DREAM-MEDIATED REGULATION OF GCM1 IN THE HUMAN PLACENTAL TROPHOBLAST

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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Abstract

DREAM-MEDIATED REGULATION OF GCM1 IN THE HUMAN PLACENTAL TROPHOBLAST

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The trophoblast transcription factor glial cell missing-1 (GCM1) regulates asymmetric division of placental cytotrophoblast to form the differentiated syncytiotrophoblast. Reduced GCM1 expression is a key feature of the hypertensive disorder preeclampsia. *In-silico* techniques identified a novel calcium-dependent transcriptional repressor – DREAM as a regulatory candidate for GCM1. The overall objective of this thesis was to determine if DREAM regulates GCM1 expression and therefore villous trophoblast turnover. siRNA-mediated DREAM silencing in both BeWo cells and floating villous explants significantly upregulated GCM1 causing reduced cytotrophoblast proliferation. Calcium-dependency was demonstrated in both BeWo cells and floating villous explants by contrasting the effects of ionomycin and nimodipine. A direct interaction between DREAM and the GCM1 promoter was demonstrated using EMSA and ChIP assay. DREAM is a negative upstream regulator of GCM1 expression in human placenta that participates in calcium-dependent trophoblast differentiation.
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TABLE OF CONTENTS
INTRODUCTION ......................................................................................................... 1
  1.1 The Placenta .................................................................................................. 2
    1.1.1 Early stages of human placental development ........................................... 2
    1.1.2 Mature placenta at term ........................................................................... 4
    1.1.3 Placental Function .................................................................................... 5
  1.2 Placental trophoblast cell lineages ............................................................... 6
    1.2.1 Villous trophoblast layer ........................................................................... 6
    1.2.2 Factors controlling villous trophoblast turnover ......................................... 8
    1.2.3 Extravillous trophoblast cells ................................................................. 10
    1.2.4 Factors controlling EVT differentiation and invasion ............................... 11
  1.3 GCM1 ........................................................................................................ 12
    1.3.1 GCM1 structure and target genes ........................................................... 12
    1.3.2 GCM1 regulation ................................................................................... 12
    1.3.3 GCM1 function in placentation ............................................................... 13
  1.4 DREAM ....................................................................................................... 14
    1.4.1 Structure ................................................................................................. 14
    1.4.2 Expression ............................................................................................. 14
    1.4.3 Function .................................................................................................. 15
    1.4.4 Calcium dependence ............................................................................. 16
    1.4.5 Potential role of DREAM in pathophysiology of placental insufficiency syndromes ............................................................... 18
  1.5 Placental insufficiency syndromes ............................................................... 18
    1.5.1 Preeclampsia .......................................................................................... 18
      1.5.1.1 Pathophysiology of severe PE ............................................................ 19
      1.5.1.2 Treatment ......................................................................................... 20
      1.5.1.3 Prevention of PE .............................................................................. 20
    1.5.2 Intrauterine Growth Restriction ............................................................... 21
  1.6 Rational of the project .................................................................................. 21
  1.7 Models used in the study ............................................................................ 22
  1.8 Objective and aims ...................................................................................... 22

MATERIALS AND METHODS .................................................................................. 23
  2.1 In vitro models ............................................................................................. 24
    2.1.1 BeWo cells ............................................................................................... 24
      2.1.1.1 Transfection of BeWo cells ................................................................. 24
      2.1.1.2 Ionomycin and Nimodipine treatment ................................................. 24
    2.1.2 First trimester placental explants ............................................................. 25
      2.1.2.1 Floating first trimester placental explant model .................................. 25
      2.1.2.2 Cytotoxicity assays ........................................................................... 26
      2.1.2.3 Placental villous explant immunohistochemistry .................................. 26
  2.2 Semi-thin sections ....................................................................................... 27
  2.3 TUNEL assay ............................................................................................... 28
  2.4 BrdU incorporation and immunodetection .................................................... 28
2.5 Quantitative real time-PCR ................................................................. 28
  2.5.1 Tissue collection ................................................................. 28
  2.5.2 RNA extraction ................................................................. 29
  2.5.3 Reverse transcription ......................................................... 29
  2.5.4 Quantitative real time PCR .................................................. 30
  2.5.5 Primers ................................................................................. 30
2.6 Image analysis and quantification of placental tissue sections .......... 31
2.7 DNA/protein interactions ............................................................. 32
  2.7.1 Promoter analysis .............................................................. 32
  2.7.2 Chromatin Immunoprecipitation (ChIP) .................................... 32
    2.7.2.1 PCR amplification of immunoprecipitated fragments .......... 34
2.8 Electromobility Shift Assay (EMSA) .............................................. 34
  2.8.1 Probe preparation ............................................................ 34
  2.8.2 Nuclear protein extraction ................................................... 35
  2.8.3 Probe/protein binding and electrophoresis ............................... 35
  2.8.4 Transfer and detection ....................................................... 36
2.9 Statistical analysis ....................................................................... 36

RESULTS ........................................................................................................... 37
3.1 Investigation of DREAM expression pattern in the human placenta
throughout pregnancy .................................................................................. 38
  3.1.1 Gestational profile of DREAM immunolocalization in the human placenta ...................................................... 38
  3.1.2 Quantification of nuclear trophoblast DREAM staining ...................... 39
  3.1.3 DREAM mRNA levels decrease across gestation ......................... 40
3.2 Investigation of DREAM and GCM1 interaction ................................ 41
  3.2.1 Chromatin Immunoprecipitation assay ...................................... 41
  3.2.2 Electromobility Shift Assay .................................................... 42
3.3 Investigating the role of DREAM in GCM1 regulation ....................... 45
  3.3.1 siRNA mediated DREAM repression upregulated GCM1 mRNA levels in BeWo cell model ................................................. 45
  3.3.2 DREAM overexpression in BeWo cells .................................... 46
    3.3.2.1 siRNA repression of DREAM upregulates GCM1 mRNA levels in first trimester placental explants .................................................. 47
    3.3.2.2 Histological assessment of DREAM siRNA treated explants ....... 48
    3.3.2.3 Modifications to trophoblast morphology in DREAM siRNA treated placental explants (semi-thin sections) ...................... 49
    3.3.2.4 Assessment of proliferation and apoptosis in DREAM siRNA treated explants .......................................................... 50
3.4 Investigation of altered intracellular calcium concentrations on DREAM activity .................................................................... 53
  3.4.1 Intracellular calcium level modification by nimodipine and ionomycin treatment of BeWo cells .................................................. 53
  3.4.2 Intracellular calcium level modification by nimodipine and ionomycin treatment in first trimester floating placental explants ............ 54
    3.4.2.1 DREAM and GCM1 mRNA levels ........................................ 55
    3.4.2.2 DREAM subcellular localization changes with intracellular calcium levels .......................................................... 55
    3.4.2.3 Proliferation of CYT cells in response to nimodipine and ionomycin treatment .................................................. 56
3.5 Investigation of DREAM expression levels in PE ........................................... 57
  3.5.1 DREAM mRNA levels in PE ................................................................. 58

DISCUSSION ........................................................................................................ 59
4.1 Conclusion .................................................................................................... 67

FUTURE DIRECTIONS ..................................................................................... 68

REFERENCES ..................................................................................................... 72
List of tables

Chapter 2 – Material and Methods

Table 1: Antibodies and dilutions used for placental tissue Immunohistochemistry 27
Table 2: Clinical information 29
Table 3: Quantitative real time PCR sequences 31
Table 4: PCR primer sequences used in ChIP analysis 33
Table 5: 3’ end biotin labeled probes used in EMSA assay 34
**List of figures**

**Chapter 1: Introduction**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blastocyst development stages</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Placental chorionic villi</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Placental terminal villi</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Trophoblast turnover</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Chorionic villi from first trimester and term placenta</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>EVT subpopulations</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Structure and alternative transcripts of human DREAM gene</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>Calcium regulated transcriptional repression by DREAM</td>
<td>17</td>
</tr>
</tbody>
</table>

**Chapter 2: Material and Methods**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Human 1st trimester floating villous explant culture model</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>Diagrammatic representation of 5000 bp region of the human GCM1 promoter region</td>
<td>32</td>
</tr>
</tbody>
</table>

**Chapter 3: Results**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>DREAM immunofluorescence in human placenta of first, second and third trimester</td>
<td>38</td>
</tr>
<tr>
<td>12</td>
<td>DREAM immunofluorescence in first trimester EVT column</td>
<td>39</td>
</tr>
<tr>
<td>13</td>
<td>DREAM nuclear immunopositivity in trophoblast cells of human placenta across gestation</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>Relative DREAM mRNA expression levels in human placenta across gestation</td>
<td>41</td>
</tr>
<tr>
<td>15</td>
<td>ChIP analysis of 5 kb fragment of 5’ GCM1 human promoter</td>
<td>42</td>
</tr>
<tr>
<td>16</td>
<td>EMSA on nuclear fraction from DREAM overexpressing and DREAM silenced BeWo cells.</td>
<td>43</td>
</tr>
</tbody>
</table>
Figure 17: EMSA interrogating 43 bp region of GCM1 promoter
Figure 18: siRNA mediated DREAM silencing in BeWo cells
Figure 19: DREAM overexpression in BeWo cells
Figure 20: DREAM siRNA treatment of first trimester floating placental villous explants
Figure 21: Histological assessment of DREAM siRNA treated first trimester floating villous explants
Figure 22: Toluidine blue stained semi-thin sections of DREAM siRNA treated first trimester explants
Figure 23: Hrk mRNA levels in DREAM siRNA treated explants
Figure 24: TUNEL staining in DREAM siRNA treated explants
Figure 25: Proliferation assay in DREAM siRNA treated first trimester placental explants
Figure 26: DREAM and PCNA co-localization
Figure 27: Nimodipine and ionomycin treatment of BeWo cells
Figure 28: GCM1 mRNA levels in nimodipine and ionomycin treated first trimester placental floating explants
Figure 29: Nimodipine and ionomycin treatment of first trimester placental explants result in DREAM translocation
Figure 30: Altered cytotrophoblast rate of proliferation in Nimodipine and ionomycin treated first trimester placental explants
Figure 31: DREAM immunohistochemistry in preeclampsia
Figure 32: Relative DREAM mRNA expression levels in preeclampsia
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM12</td>
<td>-a disintegrin and metalloprotease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>-analysis of variance</td>
</tr>
<tr>
<td>BrdU</td>
<td>-5-Bromo-2'-deoxy-uridine</td>
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<tr>
<td>cAMP</td>
<td>-cyclic adenosine monophosphate</td>
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<tr>
<td>ChIP</td>
<td>-chromatin immunoprecipitation</td>
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<tr>
<td>CYT</td>
<td>-cytotrophoblast</td>
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<tr>
<td>DRE</td>
<td>-downstream regulatory element</td>
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<td>DREAM</td>
<td>-downstream regulatory element antagonist modulator</td>
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<td>EMSA</td>
<td>-electromobility shift assay</td>
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<td>ET-1</td>
<td>-Endothelin-1</td>
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<td>EVT</td>
<td>-extravillous trophoblast</td>
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<tr>
<td>Fgf4</td>
<td>-fibroblast growth factor 4</td>
</tr>
<tr>
<td>GCM1</td>
<td>-Glial Cell Missing 1</td>
</tr>
<tr>
<td>hCG</td>
<td>-human chorionic gonadotropin</td>
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<tr>
<td>hFBW2</td>
<td>-human F-box protein FBW2</td>
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<tr>
<td>Hrk</td>
<td>-hara-kiri</td>
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<tr>
<td>IFN-(\gamma)</td>
<td>-interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>-immunoglobulin-G</td>
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<tr>
<td>IL8</td>
<td>-Interleukin 8</td>
</tr>
<tr>
<td>IUGR</td>
<td>-intrauterine growth restriction</td>
</tr>
<tr>
<td>KCNIP</td>
<td>-potassium channel interacting protein</td>
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<tr>
<td>MP</td>
<td>-microparticles</td>
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<td>mRNA</td>
<td>-messenger RNA</td>
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<td>PE</td>
<td>-preeclampsia</td>
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<tr>
<td>PFA</td>
<td>-paraformaldehyde</td>
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<tr>
<td>PIGF</td>
<td>-placental growth factor</td>
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<tr>
<td>qRT-PCR</td>
<td>-quantitative real time-PCR</td>
</tr>
<tr>
<td>RT</td>
<td>-room temperature</td>
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<tr>
<td>SCF(^{FBW2E3})</td>
<td>-(\textit{SKP1/cullin}/F-box protein ubiquitin-protein isopeptide ligase (E3))</td>
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</tbody>
</table>
SDHA - succinate dehydrogenase
sFLT-1 - soluble fms-like tyrosine kinase-1
SYN - syncytiotrophoblast
TBP - TATA box binding protein
TGF-β1 - transforming growth factor
TNF-α - tumor necrosis factor
TUNEL - terminal transferase dUTP nick labeling
UTP - Uridine-5'-triphosphate
INTRODUCTION
1.1 The Placenta

The placenta is unique to eutharian mammals and is vital to their development. Placentas exist in many shapes and sizes depending on the uterine shape, species size, litter size and habitat (Bischof and Irminger-Finger 2005). Although there are great anatomical and functional distinctions between placentas of different species, they all share a common feature: creating an efficient transfer unit between maternal and fetal circulations (Benirschke K 2005). The development of human placenta is a very complex process, involving transcription factors, growth factors and changes in oxygen tension to mention a few. The following sections describe in detail early and late stages of placental morphogenesis, as well as function in healthy and pathological placentas.

