The role of Dlx3 in gene regulation in the mouse placenta

by Li Han

THE ROLE OF DLX3 IN GENE REGULATION IN THE MOUSE PLACENTA

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Distal-less 3 (Dlx3) is a homeodomain containing transcription factor that is required for the normal development of the mouse placenta. Moreover in human trophoblasts, DLX3 appears to be a necessary transcriptional regulator of the glycoprotein hormone α subunit gene, a protein subunit of placental-derived chorionic gonadotropin. The aim of my studies described here was to determine the role of Dlx3 in gene regulation within the placenta.

My studies initially sought to determine if Dlx3 could interact physically with other transcriptional regulators in placental trophoblast cells and how these protein-protein interactions might influence Dlx3-dependent gene expression. Yeast two-hybrid screens provide evidence that mothers against decapentaplegic homolog 6 (SMAD6) was a binding partner for DLX3. SMAD6 was found to be expressed and nuclear localized in placental trophoblasts and interacted directly with DLX3. Structure-function analysis revealed that this interaction occurred within a DLX3 domain that overlapped the homeobox, a key domain necessary for DLX3 DNA binding. SMAD6 was found to repress DLX3 transcriptional activity through inhibition of DLX3-DNA interactions at target genes.

To develop a more comprehensive understanding of the gene profile regulated by Dlx3, microarray studies were used to determine differences in gene expression within the placenta of wild type and *Dlx3* null mice at embryonic day 9.5. Loss of *Dlx3* resulted in a marked reduction in gene expression of placental growth factor (*Pgf*). To investigate the mechanism(s) of regulation of *Pgf* gene expression, 5.2 kb of the

mouse *Pgf* gene promoter was cloned and used in reporter gene assays. Over-expression of Dlx3 increased the *Pgf* promoter activity in choriocarcinoma cells, suggesting that Dlx3 may have a direct effect on transcriptional activation of the *Pgf* gene promoter.

To determine the functional importance of Dlx3 in the regulation of *Pgf* gene transcription, two Dlx3 binding sites in the mouse *Pgf* 5.2 kb promoter were identified and mutated by site-directed mutagenesis. The mutations effectively abolished the ability of Dlx3 to bind these sites. *Pgf* promoter activity was found to be reduced by the combined mutation of both Dlx3 binding sites. Thus, Dlx3 appears to be an important determinant for *Pgf* gene promoter activity and ultimately Pgf secretion in placental trophoblast cells. Since PGF is an important marker for risk of preeclampsia in women, studies in a mouse model of preeclampsia were undertaken to determine if *Dlx3* and *Pgf* were mis-regulated during gestation in this model. Indeed, both *Dlx3* and *Pgf* transcript levels were greatly reduced in early gestation in BPH/5 mice suggesting that Dlx3 and Dlx3 target genes may underlie genetic mechanisms associated with preeclampsia prior to the onset of maternal disease. To determine if similar mis-regulation of the *DLX3* gene network might occur in placentas from women with preeclampsia, we next examined *DLX3* and *PGF* transcript abundance in a preliminary cohort of patients. This preliminary study supported our findings in the BPH/5 mouse model.

Together, these data suggest that Dlx3 may be an important factor during the development of mouse placenta by regulating genes such as *Pgf*. Further, mis-regulation of this gene network may direct early changes in placental function leading to symptoms characteristic of preeclampsia.

BIOGRAPHICAL SKETCH

Li Han was born and raised in Zaozhuang, China. In 1995, Li went to Weifang Medical College to start her doctor of medicine study in Weifang, Shandong. In 1999, Li worked as a clinical intern in Weifang Medical College Affiliated Hospital. Li received her MD degree in 2000 and went to Peking University Health Science Center to study for her master degree in Cardiovascular Pathophysiology. In Peking University Health Science Center, Li studied the effect of heme oxygenase in renal hypertension under the guidance of Dr. Hai Xu. The title of her master's thesis was "The effect of heme oxygenase on cardiovascular remodeling in renal hypertension."

In January 2004, Li came to Cornell University and joined the laboratory of Dr. Roberson as a Ph.D. student in the field of Molecular and Integrative Physiology.

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AI	amelogenesis imperfecta
AP	activator protein
BMP2	bone morphogenetic protein 2
BPGM	2,3-bisphosphoglycerate mutase
C/EBPβ	CCAAT box/enhancer-binding protein β
COMT	catechol-O-methyltransferase
CL	corpus luteum
Dlx	distal-less
DMEM	Dulbeco's modified eagle's medium
E	embryonic day
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal regulated kinase
ES	embryonic stem
Esx1	Extraembryonic, spermatogenesis, homeobox-1 like protein
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2
FBS	fetal bovine serum
FGF	fibroblast growth factor
Gab1	Grb2-associated binder 1
Gcm1	glial cell missing 1
Grb2	growth factor receptor-bound protein 2
HA	hemagglutinin
hCG	human chorionic gonadotropin

HRE	hypoxia-responsive element
3βHSD	3β-hydroxysteroid dehydrogenase
IFNT	interferon-tau
IP	immunoprecipitation
JNK	c-Jun N-terminal kinase
JRE	junctional regulatory element
KPBS	potassium phosphate buffered saline
MAPK	mitogen-activated protein kinase
Mash2	mammalian achaete-scute homologue 2
2-ME	2-methoxyestradiol
MEK1	mitogen-activated protein kinase kinase 1
miRNA	MicroRNA
Mmp	matrix metalloproteinase
Msx1	msh homeobox homolog 1
NE	nuclear extract
NRP	neuropilin
NRS	normal rabbit serum
PDGF	platelet-derived growth factor
PGF	placental growth factor
PI3K	phosphatidylinositol 3'-kinase
PL	placental lactogen
PLC-γ	phospholipase C-gamma
PMSF	phenylmethylsulfonyl fluoride
Runx2	Runt-related transcription factor 2
sFlt-1	soluble fms-like tyrosine kinase
siRNA	small interference RNA

Smad6	mothers against decapentaplegic homologue 6
Sos1	son of sevenless homologue 1
TDO	tricho-dento-osseous
TGF	transforming growth factor
Thbs	Thrombospondin
Vcam1	vascular cell-adhesion molecule 1
VEGF	vascular endothelial growth factor

CHAPTER ONE

REVIEW OF THE LITERATURE

1. Introduction

As the first organ forming in the mammalian embryo, the placenta is essential for survival and growth of the fetus. It mediates implantation and forms the interface between the maternal and fetal circulation, facilitating metabolic and gas exchange as well as fetal waste disposal. In addition to the transfer of gases and nutrients, it also produces hormones and growth factors that affect both the mother and the fetus. Any genetic or environmental factor that negatively influences the development of the placenta can lead to placental insufficiency and fetal growth retardation, and in more severe situations, preeclampsia, eclampsia and potentially fetal and/or maternal death. The goal of this review is to summarize details and background of the development and function of the mouse placenta and genetic determinants required for placental morphogenesis.

2. Mouse placenta morphogenesis

In mice, placental development starts at embryonic day (E) 3.5 when the trophectoderm of the blastocyst is separated from the inner cell mass [1]. As the blastocyst enters the uterus at E4.5, the trophectoderm cells distal to the inner cell mass stop dividing but continue to replicate their DNA to form trophoblast giant cells (extravillous trophoblasts in human). These giant cells secrete proteolytic enzymes such as matrix metalloproteinases (Mmp) to erode the uterine epithelia and create implantation sites for the blastocyst [2]. The trophectoderm cells immediately adjacent to the inner cell mass proliferate to generate extraembryonic ectoderm and ectoplacental cone as shown in Figure 1.1 [3]. The extraembryonic ectoderm develops into the trophoblast cells of the chorion layer and later the labyrinth (chorionic villi in human). The ectoplacental cone develops into spongiotrophoblast (column cytotrophoblast in human), a layer existing between the labyrinth and the outer

trophoblast giant cells.

During implantation, the trophoblasts of the blastocyst stimulate the transformation of the stromal fibroblast cells of the uterine endometrium into decidual cells. The stored glycogen in these cells provides nourishment for the developing embryo [4]. As development progresses, vascularity and vascular permeability are enhanced in the decidua. The predominant type of leukocyte within the decidua is the uterine natural killer cells, which is critical for appropriate endometrial angiogenesis in early implantation site development [5,6]. As the maternal interface to the embryo, the decidua participates in the exchange of nutrients, gases, and waste products during gestation. The decidua also protects embryos from the maternal immune system. Finally, the decidua has to tightly control invasion of the trophoblasts into the uterine wall [7].

At E8.5, the allantois from extraembryonic mesoderm makes contact with chorion in a process termed chorioallantoic fusion. The reciprocal interaction between allantoic mesoderm and chorionic trophoblast regulates subsequent placental development. The chorion begins to fold to form the villi, creating a space into which fetal blood vessels grow from the allantois [8]. Trophoblasts along with the associated fetal blood vessels undergo extensive villous branching to form the labyrinth. At this time, the chorionic trophoblast cells differentiate into two labyrinth cell types, multinucleated syncytiotrophoblast cells surrounding the fetal capillary endothelium and mononuclear trophoblast cells lining the maternal blood sinuses.

Invasive trophoblasts migrate to the maternal uterine spiral arteries and the trophoblasts replace the endothelial cells lining the spiral arteries and help dilate these blood vessels in a process referred to as spiral artery remodeling [9]. In rodents and primates, the uterine epithelia are eroded and the endothelial cells of the maternal capillaries are broken down such that maternal blood comes into direct contact with

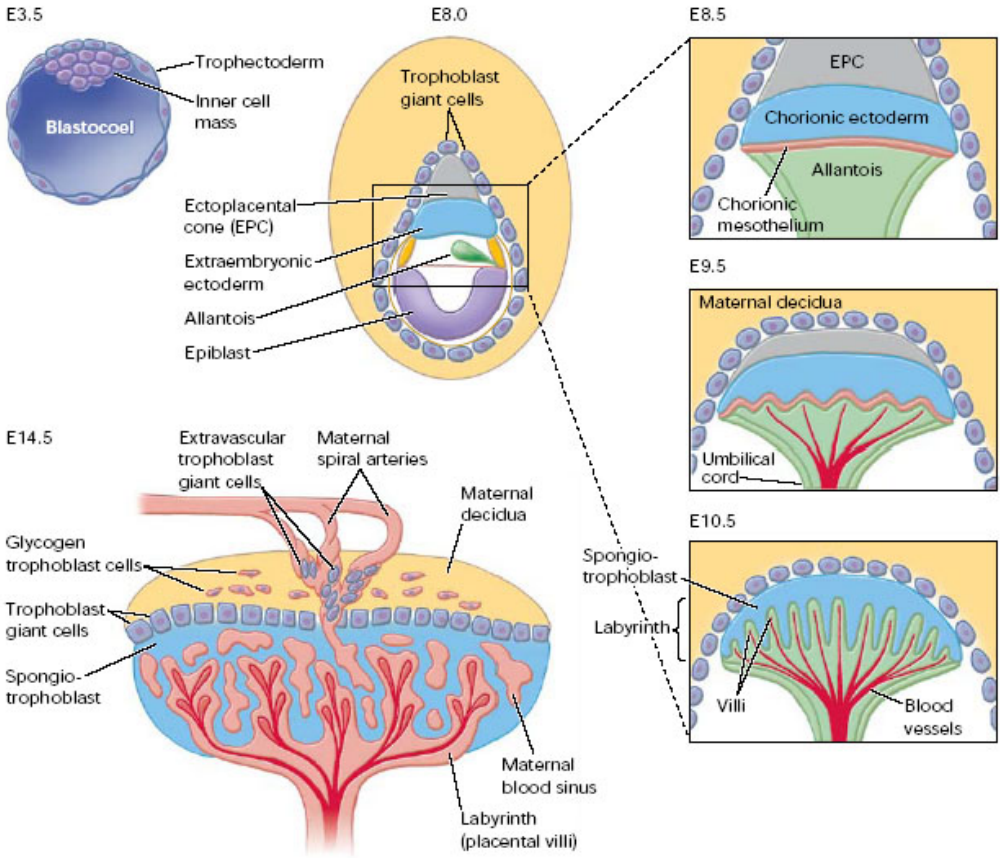

Figure 1.1 Structure and development of mouse placenta.

From Erica Watson & James Cross. Physiology. 2005, 20: 180-193.

the trophoblast surface of the villi. This ensures the material exchange between the two blood systems of the mother and the fetus without direct mixing of blood in a classical hemochorial placenta [3]. The developing fetus is connected to the placenta by the umbilical artery and vein.

By E14.5, the well developed mouse placenta is composed of three major layers: 1) the labyrinth, an inner layer of the placenta which is composed of highly branched villi; 2) the spongiotrophoblast, a middle junctional region which attaches the fetal placenta to the uterus. In this layer, trophoblasts invade into the uterine wall and maternal vessels; 3) the maternal decidua which is the outer layer including decidua of the uterus and maternal blood vessels that bring blood into or out of the implantation site [3].

Until birth at approximately E19.5, the trophoblast and fetal vasculature generate more extensively branched villi within the labyrinth. To maximize nutrient transport, maternal blood flows within the placental labyrinth are arranged in a countercurrent direction to the fetal blood flows [10]. If the labyrinth is not appropriately vascularized with branching and dilation, placental perfusion can be impaired leading to insufficient oxygen and nutrient exchange and growth restriction of the fetus.

3. Function of the placenta

The function of placenta is to exchange gases, nutrients and waste products between the mother and the fetus. In addition to material exchange, the placenta has many endocrine functions. Several hormones and growth factors are produced by the placenta such as placental lactogen (PL) in rodents [11], human chorionic gonadotropin (hCG) in primates [12], vascular endothelial growth factor (VEGF) [13] and placental growth factor (Pgf) [13,14].

In mouse placenta, trophoblast giant cells produce PL. The protein structure and

function of PL is similar to prolactin and growth hormone. PL maintains the function of corpus luteum (CL) in mice. PL can modulate fetal and maternal metabolism during pregnancy. This hormone can stimulate lipolysis in the mother to provide fuel for the growing fetus. PL can lead to insulin resistance and carbohydrate intolerance in the mother [15].

In human placenta, hCG is produced and secreted initially by the trophectoderm at first and later by syncytiotrophoblasts [16]. This glycoprotein hormone can be detected in the maternal serum 9-10 days after ovulation, and its concentration increases exponentially soon thereafter, peaks at 10 weeks gestation and declines to a low plateau that persists throughout the remainder of pregnancy [17]. By chemical structure, hCG is a glycoprotein with two subunits. The α subunit is same to luteinizing hormone, follicle-stimulating hormone and thyroid stimulating hormone. The β subunit is specific to hCG. Because of its early presence during the pregnancy, the β subunit of hCG has been widely used as a marker for detection of early pregnancy in women. hCG is required for the maintenance of progesterone secretion from the ovarian CL through engagement of the LH receptor on cells of the CL [18]. Progesterone enhances the uterus with a thick lining of blood vessels and capillaries to sustain the growing fetus as well as inhibits the production and release of more oocytes. Furthermore, hCG may play a paracrine role in the uterine endometrium to facilitate uterine receptivity during implantation in primates [19]. hCG inhibits the immune cells of the mother and facilitates the immune tolerance of the pregnancy, therefore regulating endometrial receptivity [20,21] and protecting the fetus from the maternal immune system during the first trimester [22]. For example, hCG-treated endometrial cells induced an increase in T cell apoptosis [23]. Thus, hCG supports fetal development through maintaining CL function and production of progesterone; facilitating trophoblast invasion; and can serve a paracrine regulator of the uterine

endometrium.

Secreted factors like PL and hCG serve to regulate CL function and endocrine mechanisms related to implantation; however, normal development and function of the placenta require invasion of the maternal decidua by trophoblasts, followed by abundant and organized blood vessel growth. As such, vascular insufficiencies during placentation have been demonstrated to contribute to a number of obstetrical complications such as preeclampsia. Consistent with the research described in this dissertation, the following sections focus on pro-angiogenic factors such as VEGF and Pgf that control and direct placental vascular function.

3.1 Vascular endothelial growth factor (VEGF)

VEGF is a specific endothelial growth factor that stimulates endothelial cell growth and migration and increases vascular permeability [24]. VEGF is expressed and secreted by various cell types including endothelial cells, skeletal and cardiac muscles, hepatocytes, osteoblasts, neutrophils, macrophages and keratinocytes. Mouse embryos with functional inactivation of one *VEGF* allele die at E11-12 during pregnancy due to abnormal blood vessel development [25,26]. The placentas of these mouse embryos look more condensed and show degenerating endothelial cells in fetal capillaries [26]. During embryogenesis, VEGF regulates blood vessel size and density. In postnatal life, VEGF appears to maintain endothelial cell integrity and promotes bone formation through recruitment of osteoblasts and chondroblasts [27].

VEGF mediates its effects by interacting with three membrane-associated tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1), VEGFR-2, VEGFR-3 and two co-receptors of neuropilin (NRP) [28,29]. VEGFR-1 is also known as fms-like tyrosine kinase (Flt-1); VEGFR-2 is known as fetal liver kinase (Flk-1), and in human, it is named kinase insert domain-containing receptor (KDR) [30]. All three receptors are

composed of an immunoglobulin-like extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain [31,32].

Engagement of VEGFR-2 activates a number of signaling transduction pathways to mediate different endothelial cell functions such as survival, proliferation, migration and vascular permeability in a pathway-specific fashion as shown in Figure 1.2. For example, VEGF regulates endothelial cell survival through the phosphatidylinositol 3'-kinase (PI3K) signaling pathway [33]. Phosphorylation of VEGFR-2 is essential for VEGF-dependent endothelial cell proliferation via activation of phospholipase C-gamma (PLC-γ), protein kinase C (PKC) and downstream extracellular signal regulated kinase (ERK) pathway [34]. VEGFR-2 activation also triggers the activation of c-Jun N-terminal kinase (JNK), which mediates actin reorganization in endothelial cells leading to cell movement [35].

Disruption of genes encoding VEGFRs results in severe abnormalities of blood vessel formation in mice. Embryos lacking the *VEGFR-1* gene die at midgestation due to impaired development of functional blood vessels from the endothelial cells within the embryo [36], which is consistent with deletion of one *VEGF* allele [25]. Disruption of the encoding gene of *VEGFR-2* is embryonic lethal between E8.5 and E9.5 because of defects in the differentiation of endothelial cells and formation of blood vessels within the embryo [37]. Consistent with this model, blockade of VEGFR-2 by using an immuno-neutralizing antibody in mice, results in dead and partially absorbed embryos and pale placentas at E8.0 of pregnancy. Thus, this indicates that VEGFR-2 is required for vascular development in both embryo and placenta [38].

VEGF has been shown to stimulate angiogenesis under many pathological conditions including diabetic retinopathy, wound healing and tumor growth [24]. VEGF is up-regulated in many tumors such as colon cancer, breast cancer, renal cell carcinoma and bladder cancer [39]. Plasma VEGF is often elevated in cancer patients

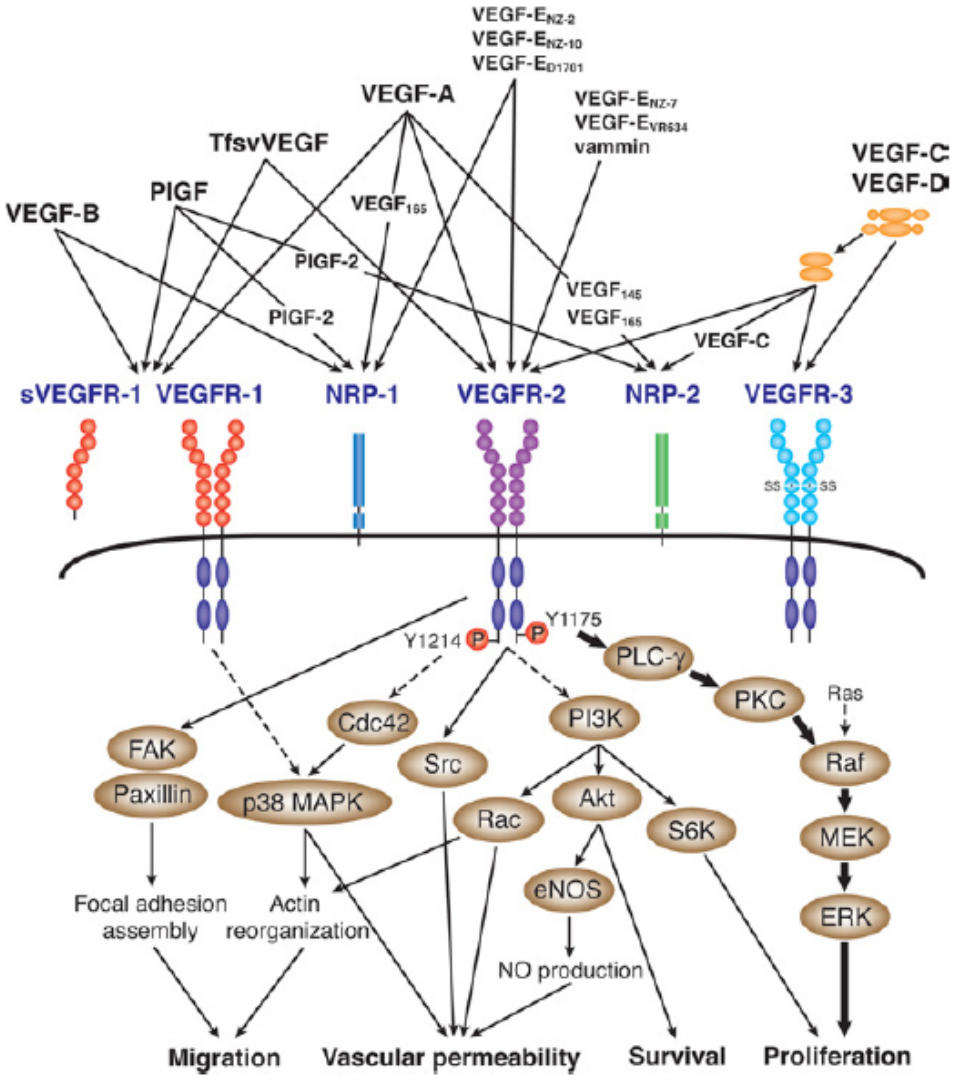

Figure 1.2 VEGF family members, receptors and VEGFR-2 signaling pathways. From Hiroyuki Takahashi & Masabumi Shibuya. Clinical Science. 2005, 109: 227-241.

and it is a marker of poor clinical outcome [40,41]. Blocking VEGF pharmacologically reduced tumor angiogenesis and growth in preclinical studies. A humanized monoclonal antibody to VEGF named bevacizumab has been approved for treatment of many cancers including metastatic colon cancer [42], non-small cell lung cancer [43,44] and metastatic breast cancer [45,46]. Bevacizumab has also been approved for use in metastatic renal cell carcinoma [47] and glioblastoma multiforme [48], a type of brain cancer.

3.2 Placental growth factor (PGF)

PGF is a member of VEGF family. It is an angiogenic growth factor that is expressed in the placenta, primarily by trophoblasts [49-51]. In addition to the placenta, PGF is expressed in other tissues including microvascular endothelial cells, human umbilical vein endothelia, bone marrow, uterine natural killer cells and keratinocytes [14]. Alternative splicing of the human *PGF* transcript generates four isoforms: PGF-1, 2, 3 and 4, all generated from a single gene [49,52,53]. Differences among the four isoforms include the insertion of a heparin-binding domain in PGF-2 and 4 that results in increased association with the cell membrane [14]. In mouse, only one *Pgf* mRNA encoding the equivalent of human PGF-2 has been identified [54]. PGF-2 specifically binds to VEGFR-1 with high affinity, and does not associate with VEGFR-2 and 3 [55]. PGF-1 shares approximately 42% amino acid sequence identity with VEGF [56] and both have significant structural similarity with platelet-derived growth factor (PDGF) and transforming growth factor (TGFβ) [57]. These growth factors are characterized by a common motif of eight conserved cysteine residues that form a cysteine knot structure, which is involved in the dimerization of the growth factors [56,58]. Although the three-dimensional protein structures of human PGF-1 and VEGF are remarkably similar, half of the residues located on the receptor-binding

domain are not conserved between these two growth factors [56].

In comparison to VEGF, the role of Pgf in neovascularization is less clear. In mice, Pgf stimulates angiogenesis, leukocyte infiltration, tumor growth, stromal cell migration and revascularization of ischemic tissues [59]. Pgf enhances the survival, growth and migration of endothelial cells in vitro and promotes vessel formation in certain model systems [60-62]. For example, mice with overexpression of Pgf exhibit increased vascularization and vessel permeability [62].

In endothelial cells and trophoblasts, PGF stimulation results in phosphorylation of different tyrosine residues on VEGFR and activation of different target genes as compared to VEGF stimulation [63,64]. In trophoblast cells, the downstream signal transduction pathways activated by PGF include PLC-γ and JNK [65,66]. Interestingly, embryogenesis appears to be normal in *Pgf* knockout mice, suggesting that its role in placental development is redundant or there are fundamental differences in the function of human and mouse Pgf [63].

The *Pgf* knockout mouse reveals important functions of Pgf, independent of other angiogenic growth factors [63]. Pgf is up-regulated under certain pathological conditions such as ischemia, wound healing and tumor formation [61]. The *Pgf* knockout mice show significantly impaired angiogenesis during pathological conditions such as ischemia, inflammation, wound healing and tumor formation [63]. Interestingly, inhibitors of Pgf reduce pathological angiogenesis in various cancers without affecting healthy blood vessels [38]. One of the PGF inhibitors named TB403, a humanized monoclonal antibody against PGF, is currently in clinical trial in cancer patients [67]. The pharmacological use of PGF inhibitor in conjunction with VEGF inhibitor enhances the anti-tumor activity compared to the VEGF inhibitor alone[38] and decreases dose of the VEGF inhibitor for tumor treatment, therefore reducing side effects of the VEGF inhibitor such as thrombosis, hypertension, microvascular

pruning in healthy organs and interruption of pregnancy [68].

Both VEGF and PGF are angiogenic factors and both have important roles in pathological angiogenesis. Combination of VEGF and PGF increases ischemic myocardial angiogenesis in a mouse model which is resistant to VEGF alone [64]. In cultured human umbilical vein endothelial cells, VEGF actually induces the production of PGF protein through a post-transcriptional mechanism dependent on PKC signaling [69]. PGF is, in turn, able to up-regulate the expression of VEGF [61]. PGF synergistically enhances VEGF-induced angiogenesis and vascular permeability [55]. Some of the mechanisms underlying PGF effects on the vascular endothelial cells include the following: 1) PGF has been shown to displace VEGF from VEGFR-1, thereby increasing the fraction of VEGF available to activate VEGFR-2; 2) PGF activates VEGFR-1, leading to intermolecular transphosphorylation of VEGFR-2 and enhancing the response of VEGFR-2 to VEGF; 3) PGF can induce downstream signal pathways through VEGFR-1, independently of cross talk with VEGFR-2; and 4) PGF can form heterodimers with VEGF and these heterodimers appear to stimulates angiogenesis by inducing the formation of VEGFR-1/ VEGFR-2 heterodimer receptor [64]. Collectively, these studies demonstrated important interactions between PGF and VEGF in controlling blood vessel formation and physiology.

3.3 Pro-angiogenic factors and placental diseases

Many obstetric complications, such as preeclampsia, are associated with abnormal trophoblast function and inadequate or dysfunctional vasculature during placental development. Preeclampsia affects approximately 5% of all pregnancies and is manifested by maternal hypertension and proteinuria after 20 weeks of pregnancy [70]. Preeclampsia may eventually progress to glomerular malfunction, thrombocytopenia, liver and brain edema, and associated life-threatening seizures [71]. Circulating levels of

PGF in maternal serum fluctuate during normal human pregnancy. For example, PGF increases during the first two trimesters, peaks at 29-32 weeks of pregnancy and decreases thereafter [72]. In women with preeclampsia and those who subsequently develop the disorder, the level of PGF in plasma follows the same secretion pattern; however, the total levels of PGF are shown to significantly decrease compared to normal pregnancy [14,72]. PGF has been used as an important predictor of subsequent development of preeclampsia in pregnant women [72,73].

Soluble FLT-1 (sFLT-1) is a variant splicing isoform of the VEGF receptor FLT-1, where sFLT-1 lacks the transmembrane and cytoplasmic domains [74]. As such, sFLT-1 acts as a potent VEGF and PGF antagonist. Serum sFLT-1 concentrations have been shown to be increased in preeclampsia patient thus resulting in reduced circulating concentrations of free or bio-available PGF and VEGF [14,75-77]. As discussed earlier, during normal pregnancy, cytotrophoblasts invade maternal spiral arteries and transform from an epithelial to an endothelial phenotype. This vascular remodeling increases the blood flow and the supply of oxygen and nutrients to fetus by the end of the first trimester [78,79]. In the placenta of preeclamptic women, inadequate cytotrophoblast invasion of maternal spiral arteries causes reduced blood flow and placental insufficiency resulting in significant placental hypoxia [80]. Placental hypoxia promotes increased release of sFLT-1 into maternal circulation, which reduces the circulating VEGF and PGF levels below a critical threshold required for maintaining the vasculature in the mother. The resultant systemic endothelial dysfunction causes maternal syndrome characterized by hypertension and proteinuria [75,77].

3.4 Regulation of VEGF and PGF

The VEGF gene is tightly regulated at the transcriptional level. Hypoxia is the major regulator via binding of hypoxia-inducible transcription factors to the hypoxia-

responsive element located in the VEGF promoter [81]. Some growth factors can induce VEGF transcription such as PDGF [82], TGFβ [83], epidermal growth factor (EGF) [84] and insulin-like growth factor (IGF-1) [85]. In addition, cytokines such as IL-10 and IL-13 inhibit the release of VEGF [86]. Unlike VEGF, some studies have shown that PGF is not hypoxia-inducible in trophoblast cells [51] and mouse cornea [87], although this is controversial since other studies have reported that hypoxia can up-regulate *Pgf* expression during angiogenesis in fibroblasts [88]. Thus, it is not fully understood whether hypoxia has a generalized effect on Pgf expression or if this is indeed tissue and/or cell type specific. In bovine retinal endothelial cells, high glucose increases expression of Pgf at both the mRNA and protein level [89]. Pgf has been shown to be up-regulated by forkhead/winged helix transcription factor FoxD1 in epithelial cells. FoxD1 binds to a conserved site in the *Pgf* promoter and activates its transcription [90]. In human trophoblast cells, glial cell missing 1 (GCM1) has been shown to be a critical regulator of *PGF* gene transcription. In those studies, overexpression of GCM1 increased human *PGF* promoter activity [91]. Lastly, changes in chromatin structure to epigenetic regulatory mechanisms can impact *PGF* gene regulation. *PGF* expression is reduced through methylation of its promoter in human cell lines of lung and colon cancers, while demethylation of the *PGF* promoter appeared to restore its transcription in these tumor cells [92].

In summary, VEGF and PGF are key angiogenic stimulators during normal development as well as in a variety of pathological conditions such as wound healing, ischemia and tumor formation. In diseases such as preeclampsia during human pregnancy, angiogenic balance appears to be an important determinant of disease outcome. In this situation the balance between VEGF and PGF may be antagonized by sFLT-1, this having a crucial impact on angiogenic potential within the developing placenta. Studies in this dissertation describe important mechanistic data suggesting

that regulation of the *Pgf* gene in mouse and likely in human trophoblasts required the trophoblast-specific transcription factor, Dlx3 (described in more detail below). Further, disruption of normal *DLX3* and *PGF* expression appears to be an important factor associated with preeclampsia.

4. Differences and similarities between mouse and human placenta

Mouse placenta differs from human placenta in its morphogenesis and endocrine functions as shown in Figure 1.3. For example, villi of mouse and human are composed of different trophoblast cell layers. Mice have three trophoblast cell layers, two syncytial layers and a single mononuclear cell layer while humans have a single syncytial layer. Placental villi are grouped into round structures called cotyledons. Mice have a single cotyledon whereas humans have multiple cotyledons [2]. In mouse, the pituitary gland is required for pregnancy and progesterone produced by the CL is essential throughout gestation. In early gestation, pituitary-derived prolactin regulates CL function. Later, trophoblast giant cells in the mouse placenta secrete PL supporting CL production of progesterone. In human, hormones from the pituitary gland are not required for the initiation and maintenance of pregnancy. Progesterone is produced first by the CL which is dependent on hCG and later progesterone is primarily produced by the placenta [93].

Although there are some anatomic and physiologic differences between mouse and human placentas, their overall structures and the molecular mechanisms underlying placental development appear to be quite similar [2]. The outer layer of the mouse placenta is composed of trophoblast giant cells whose function is similar to invasive extravillous cytotrophoblast cells in humans. The function of the middle layer of mouse placenta, the spongiotrophoblast, is analogous to the column cytotrophoblast that anchor the villi of the human placenta. The labyrinth layer of the mouse placenta

is similar in function to the chorionic villi of the human placenta [2]. In both species, the villi are covered by syncytiotrophoblast cells that lie in direct contact with maternal blood. The key regulatory molecules during mouse placental development are expressed and functional in human placenta as well. Comparative analysis of the molecular similarities between the mouse and human placenta shows that over 80% genes known to cause placental phenotypes in mouse are expressed in human placenta [94]. Because of the structural and molecular similarities between the mouse and human placentas, genetic studies in mice have provided important insights into the molecular bases of human placental disorders including preeclampsia in the mother and intrauterine growth retardation in the fetus.

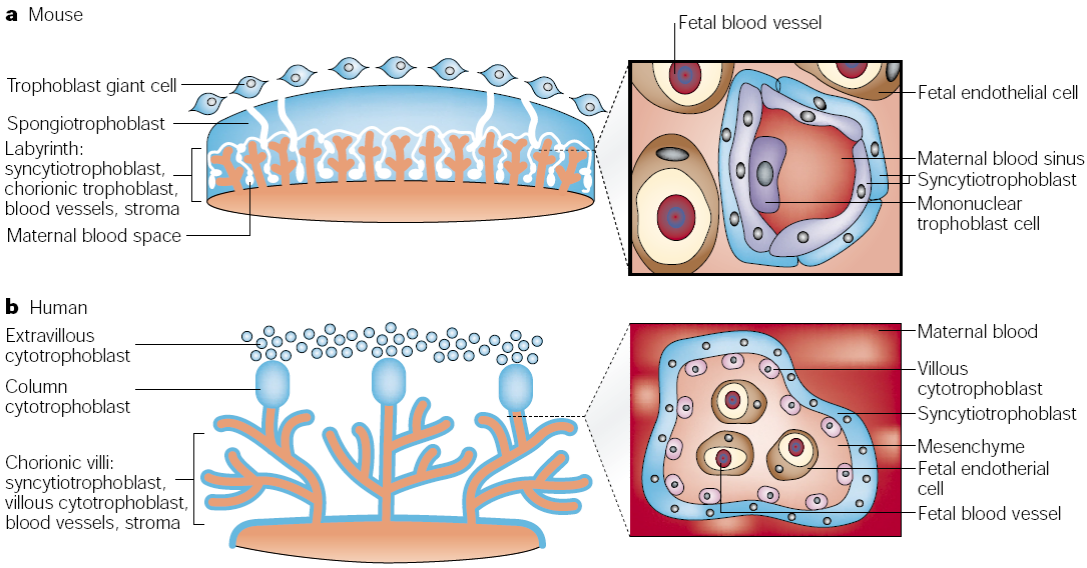

Figure 1.3 Comparative anatomies of the mouse and human placenta.

From Janet Rossant & James Cross. Nat. Rev. Genet. 2001, 2: 538-548.

5. Targeted mutations and knockouts affecting mouse placental development

The differentiation of trophoblast cell subtypes is regulated by distinct molecular mechanisms. A great number of knockout and transgenic mouse models have been generated to define genetic determinants required for developmental decisions in placental morphogenesis. For example, knockouts of extracellular matrix components, numerous receptors, signaling molecules or transcription factors have been shown to compromise placental morphogenesis. The following section describes key knockout models to illustrate the importance of these factors.

5.1 Extracellular matrix components

The extracellular matrix components, vascular cell-adhesion molecule 1 (Vcam1) and integrin α4 are required for chorioallantois fusion [95]. Vcam1 is expressed at the tip of the allantois and binds to its receptor integrin α4 that is expressed on the basal surface of the chorion [96]. The majority of *Vcam-1* deficient mouse embryos die at an early gestational age due to the failure of allantois fusion to the chorion, resulting in abnormal placental development [95]. In the minority of Vcam-1 deficient embryos, the allantois fuses with the chorion but the allantoic mesoderm is not distributed over the entire chorionic surface and formation of vascular structure is found to be abnormal [95]. Knockout of *integrin α4* in mice leads to embryonic death due to failure of fusion of the allantois with the chorion during placental development and abnormal development of epicardium and coronary vessels [96].

5.2 Fibroblast growth factor (Fgf), Fgf receptor and downstream signaling molecules

Studies in mice have shown that the main signaling pathway involved in the interaction between the trophoblast and inner cell mass was the Fgf signaling pathway.

Fgf4 is expressed by the inner cell mass at the blastocyst stage and subsequently in the embryonic ectoderm [2]. Fgf4 is required for the proliferation and maintenance of trophoblast stem cell populations [97]. *Fgf4* knock out mouse embryos die at the time of implantation and poor development of all embryonic cell lineages is evident in these embryos[98,99].

All four Fgf receptors (Fgfr) are expressed at the blastocyst stage of mouse development and Fgfr2 is expressed in trophoblast cells immediately adjacent to the embryonic ectoderm [100,101]. Disruption of *Fgfr2* results in embryonic death a few hours after implantation and arrest of trophoblast differentiation [102]. Fgfr signaling has also been demonstrated to be essential for chorioallantoic fusion and branching as well. Deletion of the immunoglobin-like domain of the Fgfr2 extracellular domain causes the mouse to die between E10-11 due to failure to form the chorioallantoic fusion and severe labyrinth defects [103]. In addition to Fgfr2, mice deficient in Egf receptor also shows labyrinth defects [104].

Many signal transduction components that act downstream of these growth factor receptors have been implicated during mouse embryogenesis. For example, growth factor receptor-bound protein 2 (Grb2) is a signaling adaptor protein downstream of Fgf. *Grb2* null embryos die after implantation because of defects in inner cell mass survival and endoderm differentiation [105]. Other signal transduction components downstream of Fgf including Grb2-associated binder 1 (Gab1), son of sevenless homologue 1 (Sos1), mitogen-activated protein kinase (MAPK) kinase 1 (Mek1) and Erk have been linked to the morphogenesis of placental labyrinth layer. The thickness of the labyrinth layer of the placenta in *Gab1* knockout mice is shown to be severely reduced [106]. The *Sos*$^{-/-}$ mouse placental labyrinth layer is also very thin and highly disorganized and vasculature in the labyrinth is incomplete [107]. Targeted disruption of the mouse *Mek1* gene results in embryonic lethality at E10.5 because vascular

endothelial cells in the labyrinth layer markedly decrease, leading to the reduced vascularization of the developing placenta [108]. *Erk2* knockout mouse embryos die at E11.5 due to the abnormality of the labyrinth layer of the placenta [109]. Interestingly, *Erk1* null mice appear to be completely normal with regard to placental function suggesting that during development, Erk1 and Erk2 do not appear to have redundant roles in the mouse [110,111].

