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Functions Of The Homeobox Gene Dlx4 In Controlling Inflammatory Signaling And Metastasis Of Epithelial Ovarian Cancer

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A

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DEDICATION

I would like to dedicate this thesis to my family for their unconditional love, support and encouragement.

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FUNCTIONS OF THE HOMEOBOX GENE *DLX4* IN CONTROLLING INFLAMMATORY SIGNALING AND METASTASIS OF EPITHELIAL OVARIAN CANCER

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Epithelial ovarian cancer (EOC) accounts for the most number of deaths among women with gynecological malignancies in the United States. Approximately 80% of EOC patients are diagnosed with disease that has disseminated beyond the confines of the ovaries. The five year survival rate for patients with advanced stage EOC is less than 30% and the recurrence of chemoresistant disease is high. Identifying the mechanisms that control peritoneal metastasis of EOC is therefore critical for improving treatment of advanced stage disease. The homeobox gene *DLX4* encodes a transcription factor that is absent from most normal adult tissues. Previous studies from our laboratory have identified that DLX4 is highly expressed in advanced stage EOC and is strongly associated with reduced survival. The underlying hypothesis of my study is that DLX4 promotes peritoneal dissemination of EOC. The overall goal of my study is to determine the role and mechanisms of DLX4 in controlling peritoneal metastasis of EOC. My specific aims are: 1) to determine whether DLX4 promotes peritoneal dissemination of EOC, and 2) to identify the mechanisms by which DLX4 controls tumor–peritoneum interactions. Firstly, my studies have identified that DLX4 promotes EOC dissemination by inducing expression of the cell adhesion molecule CD44 which is a major

receptor for hyaluronan, a glycosaminoglycan that is expressed on mesothelial cells lining the peritoneal cavity and abdominal organs. Secondly, my studies have identified that DLX4 induces CD44 expression by activating the pro-inflammatory cytokine interleukin 1-beta (IL-1β) which in turn stimulates the nuclear factor kappa B (NF-κB) signaling pathway. This study provides insights into the mechanisms of peritoneal metastasis of EOC and raises the possibility that targeting inflammatory signaling could be a strategy for treatment of advanced stage EOC.

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LIST OF ABBREVIATIONS

Ab	Antibody
BAFF	B-cell activating factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CA-125	Cancer Antigen-125
CD40L	CD40 ligand
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FIGO	International Federation of Gynecology and Obstetrics
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HA	Hyaluronic acid
HDAC1	Histone deacetylase 1
HE	Hematoxylin eosin
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor-1
hMLH1	Human MutL homolog 1
IBC	Inflammatory breast cancer

IFN-γ	Interferon-γ
IκB	Inhibitor of NF-κB
IκBα-DN	IκBα-dominant negative
IKK	IκB kinase
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
iNOS	Inducible nitric oxide synthase
i.p.	intraperitoneal
IP	Immunoprecipitation
MMP-1	Matrix metalloproteinase-1
NF-κB	Nuclear factor of kappa B
NHEJ	Non-homologous end joining
PBS	Phosphate-buffered saline
PerCP	Peridinin-chlorophyll-protein complex
PIN	Prostatic intraepithelial neoplasia
PRC2	Polycomb Repressive Complex 2
RANKL	Receptor activator of NF-κB ligand
SDS	Sodium dodecyl sulfate
TNF-α	Tumor necrosis factor-α
VEGF-A	Vascular endothelial growth factor-A

CHAPTER 1: INTRODUCTION

A. BIOLOGY OF OVARIAN CANCER

1. Classification and Clinical Staging of Ovarian Cancer

Ovarian cancer accounts for the highest lethality among women with gynecologic malignancies in the United States [1]. Ovarian cancers are classified as 1) epithelial tumors, 2) sex cord stromal tumors and 3) germ cell tumors. Epithelial ovarian cancers (EOC) constitute about 60% of all ovarian tumors and 90% of malignant tumors [2]. It has been traditionally thought that EOC originates from the ovarian surface epithelium. However, recently, the fallopian tube and some other epithelial tissues have been reported as origins for EOC [3, 4]. EOC is a heterogeneous disease and is categorized into different subtypes based on their histologic features [2]. These histologic subtypes exhibit different types of mutations (Table 1). The serous subtype is the most common form of EOC (Figure1) [5, 6]. The stage of the disease, i.e. its spread within the ovaries or to other parts of the body is evaluated during surgery (Table 2) [7]. Due to a lack of distinctive symptoms and specific diagnostic biomarkers, more than 60% of EOC patients are diagnosed with disease that has already spread throughout the peritoneal cavity (Figure 2) [8]. In contrast, only 15% of EOC patients present with ovarian-confined disease at the time of initial diagnosis (Figure 2) [8]. The five-year survival rate for women with advanced-stage EOC is less than 30% as compared to over 90% for women with localized disease (Figure 2) [8]. The majority of patients with advanced-stage EOC who undergo tumor-debulking surgery and conventional platinum-taxane chemotherapy relapse within 18 months [9].

Table 1: Common mutations identified in the histologic subtypes of EOC

Histologic subtype	Mutations	References
Serous	**High grade serous:** *TP53* *CDKN2A* *BRCA1/2* **Low grade serous:** *BRAF* *KRAS* *ERBB2*	10-12
Endometrioid	*CTNNB1* *PIK3CA* *PTEN* *ARID1A*	5, 13
Mucinous	*KRAS*	5, 14
Clear cell	*HNF1B* *PTEN* *ARID1A* *PIK3CA*	5, 15-17

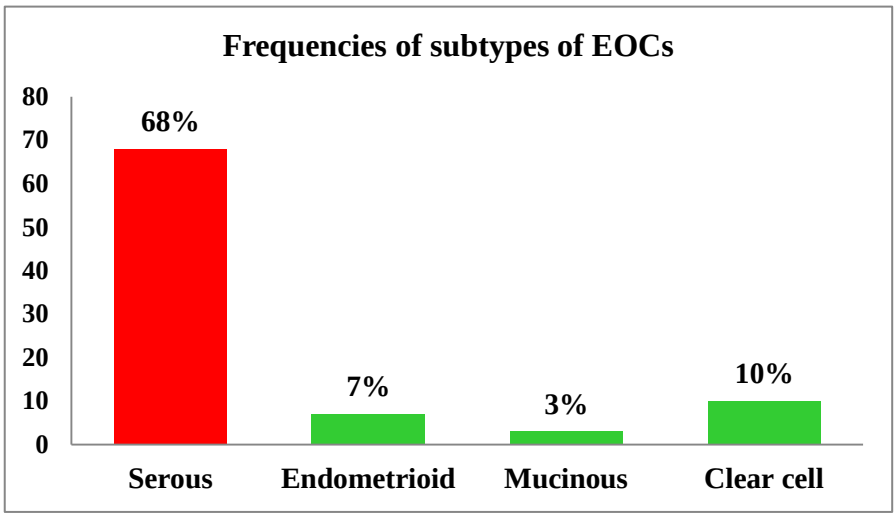

Figure 1. Relative frequencies of subtypes of EOC

Relative frequencies of subtypes of EOCs as reported by two independent studies [5, 6].

Table 2: International Federation of Gynecology and Obstetrics (FIGO) staging of EOC[7]

FIGO Stage	Characteristics of the tumor
I	Tumor is confined to the ovaries
II	Tumor involves one of both ovaries with extension and/or implantation on pelvic organs e.g. uterus and/or fallopian tubes
III	Tumor involves one or both ovaries with disease that has spread to the peritoneum outside the pelvis and/or to retroperitoneal lymph nodes
IV	Distant metastases to liver, spleen and extra-abdominal organs with development of pleural effusions

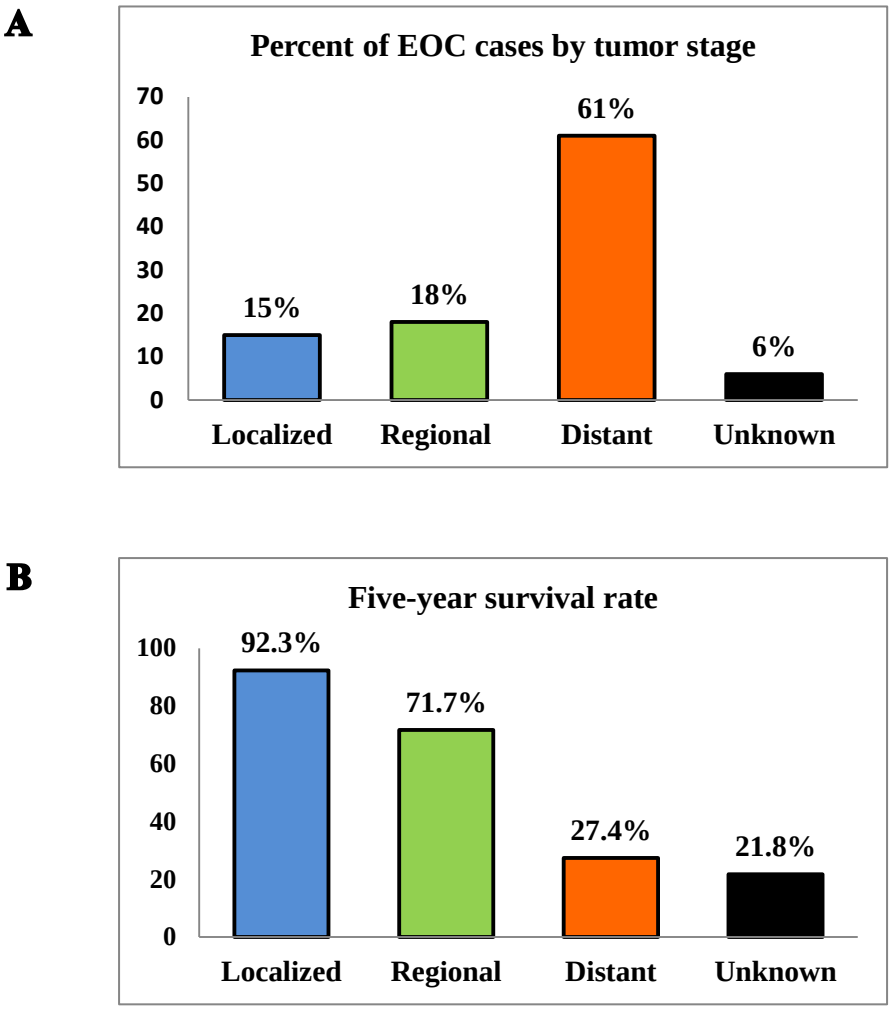

Figure 2. Stage distribution of EOC and five-year survival rate by stage [8].

(A) Percentage of EOC cases by tumor stage as reported by SEER, where localized disease corresponds to FIGO Stage I, regional disease corresponds to FIGO stage II and distant disease corresponds to FIGO Stages III and IV. **(B)** Five-year survival rate by tumor stage.

Currently, the most commonly used biomarker for EOC is the glycoprotein cancer antigen-125 (CA-125) [18]. Serum levels of CA-125 are found to be elevated in approximately 80% of advanced-stage EOC cases and is used for post-operative monitoring [18, 19]. However, measurement of CA-125 is not highly sensitive for detection of early stage EOC. CA-125 levels are also elevated in other conditions such as pregnancy, endometriosis, menstruation and other malignancies like pancreatic, breast, lung, and gastric cancers [20]. There is, therefore, a critical need for the identification of novel diagnostic biomarkers for early detection of EOC and new molecular targets for effective treatment against highly metastatic and chemoresistant EOC.

2. Biology of dissemination of EOC

Unlike most other types of solid tumors, EOC does not typically metastasize via lymphatic or hematogenous routes [21]. In the initial stage, the tumor is confined to one or both the ovaries. As the disease progresses, the ovarian capsule is disrupted and the tumor first spreads by directly extending to adjacent organs, for example, uterus and fallopian tubes (Figure 3) [22]. Distal dissemination of EOC occurs primarily via intraperitoneal seeding (Figure 4). Exfoliated tumor cells are transported by the circulating peritoneal fluid and undergo implantation on the mesothelial linings of the peritoneal cavity wall and abdominal organs (Figures 3,4) [22]. This peritoneal carcinomatosis is commonly associated with formation of ascites [22].

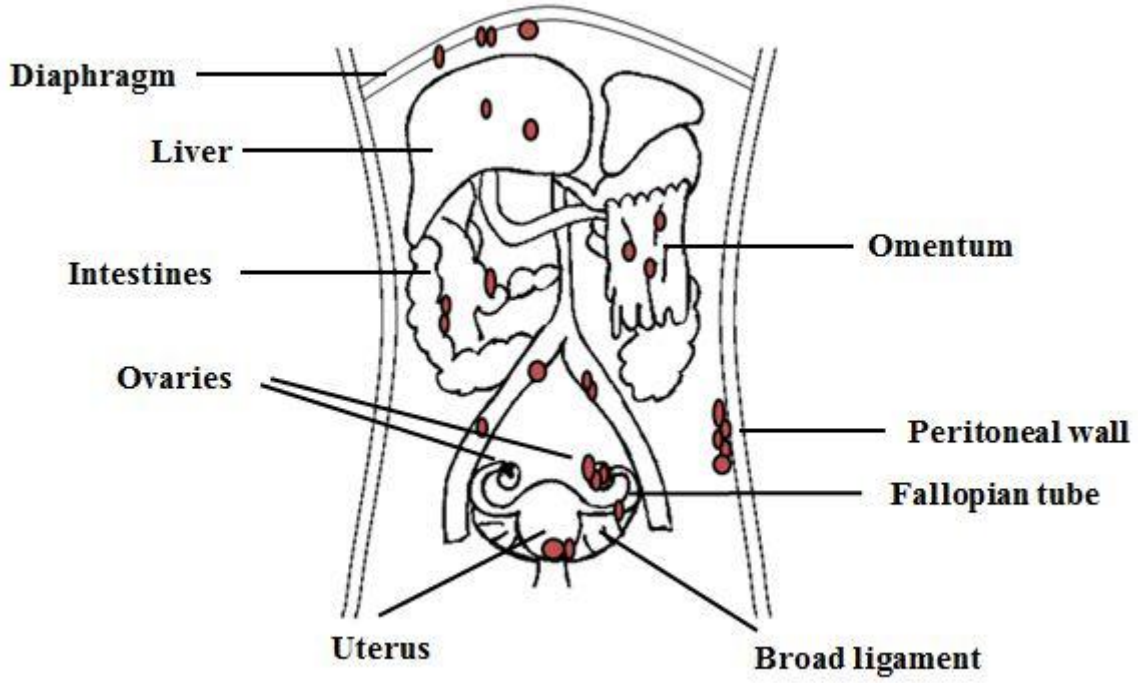

Figure 3. Pattern of spread of EOC

In the early stage, the tumor (shown as red masses) is confined to one or both the ovaries. Once the ovarian capsule is disrupted, the tumor spreads by direct extension to adjacent organs. Exfoliated tumor cells are then transported by the circulating peritoneal fluid and implant on the mesothelial linings of the pelvic cavity wall and abdominal organs. The omentum is the most common implantation site [22].

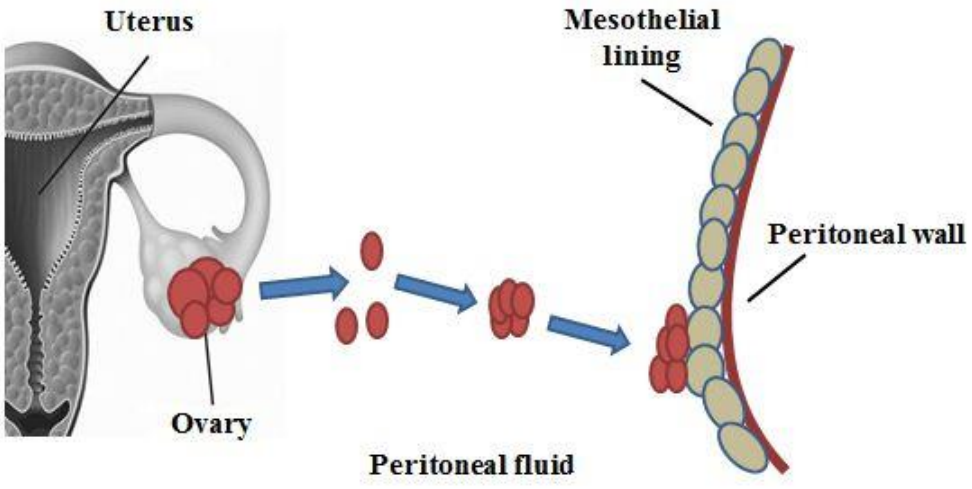

Figure 4. Model of peritoneal seeding of EOC

Distal spread of EOC cells (shown in red) occurs via 'seeding' of the peritoneal cavity. EOC cells are exfoliated into the peritoneal fluid and form multicellular aggregates to escape anoikis. Surviving EOC cells are transported by the circulating peritoneal fluid and then implant on the mesothelial linings of the peritoneal cavity [22].

