March 2019

Investigating the Role of the Chromosome 19 MicroRNA Cluster in Human Trophoblast Differentiation and Infantile Hemangioma

Ezinne Fransess Mong

*University of South Florida, fransess@health.usf.edu*

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Investigating the Role of the Chromosome 19 MicroRNA Cluster in Human Trophoblast Differentiation and Infantile Hemangioma

By

Ezinne Francess Mong

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in Molecular Pharmacology and Physiology
Department of Molecular Pharmacology and Physiology
College of Medicine
University of South Florida

Major Professor: Hana Totary-Jain, Ph.D.
Jerome Breslin, Ph.D.
Caralina Marin De Evsikova, Ph.D.
Joshua Scallan, Ph.D.

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March 21, 2019

Keywords: Placenta, stem cells, differentiation, epigenetics

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<th>Description</th>
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<tbody>
<tr>
<td>EVT</td>
<td>Extravillous trophoblast cells</td>
</tr>
<tr>
<td>CT</td>
<td>Cytotrophoblast cells</td>
</tr>
<tr>
<td>ST</td>
<td>Syncytiotrophoblast cells</td>
</tr>
<tr>
<td>VT</td>
<td>Villous trophoblast cells</td>
</tr>
<tr>
<td>C19MC</td>
<td>Chromosome 19 microRNA cluster</td>
</tr>
<tr>
<td>LIN28A</td>
<td>Lin-28 Homolog A</td>
</tr>
<tr>
<td>LIN28B</td>
<td>Lin-28 Homolog B</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to Epithelial Transition</td>
</tr>
<tr>
<td>EndMT</td>
<td>Endothelial to Mesenchymal Transition</td>
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<tr>
<td>SNAI1/SNAIL</td>
<td>Snail Family Transcriptional Repressor 1</td>
</tr>
<tr>
<td>SNAI2/SLUG</td>
<td>Snail Family Transcriptional Repressor 2</td>
</tr>
<tr>
<td>CDH2</td>
<td>Cadherin 2, Type 1, N-Cadherin (Neuronal)</td>
</tr>
<tr>
<td>TWIST1</td>
<td>Twist Family BHLH Transcription Factor 1</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Serpin Family E Member 1</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-Binding Protein 4</td>
</tr>
<tr>
<td>FGF4</td>
<td>Fibroblast Growth Factor 4</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose Transporter Type 1</td>
</tr>
<tr>
<td>IH</td>
<td>Infantile hemangioma</td>
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<tr>
<td><strong>Gene</strong></td>
<td><strong>Description</strong></td>
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<td>-----------------------------------------------------</td>
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<tr>
<td>SOX2</td>
<td>SRY-Box 2</td>
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<td>NANOG</td>
<td>Nanog Homeobox</td>
</tr>
<tr>
<td>MYC</td>
<td>MYC Proto-Oncogene, BHLH Transcription Factor</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>TE</td>
<td>Trophectoderm</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>Tead4</td>
<td>TEA Domain Transcription Factor 4</td>
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<tr>
<td>CDX2</td>
<td>Caudal Type Homeobox 2</td>
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<tr>
<td>aPKC</td>
<td>Atypical Protein Kinase C-Lambda/Iota</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming Growth Factor Beta 1</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>Transforming Growth Factor Beta 2</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>Transforming Growth Factor Beta 3</td>
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<td>E-cadherin</td>
<td>Cadherin 1, Type 1, E-Cadherin (Epithelial)</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor 1 Subunit Alpha</td>
</tr>
<tr>
<td>TCF3/4</td>
<td>Transcription Factor 3</td>
</tr>
<tr>
<td>Smad2</td>
<td>SMAD Family Member 2</td>
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<tr>
<td>Smad3</td>
<td>SMAD Family Member 3</td>
</tr>
<tr>
<td>Smad4</td>
<td>SMAD Family Member 4</td>
</tr>
<tr>
<td>(PI3K)/AKT</td>
<td>Phosphatidylinositol-4,5-Bisphosphate 3-Kinase/ AKT</td>
</tr>
<tr>
<td></td>
<td>Serine/Threonine Kinase</td>
</tr>
<tr>
<td>FOSL1</td>
<td>FOS Like 1, AP-1 Transcription Factor Subunit</td>
</tr>
</tbody>
</table>
MMP1  Matrix Metallopeptidase 1
MMP3  Matrix Metallopeptidase 3
MMP10 Matrix Metallopeptidase 10
PECAM-1 Platelet and Endothelial Cell Adhesion Molecule 1
VCAM-1 Vascular Cell Adhesion Molecule 1
EGF  Epidermal Growth Factor
VEGF  Vascular Endothelial Growth Factor
PDGF  Platelet Derived Growth Factor
CSF-1  Colony Stimulating Factor 1
IGF-1  Insulin Like Growth Factor 1
IGF-2  Insulin Like Growth Factor 2
TNFα  Tumor Necrosis Factor Alpha
INFγ  Interferon Gamma
CXCL16 C-X-C Motif Chemokine Ligand 16
CXCR6 C-X-C Motif Chemokine Receptor 6
CXCL6 C-X-C Motif Chemokine Ligand 6
CXCL14 C-X-C Motif Chemokine Ligand 14
miRNA microRNA
UTR  Untranslated region
BAC  Bacterial Artificial Chromosome
PHT  Primary Human Trophoblast cells
VSV  Vesicular Stomatitis Virus
CNS-PNETs Central Nervous System Primitive Neuroectodermal Tumor
ETMR  Embryonal Tumor with Multilayered Rosettes
ETANTR  Embryonal Tumor with Abundant Neuropil and True Rosettes
TTYH1  Tweety Family Member 1
IGF2BP1  Insulin Like Growth Factor 2 MRNA Binding Protein 1
IGF2BP3  Insulin Like Growth Factor 2 MRNA Binding Protein 3
HMGA2  High Mobility Group AT-Hook 2
CSD  Cold Shock Domain
Zcchc11  Zinc Finger CCHC Domain-Containing Protein 11
GWAS  Genome-Wide Association Study
mTOR  Mechanistic Target Of Rapamycin Kinase
CDK4  Cyclin Dependent Kinase 4
TNFα  Tumor Necrosis Factor
SYN-1  Syncytin 1
mTGC  Mouse Trophoblast Giant Cells
hCG,  Human Chorionic Gonadotropin
ERVW-1  Endogenous Retrovirus Group W Member 1, Envelope
CNS  Central Nervous System
EPC  Endothelial Progenitor Cells
HemSC  Hemangioma Stem Cells
TIMP-2  TIMP Metalloproteinase Inhibitor 2
ICAM-1  Intercellular Adhesion Molecule 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-Dr</td>
<td>Human Leukocyte Antigen – DR isotype</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal Cell-Derived Factor 1</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>EPAC</td>
<td>Exchange Protein Activated by cAMP</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide synthase</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Nonobese Diabetic/Severe Combined Immunodeficiency mouse</td>
</tr>
<tr>
<td>ACTA2</td>
<td>Actin, Alpha 2, Smooth Muscle, Aorta</td>
</tr>
<tr>
<td>TUBB3</td>
<td>Tubulin Beta 3 Class III</td>
</tr>
<tr>
<td>PSG4</td>
<td>Pregnancy Specific Beta-1-Glycoprotein 4</td>
</tr>
<tr>
<td>GATA4</td>
<td>GATA Binding Protein 4</td>
</tr>
<tr>
<td>HLAG</td>
<td>Major Histocompatibility Complex, Class I, G</td>
</tr>
<tr>
<td>iPSC-CT</td>
<td>Induced Pluripotent Stem Cell Derived Cytotrophoblasts</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone Morphogenetic Protein 4</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc Finger E-Box Binding Homeobox</td>
</tr>
<tr>
<td>PAR-clip</td>
<td>Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RRID</td>
<td>Research Resource Identifiers</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>CCHC</td>
<td>Cysteine Cysteine Histidine Cysteine domain</td>
</tr>
</tbody>
</table>
Abstract

Trophoblast differentiation and invasion is essential for normal implantation and establishment of the maternal-fetal interface, which allows for proper nutrient exchange and support of the fetus. For this to occur, cytotrophoblasts must undergo an epithelial to mesenchymal transition and differentiate into migratory and invasive extravillous trophoblasts (EVTs) that invade the maternal decidua and myometrium. Trophoblast differentiation, migration and invasion is highly regulated by a complex network of signaling pathways, adhesion molecules and transcription factors and is important for the remodeling of maternal spiral arteries from low flow, high resistance to high flow, low resistance vessels to allow optimal perfusion of the fetus and the placenta. Cytotrophoblasts also differentiate into syncytiotrophoblasts which form the outer layer of the placenta and are important for fetal-maternal gas exchange.

The chromosome 19 miRNA cluster (C19MC) and the RNA binding protein Lin28 are highly expressed in embryonic stem cells, the placenta and certain cancers. C19MC is the largest human miRNA cluster that spans over 100 kb. It is primate specific and contains 46 miRNA genes flanked by Alu elements. C19MC is regulated by genomic imprinting. The expression of C19MC often correlates closely with the RNA binding protein LIN28. LIN28 was originally discovered as a regulator of developmental timing due to its inhibitory effects on the expression of the let-7 miRNA family. Two paralogs, LIN28A and LIN28B exist in vertebrates, which were shown to regulate thousands of
mRNAs transcripts involved in cellular processes including stem-cell self-renewal, cell differentiation, proliferation, migration, and cellular metabolism.

The role of C19MC miRNAs in the regulation of EMT, trophoblast differentiation and somatic cell reprogramming has not been fully determined. In this study, we hypothesized that hypoxic conditions during implantation and placentation downregulate C19MC expression, which in turn induces EMT genes, resulting in trophoblast differentiation toward EVTs.

In this study we found that C19MC is highly expressed in villous cytotrophoblasts but not in differentiated EVTs or decidual cells in human first trimester placenta tissues. CRISPR/Cas9 mediated overexpression of C19MC inhibited the expression of EMT markers SNAI1, CDH2, TWIST1 and SERPINE1, induced the expression of reprogramming factors OCT4 and FGF4, and enhanced the reprogramming of human fibroblasts to iPSCs. Importantly, hypoxic conditions decreased the expression of C19MC and increased the expression of EMT markers in iPSCs. This study demonstrates that overexpression of C19MC induces epithelial cell phenotype and enhances cell reprogramming. Furthermore, exposure to hypoxic conditions induces EMT and trophoblast differentiation at least in part by inhibiting C19MC and reprogramming factors.

Recently, C19MC was reported to be highly expressed in infantile hemangiomas (IH). IH are benign tumors of infancy characterized by rapid growth phase followed by spontaneous involution. During the proliferative phase, immature glucose transporter-1
(GLUT1) positive endothelial cells form highly disorganized blood vessels. IH lesions also highly express C19MC and the stem cell reprogramming factors Oct4, Sox2, Nanog and Myc. When GLUT1 and C19MC expressing endothelial cells isolated from proliferating IH are grown in culture, they lose expression of both GLUT1 and C19MC and undergo an endothelial to mesenchymal transition. Propranolol, a beta-adrenergic receptor blocker, has been shown to trigger early IH tumor involution and has subsequently become first line treatment for IH patients. The levels of circulating C19MC correlated with tumor phase and with propranolol treatment. Some studies have shown considerable similarities between IH and placental transcriptomes suggesting a placental origin for proliferating endothelial cells in IH. On the other hand, other studies have proposed that IH originate from Hemangioma stem cells. This theory is supported by studies which show that human CD133+ hemangioma stem cells recapitulate the entire IH life cycle when implanted subcutaneously into immunocompromised mice.

The expression of C19MC miRNAs have been strongly correlated with the expression of the RNA binding protein LIN28. Although LIN28/let-7 axis plays a central role in the regulation of stem cell self-renewal and tumorigenesis, the role of LIN28B/let-7 signaling in IH pathogenesis has not yet been elucidated. We hypothesized that the LIN28/let-7 signaling pathway is dysregulated in IH and is modulated by propranolol treatment during IH involution. We found that LIN28B is highly expressed in proliferative IH compared to involuted IH and propranolol treated IH. The increase in LIN28B correlated with C19MC expressed and inversely correlated with let-7. Overexpression of LIN28B in HEK293 cells increased the expression of miR-516b, a C19MC miRNA. Moreover, iPSCs treated with propranolol decreased the expression of C19MC miRNAs.
and LIN28B and increased the expression of let-7. Importantly, propranolol treatment decreased iPSC proliferation and increased the expression of EMT markers. These results describe for the first time, the role of the LIN28/let-7 switch in IH pathogenesis and identifies a novel mechanism by which propranolol induces IH involution.
Chapter 1

Placental Development

Establishment of the trophectoderm and inner cell mass

Successful fertilization results in the formation of the zygote, a diploid cell formed from the fusion of two haploid gametes. The zygote is totipotent and can give rise to the entire embryo and the extra embryonic tissue. It utilizes a stockpile of RNAs and proteins from the oocyte to drive their initial development. At the 2-cell stage, a mechanical separation of the blastomeres occurs. In humans, at the 4- to 8-cell stage, a series of genetic and epigenetic reprogramming and identical cellular divisions results in a major wave of embryonic genome activation\(^1\). Once they reach the compacted 8-cell stage, the cells become polarized. Consequently, symmetrical and asymmetrical divisions occur during the transition from 8-cell to 16-cell stage, when the first cell fate determination of embryogenesis begins, known as the blastocyst stage. During this process, the outer cells remain polarized and develop into the bipotent trophectoderm (TE), while the inner cells lose polarity and develop into the pluripotent inner cell mass (ICM)\(^2\). TE differentiate into trophoblasts from which the placenta is derived whereas the ICM generates the three germ layers, mesoderm, endoderm and ectoderm that ultimately gives rise to the fetus.
During embryonic genome activation, a dramatic reprogramming of gene expression occurs. The master transcriptional regulator Oct4, is highly expressed in the oocytes, blastomeres and in the ICM, and controls pluripotent cell self-renewal. Targeting OCT4 in human zygotes using an inducible human embryonic stem (ES) cell-based CRISPR–Cas9 system resulted in a poorly formed ICM and subsequent embryo collapse. In vitro, Oct4 knockout embryos develop to the blastocyst stage and extraembryonic TE lineage but were unable to form pluripotent ICM cells. Sox-2, is another transcription factor required for ICM development. Together with Oct4 it binds to a powerful enhancer of Fgf4, and induces its expression. This regulation is important as Fgf4 is a key signaling factor that drives cellular specification within the ICM and also provides paracrine signaling that stimulates growth and proliferation of the developing TE. Loss of Sox-2 impairs Oct4 binding to its target genes and leads to developmental defects. Nanog is another transcription factor that is critical for the development of the cells of the ICM. Nanog deficient embryos are unable to generate epiblast and only produce parietal endoderm-like cells. In the absence of Nanog, pluripotency cannot be established and the cells of the ICM are unable to progress beyond an intermediate state. Therefore, these embryonic transcriptional factors are important parts of a regulatory circuit that coordinate the expression of many downstream genes required during normal development.

The development of the TE is contingent on an entirely different set of transcription factors such as Cdx2 and Tead4. Cdx2 is expressed in the TE and reinforces cell specification to the TE layer. Homozygous deletion of the Cdx2 gene leads to death of the embryo around the time of implantation. Cdx2 mutants are able to
form the blastocoel but cannot maintain epithelial integrity and show a failure of implantation\textsuperscript{12}. Cdx2 is also important for downregulating the expression of Oct4 and Nanog and maintaining the spatial restriction of these proteins\textsuperscript{12}. Furthermore, Cdx2 promotes symmetrical divisions which allocates more cells to the TE and reinforces cell polarity by upregulating aPKC\textsuperscript{13}. Tead4 is also important for TE formation. Tead4 knockout embryos do not produce trophoblast stem cells or TE and this knockout results in a preimplantation lethal phenotype \textsuperscript{14}. These embryos did not express TE specific genes such as Cdx2 but expressed genes that are specific to the ICM such as Oct4, indicating that Tead4 is upstream of Cdx2 \textsuperscript{15}.

Recently, LIN28 which belongs to a class of RNA binding proteins, has also been shown to be important for development of the ICM and TE. LIN28 is highly expressed in embryonic stem cells (ESC) and early embryonic tissue, but declines upon development\textsuperscript{16,17,18}. Mice embryos, highly express LIN28 in both ICM and TE cells, in all three germ layers and the extraembryonic layer\textsuperscript{17,19}. In addition, LIN28 depletion from mouse zygotes prevents the transition from the 2-cell to 4-cell stage indicating that LIN28 plays an important role in early embryo development\textsuperscript{19}.

**The placenta**

The placenta is a highly complex organ that primarily serves as the interface between mother and fetus. It’s importance in fetal development cannot be overstated as it performs a variety of essential functions including digestive, respiratory, excretory, immune and endocrine systems. The placenta undergoes major structural changes between the first trimester during organogenesis and the remainder of pregnancy \textsuperscript{20}.  


**Trophoblast differentiation in early pregnancy**

Trophoblasts are the first cells to differentiate from the fertilized egg during the first stage of pregnancy. They are derived from the outer layer of the blastocyst which develops into a large portion of the placenta and primarily provide nutrients to the embryo. Trophoblasts adhere to the uterus and initiate implantation and so are very important for initiation and maintenance of a successful pregnancy. Trophoblasts differentiate into two major lineages: syncytiotrophoblasts (STs) and extravillous trophoblasts (EVTs), while maintaining a stem population of villous cytotrophoblasts (CTs) throughout the course of pregnancy.

STs are specialized epithelial cells that cover the chorionic villi and are in direct contact with maternal blood. They are directly responsible for maternal-fetal gas exchange and nutrient transport during pregnancy. They also perform endocrine functions including the production of growth factors and hormones, protect the fetus from infections, xenobiotics and drugs and regulate fetal programming\(^{20, 21}\).

EVTs are derived from proliferating CTs in the placental cell columns and comprise of endovascular and interstitial invasive trophoblasts. The interstitial trophoblasts migrate to and invade the maternal uterine tissue to anchor the placenta to the uterus. The endovascular trophoblasts on the other hand, migrate to the maternal uterine spiral arteries and facilitate their remodeling into high-flow, low-resistance vessels that ease placental perfusion and nutrient uptake. This conversion of maternal spiral arteries ensures sufficient blood flow and must occur by the end of the first trimester of a successful pregnancy.
During the first stages of pregnancy the placenta develops in a relatively hypoxic environment. The partial pressure of oxygen in the placenta is significantly lower than in the endometrium between 8 and 10 weeks. During this time, EVT plugs block the maternal spiral arteries and prevent maternal blood flow from entering the intervillous space. This indicates that placental and fetal development in the early stages largely takes place in hypoxia. At the end of the first trimester, hypoxia in the placental bed is abated by the displacement of trophoblast plugs which exposes the placenta to maternal blood flow and establishes the maternal-placenta circulation.

Deficiencies in trophoblast migration and invasion result in several diseases of pregnancy such as preeclampsia or placenta accreta. EVT invasion requires cellular transition from an epithelial phenotype to a mesenchymal phenotype which is essential for efficient implantation and placentation.

**Epithelial to mesenchymal transition (EMT)**

EMT refers to the process by which polarized epithelial cells undergo biochemical changes to gain mesenchymal cell characteristics which include enhanced migratory capacity, invasiveness, increased production of extra cellular matrix (ECM) components and greater resistance to apoptosis. A total EMT is characterized by the degradation of the underlying basement membrane and the migration of the resulting mesenchymal cell to a more distant site.

EMTs have been classified into three distinct subtypes. Type 1 EMTs are associated with organ development, implantation and embryo formation. It is highly
essential for the movement of cells in the embryos during development as cells differentiate and move between epithelial and mesenchymal states\textsuperscript{24, 25}. Type 2 EMTs are associated with fibrosis and wound healing and begin as part of a repair mechanism that generates fibroblasts and related cells following trauma or injury. These EMTs are associated with inflammation and are discontinued as soon as inflammation is abated. Continued inflammation elicits a continuous EMT response and can lead to tissue fibrosis and organ damage. Type 3 EMTs are associated with cancer cells which gain the ability to metastasize to and invade secondary tumor sites. Type 3 EMTs in cancer cells may be partial or complete with cells often maintaining both epithelial and mesenchymal characteristics. Although EMTs can be classified into these 3 distinct types, they are all governed by a common set of transcription factors that regulate the expression of downstream genes and miRNAs that results in the production of enzymes that degrade the ECM and enable detachment\textsuperscript{24} (Figure 1).
Figure 1. Epithelial to mesenchymal transition (EMT).
EMT involves the transition between polarized epithelial cells to migratory mesenchymal cells that secret ECM components. Common epithelial and mesenchymal cell markers are listed below each cell type. EMT can also generate cells which display an intermediate phenotype defined by the possession of both epithelial and mesenchymal cell properties.
Molecular regulation of EMT during early placental development

EMT is regulated by well-orchestrated series of molecular processes that include signal induction and transduction within the cell through key transcription factors. Many EMT inducers have been identified to date including hypoxia and the transcription factors SNAIL, SLUG and TWIST. The TGFβ and Wnt signaling pathways also regulate key transcription factors that induce phenotypic changes characteristic of EMT.26-28

The transcriptional repressors SNAIL and SLUG, members of the SNAIL family of zinc-finger proteins play important roles in EMT. SNAIL has been shown to be a potent inducer of EMT primarily through the repression of the epithelial marker E-Cadherin.29-31 SNAIL binds directly to E-boxes in the E-cadherin promoter to repress its expression.31 SNAIL has also been shown to directly repress the expression of the tight junction adhesion claudin and occcludin genes important for the maintenance of epithelial cell architecture.32 In villous explant cultures, low oxygen conditions increased the expression of SNAIL which led to a decrease in E-cadherin, increase in integrin α5 and a significant increase in EVT invasion.33 The increase in expression of SNAIL expression in low oxygen condition was due to direct binding of HIF-1α to its promoter.34 These findings suggest that low-oxygen tensions in early pregnancy lead to the upregulation of SNAIL, which causes a decrease in the expression of epithelial adhesion and junctional proteins thus promoting EMT and an increase in EVT cell migration and invasion. Like SNAIL, SLUG also directly represses the expression of E-cadherin in epithelial cells and breast cancer cells and induces EMT.35,36 Choriocarcinoma-derived JEG3 cells treated with cyclosporin A, an
immunosuppressive agent, showed increased SLUG expression, decreased E-cadherin expression and increased invasiveness\textsuperscript{37}. These studies indicate that SLUG and SNAIL likely promote EMT through the same molecular mechanisms.

