DISTAL-LESS 3 ACTS AS A GLIAL CELL MISSING-1 REPRESSOR IN REGULATING PLACENTAL GROWTH FACTOR EXPRESSION IN HUMAN TROPHOBLAST CELLS

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Sha Li

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DISTAL-LESS 3 ACTS AS A GLIAL CELL MISSING-1 REPRESSOR IN REGULATING PLACENTAL GROWTH FACTOR EXPRESSION IN HUMAN TROPHOBLAST CELLS

Sha Li, Ph. D.

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Placental growth factor (PGF) is highly expressed within the placenta and plays important roles in trophoblast development and placental angiogenesis. Serum levels of PGF are increased throughout pregnancy under normal conditions but are dysregulated in human pregnancies complicated with preeclampsia (PE); hence, circulating PGF has been proposed as a biomarker to predict PE. Therefore, understanding the regulation of *PGF* is highly desirable for unraveling pathogenesis of PE and developing potentially therapeutic strategies for the treatment of PE in women.

In this study we described regulation of *human PGF* (*hPGF*) in the JEG-3 human trophoblast cell line by the transcription factors Distal-less 3 (DLX3) and Glial-cell missing-1 (GCM1). For the first time, we demonstrated that DLX3 induced *hPGF* expression through acting directly on the *hPGF* promoter. Interestingly, when expressed with another *hPGF* regulator GCM1, DLX3 showed inhibitory effects on the transactivation-stimulating activity of GCM1, resulting in an antagonistic regulation on *hPGF* expression. Nevertheless, siRNA-mediated knockdown studies indicated both DLX3 and GCM1 were required for *hPGF* expression. These studies

went on to identify a sequence on the *hPGF* promoter that was crucial for both DLX3- and GCM1-induced *hPGF* expression; chromatin-immunoprecipitation revealed occupancies of both factors at this novel *cis* element. Deletion mutation studies of this sequence suggested this element also controlled the basal expression of *hPGF*.

To further characterize the transcriptional inter-play between DLX3 and GCM1, we performed mammalian one hybrid analyses where DLX3 was shown to physically interact with GCM1. Consistent with this finding, reciprocal immunoprecipitation studies in JEG-3 cells demonstrated these two factors physically interacted. In addition, functional domain analyses of DLX3 suggested DLX3 associated with GCM1 through its DNA binding homeodomain, whereas the DLX3 amino and carboxyl domains were likely to facilitate and stabilize this interaction.

Taken together, our studies have demonstrated that DLX3 works in concert with GCM1 to regulate *hPGF* expression in the human trophoblast cell line. We further identified a functional interaction of the DLX3/GCM1 transcription pair, which might act as a fine tuning mechanism to tightly control *hPGF* from overexpression, thereby potentially avoiding excess angiogenesis and expansion of the placenta.

BIOGRAPHICAL SKETCH

Sha Li was born in Lijiang, Yunnan, China in 1987. She pursued her undergraduate education in the University of Science and Technology of China, Hefei, Anhui, China. In 2009, she received her BS in Biotechnology with the President Honor and National Honor. Her undergraduate research work was studying the requirement of the SNAIL protein in Heregulin-β1 induced Epithelial-Mesenchymal transition (EMT) in breast cancer cell line SKBR3 and potential involvement of PI3K/Akt pathway in this process.

Sha was enrolled as a Ph.D. student in the field of Molecular and Integrative Physiology in College of Veterinary Medicine at Cornell University in fall 2009. After completing her rotations, she joined the lab of Dr. Mark Roberson in fall 2010.

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Last but not least, I would like to thank my husband Kai Ma and our beloved daughter Summer Ma.

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LIST OF ABBREVIATIONS

ANG	Angiopoietin
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
cAMP	Cyclic AMP
ChIP	Chromatin-immunoprecipitation
CTB	Cytotrophoblast(s)
DLX3	Distal-less 3
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ETS2	V-ets erythroblastosis virus E26 oncogene homolog 2
EVT	Extravillous trophoblast(s)
GCM1	Glial cell missing-1
hCG	Human chorionic gonadotropin
hCGα	Human chorionic gonadotropin alpha subunit
hCGβ	Human chorionic gonadotropin beta subunit
HIF	Hypoxia-inducible factor(s)
IGF-II	Insulin-like growth factor-II
IFNT	Interferon-tau
IP	Immunoprecipitation
IUGR	Intrauterine growth retardation
KDR	Kinase insert domain receptor

KO	Knockout
MTF-1	Metal-responsive transcription factor 1
MMP9	Matrix metallopeptidase 9
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor-like region(s)
PE	Preeclampsia
PGF	Placental growth factor
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
qPCR	Quantitative real-time PCR
TGF-β	Transforming growth factor beta
Flt-1	Fms-related tyrosine kinase 1
sFlt-1	Soluble fms-related tyrosine kinase 1
STB	Syncytiotrophoblast(s)
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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CHAPTER ONE. LITERATURE REVIEW

1.1 Placenta and Preeclampsia

1.1.1 Introduction to the placenta

In eutherian mammals, successful pregnancy is dependent on the placenta, a vascular structure formed at the fetal-maternal interface to bring two isolated circulatory systems together so gas and nutrient exchange and endocrine and immunological communication can occur (Bauer *et al*., 1998; Gude *et al*., 2004). In addition to the major transport function, the placenta is substantial in several aspects to support the growth of the fetus and to maintain pregnancy: First, it serves as a barrier to protect the conceptus from xenobiotics, infections and maternal diseases (Audus *et al*., 1999). The human placental barrier is mainly composed of a single rate-limiting layer of multinucleated syncytiotrophoblasts (STB) (Audus *et al*., 1999), fetal mesenchymal cells, endothelium and basal lamina (Enders & Blankenship, 1999). This barrier is "leaky" and only allows free transport of lipid-soluble and low-weight molecules (Audus *et al*., 1999). Second, the placenta acts as an endocrine organ to produce and release hormones into both the maternal and fetal circulation (Gude *et al*., 2004). Synthesis of placental specific hormones including human chorionic gonadotropin (hCG), progesterone, estrogen, placental lactogen, placental growth hormone and others are shown to affect pregnancy recognition and maintenance, maternal metabolism, fetal growth and parturition (Gude *et al*., 2004). Moreover, the placenta exhibits specific immunological features and thus is able to mediate the maternal-fetal tolerance to allow residence of the fetal-placental allograft within the

maternal uterus (Trowsdale & Betz, 2006; Moffett & Loke, 2006). Importantly, growth and functions of the placenta are precisely regulated and coordinated to accommodate the increasing metabolic demands of the developing fetus as gestation progresses (Gude *et al*., 2004; Garnica & Chan, 1996).

1.1.2 Trophoblast and placentation

The transport, protective, endocrine and immune-tolerant functions of the placenta could be all attributed to cells of a common origin—trophoblasts (Rossant & Cross, 2001). Trophoblasts are the most important non-endothelial cell type within the placenta (Rossant & Cross, 2001). They actively engage in placental morphogenesis, driving the sequential formation of placental anatomy and multiple physiological processes within the placenta (Moffett & Loke, 2006). The source of trophoblasts can be traced back to the blastocyst stage of the developing embryo where two distinct cell lines are present: the inner cell mass giving rise to the embryo and the trophectoderm surrounding the blastocyst that later develops to trophoblast cell lineages (Moffett & Loke, 2006; Rossant & Cross, 2001).

As pregnancy progresses, trophoblasts differentiate into several trophoblast subtypes that populate different layers of the placenta (Red-Horse *et al*., 2004; Ji *et al*., 2013). The differentiation of trophoblasts can be generally categorized into two pathways (Ji *et al*., 2013): the villous (syncytial) pathway where stem cell-like mononucleated cytotrophoblasts (CTB) differentiate and fuse into multinucleated STB, and the extra villous (invasive) pathway whereby CTB differentiate to extravillous trophoblast cells (EVT) that acquire invasive properties (Red-Horse *et al*., 2004; Ji *et al*., 2013; Cartwright *et al*., 2010). STB coat the villi and come into direct

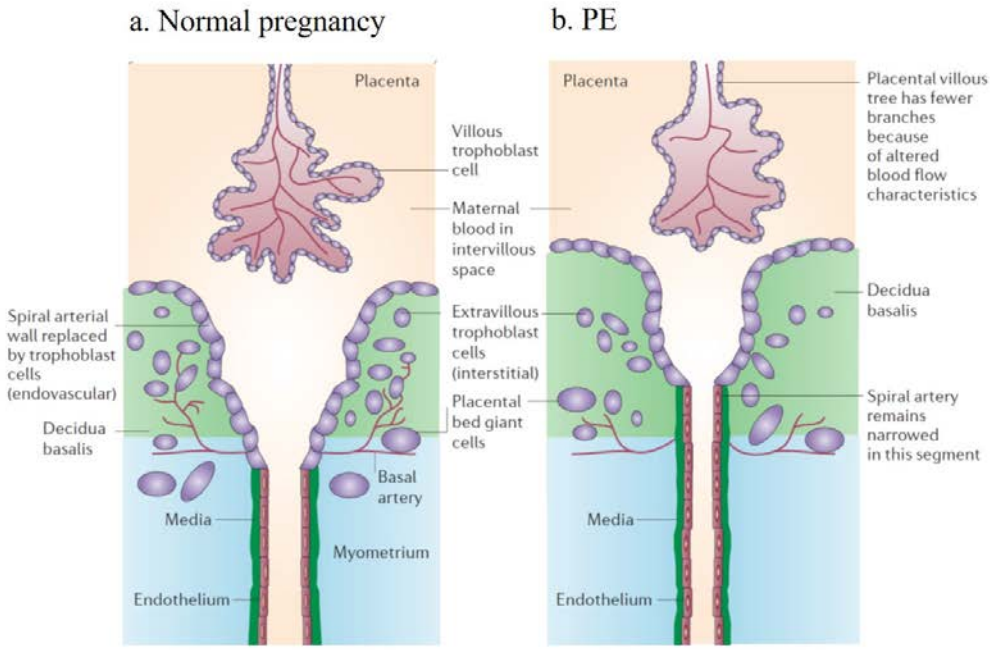

Figure 1.1 | Development of human placentae (adapted from Moffett and Loke, 2006. *Nat. Rev*). (**a**) Normal pregnancy. The placental villi are covered by the villous trophoblasts, comprising cytotrophoblasts that proliferate to generate the outer syncytial layer in direct contact with maternal blood. Extravillous cytotrophoblasts proliferate, successful invade through the decidua and transform the distal spiral arteries into wide calibre vessels that can deliver blood to the intervillous space at low pressure. (**b**) Pre-eclampsia (PE). Inadequate trophoblast-cell invasion leads to deficient transformation of the spiral arteries. The disturbed pattern of blood flow leads to reduced growth of the branches of the placental villous tree of PE.

contact with maternal blood (Figure 1.1a) (Red-Horse *et al*., 2004; Ji *et al*., 2013). They are mainly involved in utero-placental gas and nutrient exchange and hormone

production (Red-Horse *et al*., 2004; Ji *et al*., 2013). EVT, on the other hand, act at the maternal uterus for remodeling of spiral arteries to enhance uteroplacental perfusion (Figure 1.1a) (Red-Horse *et al*., 2004; Kaufmann *et al*., 2003). Interstitial EVT invade toward the maternal decidua and portions of myometrium, forming placental cotyledons, where chorion villi float in maternal vasculature/blood; Endovascular EVT break into the uterine endothelium, penetrate arterial walls and replace maternal endothelial cells, converting the narrow arteriole into dilated, inelastic wide tubes without maternal vasomotor control (Figure 1.1a) (Red-Horse *et al*., 2004; Kaufmann *et al*., 2003; Cartwright *et al*., 2010). This vascular remodeling effectively reduces vascular resistance to contribute to elevated perfusion.

The importance of normal trophoblast differentiation in placental development is underscored by a spectrum of pregnancy-related disease conditions in human. For instance, shallow invasion of EVT and incomplete maternal vascular remodeling have been found in preeclampsia (PE) (Figure 1.1b), as well as in intrauterine growth retardation (IUGR) (Lyall *et al*., 2013; Roberts & Escuder, 2012; Kaufmann *et al*., 2003), whereas excessive invasion into the myometrium is associated with placenta accrete (Adler *et al*., 2014), and uncontrolled proliferation of invasive trophoblasts results in placental carcinoma (Froeling *et al*., 2014; Lurain *et al*., 2010). Variations in the degree to which placental trophoblast lineages invade maternal tissues (from subnormal to pathophysiological) help to predict a central hypothesis of my dissertation research: **tight control of transcriptional networks responsible for invasion into the maternal compartment are necessary to obtain homeostasis in these invasive properties.**

1.1.3 Angiogenesis in the placenta

Placental vasculogenesis, the formation of *in situ* blood vessels, starts with the differentiation and formation of the endothelial progenitor cells—angioblasts in the extraembryonic mesoderm-derived allantois in very early stages of embryogenesis (Coultas *et al*., 2005). The subsequent step—angiogenesis defined by branching of newly formed vessels from pre-existing ones, further elaborates placental vasculature networks (Kaufmann *et al*., 2004). Placental angiogenesis occurs extensively in both the maternal and fetal placental compartments throughout gestation, ensuring optimal uteroplacental perfusion (maternal placental side) and efficient blood exchange within placental villi (the fetal placental side) of the placenta (Reynolds & Redmer, 2001; Kaufmann *et al*., 2004). It is a tightly regulated physiological process, and deranged vasculature is the most common placental pathology that has been identified in an array of pregnancy complications in women including PE and IUGR (Macara *et al*., 1996; Mayher *et al*., 2004; Reynold *et al*., 2006; Redman *et al*., 2005).

It is a general consensus that angiogenesis is initiated by growth factors which increase vessel permeability, promote extracellular matrix (ECM) remodeling, and stimulate endothelial proliferation and migration (Chen and Zheng, 2014). Trophoblast-derived factors including vascular endothelial growth factor (VEGF), placental growth factor (PGF), transforming growth factor *beta* (TGF-β), insulin-like growth factor-II (IGF-II), basic fibroblast growth factor (bFGF), angiopoietins (ANG) have been described in placental angiogenesis, among which the VEGF signaling plays a pivotal role (Reynolds & Redmer, 2001; Zygmunt *et al*., 2003; Kaufmann *et al*., 2004; Xuan *et al*., 2007; Reynolds *et al*., 2005). Notably, the VEGF family

member PGF is abundantly expressed by trophoblast cells and appears to be essential in modulating angiogenesis (Khaliq *et al*., 1996). In addition to amplifying the VEGF signaling, PGF recruits and activates monocytes for the release of chemokines and cytokines to further enhance angiogenesis (Selvaraj *et al*., 2003). Moreover, PGF supports the proliferation of smooth-muscle cells and fibroblasts, and thereby promotes vessel maturation (Yonekura *et al*., 1999; Bellik *et al*., 2005). Meanwhile, the placenta also produces modified forms of growth factor receptors (antiangiogenic factors) such as soluble VEGFR-1 (sFlt1) for VEGF and soluble Endoglin (sEndoglin) for TGF-β (Levine *et al*., 2004; Gregory *et al*., 2014), which are believed to serve as decoy receptors for circulating ligands (VEGF and TGF-β) and hence provide titrating feedback mechanisms to prevent placenta overgrowth and undesirable over invasion of trophoblasts into the uterus.

In addition to classical pro- and anti-angiogenic factors, the glycoprotein hCG produced by trophoblasts in early pregnancy has been shown to contribute to angiogenesis at the uteroplacental side of the developing placenta in a paracrine manner (Herr *et al.*, 2007; Berndt *et al.*, 2006). *In vitro* studies described roles of hCG in inducing sprout formation within blood vessels and preventing endothelial cell from apoptosis together with VEGF (Herr *et al.*, 2007). Both protein kinase A (PKA) and protein kinase C (PKC) signaling pathways were implicated to mediate hCG-induced angiogenesis (Herr *et al*., 2007; Berndt *et al*., 2006; Zygmunt *et al*., 2003).

1.1.4 Preeclampsia

1.1.4.1 Definition and risk factors of PE

Preeclampsia (PE) is a spontaneously occurring syndrome during pregnancy

defined by onset of maternal hypertension and proteinuria typically after 20 weeks of gestation (Chaiworapongsa *et al.*, 2014). It is a multisystem disorder targeting several organs including the kidney, liver, heart, lung and brain (Duley *et al.*, 2009). If left untreated, PE can develop to its end stage of full eclampsia, a convulsive state of life threatening complication (Duley *et al.*, 2009). PE is one of the major reasons for premature delivery (Sibai *et al.*, 2006) and a leading cause of maternal and fetal/neonatal mortality and mobility (Hutcheon *et al.*, 2011). In the United States, PE occurs in 3~5% of all pregnant women and is attributed to 18% of maternal deaths (ACOG *et al.*, 2002; Ananth *et al.*, 2013). Worldwide, PE affects up to 8% of all pregnancies, accounting for over 8,000,000 cases and at least 76,000 maternal and 500,000 infant deaths annually (Villar *et al.*, 2003; Duley *et al.*, 2009). Despite these astonishing statistics, knowledge about the pathogenesis of PE is limited (Chaiworapongsa *et al.*, 2014). In fact, current effective treatment strategies for PE have only been concentrated on relieving the maternal syndrome and have not been changed greatly for over fifty years (Chaiworapongsa *et al.*, 2014).

The development and progression of PE have been implicated to be associated with a wide spectrum of risk factors, including a previous history of PE, primiparity, multiple fetus pregnancy, family history of PE, high BMI before pregnancy, maternal age ≥40 years, and pre-existing medical conditions such as long-term hypertension, renal disease, diabetes and thrombophilia (Duckitt *et al.*, 2005; Kupferminc *et al.*, 1999). In addition, malnutrition and calcium intake deficiency have been identified as important risk factors of PE and might partially explain higher incidence of PE in developing countries (Brewer *et al.*, 1976; Hofmeyr *et al.*, 2003). A paternal

component may also be a consideration, as implicated in the "dangerous father" hypothesis that some male individuals have a higher risk of fathering PE-complicated pregnancies regardless of partners, which might be attributed to a generally maternal immune intolerance to paternal alloantigens they bring in (Dekker *et al.*, 2011; Chaiworapongsa *et al.*, 2014).

1.1.4.2 Pathogenetic mechanism of PE

The placenta is the root cause of PE, as the syndrome of PE would be resolved upon placenta removal (Roberts *et al.*, 2012). This notion is also supported by occurrence of PE in the absence of a fetus but with the presence of placental trophoblast tissue in molar pregnancies (Roberts *et al*., 2012; Huppertz *et al*., 2008). Placentae complicated with PE are featured with reduced uteroplacental perfusion and thus excess oxidative stress, secondary to incomplete remodeling of uterine spiral arteries together with shallow trophoblast invasion and reduced terminal villi volume and surface area (Sohlberg *et al*., 2014; Burton *et al*., 2009; Caniggia *et al*., 2000; Lyall *et al*., 2013; Roberts *et al*., 2012).

A two stage model has been proposed to describe PE progression: the preclinical stage characterized by failure of EVT invasion of maternal spiral arteries, leading to a hypoxic placenta. This local defect leads to the clinical stage of maternal systemic endothelial dysfunction featured with hypertension, proteinuria, abnormal blood clotting and other internal organ damage (Redman *et al*., 1991; Redman *et al*., 2005; Roberts *et al*., 2009). The link between the two stages likely involves the release of upregulated hypoxic-responsive placental factors from the ischemia placenta into

the maternal circulation that are capable of inducing the clinical manifestations of the disease (Redman *et al*., 2014; Gilbert *et al*., 2008). Factors secreted by the hypoxic trophoblasts, featured by components of the VEGF/PGF pathway have been an intensive research area as they would potentially serve as biomarkers for prediction, early diagnosis, and therapeutic targets of PE.

1.1.4.3 Potential use of PGF in PE prediction

Defective placental angiogenesis has long been considered as a pathway to PE (Wang *et al*., 2009; Dechend & Luft, 2008): an imbalance between proangiogenic and antiangiogenic factors favoring an antiangiogenic state is evidenced in both the local placentae and maternal circulations of PE-complicated pregnancies (Wang *et al*., 2009; Dechend & Luft, 2008), in which aberrant VEGF/VEGFR signaling has been well described (Levine *et al*., 2004; Wang et al., 2009; Dechend & Luft, 2008). Members of this family of secreted peptides are highly sensitive to oxygen tension. For example, expression of the antiangiogenetic factor—soluble fms-related tyrosine kinase 1 (sFlt-1), and proangiogenetic factors VEGF-A and PGF could all be regulated by hypoxic stimulus (Forsythe *et al*., 1996; Murphy *et al*., 2013; Khaliq *et al*., 1999). Therefore, it is expected that their expression would be altered in hypoxic placentae complicated with PE, which, in fact, has been confirmed in several studies (Forsythe *et al*., 1996; Murphy *et al*., 2013; Khaliq *et al*., 1999). Persuasive evidence has been gathered in support of the involvement of sFlt-1 and PGF in maternal manifested syndrome of PE: higher levels of sFlt-1 and lower levels of PGF in maternal circulations were detected in PE-complicated pregnancies compared with that of

normal pregnancies (Maynard *et al.*, 2003; Levine *et al.*, 2004). The extent of dysregulation of these factors has been associated with severity of the disease (Levine *et al.*, 2004).

Excess sFlt-1 and insufficient PGF synergistically contribute to downregulation of VEGF-dependent signaling (Kendall & Thomas, 1993). VEGF is important for maintaining the integrity of endothelial cells, especially for fenestrated endothelia in renal glomeruli, brain and liver (Esser *et al*., 1998). In addition, VEGF acts directly on the systemic vasculature to exert vasodilatory effects through nitric oxide (NO) and to promote prostacyclin-dependent vasorelaxation on vascular endothelial cells (He *et al*., 1999; Wheeler-Jones *et al.*, 1997). Interestingly, all these physiological processes and homeostasis crucial for successful pregnancies are inhibited in PE, featured by onset of maternal hypertension, proteinuria, edema and seizure, suggesting downregulated VEGF-dependent signaling in PE. Biological effects of VEGF-dependent signaling on maintaining a healthy maternal vasculature are also well supported by observations in patients undergoing antiangiogenic cancer therapy using VEGF-signaling inhibitors (Patel *et al*., 2008; Eremina *et al*., 2008). These patients develop hypertension, proteinuria and glomerular endothelial damage reminiscent of hallmarks of PE (Patel *et al*., 2008; Eremina *et al*., 2008). Expression of circulating VEGF in PE has been investigated, however, with conflicting results (Levine *et al.*, 2004). Nevertheless, downregulation of VEGF signaling caused by increased sFlt-1 and decreased PGF is likely to be a prominent component in disease pathogenesis, and the sFlt-1/PGF ratio in the maternal circulation is proposed to be used as clinically biomarkers for the prediction of PE in women (Zeisler *et al.*, 2016).

This is an important step forward in predicting the need for therapeutic engagement prior to the onset of maternal disease in these patients.

Animal models of PE further supported association of PE with insufficiencies of circulating PGF. For example, lower plasma PGF levels during pregnancy was detected in the BPH/5 mouse model of PE compared with controls (Woods *et al.*, 2011). In another animal model of PE—the RUPP rats, hPGF administration remarkable attenuated placental ischemia-induced maternal hypertension without major adverse consequences, suggesting PGF to be a potential therapeutic target for PE (Spradley *et al*., 2016).

Besides VEGF/VEGFR, other molecules and substances such as sEndoglin (Venkatesha *et al*., 2006), procoagulant factors (Rousseau *et al*., 2009), angiotensin receptor-1 (Zhou *et al*., 2008), miRNA (Luque *et al*., 2014; Chen & Wang, 2013), and placental debris (Redman & Sargent, 2000) have been implicated in PE progression. For instance, sEndoglin, the soluble form of TGF-β receptor, was found to be upregulated in maternal circulations complicated with PE (Venkatesha *et al*., 2006). It interferes with the binding of TGF-β to receptors expressed on endothelial cells, resulting in dampened signals of vasodilation and eNOS-mediated vascular relaxation (Venkatesha *et al*., 2006). Thus elevated sEndoglin levels in maternal circulations have been suggested to play a role in the onset of gestational hypertension of PE patients. Despite current understanding of several single molecular pathways involved in PE development, it is suggested that multiple mechanisms and pathways of maternal and placental origin work together to contribute to this extremely heterogeneous and complicated condition (Chaiworapongsa *et al.*, 2014), and thus

more comprehensive studies are highly desirable in the future.

1.1.4.4 Brief introduction to animal models of PE

PE seems to be restricted to humans and certain higher primates. Spontaneously occurring PE has been reported in cases of baboon twins (Hennessy *et al.*, 1997) and rhesus monkeys (Krugner-Higby *et al*., 2009). Additionally, both chimpanzee and gorillas have been reported to be susceptible to developing PE during pregnancy (Pijnenborg *et al*., 2011; Carter, 2011). However, no substantive support for a formal diagnosis of eclampsia was observed in these animals. It is speculated that the evolution of bipedalism and a large human brain that demand the deep endovascular cytotrophoblast invasion into the maternal uterus are necessary for the development of full spectrum of syndromes associated with PE in humans (Robillard *et al*., 2003; Rockwell *et al*., 2003; Rosenberg and Trevathan, 2007). A number of animal models have been put forth with the effort to recapitulate pregnancy associated disorders of PE (Erlandsson *et al*., 2016; McCarthy *et al*., 2011). To name a few, (i) the spontaneous model such as the BPH/5 mice, (ii) surgically induced models like the RUPP rats, (iii) genetically modified models such as the *Indoleamine 2,3-dioxygenase* (*IDO*) knockout (KO) mice and the *Dlx3* KO mice, or (iv) models administered virus-driving expression of exogenous genes such as the sFlt-1 overexpression model. While they are useful in testing possible causes of the syndrome and potential preventative and therapeutic strategies, they have uniformly failed to provide definitive insights into the pathogenesis of PE because of their limited applicability to the human form of the disease (Erlandsson *et al*., 2016; McCarthy *et al*., 2011). For instance, none of

them are competent in recapitulating the human placental vascular events since human placental trophoblasts are unique in regard to particular invasiveness, even compared with other primates (Pijnenborg *et al*., 2011; Carter, 2011). Furthermore, the association between PGF insufficiency and PE progression was found and tested largely based on studies in human, partly because PGF appears dispensable during pregnancy in the genetically modified animal model of PE (Carmeliet *et al*., 2001). Therefore, studies using *in vitro* human placental cell lines, human placental primary cells and *ex vivo* placental explants would serve as useful tools to complement limitations of animal models prior clinical translations.

