THESIS

REGULATION OF TROPHOBLAST STEM CELL MAINTENANCE AND DIFFERENTIATION BY LIN28 AND AP-2γ

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2010

COLORADO STATE UNIVERSITY

July 6, 2010

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY BRITTANY A. FROMME ENTITLED "REGULATION OF TROPHOBLAST STEM CELL MAINTENANCE AND DIFFERENTIATION BY LIN28 AND AP-2γ" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

REGULATION OF TROPHOBLAST STEM CELL MAINTENANCE AND DIFFERENTIATION BY LIN28 AND AP-2γ

The placenta is a unique organ essential for survival of the fetus in all eutherian mammals. Failure to develop a normal placenta in humans can lead to diseases, such as pre-eclampsia, with high morbidity and mortality for both the mother and the fetus. These diseases are thought to be caused by abnormal proliferation and differentiation of cells in the placenta. A mouse trophoblast stem (TS) cell culture system is a useful tool in studying TS cell proliferation and differentiation into trophoblast giant cells (TGCs). TS cells cultured in proliferative media (70% conditioned media, 30% TS media, FGF4, and heparin sulfate) will remain proliferative, and TS cells cultured under differentiation media (100% TS media) will differentiate into TGCs. LIN28 is a protein that regulates mRNAs and miRNAs, and is abundantly expressed in many undifferentiated tissues. AP-2γ has been shown to be essential for TS cell maintenance and TGC formation. AP-2γ null mutants display embryonic lethality at E7.5 due to a severely disrupted extraembryonic portion of the embryo. In TS cells, AP-2γ has been shown to bind to the promoter region of *Lin28*. This study investigates the hypothesis that *Lin28* and *Ap-2γ* are necessary regulators of trophoblast stem cell maintenance and differentiation into TGCs.

This study shows the pluripotency genes, *Lin28, Sox2,* and *Nr0b1,* to be differentially expressed in proliferating TS cells and differentiated TGCs. MiRNAs can be used as markers for proliferation or differentiation. 28 significantly different miRNAs

were detected between TS cells and TGCs, 18 up-regulated in TGCs and 9 down-regulated in TGCs. Expression of the miR-290 family, initially thought to be ES cell specific, was detected in proliferating TS cells suggesting TS cells have similar miRNA mediated regulation of proliferation compared to ES cells. The Let-7 family of miRNAs was found to be up-regulated in differentiated TGCs. The Let-7 family has been shown to be regulated by LIN28, where LIN28 prevents accumulation of mature Let-7 miRNAs. In this study *Lin28* was highly expressed in proliferating cells and the Let-7's are up-regulated in differentiated TGCs. *Lin28* function in TS cells was assessed by knocking down *Lin28* using shRNA lentiviral technology. *Lin28* knockdown TS cells were used to observe results of knockdown. We obtained a 78% reduction of *Lin28* mRNA, but found that loss of *Lin28* in TS cells did not affect morphology, proliferation or differentiation.

AP-2γ null TS cells grown in culture fail to differentiate morphologically into TGCs. *Lin28, Sox2,* and *Nr0b1* show no difference in expression when grown in conditions to differentiate the cells, indicating a failure of AP-2γ null TS cells to differentiate into TGCs. RO3306 is a compound used to block Cyclin-dependent Protein Kinase 1 and force endoreduplication, causing TS cells to differentiate into TGCs. AP-2γ null TS cells cannot be forced to differentiate into TGCs, and instead undergo cell death, when cultured with RO3306. Additionally, AP-2γ null TS cells express the pluripotency markers *Oct4, Stella,* and *Nanog* which only are expressed in ES cells and germ cells. MiRNA profiling of AP-2γ null TS cells indicates that cells in proliferative conditions resemble wild type counterparts, but when proliferative conditions are removed we observe an increase in expression of the ES cell specific miR-302 cluster. While there was no effect of proliferation in wild type cells, loss of *Lin28* in AP-2γ null TS cells via

lentiviral knockdown leads to a partial rescue of TGC formation. This suggests that *Lin28* must be down-regulated in order for TGC formation, and that AP-2γ regulates *Lin28* in TS cells.

Taken together these data suggest a role for *Lin28* in mouse TS cell proliferation and differentiation, where *Lin28* must become down regulated in order for differentiation into TGCs. AP-2γ has been shown to bind to the *Lin28* promoter in TS cells; this regulation enables TS cell differentiation into TGCs. This study also shows the necessity of AP-2γ for TS cell differentiation into TGCs; loss of AP-2γ leads to a more pluripotent state rather than allowing for differentiation. Loss of AP-2γ leads to expression of pluripotency markers *Oct4, Nanog,* and *Stella,* and the ES cell specific miR-302 cluster, indicating an increase in pluripotency. We conclude that AP-2γ and LIN28 are essential molecular regulators of TS cell proliferation and differentiation.

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ACKNOWLEDGEMENTS

As I begin to see the end of this journey towards a Masters degree I think back on all of the people who made this journey memorable and who have helped me along the way. There are many people whom I would like to thank and remember.

I would first off like to thank my advisors Drs. Quinton Winger and Gerrit J. Bouma. You both know you deserve more thanks than the scope of this page, but here it is. My thanks go to you Quint for taking me in without knowing me and getting me started on this project. Thank you for pushing me to succeed, being the driving force behind this degree, and not letting me make excuses. You have shown me that I am capable of achieving more than I ever dreamed was possible, even when I didn't think I could make it. I am grateful for having had the opportunity to work for you. I hope we both have learned something from this experience!

Jerry, you are truly the one who started me in my research career. Thank you for teaching me many of the lab techniques needed to accomplish this feat of research. Your personal dedication to producing excellent students and excellent research has greatly influenced me. You have always been accessible and available for me when I needed someone to help me, for any reason. Thank you for that, your eternal optimism has gotten me through many rough spots during this project.

To my other two advisors Drs. Russell Anthony and Susan Bailey I owe my deepest gratitude. Thank you for sticking with this whirlwind ride of a research project. Your support and encouragement in the face of my ever expanding project is commendable. Dr. Anthony, thank you for always being in my corner and helping me to stick up for myself. You seem to have limitless faith and confidence in me and have

always encouraged me to do my best. You have always been a wonderful teacher and mentor. I truly admire you. Dr. Bailey, you have been everything I could hope for in an outside committee member. You have helped me keep perspective on my project to ensure I cover my bases in a logical manner. Thank you for providing me with the opportunities to speak in your class and share my research with others. You are both greatly appreciated.

To my coworkers: I have learned many things from all of you. Thank you for helping me when I needed it, listening to me when I talked, and providing a new group of friends I never imagined I could have. Juliano, you will always be my "brother" even if you never learn to speak English. Thank you for always listening to me and giving me "advice", even if it was always telling me I am wrong (I usually was). Katie, thanks for all the sushi and Sonic vent sessions! Jill Seabrook, thanks for being such a good friend. Good luck carrying forward this project, you will need it! Everyone at the ARBL has provided me with an endless source of resources and help. I will miss this little science family.

Lastly, I would like to thank my family for their continued support of me in this endeavor. I love you all and could not have made it without your continued love and support. I would especially like to thank you Zach, for putting up with many late nights at the lab and hardly any free weekends. Thank you for seeing me through all of the ups and downs of this journey. You are the best!

TABLE OF CONTENTS

LISTING OF ILLUSTRATIONS

LISTING OF TABLES

Chapter I:
Review of the Literature

Introduction

The placenta is a multifaceted organ that is essential for fetal development of all eutherian mammals. This unique organ is found only in women, lining the uterus only during pregnancy. The placenta aids in nutrient transfer to, and waste removal from the fetus, and protects the fetus from the maternal immune system. In addition, the placenta is an endocrine organ secreting hormones such as progesterone, placental lactogens, and others to maintain pregnancy and help establish proper metabolic function in the mother. The placenta is formed with both maternal and fetal components. Improper development of any of these components leads to abnormal pregnancy such as pre-eclampsia (PE) or intrauterine growth restriction (IUGR). PE and IUGR have high rates of morbidity and mortality for the mother and the only available treatment is premature delivery to remove the placenta (Regnault et al., 2002). While PE and IUGR have been studied extensively over the years the underlying causes of these disorders are not well understood.

There are several transcriptional regulators known to be involved with the specification (e.g *Cdx2*, *AP-2γ, Tead4*) (Strumpf et al., 2005; Kuckenberg et al, 2010; Yagi et al, 2007), proliferation (e.g. *Eomes*, and others) (Russ et al., 2000), and differentiation (e.g. *Pl1, Pl2, Plf*) (Simmons and Cross, 2007) of trophoblast stem cells giving rise to the placenta in both humans and animals. Of the models available for studying the molecular regulation of trophoblast stem cells, murine cell culture systems

have been used extensively to study trophoblast growth and differentiation (Tanaka et al., 1998). This review describes the human placenta and the models available for studying it.

Early History of Placenta Research

Early studies of pregnancy were greatly limited by the ethical concerns around the dissection of human cadavers. Aristotle first described the human uterus as having many "cotyledons", probably what we know now are endometrial folds, which were thought to act as suckling devices for gestating fetuses (Wynn, 1977). In the middle ages it was thought that an anastomoses was formed between the growing infant's and the maternal blood streams. This thought was expanded on in the late 1700's when the brothers John and William Hunter determined that the placenta was an organ of nutrient transfer and waste removal via anastomoses within the placenta. In addition to these revelations, the Hunter brothers also demonstrated maternal blood in the intervillous space of the placenta and noted the presence of "curling" arteries seen in the maternal decidua (Wynn, 1977). Much has changed regarding our understanding of the placenta since the early investigations of its anatomy. We know now that different types of mammals have different types of placentas. Indeed different animals serve as good models to study human placentation for different reasons.

Types of Placentas

Although all eutherian mammals have a placenta, there are several types of anatomies and physiologies of different placentas. A placenta can be classified based on

shape, amount of interaction between fetal and maternal tissues, amount of tissue separating fetus and uterus, and arrangements of both maternal and fetal blood vessels and the direction of flow as can be seen in Table 1 (Benirscke and Kaufmann, 2000). There are six possible layers that can be formed between the maternal blood and the fetal blood: the fetal endothelium lining the arteries, the fetal connective tissue, the fetal epithelium (called trophoblast), the maternal epithelium, the maternal connective tissue, and lining the maternal circulation is the maternal endothelium (Grosser, 1909). In accordance with this, Grosser's original classifications of placental types are by how many layers separate the fetal blood from the maternal circulation. An epitheliochorial placenta would have all six layers separating the maternal and fetal blood flow. The placenta does not invade into the maternal uterus, but is just closely attached (Benirschke and Kaufmann, 2000). A syndesmochorial placental is found in animals where the maternal epithelium is discontinuous due to some invasion of the trophoblast. Endotheliochoiral placentas invade slightly more than a syndesmochorial placenta, where the fetal trophoblast cells invade all the way in to the maternal endothelium. The most invasive type of placenta is called the hemochorial placenta. In this type of placenta the fetal trophoblast cells invade and erode the maternal endothelium, resulting in a restructuring of the maternal blood vessels.

Shape of Attachment:

Following Grosser's classifications, placentas are further characterized based on the shape of attachment and type of fetomaternal interdigitation (Benirschke and Kaufmann, 2000). The shape of attachment comes from the interaction between the

chorionic sac (fetal trophoblast) and maternal uterus. The fetomaternal interdigitation describes the type of attachment formed. The shapes are diffuse, cotyledonary, discoid and zonary. A diffuse placenta has light interactions with the maternal interface that are found extending over the entire surface of the trophoblast (Benirschke and Kaufmann, 2000). A cotyledonary type of placenta is classified as having many highly concentrated areas of attachment. A zonary type placenta forms a ring of attachment that completely surrounds the developing fetus like a girdle. Lastly, the discoid type placenta is formed by a disc shaped zone that has the highest concentration of interaction with the maternal uterus. The discoid placenta is the most common form of placenta (Benirschke and Kaufmann, 2000).

Maternofetal Interdigitation:

The types of maternofetal interdigitation are folded, lamellar, trabecular, villous and labyrinthine (Benirschke and Kaufmann, 2000). The folded type has ridge like folds of the chorion which loosely fit into the folds of the maternal uterus. The lamellar type of interdigitation is similar to the folded type, but slightly more complex. In the lamellar type of placenta the ridges of the chorion branch in a parallel fashion but are separated by endometrial folds. The trabecular type placenta has branching folds similar to the lamellar type which sprout finger like villi. The villous type of placenta has much more extensive branching than the lamellar type, allowing for a larger surface area for nutrient exchange. The most effective type of interdigitation is the labyrinthine placenta. This type of interdigitation is only seen in discoid shaped placentas. A labyrinthine

interdigitaion is formed when the trophoblast layer is penetrated in a web-like fashion, forming channels in which the maternal blood can bathe the trophoblast.

Based on the shape, type of maternal interdigitation, and type of maternal invasion we can roughly classify all placental mammals. Pigs and horses have difuse, folded, epitheliochorial placenta. Carnivores have zonary, lamellar, endotheliochorial placentas. Ruminants have cotyledonary, villous, syndesmochorial placentas. Rodents and humans have hemochorial, discoid placentas; humans have a villous type of interdigitation and rodents have a labyrinthine type of interdigitation. Knowing about the types of placentas, we can begin to determine which animals are good models for studying human placental development and defects.

A Brief Classification of Placental Types			
Shape	**Interdigitation**	**Invasion**	**Example**
Difuse	Folded	Epitheliochorial	Horse, Pig
Zonary	Lamellar	Endotheliochorial	Dog, Cat
Cotyledonary	Villous	Syndesmochorial	Ruminant
Discoid	Villous	Hemochorial	Human
Discoid	Labyrinthine	Hemochorial	Mouse, Rat

Table 1: A summary of the common types of placentas seen in mammals based on shape, interdigitation, and invasion.

Human Placentation and Diseases of the Placenta

The mature human placenta is made up of two parts, a fetal part called the chorionic disc and the maternal part termed the basal plate (Benirschke & Kaufmann, 2000). The fetal side of the placenta at maturity will have three layers, although four layers can been observed in early placental development. In the developing placenta the

layers are the syncytiotrophoblast layer facing the maternal circulation, the underlying cytotrophoblast layer (up until about half way though gestation), endothelial lining of the fetal capillaries, and connective tissue between villi (Benirschke & Kaufmann, 2000).

Human placentation starts with the first cell specification in a morula, where the polar outer cells are destined to become trophoblast and the apolar inner cells are destined to an inner cell mass fate (Cross, 2000). This specification can be determined by day 4 after conception (Boyd & Hamilton, 1970), after which the blastocyst will start to invade the maternal endometrium by day 6 or 7 (Benirschke and Kaufmann, 2000). Once the trophoblast has invaded the maternal myometrial tissue (called decidua at this point), the proliferative stem cell population (called cytotrophoblasts), begin to differentiate into the different cell types of the placenta (Boyd & Hamilton, 1970). There are two main developmental fates which the differentiating cytotrophoblasts may take: the villous pathway or the extravillous pathway (Benirschke and Kaufmann, 2000). In the villous pathway differentiation starts to occur when proliferating cytotrophoblasts start to fuse to form binucleated syncytiotrophoblast which form a layer around the proliferating cytotrophoblast cells (Boyd and Hamilton, 1970). The underlying cytotrophoblasts continue to proliferate and will start to protrude out into the syncytiotrophoblast layer which indicates the onset of chorionic villus growth (Boyd and Hamilton, 1970). Syncytiotrophoblast cells form a continuous layer covering all villi of the human placenta (Benirschke and Kaufmann, 2000). Fully formed villi act as the primary sites for nutrient transport, endocrine production and immune support for the fetus.

In the extravillous pathway, cells destined to an extravillous trophoblast fate will differentiate into one of two types of trophoblast: the interstitial trophoblast or the

endovascular trophoblast. Extravillous trophoblast cells come from cytotrophoblast cell columns or other proliferating cytotrophoblast cells not incorporated into villi (Benirschke and Kaufmann, 2000). Extravillous cytotrophoblast cells invade the interstitium of the placenta to promote expansion of the villous region (Benirschke and Kaufmann, 2000). The interstitial trophoblast cells take on a rounded and multinucleated cell morphology as they invade deeper into the decidua, forming placental bed giant cells (Boyd and Hamilton, 1970). Placental bed giant cells are terminally differentiated extravillous cells. Other extravillous trophoblast cells will invade into the decidua and differentiate into endovascular trophoblast cells. These cells will invade into the maternal spiral arteries, lining the arteries and remodeling them to enable increased blood flow, independent of maternal vascular signals (Benirschke and Kaufmann, 2000).

Proper development, differentiation and migration of all of the cells in the placenta lead to a normal, healthy placenta with proper nutrient transfer to and waste removal from the fetus. Abnormalities in this process can lead to diseases of pregnancy such as pre-eclampsia (PE).

Pre-Eclampsia:

PE is characterized by hypertension and proteinuria after the twentieth week of gestation in women who are normally not hypertensive (National Heart Lung and Blood Institute, 2001). PE affects between 5-8% of all pregnancies (Lian and Roberts, 2002), with an increased risk seen in patients with obesity, previous episodes of PE, diabetes, vascular disease, twins or multiples, and extreme age for pregnancy (less than 20 years, or greater than 40 years) (Lian and Roberts, 2002; Karumanchi et al., 2005). PE affects

both the mother and the fetus. The only available treatment for PE is delivery of the placenta, resulting in premature delivery of the fetus. Premature delivery of the fetus has been suggested to lead to greater problems later in life such as obesity, diabetes, and increased rate of delivering premature babies (Barker Hypothesis: Barker, 1993). Effects seen in the mother include proteinuria and hypertension during the second half of pregnancy; severe effects include edema due to endothelial malfunction. In some instances edema occurs in the cerebrum resulting in headaches, vision changes and sometimes seizures (Redman and Sargent, 2005). The medical world has known about PE for hundreds of years, but little is known about the cause. It seems the placenta is central in the pathogenesis of PE as affected placentas often show signs of vascular disease and abnormal blood flow when visualized on Doppler. In addition, the maternal spiral arteries have a shallower invasion by the cytotrophoblasts leading to increased resistance of blood flow (Zhou et al., 1997; Redman and Sargent, 2005). Currently, there is no available screening method available to look for signs of developing PE. Several factors have been shown to be differentially regulated in pre-eclamptic placentas when compared to normotensive placentas. These factors include a decrease in vascular endothelial growth factor (VEGF) (Maynard et al., 2003), decrease in Angiotensin (Brosnihan et al., 2004), decrease in prostaglandin I_2 (Mills et al., 1999), decrease in Placental growth factor (PlGF) (Maynard et al., 2003), and a decrease of the VEGF receptor-1 (Flt1) (Maynard et al., 2003). Additionally, it is thought that PE can be caused by a defect in degradation of the extracellular matrix around the trophoblast at the point of invasion, or by a failure of the trophoblast to express the correct adhesion molecules (Goldman-Whol, Yagel, 2002).

Identifying major regulators of trophoblast proliferation, differentiation and migration may lead to insights into the causes of PE. However, it is not ethical to obtain tissues at earlier time points in gestation, as doing so could interrupt pregnancy leading to miscarriage. Therefore, animal models are needed to study TS cell proliferation and differentiation.

Animal Models for Placental Studies

Early stages of human placentation and cell proliferation are difficult to study due to limited availability of materials and ethical issues surrounding the collection such material from aborted pregnancies (Knofler et al., 2001). This shortage leads to the need for animal models to study placentation and cell proliferation. The ruminant model is often employed to study placental function. The ruminant has a villous placenta, similar to the human. In addition, the ruminant gestates for an extended period making it possible to have repeated sampling throughout gestation, and produces offspring with similar developmental maturity to humans (Barry et al., 2008). The ruminant model is appropriate to study nutrient transfer and blood flow rates because of the ease of repeated blood sampling (Barry et al., 2008). Perhaps one of the greatest advantages to the ruminant model is the ability to induce placental defects in the ewe (Barry, et al., 2008).

The mouse is an appropriate animal model to study human placentation because both have a hemochorial placenta and many analogous cell layers (Georgiades et al., 2001). The three layers of the mouse placenta starting closest to the fetus and moving toward the maternal deciuda are the labyrinth layer, the spongiotrophoblast, and the

junctional zone made up of trophoblast giant cells (Soares, 2006). The labyrinth layer closely resembles the "fetal placenta" in humans (Georgiades et al., 2001). Additionally, the mouse allows for the collection of many replicates (large sample size) in a short amount of time. The mouse also is appropriate due to the ability to study gene function by manipulating the genome and utilizing transgenic mice.

For the purposes of our studies the mouse provides a good model to study the transition from proliferating trophoblast stem (TS) cells to differentiated trophoblast giant cells (TGCs), using an *in vitro* culture system (Tanaka et al., 1998). Additionally, gene function can be studied using gene targeting approaches, which is not possible in human primary culture due to the inability to prevent rapid differentiation of cells. These tools are vital to the understanding of the molecular regulation of TS differentiation.

Mouse Placentation

Trophectoderm Specification

The trophoblast and embryonic stem (ES) cell lineages are the first to be established from blastomeres in the peri-implantation stage. The trophoblast is the layer of cells surrounding the inner cell mass and the blastocoel. At the 16 cell stage the blastocyst has two types of cells, the outer polar cells, and a few inner apolar cells (Kunath et, al. 2004). The polarity is important in the specification of trophectoderm and inner cell mass, but does not itself specify trophectoderm fate as moving polar cells to the inside of the blastocyst will reverse specification (Kunath, et al. 2004). *Cdx2* is one of the earliest genes up-regulated as blastomeres take on the trophoblast fate (Niwa et al., 2000). *Cdx2* is essential for trophectoderm specification, with *Cdx2* expression occurring

in the outer polar blastomeres at the time of specification. At the same time these cells turn off *Oct4* and *Nanog* expression (genes involved with the inner cell mass), while the inner cell mass continues to express the pluripotency factors *Oct4* and *Nanog*. The *Oct4/Nanog/Cdx2* relationship has been studied in great detail. Loss of *Cdx2* leads to a failure to down-regulate *Oct4* and *Nanog* leading to subsequent death of the outer cells of a blastocyst, suggesting *Cdx2* is necessary to suppress *Oct4* and *Nanog* in order to maintain a trophoblast cell fate (Strumpf et al., 2005). Additionally, loss of *Oct4* in ES cells or ICM leads to differentiation into trophoblast cell fate (Niwa et al., 2000), and loss of *Nanog* leads to differentiation into extra-embryonic endoderm (Chambers et al., 2003; Mitsui et al., 2003), again showing repression of *Nanog* and *Oct4* is essential for trophoblast formation and maintenance. *Tead4* has been shown to be an important regulator of TS cell specification by acting upstream of *Cdx2*, and has been shown to be sufficient for inducing TS cell fate (Nishioka et al., 2009). Additionally important for TS cell maintenance is *Elf5*, which is methylated in ES cells, repressing its action; but hypo-methylated in TS cells causing abundant expression (Ng et al., 2008). *Elf5* is required for maintaining TS cell fate, forming a positive feedback loop to *Cdx2* reinforcing the trophoblast cell commitment (Ng et al., 2008). *Elf5* has been demonstrated to be regulated by AP-2γ, a transcription factor essential for trophectoderm specification and, subsequently, due to the regulation of *Elf5,* for trophoblast cell maintenance (Kuckenberg et al., 2010). AP-2γ has been shown to be capable of inducing trophoblast cell fate from embryonic stem cells, even in the absence of *Cdx2* (Kuckenberg et al., 2010). These data demonstrate that AP-2γ, *Cdx2, Elf5* and *Tead4* are important genes in regulating the specification and/or maintenance of trophoblast cell fate.

Placentation in the mouse, as with other species, occurs after implantation of the blastocyst into the maternal uterus. At the time of implantation a blastocyst has an inner cell mass destined to become the embryo proper and an outer trophectoderm. The trophectoderm has two regions, the polar trophectoderm directly overlying the inner cell mass and the mural trophectoderm which encompasses the blastocoel (shown in Figure 1a). As the blastocyst implants the mural trophectoderm invades the maternal uterus and differentiates into primary giant cells (Cross et al., 1994). The polar trophectoderm begins to proliferate into a localized subset of cells, called trophoblast stem (TS) cells, which reside in an area termed the ectoplacental cone (Cross et al., 1994). Cells arising from the polar trophectoderm give rise to precursors of, and eventually all parts of the placenta via the ectoplacental cone (Cross et al., 1994). As the embryo beings to gastrulate, around E6.5 of gestation, the TS cells begin to proliferate into a flat region called the chorion and more distal portion into the secondary TGCs (Cross et al., 1994). At this point in gestation the layers of the mouse placenta are beginning to form. The mouse will develop a discoid labyrinthine hemochorial placenta consisting of three layers at maturity.

