DISSERTATION

ONCOFETAL PROTEINS REGULATE PROLIFERATION AND DIFFERENTIATION IN HUMAN PLACENTAL CELLS

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ABSTRACT

ONCOFETAL PROTEINS REGULATE PROLIFERATION AND DIFFERENTATION IN HUMAN PLACENTAL CELLS

The chromatin associated transcription factor HMGA2 is a downstream target of let-7 miRNAs and binds to chromatin to regulate gene expression, inducing rapid cell proliferation during embryogenesis. Inhibition of let-7 miRNAs by RNA binding proteins LIN28A and LIN28B is necessary during early embryogenesis to ensure stable expression of HMGA2 and proper cell proliferation. In addition to LIN28, *HMGA2* is regulated by a BRCA1/ZNF350/CtIP repressor complex. In normal tissues, the BRCA1/ZNF350/CtIP complex binds to the *HMGA2* promoter to prevent transcription. However, in many cancers the oncomiR miR-182 targets BRCA1, preventing BRCA1 translation and allowing for increased HMGA2. Little is known about the regulation of HMGA2 during early placental development therefore we hypothesized that both LIN28 and BRCA1 can regulate HMGA2 in placental cells. Using siRNA and CRISPR gene editing techniques, we found that knockdowns of both LIN28A and LIN28B increase *HMGA2* levels in ACH-3P cells. These cells also demonstrated deficiencies in cell differentiation towards the syncytiotrophoblast, secreting higher amounts of hCG and displaying upregulated *ERVW-1.* Additionally, we found that a knockout of both LIN28A and LIN28B caused a significant increase of miR-182 and a decrease in *BRCA1* which allows *HMGA2* mRNA levels to increase and protein levels to remain the same. Using chromatin immunoprecipitation, we saw binding of the BRCA1 repressor complex to *HMGA2.* We also saw a decrease in binding to *HMGA2's* promoter in the LIN28A/B knockout cells. These findings suggest a novel role for BRCA1 during early human placental development. To test this hypothesis, we used CRISPR-

Cas9 gene editing to knockout BRCA1 in the Swan71 cell line as the Swan71 cells had significantly higher *BRCA1* levels compared to ACH-3P cells. HMGA2 mRNA and protein was significantly increased in the BRCA1 KO cells compared to control cells. Chromatin immunoprecipitation was used with an antibody for ZNF350 and PCR was run using primers for the promoter region for HMGA2. We saw a loss of BRCA1 repressor complex binding to *HMGA2* in the knockout cells compared to our control cells, leading us to conclude that increased HMGA2 was due to decreased binding of the BRCA1 repressor complex. Additionally, we tested levels of apoptosis in our cells. After serum starving cells for 16 hours, we found that Caspase 3 and 7 levels were significantly higher in our BRCA1 KO cells compared to controls. This data suggests that BRCA1 is an important factor in the regulation of the oncofetal protein HMGA2 and promotes cell survival in human placental cells.

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DEDICATION

I dedicate this dissertation to my mom. Grit and determination runs through my blood and I know that comes from you. You've taught me to pick my battles, to remain graceful under pressure, and most importantly to never be bullied out of something that's important to me. Thankful for the "curse" of the Clay women and how you've encouraged my independence (even though you probably overdid it). I love you and couldn't have accomplished this without your wisdom and grace.

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CHAPTER I: REVIEW OF LITERATURE[1]

Synopsis

Early human placental development strongly resembles carcinogenesis in otherwise healthy tissues. The progenitor cells of the placenta, the cytotrophoblast, rapidly proliferate to produce a sufficient number of cells to form an organ that will contribute to fetal development as early as the first trimester. The cytotrophoblast cells begin to differentiate, some towards the fused cells of the syncytiotrophoblast and some towards the highly invasive and migratory extravillous trophoblast. The invasion and migration of the extravillous trophoblast mimics tumor metastasis. One key difference between cancer progression and placental development is the tight regulation of these oncogenic processes and the oncogenes that drive them. Often, tumor suppressors and oncogenes work synergistically to regulate cell proliferation, differentiation, and invasion in a restrained manner compared to the uncontrollable growth in cancer. This review will compare and contrast the mechanisms that drive both cancer progression and placental development. Specifically, the mechanisms that promote cell proliferation, evasion of apoptosis, cell invasion, and angiogenesis.

Introduction

During pregnancy, the female body undergoes incredible anatomic, metabolic, and physiological changes in the process of providing for the needs for a developing fetus. One of the most essential developments is the genesis of the placenta, which is critical for hormone production and gas and nutrient exchange between the mother and the fetus [1-3]. Any aberration

[1] This chapter has been written and formatted with the intention of submitting to the journal, *Molecular Reproduction and Development* in spring of 2018.

in these physiological processes can cause devastating placental pathologies like preeclampsia and intrauterine growth restriction (IUGR) [4], leading to severe pregnancy complications [5]. Preeclampsia affects 4-8% of pregnancies in the United States and is attributed as the cause behind 500,000 fetal and 75,000 maternal deaths each year [6, 7]. IUGR also affects 7-9% of newborn infants and is thought to cause up to 50% of unexplained stillbirths [8]. These pregnancy complications can also cause long-term developmental delays and health consequences including; cerebral palsy, deafness, chronic lung disease, neurodevelopmental delays, and metabolic disorders [9-11], leading to substantial health care costs and emotional burdens on families. Both preeclampsia and IUGR appear to be heritable as they both are associated with an increased likelihood of IUGR and fetal death in subsequent pregnancies of the affected mothers [11]. Additionally, IUGR often occurs frequently in women suffering from placental morbidities such as preeclampsia, hypertension, and gestational diabetes, putting the mother's life in significant danger as well as the fetus [12].

The conditions affecting fetal growth can either be placental or fetal in origin. Fetal growth is dependent upon the overall health of the fetus, the ability of the mother to metabolize and provide sufficient amounts of substrates necessary for growth, and the competency of the placenta to transport these substrates from the mother to the fetus [13]. However, impaired placental function seems to drive the most severe cases of IUGR [14]. This placental insufficiency is a common phenotype associated with both IUGR and maternal placental comorbidities including preeclampsia and hypertension [15]. Currently there is no treatment for pathologies caused by placental insufficiency other than the immediate delivery of the fetus.

While the understanding of the consequences of IUGR and preeclampsia has increased exponentially over the past few decades, there is still a need to elucidate the underlying cause

behind placental insufficiency during development. Understanding what is driving placental insufficiency during early development will be essential in the development of better diagnostic and treatment tools for the prevention and treatment of both pathologies. This review will focus on the signaling pathways typically considered to be oncogenic and their role in driving early placental development. The delicate interplay between cell proliferation and differentiation could be a key event that malfunctions early on in pregnancy, eventually leading to placental dysfunction.

Typically, when one considers oncogenes it's hard to ignore the profound effects these proteins have on adult tissues. These genes promote rampant cell proliferation in otherwise healthy tissues. Proliferative cells eventually begin to migrate towards other organ systems, invading into those tissues to form metastatic tumors. However, to only consider oncogenes as "bad" fails to consider the original purposes of these genes. These oncogenic processes are essential during early embryonic and placental development and any aberrant signaling of these genes can cause devastating effects on fetal growth. These proteins are responsible for the cancer-like processes that characterize early placental development. However, in direct contrast to carcinogenesis, the placenta uses these factors in a tightly controlled, highly regulated environment. This regulation exploits these factors so that they create a remarkably efficient organ in a short amount of time without the adverse consequences that often come with the expression of oncogenic proteins. Therefore, we propose that oncogenes instead be considered as oncofetal proteins.

This review will focus on the similarities of oncogenic processes like proliferation, escape of apoptosis, cell invasion and migration, angiogenesis, and the signaling pathways that drive these mechanisms in both cancer and placental development. Understanding these parallels

between placentation and tumorigenesis will provide insight into not only better ways to treat cancer but also understand how these processes can fail during development leading to placental insufficiency.

Human Placental Development

Placentation begins with the uterine endometrium changing its structure to prepare for implantation, a process known as decidualization [16]. The fibroblast-like cells of the endometrium transform into secretory decidual cells. These cells secrete an immunoprivileged matrix that makes up the histotroph, the substance that will facilitate both implantation and conceptus development during the initial weeks of pregnancy [17]. The histotroph also secretes factors that regulate the invasion potential of the early trophoblast cells if an embryo implants [18].

Once fertilization occurs, the zygote travels from the ampulla of the Fallopian tube to enter the endometrial cavity within three days [19]. During this journey, the zygote divides and undergoes a series of mitotic divisions to become the morula [20]. Four days after fertilization, the morula transforms from a 16-cell spherical mass to a flattened 58-cell ball of cells partitioned into a peripheral layer, the trophectoderm, that will eventually become the placenta and the inner cell mass (ICM), which will become the fetus [21]. Approximately 9 days after fertilization, the blastocyst implants into the uterine wall in a three step process called apposition, adhesion, and invasion [22]. Additionally, by day 9 the progenitor cells of the trophectoderm, the cytotrophoblast cells, have begun to form villous structures that will eventually differentiate into the two main cell types of the placenta; the weakly proliferative and fusional syncytiotrophoblast and the terminally differentiated, invasive extravillous trophoblast (EVT) [23]. The

cytotrophoblast cells proliferate rapidly and accumulate in floating villi which will differentiate to form the syncytium. This layer of cells will eventually come into contact with the maternal blood [24]. Alternatively, cytotrophoblast cells will also form anchoring villi that will eventually attach to and invade into the mother's decidualized endometrium, myometrium, and eventually her spiral arterioles [25] (Figure 1). This balance between cytotrophoblast cell proliferation and subsequent differentiation into the invasive and migratory EVT has a marked similarity to how cancer cells form tumors and metastasize.

Cell Proliferation

As the placenta begins forming one week after fertilization and must begin to facilitate nutrient and gas exchange by the end of the first trimester, rapid and substantial cell proliferation is essential. However, unlike cancer, this cell proliferation is tightly regulated and cells lose their proliferative capacity once they undergo differentiation into the invasive EVT lineage. One group of genes that are responsible for cytotrophoblast cell proliferation are growth factors and their receptors [26]. Epidermal growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and placental growth factor (PLGF), insulin like growth factor (IGF), transforming growth factor (TGF) and their subsequent receptors have all been identified in the cytotrophoblast and speculated to act in a paracrine and autocrine manner on the differentiated cells of the placenta [27-33]. These growth factors bind to their receptor tyrosine kinase receptors on cytotrophoblast cell membranes inducing self-dimerization to activate the MEK/ERK proliferation pathway and the PI3K/Akt anti-apoptosis pathway [34]. These kinase signaling cascades are potent catalysts that influence cell proliferation and survival in many cell types, including the placenta [35]. Gene editing experiments targeting the MAPK pathway in

mice was embryonic lethal by E11.5 due to severe placental defects [36]. Additionally, gene disruption of the PI3K/Akt pathway led to depleted cells of the spongiotrophoblast (cells of the junctional zone of the mouse placenta, the specific function is still unclear [37]) and decreased vascularization [38]. These data indicate a necessary role for the growth factor driven activation of the MAPK/PI3K pathways during early placental development. Interestingly, the phosphorylated forms of ERK1 and ERK2 are only detected in the proliferative cytotrophoblast cells until the end of the first trimester. This alludes to their importance in cell proliferation, losing expression once cells being to terminally differentiate [39].

Additional oncogenic downstream target of the MAPK pathway, JUN has also been implicated in early placental cell proliferation and differentiation. However, different members of the JUN family are expressed at different timepoints. Messenger RNA for *c-Jun* was found at its highest levels in early gestational placental tissue whereas *jun-B* was at its highest levels between 35 and 40 weeks [40]. The authors of this study concluded that in the placenta *c-jun* is essential for cytotrophoblast cell proliferation while *jun-B* likely plays a role in terminal differentiation.

There are also several oncogenes outside of the family of growth factors and MAPK pathways that promote cell proliferation. For example, our lab studies the LIN28-let7-HMGA2 axis. LIN28 is an RNA binding protein considered to be a key molecular factor that regulates the transition from a pluripotent, highly proliferative state to a terminally differentiated cell [41]. One of the main targets of LIN28 is the let-7 family of miRNAs. When cells are highly proliferative, LIN28 negatively regulates the let-7 family. However, as cells begin to differentiate the let-7 family becomes upregulated and can bind to the 3' UTR of *LIN28* to inhibit its translation into protein [42]. Because of this negative feedback loop, LIN28 and the let-7s are

often inversely expressed in many cancers [43]. In addition to this, increased LIN28 has been correlated with highly aggressive cancers and poor prognosis [44]. The let-7s also regulate several other oncogenes including HMGA2, c-Myc, RAS, the PI3K pathway via the IGF receptor and VEGF [44]. In placental cells, a knockdown of LIN28A led to spontaneous differentiation and syncytialization in human trophoblast cells [45]. Furthermore, a knockdown of LIN28B and a knockout of both LIN28A and LIN28B leads to trophoblast cells that are driven to differentiate towards only the syncytiotrophoblast lineage, but not extravillous trophoblast cells (manuscript in review). Collectively these data suggest that, as with pluripotent cells, LIN28 is an essential gatekeeper in trophohoblast cell proliferation and differentiation.

Cell Survival

The ability to bypass apoptosis is another signet of cancer and is essential during placentation. Again, growth receptors and receptor tyrosine kinase pathways mentioned above play an important role in cell survival, specifically IGF-1 and IGF-2 binding to IGF-1R [35, 46].The relationship between IGF-1R and the PI3K/Akt and MAPK pathways has been described as a crucial cell protectant in many different cancer cell types [47-50]. In immortalized human placental BeWo cells and in placental tissue explants both IGF1 and IGF2 rescued serum-starved cells from apoptosis [51]. Additionally, mutated IGF1-R in pregnant women leads to both intrauterine and post-natal growth restriction [52] and there is a direct correlation between IGF levels and birth weight [53].

There are two distinct mechanisms that the IGF system targets to promote cell survival; the Bcl-2 family and caspase proteins [54]. Increased Bcl-2 expression has been reported in several cancer cell lines and tumors [55-58] and leads to increased cell survival and resistance to

chemotherapy treatment [59]. Bcl-2 immunolocalization in the placenta has been described in several papers [60-62]; however its involvement in trophoblast cell apoptosis is still unclear. Soni et al. describe a gradual increase in Bcl-2 expression throughout pregnancy with maximal immunoreactivity occurring at term [63]. Ishihara et al. also suggest that based on their findings that abundant expression of Bcl-2 in term syncytiotrophoblast prevents cell death, allowing for the maintenance of placental mass near the end of pregnancy [60]. Additionally, the IGFs regulate caspase expression. Activation of IGF1-R can prevent cleavage of caspases in both cancer cells and fetal brain cells, preventing apoptosis [64, 65]. In accordance with the findings of Bcl-2 expression, there appears to be no caspase-mediated apoptosis in the syncytiotrophoblast of term villi of the placenta. There was also no response to stimulus-induced apoptosis in syncytiotrophoblast of villous explants from term placental tissue [66]. These data suggest that the syncytiotrophoblast can protect itself against apoptotic signals to continue to function and contribute to fetal growth until the end of pregnancy.

In most cell types, the transcription factor p53 antagonizes IGF signaling to promote apoptosis and cell cycle arrest [67]. Several papers report that p53 closely monitors the IGF-1/Akt pathway and, upon sensing stress, negatively regulates IGF-1/Akt to halt cell proliferation and induce autophagy [68-70]. This negative regulation occurs by p53 transactivating IGF-BP3. The family of IGF-BPs regulates ligand availability to their IGF receptors [71]. It has been shown that a p53-induced accumulation of IGF-BP3 in the extracellular medium of cells can inhibit mitogenic function of IGF-1 *in vitro* [72]. Increased IGF-BP3 leads to increased complexing to IGF-1, reducing their ability to bind IGF-1R to promote cell survival and proliferation [73]. However, over 50% of human cancers have p53 mutations, preventing it's pro-apoptotic function to promote spontaneous tumorigenesis [74]. In the placenta, increased p53

protein expression in placental villi is correlated with pre-eclampsia [75]. As excessive apoptosis in the villous trophoblast of placental villi is a characteristic of pre-eclampsia, these data suggests that upregulated p53 induces a disproportionate amount of apoptosis, leading to placental insufficiency (Figure 2).

Finally, another important anti-apoptotic factor often found in cancer is survivin [76]. Belonging to the "inhibitor of apoptosis" family, upregulation of survivin in cancers is directly correlated with apoptotic resistance, increased cell survival, and poor response to chemotherapy [77]. Survivin has also been described in the placenta and is thought to play a crucial role in cell survival and proliferation of trophoblast cells [78, 79]. Messenger RNA levels of survivin were analyzed in first, second, and third trimester placentas of pre-eclamptic women, compared to normal placentas, survivin was significantly decreased. Additionally, survivin levels were directly correlated with severity of pre-eclampsia, with levels decreasing as pre-eclampsia became more severe [80].

Cell Invasion

Human placentation is unique in that the EVT cells of the placenta invade fully into the maternal decidua to encapsulate and erode the spiral arteries, exposing the placenta to maternal blood [81]. The similarities between cell invasion of EVT cells and cancer cells are striking. However, one key difference is that trophoblast cells adhere to a tightly regulated pattern of proliferation then differentiation and invasion without metastasis into new tissues. Cancer cells proliferate rapidly, eventually seeking out other tissues to metastasize towards. Not surprisingly, many of the same factors are required for both neoplastic cells and trophoblast cells. Some of

these requirements for invasion include altered expression of cell adhesion molecules, secretion of proteinases, and epithelial-mesenchymal transition.

In non-invasive cells, there is a network of proteins that harness cells to the extracellular matrix (ECM) and to each other. However, in invasive or metastatic cells, this network is downregulated [82] which allows cells to seek out new tissues. One group of altered proteins is the integrin family. Integrins are a heterodimeric family of cell membrane proteins that are made up of at least 18 α subunits and 8 β subunits [83]. These subunits dimerize to form at least 24 different receptors, allowing them to bind to a variety of different ECM ligands. Because of this diversity, some integrins promote adhesion and some promote invasion. This review will only focus on the integrins that influence cell invasion in the placenta.

During placental development, there is a delicate balance between adhesion-promoting integrin expression and invasion-promoting integrins. This balance in early cytotrophoblast cells is regulated in large part by α5β1 and α1β1. In contrast to cancer, cytotrophoblast cells use the invasion-restraining role of α5β1 to balance the invasion-promoting role of α1β1 to tightly regulate the depth of invasion into the mother's decidua [84]. During early gestation, the proliferating cytotrophoblast cells begin to upregulate α1β1 as they differentiate to become more invasive. However, as gestation continues and invasion becomes less of a priority, expression of the α1β1 integrin complex declines [84]. Additionally in pre-eclamptic placental tissue, α1β1 immunostaining is almost nonexistent while the invasion-restraining α5β1 is still detectable at almost normal levels [85]. This suggests that the shallow invasion of uterine vasculature, a hallmark of pre-eclampsia, is at least in part caused by altered integrin expression.

Another driver of cell invasion shared between cancer and placentation is the loss of expression of the cell adhesion molecule E-cadherin. Found at the adherens junctions of

epithelial cells, E-cadherin is a potent promoter of cell-cell adhesion [86]. Known as a suppressor of invasion, decreased function of E-cadherin is directly correlated with invasion and tumor metastasis [87, 88]. E-cadherin is predominantly expressed in anchored placental villi of first and second trimester placentas, gradually becoming down-regulated as cells differentiate to become EVT [89]. In contrast, pre-eclamptic placentas have upregulated E-cadherin in interstitial and vascular trophoblasts that colonize maternal arteries [90]. Using knockout mice, it has been shown that E-cadherin is essential for early embryonic development, specifically placental development. E-cadherin $^{-/-}$ mice fail to form a trophectoderm and die at the time of implantation [91].

Finally, the metalloproteinase (MMP) family of proteins is a critical family of enzymes that facilitate invasion. In addition to degrading the ECM, MMPs also can modify cell adhesion molecules like integrins and activate cytokines to stimulate epithelial-mesenchymal transition and drive cell invasion [92]. Several MMPs, including MMP-2, MMP-3, and MMP-9 have been described in different locations in the placenta; however there is evidence to suggest that MMP-9 is the most influential proteinase during placental invasion [93, 94]. MMP-2 and MMP-9 are found at their highest levels in the extravillous cytotrophoblast between 6-8 weeks of pregnancy, appearing to facilitate trophoblast invasion into the decidua [95]. Interestingly, MMP expression isn't restricted to the invasive trophoblast cells as MMPs have been described in the endometrial stromal and natural killer cells of the uterine lining [96]. Furthermore, permissiveness to invasion by the decidua seems to be influenced by the presence of cytotrophoblast cells. This interaction between uterine and trophoblast MMPs could be regulated by the pregnancy hormone, human chorionic gonadotropin (hCG). To stimulate maternal recognition of pregnancy during the first trimester, the developing embryo secretes proteins to decidualized endometrial stromal cells,

allowing for upregulation of MMPs [97]. In immortalized JEG-3 cells and in villous tissue explants, addition of hCG to culture medium increased invasion in a dose dependent manner [98, 99]. Interestingly, these data suggest that the uterus has the ability to influence invasion, keeping this process regulated and local. This is in direct contrast to the unregulated and rampant invasion seen in metastatic cancer.

Angiogenesis

Angiogenesis is a mandatory process driving tumor pathogenesis leading to tumor metastasis and poor cancer prognosis. Alternatively, the ability to not only join existing vessels but also to create vessels in avascular tissue is an essential component of placental development. Any aberration in the signaling pathways that drive angiogenesis and vasculogenesis can lead to shallow invasion into the maternal spiral arteries, a known cause of placental insufficiency. The angiopoietin (ANG) and vascular endothelial growth factor (VEGF) families of growth factors are two critical families for vessel development in the placenta [100]. Similar to the balancing and counterbalancing effects of integrins regulating cell invasion, VEGF and placenta growth factor (PlGF) work in a synergistic fashion to promote angiogenesis in a controlled environment [101]. Both growth factors are key components that control two different types of angiogenesis, branching and non-branching. (Figure 3)

Vasculogenesis begins approximately at 21 days post-conception when mesenchymal stem cells inside the mesenchymal villi of the placenta differentiate to become hemangiogenic progenitor cells [102]. These progenitor cells eventually migrate towards the periphery of the villous columns and coalesce to form hemangiogenic cords, the primitive original vessels of the villous [103]. Eventually these cords will mature into a more sophisticated network of vessels,

differentiating into intermediate villi with capillary networks of branched vessels [104]. This process is almost totally driven by paracrine signaling of VEGF-A from the cytotrophoblast [102]. VEGF-A works through receptor tyrosine kinase receptors, VEGFR-1 and VEGFR-2, to stimulate branched angiogenesis [105]. Branching angiogenesis requires a series of steps including permeabilization of vascular tissue, degradation of the basement membrane, and increased proliferation and migration of endothelial cells. This leads to the formation of endothelial cell tubes and recruitment of pericytes to the exterior of the capillary, forming a stable vessel [103, 106]. These mechanisms lead to the creation of a network of immature intermediate villi containing superficially located capillaries lying directly beneath the trophoblast layer of the villous surface [107]. These branched vessels are responsible for the dramatic increase in villous blood vessels facilitating enhanced fetoplacental blood flow to accommodate the rapidly developing fetus [108]. Branching angiogenesis and VEGF-A expression continues to dominate placental vascularization quickly producing a multitude of vessels until approximately the 26th week of gestation [109]. At this point, villous vascularization undergoes a switch from branching to non-branching angiogenesis. At this point, the focus moves from producing more vessels to increasing the length of the existing vessels [110].