1.2 Placental growth factor

1.2.1 PGF—a member of the VEGF family

PGF is a member of the VEGF family of proangiogenic factors and endothelial mitogens (Maglione *et al*., 1991). It was initially discovered and isolated from human term placentae, and characterized to be a 149 amino acid (aa) protein (containing an 18 aa signal peptide) that shares approximate 42% sequence homology to VEGF-A (VEGF-165) (Maglione *et al*., 1991; Roy *et al*., 2006; Lohela *et al*., 2009). In particular, the platelet-derived growth factor-like regions (PDGF) of PGF and VEGF-A share near 70% sequence similarity, which include a highly conserved eight cysteine residues (cysteine-knot motif) involved in intra- and inter-chain disulfide bond formation, conferring their formation of dimers (Maglione *et al*., 1991). Consistent with the sequence similarity, biochemical studies revealed remarkable topological identity between PGF and VEGF-A at the three dimensional structure

level (Muller *et al*., 1997; Lyer *et al*., 2001). Despite these similarities, the expression pattern and function of PGF in physiological systems have been found to be quite different from that of VEGF (Tjwa *et al*., 2003).

The human *PGF (hPGF)* is located on chromosome 14 and consists of seven exons spanning 13.7 kb (Maglione *et al*., 1993; Rowen *et al*., 1999). It encodes a single nascent mRNA product that produces four different isoforms owning to alternative splicing: PGF-1, PGF-2, PGF-3 and PGF-4 of 170, 149, 169 and 221 aa lengths, respectively (Maglione *et al*., 1993, Hauser and Weich, 1993; Cao *et al*., 1997; Yang *et al*., 2003). All PGF isoforms are glycosylated proteins secreted from cells (Yang *et al*., 2003). The main difference between them is that PGF-2 and PGF-4 lack an in-frame exon (exon 6) in the 3' coding region that is present in PGF-1 and PGF-3 (Strausberg *et al*., 2002; Rowen *et al*., 1999; Li *et al*., 2003). Exon 6 encodes a highly basic 21 aa heparin binding motif at the carboxyl end that allows PGF-1 and PGF-3 to be heparin-bound (Strausberg *et al*., 2002, Rowen *et al*., 1999; Li *et al*., 2003). In mice, two PGF isoforms originated from a single gene have been described, both of which are able to bind heparin but differ in the amino terminal end due to different selections of 5´ UTR and coding sequences (DiPalma *et al*.,1996; Kawai *et al*., 2001).

PGF is conserved within vertebrates within the PDGF domain featured by the presence of cysteine knot motif important for dimerization (Yang *et al*., 2003); *in situ* hybridization studies using human PGF cDNA as the probe detected PGF expression in species including *Drosophila*, *Xenopus*, *chicken* and *mouse* (Maglione *et al*., 1993). However, this conservation does not persist beyond the coding region of *PGF*.

Specifically, the 5´ flanking region of the *hPGF* gene shares little conservation with that of other species, suggesting a unique regulatory mechanism of *hPGF* transcriptional expression (Chang, *et al*., 2008).

1.2.2 The PGF ligand, receptor and signaling

Each PGF monomer consists of two α-helixes and seven β-strands (Iyer *et al*., 2001). Receptor recognition of PGF largely depends on several conserved residues located within the β strands (e. g. Asp72 and Glu73), although the amino terminal α-helix also seems to be involved in ligand/receptor interactions (Errico *et al*., 2004; Christinger *et al*., 2004). Additionally, glycosylation of PGF has been shown to contribute to receptor binding as mutants of PGF at residues undergoing glycosylation exhibited reduced binding activities, which, interestingly, is not seen in the closely related VEGF-A (Errico *et al*., 2004).

Like other members of the VEGF family, PGF initiates diverse pathophysiological events by acting through specific VEGF-receptors (VEGFR) (Park *et al.*, 1994). VEGFRs typically consist of seven extracellular Immunoglobin-like domains, a transmembrane domain and an intracellular tyrosine kinase domain (Olsson *et al*., 2006). The binding of ligand induces receptor dimerization and bidirectional transphosphorylation, which consequently activates downstream signaling pathways via the decoration of the intracellular domain with phosphorylated tyrosine residues (Olsson *et al*., 2006). The VEGF/VEGFR system is a key component in a variety of physiological processes such as angiogenesis and vasculogenesis,

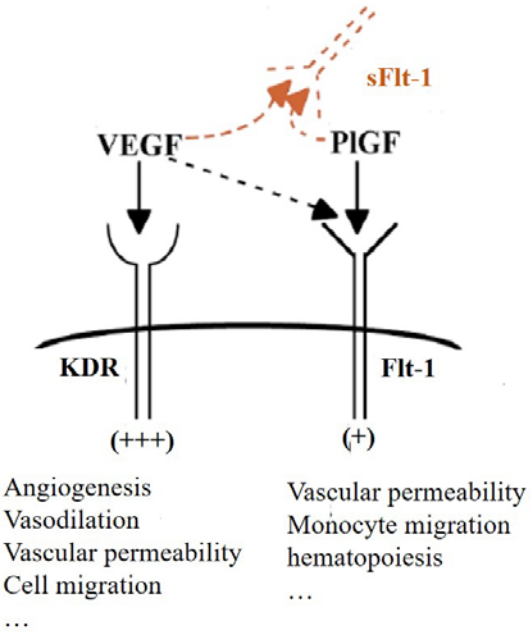

Figure 1.2 | PGF and its receptor-dependent signaling pathways (adapted from Carmeliet, P. *et al.* 2001. *Nat. Med.*). When PGF is upregulated under pathological conditions or in the physiological placenta, it stimulates KDR-dependent angiogenesis via displacement of VEGF from Flt-1 to KDR. In addition, PGF initiates multiple Flt-1-dependent downstream pathways via activation of membrane associated Flt-1. Both VEGF and PGF can be sequestered by circulating sFlt-1. Dotted lines represent lower expression/binding; solid lines denote high levels of expression/binding. The inhibitory pathway is indicated in red.

lymphangiogenesis, neurogenesis, vascular permeability induction, vasodilatation, endothelial integrity maintenance and many pathological conditions, including edema, wound healing, rheumatoid arthritis, ocular neovascularization, tumor progression, endometriosis and cardiovascular diseases (Shibuya, 2013; Olsson *et al.*, 2006; Lohela

et al., 2009).

In contrast to VEGF-A which binds to both VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1) receptors, PGF exclusively associates with Flt-1 with high affinity compared with other VEGF ligands (Figure 1.2) (Autiero *et al*., 2003; Olofsson *et al*., 1998). KDR exhibits a strong tyrosine kinase activity towards proangiogenic signals following VEGF-A stimulation, whereas Flt-1 is only weakly phosphorylated when activated and mainly acts in a mechanism of "decoy" by depriving VEGF-A of its binding to KDR (Shibuya *et al.*, 2006). Therefore, when expressed, PGF is thought to compete with and displace VEGF-A from Flt-1, allowing more free VEGF-A to activate KDR, thus amplifying KDR-dependent downstream signaling pathways of angiogenesis, vascular permeability, vasodilation, etc. (Figure 1.2) (Shibuya *et al.*, 2006; Carmeliet *et al*., 2001). Notably, although Flt-1 can be bound and activated by both VEGF-A and PGF, albeit weakly, the sites of phosphorylation within its intracellular domain differ (Autiero *et al*., 2003). This suggests that VEGF-A and PGF potentially initiate distinct Flt-1-dependent downstream pathways which may exert different biological effects. Moreover, PGF is able to form heterodimer with VEGF-A *in vivo*, resulting in KDR/Flt-1 dimerization when both receptors are expressed on cells, making the system even more complex (Eriksson *et al*., 2002; Tarallo *et al*., 2010).

In addition to being a weak receptor of angiogenesis, Flt-1 is an important regulator in hematopoiesis and inflammation through mediating PGF-induced migration and activation of hematopoietic precursors and monocytes/macrophages (Olsson *et al*., 2006; Lohela *et al*., 2009). For example, PGF has been shown to

reconstitute hematopoiesis by specifically recruiting Flt-1 expressing stem cells from the bone marrow (Hattori *et al.*, 2002). Recruitment and activation of Flt-1 expressing monocytes/macrophages by PGF have important implications in conditions associated with inflammation and cancer.

Besides being expressed as the transmembrane receptor on cells, Flt-1 could exert physiological activities beyond cellular contexts through being secreted into systemic circulation in the form of free circulating sFlt-1 which lacks the seventh immunoglobulin-like extracellular domain (Figure 1.2) (Inoue *et al*., 2000; Stepan *et al*., 2004). sFlt-1 has been proposed to serve as a means to downregulate the activities mediated by binding of ligands to the Flt-1 receptor and as an inert sink to sequester free VEGF/PGF in the serum since it is effectively functions as a decoy receptor not linked to a cellular context (Inoue *et al*., 2000; Stepan *et al*., 2004). Consistent with this notion, excess sFlt-1 has been shown to impair the endothelial system and contribute to pathogenesis of PE and chronic kidney disease in humans presumably via sequestration of biologically active VEGF and PGF away from their sites of action within the vascular system (Maynard e*t al*., 2003; Di Marco, G. S. *et al*., 2009).

PGF (PGF-1 and PGF-3) can also bind with Neuropilin-1 (Nrp-1) and Nrp-2 via their heparin-binding domains (Mamluk *et al*., 2002; Guar *et al*., 2009). Nrp-1/-2 are transmembrane receptors lacking tyrosine kinase activity, and thereby are suggested to function as additional mechanisms to modulate VEGFR-dependent signaling through sequestering PGF (and VEGF-A) (Mamluk *et al*., 2002; Guar *et al*., 2009). However, a recent study revealed that PGF could directly act through Nrp-1 independent of VEGFR to promote tumor cell survival in medulloblastoma

tumorigenesis (Snuderl *et al*., 2013).

1.2.3 Expression and functions of PGF

PGF is highly expressed in the placenta throughout gestation in humans (Maglione *et al*., 1993; Khaliq *et al*., 1996). Low levels of PGF mRNA can be detected in human thyroid, heart, brain, lung, skeletal muscle, and adipose tissue under normal physiological conditions but not in kidney and pancreas (Persico *et al*., 1999; Voros *et al*., 2005). At the cellular level, the expression of PGF is found in endothelial cells, trophoblast cells, certain lymphocytes and thyroid cells (Hauser and Weich, 1993; Yonekura *et al*., 1999; Viglietto *et al*., 1995).

First discovered to be an endothelial mitogen *in vitro* (Maglione *et al*., 1991), PGF was later found to affect a wide range of different cell types and be involved in a variety of biological events. It directly stimulates vessel growth by inducing growth, migration and survival of endothelial cells (Carmeliet *et al*., 2001; Adini *et al*., 2002; Fischer *et al*., 2007). It promotes vessel maturation by supporting proliferation of smooth-muscle cells and fibroblasts surrounding the vessel (Yonekura *et al*., 1999; Bellik *et al*., 2005). Moreover, it is able to recruit angiogenesis-competent myeloid progenitors to growing sprouts and collateral vessels (Hattori *et al*., 2002; Luttun *et al*., 2002; Rashdan & Lloyd, 2015), and induce migration and activation of monocyte-macrophage lineages to further enhance the angiogenic stimulus (Clauss *et al*., 1996; Selvaraj *et al*., 2003). The wide spectrum of paracrine and autocrine actions of PGF is directly correlated to the expression of sFlt-1 on surface of these cells.

1.2.3.1 PGF in angiogenesis

Genetically modified mice lacking functional PGF are born at a Mendelian frequency and turn out to be healthy and fertile in general (Ferrara *et al*., 2004; Carmeliet *et al*., 2001), suggesting placental PGF be dispensable for mouse pregnancies. Similarly, PGF appears less important than expected in exercise-induced angiogenesis in heart and muscle in mice (Gigante *et al*., 2004). These studies imply that PGF is redundant in vascular development and vascular maintenance in healthy adult mice under physiological conditions. Nevertheless, PGF-deficient adult mice showed abnormality in pathologically angiogenic conditions such as ischemia, inflammation, wound healing, and tumor development (Carmeliet *et al*., 2001, Luttun *et al*., 2002). In addition, they display attenuated responses to VEGF-A in pathological angiogenesis, consistent with previously described role of PGF in modulating VEGF-A-mediated angiogenesis (Carmeliet *et al*., 2001). In fact, accumulating data in humans have documented PGF as a critical regulator in postnatal angiogenesis in a number of physio-pathological conditions including tissue repair, inflammation and cancer (Cianfarani *et al*., 2006; Gargioli *et al*., 2008; Iwasaki *et al*., 2011; Maes *et al*., 2006; Oura *et al*., 2003; Van de Veire *et al*., 2010). On the other hand, gain of function studies in mice have emphasized important involvement of PGF in angiogenesis in certain tissues: PGF overexpression in skin controlled by the keratin-14 promoter led to significantly increased growth of dermal blood vessels in mice, together with increased local vascular permeability and smooth muscle maturation (Odorisio *et al*., 2002); adenovirus-driven expression of PGF in ischemic hearts and limbs elicited a strong angiogenic response with an efficacy comparable to that of VEGF-A (Luttun *et*

al., 2002). Molecular pathways mediating PGF activities within cells have been investigated, where endothelial nitric oxide synthase (eNOS) and its final by-product NO have been implicated to be functionally linked to PGF signaling (Gigante *et al*., 2006). Consistent with that, double knockout of PGF and eNOS resulted in a synergistic phenotype of ischemia that is not seen in either of the single knockout models (Gigante *et al*., 2006).

1.2.3.2 PGF in the placenta and during pregnancy

In early stages of mouse embryonic development, PGF is expressed in trophoblastic giant cells associated with the parietal yolk sac, possibly involved in coordinated vascularization with VEGF-A in the decidua and placenta (Achen *et al*., 1997). Later, PGF is abundantly found within the placenta in human umbilical vein endothelial cells (HUVEC) and its primary sites of synthesis in trophoblast lineages throughout gestation (Hauser and Weich, 1993). Immunostaining in human term placentae showed additional expression of PGF in the epithelial layer of the amnion and the maternal decidua, but little within the villous mesenchyme (Khaliq *et al*., 1996). Moreover, positive immunostaining for PGF was observed in infiltrated neutrophils and macrophages in the human placenta (Kumazaki *et al*., 2002). The expression pattern of PGF, together with the detection of Flt-1 on endothelial cells, monocytes, vascular smooth muscle cells and trophoblasts suggests both autocrine and paracrine functions of PGF within the placenta and endometrium (Barleon *et al*., 1994; Barleon *et al*., 1996; Ishida *et al*., 2001; Helske *et al*., 2001).

PGF is important for the normal growth and functions of placentae. First, it

works in concert with other members of the VEGF/VEGFR family to drive placental angiogenesis and promote vascular growth, maturation, and permeability (Torry *et al*., 2004). Second, PGF contributes to placental morphogenesis and vascular remodeling by stimulating proliferation, survival and possibly invasiveness of trophoblast cells (Athanassiades and Lala, 1998; Khaliq *et al*., 1996; Knuth *et al*., 2015; Li & Zhuang, 1997). Inhibition of the PlGF/Flt-1 signaling has been shown to increase spontaneous apoptosis rates in cultured trophoblasts, which was correlated with the presence of alleviated trophoblast apoptosis in preeclamptic placentae (Leung *et al*., 2001; Hung *et al*., 2002; Ishihara *et al*., 2002). Interestingly, the mitogenic and antiapoptotic effects of PGF are specific to Flt-1 expressing trophoblast cells, rather than endothelial cells (Angelucci *et al.*, 2001). This was found to be due to divergence in downstream signal transduction between endothelial cells and trophoblasts upon PGF stimulus: PGF-treated HUVEC were shown to activate the extracellular signal-regulated protein kinase (ERK)-1/2 pathways, whereas PGF-treated trophoblast cells had JNK (c-Jun-N-Terminal Kinase), p38, and stress activated protein kinase (SAPK) signal transduction pathways activated (Arroyo *et al*., 2004; Desai, *et al*. 1999). Differences between trophoblasts and endothelial cells in response to the PGF stimulus are likely to contribute to their coordinated integration in placental development. Additionally, although previous studies didn't detect positive roles of PGF in EVT invasion and migration (Athanassiades and Lala, 1998), a recent report has shown that PGF is protective and might be required for first-trimester trophoblast invasion, likely mediated by the 70-kDa ribosomal protein kinase I (p70) (Knuth *et al*., 2015). Such disparities of data in the literature necessitate additional research in the field to provide

a more comprehensive mechanistic view of the actions of PGF in the placenta during various stages of gestation.

Unlike in the mouse where PGF ablation doesn't appear to affect fertility (Ferrara *et al*., 2004; Carmeliet *et al*., 2001), PGF dysregulation in humans has been shown to associated with significant pregnancy-associated problems, such as PE and IUGR (Torry *et al.*, 2003; Powers *et al*. 2012; Griffin *et al*., 2015; Gutaj *et al.*, 2014). Ethical considerations, current limitations in techniques for the requirement of invasive methods, and other restrictions make it difficult to trace longitudinal expression of PGF within human placentae. Alternatively, as the secreted protein, PGF at the plasma level serves a valuable tool for this purpose. Studies monitoring serum/urine PGF in maternal circulations detected a highly dynamic change of placental PGF expression throughout gestation. Circulating levels of PGF are undetectable under normal conditions in non-pregnant adults. It remains low in early pregnancy, and gradually increases to a significant amount around 9~10 weeks of gestation, reaches the peak around 27~30 weeks, and thereafter decreases till week 40 (Pramatirta *et al.*, 2015, Levine *et al.*, 2004, Taylor *et al.*, 2003). Importantly, measurement of circulating PGF has been shown to be an attractive tool for diagnosis, prediction and prognosis of PE, as insufficient circulating PGF can be observed as early as around mid-gestation in pregnancies at risk of PE which typically develops after mid-gestation (Kumazaki *et al.*, 2002, Levine *et al.*, 2004). Increasing studies have proved the great value of using the sFlt-1/PGF ratio as a biomarker to screen patients at risk of PE (Zeisler *et al.*, 2016; Saleh *et al.*, 2016; Álvarez-Fernández *et al.*, 2015; Hund *et al.*, 2015; Bian *et al.*, 2015; Stepan *et al.*, 2015). The sFlt-1/PGF ratio

has shown prediction potential as early as in the first trimester, but had a higher accuracy from the second trimester (Bian *et al.*, 2015, Hassan *et al.*, 2013), and thereby the time-dependent slope of the sFlt-1/PGF ratio through repeated measurements would allow early screening to rule out patients of normal pregnancies from those at the risk of PE more efficiently (Schoofs *et al.*, 2014). Second, the sFlt-1/PGF value has also been shown to be associated with severity of the disease, allowing relatively rapid and accurate identification of women at high risk for imminent delivery (Verlohren *et al.*, 2012). Clinically, the sFlt-1/PGF ratio significantly complements the Doppler ultrasonography techniques for uterine perfusion detection and current diagnosis standard of hypertension and proteinuria in screening algorithms of PE (Saleh *et al.*, 2016; Álvarez-Fernández *et al.*, 2015; Stepan *et al.*, 2015). In addition to predict PE, low levels of circulating PGF are also linked to IUGR, and perhaps reflect an unhealthy status of placental development in these pregnancy-specific conditions (Benton *et al.*, 2012; Sibiude *et al.*, 2012).

1.2.3.3 PGF in inflammation and cancer

Angiogenesis is a prominent feature of inflammation. Regulators of angiogenesis are found to play key roles in a wide spectrum of inflammatory conditions in humans such as atherosclerosis, rheumatoid arthritis, and cancer (Imhof & Aurrand-Lions, 2006). PGF is one of these factors. It has been shown that PGF modulates inflammatory responses and promotes angiogenesis indirectly by inducing the recruitment and maintenance of monocytes/macrophages, mural cells, and bone marrow-derived precursor cells to pathological destinations. Molecular mechanisms

involved in PGF-induced monocyte recruitment and procoagulant activity appear to be mediated by Flt-1 receptors expressed on cell surface and downstream via Phosphoinositide 3-kinase (PI3K) /AKT and ERK-1/2 pathways, leading to expression and release of proinflammatory cytokines and chemokines (Clauss *et al.*, 1996; Sawano *et al.*, 2001; Selvaraj *et al.*, 2003). In mice, PGF overexpression in the epidermis resulted in cutaneous inflammatory response with inflammatory angiogenesis and edema formation, whereas PGF deficiency attenuated oxazolone-induced cutaneous inflammatory responses, suggesting PGF was both sufficient and necessary for the initiation and maintenance of the cutaneous inflammatory response (Oura *et al.*, 2003). PGF is also implicated in chronic inflammatory diseases, as enhanced PGF expression and signaling have been seen in atherosclerosis and rheumatoid arthritis (Roncal *et al.*, 2010; Khurana *et al.*, 2005; Luttun *et al.*, 2002; Marrelli *et al.*, 2011; Yoo *et al.*, 2009; Matsui *et al.*, 2015), and elevated circulating levels of PGF have been reported in patients with atherosclerotic or ischemic heart disease (Glaser *et al.*, 2011; Marković *et al.*, 2010).

The VEGF family and their receptors are generally held to be major mediators of tumor angiogenesis (Fantozzi *et al.*, 2014). Most types of human cancer cells have been shown to express VEGF-A, often at elevated levels, and targeted therapies against VEGF-A/KDR in tumorigenesis have shown therapeutic success in certain types of cancer (Sitohy *et al.*, 2012; Kerbel *et al.*, 2008). However, these therapies pose challenging problems: first, a substantial fraction of tumors tends to develop resistance to current anti-VEGF-A/VEGFR therapies, partly due to upregulated PGF expression under selective pressure by VEGF-A/VEGFR inhibition (Welti *et al.*,

2013; English *et al.*, 2009; Bergers & Hanahan, 2008). Second, as VEGF-A is essential for maintaining integrity of quiescent endothelial cells and therefore physiologic homeostasis of many organ systems, inhibition of the VEGF-A/KDR signaling would bring in serious adverse effects on these organs and lead to life-threatening conditions in a subset of cancer patients including hypertension, proteinuria, disturbed wound healing, hypothyroidism, hemorrhage and thrombotic events (Kamba & McDonald, 2007; Chen & Cleck, 2009). Alternatively, the PGF/Flt1 pathway has been increasingly implicated to be a promising target in cancer therapeutic development as a means of supplementing and enhancing the efficacy of anti-VEGF therapy (Torry *et al.*, 2009; Loges *et al.*, 2009; Verheul *et al.*, 2007), as the PGF/Flt-1 signaling has been proposed as a major route for angiogenic rescue after anti-VEGF therapy (Kerbel *et al.*, 2008). The advantage of PGF is that it is highly upregulated during inflammation and angiogenesis in tumors but expressed minimally in most tissues under normal conditions (Adini *et al.*, 2002; Takahashi *et al.*, 1994; Marrony *et al.*, 2003; Wei *et al.*, 2009). Therefore, the anti-PGF therapy would be expected to bring in less side effects of interfering with normal physiological processes. Changes in PGF levels in sera and tumor tissues have been shown to correlate with tumor progression, reoccurrence and patient survival of various cancers (Wei *et al.*, 2005; Zhang *et al.*, 2005; Chen *et al.*, 2004; Parr *et al.*, 2005; Ho *et al.*, 2007; Chen et al., 2013; Huang *et al.*, 2014). This is supported by studies in mice where monoclonal antibodies against PGF not only inhibited the growth and metastasis of tumors including those resistant to VEGFR inhibitors, but also caused fewer side effects (Fisher *et al.*, 2007). Moreover, the study showed PGF antibodies

worked in concert with VEGF-A antibodies in targeting tumor progression (Fisher *et al.*, 2007). However, conflicting data have been presented by studies of others, in which PGF was believed to block aggravated tumor growth and angiogenesis, and that PGF overexpression might be beneficial for cancer treatment (Bais *et al.*, 2010; Xu *et al.*, 2006). This discrepancy might be involved in differences in Flt-1 expression and complex PGF/VEGF-A/VEGFR interactions in specific tumor contexts (Yao *et al.*, 2011). Admittedly, the underlying mechanisms are still unclear, warranting further investigation.

Taken together, PGF is increasingly recognized as a promising candidate for inflammation and cancer therapeutic targets. Further studies will shed new light on how PGF can be therapeutically manipulated for the treatment of different forms of human diseases associated with inflammation and angiogenesis.