2.1. Implantation of EOC cells on to the peritoneum

The ability of EOC cells to 'seed' the peritoneal cavity is a hallmark of this disease. The attachment of EOC cells to the peritoneal surfaces is a key rate-limiting step. It is thought that attachment of EOC cells to the peritoneum is mediated via two primary mechanisms: 1) attachment to the mesothelial cells lining the peritoneal cavity and 2) attachment to the sub-mesothelial extracellular matrix (ECM) [23-26]. Mesothelial cells, that are derived from the mesoderm, form a monolayer that lines the surface of body's three serosal cavities: pleural, pericardial and peritoneal [27]. The mesothelial cell lining functions as a protective layer and a non-adhesive surface that facilitates movement of other tissues and plays an essential role in immune and inflammatory responses [27]. Attachment of EOC cells to mesothelial cells is mediated by a repertoire of ligands and receptors. The EOC biomarker CA-125 can mediate tumor cell implantation by binding the glycosylphosphatidyl inositol (GPI)-linked protein mesothelin that is expressed on mesothelial cells [28, 29]. Integrins are cell surface protein complexes composed of α and β subunits that have been reported to facilitate attachment of EOC cells to mesothelial cells and also to the sub-mesothelial ECM [30]. Several groups have demonstrated that β1 integrin mediates binding of EOC cells to peritoneal mesothelial cells [31-33]. On the other hand, several studies have reported that integrins such as β1 and αvβ3 promote EOC metastasis by binding to components of the ECM such as collagen type I, fibronectin, laminin and vitronectin [34-37]. A study by Iwanicki and colleagues demonstrated that spheroids of EOC cells can gain access to the sub-mesothelial ECM by displacing mesothelial cells that line the peritoneal organs by utilizing myosin-generated traction force that is dependent on activation of α5β1 integrin and talin I [38].

Cadherins constitute a gene super-family of membrane glycoproteins that facilitate cell-cell adhesion via homophilic interactions. Substantial evidence indicates that alteration in cadherin expression, often termed 'cadherin switching' drives tumor progression [39, 40]. The most well-characterized form of cadherin-switching involves upregulation of N-cadherin and P-cadherin and downregulation of E-cadherin [39, 40]. Loss of E-cadherin expression and increase in expression of N-cadherin in advanced stage EOC correlates with poor prognosis in patients [41]. High expression of P-cadherin has also been found to correlate with reduced overall survival of EOC patients [41]. P-cadherin facilitates formation of multi-cellular aggregates of EOC cells, inhibits anoikis and also promotes attachment of EOC cells to peritoneal mesothelial cells [42].

The most-studied cell adhesion molecule that promotes attachment of EOC cells to the mesothelial lining is the cell-surface glycoprotein CD44. CD44 proteins are encoded by a highly conserved gene and are present in different isoforms [43]. This heterogeneity among the different isoforms is due to differential glycosylation and alternative splicing of exons [43]. The smallest isoform CD44s is the most common isoform and is present on the membrane of most vertebrate cells. However, unlike CD44s, variant isoforms of CD44 are only expressed on some epithelial cells during embryogenesis, lymphocytes and in certain cancers [44, 45]. CD44v7 has been reported to promote metastasis of pancreatic adenocarcinomas [46]. Another study reported that CD44v6 interacts with vascular endothelial growth factor-A (VEGF-A) and hepatocyte growth factor (HGF) to promote tumor angiogenesis [47]. Standard and variant isoforms of CD44 have been detected in EOCs [48]. Expression of CD44s in EOC is an independent predictor of survival and its expression correlates with poor outcomes and decreased overall survival [49-51]. CD44 is a major receptor for the glycosaminoglycan,

hyaluronic acid (HA) that is synthesized by mesothelial cells lining the peritoneal cavity [52, 53]. CD44 facilitates attachment of EOC cells to peritoneal mesothelial cells by binding to HA [24, 53, 54]. A study by Strobel and colleagues demonstrated that a CD44 neutralizing antibody (Ab) significantly inhibited EOC cell implantation on peritoneal mesothelial linings in intraperitoneal (i.p.) xenograft models [55]. However, the mechanisms that induce expression of CD44 in EOC cells are poorly understood and require further investigation.

2.2. Inflammatory signaling in EOC

A second hallmark of EOC is the formation of ascites [56]. Accumulation of fluid in the peritoneal cavity stems from increased vessel permeability [57, 58]. VEGF-A is widely recognized as the causative factor of ascites formation. High levels of VEGF-A have been demonstrated to induce vascular permeability and stimulate formation of ascites in EOC [59-62]. Ascitic fluid contains a wide range of growth factors, inflammatory cytokines and chemokines that function via autocrine and paracrine mechanisms and promote tumor progression [63]. Pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and interleukin-1β (IL-1β) are present in ascites of EOC patients and are associated with poor outcomes in patients [64]. IL-6 promotes EOC cell growth and also stimulates chemoresistance and tumor angiogenesis [65-68]. Elevated levels of IL-8 also promote angiogenesis in EOC [69]. Elevated levels of IL-1β in ascites of EOC patients significantly correlate with poor histopathological grade and reduced progression-free survival [70]. Normal and malignant EOC cells as well as activated stromal immune cells are the major sources of IL-1β [71]. Constitutive secretion of IL-1β by EOC cells promotes tumor invasion by inducing expression of matrix metalloproteinase-1 (MMP-1) and tumor angiogenesis by stimulating production of VEGF [72, 73]. IL-1β promotes inflammatory

signaling in tumors via activation of nuclear factor kappa B (NF-κB) signaling pathway [74]. Secretion of IL-1β by EOC cells downregulates expression of p53 in stromal fibroblasts and stimulates NF-κB signaling which in turn leads to an increase in the expression of other molecules such as IL-8, IL-6 and VEGF [75]. However, the mechanisms by which IL-1β promotes peritoneal metastasis of EOC are not clear.

2.3. NF-κB signaling in EOC

The NF-κB signaling pathway is activated in many types of tumors. A central component of the pathway comprises a family of five transcription factors: p50, p52, p65 (RelA), c-Rel and RelB [76]. There are two different pathways for activation of NF-κB signaling: canonical and non-canonical. The canonical signaling pathway is triggered by microbial products and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1 leading to activation of RelA or c-Rel, which form dimers with p50 [77]. The dimers interact with inhibitors of NF-κB family of proteins (IκBα, IκBβ, and IκBε) in the cytoplasm and are in an inactive state. When the canonical pathway is triggered, the IκB proteins get phosphorylated by the IκB kinases (IKKs) on conserved serine residues and in turn IκB proteins undergo degradation by the ubiquitin-proteasome pathway and release the NF-κB dimers. The dimers then translocate to the nucleus and function as transcription factors to activate target genes (Figure 5) [77]. The non-canonical signaling pathway is activated by TNF-family cytokines like B-cell activating factor (BAFF), lymphotoxin B, CD40 ligand (CD40L) and receptor activator of NF-κB ligand (RANKL), which results in the activation of RelB/p52 complexes. Whereas the canonical pathway is typically regulated by the IKKβ and IKKγ subunits, the non-canonical pathway is regulated by IKKα which phosphorylates and processes p100, the precursor form of p52 (Figure 5) [77, 78]. Although NF-κB signaling is

widely activated in solid tumors, there are no known oncogenic mutations in members of the NF-κB family. The activation of this pathway, therefore, has largely been attributed to inflammatory signals in the tumor microenvironment [79].

The ovarian tumor microenvironment contains a wide variety of pro-inflammatory cytokines that are secreted by tumor cells and stromal cells. High levels of TNF-α, IL-1α, IL-1β and IL-6 have been detected in EOC tissues as compared to levels in normal ovarian tissues [80-82]. Several studies have demonstrated that high levels of cytokines such as TNF-α and IL-1β in EOC promote tumor progression by activation of NF-κB signaling [75, 83]. For example, a study by Kulbe and colleagues identified that TNF-α, through its activation of NF-κB, upregulates expression of the chemokine receptor CXCR4 which in turn interacts with its ligand CXCL12, and promotes increased tumor growth and migration [83]. Activation of the NF-κB pathway in turn induces downstream target genes that encode ligands that activate the pathway such as TNF-α and IL-1β, and also other target genes encoding IL-6, IL-8, VEGF and MMPs [74]. This NF-κB signaling loop leads to activation of chronic signaling in tumors. A number of independent studies have demonstrated a strong association between activation of NF-κB transcription factors in EOC and poor outcomes in patients [84, 85]. Higher expression of NF-κB transcription factors such as p50 and p65 has been detected in advanced-stage EOCs as compared to borderline and benign tumors or normal ovaries [85]. Levels of phosphorylated p65 in EOC strongly correlate with advanced disease stage and high tumor grade [86, 87]. However, the mechanisms by which chronic NF-κB signaling promotes peritoneal dissemination of EOC are poorly understood.

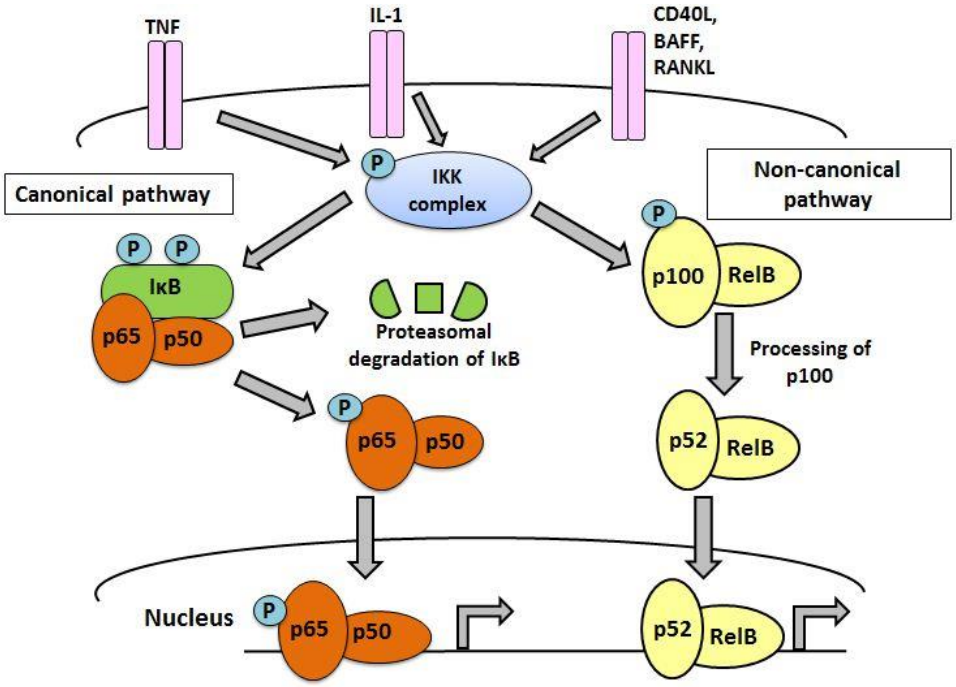

Figure 5: NF-κB signaling pathway

The canonical NF-κB signaling pathway is triggered by proinflammatory cytokines such as TNF-α and IL-1β whereas the non-canonical NF-κB signaling pathway is triggered by cytokines such as lymphotoxin B, CD40L, BAFF and RANKL. These signals lead to activation of the IKK complex (shown in blue). In the canonical pathway, the IKK complex phosphorylates IκB (shown in green) at two serine residues, which signals it for degradation and release of the phosphorylated NF-κB dimers (shown in orange). The NF-κB dimers then translocate to the nucleus and induce transcription of target genes [88]. Activation of the IKK complex in the non-canonical pathway leads to phosphorylation and degradation of p100, releasing p52-RelB heterodimers (shown in yellow). The heterodimers then translocate to the nucleus and activate transcription of target genes [89].

B. HOMEOBOX GENES

1. General overview of homeobox genes

Homeobox genes consist of a large super-family of approximately 200 vertebrate genes that play essential roles in body plan specification and development of virtually all organ systems during embryogenesis [90, 91]. In adults, homeobox genes regulate tissue homeostasis and regeneration, differentiation of hematopoietic progenitors and vascular remodeling [92, 93]. Homeobox genes were first identified due to their mutations in *Drosophila* that caused formation of body segments in the wrong context. For example, loss-of-function mutations in the *Antennapedia* gene in *Drosophila* results in development of ectopic antennae instead of legs [94]. Similarly, in humans, aberrant expression of homeobox genes can cause developmental defects like malformation of limbs or other sensory defects [95, 96]. Homeobox genes encode proteins termed 'homeoproteins' that primarily function as transcription factors [97, 98]. Homeoproteins consist of a highly conserved DNA binding domain that is known as the 'homeodomain'. This homeodomain forms three alpha-helices which binds DNA elements having TAAT core motifs (Figure 6) [99].

Figure 6. Structure of the homeodomain

Transcription factors encoded by homeobox genes consist of a highly conserved 61 amino acid DNA binding domain termed the 'homeodomain'. The homeodomain forms three α-helices (colored boxes) that bind DNA elements containing TAAT core motifs [99].

2. Genomic organization of homeobox genes

In mammals, homeobox genes are classified into different families based on the similarities in their homeodomains and functional motifs [100]. Mammalian homeobox gene families are named after their homologs in *Drosophila*. For example, members of the mammalian gene families *DLX*, *CDX*, and *OTX* are named after homologous *Drosophila* gene families *distal-less*, *caudal*, and *orthodenticle* respectively [101]. Eventhough many homeobox genes in mammals are dispersed throughout the genome, members of *HOX* and *DLX* gene families are arranged in clusters [102, 103]. The 39 members of the mammalian *HOX* gene family are grouped in clusters on four different chromosomes (Figure 7) [104, 105]. Likewise, the six members of the mammalian *DLX* gene family are arranged in bigene clusters and are located upstream of the *HOX* gene clusters (Figure 7) [105, 106]. It has been postulated that clusters of *HOX* and *DLX* gene families derived from gene duplication during evolution [103, 107].

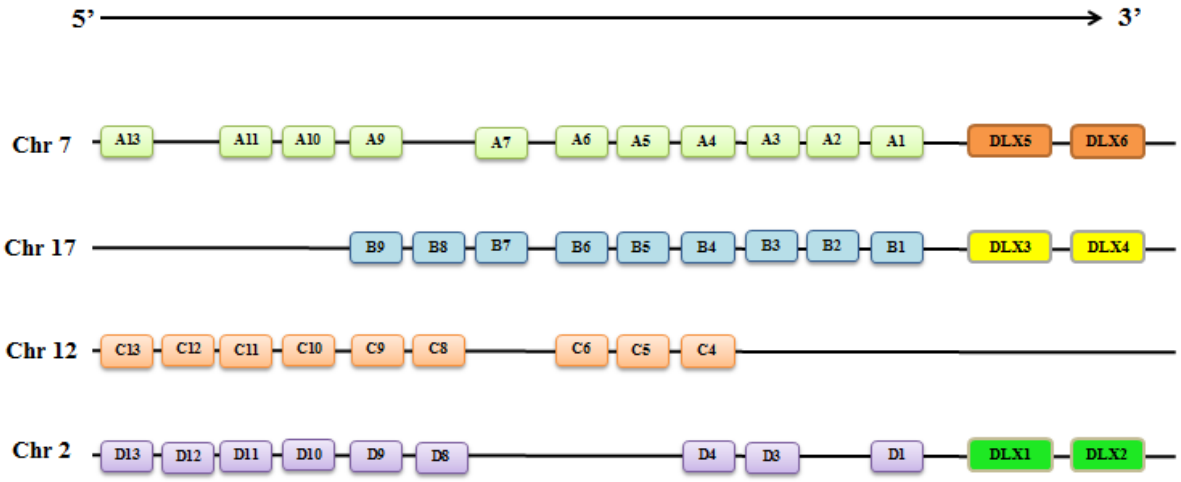

Figure 7. Clusters of *HOX* and *DLX* gene families

The 39 members of the mammalian *HOX* gene family are organized in clusters on 4 different chromosomes. The 6 members of the DLX gene family are arranged in bigene clusters, located upstream of the *HOX* loci [105, 106].

