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THE ROLE AND MECHANISM OF THE HOMEOBOX GENE HOXA9 IN THE GROWTH OF EPITHELIAL OVARIAN CANCER

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A

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DOCTOR OF PHILOSOPHY

by

Song Yi Ko, M.S.

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Dedication

This dissertation is dedicated to my family for their love, encouragement and support.

Thank you

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First of all, I would like to express my deepest gratitude to my great advisor, Dr. Honami Naora, for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. She has been extremely supportive and has given me the freedom to pursue various projects without objection. I would also like to thank my former and current committee members, Dr. Elsa R. Flores, Dr. Hui-Kuan Lin, Dr. Michael Van Dyke, Dr. Miles F. Wilkinson, Dr. Ju-seog Lee, Dr. Mong-Hong Lee, and Dr. Yasuhide Furuta for their valuable guidance, advice and encouragement. I would like to offer my special thanks to all my collaborators, Dr. Ernst Lengyel (University of Chicago), Dr. Frank Marini (Wake Forest University). My research would not have been possible without their helps.

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THE ROLE AND MECHANISM OF THE HOMEOBOX GENE *HOXA9* IN THE GROWTH OF EPITHELIAL OVARIAN CANCER

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Supervisory Professor: Honami Naora, Ph.D.

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death among women in the United States. The high lethality of EOCs stems from rapid peritoneal involvement. EOCs frequently colonize peritoneal surfaces that overlie connective and adipose tissues. However, the mechanisms that enable ovarian cancer cells to readily adapt to the peritoneal environment are poorly understood. *HOXA9*, a homeobox gene that is normally expressed in the developing female reproductive tract, is aberrantly expressed in EOCs and controls the morphologic features of these tumors. The differentiation pattern of a tumor is an important determinant of its clinical behavior and prognosis. The overall goal of this project is to determine the clinical significance of HOXA9 and its mechanisms in the biological behavior of EOC. In this study, I demonstrated that expression of HOXA9 is strongly associated with poor outcomes in EOC patients and in mouse xenograft models of EOC. Whereas HOXA9 promoted EOC growth *in vivo*, HOXA9 did not stimulate autonomous tumor cell growth *in vitro*. On the other hand, HOXA9 was found to be associated with increased abundance of cancer-associated fibroblasts (CAFs) in mouse xenograft models of EOCs and with a CAF-like gene signature in human tumors. However, HOXA9 did not induce CAF-like features in EOC cells. Expression of HOXA9 in EOC cells induced normal adipose and bone marrow-derived mesenchymal stem cells (MSCs) as well as normal peritoneal fibroblasts to express markers of CAFs and to stimulate growth of EOC and endothelial cells. These effects of HOXA9 were due in substantial part to its

transcriptional activation of TGF-β2 that acted in a paracrine manner on peritoneal fibroblasts and MSCs to induce CXCL12, IL-6 and VEGF-A expression. These results demonstrate that HOXA9 promotes progression of EOC by 'educating' the stroma to become permissive for tumor growth.

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LIST OF ABBREVIATIONS

EOC	Epithelial ovarian cancer
CAF	Cancer-associated fibroblast
MSC	Mesenchymal stem cell
OSE	Ovarian surface epithelium
EMT	Epithelial–mesenchymal transition
TGF-β	Transforming growth factor-β
IL-6	Interleukin-6
VEGF	Vascular endothelial growth factor
αSMA	alpha-smooth muscle actin
FAP	Fibroblast activation protein
GFP	Green fluorescence protein
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
FBS	Fetal bovine serum
shRNA	Small hairpin RNA
ELISA	Enzyme-linked immunosorbent assay
DAPI	4', 6-diamidino-2-phenylindole
qRT-PCR	Quantitative RT-PCR
IP	Immunoprecipitation

CHAPTER 1: INTRODUCTION

A. BIOLOGY OF EPITHELIAL OVARIAN CANCERS (EOCs)

1. Clinical significance of EOCs

Ovarian cancer is the most common cause of death among women with gynecologic malignancy in the United States.[1] Ovarian cancers can arise from three ovarian cell types; epithelial cells, stromal endocrine cells, and germ cells. More than 90% of malignant ovarian cancers are epithelial ovarian cancers (EOCs).[2] Traditionally it has been believed that EOCs are originated from ovarian surface epithelium or inclusion cysts. Recently, the fallopian tube has been reported as an origin of some EOCs.[3, 4] EOCs are categorized by stage, which describes how far the cancer has spread, and by grade which indicates how malignant the cancer is (Table 1).[5] Because of the lack of distinctive symptoms and reliable biomarkers, most EOC patients are diagnosed with late stage disease. Although more than 90% of EOC patients who have ovarian-confined disease (stage I) survive five years after diagnosis, more than 60% of EOC patients are diagnosed at an advanced stage and only 30% of these advanced stage patients will survive five years after diagnosis (Table 2).[2] Despite therapeutic advances, the optimal first-line treatment in EOCs is surgical debulking and several cycles of intravenous or intraperitoneal chemotherapy with carboplatin and paclitaxel. However, most patients with advanced disease ultimately relapse and recurrent EOCs tend to be resistant to therapy. Identification of biomarkers for early detection is clearly critical to improve survival of EOC patients, but currently available biomarkers for EOCs are very limited. The most commonly used marker for EOCs is serum CA-125. Since elevated serum CA-125 level is detected in more than 80% of patients with advanced EOC,

Table 1. The International Federation of Gynecology and Obstetrics (FIGO) grading and staging system for EOCs[5]

FIGO system		Characteristics
Grade	1	Well-differentiated (<5% solid growth within a tumor)
	2	Moderately-differentiated (5 to 50% solid growth within a tumor)
	3	Poorly-differentiated (>50% solid growth within a tumor)
Stage	I	Disease confined to the ovaries
	II	Disease spread confined to the pelvis
	III	Disease confined to the abdominal cavity, including surface of the liver, omentum or bowel
	IV	Spread to liver parenchyma, lung, or other extra-abdominal sites

Table 2. Stage distribution and 5-year relative survival by stage at diagnosis (for 2003-2009) [2]

FIGO stage	Stage distribution (%)	5-year relative survival (%)
Stage I	22.1	89.3
Stage II	7.8	65.5
Stage III	35.3	33.5
Stage IV	31.7	17.9
Unknown	3.1	29.5

it has been routinely used for initial diagnosis of EOC and also for monitoring the response to treatment.[6,7] However, it has low sensitivity for the diagnosis of early EOCs. In addition, it is not specific for EOCs. Serum CA-125 will also be increased with various benign conditions such as endometriosis as well as other types of cancers including endometrial and pancreatic cancers.[8]

2. Dissemination of EOCs

EOCs have a unique pattern of dissemination that differs from classic metastasis of other epithelial tumors. Unlike many other epithelial tumors, EOC rarely spreads via the vasculature.[9] Although EOC can initially spread by directly extending to adjacent organs (e.g. fallopian tubes and uterus), most EOCs spread by intraperitoneal seeding (Figure 1).[9, 10] Initially, EOCs are limited to one or both ovaries within the intact ovarian capsule. Once the ovarian capsule is disrupted, tumor cells are passively exfoliated by the flow of peritoneal fluid. Tumor cells are transported by the circulating peritoneal fluid throughout the peritoneal cavity and then implant at secondary sites. Major implantation sites are the omentum, peritoneum, mesentery, and broad ligament (Figure 2).[9]

Figure 1. Model of EOC dissemination

Unlike classic metastasis of other epithelial tumors, most EOCs spread by intraperitoneal "seeding". Initially, EOC cells (shown in the figures as red circles) are limited to one or both ovaries within the intact ovarian capsule. Once the ovarian capsule is disrupted, tumor cells are passively exfoliated by the flow of peritoneal fluid (step 1). Exfoliated tumor cells form aggregates to avoid anoikis (step 2). Surviving tumor cells are transported by peritoneal fluid and then implant at secondary sites (step 3).

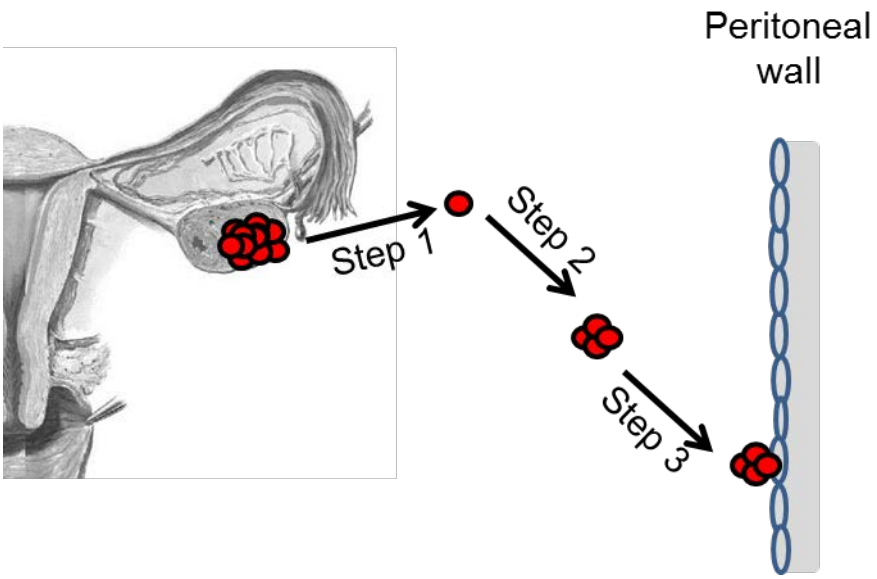

Figure 2. Pattern of spread of EOC

At its earliest stage (stage I), the tumor (shown in the figure as red masses) is limited to one or both ovaries. Once the tumor spreads beyond the ovaries, tumor cells directly extend to adjacent tissues such as the uterus, the fallopian tubes, the mesothelial lining of the pelvic cavity (peritoneum), and the broad ligament (a fold of peritoneum that supports the uterus) (stage II). Exfoliated tumor cells are transported by peritoneal fluid and implanted on the peritoneum and the mesothelial linings of abdominal organs (serosa) (stage III). Nests of tumor cells are commonly observed on the omentum (a peritoneal fold connected to the stomach and suspended over the intestines), the mesentery (a peritoneal fold anchoring the intestines to the posterior abdominal wall; not shown), and the diaphragm. Ascites is commonly associated with intraperitoneal dissemination.

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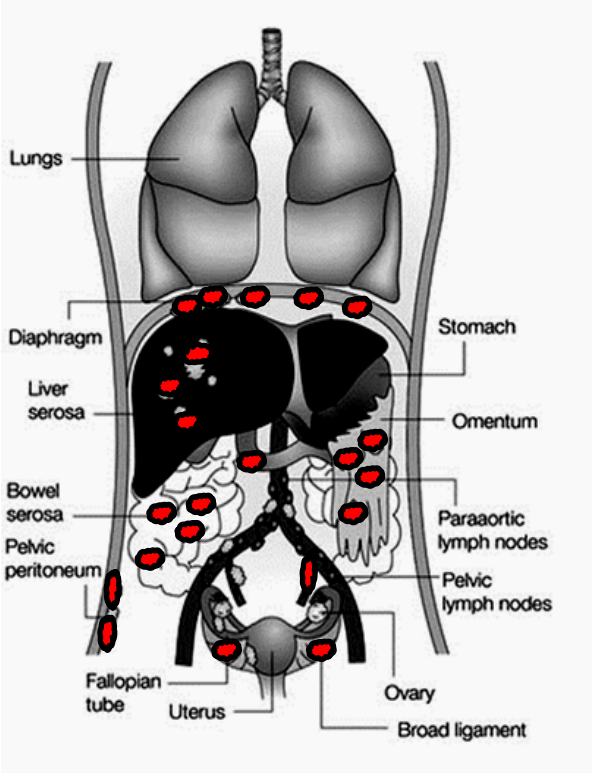

3. Subtypes of EOCs

EOC is not a single disease, but rather comprises a heterogenous group of tumors. EOCs are classified into subtypes by their histologic features, with the major subtypes being serous, endometrioid, mucinous and clear cell (Figure 3). Each subtype has distinctive morphologic and genetic features. Serous EOC, which is the most common histologic subtype, exhibits papillary structure and resembles cancers of the fallopian tube (Figure 4).[11] Serous cancer is divided into low-grade and high-grade subtypes. Although they are termed as 'low-grade' and 'high-grade' serous EOCs, low-grade serous EOCs develop through different genetic programs from high-grade serous EOC. Low-grade serous EOCs are characterized by the mutations of *KRAS, BRAF*, and *ERBB2* (Table 3).[12-14] In contrast, high-grade serous EOCs are characterized by *TP53* mutation, without mutations of *KRAS, BRAF*, and *ERBB2* (Table 3).[15, 16] Therefore low-grade serous EOCs and high-grade serous EOCs are considered as different subtypes of EOCs. Endometrioid EOCs resemble cancers of the uterine corpus, whereas mucinous EOCs are composed of endocervical-like or intestinal-like cells (Figure 4). Clear cell EOCs are composed of cells with clear cytoplasm containing glycogen. The main genetic alterations often found in each subtype are summarized in Table 3. However, the role of these genetic alterations in the unique morphologic features of the EOC subtypes has not been conclusively demonstrated.