1.2.4 Regulation of *PGF*

Current understanding of the regulation of *PGF* expression is limited. Previous studies have described the modulation of *PGF* expression at the epigenetic level: the *PGF* promoter was found hypermethylated in human lung and colorectal tumor tissues and cell lines, which might contribute to low *PGF* expression in these cells (Xu & Jain, 2007). In addition, environmental stimuli such as fluid shear stress and oxygen tension have been reported to affect *PGF* expression (Rashdan &Lloyd, 2015; Torry *et al*. 2009). Coronary artery endothelial cells exposed to increased fluid shear stress were shown to upregulate *PGF* expression, which was important for the recruitment of monocytes and hence arterial remodeling (Rashdan &Lloyd, 2015). This process was

proposed to be mediated by the NADPH oxidase 4-derived reactive oxygen species, suggesting oxygen tension-involved signaling pathways to be an important component of *PGF* regulation (Rashdan &Lloyd, 2015).

The expression of *PGF* has been shown to be regulated by oxygen tension. The response, however, is quite different from VEGF-A, and appears to be cell context dependent. *PGF* has been reported to be downregulated in placental trophoblast cells under hypoxic conditions (Khaliq *et al.* 1999; Gobble *et al*. 2009), but upregulated in human myocardium, cultured neonatal rat cardiomyocytes, transformed mouse embryonic fibroblasts (mEFs), NIH 3T3 cells and HEK 293 cells (Green *et al*., 2001; Torry *et al*., 2009) by hypoxia. These observations suggest distinct pathways governing *PGF* expression in trophoblast and non-trophoblast cells. Hypoxia-inducible factors (HIFs), the main mediators of hypoxic stimuli, have shown to bind to hypoxia-responsive element (HRE) sequences at *VEGF-A* promoters upon hypoxia stimuli and mediate hypoxia-induced *VEGF-A* upregulation (Forsythe *et al*., 1996). It was initially believed that HIFs might engage in hypoxia-induced PGF responses via similar mechanisms. However, this appeared not be the case. First, studies showed lack of functional HRE element at the *PGF* promoter (Green *et al*., 2001). Second, while HIF-1 was shown to upregulate *PGF* expression in endothelial cells involving a histone hyperacetylation mechanism (Tudisco *et al*., 2014), overexpression of HIF-1 had no effect on *PGF* expression in trophoblast cell lines (Gobble *et al*. 2009). Alternatively, the transcription factor Metal-transcription factor 1 (MTF-1) and the complex nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappa B) have been implicated to mediate changes in *PGF* expression in response to hypoxia

(Nishimoto *et al*., 2009; Cramer *et al*., 2005).

In addition to hypoxia, Torry *et al.* (2008) demonstrated that overexpression of the transcription factor Glial cell missing-1 (GCM1) in trophoblast cell lines activated the *PGF* promoter in a luciferase reporter system. The author proposed a putative GCM1 motif within the -828/-698 (nucleotide distance relative to the translation start site) region that was reported to be critical for GCM1-induced PGF transactivation (Chang *et al*., 2008). As is described in Chapter two and three of this dissertation, **we test their hypothesis and confirm that GCM1 positively regulates *hPGF* expression in the JEG-3 human trophoblast cell line. In addition, we identify the transcription factor—Distal-less 3 (DLX3), to be another important regulator of *hPGF* expression. We find DLX3 works in concert with GCM1 in an antagonistic manner to regulate *PGF*. Our studies support a novel region (-369/-320 from TATA box) within the *PGF* promoter where DLX3 and GCM1 bind and act on.** We demonstrate that this 50 base pair region is crucial for the basal and DLX3/GCM1-induced expression of PGF. At last, we are able to detect a physical interaction of the DLX3/GCM1 transcription factor pair, and characterize domains of DLX3 important for interaction with GCM1. Interestingly, we find that binding with DLX3 appears to repress GCM1's regulatory activity in *hPGF* expression, suggesting a fine tuning mechanism of DLX3 on *hPGF* expression in human trophoblast cells.

1.3 Distal-less three

1.3.1 The Dlx gene family

The homeodomain (HD) transcription factor encoded by *Distal-less* (*Dll*),

initially characterized in *Drosophila,* is required for proximodistal patterning in insect limbs and limb-derived structures including legs, wings, antennae, mouthparts and the analia (Cohen *et al.,* 1989; Cohen *et al.,* 1991). When expressed ectopically, *Dll* can induce new appendage formation in flies (Gorfinkiel *et al.,* 1997). The vertebrate homologs of *Dll*—the Dlx genes, share the highly conserved HD region with Drosophila *Dll* and have been implicated to play fundamental roles in a variety of developmental processes ranging from neurogenesis to skeleton differentiation (Anderson *et al.,* 1997a; Merlo *et al.,* 2002; Depew *et al.,* 2005; Jeong *et al.,* 2008). In mouse and human genomes, the Dlx family comprises six members grouped into three convergently transcribed pairs linked to a near Hox gene cluster along different chromosomes (Scherer *et al.,* 1995; Stock *et al.,* 1996; Nakamura *et al.,* 1996; MacGuinness *et al.,* 1996; Panganiban *et al.,* 2002).

Like many other homeobox genes, the Dlx genes are characterized by highly restricted spatio-temporal expression throughout development (Zerucha *et al.,* 2000). Pairs of Dlx genes exhibit similar patterns of expression that are conserved in their mammalian counterparts (Simeone *et al.,* 1994; Qiu *et al.,* 1997; Chen *et al.,* 1996; Robledo *et al.,* 2002). In mice, temporal and spatial expression of Dlx1, Dlx2, Dlx5 and Dlx6 in the forebrain have been observed and are essential for development of the central nervous system (CNS) (Price *et al.,* 1991; Liu *et al.,* 1997; Bulfone *et al.,* 1998; Eisenstat *et al.,* 1999; Yang *et al.,* 1998). Expression of all Dlx genes in surface ectoderm are important for epidermis and body appendage outgrowth such as the nails, hair, ectodermal and mesenchymal compartments of developing teeth (the enamel of the teeth), apical ectodermal ridge of the limb buds and genital tubercle (Dolle *et al.,*

1992; Robledo *et al.,* 2002; Merlo *et al.,* 2002). In late stages of development, Dlx genes are expressed in the skin and differentiating skeletal tissues (Depew *et al.,* 2002; Francis-West *et al.,* 2003). Expression of Dlx genes are partially overlapping, especially for tightly linked pairs (Robledo *et al.,* 2002; Morasso *et al.,* 2005). Targeted depletion studies in mice indicate both redundant and distinct functions of the Dlx genes (Morasso *et al.,* 2005).

1.3.2 Biochemical features of DLX3

1.3.2.1 Structure of DLX3

The human DLX3 is a 287 aa protein with the HD located between 130–189 aa (Bryan & Morasso, 2000). The 60 aa HD is highly conserved in structure throughout evolution (Scott *et al*., 1989; Bürglin & Affolter, 2015). The basic structure of HD is described as a globular domain with three alpha helices arranged in a helix-turn-helix pattern (Scott *et al*., 1989; Gehring *et al*., 1994). Helices I and II lie parallel to each other and overhang the third helix, making contacts with phosphate groups to stabilize HD-DNA interaction (Scott *et al*., 1989; Gehring *et al*., 1994; Bürglin & Affolter, 2015). The third helix, referred to as the ‘recognition helix’, lies in the major groove of DNA to make specific intermolecular contacts and thereby confers the DNA-binding specificity of HD proteins (Scott *et al*., 1989; Gehring *et al*., 1994). Additional base-specific recognitions are made by the amino terminal arm of HD—the loop between helices I and II and part of helix II, via contacting with the adjacent minor groove (Scott *et al*., 1989; Gehring *et al*., 1994; Bürglin & Affolter, 2015). Several conserved arginine residues found in HD contribute to sequence preference of the TA

rich-core of the consensus binding sites of homeoproteins (Gehring *et al*., 1994; Rohs *et al*., 2009).

The presence of the DNA binding HD in DLX3 suggests DLX3 serves as a transcription factor and likely regulate target gene expression by acting on promoter or enhancer regions. The optimal binding sequence for DLX3 was determined to be 5´-(A/C/G) TAATT(G/A) (C/G)-3´ by mobility shift assays using *in vitro* bacterially manufactured *Xenopus* DLX3 protein (Feledy *et al*., 1999a). This consensus binding sequence is centered on a TA-rich core, consistent with previously described features of typical HD binding sites. Notably, this sequence closely resembles the optimal binding site 5´-CTAATTG-3´ of the transcriptional repressor Msx I (Catron *et al*., 1995), suggesting potential competition for a common consensus sequence between the two classes of HD-containing proteins. However, the case of DLX and Msx is more complicated as DLX and Msx can form heterodimers through HD-HD interactions, resulting in functional antagonism of reciprocal inhibition of transcription activities (Zhang *et al*., 1997).

As a transcription factor, DLX3 was found to be nuclear localized (Bryan & Morasso, 2000). A bipartite nuclear localization signal (NLS) (124 –150 aa) has been ascribed to be crucial for nuclear transportation and transcription activity of DLX3 (Bryan & Morasso, 2000). Besides the HD and NSL, transactivation domains of DLX3 have also been described, consisting of two subdomains located on either side of HD (Bryan & Morasso, 2000; Feledy *et al*., 1999a). Studies showed specific sequences at the amino terminus (1–43 aa) and carboxyl terminus (189–220 aa) were responsible for DLX3's transactivation-stimulating activity (Bryan & Morasso, 2000).

1.3.2.2 DLX3 as transcription factor

DLX3 is generally reported as a transcriptional activator. Overexpression of DLX3 in *in vitro* cell lines induced strong activation of a model target gene construct containing DLX3 binding sites upstream from the TATA element (Feledy *et al*., 1999a). In mammalian systems, DLX3 has been reported to actively regulate expression of target genes in a number of tissues. For instance, DLX3 induces osteocalcin, Runx2 and osteoactivin during bone differentiation (Hassan *et al.,* 2004; Hassan *et al.,* 2006; Singh *et al.,* 2012). In addition, previous work in our lab identified positive regulatory roles of DLX3 in expression of placental glycoprotein hormone α subunit gene (hCGα) and matrix metalloproteinase 9 (MMP9) (Roberson *et al*., 2000; Berghorn *et al*., 2006). In this dissertation we describe transcriptional regulation of DLX3 on the *hPGF* gene which encodes an important placenta-specific factor linked to human disease. On the other hand, DLX3 has also been reported to function as a negative regulator of gene transcription. During *Xenopus* development, DLX3 expression in embryonic ectoderm appears to repress a specific class of neural genes, which was proposed to contribute to define the rostral border of the central nervous system (Feledy *et al*., 1999b). The transactivation/repression activities of DLX3 might be dependent on different cellular contexts and physiological states, and be influenced by its binding partners.

An earlier study showed that the transcription activity of DLX3 relied on two transcription domains separated by HD, and loss of either subdomain resulted in complete loss of DLX3 function (Bryan & Morasso, 2000; Feledy *et al*., 1999a). Herein, we confirm that the transcription activity of DLX3 in regulating *hPGF*

expression involves in both the amino and carboxyl domains. However, we find loss of either one of the two domains still retains robust transcription-stimulating activity of DLX3. In fact, the HD, together with either the amino or carboxyl domain alone, would supports even higher transcriptional activities compared with that of full length DLX3. This suggests redundancies of the amino and carboxyl domains in conferring the transcriptional activity of DLX3. Based on previously reported studies and our preliminary data, we further predict that the amino and carboxyl domains might have additional functions besides serving as transactivation domains, such as conferring target sequence specificity, providing post-translational modification sites, acting as scaffolds for recruitment of other transcription factors or cofactors which is discussed in Chapter Four.

1.3.3 Expression pattern and functions of *DLX3*

The expression pattern of *Dlx3* is different from those of other members of *Dlx* family in that it is undetectable in the forebrain (Boncinelli *et al.,* 1994). *Dlx3* is expressed in derivatives of non-neural surface ectoderm and a series of structures derived from epithelial-mesenchymal interaction throughout development, such as the hair follicles, dental and mammary gland epithelium, the external respiratory epithelium of the naris, taste bud primordia and the apical ectodermal ridge of limb buds (Robinson *et al.,* 1994; Dirksen *et al.,* 1994; Morasso *et al.,* 1995). Interestingly, *Dlx3* is also richly expressed in the mouse and human placentae and has been implicated to be crucial in placental development (Morasso *et al.,* 1999; Chui *et al.,* 2010). Functional importance of DLX3 in these tissues is underscored by a number of

disorders associated with *DLX3* mutation or dysregulation in humans.

The autosomal dominant tricho-dento-osseous (TDO) syndrome is characterized by abnormal hair at birth, dysplastic nails, enamel hypoplasia, taurodontism, and increased density and thickness of craniofacial bones (Lichtenstein *et al.,* 1972; Price *et al.,* 1998a; Price *et al.,* 1998b). This disease was found to be caused by a common *DLX3* gene mutation (c.571_574delGGGG) in multiple families affected with the TDO syndrome. The four nucleotide deletion lies downstream of the homeobox in the coding region of *DLX3* and is predicted to result in a truncated protein with an altered carboxyl terminus. Similarly, another mutation in *DLX3* gene (c.561_562delCT) was identified in amelogenesis imperfecta with taurodontism (AIHHT), which presents an attenuated phenotype of TDO (Dong *et al.,* 2005; Wright *et al.,* 2008). Other mutations in the *DLX3* gene have been reported in various geographic regions, all associated with TDO-like disorders in affected individual and families (Nieminen *et al.,* 2011; Li *et al.,* 2015). Interestingly, even single nucleotide polymorphisms (SNPs) in *DLX3* (rs2278163) have been suggested to associate with dental caries in Japanese children (Ohta *et al.,* 2015). A recent study has described target genes of DLX3 in amelogenesis to include enamel matrix protein (EMP) genes *Amelx*, *Enam*, *Klk4*, and *Odam* (Zhang *et al.,* 2015). These collectively indicate the requirement of fully functional DLX3 in morphogenesis of epidermal derivatives (hair) and other ectodermal derivatives (teeth and craniofacial skeleton).

The abnormal intramembranous bone formation in the skull featured in TDO suggests a potential role of DLX3 in general osteogenesis and bone homeostasis. This is supported by the mouse model in which conditional knock out of Dlx3 in

mesenchymal cells and osteoblasts resulted in increased bone mass throughout the life span of the animal, owning to upregulated osteoblast activity and bone matrix gene expression (Isaac *et al.*, 2014). Studies in this area have identified target genes of DLX3 critical for bone differentiation including osteocalcin, Runx2 and osteoactivin (Hassan *et al.*, 2004; Hassan *et al.*, 2006; Singh *et al.*, 2012).

DLX3 also plays important roles in epidermal stratification. In mice, *Dlx3* expression is extinguished everywhere except in the skin by the end of embryonic development, where it is observed in stratified epidermis and in the matrix cells of hair follicles (Morasso *et al.*, 1994). Both ectopic expression of *Dlx3* in the basal cell layer of mature skin and epidermis-specific loss of *Dlx3* were associated with adverse outcomes: the former led to disrupted epidermal proliferation and differentiation, and the latter was linked to IL-17–associated skin inflammation, indicating that stringent control of DLX3 expression at the spatial-temporal level is required for normal epidermal development and homeostasis (Morasso *et al.*, 1996; Hwang *et al.*, 2010). In addition, DLX3 has been found to modulate keratinocyte cell cycle control in coordination with the tumor suppressor p53, and loss of *DLX3* expression in skin is likely to contribute to progression of squamous cell tumors in humans (Palazzo *et al.*, 2015).

DLX3 has also been implicated in hematopoiesis. Transcriptional silencing of *DLX3* in B cells was specifically found in the MLL-AF4 rearranged subtype of acute lymphoblastic leukemia (ALL) in children (Campo Dell'Orto *et al.*, 2007). This silencing is likely to be attributed to hypermethylation of large CpG islands on 5´ flanking region of *DLX3*, and thereby the identified hypermethylation pattern of *DLX3*

promoter has been proposed as an epigenetic marker for subtype screening of the disease (Campo Dell'Orto *et al.*, 2007).

1.3.4 DLX3 in the placenta

1.3.4.1 Expression of DLX3 in the placenta

In normal mouse placentae, *Dlx3* is expressed in the ectodermal components (ectoplacental cone and chorionic plate) by E8.5, a region that gives rise to both the labyrinth and spongiotrophoblast layers of the placenta (Morasso *et al.*, 1999). Expression of *Dlx3* is maintained through midgestation with abundant transcripts to be detected in the labyrinthine layer at E10.5, and continues but diminishes through E15.5 (Berghorn *et al.*, 2005). *Dlx3* displays highly spatio-specific expression in the labyrinth compartment—the murine equivalent of the villous placenta where major blood exchange occurs, but does not appear to express in trophoblast giant cells in mouse (Morasso *et al.*, 1999). On the other hand, *Dlx3* null mice exhibit embryonic lethality between E9.5 to E10 due to placental failure (Morasso *et al.*, 1999). The *Dlx3* null placenta is characterized by significantly compromised morphogenesis of the labyrinth layer and abnormally formed fetal and maternal vascular network (Morasso *et al.*, 1999). These studies highlighted previously unrecognized functions of DLX3 during placental development.

Qualitative immunohistochemistry analyses in human placenta tissues have revealed spatially specific distribution of DLX3 in villous CTB, STB, EVT in the proximal regions of the CTB columns and endothelial cells surrounding the fetal capillaries in human placentae (Chui *et al.*, 2010). Work from colleagues in the

Roberson lab demonstrated that DLX3 was nuclear localized in both CTB and STB within microvilli of first trimester human placenta at a time of peak hCG synthesis and secretion (Roberson *et al.,* 2001). The detection of DLX3 in human first trimester and term placental tissues suggests its sustained expression throughout gestation (Chui *et al.,* 2010). Abundant DLX3 is detected in the cultured human placental trophoblast cell line JEG-3, and is found to localize to the nuclear compartment, consistent with its role as a transcriptional regulator (Holland *et al.,* 2004). Interestingly, *DLX3* expression was found to be increased in placentae complicated with IUGR (Chui *et al.,* 2012) but decreased in those affected with PE (Murthi *et al.,* 2004), suggesting the involvement of *DLX3* dysregulation in these pregnancy-associated disease conditions in human.

1.3.4.2 Functions of DLX3 in the placenta

DLX3 has been implicated in several important processes during placental development by regulating expression of placental specific genes. Our work in this area has demonstrated that DLX3 has a positive regulatory effect on basal expression of the *hCG α subunit gene* (*hCGα*) through binding to the junctional regulatory element (JRE) at the gene promoter (Roberson *et al.,* 2001). HCG is a heterodimeric glycoprotein produced by trophoblasts and secreted predominantly into the maternal circulation (Cole, 2010). It is remarkably produced in early pregnancy and acts as a luteotropin effectively prolonging the life of the corpus luteum for progesterone production, which is crucial for uterine preparation for implantation and early pregnancy recognition (Cole, 2010). In addition, an early study reported upregulation

of the placental specific *steroidogenic enzymes 3β-hydroxysteroid dehydrogenase/isomerase* (*3β-HSD*) by DLX3 in *in vitro* cell lines (Peng *et al.*, 2002). Because 3β-HSD is essential for biosynthesis of all steroid hormones including progesterone and estrogen within placentae, and thus is critical for maintenance of pregnancy, this study potentially supported a direct connection between DLX3 and placental steroid hormone production (Peng *et al.*, 2002). However, further analyses using *in situ* hybridization in the mouse placenta revealed differentially spatial distributions of *Dlx3* and *3β-HSD* expression, where *Dlx3* expression was localized within the labyrinth region and *3β-HSD* was restricted in trophoblast giant cells, suggesting the proposed transcription connection between DLX3 and *3β-HSD* is unlikely to be physiologically important (Berghorn *et al.*, 2005). Additional candidate target genes of DLX3 have been proposed to include *syncytin*, *hCG*β (Chui *et al.*, 2011), *GATA2* and *PPAR*γ (Chui *et al.*, 2013), which, however, based on very preliminary data obtained in human placental cell lines.

DLX3 has also been shown to involve in trophoblastic expression of *bovine interferon-tau* (*IFNT*) in ruminants (Ezashi *et al*., 2008). IFNT is a peptide hormone essential for early pregnancy maintenance by acting locally on the uterine endometrium in a paracrine manner (Spencer & Bazer, 2004). DLX3 was shown to transactivate the bovine *IFNT* gene promoter in human trophoblasts (Ezashi *et al*., 2008). Additionally, DLX3 together with another transcription factor v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) was shown to induce synergistic transactivation of the *IFNT* promoter in human and bovine trophoblast cell lines (Ezashi *et al*., 2008).

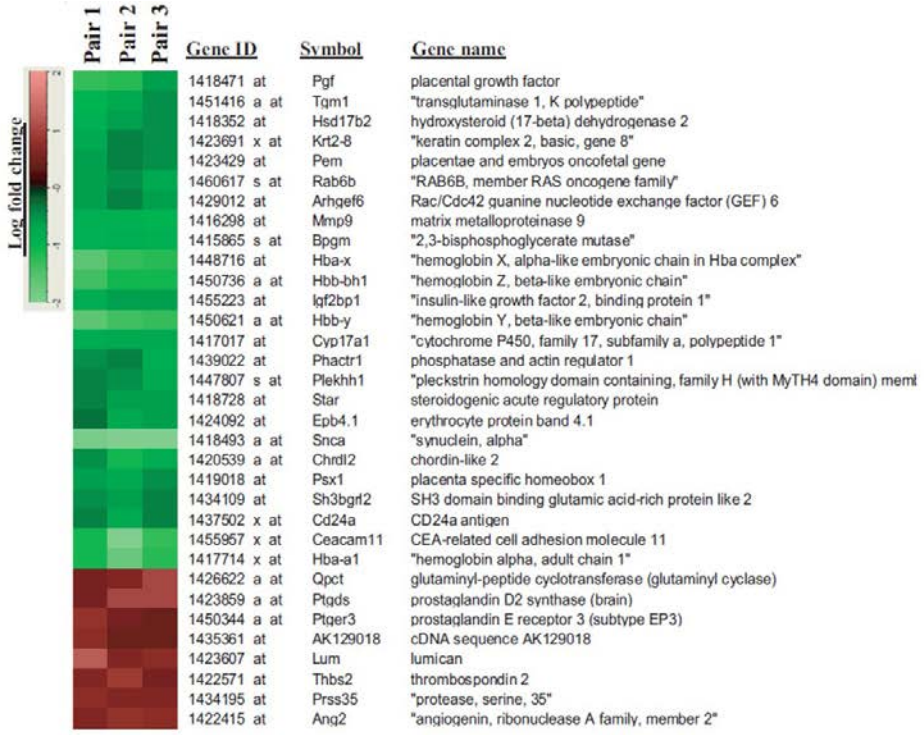

Figure 1.3 | Dlx3-dependent transcriptome in mouse placentae (adapted from Han, L. *et al.* 2007. *Endocrinology*). Microarray assays performed in placentae of *Dlx3* null and wild-type mice, and revealed eight significantly upregulated genes (red) and twenty-five significantly repressed genes (green), including *Pgf* and *Mmp9* in *Dlx3* null placentae. *Colored pixels* represent the magnitude of the gene response (log fold change).

In order to obtain a more comprehensive understanding of the gene profile regulated by DLX3, the Roberson lab has described a *Dlx3*-dependent transcriptome by performing microarray analyses in placentae of *Dlx3* null ($Dlx3^{-/-}$) and wild-type mice (Han *et al.*, 2007). The array analyses identified thirty-three genes that were

differentially expressed in *Dlx3*$^{-/-}$ placentae at statistically significant levels compared with the wild-type placentae: eight genes were down-regulated and twenty-five were upregulated (Figure 1.3) (Han *et al.*, 2007). Interestingly, *Mmp9* and *Pgf* were among the cohort of genes downregulated with *Dlx3* ablation, and both of them had been previously described in placental morphogenesis and angiogenesis.

The gelatinase MMP9 actively engages in uterine ECM remodeling and facilitates trophoblast invasion by degrading ECM components including collagens, gelatin and elastin (Kessenbrock *et al.*, 2010). It is richly expressed by uterine natural killer cells, decidual cells, and trophoblasts in human placentae (Anacker *et al.*, 2011; Cohen *et al.*, 2006). Importantly, altered expression of *MMP9* has also been reported in PE-placentae in humans (Lalu *et al.*, 2007, Qiao *et al.*, 2007, Rahimi *et al.*, 2013). *Mmp9* ablation in mice resulted in IUGR and increased rate of embryonic death due to deficiencies in trophoblast differentiation and invasion. In support of the association between dysregulated *MMP9* and PE development in humans, pregnant *Mmp9*$^{-/-}$ mice bearing *Mmp9*$^{-/-}$ embryos exhibited clinical features of PE including proteinuria, elevated blood pressure and kidney pathology (Plaks *et al.*, 2013). Colleagues in the Roberson lab have investigated mechanisms controlling *MMP9* expression in human placental trophoblast cells and identified two DLX3 binding sites at the *MMP9* promoter that were critical for DLX3-dependent *MMP9* gene transcription (Clark *et al.*, 2013), indicating the involvement of DLX3 in modulating trophoblast invasion and ECM degradation via regulating expression of *MMP9*.

PGF is a proangiogenic growth factor extensively expressed in placentae

(discussed in section 1.2). Dysregulation of *PGF* is linked to PE development and progression. Previous work in our lab has suggested a role of *Dlx3* in *Pgf* expression in mice (Han *et al.,* 2007). Inspired by these studies, this dissertation presents experimental work testing the hypothesis that DLX3 is an upstream regulator of *hPGF* in human placental trophoblast cells. I find DLX3 binds and activates the *hPGF* promoter to upregulate its expression. Loss- and gain- of function studies in JEG-3 cells indicate DLX3 is both sufficient and required for *hPGF* expression. I further describe the critical sequence on the *hPGF* promoter which DLX3 binds with and acts on. In addition, I demonstrate that DLX3 collaborates with another placental specific transcription factor—GCM1 in regulating *hPGF* expression. My study provides important insights into fundamental understanding of molecular events controlling placental *hPGF* expression and potentially therapeutic strategies for pregnancy-related disorders associated with reduced PGF expression by targeting DLX3 and GCM1.