**SNAIL and SLUG** also activate a key regulator of EMT, the Wnt/\(\beta\)-catenin signaling pathway, which play important roles in every aspect of embryonic development, stem cell maintenance, EVT differentiation and invasion cell differentiation\textsuperscript{38}. SNAIL and SLUG initiate EMT by promoting the formation of the \(\beta\)-catenin/TCF complex, which increases the binding to the TGF\(\beta\)3 promoter to increase its transcription\textsuperscript{39}. Expression of TCF3/4 is significantly increased in invading trophoblasts and treatment of primary cytotrophoblasts with Wnt3A significantly increased trophoblast migration and invasion through matrigel\textsuperscript{26}. Furthermore, silencing of TCF-4 in primary cytotrophoblasts caused a decrease in the expression of EMT markers SNAIL, integrin \(\alpha1\) and integrin \(\alpha5\)\textsuperscript{40}.

The multifunctional cytokine, transforming growth factor \(\beta\) superfamily (TGF\(\beta\)), has been shown to have contradictory effects on cell behavior\textsuperscript{41}. Although TGF\(\beta\) can act as a tumor suppressor during the early stages of cancer development, TGF\(\beta\) can also function as a potent inducer of EMT\textsuperscript{42}. TGF\(\beta\) activates the transcription factors Smad2 and Smad3 which bind Smad4 and together promote the transcription of SNAIL and initiation of EMT\textsuperscript{43, 44}.

**TWIST** is another transcriptional regulator of EMT. TWIST plays an essential role in the regulation of embryonic morphogenesis and tumor metastasis. Increasing the expression of TWIST in epithelial cells led to a loss of E-cadherin, cell-cell adhesion and
the activation of mesenchymal cell markers vimentin, fibronectin, smooth muscle actin and N-cadherin\textsuperscript{45}. TWIST is highly expressed in EVTs and siRNA mediated silencing of the TWIST gene resulted in a decrease in N-cadherin and significantly reduced the invasive capacity of the EVT cell line HTR8/SVneo\textsuperscript{46}. In addition, TWIST modulates the expression of E-cadherin and regulates syncytium formation in choriocarcinoma-derived BeWo cells\textsuperscript{47}.

Hypoxia is also a potent inducer of EMT. Studies have shown that hypoxia promotes EMT in a different variety of cancers and cell types\textsuperscript{48}. Hypoxia induced EMT is mediated by the activation of the Hypoxia Inducible Factor (HIF) pathway. Activation of the transcription factor HIF-1\(\alpha\) leads to the upregulation of EMT transcription factors such as SNAIL, SLUG and TWIST in different cancers\textsuperscript{49-51}. Furthermore, hypoxic conditions activate EMT signaling pathways including TGF\(\beta\) and Wnt signaling which further promote EMT\textsuperscript{48}. These studies indicate that hypoxia induces EMT by simultaneously activating multiple transcription factors and signal transduction pathways.

During normal placental development, EVTs lose their organized epithelial phenotype and transition into a more migratory and invasive mesenchymal phenotype that facilitates the deep infiltration of the maternal decidua and blood vessels. Furthermore, some of these invasive cells have the capacity to terminally differentiate into giant cells of the placental bed and the myometrium\textsuperscript{52}. Although the regulatory networks underlying EMT in trophoblasts are not very well understood, numerous similarities can be found in the extensive studies of EMT in cancer\textsuperscript{27}. Interestingly, EVTs
share many phenotypic characteristics with cancer cells including rapid proliferation, migration, ability to invade surrounding tissues, and resistance to the innate immune system\textsuperscript{53, 54}. Studies have shown that trophoblasts and cancer cells express many of the same growth factors, proto-oncogenes, enzymes, hormones and adhesion molecules that are likely responsible for the similar characteristics of both cell types. Furthermore, activation of the phosphatidylinositol 3'-kinase (PI3K)/AKT signaling pathway has been shown to be central to many of the mechanisms through which trophoblasts and cancer cells achieve their proliferative, migrative and invasive properties\textsuperscript{53}. A major difference between both cell types is that EVT invasion is restricted to the decidua and the first third of the myometrium suggesting tight spatio-temporal control of trophoblast behavior by the uterine microenvironment\textsuperscript{55}. Furthermore, EVT ability to undergo terminal differentiation significantly restricts their tumorigenic potential. Importantly, disruption of trophoblast invasion or migration can lead to serious consequences during pregnancy. For example, shallow invasion is a defining feature of preeclampsia and fetal growth restriction, whereas abnormally deep invasion is characteristic of placenta accreta and choriocarcinomas which are associated with uncontrolled EVT invasion\textsuperscript{56, 57}.

\textit{Regulation of trophoblast migration and invasion}

Trophoblast migration and invasion into the maternal uterine tissues is essential during pregnancy and fetal development. Invasive trophoblasts have numerous abilities including hormone and cytokine production and communication with maternal immune cells\textsuperscript{58}. In addition, invasive trophoblasts exhibit high plasticity which enables them to anchor the placenta to maternal tissues, replace endothelial cells of the maternal spiral
arteries and modulate angiogenesis and lymphangiogenesis. Deficiencies in trophoblast migration and invasion can lead to potentially serious complications during gestation such as early miscarriages, preeclampsia, intrauterine growth restriction, placenta accreta, premature birth, and maternal or fetal death. Studies aimed at identifying molecular mechanisms that control this process have identified several molecular pathways that control trophoblast migration and invasion under physiological conditions and in disease\textsuperscript{69-61}.

Regulation of trophoblast invasion in humans is tightly controlled by cytokines and growth factors by the activation of the phosphatidylinositol-3-kinase-serine-threonine (PI3K/Akt) signaling pathways\textsuperscript{62}. Disruption of PI3K/AKT using inhibitors altered the invasive trophoblast gene expression profile and impaired trophoblast invasion. This regulation has been shown to be mediated by the nuclear protein FOSL1, a downstream effector of the PI3K/AKT signaling pathway\textsuperscript{63}. Knockdown of FOSL1 disrupts the invasive trophoblast gene expression phenotype, abolishes trophoblast invasion, and is associated with the inhibition of matrix metalloproteinase protein (MMP1, MMP3 and MMP10) expression\textsuperscript{63}. Interestingly, secretion of PI3K/AKT signaling activators is not exclusive to trophoblast. The decidua, uterine macrophages and uterine natural killer cells also secrete PI3K/AKT activators suggesting a complex network of activators and mediators that regulate trophoblast invasiveness\textsuperscript{64}.

CTs in cell columns show decreased E-cadherin and high VE-(endothelial) cadherin, platelet-endothelial adhesion molecule-1 (PECAM-1), vascular endothelial adhesion molecule-1 (VCAM-1), and alpha-4-integrins\textsuperscript{65}. Some studies suggest that
defective trophoblastic invasion of maternal spiral arteries result from inadequate EMT and vascular adhesion molecule expression. Invading trophoblasts from preeclamptic placentas did not sufficiently express integrin, cadherin and Ig superfamilies suggesting an association between preeclampsia and CT failure to assume the appropriate vascular phenotype\textsuperscript{65, 66}. However, other studies have reported conflicting results\textsuperscript{67, 68}. During implantation, CT differentiation into EVT\textsubscript{s} is also accompanied by a change in the expression of adhesion molecules at the cell surface. Depending on their location, EVT\textsubscript{s} express different members of the integrin family which determine their interaction with collagens and laminin\textsuperscript{69}. These changes are associated with the EMT exhibited by EVT\textsubscript{s}\textsuperscript{58}.

The TGFβ signaling pathway is also an important regulator of trophoblast invasion. Of the 3 TGFβ isoforms that exist, TGFβ1 and TGFβ2 are thought to be the most important at the maternal-fetal interface. TGFβ2 but not TGFβ1 is expressed by EVT\textsubscript{s} within the placental bed while extracellular TGFβ1 and cytoplasmic TGFβ2 were detected in the decidual layer\textsuperscript{70}. TGFβ1 has been shown to inhibit cell invasion and promote intercellular adhesion\textsuperscript{71}. Moreover, another study showed that TGFβ1 treatment of the EVT cell line HTR8/SVneo decreased cell invasion and decreased the expression of VE-cadherin. This study also showed that the TGFβ1 induced decrease in VE-cadherin expression and subsequent decrease in cell invasion is mediated by SNAI\textsubscript{L}\textsuperscript{72}. These findings suggest that TGFβ plays a role in regulating the extent of trophoblast invasion of the maternal endometrium\textsuperscript{73}. 
Growth factors and cytokines have also been shown to directly influence human trophoblast behavior. Growth factors, such as EGF, VEGF, PDGF, CSF-1, IGF-1 and IGF-2, act at the fetal-maternal interface to stimulate trophoblast cell proliferation, adhesion and invasion\textsuperscript{74-76}. While other inhibitory proteins, such as the TGFβ family, endostatin, TNFα, INFγ and fetuin-A, inhibited trophoblast invasion and migration in vitro\textsuperscript{77-80}. Cytokines such as CXCL16, CXCR6, CXCL6 and CXCL14 have also been reported to regulate trophoblast migration and invasion\textsuperscript{81-83}. Taken together, trophoblast invasion and migration are a tightly regulated process that is governed by a complex regulatory network which when dysregulated, can lead to pathological outcomes in pregnancy.

**Diseases related to impaired placentation**

Preeclampsia is a disease typically diagnosed after 20 weeks of pregnancy. Clinical characteristics of preeclampsia include severe hypertension (systolic blood pressure above 140 mmHg and diastolic blood pressure above 90 mmHg), proteinuria, maternal edema and seizures. This disease is associated with shallow trophoblast invasion and impaired conversion of maternal spiral arteries\textsuperscript{20}. Insufficient maternal artery remodeling compromises the blood flow to maternal-fetal interface and leads to hypoxic conditions for the developing fetus. A study proposed that the insufficient invasion and impaired spiral artery remodeling in preeclampsia is caused by the failure of invading CTs to mimic a vascular phenotype\textsuperscript{66}. This study showed that invading CTs in placenta biopsy specimen from preeclampsia do not properly express many adhesion molecules including integrins, cadherins and Ig family members\textsuperscript{66}. Other studies
showed that macrophages reside in excess in the placental bed from preeclamptic pregnancies and can limit trophoblast invasion through apoptosis. Metabolic diseases such as diabetes and thyroid disorders can also adversely affect trophoblast invasion and predispose mothers to preeclampsia. A study showed that excess glucose reduced angiogenesis and migration and increased inflammation in first trimester trophoblast cells. A study in rats showed that maternal hypothyroidism reduced trophoblast migration and invasion and altered the immune profile in the placenta.

These studies suggest that deficiencies in a number of mechanisms that control trophoblast migration and invasion can lead to the development of preeclampsia during pregnancy.

Excessive trophoblastic invasion on the other hand, can also lead to placental dysfunction. Placenta creta (accreta, increta and percreta) develop when the anchoring villi and EVTs invade beyond the initial third of the myometrium to reach the perimetrium or extra-uterine tissues and organs. Classification as accreta, increta or percreta depends on the extent of invasion, with placenta percreta being the most severe form of the disease. This condition is associated with impaired decidual formation which permits trophoblast hyper invasion into the myometrium. Despite the increased invasion, placenta creta is still associated with incomplete vascular remodeling of maternal spiral arteries. Placenta creta can cause heavy bleeding during pregnancy and childbirth.

Finally, a study of placental beds from late miscarriages showed that late sporadic miscarriage is associated with decreased endovascular and interstitial
trophoblast populations, decreased trophoblast invasion and impaired remodeling of maternal spiral arteries\textsuperscript{88}. Miscarriages can occur at the beginning and end of pregnancy and more studies are needed to determine the underlying causes of this condition.
Chapter 2

The Chromosome 19 MicroRNA Cluster

MicroRNAs (miRNAs) are short 22 nucleotide long RNAs that post-transcriptionally regulate gene expression by degrading or inhibiting translation of their target mRNAs. They perform their gene regulatory function by base pairing between the miRNA seed sequence (nucleotide position 2-8 of the mature miRNA) and complementary sequences in the 3' untranslated regions (UTRs) of their targets. miRNAs have been shown to regulate many developmental and physiological processes including embryonic stem cell differentiation, cell proliferation, migration, invasion and stem cell reprogramming.

The chromosome 19 miRNA cluster (C19MC) is the largest miRNA cluster discovered in the human genome. It is located in an imprinted region on chromosome 19 and its entire genomic region is conserved in but not beyond primates. The C19MC cluster is composed of 49 miRNA precursors, interspersed with Alu repeats, which generate 53 unique mature miRNAs. These miRNAs are highly similar, with many of them sharing the same seed sequence, indicating that they regulate common sets of genes. In fact, common seed comparison analysis showed that C19MC miRNAs likely regulate genes involved in embryonic development and tumorigenesis.

C19MC is physiologically expressed in ESCs and the placenta. DNA methylation profiling of the C19MC locus identified a differentially methylated CpG rich
promoter region approximately 17 kb upstream of the first C19MC miRNA (Figure 2). C19MC miRNAs are located at the intronic regions of a large RNA polymerase II non-coding transcript and are processed by the DGCR8-Drosha microprocessor complex\(^9^3\). In addition, chromatin immunoprecipitation and in vitro transcription assays showed that Pol III is also associated with and transcribes some miRNAs of the C19MC\(^1^0^0\).

**C19MC in stem cells and cell reprogramming**

C19MC is expressed in ESCs and is part of a unique ESC miRNA signature that includes miR-302, miR-17, miR-106 and miR-371 clusters\(^9^9,\,1^0^1\). Bioinformatics analysis showed that 16 of the C19MC miRNAs share the same “AAGUGC” seed sequence with members of the miR-302 cluster that target genes involved in signaling pathways related to the maintenance of stemness\(^9^9,\,1^0^2\).

Recently, a study showed that the miR-302/367 cluster rapidly and efficiently reprogram mouse and human somatic cells to an iPSCs without the use of exogenous reprogramming factors\(^1^0^3\). Moreover, combination of stem cell enriched miRNAs with the reprogramming factors increased the efficiency of somatic cell reprogramming to iPSCs\(^9^1\). Similarly, miR-524, a C19MC miRNA, was also shown to increase the efficiency of reprogramming when co-expressed with reprogramming factors\(^1^0^4\). This study showed that miR-524 promotes cell reprogramming by increasing cell proliferation, decreasing apoptosis, promoting MET and increasing the expression of pluripotency genes.
C19MC in the placenta

C19MC miRNAs are highly expressed in the placenta and have been reported to play important roles in placental development. Laser-capture microdissection experiments in first trimester and term human placentas showed that C19MC miRNAs are abundantly expressed in villous trophoblasts (VT) but are reduced in invasive EVTs\textsuperscript{105}. To investigate the role of C19MC in trophoblast invasion, bacterial artificial chromosome (BAC) was employed to overexpress C19MC miRNAs in the EVT-derived cell line HTR8/SVneo. This study showed that overexpression of C19MC inhibited cell migration\textsuperscript{105}. Furthermore, ectopic expression of several individual C19MC miRNAs also inhibited the migration and invasion of EVTs\textsuperscript{106-108}. MiRNAs of the miR-515 family, that belong to C19MC, were significantly down-regulated during human ST differentiation. Importantly, in placentas from women with preeclampsia, these miRNA were significantly up-regulated\textsuperscript{109}. Therefore, C19MC plays an important role the regulation of trophoblast differentiation and optimal trophoblast migration and invasion essential for proper implantation.

Another important role of C19MC miRNAs in the placenta is the protection against viral infections. Primary human trophoblasts (PHT) which express high levels of C19MC, have been shown to be resistant to infection by a number of viruses including coxsackievirus B3, poliovirus, vesicular stomatitis virus (VSV), vaccinia virus, human cytomegalovirus and herpes simplex virus\textsuperscript{110}. Furthermore, PHT derived exosomes were sufficient to confer viral resistance to VSV infection in recipient cells\textsuperscript{110}. Further confirming these results, exposure to PHT conditioned medium, stable transfection of a C19MC BAC or miRNA mimics into non-trophoblastic cells sufficiently attenuated viral
infection\textsuperscript{110, 111}. C19MC has been shown to confer viral resistance primarily through the
induction of autophagy in recipient cells, since inhibition of autophagy ablates the
antiviral response\textsuperscript{110, 112}.

Altered expression of C19MC miRNAs has been reported in preeclampsia. Numerous studies reported increase in C19MC miRNAs in placentas from severe preeclampsia compared to placentas from normal pregnancies\textsuperscript{106, 108, 109, 113, 114}. In addition, circulating C19MC miRNAs are increased in plasma from preeclamptic patients compared to plasma from patients with normal pregnancies indicating that they can be used as non-invasive diagnostic biomarkers for the disease\textsuperscript{114, 115}. Furthermore, expression of the C19MC miRNA, miR-517a, in first trimester plasma samples showed high predictive value for identifying patients that subsequently developed preeclampsia\textsuperscript{116}.

**C19MC in benign tumors and cancers**

In addition to its physiological expression in stem cells and the placenta, C19MC is also found in few benign tumors and cancers. Chromosomal abnormalities at 19q13.4 were reported in mesenchymal liver hamartoma which is a rare benign tumor of childhood\textsuperscript{117} and in a subgroup of benign thyroid adenomas\textsuperscript{118}. C19MC miRNAs have also been reported to be overexpressed in another benign tumor of infancy, infantile hemangioma (IH)\textsuperscript{119}. In IH, but not other vascular malformations, C19MC miRNAs were localized to the endothelial cells and circulating C19MC miRNAs were detected in the plasma of IH patients. Therefore, circulating C19MC miRNAs may be serve as a noninvasive tool to diagnose IH and monitor treatment response\textsuperscript{119}. 
Amplification of the C19MC was first reported in central nervous system primitive ectodermal tumors (CNS-PNETs) including the deadly embryonic tumors with multilayered rosettes (ETMRs), embryonal tumors with abundant neuropil and true rosettes (ETANTR), medulloepitheliomas, ependymoblastomas, and PNETs with atypical histologic features\(^{120-123}\). Integrated genetic and epigenetic analyses of ETMR samples found that the amplification is driven by the fusion of the C19MC amplicon to the TTYH1 gene, which encodes a chloride channel that is highly expressed in neural tissue \(^{121}\). Importantly, amplification in C19MC correlated closely with upregulation of the RNA-binding pluripotency gene, LIN28 in CNS-PNETs\(^{124}\). However, the relationship between C19MC and LIN28 remain to be completely elucidated.

Studies have also shown that C19MC is highly expressed in triple negative breast cancers and hepatocellular carcinomas\(^{125-127}\). Furthermore, C19MC miRNAs are expressed in parathyroid tumors and correlated with aggressive tumor behavior\(^{128}\). Therefore, C19MC miRNAs can be amplified by chromosomal rearrangements and contribute to the pathogenesis of both benign and malignant neoplasms.
Figure 2. Simplified schematic representation of the C19MC locus and its surrounding genes on chr19q13
Open and closed lollipops indicate unmethylated and methylated CpG regions respectively and represent the differentially methylated region located approximately 17kb upstream of C19MC. Arrows indicate transcription direction.
Chapter 3
LIN28 in Stem Cells and the Placenta

The RNA binding protein LIN28

LIN28 is an important RNA-binding protein that is highly expressed in embryonic stem cells. It is a master regulator of development, differentiation and metabolism\textsuperscript{129}. Vertebrates have two LIN28 paralogs, lin-28A and lin-28B, both containing a unique RNA-binding domain; the N-terminal cold shock domain (CSD) and a pair of cysteine cysteine histidine cysteine-type zinc fingers that form a zinc knuckle domain (ZKD) at the C-terminal. The human LIN28A and LIN28B share approximately 73\% sequence homology\textsuperscript{130}, however they differ in a few important ways\textsuperscript{130}. The LIN28B protein has an extended C-terminus tail of approximately 41 amino acids longer than LIN28A, which contains a nuclear localization signal\textsuperscript{131} (Figure 3A). However, both LIN28A and LIN28B can localize in the cytoplasm and the nucleus\textsuperscript{130, 132}.

Photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation experiments showed that LIN28A and LIN28B proteins bind to thousands of mature mRNAs at defined sites\textsuperscript{133}. Given the overlap of LIN28A and LIN28B RNA binding domains, they act redundantly. Among the strongest LIN28 binding mRNAs that showed >10\% average increase in transcript levels were the nuclear RNA binding proteins, confirming a possible role in modulation of mRNA splicing\textsuperscript{134}. In addition, histones, cell cycle, insulin-PI3K-mTOR signaling pathway and the type-2 diabetes associated genes
IGF2BP1 to IGF2BP3 and HMGA2 were among the direct LIN28 targets. However, the most distinct mRNA that was regulated by LIN28B was LIN28B itself, which forms a feed-forward pattern that maintain its own levels of expression and stem cells properties\textsuperscript{133}.

