by the placenta.

Although many times IUGR can be attributed to environmental and/or genetic factors, there are a significant number of IUGR cases that remain idiopathic. Of those incidences when IUGR can be related to a specific cause, it is often a maternal vascular disease, such as hypertension or thrombophilia (a congenital condition of overactive coagulation), that decreases placental blood flow (Baschat et al., 2007). A diagnosis of IUGR has been associated with increased levels of interleukin-6 (IL-6) in placental lysates taken at term, as well as a decrease in umbilical cord serum levels of adiponectin (Street et al., 2009). While such factors may play a role in the etiology of IUGR, they may also be a manifestation of an underlying cause for a maternal-placental environment that is suboptimal for fetal growth. Inadequate maternal nutrition, and especially limited protein intake, is widely recognized as a risk factor for IUGR (Baschat et al., 2007). In addition, maternal factors such as drug use, including smoking and alcohol use, have been implicated in the onset of IUGR. This is thought to occur not only through an associated inadequate intake of nutrients, but also as an independent factor (Baschat et al., 2007).

The mother however is not always the sole culprit responsible for IUGR. Chromosomal abnormalities, intrauterine infections, and congenital malformations also account for a fraction of IUGR cases (Baschat et al., 2007). Increased demands on the placenta, such as in twin pregnancies, may also result in IUGR. The majority of IUGR cases arise from placental insufficiency resulting from a malformed placenta. Placental insufficiency can be due to a large number of placental malformations including premature placental abruption (detachment of the placenta from the uterine wall), persistent villous immaturity, development of only one

umbilical artery, placenta accreta (overly deep invasion of the placenta and attachment to the uterine myometrium), or complicating diseases, such as preeclampsia (Benirschke and Kaufmann, 1990).

A diagnosis of IUGR has been suggested to be associated with a number of alterations at the level of the placenta affecting the movement of nutrients to the fetal compartment. Two members of the triglyceride lipase gene (TLG) family, endothelial lipase (EL) and lipoprotein lipase (LPL), have recently been shown to be altered in cases of IUGR (Gauster et al., 2007). The TLG family is involved in the release of fatty acids from lipoproteins after they have been transported across the cell membrane. The authors of this study identified a decrease in EL, the predominant TLG expressed on trophoblasts and placental endothelial cells, and an increase in LPL expression. These data suggest altered efficiency of fatty acid transport may play a role in the etiology of IUGR. In addition, a decrease in the expression of Na^+/K^+ ATPase on the microvillous membrane of syncytiotrophoblast is reported to be associated with IUGR (Johansson et al., 2003). This could lead to a decrease in a major mechanism of nutrient transfer in the syncytiotrophoblast that relies on a sodium gradient for transport across the membrane.

In addition to reduced and abnormal weight gain by the fetus affected by IUGR, the condition may represent a number of underlying problems affecting normal development of the fetus. These include cardiovascular, metabolic, endocrine and hematologic abnormalities (Baschat et al., 2007). Insufficient oxygen and glucose uptake through the placenta will mobilize the fetal liver to release glycogen stores. If this is continued, it will eventually lead to a reduction in amino acid transport across

the placenta, which will stimulate the breakdown of muscle in the fetus. A decrease in glucose and amino acids will also down-regulate fetal production of insulin and IGF-1 and -2, while the hormone associated with stress, cortisol, increases (Baschat et al., 2007). Persistence of these metabolic and endocrine changes may program the fetus toward a propensity for altered metabolic responses later in life.

As is the case for many diseases of pregnancy, IUGR has also been associated with the manifestation of diseases later in the life of the offspring. These include an increased risk of type 2 diabetes mellitus, hypertension, and cardiovascular disease (Valsamakis et al., 2006). Using a sheep model, Duffield and colleagues have demonstrated that IUGR can alter adipocyte growth and gene expression after birth (Duffield et al., 2009). These data may have important implications relating to obesity and the associated negative health outcomes in children diagnosed with IUGR during gestation.

Preeclampsia

Preeclampsia is a disease of pregnancy characterized by maternal hypertension and proteinuria, thought to be due to shallow placentation. It affects up to 3% of women, and, left untreated, can result in serious complications for mother and child, including, in rare instances, death (Redman and Sargent, 2005). Risk factors for the condition include preexisting insulin resistance, previous diagnosis of preeclampsia, a high BMI, high blood pressure, nulliparity, a twin pregnancy, a family history of preeclampsia, advanced maternal age, and large amounts of physical activity (Østerdal et al., 2009, Solomon and Seely, 2001, Duckitt and Harrington, 2005). In addition, a women's risk of developing preeclampsia decreases with

previous exposure to the paternal antigens, either through a previous pregnancy or a long period of co-habitation (Lie et al., 1998, Trupin et al., 1996). Long-term consequences for mother and child have been suggested as well, and multiple studies have demonstrated an increased risk of cardiovascular disease and death in mothers that had a preeclamptic pregnancy, although this may be attributable to an increased prevalence of metabolic and/or hypertensive diseases in the study populations as well (Irgens et al., 2001, Manten et al., 2007). The only known cure for the disease is delivery of the placenta, although treatments such as antioxidant vitamins, hypertension medications, and magnesium sulfate (to treat maternal seizures in cases of severe preeclampsia) have been suggested as possible mechanisms to treat the condition without the requirement of delivery of the child (Sibai, 2003, Redman and Sargent, 2005). Like gestational diabetes mellitus, preeclampsia is typically a disease of the later stages of pregnancy. However, it can be diagnosed anytime after the 20^{th} week of gestation, and the disease tends to be more severe the earlier in gestation it is diagnosed.

Preeclampsia arises following the onset of endothelial dysfunction, whether of maternal or placental in origin (Redman and Sargent, 2005). It is thought to be the result of a hypoxic placental environment and oxidative stress caused by insufficient invasion and spiral artery remodeling by extravillous trophoblast cells (Jauniaux et al., 2003). As a result, supplementation of preeclamptic women with high levels of the potent antioxidants vitamins C and E has been studied. Initial trials suggested a positive outcome, with significantly less at risk women developing preeclampsia when given vitamin C and E supplements for the last half of pregnancy (Chappell et

al., 1999). However, a subsequent larger clinical trial found that women given supraphysiological doses of vitamins C and E during pregnancy do not have a lower risk of preeclampsia, and the rate of low birthweight babies in this cohort actually increased with vitamin supplementation (Poston et al., 2006). In addition, subsequent *in vitro* analysis of the effects of high levels of vitamins C and E on trophoblast cells show a diminished production of hCG coupled with increases in TNF-α production (Aris et al., 2008).

Preeclampsia is thought to be a by-product of insufficient trophoblast invasion of the maternal endometrium, leading to poor remodeling of the spiral arteries and a subsequent inability of the maternal blood supply to keep up with the increasing demands for oxygen as the placenta grows (Redman and Sargent, 2005). Increases in the amounts of soluble fms-like tyrosine kinase receptor-1 (sFlt-1) and soluble endoglin in serum and amniotic fluid from preeclamptic patients has been described (Levine et al., 2006, Levine et al., 2004, Jeyabalan et al., 2008, Masuyama et al., 2007, Wang et al., 2010). In addition, culture of trophoblast cells from normal and preeclamptic women show increased amounts of sFlt-1 and sEndoglin secretion from preeclamptic trophoblasts compared to healthy control cells (Gu et al., 2008). Both sFlt-1 and sEndoglin are antiangiogenic, and thus are hypothesized to block the angiogenic effects of vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and transforming growth factor (TGF)-β, consequently leading to the characteristic maternal hypertension of preeclampsia. It has been suggested that signaling involving calcineurin and angiotensin II receptor from activated T cells can lead to the overproduction of sFlt-1 as well (Zhou et al., 2008).

Due to the inflammatory environment often associated with preeclampsia, many researchers have investigated a variety of pro-inflammatory stimulators within the context of preeclampsia (Visser et al., 2007). Treatment of pregnant rats with polyinosinic:polycytidylic acid (poly I:C), an activator of toll-like receptor 3 (TLR3), results in elevated systolic blood pressure in these rats compared to controls, suggesting TLR3 activation may play a role in the preeclamptic phenotype (Tinsley et al., 2009). Other suggestions include an excessive turnover of the syncytiotrophoblast, leading to a large number of synctial knots being released into the intervillous space and a subsequent inflammatory response (Gauster et al., 2009). In contrast, impaired cellular fusion, leading to increased apoptosis rates in cytotrophoblasts has also been suggested to lead to the inflammatory response in preeclampsia (Langbein et al., 2008). Increased decidual production of IL-6, perhaps as part of the pro-inflammatory response commonly associated with preeclampsia, has also been described (Lockwood et al., 2008).

Insulin-like growth factors (IGFs) are known to have profound effects on glucose mobilization and metabolism by the fetoplacental unit during gestation. Studies suggest that the syncytiotrophoblast and maternal decidua can increase expression of the protease that cleaves IGF-binding proteins (PAPPA2) during preeclampsia, thus increasing availability of IGFs for signaling at the placenta in an effort to increase glucose and amino acid transfer and metabolism (Nishizawa et al., 2008, Winn et al., 2009).

Despite these findings, the etiology of preeclampsia remains unclear, and a variety of potential instigators of the condition have been proposed. Some of the most

compelling evidence regarding the development of preeclampsia however involves the recognition of HLA-C, expressed by extravillous trophoblasts by specific KIR haplotypes on uNK cells. These data suggest that women with low to no expression of the "AA" type carrying a fetus expressing trophoblastic HLA-C2 are at a significantly higher risk of developing preeclampsia (Hiby et al., 2004).

The maternal role in the manifestation of preeclampsia is most commonly attributed to vascular disorders or longstanding diseases such as hypertension (Ness and Roberts, 1996). As a result, the importance of careful monitoring of women who present with diseases such as hypertension or metabolic syndrome is paramount to catching the onset of preeclampsia early (Davis et al., 2007). Aberrant production of a number of adipokines, most notably leptin and adiponectin, have been associated with preeclampsia (Herse et al., 2009, Masuyama et al., 2007), although these data remain controversial (Bienertová-Va et al., 2008). Interestingly, Siglec-6, a transmembrane protein that has been shown to bind leptin, has also been shown to be increased in trophoblasts from preeclamptic patients (Winn et al., 2009).

It has been suggested that the driving force behind poor placentation outcomes resulting in IUGR and/or preeclampsia is an underlying maternal condition of metabolic syndrome (Ness and Sibai, 2006). This may certainly be one component in the manifestation of preeclampsia, however other data suggests IUGR and preeclampsia can arise from two different mechanisms. Trophoblasts taken at term from normal, IUGR, preeclamptic, or preeclamptic with IUGR placentas placed in long-term culture have been reported to have significantly different degrees of syncytialization and hCG production (Newhouse et al., 2007), with trophoblasts

affected by preeclampsia having low levels of hCG production. These data would certainly suggest that IUGR and preeclampsia arise from different mechanisms, as the trophoblast cells display behaviors distinct from one another even in an isolated *in vitro* environment.

The lack of a solid understanding regarding the origins of preeclampsia, coupled with the fact that there remains no known cure, has led many researchers to attempt to find markers for the onset of preeclampsia that physicians may use to screen at-risk mothers (Farina et al., 2009, Founds et al., 2009, Okazaki et al., 2007, Sasaki et al., 2002). To date, although a number of genes, mostly involved in invasion and inflammation have been suggested, there remains no established method for predicting the onset of preeclampsia in pregnant women.

3. The Impact of Other Physiological Systems on the Placenta

The fact that pregnancy does not occur in a vacuum, mediated solely by the fetus and the placenta, makes it one of the most interesting, albeit complicating, systems to study. All physiological systems of the mother can profoundly influence the health of the pregnancy, just as the fetoplacental unit can exert a powerful influence over physiological systems of the mother. In this section I will focus on two such systems as they relate to human pregnancy, as our data implicates the hormone adiponectin to mediate the actions of both of these systems on sycytialized trophoblast cells.

Immune System

As the uterine endometrium transforms to decidua, a large number of leukocytes invade the tissue (King, 2000). These are predominantly uterine NK cells, along with macrophages and a small number of T cells, with a notable absence of B cells which are characteristic of an adaptive immune response (Moffett-King, 2002, King et al., 1998). As gestation progresses however, uNK cells become fewer in number, and by term they are absent from the decidua. This pattern suggests uNK cells are crucial mediators of the invasion process. The $CD56^{high}CD16^{-}$ uNK cells are distinct from the $CD56^{+}CD16^{+}$ NK cells found in circulation, and thus play a specific and unique role during implantation and invasion of the human blastocyst. The uNK cells express specific killer inhibitory receptor (KIR) haplotypes that will specifically recognize HLA-C expressed by extravillous trophoblasts and the inhibitory receptor for HLA-E (also expressed by extravillous trophoblasts). In addition, their production of the cytokines colony stimulating factor 1 (CSF1), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon γ (IFNγ) and tumor-necrosis factor (TNF) also may influence the behavior of extravillous trophoblast cells (Moffett-King, 2002).

The developing placenta also influences uNK cells, as hCG has been shown to increase proliferation of these cells through binding to the mannose receptor (CD206), expressed on the surface of uNK cells (Kane et al., 2009). In addition, placental growth factor (PlGF), a growth factor produced by a variety of cell types at the maternal-fetal interface, promotes uNK cell proliferation. Expression of PlGF is reduced during cases of preeclampsia, and loss of PlGF results in an increase in uNK cell maturation (Tayade et al., 2007). uNK cells themselves also produce angiogenic

molecules including PlGF as well as vascular endothelial growth factor (VEGF) and angiopoietin 2 (Moffett-King, 2002). As a consequence, uNK cells may play an important role in vascular remodeling of the placental bed, and their misregulation and/or production of these growth factors may have important implications in preeclampsia.

Another cell type that promotes immune tolerance of the fetus is the unique subset of T cells known as T regulatory cells (Tregs). These cells are found in higher abundance in the decidua during pregnancy than in peripheral blood, and have been shown to be reduced in cases of spontaneous abortion (Yang et al., 2009, Tilburgs et al., 2006, Sasaki et al., 2004). They were initially identified through their role in promoting maternal tolerance during pregnancy in studies using mouse models deficient in Tregs. When mated in an allogeneic breeding scheme, these mice aborted the fetuses, however syngeneic pregnancies progressed normally (Aluvihare et al., 2004).

The expansion of T regulatory cells during the early stages of pregnancy has been suggested to occur through a variety of methods. Although controversial, Tai and colleagues have provided evidence for Treg expansion by treatment with physiologically relevant doses of estrogen (Tai et al., 2008). However, these data have not been corroborated by others (Zhao et al., 2007). The subset of regulatory T cells recruited to the maternal-fetal interface is suggested to be specific for paternal alloantigens (Mjösberg et al., 2007, Tilburgs et al., 2008). Other groups therefore have suggested that the proliferation of paternally specific Tregs occurs soon after semen exposure (Leber et al., 2010). The migration of these cells to the decidua

appears to largely be mediated via production of an assortment of chemokines produced by cells at the maternal-fetal interface including macrophages, dendritic cells and T cells (Leber et al., 2010). In addition, hCG has been shown to attract Tregs, which express the LH/CG receptor (Schumacher et al., 2009).

T regulatory cells seem to be unique to pregnancy, as their transfer from a pregnant mouse to a CBA/J x DBA/2J abortive mouse model rescued the pregnancy if transferred during the very early stages of pregnancy, however pregnancy failed when T regulatory cells from a non-pregnant animal were transferred (Zenclussen et al., 2005). Once T regulatory cells have been activated through antigen presentation by macrophages or dendritic cells, they gain regulatory properties (Saito et al., 2007). The Treg population can then promote tolerance either through contact with other cells or by producing specific cytokines such as transforming growth factor β (TGFβ) and interleukin-10 (IL-10), which have been shown to be beneficial to the maintenance of pregnancy (Saito et al., 2007, Thaxton and Sharma, 2010, Leber et al., 2010).

In addition, T regulatory cells in the placenta express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which in turn may enhance the production of IFNγ by decidual leukocytes. The production of IFNγ may increase production of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) by dendritic cells or macrophages, a phenomenon which promotes fetal tolerance by metabolizing tryptophan and preventing T cell activation (Saito et al., 2007). This complex mechanism is supported by the observation that IDO is decreased in cases of miscarriage (Miwa et al., 2005). In addition, recent evidence suggests galactin-1,

expressed by Tregs, promotes apoptosis of activated T cells, the expansion of Tregs, and is important in mediating IL-10 induced immune tolerance (Leber et al., 2010). Despite the abundance of reports showing the importance of Treg cells in promoting healthy immune tolerance during pregnancy, many obstacles remain before this knowledge can be of practical importance in preventing spontaneous miscarriage in a human population (Leber et al., 2010). In addition, the amount of redundancy in the physiological tolerance of pregnancy makes the system much more complicated than can be explained by one specific cell type.

In addition to the presence of unique cell types such as uNK and Treg cells, the maternal-fetal interface utilizes a variety of different cell surface markers on trophoblast and resident immune cells in the decidua in order to promote immunological tolerance. The interaction between these immunomodulatory proteins elicits specific tolerogenic events.

Members of the B7 family have been shown to mediate cell-cell interactions, and include B7-1 and B7-2, as well as B7-H1, B7-H2, B7-H3, B7-H4, B7-H6 and B7-DC (Petroff and Perchellet, 2010). Of these, B7-DC is expressed by the syncytiotrophoblast, but only during the early stages of pregnancy, while B7-H2 and B7-H3 are expressed by extravillous trophoblasts throughout gestation (Petroff et al., 2005). The B7-H1 molecule is expressed by both syncytiotrophoblast and extravillous trophoblasts cells, with a more robust presence as pregnancy continues (Petroff et al., 2003). In contrast, B7-2 appears to be present only in fetal macrophages, and is absent from trophoblast cells (Petroff et al., 2003). B7-1, B7-H4, and B7-H6 appear to be absent from the maternal-fetal interface all together (Petroff et al., 2003, Petroff

and Perchellet, 2010). The varied expression pattern of this family of molecules makes it probable that they play a multitude of functions at the maternal-fetal interface. Although evidence exists for B7-H1 inhibition of production of the pro-inflammatory molecules, IFNγ and TNFα by T cells (Taglauer et al., 2008), the role of the various members of the B7 family of molecules in maternal immune tolerance largely remains to be eludiated.

In addition to specific expression of cell surface proteins, the trophoblasts produce a number of unique soluble factors, such as HLA-G and members of the TNFα superfamily that serve immunomodulatory roles at the maternal-fetal interface. The HLA-G gene consists of seven different isoforms, which arise from alternative splicing (Hunt, 2006). Expression of the different isoforms have been shown to be regulated by factors important for dictating trophoblast fate, including oxygen concentration (differentiation to extravillous trophoblast, HLA-G1, -G2, -G5, and -G6 expression) and epidermal growth factor (differentiation to syncytiotrophoblast, HLA-G5) (Hunt, 2006). Of these, both HLA-G5 and HLA-G6 are soluble isoforms that circulate in the mother's blood throughout pregnancy (Hunt, 2006). The effects of HLA-G on the immune system generally promote immune tolerance, and include regulation of cytokine production, regulation of NK actions including migration and killing, suppression of T cell killing, and changes in dendritic cell stimulation (Hunt, 2006). An abundance of data allows HLA-G to be widely regarded as an important mechanism in the suppression of the maternal immune system.

Trophoblast cells of the human placenta also produce a number of members of the TNF superfamily, including eight members that have been shown to induce

death of activated immune cells; TNFα, lymphotoxin (LT)α, LTβ, Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), TNF-like weak inducer of apoptosis (TWEAK), tumor necrosis factor superfamily member 14 (TNFSF14/LIGHT), and tumor necrosis factor superfamily member 9 (TNFSF9/4-1BBL) (Phillips et al., 2001). At least one of these, TRAIL, has been shown to induce apoptosis in cells found at the maternal-fetal interface, such as macrophages, but not trophoblast cells (Phillips et al., 1999). This may be due to the nuclear localization of its receptor in trophoblast cells (Bai et al., 2009). All told, the mechanisms utilized at the maternal-fetal interface to avoid the mother's immune system are varied and highly redundant. Many other mechanisms, and likely some not yet discovered, also play a part in this complex interplay between mother and offspring.

Adipose Tissue

With the rise in the obesity rate in the developed world, the important functions adipose tissue plays on whole-body physiology have garnered increased attention. Obesity is one of the primary risk factors for a host of diseases including atherosclerosis, type 2 diabetes mellitus, hypertension, cardiovascular disease, stroke, and osteoarthritis. Indeed, obesity-related diseases are among the leading causes of death in the United States (Heron et al., 2009). Additionally, maternal obesity prior to pregnancy has been identified as a risk factor for a host of birth defects including spina bifida, heart defects, diaphragmatic hernia, and limb reductions (Waller et al., 2007). Interestingly, approximately 10-25% of obese individuals do not develop metabolic diseases related to obesity, and are essentially healthy (Bluher, 2010). This phenomenon may be to due to a propensity in individuals to accumulate fat stores

either subcutaneously or viscerally, and underscores the important role adipose tissue plays in mediating the physiological effects associated with over-consumption of calories.

Adipose tissue is a heterogeneous tissue, comprised of adipocytes, preadipocytes, macrophages, and vasculature. A host of hormones and cytokines, collectively known as adipokines, are secreted by adipocytes and the surrounding stroma. The behavior of adipose tissue (i.e. adipose tissue dysfunction), as well as the site of adipose deposition (visceral vs. subcutaneous), appear to influence the overall health of obese men and women. Those individuals with high visceral fat mass, regardless of BMI or total fat mass, have been shown to be much more likely to develop insulin resistance (Bluher, 2010). This is thought to be largely due to the enhanced ability of subcutaneous fat to expand, whereas visceral fat will reach its full potential for expansion relatively quickly, leading to ectopic lipid deposition in the skeletal muscle, liver, and β cells of the pancreas (Bluher, 2010). Additional evidence exists for fundamental differences between subcutaneous and visceral fat. Many of the pro-inflammatory cytokines associated with adipocyte dysfunction are predominantly made by visceral fat, whereas subcutaneous fat has a different production profile of adipokines (Bluher, 2009, Chudek and Wiecek, 2006). Visceral fat area is also positively correlated with the amount of triglycerides, free fatty acids, and insulin in the plasma, and negatively correlated with insulin sensitivity parameters such as rate of glucose uptake and serum levels of testosterone and sex hormone binding globulin (SHBG) (Bluher, 2009).

As reviewed by Guilherme and colleagues (Guilherme et al., 2008), adipose tissue plays a key role in modulating skeletal muscle and hepatic sensitivity to insulin. Adipose tissue helps to maintain insulin sensitivity through the storage and regulated release of lipids for energy metabolism during periods of low glucose, as well as maintenance of a specific adipokine milieu in circulation. In the case of obesity, adipose tissue initiates an inflammatory response, causing the release of fatty acids and triglycerides into circulation, with subsequent storage in skeletal muscle, resulting in impaired response to insulin (reviewed in (Guilherme et al., 2008)). High levels of circulating free fatty acids have also been linked with the development of insulin resistance (Guilherme et al., 2008). As the skeletal muscle is exposed to more fatty acids, either by direct uptake from the circulation or intracellular release of ectopic stores, the myocyte will begin to utilize them as its energy source, resulting in decreased glucose uptake into the cell and insulin resistance (Guilherme et al., 2008).

The problem is compounded by the recruitment of macrophages to the adipose tissue, resulting in the induction of a pro-inflammatory state and production of cytokines such as TNFα. These cytokines will impair the adipocyte's ability to store triglycerides, promoting the release of more fatty acids into circulation, ultimately resulting in adipocyte dysfunction (Guilherme et al., 2008). In addition, the circulating adipokine balance is disrupted, exacerbating the inability of skeletal muscle and hepatocytes to respond to insulin.

The number of adipocytes in an individual is set early on in life, and the rate of turnover is not altered by BMI status (Spalding et al., 2008). Therefore, as an adult individual puts on weight, the size of the adipocyte increases, as opposed to the

number of individual adipocytes. As these cells become increasingly swollen with lipids their production of monocyte chemoattractant protein-1 (MCP-1) is increased, leading to an infiltration of macrophages and initiation of a pro-inflammatory state (Guilherme et al., 2008). Moreover, the size of the adipocyte can be an important determinant in adipokine production by that cell (Bluher, 2009), a phenomenon which will exacerbate the metabolic abnormalities initiated by adipocyte dysfunction.

Furthermore, it is currently thought that as adipocytes swell and adipose tissue enlarges, pockets of adipocytes that are relatively distant from the vascular supply experience a certain degree of hypoxia (Trayhurn et al., 2008). This in turn leads to alterations not only in the adipocyte production of angiogenic factors, but also pro-inflammatory mediators, specific adipokines, as well as changes in metabolic responses to hypoxia, such as the glucose transporter, GLUT1 (Trayhurn et al., 2008). Adipocyte dysfunction and inflammation not only contributes to the release of fatty acids from the cell, it alters the expression of key genes, such as peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor known for its role in adipogenesis and normal adipocyte function (Guilherme et al., 2008).

Adipose tissue is not simply a storage facility for lipids, but is also now appreciated for its potent endocrine role. The variety of systems and molecular pathways influenced by adipokines is continually amended as identification and examination of each adipokine increases. Broadly, the adipokines appear to be involved in regulating energy homeostasis, and thus have predominately been studied for their effects on glucose metabolism and insulin responsiveness.

The ever-expanding list of adipokines includes traditional metabolic hormones such as leptin and adiponectin, as well as more traditional cytokines such as TNFα and interleukin-6 (IL-6). In addition, a large number of novel factors are now known to be produced by adipose tissue, including retinol binding protein 4 (RBP4), resistin, apelin, fasting induced adipose factor (FIAF) and visfatin. While many of these factors support the adipose tissue itself, such a large variety of secretory proteins emphasize the importance adipose tissue plays in whole-body physiology. Adipose tissue is known to communicate with a number of different organs in the body such as the brain (e.g. via leptin and adiponectin), the immune system (e.g. IL-6, TNFα, transforming growth factor β (TGFβ), IL-8, IL-1β, IL-10, MCP-1, resistin), and the vasculature (e.g. vascular endothelial transforming growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1) (Trayhurn et al., 2006).

The influence of adipokines extends to the reproductive system and pregnancy. Not only does adipose tissue have profound effects on pregnancy through its key role in maintaining energy homeostasis and reserves in order to support the developing child, many adipokines have also been shown to be altered during pregnancy and disease states of pregnancy.

Due to the frequency in which obesity is correlated with polycystic ovary syndrome (PCOS), many have investigated a possible role for various adipokines in the development of this condition. Resistin is one adipokine that has been implicated in the development of PCOS, with the insulin sensitizer, rosiglitazone (a PPARγ agonist), being shown to decrease resistin levels in PCOS patients (Majuri et al., 2007). In normal menstrual cycles, both leptin and resistin levels are higher during

the luteal phase, although the physiological significance of this is not yet clear (Asimakopoulos et al., 2009). Other adipokines, such as RBP4, have been shown to be associated with gonadotropin levels (Makimura et al., 2009).