Labyrinth Layer

The layers of the mouse placenta from fetal side to maternal side are the labyrinth layer, the spongiotrophoblast layer, and the outer layer of trophoblast giant cells (Figure 1b). The labyrinth layer of the mouse placenta consists of several cell types arising from the chorionic plate (Cross, 2000). The labyrinth layer is highly vascularized and is the main site of nutrient transfer (Cross et al., 1994). Cells inside the labyrinth layer include

two layers of multinucleated syncytiotrophoblast cells, which form the barrier between the maternal blood sinuses and the fetal capillaries. Additionally there are mononuclear trophoblast cells that line the lumen of the maternal blood sinuses and have an unknown function, but do express placental lactogen II (*Pl2*) which is considered a marker for TGCs. Lastly there are densely packed cuboidal trophoblast cells that persist late into placental development expressing the ectoplacental cone marker *Eomes* (Cross, 2000).

Spongiotrophoblast

Intermixed at the maternal edge of the labyrinth layer is the spongiotrophoblast layer of cells. Little is known about the function of this layer, although it is thought to provide structural support for villous like structures in the adjacent labyrinth layer (Cross et al., 2002). The spongiotrophoblast precursor cells arise from the ectoplacental cone and spongiotrophoblast cells can become trophoblast giant cells. It appears, however, that the two types of cells are specified independently of each other as mutations can cause a decrease in one without the other (Simmons and Cross, 2005). For example, loss of *Mash2* leads to a decrease of spongiotrophoblast and an increase in TGC formation (Guillemot et al., 1994), but loss of *Hsf1* leads to a decrease in spongiotrophoblast without effecting TGC formation (Xiao et al., 1999). The spongiotrophoblast layer is not homogeneous, in fact late in gestation some cells from this layer become glycogen trophoblast cells, migrating into the maternal decidua (Adamson et al., 2002). Glycogen trophoblast cells arise after E12.5 and can migrate much deeper into the maternal decidua than trophoblast giant cells (Adamson et al., 2002). These cells are the main source of IGF-2 (a hormone involved in regulating growth of the placenta) in the placenta, although little is known about the molecular regulation of glycogen trophoblast cells.

Trophoblast Giant Cells (TGCs)

The most distal layer to the fetus is the layer containing TGCs. These cells terminally differentiate and then go through a process of endoreduplication, which is a rare process involving many rounds of DNA replication without any cell division resulting in high ploidy level (Gardner & Davies, 1993; MacAuley et al., 1998). TGCs begin to differentiate early in placental formation and persist throughout gestation. These cells are in contact with maternal tissue at all stages of development and appear to mediate invasion into the maternal decidua (Cross, 2000). TGCs assist in adapting the maternal physiology by secreting luteotrophic and lactogenic hormones (Cross et al., 2002) as well as angiogenic factors and vasodilators, likely promoting local blood flow to the implantation site and placenta (Carney et al., 1993; Voss et al., 2000, Yotsumoto et al., 1998). TGCs have many different subtypes which have largely been characterized by *in vitro* studies, which will be discussed later in this review.

Studying the placenta *in vivo* only allows for glimpses of developmental events at specific time points in gestation. However, it is difficult to separate the placental layers to study individual cell types. A murine trophoblast cell culture system has been established to study proliferating TS cells and their differentiation into all of the cell layers of the placenta (Tanaka et al., 1998).

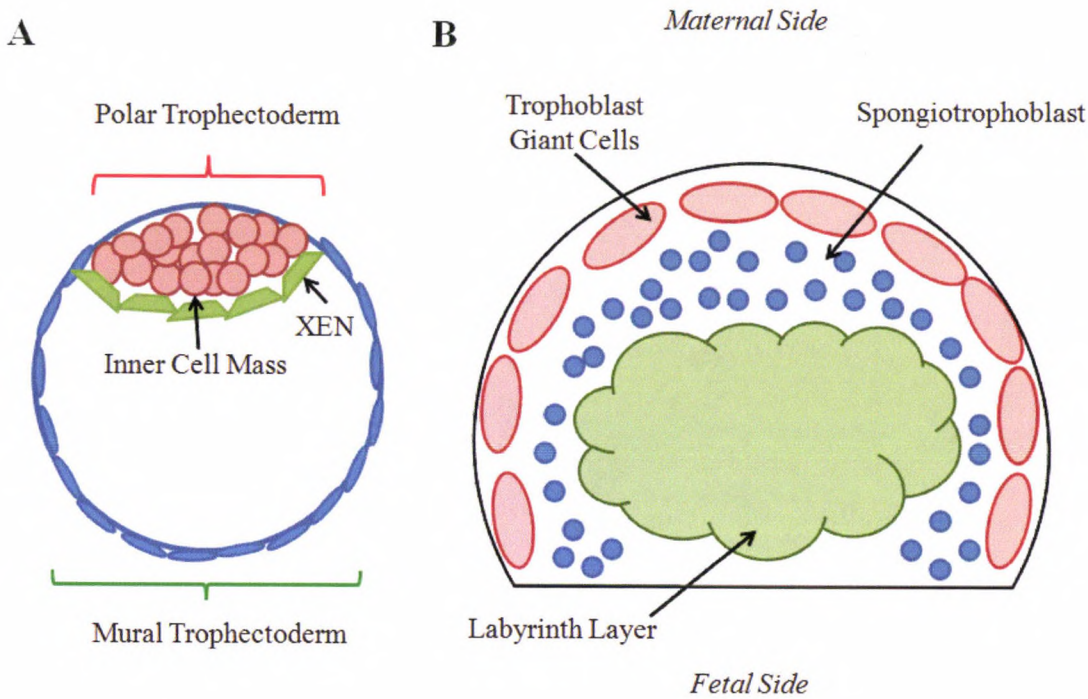

Figure 1: (A) Schematic illustration of a blastocyst indicating the inner cell mass, polar trophectoderm, extra-embryonic endoderm (XEN) and mural trophectoderm. (B) A schematic illustration of a mature mouse placenta with three layers: the labyrinth layer, spongiotrophoblast, and trophoblast giant cell outer layer.

Mouse Trophoblast Cell Culture

TS cells can be derived in culture from pre-implantation embryos and differentiate into TGCs under certain culture conditions. TS cells will proliferate when cultured on a feeder layer of embryonic mouse fibroblasts (EMFI) or 70% EMFI conditioned media, with Fibroblast Growth Factor 4 (FGF4) and heparin sulfate added (Tanaka et al., 1998). This system mimics *in vivo* conditions, where the inner cell mass secretes FGF4 to the overlying trophectoderm layer. TS cells will continue to proliferate under the influence of FGF4, heparin sulfate and conditioned media indefinitely, showing no morphological or molecular changes after 50 passages (Tanaka et al., 1998).

Culturing TS cells with Leukemia Inhibiting Factor (LIF), which is sufficient for ES cell maintenance, is not sufficient for TS cell proliferation, leading to TGC formation (Tanaka et al., 1998).

Removal of FGF4 and conditioned media result in TGC formation after six days of culture (Tanaka et al., 1998). TGC formation is the favored pathway over proliferation of TS cells, which leads to the occurrence of spontaneous TGC formation even in TS cells cultured with FGF4, heparin sulfate and conditioned media (Tanaka et al., 1998). In addition to removal of FGF4 and conditioned media from culture conditions, other methods have been used to induce TGC formation. Retinoic acid is a strong inducer of TGC formation, and can cause abundant TGC formation even in the presence of FGF4 and conditioned media when added exogenously (Yan et al., 2001). Additionally, diethylstilbesterol induces TGC formation and overrides FGF4 and conditioned media (Tremblay et al., 2001). Another compound that forces TGC formation, even in the presence of FGF4 and conditioned media, is RO3306. RO3306 is a compound that inhibits Cyclin-dependent protein kinase1 (CDK1), the gatekeeper of cell division, which causes endoreduplication (Ullah, et al., 2008).

TGCs formed in culture mimic secondary TGCs found *in vivo.* These secondary TGCs, *in vivo,* are thought to originate from the ectoplacental cone due to the early expression of ectoplacental cone markers *Mash2* and *Tpbpa* followed by later expression of TGC markers (Carney, et al., 1993; Scott et al., 2000). TGCs are largely characterized by a giant cell phenotype with a large, highly polyploid nucleus. These giant cells are highly invasive and *in vivo* play a role in implantation and decidualization (Zybina et al., 2000). The placental lactogen family is one of the earliest markers of TGC lineage

(Simmons, et al. 2007). TGC markers include placental lactogen 1, *Prl3d1* (formally *Pl1*), placental lactogen 2, *Prl3b1* (formally *Pl2*), and proliferin, *Prl2c2* (formally *Plf*) (Lee et al., 1988; Faria et al., 1990, 1991; Yamaguchi et al., 1992, 1994; Carney et al., 1993; Hamlin et al., 1994). These genes increase in expression levels as TGCs differentiate from TS cells both *in vivo* and *in vitro*. Differentiation of TS cells into TGCs is considered a default pathway. Factors, such as FGF4 and heparin in culture media prevent TS cell differentiation, and are in place to maintain TS cell proliferation.

Studying TGCs *in vitro* has provided evidence that there is a mixed population of giant cells (Simmons et al., 2007). A total of four different TGCs, each with a distinct localization *in vivo*, can be identified using different markers *in vitro*. All of the subtypes are polyploid and post mitotic (Simmons et al., 2007) and localize to distinct regions within the placenta. Parietal trophoblast giant cells (P-TGC), which arise from the region separating the ectoplacental cone from the maternal decidua, only express *Prl3d1*. Sinusoidal TGCs (S-TGCs), the cells lining the maternal sinusoids, only express *cathepsin Q* (*Ctsq*). Cells lining the canal spaces (C-TGCs) express both *Prl2c2* and *Prl3b1*, and giant cells lining the spiral arteries only express *Prl2c2*.

Endoreduplication

TGCs have large polyploid nuclei that are the result of a process called endoreduplication. Endoreduplication involves many replications of DNA without cell division. While endoreduplication occurs simultaneously with differentiation, endoreduplication and differentiation are exclusive of each other (Geng et al. 2003; Parisi et al. 2003). Endoreduplication is caused by dissociating the S phase from the M phase. Dissociating the S phase from the M phase is accomplished by p57, a G1/S

CDK1/CyclinB inhibitory protein (Hattori et al. 2000). Increased accumulation of p57 upon the completion of S phase along with a decrease in G1 phase is thought to promote the alternation of G/S phases during the endocycle (Hattori et al., 2000). In TS cells, endoreduplication is blocked by FGF4 signaling. FGF4 prevents the accumulation of p57 and p21, leading to a normal cell cycle (Ullah et al., 2008). The initiation of endoreduplication in TS cells is mediated by the release of *Hand1* from the nucleus, which triggers a series of downstream targets that begin endoreduplication (Martindil et al., 2008). Endoreduplication can be triggered manually by adding the compound RO3306 to TS cells (Ullah et al., 2008). RO3306 is a CDK1 inhibitor, which will allow for endoreduplication to occur in cells capable of endoreduplication, but will cause cell death in cells that can not undergo endoreduplication (Ullah et al., 2008). In the case of TS cells, this is accomplished by blocking CDK1, allowing for accumulation of p57 and p21 (Ullah et al., 2008).

Other key regulators of TS cell proliferation are yet to be elucidated. A recent microarray profiling experiment performed on proliferating TS cells and differentiated TGCs has identified several novel genes differentially expressed between TS cells and TGCs (Winger and Cooney, unpublished data). Among these genes are several transcription factors and pluripotency inducers expressed in ES cells and induced pluripotent (iPS) cells, including *Lin28* (Winger and Cooney, unpublished data).

Lin28: a Multifaceted Protein Involved in Many Stem Cell Systems

Lin28 has been the source of much interest in pluripotency since its discovery in inducing a pluripotent, ES-cell like fate from an embryonic fibroblast cell (Takahashi and Yamanaka, 2006). *Lin28* was one of the original factors found to be capable of inducing pluripotency from fibroblasts, leading to the ability to differentiate into the three germ layers and form teratomas (Takahashi and Yamanaka, 2006). *Lin28* has been shown to be a regulator of ES cell proliferation, where loss of *Lin28* leads to a decrease in proliferation rates, and overexpression of *Lin28* leads to increased proliferation (Xu et al., 2009).

Lin28 is highly homologous among animals, including mammals (Yang and Moss, 2003). *Lin28* is expressed during both mouse and human development, in embryonic stem cells, and in many cancers. Early in mouse development (E6.5) LIN28 is present in all three germ layers and the trophoblast. By E9.5, LIN28 expression is restricted to only a few tissue types, including most epithelia tissues, myocardium and neuroepithelium. In adult mice, LIN28 can be found in the kidney in the loop of Henle and the collecting duct, and most prominently in skeletal muscle and cardiac muscle (Yang and Moss, 2003). It also is transiently expressed in the intestinal epithelium, and depending on the period of the estrous cycle is transiently expressed in mammary tissue (Yang and Moss, 2003). Additionally, LIN28 has been identified in the adult testes (West et al., 2009), in germ cells and embryonically, in primordial germ cells (PGCs) during specification. *Lin28* acts in PGC specification by inhibiting Let-7 miRNA

accumulation and allowing for *Blimp1* induction of PGCs (West et al., 2009; Matzuk, 2009).

The *Lin-28* gene encodes a 25 kD protein initially discovered in the nematode *C. elegans*. Lin-28 is a heterochronic gene affecting early development in *C. elegans,* this gene is expressed in the first larval stage in many tissue types including muscle and nerves (Moss and Tang, 2003). Expression is down regulated after the first larval stage (Moss and Tang, 2003). Mutations in *Lin-28* lead to impaired development starting in the second larval stage. Loss of function mutations lead to an accelerated development, causing some developmental events to be skipped. Gain of function mutations cause a delayed development (Yang and Moss, 2003).

The LIN28 protein has two RNA binding domains: a cold shock domain and a retroviral CCHC zinc finger RNA binding domain. LIN28 plays a role in binding mRNA and increasing translational efficiency of IGF-2 in skeletal muscles (Polesskaya, et. al, 2007). Protein is localized primarily to the cytoplasm, but has also been detected in the nucleus and nucleolus. Under stress, LIN28 is sequestered into P-bodies and stress granules (Balzer and Moss, 2006). When the CCHC domain is mutated, LIN28 becomes sequestered to the P-bodies, a site of miRNA processing. If both RNA binding domains, the CCHC, and the cold shock domain are mutated, LIN28 becomes trapped in the nucleus, suggesting LIN28 must be bound to an RNA transcript in order to have proper nuclear shuttling (Balzer and Moss, 2006).

The LIN28 protein is known for its interaction with the Let-7 family of microRNAs (miRNAs). Opposite expression patterns between LIN28 and Let-7 are observed in many cell types as they differentiate, with high *Lin28* expression in

undifferentiated cells and high Let-7 expression in differentiated cells (Viswanathan et al., 2007). *Lin28* is expressed almost exclusively in undifferentiated cells, whereas the Let-7 family is expressed highly in differentiated cell types. LIN28 regulates the Let-7 family at a post transcriptional level via terminal uridylation acting downstream of Drosha processing, making pre-Let-7 transcripts unreadable by Dicer and subject to degredation (Heo, et. al, 2008). Further, it is known that LIN28 can recruit a poly(A) polymerase, TUT4, to aid in the process of pre-miRNA uridylation (Heo, et. al, 2009).

While *Lin28* has been identified in the trophectoderm in E6.5 mouse embryos, a role for *Lin28* in the trophectoderm has yet to be elucidated (Yang and Moss, 2003). It is possible that *Lin28* has a role in increasing translation of IGF-2 in the developing placenta, similar to what is observed in skeletal muscle (Polesskaya, et. al, 2007). This study investigates a role for a *Lin28* interaction with miRNAs in the placenta. MiRNAs regulate many elements of differentiation, and recently have been shown to play a role in maintaining proliferation in ES cells (Wang et al., 2007).

MicroRNAs (miRNAs) in Proliferating and Differentiating Stem Cells

MicroRNAs (miRNAs) are small, approximately 22 base pair (bp), non-coding RNAs involved in mRNA degradation or translational repression. MiRNAs act to repress genes by acting at the 3' UTR of untranslated genes. The first miRNAs *lin-4* and *let-7*, were discovered in the nematode *C. elegans* (Roush, 2008). It is known now that over 6,000 miRNAs exist in a largely conserved fashion.

MiRNAs biogenesis was initially discovered when studying *lin-4* and *let-7* function in *C. elegans* during developmental timing events (Lee et. al, 1993; Rienhart et al., 2000). Northern blot analysis of most miRNAs shows bands at ~70bp and ~22bp, indicating miRNAs are formed from a longer precursor RNA (Lee et. al, 1993; Reinhart et al., 2000). Precursor RNAs are formed in the nucleus in a stem-loop structure called primary miRNAs (pri-mRNAs) that exist in either polycistronic (one stem-loop) or monocistronic (multiple stem-loops) forms which can be very large (Y.Lee et. al, 2002). Pri-miRNAs are cleaved by the RNAse polymerase II enzyme Drosha and its partner DCGR8 into precursor miRNAs (pre-miRNAs). Pre-miRNAs have a characteristic stem-loop structure and are approximately 70 bps in lenght. Pre-miRNAs can be shuttled out of the nucleus as they are found in both the nucleus and the cytoplasm. Pre-miRNAs are further processed by the RNAse Polymerase III Dicer into the mature 22 bp miRNAs (Hammond et al., 2000; Ketting et al., 2001; Knight and Bass, 2001). Mature miRNAs associate with Argonaut proteins to form an RNA induced silencing complex (RISC) which, when associated with target mRNAs, cause translational silencing or transcript degradation.

MiRNA targeting is determined by six base pairs at the 3' end of the miRNA referred to as the seed sequence (Jackson et al., 2003). It has been shown that bps 2-8 in the 3' region are involved with post transcriptional repression, and that these bps have perfect or imperfect complimentarity to the 5' end of the target mRNA sequence of the 3' UTR (Lai, 2002; Bartel 2004). Once binding occurs between the miRNA seed sequence and the target mRNA, the double stranded RNA will enter the RISC complex (Bartel, 2004). Perfect matches between the seed sequence and the target mRNA will lead to

transcript degradation. Translational repression is not well understood, and is thought to occur when there is imperfect matching of the seed sequence and target mRNA, although miRNAs also have been shown to act as post translational modifiers in actions such as de-adenylation, de-capping, mRNA sequestering and mRNA storage in processing bodies (p-bodies) (Fazi and Nevi, 2008). It is clear that miRNAs provide an eloquent level of gene regulation, although they have been studied little in a tissue or organ specific manner until recently.

ShRNAs are similar to miRNAs, as they are both small non-coding RNAs used to cause mRNA degredation. ShRNAs are recombinant, man-made sequences that act retro-virally to incorporate into the genome. This process allows the shRNA to take over the cellular machinery to process the targeting RNA and make mature shRNA. The mature shRNA will then bind to target sequences to cause mRNA degredation.

MiRNAs in Embryonic Stem Cells

MiRNAs have been shown to play a role in embryonic stem (ES) cell maintenance and differentiation. Loss of Dicer or DCRG8 results in a proliferation defect in ES cells, suggesting a major role for miRNAs or other small RNAs in ES cell proliferation and renewal (Wang et al., 2008). The miR-290 family (miR291a-3p, miR291b-3p, miR294, and miR295) has been implicated in ES cell renewal using Dicer and DCRG8 knockout ES cells, having a severe effect on the G1/S transition resulting in a failure to proliferate (Wang et al., 2008). In DCRG8 knockout ES cells it has been shown that adding back members of the miR-290 family will rescue this loss of proliferation, and over expression of these miRNAs will lead to increased proliferation

(Wang et al., 2008). Blelloch has coined the miR-290 family ES cell specific cell cycle regulators (Wang et al., 2008). In addition to the miR-290 family, another cluster of miRNAs has been implicated in ES cell maintenance. The miR-302 gene encodes for a cluster of miRNAs: miR302a, miR302b, miR302c, miR302d, and miR367 that are specifically expressed in ES cells and embryonal carcinomas (Suh et al., 2004). It has been shown that the miR-302 cluster is activated by transcription factors *Sox2*, *Oct4,* and *Nanog* (Card et al., 2008). When over expressed in non-pluripotent cells the miR-302 cluster causes an increase in the number of cells in S-phase, and a decrease in the number of cells in G1-phase, indicating these miRNAs target Cyclin D1, which has a complementary seed sequence, and regulate cell cycle progression (Card et al., 2008). The miR-290 family and the miR-302 cluster have been shown to be imperative for ES cell proliferation and cell cycle regulation.

Conversely, the Let-7 family of miRNAs has an opposite role to the miR-290 and miR-302 clusters. The Let-7 family is a highly conserved group of miRNAs (Pasquinelli et al., 2000). There are 8 Let-7 members in the mouse coming from 10 precursor miRNAs (two for Let-7a) (Roush and Slack, 2008). The Let-7 family of miRNAs are transcribed separately, with only one conserved cluster (Let-7a, d, and f) which are transcribed together (Roush and Slack, 2008). The Let-7s are highly expressed in many differentiated tissues, but absent in ES cells and undifferentiated cell precursors. A major function of the Let-7 family is to promote differentiation by repressing target oncogenes and pluripotency factors (Hatfield and Ruohola-Barker, 2008). Major targets of the Let-7 family include *Hmga2* and *Lin28*, although there are over 900 predicted targets for the

Let-7 family (www.targetscan.org). The Let-7 family is known to be repressed and regulated by *Lin28* in many tissues, including ES cells (Viswanathan et al., 2007).

MiRNAs in Pregnancy and TS Cells

There are placental miRNAs that are differentially expressed in normal pregnancies and pre-eclamptic pregnancies (Pineles et al., 2007). Placental samples were taken from pre-eclamptic placentas at the time of delivery and compared to control samples taken from Caesarian section delivered normal placentas (Pineles et al., 2007). From these samples it was found that miR-210 and miR-182 were significantly elevated in PE placentas when compared to controls (Pineles et al., 2007). Further studies on PE vs. control placentas showed that more miRNAs were differentially expressed in PE placentas, with 11 miRNAs higher in PE placentas, and 23 lower in PE placentas (Zhu et al., 2008).

In addition to miRNAs expressed in placentas, there have been recent insights into miRNAs expressed in TS cell specification from ES cells (Viswanathan et al., 2009). ES cells were profiled for miRNA expression and subsequently forced to a trophectoderm fate. TS cells were induced from ES cells using i-Ras induction, and were profiled for miRNA expression and compared to the ES cells (Viswanathan et al., 2009). There was an increase in expression of miR-21 and miR-155 in TS cells, and a decrease in miR-363 in TS cells (Viswanathan et al., 2009). This study also examined blastocyst derived TS cells as they proliferate and differentiated into TGCs, and found that there were differentially expressed miRNAs, including an increase of expression of the Let-7 family in differentiated TGCs (Viswanathan et al., 2009). MiRNAs and novel trophoblastic

pluripotency factors will undoubtedly play a role in TS cell proliferation and differentiation.

AP-2γ Plays a Role in the Mammalian Trophoblast

There are five members of the activating protein 2 (AP-2) family of transcription factors in both the mouse and human genome. These are AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ε (official gene symbols: *Tcfap2a-e*). These proteins are characterized by their DNA binding domain, a helix-span-helix at the C-terminus, and a proline/glutamine rich domain at the N-terminus (Eckert, et. al, 2005). The most common AP-2 binding site is a GC rich palindromic sequence of 5'- GCC (N3) CCG-3'.

Of these five family members all except AP-2δ are expressed in pre-implantation embryos (Winger et al., 2006), and only AP-2γ is necessary for extra-embryonic cell development (Auman et al., 2002). AP-2α deficient mice have no placental phenotype (Brewer et al., 2002), and although expression of AP-2α is normally found in the trophectoderm, its absence is inconsequential for placental development. AP-2γ is the AP-2 family member expressed earliest in the trophectoderm with expression beginning at E6.5 and continuing throughout development, where it is localized to all layers of the placenta (Auman et al., 2002). Loss of AP-2γ causes embryonic lethality by E8.5 due to failure of the embryo to implant. This defect was observed by the presence of a disrupted maternal-fetal interface and a small, non-invasive ectoplacental cone in the decidual region (Auman et al., 2002). Blastocyst outgrowths deficient in AP-2γ had very little

growth and a significantly lower number of TGCs formed compared to littermate controls (Auman et al., 2002). Recently, AP-2γ has been shown to be capable of inducing a TS cell fate, independent of *Cdx2* expression, when over-expressed in embryonic stem cells (Kuckenberg, et al., 2010). AP-2γ up-regulates *Elf5,* a factor necessary for maintenance of trophoblast cell fate (Ng et al., 2008). Additionally, AP-2γ has been shown to bind to the promoter regions of many transcription factors that are known to be important for ES cell and TS cell development including the TS transcriptional regulators *Eomes*, *Cdx2*, and others (Kidder and Palmer, 2010).