**The LIN28/let-7 bi-stable switch**

LIN28 was originally identified in *C. elegans* during mutagenesis screening for genes that control developmental timing\textsuperscript{135}. LIN28 gain of function mutations promoted self-renewal and delayed hypodermal and vulval stem cell differentiation, whereas LIN28 loss of function mutations accelerated vulval stem cell differentiation\textsuperscript{16}.

LIN28 is highly expressed during embryogenesis and early larval development but was post transcriptionally suppressed as cells differentiated and disappeared by adulthood. The first two discovered miRNAs, lin-4 and let-7, were found to posttranscriptional repress LIN28 by directly targeting complementary sequences in its 3' UTR. Let-7 miRNAs are highly conserved and were originally discovered in a genetic screening for *C. elegans* heterochronic mutants\textsuperscript{136}. These miRNAs are important during development, where repression by LIN28 is critical for maintaining pluripotency and preventing differentiation. Loss of function of let-7 homologous mimicked LIN28 gain of function phenotype highlighting their opposite roles in regulating differentiation.

In the mammalian genome, LIN28 is highly expressed in embryonic stem cells (ESCs), in which pri-let-7 transcripts, but not mature let-7, were also highly expressed\textsuperscript{137, 138}. These findings led to the discovery that LIN28A and LIN28B directly bind to pri-let-7 transcripts and inhibit its maturation through distinct mechanisms that
are also conserved in C. elegans\textsuperscript{139-144}. Mutagenesis studies have shown that both the CSD and CCHC domains are required for LIN28A protein binding to the terminal loop region of pre-let-7 in cell-free assays to block let-7 processing and inhibit its maturation\textsuperscript{143, 145}. Subsequently, a G-rich element (GGAG, GAAG, or AGGG) located at the 3’ end of the loop region of pri-let-7 was shown to confer LIN28 protein binding and resulted in inhibition of either DROSHA\textsuperscript{143} or DICER1 RNase III processing\textsuperscript{139, 146, 147}. The same GGAGA motif was also present in 28% of mRNAs targets of LIN28A identified by the LIN28A-HiTS-CLIP experiments\textsuperscript{134, 144}. LIN28A also recruits terminal uridylytransferase, Zcchc11, which leads to decay of the oligouridylated pre-let-7 miRNAs\textsuperscript{146, 147}. In contrast, LIN28B mediated repression of let-7 is Zcchc11 independent owing to the primary subcellular localization of LIN28B to the nucleoli and sequestering it in the nucleoli thus preventing processing by the microprocessor complex\textsuperscript{148}. This data is strengthened by experiments which showed that depletion of Zcchc11 inhibited tumorigenicity in LIN28A but not LIN28B expressing tumor xenografts and cancer cell lines\textsuperscript{148}. Therefore, LIN28 and let-7 form an evolutionarily conserved double negative feedback loop, also known as bi-stable switch, that governs stem cell self-renewal\textsuperscript{140, 142, 136} (Figure 3B).

**LIN28 regulates cell development and metabolism**

LIN28 also plays a role in the regulation of developmental timing, growth and metabolism. A genome-wide association study (GWAS) of height from 15,821 subjects identified LIN28B as one of 12 loci strongly associated with height, implicating the lin28/let-7 pathway as a strong regulator of human height\textsuperscript{149}. Two other GWAS studies
reported that LIN28B was strongly associated with genetic regulation of female sexual maturation and timing of human pubertal growth and development\textsuperscript{150, 151}. Engineered transgenic mice overexpressing LIN28A showed increased body size, crown-rump length and delayed onset of puberty\textsuperscript{152}. Further investigation revealed that these mice showed increased glucose metabolism and insulin sensitivity consistent with downregulation of let-7 targets Myc, Kras, Igf2bp1 and Hmga2\textsuperscript{152}. Moreover, overexpression of LIN28A or LIN28B in mice induced insulin sensitivity and resistance to high-fat-diet induced diabetes\textsuperscript{153}. This study found that LIN28 exerts this effect by regulating components of the insulin-PI3K-mTOR pathway and overexpression of let-7 or muscle-specific loss of LIN28A reverses this phenotype. Furthermore, treatment with rapamycin, an mTOR inhibitor, also inhibited the insulin sensitivity and enhanced glucose metabolism induced by LIN28A overexpression\textsuperscript{153}. Another GWAs study showed that many LIN28 mRNA targets are involved in cellular metabolism further confirming its role in regulating cellular growth and metabolism\textsuperscript{154}. Therefore, LIN28 regulates cell metabolism through de-repression of let-7 mRNA targets and by directly binding and regulating the expression of many genes that involved in cellular metabolism independently of let-7.

\textit{LIN28 in stem cells and cell reprogramming}

Stem cells are undifferentiated cells that have the capacity to become cells of multiple lineages. They are generally classified as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or adult stem cells\textsuperscript{155, 156}. ESCs are derived from the ICM of the blastocyst during embryogenesis while iPSCs are generated by
reprogramming somatic cells\textsuperscript{157, 158}. ESCs and iPSCs are pluripotent and depending on highly coordinated gene regulatory mechanisms, can differentiate into any cell type in the body\textsuperscript{159}. Adult stem cells such as neural stem cells and hematopoietic stem cells are generally found among differentiated cells in specific tissues or organs. They can differentiate into some or all of the specialized cells in the tissues or organ but cannot become any cell in the body. All stem cells have self-renewal ability that allows them to remain pluripotent as they undergo cell division.

LIN28A and LIN28B are important for the maintenance of the stem cell state through let-7 dependent and let-7 independent mechanisms. Both LIN28A and LIN28B are highly expressed in human ESCs and are highly involved in pluripotency. For example, the promoter regions of LIN28A are occupied by the ESC transcription factors Oct4, Sox2, Nanog and Tcf3, indicating that LIN28A expression is tied to expression of other pluripotency factors in stem cells\textsuperscript{160}. The increase in LIN28 expression represses let-7 miRNAs, leading to increased expression of let-7 targets such as c-Myc, Sal4, Hmga2, Igf2bps, LIN28 itself and various cell cycle regulators\textsuperscript{161}. In addition, LIN28 has been shown to associate with and regulate translation and stability of thousand mRNAs, including Oct4, Cyclin B, Cdk4, Igf3, all of which promote ESC growth and repress mRNAs important for oxidative phosphorylation, allowing for the low mitochondrial metabolism typical of the primed pluripotency state\textsuperscript{132, 162-164 165}.

Somatic cells can be reprogrammed into iPSCs by the introduction of the reprogramming factors Oct3/4, Sox2, c-Myc and Klf4\textsuperscript{157}. Another study showed LIN28A, LIN28B are not only important for maintaining stem cell pluripotency but can also be used in cell reprograming when combined with Oct4, Sox2, Nanog, eliminating the need
for c-Myc which causes cell death and differentiation of ESCs\textsuperscript{166, 165}. Overexpression of LIN28 was also shown to increase the rate of cell division and accelerated the kinetics of iPSC reprogramming\textsuperscript{167}.

**LIN28 in the placenta**

Despite the large amount of literature characterizing the function of LIN28 in stem cell and embryonic development, little is known about the role of Lin28 in the human placenta. In a recent work from our laboratory, we determined that LIN28B to be the predominant paralog to be expressed in the human placenta. Primary cultures of CTs and STs isolated from normal term placentas also showed higher expression of LIN28B compared to LIN28A\textsuperscript{168}. In situ protein localization of human placentas at term further confirmed that LIN28B is primarily expressed in the STs and CTs, as opposed to the decidual cells. We also show that LIN28B mRNA and protein levels were significantly reduced in placentas of preeclampsia complicated pregnancies compared to placentas of normal pregnancies. These data suggested that the decrease in LIN28B expression may be an underlying contributing factor to the development of preeclampsia. In first trimester human placenta, LIN28B was highly expressed in syncytial sprouts and its expression increases from proximal, non-invasive, to distal invasive EVTs, while decidual cells exhibited very little LIN28B expression. These findings further indicate its role in early EVT invasion into the maternal decidua. Overexpression of LIN28B significantly increased proliferation, migration and invasion of the EVT cell line HTR8/SVneo, whereas knockdown of LIN28B in JEG3 cells (that highly express LIN28B) decreased cellular proliferation\textsuperscript{168}. Moreover, we showed that LIN28B regulates
the expression of syncytin-1 which is involved in fusion of CTs to form the ST layer of the placenta and also regulate the integrin subunit ITGβ4 which is enriched in noninvasive trophoblasts, further confirming the role of LIN28B in regulating trophoblasts differentiation to the EVTs and STs. We also showed that LIN28B regulates the expression of the proinflammatory cytokine TNFα and the circulating peptide hormone ELABELA, which are both associated with preeclampsia. Importantly, we showed that LIN28B regulates expression of C19MC miRNAs.

Since preeclampsia is associated with shallow implantation, impaired trophoblast invasion, and remodeling of the maternal spiral arteries resulting in insufficient placental perfusion, we tested the effect of hypoxia on LIN28B expression and found that LIN28B is drastically reduced upon exposure to hypoxia. The reduction in LIN28B was associated with decreased SYN-1 expression and increased TNFα expression. These data further confirm that LIN28B may play an important role in the pathology of preeclampsia.

Other studies have previously reported that LIN28B is highly expressed in human term placentas\textsuperscript{169}. In human embryonic tissues, LIN28B expression increases during early stages of gestation and plateaus between gestational weeks 7 and 9, indicating its important role in the early stages of pregnancy\textsuperscript{170}.

A previous study has investigated the role of LIN28A in human trophoblast differentiation and invasion. They showed that LIN28A was detected in villous trophoblasts, both CTs and STs of first trimester placental\textsuperscript{171}. Knockdown of LIN28A in the human trophoblast cell line ACH-3P that highly express LIN28A, increased let-7
expression and decreased cell invasion suggesting that LIN28A is required for trophoblast invasion\textsuperscript{171}.

Another study showed that LIN28A was abundantly expressed in embryonic day 11.5 mouse placenta. LIN28A was detected in the chorioallantoic interface and labyrinth layer, with little LIN28A staining in spongiotrophoblast or differentiated mouse trophoblast giant cells (mTGCs). Decrease in LIN28A and increase in let-7 miRNA levels were also reported after differentiation of mouse trophoblast stem cell into mTGCs and in ACH-3P cells induced to syncytialize with forskolin treatment. Moreover, knockdown of LIN28A in ACH-3P cells resulted in increased spontaneous syncytialization accompanied by increase expression of ST markers hCG, LGALS13, and ERVW-1 mRNA, and also increased the responsiveness to forskolin-induced differentiation. However, unlike ACH-3p cells, knockdown of LIN28A in mouse trophoblast stem cells did not induce syncytialization\textsuperscript{172}.

**LIN28 in Cancer**

LIN28A and LIN28B are highly expressed in several primary human tumors including colon, breast, lung, and cervical cancers. Recent studies have identified LIN28 as a highly specific marker of the aggressive embryonal CNS tumor with multilayered rosettes (ETMR, previously known as ETANTR). In these tumors, the increase in LIN28 correlated closely with C19MC amplification\textsuperscript{124}.

The increase in LIN28 was also observed in human cancer cell lines and was associated with a decrease in let-7, and an increase in let-7 mRNA targets such as Hmga2, Kras and c-Myc\textsuperscript{173} (Figure 4). Increased expression of c-myc due to reduction
in let-7 is important, as c-myc transcriptionally activates LIN28 and other oncogenes to establish a positive feed-forward loop\textsuperscript{173, 173}. Studies have also shown evidence that LIN28A supports cancer progression by promoting cell cycle progression and proliferation through regulation of CDK2, CCND1 and CDC25A in cancer cell lines\textsuperscript{174}, by let-7 dependent and let-7 independent mechanisms\textsuperscript{174}. Overexpression of LIN28 in NIH/3T3 cells caused a depletion in let-7 expression and parallel increase in the abundance of c-Myc, a let-7 target. LIN28 transformed cells formed colonies in soft agar and tumors in nude mice indicating its role in enhancing metastases\textsuperscript{173}.

Gain-of-function and loss-of-function studies showed that LIN28/let-7 signaling is important for the maintenance of self-renewal with LIN28 and let-7 positively and negatively regulating cancer stem cells respectively\textsuperscript{175}. These studies provide evidence to support the role of LIN28 in cancer progression and metastasis through let-7 dependent and let-7 independent mechanisms and underscore the significance of increased LIN28 expression in aggressive tumors\textsuperscript{176}. 

Figure 3. Simplified schematic representation of LIN28A and LIN28B in normal development.
(A) Conserved domains in the human LIN28 paralogs LIN28A and Lin28B. LIN28B (250 amino acids) is slightly longer than LIN28A (209 amino acids). CSD, cold shock domain; CCHC, cysteine cysteine histidine cysteine domain; NLS, nuclear localization signal. (B) LIN28 proteins are highly expressed in undifferentiated stem cells during normal development. LIN28 blocks the maturation of let-7 miRNAs to facilitate stem cell maintenance and proliferation. During differentiation, LIN28 expression decreases and let-7 miRNAs are increased. Let-7 miRNAs decrease the self-renewal capacity of stem cells which allows lineage commitment and terminal differentiation to occur.
Figure 4. Schematic representation of Lin28 and let 7 in cancer. In cancer, LIN28 and let 7 expression are often imbalanced. More aggressive cancers show low let 7 expression and high Lin28 expression with increased self-renewal capacity and tumorigenic potential.
Chapter 4

Infantile Hemangioma

Prevalence and risk factors

Infantile hemangiomas (IH) are benign vascular tumors of childhood that affect approximately 5% of all infants\textsuperscript{177-179}. IHs are more prevalent among female infants with some studies suggesting female-to-male ratios ranging from 1.4:1 to 5:1\textsuperscript{180, 181}. Currently, the definitive explanation for higher IH incidence in female infants is unclear. Infants with IH are more likely to be white non-Hispanic, premature and be products of multiple gestations. Multivariate logistic regression showed that low birth weight is the most significant risk factor for IH. With every 500g decrease in birth weight, the risk of developing IH increased by 40\%\textsuperscript{182}. Some studies report an increased incidence of IH in infants after chorionic villous sampling\textsuperscript{92, 183} and amniocentesis\textsuperscript{184}. Other pre-birth factors associated with IH include older maternal age, pre-eclampsia and placenta previa\textsuperscript{181}. Studies have also shown a correlation between placental or perinatal hypoxia and incidence of IH\textsuperscript{185}. Other factors associated with IH include fertility drug use, erythropoietin use, breech presentation, being the first born and familial clustering\textsuperscript{180, 181, 186-189}. 

\textsuperscript{180, 186-189}
**Phases of IH development**

IHs have an atypical life cycle that involves at least two distinct evolutionary phases: the proliferation phase and the involution phase. An intermediate phase commonly referred to as a plateau phase occurs between the proliferation and involution phases in which proliferating cells and cells undergoing apoptosis are likely at an equilibrium\(^{190-193}\).

IH first present after birth as flat, raised patches with well-defined borders. The proliferation phase is rapid and involves endothelial cell proliferation and formation of disorganized blood vessels (non-leaky) composed of immature endothelial cells\(^{194,195}\). The IH enlarges and develops a rubberier consistency during this period. Tumors are often characterized by peripheral pallor and dilation of adjacent veins\(^{196}\). Painful ulceration and scarring may develop during the proliferation phase due to the speed of tumor growth. Clinical onset of IH is typically before the first 4 weeks of age and duration of proliferation varies depending on morphology\(^{181,191}\). A greater part of IH growth occurs between 1 and 2 months of age with majority of IHs reaching 80% of their eventual size by 3 months of age. Most growth of IH lesions is typically completed around 5 months of age\(^{181}\).

In most IH cases, spontaneous tumor involution starts between 6 months and 1 year of age and continues for years with most regression occurring before 4 years of age. A large retrospective study reported that involution terminated at a median of 36 months with 92% of tumors completing involution by year 4\(^{197}\). Importantly, completion of the involution phase does not always mean complete resolution of the tumor and
69% of IH cases leave behind noticeable changes in the skin, including fibrofatty residuum, dyspigmentation, scars, telangiectasia and anetoderma.

**Pathogenesis of IH**

The pathogenesis of IH is complex and poorly understood. However, several major hypotheses have been proposed. In 2000, North et al. reported intense endothelial immunoreactivity for the erythrocyte-type glucose transporter protein GLUT-1 during all phases of IH development but not in any of 66 vascular malformations, 20 pyogenic granulomas or 7 granulation tissue specimens, establishing the viability of GLUT-1 immunoreactivity as a diagnostic marker for IH\textsuperscript{198}. In a retrospective study, North et al. showed that IH tissues also have high immunoreactivity for placental molecular markers including Fc-γ receptor-IIb, merosin and Lewis Y antigen. These markers were observed in placental villi and IH but not in micro vessels of normal skin and subcutis\textsuperscript{199}. These findings suggest a close association between the placenta and IH and provide evidence to support a possible placental origin for IH. The placental origin theory suggests that progenitor cells in IH embolize from the placenta during pregnancy or birth due to placental disruption. This theory is also supported by clinical studies that show that increased prevalence of IH correlates with placental disruption arising from chorionic villus sampling, preeclampsia and placenta previa\textsuperscript{92, 183, 184}. In furtherance of this theory, a study by Barnes et al showed that the transcriptomes of the human placenta and IH are highly similar, suggesting a placental origin for the tumor\textsuperscript{200}. The level of similarity was comparable to the similarities observed between a tissue and its derived tumor. Therefore, a unifying theory of IH pathogenesis proposes that IH
originates from abnormal proliferation and differentiation of a hemogenic endothelium that exhibits a neural crest phenotype and can differentiate into mesenchymal, endothelial, and neuronal cells. These cells are thought to originate from the placental chorionic mesenchymal core which embolize to the developing fetus\textsuperscript{201}.

Other studies have presented evidence to suggest that intrinsic endothelial progenitor cells (EPC) contribute to the development of IH. This theory proposes that IH originates from clonal expansion of circulating EPCs which results in the formation of new blood vessels\textsuperscript{202}. Supporting this theory, a study showed that blood samples of infants with IH contained higher numbers of circulating EPCs\textsuperscript{203}. Bischoff et al showed that proliferating but not involuting or involuted IH tissues express an endothelial progenitor cell marker, CD133-2, providing another evidence of EPCs in IH\textsuperscript{202}. Multipotential CD133-positive stem cells (HemSC) isolated from proliferative IH showed high proliferative and clonogenic capacity and were able to differentiate into cells of multiple lineages in vitro\textsuperscript{204}. HemSCs and cord-blood EPCs are highly similar thus suggesting that proliferating immature endothelial cells in IH could originate from circulating EPC\textsuperscript{204}. HemSC implanted into immunocompromised mice generated human blood vessels after 7 weeks. Two months following implantation, the tumors showed human adipocyte differentiation and a decrease in the number of blood vessels. These results indicate that HemSCs are capable of recapitulating the unique IH life cycle\textsuperscript{204}.

In addition, the IH cytokine niche provides a conducive environment for the de-novo development of blood vessels due to the high expression of VEGFR-2 and VEGFA\textsuperscript{194, 201}. Furthermore, high expression of IGF2 promotes proliferation while the
activation of the anti-apoptotic TRAIL-OPG pathway prevents apoptosis in IH\textsuperscript{201, 205}. In addition to increased expression of VEGF, other pro-angiogenic cytokines such as bFGF, matrix metalloprotease (MMP) 9 and MMP-2 are increased while the expression of the tissue inhibitor of metalloproteinases-2 (TIMP-2) was decreased in proliferative IH\textsuperscript{195, 206, 207}. These angiogenic factors are downregulated during involution while inhibitors of angiogenesis such as interferon-β are upregulated during IH involution. During IH involution, endothelial cells highly express caspases, which are known markers of apoptosis\textsuperscript{208}. Endothelial cell apoptosis during involution is also accompanied by an increase in expression of markers of endothelial cell maturation such as ICAM-1 and HLA-Dr\textsuperscript{209}.

Low birth weight, a major risk factor for IH, is commonly caused by in utero hypoxia. Thus, hypoxia has also been hypothesized to trigger a vascular response in infants and numerous studies have highlighted its potential role in the development of IH. Tissue ischemia increases and stabilizes the expression of hypoxia inducible factor-1α and promotes the increase in expression of downstream effectors SDF-1α and VEGF-A by hypoxic endothelial cells\textsuperscript{210}. These effectors (SDF-1α, HIF-1α, VEGF-A and MMP9) have been shown to be upregulated in IH tissue and in blood from children with proliferating IH\textsuperscript{211}. These mediators facilitate the trafficking of progenitor cells to areas of tissue hypoxia\textsuperscript{212}. Hypoxia also induces the expression of GLUT-1, an important hypoxia sensor, and IGF2 which are known to be highly expressed in IH\textsuperscript{213, 214}. Therefore, tissue hypoxia which leads to neovascularization due to the upregulation of pro-angiogenic cytokines and trafficking of progenitor cells to hypoxic sites has been hypothesized to contribute to the pathogenesis of IH. This hypothesis is further
strengthened by reports of decreased blood flow in the skin preceding IH
development\textsuperscript{215} and a study that reported a synergistic effect between hypoxia and
estradiol on the upregulation of MMP-9, an important factor for EPC mobilization, in
endothelial cells in-vitro\textsuperscript{211}.