With regards to pregnancy, the adipokines have only recently begun to be evaluated. Circulating levels of leptin rise as gestation continues, and then drop to pre-pregnancy levels around parturition (Briana and Malamitsi-Puchner, 2009). In addition, leptin has been reported to be elevated in cases of preeclampsia (Haugen et al., 2006). These levels and expression pattern cannot however be attributed solely to adipose tissue, as the human placenta also produces a substantial amount of leptin (Senaris et al., 1997), presumably as a means for the placenta to regulate maternal energy metabolism. Indeed, placental production of leptin has been shown to drop in cases of gestational diabetes mellitus (Lappas et al., 2005b). Leptin is known to regulate trophoblast invasion, proliferation, and nutrient transfer (Magarinos et al., 2007, Briana and Malamitsi-Puchner, 2009, Schulz and Widmaier, 2004). The hormone has also been reported to induce pro-inflammatory cytokine production by the placenta and maternal adipose tissue (Lappas et al., 2005a). Leptin has also been shown to increase production of hCG in human cytotrophoblasts collected at term, while progesterone production was decreased in cultures (Cameo et al., 2003).

With the exception of leptin, very little is known about the role of other adipokines during pregnancy. Expression of resistin has been noted in both term human placental explants, and BeWo choriocarcinoma cells (Yura et al., 2003). Systemic resistin levels are known to increase throughout gestation in a normal pregnancy, and resistin has been positively correlated with birth weight (Cortelazzi et

al., 2007, Jansson et al., 2008, Chen et al., 2005). There is discrepancy in the literature regarding whether circulating resistin levels drop or are elevated in cases of preeclampsia (Cortelazzi et al., 2007, Haugen et al., 2006), probably due to differences in study design and disease definitions. Interestingly, treatment of BeWo cells with recombinant human resistin induced expression of matrix metalloproteinase (MMP)-2 and resulted in a concomitant decrease in tissue inhibitor of metalloproteinases (TIMP)-1 and -2 (Di Simone et al., 2006). These changes were associated with an increase in invasive behavior of BeWo cells (Di Simone et al., 2006).

Retinol binding protein 4 (RBP4) is produced by both the liver and adipose tissue. Aside from transporting retinol in the blood, it has also been recently classified as an adipokine, and associated with insulin resistance. The levels of RBP4 in women suffering from GDM have been reported to be elevated compared to healthy pregnant women (Maghbooli et al., 2010, Mazaki-Tovi et al., 2010), although these results remain controversial (Tepper et al., 2010, Ueland et al., 2008). Hypertensive pregnant women have also been reported to have elevated levels of RBP4 in circulation (Seiji et al., 2009). Similarly, the paucity of data and conflicting reports regarding levels of RBP4 during preeclampsia make it difficult to determine the influence this adipokine may have on diseases of pregnancy (Vaisbuch et al., 2009, Stepan et al., 2009). Despite these incongruities, those studies that have been done regarding adipokines and their potential influence on the placenta substantiate the hypothesis that they are important players in regulating metabolic demands of the fetus.

4. Adiponectin

Insulin Sensitivity

Adiponectin, first identified by five independent groups (Hu et al., 1996, Maeda et al., 1996, Nakano et al., 1996, Scherer et al., 1995, Arita et al., 1999), is composed of an N-terminal collagen-like domain and a C-terminal globular domain (for a review on adiponectin see (Kadowaki and Yamauchi, 2005)). The protein is highly similar to that of complement factor C1q and collagens VIII and X (Arita et al., 1999). In serum, adiponectin forms multimers through disulfide bond formation. High molecular weight (HMW) adiponectin, composed of up to 18 molecules of adiponectin bound together, is the most abundant form found in serum. However, low and medium molecular weight adiponectin, composed of trimers or hexamers, can also be found in circulation (Kadowaki and Yamauchi, 2005). In addition, full-length adiponectin can be cleaved to form a truncated globular form which appears to be more biologically active than the full-length molecule (Yamauchi et al., 2001). Adiponectin circulates at very high levels, making up approximately 0.01% of total protein in human serum (Zhu et al., 2008). Circulating levels are higher in females than males (Pajvani et al., 2003, Zoccali et al., 2002), perhaps because of the divergence in adipocity between the sexes.

The primary metabolic function ascribed to adiponectin is to maintain peripheral insulin sensitivity. This is accomplished by decreasing hepatic glucose production (Berg et al., 2001, Combs et al., 2001), increasing fatty acid oxidation in the liver (Yamauchi et al., 2001), and decreasing the amount of triglyceride accumulation in skeletal muscle (Yamauchi et al., 2001, Fruebis et al., 2001).

Although incompletely enumerated, it is thought that adiponectin elicits these broad biological effects primarily through activation of the adaptor protein APPL1 (adapter protein containing plekstrin homology domain, phosphotyrosine domain, and leucine zipper motif), which can in turn interact with a number of intracellular signaling cascades, including insulin signaling, glucose transport and fatty acid metabolism (Mao et al., 2006, Yamauchi et al., 2001).

Modulation of insulin responsiveness by adiponectin has been examined by a number of groups through both over-expression systems and genetic depletion (Yamauchi et al., 2003b, Maeda et al., 2002, Ma et al., 2002, Kubota et al., 2002, Satoh et al., 2005, Shklyaev et al., 2003, Combs et al., 2004), with the predominant outcome being a role for adiponectin in improving insulin sensitivity. Interestingly, the influence of adiponectin appears to be more profound in the liver, as one group has recently reported hepatic, but not muscular, insulin resistance in adiponectin null mice (Yano et al., 2008). In keeping with these findings, serum adiponectin is decreased in cases of obesity, a physiological state in which insulin resistance is common. A very intriguing study has recently shown that over-expression of adiponectin in the leptin null mouse (*Ob/Ob*) is sufficient to normalize glucose and insulin levels and rescue the diabetic phenotype, despite these mice remaining obese (Kim et al., 2007). These data underscore 1) the importance of adiponectin in maintaining insulin sensitivity, 2) that adipocyte dysfunction and consequent alterations in adipokine profiles are key in producing an insulin resistant phenotype, and 3) the yin/yang relationship between leptin and adiponectin.

In keeping with the pro-inflammatory state of obesity, IL-6 has been shown to inhibit the production of adiponectin in an adipocyte cell line (Fasshauer et al., 2003), as has the transcription factor, PPARγ (Maeda et al., 2001). Adiponectin has also been postulated to play a protective role in the development of diabetes (Lindsay et al., 2002), creating interest in its potential as a therapeutic device in treating type 2 diabetes. In addition to its insulin sensitizing role, the molecule has recently been shown to influence cellular differentiation (Oshima et al., 2005, Yamaguchi et al., 2008, Yamaguchi et al., 2007, Fiaschi et al., 2009), proliferation (Lee et al., 2008), and hormone production (Lu et al., 2008, Coope et al., 2008, Wen et al., 2008, Ledoux et al., 2006, Lagaly et al., 2008) in other tissues. Although adiponectin has understandably been investigated primarily with regards to obesity and diabetes (both instances in which its expression is decreased), it has been reported to be increased in cases of anorexia nervosa (Delporte et al., 2003), a physiological state in many ways opposite that of obesity.

Adiponectin is reported to elicit both pro-inflammatory and anti-inflammatory responses, dependent on the biological system and molecular weight form of adiponectin under study. Its initial beneficial effects on atherosclerosis and endothelial dysfunction suggested adiponectin was anti-inflammatory. In keeping with this hypothesis, a mouse model for collagen-induced arthritis has shown adiponectin to alleviate many of the markers of inflammation, including the production of pro-inflammatory cytokines (Ebina et al., 2009). Adiponectin has also been implicated in inducing anti-inflammatory pathways in cases of liver disease

(Mandal et al., 2010), and increase production of the anti-inflammatory cytokine, IL-10 (Wolf et al., 2004, Wulster-Radcliffe et al., 2004).

In contrast, adiponectin can stimulate production of interleukin-8 (IL-8) by rheumatoid fibroblasts in culture (Kitahara et al., 2009), and thus act as a pro-inflammatory mediator. Although the full-length form of adiponectin is commonly used as the anti-inflammatory form of adiponectin, whether adiponectin elicits a pro- or anti-inflammatory cascade has been proposed to be a result of which molecular weight form of adiponectin is under question. The high molecular weight (HMW) form of adiponectin has been implicated in mediating pro-inflammatory responses in peripheral blood mononuclear cells (PBMCs) (Song et al., 2009). Additionally, the globular form of adiponectin activates the pro-inflammatory signaling molecule, NFκB to an even greater extent than HMW adiponectin (Tomizawa et al., 2008).

Adiponectin Receptors

Two receptors for adiponectin have been identified, adiponectin receptor 1 (adipoR1) and adiponectin receptor 2 (adipoR2) (Yamauchi et al., 2003a). Both are composed of seven transmembrane domains, although they are structurally and functionally distinct from G-protein coupled receptors (Yamauchi et al., 2003a). They are members of a distinct family of receptors, known as the PAQR family, which in addition to the adiponectin receptors includes the seven transmembrane receptors through which nongenomic actions of progestins have been suggested to be mediated (Tang et al., 2005). The PAQR family of receptors, of which 11 family members have been identified as conserved in multicellular eukaryotes, show structural similarity

primarily in the membrane spanning and intracellular loop motifs, similar to what is seen in some families of GPCRs (Tang et al., 2005).

In the mouse, adipoR1 is expressed ubiquitously, but is most abundant in skeletal muscle while adipoR2 is more highly expressed in the liver (Yamauchi et al., 2003a). In contrast, both receptors appear to be expressed in relatively equal abundance in human skeletal muscle compared to liver, a pattern that is also seen in the rat (Yamauchi et al., 2003a, Satoh et al., 2005).

AdipoR1 is a high-affinity receptor for the globular form of adiponectin, while adipoR2 possesses intermediate affinity for both globular and full-length adiponectin (Yamauchi et al., 2003a). In addition, there is evidence suggesting adiponectin can bind to the cell surface molecule T-cadherin, although the functional role of this phenomenon needs to be elucidated (Hug et al., 2004). Although the hormone can bind to either receptor, and both receptors are expressed in many of the same tissues, they appear to mediate very different physiological actions. Mice devoid of adipoR1 displayed an increased propensity for adipose accumulation as well as reduced glucose tolerance, energy expenditure, and heightened leptin and plasma cholesterol levels (Bjursell et al., 2007). In contrast, when *AdipoR2*$^{-/-}$ mice where challenged with a high fat diet, they appeared resistant to glucose intolerance and displayed lower levels of cholesterol than their wildtype counterparts (Bjursell et al., 2007). Many of the classical actions of adiponectin are thought to be mediated through adipoR1, however, a detailed examination of receptor usage/function within specific tissues is currently lacking.

Signaling Mechanisms

Not only do the two adiponectin receptors have somewhat divergent expression patterns and ligand affinities, they also appear to utilize unique intracellular signaling cascades. Disruption of adipoR1 expression results in a decrease in adiponectin stimulated adenosine 5'-monophosphate-activated kinase (AMPK) activity, while knockdown of adipoR2 causes a loss of signaling through peroxisome proliferator-activated receptor (PPAR)α (Yamauchi et al., 2007). Interestingly, AMPK has recently been identified to be important in regulating differentiation in skeletal myocytes, as part of the cell's ability to respond to changes in nutrient availability (Fulco et al., 2008). Furthermore, the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is known to inhibit adipocyte differentiation *in vitro* (Giri et al., 2006, Habinowski and Witters, 2001, Hwang et al., 2005), although whether the effects of AICAR are mediated entirely through AMPK remains to be conclusively shown. In support of the suggestion that AMPK can regulate cellular differentiation, the phytochemicals genistein, EGCG and capsaicin have all been shown to activate AMPK and inhibit adipocyte differentiation (Hwang et al., 2005). Additionally, there are other reports that implicate adiponectin directly in inhibition of osteoclast formation (Yamaguchi et al., 2008, Yamaguchi et al., 2007, Oshima et al., 2005), and mediated through adipoR1 and AMPK (Yamaguchi et al., 2008).

Although the AMPK signaling pathway is widely thought of as the primary mechanism of adiponectin signal amplification, cases exist in which the AMPK pathway does not appear to be responding to adiponectin stimulation. In these

instances the mitogen-activated protein kinase, ERK1/2, pathway appears to be dominant (Maillard et al., 2010, Chabrolle et al., 2009, Lee et al., 2008).

Adiponectin also appears to initiate intracellular events through a variety of other signaling mechanisms including the insulin signaling cascade, p38 mitogen-activated protein kinase, c-Jun-N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K), Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) and NFκB pathways (Coope et al., 2008, Haugen and Drevon, 2007, Yamauchi et al., 2003a, Chabrolle et al., 2009, Tang and Lu, 2009, Tomizawa et al., 2009). Although many of the details regarding signaling initiated by adiponectin are yet to be elucidated, there is a growing body of evidence linking the adaptor protein APPL1 to adiponectin signal transduction. In cultured myocytes adiponectin-mediated activation of both AMPK and p38 MAPK appears to be mediated at least in part by APPL1 (Mao et al., 2006). Lee and colleagues have shown that APPL1 can mediate the downstream actions of the adiponectin receptor even when the receptors do not directly activate AMPK. Down-regulation of APPL1 using siRNA impaired the ability of adiponectin to activate ERK1/2 in HEK293 cells (Lee et al., 2008). In addition, APPL1 may provide the means by which adiponectin interacts with insulin signaling and glucose uptake in skeletal muscle (Mao et al., 2006).

Interaction with the GTPase Rab5 allows APPL1 to influence glucose transport in skeletal myocytes, and APPL1 has been linked to modulation of insulin-mediated Akt signaling (reviewed in (Hosch et al., 2006)). Perhaps most telling, over-expression of the APPL1 isoform, APPL2, in muscle cells interrupts adiponectin signaling (Wang et al., 2009). These authors showed that, under basal conditions,

APPL2 is associated with the adiponectin receptor at the plasma membrane, while APPL1 resides largely in the cytosol. Upon adiponectin binding to its receptor, the location of these two proteins switches, such that APPL1 is associated with the receptor and can potentiate the signal initiated by adiponectin. The same group also showed that over-expression of APPL2 in their system can lead to a down-regulation of adiponectin signaling (Wang et al., 2009). Thus, APPL2 is a negative regulator of adiponectin signaling.

Alternatively, both serine/threonine kinase 11 (STK11 or LKB1) and calcium/calmodulin-dependent protein kinase kinase (CaMKK) are postulated to mediate adiponectin's effects upstream of AMPK (Fujii et al., 2006). With regard to the placenta, adiponectin effects have been reported to be mediated through AMPK, ERK1/2, PPARγ and NFκB (Lappas et al., 2005a, Benaitreau et al., 2009). The mechanisms employed by adiponectin at the maternal-fetal interface have received scant attention however, thereby leaving a number of possible means by which adiponectin may function within the placenta.

Adiponectin in Reproduction

The role of adiponectin has also been investigated in various tissues of the reproductive system. For the most part however, the focus has been on adiponectin's ability to increase insulin sensitivity during cases of obesity-induced polycystic ovary syndrome (PCOS) (Majuri et al., 2007, Mitchell et al., 2005). Transcripts for adiponectin as well as its receptors have been reported in a variety of bovine ovarian-derived cell types, including granulosa cells, follicles of different stages, the corpus luteum, cumulus cells and oocytes (Maillard et al., 2010). However, it is important to

note that regarding the human, adiponectin production has not been identified in granulosa cells, although they were positive for both adipoR1 and adipoR2 (Chabrolle et al., 2009), and adiponectin has been found in follicular fluid of women undergoing *in vitro* fertilization (Gutman et al., 2008).

Adiponectin appears to complement the ovarian actions of a variety of hormones/growth factors without necessarily exerting the same actions independently. For instance, insulin-like growth factor (IGF)-induced granulosa cell proliferation is significantly enhanced by addition of adiponectin to the culture media, but not following adiponectin treatment in the absence of IGF (Maillard et al., 2010). Similarly, in bovine theca cells, adiponectin treatment results in a drop in luteinizing hormone (LH)-induced progesterone and androstenedione production, as well as steroidogenic enzyme gene expression (Lagaly et al., 2008). In bovine granulosa cells, the insulin-stimulated progesterone and estradiol production was significantly reduced by addition of adiponectin (Maillard et al., 2010). This phenomenon is mirrored in the pig ovary, where a drop in aromatase expression following adiponectin treatment has been reported (Ledoux et al., 2006). In contrast, in primary human granulosa cell cultures, adiponectin was reported to increase IGF-I stimulated progesterone and estradiol production (Chabrolle et al., 2009). The discrepancies in these data highlight the need for more extensive investigation as well as potential divergence between humans and potential model species.

Extremely little data has been reported regarding the effect, if any, of adiponectin on male reproduction. Adiponectin has been reported to be produced by Leydig cells of the rat testis (Caminos et al., 2008), although the data is scant. The

same group has reported adiponectin's inhibition of testosterone production in rat testicular cell cultures, both basally and in response to hCG stimulation (Caminos et al., 2008). One study examining the specific effects of the adiponectin receptors reported that *AdipoR2*$^{-/-}$ mice had smaller testes weight, atrophy of the seminiferous tubules, and aspermia when compared to their wildtype counterparts (Bjursell et al., 2007). These same animals had no change in testosterone levels (Bjursell et al., 2007).

Studies examining the effect of adiponectin on hypothalamic neurons and gonadotrope cells report adiponectin exposure causes a decrease in gonadotropin releasing hormone (GnRH; (Wen et al., 2008)) and LH secretion (Lu et al., 2008, Rodriguez-Pacheco et al., 2007). Interestingly, female infertility and an increase in systemic prolactin levels has been reported in a transgenic mouse model overexpressing adiponectin (Combs et al., 2004). Furthermore, the levels of adiponectin in follicular fluid were higher in response to treatment with luteinizing hormone (LH) (Gutman et al., 2008), suggesting a possible feedback loop between the pituitary and adipose. Such data set precedence for a role of adiponectin in various aspects of reproduction.

Adiponectin in Pregnancy

Placental expression of adiponectin remains controversial. Positive adiponectin immunoreactivity has been identified in rabbit day 6 blastocysts, although the staining pattern is much stronger in the inner cell mass (ICM) compared to the differentiating trophoblasts (Schmidt et al., 2008). Caminos and coworkers have reported adiponectin expression in both the rat and human placenta as well as

both adipoR1 and adipoR2 receptors (Caminos et al., 2005). These data have been corroborated by others, demonstrating adiponectin mRNA and protein expression in the syncytiotrophoblast of term placentas, as well as adiponectin secretion from placental explants (Lappas et al., 2005b, Chen et al., 2006). However there remain concerns as to whether the tissues and methodologies used in these studies conclusively demonstrate adiponectin expression specifically in trophoblast cells. Indeed, more recent investigations have failed to detect adiponectin gene expression in the placenta (Haugen et al., 2006, Ichida et al., 2007, McDonald and Wolfe, 2009). By one report, placental lysates that have been extensively washed to remove all intervillous blood lose their previous positive banding pattern for adiponectin by western blot (Desoye and Hauguel-de Mouzon, 2007). The controversy regarding adiponectin expression by the placenta underscores the need for focused attention on the role of adiponectin at the maternal-fetal interface.

To date, few published studies have examined the role of adiponectin at the maternal-fetal interface. These studies primarily utilized placental explant cultures collected at term. Treatment of placental explants with adiponectin resulted in an increase in secretion of the cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, as well as the prostaglandins PGE2 and PGF2α (Lappas et al., 2005a). Adiponectin administration *in vivo* caused a decrease in placental mRNA expression of the glucose transporter GLUT-3, lipoprotein lipase (LPL), adipoR2 and transforming growth factor (TGF)-β in the rat (Caminos et al., 2005). The authors postulate that adiponectin may be influencing nutrient transport and trophoblast proliferation/invasion, although these results need to be corroborated. One study

using the Jeg-3 and BeWo choriocarcinoma cell lines, reported that adiponectin inhibited proliferation of these cells (Benaitreau et al., 2009). These same authors were also unable to amplify adiponectin mRNA from choriocarcinoma cells (in agreement with our data as well as that of others). As a result, the proliferative effect of adiponectin may only be relevant to the cytotrophoblast layer of the mature placenta. A recent report using primary term trophoblast cells from healthy placentas has shown full-length adiponectin to inhibit the insulin-stimulated increase in amino acid transport (Jones et al., 2010), data which are intriguing given the dogma that adiponectin acts to sensitize tissues to insulin. Interestingly, treatment with the globular form of adiponectin in these cells failed to elicit the inhibition of insulin-mediated events (Jones et al., 2010). The discrepancy between different forms of adiponectin and their physiological actions is an area that requires more careful examination.

The remaining reports regarding adiponectin and the placenta to date have examined effectors of adiponectin release from placental explants (Lappas et al., 2005b, Chen et al., 2006), and are therefore somewhat controversial. These studies are largely not in accord with one another, and given the model system, make it difficult to determine the significance of the results. Despite this ambiguity, adiponectin circulates in μg/ml quantities and as a consequence the placenta is exposed to adiponectin via maternal blood once the mature hemochorial placenta has been established. In addition, although data regarding placental production of adiponectin is inconsistent, the literature is in agreement regarding placental

expression of both adipoR1 and adipoR2. Therefore the effects of adiponectin on the placenta remain a potentially important aspect of placental biology.

A number of studies have examined the expression of adiponectin throughout the normal progression of pregnancy, as well as in disease states such as gestational diabetes and preeclampsia. During normal gestation, maternal serum levels of adiponectin drop slightly as gestation advances (Suwaki et al., 2006, Cortelazzi et al., 2007, Catalano et al., 2006, Fuglsang et al., 2006, O'Sullivan et al., 2006, Mazaki-Tovi et al., 2007, Ramsay et al., 2003, Cseh et al., 2004). During cases of GDM however, a significant decline in circulating adiponectin levels has been observed (Cortelazzi et al., 2007, Altinova et al., 2007, Ranheim et al., 2004, Retnakaran et al., 2007, Tsai et al., 2005, Williams et al., 2004, Cseh et al., 2004, Georgiou et al., 2008, Mazaki-Tovi et al., 2009b). This decrease in serum adiponectin during GDM in many cases persists after correcting for BMI, suggesting adiponectin expression may be altered during GDM even in cases when the mother is within a normal weight range.

Furthermore, examination of adiponectin levels during the first trimester of pregnancy suggest a reduction in serum levels of adiponectin may be predictive of later development of GDM (Georgiou et al., 2008, Williams et al., 2004). These data illustrate adiponectin's intimate association with insulin levels, and suggest the adipokine may play a role in the manifestation of GDM. An exacerbated reduction in adiponectin during GDM could result in the loss of its protective effects, both systemically for the mother, as well as at the maternal-fetal interface. Although the data is intriguing, all evaluations of adiponectin and GDM are correlative studies evaluating maternal serum levels of adiponectin and pregnancy outcomes. Therefore,

the bulk of the literature serves to illustrate and justify the need for mechanistic examination of the role of adiponectin at the maternal-fetal interface.

With regards to preeclampsia, correlative studies of adiponectin levels in maternal serum and disease status comprise all of the data thus far reported. Studies have shown that in women suffering from preeclampsia adiponectin is elevated (Avci et al., 2010, D'Anna et al., 2006, Fasshauer et al., 2008, Nien et al., 2007), although not all studies are in agreement (Mazaki-Tovi et al., 2009c). Adiponectin is also higher in women diagnosed with abnormal uterine perfusion, with frequent development into IUGR or preeclampsia (Fasshauer et al., 2007). In addition, adipoR1 and adipoR2 are increased at term in women with preeclampsia (Jarvenpaa et al., 2009, Tie et al., 2009). That being said, adiponectin levels may actually be lower during the first trimester in women destined to be diagnosed with preeclampsia (D'Anna et al., 2005, D'Anna et al., 2006). The difference in adiponectin levels seem to diverge based on whether the patient is diagnosed with early- or late-onset preeclampsia, thus potentially highlighting the different pathogenesis between these two diagnoses.

There is increasing interest in the potential role adiponectin may directly have on the fetus. Thus, studies examining the level of adiponectin in amniotic fluid and umbilical cord blood are being reported with increased frequency. The levels of adiponectin in cord blood are lower in offspring from diabetic pregnancies, compared to those children from insulin sensitive pregnancies (Lindsay et al., 2003). This is also the case in the small child from a set of twins with divergent growth (Mazaki-Tovi et al., 2009a). Interestingly, the levels of amniotic adiponectin do not seem to be

correlated with those in maternal blood, suggesting the fetus is capable of making adiponectin, at least as early as mid-term (Baviera et al., 2007). These data raise the possibility that, in a scenario much like that of fetal insulin, adiponectin of fetal as well as that of maternal origin may be able to influence the behavior of the placenta. It is important to remember however, these data are in their infancy and the exposure levels of placental trophoblasts are likely to be much higher with regard to maternal adiponectin.

5. Study Significance

Pregnancy is a time of metabolic stress for the mother, exacerbated in disease states and cases of obesity. The role of adipokines in mediating physiological responses to this stress has only recently gained appreciation. Adiponectin is one such molecule known to be important in mediating insulin sensitivity, with recent evidence of a role for adiponectin outside the classical tissues associated with insulin action (i.e. liver and skeletal muscle). A multitude of studies have examined serum levels of adiponectin during various gestational complications, however few have evaluated the influence of adiponectin on the placenta. Disorders such as GDM, IUGR, and preeclampsia result in an aberrant hormonal milieu in maternal serum. This phenomenon can have profound effects on the developing fetus, as the placenta is bathed in maternal blood and thus exposed to all circulating hormones, and is the sole mediator of how the maternal environment is translated to the fetus. As one of the key hormones affected during GDM, it is vital that the effects of adiponectin on the placenta be examined thoroughly.

The goal of this study was to identify the effects, if any, of adiponectin on the human placenta. A number of clinical studies have identified a correlation between adiponectin levels in maternal circulation and GDM, preeclampsia, and/or IUGR. At initiation of this study, the only reports regarding adiponectin at the maternal-fetal interface were done in placental explants, a model system composed of many different cell types. We have utilized primary cultures of human trophoblasts collected at term in order to identify the effects of adiponectin on the trophoblast.

In the first study, **"Adiponectin Attenuation of Endocrine Function within Human Term Trophoblast Cells,"** we began by identifying that adiponectin is not produced by the placenta at term, although both the adipoR1 and adipoR2 receptors are present. Therefore, we establish that the placenta is able to respond to adiponectin in maternal circulation. Based on these findings, we establish our long-term culture conditions in order to allow the cytotrophoblast cells to syncytialize *in vitro,* as syncytialized cells will be the most likely to be exposed to adiponectin circulating in the mother's blood. Due to evidence in the literature that adiponectin can affect cell differentiation, we exposed syncytialized trophoblast cells to adiponectin throughout the culture period. We identified that adiponectin decreased gene expression for a number of hormones, one of the key functions of the syncytiotrophoblast. Chorionic gonadotropin, progesterone, and placental lactogen all dropped after 5d adiponectin exposure. We also found that these effects are not due to an inhibition of differentiation by adiponectin, indeed the cells syncytialized at least as well with adiponectin as the no treatment control cells.