5.3 Transcription factors

Fgf signaling regulates the expression of the homeobox transcription factors caudal-type homeobox (Cdx2) and eomesodermin homologue (Eomes), which are in turn required for maintaining the fate of trophoblast stem cells [112]. The formation of the spongiotrophoblast or trophoblast giant cells from the ectoplacental cone is regulated by a balance among the actions of basic helix-loop-helix (bHLH) transcription factors at the transcriptional level. For example, mammalian achaete-scute homologue 2 (Mash2) has shown to be an important regulator of trophoblast proliferation; its expression level diminished as mouse trophoblasts differentiate into giant cells. The targeted deletion of the *Mash2* gene results in an increased number of trophoblast giant cells and diminished spongiotrophoblast layer [113]. Contrary to the effect of Mash2, the other bHLH transcription factor Hand1 [114] promotes differentiation of trophoblasts into giant cells.

Gcm1 regulates the initiation of chorioallantoic branching and the differentiation of syncytiotrophoblast in mouse [115]. Gcm1 expressing cells are localized precisely at sites of primary branch points and as the branches elongate, Gcm1 expression remains at the distal tip of the branches. In *Gcm1* knockout mice, the allantois still fuses with the chorion, but subsequent steps of placental labyrinth formation appear to be inhibited, so the mouse embryos die at E10 due to absence of placental labyrinth [115].

Extraembryonic, spermatogenesis, homeobox-1 like protein-1 (Esx1) and distal less 3 (Dlx3) are other transcription factors critical for development of the labyrinth layer. *Esx1* is located on the distal arm of X chromosome [116]. The *Esx1* gene encodes a homeobox transcription factor that has been shown to be expressed only in trophoblast cells of the labyrinth. In the placenta of *Esx1* mutant mouse, the chorioallantoic branching is shown to be normal but there are obvious deficiencies in fetal blood vessel growth into the labyrinth [117]. Surprisingly, the *Esx1* knockout mouse placenta is actually larger than normal suggesting an attempt to compensate for the reduced vascularization and nutrient transport. The other transcription factor Dlx3 is also shown to be required for normal labyrinth development. *Dlx3* null mice die in uteri between E9.5-10 as a result of placenta defects that involve abnormal development of spongiotrophoblast and labyrinth layer, including defects in vascularization [118]. The focus of the research presented in this dissertation is related to the role and regulation of Dlx3. As such, a more detailed discussion of this factor is warranted.

6. Distal-less family members

In *Drosophila*, the distal-less homeodomain gene *Dll* regulates the formation of appendages such as legs and antennae [119]. Proteins of the Distal-less family are all related to this *Drosophila* distal-less homeodomain homolog [119,120]. In mammals, the distal-less family includes six members, arrayed in pairs and aligned with the *Hox* gene clusters along different chromosomes [121]. In mice and humans, *Dlx1* and *Dlx2* are linked with the *Hox D* gene cluster [122]; *Dlx3* and *Dlx4* are linked to the *Hox B* cluster [123]; and *Dlx5* and *Dlx6* are linked to the *Hox A* cluster [124]. The relationship between convergently transcribed pairs of *Dlx* family members and specific *Hox* gene clusters suggests that these homeodomain-containing transcription factors are likely involved in important aspects of developmental morphogenesis [121].

Table 1.1 Mouse mutant placental phenotypes mentioned in the context

Gene	Gene product	Placental phenotype of mutant mice
Vcam1 [95]	Adhesion molecule	Failure of chorioallantoic attachment
Integrin α4 [96]	Adhesion molecule (Vcam1 receptor	Failure of chorioallantoic attachment
Fgf4 [98,99]	Fibroblast growth factor	Failure of implantation
Fgfr2 [102,103]	Fgf receptor	Failure of chorioallantoic attachment or defective labyrinth formation
Grb2 [105]	Adaptor protein	Small labyrinth
Gab1 [106]	Adaptor protein	Small labyrinth
Sos1 [107]	MAPK pathway	Small labyrinth
Mek1 [108]	MAPK kinase	Small labyrinth
Erk2 [109]	MAPK pathway	Small labyrinth
Cdx2 [112]	Homeodomain transcription factor	Failure to maintain trophoblast stem cells
Eomes [112]	Homeodomain transcription factor	Failure to maintain trophoblast stem cells
Mash2 [113]	bHLH transcription factor	Loss of spongiotrophoblasts, increased giant cell formation
Hand1 [114]	bHLH transcription factor	Block to giant cell differentiation
Gcm1 [115]	Transcription factor	Block to chorioallantoic branching morphogenesis, no labyrinth
Esx1 [117]	Homeodomain transcription factor	Placental hyperplasia, vascularization defects
Dlx3 [118]	Homeodomain transcription factor	Small labyrinth

Dlx genes are expressed in distinct but overlapping regions within the embryo, primarily in the forebrain, branchial arches and tissues derived from epithelial/ mesenchymal interactions during development [125,126]. In mouse, all six *Dlx* genes are primarily expressed in ectodermal derivatives: the nervous system and the surface ectoderm. Dlx1, Dlx2, Dlx5 and Dlx6 were found to be expressed in the central nervous system, highly restricted to diencephalon and telencephalon [124]. In situ hybridization studies have revealed a strict temporal sequence of the expression of *Dlx* gene in the forebrain. *Dlx2* appears to be activated first, and then *Dlx1* is subsequently transcribed in a subset of the *Dlx2* expressing cells, followed by *Dlx5* and *Dlx6* [127]. *Dlx2* knockout mice show abnormal development of proximal first and second branchial arches and abnormal differentiation in the forebrain [128]. The *Dlx1/Dlx2* double mutants show a major blockage of neurogenesis within the subcortical telencephalon [129]. Targeted disruption of *Dlx5* in mouse leads to craniofacial defects, severe malformation of the vestibular organ and delayed ossification [130]. The *Dlx5/Dlx6* double mutants result in severe craniofacial and appendicular skeletal abnormalities [131].

6.1 Dlx3

In contrast to other members of *Dlx* family, *Dlx3* is not expressed in the central nervous system [125]. *Dlx3* has been shown to be tandemly and inversely arrayed with *Dlx4* on human chromosome 17, separated by approximately 17.6 kb of intervening sequence [132]. *Dlx3* and *Dlx4* show highly similar expression patterns. This concordant expression of genes suggests that they may be regulated by shared cis-control elements in their shared intermediate domains [133]. Conserved elements exist in the intermediate domain of *Dlx3-4* by sequence comparisons between mouse and human [134]. During development, Dlx3 has been demonstrated to be involved in

developmental determination of multiple tissues, including the first and second branchial arches, dental tissues, epithelial derivatives and the placenta.

Although all the *Dlx* genes are expressed in the first and second branchial arches, the expression *Dlx3* is spatially and temporally distinct from the other members. It is the latest and most distally expressed in branchial arches [126]. Expression of *Dlx3* in the branchial arch appears to affect development of odontoblasts and craniofacial structures [135]. The presence of *Dlx3* mRNA in the tooth germ has been reported during bud, cap and bell stages of dental development [125,136]. In addition, more recent studies have shown that *Dlx3* was expressed in periosteum, osteoblasts, and chondrocytes of the developing limb and in osteogenic cell lineage, suggesting that Dlx3 may play a prominent role in bone physiology [137,138].

Dlx3 is involved in the development of epithelial tissues such as skin, hair follicles, otic and olfactory placodes and limb buds [139]. In the epidermis, Dlx3 is shown to be exclusively expressed in suprabasal cells, with transcription initiating shortly after cells exit the basal layer. Ectopic expression of Dlx3 in the basal cell layer of transgenic mouse skin induces cessation of proliferation and premature differentiation of keratinocytes, supporting a role for Dlx3 during epidermal differentiation [140]. By the end of embryonic development in mice, Dlx3 expression has become restricted to the skin, where it is transcribed in the suprabasal cells of stratified epidermis and in the matrix cells of hair follicles [121]. Dlx3 is also expressed throughout hair cycle and it is a crucial regulator of hair morphogenesis, differentiation and cycling [141]. Conditional deletion of *Dlx3* in the mouse epidermis results in complete alopecia, which is thought to be caused by failure of the hair shaft to form due to abnormal differentiation of the cortex of the hair shaft [141].

In mouse placenta, *Dlx3* is exclusively expressed in the ectoplacental cone and chorionic plate by E8.5, later in trophoblasts within the labyrinth layer by E10.5 [118],

and *Dlx3* continues to be expressed in the labyrinth throughout the remainder of gestation [142]. Interestingly, *Dlx3* does not appear to be expressed in trophoblast giant cells in mouse [142]. Work from colleagues in the Roberson lab demonstrated that DLX3 was nuclear localized in both cytotrophoblast and syncytial trophoblast within microvilli of first trimester human placenta at a time of peak hCG synthesis and secretion [143]. In cultured human placental trophoblasts, DLX3 localizes to the nuclear compartment as well consistent with its role as a transcriptional regulator [144].

6.2 Function of Dlx3

The homeodomain within Dlx3 is a 60 amino acid conserved DNA binding motif that suggests that Dlx3 functions as a transcription factor and recognizes specific target sequences in the genome. Dlx3 also contains nuclear localization and transcriptional activation domains. Both mouse and human Dlx3 protein is composed of 287 amino acids with homeodomain located in residues 130-189 [145]. The sequence of Dlx3 protein is highly conserved between mouse and human with only five different amino acids and none of them is located at the homeodomain. The DNA binding domain of Dlx3 is located on the third α-helix of homeodomain which has been implicated in the direct binding with the major groove of DNA. The consensus DNA binding sequence for Dlx3, (A/C/G)TAATT(G/A)(C/G), was determined by random oligonucleotides binding selection in vitro [146]. The activation domain of Dlx3 includes two sites: residue 1-43 and residues 189-220 with the latter just downstream of the homeodomain [145].

Dlx3 is thought to be a cell-type specific transcriptional activator in placental trophoblasts. In human trophoblast cell lines, DLX3 plays a key role in regulating cell-specific expression of the α subunit of hCG, a key hormone of early pregnancy in primate placenta, as described earlier [143]. Trophoblast-derived hCG plays a critical role

in the maintenance of progesterone secretion from the corpus luteum until the placenta can produce sufficient progesterone to maintain pregnancy [18]. DLX3 binds to and transactivates the promoter of hCG gene via a cis acting element termed the junctional regulatory element (JRE). Mutations within the JRE markedly reduce basal expression of the α subunit of hCG because DLX3 cannot bind the mutant JRE [143].

In addition to regulation of the α subunit of hCG, DLX3 is required for trophoblast-specific expression of the 3β-hydroxysteroid dehydrogenase type VI (3βHSD) gene [147]. 3βHSD is an enzyme essential for the biosynthesis of all active steroid hormones including progesterone. 3βHSD is expressed in human placenta and is thought to be required for the biosynthesis of placental progesterone and thus the maintenance of pregnancy [148]. In human placental cells, transfection studies have identified two novel trophoblast-specific enhancer elements within the *3βHSD* VI gene promoter. These two enhancers bind DLX3 and activator protein (AP-2γ), a combination of cis elements and regulatory factors shared with regulation of the glycoprotein hormone α subunit promoter. Interestingly, the combined actions of DLX3 and AP-2γ appear to be necessary for the transcriptional regulation of both the glycoprotein hormone α subunit and *3βHSD* VI genes in trophoblasts, suggesting commonality in transcription factor regulatory combinations or codes directing cell-type specific gene expression [143,147,149].

DLX3 also appears to regulate the gene expression of the bovine interferon-tau (IFNT) gene in ruminants. IFNT is secreted by ruminant trophoblasts and is thought to be a critical signal for maternal recognition of pregnancy in cattle and sheep [150]. IFNT in ruminants has similar paracrine functions to those of hCG in primates. IFNT acts locally on the uterine endometrium to promote uterine implantation by increasing protein synthesis in endometrial glands [151]. DLX3 transactivates the bovine *IFNT* promoter in human trophoblasts. In both human and bovine trophoblast cell line,

DLX3 is coexpressed with v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2), the key regulator of *IFNT* expression. ETS2 and DLX3 have been shown to induce synergistic activation of *IFNT* promoter and deletion of the ETS2 binding site within the *IFNT* gene promoter eliminates the ability of DLX3 to transactivate the *IFNT* gene [152].

Loss of *Dlx3* in mice is associated with reduced expression of another homeobox gene, *Esx1*. The *Esx1* gene encodes a homeobox transcription factor that appears to be expressed in visceral yolk sac, the ectoderm of the chorion, and trophoblast cells of the labyrinth in the mouse. In adult tissues, *Esx1* expression was shown to be restricted to germ cells in testes [116]. Thus, Esx1 has an important role in the differentiation of specific extraembryonic tissues and also in male germ cell development. Based on knockout studies, Dlx3 was identified as an important transcriptional regulator of *Esx1* gene promoter activity [118]. While links to the *Dlx3* gene program make important connections to *Esx1*; little is known about the larger group of Dlx3 responsive genes within the placenta. This dissertation focuses on microarray analyses and transcript profiling in comparing the placentas of wild type and *Dlx3* null mice.

Dlx3 is expressed in tissues in addition to placenta, such as epidermis, bone structure in mouse and human and Xenopus tissues. Dlx3 is an important regulator of epidermal differentiation. In normal epidermis, the profilaggrin and Dlx3 gene are co-expressed in the granular layer which is the latest stage of epidermal differentiation. Ectopic expression of Dlx3 in the mouse basal cell layer causes the reduced expression of profilaggrin in the granular cells but increased expression in basal cells. Gel mobility shift experiment demonstrates the presence of a binding site for DLX3 within the proximal promoter region of the human profilaggrin gene [140]. P63, a member of p53 family, is essential for the development and maintenance of the stratified epidermis. Dlx3 and p63 colocalize in the embryonic ectoderm throughout embryo

development, and Dlx3 expression is reduced in the embryos of *p63* knock out mouse. P63 binds to the promoter of *Dlx3* gene and transactivates *Dlx3* gene expression in mouse keratinocytes [153]. Once increased by p63, Dlx3 activates Raf1 kinase which mediates the phosphorylation of p63. This phosphorylation causes the degradation of p63 and thus the feedback regulatory loop is formed between p63 and Dlx3 [154].

Dlx3 has been shown to express in pre-osteoblasts, osteoblasts and osteocytes in bones [138]. Runt-related transcription factor 2 (Runx2) is the earliest transcription factor that is essential for the formation of osteoblasts in response to bone morphogenetic protein 2 (BMP2) [137]. BMP2 induces *Runx2* gene transcription through activation of DLX3 protein expression [155]. *Dlx3* is up-regulated in differentiated osteoblasts and osteocytes, which implies that *Dlx3* also plays a role in the late stage of osteoblast differentiation [156].

In these cases, Dlx3 acts as a transcriptional activator. However, Dlx3 can also serve as a negative regulator of gene transcription in amphibians. During Xenopus development, Dlx3 is activated at the beginning of gastrulation and it is not expressed in the dorsal region of the embryo, which is the future neural plate. Dlx3 represses the activation of genes which are specific to the nascent central nervous system through blocking the activation of zinc finger proteins, Zic3. By the neurula stage, Dlx3 is essentially expressed specifically in epidermis [157].

The activation/repression ability of Dlx3 may depend on different cell contexts and physiological states by interacting with variable heterodimeric partners. This dissertation examines the binding partners of DLX3 in human trophoblast cells and demonstrates that a DLX3 partner is mothers against decapentaplegic homolog 6 (SMAD6).

6.3 Importance of Dlx3

The importance of Dlx3 arises from its function in different biomedical systems of disease states. *Dlx3* null mice die in utero by E10 due to placental failure [118]. This is failures due to abnormal development/morphogenesis of the labyrinth layer in the mouse placenta. The early death of *Dlx3* knock out mice makes it difficult to study Dlx3 function in later development; however, there is additional genetic evidence in humans implicating the function of DLX3 in hair, tooth, and craniofacial morphogenesis [138].

Mutation in human *DLX3* gene has been linked to tricho-dento-osseous (TDO) syndrome, an autosomal dominant disorder manifested by kinky, curly hair at birth, enlarged pulp chambers in the teeth (taurodontism), enamel defects that cause tooth loss by adulthood and increased thickness and density of the cranial bones [158,159]. Consistent with these observations, transgenic mice with the human TDO mutant have markedly increased trabecular bone volume and bone mineral density in the femoral bone [160]. The TDO defect is a four nucleotides deletion just downstream of the DNA-binding homeodomain in DLX3 [158]. This deletion causes a frameshift mutation resulting in a premature stop codon and ultimately the deletion of the carboxy-terminal 32 amino acids of DLX3 protein [159,161]. The mutated DLX3 TDO has a dominant negative effect on wild type DLX3 in that the TDO mutant appears to be unable to bind DNA alone but can form a heterodimer with wild type DLX3 and subsequently inhibits wild type DLX3 transcriptional activity [162].

Amelogenesis imperfecta (AI) with taurodontism in human has similar phenotypic characteristics to TDO. AI is an autosomal dominant trait leading to dental enamel defects and enlarged pulp chambers. The mutation is a deletion of two nucleotides in the *DLX3* coding sequence that causes a frameshift altering the last two amino acids of the homeodomain. This results in a mutant protein that is 88 amino

acids shorter than the normal DLX3 protein [163]. The carboxyl terminal change downstream of the AI mutant is not the same as the TDO mutant.

In addition to TDO and AI, DLX3 mutations have been associated with some forms of craniofacial abnormalities, including cleft palate [164]. The putative involvement of DLX3 in the occurrence of cleft palate is also supported by the mouse model deficient in endothelin-1 receptor, which results in cleft palate and hypoplasia of the mandible [165-167]. In this model, Dlx3 expression is shown to be dependent upon endothelin-1 through a Gαq/Gα11-dependent signaling mechanism. In the *G*αq/*G*α11-deficient mouse, Dlx3 is also specifically down-regulated, supporting the speculation of the importance of Dlx3 in cranio-facial morphogenesis [168].

Collectively, these studies of TDO, AI and craniofacial abnormalities underscore the biomedical significance of studying Dlx3, its mechanism of action and downstream gene program. The focus of this dissertation is to dissect the role of Dlx3 in gene regulation in the mouse placenta.

6.4 Regulation of Dlx3 gene expression

The expression of Dlx3 has been shown to be regulated by a number of growth factors and hormones including BMP2, endothelin-1 and transcription factors for example CCAAT box/enhancer-binding protein β (C/EBPβ) and Msh homeobox homolog 1 (Msx1). The following sections briefly described studies that defined the role of these factors in the regulation of Dlx3 transcript and protein abundance.

BMP2

Expression of Dlx3 was demonstrated to be BMP2-dependent in early gastrulation [169] and for cellular differentiation of both keratinocytes [141] and osteoclasts [169]. BMP2 is expressed in proliferative basal and differentiated suprabasal

keratinocytes. In cultured keratinocytes, the transcription of Dlx3 is induced within 12 hours and remains activated up to 48 hours by BMP2 treatment *in vitro* [141]. This transactivation is appeared to be mediated through a member of the Smad family of transcriptional regulators. Smad proteins can be subdivided into three classes: regulated Smads (Smad 1, 2, 3, 5 and 8), common Smads (Smad 4) and inhibitory Smads (Smad 6 and 7) [170-172]. Smads integrate BMP action following BMP2 binding to its type I and II receptors, Smad1 becomes phosphorylated. Phosphorylated Smad1 then interacts with common Smad4 and subsequently the Smad dimer translocates into the nucleus. Finally, the Smad1/4 complex activates BMP2-specific target genes such as Dlx3 [173]. In osteoblasts, DLX3 is shown to be continuously up-regulated during BMP2 induced osteogenesis. BMP2 induced DLX3 also activates gene transcription of Runx2, the transcription factor essential for the formation of osteoblasts [155].

Endothelin-1

Endothelin-1 is expressed in the epithelium of pharyngeal arches. Activation of endothelin-1 has shown to be required for proper development of craniofacial structures [166]. As indicated earlier, targeted deletion of the endothelin-1 ligand or its receptor causes cleft palate and hypoplasia of the mandible. Dlx3 expression is shown to be significantly reduced in endothelin-1 receptor knock out mice [165]. Gαq/Gα11 signaling pathway is the downstream signal of endothelin-1. In the *G*αq/*G*α11 deficient mouse, Dlx3 is specifically down-regulated in pharyngeal arches [168]. These studies implied that Dlx3 is dependent upon endothelin-1 through Gαq/Gα11-mediated mechanism.

CCAAT box and C/EBPβ

In mouse epidermis, a CCAAT box enhancer located within the 5'-flanking sequences of the *Dlx3* promoter appears to be responsible for the majority of the *Dlx3* promoter activity. Additionally, the Sp1 binding site located immediately upstream of

transcription start site acts as a positive regulatory element of the *Dlx3* promoter, independent of the CCAAT box [174]. The Roberson lab has also shown that the CCAAT box enhancer is required for regulation of basal expression of the *Dlx3* gene promoter in choriocarcinoma cells, a model for human placental trophoblasts [174]. In placental cells, the CCAAT box sequence specifically binds C/EBPβ. Interestingly, Dlx3 and C/EBPβ were shown to be coexpressed in human primary trophoblasts derived from normal term pregnancy [144]. These studies suggested a central role for C/EBPβ in the basal regulation of the *Dlx3* promoter in placental trophoblasts.

Msh homeobox homolog 1 (Msx1)

The other homeodomain-containing transcription factor family, Msx, is closely related to Dlx family. Dlx proteins have been shown to be transcriptional activators, while Msx proteins appear to function as transcriptional repressors that antagonize Dlx function [175]. The Msx1 protein binds a DNA sequence containing TAAT, which is also recognized by the Dlx3 homeobox [157]. Thus, the preferred DNA binding sites for Dlx3 and Msx1 are essentially the same [157]. During mouse embryogenesis, the expression patterns of *Msx1* and *Dlx3* genes overlap in the limb bud and during craniofacial development suggesting that this putative antagonism may be essential to these developmental processes [175]. In the adult mouse, Dlx3 and Msx1 protein have also been shown to be coexpressed in the differential layers of epidermal tissues. In this scenario, the transcriptional activity of Dlx3 is thought to be regulated by the transcriptional repressor protein Msx1 [175]. Msx1 can form a heterodimer with Dlx3 therefore competing for DNA binding and effectively inhibiting the function of Dlx3 [145].

This dissertation investigates proteins within the human placental cells that can interact with DLX3 and then may further affect its transcriptional activity. The binding

partner of DLX3 is SMAD6. This dissertation also examine the mechanisms of SMAD6 regulating the transcriptional effect of DLX3 on its target genes such as *Esx1* and *Pgf*.

7. Summary

During mammalian embryogenesis, the placenta provides maternal nutrition, facilitates fetal respiration, eliminates fetal waste, and protects the fetus from maternal immune system. The placenta is also an important source of hormones and growth factors that are essential for normal embryonic development and maintenance of the reproductive cycles necessary for successful and productive pregnancy. Dlx3 plays an important role in the development of placental labyrinth in the mouse and the regulation of hormones critical to pregnancy (such as hCG) in the primate. My hypothesis in this dissertation is that Dlx3 plays a critical role in placental development by regulating its related genes. The research described in Chapter 2 sought to examine the mechanisms associated with DLX3 as a transcriptional activator by characterizing binding partners of DLX3 in trophoblast cells. In Chapter 3, I described the gene profile regulated by Dlx3 in the mouse placenta and *Pgf* is one of the Dlx3 dependent genes. Specific control of *Pgf* gene expression may be of particular importance in disease situations like preeclampsia, where PGF appears to be a significant component and a marker of the progression of the disease. In Chapter 4, I analyzed the role of Dlx3 in *Pgf* gene regulation in trophoblast cells. My studies demonstrated that Dlx3 directly affects transcriptional regulation of Pgf. By unraveling Dx3-dependent mechanisms related to placenta development in the mouse, we may better understand the human placenta and diseases such as preeclampsia in women and fetal growth retardation that compromise both maternal and fetal health.

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CHAPTER TWO

SMAD6 REPRESSES DLX3 TRANSCRIPTIONAL ACTIVITY THROUGH INHIBITION OF DNA BINDING

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Figure 2.1 was kindly provided by Dr. Kathie A. Berghorn. Figures 2.2, figure 2.3 & figure 2.4 were kindly provided by Ms. Patricia A. Clark.

Abstract

Dlx3 is a homeobox-containing transcription factor required for normal placental development in mice. Here we demonstrate that DLX3 interacts with SMAD6, a member of a larger family of transcriptional regulators generally thought to regulate transforming growth factor β/bone morphogenetic protein signaling. Immunocytochemical and immunoprecipitation studies demonstrate overlapping nuclear localization and physical interaction between DLX3 and SMAD6 in human choriocarcinoma cells and in differentiated trophoblasts from human placenta. *In vitro* protein interaction studies mapped the SMAD6 interaction domain within DLX3 to residues 80-163, a region of DLX3 that includes a portion of the homeodomain. DLX3 and DLX4 share homology within this region, and DLX4 was also found to bind SMAD6. Using the *Esx1* gene promoter as a model for a Dlx3-responsive gene, studies demonstrate two near consensus Dlx3 binding sites within the proximal 2.3 kb of the transcription start site. Interestingly, binding of DLX3 to one of these two sites was inhibited by interaction with SMAD6. Consistent with this result, expression of an *Esx1* promoter luciferase reporter was increased by overexpression of DLX3; this effect was reversed with co-expression of SMAD6. Further, small interfering RNA mediated knockdown of endogenous SMAD6 increased DLX3-dependent expression of the *Esx1* gene promoter. Thus, SMAD6 appears to functionally interact with DLX3, altering the ability of DLX3 to bind target gene promoters. SMAD6 appears to play a modulatory role in the regulation of DLX3-dependent gene transcription within placental trophoblasts.

Introduction

The Dlx family of transcriptional regulators includes six members in mammals, arrayed in pairs and aligned with the *Hox* gene clusters along different

chromosomes [1,2]. *DLX3* is tandemly arrayed with *DLX4* on human chromosome 17 and is involved in developmental determination of multiple tissues, including the first and second branchial arches, teeth, bone, and multiple epithelia, including the skin, mammary gland primordia, and the placenta [3]. The relationship between convergently transcribed pairs of Dlx family members and specific *Hox* gene clusters has suggested that, although independent of *Hox* gene expression patterns, these homeodomain-containing transcription factors are clearly involved in important aspects of developmental morphogenesis (reviewed in Ref. [2]). The importance of Dlx3 during development and in the adult arises from several different observations of disease states. Mutations in *DLX3* are believed to be causally related to TDO, a genetic disorder manifested by taurodontism, hair abnormalities, and increased bone density in the cranium [4-6]. The defect in *DLX3* leading to TDO appears to be associated with a four-nucleotide deletion just downstream of the homeodomain, resulting in a premature truncation of the protein. AI with taurodontism has similar characteristics as TDO and has also been associated with mutations with *DLX3* in some families investigated, albeit distinct from the four nucleotide deletion/mutation described above [7]. AI is an autosomal dominant trait leading to dental enamel defects and enlarged pulp chambers and has been associated with a two-nucleotide deletion within the homeodomain of *DLX3*. This deletion again results in a frameshift and premature truncation of DLX3 in the carboxyl terminus, primarily downstream of the homeobox. In addition to TDO and AI, *Dlx3/Dlx4* have been identified in the gene interval thought to be involved in some forms of craniofacial abnormalities, including cleft palate [8]. The putative involvement of Dlx3 in the occurrence of cleft palate is also supported by the murine model deficient in endothelin-A receptor, which results in cleft palate and hypoplasia of the mandible [9-11]. In this model, *Dlx3* expression is thought to be dependent upon endothelin-1 through a Gαq/Gα11-dependent

mechanism. In the *G*αq/*G*α11-deficient mouse, *Dlx3*, among other factors, is specifically down-regulated, supporting the speculation of the importance of Dlx3 in cranio-facial morphogenesis [12]. Thus, the role and importance of Dlx3 in morphogenic aspects of development and in epithelial differentiated function is rather far reaching.

The *Dlx3* null mouse dies *in utero* by E10 due to putative placental failure [13]. This was associated with a failure in the development/morphogenesis of the placental labyrinth compartment of the murine placenta. Further, genetic loss of *Dlx3* was correlated with reduced expression of an additional homeobox factor, Esx1, suggesting that Dlx3 may be an important transcriptional regulator of *Esx1* promoter activity. Studies from our laboratory identified DLX3 as a cell type-specific transcriptional activator in placental trophoblasts. DLX3 binds to and transactivates the promoter for the glycoprotein hormone α subunit gene via a cis-acting element required for full basal activity of this gene [14]. The glycoprotein hormone α subunit is a subunit of the heterodimeric glycoprotein hormone, CG. Trophoblast-derived CG has been identified in primates and equine and appears to play a critical role in the maintenance of early pregnancy in women, providing early gonadotropic support to the CL and maintenance of progesterone production [15-17]. Both for the case of the α subunit promoter and regulation of *Esx1* in the *Dlx3* null mouse, Dlx3 appears to function as a putative transcriptional activator. However, it has also been proposed that Dlx3 can serve as a negative regulator of gene transcription in amphibian models [2]. This apparent activation/repression capability may be due to variable heterodimeric partners of Dlx3 (as proposed in Ref. [2]), dependent upon cell context and physiological state. This observation was the impetus for us to examine potential binding partners of DLX3 in the context of the human placenta. The present studies identify SMAD6 as a binding partner for DLX3 using a yeast two-hybrid screen of a human term placental cDNA library. DLX3 and SMAD6 are co-localized in the

nucleus of cells of trophoblast origin including cytotrophoblasts and syncytial trophoblasts from fully differentiated human term placenta. Interaction between DLX3 and SMAD6 alters the DNA binding properties of DLX3 such that SMAD6 serves as a negative regulator of DLX3-dependent gene transcription of the *Esx1* promoter.

Materials and Methods

Plasmids and cDNAs

All plasmids used in these studies were prepared by two cycles through cesium chloride using standard protocols. Expression vector for human DLX3 was generously provided by Dr. Maria Morasso (National Institutes of Health, Bethesda MD). A series of deletion mutants of the *DLX3* cDNA were constructed by PCR. To facilitate cloning into the pKH3 vector (generously provided by Dr. Jun-Lin Guan, Cornell University, Ithaca, NY), EcoR I and Cla I restriction sites were added to the forward and reverse primers, respectively. The forward primers used in these reactions were as follows: forward 1, 5'-TCAGGAAT TCAAATGAGCGGCTCCTTCGATCGC-3', forward 40, 5'-TCAGGAATTCAACTGGGC TATTACAGCGCTCCTCAG-3', forward 80, 5'-TCAGGAATTCAATACTCGCCCAAGT CGGAATATACC-3', forward 121, 5'-TCAGGAATTCAAATGGTGAACGGCAAGCCCAAAAAG-3', and forward 195, 5'-TCA GGAATTCAACTGGAACACAGCCCCAACAACAGT-3'. The reverse primers used in these reactions were as follows: reverse 128, 5'-GTACATCGATCACGGCTTTCGGACC TTCTTGGGCTTCCC-3', reverse 163, 5'-GTACATCGATCAAGCTAGCTCGGCGCGCTCAGGCAA-3', reverse 202, 5'-GTACATCGATCAACTGTATTGGGACTGTGCTCCAG-3', and reverse 287, 5'-GTACATCGATTCAGTACACAGCCCCAGGGTT-3'. PCR products were cloned initially into the pGEM T-Easy vector (Promega Corp., Madison, WI). Once verified by nucleotide sequence analysis, fragments were subcloned into the pKH3 vector for

use in studies. SMAD6 expression plasmid was a gift from Dr. Ali Hemmati-Brivanlou (The Rockefeller University, New York, NY). SMAD4 expression vector was a gift from Dr. Colin Clay (Colorado State University, Fort Collins, CO). The human *DLX4* cDNA was obtained by PCR from RNA isolated from JEG3 cells using the following primers: 5'-TCAGGAATTCAAATGACCTC TTTACCCTGTCCC-3' and 5-GTACATCGA TCACATCATCTGAGGCAG TGC-3'. The resulting *DLX4* cDNA was cloned into pKH3 and verified by nucleotide sequence analysis. *Esx1*-2.3kb promoter was obtained by PCR using mouse genomic DNA and the following primers: 5' primer (5'-GGTACCAGCACCGAGCTATCACAACCATCA-3') and 3' primer (5'-GCTAGCTACCAGCTGCTTCTCCCGTA-3'). To facilitate cloning, Kpn I and Nhe I restriction enzyme sites were engineered at the end of the 5' primer and 3' primer respectively. The PCR products were cloned into pGEM T-Easy vector. After Kpn I and Nhe I digestion, the promoter fragment was subcloned into a luciferase reporter vector. The fidelity of the construct was confirmed by nucleotide sequence analysis. PCR-based site-directed mutagenesis was used to disrupt the distal DLX3 binding site within the *Esx1* luciferase reporter. This mutation substituted a Not I restriction site for the near consensus DLX3 binding site. The mutation was confirmed using nucleotide sequence analysis. The human α subunit gene promoter luciferase reporter has been reported previously [14].

Yeast two-hybrid screen

To investigate novel protein-protein interactions, full-length DLX3 served as the bait protein with a human term placental cDNA library serving as the target. The bait, human *DLX3* cDNA was cloned into the vector pGBKT7 and transformed in the yeast strain AH109. A pretransformed human term placental Matchmaker cDNA library was in yeast strain Y187 (BD Biosciences/Clontech, Palo Alto, CA). The bait and library

plasmids were expressed as GAL4 fusion proteins. 3-amino-1,2,4-triazole was titrated (5-35 mM) using the bait strain to control background yeast growth. A concentration of 12 mM 3- amino-1,2,4-triazole was used in the library screen. The bait and library strains were mated with an efficiency of approximately 4%. The bait strain required Leu^- Synthetic Dropout (SD) minimal medium, and the library strain required Trp^- SD minimal medium. Mating was carried out in YPDA media containing 0.003% adenine hemisulfate. Following mating of the bait strain with the human placental library, yeast was initially plated on medium stringency SD media ($His^-/Leu^-/Trp^-$) plates. When colonies were of sufficient size, colonies were replicate plated on high stringency SD medium ($Ade^-/His^-/Leu^-/Trp^-$/X-α-Gal) plates to verify that they maintained the correct phenotype. A colony filter lift assay was performed to access β-galactosidase activity to identify and rank the strength of potential interactions. Once identified, yeast plasmids were isolated using disruption with glass beads and plasmids rescued/purified using the Qiagen Miniprep reagents and spin column (Qiagen Inc., Valencia, CA). Identity of the rescued plasmids was verified by nucleotide sequence analysis. The interaction between DLX3 and target genes was examined using a reconstitution assay where both plasmids were co-transformed into the AH109 yeast strain and plated on high stringency SD medium.

Preparation of JEG3 cell nuclear extracts

Subconfluent JEG3 cells were used for the preparation of nuclear extracts. Cells were washed twice with ice-cold Dulbecco's Phosphate Buffered Saline (PBS; Invitrogen Corp., Carlsbad, CA). Cells were collected by scraping in ice-cold PBS supplemented with 1:1000 dilution of protease inhibitor cocktail (referred to as protease inhibitor cocktail; Sigma-Aldrich Corp., St. Louis, MO), 5 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Cells were pelleted by

centrifugation and resuspended in a hypotonic buffer consisting of 120 mM potassium chloride, 30 mM sodium chloride, 30 mM Hepes (pH 8.0), 0.3 M sucrose, protease inhibitor cocktail, 5 mM benzamidine, and 0.2 mM PMSF and allowed to swell for 15 min on ice. Cells were lysed by douncing and nuclei were isolated by layering the broken cell lysate over a sucrose cushion (0.9 M sucrose) followed by centrifugation at 2000×g for 30 min at 4˚C. The nuclear pellet was resuspended in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 1 mM ethylenediamine tetraacetic acid (EDTA), protease inhibitor cocktail, 5 mM benzamidine, and 0.2 mM PMSF. Additional sodium chloride was added to a final concentration of 450 mM and nuclear proteins were extracted with constant rocking at 4˚C for 30 min. Nuclear extracts were clarified by centrifugation (85,000×g for 60 min) and the nuclear extract was stored in aliquots at –80˚C until later use. Protein concentrations of the nuclear extracts were determined by Bradford assay.

Immunoprecipitation (IP) from JEG3 nuclear extracts and Western blotting analysis

JEG3 cell nuclear extracts (200µg) were suspended in 1ml of 0.1% Triton X buffer (50 mM Tris (pH 7.6), 50 mM sodium chloride, 0.1% Triton X, protease inhibitor cocktail, 5 mM benzamidine, and 0.2 mM PMSF). To pre-clear the nuclear extracts, protein A/G-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to each suspension and allowed to mix for 1 h at 4˚C with gentle rocking. Following centrifugation (1200×g for 1 min) to remove protein A/G-agarose, antibodies were added at the following dilutions: normal rabbit serum at 1:1000; Dlx3 antibody at 1:1000; SMAD6 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:100. Following 2 h at 4˚C of gentle rocking, protein A/G-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added and allowed to mix for an

additional 2 h. Complexes were then washed four times with 0.1% Triton X buffer. Samples were then suspended in an equal volume of 2×SDS loading buffer (100mM Tris (pH 6.8), 4% SDS, 20% glycerol and 200 mM dithiothreitol). Protein samples were boiled for 3 min and chilled for 5 min on ice. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes by electroblotting. Membranes were blocked with nonfat dried milk (5%) in Tris-buffered saline [10 mM Tris (pH 7.6), 150mM sodium chloride] containing 0.1% Tween 20 (TBST). For western blots, the DLX3 antibody was used at 1:5000 in TBST, 5% nonfat dried milk. The reciprocal western blot from IPs using the SMAD6 antibody was not possible since the IP heavy chain IgG blocked visualization of SMAD6 on western blot due to similar molecular size. In other western blot studies, the SMAD6 antibody was used at 1:500 and the actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz CA) was used at 1:1000 dilution. Proteins bands were visualized by chemiluminescence reagents (PerkinElmer Life Sciences, Wellesley, MA).

Immunocytochemistry

JEG3 cells were cultured on glass slides or coverslips for 24 h, rinsed one time with potassium phosphate buffered saline (KPBS), then fixed for 20 min with 4% paraformaldehyde. Slides or coverslips were then stored in 70% ethanol until used. Prior to use, slides were rinsed in KPBS seven times over 1 hour. JEG3 cells were incubated with primary antibody (SMAD6 at 1:100; DLX3 at 1:500) overnight at 4˚C. Slides were again rinsed with KPBS followed by incubation with a fluorescent-conjugated secondary antibody (Alexa 594, Molecular Probes, Eugene OR; and Cy2, Jackson Immunoresearch Labs, Westgrove, PA) in KPBS-Triton X at 37˚C for 2 h. Cells were rinsed in KPBS, dehydrated through a graded series of ethanol, cleared

with xylene and coverslips attached with Krystalon (EM Science, Gibbstown NJ).