**Clinical classification of IH**

IHs can be classified based on soft tissue depth during the proliferative phase as
superficial, deep or compound IHs. Superficial IHs appear as red, raised lesions with
well-defined margins on the surface of the skin and have little to no subcutaneous
involvement\textsuperscript{192,196}. These lesions have historically been described as strawberry
hemangiomas\textsuperscript{191}. Deep IHs reside deep to the skin surface and have extensive
subcutaneous involvement. The surface of deep IHs may appear blue or unchanged.
Compound IHs combine both superficial and deep components in the same lesion\textsuperscript{196}.

Superficial IHs can be further classified as telangiectatic, abortive, non-
proliferative, nascent or arrested-growth IH. Although these lesions have little to no
proliferative phase and exhibit minimal growth, immnohistological study showed that
they display GLUT-1 immunoreactivity, confirming that they are true IHs\textsuperscript{216,217}.

Involution of abortive IHs is also more rapid, often occurring before 1 year of age
without leaving behind a fibrofatty residuum\textsuperscript{217}.

IHs can also be classified as localized, segmental, indeterminate or multifocal
based on their clinical characteristics\textsuperscript{218}. A large retrospective study by Haagstrom et al
defined localized hemangiomas as IHs that developed from a single focal point or
localized to an area without an obvious developmental configuration. Segmented hemangiomas were defined as IHs that possessed a configuration consistent with an identifiable and/or substantial portion of a developmental unit\textsuperscript{219}. Indeterminate hemangiomas were defined as those which could not be easily classified as localized or segmental while multifocal hemangiomas were defined as cutaneous lesions occurring at more than 1 anatomical site\textsuperscript{220}. The same study found that morphological subtype was the single best predictor of complications and need for clinical intervention; segmental hemangiomas were more likely to require treatment and lead to complications compared to localized hemangiomas. Indeterminate hemangiomas had a higher rate of complications and treatment than localized but lower than segmental hemangiomas\textsuperscript{220}. Complications tended to include ulceration, bleeding, airway obstruction, visual and auditory compromise\textsuperscript{220}.

\textit{Complications of IH}

Due to the IH life cycle, most lesions do not require clinical intervention. However, a significant minority of IH can develop complications that threaten vital functions, quality of life and overall health of the patient and thus require treatment. Complications attributable to IH include, ulceration, bleeding, airway obstruction, feeding impairment, congestive heart failure and hypothyroidism\textsuperscript{221}. Ulceration, the most common IH complication, refers to a breakdown of the IH skin surface and occurs in about 5\% to 21\% of IH cases\textsuperscript{222,223}. Ulcerations are more prevalent in lesions with a superficial component and are more likely to occur during the proliferative phase\textsuperscript{224}. Ulcerations can lead to bleeding, secondary infection scarring and disfigurement and
can cause significant pain. Bleeding is almost exclusive to ulcerated IH lesions and is easily controlled with pressure when it occurs in non-ulcerated IH due to surface trauma. Bleeding was reported in 41% of patients with ulcerated IH but was rarely clinically significant\textsuperscript{225}.

IH can lead to feeding impairment and cause a failure to thrive in patients with IHs involving the perioral region or airway. These feeding difficulties are often caused in by an oral sensory impairment and severe congenital laryngeal anomalies that can make it harder for the patient to breath or swallow\textsuperscript{226}. Furthermore, IH lesions that occur in the periocular region can cause visual impairment such as astigmatism, strabismus, anisometropia and lead to the development of amblyopia\textsuperscript{227, 228}. High output congestive heart failure has also been reported in a case of multiple cutaneous hemangiomas and a case of cutaneous and diffuse liver hemangiomas\textsuperscript{229, 230}. This congestive heart failure is rare and is caused by arteriovenous shunting of a large volume of blood through the tumor. Management of the heart failure usually improves the cardiac compromise and the hemangioma\textsuperscript{221}.

\textit{Medical interventions for IH}

Complications arising from hemangiomas can be life threatening and necessitate treatment. In the 1940s and 1950s aggressive irradiation and excision surgery were widely used to treat hemangiomas with varying rates of success. A study in the late 1960s reported shrinkage of IH in a patient with multiple hemangiomas and thrombocytopenia treated with systemic corticosteroid therapy\textsuperscript{231}. In the following years patients with proliferative IHs were treated with oral prednisolone and showed
successful growth arrest with no rebound growth after treatment\textsuperscript{232}. Although many studies have shown that corticosteroid therapy is efficacious for the treatment of IHs\textsuperscript{233}, concerns about adverse effects of extensive systemic steroid administration persist among physicians. Some adverse effects observed include cushingoid face, hirsutism, personality change and gastric irritation. These effects usually resolved after steroid therapy was completed\textsuperscript{234}.

interferon-\textalpha, an important cytokine for the innate immune response to viruses, has also shown efficacy for the treatment of corticosteroid therapy resistant IHs. Interferon. This therapy was associated with transient side effects including fever, neutropenia and skin necrosis\textsuperscript{235}. However, significant side effects including neurological toxicities and cortical and motor function impairment have been reported thus precluding its use as a preferred therapy for IH\textsuperscript{221}.

In 2008, Léauté-Labrèze et al reported a serendipitous finding that oral propranolol, a non-selective $\beta$-adrenergic receptor blocker, effectively inhibited the growth of IH\textsuperscript{236}. Propranolol was used to treat children with IH who also presented with increased cardiac output\textsuperscript{236}. Propranolol treatment induced rapid changes in the hemangiomas including a change in color from red to purple associated with a softening of the lesion. The lesions achieved almost complete regression with residual skin telangiectasias\textsuperscript{236}. A follow-up study reported that propranolol administered with a starting dose of 2 to 3 mg/kg per day in 2 or 3 divided doses was sufficient to produce a rapid consistent and therapeutic response in patients with minimal side effects\textsuperscript{237}. Due to its efficacy and exceptional tolerance in infants, propranolol has since become the
first-line therapeutic option for the management of IH. In 2014, the US Food and Drug Administration approved a pediatric sugar, alcohol and paraben free formulation of oral propranolol (Hemangeol: Pierre Fabre Dermatologie, Castres, France) for the systemic treatment of proliferating hemangiomas.

In 2015, a large randomized controlled clinical trial of oral propranolol in infantile hemangioma found that 88% of infants 1 to 5 months of age with proliferating hemangiomas who received propranolol treatment showed marked improvement by week 5 compared to 5% of patients who received placebo. Success was defined as total or near total resolution of the hemangioma after 24 weeks of treatment and the optimal dose was determined to be 3 mg/kg per day\textsuperscript{238}. Additional studies have also reported that oral propranolol is safe and effective for the treatment of complex and cosmetically relevant hemangiomas\textsuperscript{239}.

Although propranolol therapy for the management of hemangiomas in infants has been reported to be very safe, 26 out of 1260 patients (2.1%) reported intolerable side effects including severe sleep disturbance (17/26), severe agitation (3/26) and severe respiratory disorders (4/26) and treatment had to be discontinued for these patients. Other beta adrenergic receptor inhibitors that do not cross the blood-brain barrier such as atenolol are well tolerated with fewer CNS side effects\textsuperscript{240} and do not compromise efficacy\textsuperscript{241}. Furthermore, a retrospective noninferiority study which compared atenolol therapy to propranolol for the treatment of infantile hemangiomas found that atenolol is as effective as propranolol and is less associated with risk of bronchospasm\textsuperscript{242}. These
studies indicate that atenolol can be used as an alternative therapy for patients who experience CNS related side-effects when treated with propranolol.

**β-Adrenergic receptor blockers**

The β-Adrenergic signaling pathway mediates the fight or flight responses of the sympathetic nervous system (SNS) through the catecholamine effectors epinephrine and norepinephrine. Most major organs are innervated by SNS neural fibers which release norepinephrine into target tissues in response to various physiological and environmental stimuli. Effects of catecholamine release are mediated by α_{1-}, α_{2-}, and β-adrenergic receptor families which are differentially expressed in different tissues and perform their signaling function by coupling to G-proteins^{243}.

β-adrenergic receptors are sensitive to epinephrine and norepinephrine and their stimulation regulates broad cellular processes including growth control, muscle contraction and metabolic regulation. Stimulation by norepinephrine or epinephrine activates β-adrenergic receptor coupling to the G_{s} protein which stimulates the synthesis of cAMP by adenylyl cyclase. This increase in cAMP mediates cellular processes through 2 major effectors; protein kinase A (PKA) and exchange protein activated by adenylyl cyclase (EPAC). PKA activation regulates cell differentiation, motility, morphology and induces transcriptional alterations by phosphorylating transcription factors, whereas EPAC signaling activates the MAPK pathway thus exerting effects on cell proliferation and growth^{243, 244}.
Mechanism of action of β-Adrenergic receptor blockers in IH

Although the exact mechanism of action of propranolol in IH is still unknown, some proposed mechanisms include vasoconstriction, modulation of the renin-angiotensin system, inhibition of angiogenesis via downregulation of VEGF-A and MMPs, inhibition of nitric oxide and stimulation of apoptosis. Furthermore, all 3 β adrenergic receptor subtypes have been shown to be expressed in IH lesions. β₂ adrenergic receptor is localized mainly in endothelial cells and studies have also shown β₁ and β₂ receptor expression in hemangioma endothelial cells, hemangioma pericytes and HemSC.

Activation of β₂ receptors in endothelial cells by epinephrine induces vasodilation through a signaling cascade that involves the activation of adenylyl cyclase, the release of cAMP and the activation of PKA which leads to activation of endothelial NO synthase and downstream NO release. NO released into the blood stream leads to the activation of guanylate cyclase which induces the formation of cyclic guanosine monophosphate (cGMP) and activation of protein kinase G. This cascade culminates in the relaxation of vascular smooth muscle and vasodilation. β adrenergic receptor antagonists such as propranolol can thus cause vasoconstriction by inhibiting vasodilation caused by β₂ receptor activation. Propranolol induced vasoconstriction decreases blood flow within the hemangioma and accounts for the change in color from red to purple and the tumor softening observed shortly after treatment is started.

Propranolol has been shown to modulate growth-factor induced proliferation, migration and tube formation by inhibiting VEGF induced tyrosine phosphorylation of
VEGFR-2, MMP-2 secretion and inducing G0/G1 cell cycle arrest in endothelial cells in vitro. In addition, VEGF and bFGF are highly expressed in proliferative hemangiomas. Norepinephrine increases VEGF-A production in different normal and diseased cell types. Therefore, by blocking β adrenergic receptors, propranolol decreases VEGF-A mediated angiogenesis in IH. Indeed, propranolol treatment of IH endothelial cells leads to decreased VEGF expression, decreased cell proliferation and increased apoptosis by activating the caspase cascade and increasing the expression of the pro-apoptotic p53 gene and Bax/Bcl-xl ratio. Oral propranolol was shown to decrease peripheral serum concentrations of VEGF and eNOS in patients with IH. Another study found significant reductions in serum concentrations of VEGF, bFGF and MMP9 after 8 weeks of propranolol treatment. These findings suggest that propranolol exerts its therapeutic effects on IH lesions by modulating the expression of these pro-angiogenic cytokines.
**Gap in knowledge and purpose of this dissertation**

C19MC is the largest human cluster of miRNAs that is physiologically expressed in pluripotent stem cells and the placenta. C19MC is imprinted and is regulated by DNA methylation of an upstream CpG island. Chromosomal aberrations at 19q13.4 such as amplifications which results in activation of C19MC have been found in benign tumors and aggressive cancers. Many C19MC miRNAs share similar seed sequences and thus target many of the same mRNAs. A number of studies have shown that individual miRNAs of the C19MC play important roles in the regulation of stemness and cell differentiation, proliferation, migration, invasion and resistance to viral infections.

However, investigating the physiological and pathological role of the entire C19MC cistron in the placenta and in tumors has been challenging because of its size. Bacterial Artificial Chromosome (BAC) technology has been employed to overexpress the entire C19MC locus. Since BAC contains all the regulatory elements such as the upstream CpG island that control its expression, it achieved modest upregulation. To address this challenge and the gap in knowledge: 1) we employed the CRISPR/Sam technology that avoided the imprinted upstream CpG island to transcriptionally activate the entire endogenous C19MC cistron in different human cell lines in a highly efficient, specific and reproducible manner; 2) we investigated the physiological role of the entire C19MC cistron in trophoblast differentiation; 3) we determined the effect of C19MC on cell reprogramming; and 4) we evaluated the effect of hypoxic conditions during implantation and placentation on C19MC expression and EVT differentiation. Our findings reveal the role of C19MC in cell reprogramming and maintaining epithelial
aspects of trophoblast cells. Moreover, we show that hypoxic conditions promote EVT differentiation at least in part by decreasing the expression of C19MC miRNAs.

C19MC is highly expressed in the human placenta and numerous benign tumors and cancers. The increase in C19MC expression often correlated closely with increased expression of the RNA-binding protein LIN28. However, the molecular mechanisms that regulate C19MC and LIN28 have not been investigated. IH is a benign tumor of infancy, characterized by rapid growth followed by spontaneous involution. Although treatment is not required in a majority of cases due to natural tumor involution, IH can cause significant morbidity which necessitates treatment. The current first line IH treatment is the beta blocker propranolol, which induces rapid tumor involution soon after treatment onset. In addition, C19MC miRNAs are highly expressed in IH and decreases in circulating C19MC miRNA levels correlated with tumor phase and with propranolol treatment. Although C19MC miRNAs and LIN28 protein expression have been strongly correlated in some tumors, it has not been reported in IH. In addition, the effect of propranolol on C19MC and the LIN28/let 7 bi-stable switch has not yet been determined. To address this gap in knowledge, we analyzed the expression of LIN28B in proliferative IH, involuted IH and propranolol treated IH samples. Using sRNAseq, we also examined the expression of miRNAs differentially expressed in IH and in propranolol treated iPSCs. We show that overexpression of LIN28B in HEK293 cells induces the expression of miR-516b, a C19MC miRNA without altering the methylation status of the C19MC upstream CpG island or increasing transcription in the region, indicating that LIN28B increases the expression of miRNAs of the c19MC in a methylation independent mechanism. Moreover, the expression of
both C19MC miRNAs and LIN28B was reduced by propranolol treatment. Based on our findings, we propose that propranolol induces IH involution by altering the LIN28/let 7 balance and decreasing the expression of C19MC miRNAs.
Chapter 5

Materials and Methods

Ethical approvals and Tissue specimens

Institutional review board approval for collection of resected human hemangiomas was obtained from Columbia University College of Physicians and Surgeons (IRB #AAAA9976). The clinical characteristics of the patients used for this study are shown in Table I. Term placental tissues from normotensive patients who were delivered by cesarean section were obtained from Tampa General Hospital/ University of South Florida (IRB # 00015578). Written informed consent was obtained from all patients. De-identified foreskin was used as normal infant skin (NS) and obtained from Tampa General Hospital, Tampa Florida. Placental sections from the first trimester (7 and 8-week gestation, n = 2) and early human pregnancies (20-week gestation, n=2) were obtained from a previously banked paraffin tissue bank, under approval by Yale University Human Investigation Committee and by the institutional review board of the University of South Florida. Animal study procedures were approved by the Institutional Animal Care and Use Committee (IACUC, R IS0004309) at the Morsani College of Medicine, University of South Florida.

Cell Culture

The human iPSC line SCVI274 was a gift from Dr Joseph C. Wu at Stanford Cardiovascular Institute, Stanford University School of Medicine. iPSCs were cultured
on matrigel coated 6-well plates and maintained in Essential 8 medium (A1517001, Life
Technologies) and passaged every fourth day. HEK293 cells (Stratagene #240085) and
HeLa cells (ATCC® CCL-2™) were maintained in DMEM supplemented with 10% heat-
inactivated fetal bovine serum (Sigma-Aldrich). MCF7 cells (ATCC® HTB-22™) were
maintained in EMEM supplemented with 10% FBS and PC3 (ATCC® CRL-1435™) were
maintained in F-12K medium supplemented with 10% FBS. Normal Human Dermal
Fibroblasts (NHDF, Lonza CC-2511) were purchased and maintained in FGM-2
BulletKit (Lonza CC-3132). The HemSC line H42 was a gift from Dr. June Wu at the
Department of Surgery, Columbia University College of Physicians and Surgeons.
Briefly, freshly dissected IH tissues were digested in 0.2% collagenase A (Roche
Diagnostics, Indianapolis, IN). Suspension of single cells were selected using anti-
CD133–coated magnetic beads (Miltenyi Biotec) and cultured on fibronectin-coated (1
μg/cm²) plates with endothelial growth media-2 SingleQuot (EBM-2, CC-3156;
Cambrex) supplemented with 20% FBS 258, 259. HemSCs were maintained in EBM-2
SingleQuot media supplemented with 20% FBS. Propranolol (Sigma-Aldrich, P0884)
was reconstituted in DMSO and used at 50 μM concentration for all experiments.

*Transient transfection*

For LIN28B over-expression experiments, 10⁵ HEK293 cells per ml were seeded in 12-
well plates (Greiner Bio). The following day, the cells were transfected with pcDNA3-
FLAG-Lin28B, a gift from Narry Kim (Addgene plasmid # 51373) using Lipofectamine
2000 (Life Technologies) according to the manufacturer’s instructions. The culture
medium was changed 24 hours after transfection and total RNA was harvested at
72 hours.
**Cell proliferation**

iPSCs were grown in 6 well plates and split 1:10 at 3-day intervals. Cells were seeded at subconfluency on a matrigel-coated 96-well plate in Essential 8 media then treated with 50 μM propranolol or vehicle control for 72 hours. WST1 proliferation assays were performed as previously described.260

**Cell viability**

The viability of iPSCs after propranolol treatment was analyzed by staining of live and dead cells with calcein AM and Propidium iodide (PI), respectively, 72 hours after treatment. Cells were washed with PBS before the addition of 2 μM calcein AM and 4 μM PI solution. Fluorescence microscopy images were captured after 15 minutes.

**Design and cloning of C19MC-specific sgRNAs**

Single guide RNAs that target upstream region of C19MC were designed using the ATUM CRISPR sgRNA design tool. Two guide sequence oligos, 759-sgRNA 5’-CACCGCAAATCCTAGGCCTGCCCTG and 620-sgRNA5’-CACCGGTGAGCTGATGATCGCTCCA, were cloned into the lenti sgRNA(MS2)_zeo backbone (a gift from Dr. Feng Zhang261, Addgene #61427) using a Golden-Gate sgRNA cloning protocol and transformed into Stbl3 recombination deficient competent cells (Life Technologies, C7373-03). Ampicillin resistant clones were selected and verified by sequencing.
**Transient activation of C19MC cistron**

HEK293 (10^5 cells/well) were seeded in 12-well plates (Greiner Bio). The following day, the cells were transfected with a 1:1:1 mass ratio of sgRNA, MS2-P65-HSF1_Hygro and dCAS9-VP64_GFP (gift from Dr. Feng Zhang\textsuperscript{261} Addgene #61426 and #61422, respectively) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Culture medium was changed after 24 h. Total RNA was harvested after 72 h.

**iPSC generation**

Three micrograms of 759-sgRNA, MS2-P65-HSF1_Hygro and dCAS9-VP64_GFP in a 1:1:1 mass ratio alone or together with three micrograms of pCXLE-hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-hUL (gift from Dr. Shinya Yamanaka\textsuperscript{262} Addgene #27077, #27078 and #27080, respectively) at 1:1:1 mass ratio were electroporated into 6 × 10^5 NHDF cells using nucleofector (Lonza) and Amaxa Human Dermal Fibroblast Nucleofector Kit (Lonza, CC-2511) according to the manufacturer's instructions. Cells were seeded on 0.1% gelatin coated 6 well plates. After 3 days incubation, cells were trypsinized and re-plated at 2 × 10^5 cells onto 100-mm matrigel-coated dishes. The next day culture medium was replaced with mTeSR1 medium (STEMCELL Technologies, 85857). Colonies were counted from 20 to 30 days after plating and colonies similar to ESCs in morphology were harvested for further expansion and maintained in E8 media (Life technologies, A1517001) for at least 10 passages.
**In Vitro differentiation of iPSCs**

Human iPSCs were harvested by treating with collagenase IV for 30 minutes at 37°C. Cell clumps were transferred to ultra-low attachment plates (Fisher Scientific, 07-200-601) and maintained in DMEM supplemented with 20% FBS. Media was changed every other day. After 7 days in suspension culture, embryoid bodies were transferred to gelatin coated 8-well glass chamber slides and cultured for a further 10 days. Expression of the different germ layer markers were quantified by qRT-PCR.

Differentiation of iPSCs into CTs, STs and EVTs was performed as previously described. Briefly, iPSCs (cell line SCVI274) were treated for 2 days with EMIM medium containing KnockOut DMEM/F12 medium (Thermo Fisher Scientific, 12660012) containing 1% insulin-transferrin-selenium A (Thermo Fisher Scientific 41400045), 1x nonessential amino acid (Sigma-Aldrich M7145-100ML), 2% (wt/vol) BSA (Thermo Fisher Scientific, AM2618) 2 mM L-glutamine (Life Technologies, 25030-081) 100 ng/mL heparan sulfate proteoglycan (Sigma-Aldrich, H4777-.1MG). and then switched to EMIM medium supplemented with 10 ng/mL human BMP4 ((R&D Systems, 314-BP-010) for additional 5 days, at which point they were designated hPSC-derived CTs. For STs and EVTs differentiation, the hPSC-derived CTs were passaged using 0.25% trypsin and replated on Matrigel (Corning, 354277) coated plates (1:200) in FCM [KnockOut DMEM/F12 containing 20% (vol/vol) KnockOut serum replacement (Knockout SR; Thermo Fisher Scientific, 10828010), 1x GlutaMAX (Thermo Fisher Scientific, 35050061), and 1x nonessential amino acid, 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific ,21985023); conditioned on irradiated mouse embryonic
fibroblasts for 24 h], supplemented with 10 ng/mL human BMP4 and incubated in normoxic (21% Oxygen) and hypoxic (1% oxygen) conditions respectively for the indicated number of days.