In the second study, **"The Pro-Inflammatory Role of Adiponectin at the Maternal-Fetal Interface,"** we continued with identification of the effects of adiponectin on human term trophoblasts syncytialized in culture. Long-term (5d) adiponectin exposure increased production of the pro-inflammatory cytokines, IL-1β and IL-8. In addition, we recognized that the protein Siglec10 is up-regulated following adiponectin exposure. Siglec10 has been recently implicated in dampening the immune response to danger-associated molecular patterns (DAMPs). We have also shown that both Siglec10 and its partner, CD24, are expressed by trophoblasts. In non-treated cells, Siglec10 is expressed at low levels, and seems to be localized to the syncytiotrophoblast. These data suggest Siglec10 may be a mechanism used by the trophoblast to dampen immune responses.

In the third study, **"Signaling Cascades Initiated by Adiponectin in Trophoblast Cells,"** we investigated the mechanisms by which adiponectin elicits the effects we have previously identified. We report that adiponectin activates the ERK1/2 and JNK pathways in term trophoblasts. In addition, we observed activation of the EGF receptor within 5 minutes of adiponectin treatment. The signaling mechanisms of adiponectin are mediated through the adaptor protein APPL1, as over-expression of its inhibitory binding isoform, APPL2, abolished adiponectin signaling. Interestingly, we also showed that leptin and adiponectin are intimately linked at the maternal-fetal interface, as over-expression of leptin in term trophoblasts also abolished adiponectin signaling. These data identify possible mechanisms by which the trophoblast cell may regulate its response to adiponectin.

In the final study, **"Microarray Analysis of Adiponectin on Trophoblast Cells,"** a number of genes, including some outlined in previous chapters, are altered following 5d exposure to adiponectin in term trophoblast cells. Included in this list are numerous genes involved in the mediation of IGF bioavailability, such as IGF binding proteins and proteases. The pattern of up- and down-regulation in this set of genes seems to suggest that adiponectin decreases the amounts of available IGF at the maternal-fetal interface. In this manner, adiponectin may alter one of the fundamental mechanisms in nutrient transfer and growth at the placenta. Other sets of genes, such as 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) and type 2 (HSD11B2) are also altered according to the microarray data. These data suggest that cortisol metabolism is changed following adiponectin treatment such that higher levels of active cortisol are likely to be present in the trophoblast. The microarray has provided important data by which we may identify many of the mechanisms altered by adiponectin in the human, syncytialized trophoblast.

Taken together, these studies significantly advance the field by providing the first data regarding the effects of adiponectin on human trophoblast cells. Since initiation of this study a report has come out examining adiponectin treatment on choriocarcinoma cell lines. These data are valuable, however we have been unable to recapitulate many effects we see using primary cultures, including those delineated in this report, in choriocarcinoma cell lines. On the whole, our studies suggest adiponectin may be thought of as a "pro-maternal" hormone, meaning that it works through multiple mechanisms to interfere with functions of the syncytiotrophoblast.

In this manner, adiponectin may be one hormone working to maintain the balance between the needs of the developing fetoplacental unit and the mother.

VI: Chapter Two:

Adiponectin Attenuation of Endocrine Function within Human Term Trophoblast Cells

1. Abstract

The hormone adiponectin has been shown to be important in maintaining insulin sensitivity throughout the body, while potential effects on the placenta have not been assessed. Pregnancy constitutes a unique physiological environment in which metabolism has a profound effect on the health of both the mother as well as the developing fetus. It is imperative that a delicate balance in glucose delivery be maintained between maternal tissues and the fetal/placental unit. Adiponectin's role in regulating peripheral insulin responsiveness suggests it may be a factor in maintaining this balance during gestation as well. Examination of human cytotrophoblast cells revealed that mRNA for both adiponectin receptors, adipoR1 and adipoR2, are abundantly expressed at term. We were however, unable to reliably detect mRNA for adiponectin in primary cytotrophoblasts. Expression of both receptors was maintained following induction of syncytium formation by exogenous EGF treatment. Treatment of cytotrophoblasts with adiponectin resulted in a significant drop, as assessed by quantitative RT-PCR, in expression for a number of genes involved in the endocrine function of the placenta, including the chorionic gonadotropin subunits, placental lactogen, and some steroidogenic enzymes. Immunofluorescent staining for connexin 43 and desmoplakin in primary trophoblasts revealed that adiponectin does not inhibit syncytialization of trophoblast cells in culture. Taken together, these data describe a novel role for maternal adiponectin in regulating the placental environment. Determination of the effects of such adipokines on the maternal-fetal interface is increasingly important, as the incidence of

pregnancies complicated by gestational diabetes remains a significant health problem in developed countries.

2. Introduction

Pregnancy represents a unique stage in an organism's lifespan in which the female must be able to cope with the large energy demands placed on her by the developing fetus, while continuing to meet her own metabolic needs. Hormones such as insulin and leptin have long been known to mediate this balance, with diseases such as gestational diabetes resulting in cases when this balance is not maintained.

The relatively recent acknowledgement of adipose tissue as an endocrine organ has occurred by the discovery of adipose secretion of a number of hormones and cytokines, collectively known as adipokines. Among this diverse and growing family of molecules is the hormone adiponectin, first discovered as a factor secreted by adipocytes by four independent groups (Hu et al., 1996, Maeda et al., 1996, Nakano et al., 1996, Scherer et al., 1995). Adiponectin has subsequently been shown to act as a potent insulin sensitizer, and has thus been primarily investigated for its role in decreasing both hepatic glucose production in the liver (Berg et al., 2001, Combs et al., 2001) and triglyceride accumulation in skeletal muscle (Yamauchi et al., 2001, Fruebis et al., 2001), while simultaneously increasing fatty acid oxidation in the liver (Yamauchi et al., 2001).

Pregnancy represents a period during which a woman's metabolic homeostasis is under significant stress. In addition, a physiological inability to maintain this delicate balance can result in impaired fetal development and maternal health. It follows that the hormone adiponectin, with its important role in maintaining peripheral insulin sensitivity during basal conditions, may be of increased significance during gestation. Indeed, there is a growing body of literature citing

lower maternal serum concentrations of adiponectin in cases of gestational diabetes (Cortelazzi et al., 2007, Altinova et al., 2007, D'Anna et al., 2005, Ranheim et al., 2004, Tsai et al., 2005, Williams et al., 2004, Cseh et al., 2004, Georgiou et al., 2008). Investigation of adiponectin at the maternal-fetal interface has generated some conflicting results. Initially, adiponectin was reported to be produced by the placenta (Chen et al., 2006, Lappas et al., 2005b, Caminos et al., 2005), suggestive of an autocrine and/or paracrine mechanism employed by adiponectin within the placenta. These data however, have remained controversial (Haugen et al., 2006, Ichida et al., 2007). In addition, there has been little investigation regarding the effects of adiponectin on the placenta. Treatment of placental explants with adiponectin has been reported to result in increased secretion of proinflammatory cytokines and prostaglandins by placental explants (Lappas et al., 2005a). A specific effect of adiponectin on one of the functional units of the placenta, the trophoblast cells, has not been elucidated. We hypothesized that adiponectin has a direct effect on the trophoblast cells of the placenta, serving as an important mediator in maintaining the energy balance between mother and fetus that is vital for a successful pregnancy.

Our data suggest adiponectin can exert potent effects on trophoblast cells of the term placenta. We show significant reductions in endocrine gene expression as a result of adiponectin treatment of term trophoblast cells *in vitro*. This apparent decrease in endocrine function does not however appear to be the result of a significant impairment of syncytialization. Indeed, those cells administered adiponectin appear to undergo robust syncytialization, as evidenced by desmoplakin staining. These results provide the first evidence of a specific role for adiponectin at

the maternal-fetal interface, and provide evidence of a potential regulatory mechanism by which maternal insulin sensitivity during pregnancy is tightly maintained by changes in placental hormone production.

3. Materials and Methods

Cell isolation and culture

Human placentas were obtained under protocols approved by the institutional review board at the University of Kansas Medical Center. Cytotrophoblasts were isolated from healthy placentas delivered via cesarean at term as previously described (23). Villous tissue was dissected away from the basal plate of the placenta and subjected to treatment with deoxyribonuclease I (type IV; Sigma Chemical, St. Louis, MO) and trypsin (Invitrogen Corp., Carlsbad, CA). The resulting single-cell suspension was size fractionated by application to a Percoll (Sigma) density gradient. To ensure a homogeneous population of cytotrophoblasts, the cells underwent negative selection using an antibody recognizing human leukocyte antigen-A, -B, and -C (W6/32; Bio-Express, West Lebanon, NH) with a magnetically labeled secondary antibody (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). Cytospins of trophoblasts from each preparation were stained for cytokeratin 7 expression (N-TL 12/30; Dako, Glostrup, Denmark) to assess purity. Purified cells were placed in culture medium (Iscove's modified Dulbecco's medium with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin/ amphotericin B) and allowed to adhere to the culture dish by incubation in a humidified chamber for 4 h before receiving treatment. All cells underwent regular medium changes, with refreshment of treatment conditions every 48 h. After addition of recombinant globular adiponectin (2 μg/ml; Phoenix Pharmaceuticals, Burlingame, CA) to the culture medium, cells were allowed to differentiate spontaneously before being harvested on d 5 of culture.

For adiponectin and adiponectin receptor expression analysis, the choriocarcinoma cell lines Jeg-3, Jar, and BeWo (American Type Culture Collection, Manassas, VA) were maintained in growth medium (1x DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin). Induction of syncytialization of term cytotrophoblast cells was achieved by treatment with epidermal growth factor (EGF) for 72 h (10 ng/ml; Invitrogen), with syncytialization assessed by desmoplakin staining and RT-PCR for chorionic gonadotropin-β gene expression.

RT-PCR and quantitative real-time PCR (qPCR)

RNA from trophoblasts in culture was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Adipose tissue total RNA (Ambion; Applied Biosystems, Foster City, CA) was used as a positive control. Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers. The resultant cDNA was used in traditional PCR with *Taq* polymerase (Invitrogen) and visualized through gel electrophoresis.

qPCR was performed using SYBRgreen technology (Applied Biosystems). All gene expression data were normalized to 18S levels within the respective sample. Primers for genes of interest (Table VI-1) were designed using Primer Express 3.0 software (Applied Biosystems), with the exception of the syncytin primer (24), and synthesized by IntegratedDNATechnology (Coralville, IA). Resulting data were analyzed using the ΔΔCt method [mean Cts (cycle thresholds) for each gene were determined then subtracted from each sample's Ct for 18S RNA (ΔCt), and deviation from the no treatment control Ct for each gene was calculated (ΔΔCt)], after assuring amplification efficiency for both target and normalizing gene were equivalent.

Table VI-1. Primer Sequences for Quantitative RT-PCR.

Gene	Forward Primer	Reverse Primer
18S	5'-cgccgctagaggtgaaattct-3'	5'-cgaacctccgactttcgttct-3'
3βHSD	5'-tcagcgagatctggcgtataag-3'	5'-acccactccaccgttttctg-3'
AdipoR1	5'-acaagagcaggcgtgttcct-3'	5'-ctcagcgatagtaaagtgcatggt-3'
AdipoR2	5'-tttccctggcaaatgtgaca-3'	5'-aaaagctccagcaaccacaaa-3'
Aromatase (Cyp19)	5'-catatgatctgtctgtggcaaaagt-3'	5'-aagtggctgaggcataaatcga-3'
Chorionic gonadotropin α-subunit	5'-gaatgcacgctacaggaaaacc-3'	5'-cagcccatgcactgaagtattg-3'
Chorionic gonadotropin β-subunit	5'-acaaccccgaggtataaagcc-3'	5'-ccttggatgcccatgtcc-3'
Connexin43	5'-catcctccaaggagttcaatcac-3'	5'-gggcaccactcttttgcttaaa-3'
MMP9	5'-cccggaccaaggatacagttt-3'	5'-gtgccggatgccattca-3'
p450scc (Cyp11A1)	5'-caccttcaccatgtccagaattt-3'	5'-ctccacgttgccgagctt-3'
Placental lactogen	5'-tccttctgcttctcagactctattcc-3'	5'-ctagattggatttctgttgcgtttc-3'

Immunoblots

Proteins (15 μg) were fractionated by 8% SDS-PAGE and transferred to nitrocellulose membranes (Whatman Inc., Florham Park, NJ). Membranes were then subjected to Western blot analysis for chorionic gonadotropin-α (ME.109; Abcam, Cambridge, MA), chorionic gonadotropin-β (ab53087; Abcam), human placental lactogen (ab15554; Abcam), p450 side-chain cleavage (p450scc) (25), or aromatase (A7981; Sigma). Levels of actin (AC-15; Sigma) were monitored to assess protein loading. Primary antibodies were detected using secondary antibodies conjugated to near-infrared fluorophores (LiCor Biosciences, Lincoln, NE). Western blots were then imaged with the use of the Odyssey Infrared Imaging System (LiCor).

Immunoprecipitation

To detect chorionic gonadotropin α-subunit levels after adiponectin treatment, we immunoprecipitated the heterodimeric chorionic gonadotropin hormone from media collected from primary trophoblast cells in culture. Cell treatments were done as described above. The immunoprecipitation was performed with protein G magnetic beads (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Briefly, precleared medium from treated and nontreated cells was incubated with antibody against the β-subunit of chorionic gonadotropin (ab53087; Abcam). Protein G magnetic beads were then added. The samples were washed, resuspended in loading buffer, and used in Western blots (see above).

Radioimmunoassay

Progesterone levels were detected in media collected from adiponectin-treated and control cells using the Coat-A-Count kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA) according to the manufacturer's instructions. Briefly, progesterone calibrators and unknown medium samples were added to tubes coated with progesterone antibody. All samples were incubated with [^{125}I]progesterone for 3 h before being counted on a γ-counter. Unknown sample concentrations were determined by fitting to the standard curve generated by the progesterone calibrator samples (intraassay coefficient of variation of 2.4%).

Immunohistochemistry

To assess the spontaneous differentiation of these cells, term cytotrophoblast cells were cultured on chamber slides (Thermo Fisher Scientific, Rochester, NY) and treated with adiponectin as described above. Treated and untreated control cells were then fixed with paraformaldehyde (1%; Sigma) before being immunostained with antibodies directed against desmoplakin 1 and 2 (DP2.15; Millipore Corp., Billerica, MA) or connexin 43 (sc-9059; Santa Cruz Biotechnology, Santa Cruz, CA) and identified using a fluorescently tagged secondary antibody (Invitrogen) before being nuclear counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cells were also cultured and stained in parallel with nonspecific IgG as a negative control.

Gelatin zymography

Protease activity was assessed by application of conditioned medium to 8% acrylamide gels containing porcine gelatin. After electrophoresis, gels were washed for a total of 1.5 h in Tris-Cl with Triton X-100. Gels were then cut into strips, with

replicate samples on each strip, and incubated overnight in Tris-Cl with either EDTA [matrix metalloproteinase (MMP) inhibitor; Sigma], phenylmethylsulfonyl fluoride (serine protease inhibitor; Sigma), leupeptin (cysteine protease inhibitor; Sigma), pepstatin A (aspartate protease inhibitor; Sigma), or buffer alone. The next day, gels were stained to an appropriate contrast with Coomassie blue dye.

Data analysis

All experiments were performed with a minimum of four different cell preparations collected from distinct placentas. Statistical analysis was done using SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). Means for untreated control cells *vs.* adiponectin-treated cells were compared using the unpaired *t* test. For all experiments, $P < 0.05$.

4. Results

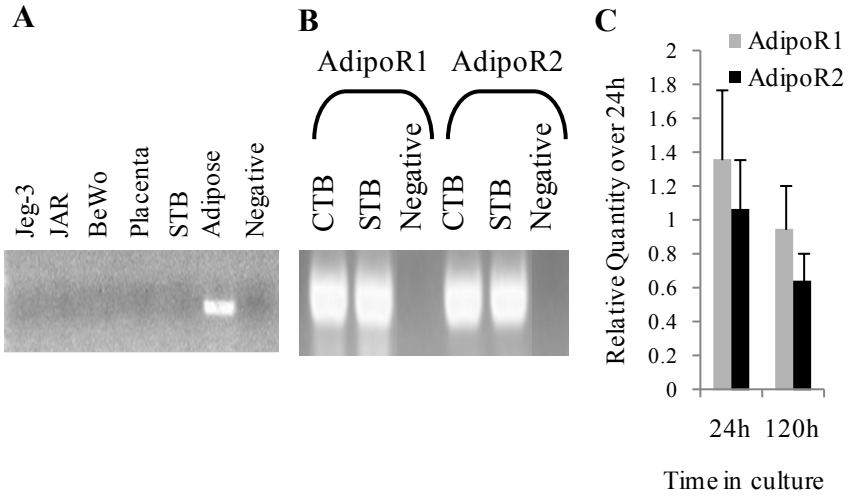
Adiponectin of a distant source can act on trophoblast cells of the placenta

To investigate the role of adiponectin at the maternal-fetal interface, we began by identifying potential sources of adiponectin during pregnancy. Despite initial reports suggesting adiponectin is produced by the placenta (Caminos et al., 2005, Lappas et al., 2005b, Chen et al., 2006), we were unable to detect a transcript for adiponectin using either RT-PCR or qPCR (Fig. VI-1a). In contrast, adiponectin was readily detected in mRNA from human adipose tissue (Fig. VI-1a). Our inability to amplify adiponectin cDNA from any of the choriocarcinoma cell lines (Jeg-3, JAR or BeWo) led us to conclude that adiponectin is not a product of trophoblast cells. To ensure adiponectin production was not restricted to a specific lineage of differentiated trophoblast, such as the syncytium, we performed RT-PCR for adiponectin using cDNA obtained from total placenta tissue as well as cytotrophoblasts induced to syncytialize via EGF treatment. Similar to our previous data, we failed to detect an adiponectin transcript, lending further evidence to the idea that the placenta is not a source of adiponectin.

In agreement with previous reports (Caminos et al., 2005), we were able to detect mRNA expression for both adiponectin receptors, adipoR1 and adipoR2, using purified cytotrophoblasts as well as cytotrophoblasts cultured with EGF (Fig. VI-1b). In addition, expression of these receptors was not significantly altered as the cells syncytialized (Fig. VI-1c). Thus, we conclude that while the placenta may not be a source of adiponectin, the trophoblast cells of the placenta are presumably able to respond to the hormone through both the adipoR1 and adipoR2 receptors.

Figure VI-1. Expression of adiponectin and its receptors in the placenta. (a) RT-PCR for adiponectin using RNA collected from human trophoblasts, placenta and adipose tissue. (b) RT-PCR for adipoR1 and adipoR2 using trophoblasts isolated at term. (c) Relative gene expression for adipoR1 and adipoR2 using quantitative RT-PCR on trophoblasts collected at term and cultured for 24 or 120 hours, mean ± SEM. Jeg-3, JAR, and BeWo, human choriocarcinoma cell lines; Placenta, term placental explants; STB, term trophoblast cells syncytialized in culture; Adipose, human adipose tissue; Negative, negative control; CTB, term cytotrophoblasts.

Figure VI-1.


A
Jeg-3
JAR
BeWo
Placenta
STB
Adipose
Negative
B
AdipoR1
AdipoR2
CTB
STB
Negative
CTB
STB
Negative
C
AdipoR1
AdipoR2
Relative Quantity over 24h
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24h
120h
Time in culture

Adiponectin treatment decreases endocrine gene expression in trophoblasts

As cytotrophoblasts remain in culture they spontaneously differentiate to form a multinucleated syncytium (Kliman et al., 1986). We examined the effect of adiponectin on this process by treating cytotrophoblasts isolated from term placentas in culture with recombinant globular adiponectin. In order to allow adequate time for spontaneous differentiation, the culture treatment was maintained for five days. Initially we treated with 0.5 or 2 μg/ml of recombinant globular adiponectin. Although we observed trends with the 0.5 μg/ml treatment, this sub-physiological dose of adiponectin failed to show significant alterations in mRNA expression (data not shown). Quantitative RT-PCR using RNA isolated from cells treated with 2 μg/ml adiponectin showed a significant drop in gene expression for numerous endocrine transcripts, including both the chorionic gonadotropin α (77.8±5.6% reduction) and β (84.5±2.0% reduction) subunits and human placental lactogen (PL-A and PL-B; 91.3±2.3% reduction; Fig. VI-2a-c).

These results were substantiated by western blot for the α and β subunits of CG, as well as PL (Fig. VI-2d). For CGβ and PL, media samples from adiponectin treated cells (5d; 2 μg/ml) were compared to media from nontreated control cells via western blot. The expression of CGα was evaluated by first performing immunoprecipitation with an antibody directed against CGβ. Western blots for CGα revealed an immunoreactive band corresponding to the heterodimeric protein, and migrated at the same position as protein detected with the CGβ antibody. Shown in Figure 2d are representative blots from one placenta. The experiment was repeated

with six different placentas, with 4/6 of these placentas displaying a similar drop in protein expression following adiponectin treatment.

Examination of the expression of specific enzymes involved in placental steroid hormone production, i.e. cholesterol side-chain cleavage enzyme (p450scc) and cytochrome p450 aromatase, also revealed a suppression in transcript levels (55.0±9.2% and 62.1±19.8% reduction respectively; Fig. VI-3a and VI-3b). Interestingly, mRNA expression of the enzyme 3β hydroxysteroid dehydrogenase (3βHSD) was unaltered by adiponectin treatment (Fig. VI-3c), suggesting divergent regulatory pathways controlling distinct steps in the steroidogenic pathway. In addition, examination of the protein levels showed a drop in both p450scc and aromatase expression following exposure to 2 μg/ml adiponectin for five days *in vitro* (Fig. VI-3d). These data are further corroborated by a drop in progesterone production, as determined by radioimmunoassay, in 4/6 placentas examined (Fig. VI-4). Hormone production is one of the key functions of the syncytium. Our data suggest adiponectin may impair this functionality, a phenomenon that could be achieved by inhibiting the differentiation process or specifically targeting the genes involved in hormone production.

Figure VI-2. Adiponectin inhibits endocrine gene expression. Quantitative RT-PCR using RNA from term trophoblasts cultured for five days in the absence (nt) or presence of adiponectin (2 μg/ml). Relative quantity of (a) chorionic gonadotropin α subunit, (b) chorionic gonadotropin β subunit, and (c) placental lactogen mRNA. (d) Protein expression of chorionic gonadotropin α and β subunits, placental lactogen. Mean ± SEM, * $P < 0.05$.

Figure VI-2.

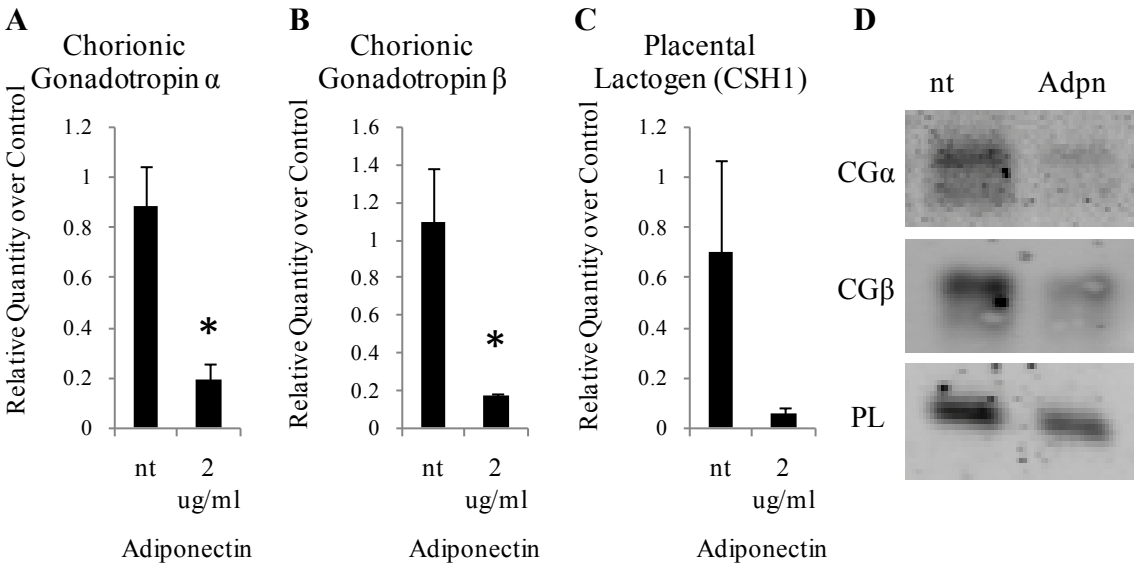

A
Chorionic Gonadotropin α
Relative Quantity over Control
1.2
1
0.8
0.6
0.4
0.2
0
*
nt
2 ug/ml
Adiponectin
B
Chorionic Gonadotropin β
Relative Quantity over Control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
nt
2 ug/ml
Adiponectin
C
Placental Lactogen (CSH1)
Relative Quantity over Control
1.2
1
0.8
0.6
0.4
0.2
0
nt
2 ug/ml
Adiponectin
D
nt
Adpn
CGα
CGβ
PL

Figure VI-3. Adiponectin regulates gene expression of steroidogenic enzymes in an enzyme-specific manner. Exposure of term trophoblast cells to adiponectin for five days *in vitro* resulted in an inhibition of gene expression for both p450 side chain cleavage and aromatase enzymes, while failing to result in a significant drop in expression of 3βHSD. Relative quantity of p450 side chain cleavage (a), aromatase (b), and 3βHSD (c) mRNA in cells cultured in the absence (nt) or presence of adiponectin (2 μg/ml). (d) Primary trophoblasts treated with adiponectin for 5 days *in vitro* display decreased levels of p450scc and aromatase proteins. Mean ± SEM, * $P < 0.05$.

Figure VI-3.