Samples of human term placenta (derived from elective caesarian section) were obtained from Cayuga Medical Center, Ithaca, NY under the guidelines and approval of the Cornell University and the Cayuga Medical Center Committees on the Use of Human Subjects in Research. Samples were collected, fixed with 4% paraformaldehyde for 48 h and transferred to 70% ethanol until processing. Tissues were paraffin embedded and 5 μm sections were obtained. Immunocytochemistry was performed as previously described [14], except that fluorescent-conjugated secondary antibodies were used as described above.

Recombinant proteins and immuno-precipitation analysis

Recombinant SMAD6, SMAD4, DLX4, DLX3 and deletions of DLX3 were prepared using a coupled transcription and translation Wheat Germ Extract System (Promega Corp., Madison, WI) following the prescribed protocol. Proteins were radioactively labeled using ^{35}S methionine (1,000Ci/mmol at 10mCi/ml; Amersham, Piscataway, NJ). A portion (10%) of each recombinant protein was saved for input analysis. Protein combinations were added at a 1:1 (by volume) mixture to a 0.1% Triton X buffer along with appropriate antibody at specified concentrations (DLX3 at 1:1000; SMAD6, Santa Cruz Biotechnology Inc., Santa Cruz, CA at 1:100; and SMAD4, Santa Cruz Biotechnology Inc., Santa Cruz, CA at 1:500). Following 2 h of gentle rocking at 4˚C, protein A/G-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added and allowed to mix for an additional 2 h. Complexes were then washed four times with 0.1%Triton X buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, the gel was fixed in 25% methanol, and 15 % glacial acetic acid for 1 h with gentle rocking at room temperature. The gel was then washed three times in 40% isopropanol solution, dried and bands visualized by

autoradiography.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were carried out as described previously [14,18] using the indicated antibodies. Reactions (without probe) were maintained at room temperature for 30 min followed by addition of ^{32}P-labeled oligonucleotide DLX3 binding site probes (the junctional regulatory element or JRE from the glycoprotein hormone α subunit promoter [14] and two putative DLX3 binding sites identified within the *Esx1* promoter). The binding reactions were maintained an additional 30 min then resolved on native polyacrylamide gels. To determine if SMAD6 could displace prebound DLX3 in EMSA, binding reactions containing DLX3 alone were allowed to incubate with probe for 30 min to reach equilibrium. SMAD6 protein was then added and the reactions were incubated for an additional 30 min. These reactions were compared to binding reactions where both DLX3 and SMAD6 were added together and incubated for the full 60 min. Following electrophoresis, the gels were dried and DNA-protein complexes were visualized by autoradiography. All DNA binding studies were conducted at least three times with similar results. The nucleotide sequences for probes were as follows (only one strand indicated): JRE: 5'-ACGTCATGGTAATTACACCAAG-3' Distal binding site: 5'-ACA AGGAGCTAATTTACTTCCT-3' Proximal binding site: 5'-TTAGGGCTCTAATTCAGACTCT-3'.

Cell culture and transient transfection studies

JEG3 cells were cultured in monolayer using Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Before transfection studies, cells were split to 35 mm dishes and sub-confluent cultures were

used. JEG3 cells were transiently co-transfected with an *Esx1* promoter luciferase reporter construct, DLX3 expression vector at a constant dose (2.0 μg) and increasing doses of SMAD6 expression vector using lipofection (FuGENE 6 transfection reagent; Roche Diagnostics Inc., Indianapolis, IN). All transfections were carried out with a constant amount of DNA by supplementing reactions with the parent vector pKH3. Luciferase activity was determined after 24 h of transfection using reagents from the Luciferase Reporter Assay System (Promega Corp., Madison, WI), and luciferase activity was standardized by total cell protein amount (luciferase/1.0 μg) as determined by Bradford assay. All transfection studies were conducted in triplicate on at least three separate occasions with similar results. Data are shown as means (n=3) ± standard errors of the mean of a representative experiment.

Preparation of stable cell lines expressing small interference RNA (siRNA)

The mammalian expression vector, pSUPER-retro-neo (pSR; OligoEngine, Seattle, WA) was used for preparation of retrovirus containing specific siRNAs and expression of siRNAs in JEG3 cells following viral infection. Each gene-specific insert targeted a 19-nucleotide sequence within the human *SMAD6* mRNA. The siRNA sequences were as follows: *SMAD6*-#1: 5'CACATTGTCTTACACTGAA *SMAD6*-#2: 5'TCAAGGTGTTCGACTTCGA *SMAD6*-#3: 5'GCCACTGGATCTGTCCGAT The plasmids were referred to as *SMAD6* siRNA #1, #2 and #3. A control siRNA vector (also prepared in pSR) was constructed using a 19-nucleotide sequence (5'TTCTCCGAAC GTGTCACGT) putatively without significant similarity to any mammalian gene sequence and therefore served as an appropriate negative control (OligoEngine, Seattle, WA). The forward and reverse strands of oligonucleotides containing the siRNAs and nonsense sequence also contained Bgl II and Hind III sites at the 5'end of the forward and reverse

oligonucleotides, respectively. The oligonucleotides were annealed and inserted into the pSR vector after digestion of the vector with Bgl II and Hind III. These siRNAs were a self-contained hairpin loop for the double stranded siRNA. All siRNA sequences were confirmed by nucleotide sequencing.

Cell culture, transfection and retroviral infection of siRNAs

HEK293 Phoenix Ampho packaging cells (American Type Culture Collection; Manassa, VA) were cultured in DMEM containing 10% FBS and were transfected with the pSUPER-retro-neo siRNA plasmids using FuGENE 6 transfection reagent (Roche Diagnostics Inc., Indianapolis IN). Forty-eight hours following transfection, the culture media containing the retrovirus for individual siRNAs and control siRNA were filtered through a 0.45-μm filter, and the viral supernatant was used for infection of JEG3 cells in the presence of 8 μg/ml polybrene. JEG3 cells were exposed to the retrovirus overnight, cell were then washed with fresh medium and allowed to recover for 24 h. Following infection, stable cell lines were selected using neomycin at 500 μg/ml for 7 days (time until untransfected cells all died) then cultures were maintained in medium containing 500 μg/ml neomycin. Transient transfections of the siRNA cell lines were carried out as described above.

Statistical analysis

Luciferase data were subjected to analysis of variance and differences between treatments were determined using Tukey's Studentized Range Test. Probability of less than 0.05 ($p<0.05$) was considered statistically significant.

Results

DLX3 and SMAD6 functionally interact in a yeast two-hybrid screen

Full-length human DLX3 was used as a bait protein in a yeast two-hybrid screen of a human term placental library. The screen included coverage of approximately 3.4×10^6 independent clones. Once colonies were identified from the original screen and library plasmids were rescued, plasmids were retransformed in a reconstitution assay with the bait vector into the AH109 yeast strain. The library plasmid resulting in the most robust interaction (as measured by β-galactosidase activity) with *DLX3* was a cDNA containing the entire coding region of *SMAD6*. Transformation of the *SMAD6* library vector alone did not support yeast growth on high stringency SD medium (Ade^-/His^-/Leu^-/Trp^-/X-α-Gal) plates in the presence of 12 mM 3-amino-1,2,4-triazole. Transformation of Dlx3 bait vector minimally supported yeast growth under the same conditions. Co-transformation of *DLX3* bait and *SMAD6* library plasmid resulted in rescue of yeast growth (Figure 2.1A).

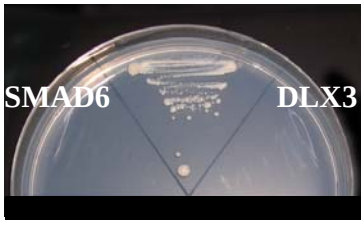
DLX3 and SMAD6 are co-localized in the nucleus in choriocarcinoma cells and in human placental trophoblasts

Initially, studies focused on examining localization of DLX3 and SMAD6 proteins in cells of trophoblast origin. DLX3 and SMAD6 were expressed and localized primarily in the nuclear compartment in JEG3 cells (Figure 2.1B), a choriocarcinoma cell line of trophoblast origin. Consistent with this observation, DLX3 and SMAD6 were nuclear localized to both cytotrophoblast and syncytial trophoblast within microvilli of fully differentiated term human placenta. Localization of DLX3 in term placenta provided additional insight into the expression pattern of DLX3 during gestation in primates. We have previously shown DLX3 in placental trophoblasts in human placenta obtained at 8 weeks gestation during peak production

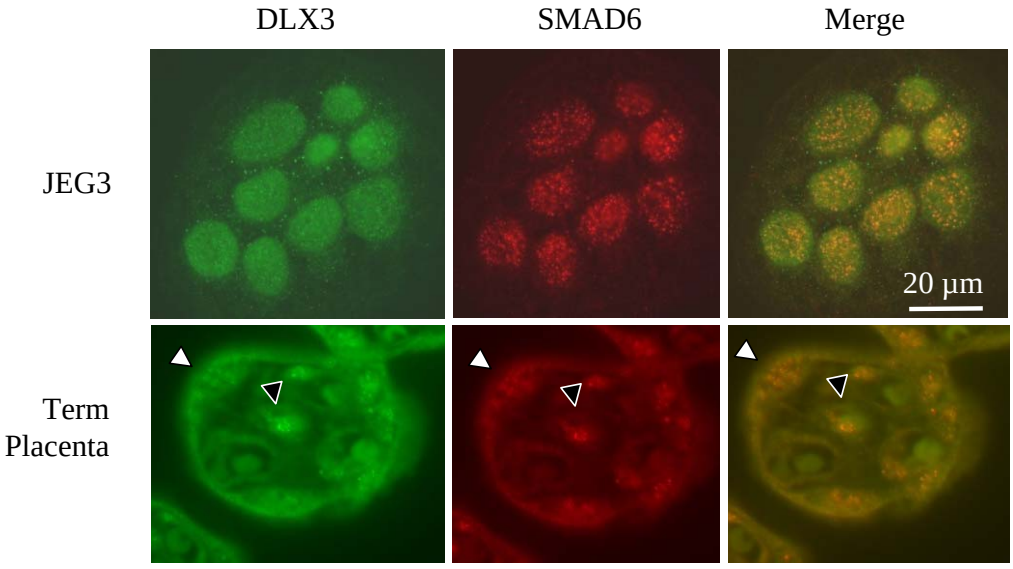
Figure 2.1. DLX3 and SMAD6 interact in yeast two hybrid and co-localize in placental trophoblasts. A. Yeast two-hybrid screen of a human term placental library was carried out using full length DLX3 as bait. Following plasmid rescue, Gal4 DNA binding domain-*DLX3* bait was co-transformed along with Gal4 activation domain-*SMAD6* into the AH109 yeast strain and plated on ($^{-}$His, $^{-}$Leu, $^{-}$Trp) media as described in Methods. **B.** JEG3 cells were plated on glass slides and fixed as described in Materials and Methods. Double labeled immunocytochemistry was used to determine localization of DLX3 (green) or SMAD6 (red). The merged images demonstrate overlapping expression patterns. Human term placenta was obtained, fixed and sectioned as described in Materials and Methods. Similar double labeled immunocytochemistry was used to localize DLX3 and SMAD6. The white arrow head identifies nuclei in syncytial trophoblasts while the black arrow head identifies nuclei in cytotrophoblasts. Bar = 20 μm.
This figure is kindly provided by Dr. Kathie A. Berghorn.

A

SMAD6 + DLX3



B



of hCG [14]. The current studies support the conclusion that DLX3 expression may be maintained within trophoblast cell populations until term in the human placenta. Since both DLX3 and SMAD6 were expressed endogenously in the JEG3 horiocarcinoma cell model, we sought to use this model for subsequent analyses. We have used JEG3 cells previously for molecular analysis of gene regulatory processes related to glycoprotein hormone and *DLX3* gene promoter expression [14,18,19]. IP studies using nuclear extracts from JEG3 cells and SMAD6 antibody revealed that DLX3 and SMAD6 interact in mammalian cells (Figure 2.2A). The reciprocal study using DLX3 antibody to IP SMAD6 was not possible due to the molecular size of SMAD6 and interference with the IgG heavy chain used in the IP studies. However, this constraint was overcome with preparation of DLX3 and SMAD6 as recombinant proteins labeled with [^{35}S] methionine. IP of recombinant DLX3 and SMAD6 with the DLX3 antibody revealed specific association with SMAD6 but not Smad4 (Figure 2.2B). Thus, the original interaction defined in the yeast system was supported by IP studies in choriocarcinoma cells that endogenously express these two proteins and *in vitro* using recombinant DLX3 and SMAD6 proteins.

Structure/function analysis of the DLX3/SMAD6 interaction interface

To further understand the mechanism of the DLX3/SMAD6 interaction, we constructed a series of deletion mutants of DLX3 (Figure 2.3A). The rationale for these mutations was predicated on existing understanding of important domains within DLX3 defined by others [20,21], centering upon the homeodomain (residues 130-189; Figure 2.3A), the centrally located DNA binding domain. DLX3 deletion mutants were prepared *in vitro* along with full-length DLX3 and SMAD6. SMAD6 IP was then used to determine the domains sufficient to support interaction with DLX3. Initially, we identified the importance of DLX3-(1-202), which bound SMAD6 at levels similar

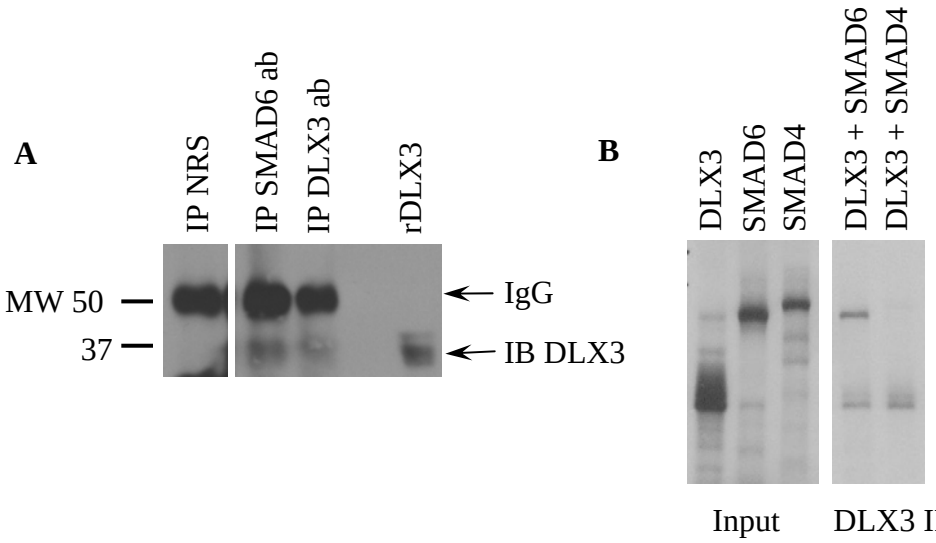
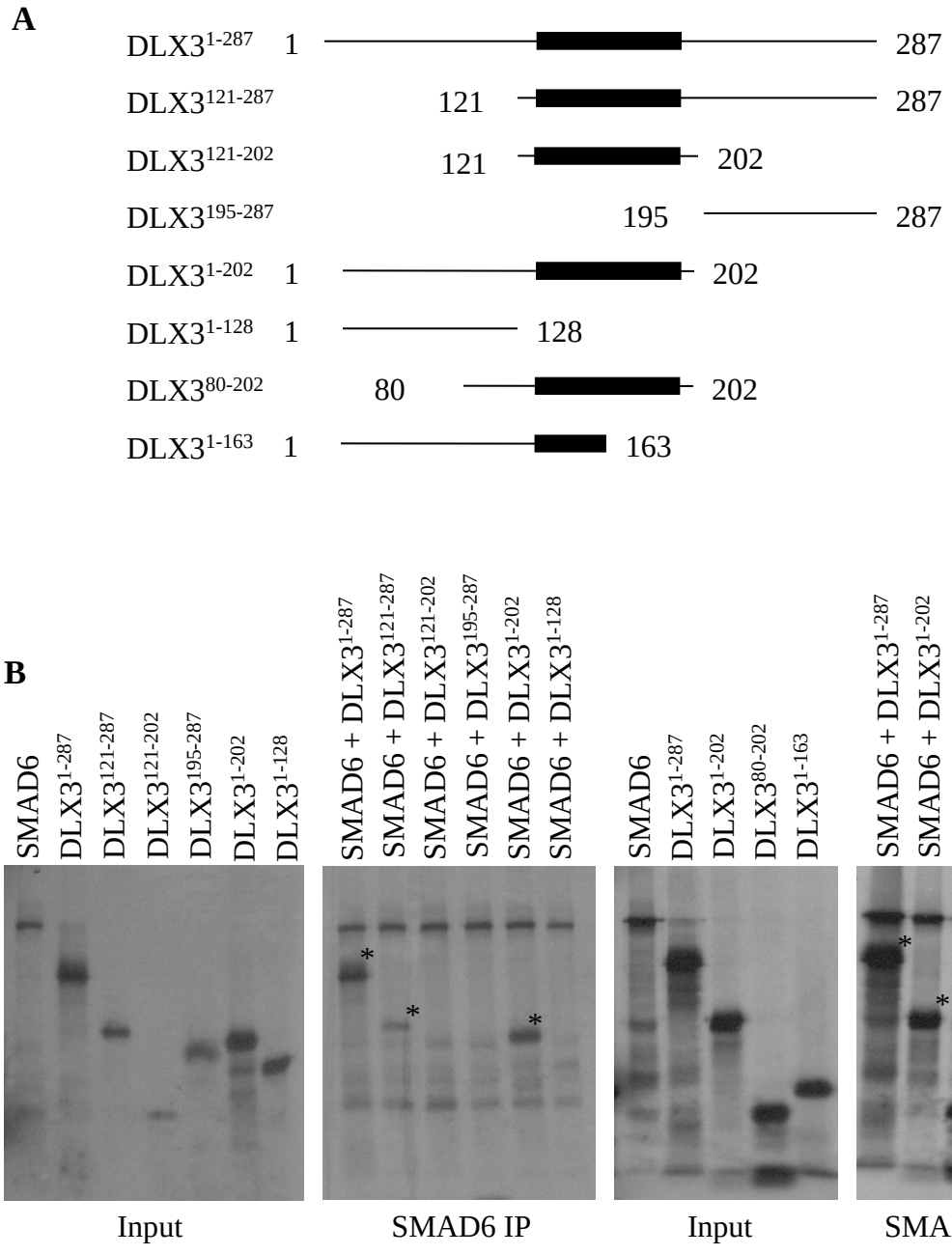


Figure 2.2. DLX3 and SMAD6 interact in the JEG3 choriocarcinoma cell model. A. JEG3 cell nuclear extracts were used in immunoprecipitation (IP) studies. IPs included the use of normal rabbit serum (NRS) or antisera (ab) directed against DLX3 or SMAD6. Recombinant DLX3 (rDLX3; without HA epitope tag) and IPs were resolved by SDS-PAGE and Western blots for DLX3 were carried out (IB DLX3). Molecular size standards (MW) are depicted to the left of the panel. **B.** Recombinant DLX3, SMAD6 and SMAD4 were produced using wheat-germ lysates in a coupled transcription/translation reaction containing ^{35}S methionine (Input). DLX3 and either SMAD6 or SMAD4 were combined followed by IP with the DLX3 antibody (DLX3 IP). The input and IPs were resolved by SDS-PAGE, the gels fixed and dried and autoradiography was used to visualize bands.

This figure is kindly provided by Ms. Patricia A.Clark.

Figure 2.3. Deletion mutants of DLX3 reveal interaction interface with SMAD6. **A.** A series of DLX3 deletions (characterized in schematic form) were prepared using PCR. The numbers represent amino acid designations for the termini of wild type DLX3 and each mutant. **B.** Wild type DLX3, SMAD6 and the DLX3 deletion series were prepared as recombinant proteins as described in Figure 2B (Input) and subjected to IP studies using the SMAD6 antibody (SMAD6 IP) and recombinant SMAD6 and the combination of deletions mutants indicated. The input and IPs were resolved by SDS-PAGE, gels were fixed and bands visualized by autoradiography. Asterisks (*) designate bands consistent with protein-protein interactions.
This figure is kindly provided by Ms. Patricia A.Clark.


A
DLX3 1-287 1 287
DLX3 121-287 121 287
DLX3 121-202 121 202
DLX3 195-287 195 287
DLX3 1-202 1 202
DLX3 1-128 1 128
DLX3 80-202 80 202
DLX3 1-163 1 163
B
SMAD6
DLX3 1-287
DLX3 121-287
DLX3 121-202
DLX3 195-287
DLX3 1-202
DLX3 1-128
SMAD6 + DLX3 1-287
SMAD6 + DLX3 121-287
SMAD6 + DLX3 121-202
SMAD6 + DLX3 195-287
SMAD6 + DLX3 1-202
SMAD6 + DLX3 1-128
SMAD6
DLX3 1-287
DLX3 1-202
DLX3 80-202
DLX3 1-163
SMAD6 + DLX3 1-287
SMAD6 + DLX3 1-202
SMAD6 + DLX3 80-202
SMAD6 + DLX3 1-163
Input
SMAD6 IP
Input
SMAD6 IP

to DLX3-(1-287) (Figure 2.3B). DLX3-(121-287) bound SMAD6 as well, suggesting that SMAD6 interaction domain required at least a portion of the homeodomain (Figure 2.3B). Based upon these results, we predicted that DLX3-(121-202) mutant would also bind SMAD6. This was not readily interpretable in our studies since putative degradation fragments/products of these recombinant deletion mutants were of similar molecular size as the DLX3-(121-287) mutant. Subsequent DLX3 deletion mutants defined a region of DLX3-(80-163) that was sufficient to bind SMAD6 *in vitro* (Figure 2.3B). These studies again supported the conclusion that at least the amino-terminal portion of the homeodomain was sufficient for interaction with SMAD6 *in vitro*. Alignment of DLX3 with DLX4 (another member of the DLX family expressed in placenta [22]) revealed approximately 37% amino acid conservation within this region (residues 80-163; Figure 2.4A), with higher levels of homology particularly clustered within the homeodomain, supporting the prediction that DLX4 may also bind SMAD6. Based upon *in vitro* studies, recombinant full-length DLX4 bound SMAD6 similar to DLX3-(1-287) (Figure 2.4B). DLX3-(195-287), previously shown not to bind SMAD6 (Figure 2.3B), was used in these studies as a negative control.

DLX3 binds to the 5'-flanking sequence of the Esx1 promoter

To begin to examine the functional significance of the DLX3/SMAD6 interaction on gene transcription, we cloned 2.3 kilobases of the 5'-flanking sequence of the Esx1 promoter. The report of the *Dlx3* null mouse provided evidence that the loss of *Dlx3 in vivo* was correlated with a loss of *Esx1* mRNA in mouse placenta [13]. Taking advantage of this observation, we identified two near consensus Dlx3 binding sites within the 2.3-kb promoter fragment. The distal site was located at -2135 and the more proximal site at -585 relative to the transcription start site of the *Esx1* promoter, as previously

Figure 2.4. DLX4 binds SMAD6. A. Alignment of DLX3 and DLX4 between residue 80 and 198 reveals regions of conserved sequence within the SMAD6 interaction domain (residue 80-163). The consensus amino acid sequence is listed in bold below the alignment. **B.** Wild type $DLX3^{1-287}$, $DLX3^{195-287}$, SMAD6 and DLX4 were prepared as recombinant proteins as described in Figure 2 (Input) and subjected to IP studies using the SMAD6 antibody (Smad6 IP) and recombinant SMAD6 and combinations of DLX3 or DLX4 as indicated. The input and IPs were resolved by SDS-PAGE, gels were fixed and bands visualized by autoradiography. Asterisks (*) designate bands consistent with protein-protein interactions.
This figure is kindly provided by Ms. Patricia A.Clark.

A

```
        80
DLX3      YSPKSEYTYGGSYRQYGAYREQPLPAQDPVSVKEEPEAEVRMVNGKPKKVRKPRTIYSSY
DLX4      SYLPRQQQLVAPSQPFHRPAEHPQ—ELEAESEKLALS LV PSQQQSLTRKLRKPRTIYSSL
consensus                      e p         s k                  k  rk p r ti yss

                                    163                                   198
DLX3      QLAA LQRRFQKAQYLALPERAELAAQLGLTQTQVKIWFQNRRSKFKKLYKNGE- VPLEHS
DLX4      QLQHLDQRFQHTQYLALPERAQLAAQLGLTQTQVKIWFQNKRSKYKKLLKQSSGEPEEDF
consensus ql   l   r f q  qyl a l pe ra  laa q l g l  tqtq v k l wfq n  rsk  kkl  k     p e
```

B

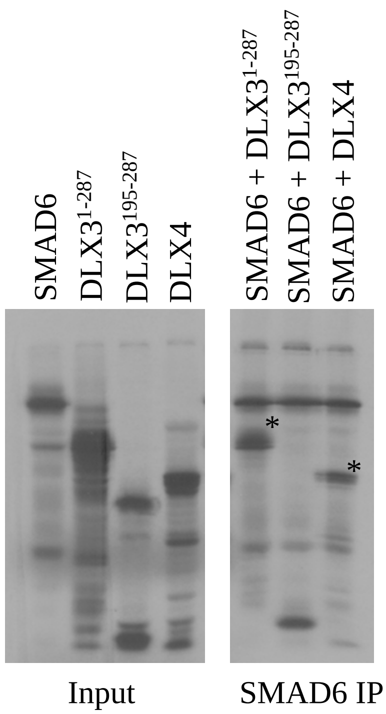
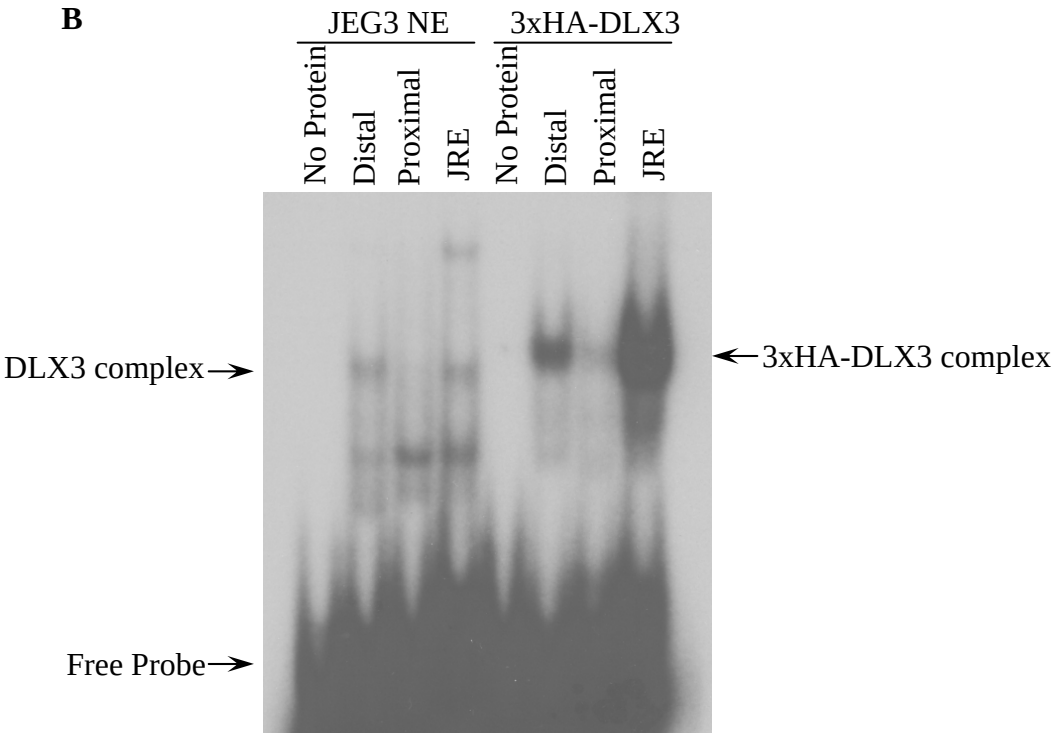


Figure 2.5. Two putative Dlx3 binding sites are present within the *Esx1* promoter. **A.** The junctional regulatory element (JRE), a consensus Dlx3 binding site from the glycoprotein hormone α subunit promoter was compared to the distal (position -2135) and proximal (position -585) binding sites from the *Esx1* promoter. The central core of the binding site is underlined. **B.** Electrophoretic mobility shift assays (EMSA) were used to compare binding complexes formed on the JRE, the distal and proximal Dlx3 binding sites within the *Esx1* promoter using JEG3 cell nuclear extract and recombinant DLX3. Equal amounts of nuclear extracts or recombinant DLX3 were added to each binding reaction. Recombinant DLX3 in these studies was epitope tagged with three copies of the HA epitope (3xHA-DLX3). Binding reactions were resolved on native polyacrylamide gels. The gels were dried and bands visualized by autoradiography. Free probe is designated by an arrow at the bottom of the gel.

A

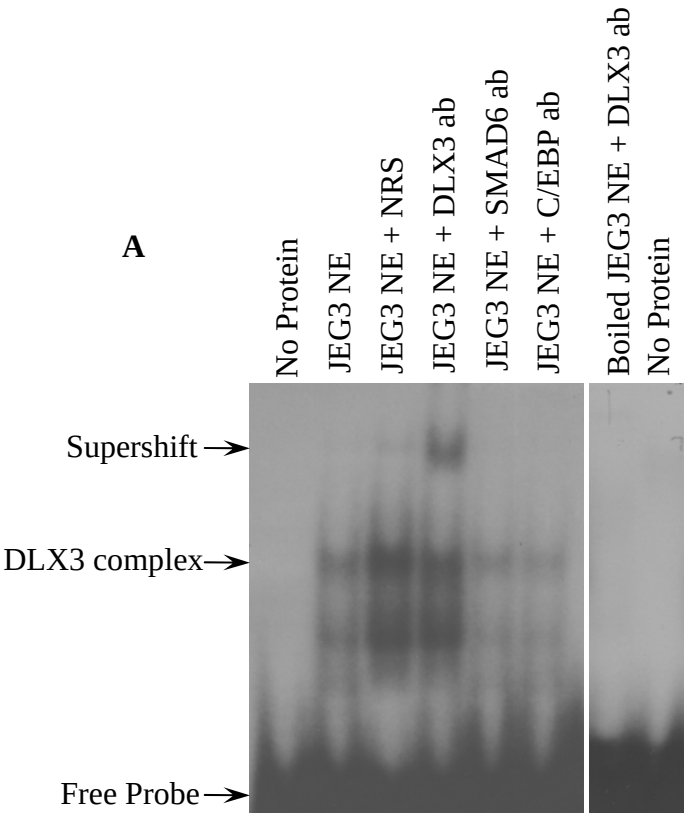
JRE ACGTCATGGTAATTACACCAAG
Distal ACAAGGAGCTAATTTACTTCCT
Proximal TTAGGGCTCTAATTCAGACTCT
Consensus (A/C/G)TAATT(G/A)(C/G)

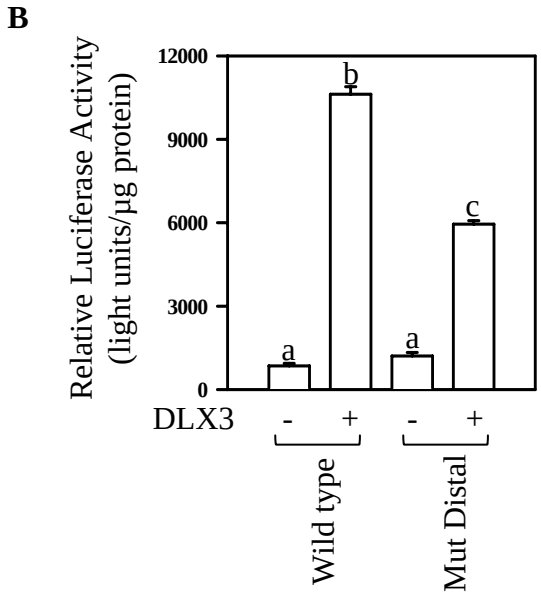
B



defined [23]. At both of these two sites, the central core of the binding site (TAATT) was conserved; however, the two nucleotides present on the 3' termini of this central core were not conserved (Figure 2.5A). These nucleotides varied from the consensus Dlx3 binding site [24] we defined in the human glycoprotein hormone α subunit promoter [14]. Using EMSAs, we compared the DNA binding complexes formed when using the distal (-2135) and proximal (-585) putative Dlx3 binding sites from the *Esx1* promoter and nuclear extracts from JEG3 cells (Figure 2.5B). As a positive control, the JRE from the α subunit promoter was used. Figure 2.5B depicts direct comparison of complexes formed with all three of these binding sites. The distal site was more similar to the JRE binding complex electrophoretic mobility when compared to the proximal site. When using recombinant DLX3 in EMSA (Figure 2.5B), all three sites were sufficient to bind DLX3; however, clear differences in the relative binding of DLX3 for these binding sites were revealed. The recombinant DLX3 was slightly larger in molecular size due to the presence of the three hemagglutinin (HA) epitopes engineered into the recombinant protein. The rank order of relative binding for recombinant DLX3 was the JRE (highest) > the distal site > the proximal site (lowest). We focused our attention on the distal DLX3 binding site within the *Esx1* gene promoter. EMSA studies using nuclear extracts and antiserum against DLX3 provided direct evidence that endogenous DLX3 can bind to this site (Figure 2.6A). The addition of the DLX3 antibody to EMSA binding reaction resulted in supershifted complexes that were not evident using preimmune normal rabbit serum (NRS). The addition of the SMAD6 or the C/EBP antiserum to these binding reactions did not appreciably alter complex formation in EMSA, suggesting that SMAD6 may not directly participate in DNA binding with DLX3 and that DLX3 antibody interactions were specific. To determine the importance of this site to DLX3-induced *Esx1* gene transcription, the distal site was mutagenized to a Not I restriction site and examined

Figure 2.6. The distal binding site of the *Esx1* promoter binds DLX3 and is necessary for full DLX3-induced gene transcription. A. Using only the distal binding site for DLX3 and JEG3 cell nuclear extract (JEG3 NE), studies examined if a DLX3 antibody would disrupt binding of endogenous proteins including DLX3 to this site. Binding reactions were resolved on native polyacrylamide gels, the gels were dried and bands visualized by autoradiography. Addition of a DLX3 antibody (DLX3 ab) to binding reactions resulted in the formation of a super shifted complex as indicated (supershift). Normal rabbit serum was used as a control for the DLX3 antibody. In some binding reactions, the JEG3 NE was boiled and then added to reactions containing the DLX3 ab as a negative control. Free probe is designated by an arrow at the bottom of the gel. **B.** Transient transfection studies were used to determine the relative importance of the distal DLX3 binding site on expression of the *Esx1* promoter luciferase reporter gene. JEG3 cells were transiently transfected with either wild type *Esx1* luciferase reporter or an *Esx1* luciferase reporter containing a mutation within the distal DLX3 binding site. Cells were cotransfected with control plasmid or a DLX3 expression vector (2 μg; designated – or +). Data are reported as relative luciferase activity standardized for protein for a representative study (n=3/treatment). The designations a, b and c indicate significant differences ($p<0.05$).


A
No Protein
JEG3 NE
JEG3 NE + NRS
JEG3 NE + DLX3 ab
JEG3 NE + SMAD6 ab
JEG3 NE + C/EBP ab
Boiled JEG3 NE + DLX3 ab
No Protein
Supershift
DLX3 complex
Free Probe

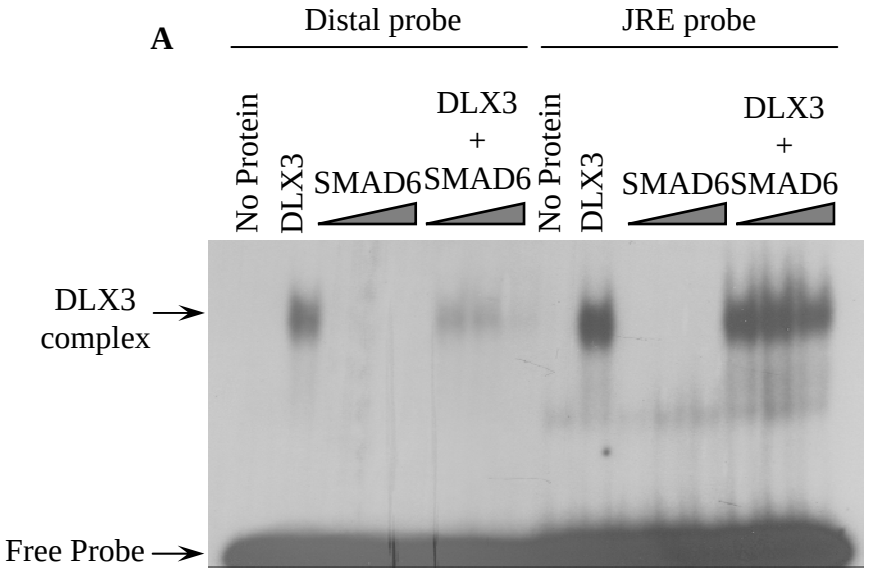

B
Relative Luciferase Activity
(light units/µg protein)
12000
9000
6000
3000
0
a
b
a
c
DLX3
-
+
-
+
Wild type
Mut Distal

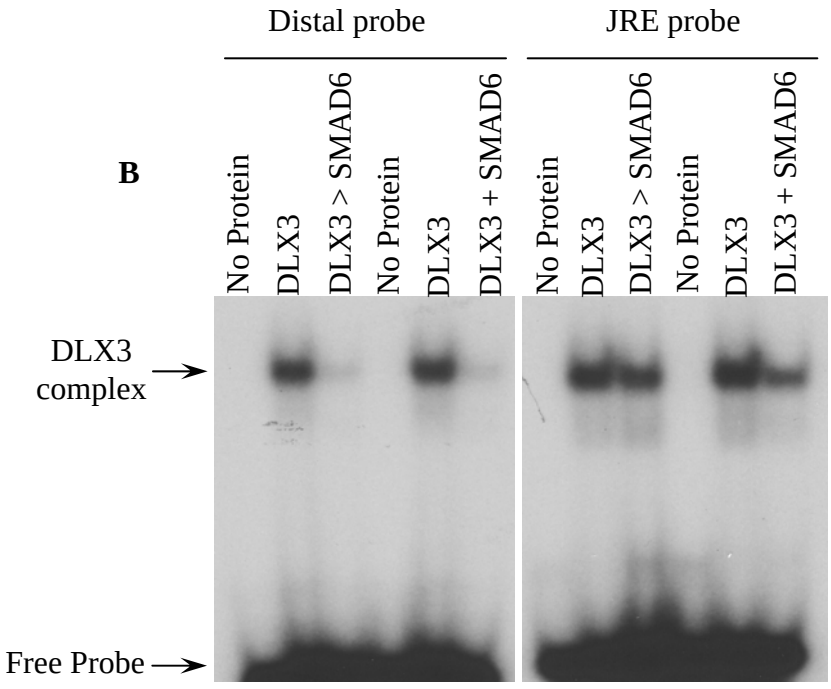
using a luciferase reporter gene approach in transient transfection studies (Figure 2.6B). The wild type *Esx1* promoter was strongly induced by DLX3. The mutation in the distal putative DLX3 binding site reduced DLX3-induced *Esx1* expression by >50% ($p<0.05$), suggesting that this site was important for promoter activity induced by DLX3.