**Teratoma formation and immunohistochemistry**

iPSCs were mixed with Matrigel and injected into each flank of NOD-SCID mice. Tumors were harvested at 4 weeks post-injection, fixed in 4% paraformaldehyde and embedded in paraffin. Sectioned tumors were immunostained with a human anti-mitochondria specific antibody (Abcam cat# ab92824) at 1:1000 dilution following the manufacturer’s protocol and nuclei were counterstained with hematoxylin.

**Alkaline Phosphatase staining**

Alkaline phosphatase histochemical staining was performed using Alkaline phosphatase detection kit (Millipore cat# SCR004) as described in the manufacturer’s instructions. Briefly, cells were fixed with 4% paraformaldehyde for 10 minutes and washed once with rinse buffer (20 mM Tris-HCl, pH 7.4 and 0.05% Tween 20). Staining solution was added to the wells, and the plates were incubated in the dark for 25 min. Bright field images were then obtained using a microscope.

**RNA isolation, RT–PCR and quantitative PCR (qPCR)**

Total RNA was isolated from human tissues and cultured cells using an RNeasy Mini Kit (Qiagen) and stored at -80°C in RNAse-free water. For RT–PCR analysis, 1 µg total RNA was reverse transcribed using random hexamer or oligodT primers and M-MuLV
reverse transcriptase (New England Biolabs) according to the manufacturer’s specifications.

For transcription assessments, cDNA products were amplified for 25 PCR cycles using previously described P0, P1, P5, P8 and P9 primer sets\textsuperscript{97} and Q5 High Fidelity DNA polymerase (New England Biolabs). PCR products were run on 1\% agarose gels and imaged with a BioDoc-It imaging system (UVP). All primers used in this study were obtained from Sigma Aldrich. To assess relative mRNA and miRNA expression levels, quantitative PCR (qRT-PCR) of cDNA products was performed using the following ThermoFisher TaqMan qRT-PCR probes: LIN28A (Hs00702808_s1), LIN28B (Hs01013729_m1), SOX2 (Hs01053049_s1), NANOG (Hs04399610_g1), OCT4 (Hs00999632_g1), CDH2 (Hs00983056_m1), SERPINE1 (Hs00167155_m1), TWIST1 (Hs01675818_s1), SNAI2 (Hs00161904_m1), ACTA2 (Hs00426835_g1), TUBB3 (Hs00801390_s1), GATA4 (Hs00171403_m1), CDX2 (Hs01078080_m1), PSG4 (Hs01923769_s1), p63 (Hs00978340_m1), HLAG (Hs00365950_g1), GAPDH (Hs02786624_g1) miR-515-5p (001112), miR-516a-5p (002416), miR-516b (001150), miR-517a (002402), miR-518c (002401), miR-519d (002403), let-7a (000377), let-7c (000379), U18 (001204). Taqman probes were used according to manufacturer’s instructions with TaqMan Fast Advanced Master Mix and QuantStudio 3 instrument (Life Technologies) as previously described\textsuperscript{264}. Data were analyzed by the \( \Delta \Delta C_t \) method: target \( C_t \) values and were normalized to GAPDH \( C_t \) values.

**DNA isolation and HpaII sensitivity assay**

DNA was isolated from cultured cells using the Blood and Tissue Kit (Qiagen) and stored at -20\(^\circ\)C in RNAse-free water. 300ng of DNA was digested with 2 µl of 10,000
units/ml HpaII (NEB) restriction enzyme or water (mock) for 12 hours. qPCR was performed using 60 ng of DNA from mock or HpaII reactions with primers designed to amplify 340bp region containing 6 HpaII restriction sites in the C19MC CpG island (F: 5’-GCGCCGGCTGCACGTCCCTTAGGAG and R:5’-CCCGCTGCCTGGAAGTATCGCCACC) and SYBR Green (SYBR Green PCR Master mix, Bio-Rad) in a QuantStudio 3 instrument (Applied Biosystems). HpaII sensitivity was calculated using the formula $[1 - 2^{-\Delta Ct}] \times 100\%$ as described\textsuperscript{265}.

**Luciferase reporter plasmid, transfection and luciferase assay**

The CDH2 3' UTR was amplified by PCR using the following primer pairs that contain the NOT1 restriction site (F: 5’ - ATGCCTGCGGCCGC TGTAGCAGTTAAAAAGAGGTAGGTG and R:5’- ATGCCTGCGGCCGC AACTTTGTAGTCTACTAGCACAGTG). PCR product and PGL2 basic (Promega, #E1641) were digested with the NOT1 restriction enzyme and ligated. The insertion orientation of the CDH2 3'UTR was confirmed by DNA sequencing. 10^4 HEK293 were plated and transfected after 24 hr with 1:1:1 mass ratio of 759 sgRNA, MS2-P65-HSF1-Hygro and dCAS9-VP64-GFP using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Culture medium was changed after 24 hours. At 48 hours from the transfection, CDH2 3' UTR and PGL4.74 (Promega cat #E6921) were co-transfected in a 4:1 mass ratio. Firefly and Renilla luciferase activity were measured after 24 hours using the Dual Glo Luciferase Reporter Assay System (Promega #E1910) and a luminometer (Cytation 3 cell imaging multi-mode Reader, BioTek Instruments).
**Immunofluorescence**

Paraffin embedded infantile hemangioma and placenta sections (10 μm) were immunostained with primary antibodies against LIN28B 40 μg/ml (Abcam Cat# ab71415, RRID:AB_2135050) and GLUT1 3.4 μg/ml (Abcam Cat# ab40084, RRID:AB_2190927) overnight at 4°C. Sections were washed 3 times in PBS and incubated with 4 μg/ml secondary antibody Alexa Fluor 488 (Life Technologies Cat# a21202, RRID:AB_141607) for GLUT1 and alexa fluor cy3 (Life Technologies Cat# a10520, RRID:AB_2534029) for LIN28B for 30 minutes at room temperature. The sections were then washed with PBS and mounted with ProLong Diamond Antifade Mountant with DAPI, (Invitogen). Confocal images were obtained using an Olympus FV 1200 instrument. Immunofluorescence intensities were measured using ImageJ software as described\(^{266}\). Fluorescence intensities were normalized to background intensities of secondary antibody only controls.

**Immunoblotting**

Immunoblot analyses were performed as previously described\(^ {267}\) using 24 ng/ml antibodies against GAPDH (Cell Signaling Technology Cat# 2118L RRID:AB_561053), 0.2 μg/μl antibodies against LIN28B (Abcam Cat# ab71415 RRID: AB_2135050) followed by IRDye 680 donkey anti-rabbit IGG secondary antibodies(0.2 μg/ml, LI-COR, 926-68073 RRID:AB_10954442). Immunoblots were imaged using the Odyssey Infrared Imaging System (LI-COR) and quantified using Image Studio software (LI-COR).
**In situ hybridization**

*In situ* hybridization for hsa-miR-517a/c, a miRNA of the C19MC, in early pregnancy placental sections was performed according to the manufacturer’s instructions. Briefly, paraffin embedded sections were deparaffinized in xylene then rehydrated by a series of graded alcohol washes. *In situ* hybridization was then performed using 40 nm 5’,3’ digoxigenin-labeled locked nucleic acid probe for hsa-miR-517a/c or scrambled negative control using the Exiqon *in situ* hybridization optimization kit according to the manufacturer protocol (ISH kit that include the scramble probe: Exiqon, 90005, miR-517a/c probe: Exiqon, 611715-360). Hybridization and post-hybridization graded SSC washes were performed at 55°C. The sections were then blocked, and the probes were detected using alkaline phosphatase conjugated sheep anti-digoxigenin Fab fragments (Roche, 11093274910). The signal was developed using NBT/BCIP (Roche, 11697471001) as a substrate that produces dark-blue indigo precipitating dye followed by nuclear counterstaining with Nuclear Fast Red (Vector laboratories, H-3403). The sections were then dried and covered for later image analysis.

**Immunohistochemistry**

Cytokeratin and vimentin immunostaining on early pregnancy placental sections was performed as previously described. Briefly, paraffin embedded sections were deparaffinized in xylene and rehydrated in a series of graded alcohol washes followed by antigen retrieval by boiling in citric acid. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂. The sections were then blocked using normal horse serum followed by incubation with the primary antibody (mouse-anti-cytokeratin,
Dako M7018, 1:600, RRID: AB_2134589). The following day cytokeratin was detected by incubation with the secondary antibody (biotinylated horse-anti-mouse, Vector Laboratories BA-2000, 3.75ug/ml, RRID: AB_2313581) along with the avidin-biotin-peroxidase complex (Vectastain ABC Kit, pk6200, Vector Laboratories). The signal was then developed using 3,3- diaminobezidine (sk-4100, Vector Laboratories) as substrate. The sections were then thoroughly washed then blocked using normal donkey serum followed by overnight incubation with the primary antibody (chicken-anti-vimentin, Abcam ab39376, 1ug/ml, RRID: AB_778827). The following day vimentin was detected by incubation with the secondary antibody (donkey-anti-chicken, Jackson ImmunoResearch 703-065-155, 1.2ug/ml, RRID: AB_2313596) along with avidin-biotin-alkaline phosphatase (Vectastain ABC-AP, Vector laboratories AK-5200). The signal was then developed using Vector Red AP substrate (Vector Red, Vector Laboratories SK-5100) followed by nuclear counterstaining with hematoxylin. The sections were then dried and covered for later image analysis.

**miRNA- and mRNA-sequencing**

Two μg of total RNA were converted into a small-RNA cDNA library according to the previously published protocol. Briefly, the RNA input for each sample was ligated to a 3’ adaptor barcoded sequence, pooled, size selected and gel purified, followed by 5’ adapter ligation and then subjected to size selection and gel purification. The cDNA library preparation was completed by second strand synthesis using SuperScript III, alkaline RNA hydrolysis, and PCR amplification for 10 cycles. mRNA libraries were prepared by utilizing the Illumina TruSeq Stranded mRNA LT protocol using 500 ng total RNA and NEB’s Protoscript II reverse transcriptase for the first-strand cDNA synthesis
according to the manufacturer’s protocol. Individual RNAseq libraries were quality controlled on an Agilent TapeStation with a High Sensitivity D1000 ScreenTape. Indexed samples were quantified using the Qubit dsDNA HS assay and pooled at equimolar concentration (10 nM). The libraries were sequenced on an Illumina NextSeq 500 sequencer 75-bp paired-end in mid-output mode in the Genomics Core Facility of The Rockefeller University.

**Bioinformatics analysis**

The miRNA read annotation for sRNAseq experiments was performed as previously described\(^{270,271}\) using a slightly updated, manually curated miRNA reference based on previous work \(^{272}\). For nomenclature of single miRNAs and definition and nomenclature of miRNA cistrons (or “pre-cursor clusters”) see Figure S1 of Akat et al. \(^{273}\). The RNAseq data was aligned to the Human Genome Build 38 using the STAR aligner\(^{274}\) (version 2.0.4j) allowing for two mismatches. Expression values (count matrices) were generated using featureCounts against gene definitions from Ensembl release 88 (GTF file) using fractional counting of multi-mapping reads.

**Statistical analysis**

sRNAseq and RNAseq data analyses were performed using the R statistical language. Differential analysis was performed using the Bioconductor package edgeR. All RT-PCR and immunoblotting data are reported as mean ± standard error of the mean (SEM). All data were tested for normality. Comparisons between two groups of normally distributed data were made by two-tailed Student’s t-tests with correction for unequal variance, while comparisons of data that was not normally distributed were done using Mann-Whitney U tests. Comparisons with more than two groups were subject to one-
way ANOVA with Dunnett’s *post hoc* tests against controls. Statistical testing was performed with IBM SPSS 24. *P* < 0.05 was considered statistically significant or as specified in the relevant tables and figure legends.
**Table 1. Clinical characteristics of patient samples obtained from Columbia University College of Physicians and Surgeons (IRB #AAAA9976)**

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Patient age</th>
<th>Sex</th>
<th>IH location</th>
<th>Treatment</th>
<th>Stage of IH</th>
<th>Preparation</th>
</tr>
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<td>25</td>
<td>1.5 months</td>
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<td>Subglottic</td>
<td>None</td>
<td>Proliferating; obstructive</td>
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<td>84</td>
<td>4 months</td>
<td>Male</td>
<td>Left cheek</td>
<td>Propranolol</td>
<td>propranolol</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>24</td>
<td>5 months</td>
<td>Female</td>
<td>Retroauricular</td>
<td>None</td>
<td>Proliferating; ulcerated</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>24</td>
<td>5 months</td>
<td>Female</td>
<td>Retroauricular</td>
<td>None</td>
<td>Ulcerating</td>
<td>Fresh</td>
</tr>
<tr>
<td>66</td>
<td>5 months</td>
<td>Male</td>
<td>Upper back</td>
<td>Propranolol</td>
<td>Proliferating; ulcerated</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>82</td>
<td>6 months</td>
<td>Female</td>
<td>trunk (left side)</td>
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<td>Paraffin embedded</td>
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<tr>
<td>62</td>
<td>7 months</td>
<td>Female</td>
<td>Scalp</td>
<td>Propranolol</td>
<td>Proliferating; ulcerated</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>63</td>
<td>9 months</td>
<td>Female</td>
<td>Scalp</td>
<td>None</td>
<td>Proliferating; bleeding</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>26</td>
<td>15 months</td>
<td>Female</td>
<td>Forehead</td>
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<td>Involuting</td>
<td>Fresh</td>
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<td>Female</td>
<td>Forehead</td>
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<td>Stable/involuting</td>
<td>Fresh</td>
</tr>
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<td>Chin</td>
<td>None</td>
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<td>Fresh</td>
</tr>
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<td>Anterior neck</td>
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<td>Involuting</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>27</td>
<td>24 months</td>
<td>Female</td>
<td>Anterior neck</td>
<td>None</td>
<td>Involuting</td>
<td>Fresh</td>
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<td>Fresh</td>
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<tr>
<td>36</td>
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<td>Fresh</td>
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<tr>
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<td>Cheek</td>
<td>Propranolol</td>
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</tr>
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<td>Fresh</td>
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</tr>
<tr>
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<td>cheek</td>
<td>None</td>
<td>Involuted</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>18b</td>
<td>84 months</td>
<td>Female</td>
<td>Trunk (left side)</td>
<td>None</td>
<td>Involuting</td>
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</tr>
</tbody>
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Chapter 6

Chromosome 19 MicroRNA Cluster Enhances Cell Reprogramming by Inhibiting Epithelial to Mesenchymal Transition

Introduction

Human embryonic implantation into the uterus requires extensive, coordinated attachment and invasion of the maternal endometrium by fetal trophoblasts. After fertilization and while still in the fallopian tube, the morula differentiates into the blastocyst, which consists of the inner cellular mass and an outer layer of epithelial cells, called trophectoderm, that eventually develops into the placenta\textsuperscript{275}. Shortly before attachment, the highly mitotic cytotrophoblast (CTs), derived from trophectoderm, differentiate into multinucleated syncytiotrophoblast (STs) or extravillous trophoblast (EVTs). The STs form the outer layer of the villous and regulate the maternal-fetal gas exchange, waste elimination and nutrient uptake. The EVT, consists of two major subtypes; interstitial EVTs and endovascular EVTs. Interstitial EVTs invade the decidua and inner myometrium to anchor the chorionic villi to the decidua and uterine wall while endovascular EVTs penetrate and remodel the maternal spiral arteries to transform them into high-flow, low-resistance vessels which facilitate placental perfusion and nutrient uptake by the fetus\textsuperscript{20, 276}.

EVT invasion is a highly regulated process and inadequate invasion can lead to placental hypoperfusion resulting in several early pregnancy complications including early pregnancy loss, fetal growth restriction, and preeclampsia\textsuperscript{277, 278}. EVT
differentiation involves extensive changes in which cells lose their cell junctions and apical-basal polarity and acquire migratory and invasive capacity, a process that requires the epithelial to mesenchymal transition (EMT). EMT is facilitated by transcription factors such as SNAIL, SLUG, TWIST and zinc-finger E-box-binding protein (ZEB) which downregulate epithelial markers and induce the expression of mesenchymal cell markers such as N-cadherin (also known as CDH2) and vimentin. Studies have shown that first trimester CTs and EVT display gene expression characteristics of epithelial and mesenchymal cells, respectively, indicating that EMT is involved in CT to EVT differentiation.

MicroRNAs (miRNA) are heavily involved in the regulation of EMT. Human and primate CTs highly express a unique cluster of miRNAs found on chromosome 19 (C19MC). This miRNA cluster spans approximately 100 kb and contains 46 miRNA genes that produce 56 mature miRNA species. It is epigenetically controlled by imprinting, with only the paternal allele expressed in the placenta. C19MC miRNAs are highly expressed in villous CTs and STs but their expression is significantly reduced in EVT. Furthermore, bacterial artificial chromosome (BAC)-mediated overexpression of C19MC attenuated cell migration and invasion phenotype by regulating enzymes and proteins that regulate cell motility indicating its role in the regulation of EVT invasion.

C19MC miRNAs are also highly expressed in embryonic stem cells (ESCs) together with other miRNA clusters including miR-302/367, miR-17/92, miR-200 and miR-371-373. Moreover, sixteen of the C19MC miRNAs share the same seed sequence with the miR-302/-372 family. Reprogramming of somatic cells into
induced pluripotent stem cells (iPSCs) relies upon ectopic expression of the OSKM transcription factors (OCT4, SOX2, KLF4 and MYC) or the OSLN factors (OCT4, SOX2, LIN28 and NANOG)\textsuperscript{157,166,285}. iPSC generation from human fibroblasts by OSKM, requires a mesenchymal-to-epithelial transition (MET) orchestrated by the suppression of pro-EMT signals and activation of a cellular epithelial program\textsuperscript{37}. Importantly, overexpression of miR302/367 cluster has been shown to induce reprogramming human somatic cells without the requirement for OSKM transcription factors\textsuperscript{103}. Furthermore, the miR302/367 cluster, miR-291-3p, miR-294 and miR-295 enhanced reprogramming efficiency when co-expressed with OSKM transcription factors\textsuperscript{91,286}. Additionally, miR-524-5p, a member of the C19MC has been shown to promote reprogramming to pluripotency in the presence of OSKM by targeting EMT related genes, promoting cell proliferation and inhibiting apoptosis\textsuperscript{287}.

To date, the role of the entire C19MC cluster in regulating EMT, trophoblast differentiation during placentation and somatic cell reprogramming is not yet fully understood. We hypothesize that C19MC is crucial for maintaining epithelial aspects of trophoblast cells and that downregulation of C19MC expression during early placentation by hypoxic conditions induces EMT and differentiation of CTs toward EVT.

**Results**

*C19MC is expressed in non-invasive CTs and STs of the placenta*

To determine the cellular localization of C19MC miRNAs in the placenta from early pregnancies, we performed *in situ* hybridizations (ISH) with probes for miR-517a/c on first trimester and early pregnancy placental sections. Control scrambled probe was
used for negative control. To distinguish trophoblasts from decidual tissue, adjacent consecutively cut sections were subjected to immunohistochemical staining for cytokeratin and vimentin, respectively. Stronger miR-517a/c expression was found in CTs, STs, and proliferative non-invasive trophoblastic cell columns (proximal cytotrophoblastic cells), in anchoring villi and C19MC expression gradually decreased in the invasive EVTs distal cytotrophoblastic cells) invading the maternal decidua. Moreover, in maternal decidual cells, miR-517a/c was detected (Figure 5A). ISH for miR-517a/c on term placental sections also showed strong expression of C19MC in villous trophoblasts (VTs) (Figure 5B). These data suggest that invasive CT differentiation is associated with inhibition of C19MC expression.

**Transcriptional activation of C19MC**

To investigate the role of C19MC in trophoblast cell differentiation, we employed the dCas9-based transcription activation system that combines a single-guide RNA (sgRNA) that contain two MS2 RNA aptamers, a catalytically inactive Cas9 variant fused to VP64 gene activation system and MS2-p65-HSF1 activation helper protein, designated as synergistic activation mediator (SAM) system\(^\text{261}\). To avoid the imprinted CpG island of the C19MC cistron located 17 kb upstream of the first miRNA of C19MC, two different sgRNAs were designed. The 620-sgRNA binds at ~579 bp upstream of the first miRNA of the C19MC, whereas 759-sgRNA binds at ~171 bp upstream of the first two miRNAs of the C19MC (Figure 6 A and B).

To determine the specificity and the extent of overexpression of the entire C19MC miRNA cistron using this method, sRNAseq analysis of HEK293 cells
transiently transfected with 759-sgRNA/SAM or 620-sgRNA/SAM for 72 hours was performed. To assess reproducibility, we performed an additional experiment using 759-sgRNA/SAM. Strikingly, sRNAseq analysis showed consistent 2- to 7984-fold upregulation of 45 miRNAs in all three independent experiments (threshold: ≥ 2-fold, FDR ≤ 0.20), 43 of which belong to the C19MC miRNA cluster (Figure 7A, Supplementary Table SI). Importantly, miRNAs of the miR-371-373 cluster that is located in close proximity to C19MC were not induced by either 759-sgRNA/SAM or 620-sgRNA/SAM. Interestingly, other ESC-enriched miRNAs such as miR-302a-5p, miR-20b, miR-200a, miR-200b, miR-200c and miR-141 were also upregulated (FDR ≤ 0.20) in HEK cells transfected with either 759-sgRNA/SAM or 620-sgRNA/SAM (Supplementary Table SI). On the other hand, no overlap was found in the downregulated miRNAs (Figure 7B, Supplementary Table SI).