In addition to being imperative for murine trophoblast development, AP-2γ also appears to have an important role in human trophoblast function. Extravillous trophoblast cells express both AP-2γ and AP-2α (Kotani, et al 2009). Interestingly, in pre-eclamptic pregnancies AP-2α and AP-2γ are significantly higher expressed in the extravillous trophoblast cells (Kotani et al., 2009). *In vitro* modeling of extravillous trophoblast is performed by HTR8/SVneo cells, which take on the invasive nature of these cells when grown on a Matrigel matrix (Graham, et al., 1991). This invasive effect is significantly suppressed when AP-2γ or AP-2α are over expressed in HTR8/HVneo cells (Kotani et al., 2009). In order to simulate pre-eclamptic conditions *in vitro,* TNF-α, a cytokine stimulated in pre-eclamptic pregnancies, was added to culture conditions. TNF-α greatly increased amounts of AP-2α and AP-2γ in both HTR8/SVneo cells and extravillous trophoblast explanted cultures, which in turn reduced the efficiency of migration (Kotani et al., 2009). Further evidence that AP-2α and AP-2γ play a role in influencing the migration of extravillous trophoblast cells was seen when AP-2γ and AP-2α were silenced by siRNA and TNF-α was added to culture yielding a 70% invasion rate

(Kotani et al., 2009). This rate was lower than a non-treated control but significantly higher than TNF-α treatment alone (Kotani et al., 2009). Therefore, these *in vitro* studies indicate that AP-2α and AP-2γ are necessary for proper trophoblast function in the human as well as in the mouse.

Summary

In summary the placenta is an essential organ for pregnancy, supplying the fetus with nutrients, acting as barrier for disease, and is a site for gas exchange. Defects in placentation can lead to diseases such as pre-eclampsia and intrauterine growth restriction in as many as 8% of pregnancies (Benirshke and Kaufmann, 2000). A better understanding of the mechanisms of trophoblast proliferation and differentiation may lead to insights into the underlying causes of PE and IUGR, potentially leading to earlier diagnosis and better treatment of these diseases. The mouse is an appropriate model to investigate placental growth as both the mouse and the human share many placental morphologies. Use of an established trophoblast stem cell culture system allows for molecular studies to investigate proliferation and differentiation of placental cell types.

This study investigates the hypothesis that LIN28 and AP-2γ have a necessary interaction which allows for TGC formation. This hypothesis will be investigated using the following aims: 1) Expression of *Lin28, Sox2, Nr0b1,* and *Igfbp7* will be investigated in proliferating TS cells and differentiated TGCs. In addition, a potential role for miRNAs will be examined by investigating miRNA expression in proliferating TS cells and differentiated TGCs. 2) LIN28 and AP-2γ function in trophoblast cells will be

determined using knockdown or knockout technologies in TS cells *in vitro*. 3) LIN28 and AP-2γ expression will be examined in human placental cells.

Aim1: Expression of Lin28, Sox2, Nr0b1, and Igfbp7 will be investigated in proliferating TS cells and differentiated TGCs. In addition, a potential role for miRNAs will be examined by investigating miRNA expression in proliferating TS cells and differentiated TGCs

a) Differential expression *Lin28, Sox2, Nr0b1,* and *Igfbp7* will be examined in TS cells vs. TGCs by real time PCR. TGCs will be formed by removal of FGF4 and by inducing differentiation using RO3306. Expected results are that *Lin28, Sox2,* and *Nr0b1* will be down-regulated with TGC formation, but *Igfbp7* will be up-regulated. b) miRNAs differentially expressed between TS cells and TGCs will be identified using real time PCR. Expected results are high *Lin28* expression in TS cells and high Let-7 miRNA expression in TGCs.

Aim 2: LIN28 and AP-2γ function in trophoblast cells will be determined using knockdown or knockout technologies in TS cells in vitro.

Aim 2 will investigate a) effects of *Lin28* knockdown in wild type TS cells will be investigated. Loss of *Lin28* is expected to have a decreased effect on proliferation as it does in ES cells. b) characterize the phenotype of AP-2γ null TS cells. c) examine expression of *Lin28, Sox2, Nr0b1* and *Igfbp7* will be in AP-2γ null TS cells grown under proliferation and differentiation conditions using real time PCR. A defect in TS cell differentiation into TGCs is expected, similar to the effect seen in AP-2γ null blastocyst outgrowths (Auman et al., 2002). Expected results are that *Lin28, Sox2, Nr0b1* and *Igfbp7* are not differentially regulated. d) miRNAs will be assessed between AP-2γ null

TS cells in proliferative and differentiation conditions using real time PCR. It is expected that AP-2γ null TS cells will have different miRNAs expressed than wild type TS cells. e) the role of *Lin28* in AP-2γ null TS cells will be investigated using gene knock-out or knock-down technologies, and it is expected that *Lin28* plays an important role in AP-2γ null TS cell proliferation or differentiation.

Aim 3: LIN28 and AP-2γ expression will be examined in human placental cells

Aim 3 will assess LIN28 and AP-2γ expression in human placental cells. It is expected that LIN28 and AP-2γ expression is conserved between mouse and human placentas.

This study aims to provide insight to the molecular regulation of TS cell proliferation and differentiation into TGCs. Results will provide information needed to focus studies on the regulation of proliferation of human placental cells. AP-2γ already has been identified as a factor involved with migration in pre-eclamptic pregnancies (Kotani et al., 2009). Further investigation into the exact molecular functions of AP-2γ will provide greater insight to the onset of PE.

CHAPTER II:

Materials & Methods

Trophoblast Stem Cell Derivation:

Trophoblast stem cells were derived from E3.5 mouse blastocysts, collected from pregnant mice and cultured in 4 well plastic dishes. Blastocysts were cultured on a feeder layer of Mitomycin-C (20mg/mL, Sigma) treated mouse embryonic fibroblasts and fed with TS media (500mL RPMI, CellGro; 130mL fetal bovine serum, HyClone; 6.3mL sodium pyruvate, CellGro; 5mL penicillin/ streptomycin/ amphorecin, CellGro; 4.5μL 100mM β-Mercapto-Ethanol, Sigma) and FGF4 (25mg/ml, Sigma). Blastocysts hatched within 24-48 hours of plating and formed outgrowths. 24-48 hours after blastocyst outgrowth formation, the cells were disaggregated with trypsin, and replated on the same well. 6-10 days after disaggregation trophoblast stem cell colonies formed. At this time TS cells were moved to new plastic dishes and cultured as described below. Wild type and AP-2γ null TS cells were derived and provided by Dr. Winger from previous experiments.

TS Cell Culture:

Cells were cultured under standard TS cell conditions (Tanaka, 1998). TS cells were grown in 70% embryonic mouse fibroblast (EMFI) conditioned media, and 30% TS

media, with 10μL FGF4 (25mg/mL, Sigma), and 10μL Heparin Sulfate (5mg/mL, Sigma) at 37°C, 5%CO_2, 5% humidity. EMFI conditioned media was collected by culturing 10mL TS media on mitomycin-C (20mg/mL, Sigma) treated mouse embryonic fibroblasts for three days. Media was collected every three days for 9 days total (3 collections). Collected EMFI conditioned media was filtered through a .1mm filter, aliquoted to 7 mL aliquots in sterile 15 mL tubes and stored at -20°C. Media was changed every 48 hours. Cells were grown to confluency before passaging. Cells were passaged by trypsinizing (0.5% Trypsin EDTA, CellGro) for 5 minutes at 37°C, trypsin was inactivated with 5mL TS media. 0.5mL of trypsinized cells were moved to a 10cm dish with 10mL new media. Cells were stimulated to differentiate by removing conditioned media, FGF4, and heparin sulfate from culture media. Cells were allowed to differentiate for six days, with media changed every 48 hours.

RNA Isolation:

Upon confluency, cells were removed from plates with trypsin and lysed in 700μL RLT lysis buffer (Qiagen). Differentiated cells were lysed in 700μL RLT lysis buffer added directly to the cell culture plates after being washed once in PBS. Cells in lysis buffer were homogenized by loading the cell suspension onto a QiaShredder column (Qiagen) for 2 minutes at 13,000 RPM. Total RNA was isolated using the RNeasy Mini Isolation Kit (Qiagen) per manufactures instructions using the DNAse treatment to remove genomic DNA. RNA was eluted in 40μL RNase Free H_20, and concentration and purity were assessed using a NanoDrop 1000 (ND1000 Spectrophotometer, NanoDrop).

Reverse Transcription:

Reverse transcription was carried out using qScript Supermix (Quanta Biosciences). Reaction volume was 20μL consisting of 16μL RNA/H_2O (1000 ng RNA total/reaction) and 4μL qScript Supermix. Reverse transcription was carried out in a thermocycler using the following cycle conditions: 5 minutes 37°C, 30 minutes 65°C, 5 minutes 95°C. cDNA was stored at -20°C.

Real Time RT-PCR:

Real time RT-PCR was used to analyze expression of *Lin28, Sox2, Nr0b1, Igfbp7, Oct4, Nanog, Stella* and the AP-2 family of transcription factors. cDNA was made from RNA isolated with Qiagen RNeasy cDNA kit. Real time PCR was performed using 100ng/ reaction (RNA equivalent) cDNA, Taqman probes (Applied Biosystems Inc.) for *Lin28, Sox2, Nr0b1, Igfbp7, Gapdh, Nanog, Stella, Oct4,* and all of the AP-2 family members and run in 384 well plates on the LightCycler 480 PCR system (Roche). A list of probes can be seen in Apendix I. Each reaction contained 10μL of 2x Taqman Mastermix (Applied Biosystems Inc.), 1μL of Taqman probe, and 9μL cDNA/H_2O. Reactions were run in duplicate using three biological replicates. Cycle settings are 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, 60 seconds at 60°C followed by a fluorescent detection at the end of the 60°C cycle. At the end of the run, the plate was held at 37°C. Data was analyzed with Excel using the comparative Ct method (Schmittgen & Livah, 2008). Data was plotted as $2^{-\Delta Ct}$ for average values

normalized with *Gapdh* (Schmittgen & Livah, 2008). Statistics were run in SAS using a one way ANOVA followed by a Tukey comparison.

miRNA Profiling

TS cells and TGCs (n=4 for each cell type) were grown to confluency on 10cm plates, collected, and total RNA was isolated using the miRVana miRNA isolation kit (Ambion). Cells were lysed in 600μL Lysis/Binding solution (miRVana, Ambion) and scraped off the plate using a sterile cell scraper. RNA was eluted in 50μL 95°C RNAse free water. RNA quality and purity was assessed using the NanoDrop (ND-1000 Spectrophotometer, NanoDrop). A total of 1μg RNA was added to each cDNA synthesis reaction. Reverse transcription was done using the QuantiMir RT kit (System Biosciences, RA420A-1) per manufacturer's directions. MiRNA PCR profiling plates (System Biosciences, miRNome microRNA Profiling kit (mouse) RA670A-1) were used to assess expression of 380 mouse miRNAs. Real time-PCR was performed by adding 5μL cDNA product in 1250μL 2x SYBR Green (Roche), 50μL Universal Reverse Primer (System Biosciences, RA670A-1) and 778 nuclease free water. Five μL master mix was added to 1μL forward primer in a 384 well plate. Plates were run on a LightCycler 480 PCR system (Roche) using the following cycle conditions: 2 minutes at 50°C, 10 minutes at 95°C, 45 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. Significant differences and fold changes were calculated using Global Pattern Recognition software (GRP Multinorm v.2.0) as previously described (Bouma, et. al 2004; Akilesh, et. al, 2003). Briefly, data are compared by repeated Student's t-test using multiple normalizers. A score of 0.4 or greater was considered to be significant.

Immunohistochemistry

Placenta and decidua were taken from E12.5 wild type mice. All samples were fixed overnight in 4% PFA. Samples were dehydrated and embedded in paraffin. Sections were cut at 5μm using a microtome (Leica RM2125RT, Leica Microsystems). Sections were deparaffinized using xylene, and rehydrated through a series of ethanol dilutions (100%, 95%, 70%, and 50%, 3 minutes each). Slides were exposed to L.A.B. reagent (Polysciences, Inc.; #24310-500) for 15 minutes for antigen retrieval. Sections were washed 3 times in PBS (3 minutes each). Sections were circled with a PAP pen and exposed to SuperBlock (ThermoScientific, #37515) blocking reagent for 45 minutes at room temperature. Rabbit anti-LIN28 antibody (Abcam, Ab46020) was added to sections at a concentration of 1:500 in SuperBlock and incubated overnight in a humidified chamber at 4°C. A no primary antibody control as well as a blocking peptide against the LIN28 antibody (Abcam #) were used as controls. Sections were washed 3 times in PBS for 5 minutes each. Biotinylated goat secondary antibody (Vector Labs, ABC kit) was prepared at the suggested concentration of 1 drop in 10mL PBS and added to slides for 45 minutes at room temperature. Slides were washed 3 times in PBS for 5 minutes each. ABC reagent (5 mL PBS, 2 drops A (mix), 2 drops B (mix), Vector Labs) was added to each slide for 30 minutes. Slides were washed in PBS 3 times for 5 minutes each. DAB (2.5 mL H_20, 1 drop buffer (mix), 2 drops DAB (mix), 1 drop H_20_2 (mix), Vector Labs) reagent was added to slides for 8 minutes. Slides were washed in PBS and counterstained with hematoxylin. Hematoxylin was rinsed off in running tap water for 5 minutes. Slides went through an acid alcohol (70% EtOH, 1% HCl) wash for

hematoxylin clarification and rinsed in running tap water. Slides were dehydrated through a series of ethanol dilutions (50%, 70%, 95%, 100%, 30 seconds each) and cleared in xylenes (30 seconds). Slides were blotted dry with a kimwipe and coverslips were mounted using Cytoseal-60 (RichardAlan Scientific, #8310-4).

Immunofluorescense:

TS cells in culture were grown in 200μL proliferative conditions on coverslip chamber slides (Lab-Tek 8-well, Nalgene/NUNC, #154534) to ~80% confluency (n=4). Cells were fixed on the coverslip in 200μL 4% PFA per well for 5 minutes. Cells were washed 3 times in PBS for 3 minutes each. 10% blocking sera was added to each well against the secondary antibody species for 1 hour at room temperature. Primary antibody goat-α-Lin28 (D-20, SantaCruz, SC-54030) was added in 10% blocking sera against secondary antibody at a concentration of 1:200 overnight at 4°C. A no primary antibody control was used. Cells were washed 3 times in PBS for 3 minutes each. Donkey anti-goat Cy-5 labeled secondary antibody was added in the dark at a concentration of 1:100 for 45 minutes at room temperature. A DAPI counterstain was added for 5 minutes at a concentration of 1:5000. Cells were washed in PBS 3 times for 3 minutes. Cells were maintained in 200μL PBS for imaging. Images were taken on a confocal microscope (Axio Observer Z1 Confocal Microscope connected to an LSM 510 META laser system). Nuclear expression was analyzed by Chi-square test on SAS, and considered to be significantly different with a p-value < 0.05.

Cyclin/CDK Inhibition

Cells were split into 6 well plates, with 10,000 cells per well. Cells were treated with 8μM RO-3306 (Alexis Biochemicals, # 270-463-M001) in 70% conditioned media, FGF4 and heparin sulfate for 4 days, changing the media every other day. A DMSO vehicle control was used in the same conditions as well as a no chemical treatment control. RO3306 was added in the same concentration to 100% TS media (minus 70% conditioned media, FGF4, and heparin sulfate). Pictures were taken every 24 hours to monitor phenotypic changes. RNA was collected from cells using the RNeasy Mini kit (Qiagen) after 4 days of treatment. Effects of RO-3306 on gene expression of *Lin28, Sox2, Nr0b1* and *Igfbp7* were identified using real time PCR.

Lentiviral Knockdown of Lin28

TS cells were plated at a density of 1000 cells/well on 24 well plates in TS media + FGF4 + heparin sulfate. Six wells were set up to enable testing of 4 shRNA Lentiviral clones targeting *Lin28*, one scramble sequence control shRNA, and one non-treated control. Cells were allowed to plate down for 24 hours. At 24 hours, 1μL/ml of Polybrene (8 mg/ml, Sigma) was added to each well, followed directly by adding *Lin28* targeting shRNA Lentiviral particles (Sigma, MISSION Lentiviral Transduction Particals, # SHCLNV) to a multiplicity of infection (MOI--see calculation below) of 500. Lentiviral particles were left in media for 24 hours to ensure infection of the shRNA. After infection, fresh TS media, FGF4, and heparin sulfate was added with 1μL/ml puromycin (1mg/ml, Sigma Aldrich, # P9620-10ml). Puromycin treatment was added to TS media, FGF4, and heparin sulfate every two days until all non-infected cells were dead (2-10

days). After puromycin selection, the surviving cells were allowed to expand to confluency at which point they were moved to 6-well plates. When cells became confluent on the 6-well plates they were removed with 500μL trypsin for 5 minutes, stopped with 1ml 30% TS media plus 70% conditioned media, FGF4, heparin sulfate, and 500μL of trypsinized cells were re-plated on fresh 6-well plates. The remaining cells were saved for RNA isolation. From each well, RNA was isolated, cDNA was made, and real time PCR was performed to evaluate *Lin28* knockdown.

Multiplicity Of Infection Calculation:

- (total cells per well) x (desired MOI) = total transducing unites needed (TU)
- (total TU needed) / (TU/ml reported by Sigma) = total ml particles to add to each well

CHAPTER III:

Pluripotency Factors Regulate TS Cell Proliferation and Differentiation

Introduction:

A healthy placenta is essential for survival of the fetus in eutherian mammals. In the mouse, the placenta is formed by cell differentiation from a subset of stem cells called trophoblast stem (TS) cells. These cells differentiate into three layers of the mouse placenta: the labyrinth layer that makes up the area of maternal/fetal nutrient exchange, the spongiotrophoblast thought to be a structural support zone as well as providing the progenitor cells of glycogen trophoblast cells, and the trophoblast giant cells (TGCs) which are thought to mediate blood flow to the placenta and invasiveness into the decidua (Cross, 2000).

TS cells grown in culture provide a good model to study mouse TS cell differentiation because proliferation and differentiation can be tightly regulated, and TS cells grown in culture can differentiate into cells representing all layers of the placenta (Tanaka et al., 1998; Simmons and Cross, 2005). TS cells grown in 70% mouse embryonic fibroblast conditioned media, with FGF4 and heparin sulfate (proliferation media) proliferate as TS cells. When those conditions are replaced by growing the cells in 100% TS media only (differentiation media), the TS cells begin to differentiate into a mixed population of cell types, but predominately become TGCs (Tanaka et al., 1998).

Much is known about the molecular regulation of proliferation and differentiation in embryonic stem cells, but less is known about these processes in TS cells. Diseases of

the human placenta, such as pre-eclampsia and IUGR, likely are due to defects in the regulation of trophoblast proliferation and differentiation. Using a mouse model to investigate potential genes involved in TS cell proliferation and differentiation will lead to a better understanding of how these processes occur in humans.

Endoreduplication is a rare process seen in trophoblast giant cells when they undergo many rounds of DNA replication without any cell division. RO3306 is a compound used to block Cyclin-dependent protein kinase1 (CDK1), the gatekeeper of cell division (Ullah et al., 2008). In TS cells, exposure to RO3306, even in proliferation media, forces TS cells to endoreduplicate and differentiate into TGCs. Treatment of cells that are incapable of undergoing endoreduplication, such as ES cells, undergo apoptosis when exposed to RO3306 (Ullah et al., 2008). Endoreduplication is a normal process of TS cells differentiating into TGCs.

Pluripotency genes expressed in TS cells were identified from preliminary microarray data on proliferating TS cells and differentiated TGCs (Drs. Quinton Winger and Austin Cooney). *Lin28*, *Sox2*, and *Nr0b1* were of interest due to their roles in pluripotent cells. *Lin28* was one of the original factors found to be required for human induced pluripotent stem cell (iPS) formation (Takahashi and Yamanaka, 2006), and since then has been implicated in pluripotency by regulating the Let-7 family of miRNAs (Viswanathan et al., 2007). *Sox2* also was an original factor found to be necessary for iPS cell induction (Takahashi & Yamanaka, 2006), and is known in ES cells to interact with *Oct4* to sustain pluripotency (Rodda et al., 2005). *Nr0b1,* also known as *Dax1,* has been shown to be essential for maintaining pluripotency in ES cells, when absent, ES cells are forced to differentiate (Nikkan et al., 2006). *Igfbp7,* also called *Mac25*, has not

been implicated in ES cell differentiation or TS cell differentiation, but is of interest because it contains an FGF-receptor binding domain and Activin binding domain, as well as a role for *Igfbp7's* in regulating angiogenic factors such as VEGF (Kato, 1999; Tamura et al., 2009).

Lin28, Sox2, and *Nr0b1* were significantly higher ($P < 0.001$) expressed in proliferative TS cells with a fold change of 79, 100, and 100, respectively, when compared to their expression in differentiated TGCs. *Igfbp7* is significantly higher expressed ($P < 0.001$) in TGCs with a fold change of 34 when compared to the proliferative TS cells.

This study investigates the hypothesis that *Lin28* is important for regulating the proliferation or differentiation of TS cells to TGCs, by interacting with the Let-7 family of miRNAs in TS cells. Gene expression patterns of *Lin28, Sox2, Nr0b1,* and *Igfbp7* were assessed using real time PCR. LIN28 protein was localized in TS cells in culture and in placental sections. Additionally, a potential interaction between Let-7 miRNA expression and *Lin28* expression in proliferating TS cells and differentiated TGCs was evaluated. This study also investigated miRNAs expression in TS cells and TGCs, and will identified miRNAs potentially involved in TS cell proliferation or differentiation. Lastly, the function of *Lin28* was assessed in TS cells by knocking down *Lin28* using lentiviral shRNA particles. These data are necessary to provide a better understanding of the regulatory mechanisms underlying proliferation in wild type TS cells.

Results:

Confirmation of Differentially Expressed Genes During TS Cell Differentiation

Real time PCR was performed using Taqman probes to confirm the differential expression of *Lin28, Sox2, Nr0b1,* and *Igfbp7* observed in a microarray expression profiling experiment. Removal of 70% conditioned media, FGF4 and heparin sulfate resulted in a formation of TGCs and a rapid and significant ($p < 0.05$) down regulation of *Lin28*, *Sox2*, and *Nr0b1* expression, as well as a significant increase in *Igfbp7* expression (Figure 2). The differences between time points analyzed and were considered to be significantly different using the ProcGLM (ANOVA) on SAS (p-value of $\alpha < 0.05$ were considered to be significantly different), and were followed up with a Tukey test.

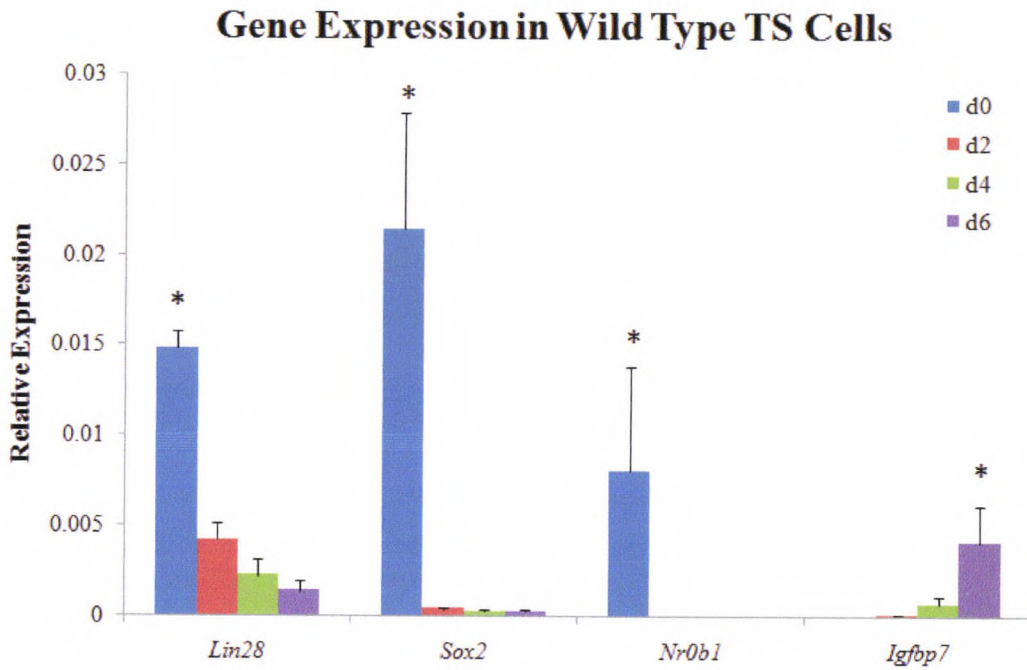

Figure 2: Expression of *Lin28*, *Sox2*, *Nr0b1* and *Igfbp7* in differentiating TS cells according to real time PCR analysis. Upon removal of proliferation media, there is a rapid and significant decrease in *Lin28*, *Sox2*, and *Nr0b1* expression. *Igfbp7* expression increases significantly as cells differentiate into TGCs. Data are plotted as relative expression to *Gapdh*. Astricks indicate a signficant difference ($p < 0.05$) between time points within a gene.