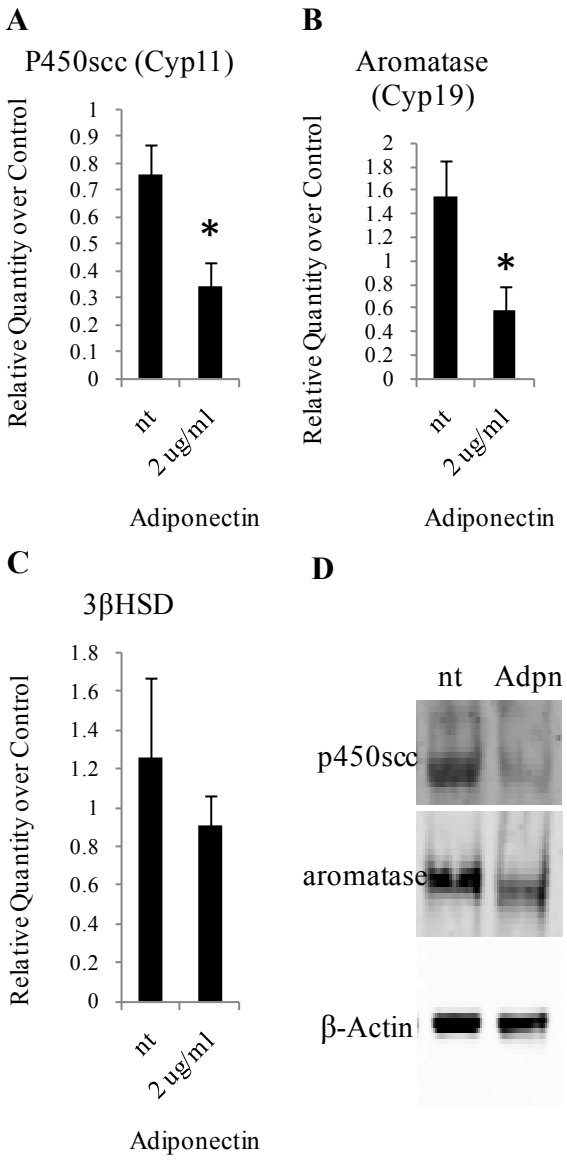
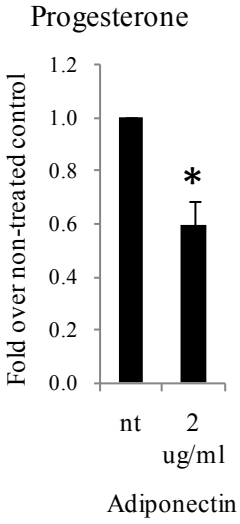

A
P450scc (Cyp11)
Relative Quantity over Control
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
nt
2 ug/ml
Adiponectin
B
Aromatase
(Cyp19)
Relative Quantity over Control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
nt
2 ug/ml
Adiponectin
C
3βHSD
Relative Quantity over Control
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
nt
2 ug/ml
Adiponectin
D
nt
Adpn
p450scc
aromatase
β-Actin

Figure VI-4. Adiponectin decreases progesterone production by trophoblast cells *in vitro*. Radioimmunoassay for progesterone was performed on media from cells treated with 2 μg/ml adiponectin for five days, as well as non-treated control cells. There was a significant drop in progesterone secretion following adiponectin treatment in four of six placentas examined. Interestingly, those two placentas that failed to show a decrease in progesterone production, also failed to show a drop in protein expression for other hormones, such as placental lactogen. * $P<0.05$.

Figure VI-4.


Progesterone
Fold over non-treated control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
nt
2 ug/ml
Adiponectin

Adiponectin does not inhibit spontaneous differentiation of trophoblasts in vitro

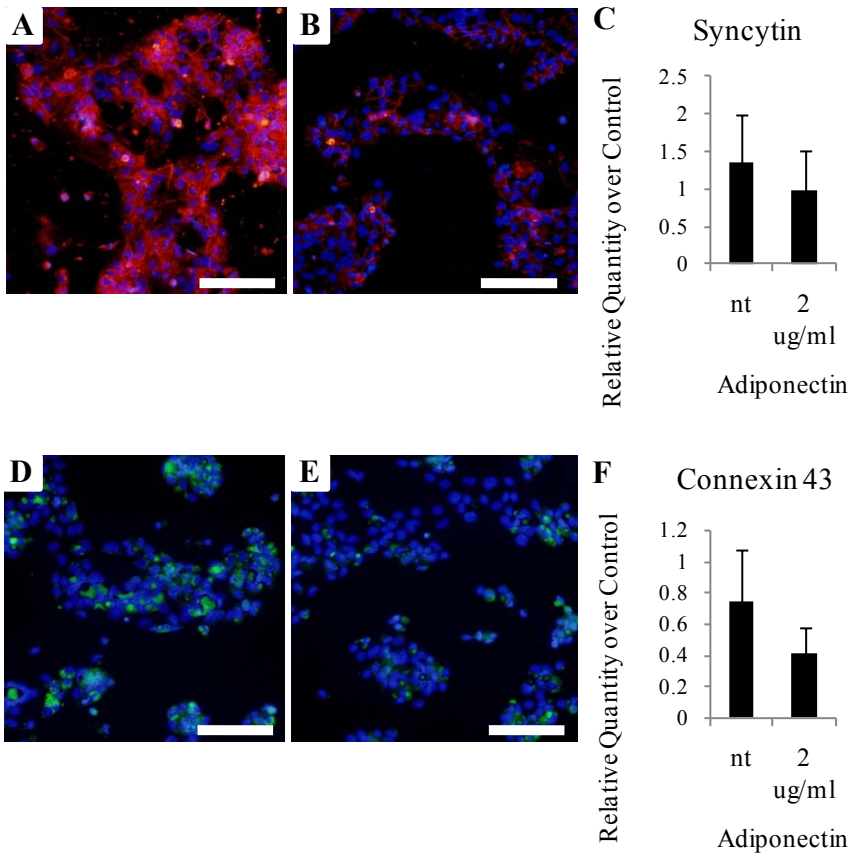
In order to examine the effect of adiponectin on the spontaneous differentiation of trophoblast cells, we cultured cytotrophoblasts from term placenta on chamber slides in the presence of recombinant adiponectin (2 μg/ml) for five days. Cells were then stained for either desmoplakin or connexin 43 in order to observe any morphological changes as a result of adiponectin treatment. The gap junction component, connexin 43, has been shown to be present between cytotrophoblasts and cytotrophoblasts in contact with syncytiotrophoblasts (Cronier et al., 2002). Both desmoplakin as well as the connexin 43 staining failed to reveal any inhibition in the spontaneous aggregation/differentiation process undergone by cytotrophoblasts (Fig. VI-5). Indeed, those cells exposed to adiponectin may even display a higher degree of differentiation than the control untreated cells, as evidenced by increased plasma membrane localization of desmoplakin (Fig. VI-5a and VI-5b) and a lower degree of connexin 43 staining (Fig. VI-5d and VI-5e). Quantitative RT-PCR for connexin 43 and the retroviral gene syncytin failed to show a significant difference in gene expression between the control cells and those treated with adiponectin for either transcript (Fig. VI-5c and VI-5f). Taken together, these data show a lack of inhibition of morphological differentiation of trophoblast cells after they have been exposed to adiponectin in long-term culture.

We also evaluated MMP expression and activity through qPCR and gelatin zymography. In contrast to the endocrine genes, we observed no significant difference in the gene expression of MMP9 (data not shown). The trophoblasts were also secreting abundant amounts of active MMP. We were however unable to detect a

significant difference in the level of MMP activity in adiponectin treated cells compared to untreated controls (data not shown).

Figure VI-5. Adiponectin does not inhibit differentiation of trophoblasts *in vitro.* Immunocytochemistry for desmoplakin and connexin 43 was performed on cells exposed to adiponectin (2 µg/ml) for five days *in vitro* in order to examine spontaneous differentiation of the trophoblast cells. Similar staining was observed between the untreated cells (a, d) and those exposed to adiponectin (b, e). (a) Control cells stained with desmoplakin and DAPI. (b) Cells treated with adiponectin and stained for desmoplakin and DAPI. (d) Control cells stained for connexin 43 and DAPI. (e) Adiponectin treated cells stained for connexin 43 and DAPI. Scale bar = 100 µm. (c) Relative quantity of syncytin mRNA using quantitative RT-PCR on control and adiponectin treated cells. (f) Relative quantity of connexin 43 mRNA in cells receiving either no treatment or adiponectin for five days in culture; mean ± SEM. nt, no treatment; 2 µg/ml, adiponectin treatment.

Figure VI-5.


A
B
C
Syncytin
Relative Quantity over Control
2.5
2
1.5
1
0.5
0
nt
2 ug/ml
Adiponectin
D
E
F
Connexin 43
Relative Quantity over Control
1.2
1
0.8
0.6
0.4
0.2
0
nt
2 ug/ml
Adiponectin

5. Discussion

Despite its relative recent characterization, adiponectin is widely regarded as an important means by which an organism regulates it metabolic status. The placenta represents an organ unique in its pivotal role in mediating the nutritive state of the developing fetus. In addition, the placenta is highly secretory, producing a number of hormones and growth factors, such as members of the growth hormone family, which have been implicated in influencing maternal energy homeostasis (Barbour et al., 2002). Both placental lactogen and placental growth hormone, produced in large amounts by the mature placenta, can act on distant targets to increase insulin resistance and thus allow for increased glucose availability to the fetal-placental unit (Barbour et al., 2002, Handwerger and Freemark, 2000). In addition, clinical evidence exists for higher placental lactogen levels in cases of gestational diabetes, particularly in instances of gestational diabetes without placental dysfunction (Ursell et al., 1973).

While a certain amount of maternal insulin resistance is necessary during pregnancy in order to ensure adequate glucose transfer to the fetus (Lain and Catalano, 2007, Reece, 2008), this system must remain under tight control. One such mechanism by which the mother may be able to control the influence of the placenta on her own insulin sensitivity is through maternal adiponectin secretion. Our data suggest a novel role for adiponectin at the level of the maternal-fetal interface whereby adiponectin may regulate hormone production by the placenta, thus reducing the amount of peripheral insulin resistance induced by many of these hormones. Such a hypothesis is supported by the abundance of evidence in the literature citing a decrease in serum adiponectin levels during cases of extreme maternal insulin

resistance (i.e. gestational diabetes mellitus) (Cortelazzi et al., 2007, Altinova et al., 2007, D'Anna et al., 2005, Ranheim et al., 2004, Tsai et al., 2005, Williams et al., 2004, Cseh et al., 2004, Georgiou et al., 2008). In addition, studies in bovine and porcine ovaries, as well as a murine hypothalamic and pituitary model, have set precedence for adiponectin having a negative impact on hormone production (Lu et al., 2008, Wen et al., 2008, Ledoux et al., 2006, Lagaly et al., 2008). The data presented herein suggests for the first time a direct effect of adiponectin on hormone production by the placenta.

Hormone production by the human placenta is a unique function of the syncytiotrophoblast cells. This subpopulation of trophoblast cells make up the outer lining of the villi, and are derived from the underlying cytotrophoblast layer. Differentiation of cytotrophoblasts to syncytiotrophoblasts in culture is commonly measured by the up regulation of endocrine genes and hormone production. Due to the significant decrease in endocrine gene expression seen following adiponectin administration to trophoblasts *in vitro*, we hypothesized that adiponectin may be inhibiting this differentiation process. Morphological analysis of cells treated with adiponectin, however, did not reveal an obvious inhibition of cellular aggregation and syncytialization when compared to the untreated controls. This suggests divergent mechanisms may exist for morphological syncytialization as compared to formation of a functioning syncytium, at least with regard to endocrine function. The cellular mechanism(s) by which adiponectin elicits these distinct effects remains to be determined.

Although we have chosen to focus on adiponectin production by the mother, it remains important to point out that the fetus is also producing adiponectin by late gestation (Lindsay et al., 2003). Our data do not preclude the possibility of significant fetal contributions to local adiponectin levels at the placenta. Nonetheless, we feel this should not distract from the overall hypothesized effect of adiponectin in helping to regulate maternal insulin sensitivity via placental hormone synthesis.

There remains a significant lack of understanding regarding the influence of adiponectin at the maternal-fetal interface. Given the proven importance of this hormone in maintaining energy homeostasis in other biological systems, there is a real need for a better understanding of the influence adiponectin may play on various functions of the placenta. Our data provide the first evidence for a significant role of adiponectin on the syncytium of the mature placenta. These studies provide a springboard from which subsequent studies may elucidate precise mechanisms by which adiponectin exerts its influence over the trophoblast cells of the placenta. Understanding this regulatory mechanism will enhance our knowledge of the physiological processes that may be affected during cases of gestational diabetes mellitus, thus allowing for more comprehensive care of both the mother and child affected by this disorder.

6. Acknowledgements

We thank Dr. Lane K. Christenson for helpful discussions and performing the progesterone assay as well as assistance in data analysis, Dr. David Albertini for technical assistance, and Dr. Michael J. Soares and Dr. Joan Hunt for critical review of the manuscript.

VII: Chapter Three:

The Pro-Inflammatory Role of Adiponectin at the Maternal-Fetal Interface

1. Abstract

A successful pregnancy is contingent on maternal tolerance of the immunologically foreign fetus. Prevalent diseases such as preeclampsia are thought to be due to an inappropriate immune response at the maternal-fetal interface. Here we report the metabolic hormone, adiponectin, to be pro-inflammatory at the maternal-fetal interface. Treatment of term trophoblasts with adiponectin (2μg/ml) for 5d increased expression of IL-1β and IL-8 mRNA, an effect that also occurred when syncytialized cells are exposed to adiponectin for only 24h. There exist a number of molecules that have been proposed to temper cellular response to pro-inflammatory mediators, including the cell surface proteins, CD24 and Siglec10. Immunohistochemistry of sections of term villi revealed abundant expression of CD24 in cytotrophoblasts and syncytiotrophoblasts, while Siglec10 was only expressed by syncytiotrophoblasts. Treatment of cytotrophoblast cells from healthy term placenta with globular adiponectin (2μg/ml) during the course of spontaneous syncytialization *in vitro* resulted in a significant increase in Siglec10 mRNA and protein levels. These data suggest the trophoblast cell is capable of upegulating Siglec10 expression as a means to counteract the pro-inflammatory effects of adiponectin. Indeed, over-expression of Siglec10 in primary trophoblast cultures results in a dampening of the cytokine gene expression response to adiponectin exposure. Our findings are novel in two respects: 1) this represents the first time the CD24/Siglec10 pathway has been implicated in a trophoblast response to a pro-inflammatory mediator, and 2) these data describe a role for adiponectin in enhancing pro-inflammatory signals in syncytiotrophoblasts.

2. Introduction

For mammalian pregnancy to be successful a complex interplay between maternal and fetal systems must take place. One crucial interaction is the maternal accommodation of nutrient demands placed on her both by her own rapidly changing body and the developing fetus. Maternal stores of adipose have been implicated in playing an important role in mediating this balance, both energetically as well as through endocrine actions. We have previously reported adiponectin, a hormone produced by maternal adipose stores, to cause a significant drop in hormone production by the placenta (McDonald and Wolfe, 2009). Although a number of epidemiological studies have identified adiponectin as being altered during metabolically stressed pregnancies (such as gestational diabetes and preeclampsia), much work remains in order to determine the effect(s) of adiponectin on the human placenta.

Another key aspect of a successful pregnancy is tolerance of the immunologically foreign fetus by the maternal immune system. The maternal-fetal interface has a number of systems in place which help to maintain its quiescent state with regards to immune activation, and this area is under intense investigation by a number of laboratories. A potential immunological role for adipokines, a group of hormones commonly associated with their potent metabolic roles, has received relatively little attention to date. A growing number of clinical studies have reported increases in term adiponectin levels in maternal serum in women diagnosed with preeclampsia (Avci et al., 2010, D'Anna et al., 2006, Davis et al., 2007, Fasshauer et al., 2008, Haugen et al., 2006, Nien et al., 2007, Ramsay et al., 2003). Although

adiponectin was originally identified as being anti-inflammatory (Wolf et al., 2004, Wulster-Radcliffe et al., 2004, Mandal et al., 2010, Ebina et al., 2009), a number of studies have recently shown it to induce pro-inflammatory cytokines under specific conditions (Song et al., 2009, Tomizawa et al., 2009, Tomizawa et al., 2008, Kitahara et al., 2009, Lappas et al., 2005a, Haugen and Drevon, 2007). Additionally, adiponectin has now been shown to activate the NFκB signaling pathway (Haugen and Drevon, 2007, Tomizawa et al., 2008, Lappas et al., 2005a), one of the most common signaling mechanisms involved in pro-inflammatory cytokine production. The pro-inflammatory responses appear to be mediated primarily through globular adiponectin (Tomizawa et al., 2009, Tomizawa et al., 2008), the form of adiponectin which has shown to be highly effective in mediating a variety of biological effects, such as fatty acid oxidation in skeletal muscle (Fruebis et al., 2001).

With regards to cytokine production at the maternal-fetal interface, globular adiponectin has been shown to increase production of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα in human placental explant cultures collected at term (Lappas et al., 2005a). Although this study suggests that adiponectin plays a pro-inflammatory role at the maternal-fetal interface, the use of explants leaves open the possibility that a cell type other than trophoblast cells is responsible for the reported cytokine production. Indeed, a number of studies have shown adiponectin to influence cytokine production by other cell types also present in placental explants, including macrophages (Wulster-Radcliffe et al., 2004) and endothelial cells (Tomizawa et al., 2009, Tomizawa et al., 2008). In order to conclusively demonstrate the effect of globular adiponectin on trophoblast cells of the human placenta, we examined the

effect of adiponectin treatment on gene expression for a variety of cytokines in isolated trophoblast cells.

Immune tolerance at the maternal-fetal interface is thought to occur through a variety of mechanisms, including regulation of HLA expression, regulatory T cells, and immunomodulatory proteins, such as members of the B7 family (Petroff, 2005, Clark et al., 2010). The amount of redundancy between mechanisms makes this system increasingly complex. In addition, a recent report has outlined a mechanism involving the cell membrane proteins, CD24 and Siglec10, in dampening immune activation initiated by damage-associated molecular patterns (DAMPs) (Chen et al., 2009). Although no data has been reported regarding a role for the CD24/Siglec10 pathway at the maternal-fetal interface, it remains possible this is another mechanism by which the human placenta regulates immune activation throughout gestation.

In this report we demonstrate that globular adiponectin is a potent inducer of pro-inflammatory cytokine production by the trophoblast cells of the human placenta. Furthermore, we provide evidence for the CD24/Siglec10 pathway as a compensatory mechanism utilized by the syncytiotrophoblast in an effort to moderate the pro-inflammatory cascade initiated by treatments such as adiponectin. These data not only demonstrate that adiponectin can be detrimental to the success of a pregnancy through its pro-inflammatory role, but also suggest a novel mechanism in trophoblast cells by which pro-inflammatory mediators may be brought under control, thus ensuring the continued success of the pregnancy.

3. Materials and Methods

Cell Isolation and Culture

Human placentas were obtained under protocols approved by the institutional review board at the University of Kansas Medical Center. Cytotrophoblasts (CTBs) were isolated from healthy placentas delivered via cesarean at term as previously described (McDonald and Wolfe, 2009). Purified cells were placed in culture media (Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin/amphotericin B) and allowed to adhere to the culture dish by incubation in a humidified chamber overnight prior to receiving treatment. All cells underwent regular media changes, with refreshment of treatment conditions every 48h. For all adiponectin treatments, recombinant globular adiponectin (2µg/ml; BioVendor, Czech Republic) was used.

RNA Isolation and Quantitative RT-PCR (qPCR)

RNA from trophoblasts in culture was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers. Quantitative real-time PCR (qPCR) was performed using SYBRgreen technology (Applied Biosystems, Foster City, CA). All gene expression data was normalized to 18S levels within the respective sample. All primers (Table VI-1) were designed using Primer Express 3.0 software (Applied Biosystems) and synthesized by Integrated DNA Technology (Coralville, IA). Resulting data was analyzed as outlined previously (McDonald and Wolfe, 2009).

Table VII-1. Primer Sequences for Quantitative RT-PCR.

Gene	Forward Primer	Reverse Primer
18S	5'-cgccgctagaggtgaaattct-3'	5'-cgaacctccgactttcgttct-3'
IL-1β	5'-cttggtgatgtctggtccatatga-3'	5'-ggacatggagaacaccacttgtt-3'
IL-8	5'-tgatttctgcagctctgtgtga-3'	5'-gggtccagacagagctctcttc-3'
Siglec10	5'-gcgggtcccaggctatg-3'	5'-ccctccggcaccatcaa-3'

Cytokine Assay

Cytokine production was measured using media samples from cells cultured for 5d in the presence of adiponectin, and compared to media from corresponding cells from the same placenta prep that were cultured in media alone. Cytokines were quantified using the Milliplex MAP kit (Millipore, Billerica, MA) and the manufacturer's protocol. Briefly, media samples were incubated with antibody-labeled beads against interleukin (IL)-8 and IL-1β overnight at 4°C. After extensive washing, amount of bound antibodies were assayed through the use of detection antibodies and streptavidin-phycoerythrin. The assay plate was read on a Luminex 200 system (Luminex, Austin, TX). Cytokine levels wer quantitated through application of a standard curve.

Immunofluorescence

Immunohistochemistry was performed on villous sections of term placentas, freshly isolated cytotrophoblasts, and trophoblasts cultured on chamber slides for 5d. Villous sections with the basal plate intact were fixed in 4% paraformaldehyde (Sigma Chemical, St. Louis, MO) for four hours and then infused with 18% sucrose solution overnight before being frozen. Frozen fixed tissue was then cut into 10μm sections on a cryostat. Cytospins from freshly isolated cytotrophoblasts were allowed to dry at room temperature overnight and stored at -20°C. Isolated cytotrophoblasts were also plated on chamber slides (Thermo Fisher Scientific, Rochester, NY) for 5d with/without adiponectin (2 μg/ml) before being fixed in 1% paraformaldehyde for 10 minutes and permeabilized in ethanol:acetic acid at -20°C for 5 minutes, with antigen unmasking in 0.5M ammonium chloride for 30 minutes.

Slides were blocked against nonspecific antibody binding with 10% (v/v) donkey serum in PBS. A primary antibody to Siglec10 (AF2130, R&D Systems, Minneapolis, MN), CD24 (ML5; BD Biosciences, San Jose, CA) or nonimmune control IgG was applied to sections and incubated overnight at 4^{o}C in a humidified chamber. Sections were washed, secondary antibody (Alexa Fluor 546 donkey anti-goat IgG or Alexa Fluor 555 donkey anti-mouse IgG, Invitrogen) applied at the appropriate concentration and incubated at room temperature for 1 hr. Sections were then washed and counterstained with DAPI (Invitrogen). Immunofluorescence was visualized using an Olympus IX71 inverted microscope.

Lentiviral Infection

Complete virus was made in 293FT cells (Invitrogen) by co-transfection with WPI vector harboring the complete cDNA for human Siglec10 and the packaging vectors, psPAX2 and pMD2.G (generous gifts from the Trono Lab). Transfections were completed using Lipofectamine 2000 (Invitrogen) and a standard transfection protocol. Briefly, vectors and lipofectamine 2000 were added to separate aliquots of Opti-MEM reduced media (Invitrogen) and incubated at room temperature for 5 min. The aliquots of Opti-MEM were then combined and allowed to form liposomes by incubation at room temperature for 20 min. 293FTs were trypsinized, the lipofectamine complexes were added to distinct 10 cm^2 culture dishes, and 6 x 10^6 cells added. Mature virus was harvested by collection of the media 48 and 72h post-transfection. Infection of term trophoblast cells was done by adding media harboring mature Siglec10WPI virus in the presence of 8μg/ml Sequabrene (Sigma Chemical).

Data Analysis

All experiments were performed with a minimum of three distinct placentas. Statistical analysis was done using SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). Differences in the mean relative quantity of gene expression in adiponectin treated compared to non-treated cells was assessed for significance through use of the Mann-Whitney Rank Sum Test. Cytokine production following adiponectin treatment was compared to levels in control cells within the sample placenta preparation using the Wilcoxon Signed Rank Test, in order to account for variation between placentas. For all experiments, $P < 0.05$.

4. Results

Adiponectin elicits pro-inflammatory cytokine production in human trophoblasts

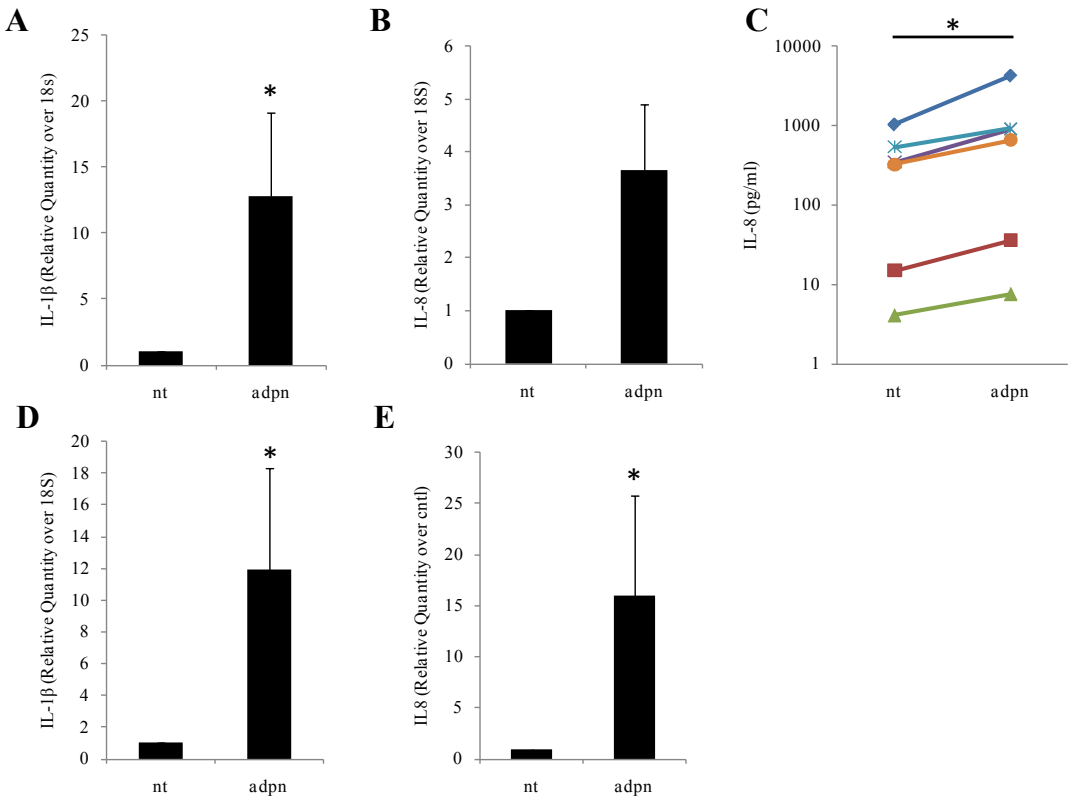
Previous data generated in our laboratory uncovered a negative effect of long-term (5d) adiponectin treatment on hormone production by trophoblast cells allowed to differentiate spontaneously *in vitro* (McDonald and Wolfe, 2009). Here, we provide evidence that within the same experimental paradigm, adiponectin also acts as a pro-inflammatory mediator, enhancing production of the cytokines IL-1β and IL-8 in trophoblast cells.

Term trophoblasts were isolated and cultured in the presence of globular adiponectin (2μg/ml) for 5d. We have previously shown in our laboratory that this is sufficient time to allow for spontaneous differentiation of the cells in culture to a syncytiotrophoblast phenotype (data not shown). Quantitative RT-PCR for the pro-inflammatory cytokines IL-1β and IL-8 show an increase in gene expression for these two cytokines in cells collected at the end of the 5d culture period and exposed to adiponectin throughout the culture period, as compared to those cultured in the absence of adiponectin (Fig.VII-1a&b). We also analyzed the production of IL-8 and IL-1β in media samples collected from 5d treatment of adiponectin. The production of IL-1β was extremely low in some of the samples, making direct comparisons between treated and untreated cells difficult (data not shown). Although IL-8 production was highly variable between placentas, the production of this cytokine was significantly increased following adiponectin treatment in all of the placenta preparations analysed (Fig. VII-1c).