3. Deregulation of homeobox genes in tumors

Substantial evidence indicates that expression of several homeobox genes is deregulated in a wide variety of tumors [97]. Deregulation of homeobox genes in cancer falls into two broad categories. The first category of homeobox genes are those that are usually expressed only in normal, differentiated adult tissues but are often downregulated in tumors and have tumor suppressive functions (Figure 8) [97, 99, 101]. Loss of expression of these homeobox genes has been attributed to epigenetic mechanisms, long non-coding RNAs and chromosomal aberrations like loss of heterozygosity [101]. For example, loss of *HOXA5* in breast cancers occurs due to promoter hypermethylation [108]. Loss of HOXA5 results in a down-regulation of p53 expression and tumor progression in breast cancers [108]. The long non-coding RNA *HOTAIR* which is located in the *HOXC* locus binds to and re-targets Polycomb Repressive Complex 2 (PRC2), a large multi-protein complex that mediates transcriptional silencing [109, 110]. *HOTAIR* re-targets the PRC2 complex to the *HOXD* locus [109]. In breast cancers, increased expression of *HOTAIR* in primary tumors is considered to be highly predictive of metastatic disease and poor outcomes [109]. The homeobox gene *NKX3.1*, which maps to chromosomal region 8p21, is expressed in fetal and adult prostate tissues and controls normal differentiation of prostatic epithelium [111, 112]. Loss of heterozygosity of *NKX3.1* occurs in approximately 60%-80% of prostate cancers [113]. Loss of *NKX3.1* induces development of prostatic intraepithelial neoplasia (PIN) [114]. Furthermore, loss of *NKX3.1* coupled with loss of the tumor suppressor gene *PTEN* and overexpression of oncogene *c-myc* promotes prostate cancer progression [115-118].

The second category of homeobox genes are those that are usually expressed in embryonic tissues but not in adult tissues. These homeobox genes are often upregulated in

tumors and have tumor promoting functions (Figure 8) [97, 99, 101]. Gain of function of homeobox genes in tumors can be caused by chromosomal aberrations like gene amplification [101]. For example, the *HOXB* gene cluster and the homeobox gene *DLX4* map to the 17q21.3-q22 region, a chromosomal hotspot amplified in about 10% of breast cancers and EOC [119-121]. *HOXB7* is also overexpressed in various other types of tumors such as pancreatic cancer, colorectal cancer and melanomas [122-124]. High expression of HOXB7 in breast cancers, EOC and melanomas drives tumor progression in part by activating transcription of the gene that encodes basic fibroblast growth factor (bFGF) [122, 125-127]. HOXB7 also promotes resistance to tamoxifen in breast cancers by inducing expression of epidermal growth factor receptor (EGFR) and its ligands, thereby activating EGFR signaling [128]. *HOXB7* and *DLX4* are over-expressed in more than 50% of breast cancers and EOC though only 10% of cases exhibit a loss of the chromosomal region containing the gene cluster [129, 130]. This highlights the need to identify more mechanisms of homeobox gene deregulation as well as downstream transcriptional targets that will aid in developing better targeted therapies.

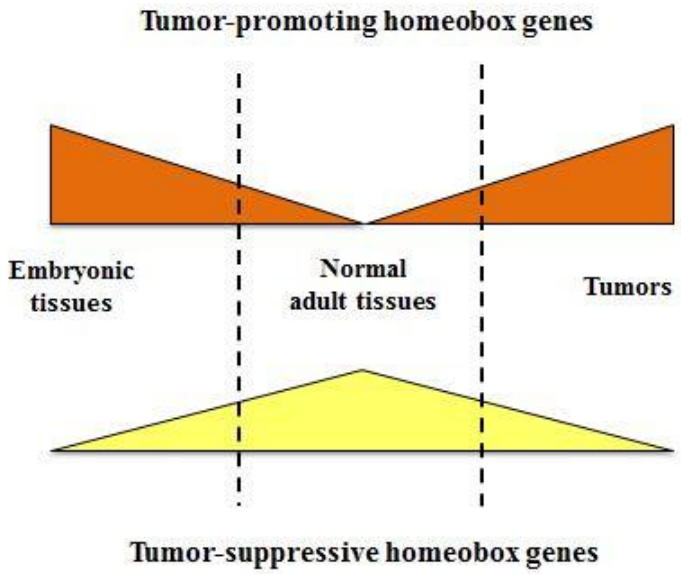

Figure 8. Trends in the aberrant expression of homeobox genes in tumors

Homeobox genes that are normally expressed only in embryonic tissues are often activated in tumors and generally have tumor-promoting functions (shown in orange). Homeobox genes that are normally expressed in normal differentiated adult tissues are down-regulated in tumors and generally have tumor-suppressive functions (shown in yellow) [101].

Table 3: Examples of significance of aberrant homeobox gene expression in tumors

Homeobox gene	Type of cancer	Expression pattern in tumors	Functional significance of deregulation in tumors	References
HOXB7	melanoma, breast, pancreatic, colorectal, ovarian		• Induces expression of EGFR and FGF2 • Promotes tumor growth and angiogenesis • Promotes EMT and chemoresistance	122-128
HSIX1	breast, pancreatic, rhabdomyosarcomas		• Promotes tumor growth by inducing cyclin A1 • Promotes lymphangiogenesis and metastasis by inducing VEGF-C and ezrin expression	131-135
HOXA9	ovarian, glioblastomas		• Promotes EOC growth by stimulating cancer-associated fibroblasts and macrophages with immunosuppressive properties • Promotes EOC dissemination via induction of P-cadherin	136-139
NKX3.1	prostate		• Loss of NKX3.1 causes PIN-like lesions in mice • Co-operates with loss of PTEN to induce prostate cancer progression	114, 116-118
HOXA5	breast		• Loss of HOXA5 promotes cell cycle deregulation and metastasis by inducing loss of p53 and promoting expression of Twist • Loss of HOXA5 induces genomic instability by down-regulating expression of human MutL homolog 1 (hMLH1)	108, 140, 141
CDX2	colorectal		• Loss of CDX2 promotes genomic instability by inducing DNA repair via non-homologous end joining (NHEJ) pathway • Loss of CDX2 deregulates cell cycle progression by down-regulating expression of cyclin dependent kinase inhibitors p27Kip1 and p21/waf1/cip1	142-144

4. Role of the homeobox gene *DLX4* in tumors

DLX4, a member of the *DLX* family of homeobox genes, is also reported as *BP1*, *DLX7* and *DLX8* [91, 145]. *DLX4* is generally expressed in endometrium, placenta, trophoblast and normal bone marrow cells but not in other normal adult tissues [146-148]. Increasing evidence indicates that *DLX4* is expressed in hematological malignancies and also solid tumors such as EOC, breast, prostate, and lung cancers (Table 4). Previous studies in our laboratory have demonstrated that high expression of DLX4 in tumors confers resistance to anti-proliferative signals mediated by TGF-β, promotes genomic instability and chemoresistance in various tumors [149, 150]. DLX4 has been reported to promote invasiveness of breast cancer by inducing expression of TWIST and inhibiting expression of E-cadherin [151, 152]. Other studies from our laboratory have found that DLX4 is absent from normal ovary and ovarian cystadenomas [129]. On the other hand, expression of DLX4 in EOC strongly correlates with advanced disease stage, high tumor grade and reduced overall survival of patients [129]. The ability of DLX4 to promote EOC growth has been attributed in part to its stimulation of tumor angiogenesis [129]. However, the mechanisms by which DLX4 promotes EOC metastasis are poorly understood.

Table 4: Examples of aberrant expression and functional significance of DLX4 in tumors

Type of cancer	Pattern of DLX4 expression in cancers	Functional significance of deregulation of DLX4 in cancers	References
Breast	Overexpressed in 80% of breast cancers	• Represses BRCA1 expression • Upregulates Twist and promotes tumor metastasis • Promotes tumor cell survival by inducing Bcl-2 and inhibiting apoptosis	130, 151-157
Ovarian	Overexpressed in ~50% of EOC and correlates with advanced disease stage and high tumor grade	• Promotes tumor angiogenesis by inducing expression of VEGF and bFGF	129
Prostate	Overexpressed in 70% of prostatic adenocarcinomas		158
Choriocarcinoma	Expressed in normal placenta and human choriocarcinoma cell lines	• Promotes tumor cell survival	159, 160
Leukemias	Overexpressed in bone marrow of 63% of acute myeloid leukemia (AML) cases	• Increases clonogenicity of leukemic cells • Induces expression of c-myc	148, 161, 162

C. HYPOTHESIS AND SPECIFIC AIMS

The high morbidity and mortality caused by EOC stems from its propensity to disseminate throughout the peritoneal cavity and the inability to detect the disease at an early, organ-confined stage. Cancer-associated ascites is enriched in growth factors and inflammatory cytokines that promote inflammatory signaling and peritoneal metastasis. However, the mechanisms by which EOC cells activate chronic inflammatory signaling and by which inflammatory cytokines promote metastasis are poorly understood. The overall goal of my study is to identify the molecular mechanisms that promote rapid metastasis of EOC.

Expression of the homeobox gene DLX4 in EOC has been found to be strongly associated with advanced disease stage and poor survival of patients. However, the significance of DLX4 in promoting metastasis of EOC is not known. My broad hypothesis is that DLX4 promotes peritoneal dissemination of EOC. Specifically, I hypothesize that DLX4 promotes peritoneal dissemination of EOC by controlling inflammatory signaling.

The specific aims of my study are:

1) To determine whether DLX4 promotes peritoneal dissemination of EOC
2) To identify the mechanisms by which DLX4 controls tumor-peritoneum interactions

CHAPTER 2: MATERIALS AND METHODS

1. Antibodies

Sources of antibodies (Abs) were as follows: DLX4 Ab (for flow cytometry, Abcam, Cambridge, MA), DLX4 Ab (for chromatin immunoprecipitation, Abnova, Taipei, Taiwan), CD44 Ab (for flow cytometry, BD Biosciences, San Jose, CA), CD44 Ab (for neutralization, Abcam), phosphorylated NF-κB p65 (Ser 536) Ab (for flow cytometry, Cell Signaling Technology, Danvers, MA). Secondary Abs were purchased from Pierce Biotechnology, Rockford, IL and BD Biosciences.

2. Plasmids

A human *DLX4* cDNA plasmid was provided by Dr. Patricia Berg (George Washington University) [162]. Flag-tagged *DLX4* was subcloned into pIRES-EGFP2 vector (Clontech, Palo Alto, CA) [149]. Flag-tagged *DLX4* was also subcloned into the retroviral vector, pRetroQ (Clontech). pGFP-VRS plasmids containing non-targeting shRNA and *DLX4* shRNAs were purchased from OriGene Technologies (Rockville, MD). *IL1B* cDNA was purchased from Origene Technologies. pGipZ lentiviral vectors containing *IL1B* shRNA and non-targeting shRNA were purchased from shRNA and the ORFeome Core Facility (University of Texas MD Anderson Cancer Center, Houston, TX). The NF-κB-luciferase reporter construct containing tandem repeats of NF-κB transcriptional response element (TRE) was purchased from SABiosciences (Frederick, MD). pBabe-GFP-IκBα dominant negative (IκBα-DN) construct was provided by Dr. William Hahn (Broad Institute of Harvard and MIT, Cambridge, MA; Addgene plasmid 15264) [163].

3. Cell culture

3.1. EOC cell lines

The EOC cell line A2780 was provided by Dr. Gordon Mills (University of Texas MD Anderson Cancer Center). The EOC cell line 2008 was provided by Dr. Zahid Siddik (University of Texas MD Anderson Cancer Center). Both cell lines were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 2mM glutamine and penicillin-streptomycin.

3.2. Primary human mesothelial cells

Cultures of primary normal human mesothelial cells were provided by Dr. Ernst Lengyel (University of Chicago). Mesothelial cells were obtained from normal omental tissues of women undergoing surgery for benign conditions [164]. Cultures were maintained in RPMI 1640 medium supplemented with 20% FBS, 2mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

4. Transfection and generation of stable lines

For generating A2780 stable lines, the retroviral constructs containing empty vector and Flag-tagged *DLX4* were used to transfect Ampho293 cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). Viral supernatants were harvested after 48 hours and used to infect A2780 cells. Stable lines were selected with puromycin (0.5μg/ml). Lipofectamine 2000 was used for transient transfections of A2780 and 2008 cells.

5. *In vitro* cell attachment assays

In vitro cell attachment assays were performed as described in Ko *et al* [30]. Mesothelial cells (30,000 per well) were seeded in 96-well plates to obtain confluent monolayers. At one day thereafter, green fluorescent protein (GFP)-expressing EOC cells (15,000 per well) were seeded onto mesothelial cells and the plates were incubated at 37ºC for one hour. For blocking experiments, EOC cells were pre-incubated with the CD44 blocking Ab or control IgG at a concentration of 10μg/ml for one hour and then seeded onto mesothelial cells. Unattached EOC cells were removed by gently washing the wells 3 times with culture media. Attached EOC cells were viewed by immunofluorescence microscopy and cells were counted in five random 200X microscopic fields per well in three independent experiments.

In other experiments, plates were coated with collagen I, fibronectin or laminin (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.5μg/well and the plates were incubated at 4ºC overnight. Plates were then washed 2 times with phosphate-buffered saline (PBS) and GFP-expressing EOC cells were seeded onto coated plates and the plates were incubated at 37ºC for one hour. Attached cells were evaluated as described above.

6. Flow cytometry

Abs were diluted in PBS containing 1% bovine serum albumin (BSA). For cell surface staining of CD44, tumor cells were incubated with CD44 Ab (1:10) for 30 minutes at 4°C, washed and incubated with peridinin-chlorophyll-protein complex (PerCP)-conjugated anti-mouse IgG. For intracellular staining of DLX4 and phosphorylated p65, tumor cells were fixed in 1% paraformaldehyde (20 minutes at 4°C) and permeabilized in 0.1% saponin (15 minutes at room temperature). Following washing, cells were incubated with Abs to DLX4

(1:20) and phosphorylated NF-κB p65 (1:500) for 30 minutes at 4°C, washed and incubated with PerCP- or phycoerythrin (PE)- conjugated secondary Abs. Staining was detected by flow cytometry (FACS Calibur, BD Biosciences).

7. ELISA

ELISA kit for IL-1β was purchased from R&D Systems (Minneapolis, MN). Cells were lysed using M-PER buffer (Pierce Biotechnology) and cell lysates were prepared as per the manufacturer's instructions. Intracellular IL-1β levels were assayed in the cell lysates as per the manufacturer's instructions and normalized to the total cellular protein content in three independent experiments.

8. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using PureLink RNA mini kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. 1 μg of RNA was used to synthesize cDNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) as per the manufacturer's instructions. Transcript levels were analyzed on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using primers listed in Table 5 and iTaq Universal SYBR® Green Supermix (Bio-Rad) as per the manufacturer's instructions. Target gene transcript levels were normalized to levels of ribosomal protein *RPL32*. mRNA levels were assayed in triplicate in two independent experiments.

Table 5: Primers for qRT-PCR

Gene		Sequence
CD44	Forward	5’- GGCTTTCAATAGCACCTTGC - 3’
	Reverse	5’- ACACCCCTGTGTTGTTTGCT - 3’
CDH3	Forward	5’- CAGGTGCTGAACATCACGGACA - 3’
	Reverse	5’- CTTCAGGGACAAGACCACTGTG - 3’
ITGB1	Forward	5’- GGATTCTCCAGAAGGTGGTTTCG - 3’
	Reverse	5’- TGCCACCAAGTTTCCCATCTCC - 3’
IL1B	Forward	5’- CCACAGACCTTCCAGGAGAATG - 3’
	Reverse	5’- GTGCAGTTCAGTGATCGTACAGG - 3’
RPL32	Forward	5’- ACAAAGCACATGCTGCCCAGTG - 3’
	Reverse	5’- TTCCACGATGGCTTTGCGGTTC - 3’

9. Chromatin Immunoprecipitation (IP)

Chromatin IP assays were performed using EZ-ChIP Chromatin Immunoprecipitation Kit (Upstate Biotechnology, Temecula, CA). Cells were crosslinked by adding formaldehyde to a final concentration of 1% for 10 minutes at room temperature. Glycine was then added at room temperature for 5 minutes to quench formaldehyde. Cells were then washed twice with 1X PBS and harvested. Cell pellets were lysed with Sodium Dodecyl Sulfate (SDS) lysis buffer (Upstate Biotechnology) and then sonicated to generate fragments of DNA of ~200-1000 base pairs in length. Sheared chromatin was first pre-cleared by incubating with protein G agarose beads for 1 hour. Pre-cleared chromatin was then incubated with Abs to DLX4 or normal IgG overnight at 4°C. Protein G agarose beads were then added and incubated for 1 hour at 4°C. The agarose-antibody/chromatin complex was washed as per the manufacturer's instructions followed by elution of protein/DNA complexes, reverse crosslinking of protein/DNA complexes at 65°C overnight and purification of DNA using spin columns. Purified DNA was used to amplify fragments of the *IL1B* promoter by PCR. PCR products were analyzed on a 3% agarose gel. The primers used for PCR amplification are listed in Table 6.

Table 6: Primers for chromatin IP assays

Gene		Sequence
IL1B	Forward	5'- GGTAGAGACCCACACCCTCA - 3'
	Reverse	5'- CATGGAAGGGCAAGGAGTAG - 3'
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Forward	5'- TACTAGCGGTTTTACGGGCG - 3'
	Reverse	5'- TCGAACAGGAGGAGCAGAGAGCGA - 3'

10. Luciferase reporter assays

Cells were plated at a density of 1-2 x 10^5 cells per well in 12-well plates and co-transfected with expression plasmids (500ng/well) and NF-κB Luc or Negative Luc reporter plasmids (100ng/well) using Lipofectamine 2000 reagent. At 24 hours after transfection, luciferase activities were assayed using the Dual-reporter assay kit (Promega, Madison, WI). Efficiencies of transfections were normalized using Renilla luciferase readings. Relative luciferase activities were assayed in three independent experiments.