Figure 3. Relative frequencies of subtypes of EOCs

Relative frequency of subtypes of EOCs based on two relatively recent population-based studies.[17, 18] Serous is the most common subtype of EOCs and followed by endometrioid and clear cell which occur with approximately equal frequency. Mucinous EOCs are less common.

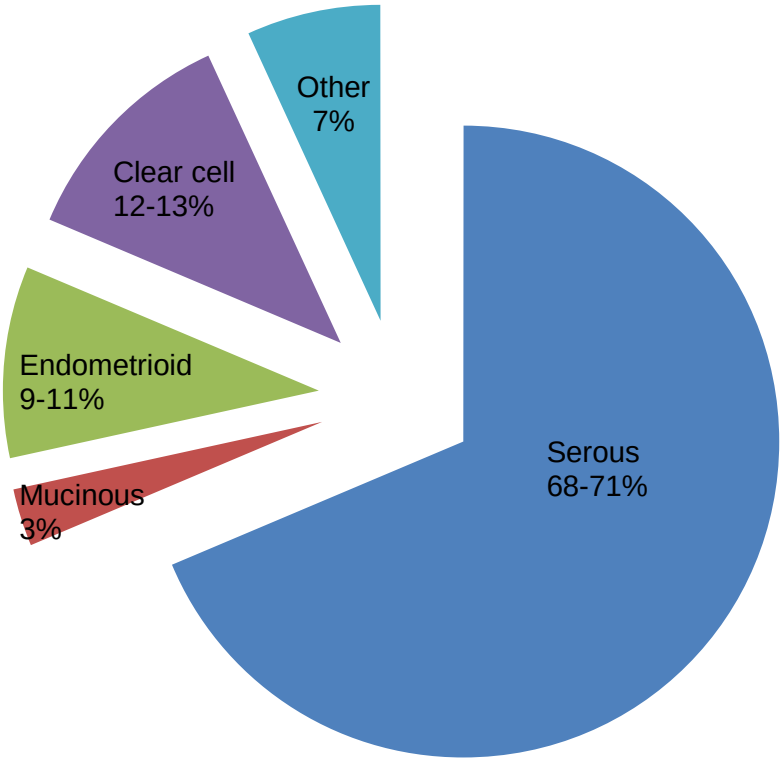

Figure 4. Müllerian-like morphological features of EOCs

(A) Hematoxylin–eosin-stained section of normal human ovary surrounded by monolayered epithelium (arrow) and containing an inclusion cyst (i.c.). **(B)** Sections of clinical specimens of EOCs: serous, with papillary features; endometrioid, with glandular features; and mucinous, with mucin-rich cytoplasm. **(C)** Sections of normal human fallopian tube, endometrium and endocervix. Bar, 50µm

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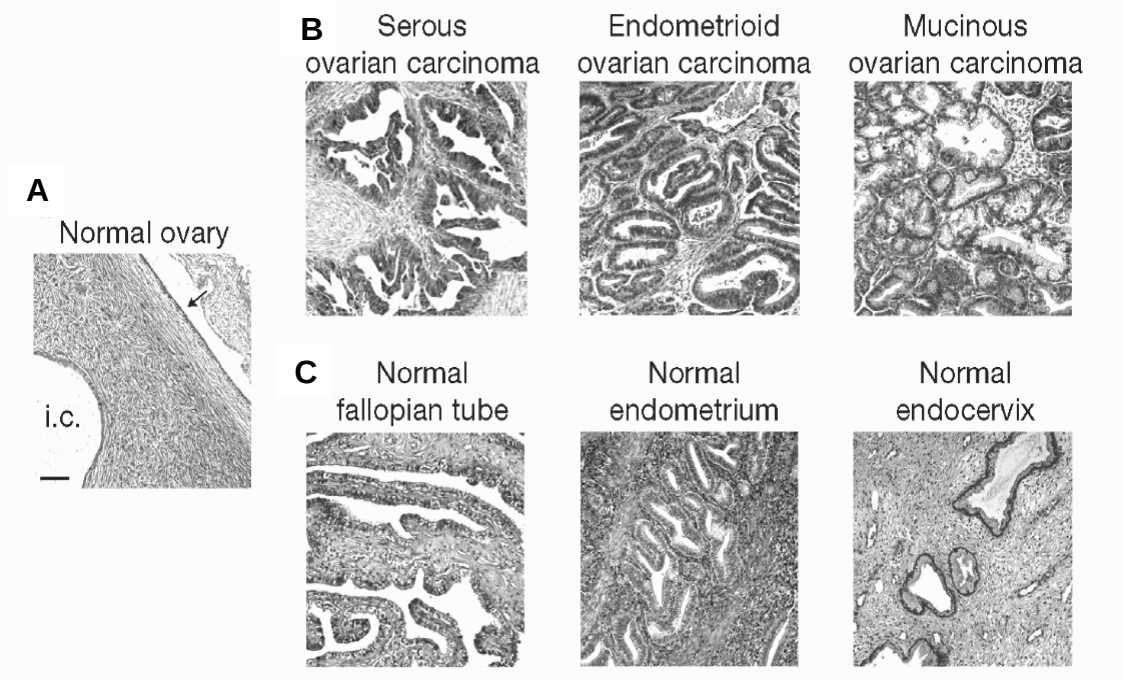


Table 3. The main molecular genetic alterations in different subtypes of EOCs

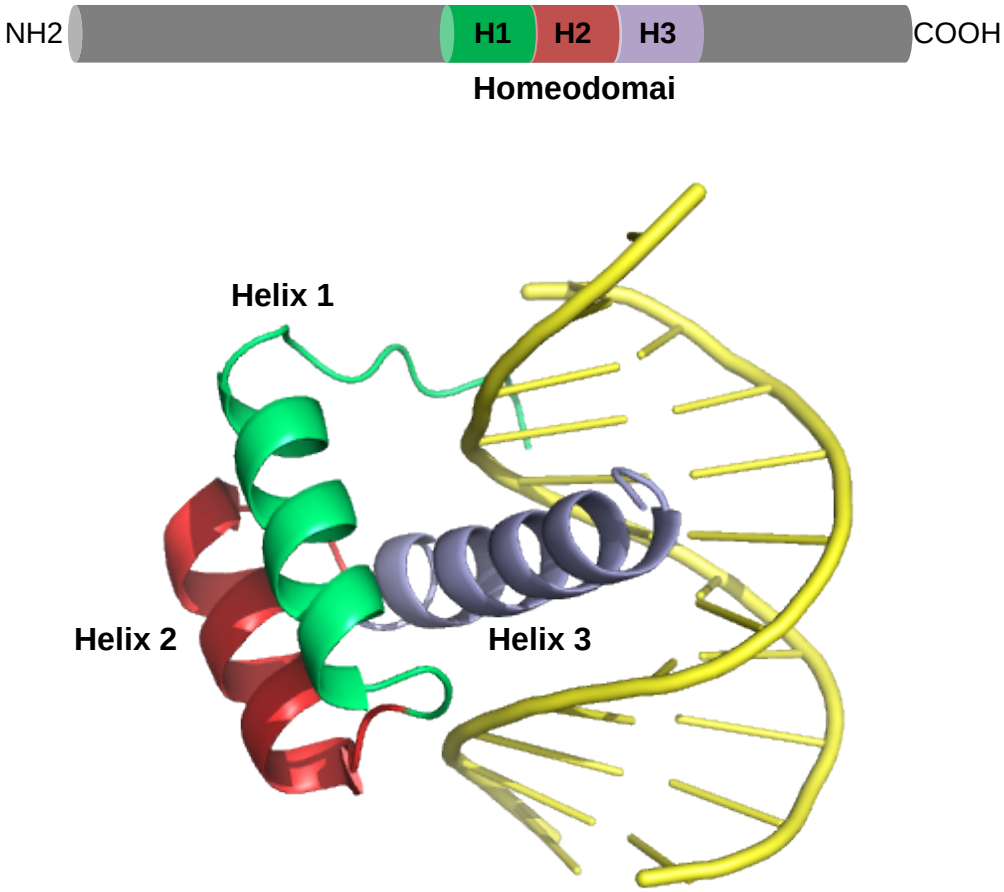
	Subtype	Gene	Frequency	References
Serous EOCs	Overall serous EOCs	***BRCA1/2*** ***PTEN*** ***PIK3CA***	<10%	[19, 20]
	High-grade	***TP53***	~80%	[15, 16]
		CCNE1	~30%	[21]
	Low-grade	***KRAS*** ***BRAF*** ***ERBB2***	~30%	[12-14]
Endometrioid EOCs		***CTNNB1***	33%	[22-25]
		PTEN	14 - 21%	[26, 27]
		PIK3CA	~20%	[20, 28]
Mucinous EOCs		***KRAS***	~50%	[29-31]
Clear cell EOCs		***PIK3CA***	48%	[28]

B. ABERRATIONS OF HOMEOBOX GENES IN HUMAN CANCER

Homeobox genes were originally identified as master regulatory genes controlling segment identity in *Drosophila*. *Antennapedia* gene is one of the most well-known examples. Loss-of-function mutations in the regulatory region of this gene induce the formation of ectopic antennae, rather than legs.[32] Conversely, gain-of-function mutations cause antennae to ectopic legs transformation.[32] Homeobox genes encode a family of transcription factors which contain a highly conserved DNA binding domain known as the homeodomain (Figure 5). This domain forms three alpha-helixes that bind DNA elements containing TAAT core motifs.[32] The homeobox gene superfamily comprises more than 200 genes that are categorized into different families based on the similarity of their homeodomains.[33, 34] Homeobox genes play essential roles in controlling skeletal patterning, limb formation, and development of various organ systems including the central nervous system and gastrointestinal tract.[35-38] Homeobox genes also play important role in adult tissues. For example, homeobox genes regulate in vascular remodeling and angiogenesis.[39] Homeobox genes also control self-renewal as well as lineage specification of hematopoietic stem cells.[40]

Figure 5. The structure of the homeodomain

Transcription factors encoded by homeobox genes are characterized by their highly conserved 61 amino acid DNA-binding domain termed the homeodomain. The homeodomain forms three alpha helixes (colored boxes) that bind DNA elements containing TAAT core motifs. Crystal structure of the Pdx1 homeodomain in complex with DNA (PDB ID: 2H1K; crystal structure was visualized by PyMOL.