The expression of 4 randomly selected C19MC miRNAs was validated by qRT-PCR, which showed significant upregulation of all 4 C19MC miRNAs (300- to 31,000-fold, p ≤ 0.05), while the expression of miR-21, a non-C19MC miRNA, was unchanged confirming the specificity of the SAM system (Figure 7C). To test reproducibility of this method, 759-sgRNA/SAM was transfected into an immortalized invasive trophoblast cell line, the HTR8/SVneo cells, which does not highly express C19MC and found that all 4 selected miRNAs were increased by 16- to 244-fold compared to GFP transfected control (Figure 7D). Furthermore, transfection of 759-sgRNA/SAM or 620-sgRNA/SAM in different human tumor cell line including MCF7 and PC3 cells successfully activated the transcription of C19MC (data not shown). These data demonstrate that the
CRISPR/SAM system can be used to selectively and consistently activate the entire C19MC cistron that spans over 100 kb.

**C19MC downregulates predicted mRNA targets**

To test whether C19MC miRNAs regulate genes involved in trophoblast differentiation, we performed RNAseq analysis of HEK293 cells transiently transfected for 72 hours with either 620-sgRNA/SAM or 759-sgRNA/SAM (Supplement Table SII). Cumulative distribution of differentially expressed mRNAs (FDR ≤ 0.20) with predicted targets of miR-512, miR-520, miR-1323 and miR-515 (TargetScan, release 7.1) showed a statistically significant (p ≤ 0.05) shift to the left, implying a dominant down-regulation of its target genes in line with activation of the C19MC cistron (Figure 8 A-D).

**C19MC target mRNAs are enriched in cell differentiation pathways**

We focused on the 369 transcripts downregulated (FDR ≤ 0.20, ≤ -1.5 -fold) in both with 759-sgRNA/SAM and 620-sgRNA/SAM transfected HEK cells. Of these 369 mRNA transcripts, 181 were predicted targets of C19MC (MirWalk 2.0^{288}) (Figure 9A). Hallmark gene set analysis revealed that these 181 genes are significantly enriched in the hedgehog signaling (HH), EMT, UV response, hypoxia and p53 pathways (FDR ≤ 3.32x10^{-5}) (Figure 9B). Surprisingly, RNAseq of HEK293 cells transiently transfected with 620-gRNA/SAM or 759-gRNA/SAM also revealed 288- or 11-fold increase respectively, (FDR ≤ 0.2) in the expression of FGF4, and a 12.9 -fold increase (FDR ≤ 0.2) in OCT4 in759-gRNA/SAM transfected cells (Supplementary Table SII). The expression of OCT4 and other pluripotency transcription factors SOX2 and NANOG
were assessed by qRT-PCR which showed that OCT4 expression was > 8 -fold increased \( (p < 0.05) \) in HEK293 cells transiently transfected with 759-gRNA/SAM but not in 620-gRNA/SAM (Figure 9C).

**C19MC inhibits the expression of EMT genes.**

To further investigate the effect of C19MC overexpression on the EMT pathway, we used the EMT RT² profiler PCR Array (Qiagen) to quantify changes in the expression of 84 EMT associated genes. The qRT-PCR array showed 2- to 4.75-fold decrease in the expression of CDH2, SERPINE1, TWIST1, SNAI2, CALD1, GSC, ITGAV, MMP3, TCF and WNT5a \( (p \leq 0.05) \) in HEK293 cells transfected with 620-sgRNA/SAM compared to control, confirming the hallmark gene set analysis (Figure 10). These data were further confirmed by qRT-PCR analysis that showed significant decrease in the expression of CDH2, SERPINE1, TWIST1 and SNAI2 in HEK293 cells transfected with 759-sgRNA/SAM or 620-sgRNA/SAM compared to control compared to control (Figure 11 A). To determine if the decrease in the expression of CDH2 was as a result of direct targeting by C19MC miRNAs, we constructed a luciferase reporter containing the 3’ UTR of the CDH2 gene. HEK293 cells transfected with 759-sgRNA/SAM and the CDH2 3’ UTR luciferase construct showed 35% decrease \( (p < 0.05) \) in luciferase activity (Figure 11B). Taken together, these results indicate that overexpression of C19MC induces the transcription of major pluripotent factors OCT4 and FGF4 and inhibits EMT by directly repressing the expression of genes that are crucial for EMT.
Figure 5. C19MC expression in the human placenta. (A-B) Representative images of *in situ* hybridization for scramble control or miR-517a/c (purple), nuclei counterstained with red and double-immunostaining for trophoblast marker cytokeratin (brown) and decidual marker vimentin (pink) of adjacent consecutively cut sections from first trimester human placenta sections (A) or term placenta (B). Scale bar represents 400μm. Original magnification 10x, Black inserts 20X, pink and purple inserts 40X.
Figure 6. Location of C19MC sgRNAs.
Location of the gRNA#759 (A) and gRNA#620 (B) relative to the upstream CpG island (Green rectangle) and the first miRNAs of the C19MC cluster as viewed on the UCSC Genome Browser screen.
Figure 7. Transcriptional activation of C19MC. (A-B) Venn diagram of upregulated miRNAs (A) and downregulated miRNAs (B) from sRNAseq data from 3 independent experiments in HEK293 cells transfected with either 759-sgRNA/SAM or 620-sgRNA/SAM for 72 hr. (C-D) qRT-PCR analysis of four representative miRNAs of the C19MC cistron or miR-21 normalized to U18, 72h after transfection of HEK293 cells with 759-sgRNA/SAM or 620-sgRNA/SAM (C) or 72h after transfection of HTR8/SVneo cells with 759-sgRNA/SAM (D). Graphs represent means ± SEM of at least 3 independent experiments containing 3 replicates each. *p < 0.05 vs. GFP transfected control cells by 2-way ANOVA with Dunnett’s post-hoc (C) or by Multiple T-tests with Holm-Sidak correction (D).
Figure 8. C19MC downregulates predicted mRNA targets

(A-D) Cumulative distribution of differentially expressed mRNAs targeted by miR-512, miR-520, miR-1323 and miR-515 in HEK293 cells transfected with 759-sgRNA/SAM compared to GFP transfected cells compared to GFP transfected cells.
Figure 9. C19MC targets mRNAs are enriched in cell differentiation pathways (A and B) Venn diagram (A) and Hallmark gene set analysis (B) of the 181 common downregulated mRNAs in HEK293 cells transfected with 759-sgRNA/SAM or 620-sgRNA/SAM compared to GFP control and predicted C19MC miRNAs target genes. (C) qRT-PCR analysis of the indicated genes normalized to GAPDH in HEK293 cells transfected with 759-sgRNA/SAM for 72 hours. Graph represents means ± SEM of at least 3 independent experiments containing 3 replicates each. * p < 0.05 vs. GFP transfected control cells (multiple t-tests with Holm-Sidak correction).
Figure 10. C19MC alters the EMT gene expression profile in HEK293 cells

Heatmap showing non-hierarchical clustering of EMT genes with dendograms indicating co-regulated genes in 620-sgRNA/SAM transfected cells and GFP control.
Figure 11. C19MC inhibits the expression of EMT genes

(A) qRT-PCR analysis of the indicated genes normalized to GAPDH in HEK293 cells transfected with 759-sgRNA/SAM or 620-sgRNA/SAM for 72 hr (B) Luciferase reporter assay of HEK293 cells transfected with 759-sgRNA/SAM and PGL2 basic plasmid containing CDH2 3’ UTR normalized to GFP control. Graphs represent means ± SEM of at least 3 independent experiments containing 3 replicates each. * p < 0.05 vs. GFP transfected control cells. (A: Two-way ANOVA with Dunnett’s post-hoc, B: multiple t-tests with Holm-Sidak correction)
**Hypoxia inhibits C19MC and increases the expression of EMT markers in iPSCs**

During the first trimester, oxygen tension plays a key role in regulating trophoblast cell proliferation and differentiation\(^\text{22, 289}\). To determine the effect of hypoxia on C19MC expression *in vitro* and avoid the spontaneous differentiation that occurs in primary human trophoblast cells\(^\text{290}\), we used iPSCs which share many stem-like characteristics with CTs and express similarly high levels of C19MC. sRNAseq analysis of iPSCs exposed to hypoxia (1% O\(_2\)) for 24 hours showed 1.9-fold decrease in the expression of C19MC (mir-498 cistron), while the hypoxia-inducible miR-210\(^\text{291}\) was 13.9-fold increased (FDR ≤ 0.20) compared to normoxia conditions (21% O\(_2\)) (Table 2). Specifically, 12 miRNAs of the C19MC were 2.1- to 292.6-fold decreased FDR ≤ 0.20 (Supplementary Table SIII), of which five miRNA were confirmed by qRT-PCR (Figure 12A).

Hallmark gene set analysis revealed that hypoxia induced the expression of genes involved in hypoxia response, EMT, and P53 pathways whereas E2F and G2M checkpoint pathways were decreased (FDR ≤ 0.2) (Supplementary Table SIV). RNAseq analysis showed 43.7- and 10-fold increase (FDR ≤ 0.20) in SERPINE1 and TWIST1, respectively in iPSC exposed to hypoxia (Supplementary Table SIV). The increase in the expression of the EMT genes, SERPINE1, TWIST1 and SNAI2 (18.6-, 1428.2-, 4.7-fold, respectively, p<0.05), was also confirmed by qRT-PCR (Figure 12B). Furthermore, qRT-PCR analysis of iPSCs exposed to hypoxia showed downregulation of the stem cell associated genes LIN28A, LIN28B, SOX2, NANOG and OCT4 (Figure 12C).
**BMP4 mediated differentiation of iPSCs to CTs induces EMT and inhibits C19MC**

Hypoxia is known to preferentially promote the initial differentiation of CTs into EVT. To determine the effect of hypoxia on C19MC and EMT genes during the CT to EVT differentiation process, we used a previously described method for iPSC differentiation. First, we differentiated iPSCs into CT (iPSC-CTs) using a low dose BMP4 treatment (Figure 13A). Following BMP4 treatment, iPSC-CTs showed a more flattened and elongated CT phenotype and expressed high levels of the CT associated genes P63 and CDX2 compared to undifferentiated iPSCs (Figure 13B and C). Moreover, iPSCs-derived CTs showed reduction in OCT4 expression compared to undifferentiated iPSC (Figure 13C). Next, we sought to determine the expression of C19MC and EMT genes during low-oxygen induced EVT differentiation. We re-plated iPSC-CTs and continued to differentiate them in FCM supplemented with BMP4 under normoxic or hypoxic conditions. In hypoxia, the cells expressed significantly reduced levels of PSG4 (an STB-associated transcript) and showed significant increase in the expression level of HLAG (an EVT marker) consistent with the observation that ST differentiation is inhibited by low oxygen conditions (Figure 13D). Interestingly, iPSC-CTs, and iPSC-CTs further cultured in normoxia or hypoxia showed an increase in the EMT genes CDH2 and SERPINE1 and loss of C19MC miRNAs miR-518c and miR-519d compared to undifferentiated iPSCs (Figure 13E and F). This is likely due to the use of BMP4, an EMT inducer, in the differentiation of iPSCs to CTs. These data show that EMT induced by hypoxia or BMP4 is associated with a significant decrease in the expression of C19MC.
Table 2. Hypoxic conditions decrease expression of the C19MC cistron in iPSCs. Data shows fold change of normalized frequency (>2-fold, FDR ≤ 0.2) C19MC.

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<th>Fold change</th>
<th>FDR</th>
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Figure 12. Hypoxia inhibits C19MC and increases the expression of EMT markers in iPSCs

(A-C) qRT-PCR analysis of the indicated miRNA normalized to U18 (A) or indicated genes normalized to GAPDH (B and C) of iPSCs cultured in hypoxia (1% O₂) or normoxia (21% O₂) for 24 hours. Graphs represent means ± SEM of at least 3 independent experiments containing 3 replicates each. * p < 0.05 vs. normoxia control cells. (A-C: Multiple t-tests with Holm-Sidak correction)
Figure 13. BMP4 mediated differentiation of iPSCs to CTs induces EMT and inhibits C19MC

(A and B) Schematic (A) and 20x phase images (B) showing two-step cytotrophoblast differentiation using low dose BMP4. iPSCs were cultured in EMIM + 10 ng/ul BMP4 for 4 days to form flattened CTB like cells. Cells were replated and further cultured in FCM + BMP4 for 4 days under normoxic and hypoxic conditions to induce STB and EVT differentiation. (C-F) qRT-PCR analysis of the indicated genes normalized to GAPDH (C-E) or the indicated miRNA normalized to U18 (F). Graphs represent means ± SEM of at least 3 independent experiments containing 3 replicates each. * p < 0.05 vs. iPSC (C: Multiple t-tests with Holm-Sidak correction, D-F: Two-way ANOVA with Dunnett’s post-hoc).
**C19MC increases efficiency of somatic cell reprogramming**

Since C19MC miRNAs are highly expressed in embryonic stem cells and increased OCT4 expression, we tested whether C19MC miRNAs induce cell reprogramming. Therefore, normal Human Dermal Fibroblasts cells (NHDFs) were transfected with 759-sgRNA/SAM alone, with three episomal plasmids that contains reprogramming factors OCT3/4, SOX2, KLF4, and LIN28 in combination with L-MYC and p53 shRNA as previously described (Y4) \(^{262}\), or with a combination of 759-sgRNA/SAM and Y4 together (759-sgRNA/SAM + Y4) (Figure 14A).

In NHDFs transfected with 759-sgRNA/SAM only, no colonies were observed (Figure 14B). Intriguingly, NHDF cells transfected with 759-sgRNA/SAM +Y4 developed a significantly higher number of colonies (an average of 17 colonies/200,000 cells) compared to cells transfected with Y4 alone which developed an average of 5 colonies/200,000 cells (p ≤ 0.05) at day 24 post transfection (Figure 14B). Moreover, the colonies were observed sooner in NHDFs cells transfected with 759-sgRNA/SAM +Y4 at 7 to 10 days post transfection compared to 14 to 20 days post transfection in cells transfected with Y4 alone (Figure 14C). The undifferentiated stage of the colonies was confirmed by alkaline-phosphatase staining at day 24 post transfection (Figure 21D). These data indicate that combination of 759-sgRNA/SAM +Y4 increases the speed and efficiency of cell reprogramming.
Figure 14. C19MC increases efficiency of somatic cell reprogramming

(A) Schematic of transfection protocol. 759, sgRNA-759/SAM; Y4, Yamanaka factors. (B) Colony counts from 759-, Y4- and 759+Y4-transfected NHDFs at day 24. (C) Colony morphology at 10 days post transfection. (D) AP staining of iPSC clones from Y4- or 759+Y4-transfected NHDFs at day 24. Graph represents colony counts (B: ANOVA with Dunnett’s post-hoc)
**C19MC iPSCs form embryoid bodies in vitro and teratomas in vivo**

To test the differentiation potential of iPSCs generated using 759-sgRNA/SAM +Y4 *in vitro*, we used a floating cultivation method for embryoid body formation. iPSCs generated using 759-sgRNA/SAM +Y4 formed spheroid shaped structures after 8 days in suspension culture (**Figure 15A**). Embryoid bodies were transferred to gelatin-coated plates and cultured for an additional 7 days. Attached cells showed various cell morphologies including beating cardiac like cells (**Supplemental video 1**). We analyzed the embryoid bodies for the expression of the germ layer markers ACTA2 (mesoderm), TUBB3 (ectoderm) and GATA4 (endoderm) by qRT-PCR and found significantly higher levels of ACTA2 and GATA4 (84- and 37- fold respectively, p≤ 0.05) compared to undifferentiated control iPSCs (**Figure 15B**).

In order to test the pluripotency of iPSCs generated using 759-sgRNA/SAM +Y4, cells were transplanted subcutaneously into dorsal flanks of SCID immunodeficient mice. Tumors were harvested four weeks after injection for histological analysis. Tumor sections were immunostained with a human mitochondria specific antibody to differentiate between implanted human cells and mouse cells and nuclei were counterstained with hematoxylin. Histological examination showed that the tumor contained various tissue structures derived from the three germ layers, endoderm, mesoderm and ectoderm (**Figure 15C-E**).
Figure 15. C19MC iPSCs form embryoid bodies in vitro and teratomas in vivo

(A) Image of embryoid bodies at 7 days in suspension cell culture. (B) qRT-PCR analysis of 3 germ layer markers in embryoid bodies compared to non-differentiated human iPS cells (clone 3-CF-6 P5). (C-E) Images of hematoxylin stained teratoma sections showing ectoderm, mesoderm and endoderm like cellular structures. Graph represents means ± SEM. * p < 0.05 (Multiple t-tests with Holm-Sidak correction)
Discussion

Trophoblast differentiation and subsequent invasion into the decidua is critical for implantation and placentation during early pregnancy. Villous trophoblasts (VTs) possess epithelial characteristics with apicobasal polarity, lateral cell junctions and basal contact with basement membrane. Upon differentiation to EVTs, they undergo EMT and lose their cell junctions and apical basal polarity and acquire migratory and invasive capacity. This study demonstrates that C19MC is primarily expressed in the non-invasive epithelial VTs and is lost as trophoblast differentiate into EVTs. Utilizing the CRISPR SAM system we were able to transcriptionally activate the entire endogenous C19MC and demonstrate its role in enhancing the epithelial stem cell state. Co-expression of C19MC miRNAs with Yamanaka factors (C19MC+Y4) in NHDFs significantly increases the efficiency of reprogramming to iPSCs. While under hypoxic condition, the expression of C19MC is reduced, which in turn induces EMT and cell differentiation.

To our knowledge, this is the first report that shows the in-situ expression of one miRNA of the C19MC cistron in first trimester human placental tissues. These images reveal that C19MC is highly expressed in the VTs comprising of CTs, STs and in the proliferative (proximal) trophoblastic cell columns in the anchoring villi. C19MC expression is gradually lost as trophoblast differentiate into EVT and is not expressed in the decidual layer of the placenta. These data are in agreement with previous report that utilized laser-capture microdissection of paraffin-embedded placental specimens followed by RT-qPCR.
To investigate the role of C19MC in trophoblast differentiation, we employed a new strategy to transcriptionally activate the whole C19MC cluster that span over 100 kb. Until recently, studying the functions of C19MC miRNAs has been challenging because of its size. Several groups have used the Bacterial Artificial Chromosome (BAC) technology, which carries the entire C19MC locus within its chromatin and all the regulatory elements (CpG islands, promoters, Alu repeats, enhancers, silencers, insulators etc.) which resulted in modest upregulation of some miRNAs of the C19MC. Furthermore, the construction, screening, transfection and selection for stable integration of BAC plasmids are time and labor intensive. Other groups have used miRNA mimics to over express some C19MC miRNAs but using this method to upregulate all 56 mature miRNAs is not feasible. In this study we used CRISPR SAM technology to transcriptionally activate the entire C19MC cistron. To avoid the imprinted upstream CpG island we designed guide RNAs to bind only a few hundred base pairs upstream of the first miRNA genes. Comparing sRNAseq analysis of three independent experiments using either 759-sgRNA/SAM or 620-sgRNA/SAM in HEK293 cells revealed that a single guide RNA is sufficient to upregulate the expression of the entire 100 kb C19MC locus. This transcriptional activation is specific to C19MC as the expression of the neighboring miR-371-373 cluster, which is located 20 kb downstream of the C19MC, was not increased, supporting a previous report that C19MC miRNAs are a product of a single transcript that gets rapidly spliced and then processed by Drosha-DGCR8 complex. Only two upregulated miRNAs that are not member of the C19MC overlapped in the 3 independent experiments, miR-139 and miR-449. Interestingly, these two miRNAs are known to be inhibitors of EMT and may be
regulated by the C19MC\textsuperscript{297,298}. Some other miRNAs that are known to stimulate cell reprogramming and do not belong to the C19MC were also upregulated in HEK293 cells transfected with either 759-sgRNA/SAM or 620-sgRNA/SAM. On the other hand, some miRNA that are known to promote cell differentiation including let-7a, miR-34a, miR-181b, miR-15a, miR-15b, miR-133a, miR-29b and miR-205, were downregulated. However, no overlap in the downregulated miRNAs was observed in the three different experiments. These non-C19MC miRNAs may be directly or indirectly regulated by the C19MC. Further studies are needed to investigate the potential crosstalk between C19MC and the other differentially expressed miRNAs. Therefore, in the present study we were able to activate the entire C19MC locus in different human cell lines in a highly efficient, specific, and reproducible manner. This new approach provides a powerful tool for studying the contribution of C19MC miRNAs during pregnancy.

In agreement with the physiological expression of C19MC miRNAs in VTs and its gradual loss in EVTs, we found that transcriptional activation of the C19MC led to significant decrease in the expression of numerous genes involved in HH signaling and EMT, many of which are C19MC predicted targets. This data is in agreements with previous study which showed that overexpression of C19MC in an EVT cell line, HTR8-SVneo, decreases migration\textsuperscript{105}. Moreover, numerus studies have shown a link between HH signaling and EMT \textsuperscript{299}. In human trophoblast, activation of HH signaling induced key EMT regulators including SNAI1 and TWIST1 \textsuperscript{300}. Therefore, the data presented here place C19MC upstream of HH and EMT signaling, suggesting that the high levels of C19MC in the villous trophoblasts maintain their stem-like epithelial phenotype and suppress HH and EMT. Whereas, loss of C19MC release the inhibition on the HH and
EMT genes which leads to acquisition of the migratory and invasive characteristics of EVT. Several studies have shown increased expression of miRNAs of the C19MC in placenta and maternal serum of PE affected pregnancies\textsuperscript{116, 301}. Other studies showed down-regulation of EMT genes correlates with preeclampsia\textsuperscript{302-305}. Therefore, maintaining optimal expression levels of C19MC may be critical for EVT differentiation and invasion and dysregulation in C19MC may result in preeclampsia or placenta accreta.