11. Statistical analysis

Statistical significance for differences in the number of peritoneal implants in mice was assessed by Mann-Whitney ***U***-Test. Statistical significance for *in vitro* assays was calculated by Student's *t*-test. All statistical analyses were performed using STATISTICA10 software (StatSoft, Inc.). *P* values < 0.05 were considered significant.

CHAPTER 3: ROLE OF DLX4 IN PERITONEAL DISSEMINATION OF EOC

A. RATIONALE

More than 60% of EOC patients are diagnosed with advanced stage disease that has spread throughout the peritoneal cavity [8]. EOC cells typically spread by shedding into the circulating fluid that transports the cells throughout the peritoneal cavity. EOC cells then implant on to the mesothelial linings of the peritoneal and abdominal organs including the mesentery, diaphragm, omentum and cavity wall (Figures 3, 4). However, the mechanisms that enable implantation of EOC cells on to peritoneal surfaces are poorly understood.

Previous studies from our laboratory have found that the homeobox gene *DLX4* is highly expressed in advanced stage EOC [129]. High expression of DLX4 correlated with disease stage and poor survival of patients [129]. However, the mechanisms by which DLX4 promotes peritoneal metastasis of EOC are not known. In this chapter, the goal of my studies is to determine whether DLX4 promotes peritoneal dissemination of EOC.

B. RESULTS

1. DLX4 promotes implantation of EOC cells onto peritoneal surfaces in i.p. mouse xenograft models

In advanced stage disease, exfoliated EOC cells are transported by the circulating peritoneal fluid (Figure 4). Thereafter, these circulating EOC cells implant onto the mesothelial cells that line the peritoneal cavity. Nests of tumor cells are frequently observed on the mesentery, diaphragm and omentum of patients (Figure 3). Our laboratory has previously generated xenografts by i.p. inoculation of female nude mice with cells of GFP-expressing A2780 EOC lines that stably express or lack DLX4 [129]. To initiate this study, I analyzed tissues of mice inoculated with vector-control A2780 cells and with DLX4-expressing A2780 cells (+DLX4) to determine the degree of implantation of EOC cells to sites within the peritoneal cavity. Significantly higher numbers of implants were observed on the mesentery and diaphragm of mice inoculated with +DLX4 A2780 cells as compared to mice inoculated with equal numbers of vector-control A2780 cells ($P < 0.01$) (Figure 9). This observation raised the possibility that DLX4 promotes attachment of EOC cells to the mesothelial linings of the peritoneal and abdominal organs.

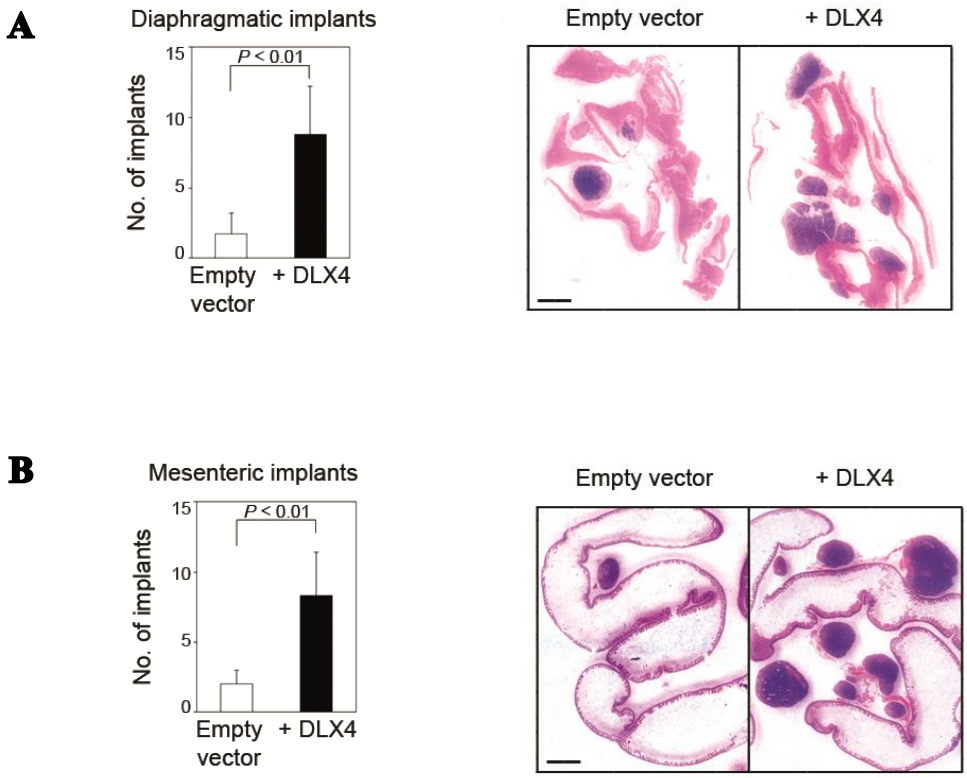

Figure 9. DLX4 increases the number of peritoneal tumor implants in i.p. mouse xenograft models

Female nude mice (n=5 per group) were inoculated i.p. with $3x10^6$ cells of vector-control and +DLX4 A2780 cell lines and sacrificed 4 weeks thereafter. Numbers of implants were counted on hematoxylin-eosin (HE) stained xenograft tissue sections from **A)** diaphragm and **B)** mesentery of mice. Bar, 2mm.

2. DLX4 promotes *in vitro* attachment of EOC cells to mesothelial cells

For this study, two EOC cell lines were used as models. For determining the effect of overexpressing DLX4, I used retrovirally transduced stable A2780 cell lines that express either empty vector or DLX4. For determining the effect of inhibiting DLX4, I used 2008, an EOC cell line that endogenously expresses DLX4 at a high level [129]. Knockdown of DLX4 in 2008 cells was carried out by using two shRNA plasmids that targeted two different sites within *DLX4* (shDLX4-A and shDLX4-B). Overexpression of DLX4 in A2780 cell lines and knockdown of DLX4 in 2008 cell lines was assayed and quantified by qPCR analysis (not included) and flow cytometric analysis of intracellular staining of DLX4 (Figure 10).

Attachment of EOC cells within the peritoneal cavity is mediated via two primary mechanisms: attachment of EOC cells to the mesothelial cells lining the peritoneal cavity and attachment to the sub-mesothelial ECM [23-26]. To determine whether DLX4 promotes attachment of EOC cells to peritoneal mesothelial cells, *in vitro* cell attachment assays were performed (Figure 11) by seeding equal numbers of GFP-expressing vector-control A2780 cells and +DLX4 A2780 cells on to confluent monolayers of primary normal human mesothelial cells. Significantly higher number of +DLX4 A2780 cells were attached to the mesothelial cells as compared to the vector-control A2780 cells ($P < 0.01$) (Figure 12A). Conversely, 2008 cells in which DLX4 was knocked down (shDLX4-A and shDLX4-B) showed significantly reduced attachment to mesothelial cells as compared to control 2008 cells (empty vector and non-targeting) ($P < 0.01$) (Figures 12B, 12C). These findings indicate that DLX4 promotes attachment of EOC cells to mesothelial cells.

To determine whether DLX4 promotes attachment of EOC cells to components of the sub-mesothelial ECM, *in vitro* cell attachment assays were performed by seeding equal numbers of GFP-expressing A2780 cells (empty vector and +DLX4) onto plates coated with ECM components, i.e. collagen I, fibronectin and laminin. No significant difference was observed between the numbers of vector-control and +DLX4 A2780 cells that attached to the components of the ECM (Figure 13). Similarly, no significant difference was observed in the numbers of DLX4-knockdown 2008 cells and control 2008 cells that attached to the ECM components (Figure 13). Together, these observations indicate that DLX4 promotes attachment of EOC cells to peritoneal mesothelial cells but not to the components of the ECM.

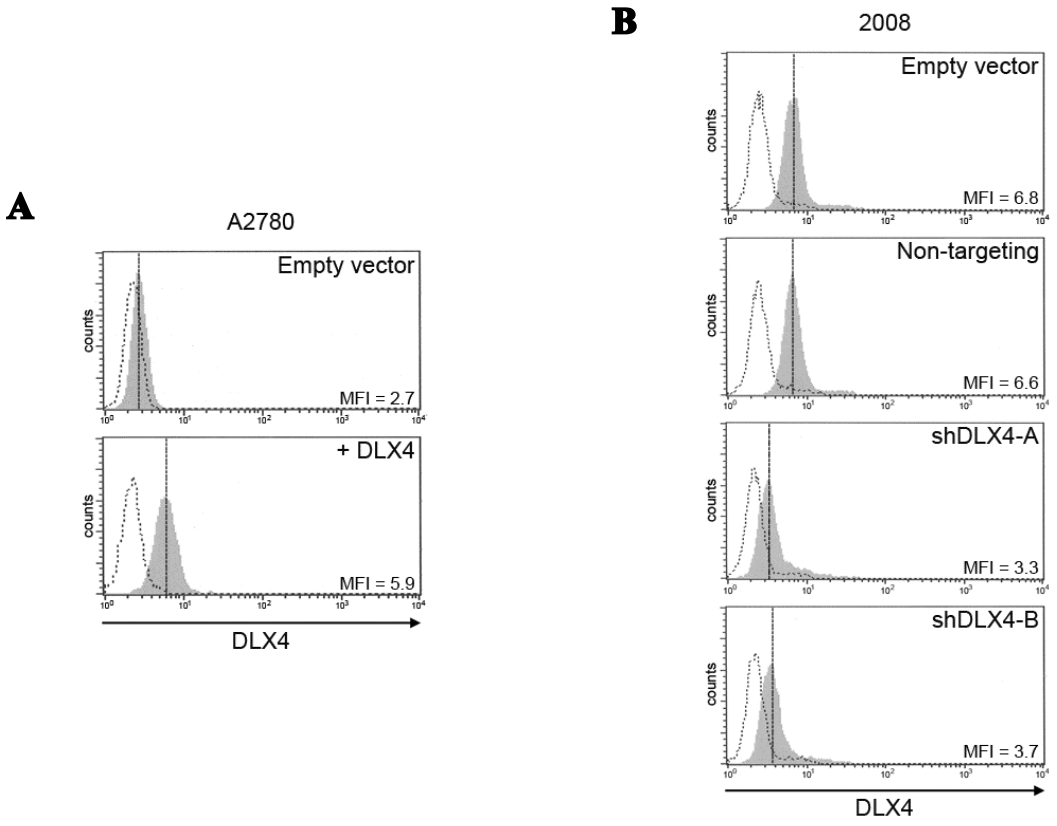

Figure 10. Overexpression of DLX4 in A2780 cells and knockdown of DLX4 in 2008 cells

Flow cytometric analysis of intracellular staining of DLX4 in **(A)** A2780 cell lines that express empty vector and DLX4 and in **(B)** 2008 cells that express empty vector, non-targeting shRNA and shRNAs that target two different sites within *DLX4* (shDLX4-A and shDLX4-B). Mean fluorescence intensities (MFI) of staining are indicated.

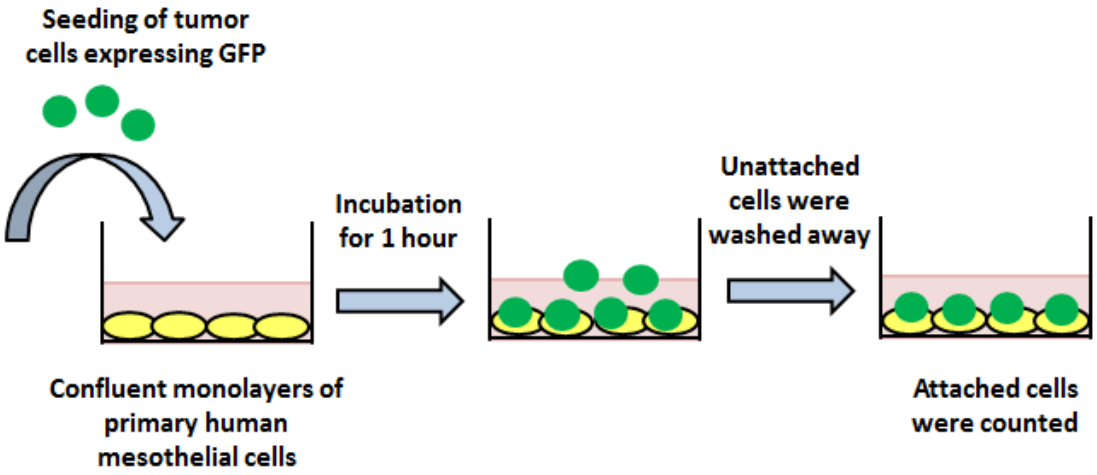

Figure 11. ***In vitro*** **cell attachment assays**

Equivalent numbers of GFP-expressing EOC cells (shown in green) were seeded onto confluent monolayers of primary normal human mesothelial cells (shown in yellow) in 96-well plates or on wells coated with collagen I, fibronectin or laminin. Cell attachment was assayed at one hour after seeding. Attached cells were viewed by immunofluorescence microscopy and counted in five random 200X microscopic fields per well. Each assay was performed in triplicate.

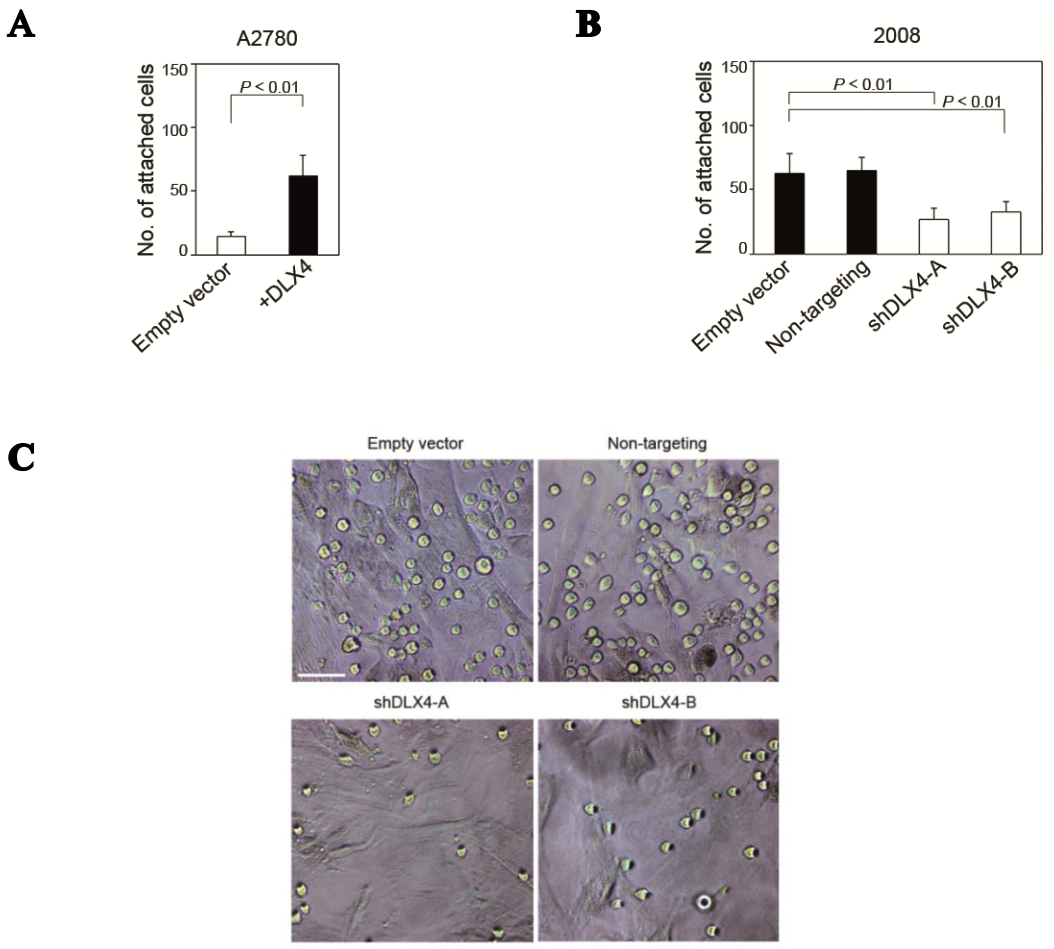

Figure 12. DLX4 promotes *in vitro* attachment of EOC cells to peritoneal mesothelial cells

Equivalent numbers of GFP-expressing EOC cells were seeded onto confluent monolayers of peritoneal mesothelial cells. After one hour, numbers of attached cells were counted in 5 random 200X microscopic fields per well. Numbers of **(A)** Attached vector-control and +DLX4 A2780 cells and **(B)** Control and DLX4-knockdown 2008 cells and **(C)** A representative picture of GFP-expressing 2008 cells attached to the monolayer of mesothelial cells. Shown in (A) and (B) are mean ± sd values of three independent attachment assays.