In addition, we examined the expression of the pro-inflammatory cytokines, IL-1β and IL-8 in term trophoblast cells after 24h of adiponectin exposure. These cells were placed in culture for 4d prior to receiving adiponectin (2μg/ml) for the final 24h of the culture period. In this way, they were allowed to differentiate *in vitro*, and are thus comparable to the cells we have previously found to have increased expression of IL-1β and IL-8 with adiponectin exposure for the entire 5d culture period. Treatment with globular adiponectin for the final 24h of the culture period resulted in an up-regulation of mRNA for both IL-1β and IL-8 (Figure VII-1d&e), similar to what was seen with adiponectin administration for the entire 5d. These data suggest that the pro-inflammatory cytokine production by trophoblasts is a direct response to adiponectin itself, rather than the result of an intermediate effecter modulated by adiponectin. Taken together, these data demonstrate that adiponectin acts as a pro-inflammatory mediator in term, syncytialized trophoblast cells.

Figure VII-1. Adiponectin up-regulates expression of the pro-inflammatory cytokines, IL-1β and IL-8 in term trophoblast cells. Quantitative RT-PCR for IL-1β (A) and IL-8 (B) in trophoblasts collected at term and cultured in the presence of globular adiponectin (2μg/ml) for 5d. Gene expression for both pro-inflammatory cytokines is up-regulated in the adiponectin exposed cells compared to no treatment control cells (n=4). Secretion of IL-8 is upregulated following 5d adiponectin treatment (n=5). Adiponectin also stimulates expression of IL-1β (D) and IL-8 (E) in term trophoblasts placed in culture for 4d prior to treatment with adiponectin for 24h (n=4). * $P < 0.05$.

Figure VII-1.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IL-1β (Relative Quantity over 18s)
25
20
15
10
5
0
*
nt
adpn
B
IL-8 (Relative Quantity over 18S)
6
5
4
3
2
1
0
nt
adpn
C
IL-8 (pg/ml)
10000
1000
100
10
1
*
nt
adpn
D
IL-1β (Relative Quantity over 18S)
20
18
16
14
12
10
8
6
4
2
0
*
nt
adpn
E
IL8 (Relative Quantity over cntl)
30
25
20
15
10
5
0
*
nt
adpn

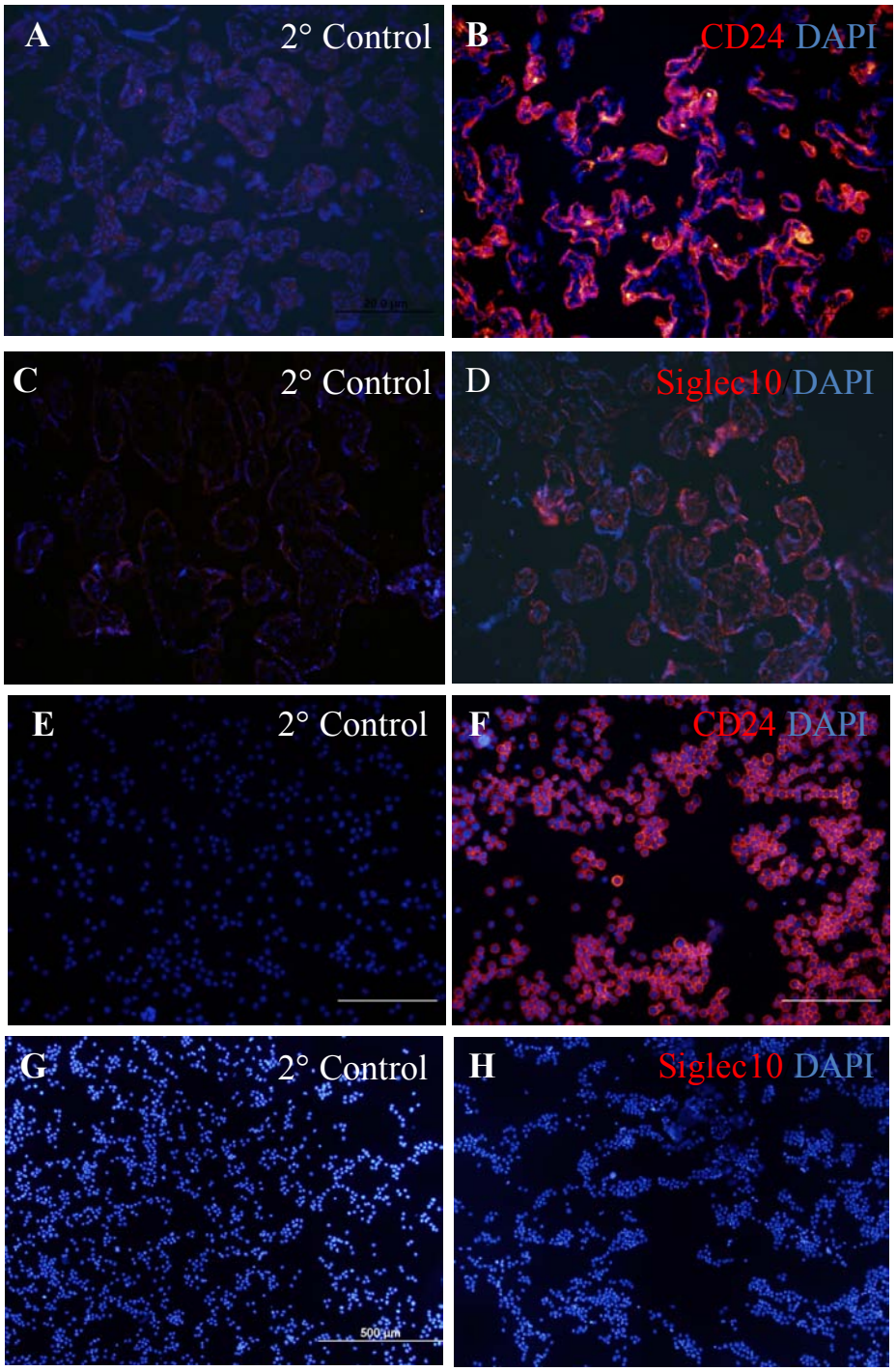
Trophoblast cells express CD24 and Siglec10 at term

A recent report has outlined a role for the cell membrane proteins, CD24 and Siglec10, as partnering to dampen a cell's inflammatory response to danger-associated molecular patterns (DAMPs) (Chen et al., 2009). We hypothesized this may represent another mechanism utilized by trophoblast cells to keep the maternal-fetal interface in a largely inactivated immune state. Towards this end, we began by looking for expression of both CD24 and Siglec10 in preparations of pure trophoblast cells.

Villous sections taken at term revealed robust expression of CD24 within the syncytiotrophoblast layer of the villous (Fig. VII-2b). Trophoblast cells collected at term from healthy placentas were also examined for the expression of both CD24 and Siglec10 proteins. Syncytiotrophoblast expressed Siglec10 protein, although at a much lower amount than that of CD24 (Fig. VI-2d). Interestingly, cytospins made from freshly isolated trophoblasts, and thus a population of >99% undifferentiated cytotrophoblasts, express robust levels of CD24, but remain devoid of Siglec10 (Fig. VI-2f&h). The dynamic expression pattern of Siglec10 is interesting, and suggests that it may fulfill a more specific role than CD24 in human trophoblasts. Consequently we chose to examine the effect of adiponectin exposure on Siglec10 expression.

Figure VII-2. Both CD24 and Siglec10 are expressed by human trophoblast cells at term. Villous sections of term placenta (A-D) are positive for both CD24 and Siglec10 protein, particularly within the syncytiotrophoblast layer. In contrast, cytospins of cytotrophoblasts freshly isolated from term placentas (E-H) show positive reactivity for CD24, but not Siglec10. Negative control for CD24 staining (a&e); CD24 immunohistochemistry (b&f); negative control for Siglec10 staining (c&g); Siglec10 immunohistochemistry (d&f). All sections were counterstained with DAPI, n=3. Scale bar = 500μm

Figure VII-2.


A
2° Control
B
CD24 DAPI
C
2° Control
D
Siglec10 DAPI
E
2° Control
F
CD24 DAPI
G
2° Control
H
Siglec10 DAPI

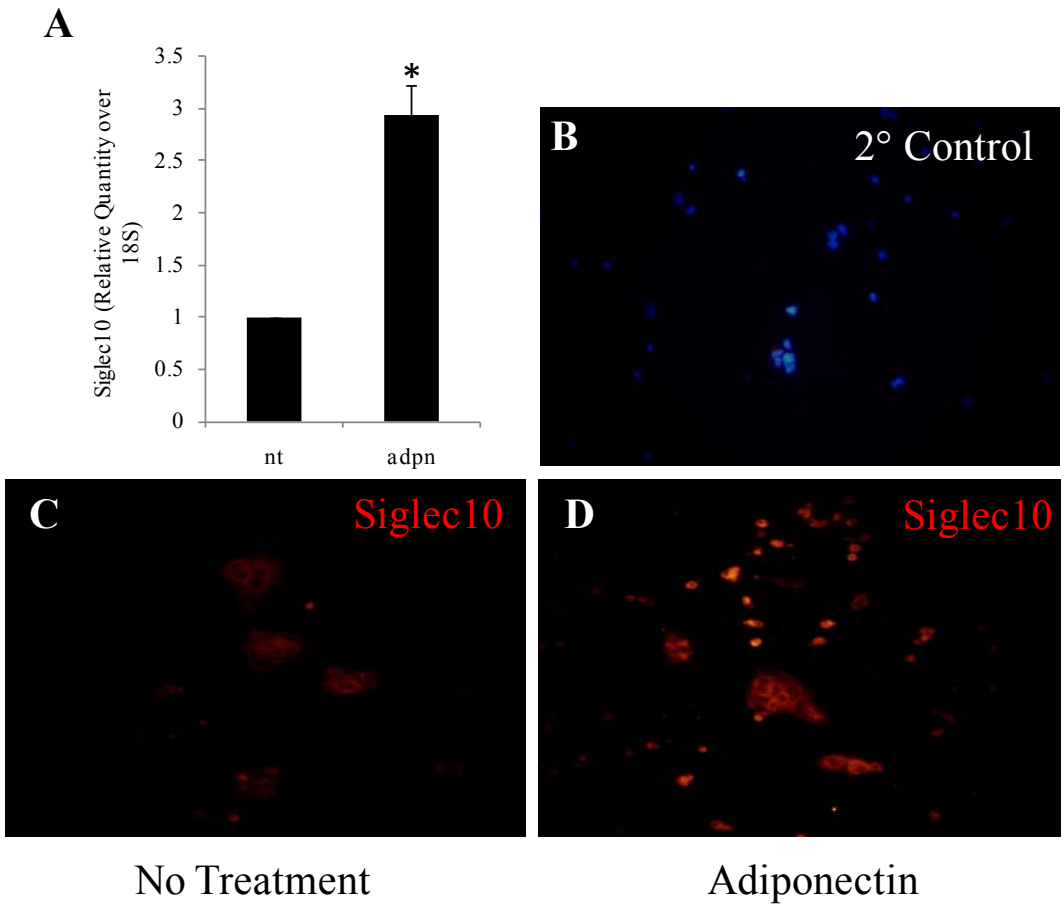
Adiponectin increases Siglec10 expression in trophoblast cells

We began our investigation of the effect of globular adiponectin treatment on term trophoblasts cells by exposing the cells to adiponectin for 5d in culture, as was done in our previous experiments. Quantitative RT-PCR reveals an increase in Siglec10 gene expression following adiponectin exposure (Fig. VII-3a). In addition, cells were plated on chamber slides, exposed to adiponectin for 5d, and stained for Siglec10. Trophoblasts cultured in the absence of adiponectin showed limited positive immunoreactivity for Siglec10 (Fig. VII-3c). Cells exposed to adiponectin throughout the culture period, however, had increased levels of Siglec10 immunoreactivity, compared to no treatment control cells (Fig. VII-3d). This phenomenon was not due to a possible effect of adiponectin on the syncytialization process, as we have previously shown that adiponectin does not inhibit syncytialization of term trophoblasts *in vitro* (McDonald and Wolfe, 2009).

These data indicate that Siglec10 expression, at both the mRNA and protein level, is increased in trophoblast cells following long-term exposure to adiponectin. Based on the reported function of CD24/Siglec10 to down-regulate the pro-inflammatory response to DAMPs (Chen et al., 2009), we hypothesized that a similar phenomenon may occur in human trophoblast cells. Furthermore, this may explain the lack of pregnancy-related phenotypes/complications following adiponectin over-expression in mouse models (Lu et al., 2008).

Figure VII-3. Adiponectin treatment increases Siglec10 expression in term trophoblast cells. Human trophoblasts collected at term and cultured for 5d in the presence of globular adiponectin (2μg/ml) show an increase in Siglec10 expression. A) Quantitative RT-PCR for Siglec10 shows an increase in Siglec10 mRNA after 5d exposure to adiponectin *in vitro.* B) Negative control for Siglec10 staining, counterstained with DAPI; C) Siglec10 staining in control term trophoblasts cultured for 5d. D) Siglec10 staining in term trophoblasts exposed to adiponectin while in culture for 5d. n=4. * $P < 0.05$.

Figure VII-3.


A
3.5
3
2.5
2
1.5
1
0.5
0
Siglec10 (Relative Quantity over 18S)
*
nt
adpn
B
2° Control
C
Siglec10
D
Siglec10
No Treatment
Adiponectin

5. Discussion

The maternal-fetal interface is physiologically unique in its ability to manipulate the maternal immune system in order to ensure continued growth and development of the immunologically-foreign fetus. Inappropriate immune activation can result in a number of adverse pregnancy outcomes, including preeclampsia, fetal growth restriction, and preterm labor/delivery. A predominantly Th1 environment at the maternal-fetal interface has been implicated in mediating the onset of these diseases (Challis et al., 2009). Furthermore, activators of trophoblast NFκB signaling can result in preeclampsia-like symptoms in a mouse model (Tinsley et al., 2009).

The hormone adiponectin was originally identified for its role in regulating blood glucose levels and contributing to insulin sensitivity. Adiponectin is produced predominantly by adipose tissue and enhances the effects of insulin through decreased gluconeogenesis in the liver, and increased insulin sensitivity and glucose uptake in skeletal muscle (Berg et al., 2001, Combs et al., 2001, Fruebis et al., 2001, Yamauchi et al., 2001). Indeed, the hormone was initially suggested as a potential treatment option for insulin resistance and/or obesity (Shklyaev et al., 2003).

Numerous studies have identified an anti-inflammatory role for adiponectin (Ebina et al., 2009, Mandal et al., 2010, Wolf et al., 2004, Wulster-Radcliffe et al., 2004). As investigation regarding the effects of adiponectin in multiple physiological systems has progressed, it has been shown to also exert pro-inflammatory cytokine production in specific diseases, particularly autoimmune diseases such as rheumatoid arthritis (Kitahara et al., 2009) and lupus (Song et al., 2009). Interestingly, the pro-inflammatory versus anti-inflammatory effects of adiponectin have been suggested to

be mediated through different forms of the hormone. High molecular weight (HMW) adiponectin has been reported to be pro- or anti-inflammatory, while the cleaved form, also known as globular adiponectin, has been implicated in mediating pro-inflammatory processes (Tomizawa et al., 2008, Haugen and Drevon, 2007).

With regards to the placenta, adiponectin has been previously shown to activate NFκB signaling (Lappas et al., 2005a). These data however were collected with the use of placental explant cultures, meaning that the pro-inflammatory effect by adiponectin could be derived from a number of different cell types present in the culture system. In this report we have evaluated the direct effects of globular adiponectin on term trophoblasts collected from healthy pregnancies. Thus, these data support the previous data suggesting adiponectin plays a pro-inflammatory role at the maternal-fetal interface, and show that this effect is mediated through trophoblasts themselves.

Trophoblast cells utilize a number of different mechanisms to evade and down-regulate the maternal immune system. Despite intense investigation, the redundancy and complexity of maternal immune regulation leaves much to be discovered. A recent paper by Chen and colleagues (Chen et al., 2009) outlined the cell surface molecules, CD24 and Siglec10, to potently down-regulate an organism's response to self antigens classified as damage-associated molecular patterns (DAMPs). Microarray analysis, performed in our laboratory, of primary human trophoblast cells cultured in the presence of adiponectin throughout *in vitro* differentiation showed elevated expression for both CD24 and Siglec10 compared to the untreated control cells (McDonald and Wolfe, unpublished data). From these data

we hypothesized that the CD24/Siglec10 system may also be functional at the maternal-fetal interface. The data reported herein provides evidence that Siglec10 is dynamically up-regulated in trophoblasts in response to strong pro-inflammatory signals such as adiponectin. Further examination of the CD24/Siglec10 pathway in trophoblasts is warranted, as this may be another mechanism by which maternal immune response to the developing fetus is dampened.

A previous report by our group identified a detrimental effect of adiponectin on trophoblast hormone production (McDonald and Wolfe, 2009). Using the same treatment paradigm as that outlined in the current report, we were able to show a decrease in the production of chorionic gonadotropin, placental lactogen, and progesterone by the cells allowed to spontaneously differentiate in the presence of adiponectin. If we think of pregnancy as a balance between the physiological needs and health of the mother versus the fetus, these data support the notion that adiponectin promotes the health of the mother, and thus works to keep the metabolic and invasive demands of the fetus and fetally-derived trophoblast cell in check. These data suggest that adiponectin is pro-inflammatory toward trophoblast cells, and thus detrimental to the ongoing success of pregnancy. Examination of the effect of adiponectin and the trophoblast response in malnourished or extremely lean women (conditions associated with elevated maternal adiponectin) is thus very important, and could have significant contributions to public health on a global scale. Additionally, these data support further investigation into the role of CD24/Siglec10 as another potential mechanism by which trophoblast cells successfully master maternal immune mechanisms.

VIII: Chapter Four:

Signaling cascades initiated by adiponectin in trophoblast cells

1. Abstract

The transfer of nutrients to the developing fetus places an enormous strain on the metabolic control mechanisms of the mother. This becomes increasingly true as gestation continues, and the fetus enters a period of high growth. Inappropriate regulation of glucose transfer to the fetus can result in the manifestation of diseases such as gestational diabetes, a condition that can have very serious implications for the present and future health of both mother and child. Hormones originating from maternal adipose tissue have recently gained interest for their potent roles in regulating metabolism and glucose homeostasis. We have previously shown that the adipocyte-derived hormone, adiponectin, can alter the hormone production of term syncytialized trophoblast cells, as well as initiate a pro-inflammatory response from these cells *in vitro*. In this report we identify that adiponectin can activate the ERK1/2 and EGF signaling pathways in term trophoblasts. However, in contrast to other reports, including one in a choriocarcinoma cell line, we do not observe activation of the adenosine 5'-monophosphate-activated kinase (AMPK) pathway following adiponectin treatment (globular adiponectin; 2 μg/ml). Furthermore, over-expression of the adaptor protein, APPL2, which functions to block the actions of APPL1 at the adiponectin receptor, results in a loss of adiponectin signaling, implying that APPL1 binding is necessary to transmit the adiponectin signal in trophoblast cells. Finally, over-expression of leptin in these cells also ablates the signal induction by adiponectin. These data substantiate the idea that leptin and adiponectin are two hormones with largely opposing functions. Thus, leptin production by the trophoblast may be one mechanism by which the placenta avoids some of the effects of maternal

adiponectin, which to this point, appear largely detrimental to the developing fetus. These data are novel in that they represent the first time adiponectin signaling has been evaluated in isolated primary trophoblast cells, and identify adiponectin stimulation of EGF signaling. In addition, leptin interference with adiponectin signaling may have important implications regarding the impact these hormones have at the maternal-fetal interface.

2. Introduction

Efficient and effective response to metabolic cues is crucial for life, but its importance is futher illuminated during pregnancy, when the placenta is responsible for integrating all external cues and mediating both maternal and fetal metabolism. The number of hormones, particularly those of pancreatic and adipocyte origin, that affect the metabolic and nutrient transfer responses of the placenta are great in number. One of these hormones, known to have a direct effect on the placenta is adiponectin.

The metabolic hormone adiponectin was originally identified as possessing potent insulin sensitizing effects, increasing insulin responsiveness in skeletal muscle, decreasing hepatic glucose production and increasing β-oxidation of fatty acids in the liver (Berg et al., 2001, Combs et al., 2001, Yamauchi et al., 2001, Maeda et al., 2002). Since its original characterization, adiponectin has been implicated in playing a role in a large variety of biological systems, many having to do with metabolic sensing.

Adiponectin was originally shown to utilize both adenosine 5'-monophosphate-activated kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)α signaling mechanisms, mediated through binding to two different receptors, adipoR1 and adipoR2, respectively (Yamauchi et al., 2003a). Since the original identification of the adiponectin receptors, however, adiponectin has also been shown to activate a diverse range of signaling pathways including the mitogen activated pathways, p38 and ERK1/2, PI3K, JAK/STAT and NFκB (Coope et al., 2008, Haugen and Drevon, 2007, Yamauchi et al., 2003a). Although downstream

signaling may vary, adiponectin receptors appear to bind the adaptor protein, APPL1 (adapter protein containing plekstrin homology domain, phosphotyrosine domain, and leucine zipper motif), which allows for downstream signaling mechanisms to be initiated (Mao et al., 2006). Interference of this interaction by the protein APPL2 has been shown to inhibit activation of downstream signaling cascades normally initiated by adipoR1 (Wang et al., 2009).

At the maternal-fetal interface various reports have shown AMPK, NFκB, ERK1/2 and PPARγ to be activated by adiponectin (Lappas et al., 2005a, Benaitreau et al., 2009). To our knowledge however, these reports were based on studies using either placental explants, and thus harboring a multitude of cell types, or choriocarcinoma cell lines, which may have very unique signaling and responses compared to trophoblast cells *in vivo.* The goal of the current study was to identify the signaling mechanisms utilized by adiponectin in trophoblast cells of the human term placenta.

We and others have previously shown that adiponectin is not produced by the human placenta, and therefore the syncytiotrophoblast, as the cellular layer in direct contact with maternal blood for most of the pregnancy, is the most likely region of the placenta to be exposed to maternal adiponectin (Ichida et al., 2007, McDonald and Wolfe, 2009, Haugen et al., 2006, Desoye and Hauguel-de Mouzon, 2007). We and others have shown that adiponectin can exert dramatic effects on the hormone production and proliferation of trophoblast cells (Benaitreau et al., 2009, McDonald and Wolfe, 2009).

Adiponectin is not the only metabolic hormone that can affect trophoblasts. Leptin, produced by the human placenta, is a known mediator of proliferation and invasion by trophoblasts (Magarinos et al., 2007, Schulz and Widmaier, 2004, Senaris et al., 1997). In general, many of the effects of leptin appear to directly oppose that of adiponectin. Additionally, leptin has been reported to cause increased production of hCG by trophoblast cells (Cameo et al., 2003), the opposite effect as what we have reported to occur following trophoblast exposure to adiponectin (McDonald and Wolfe, 2009). We expect the oppostion of leptin and adiponectin to continue at the maternal-fetal interface. Consequently, in addition to identifying the signaling mechanisms activated by adiponectin in syncytialized trophoblast cells, we also determined if leptin could modify these reponses. A lentiviral construct was used to produce high levels of leptin within these cells prior to examination of the signaling cascades initiated by adiponectin treatment. In this report we are able to identify activation of the ERK1/2, JNK, and EGF signaling cascades following treatment with adiponectin. In addition, we have found that overexpression of leptin within these cells interferes with the ability of adiponectin to initiate downstream signaling cascades.

3. Materials and Methods

Materials

The AMPK agonist, AICAR was purchased from Calbiochem (EMD Chemicals, Gibbstown, NJ). The pAMPKα, pERK1/2, pJNK antibodies were purchased from Cell Signaling Technology (Danvers, MA). The pEGFR and ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were purchased from Invitrogen (Carlsbad, CA) and LiCor Biosciences (Lincoln, NE). Lentiviral vectors, WPI, psPAX2 and pMD2.G were a generous gift from the Trono lab (Lausanne, Switzerland). The APPL2 mammalian expression vector was purchased from Open Biosystems (Thermo Fisher Scientific, Waltham, MA). All quantitative RT-PCR primers were purchased from Integrated DNA Technology (Coralville, IA).

Cell Isolation and Culture

Human placentas were obtained under protocols approved by the institutional review board at the University of Kansas Medical Center. Cytotrophoblasts (CTBs) were isolated from healthy placentas delivered via cesarean at term as previously described (McDonald and Wolfe, 2009). Purified cells were placed in culture media (Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin/ amphotericin B) and allowed to adhere to the culture dish and syncytialize *in vitro* by incubation in a humidified chamber for 5d prior to receiving treatment. All cells underwent regular media changes every 48h and were placed in culture media in the absence of serum for the last 24h prior to

receiving adiponectin treatment. In lentiviral infection experiments, cells were allowed to adhere to the culture dish by overnight incubation prior to infection.

Immunoblots

Proteins (10 μg) were fractionated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman Inc., Florham Park, NJ). Membranes were then subjected to western blot analysis for the protein of interest. Primary antibodies for pAMPKα (Thr172; 40H9), pERK (Thr202/Tyr204; E10), pJNK (Thr183/Tyr185; G9), ERK (C-14), pEGFR (Tyr 1173), and actin were detected using secondary antibodies conjugated to near-infrared fluorophores (Alexa Fluor 680 goat anti-mouse IgG, Alexa Fluor 680 donkey anti-goat IgG, Invitrogen; IRDye 800 goat anti-rabbit IgG, LiCor Biosciences). Western blots were then imaged with the use of the Odyssey Infrared Imaging System (LiCor Biosciences).

Lentiviral Production and Infection

Complete virus was made in 293FT cells (Invitrogen) by co-transfection with WPI vector harboring either the complete cDNA for human APPL2, leptin or the empty vector control, WPI and the packaging vectors, psPAX2 and pMD2.G. Transfections were completed using Lipofectamine 2000 (Invitrogen) and a standard transfection protocol. Briefly, vectors and lipofectamine 2000 were added to separate aliquots of Opti-MEM reduced media (Invitrogen) and incubated at room temperature for 5 min. The aliquots of Opti-MEM were then combined and allowed to form liposomes by incubation at room temperature for 20 min. 293FTs were trypsinized, the lipofectamine complexes were added to distinct 10 cm^2 culture dishes, and 6 x 10^6

cells added. Mature virus was harvested by collection of the media 48 and 72h post-transfection. Infection of term trophoblast cells was done by adding media harboring mature APPL2-WPI, leptin-WPI, or WPI (empty vector control) virus in the presence of 8µg/ml Sequabrene (Sigma Chemical).

RNA Isolation and Quantitative RT-PCR (qPCR)

RNA from trophoblasts in culture was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers. Quantitative real-time PCR (qPCR) was performed using SYBRgreen technology (Applied Biosystems). All gene expression data was normalized to 18S levels within the respective sample. All primers (Table VIII-1) were designed using Primer Express 3.0 software (Applied Biosystems). Resulting data was analyzed using the ΔΔCt method, as previously described (McDonald and Wolfe, 2009).