1.3.5 Regulation of DLX3

Upstream transcription factors, signal transduction pathways, and important *cis*-elements have been described to regulate the *DLX3* gene expression. One of the best known regulators of the *DLX3* gene promoter is the bone morphogenetic protein (BMP) signaling pathway which has been demonstrated to modulate *DLX3* expression during ventral ectodermal development and in several mature cell types including keratinocytes, hair follicles and osteoblasts (Park & Morasso, 2002; Hwang *et al*., 2008; Hassan *et al*., 2006; Yang *et al.*, 2014). SMADs, p38, and PKA/β-catenin

pathways have been implicated to mediate this BMP-DLX3 axis (Park & Morasso, 2002; Yang *et al*., 2014; Viale-Bouroncle *et al*., 2015). However, compared with other BMP target genes such as *Msx I, DLX3* appeared less sensitive to the BMP signaling (Beanan & Sargent, 2000). For instance, blocking the BMP signaling using BMP receptor inhibitors reduced but did not eliminate *Dlx3* expression in *Xenopus* embryonic ectoderm, suggesting redundant pathways controlling *Dlx3* expression (Feledy *et al.*, 1999a). In addition to BMPs, the fibroblast growth factor (FGF) signaling was shown to play an important role in maintaining *DLX3* expression in epidermis during limb development and regeneration, possibly by forming a positive autocrine feedback loop with DLX3 (Mullen *et al*., 1996).

Several *cis*-acting regulatory sequences essential for the expression of *Dlx* genes have been characterized. The intergenic region of the murine *Dlx3* and *Dlx4* contains shared *cis* elements that regulate their expression (Sumiyama *et al*., 2002). Proximal regions of murine *Dlx3* gene and of its *Xenopus* ortholog (*Xdll2*) that confer the maximal promoter activities have also been described (Morasso *et al.*, 1995; Park & Morasso, 1999). In addition, the p53 family member p63 was shown to act upstream of DLX3 and appeared to be essential for the development and maintenance of the stratified epidermis in mice (Radoja *et al.*, 2007). DLX3 has been proposed to serve as a negative feedback regulatory signal to promote p63 degradation through a Raf-dependent pathway (Di Costanzo *et al*., 2009).

DLX3 is also regulated at the posttranslational level. Several types of posttranslational modifications on the DLX3 amino domain have been described: phosphorylation at serine 10 by PKA was reported to increase the transcriptional

activity and stability of DLX3 (Li *et al.*, 2014); SUMOylation at K83 and K112 by SUMO1 appeared to promote DLX3 transcriptional activity (Duverger *et al.*, 2011). These post-translational modifications serve to modify functions of DLX3 independent of variations in transcriptional regulation.

Notably, protein-protein interaction could potentially influence the transcription activity of DLX3. For instance, when co-expressed, DLX3 and Msx I were found to dimerize via their HDs, which prevented DLX3 from binding to target sequence in the genome (Zhang *et al.*, 1997; Bryan & Morasso, 2005). The Roberson lab characterized additional regulation of DLX3 activity by Smad6 in human trophoblast cells. The DLX3 and Smad6 interaction was identified through a yeast two hybrid screen by using full length DLX3 as bait. Smad6 was found to bind to a region of DLX3 (80–163 aa) that includes a portion of the HD. As expected, this association negatively affected DLX3's interaction with DNA, and was thought to play a negative modulatory role in the regulation of DLX3-dependent gene transcription within placental trophoblasts (Berghorn *et al.*, 2006).

1.4 Glial cell missing-1 (GCM1)

Glial cell missing (*gcm*), also known as *glial cells deficient* (*glide*), was first described in the development of *Drosophila* nervous system. As the name implies, loss-of-function mutations in *gcm* resulted in early lethality featured by dramatic loss of glial cells in the *Drosophila* CNS (Jones *et al*., 1995, Vincent *et al*., 1996). *Gcm* and the related *gcm2*, which function redundantly in some situations, are expressed transiently during early development in precursor glial cells and appear to control

determination of cell fate in the CNS: progenitor cells expressing *gcm* differentiate to glial cells; ectopic expression of *gcm* can transform presumptive neurons into glial cells (Hosoya, 1995; Jones *et al*., 2005; Chotard *et al*., 2005; Altenhein *et al*., 2006; Altenhein *et al*., 2015). *Gcm* is also expressed in and important for differentiation of hemocytes, peritracheal cells and tendon in *Drosophila* (Bernardoni *et al*., 1997; Alfonso *et al*., 2002; Laneve *et al*., 2013; Soustelle *et al*., 2004; Cattenoz *et al*., 2016).

1.4.1 Expression of GCM1 in mammals

Two *gcm* homologs have been identified in mammals: *GCM1*/*Gcm1* and *GCM2*/*Gcm2* (Akiyama *et al.*, 1996; Nait-Oumesmar *et al.*, 2000; Kanemura *et al.*, 1999; Kim *et al.*, 1998). Expression patterns and functions of *GCM* homologs in mammals are not well-conserved from those in *Drosophila*. *Gcm1* and *Gcm2* are detectable at low levels in the CNS and appear to have little relevance to the binary switch between the neural-glial cell fate (Kim *et al.*, 1998; Altshuller *et al.*, 1998). Instead, they have been implicated in epigenetic regulation of target gene expression that are crucial for neural stem cell (NSC) maintenance in the CNS (Hitoshi *et al.*, 2011).

Mammalian GCM proteins are detected in placenta, kidney, thymus, pharyngeal pouches and parathyroid gland (Hashemolhosseini *et al.*, 2002). Importance of GCM in the development and maintaining physiological integrity of these tissues are underscored by pathological contexts including PE, hyper- or hypoparathyroidism, and parathyroid gland tumors (Chen *et al.*, 2004; Mannstadt *et al*., 2008; Nonaka *et al*., 2011). *GCM1* is highly expressed by trophoblast cells in the

mouse and human placentae and required for placental development (Basyuk *et al*., 1999; Baczyk *et al*., 2004; Chen *et al.*, 2004). It is also found in selective cell populations in thymus and the S3 segment of proximal tubes (Hashemolhosseini *et al.*, 2002). *GCM2* on the other hand, is expressed during embryonic development in the parathyroid-specific region that later gives rise to the parathyroid glands (Kebebew *et al*., 2004). *Gcm2* knockout mice or mice with *Gcm2* dysregulation suffer from hypoparathyroidism (Yuan *et al*., 2014; Günther *et al*., 2000; Grigorieva *et al*., 2010). Consistently, mutations or inactivation of *GCM2* have been found to cause hypoparathyroidism in humans (Mannstadt *et al*., 2008; Canaff *et al*., 2009).

1.4.2 Biochemical features of GCM1

The human *GCM1* encodes a 436 aa zinc finger transcription factor with a nuclear localization signal and a DNA-binding domain localized in the amino terminal 150 residues (Hospya *et al.*, 1995; Jones *et al.*, 1995). The DNA binding domain, termed the GCM motif, is well-conserved within in the GCM family and binds with high specificity to an octameric binding motif, 5′-ATGCGGGT-3′ (Hashemolhosseini & Wegner 2004; Cohen *et al.*, 2003; Akiyama *et al.*, 1996). At the protein level, the GCM domain is characterized by a novel fold composed of two subdomains of β-sheets rigidly held together by coordination of two structural zinc cations (Hashemolhosseini & Wegner 2004; Cohen *et al.*, 2003).

1.4.3 GCM1 in the placenta

1.4.3.1 Expression of GCM1 in the placenta

In situ hybridization analysis of mouse placentae at E7.5 revealed that *Gcm1* was expressed exclusively in the extraembryonic ectoderm (basal layer) of the chorion, which comes into direct contact with the allantois during chorioallantoic fusion (Basyuk *et al*., 1999). In later stages after the chorioallantoic fusion, abundant *Gcm1* mRNA is detected in the syncytial layer underlying maternal blood sinuses and CTB columns in the labyrinth layer (Basyuk *et al*., 1999). Expression of *Gcm1* is stronger in some clusters of trophoblasts compared to others, indicating trophoblast cells are heterogeneous with respect to *Gcm1* expression and potentially protein turnover (Basyuk *et al*., 1999). This pattern of *Gcm1* expression in the labyrinth is sustained until around E15.5 when it starts to decrease as parturition nears (Basyuk *et al*., 1999).

Studies have shown a generally conserved expression pattern of *GCM1* in human placentae, where it is distributed in villous CTB, CTB columns and STB (Baczyk *et al*., 2004). Consistent with its primary role of a transcription factor, the GCM1 protein was observed to localize in the nuclei of CTB and the STB layer of the villi by immunocytochemistry staining (Baczyk *et al*., 2004; Chen *et al.*, 2004). Longitudinal expression of *GCM1* in human pregnancy was also evaluated (Chen *et al.*, 2004) and revealed a relatively constant expression pattern throughout gestation with the highest level in the first trimester and decreased level in the third trimester, particularly after 37 weeks, possibly reflecting a shrinking number of *GCM1* expressing CTB which are at early stages of differentiation as gestation progresses (Chen *et al.*, 2004). Importantly, significant reductions in GCM1 protein and mRNA were observed in placentae obtained from patients with PE compared with gestational

age-matched controls, suggesting the involvement of dysregulated *GCM1* in pathogenesis of PE (Chen *et al.*, 2004). However, evidence supporting this finding is limited and further studies of *GCM1* expression in normal and disease-complicated human placentae are warranted.

1.4.3.2 Functions of GCM1 in the placenta

GCM1 is a prominent factor in the development, maintenance and turnover of the human trophoblasts and plays central roles in the regulation of key aspects of placental development. GCM1 blocks CTB proliferation by promoting cell-cycle arrest in these cells and induces their differentiation (Baczyk *et al*., 2009). In vitro, inhibition of GCM1 by siRNA increased proliferation rate of the BeWo cells (human choriocarcinoma cell line) and reduced formation of the STB compartment in human placental explants (Baczyk *et al*., 2009). Downregulation of GCM1 additionally inhibited CTB differentiation along the invasive pathway in extravillous explants on matrigel (Baczyk *et al*., 2009). Conversely, activation of GCM1 by forskolin, a pharmacological activator of adenylyl cyclase activity and the cyclic AMP (cAMP), led to decreased mitosis and increased syncytialization of CTB in the floating villous explant model (Baczyk *et al*., 2009). Notably, decreased GCM1 expression is correlated with defects in both extravillous and villous trophoblast lineages observed in PE-complicated placentae (Baczyk *et al*., 2009; Chen *et al*., 2004).

GCM1 plays a pivotal role in placental development by regulating the expression of several essential genes in trophoblasts cells for proper differentiation and functions of trophoblast lineages. GCM1 was first identified to regulate the

placental expression of the aromatase gene, which is responsible for estrogen biosynthesis (Yamada *et al*., 1999). Remarkably, GCM1 is the first transcription factor described to be capable of inducing STB formation from CTB. It is now known that this induction is largely attributed to activation of downstream gene targets of GCM1—*syncytin* genes in CTB (Liang *et al.*, 2010). The syncytin fusogenic proteins and their receptors are central regulators mediating CTB fusion into the syncytial layer (Esnault *et al*., 2008; Liang *et al*., 2010; Yu *et al*., 2002). By acting directly on the gene promoters, GCM1 upregulates *syncytin2* and its receptor *MFSD2A*, likely involved a demethylation mechanism (Liang *et al*., 2010). *High-temperature requirement protein A4* (*HtrA4*) is another gene regulated by GCM1 in placenta (Wang *et al.*, 2012). *HtrR4* encodes a serine protease that cleaves fibronectin and hence involves in uterine ECM remodeling to facilitate EVT invasion during placentation (Wang *et al.*, 2012). Moreover, the HtrR4 protein has been showed to interact with the syncytin-1 protein and suppress cell-cell fusion (Wang *et al.*, 2012). Therefore, by upregulating *Htr4A*, GCM1 promotes invasion event other than the fusion event in EVT, contrasting with its fusion-induction function in CTB via regulating *syncytin* expression (Wang *et al.*, 2012). GCM1 has also been implicated in hCG synthesis by STB. Cheong and colleagues identified a GCM1-binding site (GBS1) on the *hCGβ* promoter which was activated upon GCM1 association. Production of hCG, in turn, stimulated the expression of GCM1 target genes and promoted placental cell fusion through cAMP and PKA activation, as inhibitors of PKA blocked hCG-induced trophoblast differentiation (Pidoux, G. *et al*., 2007; Shi *et al*., 1993). Therefore, it appears that a positive feedback loop between GCM1 and

hCG modulates hCGβ expression and trophoblast differentiation in the human placenta (Cheong *et al*., 2015).

The expression pattern and functions of GCM1 in placenta were highlighted in *Gcm1* deficient mice models (Anson-Cartwright *et al*., 2000; Schreiber *et al.*, 2000; Bainbridge *et al*., 2012). Complete loss-of-function of *Gcm1* in mice leads to embryonic lethality around E10 due to malformation of the placental labyrinth layer, featured with blocked branching of the chorioallantoic interface and absence of STB lineage (Anson-Cartwright *et al*., 2000; Schreiber *et al.*, 2000). Heterozygous *Gcm1* mutants are fertile, but exhibit abnormal trophoblast differentiation and fetoplacental vascularity, increased syncytial necrosis and dysregulation of *syncytin* and *sFlt1* genes (Bainbridge *et al*., 2012). Interestingly, wild-type mothers carrying *Gcm1* heterozygous fetuses developed late gestational hypertension—one of the hallmarks of PE in humans (Bainbridge *et al*., 2012).

1.4.4 Regulation of GCM1

Like DLX3, expression of *GCM1* and activities of GCM1 are regulated at multiple levels. It has been well-characterized that the cAMP-PKA pathway controls *GCM1* expression at both transcriptional and post-transcriptional levels. At the transcriptional level, cAMP-PKA upregulates *GCM1* by promoting the binding of the cAMP response element-binding protein (CREB) to the CREB binding site on the *GCM1* promoter region (Schubert *et al*., 2008). OASIS, another member of the CREB family, mediates PKA-induced *GCM1* transcription by the similar mechanism (Schubert *et al*., 2008). Consequently, overexpression of CREB or OASIS in

choriocarcinoma cells was shown to lead to placental cell fusion (Knerr *et al*., 2005; Schubert *et al*., 2008). Interestingly, CREB and OASIS exhibit sequential expression pattern during mouse placental development, suggesting a highly organized order of GCM1 transcriptional regulation throughout placentation (Schubert *et al*., 2008). PKA also stimulates the association of GCM1 with the CREB-binding protein (CBP), which acetylates GCM1 at the transactivation domain, leading to increased GCM1 stability against ubiquitination and a concomitant increase in transcriptional activity (Chang *et al*., 2005). Another GCM1 binding partner, histone deacetylase 3 (HDAC3), has been shown to interfere with CBP/GCM1 interaction and thereby counteracts the acetyl-transferase coactivator activity of CBP on GCM1 (Chuang *et al.*, 2006). Activation of the PKA signaling was shown to stimulate disassociation of HDAC3 from GCM1 and to enhance binding of CBP with GCM1 (Chuang *et al.*, 2006). In addition, PKA-induced phosphorylation of GCM1 at Ser47 could be recognized by the desumoylating enzyme SENP1, which subsequently desumoylates and increases transcriptional stimulating activity of GCM1 (Chang *et al.*, 2011). Taken together, the PKA signaling pathway both regulates transcriptional expression and post-translational modifications of GCM1, thereby increasing the expression, stability, transcriptional activity of GCM1.

The PKC signaling pathway is another important player in GCM1 regulation (Yasui *et al*., 2012). Unlike PKA, PKC appears to modulate GCM1 activity largely at the post-translational level through ERK-dependent phosphorylation of GCM1 at key serine residues S328, S378 and S383 (Yasui *et al*., 2012). It has been suggested that PKC-induced phosphorylation would alter the interaction of GCM1 with its binding

partner(s) and thereby remove protection from degradation, leading to a decrease in GCM1 stability (Yasui *et al*., 2012).

Aside from CBP and HDACs, accumulating evidence has implicated the influence of other binding partners on transactivation-stimulating activity and stability of GCM1. For instance, the placental specific transcription factor GATA3 was reported to bind with GCM1 (Chiu & Chen, 2016). While their interaction did not affect DNA binding of GCM1, GATA3 appeared to repress the transcription activity of GCM1, as placental cell lines of GATA3 knockdown showed elevated expression of the GCM1 target genes and increased invasiveness (Chiu & Chen, 2016).

Oxygen tension has been shown to downregulate GCM1 activity through inhibiting the cAMP-PKA pathway and activating the GSK-3β signaling (Loberg *et al.*, 2002; Chiang *et al*., 2009); the latter was shown to phosphorylate GCM1 at a site that promotes its ubiquitination and degradation via E3 ligase (Chiang *et al*., 2009).

1.5 Summary

The placenta is the least studied and understood organ in the human body. Investigation in the placenta-originated human diseases, such as PE, is highly desirable. Downregulation of *hPGF* is associated with PE development and hPGF has been proposed as a prominent target for prediction, diagnosis and potentially therapeutic management of PE in women. The key question in the area is what controls *hPGF* expression within placentae of normal pregnancies and how *hPGF* is dysregulated in placentae complicated with PE. Answers to this question will not only elaborate our understanding of PE pathogenesis, but also provide mechanisms

potentially translatable to clinical application. Herein my dissertation, I have described mechanisms of regulating placental *hPGF* expression by two important placental specific transcription factors—DLX3 and GCM1, in the JEG-3 trophoblast cell line. For the first time, I demonstrate that DLX3 is a competent and required factor for *hPGF* expression. I also identify an interaction between DLX3 and GCM1 by detecting their formation of a functional complex on the *hPGF* promoter in regulating *hPGF* expression in an antagonistic manner. Focusing on transcription regulation of *hPGF*, I have identified a *cis* acting sequence on the *hPGF* promoter that appears to mediate both the basal expression of *hPGF* and DLX3- and GCM1-induced upregulation of *hPGF*. I have also explored molecular and biochemical mechanisms underlying the DLX3 and GCM1 interaction, where I find inhibitory effect of DLX3 on the transcription activity of GCM1. Structural studies focusing on DLX3 reveal the central role of the DLX3 homeodomain in mediating the binding of DLX3 with GCM1, and the inhibitory effect of DLX3 on GCM1 activity. While not being essential, the DLX3 amino and carboxyl domains appear to facilitate the homeodomain in GCM1 binding and repression. Investigations and observations made in this dissertation provide a potentially important mechanism for the stringently controlled *hPGF* expression under physiological conditions within the placenta at the spatial-temporal level, where it requires collaborative inputs from both DLX3 and GCM1. In addition, my study uncovers a novel function of DLX3 in trophoblasts as an inhibitory modulator of the transactivation-stimulating activity of GCM1 in regulating *hPGF* expression. The inhibition of DLX3 on GCM1 might serve as a fine tuning mechanism to control *hPGF* expression at a normal level, thereby preventing placenta

from overgrowth. Moreover, our studies suggest a potential therapeutic strategy of inducing *hPGF* expression by manipulating specific activities of DLX3 and GCM1 as well as their interaction in human trophoblast tissues, providing insights into further prediction and treatment of PE.

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CHAPTER TWO. DISTAL-LESS 3 AND GLIAL CELL MISSING-1 FUNCTIONALLY COLLABORATE TO REGULATE PLACENTAL GROWTH FACTOR EXPRESSION IN HUMAN TROPHOBLAST CELLS

Sha Li and Mark S. Roberson

Department of Biomedical Sciences, College of Veterinary Medicine,

Cornell University, Ithaca, NY

Authors contributions:

S. Li: graduate student responsible for constructing the *hPGF* promoter reporter, and performing deletion mutagenesis, luciferase assays, qPCR, WB, siRNA, and ChIP studies; prepared figures and wrote initial draft of the manuscript.

Dr. M.S. Roberson: investigator and corresponding author; responsible for oversight of the entire project and final manuscript.

2.1 Abstract

Human placental growth factor (*hPGF*) is abundantly expressed by trophoblast cells in human placentae and is important for trophoblast proliferation and placental vascular development. Circulating levels of hPGF in maternal sera are dynamically upregulated throughout gestation in normal pregnancies, whereas lower levels of plasma hPGF are associated with preeclampsia (PE). Therefore, hPGF has been proposed as a biomarker to predict PE. However, mechanisms of *hPGF* expression regulation in the human placenta remain poorly understood. In this study, we demonstrate that transcription factors Distal-less three (DLX3) and Glial cell missing-1 (GCM1) upregulate *hPGF* expression through binding to a regulatory region at the *hPGF* promoter in the JEG-3 human trophoblast cell line. This regulatory region is also found to be essential for basal transactivation of *hPGF*. Surprisingly, co-overexpression of DLX3 and GCM1 contributes to an antagonist regulation of *hPGF* expression. Further, single depletion of DLX3 or GCM1 via small interfering RNA-mediated knockdown reduces endogenous *hPGF* expression whereas dual depletion of DLX3 and GCM1 leads to additive reduction, suggesting that both DLX3 and GCM1 are required for *hPGF* expression. Finally, ChIP-qPCR reveals colocalization of DLX3 and GCM1 at the identified regulatory region on the *hPGF* promoter. Taken together, our study provides important insights into regulation of *hPGF* expression through the functional collaboration of DLX3 and GCM1.

2.2 Introduction

Successful pregnancies in eutherian mammals rely on formation and maintenance of a functional placenta at the fetal-maternal interface (Moffett & Loke, 2006). The establishment of the placenta in turn is dependent on stringently controlled processes of placental angiogenesis and trophoblast development that are closely linked (Cross *et al*., 2002). The VEGF family member placental growth factor (PGF), plays important roles during placental development as a proangiogenetic factor to stimulate placental angiogenesis and a growth factor to support trophoblast cell proliferation, differentiation and survival (Athanassiades *et al*., 1998; Torry *et al*., 2003; Knuth *et al*., 2015). *HPGF* is expressed at minimal levels in most tissues under normal conditions, but abundantly expressed by trophoblast cells within the placenta (Persico *et al*., 1999; Voros *et al*., 2005). As a secreted protein, hPGF is found to be dynamically upregulated in the maternal circulation during normal pregnancies, with the peak at around 27~30 gestational weeks (Clark *et al*., 1998; Taylor *et al*., 2003). Importantly, low levels of plasma hPGF during gestation have been linked to pregnancy-specific diseases in human, such as preeclampsia (PE) and intrauterine growth retardation (IUGR) (Kumazaki *et al*., 2002; Levine *et al*., 2004; Benton *et al*., 2012). This deficiency in circulating PGF can be detected in PE-complicated pregnancies several weeks before clinical onset of maternal syndrome featured by hypertension and proteinuria, suggesting a role for PGF in pathogenesis of PE (Sibiude *et al*., 2012; Chappell *et al*., 2013). In support of this, the BPH/5 animal model of PE was described to exhibit dysregulated PGF in maternal sera during pregnancy (Woods *et al*., 2011). In fact, the ratio of sFlt-1 (the soluble form of PGF

receptor) and hPGF in maternal circulations has been proposed to be a prominent biomarker to predict PE in women (Zeisler *et al*., 2016; Chappell. *et al*., 2013). Importantly, it has been reported that hPGF administration abolished ischemic placenta-induced hypertension in the RUPP rat model of PE, suggesting PGF to be a potential target for the treatment of PE in women (Spradley *et al*., 2016).

Therefore, understanding the regulation of *hPGF* in normal pregnancies and its aberrant expression in PE is highly desirable to unravel pathogenesis as well as potential prevention, early diagnosis and treatment of this disease. However, current knowledge in this area is limited. Previous studies indicated that hypoxia could upregulate *PGF* in cardiomyocytes and fibroblasts (Green *et al*., 2001; Torry *et al*., 2009), but downregulate it in trophoblast cell lines (Khaliq *et al*., 1999; Gobble *et al*., 2009), suggesting distinct pathways governing *PGF* expression in trophoblast and non-trophoblast cells. The mechanism by which hypoxia regulates *PGF* expression is largely unclear. However, it does not appear to be mediated by direct actions of hypoxia-inducible factors (HIFs) on the *hPGF* promoter (Green *et al*., 2001; Gobble *et al*., 2009). On the other hand, the placental specific transcription factor Glial cell missing-1 (GCM1) has been implicated in transactivation of the *hPGF* promoter in trophoblast cell lines (Chang *et al*., 2008). GCM1 plays crucial roles in trophoblast differentiation and is known for regulating expression of the *syncytin* genes, which encode fusogenic proteins that are sufficient and required for cytotrophoblasts (CTB) to fuse into multinucleated syncytial trophoblast cells (STB) (Liang *et al*., 2010; Yu *et al*., 2002). The resulting formation of the placental syncytial layer is essential for blood exchange and hormone production within the placenta (Liang *et al*., 2010). In

accordance with that, *Gcm1* knockout mice are embryonic lethal due to placental failure featured by the absence of syncytial formation (Anson-Cartwright *et al*., 2000; Schreiber *et al.*, 2000). In the present study, we confirmed the transactivating role of GCM1 on the *hPGF* promoter in the JEG-3 human trophoblast cell line. In addition, we identified an important GCM1 binding site on the *hPGF* promoter that was responsible for GCM1-induced *hPGF* expression. Furthermore, we found that GCM1 formed an functional antagonism with another placental specific transcription factor—Distal-less three (DLX3) in regulating *hPGF* expression.