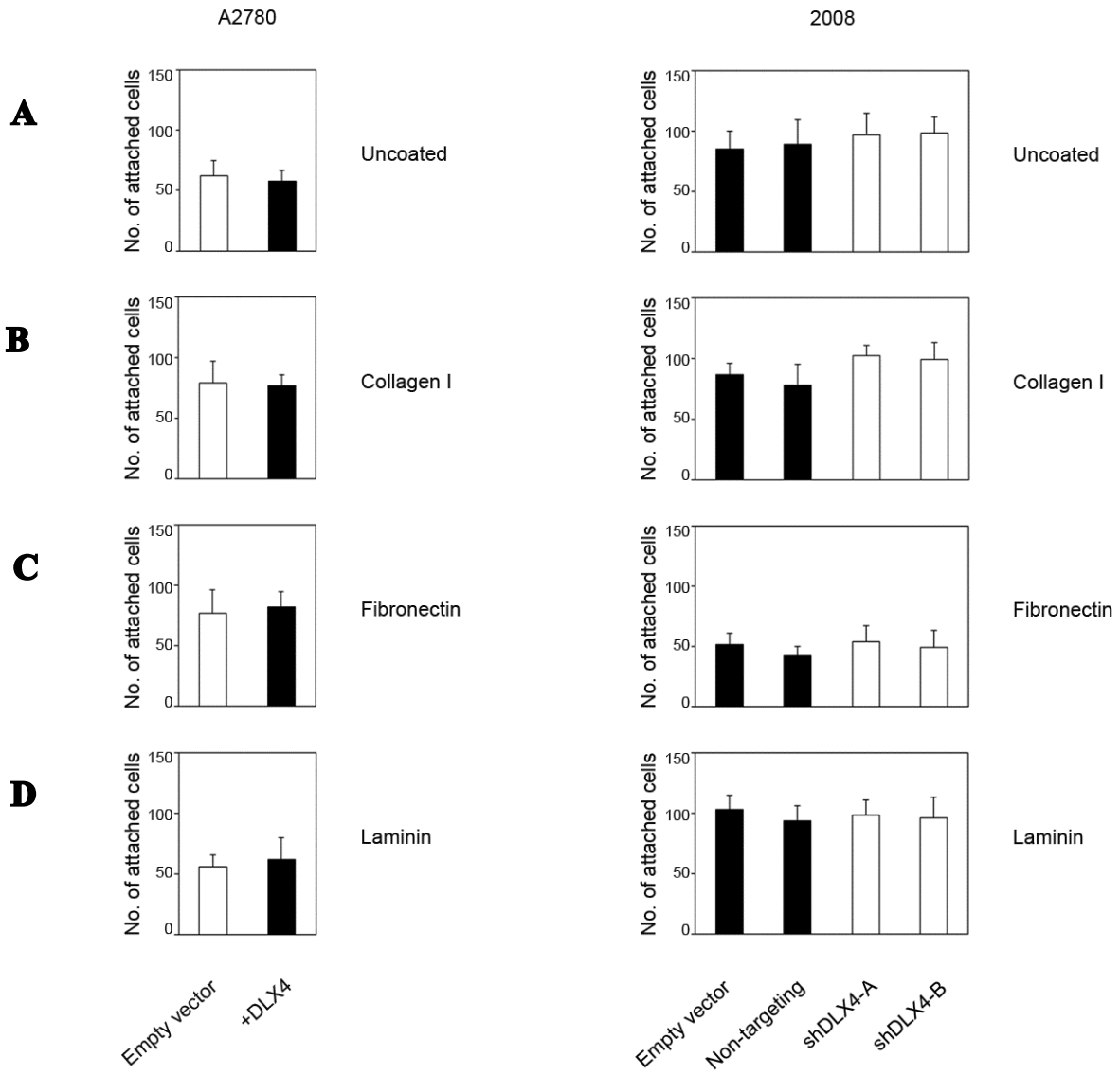

Figure 13. DLX4 does not mediate attachment of EOC cells to components of the ECM

Equivalent numbers of vector-control and +DLX4 A2780 cells and control and DLX4-knockdown 2008 cells were seeded on wells that were **(A)** uncoated or coated with **(B)** collagen I, **(C)** fibronectin and **(D)** laminin. Numbers of attached cells were counted after 1 hour in five random 200X microscopic fields per well. Shown are mean ± sd values of three independent attachment assays.

3. DLX4 induces expression of CD44 in EOC cells

Attachment of EOC cells to the mesothelium lining the peritoneal cavity is mediated by various cell adhesion molecules such as P-cadherin, β1 integrin and CD44 [24, 32, 33, 42]. Because DLX4 is a transcription factor, DLX4 might promote attachment of EOC cells to mesothelial cells by inducing expression of these cell adhesion molecules. To investigate this possibility, I initially performed qRT-PCR to evaluate mRNA levels of candidate genes that encode cell adhesion molecules when DLX4 was overexpressed or knocked down. When DLX4 was overexpressed in A2780 cells, there was more than a 10-fold induction in *CD44* mRNA levels as compared to levels in vector-control A2780 cells (P <0.001) (Figure 14A). However, there was no induction in mRNA levels for P-cadherin (*CDH3*) or β1 integrin (*ITGB1*) (Figures 14B, 14C). Conversely, when DLX4 was knocked down in 2008 cells, there was a significant reduction in *CD44* mRNA levels (P < 0.01) (Figure 14A). No reduction in *CDH3* or *ITGB1* mRNA levels was observed after knockdown of DLX4 (Figures 14B, 14C).

To further confirm the induction of CD44 by DLX4, I analyzed cell surface levels of CD44 protein by flow cytometry in vector-control and +DLX4 A2780 cells and in control and DLX4-knockdown 2008 cells. This analysis showed an induction in CD44 levels when DLX4 was overexpressed in A2780 cells (Figure 15A) and a reduction in CD44 levels when DLX4 was knocked down in 2008 cells (Figure 15B). These findings indicate that DLX4 induces expression of CD44.

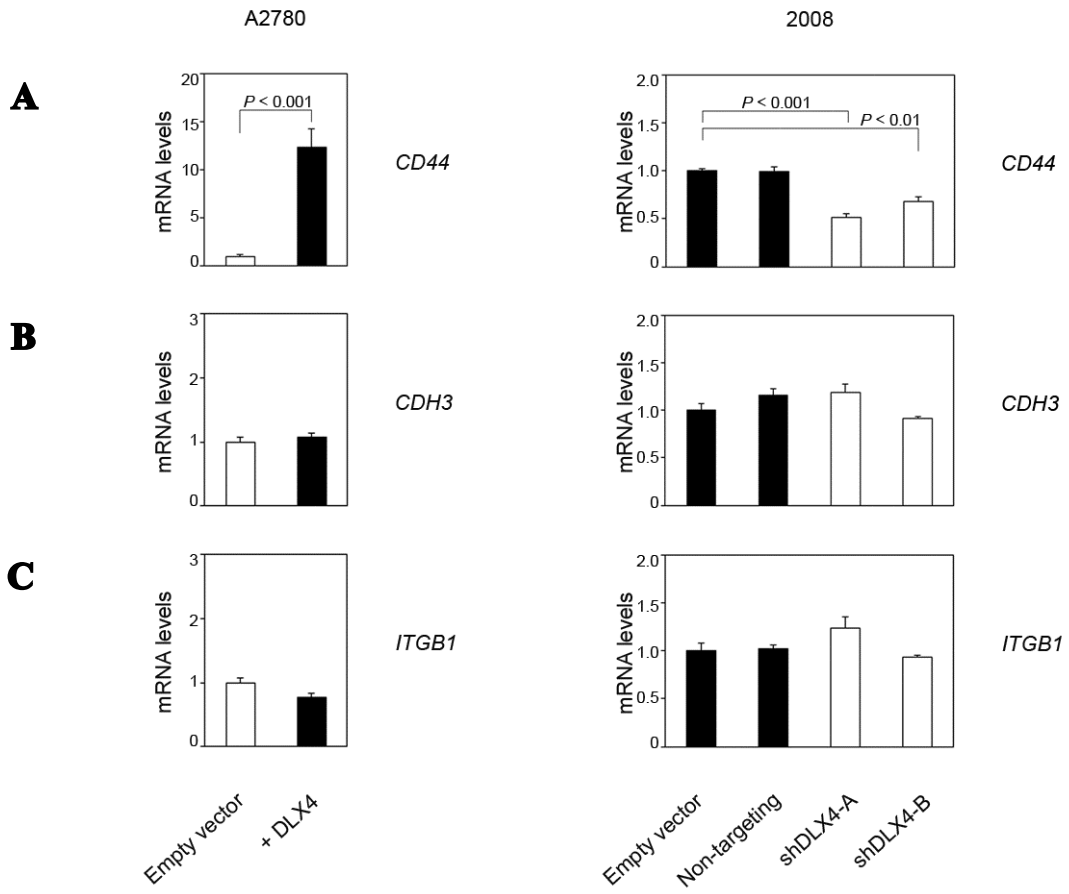

Figure 14. DLX4 induces expression of CD44 but not P-cadherin or β1 integrin

A2780 cells that expressed empty vector or DLX4 and 2008 cells that expressed empty vector, non-targeting shRNA or shRNAs for *DLX4* (shDLX4-A and shDLX4-B) were assayed by qRT-PCR for mRNA transcript levels of **(A)** *CD44*, **(B)** *CDH3* (encoding P-cadherin) and **(C)** *ITGB1* (encoding β1 integrin). All samples were analyzed in triplicates in two independent experiments. Error bars represent standard deviation.

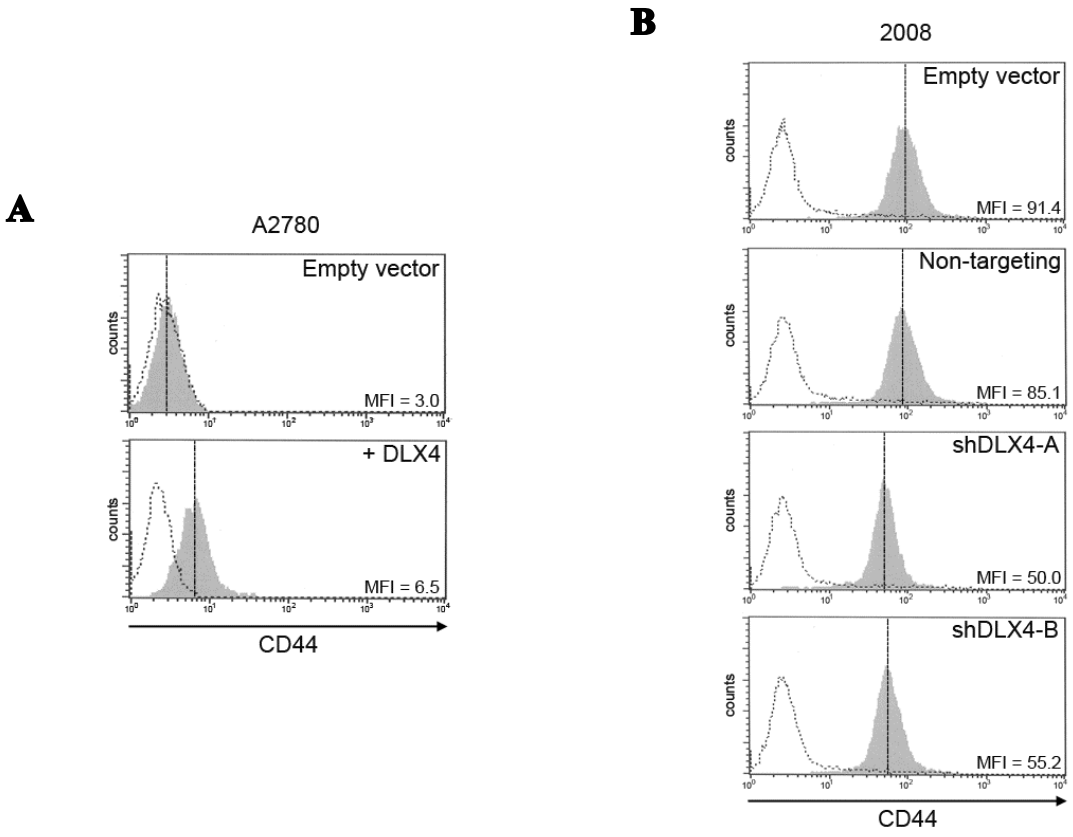

Figure 15. DLX4 induces expression of CD44

Cell surface levels of CD44 were assayed by flow cytometry in **(A)** A2780 cells that expressed empty vector or DLX4 and in **(B)** 2008 cells that expressed empty vector, non-targeting shRNA or shRNAs against *DLX4* (shDLX4-A and shDLX4-B).

4. DLX4 promotes attachment of EOC cells to peritoneal mesothelial cells via CD44

The glycoprotein CD44 is a major receptor for HA that is synthesized by peritoneal mesothelial cells [52, 53]. Binding of CD44 to HA promotes attachment of EOC cells to mesothelial cells [24, 53, 54]. Because my findings indicated that DLX4 promotes attachment of EOC cells to mesothelial cells and also induces CD44, I evaluated whether DLX4 promotes attachment of EOC cells to mesothelial cells via CD44. *In vitro* cell-attachment assays were performed in which vector-control and +DLX4 A2780 cells were pre-incubated with a neutralizing Ab against CD44 prior to seeding onto mesothelial cells (Figure 16). The Ab was directed against an epitope close to the HA-binding domain of CD44 (Figure 16). Treatment with this neutralizing CD44 Ab blocked the attachment of +DLX4 A2780 cells to the peritoneal mesothelial cells, whereas treatment with control IgG had no effect (Figure 17). In contrast, treatment with CD44 Ab had no significant effect on the ability of vector-control A2780 cells to attach to mesothelial cells (Figure 17). This observation was consistent with my previous findings that vector-control A2780 cells express very low levels of CD44 (Figures 14A, 15). Together, these findings indicate that DLX4 promotes attachment of EOC cells to peritoneal mesothelial cells via CD44.

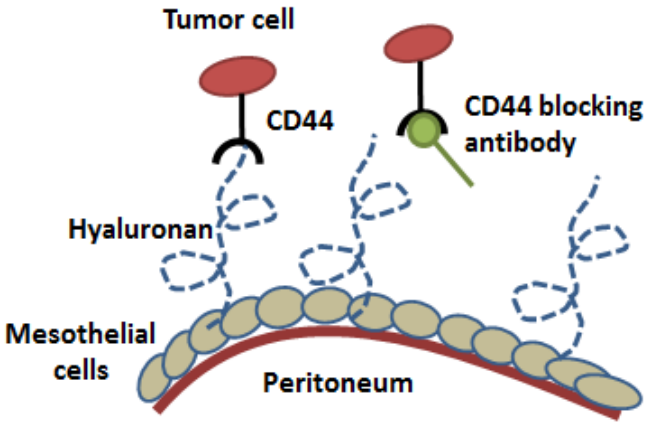

Figure 16. Mechanism of CD44-mediated attachment of EOC cells to mesothelial cells

The cell adhesion molecule CD44, which is present on EOC cells, is a major receptor for HA that is synthesized by mesothelial cells [52, 53]. Interaction of CD44 and HA facilitates implantation of EOC cells to mesothelial cells lining the peritoneal cavity [24, 53, 54]. The CD44 blocking Ab is directed against an epitope very close to the HA-binding domain of CD44 and prevents binding of CD44 to HA [165].

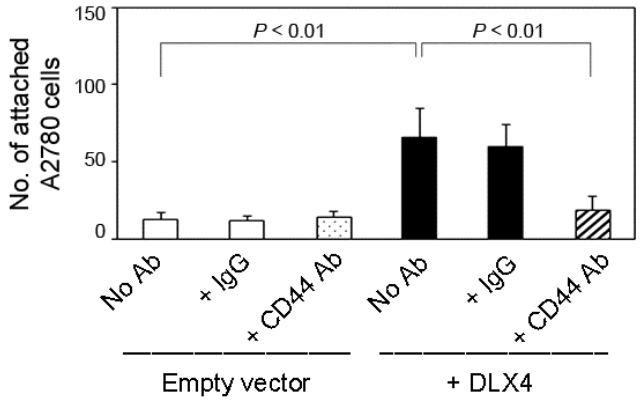

Figure 17. Neutralization of CD44 blocks the ability of DLX4-expressing EOC cells to attach to mesothelial cells

GFP-expressing empty vector and +DLX4 A2780 cells were pre-incubated with CD44 blocking Ab or normal IgG for 1 hour and then seeded onto confluent monolayers of mesothelial cells. Attachment of A2780 cells to the mesothelial monolayer was assayed at 1 hour after seeding. Attached cells were counted in 5 random 200X microscopic fields. Shown are mean ± sd values of three independent attachment assays.