1.1.1 Early stages of human placental development

The most important stages of placental development occur within the first few weeks after fertilization. Adequate placentation is essential to the progression of a successful pregnancy. Failure at this stage of development can have negative consequences for both the fetus (e.g. intrauterine growth restriction (IUGR)) and the mother (e.g. preeclampsia) and in extreme instances cause spontaneous loss or stillbirth.

The trophoblast is the first lineage to differentiate following fertilization since in humans, placental formation precedes gastrulation. Trophoblast cells are observed in the trophectoderm layer of the blastocyst 3 days post fertilization. After 5 days of fertilization the blastocyst contains approximately 60 cells. This spherical structure includes an inner cell mass of approximately 20 cells (future embryo) surrounded by a sphere of 40 trophoblast cells – destined to form the placenta (Hardy, Handyside et al. 1989) Trophoblast cells play a key role in three distinctive phases of blastocyst implantation: apposition, adhesion and embedding (Figure1).
Apposition refers to the first connection established between the blastocyst and the maternal endometrium. The direct contact with the decidua occurs at a specific location within the blastocyst, where trophoblast cells are exposed from beneath, the partially-lysed zona pellucida. Adhesion denotes a stronger attachment of the blastocyst to the endometrium through protrusions from trophoblast cells. Finally, embedding is accomplished when invading trophoblast cells differentiate into syncytiotrophoblast (SYN) (Walter B. 2004). The rapidly expanding SYN creates processes via lacunar formation to form chorionic villi which continue to branch while invading the maternal decidua. Only those trophoblast cells in direct contact with the maternal decidua have fusogenic properties, the underling cytotrophoblast (CYT) cells remain unfused and undifferentiated, serving as stem cells for the syncytial layer (Figure 2A).
Cell originating from extra-embryonic mesenchyme infiltrate the chorionic villi, carrying branches of the umbilical vessels with them; thus establishing fetal circulation (Benirschke K 2005) (Figure 2B).

The principal structure of haemochorial human placenta is determined in the first trimester of pregnancy (Reynolds, Borowicz et al. 2005). By 12-13 wks of pregnancy both the maternal-placental and the fetoplacental circulations are established. The outer epithelial layer of the placenta (SYN) is then bathed in maternal blood. Throughout gestation the placenta continues to grow, increasing in size and most importantly in surface area for efficient transport of nutrients and waste across the placenta (Benirschke K 2005).

1.1.2 Mature placenta at term

During the second and third trimesters of pregnancy the human placental villi continue to grow and branch. Eventually capillary growth in the mesenchyme exceeds the growth of the villi and the capillaries form loops and bulge through the trophoblast cells resulting in a unique grape like structure within the placental
tree called terminal villi. By the end of gestation these structures comprise of 40% of the villous volume. Their development is crucial to proper placental function as they are the site of the fetomaternal exchange (Benirschke K 2005) (Figure 3).

The placenta is anchored to the decidua by invasive extravillous trophoblast cells. These cells differentiate and invade the decidua and maternal vasculature during the first and second trimesters, increasing blood flow in the intervillous space. Thus the mature healthy placenta is bathed in the pool of maternal blood and the fetomaternal exchange of waste and nutrients occurs within the terminal villi where the maternal blood is separated from fetal blood by only a very thin continuous layer of SYN, a discontinuous layer of CYT and the endothelial cell layer of the fetal capillaries (Figure 5).

1.1.3 Placental Function

The placenta sustains the developing fetus throughout gestation. Major functions of this organ include: nutrition, respiration and excretion. Fetal and maternal blood is in close contact over a large surface area thus allowing for
efficient transport of nutrients and oxygen from the maternal circulation and carbon dioxide and waste from the fetal circulation. Depending on the molecule, transfer across the epithelial layer is either passive or active. The placenta also possesses metabolic and endocrine activity synthesizing glycogen, cholesterol and fatty acids as well as secreting hormones such as: estrogens, progesterone, placental lactogen, placental growth factor and hCG (Benirschke K 2005). These molecules, synthesized in the SYN layer, are fundamental to proper placental development and function and thus sustained pregnancy.

A healthy placenta is critical for growth and survival of the fetus, and failure of proper placental development can lead to severe perinatal diseases, including preeclampsia (PE) and IUGR. Before describing placental pathologies, however, it is important to introduce various trophoblast subpopulations and their development, as the perturbations of placental function are the result of improper trophoblast turnover.

1.2 Placental trophoblast cell lineages
The human placenta contains two major lineages of trophoblast cells with different biological activities: villous cytotrophoblast (VCT) and extravillous trophoblast (EVT) (Scifres and Nelson 2009). The rapidly expanding chorionic villous trees of placenta are covered by an epithelial villous trophoblast compartment, comprising of VCT beneath a continuous multinucleated layer of SYN. EVT cells invade into the decidua and remodel maternal spiral arteries increasing blood flow into the developing placenta.

1.2.1 Villous trophoblast layer
The placental epithelial layer forms a physical barrier between maternal and fetal circulations and some of its other functions include: nutrient, gas and waste exchange. This layer is composed of CYT and overlying SYN. The dynamic trophoblast layer undergoes tightly regulated proliferation, differentiation, fusion and apoptosis events throughout the entire gestation (Cross 1998) (Huppertz, Tews et al. 2001). Unlike SYN, CYT cells have proliferative abilities (Simpson, Mayhew et al. 1992) and a subset of these cells
undergoes a process of asymmetrical division donating one of its post-mitotic nuclei into the overlying SYN layer by a process of syncytial fusion (Mayhew 2001). This recurrent process permits expansion of the epithelial surface area of the placenta and maintains its continuity. Since the rate of syncytial fusion exceeds the amount that is required for villous growth, up to 3 grams of syncytial material is shed as apoptotic particles into the maternal circulation per day (Huppertz, Frank et al. 1998) (Figure 4).

In the first trimester there is a continuous layer of proliferating CYT cells and overlying continuous layer of terminally differentiated syncytium. By the third trimester of pregnancy the trophoblast layer has thinned out to form the “vasculo-syncytial membrane”. CYTs are dispersed and fetal capillaries are in close contact with maternal circulation for efficient nutrient and gas exchange. (Lewis and Benirschke, 1997) Even though CYT cells are spread out they still maintain mitotic activity and therefore, contribute a fresh supply of nuclei to the overlying SYN. This continuous process may also be important for maintenance of syncytial protein production. Recent studies have shown that limited UTP
synthesis does occur in the SYN thus suggesting de novo protein synthesis (Ellery, Cindrova-Davies et al. 2009).

Deficiency in syncytial fusion may have dramatic consequences on human pregnancy since the SYN layer must be continuously regenerated in order to serve two major functions. First, the SYN must provide high levels of energy-dependent carrier systems that promote fetal growth (Sibley, Birdsey et al. 1998) and second, SYN express several anticoagulant proteins on their surface that prevent pathologic thrombosis of maternal blood perfusing the inter-villous space (Krikun, Lockwood et al. 1994). The SYN is also first line of defense against pathogens as it secretes nitric oxide synthase (Sladek, Magness et al. 1997). Thus, disruptions of this highly regulated process of trophoblast turnover has been associated with placental pathologies such as PE and IUGR (Mayhew, Manwani et al. 2007).

1.2.2 Factors controlling villous trophoblast turnover

Appropriate trophoblast cell turnover is crucial for the proper development and function of placenta. Cell cycle arrest and labyrinthine trophoblast development in mice are regulated by the transcription factor Glial cell missing-1 (Gcm1). (Anson-Cartwright, Dawson et al. 2000). Mouse embryos lacking Gcm1 die in mid-gestation due to a failure of SYN differentiation. In humans, the homolog GCM1 has an expression pattern similar to mice (Baczyk,
Satkunaratnam et al. 2004) and plays a central role in mediating differentiation of trophoblast cells along both villous and extra-villous pathways (Baczyk, Drewlo et al. 2009). Caspases 8 and 10 have also been shown to play a role in VCT differentiation (Huppertz, Frank et al. 1999; Black, Kadyrov et al. 2004).

Only a handful of genes have been implicated in trophoblast cell-cell fusion, they include connexin 43 (Frendo, Cronier et al. 2003), ADAM12, syncytin 1 and 2 (Huppertz, Bartz et al. 2006). It should be noted that GCM1 was shown to directly bind to and regulate syncytin 1 and 2 expression (Lin, Lin et al. 2005).

hCG is produced in SYN, however it has been shown to play an important autocrine/paracrine function in CYT cells by promoting their differentiation and fusion (Ho, Douglas et al. 1997).

More recently, potassium (K\(^+\)) channels have been implicated in cytotrophoblast hCG secretion and differentiation in vitro (Williams, Fyfe et al. 2008). The function and expression of K\(^+\) channels has been poorly investigated in placental trophoblast; however, there are reports, in other tissues, that K\(^+\) channels can regulate cell proliferation, apoptosis (Lang, Foller et al. 2007) and cell fusion (Cooper 2001) as well as hormone secretion (Leung, Kwan et al. 2007). Since voltage-gated K\(^+\) channels can be inhibited by hypoxia (Archer, Weir et al. 2000) and oxidative stress (Gutterman, Miura et al. 2005) their function and expression merits further investigation in placental pathologies.

Primary human CYT cells in culture spontaneously differentiate and fuse to form SYN. The phenomenon of spontaneous primary CYT fusion into SYN is positively correlated with calcium uptake and up regulation in calcium channel expression (Moreau, Hamel et al. 2002).

CYT proliferation, on the other hand, can be induced with stimulation by IGF acting via the IGFRI receptor (Forbes, Westwood et al. 2008) and by treatment of FGF4 acting via the FGFR2 receptor (Baczyk, Dunk et al. 2006). The continuous proliferation of VCT drives the growth of the placental villi, while CYT differentiation ensures maintenance and renewal of the syncytial layer. Proper balance of proliferation and differentiation is a key to successful placentation.
1.2.3 *Extravillous trophoblast cells*

Human placenta also contains a second lineage of trophoblast cells termed EVT. This heterogeneous population of cells forms columns which extend from the tips of the placental villi towards the maternal decidua. At the base of the columns the EVT cells possess a proliferative phenotype but more distal cells of the column exhibit an invasive phenotype. A sub-population of the distal EVT cells separates from the organized columns and invades the maternal decidua and are thus termed interstitial EVT. A second sub-population of EVT cells – endovascular EVT, invade the narrow, muscular lumens of the maternal spiral arteries transforming them into large vessels by replacing their endothelium and smooth muscle (Pijnenborg, Vercruysse et al. 2006). Unlike the VCT cells the EVT subpopulations do not undergo cell – cell fusion but rather a unique process of endoreduplication (Zybina 1983). This phenomenon is similar to giant cells in mice but much less pronounced (MacAuley, Cross et al. 1998).

The role of the differentiated EVT cells is to anchor the placenta to the uterine wall and more importantly to remodel maternal spiral arteries in order to increase blood flow to the developing placenta (Robson, Simpson et al. 2002). Arrest of EVT proliferation ensures that these cells do not invade too deeply into the myometrium.

**Figure 6. EVT cell subpopulations.** Proximal EVT cells (light green) are proliferative. Distal EVT cells detach from the cell column and become either interstitial EVT (inEVT) or they invade spiral artery and differentiate to endovascular EVT (enEVT). Image modified from J. Wright (Master of Science thesis U of T 2008).
1.2.4 Factors controlling EVT differentiation and invasion

A recent 2008 trophoblast invasion workshop report summarized the mechanisms regulating EVT differentiation and invasion (Knofler, Simmons et al. 2008). Specifically, both canonical and non-canonical Wnt pathways as well as EGF signaling pathway play a crucial role in EVT differentiation and invasion. Additionally, the report suggested that cytokines secreted by uterine natural killer cells such as TGF-β1, TNF-α and IFN-γ can inhibit EVT invasion while IL8 can enhance it.

Since individual EVT cells have been shown to invade as far as the superficial layer of the myometrium (Kurman 1991), not surprisingly invading EVT cells depend on matrix-degrading metalloproteinases (MMPs) and plasminogen activators (Kurman 1991), (Fisher and Damsky 1993) in order to degrade decidual extracellular matrix (ECM). In addition to ECM degradation, EVT cells are also capable of adhering to the matrix with help from cell adhesion molecules (CAMs). The major function of CAMs is to facilitate the EVT movement through the decidua (Fisher and Damsky 1993). Indeed, proximal cells of the EVT column express the fibronectin receptor α5β1 integrin while invasive EVT cells express the collagen IV receptor α1β1 (Benirschke K 2005). The study by Nicola et al., identified the crucial role of intracellular calcium and calpain (calcium dependent proteases involved in cell locomotion) on prostaglandin EP1 receptor mediated migration of the first trimester human EVT (Nicola, Timoshenko et al. 2005). Another molecule implicated in EVT migration is endothelin-1 (ET-1), typically associated with hypertension. Importantly, Chakraborty et al., showed that ET-1 treatment of human EVT cell line results in rapid phosphorylation of MAP kinase (ERK1/2) (Chakraborty, Barbin et al. 2003).