This study reveals that transient activation of C19MC increased the expression of FGF4, which is essential for mammalian embryogenesis. Until now, FGF4 was known to be secreted from the inner cell mass in a paracrine fashion and stimulate the growth and proliferation of trophectoderm during early blastocyst development\textsuperscript{4}. The increase in FGF4 by C19MC in HEK293 cells described here is very significant because it suggests that VTs that express high levels of C19MC, may also express FGF4 and provide optimal signaling during embryonic development. We also show that activation of C19MC increased OCT4 expression, in agreement with previous study that used a lentiviral vector encoding mir-524 precursor, a member of the C19MC\textsuperscript{287}. The mechanisms by which C19MC induce the expression of OCT4 and FGF4 is not yet clear. It might be due to inhibition of transcriptional suppressors or by binding to the promoter regions and inducing gene expression\textsuperscript{306, 307}. Further studies are needed to determine the mechanism by which C19MC increase the expression of OCT4 and FGF4.

Transient expression of C19MC and Y4 resulted in a significant increase in the number of iPSC colonies that formed embryoid bodies and teratomas, whereas
activation of C19MC alone was unable to do so. Activation of C19MC using the CRISPR/SAM approach exerted a profound inhibition of genes involved in the p53 pathway in addition to the HH signaling and EMT. These data are in agreement with previous studies showed that suppression of the p53 pathway increased the efficiency of human iPSC generation\textsuperscript{308}. Moreover, some miRNAs of the C19MC share the same seed sequences with miRNAs of the miR-302/-372 families which have been shown to enhance reprogramming by promoting MET\textsuperscript{286}. Lentiviral vector encoding mir-524 precursor was also unable to reprogram the normal fibroblast cells, however, when co-infected with OSKM factors-encoding lentiviruses, it enhanced the reprogramming process\textsuperscript{287}. The findings of the present study suggest that expression of high levels of C19MC in VT may play an important role in sustaining a stem cell environment to support the placentation and thereby the developing fetus.

The early placental environment is very hypoxic as intervillous circulation is not established until after the first trimester\textsuperscript{22, 289}. EVTs migration, invasion into the maternal decidua and remodeling of the maternal spiral arteries are important steps for proper perfusion of the placenta, which is necessary for a successful pregnancy\textsuperscript{27}. Changes in oxygen tension within the inter-villous space are stringently controlled and play a role in trophoblast differentiation, migration and invasion that are necessary for vascular remodeling during pregnancy\textsuperscript{309}. The present study shows that at the top five gene set analysis targeted by C19MC cistron are genes that are normally up-regulated in response to hypoxia. These data suggest that by expressing high levels of C19MC, VTs become more tolerant to hypoxia. Previous work has used primary human trophoblast (PHT) cells to test the effect of hypoxia (<1% oxygen) on C19MC expression. However,
PHT cells underwent spontaneous differentiation when cultured in vitro over 72 hours and the expression levels of C19MC miRNA were not reliably detectable\textsuperscript{284}. Therefore, it is not possible to determine the effects of hypoxia on C19MC using PHT cells. Since CTs share many stem cell-like characteristics and to avoid the spontaneous differentiation of PHTs, in this study we used undifferentiated iPSCs and found that 24hr exposure to hypoxia (1% oxygen) significantly decrease in the expression of different miRNAs of the C19MC and several reprogramming factors, while the expression of EMT related genes were increased. These results are in line with previous studies that reported that hypoxia is a major inducer of EMT\textsuperscript{310-312}. Hypoxic conditions also decreased the expression of OCT4 in agreement with studies that show that repression of OCT4 induces loss of pluripotency and dedifferentiation of ESC cells derived from the inner cell mass toward the trophectoderm\textsuperscript{313}.

The data presented here suggest that hypoxic conditions during early placentation reduce C19MC expression and induce EMT and differentiation of CTs into EVT. The mechanism by which hypoxia reduces the expression of C19MC miRNA are not yet known. De novo methylation may be one potential mechanism. In fact, previous study has shown that tumor hypoxia reduced the activity of oxygen-dependent ten-eleven translocation (TET) enzymes that catalyze DNA demethylation of tumor suppressor genes\textsuperscript{314}. Hypoxia may also regulate C19MC miRNA biogenesis and activity\textsuperscript{315}. Further studies are needed to elucidate the mechanisms by which hypoxia reduce C19MC expression.

We also show that the expression levels of miRNA of the C19MC cistron were significantly reduced when iPSCs were differentiated into bipotential stem-like CTs and
when these cells were subsequently terminally differentiated into STs and EVTs. Furthermore, EMT genes SERPINE1 and CDH2 were increased at both stages of differentiation. Although the decrease in C19MC miRNAs and increase in EMT genes in iPSC-CTs was unexpected, it is consistent with the induction of EMT by BMP4 used in the differentiation protocol. BMP4 has been shown to promote EMT and mesodermal commitment in stem cells, induce EMT in ovarian cancer cells, and induce an EMT-like response in C3H10T1/2 pluripotent stem cells\textsuperscript{293-295, 313}. Since C19MC is highly expressed in normal human trophoblasts, it is notable that the iPSC-CTs we generated showed reduced expression of C19MC\textsuperscript{105}. This data indicates that trophoblasts generated using this protocol are atypical as they do not fully recapitulate the gene expression profile of normal trophoblasts. This limitation can affect the utility of these cells for downstream studies. These results therefore suggest that downregulation of C19MC is strongly associated with the induction of EMT by hypoxia or other means such as low dose BMP4 treatment. Although our data shows a strong association between C19MC and EMT, C19MC knockdown experiments in epithelial cells are required to prove that C19MC regulates EMT. However, due to the size of the cluster, we have not been able to successfully knock down its expression in vitro.

Taken together, the data presented in this study establish the utility of CRISPR/SAM technique as a powerful tool to investigate the role of C19MC in physiology of human placental. Employing this robust technique enabled us to uncover the crucial role of C19MC in maintaining CTs multipotency and shows that loss of C19MC is required for EMT transition of trophoblast to gain invasive phenotype.
Chapter 7

Modulation of LIN28B/Let-7 Signaling by Propranolol Contributes to Infantile Hemangioma Involution

Introduction

Infantile hemangiomas (IH) are highly vascularized benign tumors diagnosed in 3-10% of children before they are one year old\textsuperscript{316}. IH lesions have a unique pattern of growth in which the initial phase of rapid proliferation is followed by slow spontaneous involution that leaves behind a fibro-fatty residuum\textsuperscript{221}. During the proliferative phase, immature endothelial cells, positive for glucose transporter-1 (GLUT1), form aberrant blood vessels, rich in α-smooth muscle actin-positive pericytes and mast cells\textsuperscript{194}. This endothelial GLUT1 expression distinguishes IH from other types of vascular tumors and vascular malformations\textsuperscript{198, 317}. Stem cell reprogramming factors OCT4, SOX2, NANOG and MYC are also highly expressed in IH\textsuperscript{318}.

The chromosome 19 microRNA (miRNA) cluster (C19MC), referred to as miRNA cistron mir-498(46) or mir-498(46), was recently reported to be highly expressed in IH\textsuperscript{119}. Mir-498(46) is unique to primates and is the largest known human miRNA gene cluster that spans over 100 kb and contains 46 miRNA genes flanked by Alu elements\textsuperscript{283}. Expression of miRNA genes in this cluster is restricted to the placenta, embryonic stem cell (ESC) and certain tumors\textsuperscript{121, 122, 283, 319}.
While some studies have shown considerable similarity between IH and placental transcriptomes\textsuperscript{200}, which suggests a placental origin for proliferating cells in IH\textsuperscript{199}, other studies propose that IH originate from hemangioma stem cells\textsuperscript{204}. In support of the latter notion, human CD133\textsuperscript{+} hemangioma stem cells (HemSCs) implanted subcutaneously into immunodeficient mice induce GLUT1-positive microvessels, which are gradually replaced by adipocytes, thereby reproducing the IH involution phase. Importantly, when grown in culture, GLUT1-positive/mir-498(46)-expressing endothelial cells, isolated from proliferative IH, lose the expression of mir-498(46) and GLUT1 and undergo an endothelial-mesenchymal transition (EndMT), a process similar to epithelial-mesenchymal transition (EMT)\textsuperscript{119,320}.

Serendipitously, it was discovered by Léauté-Labrèze that the non-selective beta-adrenergic receptor blocker, propranolol, triggers early involution of IH\textsuperscript{236}. Consequently, propranolol has become the first line therapy for IH\textsuperscript{238}. Previous work has shown that propranolol-treated IH tumors contain significantly lower levels of mir-498(46) than proliferative IH\textsuperscript{119}. Moreover, levels of circulating mir-498(46) fall during the involution phase and correlate with the clinical response to propranolol treatment\textsuperscript{119}, suggesting the potential involvement of mir-498(46) in the pathogenesis of IH.

The self-renewal capacity of ESCs is regulated in part by a set of interactive gene products, LIN28 and the let-7 family of miRNAs. Two human LIN28 paralogs, LIN28A and LIN28B, are highly expressed in ESCs. They inhibit the post-transcriptional maturation of let-7 miRNAs and influence mRNA translation\textsuperscript{140,142}. Conversely, let-7 negatively regulates the expression of LIN28 by interacting with the 3' untranslated regions of both LIN28A and LIN28B mRNAs\textsuperscript{136}. This interaction creates a double
negative feedback loop, which is highly conserved. Ectopic expression of LIN28A or LIN28B alongside the reprogramming factors Oct4, Sox2, and Nanog\textsuperscript{157, 165, 166} has been successfully used to generate induced pluripotent stem cells (iPSCs) from fibroblasts. In addition, LIN28B overexpression has been successfully used to generate iPSCs from fibroblasts\textsuperscript{24}.

EMT is the biological process through which normally polarized epithelial cells lose their apical-basal polarity and acquire a mesenchymal cell phenotype characterized by enhanced migration and invasiveness, resistance to apoptosis and increased deposition of extracellular matrix components\textsuperscript{24}. This trans-differentiation is mediated by key transcription factors such as SNAIL, SLUG, TWIST1 and ZEB that regulate downstream epithelial markers, which among other effects, leads to decreased E-cadherin levels and increased expression of mesenchymal cell markers, such as N-cadherin and vimentin\textsuperscript{279}. iPSC generation from fibroblasts requires suppression of EMT and activation of mesenchymal-epithelial transition (MET) signals\textsuperscript{37}.

IH represents a unique model to study postnatal vasculogenesis and vessel regression. Despite the prevalence of these tumors, the complex pathogenesis of IH is poorly understood. Given the role of LIN28/let-7 axis in governing stem cell self-renewal and cell differentiation, we hypothesized that LIN28/let-7 signaling is dysregulated in IH. In this study, we demonstrate that LIN28B is highly expressed in proliferative IH samples and is downregulated both in involuted and propranolol-treated IH samples. Consistent with those observations, in vitro treatment of iPSCs with propranolol caused a decrease in LIN28B, mir-498(46) expression, decreased cell proliferation and increased the expression of let-7 family of miRNAs and EMT genes. Our results
highlight the role of LIN28 and let-7 in the pathogenesis of IH and offers a new mechanism by which propranolol induces involution of IH.

**Results**

**LIN28B is highly expressed in proliferative IH**

IH are derived from dysregulated stem cells and represent a unique tumor model characterized by a proliferative phase followed by spontaneous involution, regulated by yet unknown mechanisms. Given the key role of the LIN28/let-7 axis in governing stem cell self-renewal and cell differentiation, the expression levels of LIN28A and LIN28B were first assessed in IH samples by qRT-PCR and compared to normal infant skin (NS) and samples of normal human placentas at term. IH samples exhibited a 7- and 7500-fold increase ($p < 0.05$) in LIN28A and LIN28B, respectively, compared to NS (Figure 16A). The expression levels of LIN28A and LIN28B in IH were comparable to their expression levels in the placenta (Figure 16A).

Given the dramatic increase of LIN28B expression in IH, all subsequent experiments were focused on LIN28B. To determine if LIN28B is differentially expressed in proliferative IH compared to involuting/involuted IH, we assessed LIN28B expression by qRT-PCR and found that it was 2.7-fold higher ($p < 0.33$) in proliferative compared to involuting/involuted IH samples (Figure 16B).
Figure 16. LIN28B is highly expressed in proliferative IH.
A, B, Quantitative RT (qRT) PCR analysis of LIN28A and LIN28B (A) or LIN28B (B) expression normalized to GAPDH. IH (n=4), normal skin (NS; n=4), normal term placenta (PL, n=4), proliferative IH (n=2), and involuting/involuted IH (n=2). Graphs represent means ±SEM. *P<0.05 vs NS (A), vs involuting/involuted IH (B) by one-way ANOVA with Dunnett post hoc test (A) or 2-tailed Student t test (B).
**LIN28B is highly expressed in proliferative IH sections and is reduced upon involution**

To determine LIN28B cellular localization, dual immunofluorescence staining for LIN28B and the IH marker, GLUT1, was performed in paraffin embedded IH sections obtained from proliferative and involuted IH specimens. Term placenta samples were used as positive controls. In proliferative IH samples, LIN28B was highly expressed and co-localized with the GLUT1 positive endothelial cells as well as perivascular non-endothelial cells, whereas involuted IH samples showed weak staining for both LIN28B and GLUT1 (Figure 17 A). As expected, in term placentas LIN28B and GLUT1 were co-localized in trophoblast and endothelium of chorionic villi (Figure 17A). Quantifications of LIN28B to GLUT1 immunofluorescence signal intensities showed ~2-fold increase in proliferative IH compared to involuted IH samples (Figure 17B). These data confirm the increased expression of LIN28B in proliferative IH and its localization in GLUT1-positive endothelial cells and perivascular non-endothelial cells that may display properties of facultative stem cells320.
Figure 17. LIN28B is highly expressed in proliferative IH sections and is reduced upon involution.

A, Representative immunostaining for LIN28B and GLUT1 (glucose transporter-1) of proliferative IH (n=4), involuting/involuted IH (n=5) and term placenta (n=3) samples. Nuclei were counterstained with DAPI. No positive immunostaining was observed in the negative control sections (Alexa Fluor 488 or Cy3). Scale bars: 50 μm; original magnification, ×60 and ×120 (insets).

B, Quantification of LIN28B/GLUT1 fluorescence signal ratio in proliferative and involuted IH samples. Graph represents means±SEM. *P<0.05 vs involuted IH (D) tailed Student t test.
Let 7 miRNAs are downregulated in IH

The let-7 miRNA family and numerous other miRNAs negatively regulate LIN28B by binding to its 3'UTR (Figure 18A). To determine whether the increase in LIN28B in IH samples is associated with decreased expression of these miRNAs, small RNA sequencing (sRNAseq) analysis was performed on eight IH and five NS samples. Looking at the most abundant, or highly expressed, miRNAs (i.e. consuming the top 90% of sequencing reads) in IH compared to NS samples showed 32 significantly changed miRNA cistrons (FDR 0.25), of which 21 were upregulated in IH and 11 downregulated in IH compared to NS (Table 3). Of the 21 upregulated miRNA cistrons, mir-498(46) was most extensively upregulated with a 492-fold increase (Table 3). The downregulated cistrons include miRNAs that are predicted to bind to LIN28B 3'UTR, namely let-7a, let-7c, let-7f, let-7g, let-7i, miR-19b(2), miR-506(1), miR-196a/b, miR-455-5p(1), miR-9(3), miR-124(3), miR-27a(1), miR-27b(1), miR-23b(1), miR-125b(2), miR-199b-5p(1), miR-19a(1) and miR-148a(1), which were 2- to 45.6-fold downregulated (Supplementary Table SI). Importantly, let-7, miR-196 and miR-9 bind to multiple target sites on LIN28B 3' UTR (Figure 18A). These data indicate that the upregulation of LIN28B in IH may be due, at least in part, to its decreased post-transcriptional regulation by miRNAs. Additionally, LIN28B is an established repressor of miRNA biogenesis\textsuperscript{147}, among which let-7 is the most extensively studied\textsuperscript{141}. Consequently, sRNAseq analysis of IH and NS revealed that let-7g(1), let-7c(1), and miR-200c(1) were 2.9-, 3.6- and 135-fold downregulated, respectively, in IH compared to NS (Supplementary Table SI). To determine whether additional members of the let-7 family were affected, yet did not pass the sRNAseq analysis filters, qRT-PCR analysis
was performed for randomly selected members let-7a and let-7c. As expected, both were downregulated ($p < 0.05$) in IH compared to NS (Figure 18B).

**LIN28B activates miR-498(46) miRNAs independently of the upstream CpG-island**

The expression of mir-498(46) is controlled by methylation of an upstream CpG-rich promoter region that includes a transcription start site located ~17 kb upstream of the first miRNA gene. To test whether the increase in mir-498(46) expression found in IH samples is due to transcriptional activation of the upstream CpG-rich island, we performed qRT-PCR using primer sets designed to amplify regions downstream of the CpG-island, as previously described. The trophoblast-derived choriocarcinoma BeWo cell line, which endogenously expresses mir-498(46), was used as a positive control. IH samples and BeWo cells displayed active transcription starting at the ~17kb upstream mir-498(46) CpG-related promoter region, whereas no transcripts were found in NS samples (Figure 19A). This indicates that the promoter region upstream of mir-498(46) is transcriptionally active in IH.

Given that LIN28B expression is 7000-fold higher in IH than NS, we tested for a possible positive regulation of mir-498(46) miRNAs by LIN28B. To that end, HEK293 cells were transiently transfected with a LIN28B-coding plasmid, and the expression levels of LIN28B and of six randomly selected miRNAs of mir-498(46) miRNAs were measured after 72 h by immunoblotting and qRT-PCR, respectively. Interestingly, overexpression of LIN28B enhanced the expression of miR-516b by more than 2.6-fold ($p < 0.05$) but did not significantly alter the expression of miR-515-5p, miR-516a-5p, miR-517a, miR-518c and miR-519d (Figure 19B).
To test whether LIN28B affects the methylation of the ~17kb CpG- island upstream of mir-498(46) which activates its transcription, we performed an HpaII-sensitivity assay followed by qPCR. Overexpression of LIN28B did not induce a significant increase in HpaII sensitivity of the CpG island (Figure 19C) or induce transcriptional activation downstream of the CpG island (Figure 19D). These data indicate that LIN28B induces the expression of mir-498(46) independently of the ~17kb upstream promoter region.

Propranolol inhibits LIN28B in IH

The standard of care propranolol treatment of patients with IH induces rapid IH involution. To determine whether propranolol affects the expression of LIN28B in IH, we performed dual immunofluorescence staining of LIN28B and the IH marker, GLUT1, in paraffin embedded IH sections obtained from propranolol-treated proliferative IH and compared them to untreated proliferative IH samples. Both LIN28B and GLUT1 were markedly decreased in propranolol-treated proliferative IH compared to untreated proliferative IH (Figure 20A). In addition, quantification of LIN28B to GLUT1 immunofluorescence signal intensities showed ~2-fold increase in proliferative IH compared to propranolol treated IH samples (Figure 20B).
Table 3. Expression differences of highly expressed miRNA cistrons in IH vs. NS by sRNAseq. Highly expressed cistrons were defined as being within the top 90% RNAseq reads in any of the 8 IH and 5 NS samples. Shown are cistrons with a false discovery rate of less than 25% in the differential analysis.