Non-branching angiogenesis is driven by another member of the VEGF family of proteins, PlGF. Whereas VEGF-A and VEGFR-2 are expressed at high levels during early pregnancy, waning as pregnancy advances [110]; PlGF is expressed at relatively low levels during the first trimester of pregnancy but increases at 11-12 weeks and reaching peak levels at week 30 of pregnancy [111]. PlGF is thought to have an antagonistic effect on VEGF-A, forming a heterodimer that prevents VEGF-A from activating either VEGF1-R or VEGF2-R [112]. At peak PlGF expression, the immature intermediate villi begin to form the mature intermediate

villi. Non-branching angiogenesis leads to the formation of long, thin vessels found at the tips of the villous. These vessels continue to grow in length, eventually surpassing the boundaries of the mature intermediate villi to form terminal villi. Each terminal villous has a thin trophoblast layer covering only one or two capillary coils [113]. These villous structures are critical for diffusional gas exchange from mother to fetus [109] (Figure 2).

Similarly to cancer, both VEGF and PlGF are regulated by hypoxia. In tumors, hypoxia has been shown to upregulate both VEGF and VEGFR expression [114-116]. As with tumorigenesis, hypoxia is necessary in early placental development. During the first trimester, placental development occurs in a low-oxygen environment due to the absence of access to maternal circulation [117]. These conditions are considered key to stimulating placental vasculogenesis. In placental fibroblasts, hypoxia upregulates both VEGF mRNA and protein [118]. Additionally, PlGF is regulated by low oxygen conditions, albeit in an opposite fashion to VEGF. Human placental cells exposed to low oxygen conditions had decreased PlGF mRNA and protein [109]. Abnormal oxygen levels during early placental development are thought to lead to altered VEGF/PlGF expression leading to pre-eclampsia. For example, in the instances of pre-placental hypoxia where mother, placenta, and fetus are hypoxic (due to high altitude or anemia) there is an increase of VEGF and branched angiogenesis [119]. This phenomenon is also seen in uteroplacental hypoxia, where maternal oxygen levels are normal but there is impaired oxygen circulation throughout the placenta and fetus [120]. However, in instances of post-placental hypoxia where the mother has normal oxygen levels but the fetus is hypoxic, the placenta may become hyperoxic leading to inappropriate levels of oxygen during early development, causing increased levels of PlGF and increased non-branching angiogenesis [121]. This early onset

placental hyperoxia often leads to the most severe form of pre-eclampsia, with increased adverse outcomes and fetal mortality [121].

Genomic Instability

Genomic instability is widely acknowledged as a hallmark of cancer. Ranging widely from nucleotide mutations to alternations in chromosome number or structure (known as chromosome instability), genomic instability can have major deleterious effects on normal cells [122]. However, some degree of instability appears to be tolerated by cells and has been documented in human embryos. One study analyzed blastomeres from women under 35 years of age that had undergone *in vitro* fertilization (IVF). Upon analysis, researchers found that 70% of all embryos had some chromosomal genomic abnormality. Additionally, only 9% of the embryos analyzed had a 100% occurrence of diploid blastomeres [123]. This suggests that genomic instability is prevalent in human embryos and potentially explains the low levels of fertility in women compared to other species. Another study analyzed levels of aneuploidy in fertilized oocytes, cleavage stage embryos, and blastocyst stage embryos. There was a large increase in aneuploidy between the fertilized oocyte stage and cleavage stage embryos. As embryos developed to the blastocyst stage, there was a significant decrease in the aneuploidy rate (83% aneuploidy in cleavage stage versus 58% in blastocyst stage). However, while there was a decrease in rates of aneuploidy, there were still high levels of overall chromosomal abnormality [124]. These data suggest that, as with tumors, for rapid placental development to occur a lapse in the cell-cycle checkpoint machinery must occur. Additionally, it has been suggested that this genomic instability actually provides an advantage for embryo implantation [125].

In addition to aneuploidy, extravillous trophoblast cells of the placenta are also polyploid [126]. These cells are analogous to murine trophoblast giant cells that are also invasive. However, rodent trophoblast giant cells have ploidy levels that can reach up to 1024 N compared to the 4-8 N recorded in extravillous trophoblast cells [127]. These cells become polyploid through a process known as endoreduplication, where cells undergo mitosis but fail to divide after DNA replication. Endoreduplication is another phenomenon that occurs in cancer to promote genomic instability [128]. It has been proposed that endoreduplication occurs during times of genomic instability to increase tissue mass while cell proliferation is decreased to prevent propagation of cells with damaged chromosomes [129]. In the placenta, extravillous trophoblast cells invade into the decidua as two different cell types, interstitial cytotrophoblast cells (iCTBs) and endovascular cytotrophoblast cells (eCTBs). The iCTBs are the cells that invade into the decidua, moving as deep as the first third of the myometrium. Once at the myometrium, these cells undergo a final step of differentiation where they undergo endoreduplication to become multinucleated [130]. Similarly to how damaged cells undergo endoreduplication to increase size, it is thought that iCTBs undergo endoreduplication to further penetrate into the myometrium of the uterus.

Finally, even with less priority attributed to cell-cycle checkpoints and DNA repair, there must be some regulation of DNA repair in the placenta for it to develop into a proper functioning organ. Our lab is currently focused on the regulation of DNA repair and genome stability in trophoblast cells by the tumor suppressor BRCA1. BRCA1 is a multifunction protein involved in many different aspects of cell cycle regulation including; regulation of transcription of several proliferation factors, homologous recombination of double-stranded breaks (DSBs), cell-cycle checkpoint regulation, and chromatin remodeling [131]. BRCA1 works to repair DNA damage

by acting as a scaffolding protein for other DNA repair proteins and also promotes strand-invasion by interacting with the recombinase protein, Rad51 [132, 133]. Additionally, BRCA1 forms a repressor complex with CtIP and ZNF350. This repressor complex binds to promoter regions of several oncofetal proteins to prevent transcription [134]. One oncofetal proteins target already discussed in the "cell proliferation" section is HMGA2. In addition to promoting cell proliferation, increased levels of HMGA2 causes genomic instability by preventing non-homologous end-joining as well as delaying clearance of γ-H2AX, a marker for DSBs, [135]. $BRCA1^{-/-}$ knockout mice are embryonic lethal before embryonic day 7.5 due to dramatic decreases in cell proliferation and poor differentiation of the extraembryonic tissue. These knockout embryos have a complete loss of diploid trophoblast cells with an overabundance of trophoblast giant cells [136]. Interestingly, mouse trophoblast giant cells are polyploid and are potentially accustomed to levels of genomic instability through endoreduplication, which is necessary for trophoblast giant cell function.

Unfortunately, this question will be hard to prove using today's current models of trophoblast cell development. Trophoblast cells derived from first trimester placentas are very difficult to obtain. Additionally these cells are hard to culture, making alternative model systems to study trophoblast development essential. Immortalized cell lines are extensively used as a model for trophoblast development and differentiation. However, these cells present their own shortcomings that make them less than ideal candidates for use. These shortcomings are especially apparent when it comes to studying DNA damage and genomic instability. For example, cytogenetic analysis of the extravillous first trimester Swan71 cell line immortalized with hTert revealed that these cells were near pentaploid in karyotype [136]. This is almost certainly due to chromosomal missegregation during mitosis, leading to a heterogeneous

population of aneuploid cells. Additionally, when our lab began using this cell line to investigate BRCA1 in human trophoblast cells we found high levels of markers for DNA damage. We created a BRCA1 knockout trophoblast cell line using CRISPR-Cas9 genome editing to investigate levels of DNA damage by immunostaining for markers of double and single-stranded breaks. Surprisingly, the level of DSBs, as evidenced by immunostaining for γ-H2AX, was indistinguishable between BRCA1 knockout cells (BrKO) and wild-type Swan71 cells (Figure 4). This high level of double-stranded breakage was confirmed using another marker for DSBs, 53BP1 (data not shown). These data corroborate the idea that immortalized cells suffer from cellular crises when cultured *in vitro*, resulting in microsatellite and chromosomal instability. Due to this propensity towards genomic instability in culture, immortalized cells are unlikely to provide insight into the role of genomic instability during early placental development. Additionally, this genomic instability of immortalized cells leads to a higher propensity for these cells to behave as cancer cells, no longer regulated in the controlled manner that characterizes trophoblast cells. This creates a need for a better model system to investigate the regulation of oncogenic processes during trophoblast development.

Conclusion

While understanding the consequences of fetal growth restriction has increased exponentially over the past few decades, there is still a need to elucidate the underlying cause behind placental insufficiency during development. Understanding what is driving placental insufficiency during early development will be essential in the development of better diagnostic and treatment tools for the prevention and treatment of IUGR. The ability of placental cells to divide rapidly, differentiate, invade and migrate into tissues, and eventually create its own

vascular network makes it an ideal system to gain insight into cancer biology and tumor metastasis. Alternatively, as placental pathologies like intrauterine growth restriction (IUGR) and pre-eclampsia are multi-faceted disorders with no known cause, better understanding the molecular mechanisms that drive oncogenic processes will provide better insight into how the early placenta develops. Pre-eclampsia and IUGR are rarely diagnosed until after 20 weeks of gestation, significantly later than pathogenesis begins. Therefore it is critical to start thinking of oncofetal proteins in their original roles, namely as drivers of cell proliferation, differentiation, invasion, and cell survival during early embryogenesis and placental development. Studying how oncofetal proteins drive placentation is essential to facilitate the process of providing better diagnostics for earlier screenings as well as treatment, ensuring the proper care for healthier babies and happier mothers.

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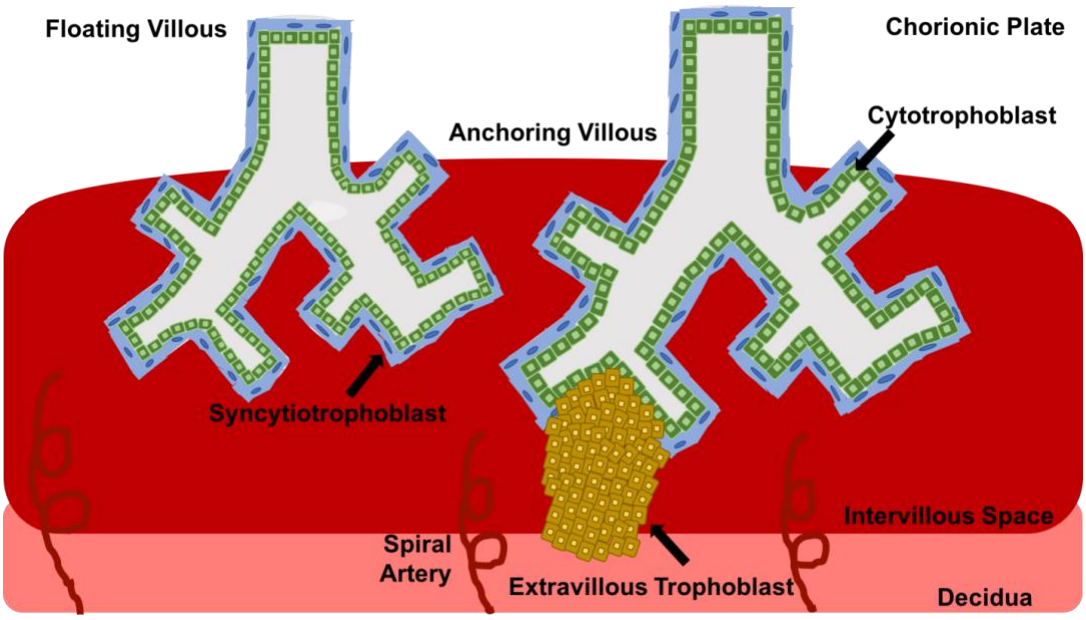

Figure 1: Early Placental Development. The progenitor cells of the placenta, the cytotrophoblast proliferate rapidly during the first trimester of pregnancy. During this time they also differentiate to become part of the syncytiotrophoblast layer that fuses and becomes the of the placenta that comes into contact with the maternal blood. Additionally, cytotrophoblast cells differentiate to become part of the extravillous trophoblast, the cells that invade into the mother's endometrium, seeking out her spiral arteries.

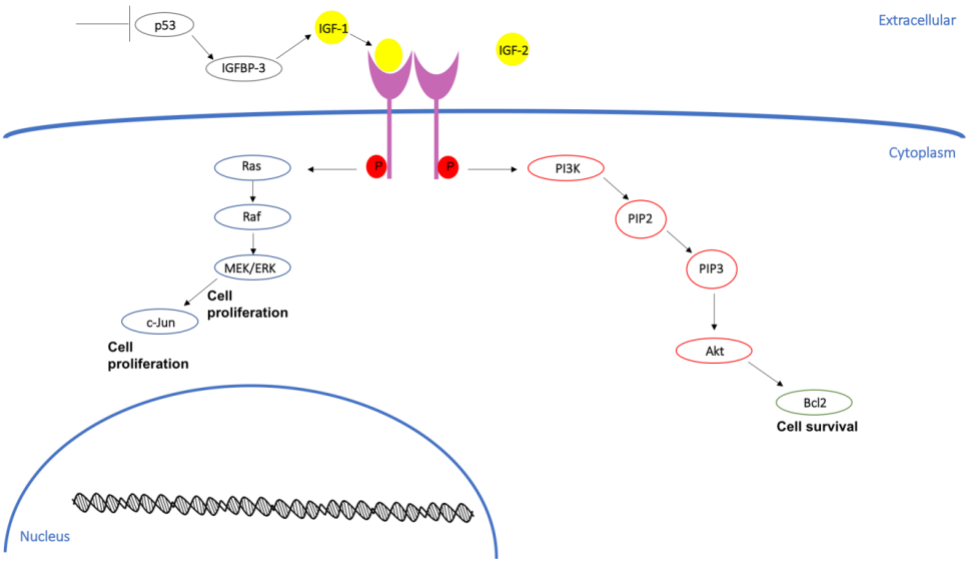

Figure 2: IGF signaling in the placenta. IGF regulates cell proliferation and survival in placenta cells through several mechanisms. Both IGF-1 and IGF-2 bind to the IGF-1R to stimulate the MEK/ERK pathway and the PI3K pathway to promote cell proliferation and evasion of apoptosis. Additionally, downregulation of p53 leads to higher levels of IGF's allowing for more proliferation and cell survival.

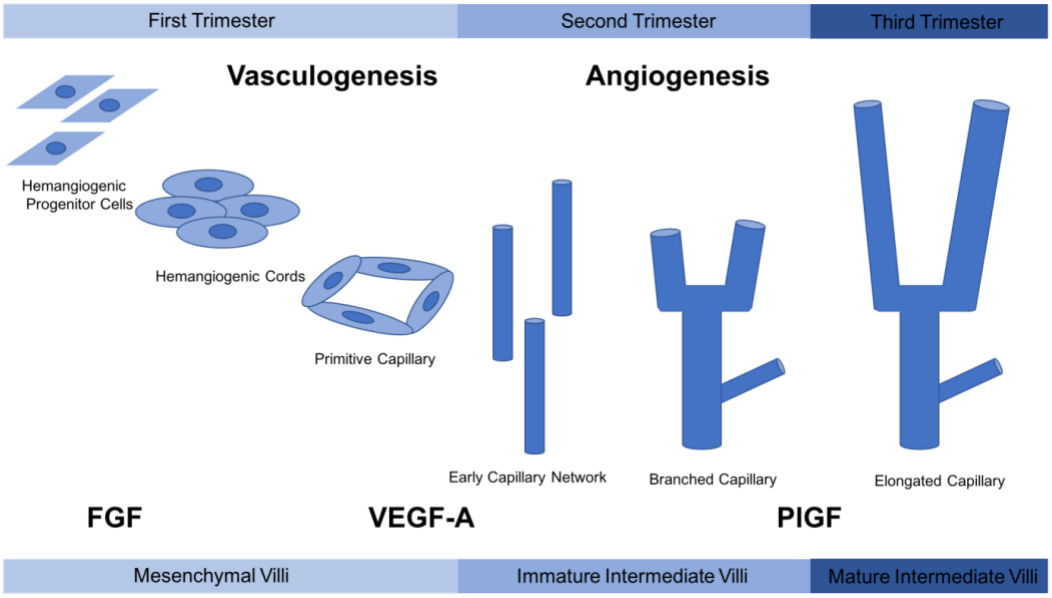

Figure 3: Angiogenesis in the placenta. During the first trimester FGF promotes vasculogenesis by promoting the differentiation of mesenchymal stem cells into hemangiogenic progenitor cells. These cells aggregate to form hemangiogenic cords and eventually primitive capillaries. VEGF-A promotes the angiogenesis of these capillaries through branching angiogenesis. As pregnancy progresses, PlGF is upregulated leading to non-branching angiogenesis and elongated capillaries.

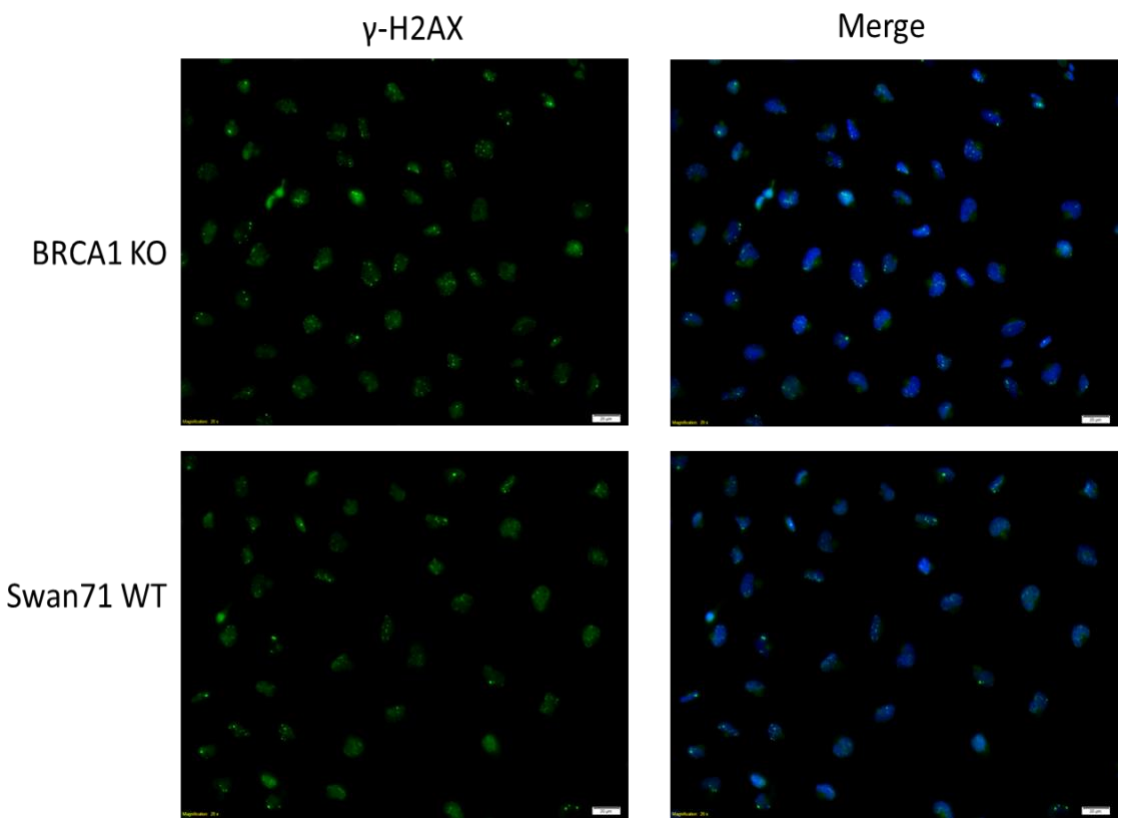

Figure 4**:** γ-H2AX in BrKO and WT Swan71 cells
Immunostaining for γ-H2AX (green) and merged with DAPI (blue) in BRCA1 knockout cells and wild-type Swan71 cells imaged at 20x magnification.

CHAPTER II: HMGA2 IS REGULATED BY LIN28 AND BRCA1 IN HUMAN PLACENTAL CELLS[2,3]

Synopsis

The chromatin associated transcription factor HMGA2 is a downstream target of let-7 miRNAs and binds to chromatin to regulate gene expression, inducing rapid cell proliferation during embryogenesis. Inhibition of let-7 miRNAs by RNA binding proteins LIN28A and LIN28B is necessary during early embryogenesis to ensure stable expression of HMGA2 and proper cell proliferation. In addition to LIN28, *HMGA2* is regulated by a BRCA1/ZNF350/CtIP repressor complex. In normal tissues, the BRCA1/ZNF350/CtIP complex binds to the *HMGA2* promoter to prevent transcription. However, in many cancers the oncomiR miR-182 targets BRCA1, preventing BRCA1 translation and allowing for increased HMGA2. Little is known about the regulation of HMGA2 during early placental development therefore we hypothesized that both LIN28 and BRCA1 can regulate HMGA2 in placental cells. Using siRNA and CRISPR gene editing techniques, we found that knockdowns of both LIN28A and LIN28B increase *HMGA2* levels in ACH-3P cells. These cells also demonstrated deficiencies in cell differentiation towards the syncytiotrophoblast, secreting higher amounts of hCG and displaying upregulated *ERVW-1*. Additionally, we found that a knockout of both LIN28A and LIN28B caused a significant increase of miR-182 and a decrease in *BRCA1* which allows *HMGA2* mRNA levels to increase and protein levels to remain the same. Using chromatin immunoprecipitation,

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we saw binding of the BRCA1 repressor complex to *HMGA2*. We also saw a decrease in binding to *HMGA2's* promoter in the LIN28A/B knockout cells. These findings suggest a novel role for BRCA1 during early human placental development.