1. Genomic organization of mammalian homeobox genes

Whereas most homeobox gene families such as the *Nkx, Dlx* and *Msx* families contain between two and nine genes, the *HOX* family which is the most extensively studied homeobox gene family has 39 members in mammals. Members of *HOX* gene family are organized in clusters. Eight *HOX* genes in *Drosophila* are organized in a single chromosome. However, the 39 members of the *HOX* family in mammals are grouped in clusters of nine to thirteen genes on four different chromosomes (Figure 6).[41] Since these four copy of *HOX* gene clusters (called *HOXA-D*) have been postulated to derive from gene duplication during evolution.[42] *HOX* paralogs which occupy the same relative positions on a given chromosomal cluster (e.g., *HOXA9*, *HOXB9, HOXC9* and *HOXD9*) share greater homeodomain sequence similarity than do different members of a single cluster. During embryonic development, *HOX* genes are activated sequentially relative to their physical positions along the four genomic clusters. *HOX* genes at the 3' end of the clusters are generally expressed early in development and in anterior regions, whereas those at the 5' end of clusters are expressed later and in more posterior regions.[43] This expression pattern is responsible for controlling segmental identity and morphology on the anterior-posterior axis. Because of sequence similarities as well as similarities in expression patterns, *HOX* paralogs exhibit some functional redundancy.

Figure 6. Conserved genomic organization of *HOX* genes

(A) *Drosophila* has eight *HOX* genes, arranged in a single cluster, whereas human beings have 39 *HOX* genes arranged in four clusters that are located on different chromosomes. Vertebrate *HOX* groups 1, 2, 4, 5, 6, 7, and 8 are most similar to drosophila *labial (lab), proboscipedia (pb), deformed (Dfd), sex combs reduced (Scr), antennapedia (Antp), ultrabithorax (Ubx), and abdominal-A (Abd-A),* respectively. Group 3 is probably specific to vertebrates. Groups 9 to 13 are all related to *drosophila abdominal-B (Abd-B).*

(B) In both invertebrates and vertebrates, *HOX* genes control patterning of the embryo along the anterior-posterior axis.

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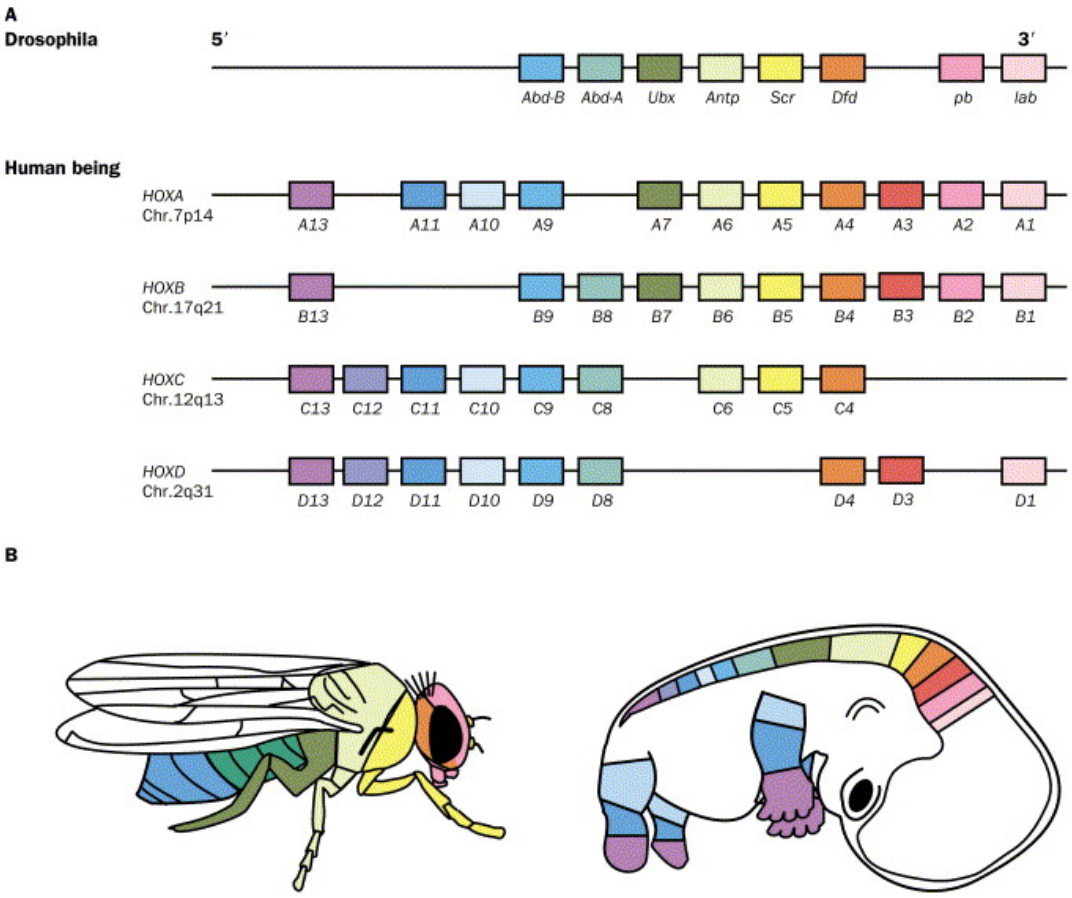

2. Aberrant expression of homeobox genes in cancers

Homeobox genes have been reported to be expressed aberrantly in a wide variety of cancers. Some examples of aberrant expression of homeobox genes in cancers are summarized in Table 4. The expression pattern of homeobox genes in cancers can be divided into two broad categories.[44] First, homeobox genes that are expressed only during embryonic development and not in adult tissues are often up-regulated in cancers. For example, *PAX2* is normally expressed during kidney development but not in adult kidney. But it is reactivated in renal cancer.[45-48] Conversely, homeobox genes that are expressed in normal adult tissues tend to be down-regulated in cancers. *Nkx3.1,* which control morphogenesis of the prostate, is a good example. *Nkx3.1* is expressed in fetal and adult prostate tissues,[49] but it is frequently deleted in prostate cancers.[50] Although several *HOX* genes are reported to be activated by chromosomal translocation in leukemia,[51] the mechanisms that induce aberrant expression of homeobox genes in solid tumors are largely unknown.

Table 4. Examples of deregulated homeobox genes in tumors

Gene	Tumor type	Deregulation in cancer	Effect	References
Nkx 3.1	Prostate Cancer	↓	Homozygous/heterozygous *NKX3.1* mutant mice develop PIN.	49, 50, 52, 53
CDX2	Colon cancer	↓	Overexpression promotes differentiation of intestinal cells, while leading to reduced proliferation and tumorigenicity. Heterozygous mutant mice are predisposed to colon cancer.	54-56
HOXB13	Prostate cancer	↓	Loss of expression inhibits differentiation; allows transactivation of AR and proliferation.	57, 58
HOXA5	Breast cancer	↓	Loss of expression correlates with loss of p53 expression. *HOXA5* is a transactivator of the p53 promoter.	59
HOXB7	Melanoma Ovarian Breast cancer	↑	Promotes tumor growth and angiogenesis by inducing expression of FGF-2. Induced EMT and promotes DNA repair.	60-63
HSIX1	Breast cancer	↑	Overexpression abrogates the G2 cell cycle checkpoint in response to X-ray irradiation.	64
PAX2	Renal cancer	↑	Expressed in renal cell carcinomas. Promotes cancer cell survival.	46-48

3. Significance of homeobox genes in EOCs

EOCs have been thought to arise from the simple monolayered ovarian surface epithelium (OSE). However, the major EOC subtypes exhibit morphological features that resemble those of the Müllerian-duct-derived epithelia (Figure 4). This has led to considerable speculation that EOCs originate from Müllerian epithelia rather than the OSE. An alternative hypothesis has been that the Müllerian-like morphological features of EOCs involves inappropriate activation of genes that normally regulate the differentiation of the Müllerian ducts. Study of Cheng *et al.*[65] has implicated aberrant *HOX* gene expression as being responsible for the morphologic features of EOCs. The mammalian *Hoxa9, Hoxa10* and *Hoxa11* genes are uniformly expressed along the axis of the Müllerian ducts prior to differentiation, but their expression becomes spatially restricted in the fallopian tubes, uterus, and lower uterine segment, respectively.[65-68] This Müllerian *HOX* gene program is not expressed in normal OSE, but is recapitulated in EOCs according to the patterns of Müllerian-like differentiation of these tumors. Moreover, differential activation of *HOX* genes in transformed OSE cells induces tumors with distinct Müllerian-like features. Transformed mouse OSE cells expressing *Hoxa9* formed serous-like tumors, whereas those with *Hoxa10* and *Hoxa11* formed endometrioid-like and mucinous-like tumors, respectively (Figure 7).[65] Other homeobox genes that control urogenital patterning also appear to be associated with the morphologic features of EOCs. It has been reported that *PAX2* and *PAX8* are prevalently expressed in non-mucinous EOCs, whereas *CDX2* expression is restricted to mucinous EOCs.[69-74] However, the significance of aberrant expression of homeobox genes to the progression and clinical behavior of EOCs is unknown.

Figure 7. Control by *HOX* genes of Müllerian differentiation

The ovarian surface epithelium and Müllerian ducts derive from the embryonic coelomic epithelium. As the Müllerian ducts differentiate to form the fallopian tubes, uterus, cervix, and upper vagina, *HOX* gene expression becomes spatially restricted. These patterns of *HOX* expression are recapitulated in serous, endometrioid, and mucinous EOCs according to their patterns of Müllerian-like differentiation.

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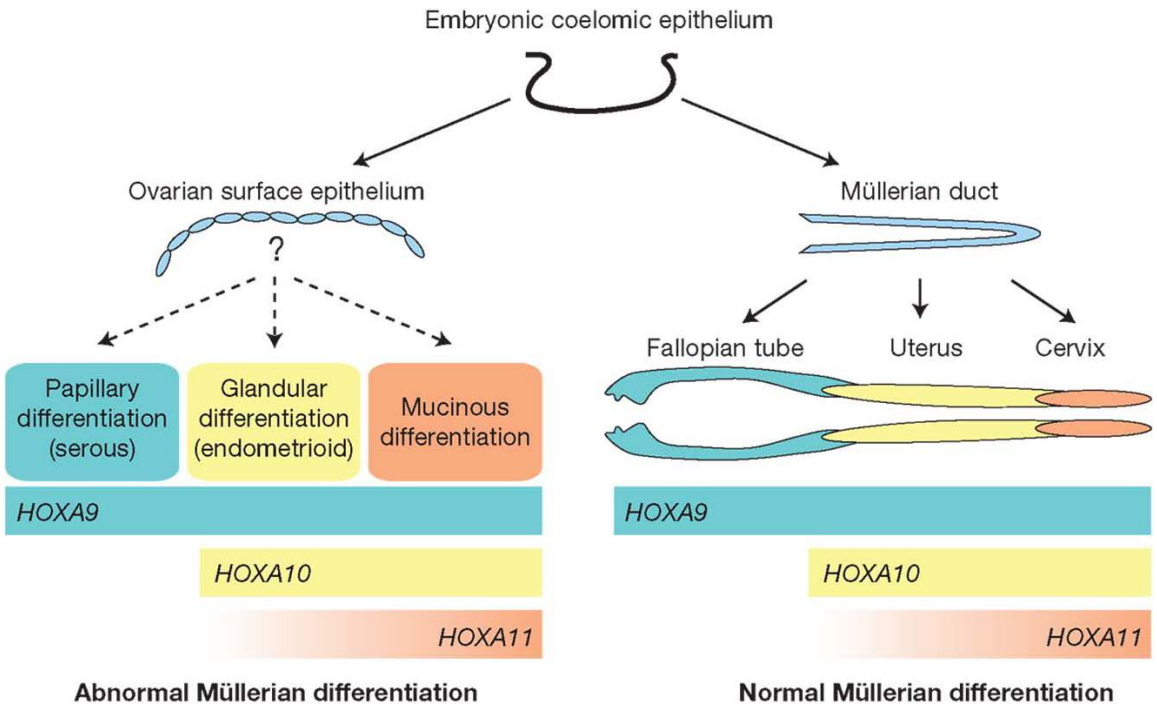

C. CANCER-ASSOCIATED FIBROBLASTS (CAFs)

It is increasingly recognized that the crosstalk between tumor cells and surrounding stroma cells is important for tumor progression. Tumor progression is dependent on angiogenesis[75-77] and immune system.[78, 79] Fibroblasts comprise the non-vascular, non-epithelial and non-inflammatory component of the connective tissue[80] and have also been recognized as a prominent modifier of tumor progression.