The homeodomain (HD)-containing protein DLX3 is highly expressed in some trophoblast cell lineages. Like Gcm1, Dlx3 loss-of-function mutation in mice leads to embryonic lethality at midgestation characterized by placental deficiency in syncytial layers (Morasso *et al*., 1999). Our previous work in this area identified several placental specific target genes of DLX3, including the α subunit gene of human chorionic gonadotropin (hCGα) and matrix metalloproteinase 9 (MMM9) (Roberson *et al*., 2001; Clark *et al*., 2013). Rich production of the heterodimer glycoprotein hCG by trophoblast cells is important for early recognition and preparation of pregnancy, and is the basis for urine pregnancy test (Cole, 2010). The gelatinase MMP9 facilitates trophoblast invasion through remodeling extracellular matrix (ECM) by degrading ECM components including collagens, gelatin and elastin (Plaks *et al*., 2013). To elaborate DLX3-involved gene network and obtain a more comprehensive interpretation of DLX3-dependent transcriptome, we have performed microarray analyses in placentae of Dlx3 null and wild-type mice, where we found loss of Dlx3 resulted in the marked down regulation of *Pgf* (Han *et al*., 2007).

In this study, we demonstrate that both DLX3 and GCM1 are sufficient and required for upregulating *hPGF* in JEG-3 cells by performing overexpression and siRNA-mediated knock down studies. The *hPGF* promoter luciferase reporter assays indicate that DLX3 and GCM1 induce *hPGF* expression by transactivating the *hPGF* promoter. Moreover, deletion mutagenesis studies on the *hPGF* promoter reveal a critical *cis* acting sequence (-369 to -320, distance relative to the TATA box in nucleotides) that mediates DLX3- and GCM1-dependent *hPGF* expression. Additionally, the identified *cis* acting sequence appears important for the basal expression of *hPGF*, as deletion of this sequence dampens basal activation of the *hPGF* promoter luciferase reporter in JEG-3 cells. Chromatin-immunoprecipitation (ChIP) assays demonstrate occupancies of both DLX3 and GCM1 on the identified *hPGF* promoter sequence. Interestingly, while both DLX3 and GCM1 are transactivators of *hPGF*, their combination results in an antagonistically regulatory effect. This antagonism appears to be specific to *hPGF*, as it is not seen in cases of the DLX3 target gene *hCGα* and GCM1 target gene *ERVFRD-1* (the human homolog of *syncytin2*). Collectively, we demonstrate that DLX3 is an important transcriptional activator of *hPGF* in JEG-3 cells and confirm that of GCM1 in a similar way. We identify a novel regulatory mechanism of *hPGF* expression through the collaboration between DLX3 and GCM1. This is also the first report describing inter-play between DLX3 and GCM1 in gene regulation.

2.3 Results

2.3.1 DLX3 induces *hPGF* expression.

To explore possible regulatory functions of DLX3 on *hPGF* expression, a human DLX3 expression plasmid was transiently transfected into JEG-3 human choriocarcinoma cells as a model system for human placental trophoblasts. Overexpression of DLX3 resulted in significant and dose-dependent increases in *hPGF* expression at both mRNA and protein levels (Figure 2.1a, b). We then focused on the effect of DLX3 on *hPGF* transcription. A fragment of the *hPGF* promoter (-4560 to +400, distance relative to the TATA box in nucleotides) was cloned into a luciferase reporter plasmid and transiently transfected into JEG-3 cells in the absence or presence of DLX3. As is shown by Figure 2.1c, DLX3 overexpression activated the *hPGF* promoter in a dose–dependent manner. Expression of exogenous DLX3 was confirmed by western blot analysis making use of the hemagglutinin (HA) epitope tag on the overexpressed DLX3. Taken together, our data indicate that DLX3 positively regulated *hPGF* expression in JEG-3 cells through acting on the *hPGF* promoter.

2.3.2 DLX3 represses GCM1-induced *hPGF* expression.

Previous study has shown upregulation of the *hPGF* promoter activity by GCM1 overexpression in human trophoblast cell lines in a luciferase system (Chang *et al*., 2008). This led us to ask how *hPGF* is regulated in the context of the combined actions of DLX3 and GCM1. We speculated that co-expression of both transcription factors might better recapitulate the complicated *in vivo* physiological environment since all DLX3, GCM1 and *hPGF* are actively expressed in the same trophoblast

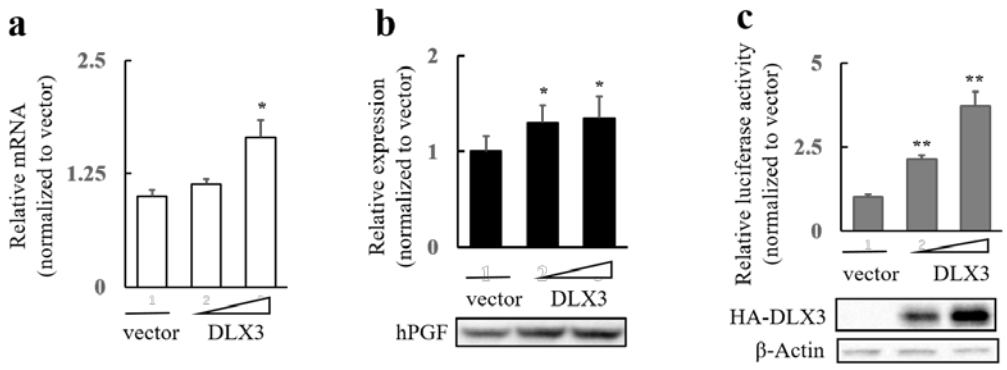

Figure 2.1 | DLX3 induces *hPGF* expression. JEG-3 cells (per 35-mm well) were transfected with 0.5 μg or 1 μg DLX3 expression plasmid, and subsequently collected and analyzed for changes in endogenous (**a**) mRNA levels of *hPGF* by qRT-PCR or (**b**) secreted levels of the hPGF protein in culture media by western blot. Bar chart in (b) showed the relative expression based on pixel intensity and exposure conditions in the western blot film below, using the Image Lab software (Bio-Rad). (**c**) JEG-3 cells transfected with indicated plasmids for 12 h were then transfected with 0.2μg *hPGF* promoter luciferase reporter plasmid, and harvested 24 h later for luciferase assay. A 5′-upstream region of *hPGF* promoter of –4560 to +400 nucleotide distance relative to the TATA box was used in the study. Lower panel: expression of exogenous DLX3 was detected by western blot using the HA antibody, with β-Actin used as a endogenous reference for loading control. Results are the mean±s.d. from three independent experiments in triplicates and asterisks denote a statistically significant difference compared with values of vector control (**P*<0.05, ***P*<0.01, ****P*<0.001).

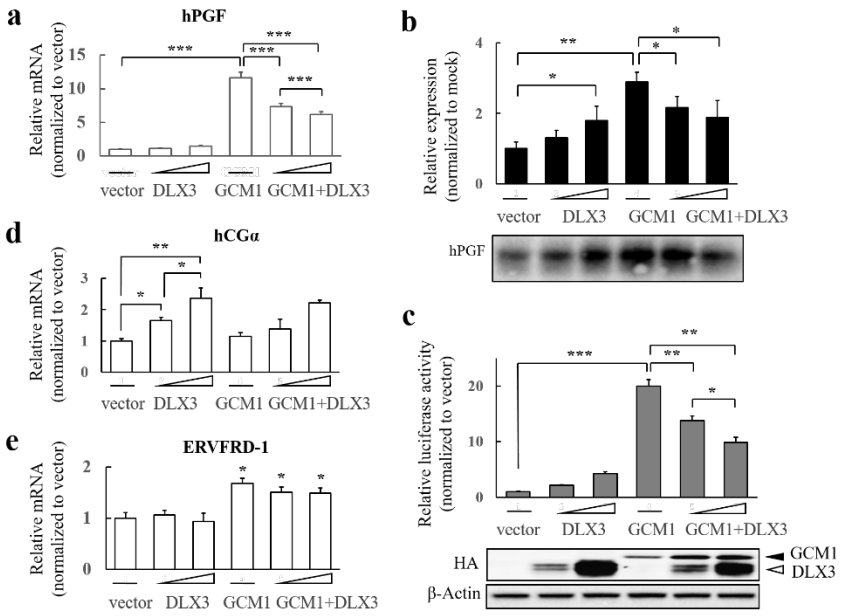

Figure 2.2 | DLX3 and GCM1 collaboratively regulate *hPGF* expression in an antagonistic manner. JEG-3 cells (per 35-mm well) were transfected with indicated plasmids and subsequently collected for the detection of changes in endogenous mRNA levels of (**a**) *hPGF*, (**d**) *hCGα* and (**e**) *ERVFRD-1* by qRT-PCR or (**b**) secreted levels of the hPGF protein by western blot in the culture media. The upper panel bar chart in (**b**) represents relative expression of the hPGF protein based on pixel intensity and exposure conditions in western blot films (representative blot film shown below) using the Image Lab software. (**c**) Relative transactivation of the *hPGF* promoter induced by overexpression of DLX3 or GCM1, or both. Luciferase assays were performed in JEG-3 cells transfected with indicated plasmids for 12 h and then with 0.2μg of the *hPGF* promoter (–4560 to +400) luciferase reporter plasmid, and harvested 24 h later. Western blot films on the lower panel detected overexpression of HA-DLX3 and HA-GCM1 using the HA antibody, with β-Actin used as a endogenous reference for loading control. Results are the mean±s.d. from three independent experiments in triplicates and asterisks denote a statistically significant difference compared with values of vector control (**P*<0.05, ***P*<0.01, ****P*<0.001).

populations within human placentae (Chui *et al.*, 2010; Baczyk *et al*., 2004; Khaliq *et al*., 1996). To address this question, we first assessed changes in *hPGF* expression at the mRNA and protein levels in JEG-3 cells overexpressing DLX3 or GCM1, or both. We observed that GCM1 alone was a stronger inducer of *hPGF* compared to DLX3 alone, albeit both significantly increased *hPGF* expression (Figure 2.2a, b). Interestingly, when combined, DLX3 and GCM1 formed a functional antagonism in a DLX3 dose-dependent manner (Figure 2.2a, b). The effect of DLX3 and GCM1 co-overexpression on the activity of the *hPGF* promoter was also assessed in the -4560 *hPGF* promoter luciferase system. Consistent with observations in endogenous *hPGF*, DLX3 was found to be a relatively modest transactivator compared to GCM1, and the combination of DLX3 and GCM1 antagonistically activated the *hPGF* promoter in a DLX3 dose-dependent manner (Figure 2.2c). These data supported that the functional antagonism between Dlx3 and GCM1 was at the transcriptional level. In contrast, the functional antagonism of the DLX3/GCM1 pair in gene regulation was not observed in DLX3 gene target *hCG*α and GCM1 gene target *ERVFRD-1*, indicating the inhibitory effect of DLX3 on GCM1 was likely to be *hPGF* specific (Figure 2.2d, e). Overexpression of DLX3 and GCM1 was confirmed by western blot analyses using the HA antibody since both exogenous proteins were HA-tagged (Figure 2.2c).

2.3.3 Depletions of DLX3 and GCM1 silence *hPGF* expression.

Studies thus far take advantage of overexpression; however, this holds the strong caveat that expression levels in these studies may be supraphysiological. Thus we next sought to examine regulatory roles of DLX3 and GCM1 on *hPGF* expression

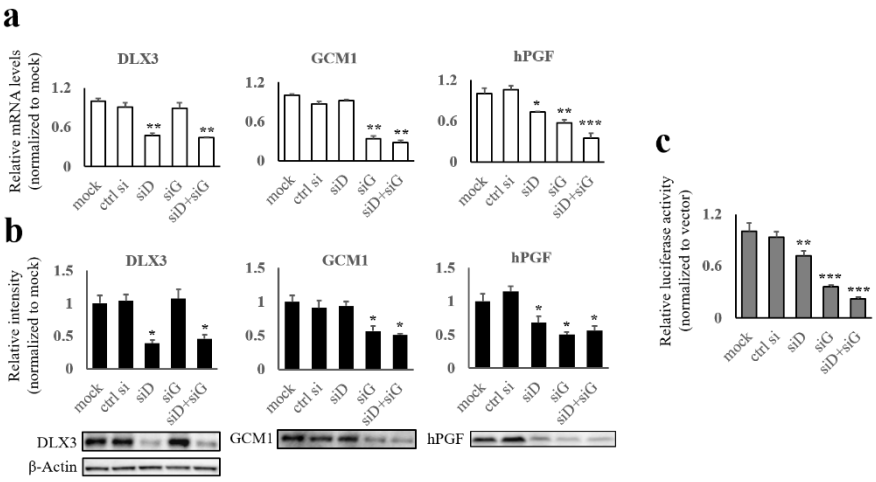

Figure 2.3 | Both DLX3 and GCM1 are required for *hPGF* expression. (**a**) JEG-3 cells were transfected twice with the pool of four siRNA mixtures against DLX3 or GCM1, or with both for 24 h, and collected for the detection of endogenous *hPGF* at the transcript level by qRT-PCR (**a**) or in the culture media at the protein level by western blot (**b**). Scramble siRNA of random sequences were used as the negative control. Changes in endogenous DLX3 and GCM1 at both the transcript and protein levels were also examined to validate knockdown efficacy. The upper bar chart in **b** showed relative changes in expression normalized to the mock control cells by analyzing the pixel intensity of bands and the exposure condition of western blot films using the Image Lab software. β-Actin was used as the endogenous loading control. Lower panel in **b** were representative western blots. (**c**) Decreased *hPGF* promoter activities were detected by luciferase assays in JEG-3 cells transfected with indicated siRNAs and the *hPGF* promoter (–4560 to +400) luciferase reporter plasmid for 24 h. Results are the mean±s.d. from three independent experiments in triplicates and asterisks denote a statistically significant difference compared with values of the mock control (**P*<0.05, ***P*<0.01, ****P*<0.001).

under siRNA-mediated knockdown conditions directly addressing the requirement for DLX3 and GCM1 in *hPGF* expression in JEG-3 cells. Synthesized interfering RNAs targeting human DLX3 or GCM1 were transiently transfected to JEG-3 cells to suppress endogenous DLX3 or GCM1 expression. Depletion of either DLX3 or GCM1 alone was able to significantly decrease *hPGF* expression at both mRNA and protein levels (Figure 2.3a, b). Dual depletion of DLX3 and GCM1 further reduced *hPGF* expression in an additive manner (Figure 2.3a, b). Consistent with these observations, luciferase reporter assays showed significant reduction in activities of the *hPGF* promoter in JEG-3 cells of DLX3 or GCM1 knockdown, and an additive reduction in cells of dual depletion (Figure 2.3c). Scrambled siRNAs of random sequences were used as the negative control. Knock down efficacies of DLX3 and GCM1 by siRNAs were confirmed at both transcript and protein levels (Figure 2.3a, b).

2.3.4 DLX3 occupies multiple sites at the *hPGF* promoter.

Based on our discovery of DLX3-dependent upregulation of the endogenous *hPGF* by activating the *hPGF* promoter, we hypothesized that DLX3 may bind to the *hPGF* promoter and hence control gene transcription. In silico sequence analysis of the *hPGF* promoter revealed eight sites or site clusters containing nucleotide sequences with high affinity to a previously identified DLX3 binding core motif (Feledy *et al*.,1999): -4490, 5'-TCAATTAA-3'; 3290, 5'-ATAATTAA-3'; -2729, 5'-TTAATTTT-3'; -2557, 5'-AAAATTAAAATTAG-3'; -2304, 5'-TAAATTAA-3'; -2137, 5'-AAAATTAAAATTAAAATTAC-3'; -1986, 5'-CTAATTC-3'; -1718, 5'-

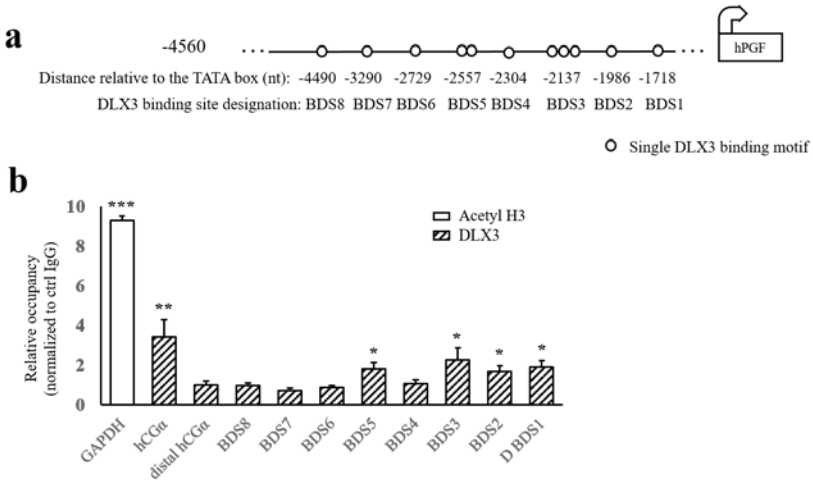

Figure 2.4 | DLX3 occupies multiple sites at the *hPGF* promoter. (**a**) Schematic illustration of distributions of eight putative binding sites (BDS) for DLX3 at the *hPGF* promoter identified by sequence analysis. The number below each BDS represents its location at the promoter relative to the TATA box in nucleotides (nt). (**b**) Relative occupancies of endogenous DLX3 at eight loci of putative DLX3 BDS on the *hPGF* promoter in JEG-3 cells were assayed by ChIP-qPCR using the DLX3 antibody. The acetyl histone H3 antibody (detecting acetyl histone H3 occupancy on the *GAPDH* promoter) and normal rabbit IgG were used as the positive and negative antibody respectively to control chromatin epitope specificity. A DLX3 BDS containing locus on the *hCGα* promoter and a random distal locus were used as the positive and negative locus respectively to control the specificity of the DLX3 antibody. Relative occupancy is normalized to control IgG and the distal locus. Data are mean±s.d of two independent experiments of in triplicates and asterisks denote a statistically significant difference compared with values of the distal *hCGα* control (**P*<0.05, ***P*<0.01, ****P*<0.001).

CAAATTAA-3' (Figure 2.4a). ChIP-qPCR assays were performed for the detection of DLX3 occupancies on these potential binding sites (BDS), and demonstrated constitutive enrichment of endogenous DLX3 binding on four BDS located near the TATA box on the *hPGF* promoter (Figure 2.4b). Enrichment of acetyl H3 on constitutively activated *GAPDH* promoter was used as the positive control for immunoprecipitation, whereas enrichment of DLX3 on its previously characterized binding site on the *hCGα* promoter (Roberson *et al*., 2001) served as the positive control to indicate specificity of the DLX3 antibody used in the studies (Figure 2.4b). A distal region of the *hCGα* gene was used as the negative control locus for background. Data were normalized to the control IgG for each IP and presented as fold changes relative to the negative locus control.

2.3.5 A critical region (-369 to -320) on *hPGF* promoter is required for basal and DLX3/GCM1- induced expression of *hPGF*.

Similarly, we identified several sequences within the *hPGF* promoter that share relatively high homology (87.5%) with the previously reported optimal GCM1 binding motif (Akiyama *et al*., 1996; Schreiber *et al*., 1998): -3591, 5'-TCCCCGCATA-3'; -2678, 5'-CAGGCGGGTCT-3'; -2244, 5'-TGAGGCGGGA-3'; -1046, 5'-ACCCCGCTTA-3'; -1021, 5'-CCCCCGCCTC-3'; -414, 5'-AGACCCGCAAA-3'; -292, 5'-GCCCCGCAG-3'; -42, 5'-ATGCAGGC-3'. To test the functional importance of the identified four DLX3 BDS as well as putative GCM1 BDS in regulating *hPGF* expression, serial deletion constructs of the *hPGF* promoter from -4560 to -146 were generated by PCR and incorporated in the same luciferase

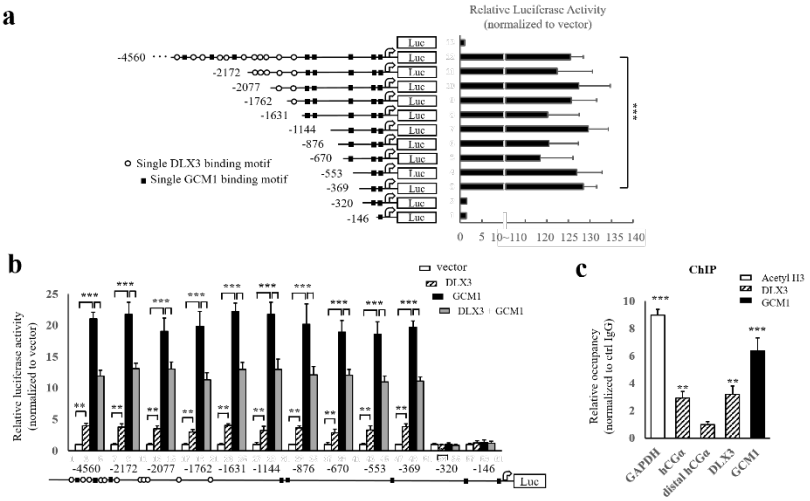

Figure 2.5 | A key promoter sequence (-369 to -320) is required for basal and DLX3/GCM1-induced *hPGF* expression. (**a**) Constructs of truncated *hPGF* promoter incorporated into the luciferase reporter schematically illustrated on the left in series were individually transfected into JEG-3 cells at equimolar amounts of 0.2 μg of the -4560 construct per 35-mm well, which were subject to luciferase assays for the detection of the basal activation of each truncated promoter. Luciferase activity is normalized to the empty luciferase vector. (**b**) JEG-3 cells (per 35-mm well) were transfected with 1 μg DLX3 or 0.5 μg GCM1 expression plasmid, or both for 12 h, and then followed by a second transfection with designated mutated constructs at equimolar amounts of 0.2 μg of the -4560 construct for 24 h and then collected for luciferase assays. (**c**) ChIP-qPCR was performed for the detection of occupancies of DLX3 and GCM1 on the promoter locus containing the identified critical sequence responsible for basal *hPGF* expression. Data are mean±s.d of three (**a, b**) or two (**c**) independent experiments of in triplicates and asterisks denote a significant difference compared with values of vector control (*P<0.05, **P<0.01, ***P<0.001).

reporter vector, as is schematically shown in Figure 2.5a. They were firstly transfected into JEG-3 cells for the detections of basal activation of each truncated promoter by luciferase assays. The -369 to -320 region was identified to be required for basal *hPGF* expression (Figure 2.5a).

Next, we detected the response of each truncated promoter to DLX3 and GCM1 overexpression in JEG-3 cells. As shown in Figure 2.5b, none of the DLX3 BDS suggested by ChIP-qPCR were found to be functionally indispensable in DLX3-induced *hPGF* upregulation. Conversely, the -369 to -320 region of the proximal promoter that lacks any known consensus binding sequences of DLX3 and GCM1 was important for DLX3- and GCM1-induced *hPGF* transactivation (Figure 2.5b). Moreover, this -369 to -320 region was also critical for the antagonistic regulation of *hPGF* by the DLX3/GCM1 pair (Figure 2.5b). This led us to ask whether DLX3 and GCM1 might colocalize at this region. Thus, ChIP-qPCR assays were performed using DLX3 and GCM1 antibodies and indeed revealed highly significant occupancies of both DLX3 and GCM on the -369 to -320 region (Fig. 5c).

Taken together, we identified a critical *cis* acting sequence at -369 to -320 on the *hPGF* promoter, which was essential for mediating the basal activation of *hPGF*, the DLX3-/GCM1-dependent *hPGF* transactivation, and the DLX3/GCM1 antagonistic regulation of *hPGF*. These together with the finding that both DLX3 and GCM1 were enriched in the region suggest (i) the DLX3-/GCM1-controlled *hPGF* expression might serve as a central mechanism for *hPGF* expression in trophoblast cells; (ii) the transcriptional inter-play between DLX3 and GCM1 might involve physical interaction.

2.4 Discussion

Our studies demonstrate that *hPGF* expression in JEG-3 cells is regulated by DLX3 and GCM1, both of which act as transcriptional activators by binding to a common region on the *hPGF* promoter. We show that in addition to upregulating *hPGF* expression, DLX3 appears to act as a repressive modulator of GCM1 which exhibits stronger transactivation-stimulating activity of *hPGF*. In addition, this dialog between DLX3 and GCM1 in gene regulation seems to be specific to their common target of *hPGF*. We also provide evidence that our identified -369 to -320 region responsible for the DLX3/GCM1-induced *hPGF* upregulation also controls basal expression of *hPGF*, suggesting central roles of these two factors in *hPGF* expression regulation in trophoblast cells.

Based on the antagonistic inter-play between DLX3 and GCM1 and their colocalization on the *hPGF* promoter, it is appealing to postulate a competition mechanism of *hPGF* regulation. In this model, the relatively modest transactivator DLX3 and the stronger transactivator GCM1 compete for the same binding site(s) on the *hPGF* promoter to exert transactivation. Therefore, DLX3 overexpression would result in a transcriptional switch toward the DLX3-dominated modest upregulation of *hPGF* expression in a DLX3 dose dependent manner, leading to the functional antagonism phenomenon. However, this postulation predicts that reduced dosage of DLX3 would lead to GCM1-dominated drastic upregulation of *hPGF* expression, which conflicts with our observations from knockdown assays where either DLX3 and GCM1 depletion significantly reduced *hPGF* expression in JEG-3 cells. An alternative mechanism suggests a potentially inhibitory effect of DLX3 on the transcriptional

activity of GCM1, which implies a physical interaction between them. However, this mechanism requires cautious interpretation, since overexpression of DLX3 did not influence transcriptional activity of GCM1 on *ERVFRD-1*. It is possible that their interaction might be dependent on or facilitated by their binding sequence(s) on the *hPGF* promoter. Notably, the identified *hPGF* promoter region where GCM1 and DLX3 bind lacks any known DNA motifs recognized by the DLX3 homeodomain or the GCM1 domain, both considered DNA binding domains. Additional binding partners might engage in and contribute to the DNA-binding and transactivation-stimulating activities of DLX3 and GCM1 in regulating *hPGF* expression. These will be explored in our future studies.