Our own study showed a crucial role of GCM1 in EVT differentiation. Silencing of GCM1 in a first trimester EVT explant model inhibited invasive trophoblast outgrowth formation without inhibiting CYT proliferation (Baczyk, Drewlo et al. 2009). These data suggested that GCM1 is a key placental transcription factor that acts in both the villous and EVT pathways to allow cells to exit the cell cycle and differentiate to either SYN or invasive EVT. As such,
dysregulation of GCM1 is likely to contribute to the pathologies of pregnancy. The regulation and function of GCM1 are discussed in the following section.

1.3 **Glial Cell Missing 1**

GCM1 belongs to a small family of unique zinc binding transcriptional regulators; it was originally identified in *Drosophila*, guiding the development of neuronal precursor cells into glial cells (Hosoya, Takizawa et al. 1995). In mammals GCM1 regulates placental labyrinth formation (Anson-Cartwright, Dawson et al. 2000) and human trophoblast differentiation (Baczyk, Drewlo et al. 2009), whereas the closely homologous GCM2 is involved in parathyroid gland development (Gunther, Chen et al. 2000). Other than placental tissue, GCM1 is also expressed in mouse kidney and in thymus (Hashemolhosseini, Hadjihannas et al. 2002).

1.3.1 **GCM1 structure and target genes**

The GCM1 transcription factor consists of about 500 amino acid residues. The N-terminal (about 150 residues) is a highly conserved region among different species, as it contains a unique GCM1 binding motif. The transactivating regions are located in a very poorly conserved region of the C-terminal of the protein (Schreiber, Sock et al. 1997).

The GCM1 protein binding motif (A/G)CCC(T/G)CAT (or its complement) (Schreiber, Enderich et al. 1998) has been identified but to date only a few GCM1 targets have been recognized, namely aromatase, leptin, syncytin and PlGF (Schreiber, Sock et al. 1997; Yamada, Ogawa et al. 1999; Yu, Shen et al. 2002; Chang, Mukherjea et al. 2008). It should be noted that all of these molecules exhibit specific syncytial expression. A possible interaction between GCM1 and Pitx2 in mouse placenta and kidney has also been reported (Schubert, Kardash et al. 2004).

1.3.2 **GCM1 regulation**

GCM1 expression and activity can be regulated at many levels. For instance, GCM1 transcriptional activation via protein kinase A (PKA) and CREB
binding protein (CBP) (forskolin or cAMP treatment) has been well demonstrated (Chang, Chuang et al. 2005) (Baczyk, Drewlo et al. 2009). GCM1 activity can also be regulated by post-translational modifications, protein-protein interactions and autoregulation. In particular, histone acetylation can prolong GCM1 protein stability and function (Chang, Chuang et al. 2005) whereas histone deacetylase 3 binding to GCM1 negatively regulates GCM1 activity (Chuang, Chang et al. 2006). GCM1 protein has a short half-life of only about 30 min to 2 hrs (Tuerk, Schreiber et al. 2000) as it is ubiquitinated by hFBW2 and degraded by 26S proteasome (Yang, Yu et al. 2005). In addition, hypoxic conditions result in the specific phosphorylation of GCM1 promoting its ubiquitination and degradation via SCF<sub>FBW2</sub> E3 ligase (Chiang, Liang et al. 2009). Positive autoregulation of its own promoter has been described in <i>Drosophila</i> (Miller, Bernardoni et al. 1998) and humans (Chiang, Liang et al. 2009).

### 1.3.3 GCM1 function in placentation

Recently, we demonstrated that GCM1 controls the balance between VCT proliferation and differentiation. Silencing of the gene increases VCT proliferation at the expense of differentiation into SYN whereas forskolin stimulated GCM1 expression induces syncytialization at the expense of CYT proliferation (Baczyk, Drewlo et al. 2009). Furthermore, GCM1 has been shown to regulate a number of SYN specific proteins such as Syncytin 1 and 2 (Yu, Shen et al. 2002). It also has divergent expression patterns in placental pathologies such as severe preterm IUGR (increased) and PE (decreased) (Chen, Chen et al. 2004) (our unpublished observations), but its regulation is poorly understood. Recently, our <i>in silico</i> GCM1 promoter analysis identified a novel calcium dependent transcription repressor – DREAM, as a potential candidate involved in GCM1 regulation. Thus, the focus of this thesis is to determine the role of DREAM in regulation of GCM1 expression and villous trophoblast turnover in human placenta.
The next sections will summarize the current knowledge of the DREAM transcription factor and its possible implications to human placental development and function.

1.4 Downstream regulatory element antagonist modulator (DREAM)

1.4.1 Structure

DREAM (also know as KCNIP-3 and calsenilin) belongs to a family of small neuronal calcium sensors (Burgoyne and Weiss 2001). It contains 256 amino acids and 9 exons (mass of 29kDa) however, the human placenta expresses only a specific spliced transcript variant containing 230 amino acids and 8 exons. Both isoforms encode a protein containing four calcium binding domains (Carrion, Link et al. 1999) (Figure 7).

![Diagram of DREAM Structure and Alternative Transcripts](image)

**Figure 7. Structures and alternative transcripts of human DREAM gene.** Note that a caspase 3 cleavage site is present in the N-terminal of the protein. Appears here with the permission from the journal (Pruunsild and Timmusk 2005).

1.4.2 Expression

Gene expression analysis revealed DREAM expression in human brain, thyroid, thymus and testis (Carrion, Link et al. 1999), human placenta, kidney,
heart, and stomach (Pruunsild and Timmusk 2005). Most studies focus on DREAM’s role and function in brain as DREAM has been shown to regulate Alzheimer’s disease-associated proteins, presenilins (Buxbaum, Choi et al. 1998). DREAM is expressed in different cellular compartments (nucleus, cytoplasm and cell membrane) and thus is involved in three distinct biological pathways. This thesis focuses on DREAM’s function as a calcium dependent transcriptional regulator.

1.4.3 Function

Originally, DREAM was discovered to interact with presenilin-2 in a two-hybrid screen, and thus was named calsenilin (Buxbaum, Choi et al. 1998). DREAM processes presenilin 1 and 2 to modulate amyloid β-peptide levels (Buxbaum, Thinakaran et al. 1998) (Cheng, Pitcher et al. 2002).

DREAM is also referred to as potassium-channel interacting protein (KCNIP-3) because it interacts with A-type voltage-gated potassium channels (Kv4.2) in a calcium dependant fashion. This interaction regulates trafficking of Kv4.2 to the cell membrane and thus their gating properties (An, Bowlby et al. 2000). G protein-coupled receptor 2 (GRK2) phosphorylates DREAM at Ser-95 to inhibit Kv4.2 potassium channel activity. This phosphorylation, however does not affect DREAM repressor activity (Ruiz-Gomez, Mellstrom et al. 2007).

Thirdly, DREAM is a calcium-dependent transcriptional repressor. The prodynorphin gene was the first described target of DREAM’s negative regulation (Carrion, Link et al. 1999). Prodynorphin is an opioid hormone expressed in striatum (part of cerebrum) that is involved in pain sensing, learning and memory. It is also expressed in endometrium where it is thought to regulate cell differentiation and tissue remodeling as well as modulating local immune responses during early pregnancy (Gravanis, Makrigiannakis et al. 2002).

Finally, DREAM plays a role in the inhibition of apoptosis through regulation of c-fos (Carrion, Mellstrom et al. 1998) and the proapoptotic protein gene Hrk (Sanz, Mellstrom et al. 2001). More recently, Matsuda et al., showed that DREAM negatively regulates calcitonin, a polypeptide produced in the
thyroid that is involved in regulation of calcium homeostasis (Matsuda, Yamamoto et al. 2006).

DREAM knock out mice do not have any apparent physical abnormalities, though they do exhibit decreased sensitivity to pain (Cheng, Pitcher et al. 2002) as well as enhanced memory (Alexander, McDermott et al. 2009). Regrettably, the only two organs analyzed in the knock out mice were brain and heart; placental pathology was not noted.

1.4.4 Calcium dependence

DREAM is the first known calcium binding transcriptional regulator and thus its function in human placenta is very intriguing. Over gestation, the placenta actively transports up to 30 grams of maternal calcium into the fetal circulation to meet the needs of fetal skeletal development (Brunette 1988). Calcium binds directly to DREAM changing its conformation and ability to bind to the Downstream Regulatory Element (DRE) site; furthermore, mutations of the calcium binding EF hands (helix-loop-helix calcium binding motif) impede the DREAM response to calcium (Carrion, Link et al. 1999).

DREAM contains four EF-hands but, just like other members of the recoverin family, it is only capable of binding calcium to 3 EF hands (EF 2-4) (Ikura, Osawa et al. 2002). Magnesium has also been found to interact with DREAM protein. Under low physiological calcium concentrations and high DREAM protein concentration (at least 20 μM to 200 μM) DREAM forms a tetramer that is capable of binding to DRE sites within its promoter targets. In a high calcium environment, calcium binds to DREAM and changes its conformation to a dimer. The DREAM dissociation constant is >10μM intracellular Ca^{2+}. These dimers are stable at very low protein concentrations but dissociate from the promoter and leave the nucleus for cytoplasmic targets (Ikura, Osawa et al. 2002) (Figure 8).
In view of the fact that DREAM is an active transcriptional repressor in its tetrameric form Osawa et al., investigated the binding of DREAM to the DRE sites. Their investigations found that each DREAM tetramer binds to four closely located DRE sites. Furthermore, they speculated that one of the DRE sites has the highest affinity for DREAM (preferential and probably biologically active), while the other three DRE binding sites have much lower affinity for DREAM (Osawa, Tong et al. 2001).

The C-terminal residues (65-256) of the DREAM molecule are essential for calcium-dependent transcriptional regulation whereas N-terminal regions are not. Interestingly, DREAM contains a caspase-3 cleavage site at residues 61-64, thus suggesting that DREAM might be an active transcriptional repressor even following caspase-3 cleavage (Choi, Zaidi et al. 2001) (Figure 7). In the placenta, caspase-3 cleavage is associated with commitment of CYT cells along an apoptotic pathway (Huppertz, Frank et al. 1998).

Figure 8. Calcium regulated transcriptional repression by DREAM. Under low physiological calcium concentrations (left half of the schematic) the tetrameric form of DREAM binds to DRE sequences within a promoter region. At high calcium concentrations (right half), calcium binds to DREAM changing its conformation to a dimer resulting in its dissociation from the promoter thus de-repressing transcription. Modified from Ikura et al., 2002 (Ikura, Osawa et al. 2002).
1.4.5 Potential role of DREAM in pathophysiology of placental insufficiency syndromes

To date the role of DREAM has not been investigated in the placenta but its possible function in GCM1 transcriptional regulation and specifically its calcium dependence merits further examination in healthy and pathological pregnancies. As noted in the earlier sections of this introduction, placental pathologies are associated with altered GCM1 expression levels and there are also reports of changes in calcium homeostasis in these pathologies. PE in particular is associated with symptoms such as hypocalcaemia and hyperuricaemia (Punthumapol and Kittichotpanich 2008). Whereas, IUGR babies have reduced bone mineralization in infancy and osteoporosis in adulthood and this phenomenon may be related to the alterations in ATP dependent calcium transport (Strid, Bucht et al. 2003). In view of the above, we believe that DREAM might play an important role in pathophysiology of placental insufficiency.

The following sections discuss pathophysiology of the placental-related disorders as well as the contributions of abnormal VCT turnover and EVT invasion in clinical conditions associated with poor pregnancy outcomes.

1.5 Placental insufficiency syndromes

Placental insufficiency refers to inadequate blood flow to the baby (shallow EVT invasion and spiral artery remodeling) and/or failure of the placenta to adequately supply the growing fetus (poor chorionic villi development). Collectively these common complications of pregnancy affects up to 25% of all gravid women. (www.mtsinai.on.ca/care/placenta-clinic). Certain medical conditions or habits can increase the risk of placental insufficiency, including: diabetes, high blood pressure, obesity, age and smoking.

1.5.1 Preeclampsia

One of most common placental insufficiencies is PE. PE is a life-threatening disorder that can only be effectively reversed by removal of the
placenta, thus often requiring preterm delivery of the fetus. Some of the clinical features of preeclampsia include hypertension, proteinuria, edema and seizures. Most cases (80% of affected women) are termed “late-onset” or “maternal” PE, where induction of labor near term will result in a healthy baby, though the maternal disease may progress before eventual recovery. The second more severe phenotype termed “early-onset” or “placental” PE is associated with high perinatal mortality and morbidity and is often accompanied with IUGR (Redman and Sargent 2005).