Table VIII-1. Primer Sequences for Quantitative RT-PCR

Gene	Forward Primer	Reverse Primer
18S	5'-cgccgctagaggtgaaattct-3'	5'-cgaacctccgactttcgttct-3'
APPL2	5'-ctccatagacgcgctgcat-3'	5'-cccccagactcgctctttact-3'
Leptin	5'-ggttgcaaggcccaagaa-3'	5'-acatagaaaagatagggccaaagc-3'

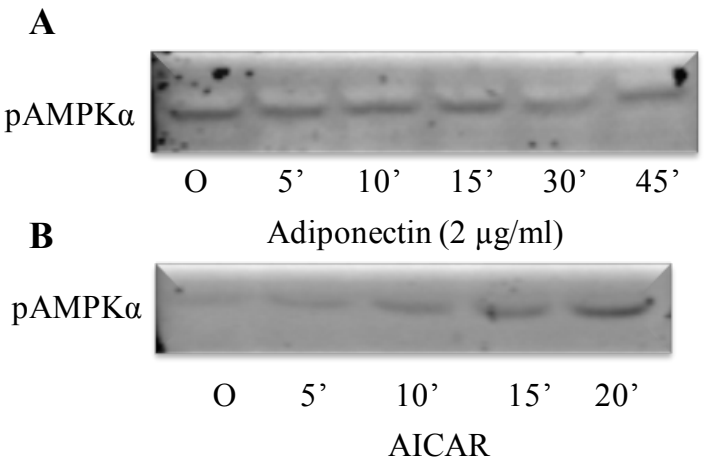
4. Results

Adiponectin does not activate AMPK in the human trophoblast

The AMP-activated protein kinase (AMPK) signaling pathway has been implicated as one of the primary means by which adiponectin elicits intracellular responses on target cells. Therefore, we initiated our investigations into the signaling mechanisms utilized by adiponectin in human trophoblast cells by examining the AMPK pathway. Despite treatment with adiponectin over a broad range of time points, we were unable to observe activation of AMPK by adiponectin (Fig. VIII-1a). We also treated the cells with aminoimidazole carboxamide ribonuclotide (AICAR), an AMPK agonist, as a positive control. As expected, AICAR activates AMPK within 10-15 minutes of treatment (Fig. VIII-1b). From these data we conclude that the effects of adiponectin must be mediated through alternate mechanisms in syncytialized human trophoblast cells.

Figure VIII-1. **Adiponectin does not stimulate AMPK in human term trophoblast cells.** Despite its well characterized activation of AMPK in liver and skeletal muscle, adiponectin does not appear to initiate activation of the AMPK signal cascade in human trophoblast cells collected at term. Western blot for AMPK following treatment with globular adiponectin (2μg/ml) for 5, 10, 15, 30, or 45 min (A). In contrast, the AMPK agonist, AICAR, stimulated AMPK activation within 10 minutes of treatment, with increasing levels of activation throughout 20 minutes (B). n=3.

Figure VIII-1.

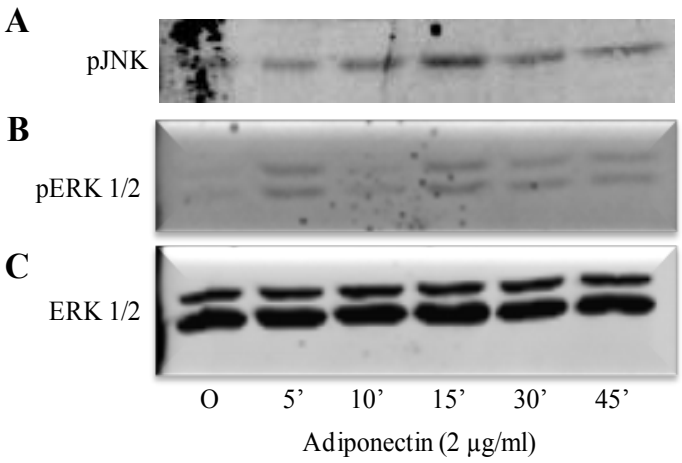


Adiponectin activates the ERK1/2 and JNK signaling cascades in human trophoblast cells

Despite a lack of activation of either AMPK or its downstream signaling molecule, ACC (data not shown), globular adiponectin did appear to cause an increase in the activated forms of two members of the MAPK family of signaling molecules, ERK1/2 and JNK (Fig. VIII-2). Activation in JNK can be seen by an increase in the phosphorylated form of JNK within 15 minutes of adiponectin treatment. In contrast, the activation of ERK1/2 appeared to occur in a biphasic pattern, with an increase in phosphorylated ERK1/2 with 5 minutes of adiponectin treatment, and then again at 15 minutes. The second activation appeared to be more sustained, as ERK1/2 was still phosphorylated following 30 minutes of adiponectin treatment. These data suggest that adiponectin is activating ERK1/2 through two different mechanisms, 1) directly through the rapid activation of its own receptor followed by 2) a secondary delayed activation of phosphorylated ERK1/2 through different pathway 15 minutes after adiponectin treatment. To investigate this further we examined activation of candidate signaling mechanisms known to activate ERK1/2 in trophoblast cells.

Figure VIII-2. **Adiponectin activates ERK1/2 and JNK signaling cascades in term trophoblast cells.** Treatment with globular adiponectin (2μg/ml) results in activation of members of the MAPK family of signaling pathways, including ERK1/2 and JNK. An increase in the activated form of JNK is apparent within 10 minutes following adiponectin treatment (A). ERK1/2 is activated both at 5 minutes and again at 15 minutes in trophoblasts collected at term (B). Total ERK1/2 levels were measured to ensure equal loading (C). n=3.

Figure VIII-2.

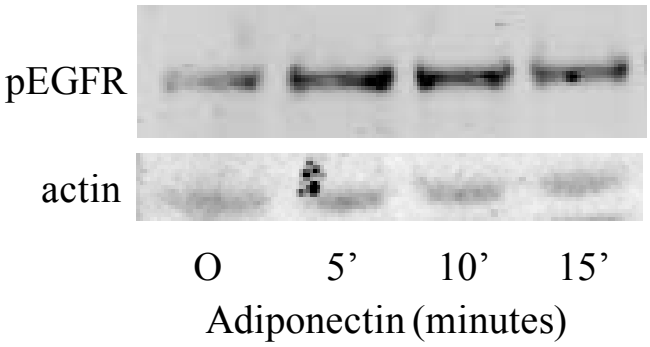


Adiponectin activates EGF receptor signaling in human trophoblast cells

Epidermal growth factor (EGF) is known to exert dramatic effects on human trophoblast cells, mediated in part through activation of the ERK1/2 signaling pathway (Takeuchi and Ito, 2010). Based on the biphasic activation pattern we observed following adiponectin exposure, we hypothesized that adiponectin may also be working through the EGF signaling pathway, resulting in a more robust ERK1/2 activation. Treatment of trophoblast cells with adiponectin revealed an increase in levels of phosphorylated EGF receptor (EGFR) after 5 minutes (Fig. VIII-3). This is in keeping with the idea that EGF signaling may be responsible for the secondary activation of ERK1/2 we see following 15 minutes of adiponectin exposure. These data are novel, in that to our knowledge adiponectin working in this manner through another signaling mechanism has not been reported in the literature.

Figure VIII-3. Adiponectin activates EGFR in syncytialized trophoblast cells. Treatment of primary trophoblast cells allowed to syncytialize *in vitro* display activation of the EGF receptor following 5 minutes of exposure to adiponectin. Cells were cultured for 5d prior to receiving adiponectin. Whole cell lysates show an increase in phosphorylated EGFR, the active form of the receptor, after adiponectin treatment for 5 minutes. This activation is transient, as the levels of phosphorylated EGFR go back down to control levels by 10 minutes of adiponectin treatment. Actin levels are assessed as a loading control. n=3.

Figure VIII-3.

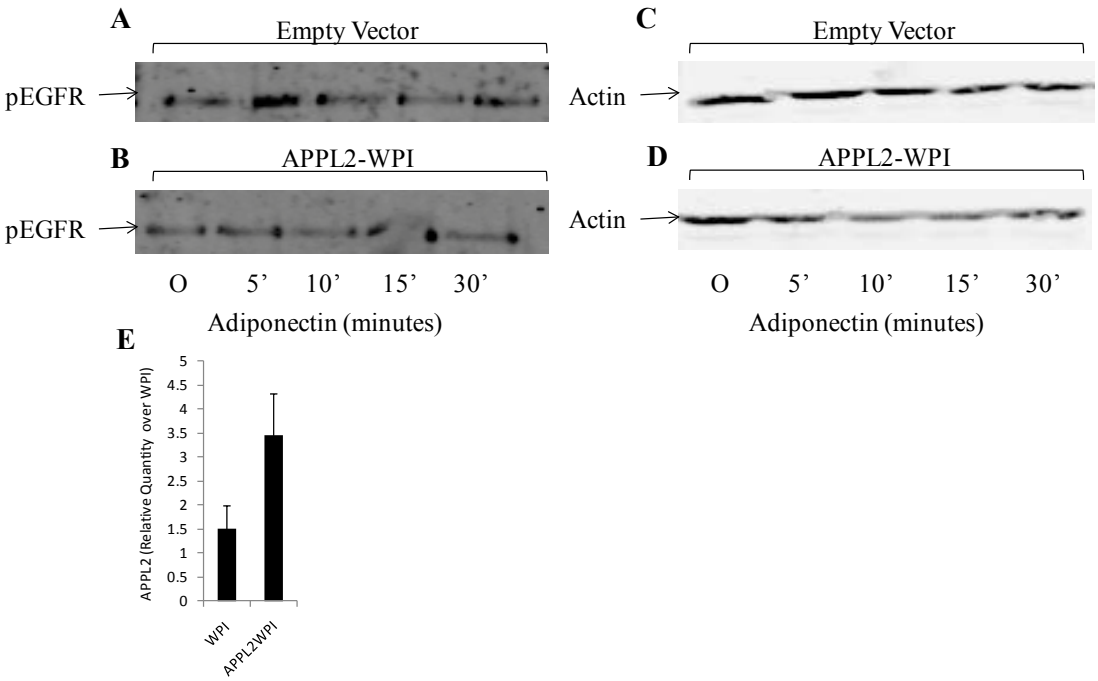


Adiponectin signaling in trophoblast cells requires the adaptor protein, APPL1

Previous reports have demonstrated the importance of APPL1 binding to the adiponectin receptor in order to perpetuate downstream signaling. Binding of the sister protein, APPL2, to the adiponectin receptor prevents adiponectin receptor activation, and thus quenches any intracellular signaling mediated by adiponectin (Wang et al., 2009). In order to investigate whether this phenomenon holds true in the trophoblast, we interfered with the binding of APPL1 to its receptor by using a lentiviral construct to over-express APPL2 in primary cultures of term trophoblasts. Over-expression of the APPL2 protein in these cells caused a loss of activation of the signaling molecules previously seen with adiponectin (i.e. EGFR, Fig. VIII-4). These data indicate that the APPL1 protein is indeed necessary to bind to the adiponectin receptor in order to elicit downstream signaling events in the human trophoblast. This is in keeping that what has been reported in skeletal muscle (Wang et al., 2009).

Figure VIII-4. **Adiponectin signaling in trophoblast cells requires APPL1.** Over-expression of the complimentary protein to APPL1, APPL2, in primary trophoblast cells results in a loss of downstream signaling events following treatment with adiponectin. Western blot for the activated form of EGFR, phosphorylated EGFR, in primary trophoblast cells over-expressing APPL2 reveals no increase in pEGFR protein following a 5, 10, or 15 minute treatment with adiponectin. This is in contrast to the increase in activation seen within 5 minutes of adiponectin treatment in cells infected with empty vector control. Actin levels were assessed as a loading control (C&D). Overexpression of APPL2 was measured through qPCR (E). Blot is a representative sample, n=3.

Figure VIII-4.

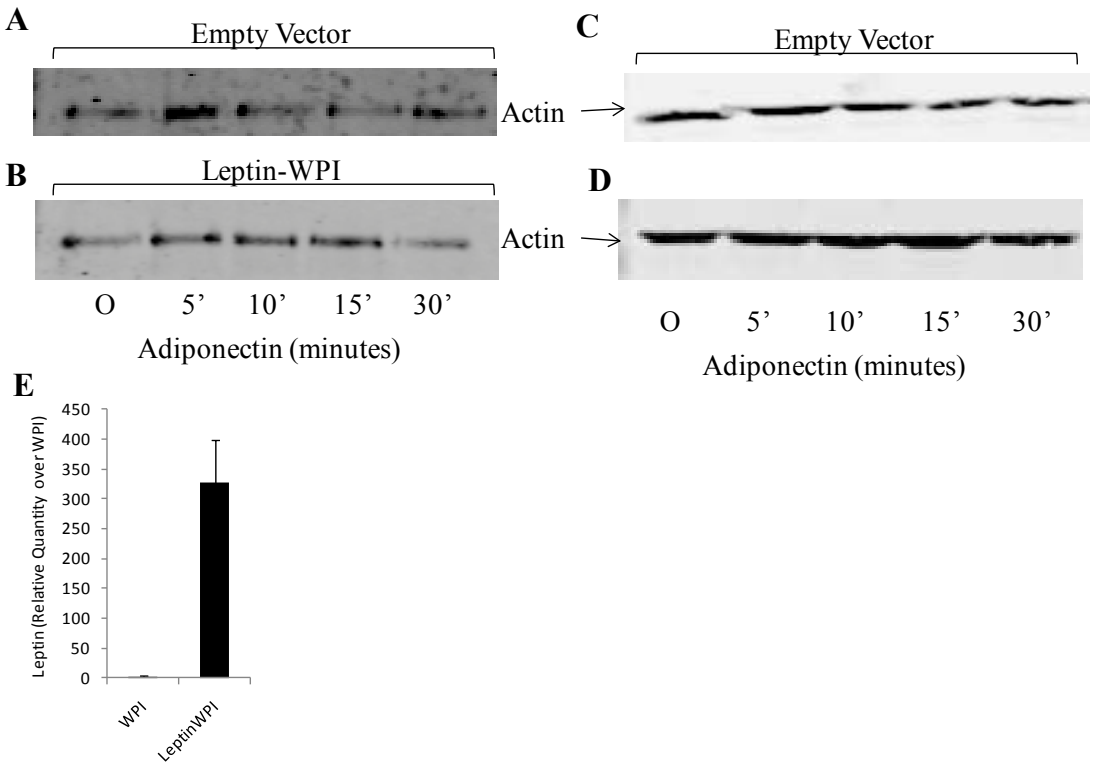


Leptin production can inhibit adiponectin signaling in trophoblast cells

Leptin and adiponectin are known to have opposing actions in a variety of systems. We hypothesized that leptin and adiponectin may function in a similar manner at the trophoblast cell. Although the human placenta is known to produce leptin (Cameo et al., 2003), in our hands primary human cytotrophoblasts collected at term produce very low levels of leptin (data not shown). In order to investigate the effects of leptin on adiponectin signaling we employed a lentiviral-mediated over-expression system to induce large amounts of leptin production by the trophoblasts themselves. Interestingly, induction of leptin by these cells severely attenuated the activation of the signaling cascades we have identified adiponectin to utilize in syncytialized human trophoblast cells (Fig VIII-5). These data indicate that leptin may block the actions of adiponectin in trophoblast cells.

Figure VIII-5. **Adiponectin-induced activation of EGFR is blocked by leptin production in trophoblast cells.** Over-expression of leptin blocks adiponectin-induced signaling in primary cytotrophoblasts syncytialized in culture. Western blots for pEGFR revealed activation following a 5 minute exposure to adiponectin in both non-infected cells (data not shown) and cells infected with the empty vector control (A). In contrast, cells over-expressing leptin were refractory to activation by adiponectin at 5, 10, and 15 minutes (B). Actin levels were assessed as a loading control (C&D). Overexpression of leptin was measured through qPCR (E). Blot is a representative sample, n=3.

Figure VIII-5.



5. Discussion

Investigation into the role adiponectin may play at the maternal-fetal interface is still in its infancy. Although data to support the idea that adiponectin exerts important effects during pregnancy are being reported with increasing frequency, only a couple of studies have investigated the mechanism(s) by which adiponectin may be performing these actions. These studies have been performed in placental explants, which contain many different cell types found at the maternal-fetal interface, and in choriocarcinoma cell lines, cells that do not always display the same characteristics as cultured primary trophoblast cells. Indeed, we have been unable to observe effects of adiponectin on Jeg-3, JAR, or BeWo choriocarcinoma cell models using the same doses and treatment paradigms as we have reported in our findings with primary trophoblast cell cultures (data not shown). Nevertheless, these published reports represent a good basis from which to hypothesize which signaling mechanisms may mediate the effects of adiponectin in human trophoblast cells, and have demonstrated that adiponectin can signal through AMPK, ERK1/2 and PI3K, as well as the transcription factors, NFκB and PPARγ (Lappas et al., 2005a, Benaitreau et al., 2009).

Adiponectin has been thought to signal primarily through the AMPK pathway (Yamauchi et al., 2003a). Amplification of the adiponectin signal by means of AMPK has been proposed to increase glucose transport in skeletal muscle, both through enhancing sensitivity to insulin signaling, and translocation of the glucose transporter, GLUT4, to the plasma membrane in a manner independent of insulin (Fujii et al., 2006). In addition, AMPK has been implicated in a variety of other cellular processes,

including tight junction formation in BeWo cells, decreased apoptosis in blastocysts, and inhibition of adipocyte differentiation (Hwang et al., 2005, Egawa et al., 2008, Eng et al., 2007, Giri et al., 2006, Habinowski and Witters, 2001, Yamaguchi et al., 2008). Despite the many reports linking adiponectin to the AMPK signaling pathway, as well as one in choriocarcinoma cells, we have been unable to observe AMPK activation following adiponectin treatment in primary term trophoblast cells.

The MAPK pathways, ERK1/2 and p38 have been linked to adiponectin signaling in other biological systems (Benaitreau et al., 2009, Haugen and Drevon, 2007, Lee et al., 2008). Indeed, there is data to suggest that adiponectin does not always activate the AMPK pathway, and in such instances ERK1/2 signaling may be the primary conductor of adiponectin signaling (Maillard et al., 2010). Other reports have shown the JAK/STAT and PI3K pathways to be activated via adiponectin (Coope et al., 2008). Our data are in agreement with the previous reports of adiponectin signaling through ERK1/2 in the placenta, however, we also observe an induction of another MAPK, c-Jun N-terminal kinase (JNK), in response to adiponection exposure. In addition, we identified activation of the epidermal growth factor (EGF) receptor following adiponectin treatment. To our knowledge, this phenomenon has not been reported previously. Indeed, an opposite phenomenon, where adiponectin binds to heparin-binding EGF (HB-EGF) and precludes it from activating its receptor has been reported (Wang et al., 2005).

The adiponectin receptor has been shown to require binding of the adaptor protein APPL1 in order to perpetuate adiponectin's signal (Mao et al., 2006). Interaction of APPL1 with the GTPase Rab5 has been shown to mediate signaling

events downstream of the adiponectin receptor (Mao et al., 2006). Indeed, experimental downregulation of APPL1 can impair downstream activation of signaling pathways, specifically activation of ERK1/2 (Lee et al., 2008). Furthermore, APPL1 is known to form a dimer with its isoform, APPL2. Over-expression of APPL2 in myotubes leads to a downregulation of adiponectin signaling by interfering with APPL1 binding to the adiponectin receptor (Wang et al., 2009). Our data suggest this is also the case in human trophoblast cells, as over-expression of APPL2 in these cells also resulted in a loss of signaling by adiponectin. Thus, although the downstream signaling mediators for adiponectin may not include the common AMPK pathway, the requirement for APPL1 binding to the adiponectin receptor appears to be conserved, suggesting this portion of the signaling mechanism is required, and not unique to a specific cell type(s).

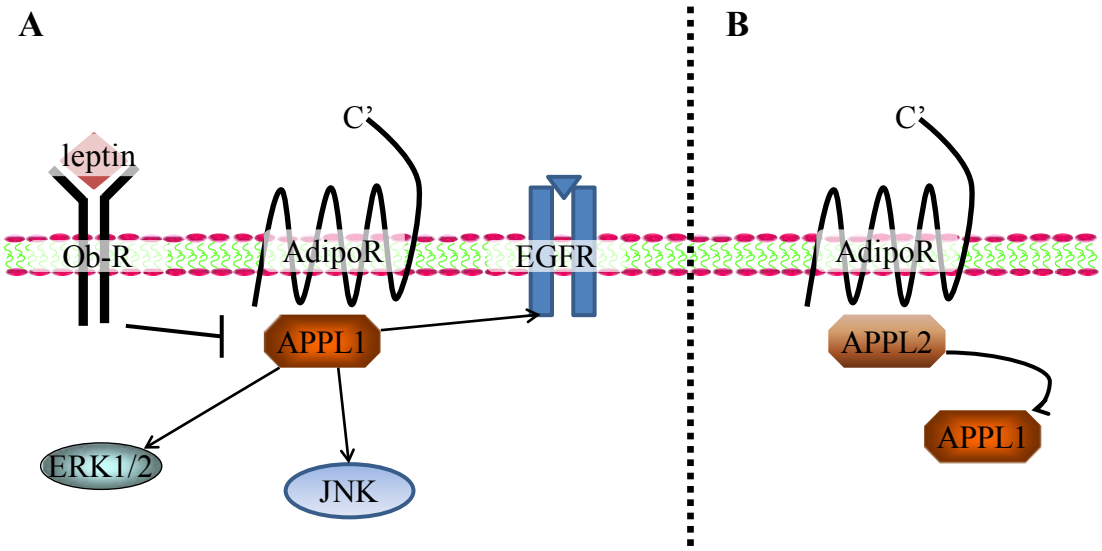
Another adipokine whose role at the maternal-fetal interface has been studied in much more depth than that of adiponectin is the hormone leptin. Interestingly, increased amounts of maternal leptin have been shown to result in the maintenance of an increased number of pups to term in hamsters (French et al., 2009). From these data the authors conclude that leptin acts as signal to the mother, telling her that sufficient energy stores are met and she might support more offspring (French et al., 2009). Indeed, in an artificial system such as performed in this study, leptin appears capable of pushing the mother into releasing more energy stores to the fetoplacental unit, to the point of it being detrimental to her own health (French et al., 2009). This seems to be in keeping with our own data and associated hypothesis, suggesting adiponectin functions largely to promote the mother's interests during pregnancy, and

therefore, in the words of French and colleagues, could be said to decrease maternal investment in gestation. Our data showing the attenuation of adiponectin signaling by leptin represents a possible mechanism by which the trophoblast may temper some of the actions of adiponectin, which we have previously reported to not necessarily be beneficial to continued growth of the fetus (see Chapters 2 and 3). Physiologically, as leptin levels increase in a fed state, adiponectin levels decrease, setting the stage for these two hormones to exert opposing actions. Indeed, Fang and coworkers have reported that leptin can have direct effects on adiponectin-mediated signaling, resulting in a blockade of adiponectin-stimulated glucose uptake in myotubes (Fang et al., 2009). These data are in accordance with our findings that over-expression of leptin in trophoblast cells (a cell type that makes leptin *in vivo*) can attenuate adiponectin-stimulated intracellular signaling.

The data reported herein provide evidence for the first time that adiponectin signaling in trophoblast requires the adaptor protein APPL1 in order to activate signaling cascades that include ERK1/2, JNK, and EGFR (Fig. VIII-6). In addition, production of leptin by these cells interferes with the ability of the trophoblast to respond to adiponectin. This may prove useful to the syncytiotrophoblast given the largely detrimental effects adiponectin has on this cell type (McDonald and Wolfe, 2009).

Figure VIII-6. Model of adiponectin signaling in the syncytiotrophoblast at term. Adiponectin, binding to its receptor on the surface of syncytiotrophoblast, can initiate signaling through ERK1/2 and JNK. In addition, treatment with adiponectin can activate the EGF receptor. These actions are mediated through the binding of APPL1 to the adiponectin receptor, as interference by APPL2 causes a loss of downstream signal activation. Additionally, leptin can work through its receptor, Ob-R, to inhibit signaling by adiponectin.

Figure VIII-6.



IX: Chapter 5:

Microarray Analysis of Adiponectin Treatment on Trophoblast Cells

1. Abstract

In the relatively short period since its discovery, the hormone adiponectin has been implicated in the regulation of a number of cellular functions including glucose metabolism, lipid oxidation, hormone production, differentiation, proliferation, and inflammation. The range of functions serves to underscore adiponectin's biological importance. In addition, the role of adiponectin has broadened to include a range of tissue types. As the developed world continues to become increasingly obese, instances in which adiponectin production is low are becoming more and more frequent. It is thus vital to understand the role of this hormone on many different physiological systems, so that we may be better able to anticipate and treat conditions that arise as a consequence of an obese state. In order to identify functions altered by adiponectin in human trophoblast cells on a global scale, we performed microarrays on isolated cytotrophoblasts exposed to adiponectin for 5d as they syncytialized *in vitro.* The data show that the expression of a number of different genes was significantly affected by adiponectin treatment. The majority of these genes were repressed following adiponectin exposure, however, a smaller but sizable group were up-regulated in trophoblasts by adiponectin. These data not only support our previous findings regarding the effects of adiponectin on isolated trophoblast cells, but also allow for identification of new mechanisms that are regulated by adiponectin. These include processes such as insulin-like growth factor (IGF) bioavailability, cortisol production and metabolism, chemotaxis, and cholesterol metabolism. Completion of this study has significantly advanced our understanding of the range of effects adiponectin may have on the human placenta. These data may be of particular interest

in cases of altered adiponectin levels, such as during maternal metabolic syndrome. Our findings in conjunction with the large number of studies outlining alterations in circulating levels of adiponectin during diseases of pregnancy (i.e. gestational diabetes and preeclampsia) support the hypothesis that adiponectin may be mediating some of the effects of these diseases locally at the maternal-fetal interface.

2. Introduction

With continued investigation, adiponectin is being found to have a broad range of effects on many different tissues throughout the body. No longer is adiponectin limited to the maintenance of insulin sensitivity and metabolic homeostasis, but it has also been shown in recent years to affect cell proliferation and differentiation, inflammatory responses, and hormone production. Additionally, the response to adiponectin appears to be highly dependent on the tissue under investigation. Adiponectin also appears to interact with many other growth factors and hormones (e.g. insulin, IGF-I and –II, EGF, GnRH, LH) to "fine-tune" the responses of a target cell.

Despite the multifaceted nature of the hormone, the metabolic function for which it was initially identified remains of great importance, and has broad implications on numerous biological systems. It follows therefore that adiponectin may profoundly influence metabolism within the placenta. Despite this, extremely little has been reported regarding the role of adiponectin on placental metabolism. We hypothesized that adiponectin would alter the expression of transporters and enzymes involved in metabolism that are expressed by the syncytiotrophoblast. In order to investigate this in an unbiased manner, we performed microarray analysis of isolated trophoblasts after they had been exposed to adiponectin in a long-term culture system. We did indeed find many alterations in gene expression following adiponectin treatment, including some of which we have already reported (see Chapters 2, 3, and 4). While expression of some transporters was altered, we also observed changes in

various hormones and growth factor-related pathways. The results of these microarrays will be discussed herein.