We then sought to determine the impact of SMAD6 on DLX3 DNA binding. Reconstitution EMSAs using recombinant DLX3 and SMAD6 demonstrated that the DLX3/SMAD6 interaction effectively reduced/ blocked association of DLX3 with the distal DLX3 binding site within the *Esx1* promoter (Figure 2.7A). In this experiment, SMAD6 alone did not form a complex with the distal DLX3 binding site. When SMAD6 was titrated into the binding reactions containing DLX3, the DLX3 binding complex was diminished in a dose-dependent manner. Using the JRE as probe, a similar titration of SMAD6 protein reduced DLX3 binding, albeit to a lesser extent compared to the distal site of the *Esx1* promoter. Studies then focused on determining if prebound DLX3 could be displaced by SMAD6 in EMSA binding reactions (Figure 2.7B). Binding reactions compared addition of DLX3 concurrent with SMAD6 and reactions where DLX3 binding was allowed to reach equilibrium then SMAD6 was added. These studies revealed that for the distal probe, SMAD6 competed for DLX3 binding regardless of order of protein addition. Similar levels of competition were not observed using the JRE (stronger relative binding), suggesting that SMAD6 can compete for DLX3 binding particularly on gene targets that have relatively weaker DLX3 binding sites, like those characterized within the *Esx1* gene promoter.

To assess the functional consequences of the DLX3/SMAD6 interaction on the *Esx1* promoter, we again used the *Esx1* promoter-luciferase reporter construct (Figure 2.8). Based upon the binding studies described above, our prediction was that SMAD6 overexpression would probably repress expression of the *Esx1* promoter induced by

Figure 2.7. SMAD6 attenuates DLX3 DNA binding. A. To examine the effects of SMAD6 on DLX3 binding, recombinant DLX3 and SMAD6 were prepared *in vitro* and used in electrophoretic mobility shift assays (EMSA) with the distal DLX3 binding site within the *Esx1* promoter and the JRE. As observed earlier, DLX3 bound this site while increasing doses of SMAD6 (as represented by the grey triangle) did not bind. Binding reactions containing a constant dose of DLX3 and increasing doses of SMAD6 resulted in a dose dependent reduction in DLX3 binding for the distal site and to a lesser extent for the JRE. **B.** EMSA studies were carried out to determine if order of protein addition had an impact on the ability of SMAD6 to interfere with DLX3 binding. Using the distal and JRE probes, binding reactions were assembled where DLX3 was added and allowed to reach equilibrium (DLX3) then SMAD6 was added (DLX3 > SMAD6). This was compared to binding reactions where DLX3 and SMAD6 were added concurrently (DLX3 + SMAD6). For both A. and B., free probe and DLX3 complexes are designated by arrows.


A
Distal probe
JRE probe
No Protein
DLX3
SMAD6
DLX3 + SMAD6
No Protein
DLX3
SMAD6
DLX3 + SMAD6
DLX3 complex
Free Probe


B
Distal probe
JRE probe
No Protein
DLX3
DLX3 > SMAD6
No Protein
DLX3
DLX3 + SMAD6
No Protein
DLX3
DLX3 > SMAD6
No Protein
DLX3
DLX3 + SMAD6
DLX3 complex
Free Probe

DLX3. Using transient transfection in JEG3 cells, overexpression of DLX3 and SMAD6 resulted in increased levels of ectopically expressed DLX3 and SMAD6 (Figure 2.8A). Further, DLX3 overexpression increased *Esx1* luciferase activity ($p<0.05$; Figure 2.8B). Titration of SMAD6 into this system resulted in reduced basal activity of the Esx1 reporter gene ($p<0.05$). Consistent with our prediction, o-transfection with increasing doses of SMAD6 expression vector along with DLX3 resulted in a marked inhibition ($p<0.05$) of DLX3-induced *Esx1* promoter activity. DLX3-induced activation of the α subunit gene promoter was also reduced by overexpression of SMAD6 (data not shown). In an effort to determine the specificity of SMAD6 action on gene transcription in general, we examined the effects of SMAD6 on the trophoblast Kunitz domain protein (TKDP) 1 (Figure 2.8C) and Rous sarcoma virus promoters (data not shown). *TKDP1* promoter expression [25,26] is trophoblast-specific but not dependent upon DLX3 expression (Figure 2.8C). The Rous sarcoma virus promoter is constitutively active in trophoblast cells. In contrast to the effects of SMAD6 on DLX3-induced expression of the *Esx1* and α subunit promoter, SMAD6 had no appreciable effect on the *TKDP* promoter and actually increased expression of the Rous sarcoma virus promoter in a dose-dependent manner. These studies support the conclusion that SMAD6 does not confer global non-specific transcriptional repression and probably the effects of SMAD6 on *Esx1* and α subunit promoter activity are specific.

siRNA-mediated knockdown of SMAD6 resulted in increased expression of the Esx1 gene promoter.

Whereas overexpression studies can be informative, interpretation of such studies must be cautious. To examine the effects of SMAD6 on DLX3-dependent gene expression, we developed stable cell lines expressing specific *SMAD6* siRNA hairpin

Figure 2.8. SMAD6 attenuates DLX3-dependent promoter activation in JEG3 cells. A. To determine the levels of ectopic expression of DLX3 and SMAD6 in overexpression studies, JEG3 cells were transiently transfected with expression vectors for DLX3 (+DLX3; 2 μg) or SMAD6 (+SMAD6; 2 μg). Whole cell lysates were prepared and western blot analyses were carried out using the DLX3 and SMAD6 antibodies. **B.** Transient transfection studies in JEG3 cells were used to investigate the functional relevance of SMAD6 on DLX3-dependent gene expression. Co-transfection of the *Esx1* gene promoter-luciferase reporter with DLX3 (constant dose, 2 μg) and increasing doses of SMAD6 expression vector (0, 0.5, 1.0 and 2.0 μg; represented by the grey triangle) resulted in a dose dependent decrease in expression of the *Esx1* luciferase reporter. **C.** Similar studies were carried out using the *TKDP-1* gene promoter luciferase reporter. As a control for DLX3 action, the *Esx1* promoter was also used. *TKDP-1*-Luc activity was unaffected by DLX3 or increasing doses of SMAD6 expression vector. Data for all panels are reported as relative luciferase activity standardized for protein from representative studies (n=3/treatment). For B. and C. The designations a, b and c indicate significant differences ($p<0.05$) within experiment; ns = not significantly different.

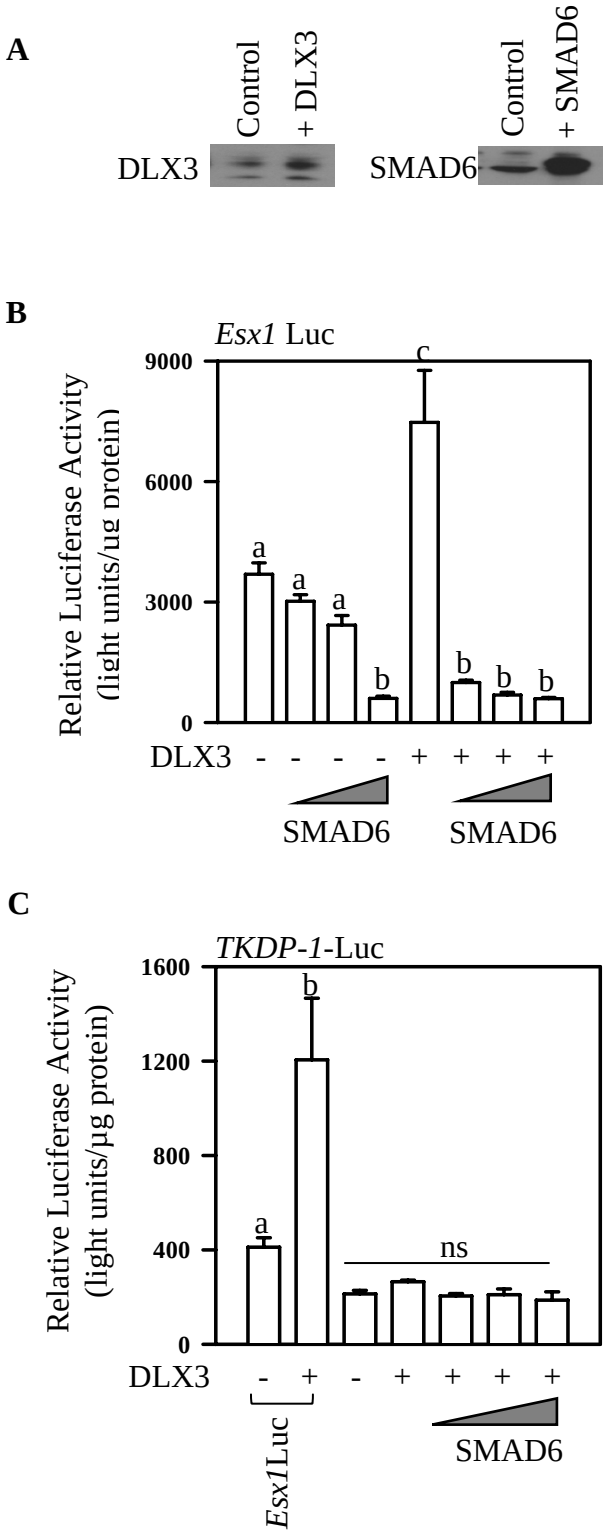
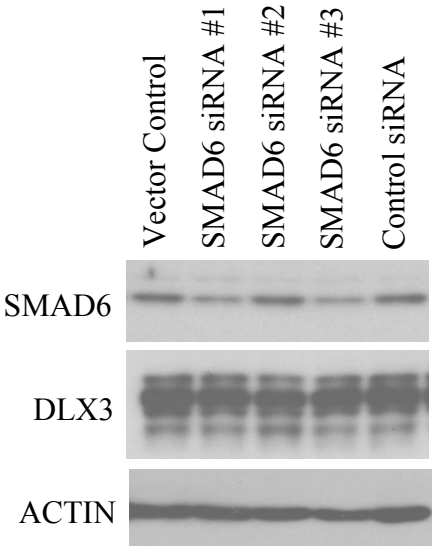
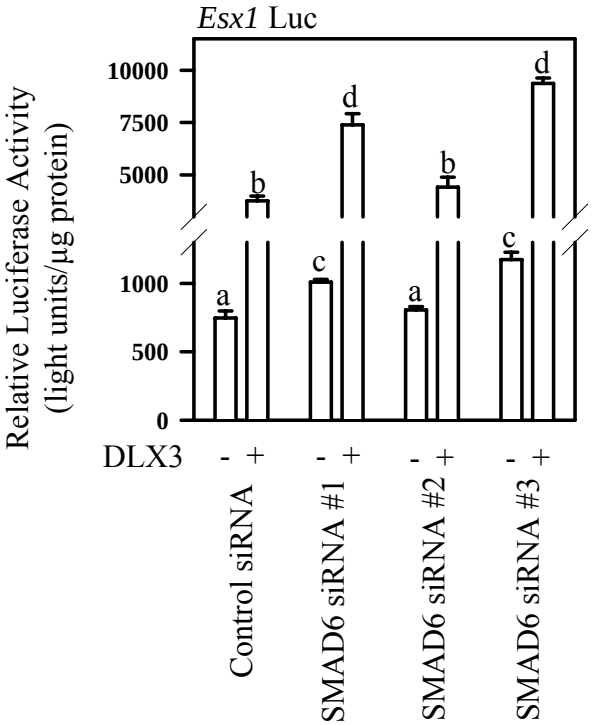

A
Control
+ DLX3
DLX3
Control
+ SMAD6
SMAD6
B
Esx1 Luc
Relative Luciferase Activity
(light units/ug protein)
9000
6000
3000
0
a
a
a
b
c
b
b
b
DLX3 - - - - + + + +
SMAD6
SMAD6
C
TKDP-1-Luc
Relative Luciferase Activity
(light units/µg protein)
1600
1200
800
400
0
a
b
ns
DLX3 - + - + + + +
Esx1Luc
SMAD6

Figure 2.9. siRNA-mediated knockdown of SMAD6 results in enhanced DLX3-dependent expression of the *Esx1* Luc reporter. A. Stable cell lines were selected using neomycin resistance following infection with retrovirus expression empty vector (Vector Control), three separate, independent siRNAs specific for *SMAD6* (*SMAD6* siRNA #1-3) or a control siRNA (Control siRNA). Following selection, cell lysates were prepared and western blot analysis was used to determine SMAD6 protein levels. The cell lines were also assayed for DLX3 and ACTIN using specific antibodies. SMAD6 protein levels were reduced approximately 57% with siRNAs #1 and 3. **B.** The Control siRNA and *SMAD6* siRNA#1-3 cell lines were used in transient transfection studies using the Esx1 Luc reporter in the absence or presence of DLX3 expression vector (2 μg). Data are reported as relative luciferase activity standardized for protein from representative studies (n=3/treatment). The designations a, b, c and d indicate significant differences ($p<0.05$).

A


Vector Control
SMAD6 siRNA #1
SMAD6 siRNA #2
SMAD6 siRNA #3
Control siRNA
SMAD6
DLX3
ACTIN

B


Esx1 Luc
Relative Luciferase Activity
(light units/µg protein)
10000
7500
5000
1000
500
0
a
b
c
d
DLX3
- +
Control siRNA
SMAD6 siRNA #1
SMAD6 siRNA #2
SMAD6 siRNA #3

loops (Figure 2.9A). Two of the three siRNA cell lines examined were found to have specific reductions in SMAD6 protein expression (*SMAD6* siRNAs #1 and #3; 57% reduction compared to control siRNA) but not expression of DLX3 nor ACTIN (internal controls; Figure 2.9A). Transfection studies in the control siRNA and the *SMAD6* siRNA cell lines using the *Esx1* luciferase reporter demonstrated that basal activity of this promoter was elevated ($p<0.05$) in the siRNA#1 and siRNA#3 cell lines but not in the control and siRNA#2 cell lines. In response to DLX3, *Esx1* luciferase promoter activity was enhanced ($p<0.05$) in *SMAD6* siRNA#1 and siRNA#3 cell lines but not in control or siRNA#2 cell lines [control siRNA (5.0-fold) *versus* siRNA#1 (7.3-fold) and siRNA#3 (8-fold)] in a manner highly correlated with the percentage loss of SMAD6 in these cell lines (Figure 2.9B). Thus, consistent with the overexpression studies, loss of endogenous SMAD6 was highly correlated with enhanced DLX3-dependent transcription of the *Esx1* gene promoter.

Discussion

The present studies provide novel evidence for the functional association between DLX3, a distal-less class homeobox factor required for normal placental development in the mouse, and an inhibitory Smad, SMAD6. The role of DLX3 as a transcriptional activator is clear based upon studies in mammalian systems, such as the glycoprotein hormone α subunit gene promoter [14], the expression of the homeobox factor *Esx1* in the *Dlx3* null mouse [13], and the role of Dlx3 in the regulation of the osteocalcin gene [27]. Moreover, Dlx3 has been linked to transcriptional activation as well as transcriptional repression in Xenopus (reviewed in Ref.[2]). The ability of Dlx3 to serve in several different ways as a transcriptional modulator may be best explained by the presence of variable tissue- and cell type-specific binding partners. The present study provides important evidence that DLX3 and SMAD6 form a complex in a yeast system, in

mammalian cells in culture and *in vitro* using recombinant proteins. DLX3 and SMAD6 also display overlapping expression in the nucleus of differentiated human term placental trophoblasts, suggesting that a functional interaction between these two proteins may be important *in vivo*. The finding that one DLX3 interacting partner is an inhibitory Smad may also reflect important cross-talk between the transforming growth factor (TGF) β/bone morphogenetic factor (BMP) signaling system and important developmental determinants that require DLX3.

Smad proteins are transcriptional regulators that can be subdivided into essentially three classes (reviewed in Refs. [28-30]). Regulated or R-Smads (Smad 1, 2, 3, 5 and 8) are substrates of the serine/threonine kinase catalytic activity of TGFβ/BMP type I receptors. Phosphorylation of R-Smads promotes the association with a common or C-Smad (Smad4) and subsequent nuclear translocation of the R- and C-Smad complex to affect TGFβ/BMP/Smad-specific target genes. Others have demonstrated an interaction between Smad4 and Dlx1 in the context of cytokine regulation of hematopoietic cells [31]. A third unique subclass of this family is the inhibitory or I-Smads (Smad 6 and 7). In the context of TGFβ/BMP signaling, I-Smads essentially oppose the activity of the stimulatory R- and C-Smads at several levels. I-Smads have been shown to compete for R-Smad binding to the type I TGFβ/BMP receptor, providing an inhibitory mechanism for R-Smad activity [32]. In addition, Smad6 has been shown to interfere with the ability of Smad1 to form a complex with Smad4, independent of Smad1 phosphorylation state [33]. I-Smads also form complexes with Smurfs, E3 ubiquitin ligases that appear to be involved in I-Smad nuclear export and marking target proteins (for example the TGFβ/BMP receptors and I-Smads themselves) for degradation by the proteosome, again contributing to an inhibitory action [28-30].

In the nuclear compartment, Smad6 appears to recruit the transcriptional co-

repressor C-terminal binding protein to target genes related to BMP action [34]. Based upon the present studies, DLX3 and SMAD6 were localized to the nucleus of placental trophoblasts independent of apparent TGFβ/BMP signaling. Smad6 has been shown to serve as a transcriptional co-repressor following interaction with another homeodomain transcription factor, *Hox* c-8 [35,36]. In these studies, Smad6 complexed with *Hox* c-8 appeared to be sufficient to recruit histone deacetylase activities to *Hox* c-8-dependent genes thus repressing transcription via altered organization of chromatin structure. Our studies suggest that the interaction between DLX3 and SMAD6 may utilize an entirely different mechanism for transcriptional repression in human placental cells. SMAD6 interaction clearly resulted in a reduction of DLX3 DNA binding activity, probably via steric interference. Our results demonstrate that the SMAD6 interaction interface includes the first two α helices of the homeodomain. SMAD6 interaction would then potentially interfere with DLX3 DNA binding, since these two α helices are central to DNA binding as well. This mechanism potentially alleviates a need for recruitment of transcriptional co-repressor activities, since a DLX3-SMAD6 complex association may preclude association of DLX3 with specific target gene promoter elements. Increased SMAD6 protein levels using overexpression inhibited DLX3-dependent gene expression. Conversely, reductions in the expression of endogenous SMAD6 using siRNA knockdown provides important evidence that DLX3-dependent gene expression may be particularly sensitive to relatively modest changes in SMAD6 protein levels in placental cells. These complementary approaches underscore the potential importance and sensitivity of DLX3-dependent genes to SMAD6 regulation. This may be largely true of Dlx3 target genes with binding sites that have low relative binding affinity, like *Esx1*.

The Dlx3 homeodomain is centrally located and serves as a DNA binding domain, one of the defining characteristics of all homeobox transcription factors [37,38]. The

domain structure of Dlx3 has been examined in studies defining a bipartite nuclear localization signal (residues 124-150) in a region adjacent to and including a portion of the homeodomain; transcriptional activation domains have been ascribed to residues 1-43 and 189-220; and Ser^{138} within the homeodomain is an apparent substrate for protein kinase C isozymes [20,21,24]. The deletion mutagenesis carried out in the present studies defined residues 80-163 within DLX3 as the SMAD6 interaction domain *in vitro*. This region of DLX3 contains the first full α helix and approximately two-thirds of helix 2 within the homeodomain. In addition, this interaction interface contains Ser^{138}, a substrate for PKC activity. Several possibilities exist for how DLX3-SMAD6 association may alter DNA binding. Perhaps the most obvious is a steric interference model, since the interaction interface includes a portion of the homeodomain. In addition, the presence of Ser^{138} in this interaction domain lends itself to potentially interesting speculation. The equivalent of this serine residue is conserved in all six Dlx family members [1], consistent with the observation that, like DLX3, DLX4 is also a SMAD6 interacting protein. Using recombinant Dlx3, Morasso and co-workers demonstrated that phosphorylation at Ser^{138} by PKC (most strongly by PKCα) resulted in partial inhibition of Dlx3 DNA binding [21], consistent with the potential effects of SMAD6 on DNA binding. This supports speculation that phosphorylation of Dlx3 at Ser^{138} may alter or increase Smad6 binding leading to reduced DNA binding at Dlx3 target genes. Studies are currently underway to address this possibility.

R- and C-Smads appear to be constitutively expressed in most cell types whereas I-Smads are subject to regulation by a number of growth factors, such as EGF, TGFβ and BMPs. In many cases the effects of these growth factors are mediated through R- and C-Smad-dependent transcriptional mechanisms. This has led to a hypothesis implicating an intracellular negative feedback loop, whereby positive TGFβ/BMP

signals are modulated over time by accumulation of induced I-Smads (reviewed in Ref. [39]). Interestingly, Dlx3 expression patterns during Xenopus development depend in part upon BMP signaling gradients [40,41]. Inhibition of these gradients using the BMP receptor antagonist chordin resulted in a dose-sensitive inhibition of Dlx3, Dlx5 and Dlx6 mRNA expression. Dlx3 expression in mouse keratinocytes was also reported to be subject to regulation by BMPs [42] in a manner potentially coordinate with I-Smad expression. This supports speculation that not only might BMP-regulated Smad6 expression serve as a negative feedback mechanism controlling the duration of Smad signaling but increased BMP-dependent Smad6 expression may lead to important modulation of Dlx3-dependent gene expression. BMPs have been shown to regulate early embryogenesis during the perimplantation period, such that these types of ligands are probably present and important during placental morphogenesis [43].

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CHAPTER THREE

ANALYSIS OF THE GENE REGULATORY PROGRAM INDUCED BY THE HOMEOBOX TRANSCRIPTION FACTOR DISTAL-LESS 3 IN MOUSE PLACENTA

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Abstract

Dlx3, a homeodomain transcription factor, is essential for placental development in the mouse. The *Dlx3*$^{-/-}$ mouse embryo dies at E9.5-10 due to placental failure. To develop a more comprehensive understanding of the gene profile regulated by Dlx3, microarray analysis was used to determine differences in gene expression within the placenta of *Dlx3*$^{+/+}$ and *Dlx3*$^{-/-}$ mice. Array analysis revealed differential expression of 401 genes, 33 genes in which signal to log ratio values of null/wild type were lower than -0.5 or higher than 0.5. To corroborate these findings, quantitative real-time PCR was used to confirm differential expression for 11 genes, nine of which displayed reduced expression and two with enhanced expression in the *Dlx3*$^{-/-}$ mouse. Loss of Dlx3 resulted in a marked reduction (>60%) in mRNA expression of *Pgf*, a member of the vascular endothelial growth factor family. Consistent with these results, Pgf secretion from placental explants tended to be reduced in the *Dlx3*$^{-/-}$ mice compared with wild type. To investigate mechanisms of Dlx3 regulation of *Pgf* gene transcription, we cloned 5.2 kb of the *Pgf* 5' flanking sequence for use in reporter gene assays. Expression of the *Pgf* promoter luciferase reporter containing at least three Dlx3 binding sites was increased markedly by overexpression of Dlx3 supporting the conclusion that Dlx3 may have a direct effect on *Pgf* promoter activity. These studies provide a novel view of the transcriptome regulated by Dlx3 in mouse placenta. Dlx3 is specifically required for full expression and secretion of Pgf *in vivo*. Moreover, *in vitro* studies support the conclusion that Dlx3 is sufficient to directly modulate expression of the *Pgf* gene promoter in placental cells.

Introduction

The complex process of determination of trophoblast cell lineages within the placenta is integral to the successful establishment of the maternal-fetal interface.

Remarkably, these tissues functionally give rise to cell populations that control fetal nutrient and gas exchange, participate in maternal-fetal immunological dialog and are critical sources of endocrine factors that communicate to the mother the presence of the conceptus. Failures associated with establishment of the maternal/fetal interface can result in fetal mortality or lead to intrauterine fetal growth abnormalities associated with fetal morbidity and the potential to contribute to the adult onset of diseases such as type 2 diabetes (recently reviewed in Refs [1-3]). Thus, a more comprehensive understanding of the genetic mechanisms that underlie the development and morphogenesis of the placenta will provide the foundation for understanding mechanisms of fetal morbidity and mortality during early pregnancy and potentially a better understanding of aspects of the origins of adult disease.

A great number of mouse knockout models have been instrumental in defining genetic determinants required for developmental decisions during placental morphogenesis. These have been recently reviewed in elegant detail [4]. For example, knockouts of numerous receptors, signaling molecules and transcription factors have been shown to compromise syncytial trophoblast differentiation and morphogenesis of the placental labyrinth, a key site within the mouse placenta of interdigitations between fetal vascular beds and maternal blood spaces [5]. Our laboratory has developed a keen interest in one of these transcription factors, Dlx3, based on two important observations. First, in human trophoblast cell lines DLX3 plays a critical role in the basal regulation of the glycoprotein hormone α subunit gene, a key subunit of the heterodimeric placental borne hormone, CG [6]. CG is expressed exclusively in primate and equine trophoblasts and is an important luteotropin during early human pregnancy. CG also appears to be an important ligand in the regulation of endometrial receptivity [7-9] through changes in MAPK signaling activity to affect morphology and function of epithelial and stromal compartments of the receptive endometrium [10]. Second, in

mouse models, disruption of the *Dlx3* loci in knockout experiments resulted in fetal death at E9.5-10 due to failed placental labyrinth development [11]. Dlx3 expression is largely restricted to trophoblasts within the labyrinth of the mouse placenta, and loss of Dlx3 appears to affect the expansion of the labyrinth during midgestation [12]. Thus, Dlx3 appears to be playing a critical role in facilitating biosynthesis of a key placental hormone that serves as a luteotropin and a modulator of uterine receptivity in primates as well as being instrumental in facilitating organizational aspects of the mouse labyrinth morphogenesis and the establishment of the maternal-fetal interface.

In the present studies, we have furthered our understanding of Dlx3 expression within the mouse placenta. We have identified a rather large Dlx3-dependent transcriptome by microarray analyses comparing $Dlx3^{+/+}$ and $Dlx3^{-/-}$ mice. *Pgf* transcript levels and secretion appear to be markedly reduced by the loss of Dlx3 in these studies. The present studies provide important new evidence that Dlx3 may directly affect transcriptional regulation of *Pgf* through interactions of Dlx3 and the *Pgf* gene promoter in choriocarcinoma cells.

Materials and Methods

Histological staining of mouse placental sections

Implantation sites from $Dlx3^{+/+}$ and $Dlx3^{-/-}$ placentas at E9.5 were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were then cut and stained in hematoxylin and eosin to characterize relative differences in labyrinth expansion and morphogenesis.

Microarray

All experimental paradigms using animals in this research were approved by the Cornell University Institutional Animal Care and Use Committee. Male and female

Dlx3$^{+/-}$ (heterozygous) mice were time mated and killed 9.5 d after conception. Gravid uteri were dissected free and fetal tissues were harvested for genotyping as previously described [12]. The placental disks (with some maternal decidua) were carefully removed and immediately frozen in liquid nitrogen pending completion of genotyping. Total RNA was extracted by homogenizing dissected, thawed tissues in Trizol reagent (Invitrogen, Carlsbad, CA) using a TissueLyser (Qiagen, Valencia, CA). The RNA was examined for quality and degradation through an Agilent BioAnalyzer by the microarray core facility (Cornell University, Ithaca, NY). Double-stranded cDNA was synthesized from mRNA and *in vitro* transcription reactions were then used to produce biotin labeled cRNA from the cDNA using GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA). The labeled cRNA was hybridized with GeneChip mouse genome 430 2.0 Array (Affymetrix, Santa Clara, CA) and scanned by GeneArray 3000 scanner (Affymetrix, Santa Clara, CA). The raw array data of three pairs of *Dlx3*$^{-/-}$ versus *Dlx3*$^{+/+}$ samples were analyzed using Affymetrix GCOS software to obtain signal, signal log ratio values, change call and associated *p* values, using the wild-type sample in each pair as baseline. Paired *t* test was performed on signal to log ratio values versus mean of zero for each gene. Multiple test correction was not used because of the heterogeneous nature of the tissue samples, the relatively small differences in gene expression levels observed between samples, and the limited number of replicates that were performed. We therefore chose an arbitrary *p* value of 0.05 to generate an initial set of genes for further validation. A set of 401 differentially expressed genes were selected which had *t* test *p* value of less than 0.05 and maximum signal value of at least 512. Gene networks were identified by database analyses (Ingenuity Pathway Analysis; Ingenuity Systems, Redwood City, CA) using all 401 identified genes in the array analysis.

Quantitative real-time PCR

Quantitative (q) real-time PCR using probe based chemistry was used to confirm differential expression of genes identified by array experiments. Briefly, a total of 9.0 µg of total mRNA from individual *Dlx3*$^{+/+}$ and *Dlx3*$^{-/-}$ mouse placentas (n=3/genotype) was reversed transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) with uracil-*N*-glycosylase was used to set up PCRs containing forward and reverse primers, amplicon specific probes and 20.0 ng of cDNA template in a total volume of 20.0 µl. All PCRs were performed in triplicate. Forward and reverse primers flanking an exon junction were designed by ProbeFinder version 2.10 (Roche Applied Science, Indianapolis, IN) and synthesized by Integrated DNA Technologies Inc (Coralville, IA). The amplicon specific fluorogenic probes used were identified from the Roche universal probe library that were labeled with 6-carboxy fluorescein (FAM) and 6-carboxy-tetra-methyl rhodamine (TAMRA) for the 5' reporter and 3' quencher, respectively. The oligonucleotide sequences of primers and identification number for the corresponding Roche mouse universal probe library are listed in Table 3.1. PCRs were performed on an ABI 7500 real-time PCR system and analyzed with sequence detection software version 1.3 (Applied Biosystems). Negative controls were run with each sample set, in which the template was replaced by PCR-grade water. *β-actin* was used as the endogenous control for normalization of cDNA input. Relative quantification of gene expression was determined by the $2^{-(\Delta\Delta Ct)}$ method after first determining that the amplification efficiency of all targets and *β-actin* was identical [13]. We also used commercially available ABI TaqMan gene expression assays to quantify synuclein-α (*Snca*) expression levels as well as confirm the specificity of the Roche probe sets to the hemoglobin gene family members *Hba-a1*, *Hba-x*, and *Hbb-y* (listed in Table 3.2).

Table 3.1. Primers and probes used for qPCR analysis

Gene symbol	GeneBank accession no.	Forward primer	Reverse primer	Roche Probe no.
Pgf	NM_008827	ctgggttggctgtgcatt	ggcaccacttccacttctgt	03
Hba-a1	NM_008218	gagctgaagccctggaaag	tggcttacatcaaagtgaggaa	60
Hba-x	NM_010405	tgagagagctatcatcatgtcca	ggggtagctgcagaagagc	07
Hbb-y	NM_008221	cttgccatcatggtgaacttt	ggtccatgggtacacaacaa	73
Hbb-bh1	NM_008219	tggatcctgagaacttcaagc	attggccactccaatcacc	29
Mmp9	NM_013599	acgacatagacggcatcca	gctgtggttcagttgtggtg	19
Ceacam11	NM_023289	aggggatcctgctcacagt	tctgggtggtagttggaatca	62
Psx1	NM_008955	gggtgtggatgaatgtgatg	gcactctcctgtttctctgg	91
Lum	NM_008524	ggatggcaatcctctcactc	tcatttgctacacgtagacactcat	16
Thbs2	NM_011581	ggctttctgggcaatggta	atatctgtgaccacagcacactc	85

Table 3.2. Applied Biosystems TaqMan gene expression assays

Gene symbol	Gene name	GeneBank accession no.	Assay ID
Snca	Synuclein-α	NM_009221	Mm00447333_m1
Hba-a1	Hemoglobin-α, adult chain 1	NM_008218	Mm00845395_s1
Hba-x	Hemoglobin X, α-like embryonic chain in Hba complex	NM_010405	Mm00439255_m1
Hbb-y	Hemoglobin Y, β-like embryonic chain	NM_008221	Mm00433936_g1

Northern blotting

The *Pgf* cDNA was amplified from E9.5 mouse placenta using PCR, the PCR product was cloned into pGEM T-Easy vector (Promega Corp., Madison, WI) and nucleotide sequence analysis was used to confirm the *Pgf* cDNA. Male and female *Dlx3*$^{+/-}$ (heterozygous) mice were time mated and euthanized 9.5 d after conception. Gravid uteri were dissected free and embryos were harvested for genotyping as described above. The placental disk (with some maternal decidua) was removed and immediately frozen in liquid nitrogen pending completion of genotyping. Total RNA was extracted from E9.5 placenta using Trizol reagent (Invitrogen, Carlsbad, CA).

RNA samples (15 μg) from different genotypes were separated in conventional denaturing formaldehyde-containing agarose gel electrophoresis, ribosomal bands documented, and then RNA transferred to nylon membranes using capillary action. The membranes were prehybridized in Church's hybridization buffer [7% (wt/vol) sodium dodecyl sulfate, 0.5 M sodium phosphate buffer (pH 7.2) and 10 mM EDTA] overnight. Radiolabeled [^{32}P] *Pgf* cDNA probe was prepared by random primer labeling to high specific activity and then added to the hybridization buffer. Hybridizations were carried out at 65°C overnight. Membranes were then washed under high stringency and bands were visualized by autoradiography. Densitometry analyses of band intensity were performed using ImageJ (National Institutes of Health, Bethesda, MD).

Placental explant studies and Pgf ELISA

Male and female *Dlx3*$^{+/-}$ (heterozygous) mice were time mated and pregnant females were killed 9.5 d after conception. Gravid uteri were dissected free and embryos were harvested for genotyping as described above. The placental disks with some maternal decidua [*Dlx3*$^{+/+}$ (n=5) and *Dlx3*$^{-/-}$ (n=7)] were removed and immediately placed into explant culture as described previously (12). Media samples were collected at time 0 and after 3 h in this culture system. Media samples were then analyzed for secreted Pgf protein levels using a mouse Pgf ELISA based immunoassay commercially available (R & D Systems, Minneapolis, MN) as per the manufacturer's instructions. Media samples were assayed in duplicate in two separate assays. In media samples collected at time 0, Pgf concentrations were below detectable limits of the assay. According to the manufacturer, interassay coefficient of variation for this assay was 5.6%. Within-assay variation was reported as 9.5%. The sensitivity of this assay (minimum detectable dose) was 1.49 pg/ml.

Cloning the Pgf promoter sequences

Mouse *Pgf* (5.2 kb) promoter was obtained by PCR using mouse genomic DNA and the following primers: 5' primer, 5'-GGTACCGATGAGAGAGGGCGGAAGAGAA-3' and 3' primer, 5'-GGTACCTTCAAGGCACAATCACCATGC-3'. To facilitate cloning, a Kpn I restriction enzyme site was incorporated at the end of both 5' and 3' primers. The PCR product was cloned into pGEM T-Easy vector (Promega Corp., Madison, WI). The nucleotide sequence was verified by sequence analysis. After Kpn I digestion, the promoter fragment was subcloned into a luciferase reporter vector and orientation confirmed using restriction analysis. The *Pgf* promoter-luciferase reporter gene plasmid was prepared by two cycles through cesium chloride using standard protocols.

Cell culture and transient transfection studies

JEG3 cells were cultured in DMEM supplemented with 10% FBS. Before transfection studies, cells were split to 35-mm dishes and subconfluent cultures were used in all experiments. The *Pgf* promoter-luciferase reporter (*Pgf*-Luc) containing 5.2 kb 5' flanking sequences as described above was used in these studies. The *Pgf*-Luc reporter was transiently cotransfected into JEG3 cells with increasing doses of Dlx3 expression vector (pKH3-Dlx3) using FuGENE 6 transfection reagent (Roche Diagnostics Inc., Indianapolis, IN) as per the manufacturer's instructions. All transfections were carried out with an equivalent amount of total DNA by supplementing reactions with the parent vector (pKH3). Twenty-four hours after transfection, luciferase activity was determined using reagents from the dual-luciferase reporter assay system (Promega Corp., Madison, WI). Luciferase activity was standardized by total cell protein content as determined by Bradford assay. All transfection studies were performed in triplicate on at least three separate occasions

with similar results. Data are shown as means (n=3) ± SEM of representative experiments. To determine ectopic DLX3 expression level in transfected cells, Western blot analysis was performed on cell lysates obtained for luciferase assay. Equal protein amounts of cell lysate were resolved by SDS-PAGE and transferred to nitrocellulose membranes using electroblotting. Membranes were blocked using Tris-buffered saline [10 mM Tris (pH 7.6) and 150 mM NaCl] containing 0.1% Tween20 (TBST) and 5% nonfat dried milk. After 1h of blocking, membranes were incubated with a rabbit polyclonal DLX3 antiserum or β-ACTIN as previously described [13], and protein bands representing ectopic expression were visualized using enhanced chemiluminescence (Perkin Elmer, Boston, MA).

Identification of putative Dlx3 binding sites within the Pgf 5' flanking sequences and DNA binding assays

Inspection of the *Pgf* 5' flanking sequences revealed three putative Dlx3 binding sites based upon comparison to the consensus binding site sequence A/C/GTAATT G/AC/G. These sites were located at positions -4297, -3061 and -1716 relative to the start site of transcription. The site at position -4297 was a consensus site, whereas the sites at positions -3061 and -1761 were near consensus (a single nucleotide mismatch in the 3' termini of the site). Oligonucleotides (top and bottom strands) were synthesized for each of these sites, annealed and labeled for use in EMSAs. Recombinant DLX3 was prepared by *in vitro* transcription and translation using a wheat germ lysate kit commercially available (Promega, Madison, WI) for use in EMSA studies. Nuclear extracts were prepared from JEG3 choriocarcinoma cells and used in EMSA as described previously [13,14].

Statistical analyses

Statistical methods for the array analyses are described above. Where appropriate for Pgf secretion data and all luciferase data, paired *t* tests were used to examine statistical differences between means. Statistical significance is reported for each experiment.