Regulation of Lin28, Sox2, Nr0b1 and Igfbp7 by FGF4 During TGC Formation

Figure 2 shows the mRNA levels of *Lin28, Sox2, Nr0b1,* and *Igfbp7* in TGCs formed by removal of proliferation media (70% conditioned media, FGF4 and heparin sulfate). To assess if gene expression levels for *Lin28, Sox2, Nr0b1,* and *Igfbp7* are influenced by the presence of FGF4 in TGCs, we used an alternate method of inducing TGC differentiation in the presence of FGF4. There are several methods of inducing TGC formation in the presence of 70% conditioned media, FGF4 and heparin sulfate. These include adding retinoic acid, diethylstilbestrol, or RO3306, a compound that promotes endoreduplication (Yan et al., 2001; Tremblay et al., 2001; Ullah et al., 2008).

RO3306 was chosen due to use in concurrent studies being performed on the TS cells. RO3306 compound was applied to TS cells in the presence of 70% conditioned media, FGF4 and heparin sulfate to induce endoreduplication. Cells growing under proliferative conditions took on a giant cell morphology when treated with RO3306 (Figure 3).

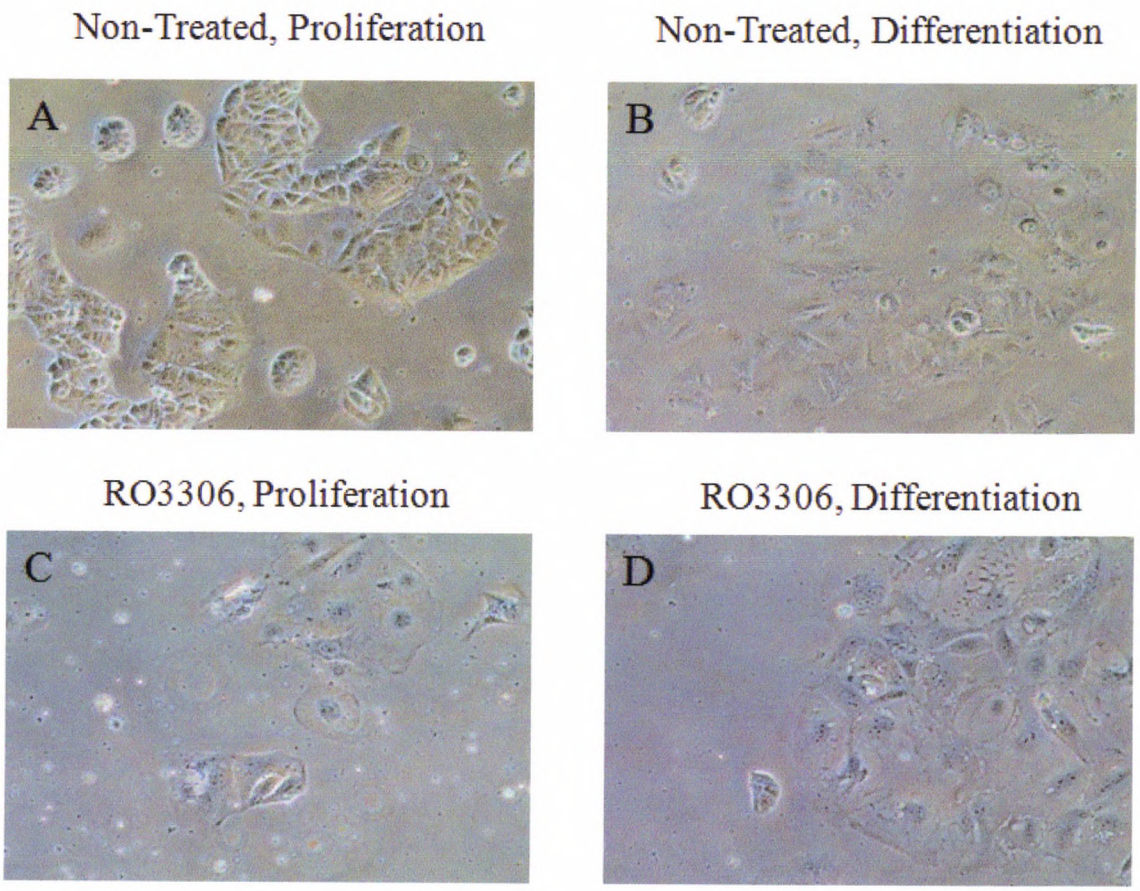

Figure 3: Morphology of cells treated with and without RO3306 under proliferation conditions (70% conditioned media, FGF4, heparin sulfate). A) Non-treated cells grown in proliferation media. B) Cells grown in differentiation media without RO3306 added being to form TGCs. C) Cells grown in proliferation media with RO3306 added become TGCs. D) Cells grown in differentiation media with RO3306 become TGCs at a faster rate than removal of proliferation media alone.

Cells were collected after 4 days exposure to RO3306 and examined for expression of *Lin28*, *Sox2*, *Nr0b1*, and *Igfpb7*. Cells had to be collected after 4 days because prolonged exposure to RO3306 caused cell death. TGCs take six days to form,

these cells were collected at day 4 of proliferation media removed, and have not fully formed TGCs (Figure 3D).

When TS cells were differentiated by removal of FGF4, there was a significant decrease in expression of *Lin28, Sox2,* and *Nr0b1*, and a significant increase in expression of *Igfbp7*. TS cells were differentiated by adding RO3306 to cells grown in proliferation conditions to determine if forced differentiation in the presence of FGF4 signaling caused changes in expression of *Lin28, Sox2, Nr0b1* or *Igfbp7* similar to TGC formation by removal of FGF4. As expected, we found that *Lin28* expression was significantly decreased when cells were differentiated into TGCs with RO3306 ($p < 0.02$). Surprisingly, *Sox2* and *Nr0b1* were not down-regulated when TGC formation was forced using RO3306. Our results show that, contrary to our predictions, *Sox2* and *Nr0b1* expression in TGCs differentiated with RO3306 have a significantly higher expression than in TGCs differentiated by removal of FGF4. *Igfbp7* showed a trend towards increased expression in RO3306 differentiated TGCs, but there was no significant difference in expression when compared to TS cells grown in proliferation media only.

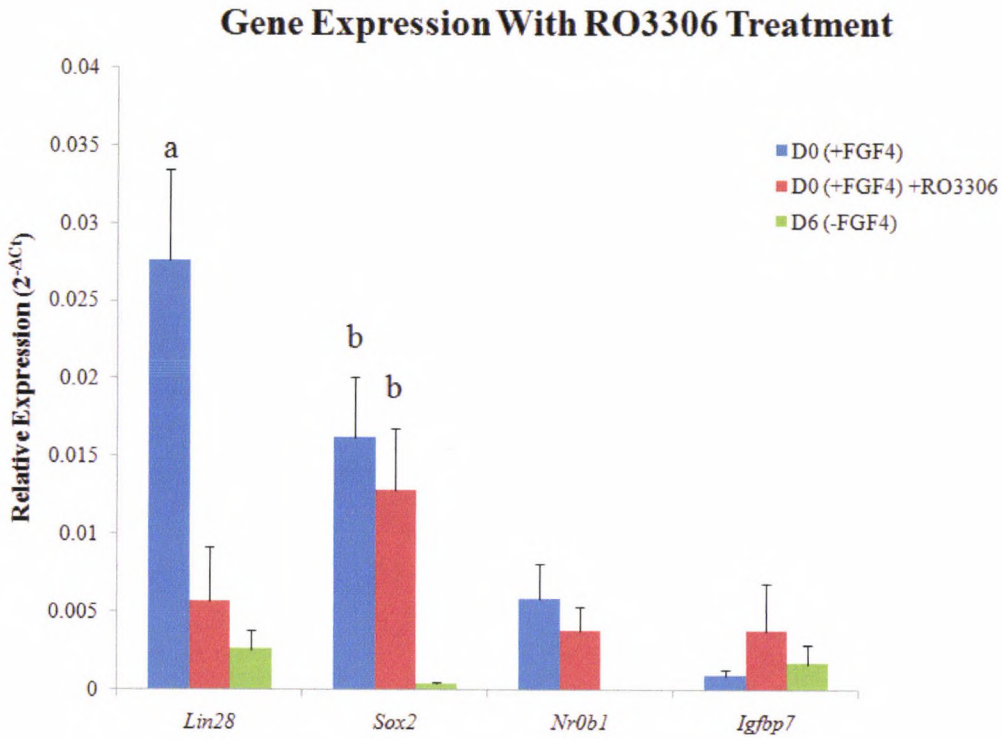

Figure 4: Gene expression of *Lin28, Sox2, Nr0b1,* and *Igfbp7* in cells growing under proliferative conditions (70% conditioned media, FGF4, heparin sulfate), proliferative conditions with RO3306, and non-treated cells differentiated by removal of proliferative conditions. Data are plotted as relative expression to *Gapdh*. a and b indicate significant ($p < 0.05$) changes between growth conditions within a gene.

Immunohistochemial Localization of LIN28

Lin28 was of interest due to its involvement in iPS cells, ES cells, and the interactions between *Lin28* and Let-7 miRNAs. Furthermore, *Lin28* expression decreases when TS cells differentiate into TGCs. LIN28 localization in proliferating and differentiating TS cells was performed using immunohistochemistry to determine if protein expression in TS cells and TGCs confirmed mRNA expression. As expected, LIN28 was localized predominantly in the cytoplasm of proliferating TS cells. Contrary to our predictions of LIN28 expression being absent in TGCs, LIN28 was still expressed

in TGCs, but expression had changed to a predominantly nuclear localization (Figure 5). 7% (± 2%) of proliferating TS cells were found to have a nuclear localization, while 44% (± 11%) differentiated TGCs had nuclear localization. Chi-square analysis shows a significant increase in nuclear localization in the TGCs compared to proliferating TS cells ($p < 0.001$).

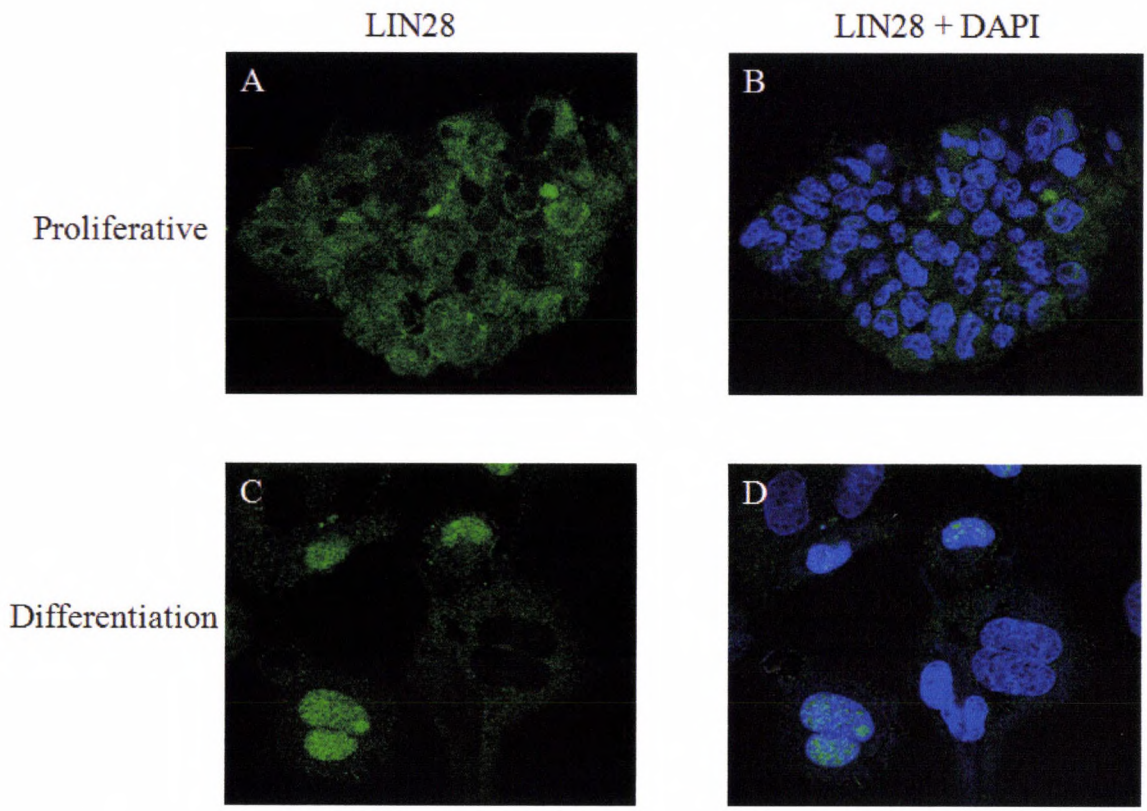

Figure 5: Expression of LIN28 protein in wild type proliferating TS cells and differentiated TGCs. A) TS cells grown in proliferation media have a predominately cytoplasmic localization of LIN28. B) Same picture as A with DAPI staining the nuclei. C) TGCs grown in differentiation media have increased nuclear expression of LIN28 when compared to proliferating TS cells. D) Same image as C with DAPI staining the nuclei. Green stain is LIN28, blue stain is the chromatin marker DAPI

To examine LIN28 expression *in vivo*, wild type mouse placentas were collected at E12.5 and processed for immunohistochemistry. Previously, LIN28 was localized to the ectoplacental cone and surrounding primary trophoblast giant cells at E7.5 (Moss, 2003). At E12.5 the placenta is mature and has all of the layers. LIN28 is expressed in TGCs and labyrinth layer of the placenta (Figure 6a, c & e). LIN28 protein was not expressed in the spongiotrophoblast. The maternal decidua also has positive staining for LIN28 (Figure 6a & c). Controls showed no LIN28 expression, and were stained with hematoxylin (Figure 6b, d & f).

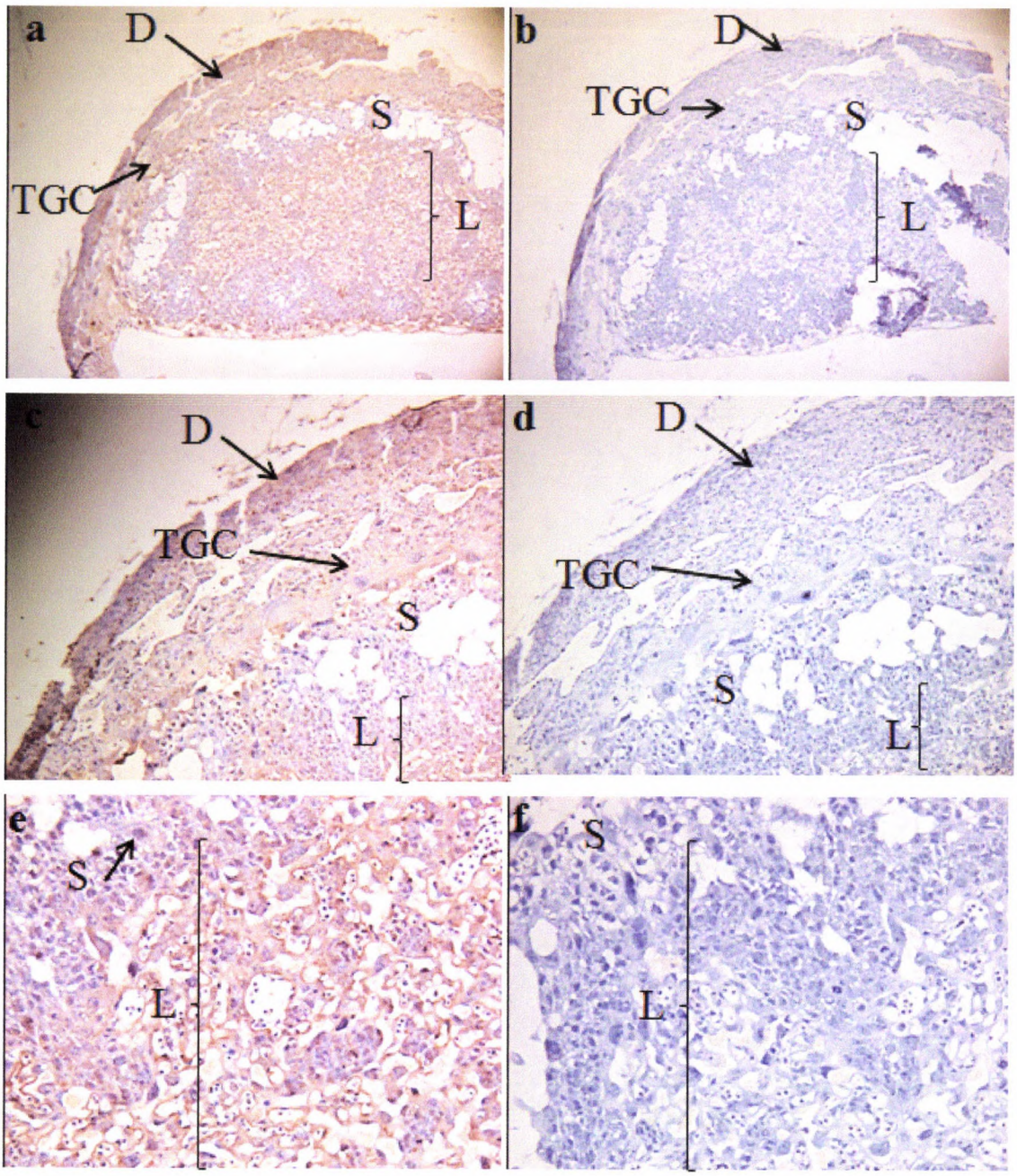

Figure 6: Localization of LIN28 in E12.5 mouse placenta. (a) Expression is localized to the TGCs and labyrinth layer. Staining for LIN28 is brown, nuclei are blue (hematoxylin). (b) Control of (a), stained with hematoxylin. (c) Staining is evident in the TGC layer but not in the spongiotrophoblast below. (d) Control of (c) stained with hematoxylin. (e) higher magnification (20x) indicates LIN28 is present in the labyrinth layer. (f) Control of (e) stained with hematoxylin. (a & b) expression at 4x magnification; (c & d) 10x magnification; (e & f) 20x magnification. TGC- trophoblast giant cell, L- labyrinth, D- decidua, S- spongiotrophoblast

Identification of miRNAs in TS Cells and TGCs

MiRNAs play a major role in ES cell proliferation and differentiation. To investigate the role of miRNAs in TS cell proliferation and differentiation, real time PCR was used to examine the expression of 380 known miRNAs and 3 (housekeeping) small RNAs; U6, RNU43, and RNU6. Significantly differentially expressed miRNAs were analyzed using Global Pattern Recognition (GPR) software; GPR analysis considers miRNAs significantly differentially expressed with a score of 0.40 or higher (Bouma et al., 2004; Akilesh, et. al, 2003). 28 miRNAs were found to be significantly different in expression between proliferating TS cells and differentiated TGCs. 19 miRNAs were significantly up-regulated in TGCs, while 9 were significantly down-regulated in TGCs (Table 2). The complete GPR analysis results of miRNAs in proliferating TS cells vs. differentiated TGCs can be seen in Appendix II.

The Let-7 family of miRNAs are all significantly higher expressed (GPR score > 0.40) in wild type differentiated TGCs (Table 2). We expected to see this increase of expression of Let-7 miRNAs in TGCs due to low expression of *Lin28* in TGCs. LIN28 is a known regulator of mature *Let-7* accumulation in ES cells. The Let-7 family of miRNAs are known to be highly expressed in many differentiated tissues, and have been shown to initiate differentiation.

Among the miRNAs expressed significantly lower in TGCs and higher (GPR score > 0.40) in proliferating TS cells is the miR-290 family (Table 2). The miR-290 family has been implicated in ES cell cycle regulation by regulating the G1/S transition to promote cell proliferation (Judson et al., 2009). Additionally, miR-130a and miR-323-5p

are expressed higher in proliferating TS cells. A list of potential targets, as they pertain to TS cells and TGCs, is given in Appendix III.

miRNAs Differentially Expressed in TGCs	
miRNA	**Fold Change**
miR-376b	36.0
miR-98	17.4
let-7f	17.2
let-7g	14.6
let-7d	13.4
let-7c	10.7
let-7i	10.5
let-7e	9.4
miR-28	9.3
miR-10b	9.3
miR-322	8.8
let-7a	7.7
miR-30a	5.9
miR-451	5.4
miR-22	4.9
miR-183	4.1
miR-370	3.6
miR-221	3.3
miR-29a	3.1
miR-323-5p	-5.0
miR-130a	-5.0
miR-290-3p	-5.7
miR-290-5p	-5.8
miR-295	-6.0
miR-291a-3p	-6.7
miR-294	-7.1
miR-292-3p	-7.7
miR293	-9.4

Table 2: MiRNAs significantly differentially expressed in proliferating TS cells vs. differentiated TGCs were analyzed using GPR software. MiRNA name and fold change are shown in this table. Positive fold change indicates significantly (GPR > 0.4) higher expressed in differentiated TGCs, negative fold change indicates significantly lower expressed in TGCs. Potential targets of these miRNAs, as related to TS cells or TGCs are given in Appendix III.

Lin28 Knockdown in Trophoblast Stem Cells

The role of *Lin28* was assessed in TS cells by using lentiviral shRNA silencing particles. TS cells were transfected with particles (MOI= 500) targeting *Lin28,* and a non-coding scramble sequence was used as a transfection control. Transfected TS cells were selected to have lentiviral insertion into the genome using puromycin selection. *Lin28* knockdown was assessed by real time PCR, which demonstrated a 78% knockdown when compared to the non-coding scramble transfection control (Figure 7). Cells with *Lin28* knockdown were maintained in FGF4, heparin sulfate and 70% conditioned media over eight passages with no noticeable effect on proliferation. Additionally, *Lin28* knockdown cells maintained their ability to differentiate into TGCs (Figure 7). Non-coding scramble transfected TS cells also had normal morphology and no effect was seen on proliferation or differentiation.

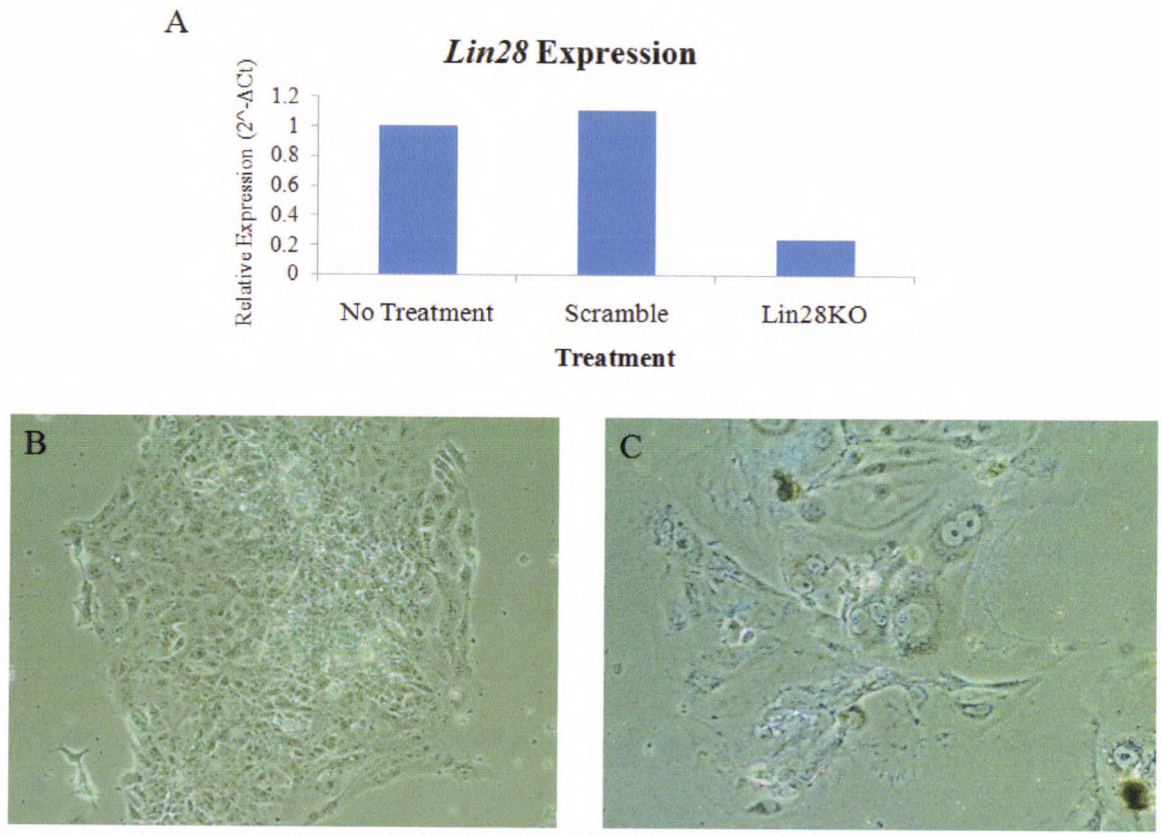

Figure 7: A) Expression of *Lin28* in non-treated cells, non-coding lentiviral control, and *Lin28* knocked down TS cells. B) Morphology of wild type TS cells with *Lin28* knockdown grown under proliferative conditions C) Morphology of *Lin28* knockdown TGCs grown in differentiation media.