C. CONCLUSION

My studies in Chapter 3 demonstrate that overexpression of DLX4 in EOC cells induces CD44 expression and promotes attachment of EOC cells to mesothelial cells. Conversely, knockdown of DLX4 in EOC cells down-regulates expression of CD44 and reduces attachment of EOC cells to mesothelial cells. Furthermore, blocking CD44 in DLX4-overexpressing EOC cells hinders their ability to attach to mesothelial cells. Together, these findings indicate that DLX4 promotes attachment of EOC cells to peritoneal mesothelial cells by inducing expression of CD44. The molecular mechanisms by which DLX4 induces expression of CD44 in EOC cells will be discussed in Chapter 4.

CHAPTER 4: MECHANISMS OF DLX4 IN PERITONEAL DISSEMINATION AND INFLAMMATORY SIGNALING IN EOC

A. RATIONALE

Formation of ascites is one of the hallmarks of advanced-stage EOC [56]. Ascites of EOC patients contains a wide variety of pro-inflammatory cytokines such as IL-8, IL-6 and IL-1β that stimulate tumor progression[63, 66, 67, 69, 72, 73]. Expression of these cytokines is associated with poor prognosis of EOC patients [64]. However, the precise mechanisms by which inflammatory signaling in EOC contributes to peritoneal dissemination of tumors is poorly understood.

Studies in Chapter 3 demonstrated that DLX4 promotes the attachment of EOC cells to peritoneal mesothelial cells (Figure 12). My studies identified that this stimulatory effect of DLX4 is mediated by its ability to induce expression of CD44 on the surface of EOC cells (Figures 14A, 15, 17). In this chapter, the goal of my studies is to identify the mechanisms by which DLX4 induces expression of CD44 in EOC cells.

B. RESULTS

1. DLX4 induces expression of IL-1β

Because DLX4 primarily functions as a transcription factor, I initially hypothesized that DLX4 might directly activate *CD44* transcription. However, no potential DLX4 binding sites were identified in the *CD44* promoter region by performing analysis of transcription factor binding sites (Genomatix). Several studies have demonstrated that the pro-inflammatory cytokine IL-1β promotes expression of CD44 in other inflammatory diseases such as arteriosclerosis and rheumatoid arthritis [166, 167]. Other studies have identified that IL-1β levels are elevated in EOC patients [70]. I therefore hypothesized that DLX4 induces expression of CD44 in EOC cells by stimulating production of IL-1β.

To determine whether DLX4 induces expression of IL-1β in EOC cells, I evaluated the expression of IL-1β when DLX4 was overexpressed or knocked down. *IL1B* mRNA levels were significantly higher in +DLX4 A2780 cells as compared to the vector control A2780 cells ($P < 0.001$) (Figure 18A). Conversely, *IL1B* mRNA levels were significantly lower in DLX4-knockdown 2008 cells as compared to control 2008 cells ($P < 0.001$) (Figure 18A). Similarly, IL-1β protein levels were induced when DLX4 was overexpressed in A2780 cells and were reduced when DLX4 was knocked down in 2008 cells (Figure 18B). These results demonstrate that DLX4 induces expression of IL-1β in EOC cells.

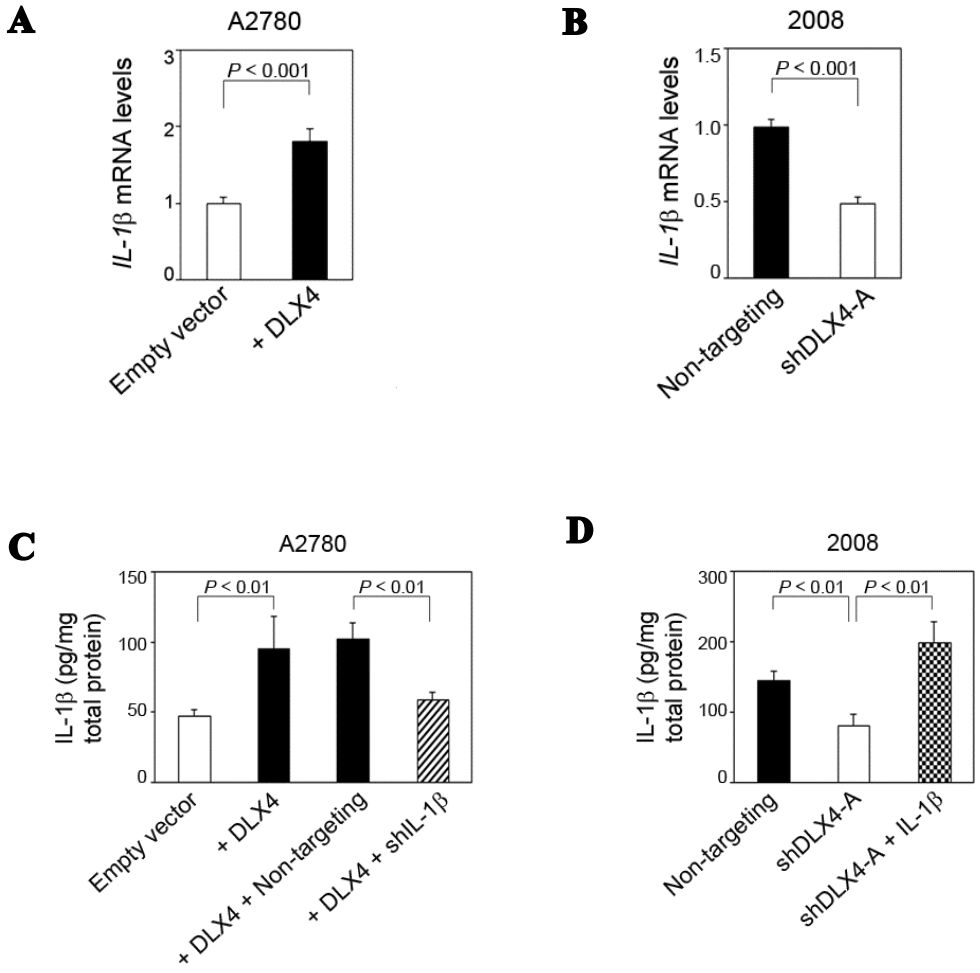

Figure 18. DLX4 induces expression of IL-1β

mRNA transcript levels of *IL1B* were assayed by qRT-PCR in **(A)** empty vector control and +DLX4 A2780 cells and **(B)** control (non-targeting) and DLX4-knockdown (shDLX4-A) 2008 cells. Shown are mean ± sd values of three independent assays.

Protein levels of IL-1β were assayed by ELISA in **(C)** empty vector control, +DLX4 and +DLX4 A2780 cells transfected with non-targeting shRNA and *IL1B* shRNA and **(D)** control (non-targeting), DLX4-knockdown (shDLX4-A) and DLX4-knockdown 2008 cells that were reconstituted with *IL1B.* Shown are mean ± sd values of three independent assays.

2. *IL1B* is a direct transcriptional target of DLX4

DLX4 is primarily known to function as a transcription factor. I therefore hypothesized that *IL1B*, the gene that encodes IL-1β, is a direct transcriptional target of DLX4 in EOC cells. A putative DLX4 binding motif was identified in the human *IL1B* promoter (Figure 19A). Binding of endogenous DLX4 to the binding site detected on the *IL1B* promoter was demonstrated by chromatin IP assays in 2008 cells (Figure 19B).

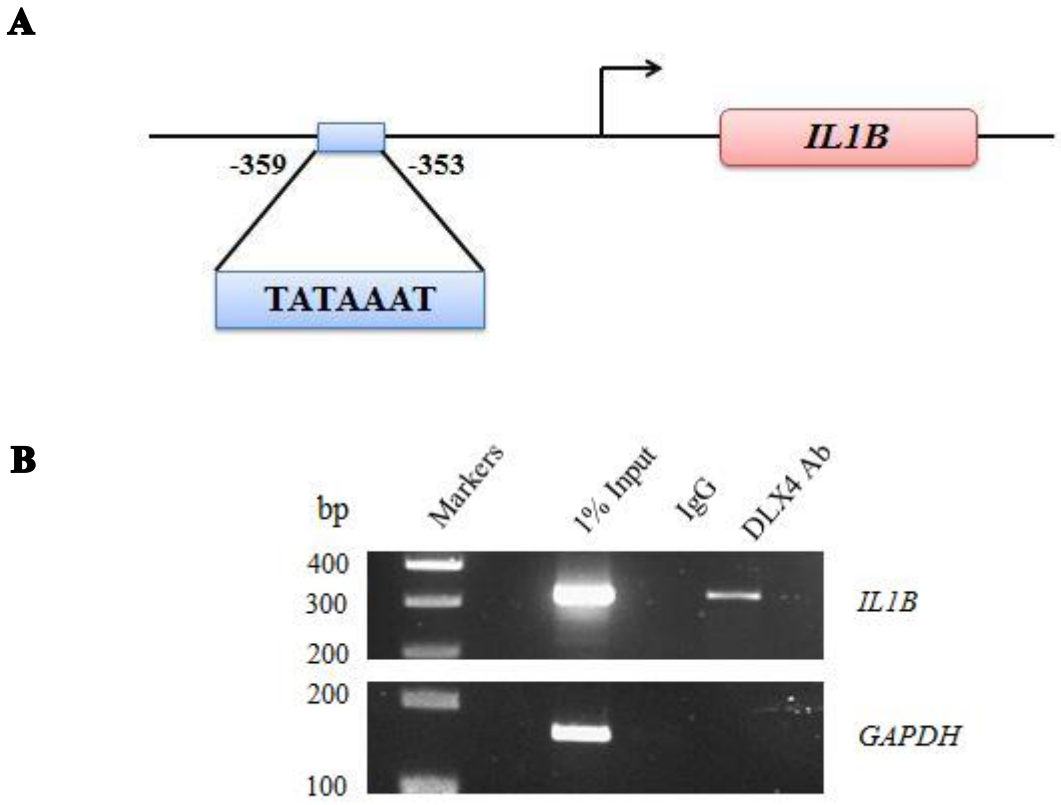

Figure 19. DLX4 directly binds to *IL1B* promoter

(A) Representation of the *IL1B* promoter with a putative DLX4 binding site (TATAAAT) located between nucleotides -353 to -359. **(B)** Chromatin immunoprecipitation analysis of the interaction of endogenous DLX4 in 2008 cells with the putative binding site identified on the *IL1B* promoter. Input DNA corresponds to 1% of the chromatin solution before immunoprecipitation. Immunoprecipitation was performed with DLX4 Ab and control IgG. Purified DNA was amplified with primers specific to *IL1B* promoter region and *GAPDH*.

3. DLX4 induces NF-κB transcriptional activity in EOC cells

IL-1β is known to induce canonical NF-κB signaling in tumors [74]. Because my studies demonstrated that DLX4 induces expression of IL-1β in EOC cells, I investigated whether DLX4 promotes NF-κB transcriptional activity in EOC cells. To accomplish this, I assayed luciferase activity of a reporter construct driven by a synthetic promoter containing tandem repeats of NF-κB binding sites (Figure 20A) in cells in which DLX4 was overexpressed or knocked down. Overexpression of DLX4 in A2780 cells induced a 3-fold increase in NF-κB transcriptional activity ($P < 0.001$) (Figure 20B). Conversely, knockdown of DLX4 in 2008 cells reduced NF-κB transcriptional activity by 50% ($P < 0.01$) (Figure 20B).

When canonical NF-κB signaling is activated, NF-κB transcription factors p65, p50 and c-Rel translocate to the nucleus, phosphorylate, form dimers and activate NF-κB target genes (Figure 5) [77, 88]. High level of phosphorylated p65 in EOC is associated with advanced disease stage in patients [85-87]. In subsequent studies, I evaluated levels of phosphorylated p65 in EOC cells when DLX4 was overexpressed or knocked down by flow cytometric analysis of intracellular staining. Higher levels of phosphorylated p65 were detected in +DLX4 A2780 cells as compared to the vector-control cells (Figure 21A). Conversely, lower levels of phosphorylated p65 were detected in DLX4-knockdown 2008 cells as compared to control 2008 cells (Figure 21B). Together, these results demonstrate that DLX4 induces NF-κB transcriptional activity and levels of phosphorylated NF-κB transcription factor p65 in EOC cells.

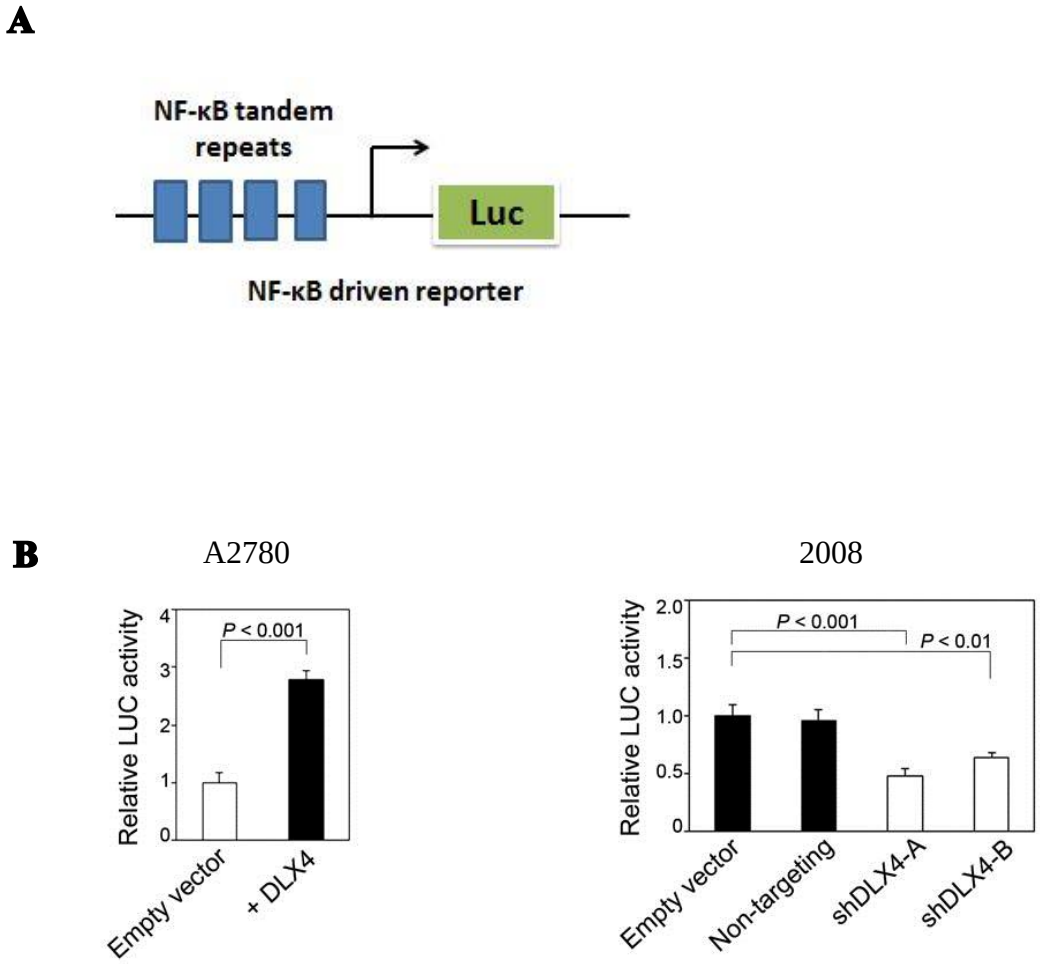

Figure 20. DLX4 induces NF-κB signaling in EOC cells

(A) An illustration of the NF-κB-driven luciferase reporter construct containing tandem repeats of NF-κB transcriptional response elements. **(B)** Luciferase reporter assays were performed to assay NF-κB transcriptional activity in empty vector and +DLX4 A2780 cells and 2008 cells transfected with empty vector, non-targeting shRNA and shRNAs against *DLX4* (shDLX4-A and shDLX4-B). Shown are relative luciferase activities in three independent experiments. Error bars represent standard deviation.