1.5.1.1 Pathophysiology of severe PE

It is generally accepted that inadequate placentation is a predisposing factor of pathophysiology of the early onset type of PE. Early in placental development, failure of maternal spiral artery remodeling by EVT cells results in placenta hypoxia. This leads to the release of proinflammatory cytokines and antiangiogenic factors such as sFLt-1 (Dimitrakova, Dimitrakov et al. 2004) that damage maternal circulation at the midpoint of pregnancy. On the other hand, expression of proangiogenic vascular endothelial growth factor VEGF and placental growth factors (PIGF) is decreased (Torry, Wang et al. 1998) (Wikstrom, Larsson et al. 2007). As the pregnancy progresses, the vascular damage escalates, thereby worsening the symptoms of PE and ultimately requiring premature delivery of the baby.

Histologic examination of placental villi from PE patients reveal syncytial bridges (Tenney-Parker changes) suggesting trophoblast hyperplasia (Kingdom and Kaufmann 1997), induction of CYT proliferation (Arnholdt, Meisel et al. 1991; Brown, Lacey et al. 2005) and increased syncytial shedding. SYN may shed as microparticles (MP) or exosomes (Redman and Sargent 2008). MP shedding is a calcium-dependent physiological phenomenon associated with membrane remodeling and plays a role in cell to cell communication, modulation of inflammation, angiogenesis and clotting function (Redman and Sargent 2008). In PE, in particular, SYN MP can damage endothelial cells as well as enhance systemic inflammatory responses (Redman and Sargent 2008).
A recent review by Burton and Jones, rejected the long standing hypothesis of excessive syncytial, apoptotic shedding in PE (Burton and Jones 2009). Firstly, they point out that syncytial knots are a physiological phenomenon in term placenta found on 10-30% of term villi. Their occurrence is only slightly increased in PE but strongly increased in senescent 42 week placentas (Fox 1965). Furthermore, approximately 100,000 non-apoptotic syncytial sprouts (often mistaken for syncytial knots) enter the maternal circulation each day without an apparent maternal reaction. Secondly, they show evidence of limited transcriptional activity within the SYN layer previously assumed transcriptionaly inactive (Ellery, Cindrova-Davies et al. 2009). The authors thus propose that the "degenerating trophoblast" material shedding into the maternal circulation is necrotic as apposed to apoptotic in nature and is only shed under pathologic conditions thus contributing to the pathophysiology of PE.

1.5.1.2 Treatment

Currently, the only successful treatment of PE is the early delivery of the fetus and the placenta. In most severe cases patients are treated with magnesium sulfate at the onset of labour and continued for 24 hrs postpartum (Witlin and Sibai 1998). Calcium ion transport can also be prevented with the use of a calcium channel blocker, Nifedipine (Magee, Cham et al. 2003); both magnesium sulfate and Nifedipine are thought to induce vasodilatation.

1.5.1.3 Prevention of PE

Use of low-dose aspirin (60-150mg/d) in 14 randomized trials proved to reduce the risk of PE (14 trials, OR 0.86, 95% CI, 0.76-0.96) but did not improve the birth weight of the babies (Coomarasamy, Honest et al. 2003). Results from randomized control trials of vitamin C and E supplementation were disappointing showing no significant improvement in the rates of PE (Kontic-Vucinic, Terzic et al. 2008). Calcium supplementation on the other hand has been shown to prevent PE and its related symptoms. Calcium reduces the risk of PE (12 trials, RR 0.48, 95% CI 0.33 to 0.69) and high blood pressure (11trials, RR 0.70, 95%
Cl 0.57 to 0.86) (Hofmeyr, Atallah et al. 2006). More recently, the benefits of heparin treatment on the prevention of the disease are being investigated (Rey, Garneau et al. 2009) and ongoing trial (HEPRIN) at Mount Sinai Hospital, Toronto, Canada.

1.5.2 Intrauterine Growth Restriction

Like PE, IUGR is also a devastating placental pathology but it is given far less attention and thus our understanding of the disease is limited. It is a fact however that, IUGR babies weighting less then 5th % for their gestational age are predisposed to lifelong risks of hypertension, cardiovascular and renal disease (Murphy, Smith et al. 2006). Histopathological examinations of IUGR placentas revealed abnormalities in uterine spiral artery remodeling, increased apoptotic syncytial knot presence, reduced CYT proliferation, thus compromising SYN functions (reviewed in (Scifres and Nelson 2009). IUGR placentae are also characterized by smaller size, reduced number of terminal villi (Krebs, Macara et al. 1996) (Toal, Keating et al. 2008) further reducing the feto/maternal surface area required for nutrient and waste exchange. IUGR placentae are not adept at sustaining the growing fetus frequently resulting in premature delivery to avoid in utero demise.

PE and IUGR are two divergent pathologies with alterations in VCT turnover and EVT invasion. Furthermore our recent (unpublished) results indicate increased GCM1 expression levels in IUGR in contrast to PE.

1.6 Rational of the project

GCM1 is the only known transcription factor regulating both villous and extravillous trophoblast development, and both GCM1 and its target genes have altered expression in placental pathologies. Thus increased understanding of its transcriptional regulation is of key importance to the placental development field. My in silico analysis of the GCM1 promoter revealed the existence of DRE binding sites; the calcium-dependent transcriptional repressor DREAM is known to bind to these sites. Additionally, the importance of calcium signaling, calcium
transport across the placenta and its positive effects in PE prevention trials also suggest DREAM to be an excellent candidate for further investigation in this thesis.

1.7 Models used in the study

Two in vitro models were used in this study: the choriocarcinoma derived cell line, BeWo and first trimester floating placental explants. BeWo cells are a useful tool for studying syncytialization as they are the only trophoblast cell line capable of cell-cell fusion; furthermore fusion can be enhanced with addition of forskolin, cAMP or ionomycin, all acting via protein kinase A. Additionally these cells are relatively easy to maintain and transfect unlike the second model – first trimester placental explants. Our recent results (Baczyk, Dunk et al. 2006; Baczyk, Drewlo et al. 2009) demonstrate it to be an excellent in vitro/in vivo model to study trophoblast turnover in their three dimensional environment with connection to the stroma.

1.8 Objective and aims

The overall objective of this project is to determine the role of DREAM in GCM1 expression and villous trophoblast turnover in human placenta. Specific aims include:

1. Investigation of DREAM expression during human placenta development
2. Investigation of DREAM and GCM1 interaction
3. Investigation of DREAM’s role in GCM1 regulation
4. Investigation of altered intracellular calcium concentrations on DREAM activity
5. Investigation of DREAM expression levels in preeclampsia
2.1 In vitro models

2.1.1 BeWo cells

The human choriocarcinoma cell line BeWo was purchased from ATCC, (Manassas, VA). Only cells between passages 2-20 were used for the experiments. The trophoblastic cell line was maintained in F12K medium (ATCC) supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2.5 µg/ml fungizone (Invitrogen, Burlington, ON), in atmospheric O₂/5% CO₂ at 37°C.

2.1.1.1 Transfection of BeWo cells

For DREAM siRNA treatment or DREAM overexpression assays, 100,000 BeWo cells per well in 1 ml of regular media were plated into 12 well plates (Sarstedt, Montreal, QU) 24 hours prior to transfection. Transfections were performed in serum and antibiotic free OPTI MEM medium (Invitrogen). 50 nM of DREAM siRNA, 50 nM non-silencing control (Cat # sc-42398, sc-37007; Santa Cruz Biotechnology, Santa Cruz, CA) or 1 µg of DREAM overexpression plasmid (pcDNA3.1 V5-His-TOPO vector; kind gift from Dr. Timmusk, Tallinn, Estonia) was transfected into the cells with 2 µl of Lipofectamine transfection reagent (Invitrogen) in a total volume of 260 µl for 5 hrs after which time 500 µl of F12K medium with 20% FBS was added. 24 hrs post transfection media was replaced with 1 ml of regular media. At the conclusion of the experiments RNA was extracted from the cells using RNeasy kit (Qiagen, Mississauga, ON) according to the manufactures recommendations.

2.1.1.2 Ionomycin and Nimodipine treatment

Ionomycin is an ionophore, commonly used in research to increase intracellular calcium levels. 1000 µM stock of ionomycin (USB Corporation, Cleveland, OH) was prepared in water and stored at 4°C. Nimodipine binds to and blocks L-type voltage-gated calcium channels, 1000 µM stock (Calbiochem, La Jolla, CA) was prepared in 70% ethanol and stored at -20°C. For treatment of
BeWo cells and placental explants, compounds were added to the media at the final 1 μM concentrations. Water and 0.007% ethanol were used as vehicle control for ionomycin and nimodipine respectively.

2.1.2 First trimester placental explants

Placental samples from first and second trimester were obtained from Morgentaler Clinic, Toronto, Canada, following a voluntary legal termination of pregnancy. Research Ethics Board approval was obtained for this study and all patients gave written informed consent. Termination of pregnancy samples were ultrasound-dated and confirmed on conceptus or fetal measurements but no additional information was made available. Tissue was collected into ice-cold HBSS+/+ and transported on ice. All experiments were carried out within 4-6 hrs from the time of sample collection.

2.1.2.1 Floating first trimester placental explant model

Individual clusters of 8-12 week gestation villi (20-30 mg wet wt) were dissected in sterile cold PBS+/+, under a microscope. The proximal stem villi were inserted into the underside of sterile polystyrene cubes so as to float the villous trees in 750 μl of serum-free media (DMEM/F12) with 1% liquid media supplement ITS+1 (Sigma, St Louis, MO, USA), 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine, 100 μg/ml gentamycin and 2.5 μg/ml fungizone. These floating villous explants were maintained in physiological 8% ambient oxygen (40 mmHg)/5% CO₂ at 37°C (Figure 9) (Miller, Genbacev et al. 2005).

Figure 9. Human floating 1st trimester villous explant culture model. Floating villous explant as seen under the dissecting microscope (A) and diagrammatic representation of explant culture set up (B). Adopted from (Miller, Genbacev et al. 2005).
For DREAM siRNA treatment, floating villous explants were incubated in the presence of 100 nM siRNA or 100 nM non-silencing control (Santa Cruz Biotechnology) for up to 2 days.

2.1.2.2 Cytotoxicity assays

Cellular toxicity in treated explants and BeWo cells was assessed with CytoTox96 assay (Promega, Nepean, ON) measuring LDH release into the media. No significant differences were found between and treated and control groups (data not shown).

2.1.2.3 Placental villous explant immunohistochemistry

Placental explants were fixed in 1 ml of 4% PFA for 2 hr at room temperature (RT). Following 3 x 15 min washes in PBS the placental samples were dehydrated in ethanol series. Samples were sequentially rocked in 70%, 80%, 90%, 95% and two times in 100% ethanol for 30 min at RT; then placed in xylene for 10 min. Finally samples were immersed in warm paraffin 3 x 30 min and then embedded in paraffin. Embedded explants were sectioned to 5 μM and adhered to Superfrost Plus Plus slides (Fisher, Ottawa, ON). Prior to immunohistochemistry slides were cleared in xylene and rehydrated in descending gradient of ethanol series, followed by PBS washes. Slides were incubated in 0.3% H₂O₂ in methanol for 30 minutes to quench endogenous peroxide activity. Antigen retrieval was accomplished with high heat 10 mM sodium citrate buffer (pH 6) treatment for 45 min. Following PBS washes (2x 5 min) slides were incubated at RT with blocking solution (DAKO, Mississauga, ON) for 1 hr. Primary antibodies (outlined in Table 1) were incubated on sections overnight at 4°C. Secondary biotinylated antibodies (1:300) were incubated on the slides for 1 hr at RT, following PBS washes (3X5 min), slides were incubated with Streptavidin-HRP (1hr, RT) followed by DAB detection (Vector, Burlington, ON). Following wash in tap water, the tissue was lightly counterstained with Harris’s Hematoxylin. Tissue sections were dehydrated by sequential immersion
of slides in ascending ethanol series. Following xylene clearing, slides were cover-sliped with Cytoseal mounting media (Cole-Parmer, Vernon Hills, IL).

For fluorescent immunohistochemistry, secondary antibodies and Streptavidin used were conjugated to Alexa 488 and/or Alexa 546 (Invitrogen). Slides were incubated with the secondary antibodies (1:300) and DAPI nuclear stain (5 μg/1ml) for 1 hr at RT in the dark. Following PBS washes (3X 5min) slides were cover-sliped using IMMU-MOUNT mounting media (Thermo Scientific, PA) and stored at 4°C.

Chromogenic images were captured using a Leica DMRX microscope, Sony DX970 colour video camera and Northern Eclipse (version 8.3) software. Fluorescent images were captured using a Sony Interline iCX285ER progressive scan camera and an Olympus 1X70 microscope. Images were collected using Resolve3D Image acquisition software and deconvolved using Deltavision softWoRx 2.50 software.