3. Materials and Methods

Cell Isolation and Culture

Human placentas were obtained under protocols approved by the institutional review board at the University of Kansas Medical Center. Cytotrophoblasts were isolated from healthy placentas delivered via cesarean at term as previously described (23). Villous tissue was dissected away from the basal plate of the placenta and subjected to treatment with deoxyribonuclease I (type IV; Sigma Chemical, St. Louis, MO) and trypsin (Invitrogen Corp., Carlsbad, CA). The resulting single-cell suspension was size fractionated by application to a Percoll (Sigma) density gradient. To ensure a homogeneous population of cytotrophoblasts, the cells underwent negative selection using an antibody recognizing human leukocyte antigen-A, -B, and -C (W6/32; Bio-Express, West Lebanon, NH) with a magnetically labeled secondary antibody (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). Cytospins of trophoblasts from each preparation were stained for cytokeratin 7 expression (N-TL 12/30; Dako, Glostrup, Denmark) to assess purity. Purified cells were placed in culture medium (Iscove's modified Dulbecco's medium with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin/ amphotericin B) and allowed to adhere to the culture dish by incubation in a humidified chamber for 4 h before receiving treatment. All cells underwent regular medium changes, with refreshment of treatment conditions every 48 h. After addition of recombinant globular adiponectin (2 μg/ml; Phoenix Pharmaceuticals, Burlingame, CA) to the culture medium, cells were allowed to differentiate spontaneously before being harvested on d 5 of culture.

Microarray Analysis

Isolated trophoblasts from three distinct placentas were placed in culture for 5d. Cells from each preparation were cultured with media alone (control) or with the addition of 2 μg/ml globular adiponectin beginning on d1 of the culture period (adpn). RNA quality and quantity were assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) prior to use on the microarrays. Preparation of the biotin-labeled targets for the 3' expression analysis was performed using the GeneChip 3' IVT Express Kit (Affymetrix). Microarrays were performed using Affymetrix HG U133 Plus 2.0 gene chips (Affymetrix, Santa Clara, CA) and the GeneChip system (Affymetrix). Results from the microarrays were analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies). Only genes that were flagged "present" in at least one of the treatment conditions (treatment versus control) were analyzed. Expression data was normalized to the 50^{th} percentile of gene expression for each individual sample to control for chip-to-chip variation before being normalized to the median of the control samples for each cytotrophoblast isolation. Statistical analysis was performed using one-way ANOVA, with a Benjamini and Hochberg False Discovery Rate of 0.05. Changes in gene expression between adpn and control samples ≥2-fold and with $P< 0.05$ were considered significant.

RNA Isolation and Quantitative RT-PCR

RNA was isolated from placentas unique from those used in the microarray and quantitative RT-PCR done on specific transcripts which showed a significant difference in gene expression on the microarray, in order to validate the findings of the microarray. RNA from trophoblasts in culture was isolated using Trizol

(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers. Quantitative real-time PCR (qPCR) was performed using SYBRgreen technology (Applied Biosystems, Foster City, CA). All gene expression data was normalized to 18S levels within the respective sample. All primers (Table VIII-1) were designed using Primer Express 3.0 software (Applied Biosystems) and synthesized by Integrated DNA Technology (Coralville, IA). Resulting data was analyzed using the ΔΔCt method, as has been previously described (McDonald and Wolfe, 2009). All experiments were performed with four distinct placentas. Statistical analysis was done using SigmaStat 3.1 software (Systat Software, Inc., San Jose, CA). Means for untreated control cells versus adiponectin treated cells were compared using the Mann-Whitney Rank Sum Test. For all experiments, $P < 0.05$ was considered significant.

Table IX-1. Primer Sequences for Quantitative RT-PCR

Gene	Forward Primer	Reverse Primer
18S	5'-cgccgctagaggtgaaattct-3'	5'-cgaacctccgactttcgttct-3'
ADAM12	5'-gggcaagaaggcataaaagaga-3'	5'-ctcggttgtctgccacgat-3'
ALPP	5'-gtgcccggtatgtgtggaa-3'	5'-ggctcaaagagacccatgagat-3'
CD24	5'-ggtgccctgcagtcaacag-3'	5'-cttgccacattggacttcca-3'
CGB	5'-acaaccccgaggtataaagcc-3'	5'-ccttggatgcccatgtcc-3'
CSH1	5'-tccttctgcttctcagactctattcc-3'	5'-ctagattggatttctgttgcgtttc-3'
CYP11A1	5'-caccttcaccatgtccagaattt-3'	5'-ctccacgttgccgagctt-3'
CYP19A1	5'-catatgatctgtctgtggcaaaagt-3'	5'-aagtggctgaggcataaatcga-3'
FURIN	5'-cggaaagtgagccactcatatg-3'	5'-gaggatgtcgatgatgcacttc-3'
GH2	5'-gaaaacgcagcagaaatctaaccta-3'	5'-acgggctccagccatga-3'
HSD11B2	5'-gcgctactcatggacacattca-3'	5'-ccacgtttctcactgactctgtct-3'
IGFBP3	5'-cgccagctccaggaaatg-3'	5'-tgccctttcttgatgatgattatc-3'
INSL4	5'-tggacgtcccaaagaaatgg-3'	5'-gctctggtgacaaattaggaatga-3'
LDLR	5'-ggctgcgttaatgtgacactct-3'	5'-gcactctttgatgggttcatctg-3'
PAPPA	5'-accaaaaaggtcaccccattc-3'	5'-ccggttgggtgctaaggat-3'
PAPPA2	5'-gattgaggtcctacgggtgttt-3'	5'-gagtcttgtgggttccaagtgtt-3'
PGF	5'-gttcagcccatcctgtgtct-3'	5'-ttaggagctgcatggtgaca-3'
Siglec10	5'-gcgggtcccaggctatg-3'	5'-ccctccggcaccatcaa-3'

4. Results

Microarray Analysis

A total of 195 transcripts were significantly down-regulated and 62 were up-regulated by a factor of ≥2.0 following adiponectin treatment (Tables VIII-2 and VIII-3). One of the most prominent groupings of genes that were down-regulated with adiponectin treatment are related to the endocrine function of the cells. These findings are in keeping with our previous findings (see Chapter 2). Although there are many more genes significantly down-regulated than vice versa, many interesting genes relating to immune response are higher following adiponectin treatment. These include Siglec10 and CD24 (see Chapter 3), as well as B7-DC, B7-H4, and chemokines CCL5 and CCL18. We have validated the results of the microarray through quantitative RT-PCR for a number of genes (Table VIII-4). These genes were chosen to include a variety of functions, as well as genes that were both up- and down-regulated with adiponectin. Additionally, the genes *CGB, CSH1, CYP11A1, CYP19A1* and *SIGLEC10* have also been validated at the protein level (see Chapters 2 and 3).

Table IX-2. **Genes down-regulated following adiponectin treatment.**

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
233857_s_at	ankyrin repeat and SOCS box-containing 2	ASB2	AK002049	-17.64	0.001
201785_at	ribonuclease, RNase A family, 1 (pancreatic)	RNASE1	NM_002933	-10.68	0.035
205387_s_at	chorionic gonadotropin, beta polypeptide	CGB	NM_000737	-8.936	0.000
231454_at	Placenta-specific 4	PLAC4	R31094	-8.078	0.015
206886_x_at	growth hormone 1	GH1	NM_022560	-7.884	0.021
210997_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	M77227	-7.529	0.001
206605_at	26 serine protease	P11	NM_006025	-7.422	0.004
208069_x_at	growth hormone 1	GH1	NM_022561	-7.286	0.019
205840_x_at	growth hormone 1	GH1	NM_000515	-6.967	0.045
230550_at	membrane-spanning 4-domains, subfamily A, member 6A	MS4A6A	AA045175	-6.877	0.000
205158_at	ribonuclease, RNase A family, 4	RNASE4	NM_002937	-6.557	0.000
211508_s_at	growth hormone 2	GH2	AF006060	-6.372	0.017
206885_x_at	growth hormone 1	GH1	NM_022559	-6.366	0.017
210880_s_at	embryonal Fyn-associated substrate	EFS	AB001467	-6.364	0.001
1555880_at	HERV-FRD provirus ancestral Env polyprotein	HERV-FRD	AK075092	-6.196	0.001
227915_at	ankyrin repeat and SOCS box-containing 2	ASB2	AI872284	-6.046	0.001
207770_x_at	chorionic somatomammotropin hormone 2	CSH2	NM_022644	-5.845	0.021
219666_at	membrane-spanning 4-domains, subfamily A, member 6A	MS4A6A	NM_022349	-5.703	0.001

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
208341_x_at	chorionic somatomammotropin hormone 2	CSH2	NM_022646	-5.475	0.022
208068_x_at	growth hormone 1	GH1	NM_022562	-5.471	0.020
211151_x_at	growth hormone 1	GH1	AF185611	-5.301	0.019
208356_x_at	chorionic somatomammotropin hormone 1 (placental lactogen)	CSH1	NM_022642	-5.18	0.022
206475_x_at	chorionic somatomammotropin hormone 1 (placental lactogen)	CSH1	NM_022640	-5.142	0.024
208342_x_at	chorionic somatomammotropin hormone 2	CSH2	NM_022645	-5.078	0.029
208293_x_at	chorionic somatomammotropin hormone-like 1	CSHL1	NM_022581	-5.077	0.019
210503_at	melanoma antigen family A, 11	MAGEA11	BC004479	-5.026	0.001
208294_x_at	chorionic somatomammotropin hormone-like 1	CSHL1	NM_022578	-5.009	0.022
203807_x_at	chorionic somatomammotropin hormone 2	CSH2	NM_020991	-4.922	0.025
208357_x_at	chorionic somatomammotropin hormone 1 (placental lactogen)	CSH1	NM_022641	-4.899	0.031
202493_x_at	chorionic somatomammotropin hormone 1 (placental lactogen)	CSH1	NM_001317	-4.733	0.023
211739_x_at	chorionic somatomammotropin hormone 1 (placental lactogen)	CSH1	BC005921	-4.62	0.024
239157_at	hypothetical protein LOC221584	LOC221584	AL521521	-4.532	0.001

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
231489_x_at	Transcribed locus, weakly similar to NP_061913.2 elongation protein 4 homolog; PAX6 neighbor gene; chromosome 11 open reading frame 19 [Homo sapiens]		H12214	-4.53	0.001
207285_x_at	chorionic somatomammotropin hormone-like 1	CSHL1	NM_001318	-4.469	0.026
235336_at	CDNA FLJ35202 fis, clone PLACE6018287		BG436225	-4.437	0.009
242629_at	Transcribed locus, moderately similar to XP_508230.1 PREDICTED: zinc finger protein 195 [Pan troglodytes]		BF056092	-4.219	0.004
244750_at	Transcribed locus		R63757	-4.184	0.048
227519_at	placenta-specific 4	PLAC4	R53820	-4.18	0.016
209993_at	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	AF016535	-4.121	0.003
227123_at	RAB3B, member RAS oncogene family	RAB3B	AU156710	-4.12	0.007
205958_x_at	chorionic somatomammotropin hormone-like 1	CSHL1	NM_022579	-4.051	0.020
210353_s_at	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	SLC6A2	M65105	-3.989	0.000
232268_at	ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	AU158643	-3.977	0.017
205602_x_at	pregnancy specific beta-1-glycoprotein 7	PSG7	NM_002783	-3.956	0.011
209994_s_at	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	AF016535	-3.951	0.006
204753_s_at	hepatic leukemia factor	HLF	AI810712	-3.901	0.000
209220_at	glypican 3	GPC3	L47125	-3.839	0.006
206549_at	insulin-like 4 (placenta)	INSL4	NM_002195	-3.837	0.005
239202_at	RAB3B, member RAS oncogene family	RAB3B	BE552383	-3.819	0.008
213790_at	ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	W46291	-3.807	0.013

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239146_at	chromosome 3 open reading frame 4	C3orf4	AI634844	-3.752	0.001
1559400_s_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	BG620958	-3.73	0.023
1557446_x_at	triggering receptor expressed on myeloid cells-like 3	TREML3	H53073	-3.675	0.001
201540_at	four and a half LIM domains 1	FHL1	NM_001449	-3.625	0.009
219702_at	placenta-specific 1	PLAC1	NM_021796	-3.619	0.032
208295_x_at	chorionic somatomammotropin hormone-like 1	CSHL1	NM_022580	-3.601	0.029
229049_at			AU158705	-3.565	0.041
227503_at	CDNA FLJ43100 fis, clone CTONG2003100		N26620	-3.517	0.010
223922_x_at	membrane-spanning 4-domains, subfamily A, member 6A	MS4A6A	AB013104	-3.505	0.000
204130_at	hydroxysteroid (11-beta) dehydrogenase 2	HSD11B2	NM_000196	-3.494	0.007
205924_at	RAB3B, member RAS oncogene family	RAB3B	BC005035	-3.472	0.008
1557914_s_at			AU140866	-3.459	0.009
233115_at	DEP domain containing 1B	DEPDC1B	AU151522	-3.413	0.018
235735_at	Tumor necrosis factor (ligand) superfamily, member 8	TNFSF8	AI936516	-3.37	0.000
206195_x_at	growth hormone 2	GH2	NM_022557	-3.36	0.021
203071_at	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	SEMA3B	NM_004636	-3.358	0.000
219721_at			NM_018350	-3.32	0.000

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202067_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	AI861942	-3.308	0.001
228176_at	endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	EDG3	AA534817	-3.241	0.034
224942_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	BG434272	-3.241	0.005
221577_x_at	growth differentiation factor 15	GDF15	AF003934	-3.235	0.023
204755_x_at	hepatic leukemia factor	HLF	M95585	-3.234	0.043
219748_at	triggering receptor expressed on myeloid cells-like 2	TREML2	NM_024807	-3.157	0.000
208191_x_at	pregnancy specific beta-1-glycoprotein 4	PSG4	NM_002780	-3.146	0.020
223137_at	zinc finger, DHHC-type containing 4	ZDHHC4	AF201931	-3.142	0.000
203918_at	protocadherin 1 (cadherin-like 1)	PCDH1	NM_002587	-3.1	0.007
201981_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	AA148534	-3.085	0.012
232035_at	histone 1, H4h	HIST1H4H	BE740761	-3.08	0.006
227818_at	coiled-coil domain containing 21	CCDC21	AL133609	-3.067	0.007
214750_at	placenta-specific 4	PLAC4	L13197	-3.056	0.015
213577_at	squalene epoxidase	SQLE	AA639705	-3.056	0.005
220769_s_at	WD repeat domain 78	WDR78	NM_024763	-3.038	0.001
204644_at	cytosolic ovarian carcinoma antigen 1	COVA1	AF207881	-3.011	0.001
210196_s_at	pregnancy specific beta-1-glycoprotein 1	PSG1	M33663	-2.973	0.027
219611_s_at	coiled-coil domain containing 21	CCDC21	NM_022778	-2.97	0.003
209582_s_at	CD200 antigen	CD200	H23979	-2.945	0.005

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213094_at	G protein-coupled receptor 126	GPR126	AL033377	-2.908	0.011
213685_at	Transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)	TCEB3	AA830143	-2.905	0.016
202952_s_at	ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	NM_003474	-2.904	0.012
236875_at	Transcribed locus		AI769477	-2.904	0.001
208257_x_at	pregnancy specific beta-1-glycoprotein 1	PSG1	NM_006905	-2.903	0.019
233737_s_at	hypothetical protein LOC284561	LOC284561	AK023548	-2.902	0.013
201790_s_at	7-dehydrocholesterol reductase	DHCR7	AW150953	-2.9	0.009
204943_at	ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	NM_021641	-2.9	0.013
210299_s_at	four and a half LIM domains 1	FHL1	AF063002	-2.895	0.020
218918_at	mannosidase, alpha, class 1C, member 1	MAN1C1	NM_020379	-2.884	0.006
239394_at	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	SLC6A2	AI765218	-2.869	0.000
213325_at	poliovirus receptor-related 3	PVRL3	AA129716	-2.867	0.017
204309_at	cytochrome P450, family 11, subfamily A, polypeptide 1	CYP11A1	NM_000781	-2.856	0.011
208180_s_at	histone 1, H4h	HIST1H4H	NM_003543	-2.853	0.028
237313_at	Homo sapiens, clone IMAGE:5225774, mRNA		BE856800	-2.853	0.004
209652_s_at	placental growth factor, vascular endothelial growth factor-related protein	PGF	BC001422	-2.852	0.008
207904_s_at	leucyl/cystinyl aminopeptidase	LNPEP	NM_005575	-2.844	0.020
216611_s_at	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	SLC6A2	AB022847	-2.844	0.000

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201945_at	furin (paired basic amino acid cleaving enzyme)	FURIN	NM_002569	-2.835	0.019
205193_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	NM_012323	-2.815	0.000
230614_at	Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	AI740573	-2.8	0.041
215715_at	solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	SLC6A2	BC000563	-2.791	0.003
1562086_at	CDNA clone IMAGE:5263207		BC035117	-2.782	0.004
230360_at	gliomedin	GLDN	AW006648	-2.779	0.009
239583_x_at	placenta-specific 7	PLAC7	BG354573	-2.762	0.019
227706_at	spire homolog 2 (Drosophila)	SPIRE2	AI215798	-2.738	0.001
228838_at	Full length insert cDNA clone YA77F06		BF431870	-2.718	0.027
218717_s_at	leprecan-like 1	LEPREL1	NM_018192	-2.709	0.032
213332_at	Pappalysin 2	PAPPA2	AL031290	-2.698	0.001
201286_at	syndecan 1	SDC1	Z48199	-2.676	0.004
243027_at	hypothetical protein LOC150084	LOC150084	AI824021	-2.651	0.027
202068_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	NM_000527	-2.638	0.004
240705_at	cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	AI472257	-2.632	0.016
221582_at	histone 3, H2a	HIST3H2A	BC001193	-2.632	0.009
232167_at	solute carrier family 2 (facilitated glucose transporter), member 11	SLC2A11	BE675356	-2.628	0.001
1558007_s_at	Leucine carboxyl methyltransferase 1	LCMT1	BC024748	-2.613	0.042

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201287_s_at	syndecan 1	SDC1	NM_002997	-2.613	0.024
205319_at	prostate stem cell antigen	PSCA	NM_005672	-2.609	0.034
209735_at	ATP-binding cassette, sub-family G (WHITE), member 2	ABCG2	AF098951	-2.603	0.004
231353_at			R77414	-2.595	0.006
209283_at	crystallin, alpha B	CRYAB	AF007162	-2.583	0.005
214247_s_at	dickkopf homolog 3 (Xenopus laevis)	DKK3	AU148057	-2.55	0.016
209442_x_at	ankyrin 3, node of Ranvier (ankyrin G)	ANK3	AL136710	-2.547	0.011
240463_at	Transforming, acidic coiled-coil containing protein 2	TACC2	AI458065	-2.545	0.001
226777_at	ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	AA147933	-2.541	0.009
208106_x_at	pregnancy specific beta-1-glycoprotein 6	PSG6	NM_002782	-2.53	0.042
228128_x_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	AI110886	-2.528	0.029
209278_s_at	tissue factor pathway inhibitor 2	TFPI2	L27624	-2.527	0.013
224941_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	BF107618	-2.521	0.012
203911_at	RAP1, GTPase activating protein 1	RAP1GA1	NM_002885	-2.519	0.033
215613_at	ADAM metallopeptidase domain 12 (meltrin alpha)	ADAM12	AU145357	-2.517	0.037
243648_at			AA280627	-2.51	0.046
213216_at	OTU domain containing 3	OTUD3	AL537463	-2.508	0.001
202708_s_at	histone 2, H2be	HIST2H2BE	NM_003528	-2.507	0.011
227113_at	alcohol dehydrogenase, iron containing, 1	ADHFE1	BE048349	-2.499	0.008
213148_at	hypothetical protein LOC257407	LOC257407	AW156899	-2.498	0.017

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206413_s_at	T-cell leukemia/lymphoma 1B	TCL1B	NM_004918	-2.496	0.017
1566739_at	Phospholipase C, epsilon 1	PLCE1	AK025366	-2.491	0.009
210809_s_at	periostin, osteoblast specific factor	POSTN	D13665	-2.475	0.011
213143_at	hypothetical protein LOC257407	LOC257407	BE856707	-2.467	0.011
207733_x_at	pregnancy specific beta-1-glycoprotein 9	PSG9	NM_002784	-2.465	0.030
229576_s_at	T-box 3 (ulnar mammary syndrome)	TBX3	N29712	-2.463	0.011
222760_at	zinc finger protein 703	ZNF703	BG290193	-2.462	0.016
239460_at			BF988443	-2.444	0.018
201982_s_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	NM_002581	-2.442	0.014
203697_at	frizzled-related protein	FRZB	U91903	-2.441	0.036
230854_at	hypothetical LOC400500	LOC400500	N32860	-2.43	0.007
227828_s_at	hypothetical protein FLJ13391	FLJ13391	AV700753	-2.43	0.008
1556464_a_at	hypothetical protein LOC257407	LOC257407	AF086098	-2.428	0.014
225369_at	endothelial cell adhesion molecule	ESAM	AL573851	-2.427	0.001
205042_at	glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase	GNE	NM_005476	-2.411	0.013
226623_at	phytanoyl-CoA hydroxylase interacting protein-like	PHYHIPL	AI829726	-2.406	0.019
236593_at	Similar to putative membrane-bound dipeptidase 2		BF109310	-2.403	0.001
240450_at	Transcribed locus		BF061543	-2.403	0.007
235100_at	CDNA FLJ11325 fis, clone PLACE1010383		BG435715	-2.402	0.038
219682_s_at	T-box 3 (ulnar mammary syndrome)	TBX3	NM_016569	-2.402	0.011

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235230_at	Phosphatidylinositol-specific phospholipase C, X domain containing 2	PLCXD2	AW170015	-2.394	0.018
209218_at	squalene epoxidase	SQLE	AF098865	-2.391	0.006
210358_x_at	GATA binding protein 2	GATA2	BC002557	-2.378	0.020
241929_at	CD36 antigen (collagen type I receptor, thrombospondin receptor)	CD36	AV760302	-2.377	0.019
1555299_s_at	endogenous retroviral family W, env(C7), member 1 (syncytin)	ERVWE1	AF072506	-2.374	0.017
237006_at	AF4/FMR2 family, member 1	MLLT2	AA703523	-2.361	0.017
219181_at	lipase, endothelial	LIPG	NM_006033	-2.359	0.017
212677_s_at	KIAA0582	KIAA0582	BG530481	-2.355	0.004
237411_at	ADAM metallopeptidase with thrombospondin type 1 motif, 6	ADAMTS6	N71063	-2.354	0.018
230978_at	hypothetical LOC401464	LOC401464	AI199850	-2.341	0.035
1556185_a_at	CDNA clone IMAGE:5260162		BC035072	-2.337	0.000
219049_at	chondroitin beta1,4 N-acetylgalactosaminyltransferase	ChGn	NM_018371	-2.335	0.004
223952_x_at	dehydrogenase/reductase (SDR family) member 9	DHRS9	AF240698	-2.328	0.000
232689_at	hypothetical protein LOC284561	LOC284561	AU156837	-2.328	0.005
225373_at	chromosome 10 open reading frame 54	C10orf54	BE271644	-2.325	0.002
206385_s_at	ankyrin 3, node of Ranvier (ankyrin G)	ANK3	NM_020987	-2.323	0.005
210195_s_at	pregnancy specific beta-1-glycoprotein 1	PSG1	M34715	-2.322	0.012
218660_at	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	DYSF	NM_003494	-2.3	0.017
212675_s_at	KIAA0582	KIAA0582	AB011154	-2.298	0.003

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224940_s_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	BF107618	-2.298	0.018
209583_s_at	CD200 antigen	CD200	AF063591	-2.296	0.044
214397_at	methyl-CpG binding domain protein 2	MBD2	AI827820	-2.296	0.000
230084_at	solute carrier family 30 (zinc transporter), member 2	SLC30A2	BF510698	-2.29	0.005
243423_at	Transcribed locus		AF150368	-2.289	0.017
220289_s_at	absent in melanoma 1-like	AIM1L	NM_017977	-2.284	0.022
236829_at	TATA element modulatory factor 1	TMF1	BF057855	-2.28	0.007
213343_s_at	glycerophosphodiester phosphodiesterase domain containing 5	GDPD5	AL041124	-2.273	0.029
239706_x_at	Pregnancy specific beta-1-glycoprotein 1	PSG1	BE856688	-2.271	0.000
204400_at	embryonal Fyn-associated substrate	EFS	NM_005864	-2.27	0.009
225372_at	chromosome 10 open reading frame 54	C10orf54	AK024449	-2.258	0.022
1558540_s_at	solute carrier family 2 (facilitated glucose transporter), member 11	SLC2A11	AK055523	-2.252	0.016
210141_s_at	inhibin, alpha	INHA	M13981	-2.239	0.007
201261_x_at	biglycan	BGN	BC002416	-2.238	0.008
205931_s_at	cAMP responsive element binding protein 5	CREB5	NM_004904	-2.235	0.034
1552481_s_at	mannosidase, alpha, class 1A, member 2	MAN1A2	NM_006699	-2.227	0.042
227752_at	serine palmitoyltransferase, long chain base subunit 2-like (aminotransferase 2)	SPTLC2L	AA005105	-2.222	0.017
209594_x_at	pregnancy specific beta-1-glycoprotein 9	PSG9	M34421	-2.221	0.020
243871_at	CDNA FLJ42179 fis, clone THYMU2030796		AI083557	-2.219	0.017

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232616_at	hypothetical protein LOC199725	LOC199725	AK023628	-2.214	0.001
218951_s_at	phosphatidylinositol-specific phospholipase C, X domain containing 1	PLCXD1	NM_018390	-2.206	0.002
201791_s_at	7-dehydrocholesterol reductase	DHCR7	NM_001360	-2.205	0.036
238466_at	CDNA FLJ33443 fis, clone BRALZ1000103		R43486	-2.201	0.001
214455_at	histone 1, H2bc	HIST1H2BC	NM_003526	-2.201	0.017
211826_s_at	AF4/FMR2 family, member 1	AFF1	L22179	-2.2	0.013
209966_x_at	estrogen-related receptor gamma	ESRRG	AF094518	-2.2	0.019
228918_at	CDNA FLJ32207 fis, clone PLACE6003204		AI457453	-2.199	0.000
219866_at	chloride intracellular channel 5	CLIC5	NM_016929	-2.194	0.017
205549_at	Purkinje cell protein 4	PCP4	NM_006198	-2.188	0.000
244523_at	monocyte to macrophage differentiation-associated	MMD	AW104453	-2.181	0.016
201809_s_at	endoglin (Osler-Rendu-Weber syndrome 1)	ENG	NM_000118	-2.179	0.016
219710_at	SH3 domain and tetratricopeptide repeats 2	SH3TC2	NM_024577	-2.178	0.009
204388_s_at	monoamine oxidase A	MAOA	NM_000240	-2.176	0.017
205581_s_at	nitric oxide synthase 3 (endothelial cell)	NOS3	NM_000603	-2.175	0.027
205026_at	signal transducer and activator of transcription 5B	STAT5B	NM_012448	-2.161	0.006
232748_at	pregnancy-associated plasma protein A, pappalysin 1	PAPPA	AU156721	-2.155	0.017
209454_s_at	TEA domain family member 3	TEAD3	AF142482	-2.155	0.000
217920_at	mannosidase, alpha, class 1A, member 2	MAN1A2	H97940	-2.151	0.000
239167_at	AF4/FMR2 family, member 1	MLLT2	H86858	-2.15	0.019