Results

Genetic perturbation of Dlx3 in knockout mice results in a failure to expand the placental labyrinth

In the mouse, *Dlx3* has been shown to be required for normal placental development and morphogenesis [11]. Dlx3 is expressed within the trophoblasts of the labyrinth beginning sometime after E8.5 and is clearly detectable by immunohistochemistry on E9.5 [12]. Dlx3 was not detectable in giant cell trophoblasts. Targeted deletion of *Dlx3* resulted in embryonic lethality at E9.5-10 characterized by a failure in the development of the murine placenta. Consistent with previous observations by Morasso *et al.* [11], histological examination of the murine placenta on E9.5 revealed a reduction in the thickness of the labyrinth in *Dlx3*$^{-/-}$ implantation sites, compared to wild type (Figure 3.1A).

Gene profiling of the Dlx3$^{-/-}$ *mouse placenta*

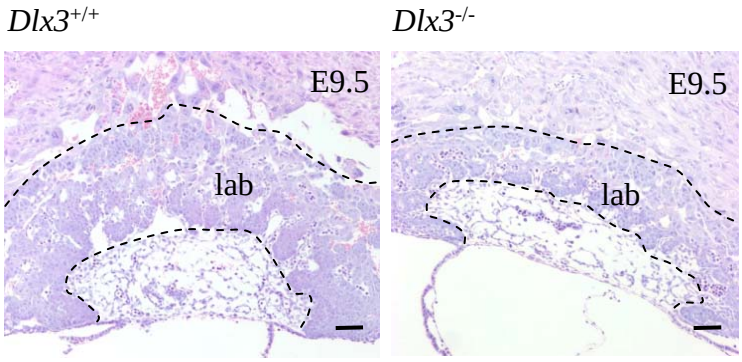
The *Dlx3*$^{-/-}$ mouse placenta is characterized by a putative failure in the expansion of the labyrinth compartment, a key developmental step effectively enlarging the fetal vascular compartment and thus the maternal-fetal interface (Figure 3.1A). To better understand the transcriptome regulated by Dlx3 within the labyrinth, we used microarray analysis of placental tissues from the *Dlx3*$^{-/-}$ mouse model. Our studies focused on the gene profile at E9.5, a time when the placenta appears to be causal in

the demise of the developing embryo [11]. Mouse Genome 430 2.0 arrays containing 45,000 probe sets analyzing the expression level of approximately 39,000 transcripts were used. From this analysis, we identified 401 differentially expressed genes (for detailed information, see Appendix 1). Of these 401 genes, RNA levels from 194 genes were shown to be reduced, whereas RNA levels from the remaining 207 genes were increased. Using the criteria of a significant paired *t* test and a signal to log ratio value of lower than -0.5 and higher than 0.5 when comparing $Dlx3^{-/-}$ to $Dlx3^{+/+}$ mice, analyses of array data demonstrated differential expression of 33 genes in three pair-wise comparisons (Figure 3.1B). RNA levels from 25 of the 33 genes were reduced, whereas the remaining eight genes were expressed at elevated levels in the placenta of $Dlx3^{-/-}$ mice, compared with $Dlx3^{+/+}$ mice.

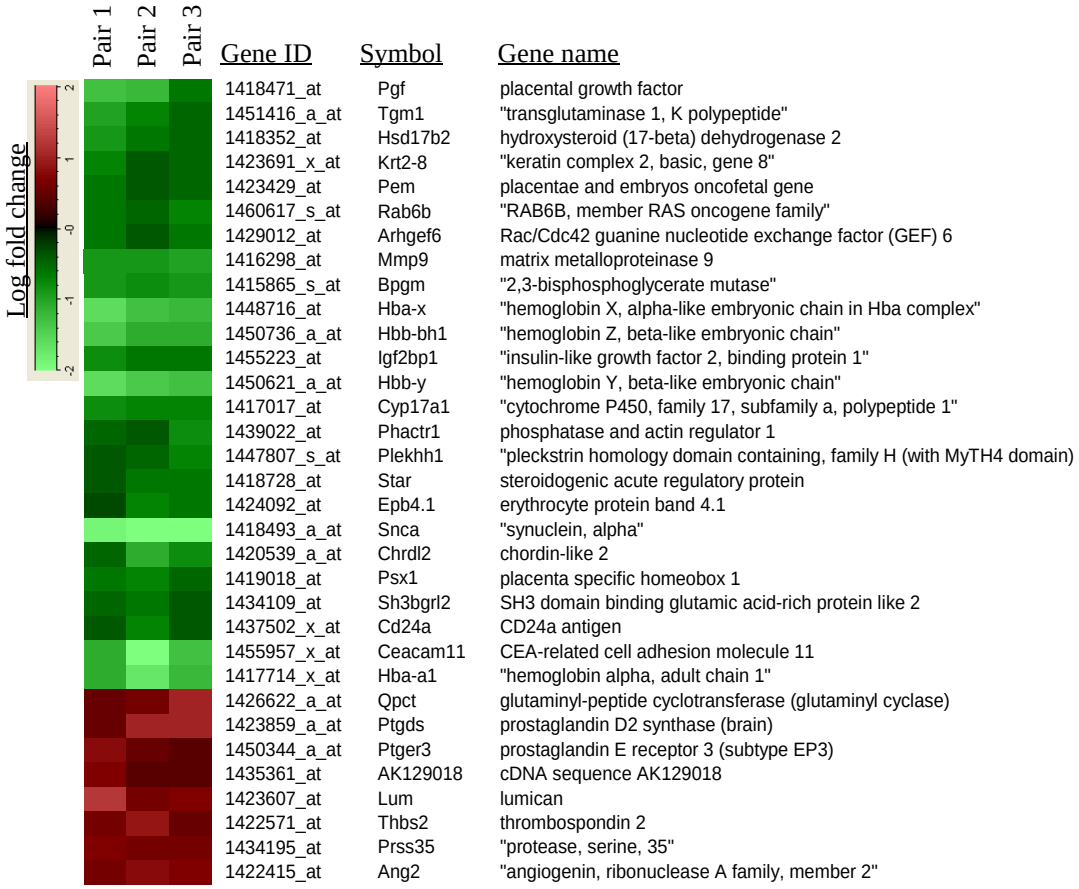
To confirm these array findings, 12 selected genes were examined by qPCR methods (Table 3.3). Selection of these genes was made from the entire set of 401 differentially expressed genes; however, the majority was selected from the list of 33 genes with the greatest fold change in transcript level (Figure 3.1B). qPCR results confirmed the fidelity of the array analysis for 11 of 12 genes with consistent fold changes in RNA levels (Table 3.3). For example, four hemoglobin genes (*Hba-a1*, *Hba-x*, *Hbb-y* and *Hbb-bh1*) examined were all reduced in the $Dlx3^{-/-}$ placenta to approximately 30-40% of wild type RNA levels, whereas lumican and thrombospondin 2 were both elevated 1.8- to 2.2-fold using these two assays. Remarkably we did not obtain consistent results for only 1 (insulin-like growth factor binding protein 5) of the 12 genes examined. We attribute this inconsistency to a lack of an effective mouse universal probe library assay in the qPCR approach rather than true differences between the assays used. Therefore the qPCR results confirm the accuracy of the array analysis in determining differential gene expression in the placenta in the absence of *Dlx3*.

Figure 3.1. Effect of Dlx3 on labyrinth morphology and gene profile of 33 differentially expressed genes in *Dlx3*$^{-/-}$ mouse placenta. A. Hemotoxylin and eosin staining of E9.5 mouse *Dlx3*$^{-/-}$ and *Dlx3*$^{+/+}$ implantation sites identifying labyrinth (lab; demarcated by dashed lines). Bar = 200 μm. **B.** Thirty-three genes from microarray analysis whose magnitude of log ratio was lower than -0.5 or higher than 0.5 are depicted. Genes were identified by Affymetrix ID (Gene ID), symbol and gene name. Each column represents a pair wise sample comparison. The signal to log ratio values of *Dlx3*$^{-/-}$/*Dlx3*$^{+/+}$ from Affymetrix GCOS software were used in the cluster analysis. The shades of green indicate repressed genes and the shades of red are induced genes. Colored pixels represent the magnitude of the gene response (log fold change).

A



B



Gene ID	Symbol	Gene name
1418471_at	Pgf	placental growth factor
1451416_a_at	Tgm1	"transglutaminase 1, K polypeptide"
1418352_at	Hsd17b2	hydroxysteroid (17-beta) dehydrogenase 2
1423691_x_at	Krt2-8	"keratin complex 2, basic, gene 8"
1423429_at	Pem	placentae and embryos oncofetal gene
1460617_s_at	Rab6b	"RAB6B, member RAS oncogene family"
1429012_at	Arhgef6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
1416298_at	Mmp9	matrix metalloproteinase 9
1415865_s_at	Bpgm	"2,3-bisphosphoglycerate mutase"
1448716_at	Hba-x	"hemoglobin X, alpha-like embryonic chain in Hba complex"
1450736_a_at	Hbb-bh1	"hemoglobin Z, beta-like embryonic chain"
1455223_at	Igf2bp1	"insulin-like growth factor 2, binding protein 1"
1450621_a_at	Hbb-y	"hemoglobin Y, beta-like embryonic chain"
1417017_at	Cyp17a1	"cytochrome P450, family 17, subfamily a, polypeptide 1"
1439022_at	Phactr1	phosphatase and actin regulator 1
1447807_s_at	Plekhh1	"pleckstrin homology domain containing, family H (with MyTH4 domain)"
1418728_at	Star	steroidogenic acute regulatory protein
1424092_at	Epb4.1	erythrocyte protein band 4.1
1418493_a_at	Snca	"synuclein, alpha"
1420539_a_at	Chrdl2	chordin-like 2
1419018_at	Psx1	placenta specific homeobox 1
1434109_at	Sh3bgrl2	SH3 domain binding glutamic acid-rich protein like 2
1437502_x_at	Cd24a	CD24a antigen
1455957_x_at	Ceacam11	CEA-related cell adhesion molecule 11
1417714_x_at	Hba-a1	"hemoglobin alpha, adult chain 1"
1426622_a_at	Qpct	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
1423859_a_at	Ptgds	prostaglandin D2 synthase (brain)
1450344_a_at	Ptger3	prostaglandin E receptor 3 (subtype EP3)
1435361_at	AK129018	cDNA sequence AK129018
1423607_at	Lum	lumican
1422571_at	Thbs2	thrombospondin 2
1434195_at	Prss35	"protease, serine, 35"
1422415_at	Ang2	"angiogenin, ribonuclease A family, member 2"

Table 3.3. Comparison of changes in transcript levels using array analysis and qPCR

Gene symbol	Gene name	Gene Bank accession number	Microarray fold change (Mean±SEM)	qRT-PCR fold change (Mean±SEM)
Pgf	Placental growth factor	NM_008827	0.50 ± 0.08	0.60 ± 0.14
Snca	Synuclein, alpha	NM_009221	0.31 ± 0.08	0.48 ± 0.06
Hba-a1	Hemoglobin alpha, adult chain 1	NM_008218	0.40 ± 0.05	0.45 ± 0.08
Hba-x	Hemoglobin X, alpha-like embryonic chain in Hba complex	NM_010405	0.39 ± 0.03	0.44 ± 0.03
Hbb-y	Hemoglobin Y, beta-like embryonic chain	NM_008221	0.37 ± 0.02	0.44 ± 0.07
Hbb-bh1	Hemoglobin Z, beta-like embryonic chain	NM_008219	0.44 ± 0.03	0.52 ± 0.14
Mmp9	Matrix metalloproteinase 9	NM_013599	0.52 ± 0.01	0.68 ± 0.12
Ceacam11	CEA-related cell adhesion molecule 11	NM_023289	0.37 ± 0.01	0.60 ± 0.06
Psx1	Placenta specific homeobox 1	NM_008955	0.66 ± 0.03	0.75 ± 0.04
Lum	Lumican	NM_008524	1.81 ± 0.24	2.03 ± 0.22
Thbs2	Thrombospondin 2	NM_011581	1.60 ± 0.14	2.16 ± 0.12

To gain further insight into how these differentially regulated genes may be organized into specific gene networks, the entire array data set (401 differentially expressed genes) was subjected to gene network analysis using Ingenuity pathway analysis (Ingenuity.com). This analysis revealed two interrelated gene networks strongly impacted by the loss of *Dlx3* (Figure 3.2). Loss of *Dlx3* resulted in the altered expression of 11 genes within a cell-to-cell signaling and interaction gene network. This network is composed of a number of important signaling ligands/receptors (Pgf and EGF receptor), signaling enzymes (the low molecular weight GTP binding protein ras, phospholipase D, protein kinase C inhibitor) and extracellular matrix-related molecules [matrix metalloproteinase 9 (Mmp9), thrombospondin 2 (Thbs2)]. This

cell-to-cell signaling and interaction gene network was linked to a second network composed of genes related to hematological disease. These two networks were linked via the putative regulation of hemoglobin genes though EGF receptor signaling. Within the hematological disease network, loss of *Dlx3* resulted in the down-regulation of five specific hemoglobin genes, some of which were embryonic/fetal hemoglobin forms (*i.e*. *Hbb-bh1*). Regulation of embryonic/fetal forms of hemoglobin in fetal, nucleated erythrocytes may be consistent with the role of Dlx3 in supporting an emerging fetal vascular compartment associated with the placental labyrinth [11,12]. Thus, changes in the Dlx3-modulated cell-to-cell signaling and interaction network affects another gene network related to hematological disease. These types of gene network relationships provide an important opportunity to examine the specific impact of related genes more broadly on the functional development and morphogenesis of the mouse placenta.

Pgf gene appears to be an important target of Dlx3 action in placental cells

Examination of our array data immediately drew our attention to changes in expression of the *Pgf* gene, a member of the VEGF family. Consistent with qPCR results, Northern blot analysis revealed a decrease in a single 1.7-kb mRNA in the placental tissue when using mouse *Pgf* cDNA as the probe (Figure 3.3A). Densitometric analysis indicated that the expression level of *Pgf* mRNA in placenta of *Dlx3*$^{-/-}$ mice was 43% of that in wild type mice (Figure 3.3B). To determine whether changes in *Pgf* transcript level were functionally coupled to reduced Pgf secretion, we performed placental explant studies [12] using implantation sites from *Dlx3*$^{+/+}$ and *Dlx3*$^{-/-}$ mice. After 3h in explant culture, concentrations of Pgf detected in explant culture medium tended to be reduced by more than 50% (P = 0.063) in *Dlx3*$^{-/-}$ placentas, compared with *Dlx3*$^{+/+}$ (Figure 3.3C) providing an indication of coupled Pgf

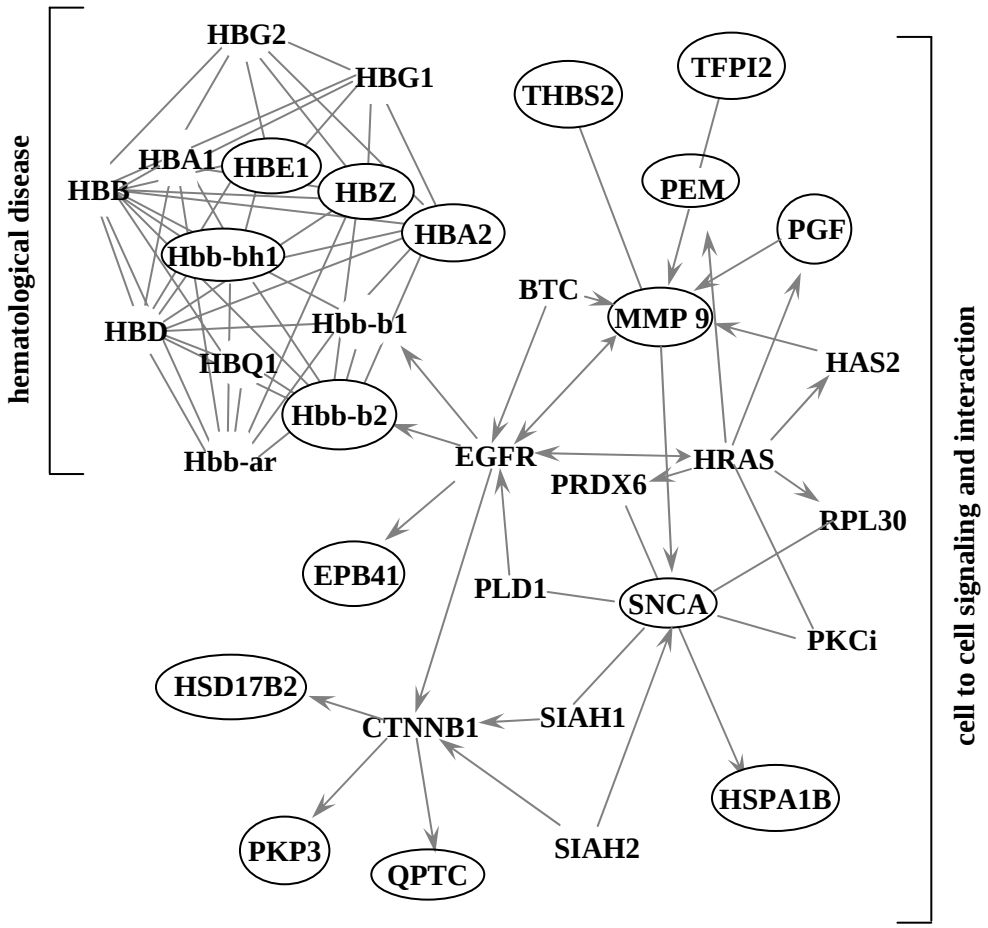


Figure 3.2. Dlx3 impact on gene networks. The microarray data (401 differentially expressed genes) were subjected to Ingenuity pathway analysis. Two networks were identified; one was related to hematological disease and the other was related to cell-to-cell signaling and interaction. The genes identified by circles are Dlx3-dependent.

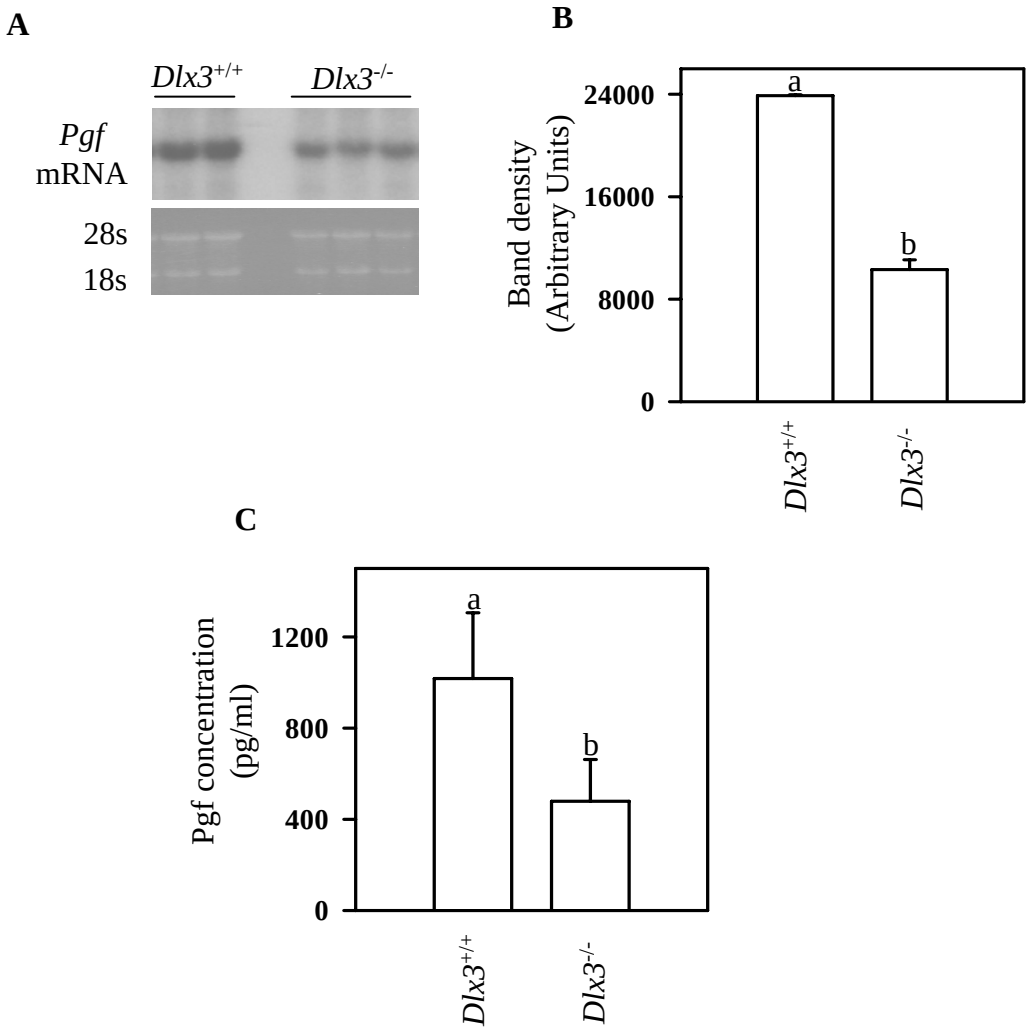


Figure 3.3. Pgf expression and secretion in placenta of *Dlx3*$^{+/+}$ and *Dlx3*$^{-/-}$ mice. **A.** Total RNA was isolated from placenta of different genotypes. RNA (15 µg) was hybridized with Pgf cDNA probe. Ethidium bromide staining of 28s and 18s ribosomal RNAs was used as the lane loading control. **B.** Band intensity from the *Pgf* Northern blots was quantified using ImageJ software and depicted as a histogram. (a and b reflect statistical differences, $p<0.05$) **C.** *Dlx3*$^{-/-}$ and *Dlx3*$^{+/+}$ implantations were prepared and placed in explant cultures for 3 hours. Media samples were assayed for Pgf using a commercially available ELISA-based assay (a and b reflect statistical tendency, $p=0.063$).

gene expression and secretion.

The Pgf gene 5' flanking region is responsive to Dlx3 overexpression in choriocarcinoma cell.

To examine the hypothesis that the *Pgf* gene promoter may be regulated by Dlx3 at the transcriptional level, we cloned a portion of the *Pgf* promoter into a luciferase reporter gene construct. The promoter fragment was based upon studies previously reported [15] and additional sequences obtained from Ensembl genome browser. Initial studies compared basal expression of the *Pgf* promoter-luciferase reporter gene activity with other promoter fragments known to be expressed in placental trophoblasts [*Esx-1* and *Dlx3* [13,14] or constitutively expressed (thymidine kinase)]. The luciferase reporter containing 5.2 kb of the *Pgf* gene 5' flanking region displayed transcriptional activity approximately 10-fold higher that the parent luciferase reporter gene. Basal *Pgf* promoter activity was greater than *Esx-1* promoter activity [13] but less than the *Dlx3* or thymidine kinase promoters [14] when transfected as luciferase reporters into the JEG3 choriocarcinoma cell line (a trophoblast cell model).

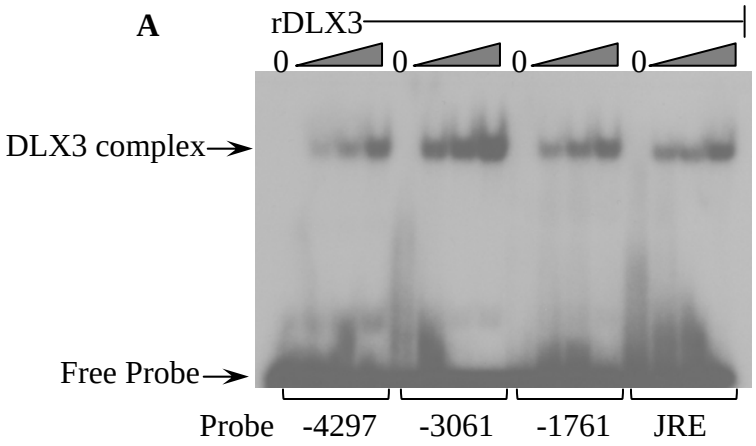
Examination of the nucleotide sequences of this *Pgf* promoter fragment revealed three consensus or near consensus Dlx3 binding sites (using A/C/G TAATT G/A C/G consensus sequence for a Dlx3 binding site). For identification of near-consensus sites, a single nucleotide mismatch outside of the central TAATT core of the binding site was considered. These sites were located on the sense strand at positions -4297 (consensus site), -3061 and -1761 (both near consensus sites). We synthesized oligonucleotide probes for the putative Dlx3 binding sites for use in EMSA using recombinant DLX3 and nuclear extracts purified from JEG3 choriocarcinoma cells to determine whether DLX3 could bind these elements *in vitro* (Figure 3.4A and B). EMSA revealed that all of these sites were sufficient to bind recombinant DLX3. The

junctional regulatory element (JRE; a consensus DLX3 binding site) from the glycoprotein hormone α subunit promoter was used as a positive control in these studies (Figure 3.4A). Binding sites at -4297 and -1761 bound recombinant DLX3 in a manner consistent with the JRE; whereas the site located at -3061 appeared to be a higher relative affinity binding site compared to the others despite being a near consensus site.

Using the -3061 binding site, we examined DNA binding complex formation using nuclear extracts from JEG3 choriocarcinoma cells (Figure 3.4B). These binding reactions resulted in the formation of discrete complexes with an electrophoretic mobility consistent with recombinant DLX3. Further using DLX3 antiserum, these studies demonstrated a DLX3-like immunoreactivity within the DNA binding complex as measured by a more retarded electrophoretic mobility often referred to as a supershift. In these studies, normal rabbit serum (NRS) and the CCAAT/enhancer-binding protein (C/EBP) antisera were used as negative controls. Similar results were observed using the -4297 and -1761 binding sites and JEG3 nuclear extracts. These studies support the conclusion that DLX3 or a highly similar epitope is present in the binding complexes formed using these sites and JEG3 nuclear extracts. Moreover, these studies support speculation that DLX3 may have a direct effect on *Pgf* promoter activity in cells of the trophoblast lineage.

To determine the effect of DLX3 on the expression of *Pgf* promoter-luciferase reporter, cotransfection studies in JEG3 choriocarcinoma cells were used. Overexpression of DLX3 resulted in a dose-dependent increase in expression of epitope tagged DLX3 (Figure 3.5A). Addition of three hemagglutinin (HA) epitopes in frame with the DLX3 coding sequence resulted in expression of HA-DLX3, which was slightly larger in molecular size relative to endogenous DLX3. Recombinant

Figure 3.4. DLX3 binds to sites within the *Pgf* gene promoter. A. EMSAs were used to determine DLX3 DNA binding to three putative DLX3 binding sites found within the *Pgf* 5' flanking region. The positions of these sites (designated as probes) were at -4297 (a consensus site), -3061 (a near consensus site) and -1761 (a near consensus site) relative to the start site of transcription for the *Pgf* promoter. The JRE from the glycoprotein hormone α-subunit was used as a positive control. Recombinant (r) DLX3 was added in increasing doses (gray triangles) and DLX3 complexes are designated by the arrow. Free probe at the bottom of the gel is also designated. **B.** EMSAs were performed using JEG3 nuclear extracts (NE) and the -3061 probe as described above. All binding reactions received the same amount of JEG3 NE. In some binding reactions, NRS, DLX3 or C/EBP antisera were added in an effort to perturb the DLX3 binding complex. NRS and C/EBP antisera served as negative controls. To identify the electrophoretic mobility of the DLX3 containing complex, rDLX3 was added as a standard. Free probe, the DLX3 complex, and the DLX3 complex bound to the DLX3 antibody (DLX3 supershift) are designated with arrows.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rDLX3
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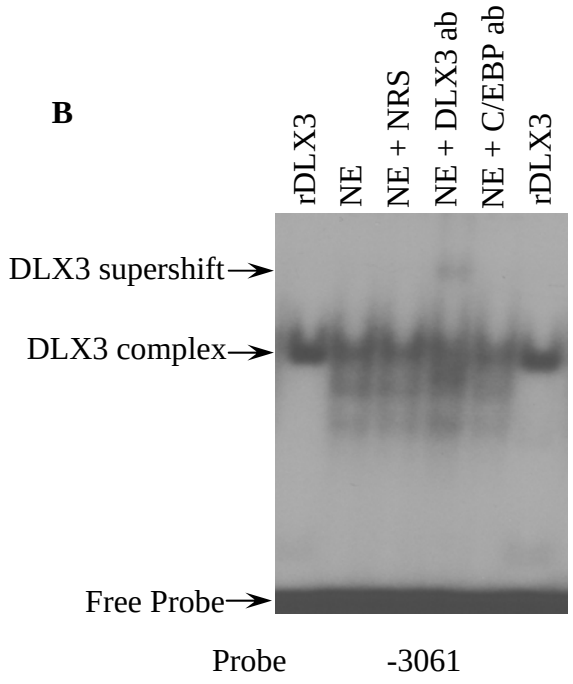
-1761
JRE


B
rDLX3
NE
NE + NRS
NE + DLX3 ab
NE + C/EBP ab
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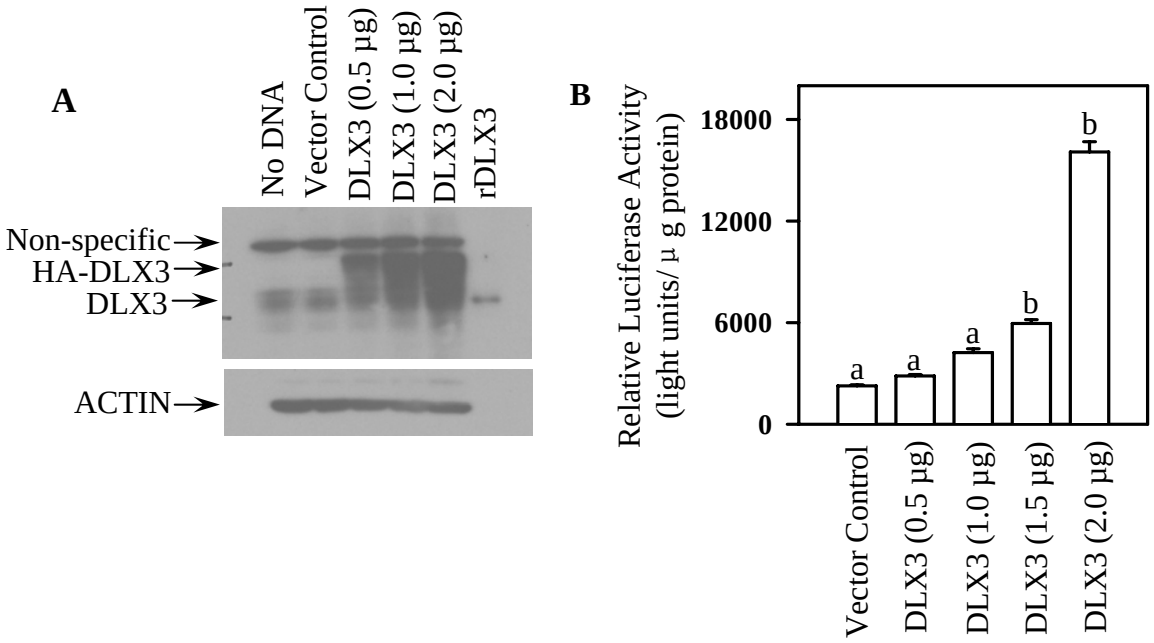

Figure 3.5. DLX3 overexpression is sufficient to activate the *Pgf* gene promoter in JEG3 cells. A. Transient overexpression studies were carried out using the vector control (2.0 µg) or increasing doses of an expression vector for HA epitope-tagged human Dlx3 (HA-DLX3). Whole cell lysates were prepared and subjected to Western blot analysis using the DLX3 antibody to determine the extent of ectopic expression of HA-DLX3. HA-DLX3 migrates slightly slower than endogenous DLX3 due to the addition of three HA epitopes. Recombinant (r) human DLX3 was used as a positive control for the Western blot. To control for lane loading, the samples were reprobed with an antibody to ACTIN. **B**. Transient transfection studies in JEG3 cells were used to determine the effect of DLX3 on *Pgf* promoter activity. Cotransfection of the *Pgf* gene promoter-luciferase reporter with increasing doses of DLX3 expression vector (0, 0.5, 1.0, 1.5, 2.0 µg) resulted in a dose dependent increase in expression of the *Pgf* luciferase reporter. Data are reported as relative luciferase activity standardized by total cellular protein content from representative studies (n=3/treatment) (a and b reflect statistical differences, $p<0.05$).

DLX3 was used as a positive control in these studies. Importantly, DLX3 overexpression increased *Pgf* promoter luciferase activity in a dose-dependent manner (Figure 3.5B). At the highest dose of DLX3 expression vector, *Pgf* promoter-luciferase reporter expression was increased greater than 7-fold. These studies provide clear evidence that DLX3 is sufficient to modulate expression of the *Pgf* promoter in cells of trophoblast origin and support the conclusion that the loss of *Dlx3 in vivo* likely impacts Pgf production at the transcriptional level.

Discussion

Dlx3 is a member of a larger group of homeobox containing transcription factors within the Dlx family [16]. Pairs of *Dlx* family members are grouped in clusters aligned with *Hox* genes on separate chromosomes in mouse and human. For example, *DLX3* and *DLX4* (also referred to as *DLX7*) are tandemly arrayed on human chromosome 17 separated by approximately 17.6 kb of intervening sequence and aligned with the *Hox* B cluster [17]. DLX3 and 4 are both expressed within placental trophoblasts [18]. The *Dlx3*$^{-/-}$ mouse dies *in utero* at E9.5-10, presumably due to failed placental development [11]. Morphologically, the labyrinth of the *Dlx3*$^{-/-}$ placenta appears compact and does not appear to enlarge at a similar rate, compared with wild type mice potentially reflecting a defect in the expansion of the fetal vascular bed (Figure 3.1A). Morasso *et al*. [11] found that defects in both yolk sac and fetal vasculature within the labyrinth were evident in the *Dlx3*$^{-/-}$ placenta; however, it remains unclear whether this is causal in the demise of the developing embryo. Placental trophoblast expression of DLX4 [18] is likely not simply redundant because the presence of Dlx4 in the *Dlx3*$^{-/-}$ mouse embryo cannot rescue the placental phenotype providing evidence for the distinct role of Dlx3 in placental morphogenesis. In primate species, DLX3 expression is conspicuous throughout gestation within syncytial and cytotrophoblasts in microvilli in human

placenta [14] and baboon placental samples obtained throughout gestation (Fazleabas, A., and M.S. Roberson, unpublished observations). This is consistent with the endocrine role of DLX3 in directing transcriptional regulation of the glycoprotein hormone α-subunit of CG, whose expression is restricted to trophoblasts of primates and horses. The mouse placenta does not produce a similar placental borne gonadotropin; as a consequence questions regarding a potential role of Dlx3 in regulating placental endocrine function have not been fully explored.

The present studies are the first to provide insight into the underlying transcriptome modulated by Dlx3 in the developing placenta. The loss of *Dlx3* impacts the expression of a functionally diverse group of genes. For example, genes affected by Dlx3 can be categorized as ligands (such as reduced expression of Pgf), cell signaling molecules (such as down-regulation of Rab6B, Rac/Cdc42 guanine nucleotide exchange factor 6 and up-regulation prostaglandin E receptor 3), regulators of cytoarchitecture (such as down-regulation of keratin complex 2 and phosphatase and actin regulator 1) and extracellular matrix related molecules (such as down-regulation of Mmp9 and up-regulation of lumican and thrombospondin 2). These changes in gene expression point to a spectrum of distinct cellular processes dependent on transcriptional activity and regulation by Dlx3. Focusing on more global changes, we identified a potential impact of loss of *Dlx3* on gene networks related to cell-to-cell signaling and interactions and a cohort of hemoglobin genes related to hematological disease. This gene network analysis lends support to the conclusion that Dlx3 may be involved in development of the fetal vascular bed consistent with a reduction in labyrinth depth. During mouse development, initial hematopoiesis begins in the yolk sac around E7.0 within extraembryonic blood islands [19]. Later (E11.0/12.0), fetal liver becomes the primary site of hematopoiesis. Because it is unlikely that the fetal hemoglobin genes observed in our array analysis are specifically expressed in

labyrinth trophoblasts (the specific site of Dlx3 expression), it seems more likely that hemoglobin mRNA expression occurs in fetal nucleated erythrocytes gradually populating the labyrinth vascular bed primarily from yolk sac at E9.5. Consistent with this notion, fetal erythrocytes were greatly reduced within the labyrinth vasculature in the *Dlx3*$^{-/-}$ mouse at E12.5 [11]. Thus it is reasonable to suspect that loss of the hemoglobin mRNAs in the *Dlx3*$^{-/-}$ placenta in our array analysis is consistent with reduced fetal vasculature and potential for reduced fetal erythrocytes within the labyrinth of the midgestation placenta.

One of the more notable gene transcripts identified by our array analysis was *Pgf*. Array analysis, qPCR and Northern analysis all consistently demonstrated a reduced expression of the *Pgf* transcript in the *Dlx3*$^{-/-}$ placenta. Consistent with coupled biosynthesis and secretion, the *Dlx3*$^{-/-}$ implantation sites tended to secrete less Pgf than wild type placentas in explant cultures, suggesting a functional consequence of reduced *Pgf* gene expression *in vitro*. Our additional studies based on the array results implicate DLX3 as a putative direct activator of the *Pgf* gene promoter in studies using a choriocarcinoma cell model. Examining the initial 5.2 kb of the mouse *Pgf* 5' flanking sequence revealed the consensus or near consensus Dlx3 binding sites (Figure 3.4). All of these sites could bind DLX3 *in vitro*, albeit with variable relative affinity and overexpression of DLX3, was sufficient to increase transactivation of the *Pgf* luciferase reporter gene (Figure 3.5). Interestingly, the extent of DLX3 overexpression necessary to induce *Pgf* promoter-luciferase reporter transcriptional activation was higher than expected. This may be related to our recent observations that the inhibitory SMAD, SMAD6, can functionally interact with DLX3 and prevent DLX3 DNA binding and thus transactivation of target genes in choriocarcinoma cells [13]. In our DLX3 overexpression studies, high doses of DLX3 expression vector leading to relatively high levels of DLX3 may have been necessary to overcome the negative

effects of SMAD6 interaction. As the dose of DLX3 increased, the stoichiometry between DLX3 and SMAD6 would be shifted in favor of unbound DLX3 and a positive transcriptional signal. Thus, our *in vivo Dlx3*$^{-/-}$ mouse implicates Dlx3 as a necessary factor for the expression and secretion of Pgf. Using *in vitro* and cell culture models, our studies provide direct evidence that DLX3 is sufficient to bind to and transactivate the *Pgf* gene promoter.