1. Origins of CAFs

Cancer associated fibroblasts (CAFs) are heterogeneous cell populations that reside in the tumor microenvironment. CAFs are distinguished from normal quiescent fibroblasts by their expression of markers such as α-smooth-muscle actin (αSMA), fibroblast-activated protein (FAP), fibroblast-specific protein-1 (FSP1/S100A4), neuron-glial antigen-2 (NG2), and PDGF β-receptor.[81] Multiple cell types have been identified as potential sources of CAFs (Figure 8).[82] Primarily they seem to be derived from local resident fibroblasts. However, it has been reported that up to 25% of CAFs originate from bone-marrow derived mesenchymal stem cells (MSCs) in mouse xenograft models of pancreatic cancer.[83] Normal epithelial cells and/or cancer cells derived from epithelial cells can be another source of CAFs by undergoing epithelial-to-mesenchymal transition (EMT).[84, 85] Additionally, some CAFs can derive from endothelial cells. In experimental mouse models, cells co-expressing αSMA or FSP1 and the endothelial cell marker CD31 have been reported. Forty percent of FSP1+ cells and 11% of αSMA+ cells also expressed CD31 in a melanoma tumor model.[86] Other cell types including tissue-resident MSCs and adipocytes have also been proposed as progenitors of CAFs (Figure 8).

Figure 8. Origin of CAFs

Local tissue-resident fibroblasts or bone marrow-derived MSCs are recruited into the developing tumor and Müllerian acquire a CAF phenotype. CAFs have also been suggested to originate from epithelial or endothelial cells through epithelial/endothelial–mesenchymal transition.

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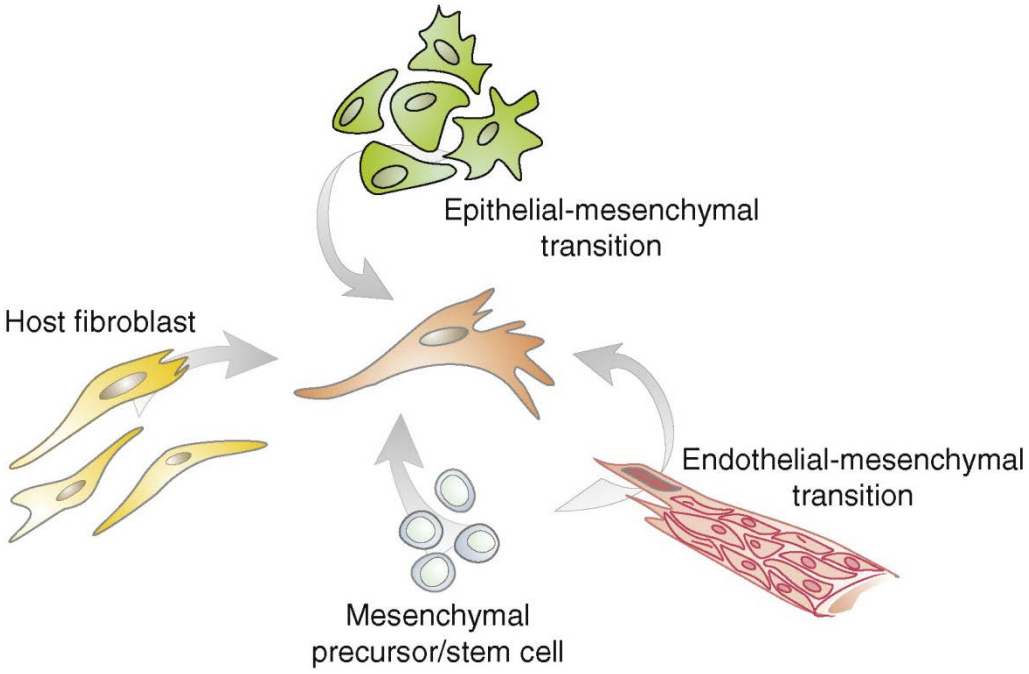

2. The role of CAFs in tumor progression

Whereas normal stromal cells tightly restrict outgrowth of epithelial cells, it has been demonstrated that CAFs promote the growth of various cancers, including pancreatic, breast and gastric cancers.[87-89] Conversely, targeting of CAFs inhibits cancer growth.[90] The interactions between CAFs, tumor cells and other host cells are dynamic, and are controlled by a complex network of mediators as described below (Figure 9).[91]

2.1. Interaction between CAFs and cancer cells

CAFs can directly stimulate cancer growth by producing growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) and IL-6.[92, 93] CAFs also stimulate migration of tumor cells. CAFs secrete extracellular matrix (ECM) degrading proteases such as metalloproteases (MMPs)[94, 95] and pro-migratory ECM component such as tenascin.[96] In addition, CAFs can promote tumor cell migration by releasing various growth factors such as TGFβ[97] which induce tumor cells to undergo EMT .[98, 99]

CAFs have also been demonstrated to induce tumorigenesis in specific contexts. Olumi *et al.* reported that non-tumorigenic, immortalized prostate epithelial cells gave rise to tumors when co-injected into mice with CAFs.[100] In contrast, no tumor formation was induced when the prostate epithelial cells were co-injected with normal fibroblasts.[100] In addition, there is evidence that CAFs promote stem cell-like features in cancer cells. When colon cancer cells were cultured with CAFs or conditioned medium from CAFs, cancer cells became more stem cell-like with increased nuclear β-catenin expression.[101]

Figure 9. Functions of CAFs

CAFs communicate with cancer cells, resident epithelial cells, endothelial cells, pericytes and inflammatory cells through the secretion of growth factors and chemokines.

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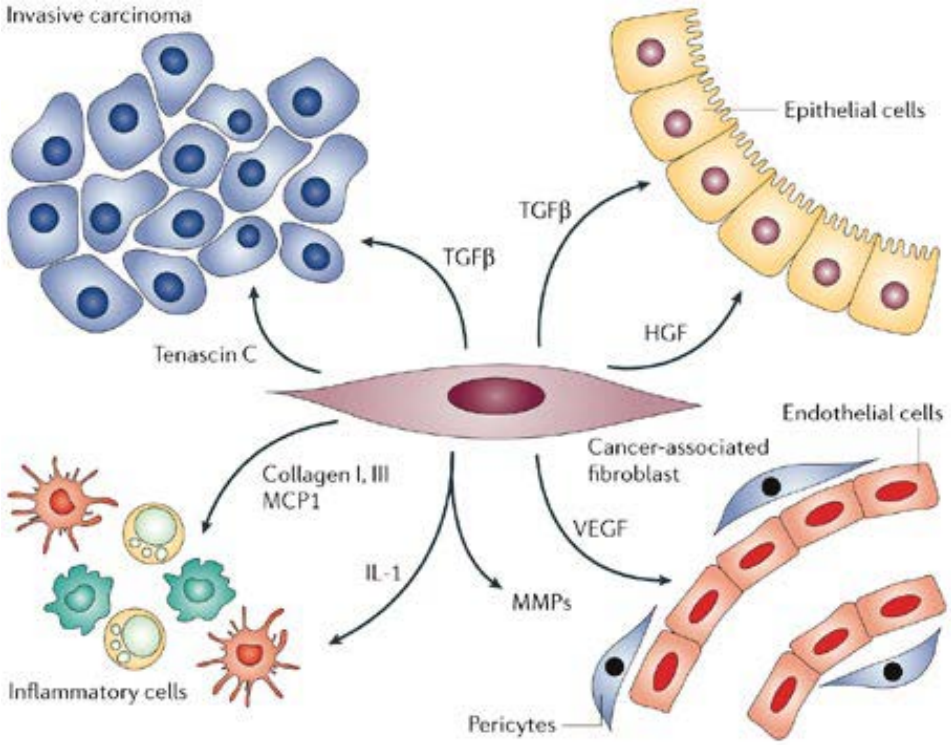


2.2. Interaction between CAFs and endothelial cells

CAFs have also been demonstrated to drive tumor growth and progression by stimulating tumor angiogenesis. Orimo *et al.* reported that Ras-transformed breast cancer cells develop larger tumors when co-injected with CAFs rather than with normal fibroblasts and attributed the enhanced tumor growth to increased tumor angiogenesis.[88] These authors also demonstrated that CAF-derived CXCL12 (also known as stromal cell derived factor-1) stimulates recruitment of bone-marrow derived endothelial progenitors to tumors.[88] CAFs are also major sources of vascular endothelial growth factor (VEGF) and MMPs, which can increase angiogenesis.[102, 103]

2.3. Interaction between CAFs and immune cells

The tumor stroma also contains a diverse variety of immune cells including macrophages and T cells. Tumor-associated macrophages (TAMs) are major components of the leukocyte infiltrate in many solid tumors. Unlike classic cytotoxic macrophages, TAMs are often characterized by anti-inflammatory functions and promote tumor growth by producing a number of cytokines and growth factors. TAM accumulation is associated with poor prognosis in patients with breast, prostate, and bladder cancers.[104-106] CAFs stimulate the accumulation of TAMs by producing monocyte chemotactic protein 1 (MCP-1, also known as CCL2) which is the most well-characterized chemotactic factor for TAMs. MCP-1 also suppresses the proliferation and activation of CD4+ T cell.[107] In addition, CAFs produce other immune-modulatory cytokines such as interferon-γ, interleukin-6, and tumor necrosis factor-α,[108] which influence the recruitment of cytotoxic T lymphocytes, natural killer cells and macrophages.

D. HYPOTHESIS AND SPECIFIC AIMS

The high lethality of EOC is due to rapid and aggressive peritoneal involvement. EOCs frequently colonize peritoneal surfaces that overlie connective and adipose tissues. However, the mechanisms that enable EOC cells to readily adapt to the peritoneal environment are poorly understood. The goal of my research is to determine the molecular mechanisms that control the aggressive behavior of EOCs.

HOXA9, a homeobox gene that is normally expressed in the developing female reproductive tract, has been previously found to be aberrantly expressed in EOCs and to control the morphologic features of these tumors. The differentiation pattern of a tumor is an important determinant of its clinical behavior and prognosis. However, the significance of HOXA9 to the clinical behavior of EOC is not known. My broad over-arching hypothesis is that HOXA9 promotes the aggressive behavior of EOC. Specifically, I hypothesize that HOXA9 promotes the aggressive behavior of this disease by modulating interactions between EOC cells and stromal cells.

The specific aims of my thesis project are:

1) To evaluate whether HOXA9 is associated with poor outcomes in EOC patients and in mouse intraperitoneal xenograft models of EOC
2) To determine whether HOXA9 modulates cellular interactions in the EOC microenvironment
3) To characterize the molecular mechanisms of HOXA9 in promoting progression of EOC

CHAPTER 2: MATERIALS AND METHODS

1. Bioinformatic and statistical analyses

Statistical analysis was performed using STATISTICA6 software (StatSoft Inc.). Values of statistical significance of data in in vitro and in vivo assays were calculated by the Student *t*-test. Data represent mean ± SEM. Correlation coefficients were determined by Spearman test. *P* values >0.05 were considered not significant. Gene expression data from EOC patients at the Peter MacCallum Cancer Center (AOCS study) (GSE9891, n = 285) were downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). Gene expression data from TCGA project (n = 567) were downloaded from the TCGA data portal site (http://tcga-data.nci.nih.gov/tcga/). Gene expression data were generated by using Affymetrix microarray platforms (U133 v2.0 for AOCS, U133A for TCGA). All data were normalized by using the robust multi-array average method.[109] Where there were multiple probe sets for an individual gene, the mean value for the given gene for each case was used. Patients were stratified according expression of a given *HOX* gene, where transcript levels for the given gene were defined as High (≥ upper quartile) and Low (≤ lower quartile). Association

of expression of a given *HOX* gene with patient survival was assessed in upper and lower quartile sub-groups by using Kaplan-Meier plot analysis and Log-rank test. Transcript levels of a given *HOX* gene were compared between cases that were categorized by clinical parameters and significance of differences between groups were estimated by using Mann-Whitney *U*-test.