Introduction

Trophoblast cells are the first cell lineage to differentiate during early embryonic development. These cells are found on the outside of the morula and differentiate to become the trophectoderm, surrounding the pluripotent cells of the inner cell mass at the blastocyst stage [1]. The differentiation of trophoblast cells into more specialized placental cell lineages is essential for proper development of the placenta. Anomalies in trophoblast cell proliferation and differentiation can lead to placental pathologies, including preeclampsia and intrauterine growth restriction (IUGR) [2-3]. IUGR affects approximately 5% of total pregnancies [4] with preeclampsia affecting 4% [5]. In addition to the immediate symptoms caused by placental dysfunction, including both fetal and maternal mortality, there are often long-term effects on human health, such as an increased probability to develop hypertension, coronary heart disease, and diabetes in adult life [6-8]. As there currently are no preventative measures available for either complication, further research is essential to elucidate the molecular events occurring in early placental development.

The Lin28-let-7-Hmga2 axis has been extensively studied in embryonic stem (ES) cells and is an important regulator of stem cell self-renewal [9]. LIN28 works through two distinct mechanisms to maintain pluripotency in ES cells; by regulating the biogenesis and maturation of the let-7 family of miRNAs and by modulating the translation of mRNAs important for maintaining an undifferentiated state [10]. LIN28 has two paralogs, *LIN28A* and *LIN28B*.

Together they work to inhibit let-7 miRNA function by preventing processing into mature miRNAs [11]. Despite the similarity between the two paralogs, LIN28A and LIN28B work through two discrete pathways to inhibit miRNA processing. LIN28A, found in the cytoplasm, binds to pre-let-7's to prevent processing by Dicer. Once bound, LIN28A recruits the TUTase Zcchc11 to oligouridylate the pre-let-7, leading to rapid degradation of the transcript [12,13]. Alternatively, LIN28B works independently of Zcchc11 by binding to and sequestering pri-let-7's within the nucleus preventing the processing by Drosha into pre-let-7 miRNA [14].

One notable effect of the repression of let-7 miRNAs is on the expression of the High-mobility group AT-hook 2 (HMGA2). HMGA2 alters chromatin structure to activate many transcription factors important for cell proliferation [15]. It is highly expressed during embryonic and fetal development before being repressed by let-7 miRNAs as embryonic cells begin to differentiate [16,17]. *Zhou et al.* defined *Hmga2* as the cause behind the pygmy phenotype in mice. *Hmga2*$^{-/-}$ mutant mice have a reduced birth weight and an adult body weight of approximately 40% of what their wild-type counterparts weigh [18]. Furthermore, HMGA2 deficient embryonic fibroblasts have severe proliferation defects [19]. These data suggest that HMGA2 expression during embryonic development is essential for proper growth.

While the let-7 miRNAs have been more extensively studied, they are not the only method of silencing HMGA2 in normal cells. The tumor suppressor protein, Breast cancer susceptibility gene 1 (BRCA1) also negatively regulates HMGA2 expression. BRCA1 forms a repressor complex with CtBP-interacting protein (CtIP) and Zinc finger protein 350 (ZNF350). This complex directly binds to a ZNF350 binding motif found on the promoter region of HMGA2 to inhibit transcription [20]. Removal of any one of these repressor proteins leads to increased expression of HMGA2 and increased cell proliferation and tumorigenesis [20]. The

oncomiR, miR-182, directly targets *BRCA1* and decreases translation [21]. Furthermore, approximately 70% of advanced ovarian cancers have increased miR-182 and HMGA2 expression and decreased BRCA1 expression, suggesting that overexpression of miR-182 can promote HMGA2 expression in cancer cells [22].

Both LIN28 and BRCA1 have been extensively characterized in breast and ovarian cancer cells. However, little is known about the regulation of HMGA2 during placental development. As early placental development mimics the hallmarks of cancer in a tightly controlled environment we hypothesized that both LIN28 and BRCA1 are important regulators of cell differentiation in the placenta. Additionally, an shRNA knockdown of LIN28A in the human trophoblast ACH-3P cell line led to spontaneous syncytialization and differentiation [23]. Additionally, HMGA2 has been found to localize to the undifferentiated trophoblast progenitor cells of the human placenta and becomes downregulated as these cells begin to differentiate [24]. Homozygous BRCA1 mutant mice are embryonic lethal by day E7.5, partially due to a poorly organized extraembryonic region. These mutants have a complete absence of diploid trophoblast cells with an inappropriate number of giant cells [25]. Interestingly, miR-182 has been reported as significantly upregulated in the placentas and serum of pre-eclamptic women compared to normotensive women [26,27]. Collectively these data suggest that regulation of HMGA2 may be an important regulator in the balance between cell proliferation and differentiation during early human placental development. In this study, we describe the regulation of HMGA2 by LIN28B and BRCA1 in human placental cells for the first time.

Materials and Methods

Cell Lines

The immortalized ACH-3P (a gift from Ursula Hiden, Medical University of Graz, Austria) cell line was used for this study. ACH-3P cells were immortalized by fusion with the cell line AC1-1. ACH-3P cells were cultured in DMEM F-12 medium (HyClone), 10% FBS (HyClone), and 1% Penicillin/Streptomycin/Amphotericin (Corning Life Sciences).

Human First Trimester Placental Samples

Human 6 (n=3), 8 (n=3), and 11.5 (n=2) week placental samples were obtained from elective terminations from anonymous, non-smoking, non-drug using patients in accordance with the protocol 10-1623H approved by the Colorado State University Institutional Review Board. Placental tissues were stored in phosphate buffered saline upon collection. Some of these samples were then transferred to ice cold 4% paraformaldehyde (Fisher Scientific) After incubation overnight at 4°C in paraformaldehyde, tissues were transferred to 70% ethanol and kept at 4°C until paraffin embedding. Other samples were frozen at -80°C until used for cellular mRNA isolation.

Real-Time PCR

Total cellular RNA was isolated from tissue and cells using a RNA Mini Kit (Qiagen). cDNA was generated from 1 ug total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was conducted using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primer sequences used are displayed in Table 1. For analysis, a PCR product for each gene was generated and cloned into the PCR-Script Amp SK(+) vector (Agilent Technologies). By amplifying this the PCR product from each gene's plasmid, a standard curve

was generated ranging from $1x10^{2}$ to $1x10^{-6}$ pg. The starting quantity (in picograms) was normalized against the starting quantity of *RPS15* mRNA [28].

Quantitative real-time PCR was also conducted using Taqman Gene Expression Assays (Applied Biosystems). In 20 uL qPCR reactions that contained 10 uL SsoAdvanced Universal Probes Supermix (Bio-Rad), 1 uL of 150 nM Taqman Gene Expression Assay (ThermoFisher), and 9 uL of cDNA template diluted to 90 uL. Reactions were incubated at 95°C for 10 minutes, then underwent 40 cycles of 95°C for 30 seconds, 59°C for 1 minute, and 72°C for 1 minute using a LightCycler480 PCR System (Roche Applied Science). Probe efficiency was determined using serial dilutions from a cDNA pool from cell samples. Standard curves from the serial dilutions were analyzed and efficiencies were calculated. Relative expression was normalized using GAPDH. Each reaction was conducted in triplicate using two controls, one RT control and one water control. Relative expression was determined for qPCR data using the comparative Ct method [29].

For miRNA analysis, total RNA was extracted using a miRNA Mini Kit (Qiagen). cDNA was generated from 500 ng of total RNA using a miScript RT II kit (Qiagen). Real-time PCR was conducted using QuantiTect SYBR Green Master Mix (Qiagen), a miScript universal primer (Qiagen), a miScript primer assay for the mature let-7 or miR-182 miRNA sequence (Qiagen), and 1 uL of cDNA diluted to 3 ng. Reactions were incubated at 95°C for 15 minutes, then 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. Relative expression of miRNA levels was normalized using *SNORD48*. Each reaction was conducted in duplicate using two controls, one RT control and one water control.

Immunofluorescence

Tissue was fixed for 24 hours in 4% PBS-buffered paraformaldehyde at 4°C, then dehydrated and embedded in paraffin wax. 5 micron sections were mounted onto charged glass microscope slides (Premiere). Sections were deparaffinized and rehydrated in successive 4 minute baths of Citrasolv (Decon Labs), 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, and distilled water. Sections then underwent antigen retrieval using 10 mM sodium citrate buffer and microwaved for 10 minutes after being brought to a boil. Sections were rinsed 3 times with PBS then blocked at room temperature using 6% goat serum in PBS for one hour. Sections were incubated in a humidity chamber overnight at 4 C in antibodies 1:100 LIN28B 1:100 HMGA2 1:100 BRCA1 or 1:200 hCG (Table 1). After washing 3 times in PBS, sections were incubated for 1 hour at room temperature in 1:1000 goat anti-rabbit AlexaFluor 488 (Abcam, ab150077) or goat anti-mouse AlexaFluor 594 (Abcam, ab150092). After 1 hour, slides were washed in PBS then dehydrated by successive baths of 50%, 70%, 90%, and 100% ethanol. Slides were then mounted with Prolong Gold containing DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). For negative controls, normal rabbit sera was used as a primary antibody (Molecular Probes).

Western Blot

Western blot analysis was used to assess cellular protein amounts. Each experiment was conducted with at least 3 replicates and repeated a minimum of three times. Cells were lysed in RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 3.5 mM SDS, 1.2 mM sodium deoxycholate, 1.6 mM EDTA, pH 8) with 10% protease/phosphate inhibitor cocktail (Sigma-Aldrich) and 1 mM phenymethanesulfonyl fluoride. The BCA protein assay kit (Pierce) was used to determine protein concentration. Absorbance was measured at λ 595 nm using a

Biotek Synergy 2 Microplate Reader (Biotek). Protein was electrophoresed in 12% Bis-Tris gels and transferred to 0.45-μm pore nitrocellulose membrane at 110 volts at 4°C for 1 hour. Membranes were then blocked in 5% milk-TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for 1 hour at room temperature then incubated with an antibody to LIN28B, LIN28A, HMGA2, or BRCA1 (Table 1) at 4°C overnight. After overnight incubation, membranes were washed 3 times for 10 minutes each wash in TBST then incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000, Abcam, ab6721) for 1 hour at room temperature. Depending on protein size, an antibody for β-actin, α-tubulin, or GAPDH (Table 2) was used to normalize protein in cell lysates. Membranes were developed using ECL Western Blotting Detection Reagent chemiluminescent kit and membranes were imaged using a ChemiDoc XRS+ chemilluminescence system (BioRad). Densitometry was performed using Image Lab (Bio-Rad). Fold change was calculated as a percent of control protein after normalization.

LIN28B Knockdown

MISSON shRNA Lentiviral Transduction particles targeting *LIN28B* (5'-CCGGGCCTTGAGTCAATACGGGTAACTCGAGTTACCCGTATTGACTCAAGGCTTTTTTG – 3') (Sigma-Aldrich, TRCN0000122599) or a nontarget control sequence (5'-CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTT-3') (Sigma-Aldrich, SHC002V) were used. ACH-3P cells were infected with either *LIN28B* mRNA-targeted lentiviral particles or scramble particles at a MOI of 50 viral particles per cell. Three days after infection, ACH-3P cells were selected by treatment with 2 μg/mL puromycin for 14 days. The degree of LIN28B KD was determined by RT-qPCR and Western blot.

LIN28A/B Double Knockout

Lentiviral particles targeting LIN28A and LIN28B were made from two separate plasmids. First, a lentiCRISPR v2 (Addgene) vector containing sgRNA for LIN28A (Oligo 1 – 5' – CACCGCTGTCCATGACCGCCCGCGC – 3'; Oligo 2- 5' – AAACGCGCGGGCGGTCATGGACAGC 3') was created. This vector was transformed into Stbl3 competent cells (ThermoFisher) and grown in LB broth. Plasmid DNA was isolated using a HiSpeed Plasmid Midi Kit (Qiagen). HEK cells were transfected using the lentiCRISPR v2 plasmid, a packaging plasmid (PAX), and an envelope plasmid (pMD2.G) in serum-free DMEM media at a total volume of 675 μL. Cells were transfected with plasmid mix, 180 μL transfection reagent Polyfect (Qiagen), and 15 mL of complete cell culture medium for 6 hours before media was changed then media was collected 72 hours later. The virus containing media was centrifuged to remove cell debris then ACH-3P cells were infected. To achieve a complete knockout of LIN28A, a second infection was required. A second lentiCRISPR v2 plasmid was created, this time containing sgRNA for LIN28B (Oligo 1 – 5' – CACCGGCTGCCGGAGCCGGCAGAGG – 3'; Oligo 2 – 5' AAACCCTCTGCCGGCTCCGGCAGCC – 3'). The plasmid and virus were grown in the same way as before. ACH-3P cells were infected and Western blotting confirmed a complete knockout of both LIN28A and LIN28B. As a control, ACH-3P cells were infected with lentivirus containing an empty lentiCrispr v2 plasmid.

Enzyme-Linked Immunosorbent Assay

LIN28B KD and scramble control ACH-3P cells were plated in six-well culture dishes with 50,000 cells per well and three replicates per treatment. 1.5 mL of cell culture medium was collected at 24, 48, and 72 hours. Concentrations of human chorionic gonadotropin (hCG) in the

cell medium were quantified using an ELISA kit specific for beta-hCG (mouse monoclonal anti-hCG conjugated to horseradish peroxidase) (ALPCO Diagnostics, Cat # 25-HCGHU-E01). Each experiment was conducted with at least three replicates and conducted twice.

Invasion/Migration Assay

Cell invasion was determined using the 24-well 8.0 μm BioCoat Matrigel Invasion Assay System (Corning). Cells were labeled with CellTracker Green CMFDA dye (Invitrogen) and seeded at a density of $1x10^4$ per well with serum-free media into the upper chamber of the system. As a control, cells were also seeded onto the uncoated Falcon FluoroBlok 24-Multiwell system (Corning). Bottom wells were filled with media containing 10% FBS and cells were incubated at 37°C, 5% CO_2. After 1, 3, 18, 24, and 48 hours of incubation, readings were taken using the Biotek Synergy 2 Microplate Reader using the 530/25-excitation filter and 590/35-emission filter. Fluorescence of invaded and migrated cells was read at wavelengths 549/565 nm. Background fluorescence was subtracted and invasion was normalized by dividing relative fluorescence intensity of the invaded cells by fluorescence intensity of migrated cells then multiplying by 100. Each experiment was performed with four replicates.

Co-immunoprecipitation

Protein was extracted from cellular lysate in the same method as for Western blotting. BRCA1 was immunoprecipitated using a polyclonal antibody (Santa Cruz, sc-1021) bound to SureBeads Protein G magnetic beads (Bio-Rad, 161-4023) in 50 μg of protein lysate. A polyclonal IgG antibody (Abcam, ab6721) was used as a negative control. After immunoprecipitation, the protein was loaded into a Western blot gel and the previously described Western blot protocol was followed using a ZNF350 antibody (Abcam, ab127895). This experiment was conducted with three replicates and conducted three times.

Chromatin Immunoprecipitation

ChIP was performed using the Abcam ChIP Kit (Abcam, ab500) following the manufacturer's protocol. Briefly, approximately $3x10^6$ cells were collected and resuspended in formaldehyde diluted with PBS. Cells were sonicated using a BioRuptor for 10 minutes on high for 30 seconds on then 30 seconds off. ZNF350 was immunoprecipitated using a ChIP grade antibody (Abcam, ab127895) in 10 μg of sample. A polyclonal IgG antibody (Abcam, ab6721) was used as a negative control. PCR and qPCR was performed for the region of the *HMGA2* promoter predicted to contain the *ZNF350* binding site. Genomic DNA and ChIP input DNA were used as positive controls for PCR. Each experiment was conducted with three replicates and repeated three times.

Statistics

To determine significance, all experimental replicates were assayed in triplicate and a Student t-test was utilized to compare between LIN28B KD and scramble control and LIN28 A/B DKO and control ACH-3P cells. All statistics were determined using Prism 7 for Mac OS X (GraphPad Software). P values less than 0.05 were considered statistically significant.

Results

LIN28B and HMGA2 in the human first trimester placenta

mRNA levels of *LIN28A* and *LIN28B* were examined in the human first trimester placenta using RT-qPCR. *LIN28B* levels were significantly higher than *LIN28A* at 6 and 8 weeks of gestation (Figure 1A). This information led us to focus on LIN28B protein immunofluorescence. Immunofluorescent imaging was used on 11.5 week placentas to localize LIN28B and HMGA2 protein. Both LIN28B (Figure 1B) and HMGA2 (Figure 1C) were

exclusively detected in the cytotrophoblast, becoming undetectable in the differentiated syncytiotrophoblast stained positive for hCG.

shRNA knockdown of LIN28B in the ACH-3P cell line

To examine the role of LIN28B in cytotrophoblast cells, ACH-3P cells were infected using lentivirus containing an shRNA construct specific for LIN28B. RT-qPCR and Western blot revealed that there was a significant decrease in mRNA and protein in cells infected with the LIN28B shRNA compared to scrambled shRNA (Figures 2A and 2B). Immunocytochemistry confirmed that LIN28B protein was decreased in the LIN28B knockdown cells compared to scramble control cells (Figure 2C).

Knockdown of LIN28B drives differentiation to syncytiotrophoblast lineage

Knockdown of LIN28B significantly increased let-7a, let-7e, and let-7f miRNAs compared to scramble control cells ($p<0.05$) (Figure 3A). Additionally, the syncytiotrophoblast marker *ERVW-1* was significantly increased whereas, the extravillous trophoblast marker *LGALS13* was decreased (Figure 3B). ELISA analysis of hCG secreted into cell media revealed that *LIN28B* knockdown cells secreted significantly more hCG into cell media at all timepoints (Figure 3C). Finally, invasion and migration of *LIN28B* knockdown cells were analyzed. There was no significant difference at any timepoint for cell invasion for the knockdown cells compared to control cells (Figure 3D). These data suggest that these cells are differentiating towards the syncytiotrophoblast lineage of the placenta but not towards the extravillous trophoblast lineage in the absence of LIN28B.

LIN28A and HMGA2 in LIN28B Knockdown Cells

RT-qPCR and Western blotting was used to analyze the mRNA and protein levels of HMGA2 and LIN28A in LIN28B knockdown cells compared to scramble control cells. Both

HMGA2 mRNA and protein were significantly upregulated (Figures 4A and 4B) in LIN28B knockdown cells compared to scramble control cells. However, upon analysis of LIN28A, we found that both LIN28A mRNA and protein were significantly upregulated (Figures 4C and 4D) in LIN28B knockdown cells compared to scramble control cells.

HMGA2 mRNA and protein in LIN82A/B Double Knockout Cells

Using a lentiviral targeting method, we used CRISPR-Cas9 to knock out LIN28A and LIN28B in ACH-3P cells (Figure 5A). There were additional let-7 miRNAs significantly upregulated in LIN28A/B knockout cells than compared to LIN28B KD, specifically the let-7s; let-7a, let-7e, let-7f, let-7g, and let-7i. (Figure 5B). Real-time qPCR and Western blotting was used to analyze HMGA2 mRNA and protein levels in the LIN28A/B knockout cells. *HMGA2* (Figure 5C) was significantly higher. However, HMGA2 protein levels (Figure 5D) were not significantly different in the LIN28A/B knockout cells compared to control cells.

Knockdown of both LIN28A and LIN28B drives differentiation to syncytiotrophoblast lineage

Using RT-qPCR, we determined that the syncytiotrophoblast marker *ERVW-1* was significantly increased whereas, the extravillous trophoblast marker *LGALS13* was decreased in the LIN28A/B knockout cells compared to controls. (Figure 6A and 6B). ELISA analysis of hCG secreted into cell media revealed that LIN28A/B knockout cells secreted significantly more hCG ($p < 0.005$) into cell media at timepoints 24, 48, and 72 hours (Figure 6C). Analysis of cell invasion indicated that the LIN28 A/B knockout cells were not significantly more invasive compared to control cells (Figure 6D), again indicating that these cells are acting more like syncytiotrophoblast cells but not extravillous trophoblast.

BRCA1 Complexes with ZNF350 and Binds to HMGA2

To investigate the increase in *HMGA2* upon downregulation of LIN28A and LIN28B, we examined alternate mechanisms for regulation of HMGA2 transcription. Co-immunoprecipitation and chromatin immunoprecipitation were used to determine if BRCA1 forms a complex with ZNF350 in trophoblast cells. Using ACH-3P cells, co-immunoprecipitation and subsequent Western blotting revealed that BRCA1 complexes with ZNF350 (Figure 7A). As ZNF350 is the protein in the BRCA1 repressor complex that binds to the *HMGA2* promoter we used chromatin immunoprecipitation to determine if this complex binds to *HMGA2* in ACH-3P cells. We found that ZNF350 binds to the ZNF350 recognition site in the *HMGA2* promoter region as evidenced by PCR following chromatin immunoprecipitation (Figure 7B). Using immunofluorescence, we determined that BRCA1 was localized solely in the syncytiotrophoblast of the human 11.5 week placenta (Figure 7C) suggesting that as cells differentiate, BRCA1 becomes upregulated.

MiR-182 and BRCA1 in LIN28B Knockdown and LIN28A/B Knockout Cells

Because miR-182 regulates BRCA1 translation leading to increased HMGA2, we analyzed miR-182 levels in the LIN28A/B knockout cells. Using RT-qPCR, we analyzed miR-182 miRNA levels in the LIN28B knockdown and LIN28A/B knockout cells. MiR-182 was not significantly increased in the LIN28B knockdown cells, (Figure 8A) potentially due to the upregulation of LIN28A. However, in the LIN28A/B knockout cells, miR-182 was significantly higher compared to control cells (Figure 8B). Similarly, in the LIN28B knockdown cells *BRCA1* was not significantly decreased (Figure 8C). However, when we analyzed *BRCA1* in the LIN28A/B knockout cells, we did see a significant decrease (Figure 8D). We also used chromatin immunoprecipitation and qPCR to determine if there was a decrease in the BRCA1

repressor complex to the promoter of *HMGA2*. Quantitative PCR determined that there was a significant decrease of binding to the HMGA2 promoter in the LIN28A/B DKO cells (Figure 8E). This removal of the BRCA1 repressor complex would allow for greater transcription of *HMGA2*.

Discussion

HMGA2 is known for its promotion of cell proliferation and growth during embryonic development [31]. HMGA2 also promotes cancer growth and tumorigenesis as transgenic expression of HMGA2 in mice led to the formation of benign tumors [32]. In embryonic stem cells and many cancers, LIN28A and LIN28B block the biogenesis of the let-7 family of miRNAs maintaining self-renewal properties and cell proliferation [33-37]. This LIN28-let7-HMGA2 axis has been described extensively in cancer. However, for the first time, we describe LIN28 in the maintenance of HMGA2 expression and cell differentiation in human placental cells.