As discussed earlier, PGF is a member of the VEGF gene family and is thought to play a role in placental and endometrial angiogenic potential [20,21]. The PGF receptor is expressed within the endothelial compartment of the labyrinth and in placental trophoblasts, suggesting a complex role of PGF in placental cell biology. In the human, alternative splicing of the *PGF* transcript generates four isoforms: PGF-1, PGF-2, PGF-3 and PGF-4 [22-25]. In mouse, only one *Pgf* mRNA encoding the equivalent of human PGF-2 has been identified [24]. Structurally related to VEGF, PGF binds to VEGFR-1 and appears to be involved in modulation of vascular permeability during pathological angiogenesis [26]. The mechanistic role of PGF and VEGFR-1 during placental development and angiogenesis has not been fully elucidated. *Pgf*$^{-/-}$ mice appear to be fertile without evidence of placental vascular pathologies [26]; however, PGF has been found to be an important marker for preeclampsia in women, a syndrome of late pregnancy characterized by maternal hypertension, proteinuria, insufficient trophoblast invasiveness, a reduction of PGF levels, and increased soluble anti-angiogenic fms-like tyrosine kinase activity during mid- to late gestation. All of these symptoms are resolved with expulsion of the placenta [27-32]. An important mouse model recapitulating many of the cardiovascular symptoms of preeclampsia has recently been described [33,34]. *Pgf* expression is markedly reduced in this mouse model of preeclampsia as well. The possibility exists that DLX3-dependent changes in PGF may be related to the molecular determinants driving the progression of events leading

to preeclampsia. Additional studies will be required to fully appreciate this possibility.

Dlx3-dependent biosynthesis and secretion of Pgf also affords a potential mechanism for the indirect actions of Dlx3 on target gene expression. Because Pgf is a secreted angiogenic/growth factor and the VEGFR-1 is expressed in cell types that do not necessarily express Dlx3, it is conceivable that Dlx3-dependent loss of Pgf would have a marked impact on other genes within the Dlx3-dependent transcriptome through activation of VEGFR-1. We would predict that some proportions of the genes identified by our array analysis are indirectly regulated by Dlx3 through Pgf-induced signaling and transcriptional activation. This may be of particular importance in situations like preeclampsia where PGF appears to be a significant component of the progression of this disease in women.

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CHAPTER FOUR

ANALYSIS OF THE ROLE OF DLX3 IN PLACENTAL GROWTH FACTOR GENE REGULATION IN TROPHOBLAST CELLS

Li Han, Patricia A. Clark, Ashley K. Woods, Jianjun Xie, Robin L. Davisson and Mark S. Roberson. Analysis of the role of Dlx3 in placental growth factor gene regulation in trophoblast cells. (in preparation).

Abstract

Distal-less 3 (Dlx3), a homeodomain containing transcription factor, is essential for chorionic gonadotropin α subunit gene transcription in primates and placental development in the mouse. The *Dlx3*$^{-/-}$ mouse embryo dies at E9.5-10 due to placental failure. Loss of *Dlx3* in the mouse also results in a marked reduction in the synthesis and secretion of Pgf, a member of the vascular endothelial growth factor family and an important marker of preeclampsia. The current studies examine the molecular mechanisms involved in Dlx3-regulated expression of the *Pgf* gene promoter. To investigate the functional importance of Dlx3 in the regulation of *Pgf* gene transcription, we identify and mutate the two putative Dlx3 binding sites in the 5.2 kb of 5' flanking sequence of the mouse *Pgf* gene. *Pgf* promoter activity is markedly increased with Dlx3 overexpression and reduced by the combined mutation of both of the Dlx3 binding sites. Thus, these putative Dlx3 binding sites are required for regulation of Dlx3-induced *Pgf* gene promoter activity in placental trophoblast cells. DNA binding studies of the two putative Dlx3 binding sites using electrophoretic mobility shift assays and recombinant DLX3 or JEG cell nuclear extracts demonstrate that DLX3 can bind these two sites *in vitro* albeit with site-specific relative affinity. The role for DLX3 is further implicated in the regulation of the *Pgf* promoter by overexpression of the DLX3 interacting protein, SMAD6. We have previously demonstrated that SMAD6 represses DLX3-dependent gene promoters by blocking DLX3-DNA binding through SMAD6-DLX3 interactions. Consistent with this notion, SMAD6 overexpression results in a dose-dependent inhibition of DLX3-induced up-regulation of the *Pgf* gene promoter. siRNA-mediated knock-down of endogenous DLX3 results in a reduction in human *PGF* mRNA levels and PGF secretion from JEG3 cells. Thus, DLX3 appears to be an important determinant for *PGF* gene promoter activity and ultimately secretion in human placental trophoblasts. Since

reduced expression of PGF is an important marker of preeclampsia, we next examined the possibility that *Dlx3* and *Pgf* are mis-regulated in a mouse model of preeclampsia using the BPH/5 mouse line. Implantation sites from control C57 and BPH/5 mice were collected at different gestational ages and transcript abundance for *Dlx3* and *Pgf* was determined. At early gestational stages, *Dlx3* and *Pgf* transcripts were reduced in the BPH/5 mouse placenta compared to controls; while at later gestational ages, both transcripts are overexpressed in the placentas of BPH/5 mouse. When similar preliminary studies were carried out using normal human placentas paired (by age, ethnicity and health status) with placentas collected from patients experiencing preeclampsia, we found that *DLX3* and *PGF* transcript abundance was reduced in the term placentas of preeclampsia patients. These combined studies lead us to conclude that Dlx3 is an important transcriptional activator of the *Pgf* gene promoter. Moreover, mis-regulation of *DLX3* is correlated with altered *PGF* gene expression during preeclampsia suggesting that *DLX3* may be a new and important early genetic determinant regulating the etiology of this critical disease.

Introduction

Dlx3, a homeodomain-containing transcription factor, is essential for placental development in the mouse [1]. Both mouse and human Dlx3 protein is composed of 287 amino acids with homeodomain located in residues 130-189 [2]. The sequence of Dlx3 protein is highly conserved between mouse and human with variation in only five different amino acids, none of which are located within the homeodomain. The *Dlx3* null embryo dies between E9.5 and E10, a time when there is remarkable morphogenesis and expansion of the placental labyrinth. The failure of *Dlx3* null embryos to survive past mid-gestation is thought to be a failure in the establishment of the fetal-maternal vascular interface within the labyrinth [1]. However, Dlx3 dependent

mechanisms involved in this process have not been fully elucidated. Our previous gene profiling analysis demonstrated that *Pgf* mRNA expression was lower in the *Dlx3* null mouse placenta at E9.5 and that Pgf secretion was reduced by the loss of *Dlx3* [3]. Thus, Dlx3 may be an important determinant for *Pgf* gene promoter activation within placental trophoblasts.

Pgf is a member of vascular endothelial growth factor (VEGF) family, consistent with its role as an angiogenic growth factor [4]. Pgf is synthesized within the placenta primarily by cytotrophoblasts and syncytiotrophoblasts [5-7]. Additionally, Pgf is expressed in other tissues including microvascular endothelial cells and human umbilical vein endothelia [4]. In women who subsequently develop preeclampsia, the level of PGF in plasma decreases significantly during gestation compared to normal pregnancy [4,8]. As such, PGF has been used as a predictor for risk of subsequent development of preeclampsia in pregnant women [8,9]. Preeclampsia affects approximately 5% of all human pregnancies and is manifested by onset of maternal hypertension and proteinuria after 20 weeks of pregnancy [10]. The BPH/5 mouse has been reported to be an important animal model for preeclampsia in women [11,12]. The BPH/5 mouse is an inbred strain that spontaneously exhibits clinical features of human preeclampsia such as hypertension and proteinuria during the third trimester of gestation [11]. The BPH/5 mouse also displays reduced serum concentrations of Pgf suggesting that mechanisms involved in the onset and progression of preeclampsia in women and BPH/5 mice may share a common basis [12].

In the present studies, we have furthered our understanding of the effect of Dlx3 on the *Pgf* promoter by studying the effects of mutation of putative Dlx3 binding sites within the *Pgf* gene promoter. Mutation of these Dlx3 binding sites reduces *Pgf* promoter activity in transfected JEG3 choriocarcinoma cells, an *in vitro* model for human trophoblasts. DLX3 is capable of binding to two sites within the mouse *Pgf*

promoter. Engagement of these sites can be functionally disrupted by overexpression of SMAD6, a known inhibitor of DLX3 binding activity. Knockdown of endogenous DLX3 was correlated with reduced the mRNA and protein expression of endogenous PGF in JEG3 cells. These results suggest that Dlx3 is an important regulator of mouse, and likely human *PGF* gene expression in trophoblast cells. Perhaps most importantly, we demonstrate that *Dlx3* is mis-regulated in the BPH/5 mouse model throughout gestation and at term in human placentas from patients with preeclampsia and these transcript profiles correlate well with altered PGF transcript levels in these models.

Materials and Methods

Cell culture and transient transfection studies

JEG3 cells were cultured in DMEM supplemented with 10% FBS. Before transfection studies, cells were split to subconfluent (approximately 60%) cultures 24 h prior to all experiments. The *Pgf* promoter-luciferase reporter containing 5.2kb 5' flanking sequences was used in these studies (referred to as Pgf-Luc). PCR-based site-directed mutagenesis was used to disrupt the two Dlx3 binding sites within the *Pgf* luciferase reporter. This mutation substituted a Not I restriction site for the near consensus Dlx3 binding site. The mutations were confirmed using nucleotide sequence analysis. The SMAD6 expression plasmid was a gift from Dr. Ali Hemmati-Brivanlou (The Rockefeller University, New York, NY). The *Pgf*-Luc reporter was transiently cotransfected into JEG3 cells with Dlx3 expression vector (pKH3-Dlx3) using GeneJuice transfection reagent (Novagen, Darmstadt, Germany) as per the manufacturer's instructions. All transfections were carried out with an equivalent amount of total DNA by supplementing reactions with the parent vector (pKH3). Twenty-four hours after transfection, luciferase activity was determined using reagents from the dual-luciferase reporter assay system (Promega Corp, Madison, WI).

Luciferase activity was standardized by total cell protein content as determined by Bradford assay. All transfection studies were performed in triplicate on at least three separate occasions with similar results. Data are shown as means ± SEM (n=3) of representative experiments.

Electrophoretic Mobility Shift Assay

EMSA was carried out as described previously [13,14]. Recombinant human DLX3 was prepared by *in vitro* transcription and translation using a commercially available wheat germ lysate kit (Promega Corp, Madison, WI) as described previously [14]. Nuclear extracts were prepared from JEG3 cells as described previously [14]. The nucleotide sequences for EMSA probes were as follows (only the forward strand indicated): -3061 binding site, 5'- GTGGGAGTAATTAAAACCTA -3'; Mutant -3061 binding site, 5'-GTGGGAGCGGCCGCAACCTA-3'; -1761 binding site, 5'-ACACAGATAATTAAGAATAC -3'; mutant -1761 binding site, 5'-ACACAGGC GGCCGCGAATAC-3'. Binding reactions (without labeled probe) were maintained at room temperature for 30 min followed by the addition of ^{32}P-labeled oligonucleotide Dlx3 binding site probes. The binding reactions were maintained an additional 30 min and then resolved on native polyacrylamide gels. Following electrophoresis, the gels were dried, and DNA-protein complexes were visualized by autoradiography. In competition studies using EMSA, DNA competitors included a 10-fold molar excess of wild type or mutant Dlx3 binding sites from the *Pgf* promoter to determine the specificity of the protein-DNA interactions. All DNA binding studies were conducted at least three times with similar results.

Preparation of cell lines expressing siRNAs

The mammalian expression vector, pSuper-retro-neo (OligoEngine, Seattle, WA)

was used for preparation and expression of DLX3 siRNA. Each gene-specific insert targeted a 19- nucleotide sequence within the human DLX3 mRNA. The siRNA sequences were as follows: siDLX3-#1: 5'-TGCTGCGGTTTCCTATTTA-3'; siDLX3-#2: 5'-GAAAGCCGCGTACGATCTA-3'; siDLX3-#3: 5'-CCTAGCCACTCAGGAATCA-3'. A control siRNA vector (also cloned into pSUPER-retro-neo) was constructed using a 19-nucleotide sequence (5'-TTCTCCGAACGTGTCACGT-3') putatively without significant similarity to any mammalian gene sequence and therefore served as an appropriate negative control. The forward and reverse strands of oligonucleotides containing the siRNA and nonsense sequence were annealed and cloned into the pSuper-retro-neo vector. All siRNA sequences were confirmed by nucleotide sequencing. JEG3 cells were transfected with the pSUPER-retro-neo siRNA plasmids using GeneJuice transfection reagent as outlined above. Following transfection, stable cell lines were selected using neomycin at 500 μg/ml for 7 days (time until untransfected cells all died) then cultures were maintained in DMEM/10% FBS medium containing 500 μg/ml neomycin.

Western blotting

Cells were collected and lysed in RIPA buffer containing protease inhibitors as described previously [15]. Protein content was determined in all samples using Bradford assay and samples were then suspended in an equal volume of 2×SDS loading buffer [100mM Tris (pH 6.8), 4% SDS, 20% glycerol and 200 mM dithiothreitol]. Protein samples were boiled for 3 min and chilled for 5 min on ice. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes by electroblotting. Membranes were blocked with 5% nonfat milk in Tris-buffered saline. For Western blots, a Dlx3 antibody was used at 1:1000 in TBST, 5% nonfat milk as described previously [14]. The β-actin antibody (Santa Cruz

Biotechnology, Santa Cruz, CA) was used at 1:1000 dilution under identical conditions as the Dlx3 antibody. Secondary horseradish peroxidase coupled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:5000 dilution. Protein bands were visualized by chemiluminescence using commercially available reagents (PerkinElmer Life Sciences, Waltham, MA).

Human PGF-1 ELISA

Stably transfected JEG3 cells expressing control or DLX3 siRNAs were split and cultured in DMEM containing 10% FBS and neomycin. After 48 h of cell culture, media were changed to serum free OptiMEM media (Invitrogen, Carlsbad, CA). OptiMEM media samples were collected at 0, 1, 4 and 8 h and all media samples were analyzed as per the manufacturer's instructions for secreted PGF protein levels using a human PGF-1 ELISA-based immunoassay commercially available (R & D Systems, Minneapolis, MN). Media samples were assayed in triplicate in two separate assays. In OptiMEM media samples collected at time 0, PGF-1 concentrations were below detectable limits of the assay. According to the manufacturer, intra-assay coefficient of variation for this assay was 5.9%. Inter-assay variation was reported as 13.6%. The sensitivity of this assay (minimum detectable dose) was typically less than 7 pg/ml.

RNA isolation

Total RNA was isolated from both C57 (control) and BPH/5 mouse placenta. Briefly, at the specified gestational ages, the placental disk was dissected free from uterine wall and decidua. Total RNA was extracted from all samples using Trizol reagent (Invitrogen, Carlsbad CA) as per the manufacturer's instructions. Human term placental tissues (without patient identifiers) were obtained from Dr. Monique Ho at University of Rochester Medical Center (Rochester, NY) under the guidelines and

approval of Cornell University and University of Rochester Medical Center Committees on the Use of Human Subjects in Research. Full thickness, radial samples from the placental disk were collected, placed in RNA preservation medium, RNALater (Qiagen, Valencia, CA) and stored at -80°C until use. Approximate 30 mg of placental tissues were disrupted by TissueRuptor (Qiagen, Valencia, CA) for 30 seconds at full speed with disposable probes to prevent contamination between samples. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RNA samples were also obtained from stable JEG3 cell lines using similar approaches with samples being homogenized by passing the lysate 6-8 times through a 20-gauge needle. For all samples, RNA quality and concentration were determined by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A ratio of A260/A280 $\geq$ 2.0 confirmed the RNA quality.

Quantitative real-time PCR

Quantitative real-time PCR using probe-based chemistry (outlined below) was used to determine differential expression of genes. Briefly, a total of 2.0 μg total RNA from mouse or human placental tissues (n = 3 each for control and BPH/5 mouse tissues; n = 6 each for control and preeclampsia) or cultured JEG3 cells (n = 3) was reversed transcribed using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA). Forward and reverse primers flanking an exon junction were designed by ProbeFinder version 2.45 (Roche Applied Science, Indianapolis, IN) and synthesized by Integrated DNA Technologies Inc (Coralville, IA). The amplicon-specific fluorogenic probes used were identified from the Roche universal probe library that were labeled with 6-carboxy fluorescein (FAM) and 6-carboxy-tetra-methyl rhodamine (TAMRA) for the 5' reporter and 3' quencher, respectively. The oligonucleotide sequences of primers and identification number for the corresponding

Roche mouse universal probe library are listed in Table 4.1. TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) with uracil-N-glycosylase, commercially available ABI TaqMan gene expression assays (containing forward and reverse primers and amplicon-specific probes) and 20.0 ng cDNA templates were used to set up PCRs in a total volume of 20 μl. All PCRs were performed in triplicate. The ABI TaqMan probes are listed in Table 4.2. PCRs were performed on an ABI 7500 real-time PCR system and analyzed with sequence detection software version 1.3 (Applied Biosystems). Negative controls were run with each sample set, in which the template was replaced by PCR-grade water. Mouse β-actin or human 18s RNA probe sets were used as the endogenous control for normalization of mouse or human placenta tissue cDNA input, respectively. Relative quantification of gene expression was determined by the $2^{-(\Delta\Delta Ct)}$ method [3].

Table 4.1 Mouse primers and Roche universal probes used for qPCR analysis

Gene symbol	GenBank accession no.	Forward primer	Reverse primer	Roche probe no.
Dlx3	NM_010055	ggggatcctataggcagtacg	cctctttcaccgacactgg	32
Pgf	NM_008827	ctgggttggctgtgcatt	ggcaccacttccacttctgt	3

Table 4.2 Human primers and TaqMan probes used for qPCR analysis

Gene symbol	Gene name	GenBank accession no.	Assay ID
DLX3	Distal-less homeobox	NM_005220	Hs00270938_m1
PGF-2	Placental growth factor	NM_002632	Hs00182176_m1

Statistical analysis

Luciferase data were subjected to analysis of variance and differences between treatments were determined using Tukey's Studentized Range Test. Human placenta

samples were subjected to analysis of paired *t*-test. Probability of less than 0.05 ($p<0.05$) was considered statistically significant.

Results

Mutation of Dlx3 binding sites reduces Pgf promoter activity

We previously reported the transcriptional activity of a 5.2 kb fragment of the mouse *Pgf* gene 5' flanking sequence, fused with a luciferase reporter gene [3]. Bioinformatic comparison of 5 kb promoter sequences of human and mouse *Pgf* genes revealed three regions of $\geq$ 75% evolutionarily conserved sequence. Two near-consensus Dlx3 binding sites (at -3061 and -1716 relative to the transcription start site) were identified within the mouse promoter. The more distal site (at -3061) is located in one of these highly conserved regions. To investigate the functional importance of these Dlx3 binding sites in regulating *Pgf* gene transcription, we mutated the two sites individually or in combination in the context of the 5.2 kb promoter of the mouse *Pgf* gene. The basal activity of either of the individual *Pgf* mutant promoters was modestly reduced compared to the wild type promoter. Basal activity of the *Pgf* promoter was reduced to approximately 50% of wild type levels by double-mutation of both Dlx3 binding sites. HA-DLX3 overexpression increased wild type *Pgf* gene promoter activity approximately 4 fold consistent with previous results [3] (Figure 4.1). HA-DLX3 overexpression was confirmed by Western blot using HA antibody (Figure 4.1; shown in the inset). When analyzed separately, the individual mutations in the *Pgf* promoter reduced the response to HA-DLX3 overexpression by approximately 30-40% compared to the wild type. The double-mutation of both putative Dlx3 binding sites nearly abrogated the response to HA-DLX3 overexpression suggesting that both sites were required for promoter activity induced by Dlx3.

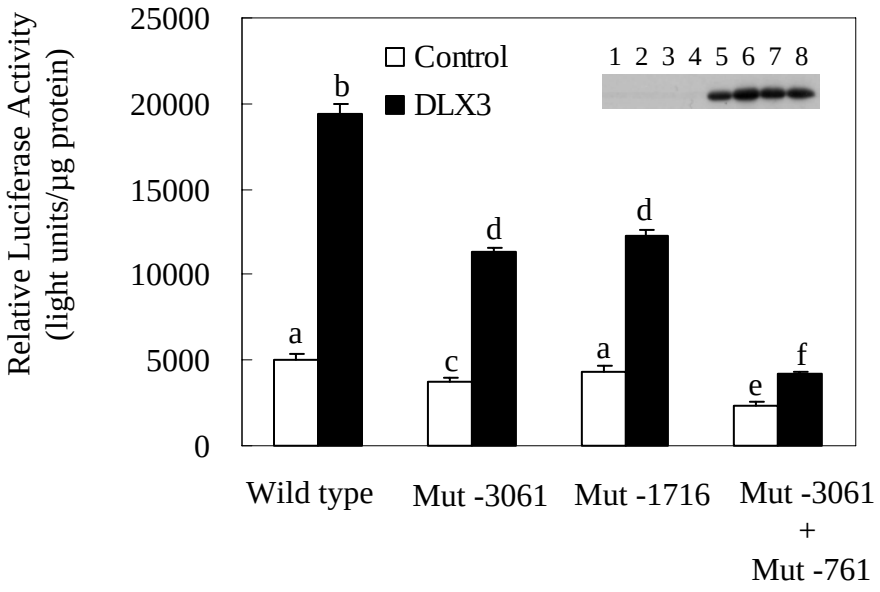

Figure 4.1. Mutation of Dlx3 binding sites reduces *Pgf* promoter activity. Transient transfection studies were used to determine the relative importance of the Dlx3 binding site on expression of the *Pgf* promoter luciferase reporter gene. JEG3 cells were transiently transfected with wild type *Pgf* luciferase reporter or *Pgf* luciferase reporter containing one or double mutations within the Dlx3 binding sites. Cells were cotransfected with control plasmid or a DLX3 expression vector. Data are reported as relative luciferase activity standardized for protein for a representative study (n=3/treatment). The designations a, b, c, d, e and f reflect statistical differences, $p<0.05$. Same numbers mean no statistical significance while different numbers mean statistical significance. The inset is a Western blot using anti-HA antibody. 1-4 lanes were cells transfected with wild type or mutant Pgf gene promoter alone; 5-8 lanes were cells with overexpression of HA tagged DLX3 protein.

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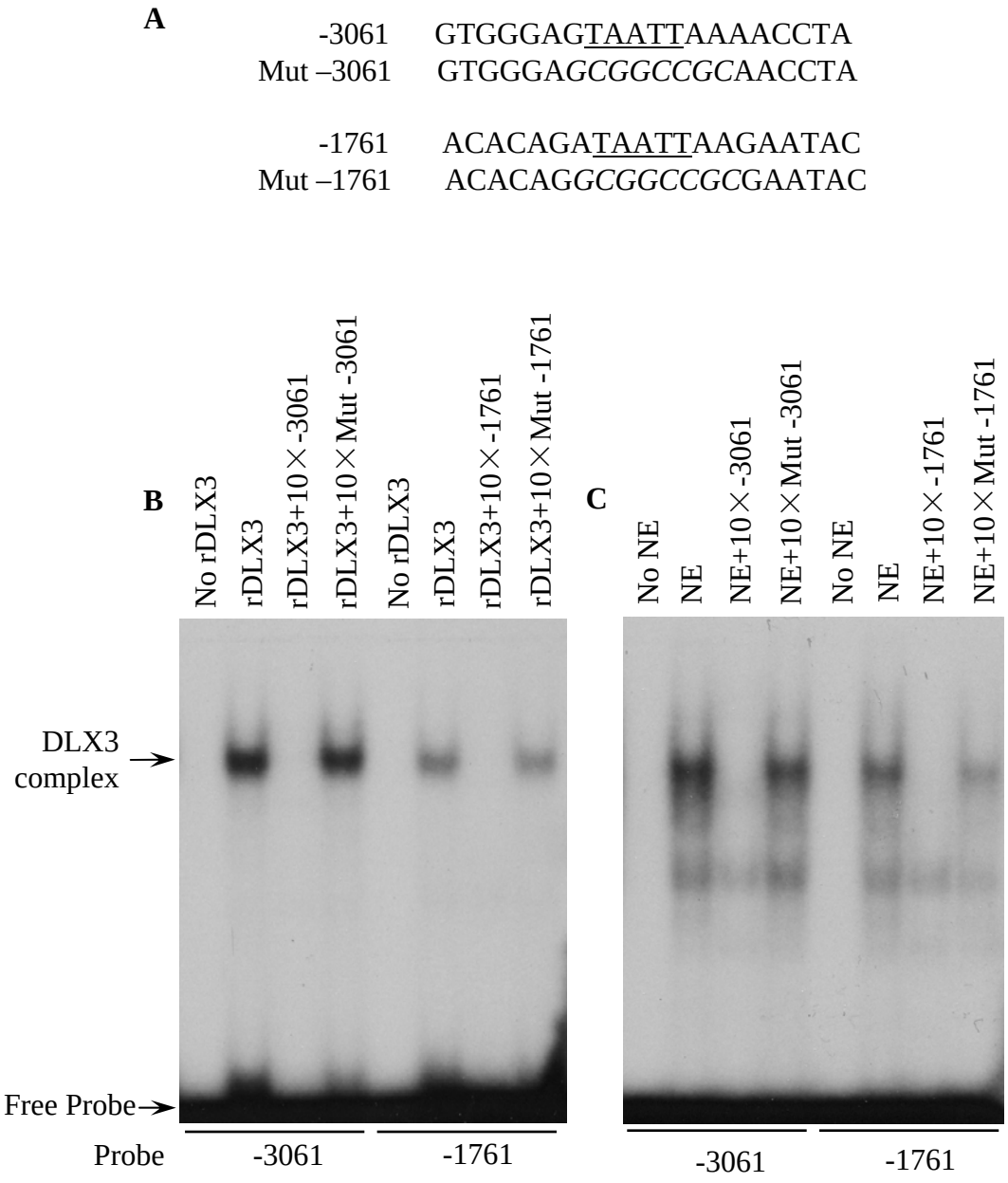
Dlx3 binds specifically cis elements within the Pgf gene promoter

Our previous studies demonstrated that the two Dlx3 binding sites within the *Pgf* 5.2 kb promoter could bind recombinant human HA-DLX3 protein and nuclear extract from JEG3 choriocarcinoma cells [3]; however, the specificity of these interactions was not examined fully. To determine whether DLX3 interaction at these sites was indeed specific, we carried out additional EMSAs with competitive binding to determine the specificity of these interactions (Figure 4.2). The core DNA sequence for DLX3 binding, (A/C/G)TAATT(G/A)(C/G), the -3061 and -1761 sites are depicted in Figure 4.2A. Mutations at these sites reflected substitution of a Not I restriction site effectively disrupting the TAATT central core of the site. EMSA were carried out using recombinant (r) human DLX3 and nuclear extracts purified from JEG3 cells. Consistent with previous reports [3], EMSA revealed that the two wild type sites were sufficient to bind DLX3. Addition of ten-fold molar excess of wild type binding site to these reactions competitively interfered with binding to the radio-labeled probe; however, addition of ten-fold molar excess of the mutant binding sites failed to compete for the binding of HA-DLX3 (Figure 4.2B and C). These results support the conclusion that the DLX3 protein (either recombinant or from nuclear extracts) binds specifically these sequences within the *Pgf* promoter.

SMAD6 inhibits DLX3-dependent Pgf promoter activation in JEG3 cells

To further corroborate the required role for DLX3 binding at these elements, we made use of a SMAD6 overexpression paradigm. SMAD6 has been shown previously to interact directly with DLX3 [14]. The DLX3-SMAD6 protein-protein interaction domain involves a region within DLX3 adjacent to the homeobox domain that is critical for protein-DNA interaction ([14]; see Chapter 2 of this dissertation). In previous studies, SMAD6 overexpression repressed DLX3 transcriptional activity

Figure 4.2. Dlx3 binding to Dlx3 binding sites within the *Pgf* gene promoter is specific. A. Two Dlx3 binding sites within the *Pgf* 5.2 kb promoter are located at position -3061 and -1761 relative to the start site of *Pgf* gene transcription. The central core of the binding site is underlined. Oligonucleotide probes for these binding sites were synthesized and used in electrophoretic mobility shift assays (EMSA). Mutations (shown in italics) within these binding sites are also shown and used in EMSA competition experiments. EMSA was used to detect binding complexes formed on the Dlx3 binding sites using recombinant human DLX3 protein (rDLX3) (**B**) or JEG3 nuclear extracts (**C**). Banding reactions were resolved on native polyacrylamide gels, the gels were dried, and bands were visualized by autoradiography. Free probe and the DLX3-DNA complex are designated with arrows at the bottom or in the middle of the gel respectively.


A
-3061 GTGGGAGTAATTAAAACCTA
Mut –3061 GTGGGAGCGGCCGCAACCTA
-1761 ACACAGATAATTAAGAATAC
Mut –1761 ACACAGGCGGCCGCGAATAC
B
No rDLX3
rDLX3
rDLX3+10×-3061
rDLX3+10×Mut -3061
No rDLX3
rDLX3
rDLX3+10×-1761
rDLX3+10×Mut -1761
DLX3 complex
Free Probe
Probe
-3061
-1761
C
No NE
NE
NE+10×-3061
NE+10×Mut -3061
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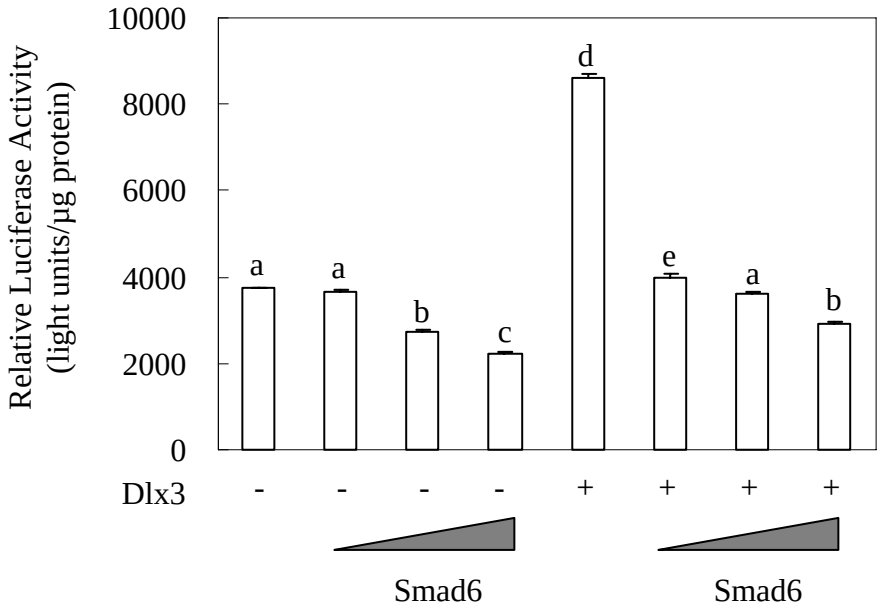


Figure 4.3. SMAD6 inhibits DLX3-dependent *Pgf* promoter activation in JEG3 cells. Transient transfection studies in JEG3 cells were used to investigate the functional relevance of SMAD6 on DLX3-dependent gene expression. *Pgf* gene promoter-luciferase reporter was cotransfected with DLX3 (constant dose, 2 µg) and increasing amount of SMAD6 expression vector (0, 0.5, 1.0, 2.0 µg). Data are reported as relative luciferase activity standardized for protein from representative studies (n=3/treatment). See previous figures for the designations a, b, c, d and e.

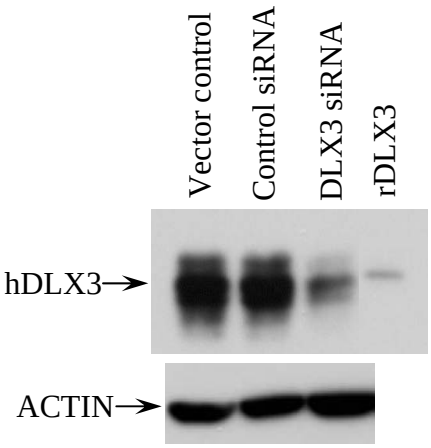
on the *Esx1* gene promoter through inhibition of DLX3 binding to DNA [14]. To investigate the effect of SMAD6 on DLX3-dependent *Pgf* promoter activation, we overexpressed SMAD6 and DLX3 in JEG3 cells along with *Pgf*-Luc reporter. In these studies, DLX3 increased *Pgf* promoter activity as previously observed. Overexpression of SMAD6 repressed basal expression of *Pgf* promoter activity in a dose-dependent manner and essentially blocked DLX3-induced *Pgf* promoter transactivation (Figure 4.3). These studies provide compelling evidence that these two DLX3 cis regulatory elements are required for DLX3-dependent gene activation and that DLX3 occupancy at these sites is central to this regulation.

Stable knockdown of human DLX3 results in suppression of the endogenous PGF gene transcript and PGF secretion

Thus far, we have investigated the role and requirement for Dlx3 on the mouse *Pgf* gene promoter activity based largely on our observations from the *Dlx3* null mouse model and microarray analyses [3]. However, PGF appears to play a critical role as an angiogenic factor within human placenta and reduced PGF in maternal circulation is an important marker of preeclampsia [8,9]. To examine the effect of DLX3 on human *PGF* gene expression, we developed stable cell lines expressing 21-nucleotide siRNAs in an effort to knock down DLX3 in JEG3 cells. Initially, we prepared three separate siRNA constructs and tested each individually in stably selected cell populations compared to a control siRNA as described in Materials and Methods. Preliminary studies indicated that DLX3 siRNA#1 and 3 were modestly effective at knocking down DLX3 protein levels while siRNA#2 was not effective. Subsequently, an additional stable cell line was prepared with siRNAs #1 and #3 co-expressed resulting in significant knockdown of DLX3 as determined by Western blot analysis (Figure 4.4A). To investigate the effect of knockdown of endogenous Dlx3

Figure 4.4. Stable siRNA-mediated knockdown of DLX3 reduces endogenous expression and secretion of human PGF in JEG3 cells. siRNA was used to knock down DLX3 in JEG3 cells. The control siRNA was constructed using a 19-nucleotide sequence without significant similarity to any mammalian gene sequence. The empty pSUPER-retro-neo vector (Vector control) and the control siRNA were used as the negative controls. **A.** DLX3 protein levels were determined by Western blot analysis in siRNA cell lines. Recombinant (r) Dlx3 protein was used as a positive control. β-ACTIN was used as the lane loading control. **B.** Mouse *Pgf* 5.2kb promoter was transfected into different siRNA cell lines. Promoter activity was measured in different siRNA cell lines. **C.** *DLX3* and *PGF-2* transcript levels were detected using q-PCR. Human 18s RNA was used as the internal control. Transcript level of *DLX3* and *PGF-2* in pSuper-retro-neo vector control was set at 1.0. **D.** Control and siRNA stable cell lines were prepared and placed in OptiMEM media, samples were collected at 1, 4, 8 hours and assayed for PGF-1 using a commercially available ELISA-based assay. See previous figures for the designations a, b, c, d, e and f.

A


Vector control
Control siRNA
DLX3 siRNA
rDLX3
hDLX3
ACTIN

B

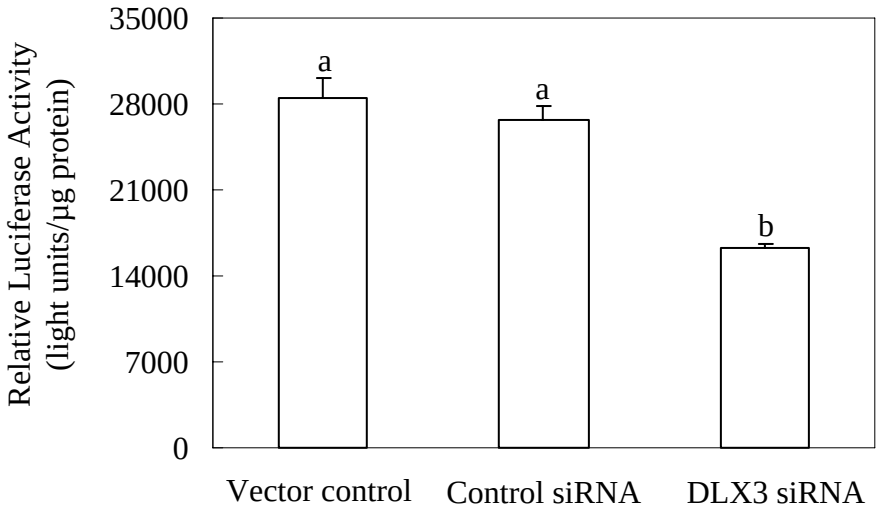
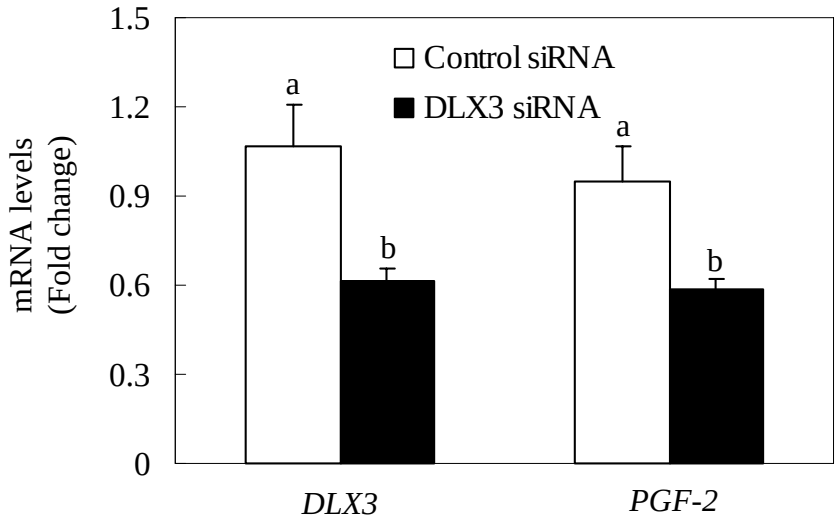
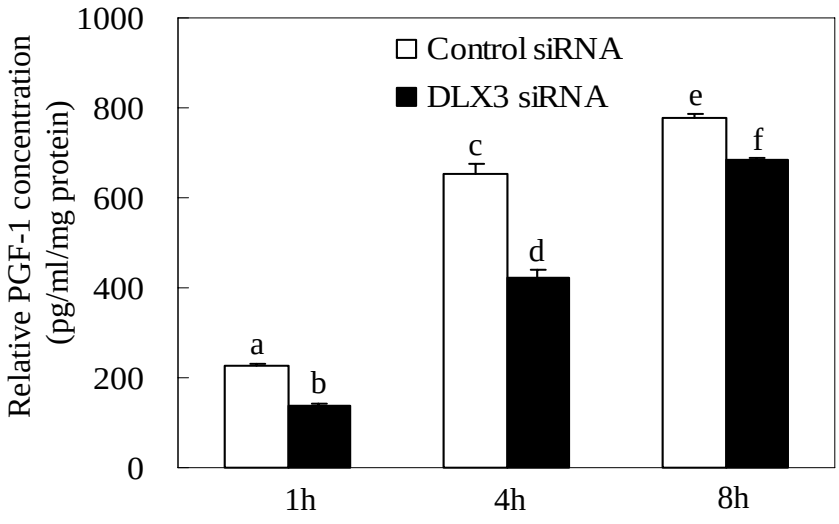

Relative Luciferase Activity
(light units/µg protein)
35000
28000
21000
14000
7000
0
a
a
b
Vector control
Control siRNA
DLX3 siRNA

Figure 4.4 (Continued)

C



D



on mouse *Pgf* promoter activity, we transfected the siRNA cell lines with Pgf-Luc and found that basal *Pgf* promoter activity was markedly reduced by knocking down DLX3 (Figure 4.4B). Then, we determined endogenous transcript levels of human *PGF* in the siRA stable cell lines using qPCR. Both *DLX3* and *PGF-2* transcript levels in control siRNA and *DLX3* siRNA were compared to pSuper-retro-neo vector control. Human *PGF* transcript level in the *DLX3* siRNA cell line was reduced to approximately 58% of the control siRNA cell line consistent with the level of siRNA-mediated knockdown of *DLX3* in this cell line (Figure 4.4C). Thus, knocking down endogenous *DLX3* reduced *PGF* mRNA expression. To determine if this *DLX3/PGF* knockdown was correlated with a reduction in PGF-1 secretion in these cell lines, cell culture media were collected at different time points (1, 4, 8 hours) from both control and *DLX3* siRNA cell lines. PGF-1 protein concentration in the media was examined using an ELISA-based assay. PGF-1 secretion was increased with increasing time of culture at both control siRNA and *DLX3* siRNA cell lines. PGF-1 protein concentration in *DLX3* siRNA cell line was significantly reduced compared to control siRNA cell line at all time points tested (Figure 4.4D). These studies provide important evidence that the human *PGF* gene may be regulated by DLX3, similar to what we observed the effect of Dlx3 on mouse *Pgf* gene transcription. This may have important implications regarding the role of PGF in the pathogenesis of preeclampsia.