2. Source of human tissue specimens

Studies using human tissue specimens and cells were approved by the Institutional Research Boards of the University of Texas MD Anderson Cancer Center and University of Chicago. Archived specimens of human EOC tissues, that were not necessary for diagnosis and were de-linked from patient-identifiers, were obtained from the gynecologic tumor banks at the University of Texas MD Anderson Cancer Center and University of Chicago with informed patient consent. All cases were Stage III/IV serous EOC. Fibroblasts were isolated from normal omental tissues of women undergoing surgery for benign conditions as previously described with informed patient consent.[110]

3. Cell culture

3.1. Primary human cells

Primary cultures of normal human omental fibroblasts were maintained in DMEM medium (Invitrogen) containing 10% FBS and MEM Non-essential amino acids. Normal human bone marrow MSCs were provided through the Tulane Center for Gene Therapy, MSC cell distribution center (Darwin Prockop, Tulane University) and cultured in α-MEM

medium (Invitrogen) containing 20% FBS. Normal human adipose MSCs isolated from lipoaspirate were purchased from American Type Culture Collection (ATCC) and cultured in MSC basal medium with growth supplement (ATCC). Immortalized, non-tumorigenic human ovarian surface epithelial cells (TNOE072) were provided by Jinsong Liu (MD Anderson Cancer Center).

3.2. Mouse cells

Stable mouse EOC **(**MOSEC) lines are described in previous studies[65] and were cultured in DMEM medium containing 10% FBS. Immortalized mouse endothelial cells[111] were cultured in DMEM containing 10% FBS. L929 mouse skin fibroblasts were purchased from ATCC and cultured in Eagle's MEM medium (Invitrogen) containing 10% FBS.

3.3. Human EOC cell lines

The serous EOC line SKOV3ip and clear-cell EOC line ES-2 were cultured in McCoy's 5A medium (Invitrogen) containing 10% FBS.

4. Transfection

pGFP-V-RS *HOXA9, TGF-β2* and non-targeting shRNA plasmids and *TGF-β2* cDNA were purchased from OriGene Technologies. SKOV3ip, ES-2 and MOSEC cells were transfected with plasmids by using FuGENE6 reagent (Roche) and selected with puromycin (0.5 μg/ml).

5. *In vitro* cell growth assays

EOC cells were seeded either directly on plastic surfaces or embedded in 2% Matrigel (BD Biosciences) in 96-well plates (2,000 per well). Cell proliferation was measured daily by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche). To assay cell viability under reduced serum conditions, EOC cells were seeded in 12-well plates ($2x10^4$ per well), cultured for 3 days in medium containing 0.2% FBS and stained with trypan blue dye. To assay colony formation, EOC cells were seeded in 6-well plates ($2x10^4$ per well) and cultured for 2 weeks in 0.3% agar. Three independent experiments were performed for each assay.

6. Stimulation of cells with conditioned media

To generate tumor-conditioned media, EOC cells ($1.5x10^6$) were seeded in 10 cm dishes and cultured in medium containing 1% FBS for 2 days. Tumor-conditioned medium was filtered, assayed by ELISA, and applied to fibroblasts and MSCs. Conditioned medium was replaced every 2 days, and cells analyzed by quantitative RT-PCR and Western blot at 5 days thereafter. For fibroblast-priming assays, fibroblasts were incubated in tumor-conditioned medium for 5 days and then washed. Fresh non-conditioned medium was added and fibroblasts cultured for 2 days. Medium conditioned by fibroblasts was filtered, assayed by ELISA, and applied to EOC cells. Growth of EOC cells were measured by the MTT assay. Endothelial cell growth was likewise assayed following culture in media conditioned by EOC cells and by primed fibroblasts. Conditioned media was depleted of CXCL12, IL-6 and TGF-β2 by Immunoprecipitation using antibodies to these growth factors. Three independent sets of each type of conditioned medium were evaluated in each assay.

7. Co-culture assay

Fibroblasts (1,000) were seeded with 1,000 cells of each GFP expressing EOC line per well in 96-well plates. Numbers of fibroblasts (GFP-negative cells) in each well were counted daily under light and immunofluorescence microscopy. Three independent experiments were performed for each assay.

8. Xenografts

All animal studies were performed at the University of Texas MD Anderson Cancer Center with approval of the Institutional Committee on Use and Care of Animals. Four-week-old female nude mice (purchased from National Cancer Institute) were used for animal studies. Mice were inoculated intraperitoneally with cells of MOSEC ($1.5x10^6$), SKOV3ip ($2x10^6$) and ES-2 ($1x10^6$) lines or subcutaneously with cells of SKOV3ip ($1x10^6$) and ES-2 ($6x10^5$) lines. For survival studies, mice were euthanized by CO_2 asphyxiation when morbid ascites had developed. Volumes of subcutaneous tumors were calculated from perpendicular measurements of tumor diameters taken daily using calipers. GFP-expressing intraperitoneal xenografts were visualized in euthanized mice under a Leica MZML III fluorescence stereomicroscope.

9. Antibodies and other reagents

Antibodies were purchased from commercial sources as follows: αSMA (Dako), CD34 (Abcam), Ki-67 (Vector Laboratories), GFP (Molecular Probes); HOXA9 antibody for western blotting (Millipore) and for chromatin immunoprecipitation (Santa Cruz Biotechnology), TGF-β2, CXCL12, IL-6 (R&D Systems), FLAG, MYC (Sigma-Aldrich),

secondary antibodies (Invitrogen). Recombinant growth factors were purchased from the following sources: TGF-β1 (Sigma-Aldrich), TGF-β2, VEGF and IL-6 (Invitrogen).

10. Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of mouse and human tissues were used for staining with hematoxylin-eosin and with HOXA9, αSMA, Ki-67, CD34 and TGF-β2 antibodies. Staining was detected by streptavidin-biotin-peroxidase and 3, 3'-diaminobenzidine (Dako). Staining using Alexa Fluor-conjugated antibodies was performed on frozen tissue sections. Evaluation of staining with each antibody is described in the figure legends.

11. Western blotting

Cell extracts were prepared by lysing cells in M-PER buffer (Pierce Biotechnology). Total cell lysates were separated by SDS-PAGE, then transferred to PVDF membrane (GE Healthcare) and blocked with 5% nonfat milk in TBS with 0.1% Tween-20 (TBS-T). Membranes were hybridized with primary antibodies overnight at 4˚C then washed with TBS-T buffer. Secondary antibody was incubated with membrane for 1 hour at room temperature. Membrane washed with TBS-T buffer was visualized with ECL western blotting detection reagent (Amersham Biosciences).

12. ELISA

ELISAs kits were purchased from R&D Systems (to detect mouse TGF-β1 and human IL-6, CXCL12, VEGF-A, TGF-β1, TGF-β2) and LifeSciences Advanced Technologies (for mouse TGF-β2). Levels of each growth factor were assayed according to the manufacturer's instruction in three independent sets of each type of conditioned medium and normalized to total cellular protein content.

13. Quantitative RT-PCR

Total RNA was extracted using PureLink RNA mini kit (Invitrogen) following manufacturer's instructions. Purified total RNA (1μg) was used to synthesize cDNA using the RT2 First Strand Kit (SABiosciences). Transcripts were analyzed by using SYBR®Green qPCR Master Mix (SABiosciences) and primers listed in Tables 5-7. Levels of target transcript were normalized to ribosomal protein *RPL32*.

Table 5. Human specific primers for quantitative RT-PCR

Gene	Sequence	
ACTA2	Forward	CTATGCCTCTGGACGCACAACT
	Reverse	CAGATCCAGACGCATGATGGCA
FAP	Forward	GGAAGTGCCTGTTCCAGCAATG
	Reverse	TGTCTGCCAGTCTTCCCTGAAG
TGFB1	Forward	GCCCTGGACACCAACTATTG
	Reverse	CGTGTCCAGGCTCCAAATG
TGFB2	Forward	AGAGTGCCTGAACAACGGATT
	Reverse	CCATTCGCCTTCTGCTCTT
VEGFA	Forward	TTGCCTTGCTGCTCTACCTCCA
	Reverse	GATGGCAGTAGCTGCGCTGATA
IL6	Forward	CAATCTGGATTCAATGAGGAGAC
	Reverse	CTCTGGCTTGTTCCTCACTACTC
CXCL12	Forward	CTCAACACTCCAAACTGTGCCC
	Reverse	CTCCAGGTACTCCTGAATCCAC
SNAI1	Forward	GCTGCAGGACTCTAATCCAGAGTT
	Reverse	GACAGAGTCCCAGATGAGCATTG
SNAI2	Forward	ATGAGGAATCTGGCTGCTGT
	Reverse	CAGGAGAAAATGCCTTTGGA
TWIST1	Forward	GGACAAGCTGAGCAAGATTCAGA
	Reverse	TCTGGAGGACCTGGTAGAGGAA
ZEB1	Forward	GGCATACACCTACTCAACTACGG
	Reverse	TGGGCGGTGTAGAATCAGAGTC
ZEB2	Forward	AATGCACAGAGTGTGGCAAGGC
	Reverse	CTGCTGATGTGCGAACTGTAGG
KLF8	Forward	CCTGAAAGCTCACCGCAGAATC
	Reverse	TGCTTGCGGAAATGGCGAGTGA

Table 5. Human specific primers for quantitative RT-PCR (continued)

Gene	Sequence	
PDGFA	Forward	CAGCGACTCCTGGAGATAGACT
	Reverse	CGATGCTTCTCTTCCTCCGAATG
PDGFB	Forward	GAGATGCTGAGTGACCACTCGA
	Reverse	GTCATGTTCAGGTCCAACTCGG
CXCL1	Forward	GCCAGTGCTTGCAGACCC
	Reverse	GATGCTCAAACACATTAG
CXCL2	Forward	CGCAGCAGGAGCGCC
	Reverse	TGGATGTTCTTGAGGTGAATTCC
TNF	Forward	CCCAGGGACCTCTCTCTAATCA
	Reverse	GCTACAGGCTTGTCACTCGG
FGF2	Forward	AGCGGCTGTACTGCAAAAACGG
	Reverse	CCTTTGATAGACACAACTCCTCTC
RPL32 control primer	Forward	ACAAAGCACATGCTGCCCAGTG
	Reverse	TTCCACGATGGCTTTGCGGTTC

Table 6. Mouse specific primers for quantitative RT-PCR

Gene	Sequence	
Tgfb1	Forward	TGATACGCCTGAGTGGCTGTCT
	Reverse	CACAAGAGCAGTGAGCGCTGAA
Tgfb2	Forward	TTGTTGCCCTCCTACAGACTGG
	Reverse	GTAAAGAGGGCGAAGGCAGCAA
Vegfa	Forward	CTGCTGTAACGATGAAGCCCTG
	Reverse	GCTGTAGGAAGCTCATCTCTCC
Il6	Forward	TACCACTTCACAAGTCGGAGGC
	Reverse	CTGCAAGTGCATCATCGTTGTTC
Rpl32 control primer	Forward	GGAGAAGGTTCAAGGGCCAG
	Reverse	TGCTCCCATAACCGATGTTTG

Table 7. Human/Mouse common primers for quantitative RT-PCR

Gene	Sequence	
HOXA9	Forward	CCCTGACTGACTATGCTTGTGGT
	Reverse	TCTCCGCCGCTCTCATTCTC
RPL32 control primer	Forward	CCTTGTGAAGCCCAAGATCG
	Reverse	TGCCGGATGAACTTCTTGGT

14. Chromatin Immunoprecipitation and reporter assays

Chromatin immunoprecipitation assays were performed using the EZ-ChIP chromatin immunoprecipitation Assay kit (Upstate Biotechnology) following manufacturer's instructions. Briefly, cells were cross-linked by adding formaldehyde to 1% final concentration at room temperature for 10 minutes. Formaldehyde was then quenched by adding glycine and then cells were harvested. Cross-linked genomic DNA was sonicated to generate DNA fragment of 200-1000 base pair in length and then performed Immunoprecipitation with normal IgG or HOXA9 antibody. DNA was purified from precipitated complexes and used to amplify fragments of the *TGF-β2* promoter. Fragments of the *Tgfb2* promoter described in the text were also amplified from mouse genomic DNA and cloned into the pGL3 luciferase reporter vector (Promega). Luciferase activities were assayed using the Dual-luciferase reporter assay kit (Promega). Primers used for chromatin immunoprecipitation and subcloning are listed in Tables 8 and 9.