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Dilution</th>
<th>Source</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>DREAM</td>
<td>1:100</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Ki67</td>
<td>1:100</td>
<td>Rabbit</td>
<td>Thermo Scientific</td>
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<tr>
<td>PCNA</td>
<td>1:100</td>
<td>Mouse</td>
<td>Cell signaling</td>
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<td>1:100</td>
<td>Mouse</td>
<td>Roche</td>
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<td>1:300</td>
<td>Goat</td>
<td>DAKO</td>
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<tr>
<td>FITC anti-rabbit</td>
<td>1:200</td>
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<td>Jackson Laboratories</td>
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Table 1. Antibodies and dilutions used for placental tissue immunohistochemistry.

2.2 Semi-thin sections

Explants were collected into 2% glutaraldehyde and sent to Pathology and Laboratory Medicine at Mount Sinai Hospital, Toronto, Canada, where they were embedded and sectioned (1μm) and toluidine blue stained. This work was performed by Mr. Doug Holmyard.
2.3 TUNEL assay

TUNEL labeling was performed in the Pathology Unit at the Centre for Modeling Human Diseases by Mr. Qiang Xu.

2.4 BrdU incorporation and immunodetection

Placental explants were cultured in the presence of 10 μmol BrdU (Roche, Germany) for the duration of the experiment. At the conclusion of the experiment, explants were fixed and processed as described in section 2.1.2.3.

For the immunodetection of BrdU, sections were dewaxed and dehydrated as described in section 2.1.2.3. Following 2 x 5 min PBS wash, slides were treated with 30 μg/ml Proteinase K (Roche) solution for 10 min at 37°C followed by treatment with 10% (v/v) solution of 10N HCl in H₂O for 10 min at room temperature. PBS washed slides were then incubated with 1:100 anti-BrdU antibody (Roche kit # 11 299 964 001) for 2 hrs at 37°C. Slides were then washed in PBS 3 x 5 min and incubated in anti-mouse biotin-labeled secondary antibody for 1 hr at room temperature (DAKO). Tissue slides were washed in PBS again and then incubated in Streptavidin-HRP (DAKO) complex for 1hr at RT. Signal was detected using DAB substrate (Vector) according to the manufacturer's instructions. Slides were counterstained with Hemotoxilin, dehydrated and cover-slipped with mounting medium.

2.5 Quantitative real time-PCR

2.5.1 Tissue collection

First and second trimester placental samples were collected from the Morgentaler Clinic into 2 ml of RNA Later buffer (Ambion, Streetsville, ON) and stored at −20°C. Third trimester and term placental samples, from patients delivering at Mount Sinai Hospital, Toronto, Canada were collected into RNA Later buffer or snap frozen and stored −70°C. Patient information is summarized in Table 2.
2.5.2 RNA extraction

Snap frozen samples were crushed using a mortal and pestle while submerged in liquid nitrogen. Crushed placental samples and RNA later stored samples were homogenized in 1 ml of TRIzol RNA extraction reagent (Invitrogen). Homogenized tissue was incubated on ice for 30 min and then centrifuged to remove cellular debris. Following addition and shaking with 100 μl of chloroform RNA was separated with 15 min 8,000 x g centrifugation step. The upper aqueous phase was collected into a new tube and precipitated by addition of 400 μl of isopropanol and -80°C overnight incubation. The following day RNA was pelleted by centrifugation (12,000 x g for 15 min). The crude RNA preparation was further purified using Qiagen isolation and purification kit (Reynolds, Borowicz et al. 74134) according to the manufacturer’s specifications. Briefly, RNA was DNase treated by passing it through a genomic DNA eliminator column. Following washes with RW1 and RPE buffers, RNA was eluted from the column using 35 μl of water. RNA quantity and quality was measured with a Nanodropper spectrophotometer (Thermo Scientific).

2.5.3 Reverse transcription

DNAse treated RNA (1μg) was reverse transcribed according to manufacturer’s instructions (Applied Biosystems, Streetsville, ON). Samples were incubated at 25°C for 10 min, 42°C for 30 min and 95°C for 5 min in the
presence of 1X reaction buffer, 1mM MgCl₂, 50 μM random hexamers, RNA inhibitor, 0.2 mM dNTPs and 0.2mM MultiScribe™ Reverse Transcriptase.

2.5.4 Quantitative real time PCR

Real time PCR was performed on an Eppendorf epgradientS Master Cycler, in triplicates in 15 µl volumes containing 10 ng of template cDNA, 7.5 µl of 2X SYBR Green PCR Master Mix (Bilban, Ghaffari-Tabrizi et al.) and 50 nM of primers. The PCR program was initiated at 95°C for 2 min, followed by 40 thermal cycles of 15 seconds at 95°C, 60 seconds at 60°C. A melting curve for primer validation and a template standard curve were performed to show template independent amplification results. To visualize amplification products PCR reaction cocktails were electrophoresed on 1.5% agarose gel in Tris-acetate/EDTA buffer. As a negative control, cDNA template was omitted from the PCR reaction. Comparative C₇ Method (ABI technical manual) was used to analyze the real time PCR. The expression of DREAM, GCM1, hrk genes was normalized to the geometric mean of SDHA and TBP genes (Bieche, Laurendeau et al. 1999) and expressed as fold change relative to non-silenced (NS) control.

2.5.5 Primers

Intron-spanning primers were designed specifically for human GCM1 and DREAM using Primer Express 2.0 software (Bilban, Ghaffari-Tabrizi et al.). TBP and SDHA sequences were obtained from Bieche et al. (Bieche, Laurendeau et al. 1999), Hrk primers were a kind gift from Dr. Jurisicova (Toronto); all sequences used are shown in Table 3.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| GCM1      | Forward 5'-ATGGCACCTCTAGCCCCTACA-3'  
|           | Reverse 5'-GCTCTTTCTTCGCTCAGCTTCAA-3' |
| DREAM     | Forward 5'-TGGAAGATAGCATCGACATTG-3'  
|           | Reverse 5'-AAGCCCCCTGTAGAGAGACTGC-3' |
| hrk       | Forward 5'-CAGGCGGAACCTTAGGAAC-3'  
|           | Reverse 5'-TCTCCAGGACACAGGGTTT-3'  |
| SDHA      | Forward 5'-TGGAACAAGAGGCTCTG-3'  
|           | Reverse 5'-CCACCCTGCATCAAATTCTG-3' |
| TBP       | Sequences are described in (Bieche, Laurendeau et al. 1999) |

Table 3. Quantitative real time PCR primer sequences.

2.6 Image analysis and quantification of placental tissue sections

Human placenta tissue from four gestation age groups 6-9 wks (n=4), 16-19 wks (n=3), 32-33 wks (n=3) and 37-38 wks (n=3) were immunostained with DREAM antibody and counterstained with Hematoxylin Solution as described in section 2.1.2.3. Utilizing a Leica DM4500B microscope an Olympus DP70 camera as well as Visiopharm Integrator System (Version 3.0.8.0, Horsholm, Denmark) 20 random images of each slide were generated. Each image was scored for nuclear trophoblastic (CYT and SYN) DREAM expression and total number of trophoblast nuclei. The results are expressed as the % of positive nuclear DREAM trophoblast nuclei per total number of trophoblast nuclei.
2.7 DNA/protein interactions

2.7.1 Promoter analysis

Five thousand base pairs 5’ region of human GCM1 promoter was subjected to analysis by Gene2Promoter Software (Version 6.3, Genomix, Germany) to identify potential binding sites. The software recognized four potential DRE binding sites within that region (See Figure 10, green bars). Detailed analysis of the promoter also revealed numerous DRE core sequences – GTCA (blue bars).

![Figure 10. Diagrammatic representation of 5000 bp region of the human GCM1 promoter region. In silico analysis predicted 4 DRE binding site (shown in green) and all other DRE core sequences are shown in blue. Five sets of primers (1-5) were designed to amplify the promoter region.]

2.7.2 Chromatin Immunoprecipitation (ChIP)

DREAM protein and GCM1 promoter interactions were investigated with the aid of commercial ChIP assay kit (Upstate, CA, USA; catalog # 17-371). The assay was performed according to the manufacture’s instructions with some modifications as described below; all of the reagents used were supplied in the kit unless stated otherwise. DREAM over-expressing BeWo cells and DREAM siRNA treated cells (see section 2.1.1.1 for the transfection conditions) were grown to 100 % confluency. Cells were then treated with 1% fresh formaldehyde (Fisher) for 10 minutes at RT in order to crosslink the proteins to the DNA. Following crosslink treatment, PBS-washed cells were lysed in SDS
lysing buffer and sonicated on wet ice (10 sets of 20-second pulses using High Intensity Ultrasonic Sonicator, 50-watt model, set to 25% of maximum power). One percent of the sonicated DNA/protein fraction was saved as an Input control for further analysis, while the remainder was subjected to immunoprecipitation with: 1) his-Tag antibody (Santa Cruz), 2) DREAM isoform 2 specific antibody (Millipore, MS), as well as 3) RNA polymerase antibody (positive control) and 4) rabbit IgG (negative control). Following a pull-down with Protein G Agarose beads, DNA/protein complexes were eluted from the beads and reversed crosslinked under high salt and high temperature conditions (65°C for 5 hrs). Proteins were degraded with Proteinase K treatment for 1.5 hrs and finally DNA was purified using the provided spin columns. The purified DNA was used as a template for PCR reactions. Anti-RNA polymerase fraction was amplified with supplied primers (positive control) and Input DNA and IgG immunoprecipitated fractions were amplified with experimental primers (Figure 10 in Introduction and Table 4).

<table>
<thead>
<tr>
<th>Amplicon number</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward 5'-GATCACCTGAGGTCAGGAGT-3'</td>
<td>527</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGACTGTCCCATGACAGA-3'</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Forward 5'-GATAGGGGTGCTGTATGATCA-3'</td>
<td>532</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTGTGACATGATGTCACACTG-3'</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Forward 5'-GACATGCCTGACATGTTAACAGT-3'</td>
<td>634</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCGACAGGGAATTAGCAATCAC-3'</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Forward 5'-AGCTACTCAGGAGGTCGAGGCA-3'</td>
<td>343</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAATATCCTGGGACACATGACG-3'</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward 5'-GCAATCTCAGCTCACGACCC-3'</td>
<td>781</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAGCAGACGCTGTTCCCTATC-3'</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. PCR primer sequences used in ChIP analysis.
2.7.2.1 PCR amplification of immunoprecipitated fragments

One μl each of input fraction, RNA polymerase immunoprecipitated fraction, DREAM and IgG immunoprecipitated fractions was subjected to PCR using either provided (positive control) or designed primers (See Table 4). PCR reactions were set up in total 20 μl volume with 1X complete PCR cocktail mix (Fermentas, Burlington, ON) and 0.5 μM of appropriate primers. The PCR program was initiated at 95°C for 3 min followed by 30 cycles of 95°C for 20 sec, 59°C for 30 sec and 72°C for 30 sec and final extension of 2 min at 72°C.

2.8 Electromobility Shift Assay (EMSA)

2.8.1 Probe preparation

DREAM and GCM1 interactions were further investigated and confirmed with the use of EMSA. Five PCR amplicons from ChIP analysis (Table 4) were 3' end biotin labeled using a kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Briefly 100 nM of DNA was biotin labeled with enzyme terminal deoxynucleotidyl transferase at 37°C for 30 min. Shorter, 43 bp, 3’ end biotin labeled probe and 6 probes containing mutations in the DRE core sequences were purchased (IDT, Skokie IL) (Table 5).

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Probe sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>TGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 1</td>
<td>TGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 2</td>
<td>TGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 3</td>
<td>TGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 4</td>
<td>CGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 5</td>
<td>CGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 6</td>
<td>CGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
<tr>
<td>Mutation 7</td>
<td>CGACAGGTACTGATCCCTCTGCTATGTGTGGCGATATGTA</td>
</tr>
</tbody>
</table>

Table 5. 3’ end biotin labeled probes used in EMSA assay. Core DRE sequences are shown in bold and introduced mutations in red.
2.8.2 Nuclear protein extraction

Nuclear proteins were extracted from BeWo cells either treated with DREAM siRNA or overexpressing DREAM plasmid. Cells were collected into a buffer containing 25 mM HEPES, 5mM KCl and 0.5 mM MgCl₂ solution and then lysed while rotating for 15 min in a buffer containing 25 mM HEPES, 5mM KCl and 0.5 mM MgCl₂ and 1% NP-40 resulting in a cytoplasmic protein preparation. To obtain the nuclear protein fraction the pellet was further lysed for 1 hr at 4°C in a buffer containing 25 mM HEPES, 10 (w/v) sucrose, 350 mM NaCL and 0.01% NP-40. Protein amount was quantified using the Bradford method.

2.8.3 Probe/protein binding and electrophoresis

BeWo cell nuclear fraction was incubated with biotin labeled human GCM1 promoter probes for 30 min at RT in a reaction containing the following components:

- Nuclear extract (5 μg) 2 μl
- 5X binding buffer* 2 μl
- Poly d(I-C) (1 μg/μL) 1 μl
- Biotin probes 50 ng/μL 1 μl
- H₂O 4 μl

* buffer contained: 50 mM Tris-HCl (pH 8.0), 750 mM KCl, 2.5 mM EDTA, 0.5 Triton-X 100, 62.5% glycerol and 1 mM DTT.