As a VEGF family member, PGF positively modulates VEGF-dependent signaling pathways of angiogenesis, vasodilation, vascular permeability, etc. (Shibuya *et al.*, 2006). Unlike VEGF, which is essential both during embryogenesis and development after birth, PGF is most prominent in postnatal angiogenesis in physio-pathological conditions such as ischemia, tissue repair and inflammation (Cianfarani *et al*., 2006; Iwasaki *et al*., 2011; Oura *et al*., 2003). Having been proposed as a biomarker in prediction and clinical screening of PE in pregnant women (Zeisler *et al*., 2016; Verlohren *et al*., 2012; Park *et al*., 2014), hPGF is implicated in pathogenesis of PE (Levine *et al*., 2004). The development of PE appears in two stages (Redman *et al*., 2014): the placental stage characterized by failure of CTB invasion of maternal spiral arterioles, leading to a placenta of under perfusion. The ischemic placenta then upregulates hypoxic-responsive placental factors which are released to the maternal circulation. This local defect leads to the second clinical phase whereby systemic

responses in the mother are presented as hypertension, proteinuria, and various internal organ impair, predominantly due to endothelial dysfunction. HPGF is a typical placental derived factor that is secreted to make contacts with the maternal endothelial system (Levine *et al*., 2004). It has been proposed to be downregulated in the hypoxic placentae in PE, leading to insufficient detection in the maternal sera of PE-complicated pregnancies (Gobble *et al*. 2009). Concomitant with its downregulation is the upregulation of the hPGF receptor—sFlt-1 (Levine *et al*., 2004; Zeisler et al., 2016). Lacking the transmembrane domain, sFlt-1 functions as a decoy sink to sequester circulating VEGF and hPGF (Stepan & Faber, 2004). Increased sFlt-1 in circulation interferes the binding of VEGF and hPGF with their receptors (VEGFRs) expressed by maternal endothelial cells, leading to reduced endothelial VEGFR-mediated vasodilation and antithrombotic effects and hence the onset of hypertension and related syndromes (Kroll *et al*., 1998; Coultas *et al*., 2005). Downregulated VEGF-signaling caused by hPGF insufficiency and sFlt-1 overexpression in PE also impairs endothelial integrity in end organs such as fenestrated endothelium in the glomerular capillaries, therefore contributing to the development of proteinuria, another hallmark of PE (Di Marco *et al*., 2009). Our studies provide important insights into the tight control of *hPGF* expression within placentae in normal pregnancies and potential pathogenesis of *hPGF* downregulation associated with PE. Previous reports have described a common distribution of DLX3, GCM1, and PGF expression within human placentae (Chen *et al*., 2004; Baczetyk *et al*., 2009; Khaliq *et al*., 1996), including the STB layer, the CTB layer beneath the STB layer, and CTB columns. These placental structures are constitutively active in trophoblast proliferation,

metabolism, and angiogenesis throughout gestation. Abundant production of hPGF in these compartments, presumably regulated by DLX and GCM1, is well correlated with roles of hPGF in promoting all of these physiological processes important for placental development and function. On the other hand, decreased DLX3, GCM1 and hPGF in human placentae complicated with PE have been reported (Chen *et al*., 2004; Murthi *et al.,* 2004; Roberson lab unpublished data; Levine *et al*., 2004), suggesting our proposed model provides potentially important mechanisms for the pathogenesis of placental *hPGF* downregulation in these patients. Interestingly, concomitantly increased expression of DLX3 and reduced levels of hPGF have been implicated in IUGR (Chui *et al*., 2012), correlated with our finding of DLX3 as the repressive modulator on GCM1-induced *hPGF* expression. All these data support the potentially pathophysiological importance of our finding in the current study.

In summary, we have demonstrated the regulation of *hPGF* expression in JEG-3 cells by DLX3 and GCM1, highlighted by a functional antagonism between these two transcription factors. We confirmed both DLX3 and GCM1 are indispensable for *hPGF* expression. We also identified the key regulatory element at the *hPGF* promoter responsible for its basal expression as well as DLX3/GCM1-induced expression. ChIP assays showed occupancies of both DLX3 and GCM1 at this site. Chapter three of this dissertation will investigate biochemical and molecular mechanism of the DLX3/GCM1 antagonism, which is involved in their physical interaction. We have proposed our model based on the *in vitro* cell system. Further studies in *in vivo* physiological environments are highly desirable to confirm our hypotheses. Nevertheless, our study suggests that the maintenance and refinement of the *hPGF*

expression within the placenta requires cooperative inputs from both DLX3 and GCM1, implicating stringently controlled placental *hPGF* expression at the spatial temporal level. In addition, our identification of the antagonistic interplay between DLX3 and GCM1 in *hPGF* regulation might serve as a fine tuning mechanism to control *hPGF* expression at a normal level, thereby preventing placenta from overgrowth. Moreover, our studies suggest a potential therapeutic strategy of inducing *hPGF* expression by modulating specific activities of DLX3 and GCM1 in human trophoblast tissues, providing insights into further prevention and treatment of PE.

2.5 Materials and Methods

Plasmid constructs. A 4960-base pair (bp) fragment of *hPGF* promoter was generated from human genomic DNA (Roche, Basel, Switzerland) by polymerase chain reaction (PCR), nucleotide-sequence verified and inserted into a firefly luciferase reporter vector (described in Han *et al*., 2007), using the following primer set (forward/reverse, (distance relative to the TATA box in nucleotides)): 5'-GCCAGACTTGGGACATTTTC-3'/5'-CCTGGGTTTCAGGGACTGAG-3' (-4560/+400). Using this 4960 basepair (bp) promoter sequence as the template, a series of truncated *hPGF* promoter luciferase reporters were generated by PCR amplification using the same reverse primer and different forward primers as the following: 2572 bp—5'-GTAGCCTGGGAGAGAGAGCA-3' (-2172), 2477 bp—5'-AGCTGCTCTGGACTGGATGT-3' (-2077); 2162 bp—5'-AGTTCCTGGAGCATAGCAGG-3' (-1762); 2031 bp—5'-GAGACCAGGAGTTCTCGACC-3' (-1631); 1544 bp—5'-

CACAGTGACCCTCCACAATG-3' (-1144); 1276 bp—5'-TGGCTGGAATGTGAGCTGTA-3' (-876); 1070 bp—5'-GCACAGTCTTCACCCACAGA-3' (-670); 953 bp—5'-CGGACACGAACAGATCACAC-3' (-553); 769 bp—5'-GCAGCGTACAGTTCCTCCTC-3' (-369); 739 bp—5'-CAGGAGGAGATGCCTGAGTG-3' (-339); 684 bp—5'-GAGGGCCTGGGAAGACTG-3' (-284); 546 bp—5'-CACGTAGGCTTGGGTTTTGT-3' (-146). All sequences were verified by nucleotide sequence analysis. To facilitate cloning, *Kpn* I and *Hind* III restriction sites were incorporated at the end of 5' and 3' primers respectively. PKH3-DLX3 plasmid was described previously (Berghorn *et al*., 2006). The pHA-GCM1 plasmid was a gift from Dr. Hungwen Chen (Academia Sinica, Taiwan).

Cell culture and reagents. JEG-3 cells were purchased from American Type Culture Collection and cultured in Dulbeco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO). Media were supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin G, and 100 μg/ml streptomycin. Cells were incubated at 37 ºC in a humidified 5% CO_2 atmosphere. For the detections of secreted hPGF, Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-3 membrane (EMD millipore, Billerica, MA) was used to concentrate cell culture media from 2 mL to a 50 μL volume. Antibodies employed in this study included rabbit anti-DLX3 (ab66390, Abcam, Cambridge, UK), goat anti-GCM1 (sc-69407X, Santa Cruz Biotechnology, Inc., Dallas, TX), mouse anti-HA (sc-7392, Santa Cruz), goat anti-PGF (sc-1880, Santa Cruz), Rabbit anti-acetyl-Histone H3 Antibody (06-599, EMD millipore), Rabbit IgG - Isotype

Control (ChIP Grade) (ab171870, Abcam) and Goat IgG - Isotype Control (ab37373, Abcam).

Plasmid transfection and RNA interference. Cells were seeded into six-well dishes with 60 to 70% confluency 24 h before transfection. Plasmid and reported gene transfections were carried out using the Polyethylenimine transfection reagant (Polysciences, Inc. Warrington, PA), per the manufacturer's protocol. RNA interference (RNAi) transfections were performed using the DharmaFECT 1 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA) in a forward transfection manner with a typical siRNA dose of 50 nM. Untransfected samples are denoted as 'mock' in the figures. The individual siRNA duplexes used (Thermo Fisher Scientific) were: *DLX3* (L-012030-00-0005), *GCM1* (L-011491-00-0005) and non-targeting control siRNA (D-001810-01-05). Cells were harvested 48 h after transfection.

Luciferase reporter assay. Indicated luciferase reporter plasmid of 0.2 μg dose was used per well of cells (in 6-well dishes). Vectors of empty inserts were used for substitution to make a total 2 μg DNA per well in each transfection. After 8 h of transfection, each well was washed with phosphate-buffered saline (PBS), and then supplemented with fresh media. Forty-eight h later cells were collected in luciferase lysis buffer (Promega, Madison, WI). Luciferase activities were read by the illuminometer (LB9501, Berthold), using 10 μL of the lysate mixed with 50 μL of the substrate in the luciferase assay system (Promega), according to the manufacturer's instructions.

Western blot. Protein samples were separated on polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% milk in Tris-Buffered Saline and 0.05% Tween 20 (TBST, pH 7.6), incubated with desired primary antibody overnight followed by a two-hour incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, and bands were detected using enhanced chemiluminescence reagents (Bio-Rad).

Quantitative real-time PCR. Total RNA was extracted from cultured cells with TRIzol (Invitrogen) according to the manufacturer's instruction. Reverse transcription was conducted using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was carried out on an Applied Biosystems ABI 7500 qPCR machine (relative Quantification setting) using TaqMan Gene Expression Assays (Applied Biosystems), and data were analyzed through the $2^{-\Delta\Delta Ct}$ method, corrected for the efficiency of each TaqMan probe and normalized to the 18S housekeeping gene. All PCRs were performed in triplicate. Negative controls in which the template was replaced by PCR-grade water were included in each run.

Chromatin immunoprecipitation and quantitative real-time PCR. Approximately ten million JEG-3 cells per IP were crosslinked with 1% formaldehyde at room temperature for 10 min and quenched with 125 mM glycine on ice for 5 min. Cells were resuspended in cell lysis buffer (0.5% Triton X-100, 75 mM KCl, 5 mM Tris–HCl, pH 8.0 and complete protease inhibitor cocktail (Roche, Basel, Switzerland)) on

ice for 15 min and centrifuged at 1,300 rpm for five minutes. Isolated nuclei were then incubated in nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0 and complete protease inhibitor cocktail) for 15 min. Solutions were sonicated with a BioRuptor Sonicator (Branson sonifier 450) on ice until chromatin were sheared to approximately 300 bp length. Next, sonicated chromatin were immunoprecipitated overnight at 4°C with Protein A/G magnetic beads (EMD millipore) and the desired antibody. Subsequently, beads were washed once with low salt wash buffer (0.1% SDS, 1% Triton X-100, 1mM EDTA, 150mM NaCl, 20mM Tris-HCl, pH 8.0), once in high salt wash buffer (o.1% SDS, 1% Triton X-100, 1mM EDTA, 500mM NaCl, 20mM Tris-HCl, pH 8.0), once in LiC Buffer (250 mM LiCl, 0.5% sodium deoxycholate, 1% Triton X-100, 1mM EDTA, 10mM Tris, pH 8.0), and once in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The chromatin was then eluted in elution buffer (1% SDS, 10 mN EDTA, 200 mM NaCl, 50 mM Tris-HCl, pH 8.0) followed by reverse crosslinking with 10 μg ml^{-1} proteinase K at 62ºC for 2h. ChIP DNA were purified through QIAquick Spin Columns (Qiagen, Hilden, Germany) and specific regions were detected by quantitative real-time PCR using SYBR green master mix (Bio-Rad, Hercules, CA) on the ABI 7500 qPCR machine (absolute Quantification setting). Oligo primers for sequence detection are as follows: DLX3 BDS1—5'-CAGGCAATACATGCCAAATG-3'/ 5'-CTCCCAAAGTGCTGGGATTA-3' (-1739/-1672); DLX3 BS2—5'-GCCGCTGAGCCTACCCTA-3'/ 5'-AATTGGGGACCATACCCTTC-3' (-2001/-1947); DLX3 BS3—5'-GCCGCTGAGCCTACCCTA-3'/5'-TCACATCCAGTCCAGAGCAG-3' (-2146/-2075); DLX3 BS4—5'-CAAGATGGCAAGACCCTGTC-3'/5'-

TGTGCTACCATGCCCAGTTA-3' (-2334/-2294); DLX3 BS5—5'-GCCACTGCACCTGGTCTAAC-3'/5'-CCTTTGGTGCAAAGAATGGT-3' (-2618/-2521); DLX3 BS6—5'-GCCTCCCGAGTAGCTAGGAT-3'/5'-GCCTGGCAAACATGGTAAAA-3' (-2769/-2694); DLX3 BS7—5'-GGCCTCCATCTTTCCATCTT-3'/5'-CAAACAGTGTGCTTGGTGCT-3' (-3315/-3227); core element—5'-AGATGCACCGCTCATGGATA-3'/5'-CCCTGTGGTAGAGAGTGGTC-3' (-442/-323); human glycoprotein hormone α subunit gene (hCGα) — 5'-TGACCTAAGGGTTGAAACAAGATAAG-3'/5'-GGAAATTCCATCCAATGATTGA-3' (-146/-65); distal region of the hCGα gene—5'-AGTTTCTTTGTGGATGAAGAGATAGACG-3'/5'-TTTTCCGAACTTCAAAGGCCCTG-3' (3233/3558); GAPDH positive control—5'-TACTAGCGGTTTTACGGGCG-3'/5'-TCGAACAGGAGGAGCAGAGAGCGA-3'.

Statistical Analysis. Data were subjected to analysis of variance and differences between treatment groups by ANOVA followed by Student's unpaired *t* test. P values of less than 0.05 ($p<0.05$) were considered statistically significant.

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CHAPTER THREE. DISTAL-LESS 3 INTERACTS WITH GLIAL CELL MISSING-1 AND REPRESSES ITS TRANSACTIVATION-STIMULATING ACTIVITY

Sha Li and Mark S. Roberson

Department of Biomedical Sciences, College of Veterinary Medicine,

Cornell University, Ithaca, NY

Author contribution:

S. Li: graduate student responsible for immunoprecipitation studies, GAL4/UAS mammalian one hybrid assays, *hPGF* promoter reporter luciferase assays and DLX3 domain functional studies, and prepared figures and wrote initial draft of the manuscript.

Dr. M.S. Roberson: investigator and corresponding author; responsible for oversight of the entire project and final manuscript.

3.1 Abstract

The homeoprotein DLX3 and GCM-domain protein GCM1 are important transcription factors regulating placental development in the mouse and human. Our previous studies demonstrate that DLX3 and GCM1 colocalize at the *hPGF* promoter and regulate *hPGF* expression in an antagonistic manner. However, mechanisms accounting for this antagonism remains unexplored. In the current study, we address this question by performing immunoprecipitation and mammalian one hybrid assays in the JEG-3 human trophoblast cell line and reveal a physical interaction between DLX3 and GCM1. Interaction with DLX3 results in inhibition of the transactivation-stimulating activity of GCM1, leading to the functional antagonism of the DLX3/GCM1 pair in regulating *hPGF* expression. In addition, functional analyses on a series of DLX3 structural domains were carried out to identify specific regions of the DLX3 protein responsible for the association with and antagonistic modulation of GCM1. We found that the DLX3 homeodomain is required but not sufficient for the DLX3/GCM1 interaction and the inhibitory effect of DLX3 on GCM1. In addition, while the amino- and carboxyl-terminal portions of DLX3 alone were without effects, their presence along with the homeodomain appeared to enhance the antagonism of DLX3 on GCM1. Interestingly, a natural occurring carboxyl domain truncated mutant form of DLX3 observed in tricho-dento-osseous syndrome (TDO) in humans retains transactivation-stimulating activity on *hPGF* and the functional antagonism with GCM1 at a level comparable with wild-type DLX3. In summary, this is the first description of a functional association between DLX3 and GCM1 leading to inhibition of the transactivation-stimulating activity of GCM1. We further identify the DLX3

homeodomain to be the critical interacting domain with GCM1, whereas the DLX3 amino and carboxyl domains provides further supports of the DLX3/GCM1 interaction. Collectively, these studies suggest that DLX3 and GCM1 together may serve to control developmental progression of the placenta through hierarchical control of target gene transactivation.

3.2 Introduction

The establishment of the fetal-maternal interface is highlighted by trophoblast cell differentiation, proliferation and invasion into the uterine endometrium (Ji *et al*., 2013; Cartwright *et al*. 2010). Concurrent with the development and expansion of trophoblast lineages, vasculogenesis and angiogenesis occur extensively within the placenta to increase blood exchange and hence meet the growing demand for nutrients and gas of the developing fetus (Kaufmann & Mayhew, 2004). Blood exchange between the fetal and maternal circulation is further enhanced by remodeling of the maternal uterine spiral arteries driven by extravillous trophoblast (EVT) invasion to reduce vascular resistance of maternal vasculature and promote fetoplacental perfusion (Ji *et al*., 2013). Understanding signaling and gene regulatory networks that control trophoblast development and function form the foundation of our knowledge about normal placentation required for a successful pregnancy. On the other hand, defects in placental development have been linked to a number of pregnancy-related complications, among which preeclampsia (PE) is a particular noteworthy one as it is a leading cause for fetal and maternal mortality and mobility worldwide (Abalos *et al*., 2013; Ananth *et al*., 2013, Lisonkova *et al*.) with limited understanding of its

pathogenesis and thus lack of effective treatment strategies. Unravelling molecular mechanisms controlling the formation of healthy placentae and targeting dysregulated pathways within sick placentae that contribute to pathogenesis of these diseases would aid in our ability to intervene in such complications of pregnancies and enhance the possibility that maternal and fetal morbidity and mortality are reduced.

A number of placental-specific transcription factors essential for normal placental development have been described based upon genetically modified mouse models, and in many instances share similar molecular mechanisms with the human placenta (Knott *et al*., 2014; Douglas *et al*., 2009). Distal-less 3 (DLX3) is among the cohort of transcription factors known to be required for normal placental development in the mouse (Morasso *et al*., 1999). DLX3 was initially described as a key regulator of epidermal, osteogenic, and hair follicle cellular differentiation during development (Morasso *et al*., 1996; Hwang *et al*., 2008). Mutations of the *DLX3* gene in humans are associated with ectodermal dysplasia featured by defects in hair, teeth and bones, as seen in disease conditions of the tricho-dento-osseous syndrome (TDO) (Price *et al*., 1998) and Amelogenesis Imperfecta of the Hypomaturation-Hypoplasia type with Taurodontism (AIHHT) (Wright *et al*., 2008). The expression and involvement of *DLX3* in placental development was first described in *Dlx3* knockout mice that are embryonic lethal due to deficiencies in placental morphogenesis, specifically the labyrinth compartment where major blood exchange occurs (Morasso *et al*., 1999). In humans, several placental specific genes have been identified as regulatory targets of DLX3. These include *human chorionic gonadotropin α subunit* (*hCG*α) (Roberson *et al*., 2001) and *matrix metalloproteinase 9* (*MMP9*), both of which are required for a

successful pregnancy in human (Clark *et al*., 2013; Anacker *et al*., 2011).

Gila cell missing-1 (GCM1) is another well-known placental specific transcription factor. It has been implicated in promoting trophoblast differentiation, especially the formation of syncytiotrophoblast (STB) layer that is crucial for blood change and hormone synthesis (Anson-Cartwright *et al*., 2000). Remarkably, GCM1 is shown to drive the expression of mouse *syncytin*s and the human homolog *ERVFRD-1*, which encode fusogenic proteins to induce cytotrophoblasts (CTB) to fuse into multinucleated STB (Liang *et al*., 2010). *Gcm1* ablation in mice leads to a phenotype similar to that of *Dlx3* null mice, with midgestational embryonic death owning malformation of placentae (Anson-Cartwright *et al*., 2000; Schreiber *et al*., 2000). Notably, a complete missing of syncytial lineage was found in *Gcm1* null placentae, highlighting a direct role of *Gcm1* in STB formation. GCM1 also promotes differentiation and invasiveness of EVT by regulating the expression of *high-temperature requirement protein A4* (*HtrA4*) which supports extracellular matrix (ECM) remodeling and hence facilitates differentiation and invasion of EVT toward the maternal uterus (Wang *et al.*, 2012). Interestingly, wild-type dams carrying *Gcm1* heterozygous fetuses developed late gestational hypertension in mice—one of the hallmarks of PE in humans (Bainbridge *et al*., 2012).

As described in Chapter two, our studies have provided evidence that DLX3 and GCM1 work in concert in regulating *hPGF* expression in the JEG-3 human trophoblast cell line. We have identified that while both DLX3 and GCM1 were transcription activators, their combination resulted in an antagonism in *hPGF* expression regulation. We further observed colocalization of DLX3 and GCM1 on a

cis acting sequence of the *hPGF* promoter which was essential for DLX3/GCM1-regulated *hPGF* expression. Based on those, it is appealing to postulate a physical interaction between DLX3 and GCM1. Investigating the molecular mechanism behind this transcriptional inter-play is highly desirable for elucidating detailed mechanism controlling *hPGF* expression. This is of great importance considering that hPGF not only plays significant roles in placental angiogenesis and trophoblast proliferation (Athanassiades *et al*., 1998; Torry *et al*., 2003), but also engages in maintenance of maternal vascular integrity through its dynamic upregulation in maternal circulation (Taylor *et al*., 2003). In association with that, reduced serum levels of hPGF can be detected weeks before clinical onset of hypertension and proteinuria in patients at risk of PE (Chappell *et al*., 2013), whereas administration of hPGF has been shown to alleviate placental ischemia-induced maternal hypertension in the RUPP rat model of PE (Spradley *et al*., 2016). Thus, careful evaluation on the regulation of *hPGF* expression would provide important insights into pathogenesis of PE and potential therapeutic strategies for early intervention of PE. In the current study, we observe direct physical interaction between DLX3 and GCM1 by immunoprecipitation (IP) and mammalian one hybrid assays. We demonstrate that association of DLX3 with GCM1 represses the transactivation-stimulating activity of GCM1, which is directly mediated by the DLX3 homeodomain. The DLX3 amino domain and carboxyl domain are proposed to facilitate or stabilize the DLX3/GCM1 association for fully repression of GCM1 by DLX3. Our study provides mechanistic explanation of the previously reported DLX3/GCM1 antagonism in *hPGF* regulation. In addition, it identifies a novel function of DLX3 on repressing GCM1 activity, providing important insights

into advanced understanding of the complex placental development at the multifactorial level.

3.3 Results

3.3.1 DLX3 physically interacts with GCM1 and inhibits the transactivation-stimulating activity of GCM1.

To investigate the molecular mechanism underlying the DLX3/GCM1 regulatory antagonism on *hPGF* expression, we tested their potential interaction by performing IP studies. Initial pilot studies examining physical interaction of endogenous DLX3 and GCM1 by IP did not detect significant signal, because GCM1 was particularly labile in trophoblasts through proteosomal degradation. As an alternative, JEG-3 cells were transfected with a GCM1 expression plasmid (pHA-GCM1) expressing the hemagglutinin (HA) epitope-tagged GCM1 in conjunction with culturing cells in the presence of the proteasome inhibitor MG132 for 24 h before collection of lysates for IP. IP was carried out using the DLX3 antibody or the control IgG antibody. The obtained imunoprecipitates were then resolved using SDS-PAGE and immunoblotting assay examined for the presence of HA-GCM1 using the HA antibody. Whole cell lysate of five percent of the total protein used in each IP was used as the input to control the overexpressed peptide signal detected in immunoblotting (IB). As shown in Figure 3.1a, HA-GCM1 was detected in protein complex immunoprecipitated using DLX3 antibody, indicating association between overexpressed HA-GCM1 and endogenous DLX3. Reciprocal study was also carried out and detected association between overexpressed HA-DLX3 and endogenous

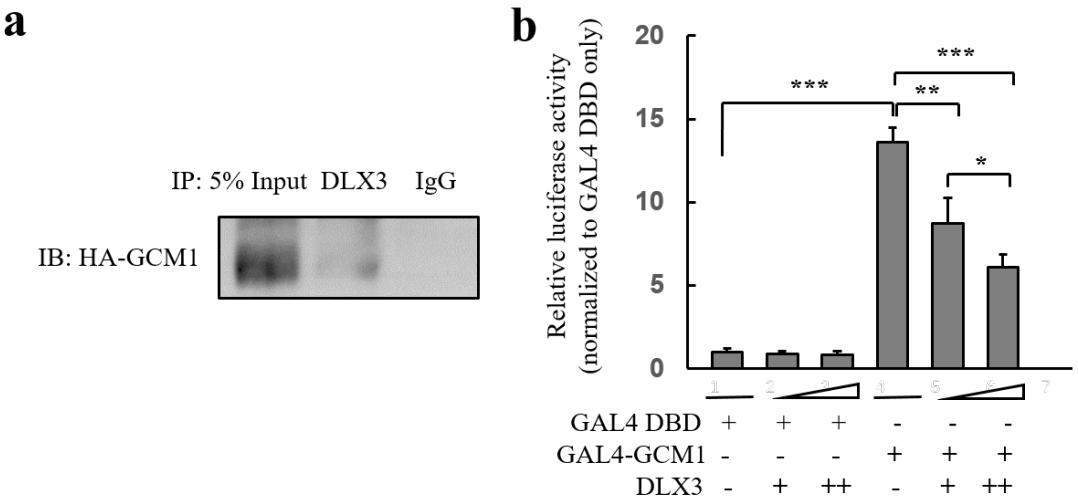

Figure 3.1 | DLX3 physically associates with GCM1 and inhibits the transactivation-stimulating activity of GCM1. (**a**) JEG-3 cells transfected with GCM1 expression plasmid for 12 h and treated with MG132 for 24 h were harvest for IP using DLX3 antibody. Immunoprecipitates were then subjected to immunoblotting (IB) against HA antibody for HA-GCM1 detection. Normal rabbit IgG was used as the negative control for IP. Independent experiment was performed three times and representative result was shown in the figure. (**b**) JEG-3 cells per 35-mm well transfected with 0.5 μg or 1 μg DLX3 expression plasmid together with 1 μg GAL4-GCM1 expression plasmid for 12 h were then transfected with 0.5 μg pGL4 luciferase reporter vector for 24 h and subsequently collected for luciferase assay. Luciferase activity is normalized to the GAL4 DBD–transfected control. Results are the mean±s.d. from three independent experiments in triplicates and asterisks denote statistically significant difference compared with values of vector control (*P<0.05, **P<0.01, ***P<0.001).