Summary of Results:

This chapter has shown confirmed differential expression of *Lin28, Sox2, Nr0b1,* and *Igfbp7* between proliferating TS cells and differentiated TGCs, showing differentially regulated set of genes between TS cells and TGCs. It was shown that in the presence of proliferation media (70% conditioned media, FGF4, and heparin sulfate), and cultured with RO3306 TS cells are forced to differentiate into TGCs. Culturing TS cells in proliferation media with RO3306 added showed *Lin28* expression significantly decreases, suggesting *Lin28* expression is not maintained by FGF4. This same experiment showed

that *Nr0b1* and *Sox2* failed to become down-regulated despite the TGC morphology, suggesting these genes may be regulated by FGF4 signaling.

Lin28 was determined to be necessary to be down-regulated for TGC formation, and thus was further investigated. Immunohistochemistry showed abundant LIN28 expression in both proliferating TS cells and differentiated TGCs. A differential localization of the LIN28 protein was found between the TS cells and the TGCs; where TGCs had significantly more cells with nuclear localization of LIN28. In E12.5 mouse placental sections, LIN28 was identified in the labyrinth layer, TGC layer, and the maternal decidua, but not in the spongiotrophoblast.

MiRNA expression was assessed in differentiating TS cells. 28 differentially expressed miRNAs were identified when comparing the proliferating TS cells to the differentiated TGCs. Among these included a significant increase in expression of the Let-7 family of miRNAs in TGCs, showing an opposite expression to *Lin28.* Also differentially expressed was the miR-290 family of miRNAs, which was significantly higher in proliferating TS cells and had previously only been identified in proliferating ES cells.

To further investigate the function of LIN28 in proliferating TS cells a lentiviral knockdown of *Lin28* was performed, obtaining a 78% decrease in expression. TS cells with *Lin28* knocked down had normal morphology and proliferated regularly. Additionally, differentiation into TGCs with *Lin28* knocked down occurred at a normal rate and formed TGCs with normal morphology. These data suggest LIN28 is a secondary regulator of proliferation in TS cells and is not necessary for proliferation, but may need to be down-regulated to allow for TGC differentiation.

Chapter IV:

Molecular Characterization of AP-2γ Null Trophoblast Stem Cells

Introduction:

There are five members of the AP-2 family of transcription factors. These consist of AP-2α-ε. All of the AP-2 family members are expressed during embryonic development (Winger et al., 2004). AP-2γ null embryos die by E7.5 due to disrupted development of the extra embryonic portion of the conceptus (Auman et al., 2002). AP-2γ null blastocyst outgrowths have a reduced rate of TGC formation in culture (Auman et al., 2002). Recently, AP-2γ over expression in ES cells was shown to be sufficient for specification and induction of TS (Kuckenberg et al., 2010). Combined with the observation that AP-2γ is required to maintain an extra embryonic cell fate, we postulated that AP-2γ is necessary for proper TS cell maintenance and development.

Using an AP-2γ null TS cell line derived in the Winger lab, this study investigates the expression of *Lin28, Sox2, Nr0b1,* and *Igfbp7* in AP-2γ null TS cells. AP-2γ has been shown to bind to the promoter region of *Lin28* in TS cells (Kidder and Palmer, 2009; Winger, unpublished data). This study investigates the hypothesis that AP-2γ null TS cells have a disrupted expression of *Lin28, Sox2, Nr0b1,* and *Ifgbp7*, leading to abnormal phenotype in these cells. This hypothesis was tested by performing real time PCR on AP-2γ null TS cells to examine *Lin28, Sox2, Nr0b1,* and *Igfbp7* expression. In addition, the role of *Lin28* was studied in TS cell differentiation using lentiviral shRNA particles in AP-2γ null TS cells.

Results:

Expression of AP-2 Family Members in TS Cells

AP-2γ is an essential factor for placental development as shown by the AP-2γ -/- knockout mouse (Auman et al., 2002). Of all the AP-2 family members, AP-2γ is the only one that, when deleted, shows a placental phenotype (Auman et al., 2002). AP-2α also has been identified in the trophoblast, although does not present a placental phenotype when deleted in mice (Brewer and Williams, 2004). Our investigation of the AP-2 family members in differentiating TS cells shows AP-2α increasing at each time point upon removal of conditioned media, FGF4 and heparin sulfate (Figure 8). We found that *AP-2γ* was highest expressed at all time points investigated. *AP-2α* was expressed at significantly lower levels than *AP-2γ* during differentiation into TGCs. (Figure 8) These data suggest that AP-2γ is the most important AP-2 member in the placenta.

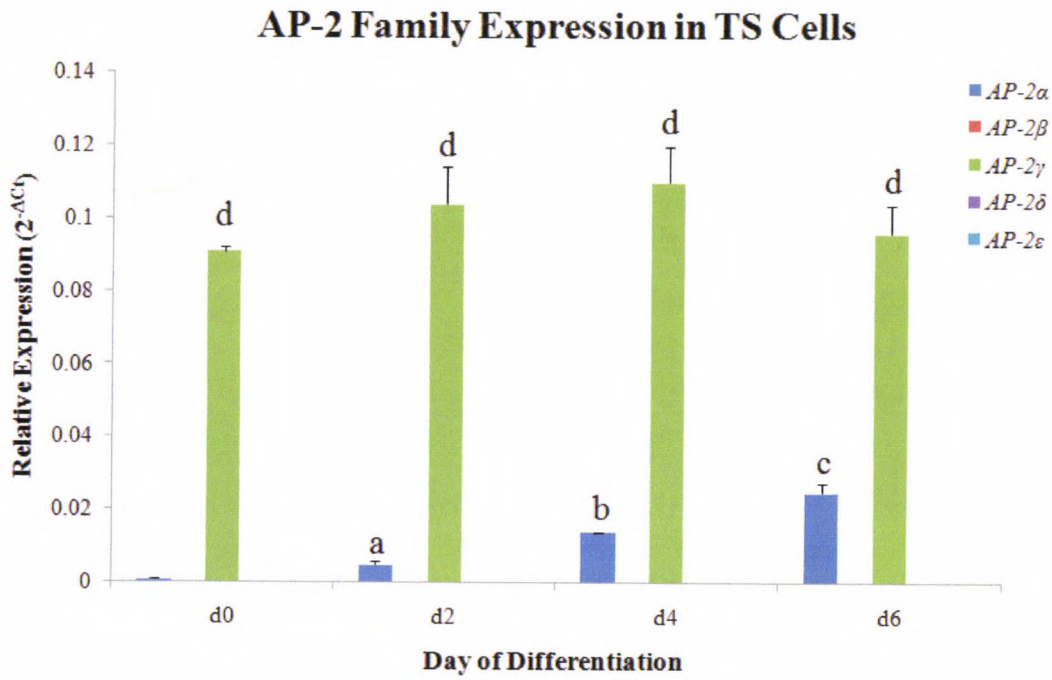

Figure 8: Expression of AP-2 family members in wild type TS cells during differentiation into TGCs. *AP-2β, δ*, and *ε* expression was not detected. *AP-2α* expression increased significantly each day throughout differentiation. *AP-2γ* is the highest expressed family member, and is expressed significantly higher than *AP-2α* at each timepoint. Data are plotted as relative expression, normalized to *Gapdh*. a-d indicate significant differences ($p < 0.05$) between genes at different days.

AP-2γ Null TS Cells Fail to Differentiate

An AP-2γ null TS cell line was developed using tamoxifen inducible Cre-recombinase mediated deletion of an AP-2γ conditional allele (AP-2γ loxP). AP-2γ null TS cells grown in proliferation media (70% conditioned media, FGF4, and heparin sulfate) morphologically resemble wild type TS cells (Figure 9). These cells grow in colonies but do not express the TS cell marker *Eomes* in levels comparable to wild type TS cells (Figure 10). When conditioned media, FGF4, and heparin sulfate are removed from the culture conditions (differentiation conditions), AP-2γ null TS cells fail to take on TGC morphology. The cells continue to proliferate and can be passaged after growth in TS media with FGF4 removed (Figure 9). In addition, AP-2γ null TS cells do not express the TGC markers *Pl1, Pl2,* or *Plf* (Figure 10).

The pluripotency genes *Lin28, Sox2,* and *Nr0b1* were investigated in AP-2γ null TS cells. AP-2γ null TS cells fail to down regulate *Lin28*, *Sox2*, or *Nr0b1* upon removal of conditioned media, FGF4 and heparin sulfate (Figure 11). *Igfbp7* expression significantly changes upon the removal of proliferation conditions; two days after removal there is a significant ($p < 0.05$) increase in *Igfbp7* expression when compared to proliferative conditions. Four days after removal of proliferative conditions there is a significant ($p < 0.05$) decrease in *Igfbp7* expression. Six days after removal of proliferative conditions there is no change in expression of *Igfbp7* when compared to proliferative conditions. These data add to the findings that AP-2γ null TS cells apparently fail to differentiate.

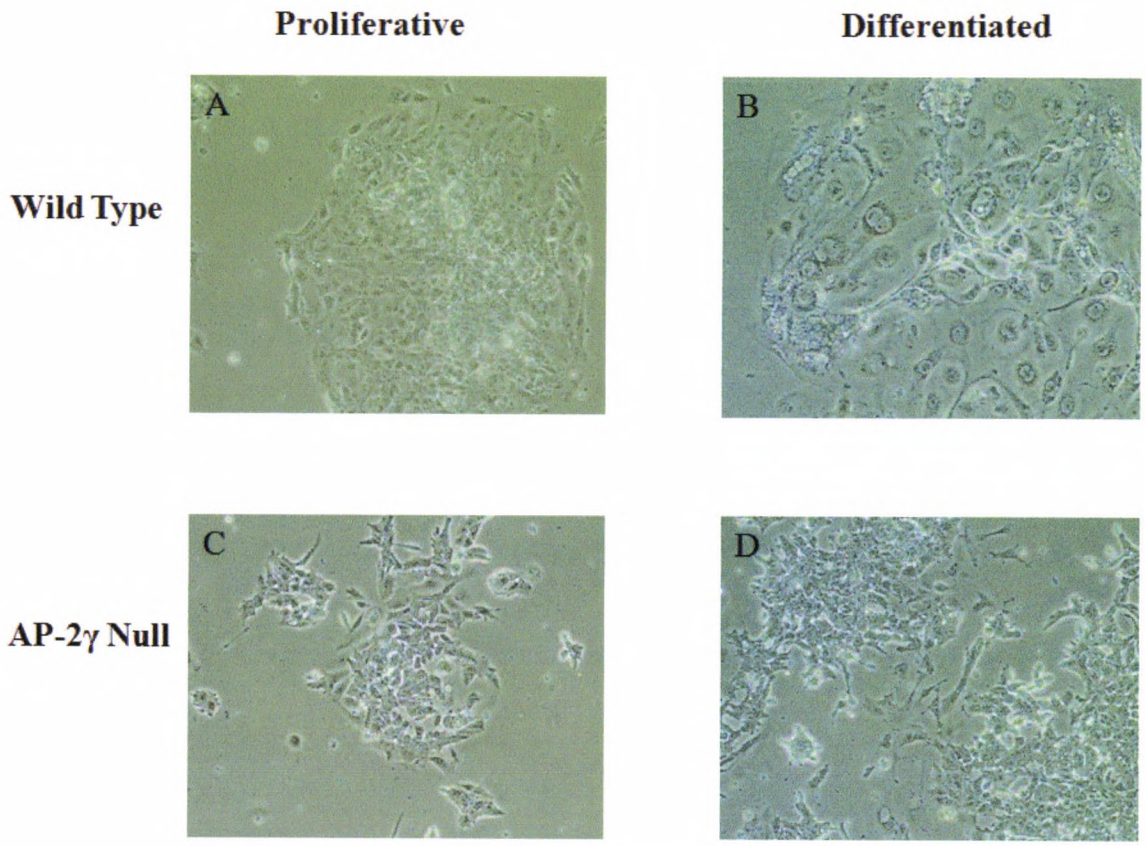

Figure 9: A) Morphology of wild type TS cells grown under proliferative conditions and B) differentiated TGCs. C) AP-2γ null TS cells under proliferative conditions morphologically resemble wild type cells. D) When 70% conditioned media, FGF4 and heparin sulfate are removed AP-2γ null TS cells fail to take on TGC morphology and continue to proliferate. All images are shown at 10x magnification.

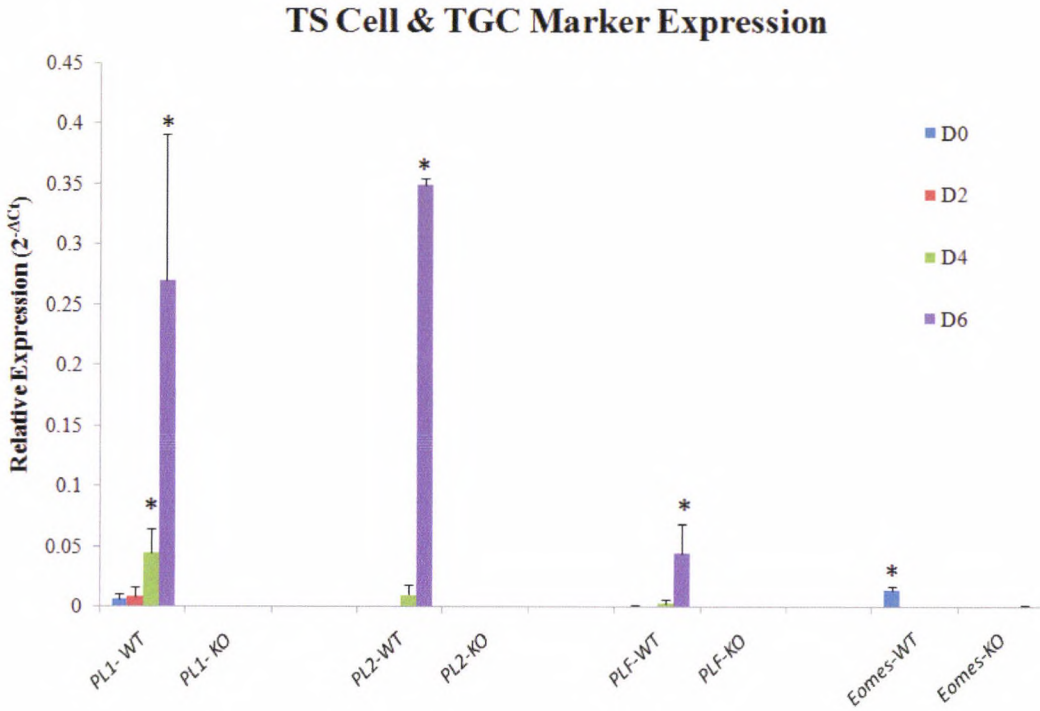

Figure 10: Expression of the TS cell marker *Eomes* and TGC markers *Pl1, Pl2,* and *Plf.* Wild type TS cells show normal expression of these markers where *Eomes* expression decreases upon the removal of FGF4, heparin sulfate, and 70% conditioned media. *Pl1, Pl2,* and *Plf* are TGC markers and their expression increases as expected as wild type TS cells differentiate into TGCs. AP-2γ null TS cells fail to express these markers. Data are plotted as relative expression compared to *Gapdh.*

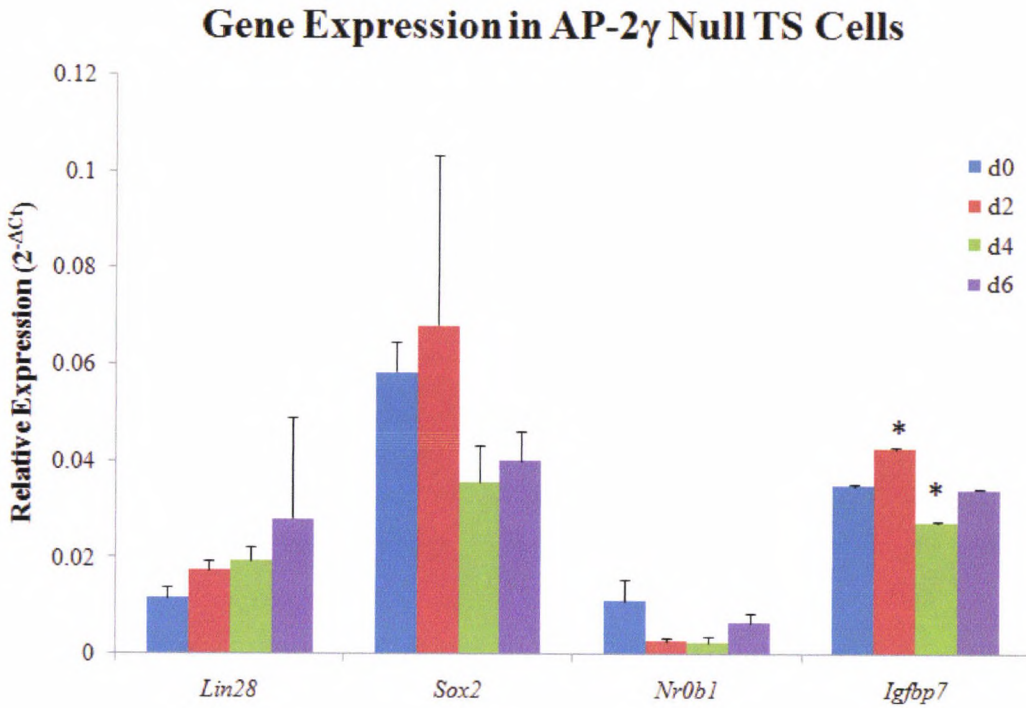

Figure 11: Expression of *Lin28*, *Sox2*, *Nr0b1* and *Igfbp7* in AP-2γ knockout TS cells. There is no significant difference in expression of *Lin28, Sox2, Nr0b1* thoughout the duration of culture. *Igfbp7* had a significantly increased expression 2 days after FGF4 removal, and a significantly decreased expression 4 days after FGF4 removal when compared to proliferative conditions at day 0. Data are plotted as relative expression to *Gapdh* and plotted using the $2^{-\Delta Ct}$ method. Astricks indicate a significant ($p < 0.05$) difference for data compared between time points within a gene.

RO3306 Treatment of AP-2γ Null TS Cells

AP-2γ null TS cells fail to differentiate when proliferation conditions (i.e. FGF4) are removed. We attempted to induce AP-2γ null TS cells to differentiate using RO3306, a compound which causes endoreduplication by blocking Cyclin-dependent protein kinase 1 (CDK1) activity. If a cell is unable to endoreduplicate, such as ES cells, the cells will undergo apoptosis (Ullah et al., 2008). AP-2γ null TS cells cultured with RO3306 failed to differentiate into TGCs, and all mutant cells exposed to RO3306 died

within 48 hours (Figure 12). AP-2γ null TS cells exposed to a DMSO vehicle control were not effected (Figure 12). Taken together these data suggests that the AP-2γ mutation alters the response of TS cells to RO3306 preventing entry into endoreduplication.

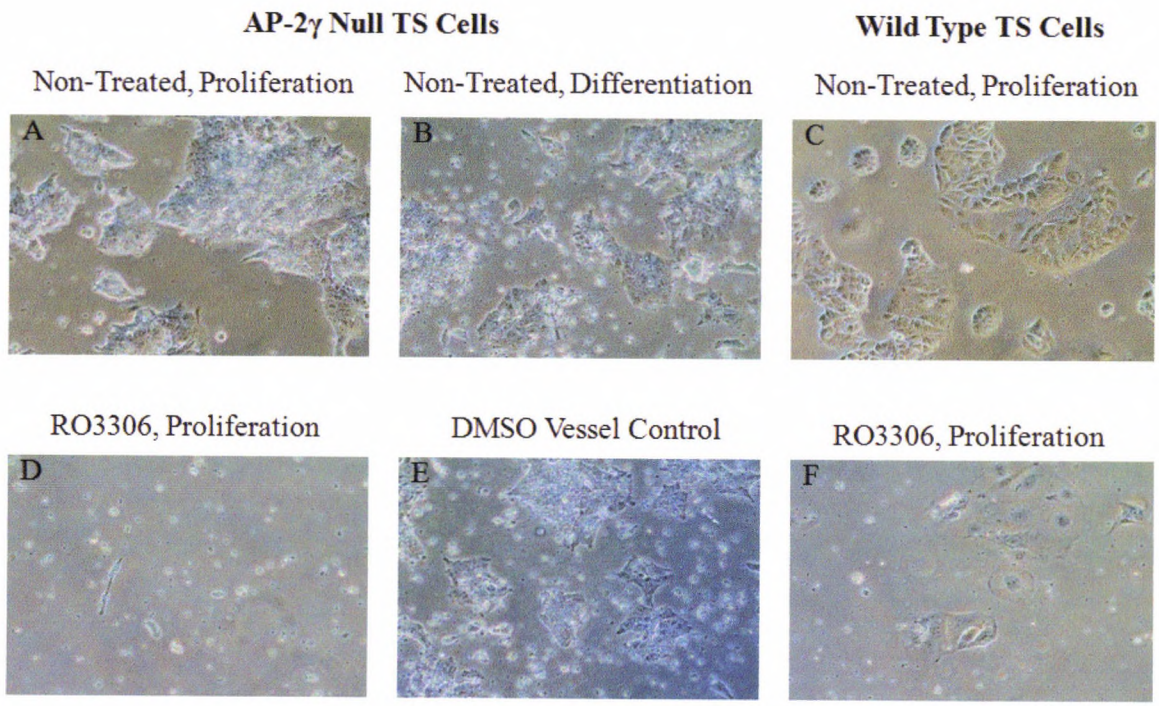

Figure 12: AP-2γ null TS cells die when exposed to RO3306. A) Non-treated AP-2γ null TS cells in proliferation media. B) Non-treated AP-2γ null TS cells in differentiation meda fail to differentiate. C) Non-treated wild type cells in proliferation media. D) AP-2γ null TS cells in proliferation media and cultured with RO3306 die, as evidenced by cell debris in the plate. E) AP-2γ null TS cells exposed to a DMSO vehicle control do not die. F) Non-treated wild type cells in proliferation media and cultured with RO3306 differentiate into TGCs.

LIN28 Expression in AP-2γ Null TS Cells

We investigated the role of *Lin28* in the regulation of AP-2γ null TS cell proliferation and differentiation. LIN28 localization was assessed using immunoflourescense on AP-2γ null TS cells grown under proliferating conditions (70%

conditioned media, FGF4, and heparin sulfate), and cells grown under differentiation conditions (100% TS media). We observed LIN28 expression in both proliferating TS cells, and cells grown under differentiation conditions. 46% (± 4%) of AP-2γ null TS cells in proliferation media had nuclear localization of LIN28, a significant ($p < 0.001$) increase compared to the wild type proliferating TS cells, where 7% of cells had nuclear localization of LIN28 (Figure 13). 44% (± 2%) of AP-2γ null TS cells grown under differentiation conditions had nuclear localization of LIN28, not different from the AP-2γ null TS cells in proliferation media, or the wild type differentiated TGCs. These data suggest that LIN28 is misregulated in AP-2γ null TS cells.