For competition with “cold” unlabeled probes, unlabeled probes were added to the reaction. For supershift with anti- DREAM and anti-HIS tag antibodies, 0.5 μg of the antibody was added to the reaction before adding labeled probe and incubated for 30 min at RT.

Five percent non-denaturating TE-polyacrylamide gel and 0.5 X TE buffer were cooled at 4°C and the gel was run at 120 V for 30 min prior to loading the gel for equilibration. Protein/probe mix was run at 100 V in the cold room at 4°C for 2 hrs.
2.8.4 Transfer and detection

Gel components were transferred to a nylon membrane (NEN Life Science Products, Boston, MA) using a semi-dry transfer apparatus (OWL HEP-1, Thermo Scientific) at 200 mA for 1 hr. Following the transfer, the bound probes were immobilized by exposing the membrane to UV crosslinking. Detection of the biotin probes was accomplished with Chemiluminescent Nucleic Acid Detection Module according to the manufacturers instructions (Pierce). Briefly, following blocking the membrane was incubated with stabilized streptavidin-horseradish peroxidase conjugate and detected with chemiluminescent substrate solution and subsequently exposed to X-ray film.

2.9 Statistical analysis

Experiments were performed in at least triplicates with three technical replicates. Unless otherwise stated, data was calculated as mean +/- standard deviation using Microsoft Excel. One-way ANOVA and the Tukey's post-hoc test were used to compare between multiple treatment groups. Data was normalized relative to the negative treatment. All statistical calculations were performed using SigmaStat® 3.1 software and p values less than or equal to 0.05 were considered significant.
RESULTS
3.1 Investigation of DREAM expression pattern in the human placenta throughout pregnancy

To date, the localization and distribution of DREAM protein has not been investigated in human placenta, thus the first aim of this project was to examine DREAM immunostaining in human placenta of first, second and third trimesters.

3.1.1 Gestational profile of DREAM immunolocalization in the human placenta

In first trimester human placenta, DREAM immunofluorescence appeared predominantly localized to nuclei of CYT cells, although a subset of SYN nuclei were also found positive. In the second trimester, DREAM immunostaining was detected in all three cell compartments; stroma and the trophoblast layer. At term the expression persisted in the stroma and trophoblast layer but appeared weaker, more diffused and predominantly cytoplasmic (Figure 11).

Figure 11. DREAM immunofluorescence in human placenta of first, second and third trimester. Confocal microscopy of PFA fixed placental tissues with nuclear stain DAPI (blue). Magnifications = 400X
First trimester EVT columns also show strong immunopositivity for DREAM (Figure 12).

![DREAM immunofluorescence in first trimester EVT column. Magnifications = 400.](image)

### 3.1.2 Quantification of nuclear trophoblast DREAM staining

DREAM plays a role in all cellular compartments; its functions in cytoplasm, cell membrane and nucleus have been reported previously (as discussed in the introduction); however this thesis evolved exclusively around DREAM’s role in transcriptional regulation of villous trophoblast layer. Thus we embarked on monitoring the nuclear DREAM expression within the trophoblast layer only. For that reason, nuclear trophoblast DREAM expression levels throughout gestation were measured by counting the number of trophoblast nuclei with positive DREAM immunostaining and expressed as a percentage of total trophoblastic nuclei. The analyses were performed in randomly selected sections using Visiopharm Integrator System (as described in section 2.6) and results are summarized in Figure 13. In first trimester (6-9 wks) almost 40% of trophoblastic nuclei are DREAM immunopositive. With the progression of gestation nuclear immunopositivity declines and is significantly lower from first trimester group in 32-33 wks and 37-38 wks groups.
3.1.3 DREAM mRNA levels decrease across gestation

DREAM mRNA levels in human placenta were quantified using qRT PCR. Four gestational groups (n = 5-11; 5 \* five placentas with 5 random sampling sites each) were analyzed using relative method, normalized to TBP and SDHA (housekeeping genes) and calibrated to the 37-38 wks gestational group. DREAM mRNA levels were significantly elevated in second and early third trimester groups as compared to first trimester and term. The highest DREAM mRNA levels were observed in 13-20 wks group (9 fold increase over late third trimester) (Figure 14).
3.2 **Investigation of DREAM and GCM1 interaction**

*In silico* analysis (Genomix software) of the 5 kb region of 5’ human GCM1 promoter identified 4 potential DREAM binding sites. My detailed analysis of the promoter region also revealed additional DRE core sequences (GTCA or reverse) as discussed in section 2.7.1 (Figure 10).

3.2.1 **Chromatin Immunoprecipitation assay**

To investigate DREAM protein and GCM1 promoter interactions, we employed the ChIP assay (see section 2.7.2, in the Methods chapter for details). DNA and protein from BeWo cells were formaldehyde cross-linked, DNA was sonicated and immunoprecipitated using positive control antibody, rabbit IgG antibody and DREAM short isoform specific antibody. Immunoprecipitated fractions were reverse cross-linked and subjected to PCR amplification with five different sets of PCR primers (Table 4) designed to amplify regions in the GCM1 promoter. PCR amplifications using primer sets 1, 4 and 5 contained amplicons suggesting DREAM binding within those regions of the promoter. The strongest amplification was observed with primer set number 4 (Figure 15). The same results were obtained in experiments performed when HIS tagged DREAM was
overexpressed in BeWo cells and immunoprecipitated with anti-HIS antibody (data not shown).

3.2.2 Electromobility Shift Assay

To further investigate the interaction between DREAM protein and GCM1 promoter EMSA was performed on nuclear extracts from the DREAM silenced and HIS-tab labeled DREAM overexpressing BeWo cells (see section 2.1.1.1 for details of the transfection procedure). GCM1 promoter probes were prepared by 3’ end biotin labeling amplicons from 5 primer sets used in ChIP analysis (See Table 4, section 2.7.2 of the methods chapter). The promoter probes varied in size from 343 bp to 781 bp. Labeled probes were also competed with “cold” probes in 1:10 ratio. Results are shown in Figure 16. Binding of DREAM to the GCM1 promoter probes can be seen in lanes 1 and 4. A similar pattern was observed by EMSA using DREAM-silenced BeWo cells though the signal was significantly less, presumably due to the reduction in DREAM expression levels.
ChIP assay and EMSA both point to interaction between DREAM protein and GCM1 promoter within a 343 bp area of region 4. *In silico* analysis did not reveal DRE sites within region 4 but careful examination (blasting for DRE core sequence) uncovered 4 core DRE binding sites located within a 43 bp area. To test whether DREAM effectively bound this discrete region within the GCM1 promoter, short biotin labeled probes specific to region 4 were purchased. In addition, a number of mutations (as outlined in Table 5, section 2.8.1) within the newly identified DRE core sites were introduced with the intention of abolishing the binding of DREAM to the GCM1 promoter. The specificity of the binding was confirmed using a "supershift assay" whereby anti-DREAM, anti-HIS tag and non-specific antibodies were added to the DNA/protein complex. Results are shown in Figure 17.

The short probe (43 bp - control) showed the same binding pattern (Figure 17A) as the 343 bp long probe (Figure 16) thus confirming that DREAM binds to this specific small region of the GCM1 promoter. The specific signal was outcompeted by using "cold probe" (1:100 and 1:1000). As expected, a much weaker signal was observed in nuclear extracts from DREAM silenced cells.
Additionally the specificity of the binding was confirmed with a “supershift” assay. Addition of anti-DREAM (B) and anti-HIS (A) tag antibodies to the binding reaction resulted in large protein/DNA/antibody complex. In fact, the complexes were too large to migrate into the gel and are seen in the wells.

To examine the affinity of DREAM protein binding to the individual 4 DRE sites, various mutations were introduced. Firstly, single mutations in individual DRE sites were created (mutations 1-4). Secondly, single (mutation 5) and double mutations (mutation 6) in all 4 of the DRE sites were introduced. Lastly, a control (mutation 7) with a single mutation outside the DRE binding sites was included (See Table 5 of methods chapter for details). Single mutations of each DRE sequence did not appear to affect the affinity of DREAM protein binding to the GCM1 promoter. Reduced affinity however, was observed with probes containing multiple mutation sites (Figure 17B). In conclusion, EMSA identified a specific 43 bp region of GCM1 promoter that strongly binds DREAM. The affinity of DREAM protein to this area of the promoter is specific and very strong as revealed by “supershift” and mutation studies.
3.3 Investigating the role of DREAM in GCM1 regulation

Thus far, this thesis has confirmed the presence of DREAM mRNA and protein in human placenta across gestations as well as direct DREAM protein interaction with a specific region of the GCM1 promoter. The goal of the next set of experiments was to investigate the role of DREAM in the regulation of GCM1 expression using functional studies in BeWo cell culture as well as first trimester floating villous explant models.

3.3.1 siRNA mediated DREAM repression upregulated GCM1 mRNA levels in BeWo cell model

Treatment of BeWo cells with a cocktail including 50 nM DREAM siRNA effectively reduced DREAM within 48 hrs of transfection. Utilizing qRT PCR it
was shown that DREAM mRNA levels were 70 % down-regulated compared with non silenced control cells by 72 hrs post transfection. At the same time, GCM1 mRNA levels exhibited significant 2.5 fold upregulation (Figure 18).

![Figure 18. siRNA mediated DREAM silencing in BeWo cells. DREAM was successfully silenced in BeWo cells (70% by 72 hrs). GCM1 mRNA levels were 2.5 fold upregulated by 72 hrs post transfection. Results were monitored with qRT PCR. N=4, *** p< 0.01](image)

### 3.3.2 DREAM overexpression in BeWo cells

DREAM was successfully overexpressed in BeWo cells using a DREAM overexpression vector (kind gift from Dr. Timmusk). 24 hr after transfection DREAM mRNA levels were 5,130 (+/- 2,520) fold upregulated as assessed by qRT PCR. The levels remained high at 48 hrs post transfection (6,170 +/- 2,130 fold). The overexpression of DREAM mRNA coincided with significant downregulation of GCM1 mRNA levels at 48 hrs post transfection (Figure 19).
3.3.3 siRNA treatment of first trimester placental villous explants

To confirm the results obtained from the BeWo cell culture model we also conducted functional studies in first trimester floating villous placental explant model.

3.1.1.1 siRNA repression of DREAM upregulates GCM1 mRNA levels in first trimester placental explants

8-12 week placental explants were cultured in the presence of 100 nM DREAM siRNA cocktail or 100 nM non silencing control for up to 48 hrs. Treatment of explants with DREAM siRNA resulted in 50% DREAM mRNA inhibition after 2 day in culture and over 3 fold GCM1 mRNA upregulation at the same time point (Figure 20).

![Figure 19. DREAM overexpression in BeWo cells. Successful DREAM overexpression in BeWo cells resulted in GCM1 mRNA repression. N=4 ***p<0.01, *p<0.05]
3.1.1.2 Histological assessment of DREAM siRNA treated explants

DREAM siRNA treated explants were examined using histological tools. DREAM immunohistochemistry was performed on the explants to additionally confirm the inhibition of DREAM expression. DREAM siRNA treated explants show much weaker immunopositivity towards DREAM antibody as compared to non silencing explants. Furthermore, in treated explants DREAM staining was predominantly localized to the cytoplasm and membranes unlike the control counterpart (Figure 21 A,B). Ki-67 (a proliferative marker) immunostaining found altered pattern of expression in the treated explants. DREAM siRNA treated explants show markedly reduced rate of Ki-67 staining in the trophoblast layer but not the stroma (Figure 21 C,D).

Figure 20. DREAM siRNA treatment of first trimester floating placental villous explants. DREAM silencing resulted in over 3 folds upregualtion of GCM1 mRNA levels. N=5, * p<0.05 *** p< 0.01

![Graph showing relative mRNA expression levels of DREAM and GCM1](image)
3.1.1.3 Modifications to trophoblast morphology in DREAM siRNA treated placental explants (semi-thin sections)

Histological assessment of DREAM treated explants revealed modifications to trophoblast morphology which was further examined using toluidine blue stained, semi-thin sections. Following two days of culture, non-silencing control explants retain a continuous single layer of CYT cells underneath an intact syncytial layer. In DREAM siRNA treated explants, the CYT layer becomes dis-continuous with some CYT showing nuclear condensation and the overlying syncytial layer contained a single to double layer of new, large and
pale euchromatic nuclei suggesting either inhibition of CYT proliferation, induction of apoptosis or accelerated SYN differentiation (Figure 22).