GCM1 later (Figure 3.6). Taken together, a physical interaction between DLX3 and GCM1 was detected in JEG-3 cells

To further confirm the interaction between DLX3 and GCM1 and to test the effect of their interaction on the transcriptional activity of GCM1, we utilized a GAL4/UAS mammalian one hybrid system. In this assay, full length human GCM1 was fused with the DNA-binding domain of the yeast GAL4 (GAL4 DBD) to yield the GAL4–GCM1 fusion protein. The pGL4.31 reporter vector containing five consensus Upstream Activating Sequences (UAS) for the GAL4 DBD (*Gal4*UAS) and a minimal adenoviral promoter upstream of the firefly luciferase gene was used to report transcriptional activation by GAL4-GCM1 with or without overexpression of DLX3. JEG-3 cells transfected with only the GAL4 DBD expression plasmid and pGL4.31 vector were used as the control to set the basal transactivation. As is shown in Figure 3.1b, both the GAL4 DBD alone and GAL4 DBD together with DLX3 overexpression showed basal transactivation, indicating a lack of interaction between GAL4 DBD and DLX3. GAL4-GCM1 alone strongly induced *Gal4*UAS transactivation, correlated well with the nature of GCM1 as transcription factor. Importantly, addition of DLX3 into the system significantly repressed GAL4-GCM1-induced luciferase reporter activity in a DLX3 dose-dependent manner, indicating the association of DLX3 with GCM1 represses the transactivation-stimulating activity of GCM1. These observations confirmed the physical interaction between DLX3 and GCM1 identified biochemically using IP and the resulting inhibition of the transactivation-stimulating activity of GCM1 by DLX3.

3.3.2 Generation of DLX3 structural domains.

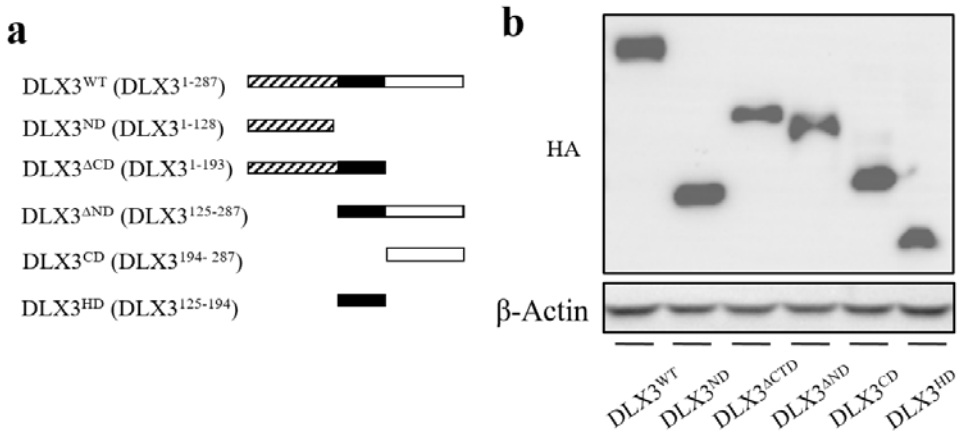

Figure 3.2 | DLX3 structural domains are expressed in JEG-3 cells. (**a**) Five DLX3 structural domain constructs were built as schematically illustrated. Striped region: amino terminal domain (ND); Black region: homeodomain (HD); White region: carboxyl terminal domain (CD); Δ: deletion; WT: wild type. (**b**) Each indicated construct was transfected into JEG-3 cells which were then treated with MG132 for 24 h and subjected to western blot analysis to detect expression of DLX3 structural domains using the HA antibody. And β-Actin was used as the endogenous loading control. Independent experiment was performed three times and representative results were shown in the figure.

We next sought to define the protein region(s) by which DLX3 physically interacts with GCM1. Five truncated mutants of DLX3, representative of its structural domains, were generated and expressed in JEG-3 cells (Figure 3.2). Studies focused

on the homeodomain (HD) which is known as the DNA binding domain (Feledy *et al*., 1999), and the amino domain (ND) and carboxyl domain (CD) separated by HD, both of which have been linked with transactivation potential of DLX3 in *Xenopus* (Feledy *et al*., 1999) (Figure 3.2a). All structural domains are epitope-tagged with triple HA. These DLX3 structural domain expression plasmids were transfected into JEG-3 cells respectively, which were then treated with MG132 to slow intracellular turnover of exogenous peptides. Western blot using the anti-HA antibody confirmed that all DLX3 domain mutants were expressed at expected molecular sizes at comparable levels (Figure 3.2b).

3.3.3 Assessment of transactivation activities of DLX3 structural domains in the *hPGF* promoter.

Transactivation activities of DLX3 structural domain mutants were assessed in the -4560 *hPGF* promoter luciferase system in JEG-3 cells (Figure 3.3). We first observed that the DLX3 ND ($DLX3^{ND}$) or CD ($DLX3^{CD}$) in isolation did not possess detectable levels of transcriptional activities, consistent with the requirement of the HD DNA-binding domain in DLX3-dependent gene regulation as a transcription factor. However, the HD in isolation ($DLX3^{HD}$) did not induce any transactivation of *hPGF* promoter either, indicating HD alone did not possess any transactivation activities. Interestingly, truncated mutants with CD or ND deleted ($DLX3^{\Delta CD}$ or $DLX3^{\Delta ND}$) not only upregulated the *hPGF* promoter activity, but also showed higher transactivation activities compared with that of wild-type DLX3 ($DLX3^{WT}$) (Figure

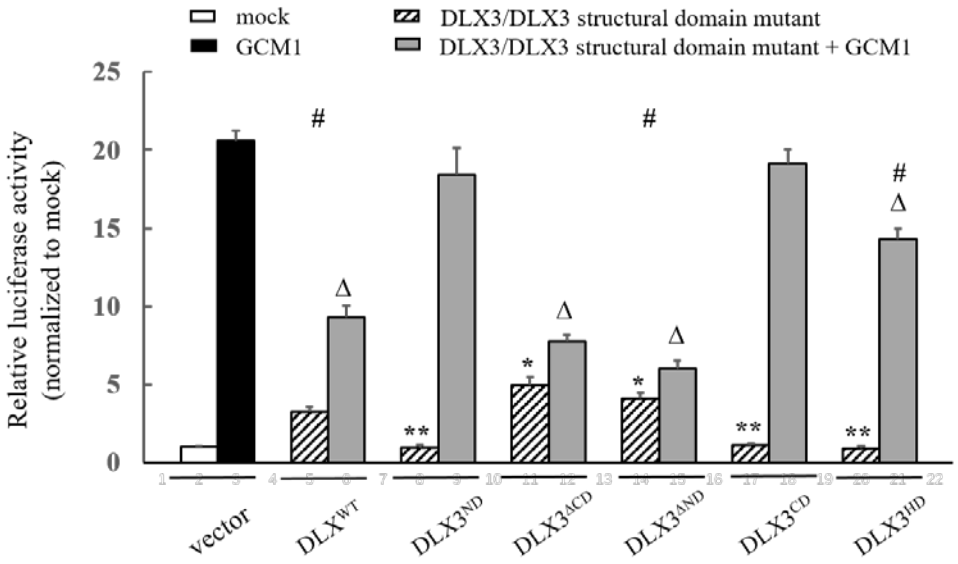

Figure 3.3 | Effects of DLX3 structural domains in transactivating the *hPGF* promoter. Luciferase assays were performed in JEG-3 cells (per 35-mm well) transfected with 0.2 μg *hPGF* promoter (-4960/+400) reporter plasmid, 1 μg of indicated DLX3 structural domain expression plasmid, with (grey bars) or without (striped bars) 0.5 μg GCM1 expression plasmid, and followed by MG132 treatment for 24 h. Luciferase activity is normalized to the mock control. Results are the mean±s.d. from three independent experiments in triplicates. Statistically significant differences: Asterisks—DLX3 structural domains compared with $DLX3^{WT}$ (**P*<0.05, ***P*<0.01); octothorpes—the GCM1/DLX3 structural domain combinations compared with the GCM1/$DLX3^{WT}$ (#*P*<0.01); triangle—the GCM1/DLX3 structural domain combinations compared with GCM1 (Δ*P*<0.01).

3.3). These observations suggested that both the DLX3 ND and CD contained transactivation activities of DLX3, and that it didn't require co-presence of the ND

and CD to exert transactivation activities of DLX3.

Studies also examined the combinational effects of the DLX3 structural domains and GCM1 in regulating the *hPGF* promoter activity (Figure 3.3). We observed that $DLX3^{ND}$ or $DLX3^{CD}$ in isolation didn't affect the transactivation-stimulating activity of GCM1 on the *hPGF* promoter, whereas $DLX3^{HD}$ alone formed mild antagonism with GCM1. However, to obtain the full antagonistic effect with GCM1 on activating the *hPGF* promoter required the co-presence of the HD and either the ND or CD, as were observe in $DLX3^{\Delta CD}$ and $DLX3^{\Delta ND}$. Collectively, our data suggested that the functional antagonism formed by DLX3 and GCM1 in transactivating the *hPGF* promoter requires co-presence of the DLX3 HD and at least one of the ND or CD domains.

3.3.4 The DLX3 HD is essential for DLX3's repression on the transactivation-stimulating activity of GCM1.

The advantage of the *hPGF* reporter system described above is examination of the responsiveness of the promoter to the combined actions of DLX3 and GCM1. However, these studies do not directly address the effects of DLX3 functional structures on GCM1 in isolation. To better address this question, studies then focused on the GAL4-GCM1/UAS mammalian one hybrid system. As is shown in Figure 3.4, only structural mutants of DLX3 containing the HD region retained inhibitory effect on the transactivation-stimulating activity of GCM1: $DLX3^{ND}$ and $DLX3^{CD}$ completely lost the inhibition; $DLX3^{HD}$ showed modest inhibition; $DLX3^{\Delta CD}$ and

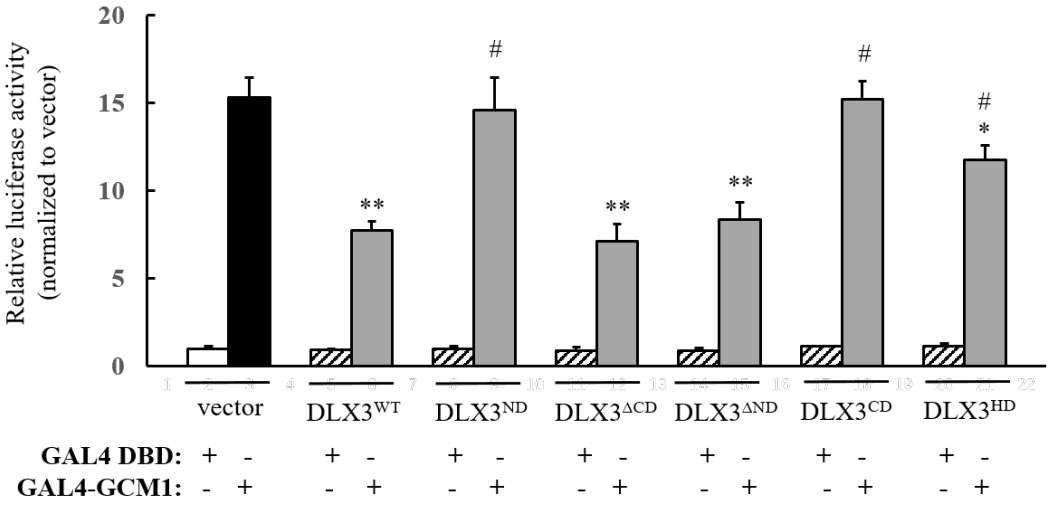

Figure 3.4 | Repressive effects of DLX3 structural domains on the transactivation-stimulating activity of GCM1. Mammalian one hybrid assays were performed in JEG-3 cells (per 35-mm well) that were firstly transfected with 1 μg indicated DLX3 structural domain expression plasmid and 1 μg GAL4-GCM1 expression plasmid for 12 h, and then transfected with 0.5 μg pGL4 luciferase reporter vector. Transfected cells were then treated with MG132 for 24 h before collection. Luciferase activity is normalized to the basal activity of the GAL4 DBD control (white bar). Results are the mean±s.d. from three independent experiments in triplicates. Statistically significant differences: asterisks—compared with values of GAL4-GCM1 alone (black bar) (*P<0.05, **P<0.01); octothorpes—compared with values of the GAL4-GCM1/DLX3^{WT} combination (#P<0.01).

DLX3 $^{\Delta ND}$ retained full inhibition compared with that of wild-type DLX3 (DLX3^{WT}). Therefore, the DLX3 HD was demonstrated to be essential for DLX3-dependent inhibition on GCM1, suggesting DLX3 is likely to interact with GCM1 directly via the HD region. Moreover, the DLX3 ND and CD appeared to be important in the functional interaction of DLX3 and GCM1 as they were required to fully restore the DLX3 inhibition on GCM1. Taken together, co-presence of HD and either the ND or CD was required for functional association of DLX3 and GCM1, characterized by the full inhibition of DLX3 on GCM1's transcriptional activity.

3.3.5 The DLX3 TDO mutant retains fully regulatory activity on *hPGF* and inhibition on GCM1

Naturally occurring mutations in DLX3 have been identified in human populations accounting for TDO and other ectodermal diseases (Price *et al*., 1998; Lee *et al*., 2008). TDO is associated with a four-base deletion downstream of homeobox in the *DLX3* coding region (c.571_574delGGGG), which is predicted to result in a truncated protein with altered carboxyl terminus (Price *et al*., 1998). Using this TDO mutant as a naturally occurring protein, studies sought to determine the differences of functional activities between the TDO mutant (DLX3^{TDO}) and wild-type DLX3 in the *hPGF* luciferase reporter assays (Figure 3.5a) and GAL4-GCM1/UAS mammalian one hybrid studies (Figure 3.5b). Based on above observations, we expect to see that DLX3^{TDO} would behave in a manner similar to DLX3^{WT} with regard to *hPGF* transactivation as well as GCM1 antagonism since the HD and ND portions of DLX3 was intact. Consistent with this prediction, DLX3^{TDO} retained full transactivation-

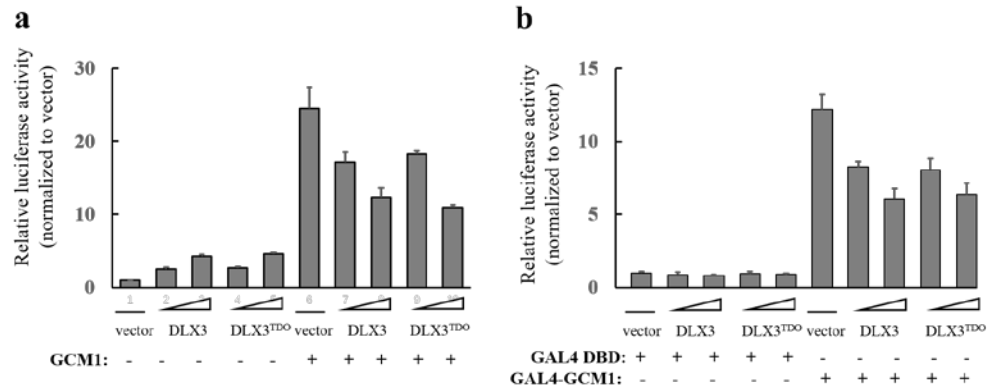

Figure 3.5 | The DLX3 TDO mutant retains full regulatory activity on *hPGF* and restriction on GCM1. (a) Luciferase assays were performed in JEG-3 cells (per 35-mm well) transfected with 0.2 μg *hPGF* promoter (-4960/+400) luciferase reporter plasmid, increasing dose of wild-type DLX3 or TDO mutant expression plasmid (0.5 μg or 1 μg), without or with GCM1 expression plasmid for 12 h and treated with MG132 for 24 h. **(b)** Mammalian one hybrid assays were performed in JEG-3 cells (per 35-mm well) that were transfected with 0.5 μg pGL4 luciferase reporter plasmid, increasing dose of DLX3 or TDO mutant expression plasmid (0.5 μg or 1 μg), without or with GAL4-GCM1 expression plasmid. Transfected cells were treated with MG132 for 24 h before collection. Luciferase activity is normalized to the control vector-transfected cells. Results are the mean±s.d. from three independent experiments in triplicates. Statistics were performed between the wild-type and TDO mutant of DLX3 groups and no significant differences were found.

stimulating activity on the *hPGF* promoter despite the lack of the wild-type carboxyl terminus. Additionally, it retained full inhibitory effect compared with $DLX3^{WT}$ on the

transactivation-stimulating activity of GCM1 in both the luciferase reporter assay and the GAL4-GCM1/UAS system assay. Collectively, these data indicated that the carboxyl region of DLX3 is less important for its functional activities in regulating *hPGF* upregulation and mediating the interaction with GCM1.

3.3.6 The DLX3 HD directly mediates physical interaction between DLX3 and GCM1.

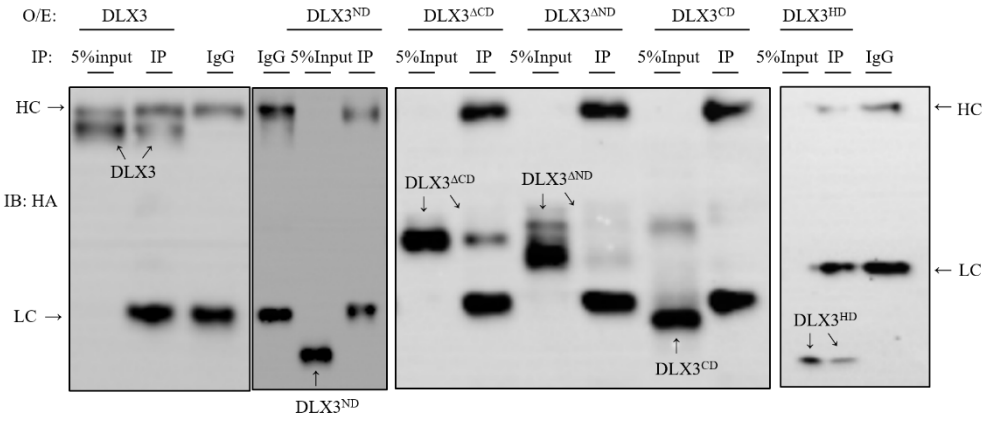

Figure 3.6 | The DLX3 homeodomain directly mediates the physical interaction between DLX3 and GCM1. JEG-3 cells were transfected with indicated DLX3 structural domain expression plasmids and treated with MG132 for 24 h, and subsequently subjected to IP using GCM1 antibody. Immunoprecipitates were analyzed on a HA immunoblot. Independent experiments were performed three times and representative blots were shown. IP: immunoprecipitated with GCM1 antibody; IgG: immunoprecipitated with the goat IgG antibody; O/E: overexpression. HC: heavy chain of denatured antibody; LC: light chain of denatured antibody.

Above studies suggest a potential interaction between the DLX3 HD domain and GCM1. To confirm that, IPs were performed in JEG-3 cells overexpressing full length DLX3 or DLX3 structural domains using the GCM1 antibody. Immunoprecipitates were resolved by SDS-PAGE and subjected to western blot analysis using the anti-HA antibody for the detections of association between HA-tagged DLX3 or DLX3 structural domains with endogenous GCM1. Whole cell lysate of five percent input of each IP was used as the overexpression control of each transfected plasmid in the blot (IB). As is shown in Figure 3.6, absence of the DLX3 HD domain as seen in $DLX3^{ND}$ and $DLX3^{CD}$ resulted in the loss of the DLX3 and GCM1 association, whereas mutants containing the HD domain ($DLX3^{WT}$, $DLX3^{HD}$, $DLX3^{\Delta CD}$ and $DLX3^{\Delta ND}$) all showed binding with GCM1. Importantly, the DLX3 HD alone was able to bind with GCM1, indicating the its essential role in mediating the DLX3/GCM1 interaction.

3.4 Discussion

Our studies have provided evidence of physical interaction between DLX3 and GCM1, which leads to transcription repression of GCM1. Functional domain analyses of DLX3 revealed that the DLX3 HD plays central roles in mediating the DLX3/GCM1 interaction and inhibiting the transactivation-stimulating activity of GCM1. Although the DLX3 ND and CD domains alone could not bind with GCM1 nor inhibit GCM1 activity, they appeared to facilitate repression of HD on GCM1 to fully recover functional activities of wild-typed DLX3 on *hPGF* regulation with GCM1 and inhibition on GCM1. In addition, transcription activity of each structural

domain was analyzed. We showed that both the ND and CD domains confered transactivation activities of DLX3. Furthermore, they transactivation activities seemed to be redundant: loss of either the ND or CD in DLX3 resulted in increased instead of ablated transactivation activities in regulating the *hPGF* promoter. Finally, we took advantage of a naturally occurring DLX3 mutant—TDO, which contains an altered CD, and revealed its transactivation activity on the *hPGF* promoter and inhibitory effect on GCM1 to be comparable with those of wild-type DLX3. These observations together with the lack of evidence of abnormal pregnancies in TDO affected patients suggest a dispensable role of the CD domain in DLX3's functional activities as a transcription factor in human placental development.

Our identification of the novel interaction between DLX3 and GCM1 in human placental cells provides implications of mechanisms by which DLX3 recognizes and regulates target gene expression within the placenta. Homeodomain proteins serve highly selective biological functions as gene expression regulators during embryonic development and postnatal stages in processes such as tissue patterning, axon guidance, and visual cortex plasticity regulation (Spatazza *et al*., 2013). Paradoxically, they are known for promiscuous DNA binding with a general preference for TA-rich sequences on promoters of target genes (Gehring *et al*., 1994). The means by which homeoproteins distinguish specific gene targets from other potential regulatory sequences in genomes often relies on cooperation with selective binding partners, including their homologs, components of the transcriptional machinery and other transcriptional factors (Mann & Affolter, 1998). Binding partners of DLX3 have been implicated to modulate the DLX3 transcription activity, where the DLX3 HD often

plays a role. For instance, DLX3 has been reported to bind with the homeodomain-containing transcriptional repressor MsxI through the HD domain, leading to the loss of its transcription activity (Bryan *et al*., 2000); whereas DLX3 and the transcription activator EST2 were shown to induce synergistic upregulation of *bovine Interferon-Tau* gene promoter (Ezashi *et al*., 2008). DLX3 was also identified to interact with the inhibitory Smad6 via its HD domain which led to repress the transcriptional activity of DLX3, presumptive owning to interrupted DNA binding of DLX3 upon Smad6 association (Berghorn *et al*., 2006). Thus, although DLX3 is generally considered a positive regulator of transcription, its recognition of specific gene targets and regulatory activity can be largely influenced by specific binding partners. Here we demonstrate that DLX3 associates with the transcription factor GCM1 via its HD domain to form a complex in the JEG-3 human placental cell line which might contribute to the selective regulation of DLX3 and GCM1 on *hPGF*. Chapter two describes that DLX3 and GCM1 both bind to a common *hPGF* promoter sequence which is responsible for the basal and DLX3/GCM1-induced *hPGF* expression. However, the precise mechanism for how these two transcription factors engage the *hPGF* gene promoter is unclear. Notably this *cis* acting element lacks consensus binding motifs for DLX3 and GCM1 that have been described previously. It is possible that novel DLX3 or GCM1 binding sites are included in this sequence. To test that, gel mobility assay focusing on the 50-basepair sequence are higher desirable. Alternatively, additional factors might be involved in coordinating direct DNA binding of a higher order complex and serving as an adaptor for the recruitment of the DLX3/GCM1 complex. Further studies such as IP and mass-spectrometry for

identification of potential binding candidates are suggested.