In summary, we have shown that upon deletion of LIN28A and LIN28B, HMGA2 levels are unaffected, potentially due to the downregulation of BRCA1 caused by increased miR-182. This results in *HMGA2* levels that cannot be downregulated by let-7 miRNA. There are no reports of miR-182 levels increasing upon the loss of LIN28 function but, interestingly, miR-182 has been reported to be elevated in the placentas of pre-eclamptic women [26]. As both LIN28 and HMGA2 are important drivers of cell proliferation, perhaps this upregulation of miR-182 serves as a rescue of cell proliferation for a placenta in the early stages of distress.

By inducing a knockdown of LIN28B protein, we found that LIN28B is necessary for keeping placental cells in a progenitor-like state. The syncytiotrophoblast marker *ERVW1* and

hCG secretion into culture media was significantly increased. The extravillous trophoblast marker *LGALS13* was decreased in cells and there was no increase in cell invasion. This suggests that a knockdown of LIN28B negatively affects differentiation towards the extravillous trophoblast lineage. Surprisingly, we found that a loss of LIN28B protein led to increased HMGA2 although there was an increase in some of the let-7s. This increase in HMGA2 is explained by increased LIN28A protein levels. *Wilbert et al.* reported that an shRNA induced knockdown of LIN28A caused an increase of LIN28B in human embryonic stem cells [38], suggesting that the two paralogs can compensate for a loss of function of the other protein.

However, when we used a lentiCRISPR targeting vector to knock out both LIN28A and LIN28B, we again found that, despite increasing levels of let-7 miRNA, *HMGA2* mRNA is significantly higher than control cells. The increase of *HMGA2* could be explained by increased miR-182 levels in the LIN28A/B knockout cells. When miR-182 is elevated, there is a reduction of the BRCA1/CtIP/ZNF350 repressor complex binding allowing for increased *HMGA2* transcription [20]. Our findings that *BRCA1* is downregulated in the LIN28A/B knockout cells suggests that this reduction of the BRCA1 repressor complex is happening in placental cells. This was confirmed by a significant decrease in the binding of ZNF350 to the promoter region of *HMGA2* in our LIN28A/B knockout cells. Decreased BRCA1 could cause increased HMGA2 transcription in the event of aberrant LIN28 expression during placentation. Therefore, we propose a novel role for miR-182 in the regulation of *HMGA2* expression to prevent cell differentiation in the human placenta. This complex formation has been reported in mammary tumorigenesis [20]. However, to our knowledge, there have been no reports of a BRCA1 repressor complex targeting *HMGA2* in trophoblast differentiation.

As placental insufficiency is a multifaceted disorder, we recognize that the BRCA1 repressor complex could potentially regulate many different aspects of this disorder. In addition to *HMGA2*, the BRCA1/CtIP/ZNF350 complex also regulates the transcription of the angiogenic factor, *Angiopoietin 1* (ANG-1) [39]. An ovine model for fetal growth restriction reported that *ANG-1* mRNA is significantly increased at D55 of gestational age before stabilizing at D90 and D135 compared to controls. *Hagen et al.* state that this increase in *ANG-1* might be an adaptive response to a pregnancy in distress [40]. Angiogenesis is an essential component of placentation and altered vasculature is a factor in most placental insufficiency pathologies. Future studies will involve investigating how BRCA1 regulates ANG1 in placental cells to further elucidate how this pathway is regulating early placental development. MicroRNAs have been suggested as potential biomarkers for diagnosis of placental dysfunction as they are relatively stable and easily found in maternal serum [41,42]. Based on the previous knowledge of miR-182's aberrant expression in the serum and placentas of pre-eclamptic women and our findings of the role of miR-182 in promoting HMGA2 expression we propose that miR-182 could potentially be a successful therapeutic biomarker for the early detection of placental dysfunction.

Table 1.

Gene	Accession Number	Fwd (5' -> 3')	Rev (5' -> 3')	Amplicon Size (bp)	Assay ID (Thermo Fisher Scientific)
LIN28A	NM_024674.5	CTTTAAGAAGTCAGCCAAGGG	TGGCATGATGATCTAGACCTC	219	
LIN28B	NM_001004317.3	TAGGAAGTGAAAGAAGACCCA	ATGATGCTCTGACAGTAATGG	175	
RPS15	NM_001018	TTCCGCAAGTTCACCTACC	CGGGCCGGGCATGCTTTACG	381	
LIN28A	NM_024674.5			143	Hs00702808_s1
LIN28B	NM_001004317.3			130	Hs01013729_m1
HMGA2	NM_001300918.1			89	Hs04397751_m1
BRCA1	NM_007294.3			59	Hs01556193_m1
LGALS13	NM_013268.2			86	Hs00747811_m1
ERVW-1	NM_001130925.1			135	Hs01926764_u1
GAPDH	NM_001256799.2			157	Hs02786624_g1

Table 2.

Protein	Antibody For Western Blot	Antibody for Immunofluorescence	Antibody for Precipitation
LIN28B	Bethyl Laboratories, A303-588A, 1:2000	Cell Signaling, #4196S, 1:100	
LIN28A	Abcam, ab46020, 1:1000		
HMGA2	Abcam, ab97276, 1:300	Abcam, ab97276, 1:100	
hCG		NeoMarkers, Ab-5, 1:200	
BRCA1	Santa Cruz, sc-1021, 1:200	Santa Cruz, sc-1021, 1:100	Santa Cruz, sc-1021, 5 µg
ZNF350	Abcam, ab25949, 1:500		Abcam, ab25949, 5 µg
β-actin	Santa Cruz, sc-47778, 1:1000		
GAPDH	Abcam, ab9485, 1:1000		
α -Tubulin	Abcam, ab176560, 1:1000		

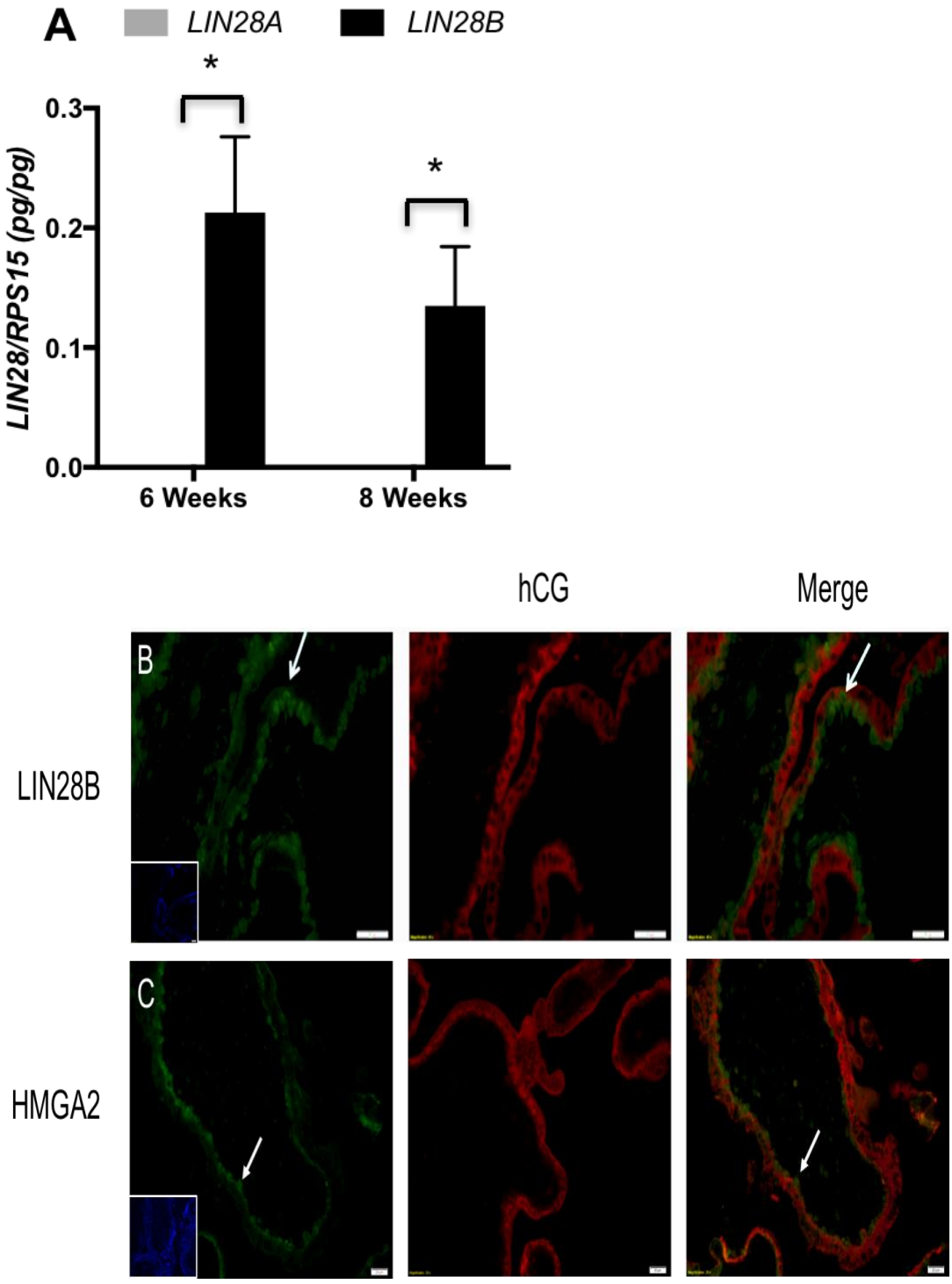

Figure 1. LIN28 and HMGA2 in the human first trimester placenta. LIN28A and LIN28B mRNA (RT-qPCR) at 6 and 8 weeks of gestation in human placentae (n=3) indicating that LIN28B is significantly higher than LIN28A during early placental development. Asterisks

indicate $p < 0.01$. Error bars represent SEM. **(A)** 11.5 week human placenta (n=2) stained for LIN28B (green) **(B)** and HMGA2 **(C)** (green) and imaged at 20x magnification. Arrows indicate LIN28B and HMGA2 are localized in the cytotrophoblast based on hCG (red) immunolocalization.

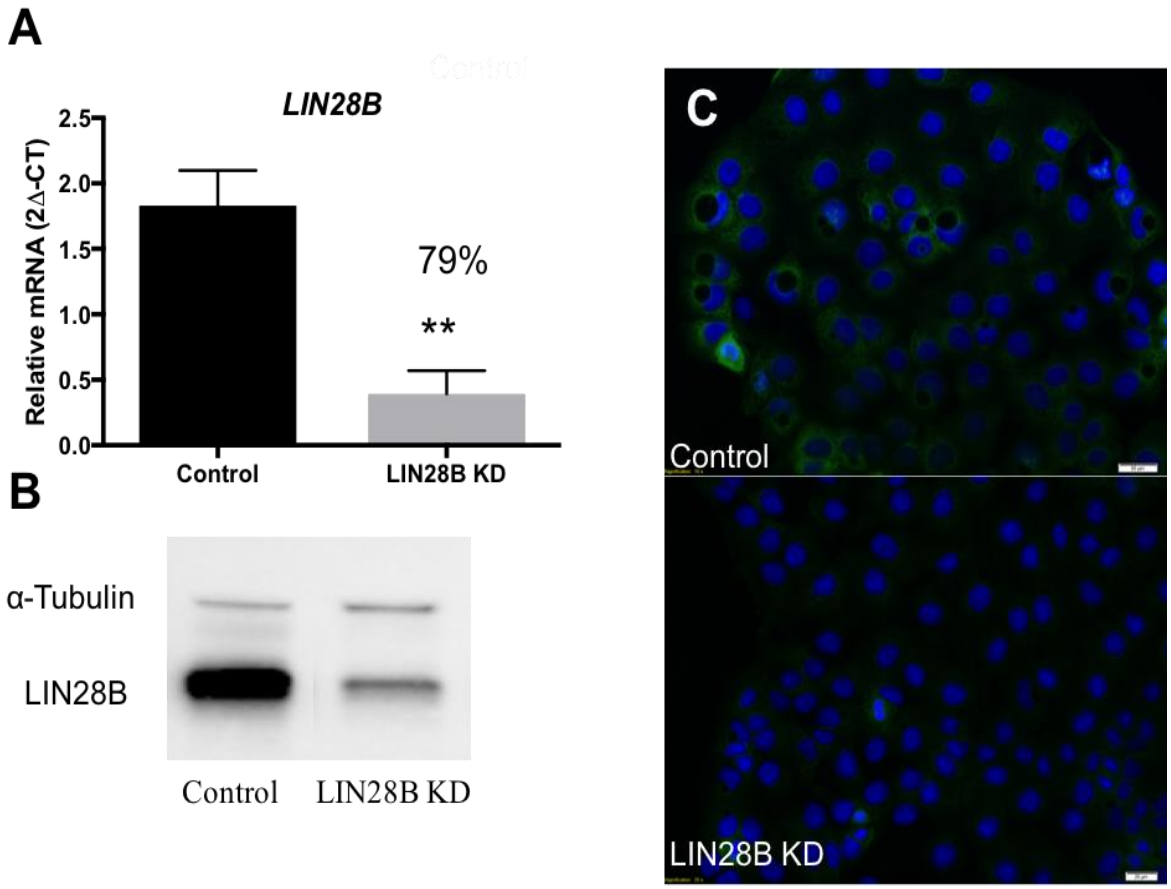

Figure 2. Short hairpin mediated knockdown of LIN28B in ACH-3P cells. **(A)** Messenger RNA and **(B)** protein levels in LIN28B KD and scramble control cells. **(C)** Immunofluorescence for protein localization of LIN28B in KD and scramble cells imaged at 20x magnification. Error bars represent SEM. Asterisk indicates $p < 0.05$

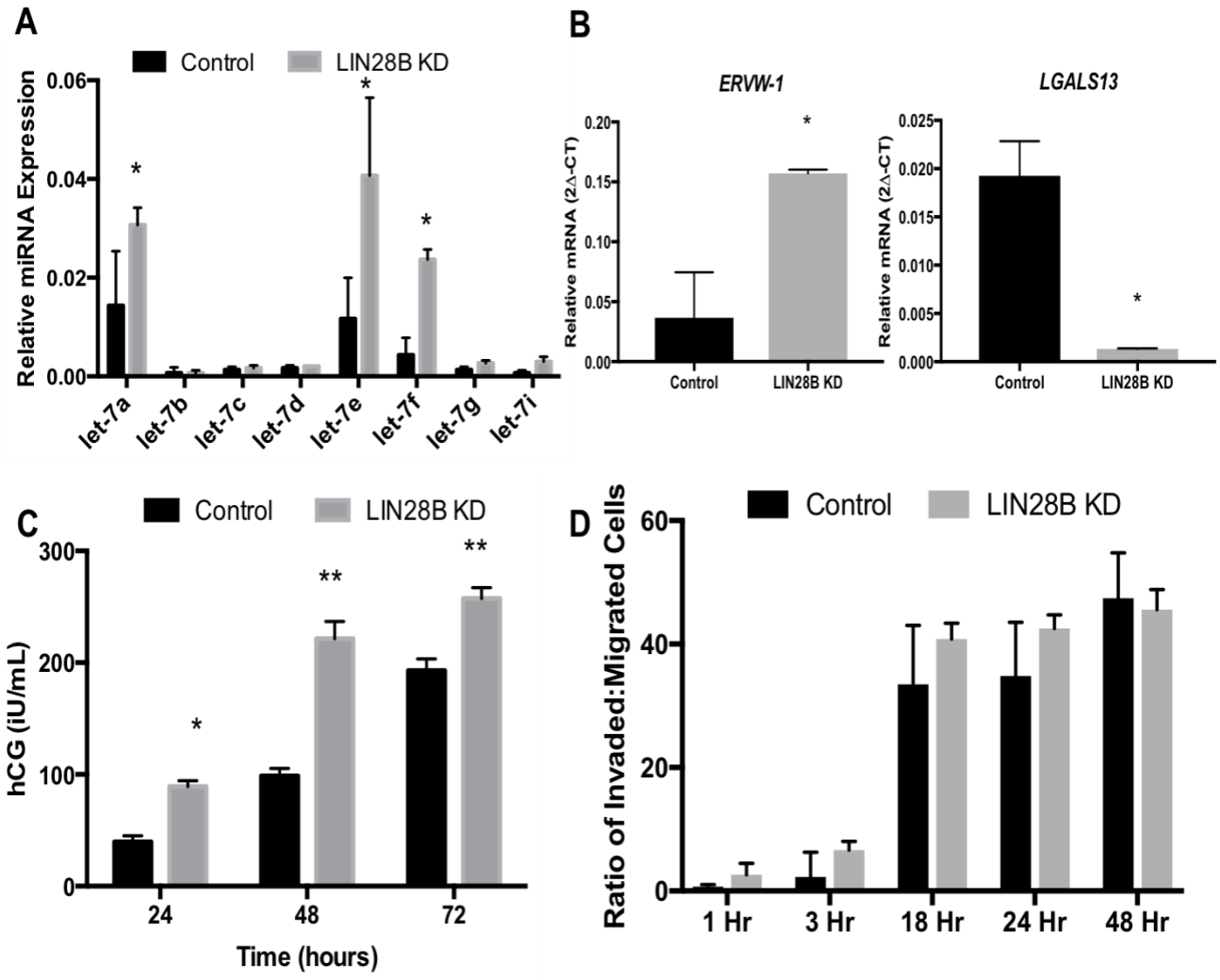

Figure 3. Knockdown of LIN28B drives cells towards syncytiotrophoblast differentiation. **(A)** Let-7 miRNA levels in LIN28B KD cells compared to control cells **(B)** *ERVW-1* and *LGALS13* in LIN28B KD cells **(C)** ELISA analysis of soluble hCG in culture medium from LIN28B KD ACH-3P cells **(D)** Ratio of invaded to migrated cells in LIN28B KD cells. One asterisk indicates $p < 0.05$, two asterisks indicate $p < 0.01$.

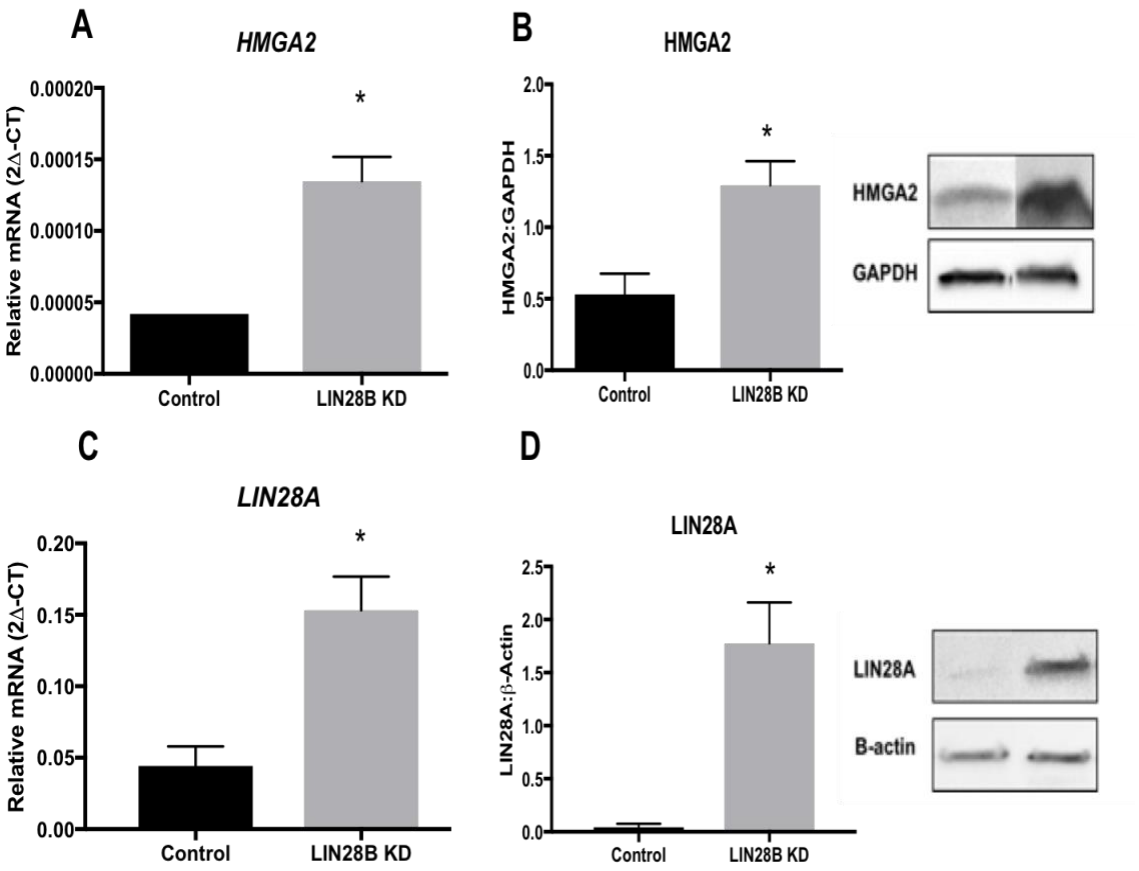

Figure 4. LIN28A and HMGA2 in LIN28B knockdown cells. **(A)** *HMGA2* mRNA and **(B)** protein and **(C)** *LIN28A* mRNA and **(D)** protein in LIN28B KD ACH-3P cells with densitometry analysis of HMGA2 and LIN28A protein bands (calculations normalized against β-Actin or GAPDH). Asterisk indicates $p < 0.05$.