3.1.1.4 Assessment of proliferation and apoptosis in DREAM siRNA treated explants

DREAM had been reported to play a role in the inhibition of apoptosis through negative regulation of pro-apoptotic protein gene hara-kiri (Hrk) (via interactions with Bcl-2 and Bcl-X(L)) in hematopoietic progenitors (Sanz, Mellstrom et al. 2001). Thus the Hrk mRNA levels were monitored in the DREAM siRNA treated explants with two objectives. First, to confirm DREAM’s functional repression following siRNA treatment, and second to assess the rate of apoptosis in these explants. Hrk mRNA levels could not be detected in the control explants; in effect, the gene could only be detected in DREAM siRNA treated explants following 48 hr of exposure (Figure 23). Relative quantitative measurements of Hrk could not be performed as the non silencing explants did
not have detectable levels of the Hrk mRNA. Products of the Hrk qRT-PCR reactions were separated on agarose gel (Figure 23).

The goal of the following experiments was to further assess the rate of trophoblast proliferation and/or apoptosis in DREAM siRNA treated explants. Hence, apoptosis level in siRNA DREAM treated and control explants were monitored with TUNEL staining which identifies the biochemical hallmark of apoptosis—internucleosomal DNA fragmentation. Very few TUNEL positive cells were detected in the time = 0 control or in the non-silencing control, while DREAM siRNA treated cells displayed a very strong TUNEL staining within the trophoblast layer (Figure 24).
The rate of CYT proliferation in the DREAM siRNA treated explants was measured using BrdU proliferation index and expressed as the % of BrdU positive trophoblastic nuclei. As compared to non silencing control (100%); 2 days treatment with 100 nM DREAM siRNA resulted in significant reduction in the rate of CYT proliferation (37 +/- 16%) (Figure 25).

Positive TUNEL staining results, reduced BrdU incorporation and the induction of expression of the pro-apoptotic gene Hrk in DREAM siRNA treated explants, all point to DREAM’s function in positive regulation of CYT proliferation. In the next set of experiments, I co-localized DREAM and a proliferation marker PCNA in healthy first and third trimester placental tissue using confocal microscopy. Dual immunofluorescence revealed their nuclear co-localization in CYT cells (Figure 26) indicating DREAM potential involvement in the regulation of CYT cell proliferation.
3.4 Investigation of altered intracellular calcium concentrations on DREAM activity

DREAM is a calcium dependent transcriptional repressor thus its activity was studied under altered intracellular calcium concentrations utilizing two chemicals that divergently alter intracellular calcium concentration; ionomycin – calcium ionophore and nimodipine – L-type voltage gated calcium channel blocker. These chemicals were employed in two culture models to alter intracellular calcium concentration.

3.4.1 Intracellular calcium level modification by nimodipine and ionomycin treatment of BeWo cells

BeWo cells were cultured in the presence 1 μM nimodipine or 1 μM ionomycin for 24 hrs. The treatment with the two chemicals did not affect DREAM mRNA levels. Nimodipine treatment, however, resulted in significant reduction (40%) in GCM1 mRNA levels whereas ionomycin treatment resulted in
significant increase of GCM1 mRNA levels (30%) demonstrating DREAM’s functional calcium dependence (Figure 27).

3.4.2 Intracellular calcium level modification by nimodipine and ionomycin treatment in first trimester floating placental explants

Ionomycin and nimodipine stimulation experiments were also performed in
first trimester placental floating explants cultured for 48 hrs.

### 3.4.2.1 DREAM and GCM1 mRNA levels

Similar to the BeWo experiments, treatment of the explants with either nimodipine or ionomycin did not influence DREAM mRNA levels. In contrast to the BeWo experiments, nimodipine treatment of the explants did not significantly reduce GCM1 mRNA levels. However, ionomycin treatment of the explants, did result in significant 3 fold up regulation of GCM1 mRNA as assessed by qRT-PCR (Figure 28).

![Figure 28. GCM1 mRNA levels in nimodipine and ionomycin treated first trimester placental floating explants. DREAM and GCM1 mRNA levels as assessed by qRT-PCR. N= 4 * p<0.05](image)

### 3.4.2.2 DREAM subcellular localization changes with intracellular calcium levels

Previous reports indicate that the transcriptional capacity of DREAM is calcium dependent. Under low calcium concentration DREAM attains a tetramer form and associates with the DRE sequences found within a promoter; whereas, in a high calcium environment DREAM changes conformation to a dimer and translocates to the cytoplasm. To further test this hypothesis we performed DREAM immunostaining in explants treated with 1 μM ionomycin or 1 μM nimodipine for 48 hrs. Immunostaining studies revealed predicted DREAM translocation in response to changes in intracellular calcium levels. In
nimodipine treated explants DREAM expression was predominantly localized in the nuclei of the CYT cells similarly to the non treated cultured control. By contrast, the calcium ionophore treated explants reveal a striking pattern of expression consistent with the translocation of the DREAM to the cytoplasm or cell membranes of CYT cells (Figure 29).

Control        Nimodipine        Ionomycin

Figure 29. Nimodipine and ionomycin treatment of first trimester placental explants results in DREAM translocation. DREAM immunostaining of 1 μM nimodipine and 1 μM ionomycin treated 9 wk placental explant for 48 hrs.

3.4.2.3 Proliferation of CYT cells in response to nimodipine and ionomycin treatment

To further investigate the effects of nimodipine and ionomycin treatment on first trimester placental explants and in particular trophoblast turnover, I performed proliferation studies. Explant culture media was supplemented with BrdU and following fixation and embedding the explant tissue, slides were subjected to anti-BrdU immunohistochemistry. The rate of proliferation was expressed as the percentage of positive trophoblastic nuclei per total trophoblast nuclei and compared to the respective controls (100%). Nimodipine stimulation of the explants for 48 hrs has no effect on the rate of trophoblast proliferation as
compared to its control. Ionomycin treatment, however significantly reduced (over 50%) the rate of trophoblast proliferation (Figure 30).

![Figure 30. Altered cytotrophoblast rate of proliferation in nimodipine and ionomycin treated first trimester placental explant. N=5 *p<0.05]

### 3.5 Investigation of DREAM expression levels in PE

GCM1 expression levels are altered in placental pathologies, PE in particular is associated with reduced levels of GCM1. As our results point to DREAM as a potential GCM1 regulator, we determined DREAM expression pattern in PE. Severe early onset PE tissues and aged matched controls were immunoassayed for DREAM. Diffused staining in the entire tissue with individual stronger nuclear expression in the trophoblast layer was observed in control placentas. In contrast, PE samples revealed very strong nuclear expression of DREAM in the trophoblast layer; and in particular in the syncytial knots, a pathologic characteristic of PE, which exhibited the most intense DREAM immunostaining (Figure 31).
In order to quantify the expression levels of DREAM in PE, qRT-PCR was performed. As compared to the age matched controls, PE placentae exhibit over 4 fold up regulation in DREAM mRNA levels confirming our immunohistochemistry observations (Figure 32).
DISCUSSION
This thesis defined the expression and function of a novel transcriptional regulator, DREAM, in the human placenta. Furthermore, involvement of this calcium dependent transcriptional factor in the control of GCM1 expression in human placenta was validated. Using a loss of function approach in two culture models, the choriocarcinoma cell line BeWo and first trimester placental explants, we were able to demonstrate DREAM negatively regulated transcription of GCM1 and thereby trophoblast proliferation. Upregulation of DREAM expression in the BeWo cell model resulted in inhibition of GCM1 mRNA expression. Placental development and function rely on a delicate balance of proliferation, differentiation and apoptosis of CYT cells. Placental insufficiencies such as IUGR and PE are associated with alteration in the villous trophoblast turnover and EVT remodeling of spiral arteries. PE and IUGR are associated with divergent expression of GCM1. GCM1 levels are reduced in severe PE placentas (Chen, Chen et al. 2004) and elevated in severe IUGR (our unpublished observations) which we have shown controls the balance of proliferation and differentiation of both villous and extravillous pathways of CYT development (Baczyk, Drewlo et al. 2009). Understanding the mechanisms that govern CYT cell survival in the human placenta, and the selective expression of GCM1 in CYT destined for syncytial fusion or in EVT remodeling of spiral arteries will improve our understanding of the early origins of severe IUGR and PE caused by so-called “placental insufficiency”. In the course of this thesis we identified a negative regulator of GCM1 expression in human trophoblast cells – DREAM.

**DREAM immunolocalization**

DREAM immunofluorescence was detected in the trophoblast layer of first trimester placentas. Second trimester placentas showed strongest DREAM immunopositivity in all three cell compartments in the trophoblast layer and stroma. DREAM expression persists to term and furthermore it is also observed in EVT columns, suggesting an important role for DREAM in human placental development and function.
The focus of this thesis was to determine the role of DREAM in the regulation of GCM1 expression and villous trophoblast turnover. Since DREAM can only impose its potential role as a transcriptional regulator of CYT cells when localized to the nucleus, we undertook a study to quantity the levels of nuclear DREAM expression in the trophoblast layer of human placenta over gestation. The study showed that trophoblast nuclear expression levels were the highest during early gestation. Correlation of these results with the rapid proliferation and expansion of villous CYT cells suggests an active role for DREAM in early placental development ensuring sustained trophoblast proliferation. During the second and third trimester the mesenchymal cell expansion results in villous tree changes to form the mature intermediate and terminal villi as endothelial cell proliferation exceeds trophoblast proliferation and the capillaries within the villi loop and coil (Benirschke K 2005). These changes correlate with the increase in mesenchymal DREAM expression and a decrease in nuclear DREAM expression in trophoblast layer in the second and third trimesters, suggesting that DREAM may also be involved in regulation of proliferation in mesenchyme. As GCM1 is not found in placental stroma, DREAM must be acting through a different mechanism. This study demonstrated that differential DREAM expression may regulate proliferation of trophoblast as well as mesenchymal cells throughout gestation, to meet the demands of the rapidly growing and changing organ.

**DREAM-mediated GCM1 regulation alters trophoblast turnover**

Functional studies in the BeWo cell culture model indicated that DREAM is a negative regulator of GCM1 expression. Additionally, the experiments performed in the human floating villous explant model demonstrated that alterations in DREAM expression impacted GCM1 mRNA levels, CYT proliferation, differentiation and apoptosis. In first trimester floating villous explants, reduced DREAM expression resulted in elevated levels of GCM1 mRNA expression, inhibition of CYT proliferation (assessed by BrdU incorporation) but did not cause degeneration of the overlying SYN following 48 hr of culture as assessed by histology (semi-thin sectioning). DREAM siRNA treatment in these explants however did induce apoptosis (evidenced by upregulation of the pro-apoptotic
protein Hrk and positive TUNEL staining in the trophoblast layer). Therefore, we speculate that a sustained (>48hrs) downregulation of DREAM expression is required to induce apoptosis and degeneration of the SYN layer. Conceivably, the reduced levels of DREAM expression in human placenta may contribute to the IUGR phenotype, characterized by a) elevated level of GCM1 (our unpublished observations), b) reduced rate of remaining CYT proliferation due to excessive CYT differentiation and c) formation of apoptotic syncytial knots (Scifres and Nelson 2009). We gained strong evidence to suggest the involvement of DREAM in the regulation of CYT proliferation and apoptosis; however future quantitative studies are required to accurately assess the level of SYN differentiation in our DREAM siRNA treated explants. We speculate that DREAM inhibits villous CYT differentiation, by repressing GCM1 expression, and thus DREAM siRNA treated explants would be expected to have accelerated rate of differentiation.

By repressing GCM1, DREAM may play a role in the retention of mitotic activity of progenitor CYT and furthermore inhibition of differentiation into SYN. This possibility is supported by the results of our dual immunohistochemistry of DREAM and proliferation marker PCNA which found their co-localization in the nuclei of CYT cells in first and third trimester placentas. The results from this thesis, suggest that nuclear localization of DREAM in CYT cells promotes their proliferation, inhibits their differentiation into SYN (via inhibition of GCM1 expression) and furthermore restrains apoptosis. DREAM siRNA treatment of first trimester explants resulted in 50% downregulation of DREAM mRNA levels but more interestingly triggered DREAM translocation from the nucleus to the cytoplasm and membrane (Figure 21 B). Ikura et al., point out that the tetrameric form of DREAM is stable only at concentrations of at least 20 μM (Ikura, Osawa et al. 2002); This suggests that our DREAM siRNA treatment of explants likely reduced DREAM protein level below 20 μM resulting in change in conformation to a dimer and translocation from the nucleus. The dimeric form of DREAM is stable at low protein concentrations (Ikura, Osawa et al. 2002) but can not function as a nuclear repressor.
Molecular mechanism of DREAM and GCM1 interaction

*In silico* analysis of the 5' region of the human GCM1 promoter identified potential DREAM binding sites (DRE). Our ChIP and EMSA studies of the GCM1 promoter directly identified a 43 bp area containing 4 DRE sites exhibiting very high affinity for DREAM protein. The transcriptional repressor activity of DREAM occurs via interaction with the promoter regions of a number of genes including, prodynorphin (Carrion, Link et al. 1999), calcitonin (Matsuda, Yamamoto et al. 2006) and thyroglobulin (Rivas, Mellstrom et al. 2004) was reported previously. Data presented in this thesis are novel in that we have identified four closely located palindromic DRE sites within a single gene (in comparison to the previously reported studies which identified only 1 or 2 DRE sites). These observations suggest that regulation of GCM1 gene expression is mediated by strong DRE-DREAM interaction within this 43 bp area located in 5' region of human GCM1 promoter.