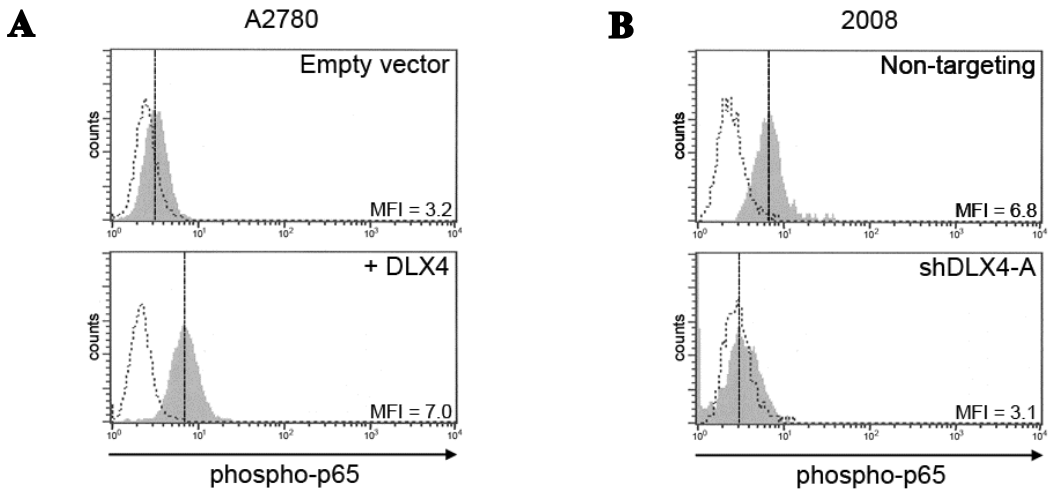

Figure 21. DLX4 induces levels of phosphorylated p65 in EOC cells

Intracellular levels of phosphorylated p65 were assayed in **(A)** empty vector and +DLX4 A2780 cells and **(B)** 2008 cells transfected with non-targeting shRNA and shRNA against *DLX4* (shDLX4-A) by flow cytometry.

4. Induction of NF-κB signaling by DLX4 in EOC cells is mediated by IL-1β

Canonical NF-κB signaling activity in tumors is triggered by several cytokines including IL-1β [74]. My studies have demonstrated that DLX4 promotes IL-1β expression and NF-κB transcriptional activity in EOC cells (Figures 18, 19, 20, 21). To determine whether induction of NF-κB activity in EOC cells by DLX4 occurs due to its induction of IL-1β, I inhibited expression of IL-1β by using shRNA against *IL1B* in +DLX4 A2780 cells. ELISA assays were performed to confirm that the shRNA reduced the IL-1β level in +DLX4 A2780 cells to the basal IL-1β level seen in vector control A2780 cells (Figure 18C). To evaluate NF-κB transcriptional activity, luciferase reporter assays were performed using the NF-κB driven reporter construct. Inhibition of IL-1β by shRNA in +DLX4 A2780 cells reduced the level of NF-κB transcriptional activity almost to the level detected in vector-control A2780 cells (Figure 22A). This finding indicates that the ability of DLX4 to stimulate NF-κB transcriptional activity is substantially mediated by its induction of IL-1β.

To confirm my findings, I investigated whether reconstitution of IL-1β can rescue the transcriptional activity of NF-κB when DLX4 is knocked down. To accomplish this, I transfected *IL1B* cDNA into DLX4-knockdown 2008 cells. ELISA assays were performed to confirm that transfection of *IL1B* cDNA in DLX4-knockdown 2008 cells restored the IL-1β level to a level comparable to that of control 2008 cells (Figure 18D). Reconstitution of IL-1β in DLX4-knockdown 2008 cells restored NF-κB transcriptional activity to a level comparable to that seen in control 2008 cells (Figure 22B). These results indicate that the induction of NF-κB transcriptional activity by DLX4 in EOC cells is mediated by its induction of IL-1β.

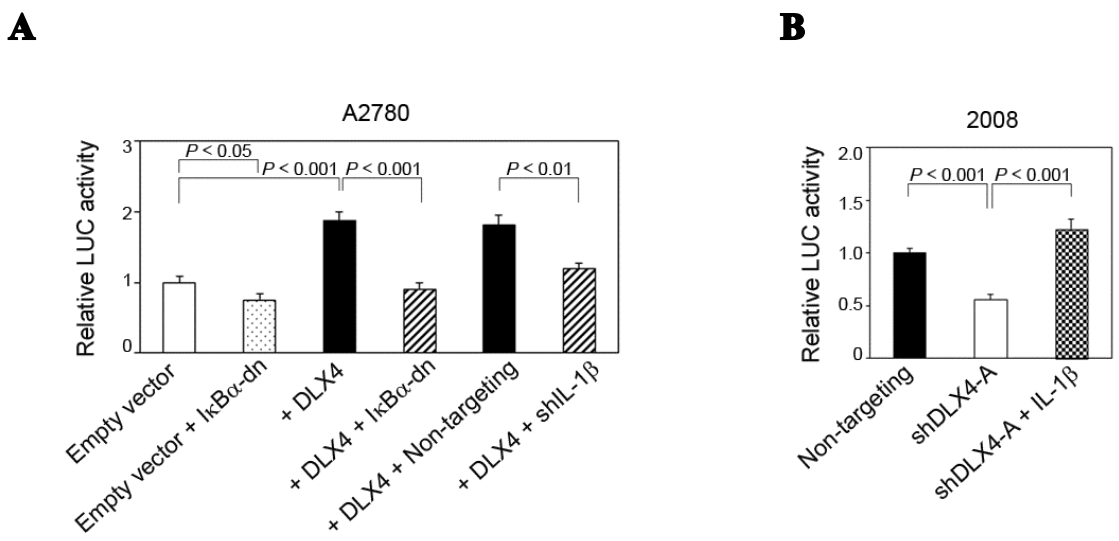

Figure 22. DLX4 induces NF-κB transcriptional activity in tumors via its induction of IL-1β

Reporter assays using the NF-κB driven luciferase construct were performed to assay NF-κB transcriptional activity in **(A)** empty vector control A2780 cells, empty vector control A2780 cells transfected with IκBα-DN, +DLX4 A2780 cells and +DLX4 A2780 cells transfected with IκBα-DN, non-targeting shRNA and shRNA against *IL1B* and **(B)** control (non-targeting) 2008 cells, DLX4-knockdown 2008 (shDLX4-A) cells and DLX4-knockdown 2008 cells after reconstitution with *IL1B* cDNA. Shown are relative luciferase activities in three independent experiments. Error bars represent standard deviation.

5. DLX4 induces CD44 in EOC cells in an IL-1β and NF-κB-dependent manner

My studies have demonstrated that DLX4 induces expression of 1) CD44 and 2) IL-1β in EOC cells (Figures 14A, 15, 18, 19). In subsequent experiments, I evaluated whether DLX4 induces expression of CD44 in an IL-1β-dependent mechanism. To accomplish this, I evaluated expression of CD44 by flow cytometry when IL-1β was knocked down in +DLX4 A2780 cells. Knockdown of IL-1β in +DLX4 A2780 cells reduced the CD44 level almost to the level seen in vector control cells (Figure 24). This finding indicates that the induction of CD44 by DLX4 is primarily mediated by its induction of IL-1β.

CD44 has been identified as a direct transcriptional target of NF-κB [168]. Because my studies also demonstrated that DLX4 induces NF-κB transcriptional activity in EOC cells via IL-1β, I evaluated whether DLX4 induces CD44 expression in a NF-κB-dependent manner. To evaluate this, I inhibited NF-κB transcriptional activity in +DLX4 A2780 cells by expressing a dominant negative IκBα construct (IκBα-DN). This construct contains serine-32 to alanine and serine-36 to alanine substitutions [163]. IκBα-DN inhibits NF-κB signaling by retaining the NF-κB transcription factors in the cytoplasm. When the canonical signaling pathway is triggered by external stimuli, IKK normally phosphorylates IκBα at serine-32 and serine-36 [169]. This phosphorylation results in proteasomal degradation of IκBα and the release of NF-κB dimers which then translocate to the nucleus and transcriptionally activate target genes (Figure 5) [169]. Mutations at serine-32 and serine-36 of IκBα prevents the phosphorylation and degradation of IκBα, thus retaining the NF-κB transcription factors in the cytoplasm and blocking NF-κB signaling (Figure 23) [169].

I initially confirmed that IκBα-DN inhibited NF-κB transcriptional activity in +DLX4 A2780 cells by performing luciferase assays with the NF-κB-driven reporter construct (Figure 22A). In subsequent experiments, I evaluated whether DLX4 induces expression of CD44 in a NF-κB dependent manner. To determine this, I evaluated expression of CD44 by flow cytometry when NF-κB transcriptional activity was blocked with IκBα-DN in +DLX4 A2780 cells. Flow cytometric analysis revealed that the CD44 level in +DLX4 A2780 cells was reduced by IκBα-DN almost to the CD44 level seen in vector control A2780 cells (Figure 24). Together, these findings demonstrate that DLX4 promotes expression of CD44 in EOC cells via induction of IL-1β expression and NF-κB signaling.

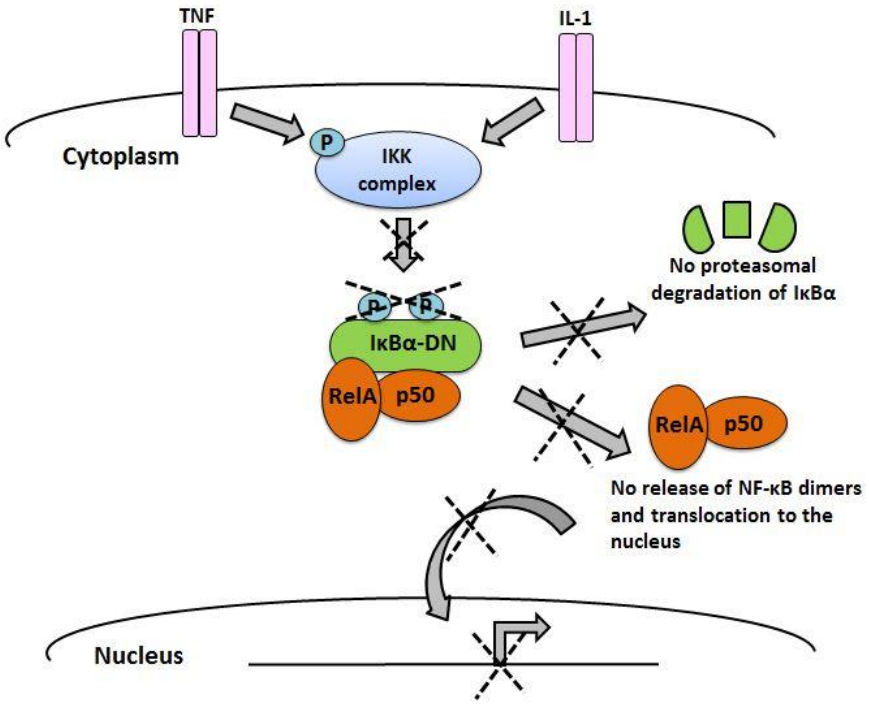

Figure 23. Mechanism of IκBα-DN in abrogating NF-κB signaling

The IκBα-DN construct contains substitution mutations of its two serine residues, 32 and 36. These mutations prevent the phosphorylation and degradation of IκBα by IKK complex. IκBα therefore retains NF-κB dimers in the cytoplasm and thereby blocks NF-κB transcriptional activity [169].

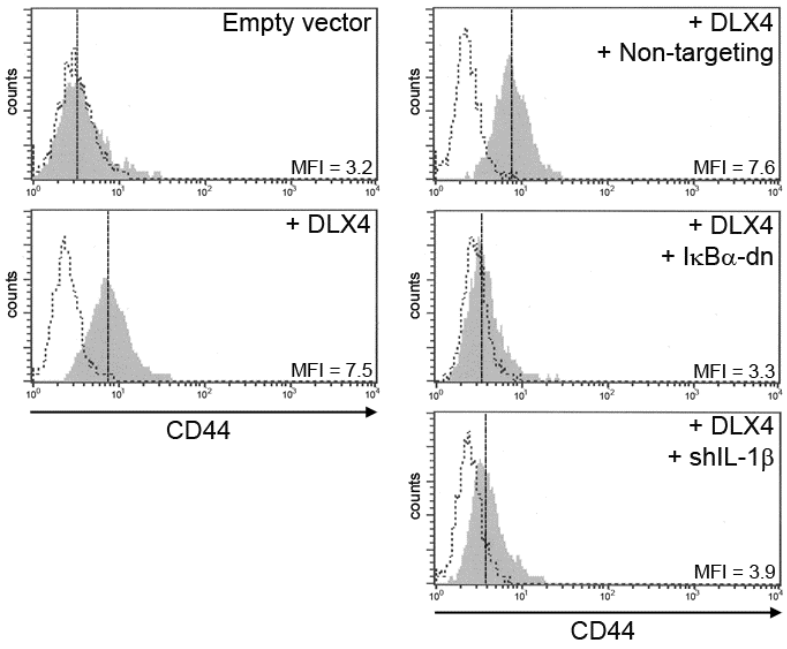

Figure 24. DLX4 induces expression of CD44 in EOC cells via IL-1β and NF-κB signaling

Cell surface expression of CD44 was assayed by flow cytometry in empty vector control A2780 cells, +DLX4 A2780 cells and +DLX4 A2780 cells transfected with non-targeting shRNA, *IL1B* shRNA and IκBα-DN.

6. Reconstitution of IL-1β in DLX4-knockdown EOC cells rescues the expression of CD44

My previous findings demonstrated that DLX4 induces CD44 expression in an IL-1β and NF-κB-dependent manner (Figure 24). To confirm my results, I evaluated whether reconstitution of IL-1β in DLX4-knockdown cells can rescue the expression of CD44. To accomplish this, I transfected DLX4-knockdown (shDLX4-A) 2008 cells with *IL1B* cDNA and analyzed CD44 levels by flow cytometry. Reconstitution of IL-1β in DLX4-knockdown 2008 cells restored the CD44 level to a level comparable to that seen in control 2008 cells (Figure 25). This finding demonstrates that DLX4 induces CD44 expression via induction of IL-1β in EOC cells.

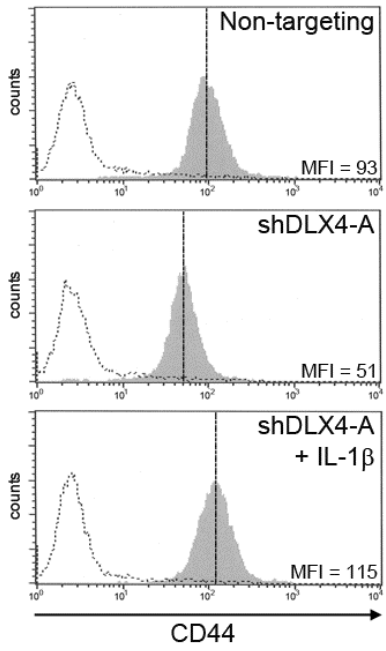

Figure 25. Reconstitution of IL-1β in DLX4-knockdown EOC cells rescues the expression of CD44

CD44 expression was assayed by flow cytometry in control 2008 cells (non-targeting), DLX4-knockdown 2008 (shDLX4-A) cells, and DLX4-knockdown 2008 cells after reconstitution of IL-1β.

7. DLX4 promotes EOC cell attachment to mesothelial cells via induction of IL-1β and NF-κB transcriptional activity

My studies demonstrated that DLX4 promotes the attachment of EOC cells to peritoneal mesothelial cells via induction of the cell adhesion molecule CD44 (Figure 17). My findings also demonstrated that DLX4 induces CD44 expression in EOC cells via induction of IL-1β (Figures 24, 25). In subsequent experiments, I evaluated whether DLX4 mediates the attachment of EOC cells to mesothelial cells in an IL-1β-dependent manner. To accomplish this, I performed *in vitro* cell attachment assays using GFP-expressing vector-control and +DLX4 A2780 cells and +DLX4 A2780 cells in which IL-1β was knocked-down. Knockdown of IL-1β in +DLX4 A2780 cells significantly inhibited the ability of these cells to attach to mesothelial cells ($P < 0.01$), whereas non-targeting shRNA had no effect (Figure 26).

Because my studies demonstrated that DLX4 induces expression of CD44 in EOC cells by stimulating NF-κB transcriptional activity (Figure 24), I evaluated whether DLX4 promotes attachment of EOC cells to mesothelial cells in an NF-κB-dependent manner. To accomplish this, I evaluated the ability of +DLX4 A2780 cells to attach to mesothelial cells when NF-κB was inhibited by IκBα-DN. Expression of IκBα-DN in +DLX4 A2780 cells reduced the ability of these cells to attach to mesothelial cells almost to the level seen in empty vector control A2780 cells (Figure 26). These results demonstrate that DLX4 promotes EOC cell attachment to mesothelial cells in an IL-1β and NF-κB-dependent manner. These findings are consistent with my previous findings that DLX4 induces expression of CD44 in an IL-1β and NF-κB-dependent manner (Figures 24, 25).