Dlx3 and Pgf gene expression is mis-regulated in BPH/5 mouse placenta.

To examine the possibility that *Dlx3* and *Pgf* transcript levels are mis-regulated in the BPH/5 mouse preeclampsia model, quantitative PCR was used to determine placental disk mRNA levels for *Dlx3* and *Pgf* at gestational day E9.5, 12.5, 14.5 and 18.5 in control C57 and BPH/5 mice. For these studies, transcript levels determined in the C57 controls were standardized to 1.0 at equivalent gestational ages for direct

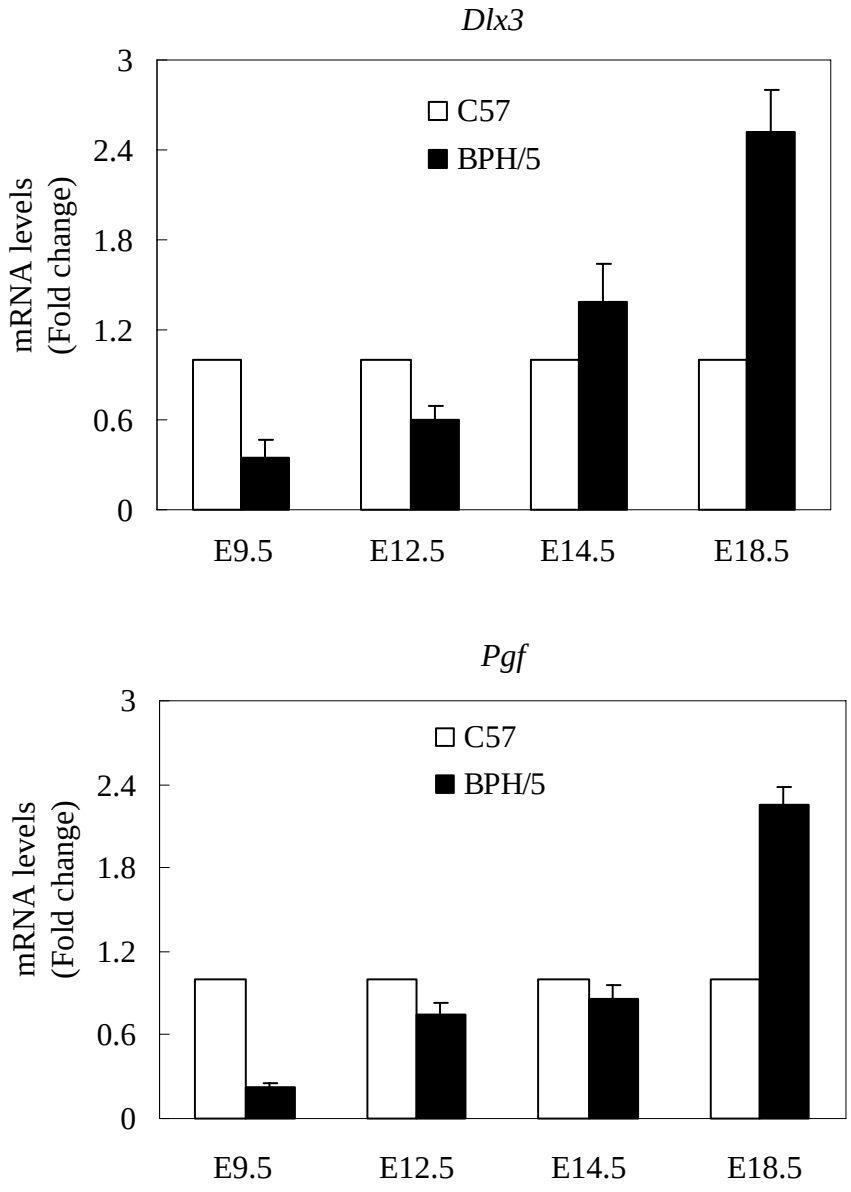


Figure 4.5. *Dlx3* and *Pgf* transcript abundance in BPH/5 mouse placenta. Quantitative PCR was used to determine placental mRNA levels for *Dlx3* and *Pgf* at gestational day E9.5, 12.5, 14.5 and 18.5 in control C57 and BPH/5 mice (n=3/group/gestational age). Transcript level of *Dlx3* and *Pgf* in C57 mouse placenta was set at 1.0.

comparison to BPH/5 transcript levels. Changes in *Dlx3* and *Pgf* transcript levels were found to follow very similar trends throughout gestation. At E9.5, *Dlx3* and *Pgf* were reduced to 20-30% of control levels. At E12.5, *Dlx3* gene expression in the placenta was reduced to 60% compared to the C57 mouse and *Pgf* expression was reduced to 75% of the control level. At E14.5, *Dlx3* gene expression was increased, while *Pgf* gene transcript was modestly reduced in BPH/5 mouse placenta. However, at E18.5, both transcripts were overexpressed 2.0-2.5 fold in BPH/5 mouse placentas compared to the C57 controls (Figure 4.5).

DLX3 and PGF-2 transcript levels tend to be reduced in term placenta of preeclampsia patients compared to control patients.

Preliminary studies of placental samples from both control (n = 6) and preeclampsia (n = 6) patients were collected by Dr. Monique Ho from University of Rochester Medical Center. For this study, the diagnosis of preeclampsia was defined as two diastolic blood pressure measurements ≥90 mm Hg measured 4 hour to 1 week apart and proteinuria of ≥300 mg on a 24 hour urine collection [10]. Control patients were pregnant women without history of hypertension of any kind. Exclusion criteria for patients in this preliminary study included evidence of intra-uterine growth retardation, prior chronic hypertension, chorioamnionitis, fetal anomaly or chromosome abnormality, multiple gestation and maternal diabetes. Control and preeclampsia patients were paired at the same gestational age, labor status, maternal ethnicity, age and parity (nullipara or multipara). Quantitative PCR demonstrated that *PGF-2* gene expression in preeclampsia patients was significantly reduced to approximately 50.2% of control patients; while *DLX3* mRNA expression was significantly reduced to about 66.9% of control patients (Figure 4.6). Taken together with the transcript profiling in the BPH/5 mouse model, these studies suggest that both

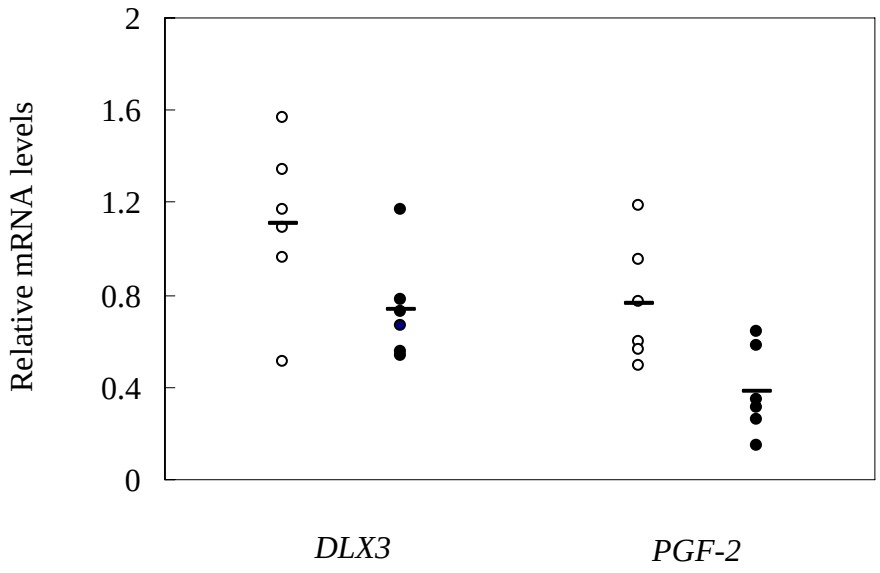


Figure 4.6. Preliminary studies of *DLX3* and *PGF-2* transcript abundance in term human placenta of control and preeclampsia patients. Quantitative PCR was used to determine term placental mRNA levels for *DLX3* and *PGF-2* in control (open circles) and preeclampsia patients (black dots) (n=6/group).

DLX3 and *PGF-2* are coordinately mis-regulated during preeclampsia.

Discussion

In mice, Dlx3 is a homeodomain-containing transcription factor that is essential for placental development. Dlx3 is exclusively expressed in ectoplacental cone and chorionic plate in mouse placenta at E8.5, and later in trophoblasts within the labyrinth layer at E10.5 [1]. Dlx3 continues to be expressed in the labyrinth throughout later gestation [15]. In primate species, DLX3 is nuclear localized to both cytotrophoblasts and syncytial trophoblast within microvilli in human placenta at approximately 8 weeks gestation and at term [16]. *Dlx3* null mouse embryos die at E9.5-10.5 due to defects in the development of labyrinth layer of the placenta [1]. Microarray analysis of the gene profile regulated by Dlx3 revealed *Pgf* is one of the genes with reduced expression in *Dlx3* null mouse placenta suggesting that Dlx3 may be an important regulator of *Pgf* gene expression [3].

Pgf belongs to a group of proangiogenic peptides within the VEGF family [4]. Few studies have been carried out to examine the regulation of *Pgf* gene expression in any cell type. Hypoxia is the major regulator of VEGF transcription via binding of hypoxia-inducible transcription factors to the hypoxia-responsive element located in the VEGF promoter [17]. Some studies have shown that, in contrast to VEGF, Pgf is not hypoxia-inducible [7,18], but other studies have reported that both hypoxia and hyperoxia can up-regulate *Pgf* expression during angiogenesis in certain tissues [19]. Thus, it is not fully understood how oxygen tension affects *Pgf* gene expression. In retinal endothelial cells, high glucose increases expression of Pgf at both the mRNA and protein level suggesting that Pgf may play a role in the retina during diabetes [20]. In epithelial cells, Pgf is up-regulated by forkhead/winged helix transcription factor FoxD1. FoxD1 binds to a conserved site in the *Pgf* promoter and activates its

transcription [21]. In human trophoblast cells, GCM1 regulates *PGF* gene transcription. Overexpression of GCM1 increases human *PGF* promoter activity [22]. In the mouse, Gcm1 plays a critical role in the developmental organization of the labyrinth layer of the placenta. The *Gcm1* knockout mouse is characterized by a distinct reduction or absence of labyrinth development [23]. In addition to specific integration of *PGF* gene expression by cell-type specific transcription factors, expression of the *PGF* gene is also regulated at the epigenetic level. *PGF* expression is reduced by promoter methylation in lung and colon cancer cell lines and demethylation at these sites restores *PGF* gene transcription [24].

Our previous results demonstrated that overexpression of DLX3 increased *Pgf* promoter activity in cultured trophoblast cells [3]. The present studies demonstrated that mutation of DLX3 binding sites within the *Pgf* promoter reduced the *Pgf* promoter activity, suggesting that these mutations interfered with DLX3-DNA interactions. Consistent with these mutagenesis studies, activation of the *Pgf* promoter by DLX3 could be inhibited by SMAD6, an inhibitory binding partner of DLX3 (see Chapter 2 of this dissertation). The repressive effect of SMAD6 is likely due to inhibition of DLX3-DNA binding. In siRNA cell line with knockdown of endogenous DLX3 expression, human PGF mRNA expression and protein secretion were reduced, consistent with the hypothesis based on the *Dlx3* null mouse model. These experiments help to confirm previous studies in mouse and perhaps more importantly provide key evidence that DLX3 is a regulator of human PGF expression in placental trophoblasts.

As discussed earlier, PGF is a VEGF family member and appears to be synthesized primarily by placental trophoblasts [5-7]. In addition to the placenta, PGF is also expressed in other tissues including microvascular endothelial cells, human umbilical vein endothelia, bone marrow, uterine natural killer cells and keratinocytes [4].

Alternative splicing of the human *PGF* transcript generates four protein isoforms: PGF-1, PGF-2, PGF-3 and PGF-4 [5,25,26]. Differences among the four isoforms include the insertion of a heparin-binding domain in PGF-2 and PGF-4 that results in increased association with the cell membrane or altered affinity for PGF receptors [4]. In the mouse, only one *Pgf* mRNA has been identified, which encodes the homolog of human PGF-2 [27]. PGF-2 specifically binds to VEGFR-1 with high affinity [28]. In trophoblast cells, the downstream signal transduction pathways activated by PGF include PLC-γ and JNK [29,30]. Interestingly, embryogenesis appears to be normal in *Pgf* knockout mice, suggesting that the role of Pgf in placental development may be redundant with other proangiogenic factors or there may be fundamental differences in the function of human and mouse Pgf in placental angiogenesis [31].

PGF has been found to be an important marker for preeclampsia in pregnant women where reduced PGF serum levels are associated with disease progression [9,32]. Serum concentrations of the soluble form of the VEGF receptor (sFLT-1) have also been shown to be increased in preeclampsia patients. Since sFLT-1 is capable of binding to free PGF and VEGF, one central hypothesis accounting for preeclampsia progression is an imbalance of angiogenic factors such as PGF cause by abnormal levels of sFLT-1 [4,33-35]. Despite considerable research effort in this area, the etiology and mechanism underlying preeclampsia are still not fully understood. Thus at present, the only effective treatment for preeclampsia is delivery of the placenta. Development of animal models that fully imitate this hypertensive disorder may broaden our understanding of this syndrome and contribute to our understanding of effective prevention and treatment [36].

Three mouse models have been described that display characteristics of preeclampsia. A transgenic mouse model has been created with females expressing human angiotensinogen and males expressing renin. After mating these two separate

models, female mice develop hypertension, proteinuria, and glomerular enlargement during pregnancy which resembles aspects of human preeclampsia [37]. The catechol-O-methyltransferase (COMT) knockout mouse has also been described as a model for preeclampsia. COMT is an enzyme that inactivates catecholamines and metabolizes hydroxyestradiol to 2-methoxyestradiol (2-ME). Levels of COMT and 2-ME are significantly lower in women with severe preeclampsia [38]. Pregnant mice deficient in COMT show a preeclampsia like phenotype such as placental insufficiency, hypertension and proteinuria [38]. While both of the mouse models have generated considerable interest in preeclampsia research, neither are spontaneous nor display a more complete repertoire of symptoms linked to this human disease.

The BPH/5 mouse is a subline derived from the spontaneous hypertensive strain BPH/2, but has a lower blood pressure than BPH/2 mouse [11]. BPH/5 mice display elevated baseline blood pressure and perhaps most importantly, hypertensive clinical features of human preeclampsia during pregnancy [11]. This finding is clearly consistent with the fact that women with elevated baseline blood pressure have an increased risk of preeclampsia [39]. The blood pressure of pregnant BPH/5 mice is stable during the first two weeks of gestation, then increases at the beginning of the third week (third trimester), peaks before delivery and returns to prepregnancy level after delivery of pups [11]. The BPH/5 mouse has late-gestational proteinuria and progressive glomerulosclerosis, which are also consistent with human preeclampsia [12]. BPH/5 mice show reduced expansion of the placenta toward the decidua starting as early as E12.5 in gestation, and decidual vessels of BPH/5 placentas have narrowed lumens and thickened walls [12]. Although both BPH/5 mice and $Dlx3^{-/-}$ mice show placental defects such as reduced vascularization within the labyrinth, there is some difference between these two mouse placentas. The BPH/5 mouse placenta has reduced junctional zone [12] (a zone between decidua and labyrinth), while the $Dlx3^{-/-}$ mouse

placenta shows reduced labyrinth [1].

Our present studies determined the relative gene expression of *Dlx3* and *Pgf* in the placenta of BPH/5 mouse through gestation. We selected early, mid and late gestational ages in an attempt to examine possible changes in gene expression prior to the initiation of overt maternal disease symptoms. The direct comparison to the C57 mouse strain as a control is based on the observation that one of the early lines that contributed to the selection of the BPH strains was C57. Our studies with these mice demonstrated that *Dlx3* and *Pgf* mRNA expression levels were markedly lower in the BPH/5 mouse placenta compared to the control mouse in the early gestational stage. Interestingly, these changes clearly occur prior to maternal disease suggesting that early deficiencies in Dlx3 and Dlx3-dependent genes may underlie key genetic mechanisms leading to later onset of maternal disease. These early changes in *Dlx3* transcript levels are also correlated with reduced *Pgf* expression, consistent with what has been observed in pregnant women who later develop preeclampsia. Women that go on to develop preeclampsia have lower serum PGF levels early in gestation (13 to 16 weeks) compared to women having normal pregnancies [8].

In mice, at later gestational ages (E18.5), our results demonstrate that both *Dlx3* and *Pgf* mRNA expression were increased compared to the C57 control females. Initially, this shift from reduced *Dlx3* and *Pgf* transcript levels to an overexpression scenario was difficult to reconcile. However, this shift may be explained by the apparent, progressive demise of BPH/5 embryos more severely affected by disease of the mother [11]. One of the original observations made in the BPH/5 mouse model was reduced litter size. More recent analyses revealed that the BPH/5 phenotype was observed to have considerable variation with more severely affected embryos actually dying *in utero*. Thus, by late gestation (E18.5), the BPH/5 fetuses that remain are apparently the least affected.

Our goal with the analyses of the BPH/5 mouse model was to determine if expression of *Dlx3* and *Dlx3*-dependent genes such as *Pgf* were altered during preeclampsia. Early gestational time points could be appropriately examined in this animal model as described above. However, our underlying interest was to determine if D*LX3* and *PGF* expression were altered in women experiencing preeclampsia. Previous studies had shown that serum PGF level in some women with preeclampsia was reduced during late gestation compared to the controls [8]. Based upon this link, we examined expression of *DLX3* and *PGF* in term placentas from control and preeclampsia patients. In our preliminary studies, patients were paired by gestational age, labor status, maternal race, maternal age and parity (nullipara or multipara) to exclude these factors that may affect disease risk and progression. As predicted by earlier studies, *PGF* expression was significant lower in term placenta from preeclampsia patients compared with control (p=0.023). Consistent with these findings, *DLX3* transcript abundance was also significantly reduced (p=0.041) suggesting the possibility of a correlation between reduced *PGF* expression and the putative regulator of *PGF* promoter activity, namely DLX3. In summary, these studies help to support the hypothesis that mis-expression of *DLX3* and the *DLX3* gene network may underlie an important genetic mechanism leading to maternal disease during pregnancy.

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CHAPTER FIVE

SUMMARY AND DISCUSSION

The placenta is a vital organ that provides a maternal-fetal interface. The placenta provides mechanisms of nutrient uptake, exchange of gases between the fetal and maternal blood supply and elimination of fetal waste products. Meanwhile, the placenta produces a host of hormones and growth factors which contribute to fetal growth and metabolism. Dlx3 is a homeobox-containing transcription factor, which is essential for normal placental development in the mouse. *Dlx3* knock out mice die at E9.5-10 due to developmental defects of the labyrinth layer in the placenta [1]. My hypothesis is that Dlx3 plays a critical role in placental development by regulating its related genes. This hypothesis is supported by the following lines of evidence:

1. Dlx3 is a placental specific transcriptional regulator for some hormones such as the α subunit of hCG and progesterone.

Previous studies from the Roberson lab have shown that Dlx3 is a cell-type specific transcriptional activator in placental trophoblasts. In human trophoblast cells, DLX3 plays a key role in regulating expression of the α subunit of hCG, a key hormone of early pregnancy in primate placenta [2]. Trophoblast-derived hCG plays a critical role in the maintenance of progesterone secretion from the CL until the placenta can produce sufficient progesterone to maintain pregnancy [3]. DLX3 binds to and transactivates the promoter of the α subunit of hCG gene via a cis acting element termed JRE. Mutations within the JRE markedly reduce basal expression of the α subunit of hCG because DLX3 cannot bind the mutant JRE [2].

In addition to regulation of the α subunit of hCG, DLX3 has also been shown to be required for trophoblast-specific expression of the 3βHSD gene [4]. 3βHSD is an enzyme essential for the biosynthesis of all active steroid hormones including progesterone. 3βHSD is expressed in the human placenta and is thought to be required for the biosynthesis of placental progesterone and thus the maintenance of pregnancy[5].

In human placental cells, transfection studies have identified two novel trophoblast-specific enhancer elements within the *3βHSD* gene promoter. These two enhancers bind DLX3 and AP-2γ, a combination of cis elements and regulatory factors shared with regulation of the glycoprotein hormone α subunit promoter. Interestingly, the combined actions of DLX3 and AP-2γ appear to be necessary for the transcriptional regulation of both the glycoprotein hormone α subunit and *3βHSD* genes in trophoblasts suggesting commonality in transcription factor regulatory combinations or codes directing cell-type specific gene expression [2,6].

2. Dlx3 regulates trophoblast invasion and placental vascularization by regulating gene expression of *Pgf* and *Mmp9*.

To develop a more comprehensive understanding of the gene profile regulated by Dlx3, microarray analysis was used to determine differences in gene expression within the placenta of $Dlx3^{+/+}$ and $Dlx3^{-/-}$ mice. The array analysis revealed differential expression of 401 genes, of which 194 genes were reduced while 207 genes were increased. The loss of Dlx3 impacts the expression of a functionally diverse group of genes. The gene network analysis revealed two interrelated gene networks strongly impacted by the loss of *Dlx3*. One network was composed of a number of important signaling ligands/receptors (Pgf and EGF receptor), signaling molecules and extracellular matrix-related molecules (see Chapter 3 of this dissertation). This cell-to-cell signaling and interaction gene network was linked to a second network composed of genes related to hematological disease. These two networks were linked via the putative regulation of hemoglobin genes though EGF receptor signaling. Regulation of embryonic/fetal forms of hemoglobin in fetal, nucleated erythrocytes may be consistent with the role of Dlx3 in supporting an emerging fetal vascular compartment associated with the placental labyrinth [1,7]. Thus, changes in the Dlx3-modulated cell-

to-cell signaling and interaction network affects another gene network related to hematological disease. These types of gene network relationships provide an important opportunity to examine the specific impact of related genes more broadly on the functional development and morphogenesis of the mouse placenta.

Examination of the array data drew our attention to changes in expression of the *Pgf* gene. Few studies have been carried out to examine the gene regulation of *Pgf*. Hypoxia [8], high glucose [9] and some transcription factors such as FoxD1[10] and Gcm1 [11] have been shown to regulate *Pgf* gene expression. To expand this understanding of *Pgf* gene regulation, our results showed that loss of *Dlx3* resulted in a marked reduction in mRNA expression and secretion of Pgf in the placenta of *Dlx3*$^{-/-}$ mice compared with the wild type mouse placenta. We investigated the molecular mechanisms involved in Dlx3-regulated expression of the *Pgf* gene promoter. We cloned 5.2 kb of the *Pgf* 5' flanking sequence for use in reporter gene assays and determined that overexpression of Dlx3 markedly increased the *Pgf* promoter activity supporting the conclusion that Dlx3 may have a direct effect on activation of Pgf promoter in placental cells. Human *PGF* mRNA and protein secretion are also reduced by knocking down endogenous DLX3 using siRNA in choriocarcinoma cells. Thus, Dlx3 appears to be an important determinant for *Pgf* gene promoter activity and ultimately secretion in placental trophoblasts.

To determine the functional importance of Dlx3 in the regulation of *Pgf* gene transcription, we mutated two putative Dlx3 binding sites in the context of 5.2 kb of 5' flanking sequence of the mouse *Pgf* gene. *Pgf* promoter activity was markedly increased with Dlx3 overexpression and reduced by the combined mutation of both of the Dlx3 binding sites. DNA binding studies using electrophoretic mobility shift assay demonstrated that recombinant DLX3 or nuclear extracts from JEG3 cells could not bind to the mutant binding sites within the *Pgf* promoter suggesting that these putative

Dlx3 binding sites are required for regulation of Dlx3-induced *Pgf* gene promoter activity in placental trophoblast cells. Studies using chromatin immunoprecipitation to determine whether Dlx3 can bind to the binding site within the *Pgf* promoter will be essential to test whether Dlx3 has a direct effect on *Pgf* promoter. This study is now in progress and will provide important evidence that Dlx3 regulates *Pgf* gene expression through directly binding on *Pgf* promoter.

In addition to *Pgf*, the gene transcript of *Mmp9* was also reduced in the *Dlx3*$^{-/-}$ mouse placenta (in Chapter 3) and at early gestational stages in the BPH/5 mouse placenta compared to the control. MMP9 is an enzyme that belongs to a larger family of MMPs, which break down extracellular matrix. MMP9 is involved in normal physiological processes, such as embryonic development and tissue remodeling, as well as in disease processes, such as arthritis and cancer metastasis [12]. Expression of MMPs by extravillous cytotrophoblasts plays an important role in placental invasion into the uterine endometrium during early pregnancy. In particular, MMP9 is strongly expressed by extravillous cytotrophoblasts during trophoblast invasion in the first trimester of gestation [13]. Aberrant expression of MMP9 by extravillous cytotrophoblasts has been linked to preeclampsia [14,15]. Studies from our lab showed that Dlx3 can regulate *Mmp9* gene expression in the placenta. Dlx3 increased mouse *Mmp9* gene promoter activity in a dose dependent manner. Furthermore, EMSA and ChIP assay demonstrated that Dlx3 could bind to the mouse and human *MMP9* gene promoter respectively (PA Clark and MS Roberson, unpublished data).

3. Dlx3 is involved in placental angiogenesis by regulating anti-angiogenesis factor thrombospondin 2 (*Thbs2*).

Thbs2 is a matricellular glycoprotein that is involved in regulation of collagen matrix formation and blockade of angiogenesis [16]. The gene expression of *Thbs2* is

upregulated in *Dlx3*$^{-/-}$ mouse placenta using microarray analysis (see Chapter 3 of this dissertation). This finding was confirmed by q-PCR. The level of secreted protein of Thbs2 in the mouse placenta was Dlx3$^{-/-}$ > Dlx3$^{+/-}$ > *Dlx3*$^{+/+}$ (PA Clark and MS Roberson, unpublished data). Thus, Dlx3 may regulate placental angiogenesis by inhibiting anti-angiogenesis factor Thbs2.

In summary, Dlx3 is a very important factor in the regulation of placenta function by regulating placental hormones, trophoblast invasion, placental angiogenesis and vascularization. The function of Dlx3 in the placenta can be further studied by the following experiments:

a) Tetraploid aggregation assay

Embryonic stem (ES) cells give rise to all the structures of the fetus and to the extraembryonic mesoderm including the yolk sac, the allantois and the fetal blood vessels of the placenta, but not to the trophoblast of the placenta [17]. However, tetraploid blastomeres, which are generated by fusing two-cell embryos, form trophoblast but not the fetus [18]. Tetraploid aggregation assay is an assay that a tetraploid embryo is aggregated with diploid ES cells. The resulting placenta has trophoblast lineages of tetraploid genotype and fetal mesoderm and vasculature of the ES genotype [18]. *Dlx3* null mouse embryos die between E9.5-10 [1]. To determine the site of action of the *Dlx3* gene, on the trophoblast or fetal component, tetraploid aggregation assay can be used. If the embryo is normal till birth when *Dlx3*$^{-/-}$ ES cells are combined with wild type tetraploid, tetraploid aggregation rescue the placental phenotype. This indicates that the primary defect lies in the trophoblast lineage. However, if no rescue of the embryonic defects, then the *Dlx3* mutation must cause defects in the development of the fetal component of the placenta.

b) Conditional deletion of Dlx3 in epiblast

Our traditional *Dlx3* knockout mouse can not distinguish the role of Dlx3 between extraembryonic tissue and embryonic tissue. The Meox2-Cre mouse is useful for determining the contribution of the placenta to the *Dlx3*$^{-/-}$ mouse embryo. Meox2-Cre is the mouse line that expresses the Cre recombinase under the control of the endogenous Meox2 promoter. This promoter drives expression of Cre throughout the epiblast following implantation [19]. It can be used to delete floxed gene alleles only in the embryo [20]. This study is now in progress and our preliminary data showed that embryos with conditional inactivation of *Dlx3* gene in the embryo survive until birth and weaning, but shows defects in hair, skin and teeth. The mice showed alopecia all over the body later in adult life (PA Clark and MS Roberson, unpublished data). Studies in Maria Morasso's lab showed that conditional deletion of *Dlx3* in the mouse epidermis results in complete alopecia, which was thought to be caused by failure of the hair shaft to form [21]. However, there was no change in the hair follicles and cell layers of the skin in our mice. One explanation exists on the caveat of the Meox2-Cre mouse that the Cre expression in the epiblast is mosaic, which means that not all cells in the epiblast express Cre. Further more studies need to be done to address the mechanisms of the skin defects in our mice.

c) Embryo transfer study

To test the effect of the heterozygous mother on the placental defects in *Dlx3*$^{-/-}$ mouse, the embryo transfer studies can be used. All the blastocysts at E3.5, generated by the mating of *Dlx3* heterozygous mouse, are transferred to pseudo-pregnant *Dlx3* wild type mother. At E9.5, the mouse embryo and placenta are collected. The placenta of *Dlx3*$^{-/-}$ mouse embryo is compared to the placenta of *Dlx3*$^{+/+}$, *Dlx3*$^{+/-}$ mouse embryo to investigate if there is still placental defect in the *Dlx3*$^{-/-}$ mouse embryo.

How is Dlx3 regulated in the placenta?

MicroRNA (miRNA)

Recent studies have focused on miRNAs, short RNA molecules, on average 22~23 nucleotides. MiRNAs are post-transcriptional regulators that bind to complementary sequences in the 3' untranslated regions of target mRNA [22,23]. MiRNA are highly conserved in eukaryotic organisms and are thought to be a vital and evolutionarily component of genetic regulation [24]. In the serum of pregnant women, miRNAs are significantly elevated and their levels are correlated with pregnancy stage, which implies that serum miRNAs can be used as promising biomarkers [25]. MiRNAs are differentially expressed in human placentas from preeclamptic pregnancies *versus* normal pregnancies [26]. Future studies should investigate whether the miRNAs specific for *Dlx3* are changed in preeclampsia. To examine this idea, we selected 5 miRNAs which potentially target on *Dlx3* including hsa-miR-19a, hsa-miR-365, hsa-miR-9, hsa-miR-506 and mmu-miR-124a. Q-PCR will be used to detect the level of these miRNAs in the placenta of both control and preeclampsia patients. These studies are currently underway in the Roberson lab.

Smad6

In Chapter 2, we examined the binding partners of Dlx3 to verify the hypothesis that Dlx3 activity may be organized through association with other nuclear regulators of transcription. We demonstrated that DLX3 interacted with the inhibitory SMAD, SMAD6, a transcriptional regulator downstream of the BMP and TGFβ signaling pathways. DLX3 and SMAD6 are colocalized in the nucleus of choriocarcinoma cells and trophoblast cells from human placenta. Our study demonstrated that Dlx3 is a transcriptional activator of gene *Esx1* by binding to *Esx1* gene promoter. SMAD6 effectively inhibited the binding of DLX3 to *Esx1* gene promoter, resulting in SMAD6

attenuating DLX3-dependent promoter activation of the *Esx1* promoter. Further, knockdown of endogenous SMAD6 by small interference RNA increased DLX3-dependent expression of the *Esx1* gene promoter. Collectively, these studies provide important evidence that DLX3 does not function alone in the context of trophoblast cell biology and is indeed influenced by interacting partners.

We then investigated the mechanism of SMAD6 regulating the transcriptional effect(s) of DLX3 on its target genes. SMAD6-DLX3 interaction clearly resulted in a reduction of DLX3-DNA binding activity, probably via steric interference. Our results also demonstrated that the DLX3 domain interacting with SMAD6 included the first two α helices of the homeodomain. Because these two α helices are central to DNA binding, SMAD6 interaction would potentially interfere with DLX3 and DNA binding. This mechanism potentially alleviates a need for recruitment of transcriptional co-repressors, since the DLX3-SMAD6 complex may preclude association of DLX3 with specific target gene promoter elements. Thus, SMAD6 repressed DLX3 transcriptional activity through inhibition of DLX3 and DNA binding. However, the binding domain of SMAD6 associating with DLX3 protein is unknown. Structure-function analysis of the SMAD6 protein will provide us with better insight into the binding region of SMAD6 with DLX3.

A number of growth factors have been shown to regulate Smad6, such as EGF, TGFβ and BMPs [27]. In many cases, the effects of these growth factors are mediated through R-Smads (Smad 1, 2, 3, 5 and 8) and C-Smad (Smad4) dependent transcriptional mechanisms. This has led to a hypothesis implicating an intracellular negative feedback loop, whereby positive TGFβ/BMP signals are modulated by accumulation of induced I-Smads [27]. Interestingly, Dlx3 expression patterns during Xenopus development depend in part upon BMP/TGFβ signaling gradients [28,29]. Inhibition of these gradients using the BMP receptor antagonist results in a dose-

sensitive inhibition of Dlx3 mRNA expression. Dlx3 expression in mouse keratinocytes is also regulated by BMP/TGFβ in a manner potentially coordinate with I-Smad expression [30]. This supports speculation that not only might BMP/TGFβ-regulated Smad6 expression serve as a negative feedback mechanism controlling the duration of Smad signaling but increased BMP/TGFβ-dependent Smad6 expression may lead to important modulation of Dlx3-dependent gene expression.

How is *DLX3* gene expression changed in human preeclampsia?

The observation that drew our attention to studying *Pgf* gene regulation was that PGF has been shown to be an important marker for preeclampsia in pregnant women. Women with preeclampsia have reduced PGF serum levels suggesting that the potential angiogenic properties of PGF may play a fundamental role in the progression of this disease [31,32]. Despite considerable research on preeclampsia, the etiology and mechanism underlying preeclampsia are still not fully understood. Thus at present, the only effective treatment for preeclampsia is delivery of the placenta, thus removing any causal factors from maternal circulation. These conclusions are based in part on studies examining molar pregnancies. Hydatidiform mole has been shown to be associated with very early-onset preeclampsia. Although there are no fetal tissues, the mother has symptoms of preeclampsia [33].

Development of animal models that fully imitate this hypertensive disorder may broaden our understanding of this syndrome [34]. The BPH/5 mouse is the inbred mouse strain that serves as an important model for preeclampsia [35]. The BPH/5 mouse displays late-gestational hypertension, proteinuria and progressive glomerulosclerosis, which are characteristics of human preeclampsia [36]. We examined whether *Dlx3* and *Pgf* were mis-regulated in BPH/5 mice. Gene transcript levels of *Dlx3* and *Pgf* were determined at different gestational ages on both control C57 and BPH/5 mice. At early

gestational stages, *Dlx3* and *Pgf* transcripts were reduced in the BPH/5 mouse placenta compared to controls; while at later gestational ages, both transcripts were overexpressed in the placentas of BPH/5 mouse. Similar preliminary studies were carried out after collecting placentas from both normal and preeclamptic human patients. We found that *DLX3* and *PGF* transcript levels were significantly reduced in the term placenta from preeclampsia patients. These combined studies lead us to conclude that Dlx3 is an important transcriptional activator of the *Pgf* gene promoter. Moreover, mis-regulation of *DLX3* was correlated with altered *PGF* gene during preeclampsia suggesting that DLX3 may be a new and important early genetic determinant regulating the etiology of this disease.

A proposed model for the role of DLX3 in the pathogenesis of preeclampsia.

The pathogenesis of preeclampsia is illustrated in the following figures (Figure 5.1A and B). Genetic factors, immune abnormalities and oxidative stress may cause placental insufficiency, which in turn leads to decreased DLX3 expression maybe through elevated expression of miRNAs for Dlx3 or activation of TGFβ/Smad6 pathway. Loss of DLX3 expression would likely lead to reduced *PGF*. The reduction in circulating PGF causes generalized endothelial dysfunction, resulting in preeclampsia characterized by hypertension, proteinuria and coagulation abnormalities. It remains unknown whether oxidative stress is the trigger (Figure5.1A) or the outcome (Figure 5.1B) of the reduced expression of DLX3 in the placenta of preeclamptic women. The mechanism in Figure 5.1 A is supported by the following evidence. Research has shown that markers of high oxidative stress are detectable through higher levels of lipid peroxidation and increased superoxide generation in the placenta of preeclamptic women [37]. Studies reported in Chapter 4 of this dissertation demonstrate that *Dlx3* transcript levels were reduced in the BPH/5 mouse placenta at

Figure 5.1. Summary of the pathogenesis of preeclampsia.

Genetic factors, immune abnormalities and oxidative stress may cause placental insufficiency, which in turn leads to reduced circulating PGF to induce hypertension, proteinuria and other complications of preeclampsia. **A.** Oxidative stress is the trigger of the reduced expression of DLX3 in the placenta of preeclamptic women. **B.** Oxidative stress is the outcome of the reduced expression of DLX3 in preeclampsia.