**Calcium-dependent DREAM activity**

Utilizing two independent culture models, we determined that alterations in intracellular calcium levels affect DREAM transcriptional function. Stimulation of BeWo cells with the L-type voltage gated calcium channel blocker nimodipine resulted in downregulation of GCM1 mRNA levels, by contrast stimulation of BeWo cells and placental explants with the calcium ionophore ionomycin resulted in significant upregulation in GCM1 mRNA levels and thus inhibition of CYT proliferation. These observations suggest that the upregulation of GCM1 expression over gestation (Baczyk, Satkunaratnam et al. 2004) and decreased rate of CYT proliferation in later gestation may be linked to increased intracellular calcium levels and thus reduced DREAM activity. A previous report has correlated elevated levels of intracellular calcium with the increased rate of CYT differentiation (Moreau, Hamel et al. 2002); however this is the first report to specifically identify the possible molecular mechanism of calcium regulated trophoblast turnover. Our results indicate that low intracellular calcium levels promote CYT proliferation whereas elevated levels of calcium, as observed in later gestation, promote CYT differentiation. Thus the changes in trophoblast
DREAM expression levels, as well as DREAM protein localization (dependent on intracellular calcium levels) that occur over gestation, might control the rate of trophoblast turnover.

**DREAM expression in PE**

Importantly, our investigation of DREAM expression in placental pathology PE, revealed significant upregulation of this transcriptional repressor. This observation correlates with the previous report demonstrating placental GCM1 downregulation in this maternal disease (Chen, Chen et al. 2004) and our *in vitro* data where downregulation of GCM1 in villi, recapitulates the features of severe PE (Baczyk, Drewlo et al. 2009). Conversely, placental GCM1 levels are upregulated in IUGR (our unpublished observation). Furthermore, high levels of DREAM protein were observed in post-mitotic syncytial knots in placentas from PE pregnancies (Figure 31). Since the syncytial layer has limited transcriptional activity (Ellery, Cindrova-Davies et al. 2009) it is possible that DREAM accumulates in the syncytial knots following CYT cell fusion. It is also possible that DREAM expression is preserved in the SYN, as a previous report indicated that DREAM retains its active transcriptional repression function even following caspase-3 cleavage (Choi, Zaidi et al. 2001).

**Significance of the study**

GCM1 activity has been shown to be regulated at the post-translational level by acetylation, phosphorylation (Chang, Chuang et al. 2005) sumoylation and ubiquitination (Chou, Chang et al. 2007) but its molecular regulation is still poorly understood. At the transcriptional level GCM1, was shown to be regulated via a functional cAMP responsive element binding motif in the promoter region upstream of the start codon (Knerr, Schubert et al. 2005). GCM1 was shown to regulate the expression of different proteins such as the placental growth factor PIGF (Chang, Mukherjea et al. 2008) a human aromatase (Yamada, Ogawa et al. 1999) and the fusion proteins Syncytin 1 and 2. (Yu, Shen et al. 2002) A GCM1 binding motif was also found in the promoter of the human chorionic gonadotrophin alpha subunit. (Yamada, Ogawa et al. 1999) These findings support a complex and important role for GCM1 in placental cellular maintenance.
and differentiation and thus merit our efforts investigating its poorly understood molecular regulation.

Understanding the mechanisms that govern CYT cell survival in the human placenta, and the selective expression of GCM1 in CYT destined for syncytial fusion or in EVT remodeling spinal arteries will improve our understanding of the early origins of severe IUGR and PE caused by so-called “placental insufficiency”. We hypothesize that the morphologic consequences of DREAM and thus GCM1 alterations in floating villi are relevant to the development of the pathologic disease states in pregnancy. In the severe form of preterm IUGR the floating villi are small, under-developed (Jackson, Walsh et al. 1995; Krebs, Macara et al. 1996) and show some evidence of reduced numbers of villous cytotrophoblast. (Macara, Kingdom et al. 1996) In the related disorder of severe early-onset PE, placentas revealed an increase in proliferative activity of CYT cells. (Arnholdt, Meisel et al. 1991; Brown, Lacey et al. 2005). These observations suggest that in these pathologies there may be a misbalance between CYT proliferation and SYN formation that could be regulated by DREAM.

Explants, in which DREAM expression was down-regulated by siRNA treatment exhibited upregulation in GCM1 mRNA levels, a reduced rate of CYT proliferation and induction of apoptosis. These parallels the observations in placentas from pregnancies complicated with IUGR and could prove a valuable model of this disease. In this study we did not quantify DREAM expression in placentas from IUGR patients but since GCM1 levels are upregulated in this pathology, we infer the levels of DREAM transcriptional repressor may be reduced.

Additional characteristics of PE and IUGR include alterations in calcium homeostasis. Consequently, DREAM, as a newly described calcium dependent transcriptional repressor, could play a very significant role in calcium signaling in the human placenta. Calcium is an intracellular messenger regulating a number of cellular functions such as proliferation, differentiation, necrosis and apoptosis. (Berridge, Lipp et al. 2000) Calcium is exceptionally versatile, exerting its function
over a wide range of concentrations with the help of ‘tool kit’ components, which include pumps, buffers, exchangers and effectors. Typically Ca\(^{2+}\) regulates cell cycle progression through a number of pathways, including regulation of ras activity (Cullen and Lockyer 2002). On the other hand, calcium-signaling is also involved in cell differentiation through calcium sensing receptors (Monteith, McAndrew et al. 2007). Finally, excessive Ca\(^{2+}\) accumulations in mitochondria are associated with necrosis and/or apoptosis (Rizzuto, Pinton et al. 2003).

It is estimated that in the SYN total cytoplasmic calcium concentration is 1000-fold higher than free calcium levels (Greer 1994). To achieve this level the placenta must contain a large pool of calcium binding proteins. Presently only a handful of such proteins have been identified in human placenta including S100 calcium binding proteins, calbindin D9k, calbindin D28k, human calcium-binding protein, oncomodulin and placental 57-kDa calcium binding protein (reviewed in great detail in (Lafond and Simoneau 2006)). We propose that DREAM with its exceptionally high affinity for calcium could also act as a calcium binding protein. Our immunohistological observations support this hypothesis as DREAM expression becomes less nuclear and more cytoplasmic with increased gestational age. To meet the increasing demands of the growing fetus and bone mineralization third trimester placentas up regulate calcium transport.

It is unclear at this point whether alterations in calcium homeostasis in placental pathologies are a result of inadequate calcium serum levels (diet), parathyroid function, placental calcium sensing and/or its transport across the trophoblast layer. Presently, no link between placental insufficiency and parathyroid function exist and only limited observations suggest that PE might be associated with hypothyroid function during pregnancy. A recent report however, proposes that elevated thyroid function in PE and increased risk of developing hypothyroidism following pregnancy is a result of high levels of sFLT-1 production, (Levine, Vatten et al. 2009), a significant prognostic indicator of PE. Interestingly, calcium supplementation has been shown to prevent PE and its related symptoms (Hofmeyr, Atallah et al. 2006). Calcium is transported across the placenta via a number of calcium channels. The presence and the function
of L-type voltage gated calcium channels has been confirmed in the human placenta (Cemerikic, Zamah et al. 1998). There is also limited evidence for the role of T-type channels within the human placenta (Yunker and McEnery 2003), the presence and/or function of which might be of great interest as they were shown to be induced by chronic hypoxia (Del Toro, Levitsky et al. 2003) (common condition of PE). Importantly this thesis demonstrated DREAM to be a clinically relevant transcription factor, in placental insufficiency disorders, since its function can be modulated pharmacologically or by diet.

4.1 Conclusion

This thesis expands on work conducted in the Kingdom and Lye Laboratories on the role of GCM1 in trophoblast turnover; in particular it elaborates upon GCM1 transcriptional regulation via the calcium-dependent transcriptional repressor DREAM. In conclusion this thesis identifies the calcium dependent transcription repressor DREAM as a key repressor of the transcription factor GCM1. DREAM exerts its actions by binding to a specific 43 bp region in the GCM1 promoter. DREAM expression is highest in the second and third trimesters of placental development correlating to the window of placental expansion and development of the functional units of the placenta - the intermediate and terminal villi. Moreover, DREAM subcellular localization changes over gestation, correlating to the changes in the total intracellular calcium concentration, from nuclear localization in the first trimester to cytoplasmic in the third. Correlation of this cellular localization with its regulation of GCM1 suggests that DREAM represses GCM1 allowing CYT proliferation in the first trimester, while its cytoplasmic localization in the last third of the pregnancy suggest it may act as a calcium binding protein. Finally this thesis demonstrates that DREAM levels are high in severe PE placentas. This finding provides a molecular explanation for the reported decrease in GCM1 and the abnormal CYT, SYN turnover in these placentas. This thesis makes a significant contribution toward advanced understanding of the regulatory mechanisms of
this important factor for proper placental establishment, development and function.
FUTURE DIRECTIONS
The data presented in this thesis describe a role for calcium-dependent transcriptional repressor – DREAM in transcriptional regulation of GCM1. ChIP and EMSA techniques utilized in this thesis identified interactions between DREAM and DRE elements within 5’ region of the human GCM1 promoter. These methods defined the site of this interaction to a minimal 43 bp sequence. This interaction is very strong but whether it is calcium dependent remains to be determined. Thus, we propose to perform EMSA on nimodipine and ionomycin treated BeWo cells to prove that indeed DREAM-DRE binding in the GCM1 promoter is calcium-dependent. Furthermore, quantitative assessment of the promoter activity is required. In such experiments, we would utilize luciferase assay where fragments (43 bp with and without introduced mutations) of GCM1 promoter region would be cloned into a luciferase reporter plasmid. Following a transient transfection of BeWo and HeLa cells, the cell lysed would be assessed for luciferase activity.

As the overall goal is to advance our understanding of GCM1 regulation, we propose to undertake DNA modifications studies of the GCM1 gene. Firstly, we will analyze single nucleotide polymorphisms (SNIP), which are known to influence the expression and function of genes. We propose to compare significant number of well defined pathology cases (PE and IUGR) and appropriate controls. We plan to interrogate the entire GCM1 gene, but we will focus our attention on a 43 bp sequence containing DREAM binding sites. Secondly, in collaboration with Dr. Weksberg, we intend to investigate epigenetic modifications of GCM1 in pathologies and healthy controls. Epigenetic modifications such as methylation can stably alter gene expression pattern by physically inhibiting the binding of transcription proteins to the gene or by recruiting additional histone deacetylases and thereby modifying and silencing chromatin.

In this thesis, the effect of DREAM siRNA treatment on the morphology and trophoblast turnover in first trimester floating villous explants was examined but quantitative measurement of the rate of CYT differentiation needs to be further assessed. Towards this goal, we propose to measure the levels of secreted hCG
(ELISA) in the media of treated BeWo cells and placental explants. Furthermore, the effects upon EVT trophoblast proliferation, differentiation and migration were not undertaken in this thesis. DREAM is expressed in EVT cells thus we hypothesize that it also plays a significant role in the development of this trophoblast cell lineage. We intend to fully characterize the role of DREAM in EVT proliferation/differentiation. Therefore EVT explants will be DREAM siRNA treated, embedded, sectioned and immunoassayed for Ki67 to assess proliferation and α5 and α1 integrins to assess the invasion. Conditioned media collected from treated explants will also be subjected to MMP ELISA to determine changes in MMP activity. In addition, calcium-dependence of DREAM should also be investigated in the EVT explant model via stimulation with calcium blocker – nimodipine and calcium ionophore – ionomycin. The explants with altered intracellular calcium concentrations would then similarly to DREAM siRNA treated explants, be examined for changes in trophoblast proliferation and differentiation. A second culture model – an invasive cell line JAR, could also be employed to further confirm the findings from the placental explant model. The cell line could also be used for DREAM over-expression studies (technically challenging in explant model).

In this thesis DREAM expression levels were only assessed at the mRNA level since the commercially available DREAM antibodies did not perform well in WESTERN blot analysis. In the near future, however; we anticipate the availability of other antibodies, especially short isoform specific, to aid with protein quantification task.

While we provide compelling evidence of elevated levels of DREAM in PE, additional studies of severe early onset IUGR placentas are required. We hypothesize that placentas from IUGR pregnancies would have reduced DREAM expression. Moreover, we intend to analyze DREAM expression and activity levels, GCM1 expression and trophoblast turnover (rate of proliferation, differentiation and apoptosis/necrosis) in placentas of patients with impaired calcium metabolism to gain deeper understanding of the role of calcium signaling in placental development and function.
The processes that mediate villous trophoblast turnover and EVT differentiation remain to be elucidated; however the results presented in this thesis and future proposed research in this area stand to contribute to our understanding of the mechanisms regulating these important events in vivo. Placental insufficiencies resulting in PE, IUGR or still birth are associated with alterations in villous trophoblast turnover and failure of EVT differentiation and invasion. Through an advanced understanding of the underlying molecular regulation that causes these events, we may be better able to devise treatment strategies for these pathologies.
REFERENCES