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219489_s_at	nucleoredoxin	NXN	NM_017821	-2.15	0.001
210387_at	histone 1, H2bg	HIST1H2BG	BC001131	-2.148	0.016
220703_at	chromosome 10 open reading frame 110	C10orf110	NM_018470	-2.139	0.007
200982_s_at	annexin A6	ANXA6	NM_001155	-2.129	0.021
219799_s_at	dehydrogenase/reductase (SDR family) member 9	DHRS9	NM_005771	-2.127	0.013
209277_at	Tissue factor pathway inhibitor 2	TFPI2	AL574096	-2.125	0.047
210879_s_at	RAB11 family interacting protein 5 (class I)	RAB11FIP5	AF334812	-2.123	0.000
206382_s_at	brain-derived neurotrophic factor	BDNF	NM_001709	-2.122	0.001
237372_at	Pregnancy specific beta-1-glycoprotein 5	PSG5	N30169	-2.116	0.003
241970_at	Poliovirus receptor-related 3	PVRL3	C14898	-2.114	0.029
230908_at	Transcribed locus		AA461490	-2.112	0.001
230973_at	SH2 domain containing 5	SH2D5	AI937119	-2.111	0.003
222378_at	Hypothetical protein FLJ43663	FLJ43663	AW973791	-2.109	0.023
209228_x_at	tumor suppressor candidate 3	TUSC3	U42349	-2.109	0.000
236028_at	Integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)	IBSP	BE466675	-2.106	0.019
206346_at	prolactin receptor	PRLR	NM_000949	-2.106	0.019
237263_at	Full length insert cDNA clone YI54D04		BF590253	-2.101	0.033
1558460_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	ABCC5	AL707614	-2.099	0.009
206374_at	dual specificity phosphatase 8	DUSP8	NM_004420	-2.098	0.001
200785_s_at	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	LRP1	NM_002332	-2.098	0.013

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219552_at	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	C9orf13	NM_024500	-2.098	0.009
203417_at	microfibrillar-associated protein 2	MFAP2	NM_017459	-2.096	0.003
1566001_at	Ankyrin repeat domain 11	ANKRD11	AK096064	-2.095	0.000
220440_at	lectin, galactoside-binding, soluble, 13 (galectin 13)	LGALS13	NM_013268	-2.093	0.006
201787_at	fibulin 1	FBLN1	NM_001996	-2.091	0.041
222513_s_at	sorbin and SH3 domain containing 1	SORBS1	N21458	-2.089	0.029
215240_at	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	AI189839	-2.085	0.001
209453_at	solute carrier family 9 (sodium/hydrogen exchanger), member 1 (antiporter, Na+/H+, amiloride sensitive)	SLC9A1	M81768	-2.079	0.026
224009_x_at	dehydrogenase/reductase (SDR family) member 9	DHRS9	AF240697	-2.078	0.034
240873_x_at	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	DAB2	R62907	-2.077	0.034
213562_s_at	squalene epoxidase	SQLE	BF979497	-2.076	0.013
204121_at	growth arrest and DNA-damage-inducible, gamma	GADD45G	NM_006705	-2.071	0.036
204664_at	alkaline phosphatase, placental (Regan isozyme)	ALPP	NM_001632	-2.069	0.017
202052_s_at	retinoic acid induced 14	RAI14	NM_015577	-2.068	0.008
202178_at	protein kinase C, zeta	PRKCZ	NM_002744	-2.052	0.009
240303_at	CDNA FLJ44282 fis, clone TRACH2003516		BG484769	-2.051	0.000
203399_x_at	pregnancy specific beta-1-glycoprotein 3	PSG3	NM_021016	-2.051	0.019

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
228485_s_at	solute carrier family 44, member 1	SLC44A1	AW165999	-2.037	0.006
205691_at	synaptogyrin 3	SYNGR3	NM_004209	-2.034	0.002
201924_at	AF4/FMR2 family, member 1	AFF1	NM_005935	-2.033	0.011
202995_s_at	fibulin 1	FBLN1	NM_006486	-2.029	0.003
240620_at	Transcribed locus		AI733442	-2.028	0.001
221766_s_at	family with sequence similarity 46, member A	FAM46A	AW246673	-2.026	0.021
205100_at	glutamine-fructose-6-phosphate transaminase 2	GFPT2	NM_005110	-2.025	0.033
1562997_a_at	Homo sapiens, clone IMAGE:5418716, mRNA		BC041484	-2.019	0.000
208881_x_at	isopentenyl-diphosphate delta isomerase 1	IDI1	BC005247	-2.014	0.016
205925_s_at	RAB3B, member RAS oncogene family	RAB3B	NM_002867	-2.013	0.011
209710_at	GATA binding protein 2	GATA2	AL563460	-2.012	0.016
205829_at	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	NM_000413	-2.012	0.019
204830_x_at	pregnancy specific beta-1-glycoprotein 5	PSG5	NM_002781	-2.012	0.005
201625_s_at	insulin induced gene 1	INSIG1	BE300521	-2.005	0.016
1559066_at	Hypothetical protein LOC158402	LOC158402	AI199398	-2.003	0.000

Table IX-3. **Genes up-regulated following adiponectin treatment.**

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
229070_at	chromosome 6 open reading frame 105	C6orf105	AA470369	5.952	0.005
205844_at	vanin 1	VNN1	NM_004666	5.435	0.025
202917_s_at	S100 calcium binding protein A8 (calgranulin A)	S100A8	NM_002964	5.076	0.000
219768_at	V-set domain containing T cell activation inhibitor 1	VTCN1	NM_024626	4.425	0.031
209924_at	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	AB000221	4.149	0.031
227697_at	suppressor of cytokine signaling 3	SOCS3	AI244908	3.788	0.007
218541_s_at	chromosome 8 open reading frame 4	C8orf4	NM_020130	3.472	0.025
32128_at	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	Y13710	3.322	0.022
232504_at	hypothetical protein LOC285628	LOC285628	AL389942	3.311	0.042
207528_s_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	NM_014331	3.049	0.025
1552807_a_at	sialic acid binding Ig-like lectin 10	SIGLEC10	AF301007	3.012	0.011
203913_s_at	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	AL574184	2.950	0.002
214084_x_at	neutrophil cytosolic factor 1 (47kDa, chronic granulomatous disease, autosomal 1)	NCF1	AW072388	2.915	0.042
208747_s_at	complement component 1, s subcomponent	C1S	M18767	2.899	0.048
205220_at	G protein-coupled receptor 109B	GPR109B	NM_006018	2.890	0.005
209921_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	AB040875	2.874	0.042

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
1554098_at	spindlin family, member 3	SPIN3	BC032490	2.841	0.042
202998_s_at	lysyl oxidase-like 2	LOXL2	NM_002318	2.703	0.000
1552626_a_at	hypothetical protein DKFZp566N034	DKFZP566N034	NM_030923	2.646	0.006
219497_s_at	B-cell CLL/lymphoma 11A (zinc finger protein)	BCL11A	NM_022893	2.611	0.042
208084_at	integrin, beta 6	ITGB6	NM_000888	2.591	0.042
1569003_at	transmembrane protein 49	TMEM49	AL541655	2.564	0.002
211548_s_at	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	J05594	2.558	0.000
220049_s_at	programmed cell death 1 ligand 2	PDCD1LG2	NM_025239	2.494	0.041
237415_at	Transcribed locus		BF508849	2.469	0.042
1405_i_at	chemokine (C-C motif) ligand 5	CCL5	M21121	2.457	0.026
221477_s_at	hypothetical protein MGC5618	MGC5618	BF575213	2.457	0.005
228745_at	Hypothetical protein FLJ13611	FLJ13611	AI376997	2.445	0.010
203535_at	S100 calcium binding protein A9 (calgranulin B)	S100A9	NM_002965	2.433	0.042
203914_x_at	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	NM_000860	2.421	0.003
202075_s_at	phospholipid transfer protein	PLTP	NM_006227	2.392	0.042
223767_at	G protein-coupled receptor 84	GPR84	AF237762	2.364	0.031
234306_s_at	SLAM family member 7	SLAMF7	AJ271869	2.364	0.029
203543_s_at	Kruppel-like factor 9	KLF9	NM_001206	2.331	0.042
206420_at	immunoglobulin superfamily, member 6	IGSF6	NM_005849	2.299	0.006
215977_x_at	glycerol kinase	GK	X68285	2.294	0.042
203922_s_at	cytochrome b-245, beta polypeptide (chronic granulomatous disease)	CYBB	AI308863	2.283	0.031

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
217167_x_at	glycerol kinase	GK	AJ252550	2.283	0.042
241068_at	DORA reverse strand protein 1	DREV1	BF510881	2.278	0.000
221578_at	Ras association (RalGDS/AF-6) domain family 4	RASSF4	AF260335	2.273	0.025
231972_at	Full length insert cDNA clone ZE08A03		AK024681	2.252	0.042
217678_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	AA488687	2.242	0.004
231696_x_at	Transmembrane protein 50B	C21orf4	AV648424	2.212	0.042
210095_s_at	insulin-like growth factor binding protein 3	IGFBP3	M31159	2.208	0.025
1553151_at	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2	ATP6V0D2	AY079172	2.203	0.042
202035_s_at	secreted frizzled-related protein 1	SFRP1	AI332407	2.193	0.042
217107_at			AL035603	2.188	0.042
208792_s_at	clusterin	CLU	M25915	2.183	0.004
224325_at	frizzled homolog 8 (Drosophila)	FZD8	AB043703	2.174	0.042
219159_s_at	SLAM family member 7	SLAMF7	NM_021181	2.165	0.027
203873_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	SMARCA1	M88163	2.165	0.042
210519_s_at	NAD(P)H dehydrogenase, quinone 1	NQO1	BC000906	2.155	0.040
1556697_at	hypothetical protein LOC285513	LOC285513	AI819722	2.132	0.006
209772_s_at	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	CD24	X69397	2.123	0.007
210904_s_at	interleukin 13 receptor, alpha 1	IL13RA1	U81380	2.123	0.025

Affymetrix ID	Gene Name	Gene Symbol	GenBank	Fold Change	P-value
204655_at	chemokine (C-C motif) ligand 5	CCL5	NM_002985	2.105	0.044
221491_x_at	major histocompatibility complex, class II, DR beta 1	HLA-DRB1	AA807056	2.079	0.036
1561430_s_at	chromosome 3 open reading frame 15	C3orf15	BC035248	2.075	0.001
230230_at	CDNA: FLJ21893 fis, clone HEP03412		AI379691	2.070	0.042
208864_s_at	thioredoxin	TXN	AF313911	2.070	0.042
203355_s_at	pleckstrin and Sec7 domain containing 3	PSD3	NM_015310	2.066	0.000
214702_at	fibronectin 1	FN1	AJ276395	2.062	0.042
216191_s_at	T cell receptor alpha locus ; T cell receptor delta locus	TRA@ ; TRD@	X72501	2.062	0.042
231697_s_at	Transmembrane protein 49	VMP1	AV660825	2.053	0.033
1569054_at	solute carrier family 1 (glial high affinity glutamate transporter), member 3	SLC1A3	BC022285	2.041	0.001
1568609_s_at	family with sequence similarity 91, member A2 ; FLJ39739 protein	FAM91A2 ; FLJ39739	AL118843	2.020	0.008
240137_at	Tudor domain containing 6	TDRD6	AI915629	2.020	0.027
227236_at	tetraspanin 2	TSPAN2	AI743596	2.016	0.042
228718_at	zinc finger protein 44 (KOX 7)	ZNF44	AI379070	2.016	0.010
212382_at	Transcription factor 4	TCF4	BF433429	2.000	0.002

Table IX-4. **Adiponectin-regulated genes identified by microarray and validated by quantitative RT-PCR.**

Gene Symbol	Function	GenBank	Fold Change Adpn/Control	
			Microarray	qPCR
ADAM12	protease	NM_003474	-2.904	-2.717[a]
ALPP	phosphatase	NM_001632	-2.069	-1.887
CD24	sialoglycoprotein	X69397	2.123	1.480
CGB	hormone	NM_000737	-8.936	-4.952[a]
CSH1	hormone	NM_022641	-4.899	-3.864[a]
CYP11A1	steroid synthesis	NM_000781	-2.856	-2.654[a]
CYP19A1	steroid synthesis	AI740573	-2.800	-3.699[a]
ERVWE1	cell fusion	AF072506	-2.374	-1.366
FURIN	proprotein convertase	NM_002569	-2.835	-2.389[a]
GH2	hormone	AF006060	-6.372	-4.248[a]
HSD11B2	steroid metabolism	NM_000196	-3.494	-2.655[a]
INSL4	insulin superfamily	NM_002195	-3.837	-3.109[a]
IGFBP3	insulin-like growth factor availability	M31159	2.208	1.659
LDLR	cholesterol metabolism	NM_000527	-2.638	-2.499[a]
PAPPA2	IGFBP protease	AL031290	-2.698	-1.747[a]
PGF	growth factor	BC001422	-2.852	-2.781[a]
PAPPA	IGFBP protease	NM_002581	-2.442	-3.254[a]
SIGLEC10	immunoglobulin superfamily	AF301007	3.012	2.938[a]
SOCS3	immune regulation	AI244908	3.788	2.403

[a] $P < 0.05$

5. Discussion

In addition to the endocrine changes and induction of an inflammatory response (see Chapters 2 and 3), adiponectin has many diverse effects on syncytialized trophoblast cells. One of the interesting mechanisms identified from these data is the IGF processing pathway. Amino acid and glucose transfer as well as the health and development of the placenta are all influenced by IGFs (Forbes and Westwood, 2008). The bioavailability of IGF is regulated by binding proteins, which sequester IGF in the serum, not allowing it to bind to its receptor on the surface of the target cell. The interaction between IGF and IGF binding proteins (IGFBPs) is disrupted through the actions of proteases which degrade specific IGFBPs and release IGF, allowing it to bind its receptor. Treatment with adiponectin in long-term culture resulted in a decrease in expression for a number of proteases and a phosphatase involved in this process including furin, disintegrin and metallopeptidase domain-containing protein 12 (ADAM12), pregnancy associated plasma protein 1 and 2 (PAPPA and PAPPA2), and placental alkaline phophatase (ALPP). Conversely, although not reaching statistical significance, there was a trend for increased expression of IGFBP3 after these cells had been exposed to adiponectin for 5d.

Increased expression of the protease PAPPA2 has been reported in cases of preeclampsia (Winn et al., 2009, Nishizawa et al., 2008), perhaps in an effort by the placenta to increase the amount of available IGF and, thus, stimulate nutrient uptake to what is often a metabolically deprived fetus. These data are in keeping with the overall hypothesis of our laboratory, that adiponectin functions to keep the demands

of the fetus in check, and in that manner may be regarded as serving the interests of the mother's metabolic system(s) over that of the fetoplacental unit.

Two steroidogenic enzymes, 11-β hydroxysteroid dehydrogenase B1 (HSD11B1) and HSD11B2, were also changed with adiponectin treatment. An increase in HSD11B1 was accompanied by a concomitant decrease in HSD11B2. These data raise an interesting phenomenon regarding potential cortisol levels in the placenta. Cortisol, one of the major stress-induced hormones, is converted from its inactive metabolite, cortisone by HSD11B1. Conversely, active cortisol is metabolized to inactive cortisone by HSD11B2. Thus, a reduction in HSD11B2 and an increase in HSD11B1 may lead to a net increase in cortisol in the syncytiotrophoblast exposed to adiponectin. Maternal levels of cortisol are approximately 10x higher than that of the fetus, thus a decrease in placental HSD11B2 may allow for increased levels of maternal cortisol to cross to the fetus (Ni et al., 2009). Increased glucocorticoids during gestation have been shown to result in a number of negative impacts on the fetus, including intrauterine growth restriction (IUGR) (Ain et al., 2005). In addition, studies in humans suggest that maternal stress and cortisol levels can alter postnatal cognitive development in the child (Davis and Sandman, 2010).

It is remarkable how many of the changes we observe with adiponectin treatment are opposite to what have been associated with preeclampsia. Genes such as PAPPA2, chorionic gonadotropin β, endoglin, and α-mannosidase, all of which drop following adiponectin treatment (Table IX-2), have been reported to be increased in placenta samples from preeclamptic patients (Winn et al., 2009). Still others are up-

regulated in both preeclampsia and with adiponectin exposure, including ankyrin repeat and SOCS box-containing 2 (ASB2) and 17βHSD (Winn et al., 2009). These data are difficult to interpret given multiple reports showing a positive correlation between circulating adiponectin levels and preeclampsia (Avci et al., 2010, D'Anna et al., 2006, Fasshauer et al., 2008, Nien et al., 2007). However, an increase in adiponectin in preeclampsia has been challenged (Mazaki-Tovi et al., 2009c). It is also important to consider the large variation in diagnostic criteria for a complex, multifactorial disease such as preeclampsia. Alternatively, the largely opposing effects of preeclampsia versus adiponectin on global gene expression may be explained by suggesting that adiponectin has the capacity to counteract many of the effects at the maternal-fetal interface initiated by preeclampsia. One must also be careful to draw too many parallels between our data and that reported with preeclampsia, as the two studies used whole placental tissue (Winn and colleagues) versus isolated trophoblasts (this report). Furthermore, it is currently unclear as to the responsiveness of placental trophoblasts to adiponectin during preeclampsia. Cells from healthy placentas may respond to adiponectin in culture in a very different manner than preeclamptic trophoblasts would *in vivo.*

Adiponectin has long been associated with inhibiting endothelial cell and macrophage activation, and as a consequence is widely considered a vasculoprotective factor (Zhu et al., 2008). It follows that adiponectin would counteract diseases such as preeclampsia, which is characterized by endothelial dysfunction. Thus, it is important to understand the effects adiponectin has, not only on endothelial cells, but also on the trophoblast. As the functional unit of the placenta,

their response to adiponectin will have important implications for the potential of adiponectin as a treatment option for diseases of pregnancy.

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X. Chapter Six:

Concluding Remarks

Only relatively recently has adipose tissue been appreciated for the profound impact it has on whole-body physiology. Its function as a storage depot for energy reserves has been expanded to include a potent endocrine role. Indeed, the number of hormones, cytokines, and growth factors, collectively termed adipokines, produced by adipose tissue is amended to include novel members on an almost weekly basis. One of these hormones is adiponectin. Adiponectin has been shown to influence metabolic homeostasis and promote insulin sensitivity in a number of different tissue types. As one of the most abundant hormones in circulation, adiponectin also has the potential to regulate many biological systems other than those traditionally appreciated for their metabolic role. The proportion of the population in the United States that are now considered overweight or obese has reached epidemic proportions. It is therefore critical that we make understanding the role of adipose tissue, and hormones such as adiponectin, a priority in this country. In addition, a woman's risk of developing specific diseases of pregnancy, such as gestational diabetes and preeclampsia, is positively correlated with her BMI. Normal pregnancy represents a state of altered maternal energy metabolism and glucose utilization. Given the importance of adiponectin in these processes, it follows that adiponectin expression and actions may play an important role in mediating the effects of metabolic stress during gestation. This is particularly true in instances of exaggerated reductions in adiponectin production, such as gestational diabetes, making adiponectin a prime candidate for an important mediator of trophoblast cells during pregnancy. The available literature regarding adiponectin and the placenta are correlative studies and thus succeeds primarily in substantiating, while not directly testing, the hypothesis

that the adipokine plays an important role at the maternal-fetal interface. Precisely what this role involves needs to be elucidated. Thus, an understanding of adiponectin's role, if any, on human trophoblast cells was the aim of these studies.

1. Adiponectin is not expressed by the human placenta.

We have utilized RT-PCR and quantitative RT-PCR in an effort to amplify a transcript for adiponectin in the human placenta. Despite the design of multiple primer sets, including those that have been published for placental adiponectin (Caminos et al., 2005), and the use of TaqMan primer/probe sets (Applied Biosystems), we have been unable to detect adiponectin gene expression. This is true in the choriocarcinoma cell lines, Jeg-3, Jar, and BeWo, as well as freshly isolated cytotrophoblasts from term, term cytotrophoblasts syncytialized *in vitro* with administration of epidermal growth factor (EGF), and RNA collected from whole placenta collected at term. Thus, we conclude that adiponectin is not produced by the human term placenta.

2. The human placenta has the capacity to respond to adiponectin.

Using RT-PCR and quantitative RT-PCR, we have been able to identify transcripts for the adiponectin receptors, adipoR1 and adipoR2. This is the case in both freshly isolated cytotrophoblasts as well as syncytialized trophoblasts. From these data we conclude that the human placenta may be able to respond to adiponectin if exposed to the hormone, presumably from maternal blood.

3. Adiponectin inhibits hormone production by syncytialized human trophoblasts.

We exposed term trophoblasts to physiological levels of adiponectin for a culture period of 5d. As hormone production is one of the primary functions of the syncytiotrophoblast, we began by assessing the production of a number of hormones following adiponectin exposure. Using qRT-PCR, we determined that adiponectin treatment resulted in a significant drop in gene expression for human chorionic gonadotropin (hCG) β, the common glycoprotein α subunit, and placental lactogen. In addition, expression of *CYP11A1 and CYP19A1,* the genes that encode the steroidogenic enzymes p450 side chain cleavage and aromatase, respectively, were also decreased. All of these changes were also observed at the protein level through the use of western blots. Furthermore, we identified a decrease in progesterone secretion from cells that had been exposed to adiponectin for the entire culture period. These data demonstrate that adiponectin exerts a potent effect on trophoblast cells. Many of these hormones, such as placental lactogen, will promote a shuttling of nutrients and glucose to the fetoplacental unit. It is therefore possible that adiponectin works to keep these demands in check by decreasing the production of these hormones.

4. Adiponectin induces a pro-inflammatory response by syncytialized trophoblast cells.

Exposure to adiponectin also elicits an up-regulation in gene expression for the pro-inflammatory cytokines, interleukin (IL)-1β and IL-8. We observed these

changes after the cells were placed in culture with adiponectin treatment for a total of 5d. Induction of the pro-inflammatory response also occurs when the cells are allowed to syncytialize spontaneously *in vitro* for 4d prior to 24h adiponectin treatment. Thus, the pro-inflammatory response to adiponectin is more likely to be a direct effect of adiponectin on the trophoblast cells. In addition, we have noted an increase in the gene expression and protein levels of the sialic acid-binding receptor, Siglec10. Siglec10 contains an immune receptor tyrosine-based inhibitory motif (ITIM), and has been proposed to function as an inhibitory receptor in the innate immune system (Munday et al., 2001). We have also shown that the syncytiotrophoblast expresses Siglec10 as well as the glycoprotein CD24, which has been suggested to function with Siglec10 to dampen the innate immune response to components released by injured cells (Chen et al., 2009). From these data we hypothesize that the trophoblast may be increasing Siglec10 expression, normally expressed at relatively low levels, in an effort to reduce the pro-inflammatory response initiated by adiponectin.

5. Adiponectin activates the MAPK pathways, ERK1/2 and JNK as well as EGFR through the actions of APPL1.

We have shown that adiponectin activates both of the mitogen-activated protein kinases (MAPKs), extracellular signal-related kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK). In addition, treatment with adiponectin elicits an increase in the levels of activated EGFR. Through lentiviral-mediated over-expression of the inhibitory binding partner, APPL2, we have also shown that the signaling capacity of

adiponectin is dependent on the adaptor protein APPL1, which will bind to the adiponectin receptor and mediate downstream signaling events.

6. Leptin attenuates the signaling actions induced by adiponectin.

By over-expressing the hormone leptin in isolated trophoblasts collected at term we are able to abolish the ability of adiponectin to signal in these cells. Leptin and adiponectin are known to initiate opposing actions in a variety of metabolic systems, and the adipose expression patterns of the two hormones mirror one another (i.e. leptin production increases with increased lipid stores, adiponectin decreases). It is thus not surprising that leptin attenuates the actions of adiponectin at the maternal-fetal interface, however this is the first time such a phenomenon has been reported.

7. Adiponectin can also elicit changes in a variety of metabolism-related genes in trophoblasts, including those involved in IGF bioavailability.

To assess the changes initiated by adiponectin treatment on syncytialized trophoblast cells, we used a global microarray approach. These data allowed us to identify a number of genes that were down-regulated following adiponectin treatment, including many of the genes already identified by our previous investigations. In addition, many genes were up-regulated by adiponectin, although only about 1/3 as many as those we determined to be reduced. One of the interesting pathways that arose from this study was the identification of a reduction in the expression of the proteases, ADAM12, PAPPA1 and 2, and the phosphatase, ALPP. All of these proteins are involved in the degradation of IGF binding proteins and the

liberation of IGF for cell signaling. In addition, from these data we have observed a trend toward an increase in the expression of IGFBP3. Taken together, these data suggest that adiponectin may be decreasing the amount of available IGF at the maternal-fetal interface, by keeping more of it sequestered by IGFBPs.

Completion of these studies has elucidated alterations in a number of important functions of the syncytiotrophoblast following adiponectin exposure. Clinical studies continue to be published highlighting the changes in circulating adiponectin levels during gestational diseases, and relating those to not only the disease state during pregnancy, but also the long-term health of both mother and child. It has now been suggested that low levels of adiponectin during pregnancy can predict pancreatic β-cell dysfunction and consequent insulin resistance postpartum (Retnakaran et al., 2010). Despite these potentially serious implications for adiponectin, we remain one of the few groups that are examining its effects on trophoblast cells specifically. Thus, the data reported herein have substantially enhanced our understanding of the role for adiponectin in trophoblast function.

In addition, these data have resulted in the generation of a number of hypotheses that may be tested in the future. Examination of the effects of adiponectin during pregnancy *in vivo* is the next logical progression from these data, and will significantly advance our understanding of the effects of too little or too much adiponectin on the placenta. Careful examination of pregnancy in adiponectin null mice, as well as utilization of the adenovirus-mediated over-expression rodent model during pregnancy may allow us to ascertain if the effects we have observed in human

primary cell cultures remains true when examined in a physiologically relevant model system with many different hormonal and environmental cues also at work.

There is an increased appreciation for the two adiponectin receptors, adipoR1 and adipoR2, in mediating distinct biological functions within the same or different cell types. It would therefore significantly enhance our understanding of adiponectin biology at the maternal-fetal interface, if we were to begin to examine the individual effects of the two receptors in trophoblast cells. This may be done through the use of lentiviral shRNA constructs, as we have utilized for some of the studies described above. It is possible that each receptor serves a unique function within the trophoblast cell, stimulating distinct signaling pathways, and thus some of the effects we have attributed to adiponectin may be specifically due to the hormone's actions through a specific receptor.

Finally, all of our data has been generated using term trophoblast cells. It would be extremely interesting to investigate the effect of adiponectin on the placenta earlier in gestation. To do this, we may be able to utilize first trimester human cell lines, or possibly even primary tissue collected from first trimester placentas. The developing placenta is a very different organ than the mature form, and therefore we expect that adiponectin may have very different effects on trophoblast cells from the first trimester placenta.

XI. Chapter Seven:

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