Table 8. Primers for chromatin immunoprecipitation

Primer	Sequence	
Mouse primer set for S1 (-1144bp~-944bp)	Forward	TGTATGCCAGCTATATCATT
	Reverse	TGGCCACGATGACTACACAG
Mouse primer set for S2 (-784bp~-584bp)	Forward	AGTTTGAGCAAGTTGAAGTA
	Reverse	CTCTGTATCACTGCTTTGAA
Mouse primer set for S3 (-524bp~-324bp)	Forward	ACAGGGCTGGATTGTAAACA
	Reverse	AGGCGTGTACACACACACAC
Mouse primer set for S4 (-174bp~+74bp)	Forward	ATGCCAGTCGCCCTCCCTTA
	Reverse	AAACCTGCTGCCAGCAGATA
Mouse primer set for S5 (+832bp~+980bp)	Forward	CAGATCAGCCACTCCGCACCG
	Reverse	GGGGGGAATCTCTCACTTTAAGG
Mouse primer set for *Gapdh*	Forward	CCATTTTGTCTACGGGACGA
	Reverse	GGCCACGCTAATCTCATTTT
Human primer set for S4 (-1007bp~-882bp)	Forward	GGTCTAAGTAACGAGAGGACTTC
	Reverse	CCAGCAGATAACATCACGATC
Human primer set for S5 (+77bp~+227bp)	Forward	CAGATCCGCCACTCCGCACCC
	Reverse	GGGAACCCTGACTTTGGCGAG
Human primer set for *GAPDH*	Forward	TACTAGCGGTTTTACGGGCG
	Reverse	TCGAACAGGAGGAGCAGAGAGCGA

Table 9. Primers for *Tgfb2* promoter construct

Primer	Sequence	
Primer set for region A	Forward	ATGCCAGTCGCCCTCCCTTA
	Reverse	GGGGGGAATCTCTCACTTTAAGG
Primer set for region B	Forward	AGGAGAAGCTAGCGAAGGGTGC
	Reverse	CGAATTGAAGCTTCCGCGGT
Primer set for region C	Forward	GGTATCGGCTAGCTTGATATCCAC
	Reverse	GACTCGCAAGCTTCCCTAGC
Primer set for mutant region C	Forward	TATGGGATCCCCCCGCCACGTGT
	Reverse	ACGTGGTTTGGGGATCCCGGCTGACGCTCTGCTCC

CHAPTER 3: HOXA9 PROMOTES GROWTH OF EOC

A. RATIONALE

EOC is the most lethal gynecologic disease in the United States. Seventy percent of EOC patients have advanced disease at the time of diagnosis and their five-year-survival rate is only 20% (Table 2). Unlike other epithelial cancers, EOC readily disperses throughout the peritoneal cavity without vasculature involvement. EOC cells are exfoliated from their primary site by the circulating peritoneal fluid and then directly implant on to secondary sites, in particular the omentum and surfaces of other abdominal organs (Figures 1 and 2).[9] However, the mechanisms that enable EOC cells to readily adapt to the peritoneal environment are poorly understood.

Cheng *et al* previously identified a broad trend in expression of Müllerian *HOX* gene program in the major subtypes of EOC according to the patterns of Müllerian-like differentiation of these tumors (Figure 7).[65] However, the functional significance of Müllerian *HOX* genes to the clinical behavior of EOC is not known. The goal of my studies in this chapter is to determine whether Müllerian *HOX* genes have any impact on the clinical outcomes of EOC patients and promote EOC growth in mouse xenograft models.

B. RESULTS

1. *HOXA9* expression is associated with poor overall survival of EOC patients

I initiated this study by evaluating the clinical significance of Müllerian *HOX* genes in EOCs in the Australian Ovarian Cancer Study (AOCS) gene expression dataset.[112] AOCS dataset is one of the most comprehensive EOC datasets and contains total 285 cases, of which 71% are serous EOCs. In this dataset, it was found that *HOXA9* transcript levels did not significantly differ between serous carcinomas of ovarian, tubal, and peritoneal origin or between serous and endometrioid EOCs, whereas *HOXA10* and *HOXA11* levels were higher in endometrioid than in serous EOCs (Table 10). These observations were consistent with previous findings of HOX protein levels in an independent cohort.[65]

To determine whether *HOXA9* expression is associated with poor survival of EOC patients, I divided all cases in AOCS dataset into three groups based on the levels of *HOXA9* transcript: *HOXA9*-High (≥ upper quartile, n=72), *HOXA9*-Low (≤ lower quartile, n=72) and *HOXA9*-Medium group (n=141). Compared to the overall survival rates of patients in the *HOXA9*-Low group, patients in the *HOXA9*-High group had significantly poor overall survival in the AOCS cohort (P = 0.0004 by Log-rank test, Figure 10A). Mortality rates of *HOXA9*-High cases were higher than of *HOXA9*-Low cases irrespective of tumor grade and disease stage (Table 11). High *HOXA9* expression was also significantly associated with poor survival in the Cancer Genome Atlas (TCGA) EOC dataset (P = 0.01, Figure 10B). In contrast, the expression level of other Müllerian *HOX* genes (*HOXA10, HOXA11)*, other neighboring *HOXA* genes or paralogs of *HOXA9* were not significantly associated with survival of EOC patients (Figure 11).

Table 10. Differences in *HOX* transcript levels between carcinoma cases in the AOCS dataset grouped by histologic subtype and primary site

Cases in the AOCS dataset[112] included serous (n=204) and endometrioid (n=20) ovarian carcinoma, and serous tubal (n=8) and peritoneal (n=34) carcinoma. *P*-value was determined by Mann-Whitney *U*-test.

Gene	Comparison	*P*-value	Transcript level
HOXA9	serous ovarian vs serous peritoneal	*P* = 0.26	No difference
	serous ovarian vs serous tubal	*P* = 0.31	No difference
	serous ovarian vs endometrioid ovarian	*P* = 0.21	No difference
HOXA10	serous ovarian vs endometrioid ovarian	*P* = 0.029	serous < endometrial
HOXA11	serous ovarian vs endometrioid ovarian	*P* = 0.005	serous < endometrial

Figure 10. High *HOXA9* expression is associated with reduced survival of EOC patients

Kaplan-Meier plot analysis of overall survival times of patients stratified by transcript level of *HOXA9* in tumors; *HOXA9*-Low (lower quartile) and *HOXA9*-High (upper quartile). *P*-value was calculated by log-rank test. **(A)** Survival analysis from the AOCS dataset (n=72 cases per group) **(B)** Survival analysis from the TCGA dataset (n=142 cases per group)

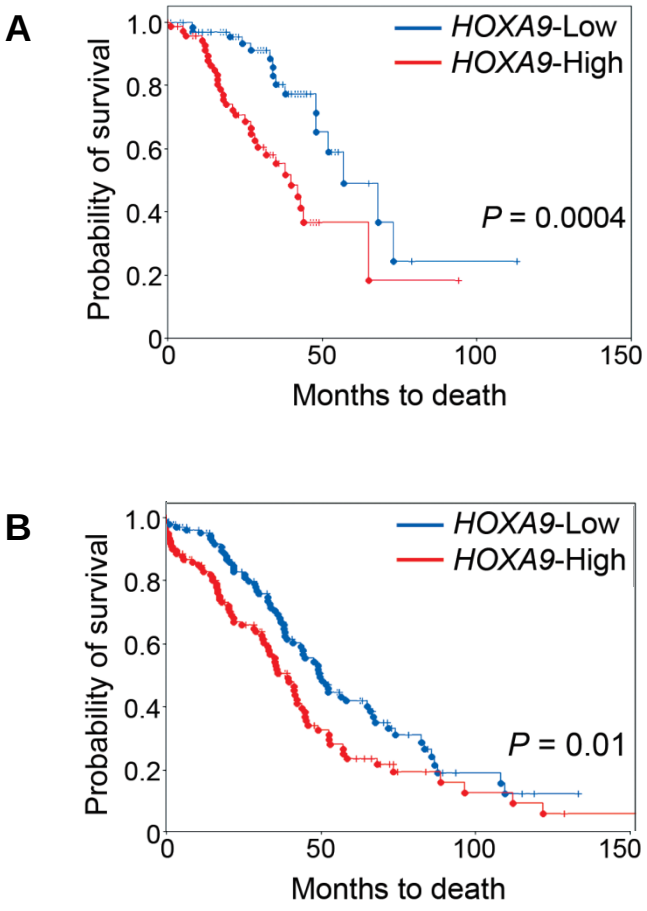
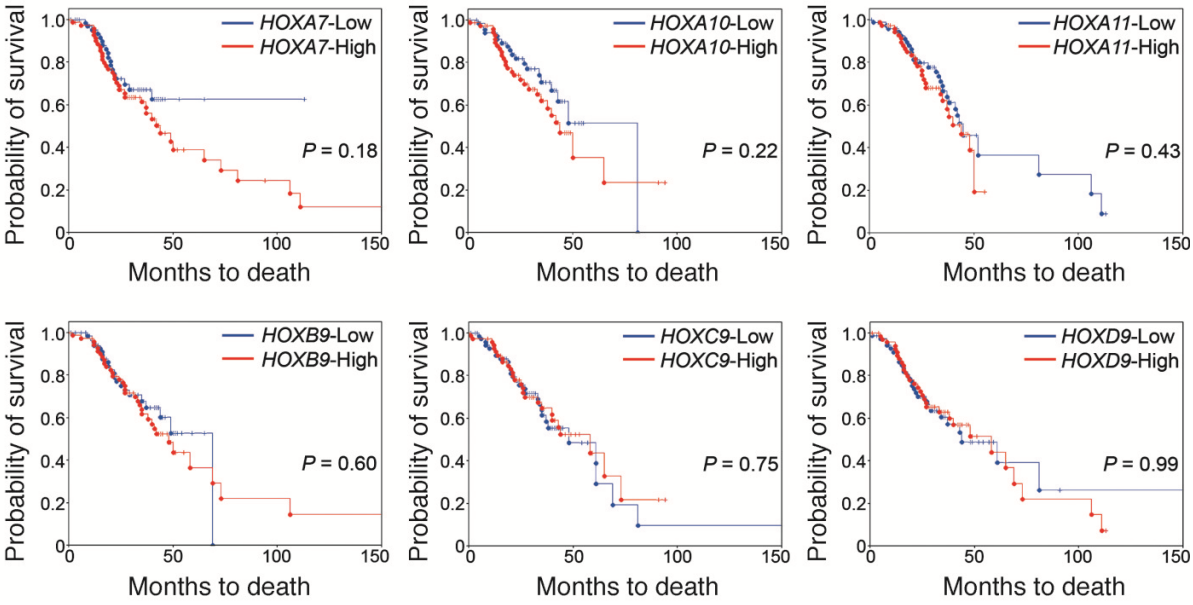


Table 11. Mortality rates of *HOXA9*-Low and *HOXA9*-High cases in the AOCS dataset categorized by tumor grade and disease stage

		***HOXA9*-Low** **Number of cases**	***HOXA9*-High** **Number of cases**
Tumor grade	Grade 1	1/10 (10.0%)	0/3 (0%)
	Grade 2	5/19 (26.3%)	10/27 (37.0%)
	Grade 3	10/41 (24.4%)	21/41 (51.2%)
Disease stage	Stage I	0/11 (0%)	1/8 (12.5%)
	Stage II	0/6 (0%)	1/4 (25.0%)
	Stage III	15/53 (28.3%)	25/53 (47.2%)
	Stage IV	1/2 (50.0%)	5/6 (83.3%)

Figure 11. Other related *HOX* genes are not associated with patient survival

Kaplan-Meier plot analysis of overall survival times of patients in the AOCS dataset stratified by transcript level of each indicated *HOX* gene in tumors. For each *HOX* gene, transcript levels were defined as High (≥upper quartile) and Low (≤lower quartile). Each group contains 72 cases.