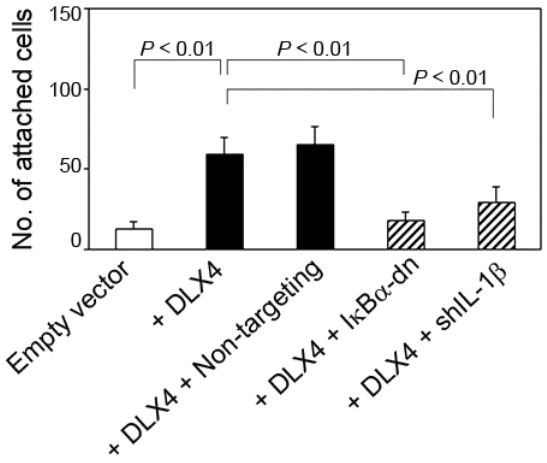

Figure 26. DLX4 promotes EOC cell attachment to mesothelial cells via induction of IL-1β and NF-κB

The ability of EOC cells to attach to mesothelial cells was assayed by *in vitro* cell attachment assays using vector control A2780 cells, +DLX4 A2780 cells and +DLX4 A2780 cells transfected with non-targeting shRNA, *IL1B* shRNA and IκBα-DN. Shown are mean ± sd values of three independent attachment assays.

8. Reconstitution of IL-1β in DLX4-knockdown EOC cells rescues the effect of DLX4

My previous findings demonstrated that DLX4 promotes attachment of EOC cells to peritoneal mesothelial cells in an IL-1β and NF-κB dependent manner (Figure 26). To further confirm these results, I evaluated the effect of reconstituting IL-1β in DLX4-knockdown EOC cells on their ability to attach to mesothelial cells. Reconstitution of IL-1β in DLX4-knockdown 2008 cells rescued the ability of these cells to attach to mesothelial cells to a level that was comparable to that of control 2008 cells (Figure 27). This finding demonstrated that DLX4 primarily promotes attachment of EOC cells to mesothelial cells via its induction of IL-1β in EOC cells.

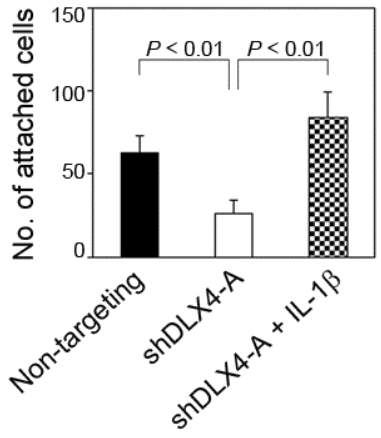

Figure 27. Reconstitution of IL-1β in DLX4-knockdown EOC cells rescues the effect of DLX4

The ability of EOC cells to attach to mesothelial cells was assayed by *in vitro* cell attachment assays using control (non-targeting) 2008 cells, DLX4-knockdown (shDLX4-A) 2008 cells and DLX4-knockdown 2008 cells that were reconstituted with IL-1β. Shown are mean ± sd values of three independent attachment assays.

C. CONCLUSION

My studies in Chapter 4 provide important insights into the mechanisms by which DLX4 promotes expression of CD44 and the ability of EOC cells to attach to peritoneal mesothelial cells. My studies have identified that DLX4 directly binds to and activates the gene encoding IL-1β in EOC cells. In addition, DLX4 induces NF-κB signaling in EOC cells via its induction of IL-1β. Furthermore, my studies demonstrate that inhibition of IL-1β and NF-κB signaling in +DLX4 A2780 cells significantly blocked the induction of CD44 and the ability of these cells to attach to mesothelial cells. Conversely, reconstitution of IL-1β in DLX4-knockdown 2008 cells rescues the expression of CD44 and the ability of these cells to attach to mesothelial cells. In summary, my results indicate that DLX4 induces expression of IL-1β and NF-κB signaling in EOC cells which in turn induce expression of CD44 and the ability of EOC cells to attach to peritoneal mesothelial cells.

CHAPTER 5: DISCUSSION

A. DLX4 PROMOTES ATTACHMENT OF EOC CELLS TO THE MESOTHELIUM LINING THE PERITONEAL CAVITY

The high lethality of EOC stems from rapid peritoneal involvement of the disease and late diagnosis. A key rate-limiting step in peritoneal metastasis of EOC is the attachment of EOC cells to the mesothelial lining of the peritoneal cavity. My studies in Chapter 3 demonstrated that high expression of DLX4 in EOC cells promotes their attachment to the peritoneal mesothelial cells (Figure 12). On the other hand, overexpression of DLX4 did not promote attachment of EOC cells to the sub-mesothelial ECM components such as collagen I, fibronectin and laminin (Figure 13). My findings demonstrate that DLX4 mediates the attachment of EOC cells by inducing expression of the cell adhesion molecule CD44 (Figures 14A, 15, 17). However, DLX4 did not induce expression of P-cadherin and β1 integrin that also mediate EOC-peritoneal interactions (Figures 14B, 14C). The inability of DLX4 to induce expression of β1 integrin is consistent with the inability of DLX4 to mediate EOC cell attachment to the components of the ECM. Notably, blocking CD44 did not completely abrogate the attachment of EOC cells to mesothelial cells (Figure 17). Interactions between EOC cells and the peritoneum are known to be mediated by multiple cell adhesion molecules [24, 32, 33, 42]. This explains the inability of the CD44 neutralizing Ab to completely abolish the attachment of +DLX4 EOC cells to mesothelial cells (Figure 17). These findings are consistent with other studies that have demonstrated that using a single neutralizing Ab against CD44, β1 integrin or P-cadherin was not sufficient to completely block EOC cell attachment to mesothelial cells [24, 33, 42, 55]. A study by Strobel and colleagues also demonstrated that combining CD44 and β1 integrin blocking Abs resulted in an additive

inhibitory effect on EOC cell attachment [33]. These findings indicate that multiple cell adhesion molecules need to be targeted simultaneously to effectively block attachment of EOC cells to mesothelial cells. The interplay between these different cell adhesion molecules can be a focus of future studies and provide insights for development of better targeted therapies.

B. DLX4 PROMOTES CD44 EXPRESSION AND EOC-MESOTHELIAL CELL INTERACTIONS BY INDUCING IL-1β EXPRESSION AND ACTIVATING NF-κB SIGNALING

IL-1β is a pleiotropic cytokine that contributes to inflammation in numerous pathological conditions such as cancer, arteriosclerosis, type II diabetes, rheumatoid arthritis, osteoarthritis and cardiovascular disorders [170]. Although many other cytokines mediate inflammatory signaling, IL-1β is known as the 'gatekeeper of inflammation' [171]. My studies in Chapter 4 demonstrated that high expression of DLX4 in EOC cells induces expression of IL-1β by direct transcriptional activation (Figures 18, 19). Inhibition of IL-1β in +DLX4 EOC cells resulted in a down-regulation of CD44 expression and reduced the ability of EOC cells to attach to mesothelial cells (Figures 24, 26). Conversely, reconstitution of IL-1β in DLX4-knockdown EOC cells restored CD44 levels and the ability of EOC cells to attach to mesothelial cells (Figures 25, 27). The ability of DLX4 to promote CD44 expression and attachment of EOC cells to mesothelial cells therefore, primarily occurs via its induction of IL-1β in EOC cells. A study by Foster and colleagues has demonstrated that induction of CD44 by IL-1β in vascular smooth muscle cells contributes to pathogenesis of arteriosclerosis [167]. Another study by Campo and colleagues has demonstrated that IL-1β and HA induce CD44 expression and inflammatory signaling in rheumatoid arthritis [166]. My

study is the first to demonstrate the significance of IL-1β in inducing CD44 expression in controlling cell-cell interactions that mediate metastasis of EOC.

IL-1β induces the NF-κB signaling pathway in a wide variety of physiological and pathological conditions [77]. My findings in Chapter 4 demonstrated that high expression of DLX4 stimulates NF-κB signaling in EOC cells via its induction of IL-1β (Figures 20, 21, 22). Moreover, inhibition of NF-κB signaling in +DLX4 EOC cells down-regulated CD44 levels and decreased the ability of EOC cells to attach to mesothelial cells (Figures 24, 26). These results indicate that DLX4 induces expression of CD44 and the attachment of EOC cells to peritoneal mesothelial cells by activating NF-κB signaling in EOC cells. My functional studies therefore support findings of clinical studies that identified a strong correlation between activated NF-κB signaling in EOC and advanced disease stage [84-87].

Substantial evidence indicates that NF-κB activates expression of a wide variety of target genes that promote tumor cell survival, tumor angiogenesis and metastasis [74]. For example, NF-κB promotes tumor angiogenesis by induction of pro-angiogenic growth factors such as VEGF, IL-6 and IL-8 in multiple types of tumors including EOC [69, 172-174]. NF-κB can also promote epithelial-mesenchymal transition (EMT) by transcriptional upregulation of Twist 1 [175]. However, NF-κB is also known to have pro-apoptotic functions in certain cell types and conditions. For example, a study by Ryan and colleagues demonstrated that p53 can induce tumor cell death via activation of MEK1 and NF-κB signaling [176]. Another study by Rocha and colleagues demonstrated that p53 downregulates cyclin D1 expression by inhibiting Bcl-3, a member of the IκB family of proteins and a transcriptional co-activator for NF-κB transcription factor p52 [177]. These authors found that p53 induces formation of p52/histone deacetylase1 (HDAC1) repressor complexes, which replaces Bcl-3/p52 activator complexes

and thereby represses transcription of the gene encoding cyclin D1 [177]. A study by Yang and colleagues demonstrated that NF-κB signaling can have dual functions in EOC [178]. On one hand, NF-κB has a tumor-suppressive functions by sensitizing EOC cells to apoptosis induced by paclitaxel and carboplatin. On the other hand, NF-κB has tumor-promoting functions in aggressive forms of EOC and contributes to chemoresistance [178]. Because DLX4 is expressed in advanced-stage and aggressive forms of EOC [129], my findings that induction of NF-κB signaling in DLX4-expressing EOC cells contributes to EOC progression are consistent with other studies that have identified a tumor-promoting role of NF-κB in advanced stage tumors.

C. MULTIPLE ROLES OF DLX4 IN TUMOR PATHOGENESIS

My studies demonstrated that DLX4 induces expression of CD44 in EOC cells and promotes their attachment to mesothelial cells by inducing expression of IL-1β and NF-κB signaling (Figure 24, 26). However, by inducing IL-1β and NF-κB signaling, DLX4 could also stimulate a variety of other processes that promote progression of EOC. IL-1β has been reported to confer chemoresistance in pancreatic carcinoma cell lines [179]. IL-1β also promotes tumor vascularization in lung cancers via induction of VEGF [180]. IL-1β and TNF-α have been implicated in the induction of hypoxia inducible factor-1 (HIF-1) expression in hepatoma cells, which in turn induces VEGF and tumor angiogenesis [181]. IL-1β, TNF-α and interferon-γ (IFN-γ) also stimulate expression of inducible nitric oxide synthase (iNOS) in various tumors [182]. Nitric oxide, the product of iNOS, plays an important role in promotion of tumor angiogenesis by regulating HIF-1 and VEGF expression in tumors [182]. Previous studies from our laboratory have demonstrated that DLX4 promotes EOC growth and angiogenesis by inducing expression of pro-tumorigenic factors such as VEGF and fibroblast growth

factor-2 (FGF-2) [129]. Since expression of VEGF is activated by IL-1β and NF-κB signaling, my findings could explain the mechanism by which DLX4 induces VEGF expression and angiogenesis in EOC.

In addition to EOC, DLX4 is overexpressed in other types of solid tumors including breast and prostate cancers and in leukemias [153, 155, 156, 158, 162]. The mechanisms by which DLX4 promotes progression of other types of tumors are not well understood. DLX4 is highly expressed in inflammatory breast cancers (IBCs), an aggressive subtype of breast cancer [156]. My findings that DLX4 stimulates NF-κB signaling raise the possibility that DLX4 might also promote the aggressive behavior of IBCs. Some specific sets of homeobox genes have been found to act similarly in different types of tumors. One such example is *HOXB7* which is overexpressed in EOC, melanoma and breast cancers [122, 125, 126]. Several studies have demonstrated that HOXB7 promotes tumor growth and angiogenesis via upregulation of FGF-2 in these different types of tumors [122, 125-127]. On the other hand, other sets of homeobox genes act differently in different types of tumors. One example is *HOXA9*. HOXA9 has been demonstrated to have tumor promoting properties in EOC and glioblastomas [136-139]. Expression of HOXA9 in EOC cells induces stromal cells to produce growth factors that support tumor growth [137]. HOXA9 also promotes peritoneal dissemination of EOC by inducing the cell adhesion molecule P-cadherin [139]. In contrast, HOXA9 has tumor-suppressive functions in breast cancers [183]. HOXA9 has been reported to prevent progression of breast cancers by maintaining BRCA1 expression [183]. My study has shown that high expression of DLX4 promotes inflammatory signaling and peritoneal dissemination of EOC via induction of IL-1β and NF-κB signaling. However, it remains to be determined whether DLX4 promotes progression of other types of tumors by stimulating

NF-κB signaling. Further investigation of the role of DLX4 in controlling NF-κB signaling in tumors will provide insights into the therapeutic targeting of the NF-κB signaling pathway.

D. THERAPEUTIC IMPLICATIONS

Because DLX4 is a transcription factor that shares functional domains in common with other family members, it may be difficult to specifically target DLX4 for therapeutic purposes. On the other hand, inhibiting the downstream effectors of DLX4, i.e. IL-1β and the NF-κB signaling pathway may be a promising strategy. Several IL-1 therapeutics such as IL-1 receptor antagonists, anti-IL-1 monoclonal Abs, soluble IL-1 receptor II (IL-1RII), IL-1β converting-enzyme (ICE) inhibitors and IL-1 ligand traps are being currently used in the treatment of rheumatoid arthritis [184]. Such novel treatments could be applied for the treatment of cancer. In addition, agents that target the NF-κB signaling pathway could be an effective therapeutic strategy [185]. For example, Bortezomib is a proteasome inhibitor that blocks NF-κB signaling and is already in use for treatment of multiple myeloma and mantle cell lymphoma [186]. Moreover, inhibition of CD44-HA interaction and EOC cell adhesion using CD44 monoclonal Abs or small molecule inhibitors could be another possible strategy for treatment of advanced-stage EOC [187, 188]. Further studies examining the effect of these inhibitors in EOC will provide insights into strategies for effective treatment for advanced stages of this disease.

E. CONCLUSION

The high mortality of EOC primarily stems from the rapid peritoneal dissemination of the disease and the inability to detect the disease at an early stage. The mechanisms that control tumor-peritoneum interactions are not well understood. My study supports a model in which DLX4 promotes CD44-mediated peritoneal attachment of EOC via induction of the pro-inflammatory cytokine IL-1β and activation of NF-κB signaling (Figure 28). Further study of the regulation and function of the NF-κB signaling pathway and its activating ligands and downstream effectors could yield important insights into possible therapeutic targets and new treatment strategies for patients with advanced-stage and chemoresistant EOC.

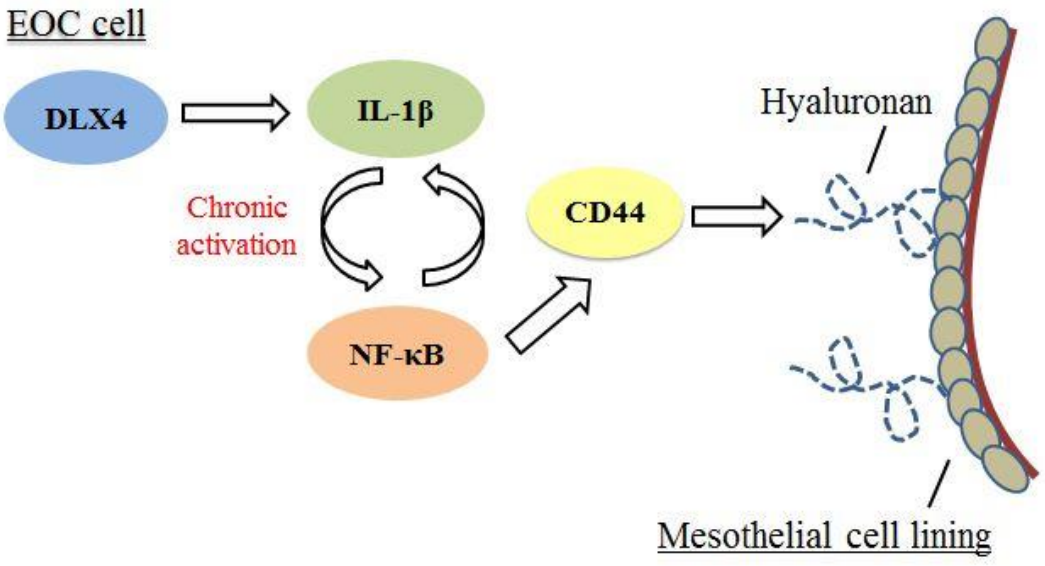

Figure 28. Model of the mechanism by which DLX4 promotes intraperitoneal dissemination of EOC

DLX4 promotes CD44-mediated attachment of EOC cells to the peritoneal mesothelial cells by inducing expression of IL-1β and activating NF-κB signaling

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