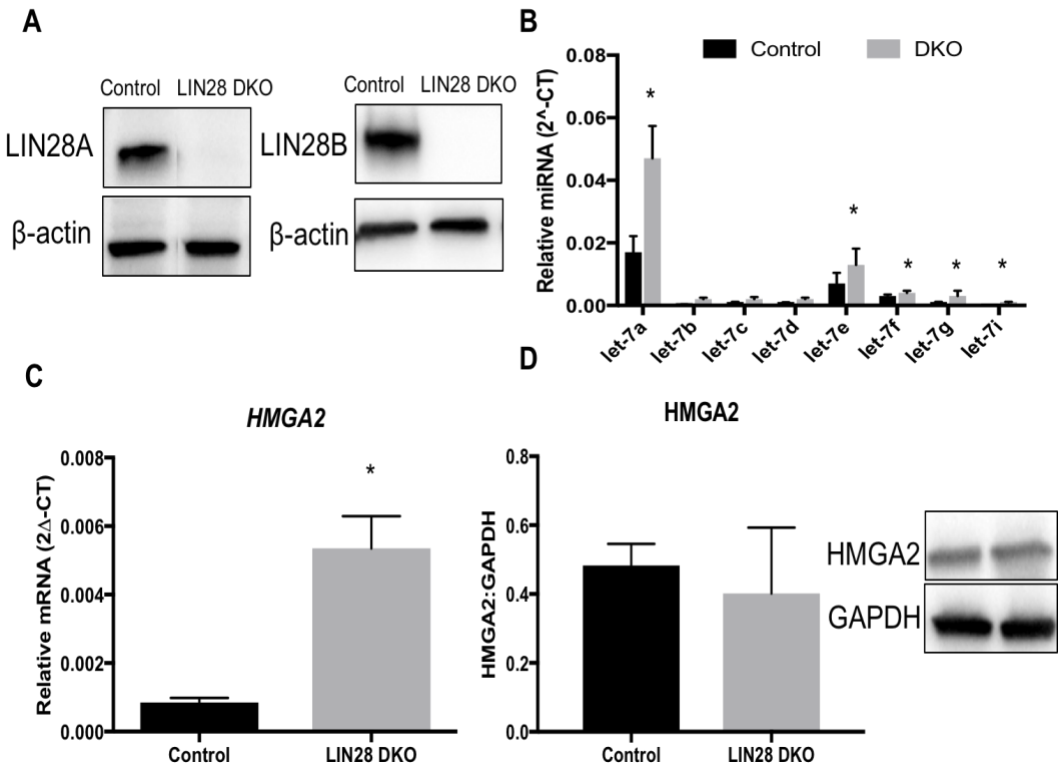

Figure 5. Let-7 miRNA and HMGA2 in LIN28 A/B double knockout cells. **(A)** LIN28A and LIN28B in LIN28 A/B double knockout cells compared to control cells. **(B)** Let-7 miRNA levels in LIN28A/B knockout cells compared to control cells**. (C)** *HMGA2* mRNA and HMGA2 protein **(D)** in LIN28A/B knockout cells compared to control cells.

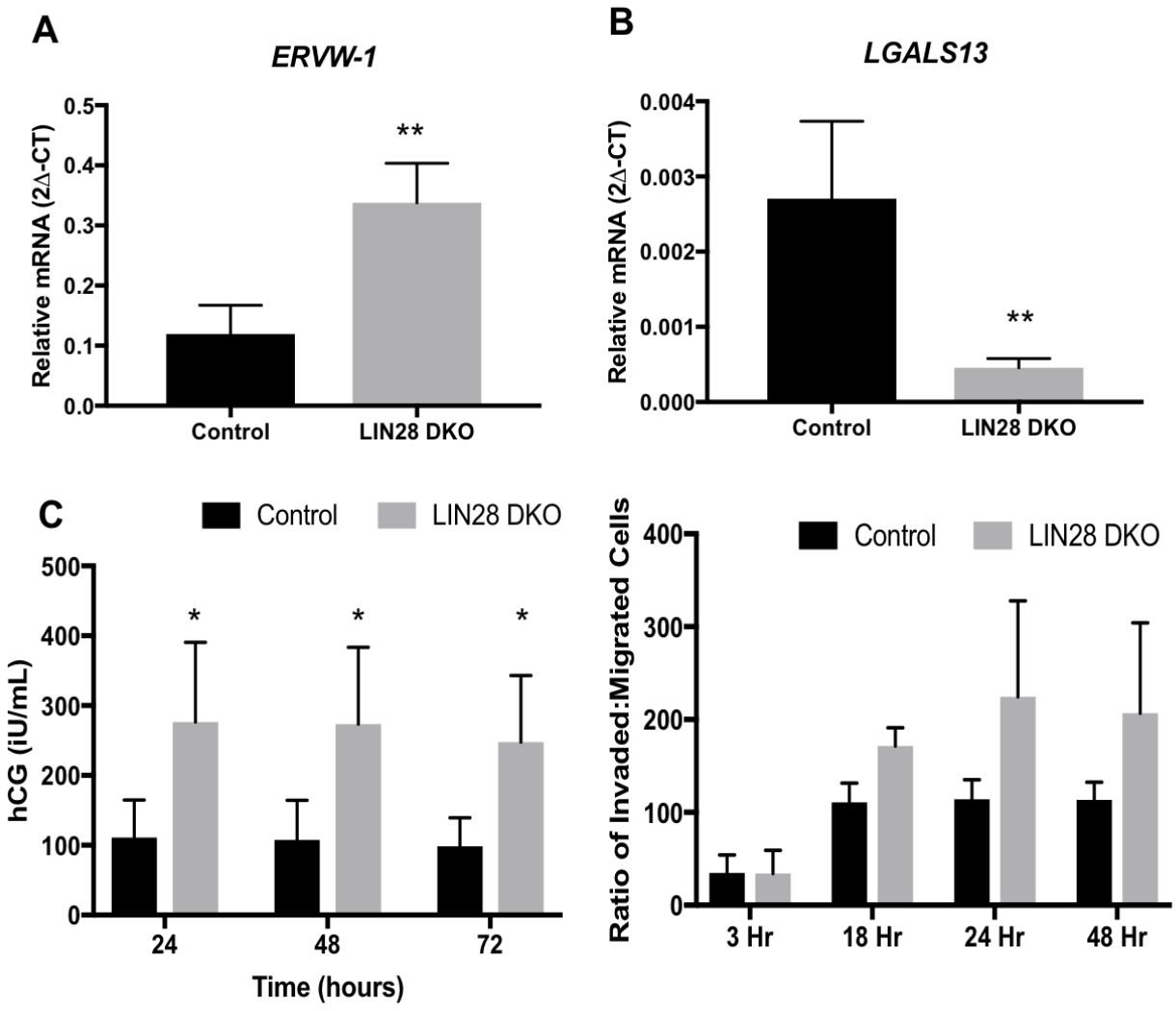

Figure 6. LIN28 A/B double knockout drives cells towards syncytiotrophoblast. *ERVW-1* **(A)** and *LGALS13* **(B)** in LIN28A/B knockout cells compared to control cells. **(C)** ELISA analysis of hCG levels from culture medium of LIN28A/B knockout cells. **(D)** The ratio of invaded to migrated cells in LIN28A/B knockout cells.

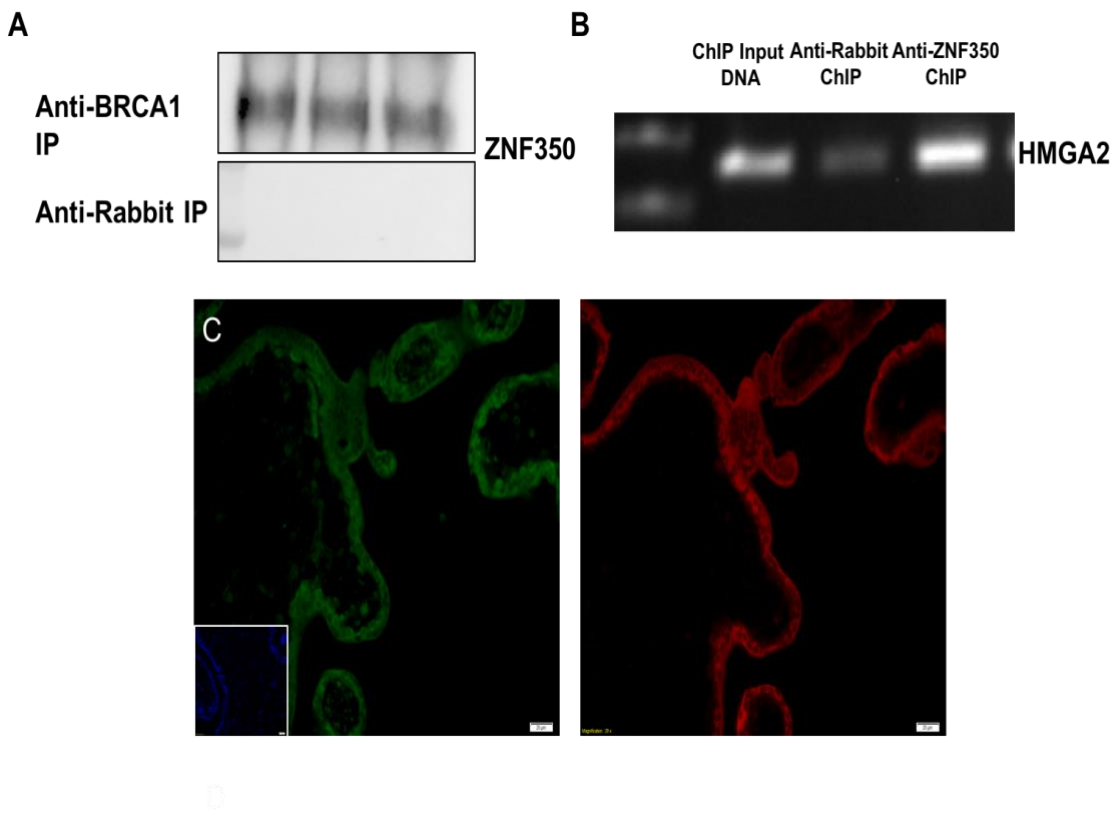

Figure 7. BRCA1/CtIP/ZNF350 repressor complex in ACH-3P cells. **(A)** Immunoprecipitation of BRCA1 from ACH-3P wild type cells (n=3) followed by Western blot detection of co-immunoprecipitated protein for ZNF350 compared to an IgG control. **(B)** PCR for the ZNF350 recognition site on the HMGA2 promoter from chromatin immunoprecipitated samples (n=3). **(C)** Human 11.5 week placenta immunostained for BRCA1 (green) or hCG (red) (n=2).

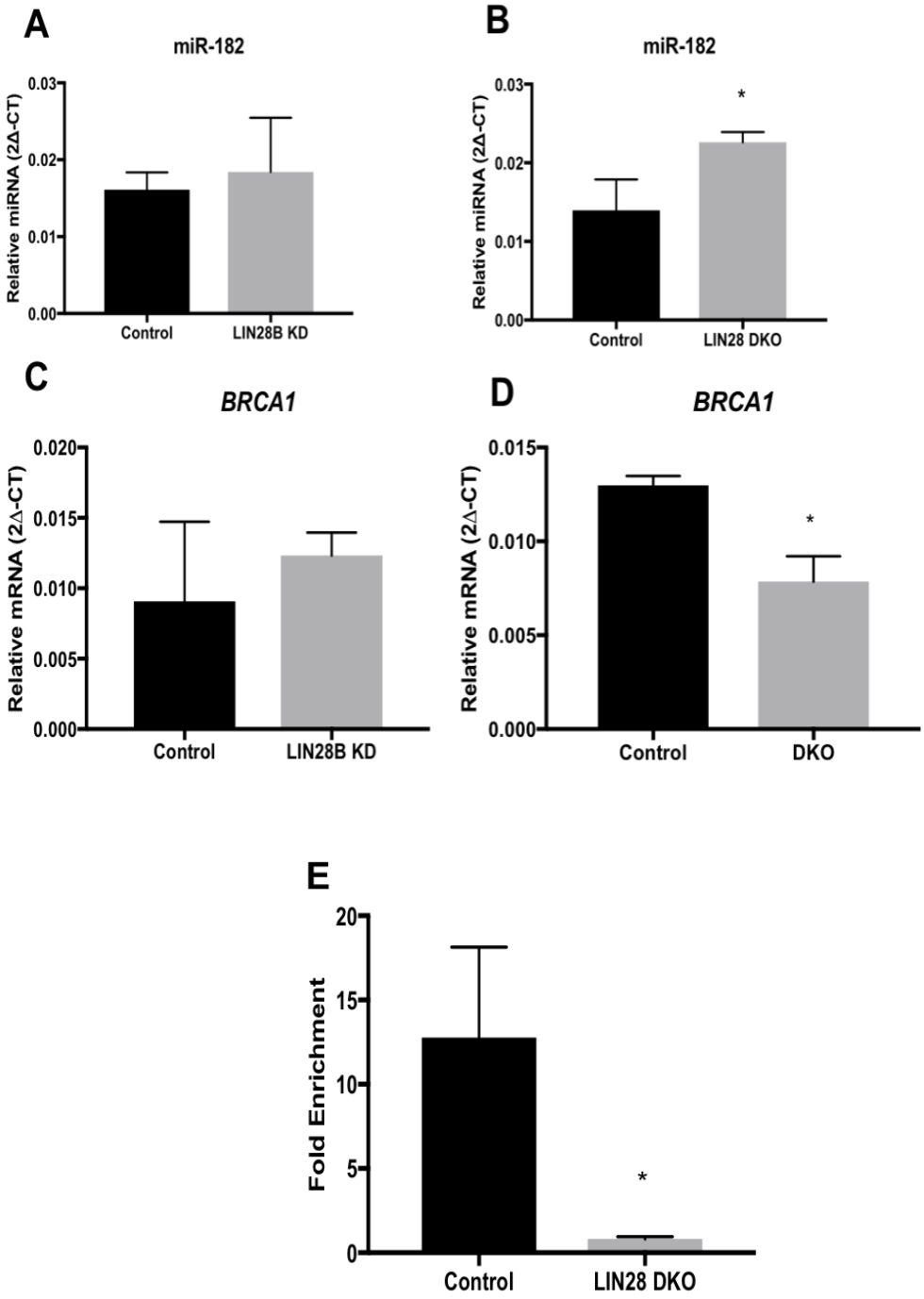

Figure 8. MiR-182 and *BRCA1* in LIN28B KD and LIN28A/B DKO Cells. **(A)** MiR-182 miRNA levels in LIN28B KD and LIN28A/B knockout **(B)** cells. **(C)** *BRCA1* mRNA in LIN28B KD and LIN28A/B knockout cells. **(D)**. **(E)** Quantitative PCR analysis for the ZNF350 recognition site

on the *HMGA2* promoter of chromatin immunoprecipitated LIN28A/B knockout cells compared to control cells.

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CHAPTER III: ALTERED BRCA1 EXPRESSION DRIVES ENHANCED HMGA2 LEVELS DURING EARLY PLACENTAL DEVELOPMENT[4]

Synopsis

Early human placental development strongly resembles carcinogenesis in otherwise healthy tissues. One key difference between cancer progression and placental development is the tight regulation of these oncogenic processes and the oncogenes that drive them. Tumor suppressors and oncogenes work synergistically to regulate cell proliferation and differentiation in a restrained manner compared to the uncontrollable growth in cancer. One example of this partnership is the regulation of the oncofetal protein HMGA2 by BRCA1. BRCA1 forms a repressor complex with ZNF350 and CtIP to bind to the promoter region of HMGA2 to prevent transcription. We determined by chromatin immunoprecipitation that BRCA1 forms this repressor complex in human trophoblast cells, suggesting a role in the placenta. Additionally, BRCA1 is an important tumor suppressor involved in DNA repair and apoptosis. Therefore, we hypothesized that BRCA1 is essential for normal trophoblast cell proliferation. To test this hypothesis, we used CRISPR-Cas9 gene editing to knock out BRCA1 in the Swan71 cell line. HMGA2 mRNA and protein was significantly increased in the BRCA1 KO cells compared to control cells. Chromatin was immunoprecipitated using an antibody for ZNF350 and PCR was for the promoter region for HMGA2. BRCA1 repressor complex binding to *HMGA2* was significantly reduced in the knockout cells compared to our control cells, leading us to conclude that increased HMGA2 was due to decreased binding of the BRCA1 repressor complex. Additionally, we found that Caspase 3 and 7 levels were significantly higher in our BRCA1 KO cells compared to controls. This data suggests that BRCA1 is an important factor in

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the regulation of the oncofetal protein HMGA2 and promotes cell survival in human placental cells.

Introduction

HMGA2 is an architectural transcription factor belonging to the High Mobility Access Group (HMGA) of proteins [1]. This family of proteins regulates transcriptional activity by modifying the architectural structure of chromatin, allowing for the promotion of cooperative binding of transcription factors to enhancer regions [2]. Typically, expressed at its highest levels in stem cells and during embryogenesis, HMGA2 levels begin to decline as cells differentiate where they remain low throughout adult life [3]. However, aberrant HMGA2 expression has been described in many cancers including but not limited to, breast [4], pancreatic [5], non-small cell lung [6], T-cell lymphoblastic leukemia [7]. HMGA2 seems to have an influence on a wide variety of tumorigenic processes, including cell proliferation, invasion, differentiation, and death [7]. In the placenta, HMGA2 has been described in the chorionic trophoblast progenitor cells, losing expression as these cells differentiate [8]. HMGA2 has also been described in the syncytiotrophoblastic and cytotrophoblast cells of testicular germ cell tumor derived choriocarcinomas [9]. However, little is known about the regulation of HMGA2 during placentation.

One mechanism that regulates HMGA2 in cancer cells is the tumor suppressor BRCA1. BRCA1, in conjunction with ZNF350 and CtIP, forms a repressor complex that binds to the promoter region of *HMGA2,* preventing transcription [10]. Removal of any of these proteins using siRNA led to decreased binding to the *HMGA2* promoter, increasing cell proliferation and invasion [10]. Due to the highly proliferative and invasive nature of human placentation, we

hypothesized that HMGA2 is necessary during early placental development, becoming negatively regulated by BRCA1 as cells begin to differentiate.

However, BRCA1's role as a transcriptional regulator is only a small part of this multi-functional protein's properties. BRCA1 is most well known as an important tumor suppressor involved in the homologous recombination pathway of DNA double stranded break (DSB) repair [11]. Upon DNA damage, BRCA1 is recruited to the site and acts as a scaffolding protein providing a binding site for many other DNA repair proteins [12]. It is essential for many steps of strand repair, including DNA-end resection [13] and the promotion of strand-invasion by interacting with the recombinase protein Rad51 [14]. Loss-of-function mutation in BRCA1 in cells leads to increased DNA damage, abnormalities in all mitotic cell cycle checkpoints, and high levels of genomic instability [15, 16]. A somatic mutation for BRCA1 in mammary epithelial cells led to highly proliferative, poorly differentiated carcinomas with high levels of genomic instability [17]. Interestingly, $BRCA1^{-/-}$ knockout mice are embryonic lethal by day 7.5 of embryogenesis. This lethality is largely in part due to a severely underdeveloped extraembryonic region, with a complete lack of diploid trophoblast cells and an overabundance of trophoblast giant cells [18]. These data suggest that BRCA1 is essential for the organization and development of the placenta.

In some cancers, the onco-miR, miR-182, targets BRCA1 to decrease translation [19]. In 70% of ovarian cancers, elevated miR-182 and HMGA2 levels and decreased BRCA1 levels were reported [20]. This suggests that miR-182 can alter BRCA1 expression leading to increased HMGA2 in cancer cells. There has been increasing evidence that microRNAs are essential mechanisms that drive placental development [21]. Mir-182 was one of the first microRNAs reported to be elevated in both the serum and placentas of preeclamptic women compared to

normotensive women [22, 23]. Based on these data, we hypothesized that miR-182 negatively regulates BRCA1 levels during pregnancy, leading to increased *HMGA2* transcription. To investigate this hypothesis, we used CRISPR-Cas9 gene editing to knock out BRCA1 in the placental cell line, Swan71. We also overexpressed stably transduced miR-182 to constitutively overexpress miR-182 and target BRCA1.

Materials and Methods

Cell Culture

The immortalized Swan71 cell line (a generous gift from Gil Mor, Yale University [24]) were cultured in DMEM F-12 Medium (HyClone), 10% Fetal bovine serum (Atlas Biologicals), and 1% Penicillin/Streptomycin/Amphotericin (Corning Life Sciences).

Human placental samples

Human 11.5 week (n=1) placental tissue was obtained from elective terminations from anonymous, non-smoking, non-drug using patients following protocol 10-1623H approved by Colorado State University. Tissue was stored in phosphate buffered saline upon collection then transferred to ice cold 4% paraformaldehyde (PFA) (Fisher Scientific). After 12 hours at 4°C, tissues were transferred from 4% PFA to 70% ethanol and stored at 4°C until paraffin embedding.

Immunofluorescence

Paraffin embedded tissue was cut into 5 micron sections and mounted onto charged glass microscope slides (Premiere). To deparaffinize, sections were treated in successive 4 minute baths of Citrasolv (Decon Labs), 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol and then rehydrated with distilled water. Antigen retrieval was then performed using 10 mM sodium

citrate buffer microwaved for 10 minutes after reaching boiling. Sections were cooled at room temperature for 1 hour then rinsed in PBS 3 times for 5 minutes each. Sections were permeabilized for 5 minutes in 0.05% Tween in PBS then rinsed in PBS 2 times for 5 minutes each. Sections were then blocked in 6% normal goat serum in PBS for one hour before put in primary antibody (EMD Millipore, MS110) at a 1:100 dilution and incubated overnight at 4°C. After incubation, slides were washed 3 times in PBS, then incubated in a goat anti-mouse AlexaFluor 488 (Abcam, ab150077) or goat anti-mouse AlexaFluor 594 (Abcam, 150092) at a 1:1000 dilution. After 1 hour, slides were washed 3 times in PBS for 5 minutes. Slides were mounted with Prolong Gold Antifade Mounting reagent containing DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). As a negative control, normal rabbit serum was used as a primary antibody (Molecular Probes). Each experiment was repeated at least three times.

Real-Time Quantitative PCR

To isolate cellular RNA a RNA Mini Kit (Qiagen) was used. Complementary DNA was generated using qScript cDNA Supermix (Quantabio) and 1 ug RNA. Samples were incubated for 5 minutes at 25°C, then 30 minutes at 42°C, and 5 minutes at 85°C. Quantitative real-time PCR was then conducted using SsoAdvanced Universal Probes Supermix (Bio-Rad) and Taqman Gene Expression Assays (Applied Biosystems). Using 20 uL total volume, 10 uL of Supermix, 1 uL of 150 nM Taqman Gene Expression Assay (ThermoFisher) and 9 uL cDNA template diluted to 11 ng were mixed together. Reactions were then incubated at 95°C for 1 minute, before undergoing 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds using a LightCycler480 PCR System (Roche Applied Science). Relative expression was normalized using GAPDH. Each reaction was conducted in duplicate with two controls, an RT control and a water control. The comparative Ct method was used to determine relative expression levels.

To extract miRNA, total RNA was isolated using a miRNA Mini Kit (Qiagen). A starting quantity of 500 ng total RNA was used to generate cDNA using a miScript RT II kit (Qiagen). RNA was incubated for 60 minutes at 37°C then incubated for 5 minutes at 95°C . For real-time PCR, QuantiTect SYBR Green Master Mix (Qiagen), a miScript universal primer (Qiagen) and a miScript primer assay for the mature miR-182 miRNA sequence (Qiagen) was used as a mix. One uL of cDNA diluted to 3 ng was added to the mix then reactions were incubated for 15 minutes at 95°C, then underwent 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Each reaction was conducted in duplicate with two controls, an RT control and a water control. MiR-182 levels were normalized against SNORD68and relative expression was determined using the comparative Ct method. Experiments were repeated three times.