2. HOXA9 promotes EOC growth in mouse xenograft models

2.1. Overexpression of HOXA9 promotes EOC growth in mouse xenograft models

To determine whether HOXA9 promotes EOC growth in mouse xenograft models, I generated mouse EOC lines (MOSEC) that stably express *HOXA9, HOXA10* and *HOXA11* (Figure 12). Female nude mice that were inoculated intraperitoneally with +HOXA9 MOSEC cells developed larger implants than mice inoculated with vector-control MOSEC cells (Figure 13). In contrast, the tumor burden of mice that were inoculated intraperitoneally with +HOXA10 or +HOXA11 MOSEC cells were not significantly different from the vector-control group (Figure 13). Consistent with these observations, mice that were inoculated with +HOXA9 MOSEC cells had significantly shorter survival times than mice inoculated with vector-control MOSEC cells (P = 0.003, Figure 14A). However, survival rates of mice that inoculated with + HOXA10 or HOXA11 MOSEC cells were similar to those of mice inoculated with vector-control MOSEC cells (Figures 14B and C). These observations were consistent with the association of *HOXA9*, but not *HOXA10* or *HOXA11*, with poor survival of EOC patients (Figure 10 and 11). The increased growth of +HOXA9 tumors as compared to +HOXA10 and +HOXA11 tumors was not due to differences in ectopic *HOX* levels between the MOSEC lines (Figure 12), nor due to non-physiological levels of ectopic expression as the Hoxa9 level in +HOXA9 MOSEC cells was within the range of HOXA9 levels detected in EOC clinical specimens (Figure 15).

Figure 12. Overexpression of *HOX* genes

MOSEC cells were transfected with empty vector and with Myc-tagged *Hoxa9*, *Hoxa10* and *Hoxa11* cDNAs. Expression level of *HOX* genes were confirmed by western blot.

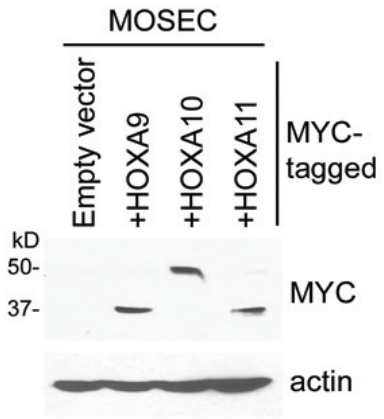


Figure 13. Growth of intraperitoneal MOSEC xenografts

Female nude mice were inoculated intraperitoneally with cells of vector-control and *HOX*-overexpressing MOSEC lines and sacrificed at 2 months thereafter. **(A)** Implants on peritoneal cavity wall and omentum are indicated. **(B)** Hematoxylin-eosin-stained tissue sections showing implants on the broad ligament. Bar, 200μm

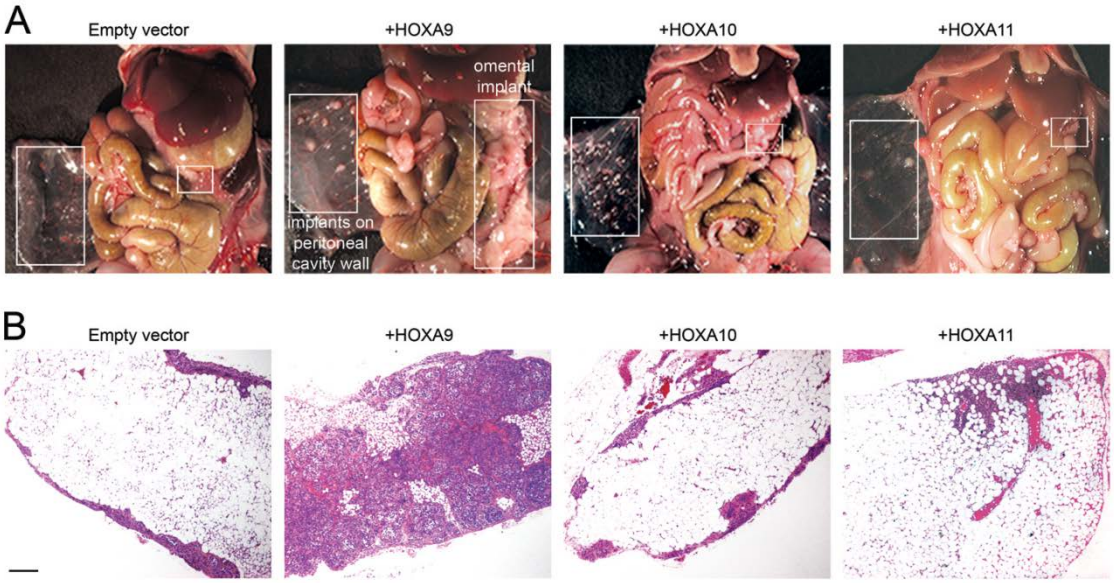


Figure 14. HOXA9, but not HOXA10 and HOXA11, promotes tumor growth in mouse xenograft models of EOC

Comparison of survival rates of female nude mice inoculated intraperitoneally with vector-control and with **(A)** +HOXA9, **(B)** +HOXA10, and **(C)** +HOXA11 MOSEC lines (n=10 per group)

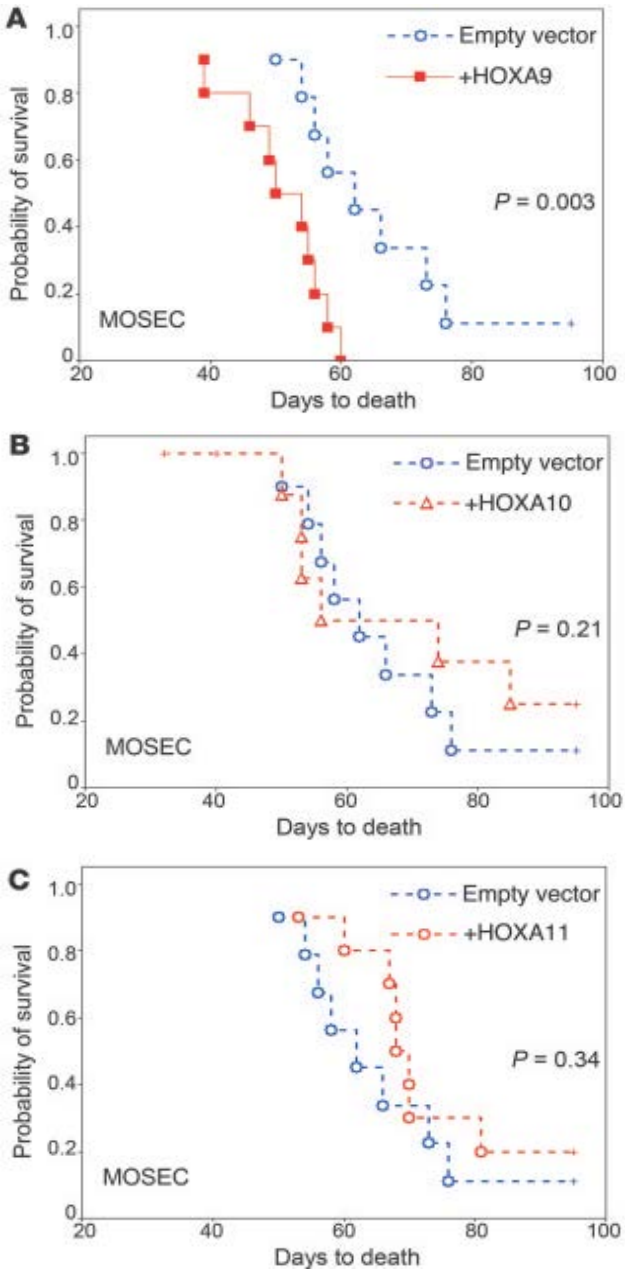
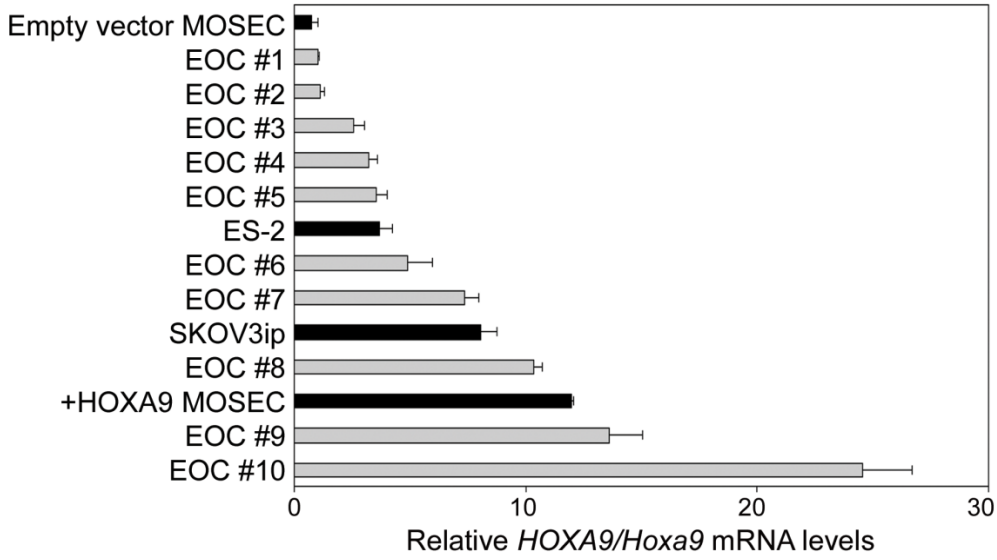


Figure 15. HOXA9 expression in EOC cells and clinical specimens

Quantitative RT-PCR analysis of *Hoxa9* mRNA levels in MOSEC lines and *HOXA9* mRNA levels in parental SKOV3ip and ES-2 lines and clinical specimens of EOC. Primers recognize both mouse *Hoxa9* (MOSEC) and human *HOXA9* (SKOV3ip, ES-2, clinical specimens).



2.2. Knockdown of HOXA9 inhibits EOC growth in mouse xenograft models

To confirm that HOXA9 promotes EOC growth, I evaluated the effect of inhibiting HOXA9 expression in human EOC lines. HOXA9 was knocked-down by using shRNAs that targeted different sites of *HOXA9* (shA9-A and shA9-B). As compared to two negative controls (empty vector and Nontargeting shRNA), two *HOXA9* shRNAs (shA9-A, shA9-B) were equally effective in knocking-down HOXA9 in SKOV3ip and ES-2 cells (Figure 16). Expression levels of endogenous HOXA9 in SKOV3ip (serous EOC) and ES-2 (non-serous EOC) cell lines were similar to those in several EOC clinical specimens (Figure 15). Knockdown of HOXA9 markedly inhibited growth of subcutaneous and intraperitoneal tumors derived from SKOV3ip and ES-2 lines (Figures 17A-D).

Figure 16. Knockdown of HOXA9 in EOC cells

SKOV3ip and ES-2 cell lines that stably express empty vector, nontargeting shRNA and shRNAs targeting different sites of *HOXA9* (shA9-A and shA9-B) were generated. Expression of HOXA9 was assayed by western blot.