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<th>Normal skin</th>
<th>Fold change</th>
<th>P Value</th>
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Figure 18. Let-7 miRNAs are downregulated in IH.
A, Predicted miRNAs target sites in LIN28B 3’ untranslated region (UTR; TargetScan Human Release 7.1). In red, downregulated miRNA in sRNAseq analysis of IH vs NS. B, qRT-PCR analysis of let-7a and let-7c expression in IH (n=4) and NS (n=4). Graph represent means ± SEM. *P<0.05 vs NS (B), by 2-tailed Student t test.
Figure 19. LIN28B activates mir-498(46) miRNAs independently of the upstream CpG-island

A, RT-PCR analysis using P0, P1, P5, P8, and P9 primer sets reveals the presence of transcripts generated upstream of mir-498(46) in infantile hemangioma (IH; n=4) but not in normal skin (NS). BeWo cells were used as positive control and H2B as reference gene. B–D, HEK293 cells were transfected with FLAG-LIN28B or GFP control vector for 72 hours followed by (B) qRT-PCR analysis of six randomly selected miRNAs of the mir-498(46) cistron normalized to U18. Inset, representative immunoblot of LIN28B and GAPDH; (C) HPAII sensitivity assay of the mir-498(46) cistron CpG island promoter region and (D) RT-PCR analysis as described in A. Data represents means±SEM of 3 independent experiments. *P<0.05 vs GFP transfected cells by Mann–Whitney U test. M indicates molecular marker.
**Figure 20. Propranolol inhibits LIN28B in IH.**

**A,** Representative immunostaining for LIN28B and GLUT1 (glucose transporter-1) of proliferative IH (n=4) and propranolol-treated proliferating IH (n=4) samples. Nuclei were counterstained with DAPI. No positive staining was observed in the negative control sections (Alexa Fluor 488 or Cy3). Scale bars: 50 μm; original magnification, ×60 and ×120 (insets). **B,** Quantification of LIN28B/GLUT1 fluorescence signal ratio in proliferative and propranolol-treated samples. Data represent the mean ± SEM of at least 3 independent experiments. *P<0.05 vs proliferative IH (B) by Student t test.
Propranolol inhibits LIN28B in iPSCs

To further investigate the effects of propranolol on the expression of LIN28B and let-7 in IH, we used the CD133+ HemSCs, which form hemangioma-like tumors when injected subcutaneously in immunodeficient mice\(^{204}\). First, we tested whether HemSCs express LIN28B and the miR-498(46) cluster in NS and IH. We also tested their expression in BeWo cells and iPSCs, which are known to express high levels of both. As negative controls, we used HUVECs that express neither LIN28B nor miR-498(46). Surprisingly, qRT-PCR revealed that compared to NS, LIN28B showed only a 2-fold increase in HemSCs, whereas in IH, iPSCs and BeWo cells LIN28B was >1000-fold \((p<0.05)\) higher (Figure 21A). As expected, negligible LIN28B expression was found in HUVECs (Figure 21A). Moreover, sRNAseq analysis revealed that miR-498(46) expression levels were 3.1-, 4.1-, 28- and 492.7- fold higher in HemSCs, HUVECs, iPSCs and IH, respectively, compared to NS (Supplementary Table SI). Lastly, the expression of LIN28B protein in HemSC and iPSCs was also assessed by Western blot analysis, which showed negligible levels of LIN28B in HemSCs (Figure 21B). Based on these findings, we proceeded to test the effect of propranolol in iPSCs, that express LIN28B, miR498(46) and beta-adrenergic receptors. After propranolol treatment for 72 h, iPSCs showed a 2.3-fold decrease \((p<0.05)\) in LIN28B protein levels (Figure 21C), but no significant differences were found at the mRNA levels (data not shown).

Propranolol induces miR-98(13) and suppresses miR-498(46) in iPSCs

To further investigate the effects of propranolol on the expression of mir-498(46) and let-7, iPSCs were treated with propranolol for 72 h and sRNAseq analysis was
performed. Compared to vehicle control, propranolol treated-iPSCs showed a 3.2-fold increase in the expression of cistron mir-98(13), which encodes 9 of 12 let-7 family miRNA genes, and a 1.6-fold downregulation of mir-498(46) (Table 4). The results were confirmed by qRT-PCR, which revealed significant reduction of mir-498(46) members miR-515-5p, miR-517a, miR-518C and miR-519d and >1. 65-fold increase in let-7a expression in iPSCs treated with propranolol for 72 hr (Figure 22A).

**Propranolol induces EMT markers in iPSCs**

Propranolol has been shown to induce early involution of IH, during which immature vascular endothelial cells undergo EndMT, transitioning to a mesenchymal phenotype with subsequent differentiation into adipocytes. To test whether propranolol induces the expression of mesenchymal markers, we performed mRNA sequencing (RNAseq) on the same propranolol and vehicle treated-iPSC samples that were used for sRNAseq. Gene set enrichment analysis performed on RNAseq data showed an enrichment of genes defining EMT (FDR 0.00046) in propranolol treated iPS cells compared to control (Supplementary Table S II). Gene set analysis also showed upregulation of the pathways for adipogenesis and TGF-β signaling (FDR 0.09 and 0.06 respectively, Supplementary Table SII). Among the most highly upregulated genes, two mesenchymal cell markers, SERPINE1 and TWIST1, showed a 12- and 2.9-fold increase (FDR 1.06 x 10^{-6} and 0.004), respectively (Supplementary Table SII). Upregulation of SERPINE1 and TWIST1 was further confirmed by qRT-PCR, which showed 8.4- and 5-fold increased expression (p<0.05), respectively (Figure 22B). Furthermore, gene set analysis of the RNAseq data also showed downregulation of E2F
targets, G2M checkpoint and mitotic spindle pathways (FDR<0.25, Supplementary Table SIII). We also examined propranolol-treated iPSCs for EMT associated morphological changes and observed that propranolol treated iPSCs exhibited a more elongated cellular shape compared to control (Figure 22C and D). Next, we tested the effect of propranolol on iPSC proliferation using the WST1 proliferation assay. Propranolol treatment induced a 33% decrease in cell proliferation without affecting cell viability (Figure 22E and F). Furthermore, gene set enrichment analysis also showed that E2F target genes and genes involved in G2/M cell cycle progression are downregulated by propranolol treatment in iPSCs (FDR 0.006 and 0.017 respectively, Supplementary Table SII) indicating that propranolol strongly inhibits proliferation.
Figure 21. Propranolol inhibits LIN28B in iPSC.
A, Quantitative RT (qRT) PCR analysis of LIN28B expression normalized to GAPDH in IH, iPSC, HemSC (line H42, from 3 independent passages), HUVEC, and BeWo cells compared with normal skin (NS). B, C, Representative immunoblot for LIN28B and GAPDH expression in (D) iPSC and HemSC line H42, from 3 independent passages and (E) iPSCs treated with 50 μmol/L propranolol or vehicle control for 72 hours accompanied by densitometric quantification of LIN28B normalized to GAPDH. Data represent the mean±SEM of at least 3 independent experiments. *P<0.05 vs NS (A), vs iPSCs (B), or vs vehicle control (C) by Student t test (B and C) or by one-way ANOVA with Dunnett post hoc test (A).
Table 4. Propranolol treatment decreases miR-498(46) expression and increases let-7 miRNA expression in iPSCs. Shown are all cistrons with and FDR < 25%. In bold mir-498(46) and mir-98(13).

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<th>Cistron (mir-)</th>
<th>Normalized frequency (%)</th>
<th>Fold change</th>
<th>P Value</th>
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Figure 22. Propranolol decreases the expression of mir-498(46) cistron and induces let-7 and EMT genes in iPSCs. iPSCs were treated with 50 μmol/L propranolol or vehicle control for 72 hours. A, B, Quantitative RT (qRT) PCR analysis of indicated miRNAs or genes normalized to U18 or GAPDH, respectively. C, D, Representative ×40 bright-field images of control and propranolol-treated iPSCs. E, F, Proliferation assay and representative fluorescence microscopy images of Calcein and propidium iodide (PI) staining for live and dead cells, respectively (Scale bars: 200 μm; ×20 magnification). Data represent the mean ± SEM of at least 3 independent experiments. *P<0.05 vs vehicle control by Student t test (A, B, and E).
Discussion

IH is a unique tumor model, characterized by a rapid proliferative phase followed by a spontaneous or propranolol-induced involution phase. The mechanisms that trigger spontaneous involution and the action of propranolol are still under investigation. The present study shows that the reprogramming factor LIN28B is highly expressed in the proliferative IH phase but is significantly decreased in involuted IH and in IH tissues from propranolol-treated patients. The high LIN28B expression in proliferative IH correlates with the expression of the ESC-enriched mir-498(46) and is inversely correlated with the expression of let-7 miRNAs. Treatment of iPSCs with propranolol reduced the expression of LIN28B and mir-498(46) and induced the expression of let-7 family of miRNAs and EMT genes. Moreover, propranolol treatment reduced iPSC cell proliferation.

The increase in LIN28B and reduction in let-7 reported in this study highlight the role of stem cells in the pathology of IH. Although HemSCs, which are 0.2% of the proliferative hemangiomma cell population, are believed to be the cellular origin of IH, our data show that these cells do not express LIN28B and miR-498(46) in tissue culture. However, our in vivo data show that LIN28B is localized in GLUT1 positive endothelial cells that were previously characterized as stem cell-like. These cells formed colonies that could be induced to re-differentiate into endothelial cells, pericytes/smooth muscle cells or adipocytes. Interestingly, when these GLUT1 positive endothelial cells were cultured for 3 weeks they converted to a mesenchymal phenotype and lost GLUT1 and mir-498(46) expression. It would be of interest to test whether freshly isolated HemSCs express LIN28B and mir-498(46) or implantation of
HemSC into immunodeficient mice restores LIN28B and mir-498(46) expression. Nevertheless, increased expression of stem cell transcription factors OCT4, SOX2, NANOG and MYC have been reported in IH. In addition, our in vivo data also show that in proliferative IH LIN28 was not limited to GLUT1 positive cells but was also expressed in perivascular non-endothelial cells, which are essential for the maintenance of IH vessels stability.

The increase in LIN28B in IH reported here may be due to transcriptional and/or post transcriptional activation. The reprogramming transcription factors such as MYC has been shown to transactivate LIN28B. Although little is known about the transcriptional activation of LIN28B, posttranscriptional regulation by miRNAs such as let-7 and other miRNAs has been extensively studied. Furthermore, LIN28B was reported to bind its own mRNA, increasing its stability and protein abundance. Thus, our sRNAseq results are in line with previous reports and highlight the central role of the LIN28B/let-7 switch in governing stem cell self-renewal in the proliferative phase of IH.

We also showed that the ESC-enriched miRNA cluster mir-498(46) is highly expressed in IH. This cluster of miRNAs is normally imprinted, with only the paternally inherited allele expressed in the placenta. The overexpression of mir-498(46) seen in IH could be regulated by methylation of the upstream CpG island or by chromosomal rearrangements such as amplifications of the corresponding chromosome 19 region, as found in embryonal tumor with multilayered rosettes (ETMRs), primitive neuroectodermal tumors (PNETs) and in thyroid adenomas. Given the ability of IH to undergo spontaneous involution, the elevated expression of mir-498(46) in proliferative IH is unlikely to be due to DNA amplifications or translocations, but rather,
due to epigenetic modification. We therefore proceeded to investigate the previously identified promoter region and transcription start site that overlap an annotated CpG island located ~17 kb upstream of the first miRNA gene of mir-498(46). Although our findings show that, unlike NS, IH samples exhibited active transcription starting from the upstream CpG island, similar to that seen in the placenta, they do not preclude the existence of additional active promoter regions within mir-498(46). In fact, mir-498(46) carries numerous CpG dinucleotide islands extensively interspersed with Alu-rich repeats, which account for over 50% of the 100-kb sequence. Numerous studies showed that Alu methylation is highly dynamic and Alu-rich regions can function as independent promoters for RNA polymerase II and RNA polymerase III in both mesenchymal stem cells and cancer stem cells.

Although LIN28 is a well-established inhibitor of miRNA maturation, the present study revealed that overexpression of LIN28B increased the expression of miR-516b of mir-498(46). This is especially noteworthy given that LIN28B can be used along with other reprogramming factors to generate pluripotent stem cells enriched with mir-498(46). In fact, a recent study showed that LIN28A activates gene expression by binding directly to a consensus DNA sequence at promoter regions and recruiting the CpG demethylase TET1. Previous study showed that both mesenchymal stem and cancer stem cells exhibit disperse expression patterns of miRNAs of the mir-498(46) cluster rather than a bloc expression regulated by the upstream promoter. Accordingly, our data show that overexpression of LIN28B did not affect the methylation of the upstream CpG-island or increase the transcription in that region indicating the existence of downstream promoter regions. LIN28B may bind to the Alu repeats within
mir-498(46), which may function as independent RNA polymerase II promoters and activate transcription by recruiting TET1. In fact, analyses of previously published PAR-CLIP data show that LIN28B binds numerous Alu repeats located in the mir-498(46)\textsuperscript{133}. This unexpected result points to a potential binding of LIN28B to the CpG-rich Alu repeats to activate transcription. Moreover, LIN28 is a potent RNA-binding protein that regulates splicing factor abundance\textsuperscript{134}, and therefore may induce processing of the transcript of mir-498(46) cistron.

In the last decade, propranolol has become the preferred treatment for morbid proliferating IH. To date, more than 500 published articles describe the observed propranolol-induced involution of IH and the various hypotheses regarding its mechanisms of action. Here we propose a new model through which propranolol triggers rapid IH involution. We demonstrate that propranolol induces a shift in LIN28B/let-7 balance to favor cell differentiation and senescence. This model is based on the data presented here, which show that propranolol reduced the expression of LIN28B and increased the expression of let-7 in IH samples in vivo and in iPSCs in vitro. Furthermore, we show that propranolol reduces the proliferation of iPSCs and initiates the conversion to mesenchymal phenotype as evidenced by the increase in SERPINE1 and TWIST1 expression, while reducing the expression of miRNAs of the mir-498(46) cistron. RNAseq and gene set analyses confirmed the downregulation in E2F targets, G2M checkpoint and mitotic spindle pathways and showed upregulation in EMT, adipogenesis, and TGF-β signaling pathways. To our knowledge, this is the first report to show that propranolol reduces the proliferation of iPSCs and induces EMT and adipogenesis. These profound effects of propranolol may be due in part to the reduction
in LIN28B that binds and regulates mRNAs of cell cycle regulators\textsuperscript{133} and/or as a consequence of the increase in let-7 miRNAs, which regulate Rb1/E2F genes\textsuperscript{328}. The induction of EMT genes by propranolol is in agreement with a previous study that showed that activation of adenyl cyclase and elevation in cAMP and EPAC signaling replaced the need for OCT4 for iPSC generation. This cAMP-dependent reprogramming increased cellular division rate and induced genes involved in MET\textsuperscript{329}. Therefore, by reducing cAMP levels, propranolol may be inducing EMT and thus accelerating IH involution. Of note, propranolol has been shown to induce adipogenesis in hemangioma stem cells\textsuperscript{330, 331}. Lastly, the reduction in miRNAs of the mir-498(46) cistron in propranolol treated iPSCs reported here is in agreement with previous work that showed that propranolol treated IH tissues contained significantly lower levels of mir-498(46) miRNAs than did proliferative, untreated IH\textsuperscript{119}.

The LIN28/let-7 axis is implicated not only in pluripotency but also in tumorigenesis, especially in cancer stem cells, which are resistant to chemotherapies and promote metastasis\textsuperscript{129, 332}. In fact, numerous pre-clinical and clinical studies provide evidence that propranolol increases chemosensitivity and reduces the metastatic rates in multiple cancer types\textsuperscript{333}. The present work highlights the role of the LIN28B/let-7 switch in IH pathogenesis and propranolol induced IH involution. This study may also have therapeutic implications for IH and for cancers in which the LIN28/let-7 pathway is imbalanced.
Chapter 8
Limitations and Overall Conclusions

Limitations

To study the role of C19MC in trophoblast differentiation, we employed a CRISPR/SAM based method that utilizes guide RNAs targeted to a region directly upstream of the first C19MC miRNA. Although transient transfection of CRISPR/SAM components in HEK293 cells induced robust C19MC expression and yielded valuable data, it is not sufficient to study the long-term effects of C19MC overexpression. Further studies using stable lentiviral vectors will be necessary to determine the long-term effects of C19MC overexpression. Furthermore, transient and stable C19MC knockdown experiments are required to provide definite proof that C19MC regulates EMT. Due to its large size, we have been unable to successfully knockdown its expression.

Another potential limitation to this study is the use of a low dose BMP treatment protocol for the differentiation of iPSCs to iPSC-CTs. Our data shows that C19MC miRNAs are decreased and EMT makers are increased upon iPSC differentiation to CTs. Although this data further strengthens the hypothesis that the EMT process is associated with a decrease in C19MC miRNA expression, use of a differentiation protocol which does not itself induce EMT would further clarify the role of C19MC.
To test the effect of propranolol on C19MC miRNAs and the LIN28B/let 7 signaling pathway, we used iPSCs which express high levels of C19MC and LIN28. However, GLUT1 positive hemangioma endothelial cells isolated from proliferative IH would have been the ideal cells to use in these studies as they are directly relevant to the pathophysiology of IH. These cells have also been characterized as stem like. Unfortunately, although the hemangioma endothelial cells express GLUT1 and C19MC when freshly isolated, they have been shown to convert to a mesenchymal phenotype and lose GLUT1 and C19MC expression after 3 weeks in culture. In addition to the culturing constraints which have the ability to confound our study, we did not have access to these cells. We therefore used iPSCs which allowed us the opportunity to generate data that have implications beyond IH. Another limitation to our study is the limited access to propranolol treated and involuted IH tissues. Since propranolol causes a rapid involution, surgical intervention is not needed. Thus, we could not directly compare the expression of C19MC and EMT markers in proliferative and propranolol treated IH. Measuring changes in EMT markers in propranolol treated IH could potentially add translational relevance to our study.

**Overall conclusions**

In this study, we sought to determine the role of C19MC in placental trophotroblast differentiation. First, we show in situ that C19MC is highly expressed in VTs but not in the invasive EVTs and decidua of first trimester human placentas. To test the effect of C19MC miRNAs, we designed a new method to transcriptionally activate the entire C19MC cistron. Using this highly efficient method, we showed that overexpression of
C19MC in HEK293 cells decreased the expression of EMT markers and increased the expression of stemness genes OCT4 and FGF4. This indicates that these miRNAs play a role in the maintenance of the epithelial phenotype and stemness in stem cells and VTs. Hallmark gene set analysis also showed that C19MC targets many mRNAs involved in cell differentiation pathways including EMT and Hedgehog signaling pathways.

Since the early placental environment is hypoxic, we tested the effect of hypoxia on C19MC miRNA expression in vitro. Our results show that hypoxia decreases C19MC and increases EMT gene expression. Although the mechanism by which hypoxia decreases the expression of C19MC miRNAs is unknown, the data suggests that hypoxia facilitates trophoblast differentiation during early pregnancy by decreasing C19MC thus promoting EMT. In addition, co-expression of C19MC miRNAs along with Yamanaka factors resulted in a significant increase in number of stem cell colonies and overall efficiency of reprogramming. This is likely due to an increase in expression of the pluripotency factor OCT4 and FGF4 induced by C19MC overexpression. Taken together, this study establishes the utility of the CRISPR/SAM technique as a tool to investigate the role of C19MC in placental physiology. This study also establishes the important role of C19MC in the maintenance of stemness and shows that loss of C19MC miRNAs is essential for EMT and trophoblasts and stem cells differentiation. Since trophoblast migration and invasion is extremely important for optimal placentation and fetal growth, dysregulation of C19MC can have detrimental effects during pregnancy.
We also investigated the roles of C19MC and LIN28 in IH. We presented evidence that LIN28B is the paralog that is highly expressed in IH. We also show in human tissue sections, that LIN28B is highly expressed in proliferating IH but is reduced in involuting IH and propranolol treated IH tissues. We also show that the high Lin28 expression in proliferative IH is accompanied by a decrease in let 7 miRNA expression consistent with the evolutionarily conserved negative feedback loop described in the literature. Furthermore, overexpression of LIN28B in HEK293 cells was sufficient to induce the expression of miR-516b, a C19MC miRNA without altering the methylation status of the CpG island upstream of C19MC or increase transcription in the region. These data indicate that LIN28B increase miRNAs of the c19MC in a methylation independent mechanism. This study also shows that propranolol has a profound effect on C19MC and LIN28B expression. iPSCs treated with propranolol showed a marked decrease in the expression of C19MC and LIN28B and an increase in EMT markers. These results demonstrate that propranolol induces IH involution by decreasing the expression of C19MC, inducing EMT, and altering the balance of the Lin28B/let-7 signaling pathway.
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Appendix A

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USF IRB approval for collection of human placentas

2/12/2014

John Tsibris, Ph.D.
Obstetrics and Gynecology
12901 Bruce B. Downs Blvd
MDC-18
Tampa, FL 33612

RE: Expedited Approval for Initial Review
IRB#: Pro00015578
Title: The Characterization of the Vascular Endothelial Growth Factor (VEGF) Receptor-2 Network in Human Placentas Complicated by Preeclampsia

Study Approval Period: 2/11/2014 to 2/11/2015

Dear Dr. Tsibris:

On 2/11/2014, the Institutional Review Board (IRB) reviewed and APPROVED the above application and all documents outlined below:

Approved Item(s):
Protocol Document(s):
Data Collection Form
Tsibris Research protocol on VEGFR2 - Revised FINAL DRAFT.docx

*Please use only the official IRB stamped informed consent/assent document(s) found under the "Attachments" tab. Please note, these consent/assent document(s) are only valid during the approval period indicated at the top of the form(s).

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45CFR46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review category:
(2) Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:
(a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the
amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more
frequently than 2 times per week; or (b) from other adults and children, considering the age,
weight, and health of the subjects, the collection procedure, the amount of blood to be collected,
and the frequency with which it will be collected. For these subjects, the amount drawn may not
exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more
frequently than 2 times per week.

(5) Research involving materials (data, documents, records, or specimens) that have been
collected, or will be collected solely for nonresearch purposes (such as medical treatment or
diagnosis).

Per CFR 45 Part 46, Subpart D, this research involving children was approved under the
minimal risk category 45 CFR 46.404: Research not involving greater than minimal risk.

Per CFR 45 Part 46, Subpart B, this research involving pregnant women was approved
under risk category 45 CFR 46.204.

As the principal investigator of this study, it is your responsibility to conduct this study in
accordance with IRB policies and procedures and as approved by the IRB. Any changes to the
approved research must be submitted to the IRB for review and approval by an amendment.

We appreciate your dedication to the ethical conduct of human subject research at the University
of South Florida and your continued commitment to human research protections. If you have
any questions regarding this matter, please call 813-974-5638.

Sincerely,

E. Verena Jørgensen, M.D., Chairperson
USF Institutional Review Board
Supplementary materials

Supplementary tables SI, SII, SIII and SIV for chapter 6 can be accessed here:
https://usf.box.com/s/jwd2rvxqrmy9pa1bemwsreq2fmrvdkb4

Supplementary tables SI, SII for chapter 7 have been previously published and can be accessed here: https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.118.310908