Western blotting

To assess cellular protein levels, western blot analysis was used. RIPA buffer (20 mM Tris, 137 mM NaCL, 10% glycerol, 1% nonidet P-40, 3.5 mM SDS, 1.2 mM sodium deoxycholate, 1.6 mM EDTA, pH 8) was used to lyse cells. 300 uL of RIPA buffer was added to cell pellets, then cells were sonicated on ice for 10 seconds then centrifuged for 12 minutes at 4,000 g. Supernatant was removed and placed in a fresh tube with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) added at a 1:100 ratio. Protein concentration was determined using a BCA protein assay kit (Pierce). Protein and the BCA reagents were added at a 1:1 ratio, incubated for 30 minutes at 37°C, then absorbance was measured at λ 595 nm using a Biotek Synergy 2 Microplate Reader (Biotek). Protein was then loaded and electrophoresed in 4-15% Bis-Tris gels (Bio-Rad) at 125 V. Protein was then transferred to 0.45-µm pore nitrocellulose membranes at 120 volts at 4°C for 1 hour and 45 minutes. To block, membranes were incubated in 5% milk-TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween20, pH 7.6) for 1 hour at room

temperature. Blots were then incubated in BRCA1 (EMD Millipore, MS110) antibody at a 1:250 dilution or HMGA2 (Abcam, ab97276) at a 1:200 dilution at 4°C overnight. After incubation, blots washed 3 times for 15 minutes in TBST then incubated with a horseradish peroxidase-conjugated secondary antibody (Abcam, ab6721) at a 1:1000 dilution for 1 hour at room temperature. Membranes were then washed 3 times for 15 minutes each wash in TBST then developed using an ECL Western Blotting Detection Reagent chemiluminescent kit. Membranes were imaged using a ChemiDoc XRS+ chemilluminescence system (BioRad). β-actin (Santa Cruz, sc-47778) was used to normalize protein in cell lysates and densitometry was performed using Image Lab (Bio-Rad). Fold change was calculated as a percent of control protein after normalization. Each experiment was performed with 3 replicates and repeated at least 3 times.

Chromatin Immunoprecipitation

ChIP was performed with the Abcam ChIP Kit (Abcam, ab500) following manufacturer's protocol. Cells were crosslinked using 4% formaldehyde diluted in PBS. Cells were then sonicated on ice using a BioRuptor (Diagenode) for 10 minutes on high for 30 seconds on and 30 seconds off. ZNF350 (Abcam, ab127895) was immunoprecipitated from 10 µg of sample. As a negative control, a polyclonal IgG antibody (Abcam, ab6721) was used. Next, PCR was performed using primers for HMGA2 promoter region containing the *ZNF350* binding site: (Fwd: 5'–CCCAGCCCTATCACCTGATC–3', Rev: 5'– CCTCCTTTGCTTTCCGACTG–3). As positive controls, both genomic DNA and ChIP input DNA were used. Each experiment was performed using three replicates and repeated at least three times.

Knockout of BRCA1

BRCA1 CRISPR guide RNA (TGCTAGTCTGGAGTTGATCA) inserted into the pLentiCRISPR v2 plasmid vector [25] (GenScript) were used to knock out BRCA1 in the

Swan71 cells. Lentiviral particles were generated after transfection into human embryonic kidney (HEK) cells. HEK cells were transfected using a transfection mix containing the plentiCRISPR plasmid, a packaging plasmid (PAX), an envelope plasmid (pMD2.G), and the transfection reagent Polyfect. Cells were transfected for 12 hours then the cell culture medium was changed. Cells were incubated for 72 hours before culture medium was collected and lentivirus was purified. Swan71 cells were then infected at a MOI of 2 viral particles per cell. As a control, Swan71 cells were also infected with lentivirus containing an empty vector control. Cells were treated with 2 μg/mL of Puromycin 72 hours after infection to ensure integration of the plentiCrispr construct.

Overexpression of miR-182

MISSION Lenti microRNA transduction particles targeting miR-182 (Mature sequence – UUUGGCAAUGGUAGAACUCACACU) (Sigma-Aldrich, HLMIR0275) or a nontarget control sequence (Sigma-Aldrich, NCLMIR001) were used. Swan71 cells were infected with lentiviral particles at a MOI of 5 viral particles per cell. Three days after infection, cells were selected with addition of 2 μg/mL of puromycin.

Measurement of Apoptosis

A Caspase-Glo 3/7 Assay (Promega) was used to determine levels of apoptosis in cells. Cells were plated in black-walled, black bottom 96 well plates (Corning) at a density of 500 cells per well. Apoptosis was induced by serum starving cells in 0.5% FBS culture medium for 16 hours. After 16 hours, 100 μL of Caspase-Glo 3/7 reagent was added to each well and incubated at room temperature for 1 hour. Luminescence was determined using a BioTek plate reader. The assay was performed with 4 replicates. Data was normalized using a media only control well luminescence.

Enzyme-Linked Immunosorbent Assay

Cells were plated in at a density of 50,000 cells per well in a 6-well dish. At 24, 48, and 72 hours after plating, 1 mL of cell culture medium was collected and concentrations of human chorionic gonadotropin were quantified using a kit for beta-hCG (mouse monoclonal anti-hCG conjugated to horseradish peroxidase) (ALPCO Diagnostics, 25-HCGHU-E01). Three replicates were used for each experiment and each experiment was conducted twice.

Invasion/Migration Assay

The BioCoat Matrigel Invasion Assay System (Corning) was used to determine cell invasion. Cells were first dyed using CellTracker Green CMFDA dye (Invitrogen) and incubated for 45 minutes. After incubation, cells were plated onto the wells at a density of $1x10^4$ with into the upper chamber of the system. The bottom chamber of the system was filled with media containing 10% FBS as a chemoattractant. As a control, cells were also seeded onto uncoated Falcon FluoroBlok 24-Multiwell plates (Corning). Cells were incubated at 37°C in 5% CO_2 and invasion was assessed at 3, 18, 24, and 48 hours after plating. To read, the Biotek Synergy 2 Microplate Reader was used with 530/25-excitation filter and 590/35-emission filter. Fluorescence was read at wavelengths 549/565 nm. Background fluorescence was subtracted and invasion was normalized by dividing the relative fluorescence intensity of invaded cells by intensity of migrated cells then multiplied by 100. Each experiment was performed with 4 replicates and repeated.

Statistics

All statistics were determined using Prism 7 for Mac OS X (GraphPad Software). Student t-tests were used to compare between cell lines and all experimental replicates were assayed in triplicate. P-values less than 0.05 were considered statistically significant.

Results

BRCA1 in the human placenta

Human first trimester 11.5 week (n=1) (Figure 1A) and term (Figure 1B) placental tissue immunostained for BRCA1 at a 20x magnification. In the 11.5-week placenta, BRCA1 seemed to be most highly expressed in the syncytiotrophoblast but appears to be in all cell types. At term, BRCA1 is expressed in all cell types of the placenta.

BRCA1 and miR-182 in human placental cells

Messenger RNA levels of *BRCA1* and miR-182 were analyzed in the immortalized placental Swan71 and ACH-3P cells. *BRCA1* levels were significantly higher in the Swan71 cells (Figure 2A) while miR-182 miRNA levels were significantly lower (Figure 2B) than the ACH-3P cell line.

Generation of a BRCA1 Knockout Cell Line

Swan71 cells were infected with lentivirus containing gRNA specific for BRCA1. Western blotting was used to determine BRCA1 knockout (Figure 3A).

BRCA1/CtIP/ZNF350 repressor complex regulates HMGA2 in placental cells

To investigate if knockout of BRCA1 affected transcription of *HMGA2*, chromatin immunoprecipitation was used to determine if the BRCA1/CtIP/ZNF350 repressor still bound to the *HMGA2* promoter. In control cells, we found that ZNF350 binds to the ZNF350 recognition site on the *HMGA2* promoter region, after immunoprecipitation against ZNF350 and PCR. However, in our BrKO cells, we did not see any evidence of binding to the *HMGA2* promoter region (Figure 4A). Additionally, we saw a significant increase in HMGA2 mRNA (Figure 4B) and protein (Figure 4C), suggesting that loss of BRCA1 leads to increased HMGA2 in placental cells.

Knockout of BRCA1 causes apoptosis and proliferation defects

Using a Caspase assay, we determined that BrKO cells were significantly more apoptotic than control cells (Figure 5A). These cells also had significantly lower levels of the differentiation markers for *LGALS13 and ERVW-1* (Figure 5B and 5C).

Overexpression of miR-182 leads to decreased BRCA1

Swan71 cells were also infected with lentiviral particles containing an overexpression construct for miR-182. Overexpression was determined using RT-qPCR, 182 overexpression cells had significantly higher levels of miR-182 compared to nontarget control cells (Figure 6A). We next analyzed BRCA1 protein levels and determined that BRCA1 levels were significantly lower in 182 overexpression cells (Figure 6B).

182 overexpression drives increased HMGA2

We analyzed HMGA2 levels and found that HMGA2 protein was significantly increased in 182 overexpression cells compared to nontarget control cells (Figure 7).

Overexpression of miR-182 causes apoptosis and proliferation defects

Using a Caspase assay, we determined that 182 overexpression cells were significantly more apoptotic than control cells (Figure 8).

Overexpression of miR-182 drives altered expression of tumor suppressor genes

As miR-182 is known to target several tumor suppressor genes, we used RT-qPCR to determine if miR-182 decreased any mRNA levels of these genes. *FOXO3* levels were the only mRNA levels significantly decreased ($p<0.05$) (Figure 9A) in our 182 overexpression cells but *RSU1* was trending towards decreased levels ($p<0.07$) (Figure 9B). *RECK* and *TIMP* levels were not significantly altered (Figure 9C and 9D).

Discussion

Besides regulating DNA repair and cell cycle checkpoints, BRCA1 is an important transcriptional regulator of proliferation factors. Microarray data of BRCA1 knockdown cells reveals that the BRCA1/CtIP/ZNF350 repressor complex regulates several proliferation markers, including *ANG1, bFGF, HMGA2, LIMK1,* and *RFC1* [26]. The BRCA1/CtIP/ZNF350 repressor complex has been described in human mammary epithelial cells and murine breast tumors [10]. However, to our knowledge, this is the first description of this complex in placental cells. Our data indicate that not only does this BRCA1 repressor complex form in placental cells but also that a knockout of BRCA1 leads to decreased binding to the *HGMA2* promoter, increasing both mRNA and protein levels of HMGA2. Additionally, we found that these cells were significantly more apoptotic and had downregulated markers for differentiation towards the syncytiotrophoblast and extravillous trophoblast. This tendency towards apoptosis could be caused by increased levels of HMGA2. Increased HMGA2 has been shown to induce genomic instability by interfering with non-homologous end-joining function and delaying clearance of markers of DNA damage, including γ-H2AX [27]. As creation of genomic instability induces apoptosis in many cell types [28], we believe that this increased HMGA2 could explain the increased apoptosis. Additionally, our BRCA1 knockout cells do seem to have increased levels of γ-H2AX immunoreactivity (Supplemental Figure 1) compared to control cells. Genomic instability isn't an area of cellular dysfunction that has been extensively studied in the placenta. However, there is compelling evidence that DNA damage contributes to placental pathologies, including pre-eclampsia [30-32]. This DNA damage is characterized by increased DNA double-stranded breaks, as characterized by increased levels of

γ-H2AX foci formation [31]. Future studies will revolve around understanding how BRCA1 influences DNA repair in placental cells.

We also describe the role of miR-182 in targeting *BRCA1* mRNA to allow increased *HMGA2* transcription. In breast tissue, miR-182 targets *BRCA1*, decreasing translation [33]. In our placental cells, we also found that overexpression of miR-182 decreased BRCA1 protein, leading to elevated levels of HMGA2 mRNA and protein. Similarly to the BRCA1 KO cells, we also found that these cells were significantly more apoptotic compared to nontarget control cells. This could be due to the increased amount of HMGA2 leading to genomic instability as explained above.

Finally, as noted at the beginning of this discussion, the BRCA1 repressor complex regulates many factors involved with proliferation and angiogenesis, including *ANG1*. Preeclampsia is a disorder also characterized by altered vasculature and maternal endothelial dysfunction [34] and altered expression of angiogenic factors has been documented in preeclamptic placentas [35]. We found that a knockout of BRCA1 did lead to decreased binding of the BRCA1 repressor complex to the *ANG1* promoter (Supplemental Figure 2). This suggests that BRCA1 plays an important role in many different aspects of placental development. Additional studies will be needed to better understand how BRCA1 influences angiogenesis in placental cells.

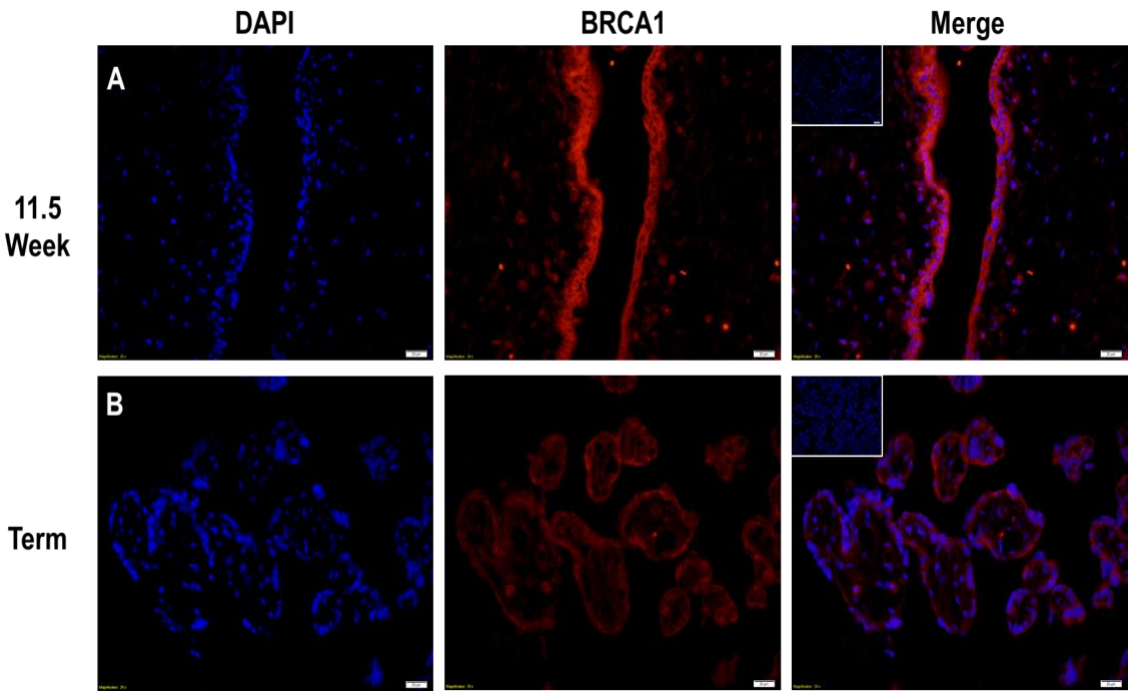

Figure 1. BRCA1 in human placental tissue. **(A)** BRCA1 immunofluorescence in 11.5 weeks of gestation (n=2) and in **(B)** term tissue (n=1) at a 20x magnification.

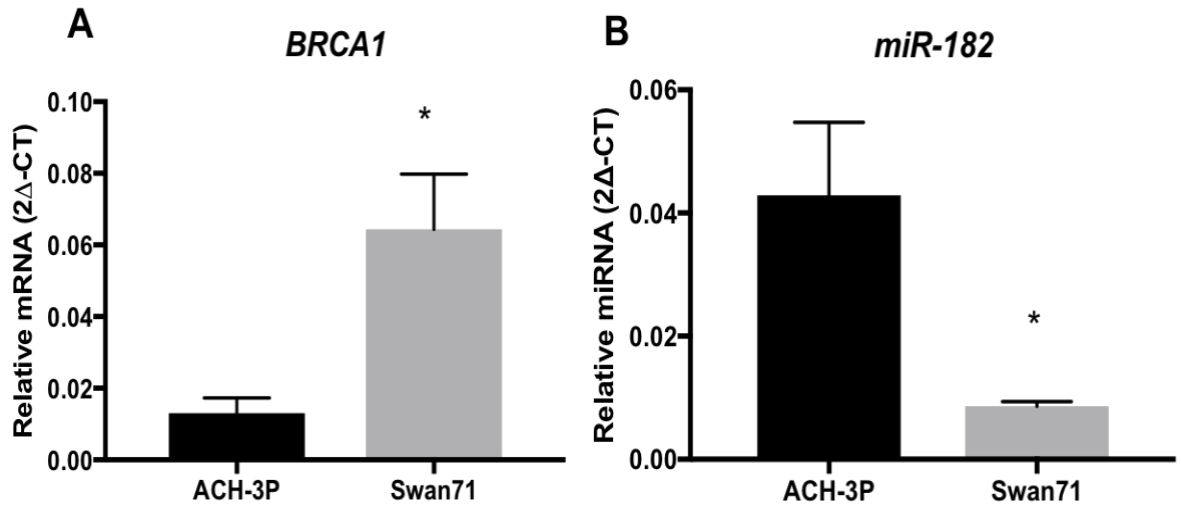

Figure 2. *BRCA1* and miR-182 in human placental cells. **(A)** *BRCA1* mRNA and **(B)** miR-182 miRNA levels in the human placental cell lines ACH-3P and Swan71. Asterisks indicate $p < 0.05$.

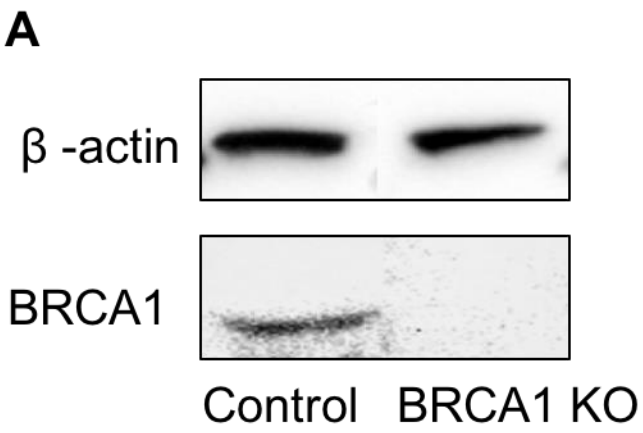

Figure 3. Generation of a BRCA1 knockout cell line. **(A)** BRCA1 protein in BRCA1 knockout cells.

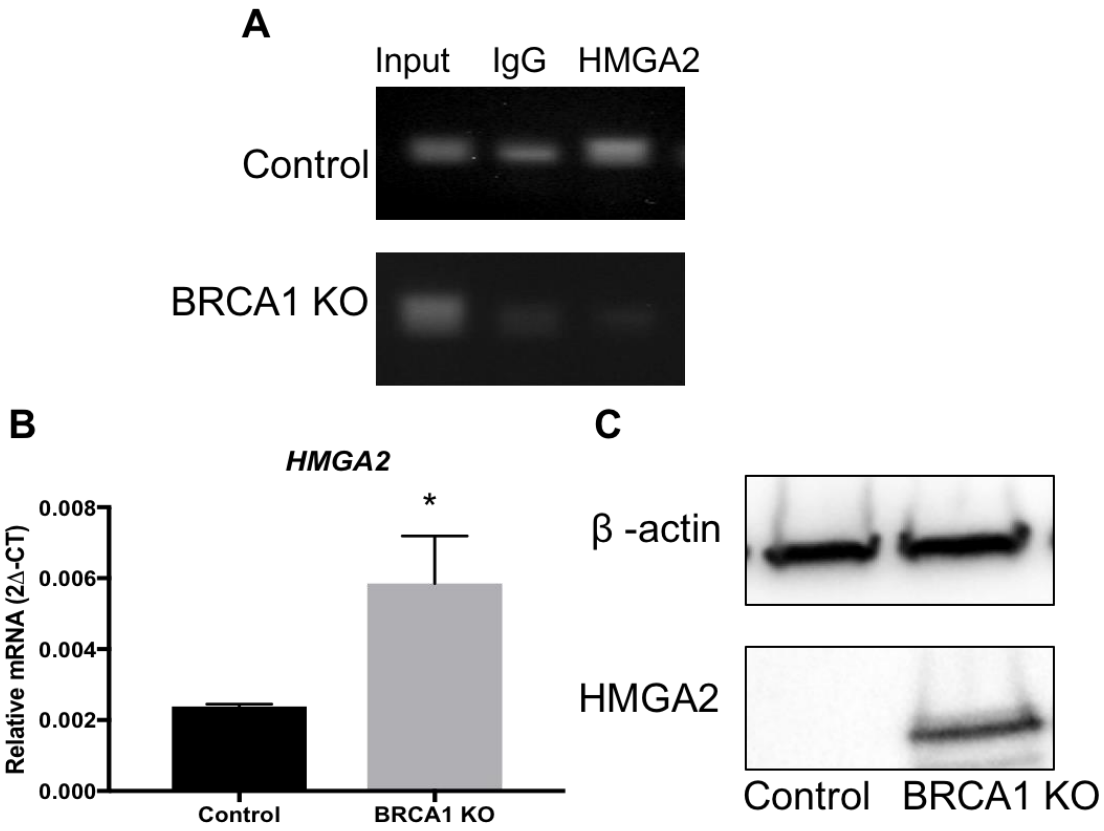

Figure 4. HMGA2 in BRCA1 knockout cells. **(A)** ZNF350 immnoprecipitated DNA with PCR for HMGA2 promoter region **(B)** *HMGA2* mRNA levels and **(C)** HMGA2 protein in BRCA1 knockout cells.