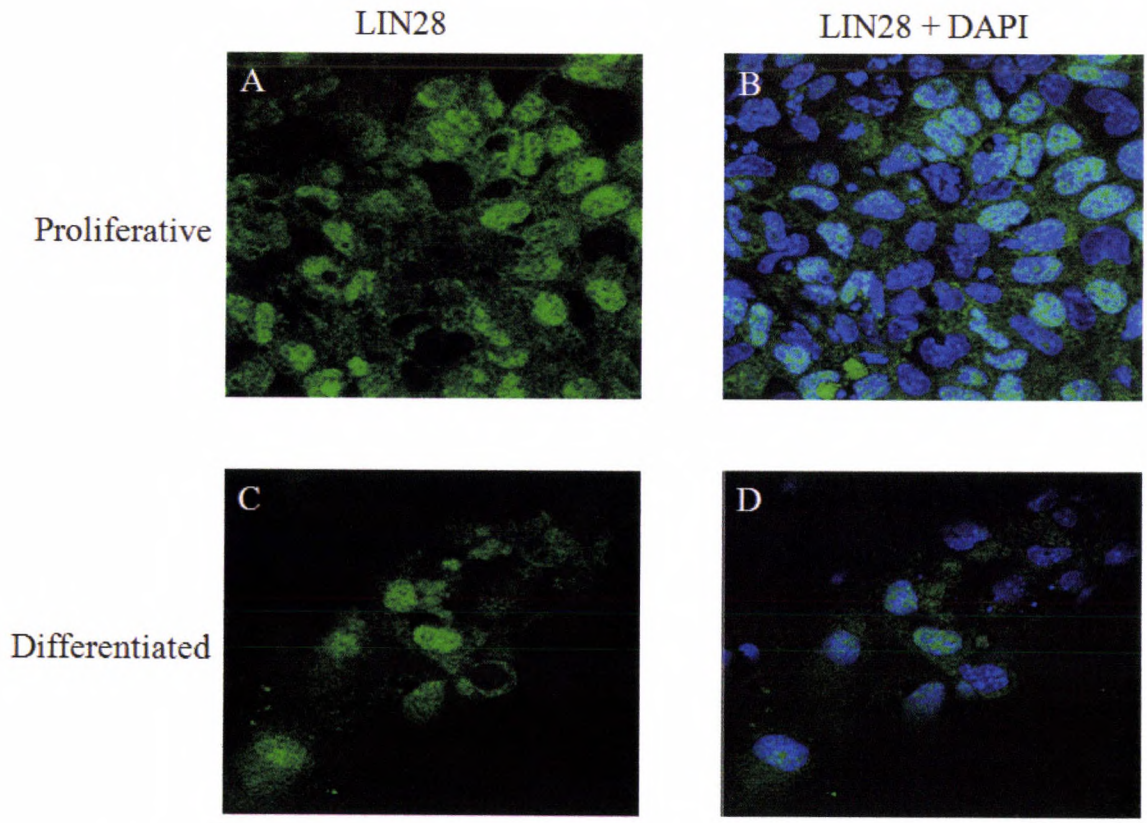

Figure 13: LIN28 staining in AP-2γ null TS cells, green staining is LIN28 and blue staining is DAPI. A) AP-2γ null TS cells grown under proliferation conditions have 46% nuclear localization of LIN28. B) AP-2γ null TS cells stained for LIN28 and DAPI. C) AP-2γ null TS cells grown under differentiation conditions have 44% nuclear localization of LIN28. D) AP-2γ null TS cells grown under differentiation conditions stained for LIN28 and DAPI.

Presence of ES Cell Markers in AP-2γ Null TS Cells

AP-2γ null TS cells fail to differentiate upon the removal of proliferative conditions, and even continue to proliferate in the absence of FGF4; with continued high expression of *Lin28, Sox2,* and *Nr0b1*. Additionally, AP-2γ null TS cells die when RO3306 is added to culture media to induce differentiation. AP-2γ null TS cells don't express TS cell markers, leading us to investigate additional pluripotency markers in AP-2γ null TS cells. We examined expression of the Pluripotency markers *Oct4, Nanog,* and

Stella in AP-2γ null TS cells in proliferative and differentiated conditions. Real time PCR analysis using Taqman probes for *Oct4, Nanog,* and *Stella* indicate a significantly ($p < 0.05$) up-regulated expression in AP-2γ null TS cells compared to wild type TS cells. Expression of *Oct4* did not change upon removal of 70% conditioned media, FGF4, and heparin sulfate from the culture conditions as seen in Figure 15. *Nanog* and *Stella* both showed a significant decrease in expression 2 days after removal of FGF4, followed by a significant increase in expression 4 days after removal of FGF4 (Figure 14).

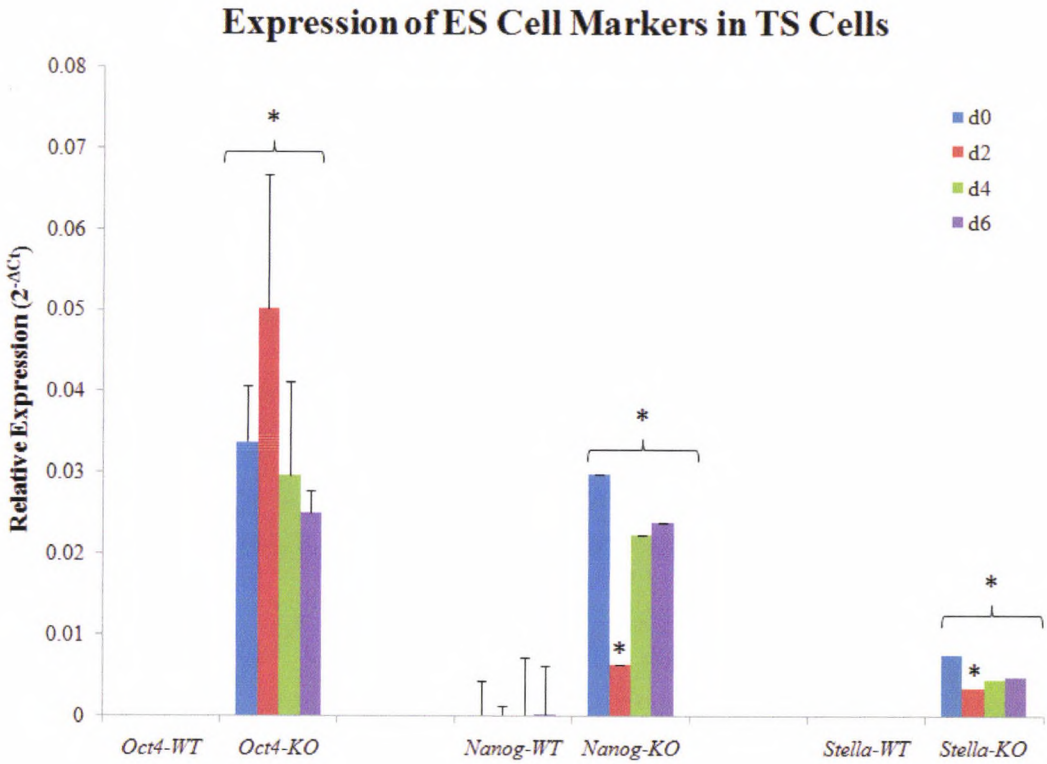

Figure 14: Expression of *Oct4*, *Nanog* and *Stella* in wild type and AP-2γ null TS cells thoughout differentiation. There is no significant change in expression of *Oct4* at each time point. *Nanog* and *Stella* both show a significant decrease in expression two days after removal of FGF4, followed by increased expression of *Nanog* at 4 and 6 days after FGF4 removal. *Oct4, Nanog* and *Stella* are ES cell markers not expressed in wild type TS cells. Data are plotted relative to *Gapdh*. Astricks indicate a significant ($p < 0.05$) difference for a gene compared within the time points.

MiRNAs Expression in AP-2γ Null TS Cells

Due to the continued elevated expression of *Lin28* in AP-2γ null TS cells, and their inability to differentiate into TGCs, a miRNA expression profile was obtained for AP-2γ null TS cells under proliferation and differentiation conditions. MiRNAs expressed in AP-2γ null TS cells (proliferative conditions) did not have any significant differences in expression compared to miRNAs expressed in wild type TS cells

(proliferative conditions). MiRNAs expressed in AP-2γ null TS cells (differentiation conditions) did not have any significant differences from wild type TS cells (proliferation conditions). There were, however, 7 miRNAs differentially expressed between the AP-2γ null TS cells in proliferative and differentiation conditions: 6 miRNAs up-regulated under differentiation conditions, and 1 down-regulated under differentiation conditions (Table 3). The up-regulated miRNAs observed when 70% conditioned media, FGF4 and heparin sulfate were removed, included miR-302b, miR-302c, and miR367 (Table 3); a set of miRNAs expressed only in ES cells and known to be regulators of *Oct4* and *Sox2;* which also regulate the miR-302 cluster of miRNAs (Card et al., 2008). Additionally, miR-302a was up-regulated in AP-2γ null TS cells under differentiation conditions with a GPR score of 0.357 and a fold change of 28. While a score of 0.40 or above is considered signficicant, miR-302a is noteworthy due to the substantial fold change and relevance to the miR-302 family. Additionally, up-regulated in the AP-2γ null TS cells under differentiation conditions were miR-99a and miR-99b, members of the miR-125 family which have been shown to be involved in ES cells. Down-regulated in AP-2γ null TS cells when cultured in differentiation conditions was miR-139-3p, which potentially targets *Elk1,* an oncogene (www.targetscan.org). A summary of miRNA expressed in wild type TS cells and AP-2γ null TS cells under proliferative and differentiation conditions is shown in Figure 15. These data show that when FGF4, a component necessary to maintain TS cell phenotype, is removed from culture conditions for AP-2γ null TS cells, AP-2γ null TS cells begin to express ES cell specific miRNAs, suggesting loss of AP-2γ in TS cells leads to a less differentiated state.

MiRNAs Differentially Expressed in AP-2γ Null TS Cells	
miRNA	**Fold Change**
miR-367	51
miR-302b	21
miR-302c	11
miR-99b	8
miR-99a	8
miR-139-3p	-6

Table 3: Differentially expressed miRNAs in AP-2γ null TS cells between proliferation and differentiation conditions. Positive fold change indicates miRNAs up-regulated under differentiation conditions, whereas negative fold change corresponds to down-regulation under differentiation conditions.

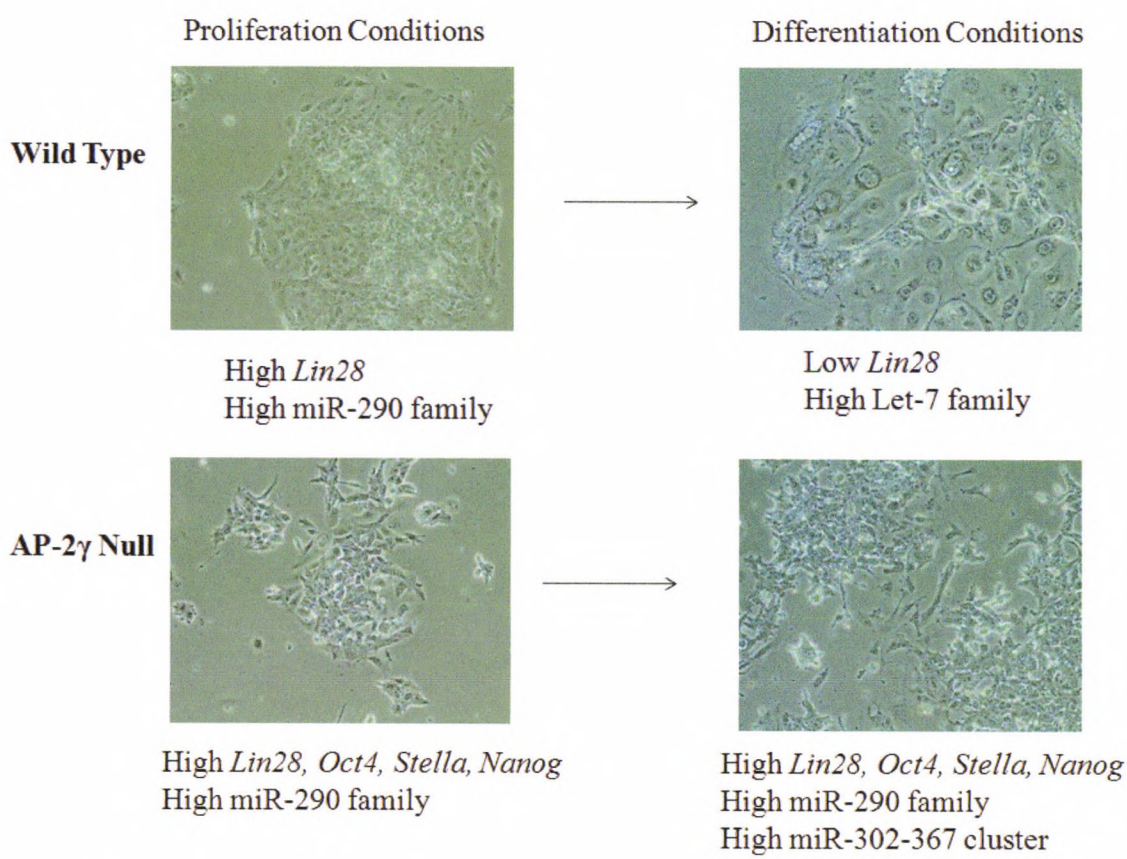

Figure 15: A summary of genes and miRNAs differentially expressed between wild type TS cells and TGCs and AP-2γ null TS cells in proliferating and differentiating conditions.

Lin28 Knockdown in AP-2γ Null TS Cells

ShRNA lentiviral particles were used to specifically target *Lin28* in AP-2γ null TS cells. A non-coding scramble sequence shRNA was used as a transfection control. Transfected TS cells were selected for genomic incorporation of the lentivirus using puromycin selection. *Lin28* expression was assessed using real time PCR and showed an 88% decrease in expression (Figure 16). AP-2γ null TS cells treated with *Lin28* lentivirus were propagated and split to three individual 6-well dishes to set up three treatments. One dish contained proliferation conditions (70% conditioned media, FGF4, and heparin sulfate); one dish contained differentiation conditions (100% TS media); and the third dish contained proliferation conditions and RO3306 to induce endoreduplication (n=3). AP-2γ null TS cells with *Lin28* knockdown had a life span of two passages. TS cells were analyzed from the time they were plated into the 6-well dishes until collection at 6 days post plating by taking pictures every two days. After 6 days, TS cells were collected for RNA analysis.

AP-2γ null TS cells with 88% *Lin28* knockdown continued to proliferate under proliferation conditions for two passages. TGC morphology was seen at the border of some colonies after two days in culture (Figure 16). Spontaneous TGC formation is common in wild type TS cells under proliferation media, but has never been seen before in this TS cell line. AP-2γ null TS cells with *Lin28* knockdown cultured under differentiation conditions also had TGC morphology at the edges of colonies and in areas where colonies were less dense (Figure 16b). Not all TS cells differentiated into TGCs in the differentiation conditions. AP-2γ null TS cells with *Lin28* knockdown cultured with

RO3306 under proliferative conditions did not die, but failed to form TGCs in culture or continue to proliferate (Figure 16).

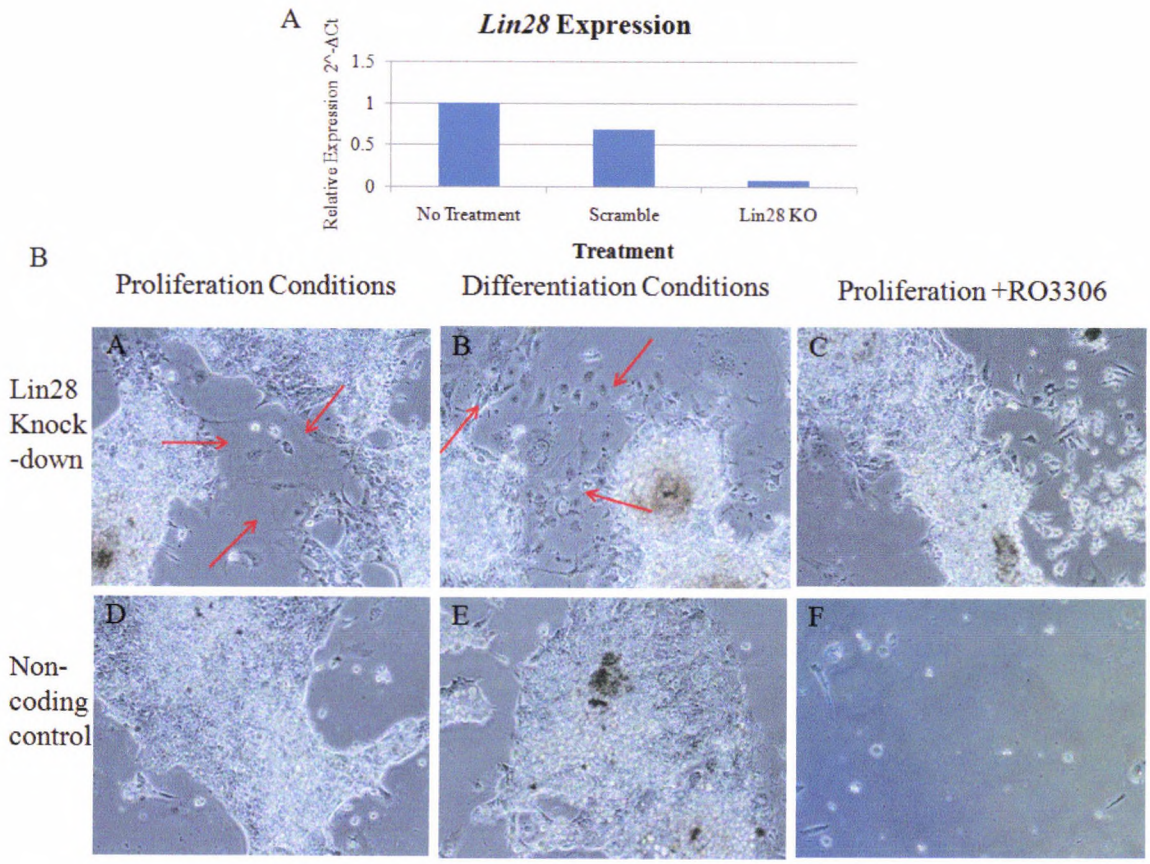

Figure 16: *Lin28* and non-coding control lentiviral transfected AP-2γ null TS cells. A) TGC formation in colonies of *Lin28* knockdown AP-2γ null TS cells cultured under proliferation conditions. B) TGC formation in *Lin28* knockdown AP-2γ null TS cells cultured under differentiation conditions. C) *Lin28* knockdown AP-2γ null TS cells cultured with RO3306 do not die immediately, but do not continue to proliferate. D, E, F) Non-coding scramble lentiviral transfected AP-2γ null TS cells act as a control cultured under (D) proliferating conditions and (E) differentiation conditions, and (F) undergo apoptosis when cultured with RO3306. Red arrows indicate TGC formation.

Summary of Results:

This chapter has shown that, in culture, *AP-2γ* is the highest expressed AP-2 family member in TS cells. Loss of AP-2γ in TS cells in culture leads to a defect in TGC

formation, and AP-2γ null TS cells continue to proliferate under differentiation conditions. TS cell and TGC marker expression was examined in AP-2γ null TS cells, showing that *Eomes, Pl1, Pl2,* and *Plf* are not expressed. *Lin28, Sox2,* and *Nr0b1* expression remains high in AP-2γ null TS cells when cultured under differentiation conditions. When AP-2γ null TS cells are cultured with RO3306 to induce endoreduplication, they undergo cell death, but are not affected when cultured with a DMSO vehicle control. An increased number of cells with nuclear localization of LIN28 in AP-2γ null TS cells was seen when compared to wild type TS cells. In addition to continued high expression of *Lin28, Sox2,* and *Nr0b1,* AP-2γ null TS cells express the pluripotency markers *Oct4, Nanog,* and *Stella.* AP-2γ null TS cells (proliferative conditions) do not have a significantly different expression of miRNAs when compared to wild type TS cells (proliferative conditions). However, AP-2γ null TS cells have a significant upregulation of the ES cells specific miRNAs, miR-302a, miR-302b, miR-302c and miR-367 when FGF4 is removed from culture conditions. In addition, up-regulated under differentiation conditions were miR-99a and miR-99b, and down-regulated was miR-139-3p. *Lin28* knockdown led to morphological changes, when *Lin28* is knocked down in AP-2γ null TS cells could differentiate into TGCs. Additionally, loss of *Lin28* in AP-2γ null TS cells led to a diminished lifespan, with cells only surviving for two passages. These data indicates AP-2γ and LIN28 together are necessary for survival of proliferating TS cells.

Chapter V:

LIN28 Expression in Human Placenta

Introduction:

While the mouse may provide us with a good model to study early placental development and molecular regulators of TS cell proliferation and differentiation, it is important to confirm our findings in human placental cells. It is impractical and unethical to collect human placental tissue during pregnancy for analysis, but samples can be collected at medical centers from planned termination of early pregnancy. Additionally, several human extravillous cell lines have been developed to study first trimester trophoblast cells. One of these cell lines is the ACH3P cell line, a fused choriocarcinoma, extravillous cytotrophoblast cell line (Hiden et al., 2007). ACH3P cells grown on plastic will proliferate, but when grown on Matrigel will take on an invasive phenotype.

Lin28 and AP-2γ have been shown to be important regulators of TS cell proliferation and differentiation in the mouse. This study investigates the hypothesis that LIN28 and AP-2γ are expressed in human placental tissue, and that LIN28 localization mimics expression in cell types observed in the mouse. LIN28 and AP-2γ expression was investigated in the ACH3P cell line while grown on plastic. Additionally, first trimester human placental villi fixed tissue sections were obtained to examine LIN28 expression (a kind gift from Dr. Anthony).

Results:

Immunofluorescence staining was obtained for LIN28 and AP-2γ localization in ACH3P cells grown on plastic. It was found that LIN28 was expressed abundantly in the cytoplasm of proliferating ACH3P cells (Figure 17A). AP-2γ was detected in the cytoplasm of ACH3P cells as well (Figure 17B).

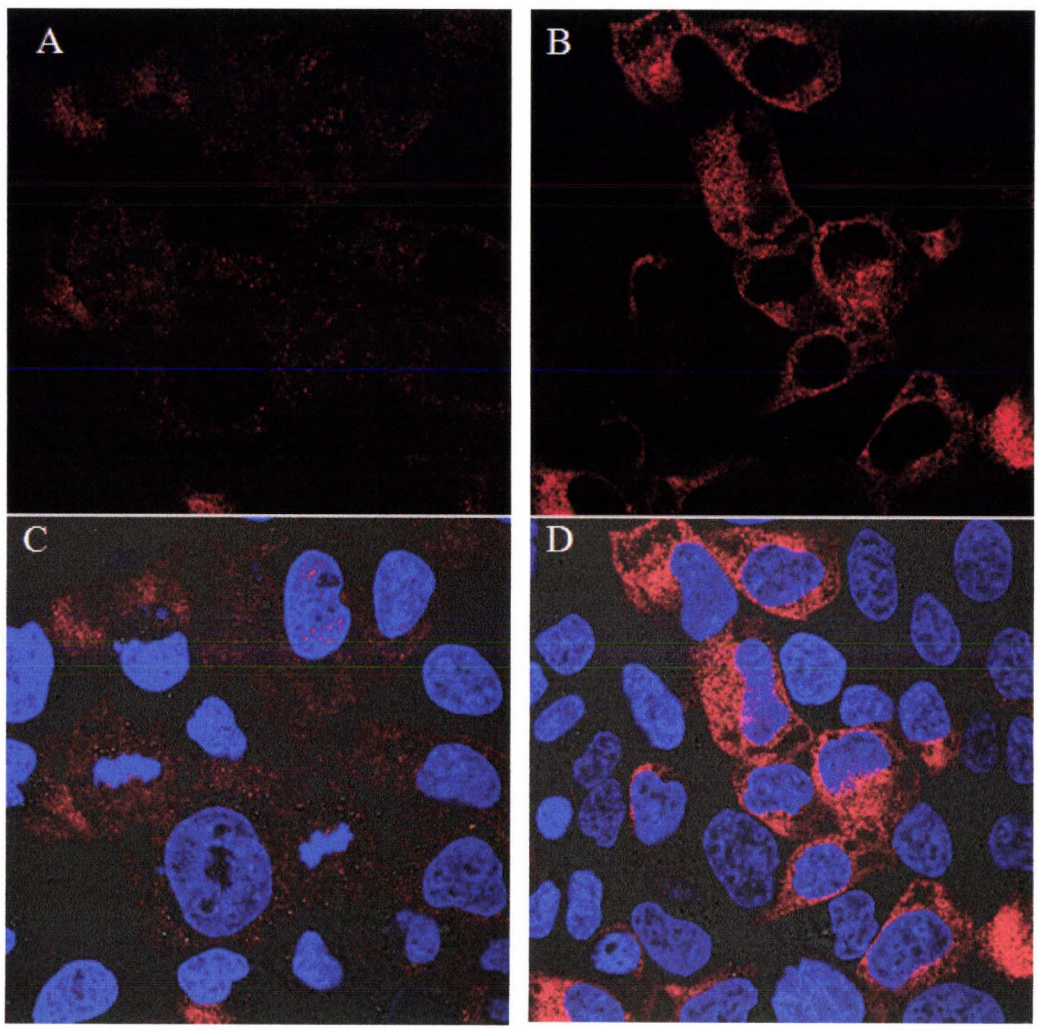

Figure 17: LIN28 and AP-2γ localization in ACH3P cells. A) LIN28 is abundantly expressed in the cytoplasm of ACH3P cells. C) LIN28 staining (red) and the nuclear marker DAPI (blue). B) AP-2γ cytoplasmic staining in ACH3P cells (red). D) AP-2γ staining (red) and the nuclear marker DAPI (blue).