A

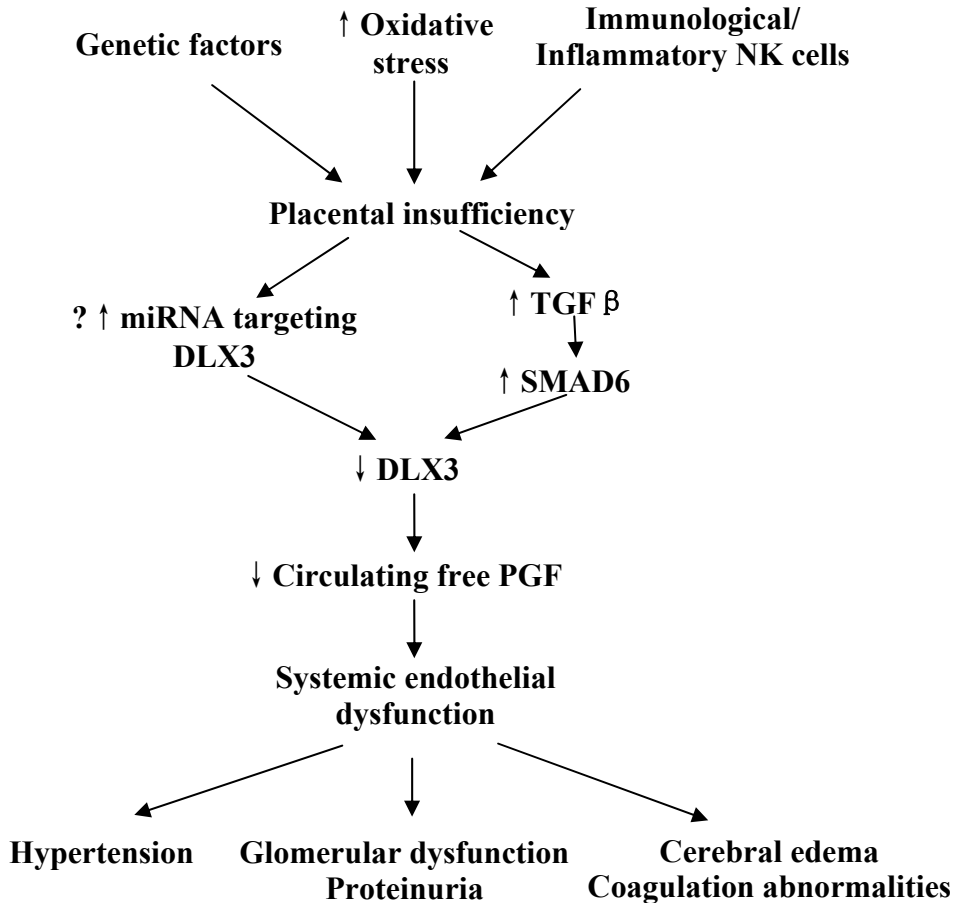
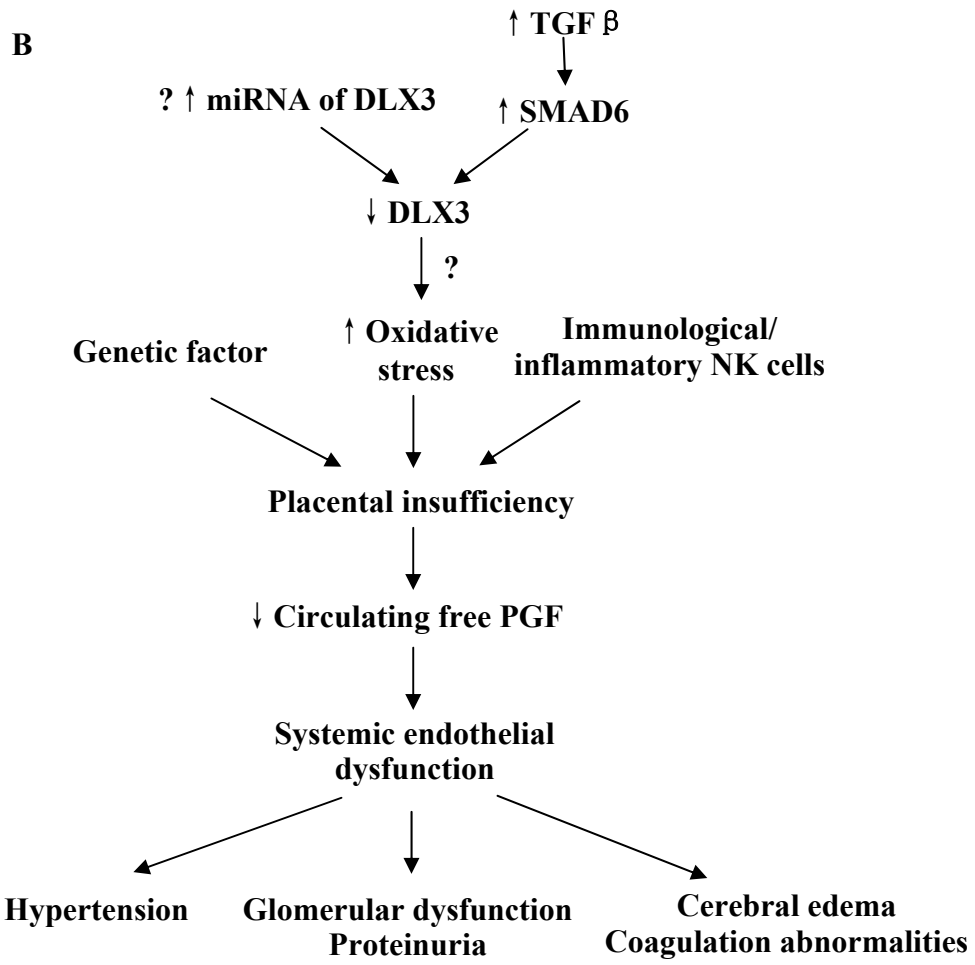

Genetic factors
↑ Oxidative stress
Immunological/ Inflammatory NK cells
Placental insufficiency
? ↑ miRNA targeting DLX3
↑ TGF β
↑ SMAD6
↓ DLX3
↓ Circulating free PGF
Systemic endothelial dysfunction
Hypertension
Glomerular dysfunction Proteinuria
Cerebral edema Coagulation abnormalities

Figure 5.1 (Continued)



early gestational stages and *DLX3* transcript level was reduced in the term placenta from preeclampsia patients. The mechanism in Figure 5.1 B is supported by other evidence. There was an increase in oxidative stress such as protein carbonyls in the *Dlx3*$^{+/-}$ placentas. *Dlx3*$^{+/-}$ mothers that were fed with tempol in the water supply during pregnancy showed reduced oxidative stress in the placenta (PA Clark and MS Roberson, unpublished data). Tempol is a membrane-permeable radical scavenger that can reduce oxidant stress-mediated placental injuries [38]. Further studies need to be done to investigate the mechanism that reduced Dlx3 expression results in an increased oxidative stress.

In summary, this dissertation examines the binding partners of Dlx3 and transcript profile regulated by Dlx3 in trophoblast cells. The longer term goal of these studies is to form the foundation for how Dlx3 and targets of Dlx3 regulate the morphogenesis of the placenta and maintenance of a competent maternal-fetal interface. By unraveling Dx3 dependent mechanisms related to placental development in the mouse, we may have a better insight of human placenta and diseases such as preeclampsia in women.

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APPENDIX I

Microarray analysis data with 401 differentially expressed genes in Dlx3$^{-/-}$ mouse placenta compared to wild type

ID	Mean Log_2(mut/wt)	Gene symbol	Gene name
1415684_at	-0.16667	Apg5l	autophagy 5-like (S. cerevisiae)
1415793_at	-0.3	Pnpo	pyridoxine 5'-phosphate oxidase
1415865_s_at	-0.86667	Bpgm	2,3-bisphosphoglycerate mutase
1415897_a_at	0.233333	Mgst1	microsomal glutathione S-transferase 1
1415928_a_at	0.166667	Map1lc3b	microtubule-associated protein 1 light chain 3 beta
1415949_at	0.333333	Cpe	carboxypeptidase E
1416022_at	0.333333	Fabp5	fatty acid binding protein 5, epidermal
1416063_x_at	-1.4	Ceacam11	CEA-related cell adhesion molecule 11
1416192_at	-0.16667	Napa	N-ethylmaleimide sensitive fusion protein attachment protein alpha
1416205_at	0.233333	Glb1	galactosidase, beta 1
1416242_at	-0.36667	Klhl13	kelch-like 13 (Drosophila)
1416298_at	-0.93333	Mmp9	matrix metalloproteinase 9
1416346_at	-0.16667	Timm8a	translocase of inner mitochondrial membrane 8 homolog a (yeast)
1416446_at	0.233333	D9Wsu20e	DNA segment, Chr 9, Wayne State University 20, expressed
1416558_at	-0.16667	Melk	maternal embryonic leucine zipper kinase
1416676_at	0.233333	Kng1	kininogen 1
1416724_x_at	0.166667	Tcf4	transcription factor 4
1416872_at	-0.16667	Tm4sf6	transmembrane 4 superfamily member 6
1416915_at	-0.16667	Msh6	mutS homolog 6 (E. coli)
1416923_a_at	0.166667	Bnip3l	BCL2/adenovirus E1B 19kDa-interacting protein 3-like
1416968_a_at	0.233333	Hsd3b7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7
1417017_at	-0.73333	Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1
1417019_a_at	-0.3	Cdc6	cell division cycle 6 homolog (S.

			cerevisiae)
1417024_at	0.166667	Hars	histidyl-tRNA synthetase
1417111_at	0.266667	Man1a	mannosidase 1, alpha
1417184_s_at	-0.86667	Hbb-b1 /// Hbb-b2	hemoglobin, beta adult major chain /// hemoglobin, beta adult minor chain
1417214_at	0.333333	Rab27b	RAB27b, member RAS oncogene family
1417346_at	0.3	Pycard	PYD and CARD domain containing
1417506_at	-0.26667	Gmnn	geminin
1417553_at	-0.33333	Plac1	placental specific protein 1
1417693_a_at	-0.33333	Gab1	growth factor receptor bound protein 2-associated protein 1
1417714_x_at	-1.33333	Hba-a1	hemoglobin alpha, adult chain 1
1417750_a_at	-1.03333	MGI:1914962	mitochondrial solute carrier protein
1417829_a_at	-0.4	Rab15	RAB15, member RAS oncogene family
1417831_at	-0.23333	Smc1l1	SMC (structural maintenance of chromosomes 1)-like 1 (S. cerevisiae)
1417843_s_at	-0.46667	Eps8l2	EPS8-like 2
1417883_at	0.233333	Gstt2	glutathione S-transferase, theta 2
1418021_at	0.333333	C4	complement component 4 (within H-2S)
1418059_at	0.366667	Eltd1	EGF, latrophilin seven transmembrane domain containing 1
1418147_at	-0.36667	Tcfap2c	transcription factor AP-2, gamma
1418171_at	0.166667	Tceal8	transcription elongation factor A (SII)-like 8
1418191_at	0.266667	Usp18	ubiquitin specific protease 18
1418206_at	-0.23333	Sdf2l1	stromal cell-derived factor 2-like 1
1418226_at	-0.3	Orc2l	origin recognition complex, subunit 2-like (S. cerevisiae)
1418281_at	-0.16667	Rad51	RAD51 homolog (S. cerevisiae)
1418293_at	0.166667	Ifit2	interferon-induced protein with tetratricopeptide repeats 2
1418352_at	-0.66667	Hsd17b2	hydroxysteroid (17-beta) dehydrogenase 2
1418432_at	0.233333	Cab39	calcium binding protein 39
1418471_at	-1.03333	Pgf	placental growth factor
1418475_at	0.266667	Scnn1b	sodium channel, nonvoltage-gated 1 beta
1418492_at	-0.33333	Grem2	gremlin 2 homolog, cysteine knot

			superfamily (Xenopus laevis)
1418493_a_at	-3	Snca	synuclein, alpha
1418547_at	0.4	Tfpi2	tissue factor pathway inhibitor 2
1418587_at	0.166667	Traf3	Tnf receptor-associated factor 3
1418699_s_at	-0.4	Fech	ferrochelatase
1418728_at	-0.53333	Star	steroidogenic acute regulatory protein
1418826_at	-0.16667	Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B
1418831_at	-0.4	Pkp3	plakophilin 3
1419018_at	-0.6	Psx1	placenta specific homeobox 1
1419136_at	0.233333	Akr1c18	aldo-keto reductase family 1, member C18
1419149_at	-0.43333	Serpine1	serine (or cysteine) proteinase inhibitor, clade E, member 1
1419247_at	0.166667	Rgs2	regulator of G-protein signaling 2
1419249_at	-0.33333	Pftk1	PFTAIRE protein kinase 1
1419325_at	0.466667	Nmu	neuromedin U
1419459_a_at	-0.16667	2610529C04Rik	RIKEN cDNA 2610529C04 gene
1419462_s_at	-0.16667	Gtl3	gene trap locus 3
1419494_a_at	-0.16667	Tpd52	tumor protein D52
1419595_a_at	0.3	Ggh	gamma-glutamyl hydrolase
1419640_at	-0.16667	Purb	purine rich element binding protein B
1419657_a_at	0.166667	C330005L02Rik	RIKEN cDNA C330005L02 gene
1419749_at	0.166667	Dnmt2	DNA methyltransferase 2
1419873_s_at	0.166667	Csf1r	colony stimulating factor 1 receptor
1419880_x_at	-0.46667		Transcribed locus
1420056_s_at	-0.43333	Ptdsr	phosphatidylserine receptor
1420113_s_at	-0.16667	2410022L05Rik	RIKEN cDNA 2410022L05 gene
1420157_s_at	-0.16667	Abcf1	ATP-binding cassette, sub-family F (GCN20), member 1
1420382_at	-0.4	MGI:2176230	apolipoprotein B48 receptor
1420476_a_at	-0.36667	Nap1l1	nucleosome assembly protein 1-like 1
1420479_a_at	-0.3	Nap1l1	nucleosome assembly protein 1-like 1
1420539_a_at	-0.8	Chrdl2	chordin-like 2
1420647_a_at	-0.43333	Krt2-8	keratin complex 2, basic, gene 8
1420973_at	-0.33333	Arid5b	AT rich interactive domain 5B (Mrf1 like)
1421034_a_at	0.4	Il4ra	interleukin 4 receptor, alpha
1421268_at	0.166667	Ugcg	UDP-glucose ceramide glucosyltransferase

1421945_a_at	-0.3	Bxdc1	brix domain containing 1
1422411_s_at	0.166667	Ear1 /// Ear2 /// Ear3	eosinophil-associated, ribonuclease A family, member 1 /// eosinophil-associated, ribonuclease A family, member 2 /// eosinophil-associated, ribonuclease A family, member 3
1422415_at	0.7	Ang2	angiogenin, ribonuclease A family, member 2
1422470_at	0.366667	Bnip3	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3
1422486_a_at	0.3	Smad4	MAD homolog 4 (Drosophila)
1422513_at	-0.36667	Ccnf	cyclin F
1422571_at	0.666667	Thbs2	thrombospondin 2
1422636_at	0.166667	Dmtf1	cyclin D binding myb-like transcription factor 1
1422771_at	-0.26667		
1422865_at	0.166667	Runx1	runt related transcription factor 1
1422943_a_at	-0.23333	Hspb1	heat shock protein 1
1423097_s_at	0.166667	Capn7	calpain 7
1423369_at	0.233333	Fmr1	fragile X mental retardation syndrome 1 homolog
1423429_at	-0.5	Pem	placentae and embryos oncofetal gene
1423450_a_at	-0.26667	Hs3st1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
1423484_at	0.366667	Bicc1	bicaudal C homolog 1 (Drosophila)
1423518_at	-0.26667	Csk	c-src tyrosine kinase
1423607_at	0.833333	Lum	lumican
1423623_at	0.166667	2810021B07Rik	RIKEN cDNA 2810021B07 gene
1423662_at	0.166667	Atp6ap2	ATPase, H+ transporting, lysosomal accessory protein 2
1423691_x_at	-0.53333	Krt2-8	keratin complex 2, basic, gene 8
1423714_at	-0.3	Asf1b	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)
1423859_a_at	0.833333	Ptgds	prostaglandin D2 synthase (brain)
1423922_s_at	-0.33333	C77668	expressed sequence C77668
1423946_at	-0.26667	Pdlim2	PDZ and LIM domain 2
1424092_at	-0.53333	Epb4.1	erythrocyte protein band 4.1
1424113_at	-0.33333	Lamb1-1	laminin B1 subunit 1
1424114_s_at	-0.16667	Lamb1-1	laminin B1 subunit 1
1424131_at	0.166667	Col6a3	procollagen, type VI, alpha 3
1424202_at	-0.16667	Seh1l	SEH1-like (S. cerevisiae)
1424254_at	0.166667	Ifitm1	interferon induced transmembrane protein 1

1424354_at	0.366667	1110007F12Rik	RIKEN cDNA 1110007F12 gene
1424465_at	-0.16667	AI413631	expressed sequence AI413631
1424512_a_at	-0.16667	Txndc9	thioredoxin domain containing 9
1424533_a_at	-0.3	3110023B02Rik	RIKEN cDNA 3110023B02 gene
1424666_at	0.233333	5430405G24Rik	RIKEN cDNA 5430405G24 gene
1424770_at	-0.26667	Cald1	caldesmon 1
1424852_at	0.3	Mef2c	myocyte enhancer factor 2C
1425028_a_at	-0.43333	Tpm2	tropomyosin 2, beta
1425163_at	-0.46667	AI661453 /// LOC224833	expressed sequence AI661453 /// hypothetical protein BC006605
1425295_at	0.466667	Ear11	eosinophil-associated, ribonuclease A family, member 11
1425391_a_at	0.233333	Osbpl5	oxysterol binding protein-like 5
1425476_at	0.366667	Col4a5	procollagen, type IV, alpha 5
1425814_a_at	0.433333	Calcrl	calcitonin receptor-like
1426001_at	-0.26667	Eomes	eomesodermin homolog (Xenopus laevis)
1426351_at	-0.23333	Hspd1	heat shock protein 1 (chaperonin)
1426361_at	-0.3	5730454B08Rik	RIKEN cDNA 5730454B08 gene
1426614_at	0.266667	Prkcbp1	protein kinase C binding protein 1
1426622_a_at	0.7	Qpct	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
1426666_a_at	-0.3	Unc84a	unc-84 homolog A (C. elegans)
1426734_at	0.166667	BC022623	cDNA sequence BC022623
1426751_s_at	-0.16667	Nup107	nucleoporin 107
1426838_at	-0.23333	Pold3	polymerase (DNA-directed), delta 3, accessory subunit
1426969_at	0.3	Trim23	tripartite motif protein 23
1427040_at	0.166667	Mdfid	MyoD family inhibitor domain containing
1427095_at	-0.63333	E030027H19Rik	RIKEN cDNA E030027H19 gene
1427123_s_at	0.233333	Copg2as2	coatomer protein complex, subunit gamma 2, antisense 2
1427199_at	-0.16667	2510002A14Rik	RIKEN cDNA 2510002A14 gene
1427236_a_at	0.166667	Mll5	myeloid/lymphoid or mixed-lineage leukemia 5
1427990_at	0.233333	Usp45	ubiquitin specific protease 45
1428187_at	0.166667	Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
1428326_s_at	0.166667	Hrsp12	heat-responsive protein 12

1428361_x_at	-0.86667		
1428367_at	0.166667	Ndst1	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1
1428657_at	0.166667	1110037N09Rik	RIKEN cDNA 1110037N09 gene
1428675_at	-0.53333		
1428760_at	0.166667	Snapc3	small nuclear RNA activating complex, polypeptide 3
1429227_x_at	-0.43333	Nap1l1	nucleosome assembly protein 1-like 1
1429294_at	-0.4	Trip13	thyroid hormone receptor interactor 13
1429787_x_at	-0.23333	Zwint	ZW10 interactor
1431059_x_at	-0.26667	Htatsf1	HIV TAT specific factor 1
1431099_at	0.3	Hoxd8	homeo box D8
1431284_a_at	-0.16667	2210013K02Rik	RIKEN cDNA 2210013K02 gene
1431302_a_at	-0.23333	Nudt7	nudix (nucleoside diphosphate linked moiety X)-type motif 7
1432417_a_at	0.166667	6330415F13Rik	RIKEN cDNA 6330415F13 gene
1433568_at	0.166667	Papd4	PAP associated domain containing 4
1433575_at	0.233333	Sox4	SRY-box containing gene 4
1433588_at	0.166667	D6Wsu116e	DNA segment, Chr 6, Wayne State University 116, expressed
1433655_at	0.166667	Rnf141	Ring finger protein 141
1433906_at	0.4	4933402J24Rik	RIKEN cDNA 4933402J24 gene
1434016_at	0.233333	D6Ertd365e	DNA segment, Chr 6, ERATO Doi 365, expressed
1434109_at	-0.5	Sh3bgrl2	SH3 domain binding glutamic acid-rich protein like 2
1434148_at	0.366667	Tcf4	transcription factor 4
1434195_at	0.633333	Prss35	protease, serine, 35
1434419_s_at	-0.23333	Tardbp	TAR DNA binding protein
1434472_at	0.166667	5031436O03Rik	RIKEN cDNA 5031436O03 gene
1434646_s_at	-0.16667	Sap18	Sin3-associated polypeptide 18
1434674_at	0.166667		
1434739_at	-0.43333	Fmr1nb	fragile X mental retardation 1 neighbor
1434856_at	0.233333		
1434930_at	-0.16667	Tpcn1	two pore channel 1
1434931_at	0.333333		
1434999_at	0.166667	Suv420h1	suppressor of variegation 4-20 homolog 1 (Drosophila)
1435091_at	-0.23333	LOC243905	hypothetical LOC243905
1435137_s_at	0.266667	1200015M12Rik	RIKEN cDNA 1200015M12

			gene
1435290_x_at	-0.16667	H2-Aa	histocompatibility 2, class II antigen A, alpha
1435416_x_at	-0.23333	Pigq	phosphatidylinositol glycan, class Q
1435494_s_at	-0.53333	5730453H04Rik	RIKEN cDNA 5730453H04 gene
1435882_at	-0.16667		
1435967_s_at	0.233333	Hibadh	3-hydroxyisobutyrate dehydrogenase
1435989_x_at	-0.4	Krt2-8	keratin complex 2, basic, gene 8
1436058_at	-0.33333	Rsad2	radical S-adenosyl methionine domain containing 2
1436506_a_at	0.266667	1110008H02Rik	RIKEN cDNA 1110008H02 gene
1436853_a_at	-1.8	Snca	synuclein, alpha
1436979_x_at	-0.16667	Rbm14	RNA binding motif protein 14
1437103_at	-0.3	C330012H03Rik	RIKEN cDNA C330012H03 gene
1437497_a_at	-0.23333	Hspca	heat shock protein 1, alpha
1437502_x_at	-0.5	Cd24a	CD24a antigen
1437515_at	-0.33333		
1437810_a_at	-1.06667	Hbb-bh1	hemoglobin Z, beta-like embryonic chain
1437902_s_at	0.266667	Rarres2	retinoic acid receptor responder (tazarotene induced) 2
1438164_x_at	0.166667	Flot2	flotillin 2
1438167_x_at	0.166667	Flcn	folliculin
1438602_s_at	0.233333	Masp1	mannan-binding lectin serine protease 1
1439022_at	-0.56667	Phactr1	phosphatase and actin regulator 1
1439045_x_at	0.366667	Mtac2d1	membrane targeting (tandem) C2 domain containing 1
1439443_x_at	-0.23333	Tkt	transketolase
1439444_x_at	0.166667	1110014C03Rik	RIKEN cDNA 1110014C03 gene
1439450_x_at	0.166667	A230046K03Rik	RIKEN cDNA A230046K03 gene
1448021_at	-1.06667		Transcribed locus
1448029_at	0.166667	Tbx3	T-box 3
1448123_s_at	0.366667		
1448160_at	-0.26667	Lcp1	lymphocyte cytosolic protein 1
1448182_a_at	-0.46667	Cd24a	CD24a antigen
1448194_a_at	-0.26667		
1448251_at	0.366667	9030425E11Rik	RIKEN cDNA 9030425E11 gene
1448306_at	0.166667	Nfkbia	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha

1448339_at	0.166667	D9Wsu20e	DNA segment, Chr 9, Wayne State University 20, expressed
1448419_at	0.3	Pop4	processing of precursor 4, ribonuclease P/MRP family, (S. cerevisiae)
1448427_at	0.166667	Ndufa6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (B14)
1448545_at	0.233333	Sdc2	syndecan 2
1448590_at	0.166667	Col6a1	procollagen, type VI, alpha 1
1448687_at	-0.23333	1110035L05Rik	RIKEN cDNA 1110035L05 gene
1448690_at	-0.33333	Kcnk1	potassium channel, subfamily K, member 1
1448716_at	-1.36667	Hba-x	hemoglobin X, alpha-like embryonic chain in Hba complex
1448765_at	0.233333		
1448890_at	0.166667	Klf2	Kruppel-like factor 2 (lung)
1449108_at	-0.33333	Fdx1	ferredoxin 1
1449119_at	-0.16667	Arih2	ariadne homolog 2 (Drosophila)
1449125_at	-0.53333	2600017J23Rik	RIKEN cDNA 2600017J23 gene
1449327_at	-1.26667	1600015I10Rik	RIKEN cDNA 1600015I10 gene
1449368_at	0.3	Dcn	decorin
1449451_at	0.333333	Serpinb11	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 11
1449556_at	0.166667	H2-T23 /// C920025E04Rik	histocompatibility 2, T region locus 23 /// RIKEN cDNA C920025E04 gene
1449591_at	0.3	Casp11	caspase 11, apoptosis-related cysteine protease
1449731_s_at	0.166667		
1450048_a_at	-0.23333	Idh2	isocitrate dehydrogenase 2 (NADP+), mitochondrial
1450344_a_at	0.566667	Ptger3	prostaglandin E receptor 3 (subtype EP3)
1450569_a_at	-0.3	Rbm14	RNA binding motif protein 14
1450621_a_at	-1.43333	Hbb-y	hemoglobin Y, beta-like embryonic chain
1450644_at	0.3	Zfp36l1	zinc finger protein 36, C3H type-like 1
1450647_at	0.166667	Hps3	Hermansky-Pudlak syndrome 3 homolog (human)
1450668_s_at	-0.16667	Hspe1	heat shock protein 1 (chaperonin 10)
1450697_at	-0.16667	Slc30a7	solute carrier family 30 (zinc transporter), member 7
1450736_a_at	-1.2	Hbb-bh1	hemoglobin Z, beta-like

			embryonic chain
1450783_at	0.233333	Ifit1	interferon-induced protein with tetratricopeptide repeats 1
1450826_a_at	-0.43333	Saa3	serum amyloid A 3
1450872_s_at	0.233333	Lip1	lysosomal acid lipase 1
1451077_at	0.366667	Rpl5	ribosomal protein L5
1451092_a_at	-0.16667	Rangap1	RAN GTPase activating protein 1
1451133_s_at	0.266667	8430437G11Rik	RIKEN cDNA 8430437G11 gene
1451210_at	-0.26667	Ppap2c	phosphatidic acid phosphatase type 2c
1451300_a_at	-0.23333	6330407G04Rik	RIKEN cDNA 6330407G04 gene
1451416_a_at	-0.73333	Tgm1	transglutaminase 1, K polypeptide
1451431_a_at	-0.16667	D2Bwg0891e	DNA segment, Chr 2, Brigham & Women's Genetics 0891 expressed
1451564_at	0.366667	Parp14	poly (ADP-ribose) polymerase family, member 14
1452012_a_at	-0.16667	Exosc1	exosome component 1
1452061_s_at	-0.16667	Strbp	spermatid perinuclear RNA binding protein
1452084_at	-0.16667	2810055E05Rik	RIKEN cDNA 2810055E05 gene
1452114_s_at	0.433333	Igfbp5	insulin-like growth factor binding protein 5
1452225_at	0.266667	2010106G01Rik	RIKEN cDNA 2010106G01 gene
1452252_at	-0.16667	3830408P06Rik	RIKEN cDNA 3830408P06 gene
1452303_at	0.166667	Arhgef10	Rho guanine nucleotide exchange factor (GEF) 10
1452307_at	-0.23333	Cables2	Cdk5 and Abl enzyme substrate 2
1452318_a_at	-0.4	Hspa1b	heat shock protein 1B
1452367_at	-0.23333		
1452428_a_at	0.233333	B2m	beta-2 microglobulin
1452543_a_at	0.433333	Scgb1a1	secretoglobin, family 1A, member 1 (uteroglobin)
1452622_a_at	0.166667	Tradd	TNFRSF1A-associated via death domain
1452714_at	0.166667	1200003E16Rik	RIKEN cDNA 1200003E16 gene
1452757_s_at	-0.8		
1452778_x_at	-0.33333	Nap1l1	nucleosome assembly protein 1-like 1
1452792_at	-0.16667	2810422M04Rik	RIKEN cDNA 2810422M04 gene

1452839_at	-0.16667	2410012M04Rik	RIKEN cDNA 2410012M04 gene
1452917_at	-0.16667	Rfc5	replication factor C (activator 1) 5
1453064_at	0.266667	5730466H23Rik	RIKEN cDNA 5730466H23 gene
1454044_a_at	0.233333	Pex3	peroxisomal biogenesis factor 3
1454967_at	0.233333		
1455915_at	0.166667	Galnt4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4
1455957_x_at	-1.46667	Ceacam11	CEA-related cell adhesion molecule 11
1455987_at	-0.16667		
1456174_x_at	0.166667	Ndrg1	N-myc downstream regulated gene 1
1456195_x_at	0.166667	Itgb5	integrin beta 5
1456292_a_at	0.3	Vim	vimentin
1456309_x_at	-0.33333	Lasp1	LIM and SH3 protein 1
1456377_x_at	-0.3	0610025L06Rik	RIKEN cDNA 0610025L06 gene
1456541_x_at	-0.16667	Atad3a	ATPase family, AAA domain containing 3A
1460194_at	0.166667	Phyh	phytanoyl-CoA hydroxylase
1460221_at	-0.16667	MGI:1929282	telomerase binding protein, p23
1460226_at	-0.26667	Trap1a	tumor rejection antigen P1A
1460592_at	-0.16667	Epb4.1l1	erythrocyte protein band 4.1-like 1
1460679_at	0.166667	Exosc4	exosome component 4
1428124_at	-0.16667	Gtf2e1	general transcription factor II E, polypeptide 1 (alpha subunit)
1428261_at	-0.16667	2310042L06Rik	RIKEN cDNA 2310042L06 gene
1428386_at	0.233333	Acsl3	UTP14, U3 small nucleolar ribonucleoprotein, homolog B (yeast)
1428411_at	0.166667	1700020I14Rik	RIKEN cDNA 1700020I14 gene
1428497_at	-0.16667	Secisbp2	SECIS binding protein 2
1429012_at	-0.53333	Arhgef6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
1429106_at	-0.23333	6330544N02Rik	RIKEN cDNA 6330544N02 gene
1429351_at	0.3	4930429H24Rik	RIKEN cDNA 4930429H24 gene
1429503_at	0.333333	2900024C23Rik	RIKEN cDNA 2900024C23 gene
1429633_at	-0.3	MGI:2443930	Mblk1-related protein-2
1430051_at	0.366667	4930486L24Rik	RIKEN cDNA 4930486L24 gene
1430927_at	0.3	2010110O04Rik	RIKEN cDNA 2010110O04

			gene
1432750_at	0.333333	2810409C01Rik	RIKEN cDNA 2810409C01 gene
1433465_a_at	-0.16667	AI467606	expressed sequence AI467606
1433501_at	0.166667	Ctso	cathepsin O
1433664_at	-0.16667	3010021M21Rik	RIKEN cDNA 3010021M21 gene
1433696_at	-0.26667	D17Ertd441e	DNA segment, Chr 17, ERATO Doi 441, expressed
1433740_at	0.233333	2610301K12Rik	RIKEN cDNA 2610301K12 gene
1433817_at	0.366667	Agpat3	1-acylglycerol-3-phosphate O-acyltransferase 3
1433922_at	-0.16667	Rab35	RAB35, member RAS oncogene family
1433976_at	0.166667	AI265725	Expressed sequence AI265725
1434225_at	0.166667	Swap70	SWAP complex protein
1434268_at	0.166667	Adar	adenosine deaminase, RNA-specific
1434441_at	0.333333		Mus musculus, clone IMAGE:4015738, mRNA
1434548_at	0.266667	Tde1	tumor differentially expressed 1
1434664_at	0.166667	2410129H14Rik	RIKEN cDNA 2410129H14 gene
1434671_at	0.166667	B230337E12Rik	RIKEN cDNA B230337E12 gene
1434716_at	0.166667		
1434957_at	0.3	Cdon	Cell adhesion molecule-related/down-regulated by oncogenes
1435092_at	0.233333	Arl4	ADP-ribosylation factor-like 4
1435238_x_at	-0.26667	2310009A05Rik	RIKEN cDNA 2310009A05 gene
1435283_s_at	0.233333	Gm967	gene model 967, (NCBI)
1435361_at	0.5	AK129018	cDNA sequence AK129018
1435423_x_at	-0.16667	4933433P14Rik	RIKEN cDNA 4933433P14 gene
1435445_at	0.366667	Ccnt2	cyclin T2
1435796_at	-0.23333	4933413A10Rik	RIKEN cDNA 4933413A10 gene
1435829_at	-0.3	B930008K04Rik	RIKEN cDNA B930008K04 gene
1435867_at	0.233333		Transcribed locus
1435890_at	0.166667	5730596K20Rik	RIKEN cDNA 5730596K20 gene
1436025_at	0.233333	A430106J12Rik	RIKEN cDNA A430106J12 gene
1436029_at	0.166667	4930533K18Rik	RIKEN cDNA 4930533K18 gene
1436122_at	-0.43333		

1436210_at	0.166667		Transcribed locus, moderately similar to XP_359179.2 similar to hypothetical protein [Mus musculus]
1436306_at	-0.23333	B430201G11Rik	RIKEN cDNA B430201G11 gene
1436367_at	0.3		Transcribed locus
1436389_at	0.166667	5730596K20Rik	RIKEN cDNA 5730596K20 gene
1436424_at	0.266667	1600020E01Rik	RIKEN cDNA 1600020E01 gene
1436568_at	0.333333	Jam2	junction adhesion molecule 2
1436920_at	0.3	C030033F14Rik	RIKEN cDNA C030033F14 gene
1437187_at	-0.46667	E2f7	E2F transcription factor 7
1437296_at	-0.3	Pkn2	protein kinase N2
1437393_at	-0.16667	AI875142	expressed sequence AI875142
1437451_at	0.233333	1110006O17Rik	RIKEN cDNA 1110006O17 gene
1437748_at	0.166667	Fut11	fucosyltransferase 11
1437869_at	-0.16667	3222402P14Rik	RIKEN cDNA 3222402P14 gene
1438030_at	0.266667	Rasgrp3	RAS, guanyl releasing protein 3
1438294_at	0.433333	Sca1	spinocerebellar ataxia 1 homolog (human)
1438420_at	0.333333	Rnpc2	RNA-binding region (RNP1, RRM) containing 2
1438454_at	0.3	B430203M17Rik	RIKEN cDNA B430203M17 gene
1438730_at	0.4	BC028801	cDNA sequence BC028801
1438884_at	0.233333	D830007B15Rik	RIKEN cDNA D830007B15 gene
1439107_a_at	0.233333	Mll5	myeloid/lymphoid or mixed-lineage leukemia 5
1439687_at	0.166667	Rab14	RAB14, member RAS oncogene family
1439699_at	0.166667	Pgr	Progesterone receptor
1439811_at	-0.46667	Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase
1439933_at	0.266667	B430316J06Rik	RIKEN cDNA B430316J06 gene
1439972_at	0.266667	Etnk1	Ethanolamine kinase 1
1440163_at	0.333333	6030490B17Rik	RIKEN cDNA 6030490B17 gene
1441320_a_at	-0.16667	AI413194	expressed sequence AI413194
1441556_at	0.466667		Transcribed locus
1441814_s_at	0.366667	2400006N03Rik	RIKEN cDNA 2400006N03 gene
1442067_at	0.366667	Ror1	Receptor tyrosine kinase-like orphan receptor 1
1442332_at	0.166667	Tgfbr3	Transforming growth factor, beta

			receptor III
1442549_at	-0.33333	Mbnl3	muscleblind-like 3 (Drosophila)
1442827_at	0.333333	Tlr4	toll-like receptor 4
1443088_at	0.5	9930031P18Rik	RIKEN cDNA 9930031P18 gene
1444018_at	0.3	B930098A02Rik	RIKEN cDNA B930098A02 gene
1445862_at	0.166667	BC031575	cDNA sequence BC031575
1447070_at	-0.16667	Wbscr1	Williams-Beuren syndrome chromosome region 1 homolog (human)
1447704_s_at	0.166667	D530033C11Rik	RIKEN cDNA D530033C11 gene
1447807_s_at	-0.53333	Plekhh1	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1
1447852_x_at	0.233333	2900002H16Rik	RIKEN cDNA 2900002H16 gene
1447897_x_at	0.4		
1447923_at	0.233333	1810026B05Rik	RIKEN cDNA 1810026B05 gene
1452788_at	-0.16667	Ppp2r5e	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform
1452810_at	-0.16667	4921521J11Rik	RIKEN cDNA 4921521J11 gene
1452984_at	0.166667	5730405I09Rik	RIKEN cDNA 5730405I09 gene
1453055_at	0.4	Sema6d	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D
1453108_at	0.333333	2810429K17Rik	RIKEN cDNA 2810429K17 gene
1453149_at	-0.16667	MGI:1917156	mitochondrial folate transporter/carrier
1453269_at	-0.3	Unc5b	unc-5 homolog B (C. elegans)
1453743_x_at	-0.3	Phf14	PHD finger protein 14
1454646_at	0.166667	E430026E19Rik	RIKEN cDNA E430026E19 gene
1454680_at	-0.23333	D5Ertd579e	DNA segment, Chr 5, ERATO Doi 579, expressed
1454740_at	0.233333	Mib1	mindbomb homolog 1 (Drosophila)
1454764_s_at	0.166667	Slc38a1	Solute carrier family 38, member 1
1455185_s_at	-0.33333	Phf16	PHD finger protein 16
1455223_at	-0.66667	Igf2bp1	insulin-like growth factor 2, binding protein 1
1455649_at	-0.63333		
1456156_at	0.266667	Lepr	leptin receptor
1456295_at	0.266667	B230114P17Rik	RIKEN cDNA B230114P17 gene

1456873_at	0.366667	Clic5	Chloride intracellular channel 5
1457465_at	-0.4	D430043L16Rik	RIKEN cDNA D430043L16 gene
1457491_at	0.233333	AA960558	expressed sequence AA960558
1457555_at	-0.23333		
1457635_s_at	0.5		
1457682_at	0.233333	9030420J04Rik	RIKEN cDNA 9030420J04 gene
1458353_at	-0.4	A230063L24Rik	RIKEN cDNA A230063L24 gene
1458440_at	-0.16667	LOC432572	similar to sperm antigen HCMOGT-1
1459476_s_at	-0.16667	Csrp2bp	cysteine and glycine-rich protein 2 binding protein
1459751_s_at	-0.16667	Ppp1r16a	protein phosphatase 1, regulatory (inhibitor) subunit 16A
1459793_s_at	0.466667		
1459981_s_at	0.366667	Rsbn1	rosbin, round spermatid basic protein 1
1460462_at	0.366667	Med18	mediator of RNA polymerase II transcription, subunit 18 homolog (yeast)
1460617_s_at	-0.6	Rab6b	RAB6B, member RAS oncogene family