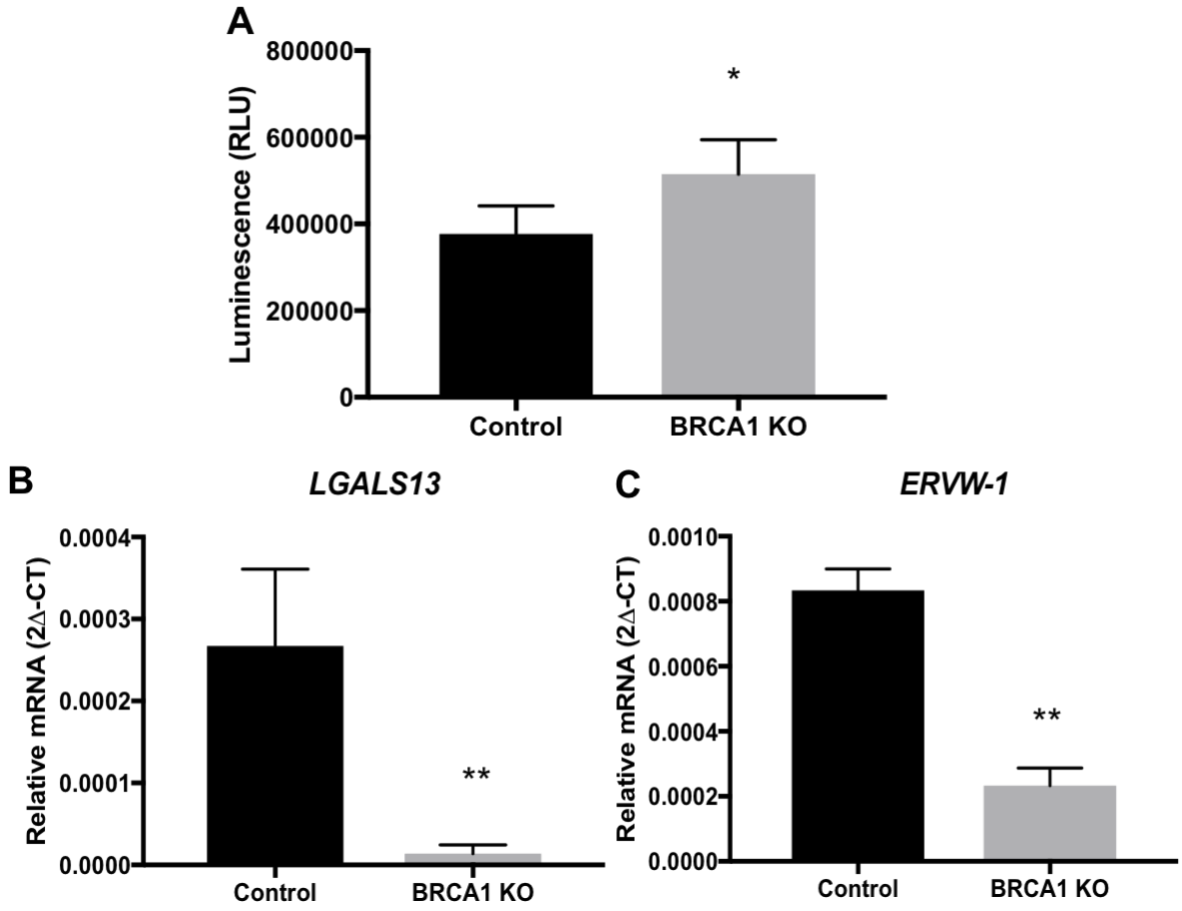

Figure 5. Apoptosis in BRCA1 knockout cells. **(A)** Levels of apoptosis in serum-starved BRCA1 knockout cells versus control. **(B)** *LGALS13* and **(C)** *ERVW-1* mRNA levels in BRCA1 knockout cells

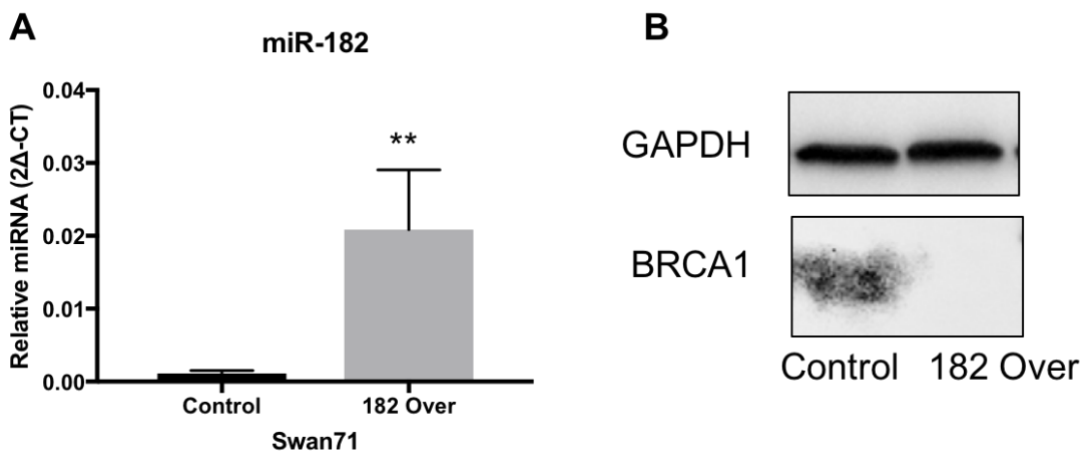

Figure 6. MiR-182 and BRCA1 in 182 overexpressing cells. **(A)** MiR-182 miRNA levels in 182 overexpressing cells and **(B)** BRCA1 in 182 overexpressing cells.

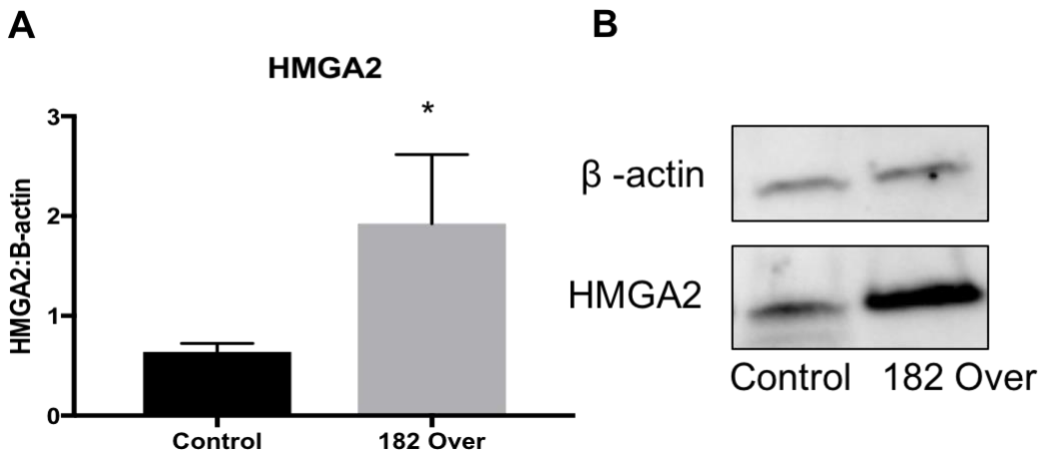

Figure 7. MiR-182 and HMGA2 in 182 overexpressing cells. **(A)** Densitometry from **(B)** western blotting for HMGA2 protein

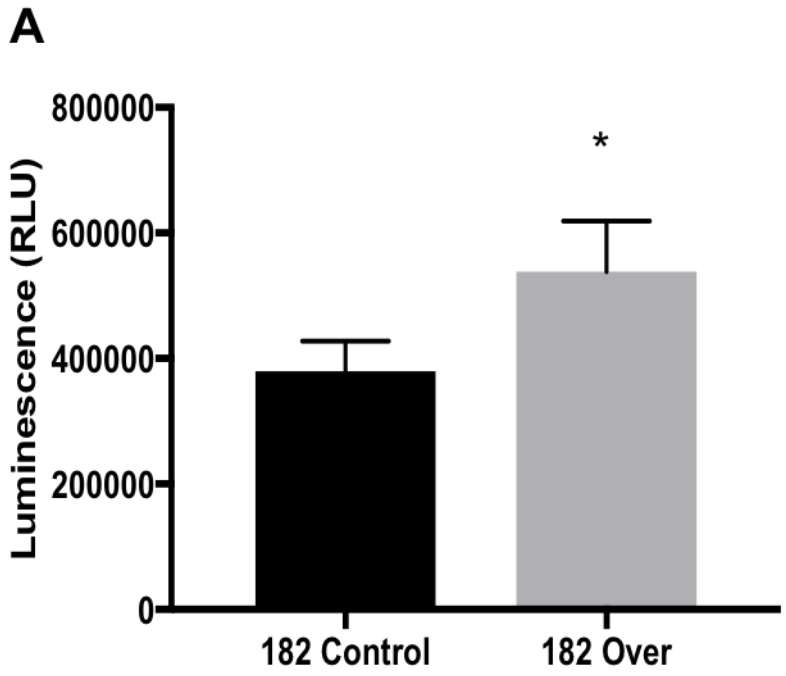

Figure 8. Apoptosis in 182 overexpressing cells. **(A)** Levels of apoptosis in 182 overexpressing cells versus control cells.

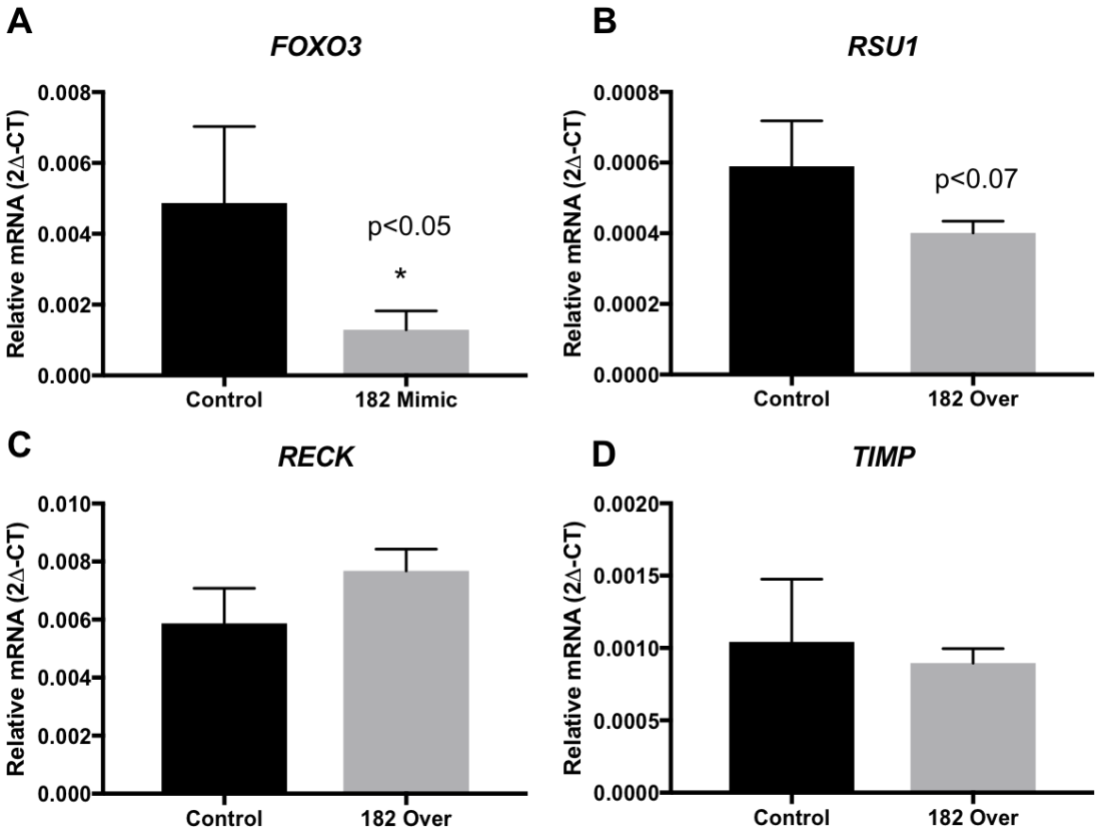

Figure 9. Tumor suppressor genes in 182 overexpressing cells. **(A)** *FOXO3,* **(B)** *RSU1,* **(C)** *RECK,* and **(D)** *TIMP* levels in 182 overexpressing cells compared to control cells.

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CHAPTER IV: LIN28B AND HMGA2 IN SHEEP TISSUE

Introduction

Understanding human pregnancy *in vivo* is a difficult task. The human placenta is hemochorial, with the placenta invading into the maternal endometrium and partially the myometrium. This allows the placenta to be in direct contact with maternal blood [1]. The other two classes of placentas, epitheliochorial and endotheliochorial exhibit some degree of separation between the chorion and maternal tissues [2]. Most animals fall into the latter two categories of placentas, with only rodents and primates having hemochorial placentas. Even then, there is a high degree of variability between levels of invasiveness. Historically the mouse has been a popular model for development and is used as a model for pregnancy research. However, despite having an invasive, hemochorial placenta, there is still some level of separation between the maternal blood space and fetal vasculature, making the mouse placenta hemotrichorial [3]. Additionally, there are several aspects of mouse gestation that makes its use as a model lacking. Mice have a gestation period of approximately 19-21 days and they produce average litters of 10-12 pups. As humans have considerably longer periods of gestation and produce primarily 1 or 2 offspring, these differences can cause disparity between results found in the mouse and the human.

For these reasons, we use the sheep as a model for human pregnancy. The sheep and human placenta do have significant differences as the sheep placenta is epitheliochorial, meaning there are six distinct layers separating the maternal membranes from the fetal vessels. Additionally, the sheep placenta is classified as multicotyledonary, meaning that the placenta is comprised of 70-120 regions of placental tissue found within the endometrium called caruncles

[2]. Alternatively, the human placenta is discoid, where the tissue is comprised of one large disk that meets the uterine wall [4]. Besides these major differences, there are some similarities that do make using the sheep an appropriate model for studying human pregnancy. For example, the villous structure of both human and sheep placentas is remarkably similar. Both types of placentas have stem, intermediate, and term villi with the fetal vasculature of these villi having similar vessels and function [5]. Additionally, the sheep has a considerably longer gestational period than the mouse, with an average of 147 days in length [6] and each pregnancy typically only produces singleton or twin births. While there are significant differences between sheep and human pregnancies, there are also strong similarities making sheep an informative model providing insight into placental development.

For this project, the sheep serves as a better model than the mouse for another large reason. Previous work in the Winger lab focused on LIN28A in mouse trophoblast stem cells as there were high levels of LIN28A mRNA and protein in the mouse placenta and mouse trophoblast stem cells [7]. However, collaborative projects between our lab and Dr. Anthony's lab determined that in both sheep and human placentas, *LIN28B* is the predominant paralog of LIN28 (data not shown). We then examined the importance of LIN28B in human placental cells which is described in Chapter II. To determine the effects of knockdown of LIN28B *in vivo*, we also used a lentiviral targeting method to knockdown LIN28B using shRNA. We hypothesized that a knockdown of LIN28B in sheep embryo would lead to severe growth and cell proliferation effects.

Materials and Methods

Generation of LIN28B Knockdown Lentivirus

The pLKO.1 plasmid (Addgene) was used to produce lentiviral particles targeting LIN28B. An oligo sequence targeting LIN28B was cloned into pLKO.1. This vector was transformed into Stbl3 competent cells and grown in Luria Bertani (LB) broth. Plasmid DNA was isolated using a HiSpeed Plasmid Midi kit (Qiagen). To determine if the sequence had successfully been integrated, PCR was run for the region of the plasmid containing the shRNA sequence and the PCR product was sent to QuintaraBio for sequencing. Upon confirmation that the sequence was successfully integrated, the plasmid was used to transfect HEK cells for generation of lentivirus. HEK cells were transfected using the pLKO.1 plasmid, a packaging plasmid (PAX), an envelope plasmid (pMD2.G), and the transfection reagent Polyfect (Qiagen). Cells were transfected for 6 hours, then the media was changed and cells were left to incubate for 72 hours. After 72 hours, the virus containing media was collected, viral particles were isolated, and was frozen at -80ºC. To determine concentration of virus, the virus was titered using the $TCID_{50}$ titer. Lentivirus was thawed then diluted 1:40. After the initial dilution, virus was diluted in 10-fold serial dilutions ranging from 10^{-2} to 10^{-7} and dilutions were added to 4 wells of a 12-well dish of HEK 293 cells. Eight ug of Polybrene per mL of media (Sigma Aldrich) were added to each well. Cells were incubated with virus for 24 hours before media was changed. 72 hours after infection, 2 ug/mL of puromycin was added to each well of cells. After most of the cells had died off in the lower dilution wells (10-12 days), the number of wells containing live cells were counted and put into the $TCID_{50}$ calculator [8].

Animal Care and Tissue Collection

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Ewes were determined to be in heat then estrus cycles were synchronized 5-7 days after observed heats using 2 intramuscular injections of 1 mL Lutalyse. Each injection was administered 4 hours apart. Forty-eight hours after the first injection, ewes were bred with intact, fertile rams. Nine days after breeding, donor ewes were euthanized using Pentasol and the uterus was flushed using DMEM F-12 (Gibco) containing 0.25% BSA. Day 9 blastocysts were recovered and infected with lentiviral particles in a mineral oil overlaid 100 uL drop containing 100,000 transducing units of lentivirus, 5 ng/uL Polybrene, and CDM-2 embryo media. Embryos were incubated in virus containing medium for approximately 4 hours transfer into recipient ewes. Six days after embryo transfer, recipient ewes were euthanized and day 15 conceptuses were flushed using DMEM F-12 and 0.25% BSA. Tissue was either snap frozen in liquid nitrogen or placed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 hours before transfer into 70% ethanol (EtOH).

Immunohistochemistry

Fixed tissue was dehydrated and embedded in paraffin wax. Sections 5 microns in width were mounted onto charged glass microscope slides (Premiere) then deparaffinized and rehydrated in successive 4 minute baths of Citrasolv (Decon Labs), 100% EtOH, 90% EtOH, 70% EtOH, 50% EtOH, and distilled H2O. Sections were then blocked in 6% goat serum in PBS for 45 minutes in a humidity chamber. After blocking, sections were transferred to primary antibody dilutions of 1:100 for either LIN28B (Cell Signaling), HMGA2 (Abcam), or BRCA1 (Santa Cruz) and incubated overnight at 4°C. Sections were then rinsed with PBS 3 times for 5 minutes each rinse before incubation in a goat anti-rabbit HRP-labeled secondary

antibody (Abcam). Sections were incubated for 1 hour at room temperature. After 1 hour, slides were washed in PBS 3 times for 5 minutes each rinse. Slides were then treated with hydrogen peroxide using a DAB kit (Vector labs). As a control, secondary only treated slides were used.

Results

LIN28B and HMGA2 in sheep placental tissue

Using immunohistochemistry, we determined that both LIN28B (Figure 1A) and HMGA2 (Figure 1B) were strongly detected in D15 sheep conceptuses. We also found that while *HMGA2* mRNA levels were detectable at D15, D50, and D135 of sheep gestation, *HMGA2* levels decline significantly as pregnancy continues (Figure 1C).

LIN28B and HMGA2 in LIN28B KD Sheep

After infecting D9 sheep blastocysts, transferring those blastocysts, and recovering those conceptuses at D15 (Figure 2), we found that *LIN28B* levels were significantly lower ($p<0.05$) in LIN28B KD sheep compared to naturally mated controls (Figure 3A). Additionally, *HMGA2* levels were trending as higher however the difference wasn't significant (Figure 3B).

Discussion

Based on data described in Chapter II, we have evidence to suggest that LIN28B was an important factor regulating cell proliferation and differentiation in placental cells. However, as cell lines are notoriously fickle, we wanted to determine if similar results could be obtained *in vivo*. Using a lentiviral vector containing shRNA specific for LIN28B, we downregulated *LIN28B* in day 9 sheep embryos. Upon collection, we found that these embryos were much smaller than naturally mated control embryos. Additionally, using real-time qPCR we were able

to confirm that *LIN28B* mRNA levels were significantly lower than control embryos. Perhaps most interestingly, we also determined that, while not significant, *HMGA2* mRNA levels also seemed to trend as higher in LIN28B knockdown embryos. These data suggest that the LIN28/HMGA2 phenomenon we observed in human placental cells also occurs in embryos. This study used a very small number of ewes and a more stringent control is necessary. However, these are strong preliminary results confirming that HMGA2 is potentially regulated by another molecular mechanism in the placenta.

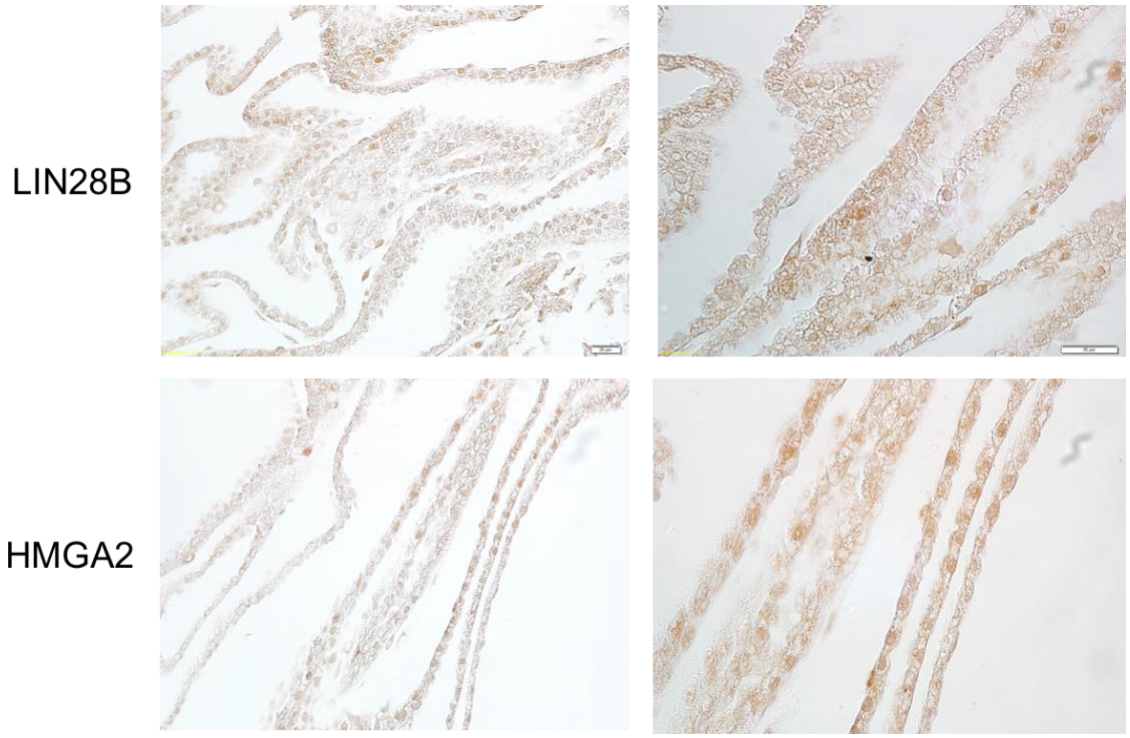

Figure 1. LIN28B and HMGA2 in gestational day 15 sheep embryos. Immunostaining depicting nuclear localization of LIN28B and HMGA2 in day 15 sheep conceptuses at 20x and 40x magnification.