Moreover, we have proposed redundant transactivation activities of the ND and CD domains of DLX3 as is seen in the DLX3 functional structure analyses and the DLX3 TDO mutant studies. However, co-presence of the ND and CD are likely to be important as they might serve additional functions such as providing residue sites for post-translational modifications of the DLX3 protein to regulate its transcription activity or stability, or facilitate with associations between DLX3 and binding partners. The increased transcriptional activities in DLX3 mutants with the ND or CD deleted are possibly due to confirmation changes of the folding of the truncated proteins, or loss of post-translational modification sites conferring transactivation inhibition of DLX3. It is interesting to note that the TDO mutant was found to be comparable with wildtype DLX3 in *hPGF* regulation and GCM1 interaction, suggesting the carboxyl terminus might be dispensable in DLX3 involved gene regulatory signaling in trophoblast cells. Notably, this is well correlated with lack of evidence of abnormal pregnancies in women affected with TDO. Combining the development defects in hair and bones in TDO patients with presumably normal pregnancies and placental formation, our studies support the notion that the DLX3 CD domain plays important roles in the development of TDO affected tissues, but may not be involved in the process of placentation. This novel idea reflects a possible mechanism controlling highly selective gene regulatory actions of DLX3 depending on the context of tissue and cell during development.

Our studies in this Chapter reveal a novel role of DLX3 in modulating the transactivation-stimulating activity of GCM1. We demonstrated that the DLX3/GCM1

interaction inhibited the transactivation-stimulating activity of GCM1, therefore exhibiting the antagonistic regulation of *hPGF* expression by the combination of DLX3 and GCM1. This modulation might serve as a fine tuning mechanism to control the expression of *hPGF* at a desirable physiological level to avoid overgrowth of the placenta. Our current study has been focusing on DLX3, whereas how the GCM1 activity is inhibited upon DLX3 association is unexplored. The GCM protein is a zinc finger transcription factor with the conserved DNA-binding domain localized at the amino terminus and the less-conserved transactivation domain at the carboxyl terminal region (Cohen *et al.*, 2003). Future study will seek to determine the molecular and biochemical mechanisms by which DLX3 exerts the inhibitory effects on GCM1.

In conclusion, for the first time we report the inter-play between DLX3 and GCM1 in gene regulation. We also identify this inter-play is dependent on their physical interaction and leads to inhibition of the transactivation-stimulating activity of GCM1. In addition, our study uncovered a novel role of DLX3 as a negative regulator of GCM1 activity. Future in vivo studies are highly desirable to test and confirm our proposed model.

3.5 Materials and methods

Plasmids and constructs. PKH3-DLX3, pHA-GCM1 and -4560 *hPGF* promoter luciferase reporter plasmids are described in previous studies (Chapter two). To generate pKH3-DLX mutant plasmids that encode epitope-tagged domain mutants of DLX3, PCR were performed using pKH3-DLX3 plasmid as the template and the following primer sets (forward/reverse): $DLX3^{ND}$ ($DLX3^{1-128}$) —5´-

CGGGAATTCAAATGAGCGG-3′/5′-
ATCGATTCACTTCTTGGGCTTCCCATTCAC-3′; DLX3$^{\Delta CD}$ (DLX3$^{1-192}$) —5′-
CCGGGAATTCAAATGAGCGG/5′-
ATCGATTCACACCTCCCCGTTCTTGTAGAG-3′; DLX3$^{\Delta ND}$ (DLX3$^{125-287}$) —5′-
GAATTCAAAAGCCCAAGAAGGTCCGAAAG-3′/5′-
GGCCATCGATTCAGTACACA-3′; DLX3^{CD} (DLX3$^{194-287}$) —5′-
GAATTCAACCGCTGGAGCACAGTCCCAATA-3′/5′-
GGCCATCGATTCAGTACACA-3′; DLX3^{HD} (DLX3$^{125-194}$) —5′-
GAATTCAAAAGCCCAAGAAGGTCCGAAAG-3′/5′-
ATCGATTCACACCTCCCCGTTCTTGTAGAG-3′. Each cDNA was verified by nucleotide sequence analyses and inserted into pHK3 vector to make HA-tagged DLX3 domain expressing constructs. To facilitate cloning, *Eco*RI and *Cla*I restriction sites were incorporated at the end of 5' and 3' primers respectively. The TDO mutant of DLX3 was generated through three rounds of PCR using the PKH3-DLX3 plasmid as the template and the following primer sets respectively. Round one: 5′-
CCGGGAATTCAAATGAGCGG-3′/5′-
AGCGGCACCTGTTCTTGTAGAGTTTCTTGA-3′
Round two: 5′-CTACAAGAACAGGTGCCGCTGGAGCACAGT-3′/5′-
GGCCATCGATTCAGTACACA-3′
Round three: 5′-CCGGGAATTCAAATGAGCGG-3′/5′-
GGCCATCGATTCAGTACACA-3′.

Antibodies and reagents. Antibodies used in this study were as follows: goat anti-GCM1 (sc-69407X, Santa Cruz Biotechnology, Inc., Dallas, TX), mouse anti-HA (sc-

7392, Santa Cruz), HRP-conjugated VeriBlot for IP secondary antibody (ab13166, Abcam, Cambridge, United Kingdom). The proteasome inhibitor MG132 (M7449) was purchased from Sigma-Aldrich and used with a final concentration of 10 μM.

Cell culture and transfection. The human choriochacinoma cell line—JEG-3 was purchased from ATCC and cultured in Dulbeco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin G, and 100 μg/ml streptomycin. Cells were incubated at 37 ºC in a humidified 5% CO_2 atmosphere. Transfection were carried out in dishes of cells with 60~80% confluency using the Polyethylenimine reagent (Polysciences, Inc. Warrington, PA) according to the manufacturer's protocol. Eight h after transfection, cells were washed with phosphate-buffered saline (PBS), and then cultured with fresh media.

Co-immunoprecipitation. JEG-3 cells were transfected with expression plasmids of interest for 12 h and treated with MG132 for another 24 h before collection. Cells were lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) freshly supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland) at 4 ºC for 1 hr. Five hundred μg of total protein per sample was used per IP assay. Each IP was incubated with 5 μg specific antibody at 4 °C for 1h with rotation. Dynabeads protein A (Invitrogen, Carlsbad, CA) was then added into samples for an additional hour. Beads were collected using a magnet, washed twice with lysis buffer and once with TPBS (0.01% Tween®-20, pH 7.4), and eluted by boiling in 2× Laemmli buffer for 5 min.

Western blot. Protein samples were separated on polyacrylamide gels, and transferred to a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% milk in Tris-Buffered Saline and Tween 20 (TBST), incubated with the HA antibody overnight followed confirmation specific horseradish peroxidase-coupled secondary antibody (ab13166, Abcam, Cambridge, United Kingdom), and bands were detected using enhanced chemiluminescence (Bio-Rad).

Mammalian one-hybrid assay. To generate the GAL4-GCM1 hybrid construct, GCM1 cDNA was amplified by PCR from the pHA-GCM1 plasmid from Dr. Hungwen Chen (Academia Sinica, Taiwan), sequence verified and incorporated downstream into a GAL4 DNA-binding domain (DBD, residues 1-147) expression plasmid regulated by a SV40 promoter. *Eco*RI and *Xba* were added to each end of GCM1 cDNA sequence to facilitate cloning. Primers used were as follows (forward/reverse): 5´-AATCCATGGAACCTGACGACTTTGA-3´/5´-TCTAGAGTCATCTCAAAGGACACAGGTT-3´. To perform the assay, JEG-3 cells were seeded in 6-well dishes overnight before transfection. They were first transfected with the GAL4-GCM1 expression plasmid, with or without the DLX3 expression or indicated DLX3 domain mutant expression plasmid for twelve h, and then transfected for a second time with the pGL4.31 luciferase reporter plasmid (Promega, Madison, WI) containing five upstream GAL4 DBD binding sites (UAS) for 12 h. After that, cells were washed with PBS and cultured in fresh media, followed by MG132 treatment for 24 h and collected for luciferase assay.

Luciferase assay. Luciferase assay was carried out as previously described (Chapter

two).

Statistics. Two-way analysis of variance (ANOVA) followed by Student t-test were used to determine statistical significance of differences between means. A P value of less than 0.05 was considered to be significant.

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3.6 Reference

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CHAPTER FOUR. DISCUSSION

4.1 Summary

In Chapter two, I have described novel findings related to the regulation of *hPGF* expression in the JEG-3 human trophoblast cell line by transcription factors DLX3 and GCM1 through acting on a *cis* acting DNA regulatory element on the *hPGF* promoter. Both DLX3 and GCM1 were shown to be required for *hPGF* expression in JEG-3 cells. While they were both transcription activators, the combined actions of DLX3 and GCM1 led to an antagonism in *hPGF* transcriptional regulation. This antagonism was later found to be attributed to an inhibitory effect of DLX3 on the transactivation-stimulating activity of GCM1, as is described in Chapter three. Interestingly, the DLX3/GCM1 regulatory antagonism appeared to be specific to *hPGF* as it was not seen in of the cases of DLX3 target gene *hCGα* and GCM1 target gene *ERVFRD*-1. Deletion mutagenesis studies identified a critical *cis* acting regulatory element located at -369 to -320 (distance relative to the TATA box in nucleotides) at the 5´ flanking promoter region of the *hPGF* gene that controls its basal expression, as deletion of this sequence resulted in dampened *hPGF* promoter activity in the luciferase reporter system. In addition, this 50 base pair sequence was enriched in DLX3 and GCM1 occupancies and showed to mediate DLX3- and GCM-induced *hPGF* transactivation, as well as the regulatory antagonism by both factors. Taken together, I propose that DLX3 and GCM1 work in concert to regulate *hPGF* expression via acting on sequence(s) within the -369 to -320 promoter region which is also essential for basal *hPGF* expression. Although speculative, this may reflect a

central mechanism for *hPGF* regulated expression in human trophoblast cells within the placental environment.

In Chapter three, I have sought to explore the molecular mechanism underlying the DLX3 and GCM1 antagonism in *hPGF* regulation. I demonstrated DLX3 and GCM1 physical interacted in JEG-3 cells by immunoprecipitation and mammalian one hybrid assays. The latter assay additionally revealed that DLX3 association with GCM1 resulted in repressed transactivation-stimulating activity of GCM1. To explore this mechanism further, I constructed a series of DLX3 truncated domain structures and tested their individual interaction with GCM1. These studies revealed that the DLX3/GCM1 interaction is mediated by the homeodomain of DLX3. Additionally, the homeodomain alone has been shown to exhibit the inhibition on GCM1. While the DLX3 amino and carboxyl termini in isolation could not bind to GCM1 nor exert inhibition on GCM1, they appeared to provide further support of the inhibitory effect of the DLX3 homeodomain on GCM1 and possibly facilitate the functional interaction between DLX3 and GCM1. This is not surprising considering previous reports that HD could serve as scaffold for the recruitment of other transcription factors and interacting partners. In fact, these are important mechanisms to modulate DNA binding specificity of homeoproteins, and to exert their transactivating or repressive effects in a context-dependent manner (Zhang *et al.*, 1997; Lee *et al*., 2006; Wang *et al*., 2011). Moreover, the amino and carboxyl regions of DLX3 have been implicated in cofactor binding and determination of target gene specificity, besides serving as transactivation domains (Prochiantz *et al*., 2014). Collectively, my study has indicated the central role of homeodomain in DLX3/GCM1 association, with a further

enhancing role of the DLX3 amino and carboxyl domains.

In summary, this dissertation investigates and demonstrates functional interaction between the transcription factors DLX3 and GCM1 in regulating *hPGF* expression in human trophoblast cell line. It reveals important novel roles DLX3 plays in human placental development and is the first description of *hPGF* regulation by DLX3 and GCM1.

4.2 Conclusions and Speculation

In conclusion, we have reported an important role of DLX3 in placental angiogenesis through regulating *hPGF* expression in a model of human trophoblast cells *in vitro*.. Moreover, we demonstrated a transcriptional inter-play between DLX3 and GCM1 in *hPGF* regulation. Whether this inter-play exerts functional regulation beyond *hPGF* is a question we are interested to address in future. However, current studies demonstrate some level of specificity of this inter-play since a similar functional interaction was not observed in cases of *hCGα* and *ERVFRD*-1. Perhaps additional regulatory element, either working in *cis* (a specific DNA sequence) or *trans* (a cofactor associated with both DLX3 and GCM1) is required to mediate the antagonistic relationship described in my research. Based on mammalian one hybrid assay which showed sequence independent association of DLX3 and GCM1, we propose possible protein candidates involved in the DLX3/GCM1 interaction such as histone deaceytalases (HDACs), as it has been implicated to bind with GCM1.

Previous work reported other *cis* acting elements on the *hPGF* promoter that had been shown to be important for *hPGF* expression. Chang and colleagues

suggested a putative binding site for GCM1 at– 42 (distance relative to the TATA box in nucleotide) that was likely to be responsible for GCM1-induced *hPGF* expression in the BeWo human choriocarcinoma cell line (Chang *et al*., 2008). Their finding of GCM1 as a critical transcription factor regulating *hPGF* expression was confirmed in our studies using the JEG-3 cell line. My studies extend their finding by identifying a critical binding site for GCM1 and DLX3 at the -369 to -320 region. This dissertation also provides evidence that the GCM1 binding site identified by Chang and colleagues may in fact play a less significant role in the regulation of *hPGF* expression, as it was not important for basal expression of *hPGF*, nor GCM1-dependent *hPGF* transactivation in the luciferase reporter system. In contrast, our studies of promoter mutagenesis, luciferase reporter assays and ChIP-qPCR analyses indicating the binding of GCM1 at the -369 to -320 region strongly support an essential role of this identified sequence in mediating the regulatory effect of GCM1 on *hPGF* expression. Future *in vitro* gel mobility shift assay would help to further address the question.

Another study by Nishimoto *et al*. implicated a requirement of a triple metal-responsive transcription factor 1 (MTF-1) binding sites (MRE) at the + 233 to + 322 of the 5´ UTR region of *hPGF* (Nishimoto *et al*., 2009). While I did not include MTF-1 in current studies, it seems that the triple MRE sites within this region might play a secondary role to our identified sequence since the deletions used in my study contained these MRE sites but loss of the -369 to -320 sequence clearly abolished basal expression of *hPGF*. It will be critical to examine possible regulatory interactions among DLX3, GCM1 and MTF-1 in future studies to extend our understanding of *hPGF* regulation.

Finally, transactivation activities of the DLX3 amino and carboxyl domains were roughly assessed in the current studies. In agreement with previous reports that both domains confer the transactivation-stimulating activity of *Xenopus* DLX3 (Feledy *et al*., 1999a), we have observed that for human DLX3, the homeodomain alone could not induce *hPGF* promoter activation but required additional presence of either the amino or carboxyl domain, or both. However, in contrast to the previous report where the author showed loss of either transactivation domain completely abolished transcription activity of DLX3 (Feledy *et al*., 1999a), my studies found that deletion of either the human DLX3 amino or carboxyl domain increases, rather than reduces or eliminates, the transactivation-stimulating activity of DLX3 on *hPGF* regulation. According to these observations, either the amino or the carboxyl transactivation domain of DLX3 was sufficient to transactivate the *hPGF* promoter. In fact, we observed that deletion of the DLX3 amino or carboxyl domain alone resulted in increased transcriptional activities, possibly due to conformation changes of the folding of the truncated proteins, or loss of post-translational modification sites conferring transactivation inhibition of DLX3. The disagreement of our data with the previous study might be due to species differences of DLX3 in the human and *Xenopus*. In addition, we included a natural occurring DLX3 mutant in our study—TDO, which is characterized by a four base pair deletion close to the 3´ region of homeobox resulting in a truncated protein with altered carboxyl terminus. We found that the transcriptional activity of TDO was comparable with that of wild-type DLX3 in regulating *hPGF* expression, suggesting the DLX3 carboxyl terminus might be dispensable in DLX3-depedent *hPGF* regulation. Notably, this is well correlated with

the lack of evidence of abnormal pregnancies in women affected with TDO. Combining the developmental defects in teeth, hair and bones in TDO patients with presumably normal pregnancies and hence healthy placentae, my studies suggest that the DLX3 carboxyl terminus plays important roles in the development of TDO affected tissues such as the teeth, hair and craniofacial bones, but might not be involved in the development and functions of placentae. This novel idea reflects a self-regulatory mechanism controlling highly selective spatio-temporal functions of DLX3 in different tissues during development that may be attributable to domain-dependent activities.

The interaction of DLX3 and GCM1 in regulation of *hPGF* has important implications *in vivo*. First, the requirement for both DLX3 and GCM1 in *hPGF* promoter expression ensures highly spatial-temporal production of hPGF within trophoblast cells that constitutively express DLX3 and GCM1. The trophoblast lineages range from undifferentiated cytotrophoblasts beneath the syncytial layer, syncytiotrophoblasts, and some anchoring cytotrophoblast columns (Chui *et al.*, 2010; Baczyk *et al*., 2004; Khaliq *et al*., 1996). Expression of *hPGF* in these cell populations is correlated well with their features of active proliferation and participation in placental angiogenesis. In contrast, low expression levels of *DLX3* and *GCM1* are found in highly differentiated EVT cells which are invasive with a reduced proliferation index and low *hPGF* production. Importantly, as GCM1 alone is a strong regulator of *hPGF* expression compared directly to DLX3, the repressive effect of DLX3 on GCM1 might serve as a fine tuning mechanism to regulate *hPGF* expression at an appropriate level, which otherwise would lead to under/over angiogenic activity

and *hPGF* induced-inflammation. In fact, aberrant angiogenesis and abnormal inflammation in placentae are closely associated with PE and IUGR, both of which have been linked with dysregulated placental *hPGF* expression. Interestingly, decreased expression of *DLX3* and *GCM1* have been found in term placentae complicated with PE, suggesting our proposed model provides a potentially important mechanism for pathogenesis of downregulation of placental *PGF* in these patients (Chen et al., 2004; Murthi *et al.*, 2004; Roberson lab unpublished data; Levine *et al.*, 2004). Studies have also reported overexpression of *DLX3* in IUGR-complicated placentae, where *hPGF* expression and angiogenesis were both inhibited (Chui *et al.*, 2012), again potentially supporting our proposed fine tuning modulation of DLX3 on GCM1-dominated *hPGF* upregulation.

4.3 Future directions

Our current conclusions have been drawn based exclusively on *in vitro* JEG-3 cells as the model system for the human trophoblast cells. It would be highly desirable to test our hypotheses and to translate our proposed model in *ex vivo* and *in vivo* systems, such as placental explants from humans and mice, animal models and potentially clinical platforms. Complete knockout (KO) of *Dlx3* or *Gcm1* in mice leads to embryonic lethality around E10 due to deficiencies in the placental labyrinth development and the resulting placental failure (Morasso *et al*., 1999; Anson-Cartwright, L *et al*.), whereas conditional KO of *Dlx3* controlled by epiblast specific Meox2 Cre ($Dlx3^{-/fl}$ $Meox2^{cresor}$) are fertile (Clark *et al*., 2012). The conditional *Dlx3* KO mice are born at normal Mendellian ratios and survive to weaning and beyond,

supporting the notion that it is the loss of *Dlx3* in the placenta that is causal in embryonic lethality in the *Dlx3* null animal. Unfortunately, current lack of trophoblast-specific Cre deleter strains makes the placental-specific genetically modified animals not feasible.

Nevertheless, the *Dlx3* heterozygous animals (*Dlx3*$^{+/-}$) has potentially great utility for future studies. These animals exhibit delayed fetal growth trajectory, associated with ischemic placentae presumably induced by excessive oxidative stress and deficiency of maternal spiral arterial remodeling (Clark *et al*., 2012). Perhaps most importantly, *Dlx3*$^{+/-}$ mothers display an increased rate of late gestational hypertension (Roberson lab unpublished paper), occurring coincidently with histopathological findings of proteinuria, reminiscent of the hallmarks of PE in human pregnancy. Additionally, whole body KO of a single *Gcm1* allele in mice (*Gcm1*$^{+/-}$) has been described and shown to be fertile (Bainbridge *et al*., 2012). Interestingly, wild-type mothers bearing *Gcm1*$^{+/-}$ conceptuses developed late gestational hypertension (Bainbridge *et al*., 2012).

These observations provide the foundation for using these *in vivo* model systems to examine roles of the *Dlx3*/*Gcm1* inter-play in placental development and supporting maternal health during pregnancy in future studies. They would also serve as important tools to investigate dosage effects of placental *Dlx3* and *Gcm1* in physiological conditions on placental development and pregnancy that could be precisely managed at the single allele level. Listed below are some specific questions we would like to address that would potentially provide significant insights into pathways leading to pathogenesis of PE.

Specific Aim1: Investigate the dosage effects of *Dlx3* and *Gcm1* on pregnancies and placental *PGF* expression *in vivo*.

I hypothesize that *Dlx3* and *Gcm1* synergistically contribute to a normal pregnancy but antagonistically upregulate *PGF* expression in *in vivo* mouse models. To test my hypotheses, $Dlx3^{+/-}$ will be crossed with $Gcm1^{+/-}$ to produce offspring with only single *Dlx3* and *Gcm1* allele ($Dlx3^{+/-}$ $GCM1^{+/-}$). Measurement related to reproductive fertility will be performed in the $Dlx3^{+/-}$ $GCM1^{+/-}$ female mice first, including pregnant frequency, embryonic lethality rate, fetus growth trajectory, gestational blood pressure, proteinuria, placental pathology and hormone profiles, etc. Contributions from the maternal and fetal sides to potential development of gestational abnormalities will be separately assessed by crossing from both gender directions. Based on previously described phenotypes of the $Dlx3^{-/-}$, $Gcm1^{-/-}$, $Dlx3^{+/-}$ and $Gcm1^{+/-}$ animals, I predict that the $Dlx3^{+/-}$ $Gcm1^{+/-}$ females carrying non wild-type embryos would develop gestational hypertension and proteinuria, associated with placental ischemia and deficiencies in labyrinth development. Additionally, these gestational specific phenotypes of the $Dlx3^{+/-}$ $Gcm1^{+/-}$ animal are expected to be more severe compared with those of the $Dlx3^{+/-}$ and $Gcm1^{+/-}$ animals. Fetal growth and viability might also be affected.

Next, levels of PGF within the placenta and in the maternal circulation will be detected by western blot, qPCR and enzyme-linked immunosorbent assay (ELISA). Circulating sFlt-1 will also be included in the tests for accurate detection of free PGF, as it might mask changes in circulating PGF. I predict *PGF* expression would be

further reduced in the *Dlx3*$^{+/-}$ *GCM1*$^{+/-}$ animals compared with those in the *Dlx3*$^{+/-}$ and *Gcm1*$^{+/-}$ animals, possibly in an additive manner, because abundant expression of both *Dlx3* and *Gcm1* at normal levels are expected to be essential for driving *Pgf* expression, and therefore loss of a single *Dlx3* allele and a single *Gcm1* allele in *Dlx3*$^{+/-}$ *GCM1*$^{+/-}$ in comparison to only single allele ablation in *Dlx3*$^{+/-}$ and *Gcm1*$^{+/-}$ animals would be expected lead to a further reduction of *Pgf* expression, as is implied by the siRNA knockdown studies in JEG-3 cells described in this dissertation. These experiments would provide important evidence to support my hypothesis that both *Dlx3* and *Gcm1* are required on *PGF* regulation within the placenta.

Furthermore, placental tissues at different stages of gestation will be collected for analyses of placental development (morphogenesis, angiogenesis, maternal uterine vascular remodeling) by histological and immunostaining studies. Experiments such as Tunel staining will be used to assess trophoblast development and survival. These studies would prove important insights into dosage effects and reveal additional downstream interactions of *Dlx3* and *Gcm1* in placental development.

Specific Aim2: Investigate whether forced expression of *PGF* would rescue the hypertensive phenotypes observed in mice deficient in *Dlx3* and/or *Gcm1*.

As outline above, both *Gcm1*$^{+/-}$ and *Dlx3*$^{+/-}$ mothers show increased rate of developing late-gestational hypertension during gestation. The question to ask is whether supplementing circulating PGF would rescue their hypertensive phenotypes during pregnancy. This hypothesis is based on the previous study of adenoviral delivery of a VEGF ligand—$VEGF_{121}$ to rescue the development of gestational

hypertension and proteinuria in the BPH/5 mouse model of PE (Woods *et al*., 2011). What's more, a recent study in the RUPP rat model of PE reported successful inhibition of placental ischemia-induced gestational hypertension by administration of hPGF (Spradley *et al*., 2016). These together with my finding of essential roles of DLX3 and GCM1 in *hPGF* expression regulation described in this dissertation form my second hypothesize that administration of exogenous PGF would rescue pregnancy-associated abnormalities of *Dlx3* and/or *Gcm1* deficient mice, including the *Dlx3*$^{+/-}$, *Gcm1*$^{+/-}$, and *Dlx3*$^{+/-}$ *Gcm1*$^{+/-}$ animals. I propose to test this hypothesis by administrating either adenoviral vectors encoding PGF that allows relative stable and long-term circulating PGF, or recombinant PGF (commercial available) repeatedly during gestation. If the former method was used, studies would be controlled by using an adenovirus encoding GFP such that liver infections would be equivalent. I predict improved pregnancy outcomes in all animals of *Dlx3* and *Gcm1* deficiency, that is, placental pathologies, fetal growth, and maternal syndrome of gestational hypertension and proteinuria would be improve upon PGF supplementation. These experiments are critical for confirming and translating the central hypothesis this dissertation has proposed into *in vivo* physiological conditions. They are also essential to support the involvement of the DLX3/GCM-PGF axis in the pathogenesis of PE, and PGF as potential therapeutic target for treatment of PE in women.

4.4 References

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