In addition human placental tissue sections were used to examine LIN28 localization. No primary antibody controls were used. LIN28 is present in the inner cytotrophoblast cell layer and the outer layer of synctiotrophoblast of the first trimester villi. (Figure 18) These results indicate that LIN28 is involved in human placental development.

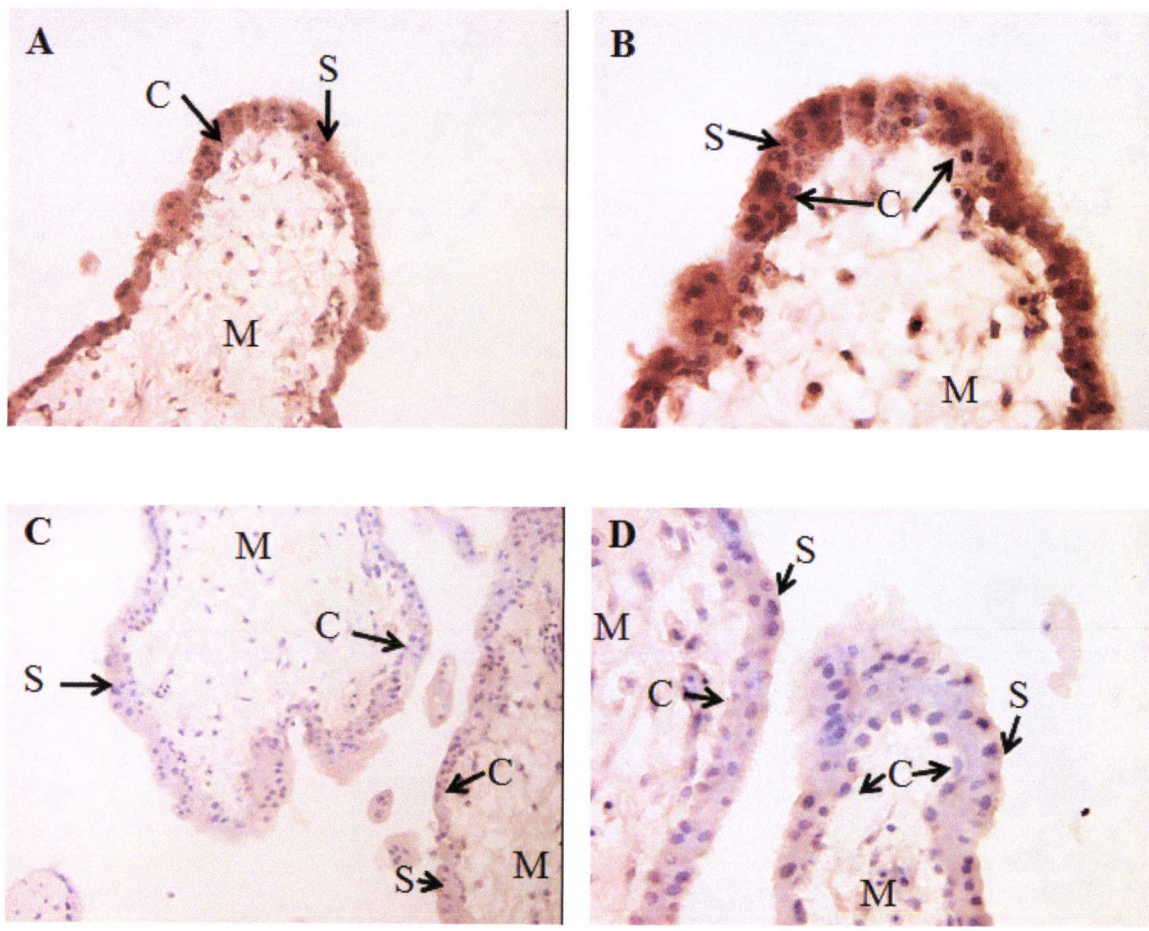

Figure 18: LIN28 localization in first trimester placental villi. Brown staining indicates LIN28 and blue staining is hematoxylin. A) LIN28 staining in placental villi. Arrows indicate cytotrophoblast and syncitiotrophoblast, along with some faint staining the mesencyhme (20x magnification). B) The cytotrophoblast and syncitiotrophoblast layers of the villi (40x magnification). C) Control for (A), stained with hematoxylin. D) Control for (B) stained with hematoxylin. C=Cytotrophoblast, S=Syncitiotrophoblast, M=Mesenchyme

Summary of Results:

This chapter has identified AP-2γ and LIN28 expression in first trimester human extravillous cytotrophoblast cells. LIN28 is abundantly expressed in the cytoplasm of ACH3P cells, an extravillous cell line used to study proliferation and differentiation of human extravillous trophoblast cells. Additionally, AP-2γ is expressed in the cytoplasm of ACH3P cells. AP-2γ has been identified to be sequestered to the cytoplasm of some cancer cells, causing suppression of its function (Aqeilan et al., 2004), and in trophoblast cells has been shown to be transported between the nucleus and the cytoplasm by actin (El-Hashash and Kimber, 2006). LIN28 was found to be localized to proliferating cytotrophoblast cells and syncitiotrophoblast cells in first trimester placental villi. LIN28 expression was expected in cytotrophoblasts because they are less differentiated. However, LIN28 expression was not expected in syncytiotrophoblast because these cells are more differentiated. These results indicate a conserved role for LIN28 and AP-2γ in human trophoblast.

CHAPTER VI:

Discussion

This study identified several pluripotency factors differentially expressed during TS cell proliferation and their differentiation into trophoblast giant cells (TGCs). The rapid and significant down regulation of *Lin28, Sox2,* and *Nr0b1* upon removal of proliferation conditions (70% conditioned media, FGF4, and heparin sulfate) suggests these genes are involved in regulating proliferation, and/or preventing their differentiation into TGCs. These findings confirm recently published expression data showing decreased expression of *Lin28* and *Sox2* in TGCs (Kidder and Palmer, 2010). Additionally, *Igfbp7*, an IGF binding protein like protein, is differentially expressed, becoming up-regulated as TS cells differentiate into TGCs. *Igfbp7*, also known as *Mac25*, is of interest because it has an FGF-receptor binding domain and an activin binding domain (Kato, 1999), and *Igfbp7* potentially could play a role in binding to and suppressing FGF4 signaling resulting in TGC formation. Additionally, *Igfbp7* has been shown to block vascular endothelial growth factor (VEGF) induced angiogenesis in vascular endothelial cells (Tamura et al., 2009). Additionally, *Igfbp7* has AP-2γ and AP-2α binding sites in its promoter region. Although this study does not investigate a specific role for *Igfbp7* in the differentiating trophoblast, it will be of interest to determine if there is a specific function in the regulation of vascularization mediated by

TGCs. Additionally, *Igfbp7* could act as a regulator to induce TGC formation by binding FGF4 and suppressing its biological activity. Expression of *Lin28, Sox2, Nr0b1,* and *Igfbp7* indicates that these genes regulate proliferation and differentiation in TS cells.

TGC formation includes many rounds of endoreduplication, which is DNA replication without cell division (Hattori et al., 2006). RO3306 is a compound that induces endoreduplication in cells that are capable of undergoing endoreduplication, such as TS cells. RO3306 forces endoreduplication in TS cells by p57 mediated restriction of Cyclin-dependent protein kinase 1 (CDK1) (Ullah et al., 2008). Forcing TS cells to endoreduplicate and become TGCs in the presence of FGF4 allowed for examination of expression of *Lin28, Sox2,* or *Nr0b1* when differentiated by a manner independent of FGF4 removal. Results of this experiment showed that only *Lin28* expression was significantly decreased ($p < 0.02$) in cells treated with RO3306 in proliferative conditions compared to non-treated proliferating TS cells. Unexpectedly, *Sox2* and *Nr0b1* were not down-regulated when TS cells are differentiated by RO3306 in the presence of FGF4. These data suggests that *Lin28* must be down-regulated in order for TS cells to differentiate into TGCs. Additionally, these data suggests that *Lin28* expression is not driven by FGF4 signaling, but *Sox2* and *Nr0b1* are.

Immunohistochemical investigation of LIN28 in TS cells was performed to localize LIN28 protein in proliferating TS cells and differentiated TGCs. In proliferating TS cells LIN28 is localized primarily to the cytoplasm of the cells. Unexpectedly, in TGCs LIN28 is still expressed in the cytoplasm, and also is expressed in the nucleus. There is a significant increase in the nuclear localization of LIN28 in TGCs compared to proliferating TS cells. LIN28 is predominantly a cytoplasmic protein (Moss, 2003),

suggesting nuclear sequestering of LIN28 in TGCs is important for TGC formation. Nuclear localization of LIN28 may be necessary to prevent LIN28 miRNA binding function, allowing for the processing and accumulation of mature Let-7 miRNAs, and differentiation into TGCs.

LIN28 expression was examined in E12.5 mouse placental sections. LIN28 expression had previously been identified in primary TGCs surrounding the embryo and in the ectoplacental cone at E7.5 (Moss, 2003). As expected, LIN28 was localized to the TGC layer of the mature placenta at E12.5, which is analogous to TGCs formed in culture. Additionally, LIN28 was localized to the labyrinth layer of the placenta and to the maternal decidua. These data suggests LIN28 is not only functioning in TGCs, but also may play a role in the labyrinth layer. IFG-2 is an important regulator of branching morphology in the labyrinth layer (Han & Carter, 2000). LIN28 has been shown to increase translational efficiency of *Igf-2* transcript in muscle cells (Polesskaya et al., 2007), and it is possible that a similar relationship occurs in the labyrinth layer of the placenta.

LIN28 is a known regulator of Let-7 miRNAs in ES cells, suppressing the accumulation of mature Let-7s and preventing differentiation (Viswanathan, 2007). The Let-7 family of miRNAs, along with other miRNAs, were examined in proliferating TS cells and differentiated TGCs. Global Pattern Recognition (GPR) software provides a conservative analysis of significant differences between two samples, as well as fold changes using multiple normalizers within the data set (Bouma, et. al 2004; Akilesh, et. al, 2003). When comparing differentiated TGCs to proliferating TS cells, GPR analysis revealed 28 miRNAs that are significantly differentially expressed. 19 of these are up-

regulated in TGCs, including 7 of the 8 members of the Let-7 family. 9 miRNAs are down-regulated in TGCs, including the miR-290 family members: miR-290-3p, miR-290-5p, miR-291a-3p, miR-292-3p, miR-293, miR-294, and miR-295. MiR-290 family members have previously been identified in ES cells and regulate ES cell proliferation at the G1/S phase, targeting p21 and CDK2/Cyclin E mRNA, promoting proliferation (Card et al., 2008; Wang and Blelloch, 2009). Discovery of the miR-290 family expression in TS cells suggests that TS cells have conserved mechanisms controlling cell proliferation to ES cells. Finding the Let-7 family up-regulated in TGCs is not surprising, as the Let-7 miRNAs are up-regulated in many differentiated tissue types, and confirms findings by others showing up-regulation of Let-7 miRNAs in differentiated TGCs (Viswanathan et al., 2009). The Let-7 miRNAs in TGCs show an opposite expression pattern to *Lin28* expression in TS cells, suggesting LIN28 is acting in proliferating TS cells to prevent the accumulation of mature Let-7's thus preventing differentiation. Other miRNAs of interest that were up-regulated in TGCs as are miR-29a, miR-22 and miR-376b, and confirms recent data by Viswanathan (Viswanathan et al., 2009). These miRNAs are involved in suppressing proliferation factors as predicted targets include several cyclins, Rab, and CDK6 (www.targetscan.org). Future studies involving inhibition or overexpression of these miRNAs will reveal their role in TGC formation.

To determine the role of *Lin28* in TS cell proliferation and differentiation, *Lin28* was knocked down using shRNA lentiviral technology. TS cells infected with shRNA against *Lin28* were selected for genomic incorporation using puromycin. Successful knockdown of *Lin28* and a non-coding scramble shRNA control were assessed by real time PCR. Effects on TS cell proliferation and differentiation were assessed by

examining morphological changes. It was expected that loss of *Lin28* would cause TS cells to differentiate into TGCs due to an accumulation of Let-7 miRNAs. *Lin28* knockdown in TS cells had no noticeable effect on proliferation or differentiation. This suggests that TS cells have additional mechanisms of preventing their differentiation into TGCs. Ongoing studies are currently being performed to determine Let-7 miRNA expression in *Lin28* knockdown TS cells. It is possible that there are alternative pathways of preventing mature Let-7 accumulation in TS cells, or that loss of *Lin28* alone is not sufficient to cause differentiation into TGCs.

AP-2γ is of known significance in trophoblast specification and maintenance (Auman et al., 2002; Kuckenberg et al., 2010). AP-2γ knockout mice have an embryonic lethality due to a severely disrupted extra-embryonic portion of the developing embryo (Auman et al., 2002). AP-2α has been identified in trophectoderm *in vivo* (Auman et al., 2002), however AP-2α knockout mice do not have a placental phenotype (Talbot et al., 1999). A tamoxifen-inducible Cre, AP-2γ floxed TS cell line was created to delete AP-2γ and study defects that occurred as a result of the deletion. All AP-2 family members were examined to determine which AP-2 family members are expressed in wild type TS cells. Analysis of all AP-2 family members in wild type TS cells as they differentiate into TGCs showed a significantly higher expression of *AP-2γ* at every time point analyzed. *AP-2α* also was present, and its expression increased as TS cells differentiated into TGCs. No other AP-2 family members were detected by real-time PCR at any time point analyzed. These data suggests that AP-2γ is the major AP-2 family member involved in TS cell proliferation and differentiation, but that AP-2α may be involved with TGC formation.

When AP-2γ null TS cells are cultured under proliferative conditions they largely resemble their wild type counterparts. These cells grow in colonies and proliferate at a rate similar to wild type TS cells. However, when 70% conditioned media, FGF4 and heparin sulfate are removed from the culture conditions, AP-2γ null TS cells continue to proliferate rather than differentiate into TGCs. Proliferation of AP-2γ null TS cells in the absence of proliferation media can be maintained for over 10 passages. Due to the defect in differentiation into TGCs, expression of *Lin28, Sox2, Nr0b1* and *Igfbp7* was examined in AP-2γ null TS cells in proliferation and differentiation media.

Real time PCR analysis revealed that AP-2γ null TS cells have sustained high expression of *Lin28, Sox2,* and *Nr0b1* after the removal of FGF4. AP-2γ null TS cells also have consistently high expression of *Igfbp7,* although without a better understanding of its function in the trophoblast, it is difficult to speculate as to the role of *Igfbp7* in AP-2γ null TS cells. When AP-2γ null TS cells are forced to endoreduplicate by adding RO3306 they undergo apoptosis. Cells which are not programmed to endoreduplicate, such as ES cells, will undergo apoptosis when exposed to RO3306 (Ullah et al., 2008). Undergoing apoptosis when exposed to RO3306 suggests that AP-2γ null TS cells have lost the ability to undergo endoreduplication.

This study demonstrated that *Lin28* must be down-regulated in order for TS cells to endoreduplicate. AP-2γ null TS cells fail to down regulate *Lin28* when proliferation conditions are removed from TS cell culture. To further assess LIN28 sub-cellular localization in AP-2γ null TS cells, immunohistochemistry was performed on AP-2γ null TS cells under proliferative and differentiation conditions. An increase in number of cells exhibiting nuclear localization of LIN28 was observed under proliferative

conditions when compared to wild type TS cells grown under the same conditions. The percent of cells with nuclear localization of LIN28 was similar to wild type TGCs, suggesting a misregulation of LIN28 at the protein level in AP-2γ null TS cells. Increased nuclear localization of LIN28 in AP-2γ null TS cells suggests an increase in intracellular trafficking of LIN28.

MiRNA expression data for AP-2γ null TS cells was obtained and showed differential expression of miRNAs under proliferation and differentiation conditions. AP-2γ null TS cells under proliferative conditions did not show any significant differences compared to wild type TS cells grown under the same conditions. However, when expression of miRNAs in AP-2γ null TS cells were compared grown under proliferative and differentiation conditions there was a significant increase in expression of the miR-302 cluster and miR-99a and b in cells grown without FGF4 (necessary for TS cell maintenance). When FGF4 was removed from AP-2γ null TS cells it resulted in significant up-regulation of miR-367, miR-302a, miR-302b and miR-302c, all members of ES cell specific miRNAs that are regulated by *Sox2*, *Oct4* and *Nanog* (Card et al., 2008). Potential targets of the miR-302 family include several growth signaling receptors, such as TGFBR2 and others (www.targetscan.org). Additionally, miR-99a and miR-99b were significantly up-regulated in AP-2γ null TS cells cultured without FGF4. These two miRNAs are part of the miR-125 cluster which is implicated in ES cell proliferation and many invasive cancers (Liu et al., 2009), and targets signaling cascades such as BMP signaling (www.targetscan.org). Findings by others have implicated a significant down-regulation of the entire miR-302 family of miRNAs when ES cells are forced to a TS cell specification using iRas induction of TS cell fate (Viswanathan et al.,

2009). The findings of this study show that the miR-302 miRNAs are significantly up-regulated when AP-2γ and FGF4 are removed from TS cells. This significant increase in ES cell specific miRNAs in AP-2γ null TS cells cultured without FGF4 suggests that FGF4 may be the last component keeping AP-2γ null TS cells in a TS cell like fate, and removal of FGF4 from the culture conditions allows for adaptation of an ES cell-like fate.

The miR-302 family is regulated by *Oct-4, Nanog,* and *Sox2* (Card et al., 2008), having already analyzed *Sox2* expression in AP-2γ null TS cells, expression of pluripotency markers *Nanog, Oct4* and *Stella* was analyzed. Analysis of ES cell markers in AP-2γ null TS cells showed a significant increase in expression of *Oct4, Stella* and *Nanog* when compared to wild type TS cells. *Oct4* and *Nanog* are the first genes to be down regulated upon TS cell specification, with expression only remaining in the inner cell mass where they are required for ES cell maintenance (Cross, 2000). Additionally, AP-2γ has been shown to repress *Nanog* expression in TS cells (Kuckenberg et al., 2010). In this study, it has demonstrated that loss of AP-2γ leads to an increased expression of *Nanog,* confirming that AP-2γ and *Nanog* have an interaction. *Stella* is a germ cell marker regulated by *Oct4* and *Nanog*, and has been identified in human ES cells as a marker of pluripotency (Zuccotti et al., 2009; Dowles et al., 2003). Increased expression of *Oct4, Nanog,* and *Stella* in AP-2γ null TS cells suggest AP-2γ null TS cells resemble ES cells in gene expression profile.

This study has demonstrated that AP-2γ null TS cells are incapable of differentiating to TGCs and when forced to endoreduplicate using RO3306 AP-2γ null TS cells undergo cell death, similar to ES cells. AP-2γ has been shown to bind to the promoter region of *Lin28* in TS cells (Kidder & Palmer, 2010; Winger, unpublished

data). To determine the interaction between AP-2γ and LIN28 and its role in TS cell differentiation into TGCs, *Lin28* was knocked down in AP-2γ null TS cells using shRNA lentiviral particles. 88% knockdown was obtained when compared to a non-coding shRNA transfection control. When cultured under proliferation conditions, AP-2γ null TS cells with *Lin28* knockdown spontaneously formed TGC-like cells at the edges of colonies. TGCs or TGC-like cells have never before been observed in AP-2γ null TS cells in over 50 passages. When AP-2γ null TS cells with *Lin28* knockdown were grown under differentiation conditions, TGC-like cells formed, although not all cells differentiated into TGC-like cells. Most astonishingly, when AP-2γ null TS cells with *Lin28* knockdown were cultured under proliferating conditions with RO3306 they did not undergo immediate cell death, but also failed to proliferate or differentiate into TGC-like cells. This failure of immediate cell death in AP-2γ null, *Lin28* knockdown cells, indicates that loss of *Lin28* in these cells allows for differentiation, but may not allow for endoreduplication to occur, evidenced by TGC morphology under differentiation conditions, but lack of TGC morphology when forced to endoreduplicate. These data suggest that *Lin28* must be down regulated in order for TGCs or TGC-like cells to form. Additionally, loss of *Lin28* in AP-2γ null TS cells led to a diminished lifespan, with cells not proliferating or surviving for more than two passages. Loss of AP-2γ or *Lin28* alone had no affect on proliferation or lifespan. This reduced ability to proliferate indicates AP-2γ and *Lin28* together are necessary for survival and proliferation of TS cells. These data also suggest that there is a necessary interaction between AP-2γ and LIN28 needed for the formation of TGCs, and in AP-2γ null TS cells this interaction is obliterated and forced removal of *Lin28* is needed to allow TGC-like cell formation.

This study has shown that *Lin28* and AP-2γ are important for TS cell maintenance and TGC formation in the mouse. However, mechanisms observed in the mouse may not always provide an accurate depiction of what is occurring in the human. It is important to identify if *Lin28* and AP-2γ have conserved roles in human placental development. This study has identified LIN28 and AP-2γ in human placental cells. Using a first trimester villous cell line, ACH3P, AP-2γ and LIN28 proteins were assessed for localization in proliferating ACH3P cells. Unexpectedly, AP-2γ was found in the cytoplasm of the ACH3P cells. AP-2γ is normally a nuclear transcription factor, but has been shown to be sequestered to the cytoplasm when being suppressed in some cancers (Aqeilan et al., 2004), and has been shown to shuttle between the nucleus and cytoplasm in trophoblast cells (El-Hashash and Kimber, 2006). Sequestering AP-2γ to the cytoplasm may be a result of the fusion of human cells with a choriocarcinoma, although further investigation into the role of AP-2γ in ACH3P cells is needed to determine if AP-2γ function is suppressed. LIN28 was identified in the cytotrophoblast and syncitiotrophoblast of the first trimester human placenta. It is possible that LIN28 is expressed in the more differentiated syncytiotrophoblast and plays a role in increasing IGF-2 translational efficiency, which is necessary for growth of the placenta. These preliminary results suggest LIN28 and AP-2γ have a conserved role in human trophoblast proliferation. Further investigation of LIN28 and AP-2γ in the human may lead to a better understand of the defects seen in proliferation and differentiation in pre-eclamptic pregnancies.

Conclusions:

This study has tested the hypothesis that there is a necessary interaction between AP-2γ and *Lin28* which allows for TS cell differentiation into TGCs, and shown a specific role for *Lin28* and AP-2γ in mouse trophoblast cells using TS cells in culture. It has been demonstrated that *Lin28* must be down regulated in order for differentiation to progress, and TGC formation to occur, and is regulated by a mechanism separate from *Sox2* and *Nr0b1*. *Sox2* and *Nr0b1* are regulated by FGF4 signaling, as shown by the maintained expression of *Sox2* and *Nr0b1* in cells differentiated by RO3306 in the presence of FGF4. In instances where *Lin28* is misregulated, such as in AP-2γ null TS cells, TGC formation is inhibited. Although it has been shown that AP-2γ binds to the *Lin28* promoter in mouse TS cells (Kidder & Palmer, 2010; Winger, unpublished data), this study reveals a necessary interaction between AP-2γ and *Lin28* in order forts cells to differentiate into TGCs. It has been verified that AP-2γ is essential for TS cell maintenance by showing that loss of AP-2γ in TS cells leads to increased expression of ES cell markers such as *Nanog, Oct4* and *Stella,* and an increase of ES cell specific miRNAs including the miR-302 cluster. Additionally, this study shows that AP-2γ and LIN28 proteins are expressed in both a human extravillous cell line and first trimester villous placental tissue, suggesting a conserved role for LIN28 and AP-2γ in human trophoblast cell proliferation and differentiation. A model is proposed in which AP-2γ must be present, and *Lin28* must decrease in expression in order for TS cells to differentiate into TGCs (Figure 19). This mechanism suggests loss of AP-2γ from TS cells leads to an ES cell-like state, where the ES cell specific markers *Nanog, Oct4, Stella,* and the miR-302 family become expressed (Figure 19). It also suggests that loss

of AP-2γ leads to failure to differentiate into TGCs, but that this loss is rescued when *Lin28* is knocked down (Figure 19).