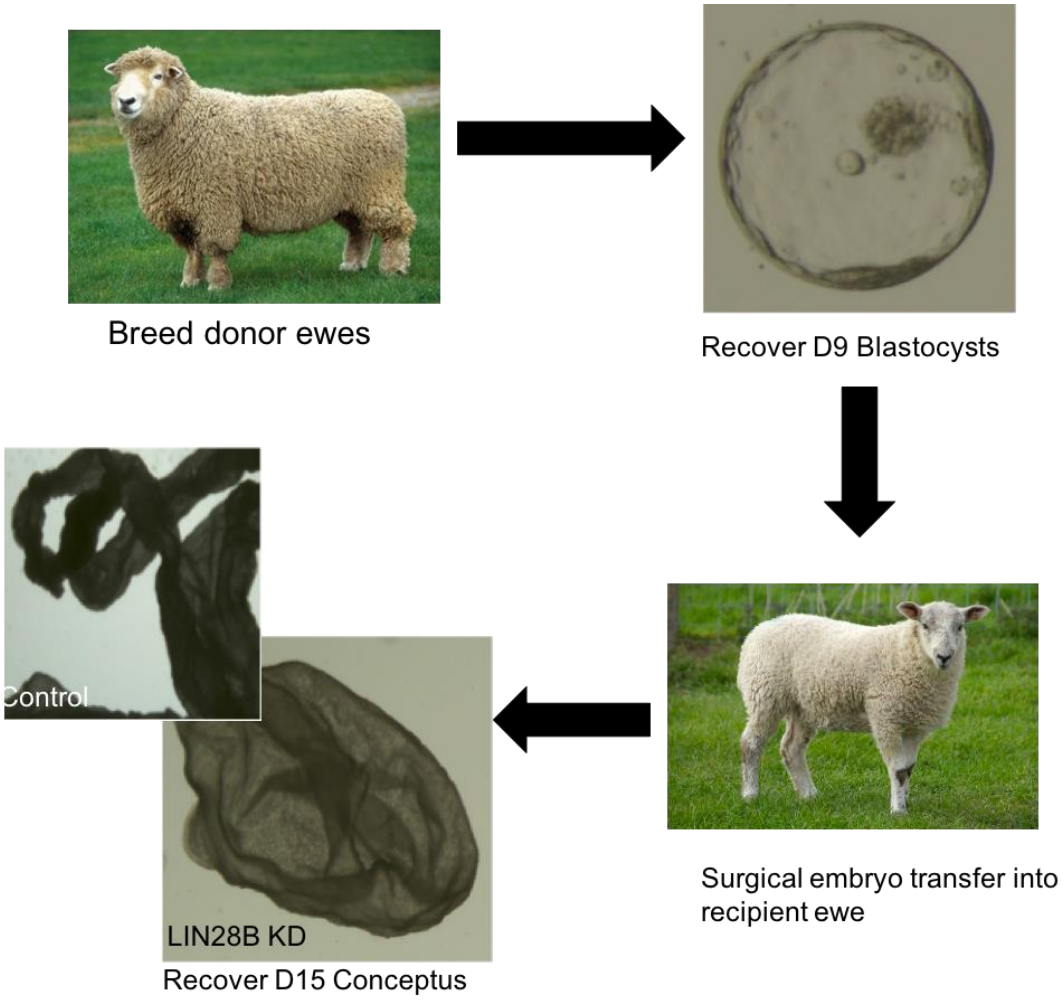

Figure 2. Embryo transfer and gene knockdown strategy. Day 9 blastocysts were recovered from donor ewes and infected with lentivirus for 6 hours before transferred into recipient ewes. Embryos were collected at day 15 of gestation and analyzed compared to naturally mated gestational day 15 embryo controls. (Figure adapted from Dr. Russ Anthony.)

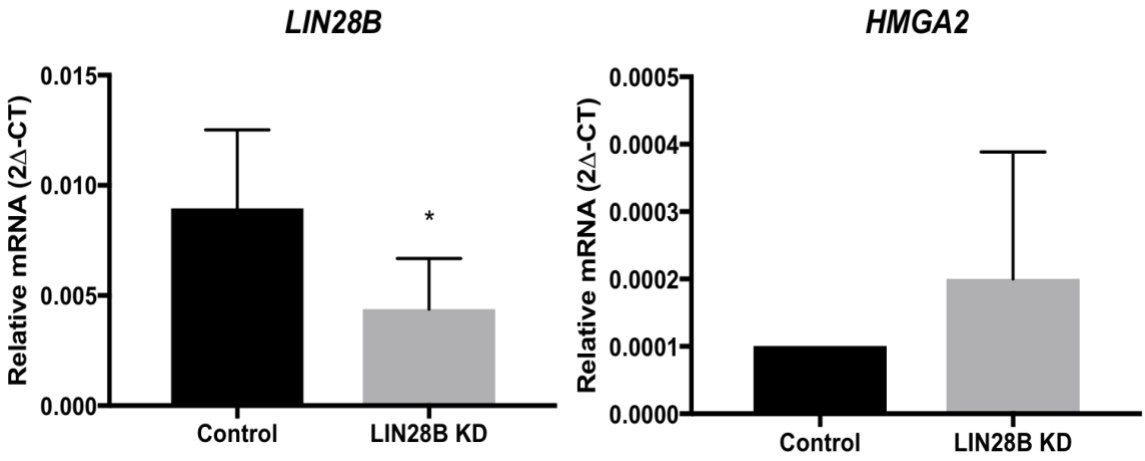

Figure 3. *LIN28B* and *HMGA2* levels in LIN28B knockdown embryos. *LIN28B* levels were significantly lower in LIN28B knockdown embryos compared to naturally mated controls. *HMGA2* levels were not significant ($p<0.07$) but trended higher compared to naturally mated controls.

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CHAPTER V: SUMMARY

The objective of this body of work was to better elucidate the molecular mechanisms that potentially regulate early placental cell proliferation and differentiation. Specifically, we focused on the regulation of the oncofetal protein, HMGA2 via the LIN28-let7-HMGA2 axis as well as by the transcriptional regulatory power of BRCA1.

There is a delicate interplay of factors that regulate cell proliferation, providing enough cells to meaningfully contribute to organogenesis, and cell differentiation. Improper cell signaling can lead to insufficient differentiation into the trophoblast cell sub-lineages, causing shallow invasion into the maternal arteries and impaired exchange of gas, nutrients, and hormones between maternal and fetal tissues. This altered placental physiology is the leading cause of placental insufficiency which causes approximately 500,000 fetal and 100,000 maternal deaths annually in the United States. In addition to contributing to maternal and fetal mortality, placental insufficiency can cause long lasting neurodevelopmental, metabolic, and developmental delays, causing significant financial and emotional strain on families. Better understanding the molecular mechanisms that drive early placental cell proliferation and differentiation will provide insight into how to better treat and prevent placental insufficiency.

We originally focused on the LIN28-let7-HMGA2 axis as it has been characterized as a key molecular pathway during early embryogenesis as well as in many cancers. While there are two paralogs of LIN28, we focused on LIN28B as there was data to suggest that LIN28B is the predominant form of LIN28 in both the human and sheep placenta. LIN28B is an RNA binding protein that binds to the let-7 family of miRNAs to prevent their processing into mature miRNAs. When cells are in a highly proliferative, undifferentiated state LIN28B levels are high

whereas let-7 levels are low. As cells begin to differentiate, LIN28B levels begin to decrease, allowing let-7 levels to rise. The chromatin modifying protein, HMGA2, is a downstream target of the let-7 miRNA family, so when cells are in an undifferentiated state HMGA2 is also highly expressed. Due to the importance of the LIN28-let7-HMGA2 axis during early embryogenesis, we hypothesized that this axis would also be a key regulator during early placental development.

Chapter II highlights that a knockdown of LIN28B does drive cells towards a more differentiated, syncytiotrophoblast fate. Cells were significantly less proliferative and secreted higher levels of hCG, however the extravillous marker *L-GAL* was significantly decreased and cells were not significantly different in their invasion potential. When we analyzed HMGA2 levels, surprisingly we found that HMGA2 mRNA and protein levels were significantly higher in the LIN28B KD cells compared to nontarget control cells. We did find that LIN28A mRNA and protein levels were also significantly increased, so we suggest that LIN28A can compensate upon decreased LIN28B levels and maintain HMGA2 function in placental cells. To investigate this hypothesis, we used CRISPR-Cas9 gene editing to knock out both LIN28A and LIN28B. Again cells acted more differentiated, but *HMGA2* mRNA was still significantly increased while HMGA2 protein levels were not significantly altered compared to empty vector control cells. This finding led us to determine that there was an alternate pathway more essential in the regulation of HMGA2 in human placental cells.

We propose BRCA1 as the regulator of HMGA2 during placental development. In breast tissue, BRCA1 forms a repressor complex with the proteins CtIP and ZNF350 to transcriptionally regulate several proliferation and angiogenic factors, including HMGA2. Using chromatin immunoprecipitation and co-immunoprecipitation we determined that this repressor complex does form and bind to the promoter region of *HMGA2* in placental cells. Additionally,

in some cancers the onco-miR, miR-182, targets *BRCA1* to prevent translation, allowing for increased cell proliferation and genomic instability. Interestingly, miR-182 was one of the first microRNAs identified to be altered in the serum and placentas of preeclamptic women. We hypothesized that miR-182 targets BRCA1 in human placental cells to regulate HMGA2. Using qPCR, we were able to determine that miR-182 was upregulated in our LIN28 double knockout cells and that *BRCA1* was significantly downregulated. These data suggest that miR-182 and BRCA1 are more essential for HMGA2 expression in human placental cells than the LIN28-let7-HMGA2 axis.

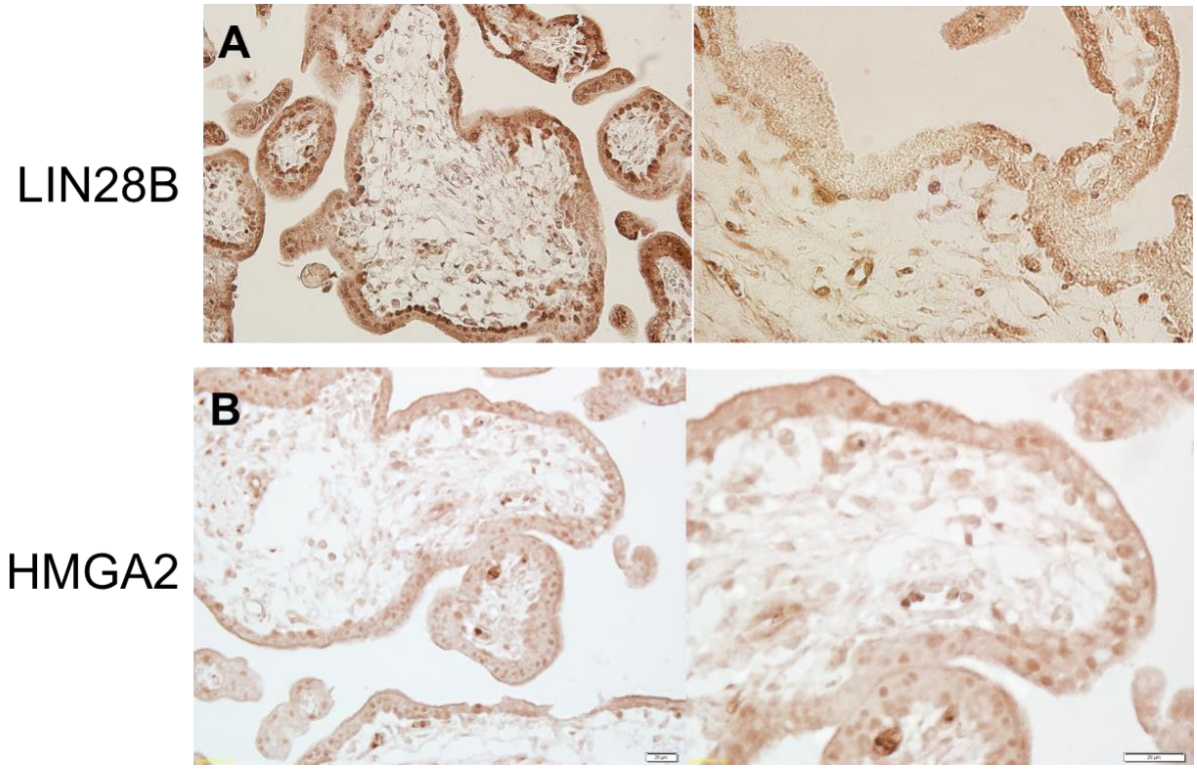
These initial findings regarding miR-182 and BRCA1 were the impetus for the experiments found in Chapter III. We used CRISPR-Cas9 to target BRCA1 in the human placental cell line, Swan71. This knockout led to decreased binding of the BRCA1 repressor complex to the *HMGA2* promoter, leading to increased HMGA2 mRNA and protein levels. Additionally, BRCA1 knockout cells were significantly more apoptotic compared to control cells, suggesting that these cells had increasingly less genomic stability. We also overexpressed miR-182 in the Swan71 cells. Overexpression of miR-182 led to significantly higher levels of HMGA2 as well as increased levels of apoptosis in these cells. Together, these data suggest that miR-182 and BRCA1 do regulate HMGA2 in human placental cells and, upon loss of BRCA1, these cells see an increase in genomic instability and apoptosis.

Finally, we recapitulated our *in vitro* LIN28B work *in vivo* using the sheep as a model. When using a lentiviral vector targeting LIN28B in gestational day 9 sheep embryos, we saw an increase in *HMGA2*, confirming the results we saw in human placental cells. We also determined that LIN28B knockdown in sheep embryos lead to severe proliferation defects and growth restricted day 15 sheep conceptuses.

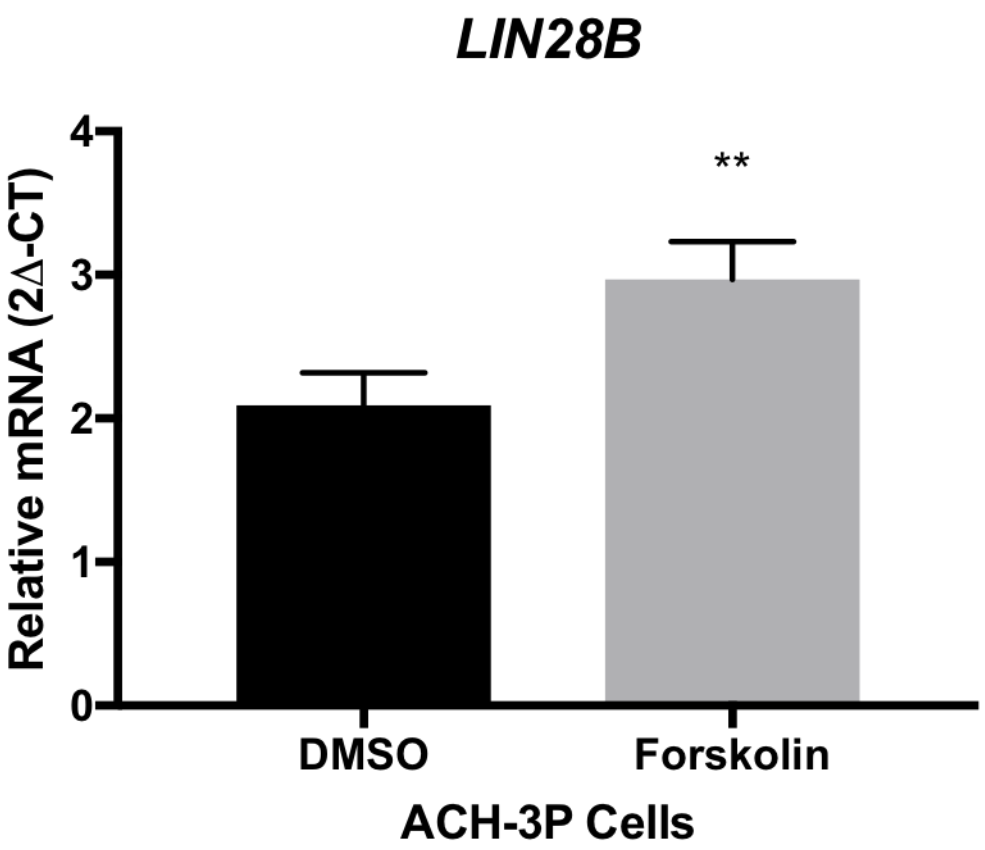
Together, these data conclude that LIN28B does play an important role in cell proliferation and differentiation during early placental development. We also have begun preliminary work in elucidating the role of BRCA1 in human placental cells. Future studies will revolve around better understanding how BRCA1 works to promote genomic stability and prevent DNA damage in cells. Better understanding these mechanisms and their role in proliferation, differentiation, and DNA repair will further our understanding of placental development and pathogenesis, potentially leading to enhanced pregnancy outcomes and improved standards of living of children and mothers affected by placental insufficiency.

APPENDIX

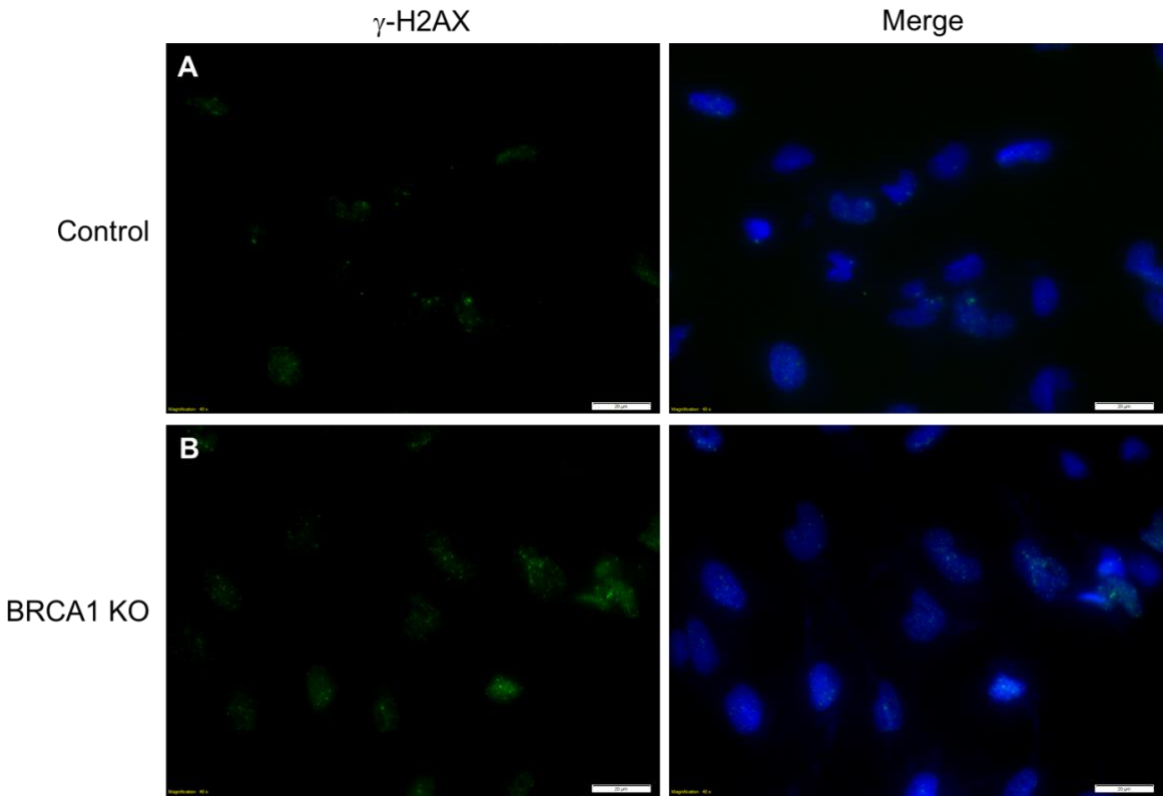
APPENDIX I: SUPPLEMENTARY FIGURES



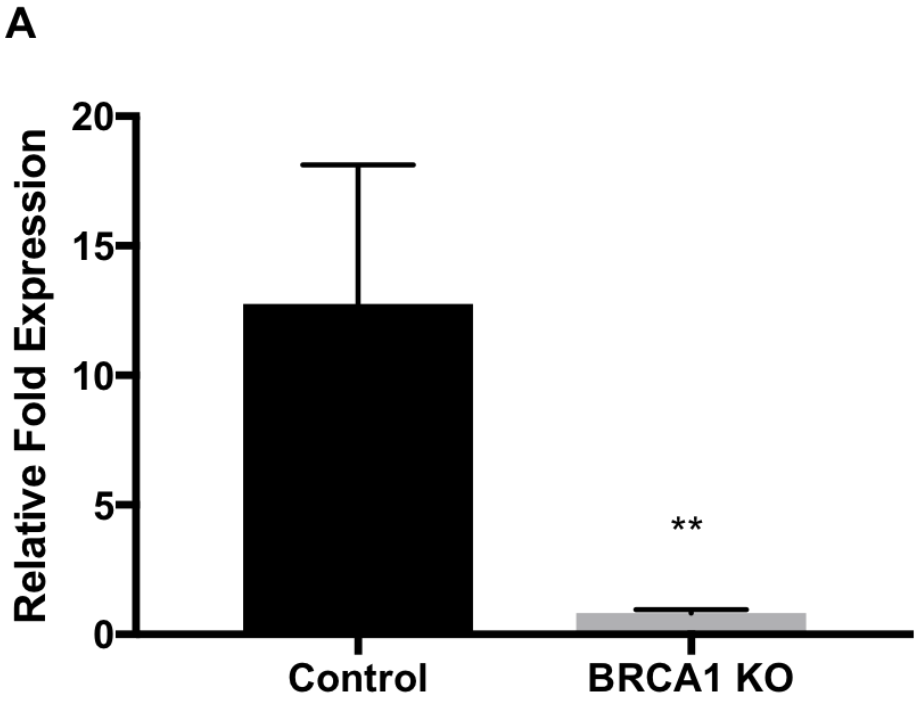
Appendix Figure 1. Immunohistochemistry for LIN28B and HMGA2. **(A)** LIN28B and **(B)** HMGA2 immunohistochemistry depicting both proteins in human 11.5 week placental tissue.



Appendix Figure 2. *LIN28B* mRNA levels in Forskolin treated cells. *LIN28B* is significantly higher ($p<0.01$) in cells treated with 40 uM Forskolin compared to DMSO treated control ACH-3P cells.



Supplemental Figure 3. γ-H2AX in BRCA1 knockout cells. γ-H2AX immunostaining in **(A)** control cells versus **(B)** BRCA1 knockout cells at a 20x magnification.



Supplemental Figure 4. Binding to the ANG1 promoter in BRCA1 knockout cells. **(A)** qPCR analysis for the ZNF350 recognition site on the *ANG1* promoter of chromatin immunoprecipitated BRCA1 knockout cells compared to control cells.