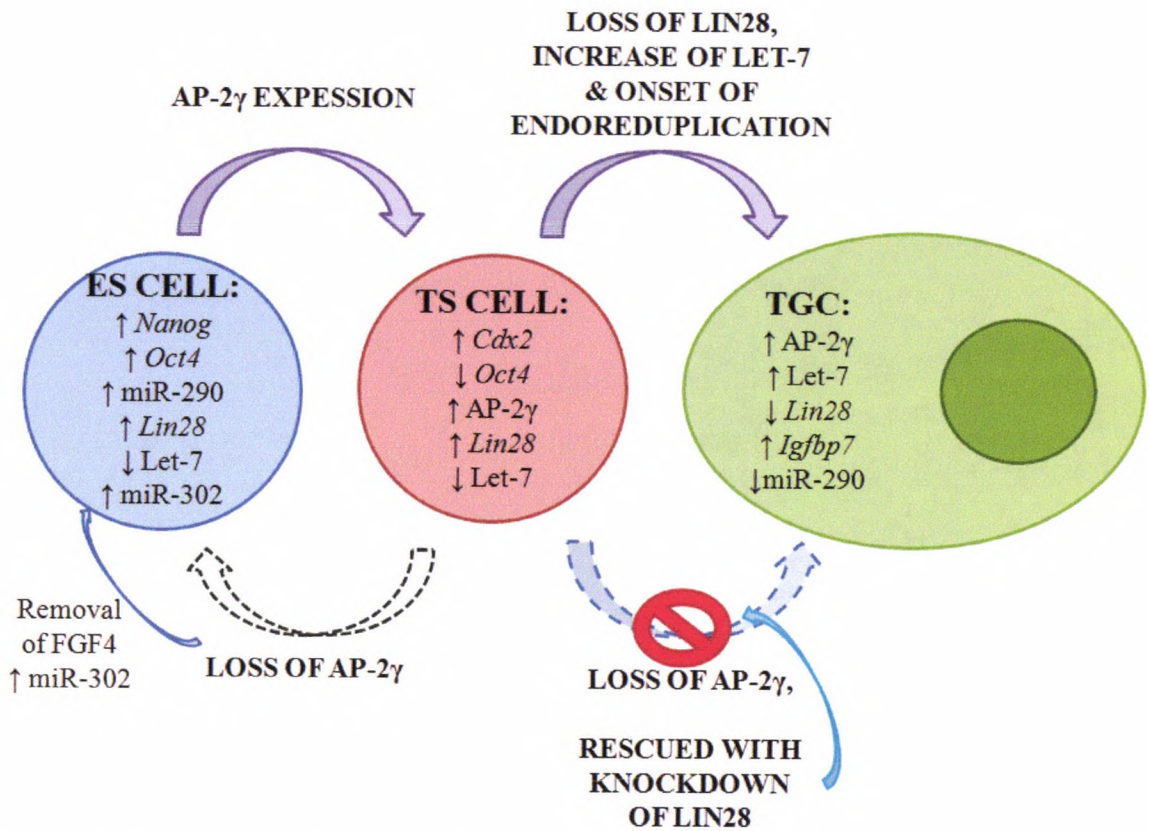

Figure 19: Proposed mechanism of TS cell differentiation and "de-differentiation" upon loss of AP-2γ. AP-2γ is necessary for the formation of TGCs; loss of AP-2γ leads to a failure to form TGCs, but TGC formation is restored in AP-2γ null TS cells with *Lin28* knockdown. Additionally, *Lin28* must decrease in expression for TGCs to form, along with an observed increase in Let-7 miRNA expression. Loss of AP-2γ in TS cells leads to a "de-differentiated" state, and includes an increase of expression of pluripotency markers *Nanog, Oct4, Stella*, and, when FGF4 is removed, the miR-302 family. Taken together this mechanism shows a necessary interaction between AP-2γ and LIN28 for the formation of TGCs.

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APPENDIX I: Taqman Probes Used in This Study

Taqman Probes Used in This Study		
Gene Name	Probe Number	Notes
Dppa3 (Stella)	Mm01184198_g1	
Gapdh	Mm99999915_g1	
Igfbp7	Mm00514987_m1	
Lin28	Mm00524077_m1	
Nanog	Mm01617761_g1	
Nr0b1 (Dax1)	Mm03053914_s1	
Pou5f1 (Oct4)	Mm03053917_g1	
Sox2	Mm00488369_s1	
Tcfap2a (AP-2α)	Mm00495574_m1	
Tcfap2b (AP-2β)	Mm00493468_m1	
Tcfap2c (AP-2γ)	Mm01242216_m1	Spans exons 2/3
Tcfap2c (AP-2γ)	Mm00493474_m1	Spans exons 5/6
***Tcfap2c* (AP-2γ)**	Mm00493474_m1	Spans exons 6/7
Tcfap2d (AP-2δ)	Mm00462523_m1	
Tcfap2e (AP-2ε)	Mm01179788_g1	

Bold indicates probe used to detect deletion of conditional allele in AP-2γ null TS cells

APPENDIX II: GPR Analysis of miRNAs in Wild Type TS Cells

miRNA	Fold Change	GPR Score
mmu-miR-376b	36.0	0.882
mmu-let-7f	17.2	0.873
mmu-miR-98	17.4	0.821
mmu-let-7d	13.4	0.807
mmu-miR-293	-9.4	0.787
mmu-let-7c	10.7	0.784
mmu-let-7g	14.6	0.781
mmu-let-7e	9.4	0.764
mmu-miR-322	8.8	0.746
mmu-miR-292-3p	-7.7	0.697
mmu-miR-28	9.3	0.695
mmu-let-7a	7.7	0.680
mmu-let-7i	10.5	0.666
mmu-miR-291a-3p	-6.7	0.657
mmu-miR-10b	9.3	0.631
mmu-miR-294	-7.1	0.620
mmu-miR-323-5p	-5.0	0.614
mmu-miR-451	5.4	0.545
mmu-miR-130a	-5.0	0.539
mmu-miR-30a	5.9	0.522
mmu-miR-370	3.6	0.519
mmu-miR-290-3p	-5.7	0.516
mmu-miR-290-5p	-5.8	0.473
mmu-miR-22	4.9	0.447
mmu-miR-221	3.3	0.429
mmu-miR-183	4.1	0.412
mmu-miR-29a	3.1	0.406
mmu-miR-295	-6.0	0.406
mmu-miR-302d	-6.2	0.386
mmu-miR-450b-5p	3.5	0.386
mmu-miR-500	2.4	0.372
mmu-miR-542-5p	3.0	0.372
mmu-miR-30d	2.7	0.363
mmu-miR-467h	-2.9	0.360
mmu-miR-23a	2.7	0.354
mmu-miR-204	4.4	0.343

mmu-miR-126-5p	-2.8	0.334
mmu-miR-343	-3.9	0.334
mmu-miR-450a-5p	2.5	0.334
mmu-miR-291b-3p	-4.8	0.331
mmu-miR-23b	2.1	0.314
mmu-miR-107	-2.6	0.314
mmu-miR-196a	-5.0	0.311
mmu-miR-495	2.1	0.303
mmu-miR-362-5p	-3.0	0.300
mmu-miR-224	5.0	0.294
mmu-miR-292-5p	-2.6	0.294
mmu-miR-151-3p	2.1	0.291
mmu-miR-126-3p	-3.1	0.288
mmu-miR-29c	2.2	0.285
mmu-miR-142-5p	2.9	0.285
mmu-miR-362-3p	-2.5	0.282
mmu-miR-10a	4.1	0.277
mmu-miR-532-3p	-2.9	0.274
mmu-miR-450b-3p	1.9	0.262
mmu-miR-466h	-2.7	0.262
mmu-miR-669d	-2.7	0.262
mmu-miR-291a-5p	-2.0	0.259
mmu-miR-363	2.3	0.254
mmu-miR-466j	-2.7	0.254
mmu-miR-215	4.8	0.251
mmu-miR-325	9.2	0.251
mmu-miR-539	8.8	0.251
mmu-miR-24	1.9	0.248
mmu-miR-155	9.2	0.248
mmu-miR-503	4.5	0.239
mmu-miR-665	-2.1	0.239
mmu-miR-182	1.8	0.236
mmu-miR-654-5p	-2.5	0.236
mmu-miR-219	1.8	0.233
mmu-miR-466d-3p	-2.3	0.231
mmu-miR-146a	2.0	0.228
mmu-miR-152	1.9	0.225
mmu-miR-337-3p	2.8	0.225
mmu-miR-193b	1.6	0.222

mmu-miR-122	-2.2	0.216
mmu-miR-146b	-2.1	0.216
mmu-miR-181a	1.8	0.216
mmu-miR-425	3.6	0.216
mmu-miR-494	1.9	0.216
mmu-miR-15b	-2.5	0.213
mmu-miR-193	1.8	0.213
mmu-miR-291b-5p	-1.9	0.213
mmu-miR-466f	-2.3	0.210
mmu-miR-132	2.7	0.202
mmu-miR-210	-1.4	0.202
mmu-miR-223	-2.2	0.202
mmu-miR-338-3p	3.6	0.202
mmu-miR-466e-3p	-2.2	0.202
mmu-miR-297b-5p	-1.8	0.199
mmu-miR-21	1.7	0.196
mmu-miR-34c	-1.6	0.196
mmu-miR-300	-2.0	0.196
mmu-miR-667	-2.1	0.193
Mouse U6 snRNA	-2.9	0.193
mmu-miR-19a	3.2	0.190
mmu-miR-33	-2.9	0.190
mmu-miR-467b	-2.6	0.190
mmu-miR-7a	1.5	0.184
mmu-miR-7b	1.7	0.184
mmu-miR-125a-3p	-1.6	0.184
mmu-miR-151-5p	1.6	0.184
mmu-miR-197	-1.6	0.184
mmu-miR-92b	-1.5	0.182
mmu-miR-181b	1.9	0.182
mmu-miR-206	-1.5	0.179
mmu-miR-297a	-1.9	0.176
mmu-miR-297b-3p	-1.9	0.176
mmu-miR-669h-3p	-2.0	0.176
mmu-miR-139-3p	-1.7	0.173
mmu-miR-328	1.3	0.173
mmu-miR-26b	6.6	0.170
mmu-miR-29b	3.8	0.170
mmu-miR-455	-1.6	0.167

mmu-miR-25	-1.4	0.164
mmu-miR-92a	1.4	0.164
mmu-miR-93	-1.3	0.164
mmu-miR-99b	1.1	0.164
mmu-miR-129-5p	-2.5	0.164
mmu-miR-185	1.3	0.164
mmu-miR-296-3p	1.5	0.164
mmu-miR-465a-5p	1.4	0.164
mmu-miR-669a	-1.9	0.164
mmu-miR-16	-1.1	0.161
mmu-miR-20b	1.9	0.161
mmu-miR-381	-1.9	0.161
mmu-miR-191	1.3	0.159
mmu-miR-220	-1.4	0.159
mmu-miR-433	-1.5	0.159
mmu-miR-466b-3p	-2.1	0.159
mmu-miR-100	-1.1	0.156
mmu-miR-208a	2.2	0.156
mmu-miR-449b	-2.3	0.156
mmu-miR-465b-3p	-1.4	0.156
mmu-miR-532-5p	-1.9	0.156
mmu-miR-543	1.8	0.156
mmu-miR-27b	2.0	0.153
mmu-miR-30c	1.5	0.153
mmu-miR-449a	-2.2	0.153
mmu-miR-34a	-1.3	0.150
mmu-miR-106b	1.0	0.150
mmu-miR-188-5p	1.5	0.150
mmu-miR-669b	-1.9	0.150
mmu-miR-376a	2.8	0.147
mmu-miR-467c	-1.8	0.147
mmu-miR-669c	-2.2	0.147
mmu-miR-431	1.2	0.144
mmu-miR-466b-3-3p	-1.7	0.144
mmu-miR-466c-5p	-2.4	0.144
mmu-miR-544	-1.5	0.144
mmu-miR-669e	-1.5	0.144
RNU43 snoRNA	-2.1	0.144
mmu-miR-103	1.3	0.141

mmu-miR-140	-1.9	0.141
mmu-miR-323-3p	1.2	0.141
mmu-miR-487b	-1.2	0.141
mmu-miR-490	-1.8	0.141
mmu-miR-541	-1.1	0.141
mmu-miR-551b	1.3	0.141
mmu-miR-125a-5p	-1.1	0.138
mmu-miR-130b	-1.6	0.138
mmu-miR-148a	-1.6	0.138
mmu-miR-298	-1.0	0.138
mmu-miR-351	4.5	0.138
mmu-miR-30b	1.9	0.135
mmu-miR-200c	1.4	0.135
mmu-miR-409-3p	-1.5	0.135
mmu-miR-133b	-1.4	0.133
mmu-miR-188-3p	1.3	0.133
mmu-miR-378	1.2	0.133
mmu-miR-467a	-3.0	0.133
mmu-miR-504	-2.0	0.133
mmu-miR-199b	-1.4	0.130
mmu-miR-205	3.6	0.130
mmu-miR-207	-1.3	0.130
mmu-miR-465c-3p	1.4	0.130
mmu-miR-466f-5p	-4.3	0.130
mmu-miR-669g	-1.7	0.130
mmu-miR-186	-1.1	0.127
mmu-miR-194	1.0	0.127
mmu-miR-331-3p	1.2	0.127
mmu-miR-380-3p	-1.2	0.127
mmu-miR-652	1.1	0.127
mmu-miR-99a	1.2	0.124
mmu-miR-125b-3p	-2.0	0.124
mmu-miR-138	1.0	0.124
mmu-miR-149	1.0	0.124
mmu-miR-195	-1.5	0.124
mmu-miR-320	-1.2	0.124
mmu-miR-484	-1.1	0.124
mmu-miR-297c	-1.3	0.121
mmu-miR-367	-2.2	0.121

mmu-miR-383	-1.7	0.121
mmu-miR-466e-5p	-2.0	0.121
mmu-miR-467f	-1.6	0.121
mmu-miR-501-3p	-1.9	0.121
mmu-miR-335-3p	-1.3	0.118
mmu-miR-135a	2.3	0.115
mmu-miR-452	-1.2	0.115
mmu-miR-467e	-1.4	0.115
mmu-miR-19b	2.5	0.112
mmu-miR-105	-1.5	0.112
mmu-miR-125b-5p	1.3	0.112
mmu-miR-128	-1.2	0.112
mmu-miR-214	-1.2	0.112
mmu-miR-434-3p	-1.4	0.112
mmu-miR-582-3p	2.5	0.112
mmu-miR-142-3p	-1.1	0.110
mmu-miR-148b	-1.3	0.110
mmu-miR-296-5p	1.9	0.110
mmu-miR-324-5p	-1.1	0.110
mmu-miR-375	-1.2	0.110
mmu-miR-471	-1.1	0.110
mmu-miR-483	1.2	0.110
mmu-miR-511	-1.5	0.110
mmu-miR-106a	-1.2	0.107
mmu-miR-192	-1.1	0.107
mmu-miR-212	1.1	0.107
mmu-miR-326	-1.2	0.107
mmu-miR-147	2.2	0.104
mmu-miR-199a-5p	-1.1	0.104
mmu-miR-339-5p	-1.8	0.104
mmu-miR-466i	-2.0	0.104
mmu-miR-499	-1.6	0.104
mmu-miR-666-5p	-2.2	0.104
mmu-let-7b	1.0	0.101
mmu-miR-202-3p	1.3	0.101
mmu-miR-488	-1.8	0.101
mmu-miR-615-3p	-1.1	0.101
mmu-miR-141	1.1	0.098
mmu-miR-335-5p	-2.3	0.098

mmu-miR-342-3p	-1.7	0.098
mmu-miR-465c-5p	-1.1	0.098
mmu-miR-505	-2.9	0.098
mmu-miR-547	2.7	0.098
mmu-miR-466a-5p	-1.3	0.095
mmu-miR-216b	1.2	0.092
mmu-miR-299	1.2	0.092
mmu-miR-350	-1.3	0.092
mmu-miR-465b-5p	1.4	0.092
mmu-miR-466c-3p	-2.2	0.092
mmu-miR-27a	-1.1	0.089
mmu-miR-145	-1.0	0.089
mmu-miR-181d	1.2	0.089
mmu-miR-217	1.9	0.089
mmu-miR-338-5p	1.3	0.089
mmu-miR-434-5p	1.0	0.089
mmu-miR-453	-1.5	0.089
mmu-miR-466k	-2.2	0.089
mmu-miR-501-5p	-1.2	0.089
Hm/Ms/Rt U1 snRNA	-1.7	0.089
mmu-miR-184	-1.7	0.086
mmu-miR-301a	-1.1	0.086
mmu-miR-302b	2.4	0.086
mmu-miR-365	2.3	0.086
mmu-miR-409-5p	-2.4	0.086
mmu-miR-423-3p	-1.4	0.086
mmu-miR-470	1.6	0.086
mmu-miR-582-5p	1.1	0.086
mmu-miR-31	-1.4	0.084
mmu-miR-139-5p	1.8	0.084
mmu-miR-384-5p	1.2	0.084
mmu-miR-450a-3p	-1.6	0.084
mmu-miR-34b-5p	-1.3	0.081
mmu-miR-124	-1.1	0.081
mmu-miR-143	5.3	0.081
mmu-miR-200a	1.5	0.081
mmu-miR-216a	1.7	0.081
mmu-miR-324-3p	-1.2	0.081
mmu-miR-374	1.3	0.081

mmu-miR-449c	-1.5	0.081
mmu-miR-574-3p	1.0	0.081
mmu-miR-668	-1.2	0.081
mmu-miR-18b	1.9	0.078
mmu-miR-26a	1.5	0.078
mmu-miR-345-5p	-1.1	0.078
mmu-miR-346	-1.1	0.078
mmu-miR-410	1.6	0.078
mmu-miR-329	-2.8	0.075
mmu-miR-342-5p	-1.1	0.075
mmu-miR-485	-1.4	0.075
mmu-miR-486	-1.1	0.075
mmu-miR-540-3p	1.4	0.075
mmu-miR-337-5p	-1.1	0.072
mmu-miR-361	-1.1	0.072
mmu-miR-369-3p	4.8	0.072
mmu-miR-421	-1.1	0.072
mmu-miR-133a	-1.1	0.069
mmu-miR-302c	-1.4	0.069
mmu-miR-412	1.0	0.069
mmu-miR-423-5p	-1.3	0.069
mmu-miR-466a-3p	-1.3	0.069
mmu-miR-466d-5p	-2.5	0.069
mmu-miR-496	-1.1	0.069
mmu-miR-546	-1.0	0.069
mmu-miR-574-5p	-1.8	0.069
mmu-miR-598	-2.4	0.069
mmu-miR-666-3p	-1.8	0.069
mmu-miR-187	1.7	0.066
mmu-miR-196b	-1.0	0.066
mmu-miR-330	-3.8	0.066
mmu-miR-345-3p	-1.2	0.066
mmu-miR-468	-1.4	0.066
mmu-miR-200b	2.0	0.063
mmu-miR-211	-1.1	0.063
mmu-miR-20a	1.2	0.061
mmu-miR-154	-1.2	0.061
mmu-miR-382	-1.1	0.061
mmu-miR-540-5p	-1.4	0.061

mmu-miR-9	2.5	0.058
mmu-miR-199a-3p	-1.1	0.058
mmu-miR-201	1.2	0.058
mmu-miR-654-3p	1.0	0.058
mmu-miR-150	1.3	0.055
mmu-miR-411	-1.2	0.055
mmu-miR-18a	1.0	0.052
mmu-miR-96	2.0	0.052
mmu-miR-101b	1.1	0.052
mmu-miR-302a	2.4	0.052
mmu-miR-469	1.1	0.052
mmu-miR-489	-1.4	0.052
mmu-miR-135b	2.4	0.049
mmu-miR-331-5p	-1.2	0.049
mmu-miR-465a-3p	1.3	0.049
mmu-miR-466f-3p	-1.6	0.049
mmu-miR-429	1.1	0.046
mmu-miR-15a	1.7	0.043
mmu-miR-222	1.8	0.043
mmu-miR-344	-1.1	0.043
mmu-miR-380-5p	-1.8	0.043
mmu-miR-497	-6.0	0.043
mmu-miR-466g	-1.5	0.040
mmu-miR-467d	-1.8	0.040
mmu-miR-134	1.2	0.037
mmu-miR-327	-1.6	0.037
mmu-miR-379	-4.8	0.037
mmu-miR-136	1.5	0.035
mmu-miR-203	2.3	0.035
mmu-miR-340-3p	2.7	0.035
mmu-miR-542-3p	8.3	0.035
mmu-miR-466l	-1.3	0.032
mmu-miR-101a	1.8	0.029
mmu-miR-208b	-1.7	0.029
mmu-miR-448	1.0	0.026
mmu-miR-466b-5p	-2.8	0.026
mmu-miR-491	1.2	0.026
mmu-miR-17	1.4	0.023
mmu-miR-34b-3p	-1.8	0.023

mmu-miR-341	1.0	0.023
mmu-miR-129-3p	-2.8	0.020
mmu-miR-467g	-1.1	0.017
mmu-miR-509-5p	-1.3	0.017
mmu-miR-30e	10.0	0.014
mmu-miR-218	1.2	0.014
mmu-miR-592	2.0	0.014
mmu-miR-190b	4.8	0.012
mmu-miR-32	-1.3	0.009
mmu-miR-144	-1.1	0.009
mmu-miR-181c	3.1	0.006
mmu-miR-127	-1.3	0.003
mmu-miR-301b	-1.4	0.003
mmu-miR-493	3.0	0.000
mmu-miR-615-5p	5.2	0.000
mmu-miR-669f	1.1	0.000
mmu-miR-1	14.6	N.S.
mmu-miR-1-2-as	1.4	N.S.
mmu-miR-137	-1.3	N.S.
mmu-miR-153	-2.7	N.S.
mmu-miR-190	3.3	N.S.
mmu-miR-202-5p	5.7	N.S.
mmu-miR-339-3p	3.5	N.S.
mmu-miR-340-5p	7.7	N.S.
mmu-miR-369-5p	-2.0	N.S.
mmu-miR-376c	1.5	N.S.
mmu-miR-377	-2.0	N.S.
mmu-miR-384-3p	1.3	N.S.
mmu-miR-463	4.3	N.S.
mmu-miR-464	-1.1	N.S.
mmu-miR-509-3p	7.8	N.S.
mmu-miR-568	-1.1	N.S.
mmu-miR-590-3p	9.2	N.S.
mmu-miR-590-5p	1.5	N.S.
mmu-miR-653	-1.1	N.S.

MiRNAs sorted by GPR score from high to low. GPR scores of 0.40 or higher are considered to be significantly different. Positive fold changes correspond to miRNAs up-regulated in TGCs, negative fold changes correspond to miRNAs down-regulated in TGCs.

APPENDIX III: Potential miRNA Targets

miRNAs Differentially Expressed in TGCs Cells		
miRNA	**Fold Change**	**Potential TSC Related Targets**
miR-376b	36.0	
miR-98	17.4	*Hmga2, Lin28, Hand1, Fgf4, Il6, Tead4*
let-7f	17.2	*Lin28, Hmga2*
let-7g	14.6	*Lin28, Hmga2*
let-7d	13.4	*Lin28, Hmga2*
let-7c	10.7	*Lin28, Hmga2*
let-7i	10.5	*Lin28, Hmga2*
let-7e	9.4	*Lin28, Hmga2*
miR-28	9.3	*Dnmt3a*
miR-10b	9.3	*AP-2γ*
miR-322	8.8	*Lin28, Hmga2*
let-7a	7.7	*Lin28, Hmga2*
miR-30a	5.9	*Lin28*
miR-451	5.4	
miR-22	4.9	*Dnmt3a*
miR-183	4.1	
miR-370	3.6	*Lin28*
miR-221	3.3	
miR-29a	3.1	*Vegfa, Igf1, Eomes, AP-2γ*
miR-323-5p	-5.0	
miR-130a	-5.0	*Tnfα*
miR-290-3p	-5.7	*CyclinG2*
miR-290-5p	-5.8	*CyclinG2*
miR-295	-6.0	*CyclinG2*
miR-291a-3p	-6.7	*CyclinG2*
miR-294	-7.1	*CyclinG2*
miR-292-3p	-7.7	*CyclinG2*
miR293	-9.4	*CyclinG2*

Potential target genes of the miRNAs differentially expressed in TS cells vs. TGCs. Positive fold change indicates up-regulated in TGCs, negative fold change indicates down-regulated in TGCs. MiRNAs without targets indicated do not have predicted targets of relevance to TS cells or TGCs as we understand them today.