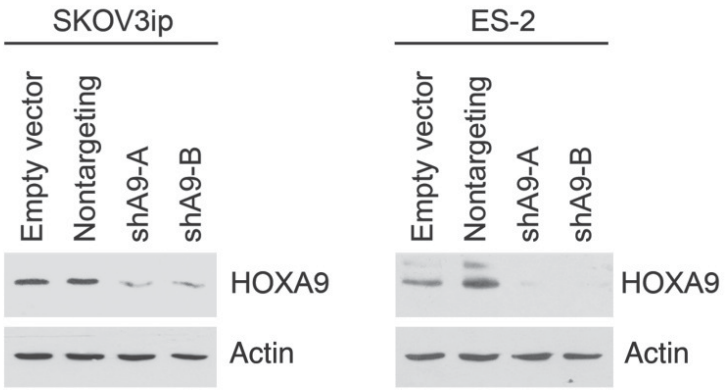
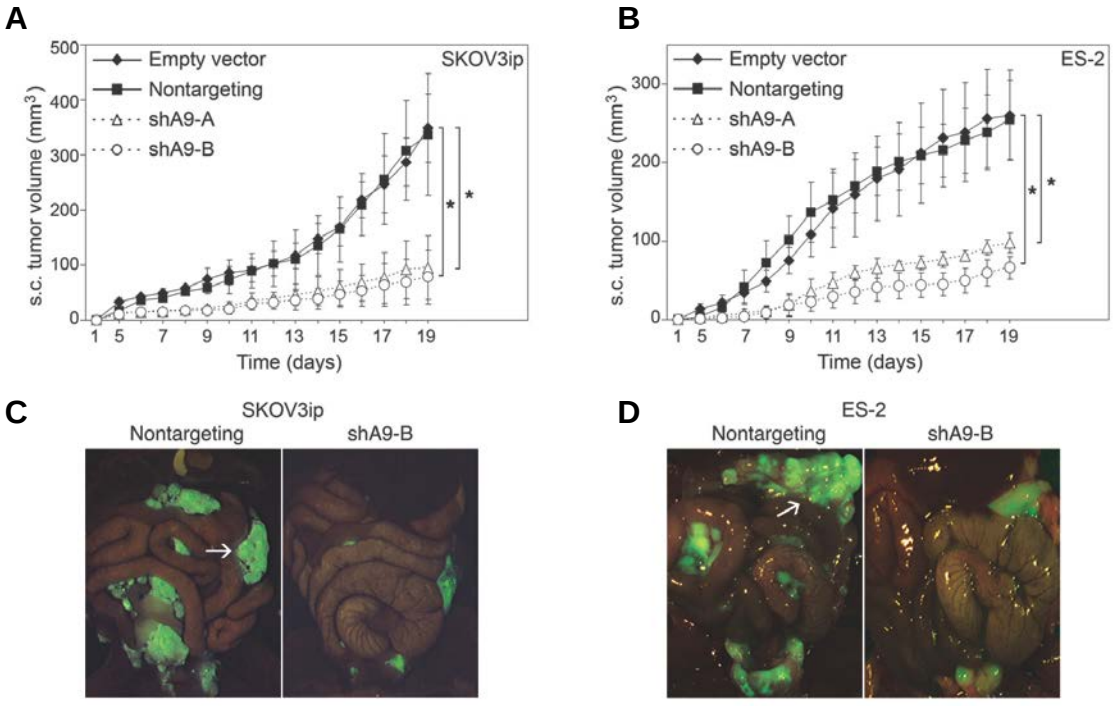


Figure 17. Knockdown of HOXA9 inhibits tumor growth in mouse xenograft models of EOC

(**A** and **B**) Growth rates of subcutaneous tumors derived from +HOXA9 control (Empty vector, Nontargeting) and HOXA9-knockdown (shA9-A, shA9-B) **(A)** SKOV3ip and **(B)** ES-2 lines (n=5 per group).* *P*<0.0005 (**C** and **D**) Mice were inoculated intraperitoneally with GFP expressing **(C)** SKOV3ip and **(D)** ES-2 lines and sacrificed at 4 weeks and 20 days, respectively. Implants were visualized under a fluorescence stereoscope. Omental implants in + HOXA9 control groups are indicated by arrows. Original magnification, ×0.8



C. CONCLUSION

The studies in this chapter demonstrate that expression of HOXA9 in EOC cells is strongly associated with poor outcomes in EOC patients and in mouse xenograft models of EOC. High *HOXA9* expression was significantly associated with poor overall survival in datasets of two independent cohorts of EOC patients. In addition, the studies showed that overexpression of HOXA9 promotes EOC growth, whereas knockdown of HOXA9 inhibits EOC growth in mouse xenograft models. In contrast, expression of other neighboring *HOXA* genes or paralogs of *HOXA9* is not associated with overall survival in EOC patients. Moreover, overexpression of HOXA10 or HOXA11 does not promote EOC growth in mouse xenograft models. Together, these findings indicate that HOXA9 promotes EOC growth and that this capability is not shared by other related *HOX* genes. The cellular and molecular mechanisms of HOXA9 that promote EOC growth will be the focus of Chapter 4 and Chapter 5, respectively.

CHAPTER 4: HOXA9 EXPRESSION IN EOC IS ASSOCIATED WITH INCREASED ABUNDANCE OF CAFS

A. RATIONALE

Tumor growth can be stimulated by a number of genetic alterations. Some genetic alterations increase tumor cell proliferation or inhibit tumor cell death by mechanisms that are autonomous to the tumor cell. Other genetic alterations can promote tumor growth by stimulating non-autonomous mechanisms, such as the interactions between tumor cells and the tumor microenvironment.

Studies in Chapter 3 demonstrated that HOXA9 expression is associated with poor survival of EOC patients and promotes EOC growth in mouse xenograft models. The goals of the studies in this chapter are 1) to determine whether HOXA9 promotes EOC growth by stimulating tumor cell autonomous or non-autonomous processes, and 2) to identify the nature of the cellular process that is controlled by HOXA9 in EOC.

B. RESULTS

1. HOXA9 promotes EOC growth *in vivo* but not *in vitro*

As discussed in Chapter 3, HOXA9 promotes EOC growth in mouse xenograft models. Consistent with these findings, significantly fewer Ki67+ tumor cells were detected in HOXA9-knockdown tumors than in control tumors ($P < 0.001$, Figure 18). This result demonstrates that HOXA9 promotes the proliferation of EOC cells *in vivo*. However, *in vitro* growth rates of HOXA9-knockdown and control EOC lines were identical, irrespective of whether these cells were cultured on plastic, in three-dimensional Matrigel cultures, or under serum-deprived or anchorage-independent conditions (Figures 19A-D). Similarly, overexpression of HOXA9 increased proliferative activity of MOSEC cells *in vivo* but not *in vitro* (Figures 20A and B). These findings indicate that HOXA9 does not stimulate EOC growth by a tumor cell-autonomous process, but rather that the ability of HOXA9 to promote tumor growth depends on interactions with host cells.

Figure 18. Effect of HOXA9-knockdown on EOC growth *in vivo*

Tumor tissues were collected from mice that were inoculated with SKOV3ip and ES-2 lines. Average numbers of Ki-67+ tumor cells per 200x microscopic field were calculated by scoring five random fields of stained tissue sections of each mice (n=5 per group).

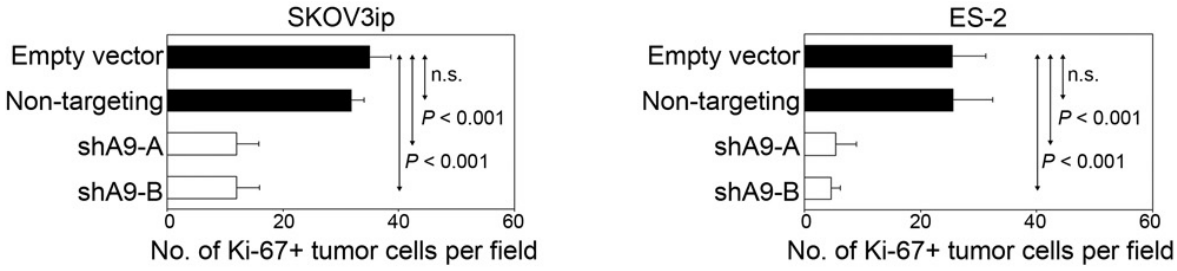


Figure 19. Effect of HOXA9-knockdown on EOC cell growth *in vitro*

(**A** and **B**) Growth rates of cell lines cultured **(A)** on plastic and **(B)** in three-dimensional Matrigel cultures were determined by MTT assay. **(C)** Numbers of viable and non-viable cells, determined by exclusion of trypan blue dye, were counted in each well at 3 days after culture under serum-deprived conditions. **(D)** Average number of colonies per 40x microscopic field at 2 weeks after culture in soft agar. No significant (n.s.) difference was found between cell lines.

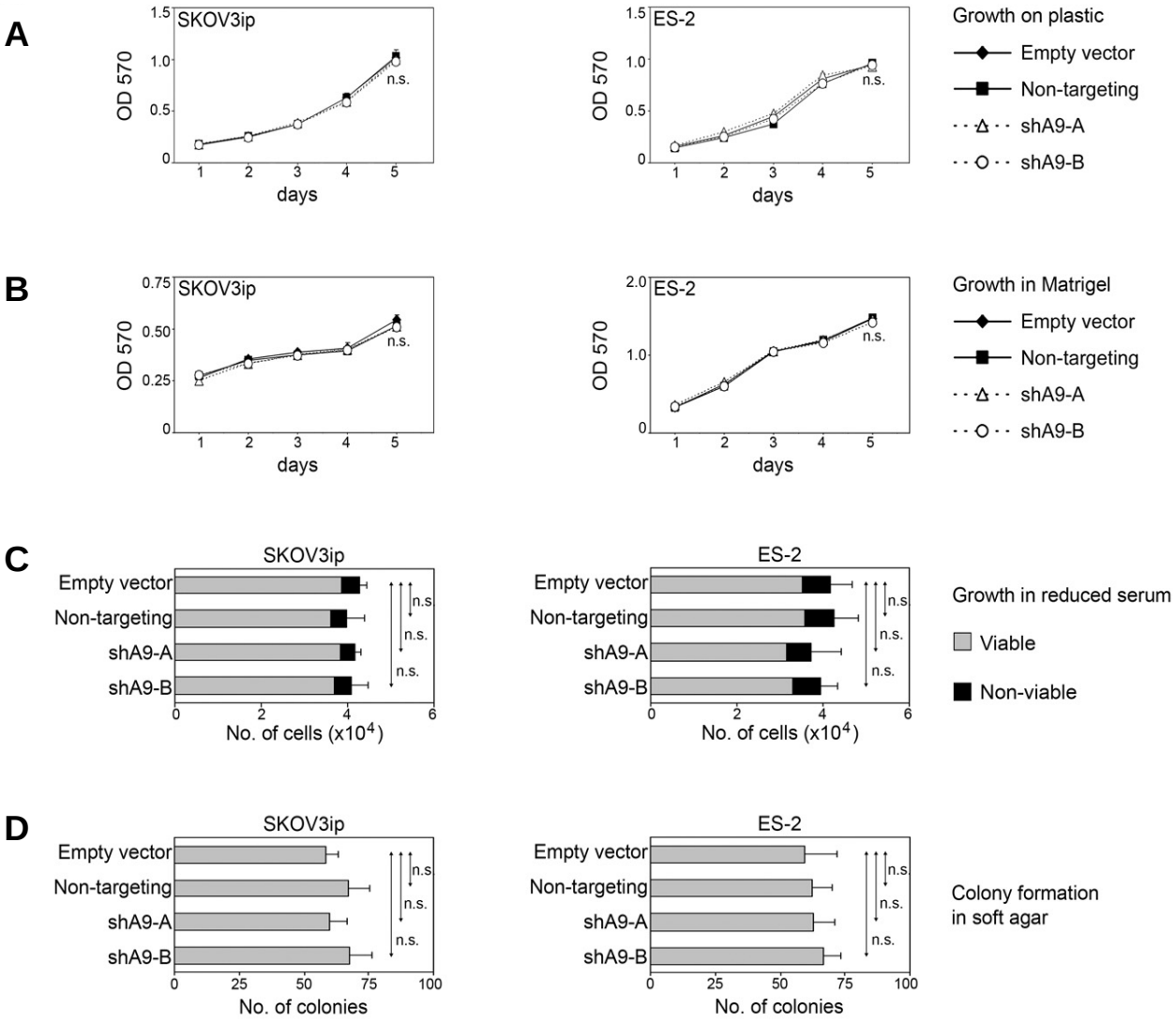
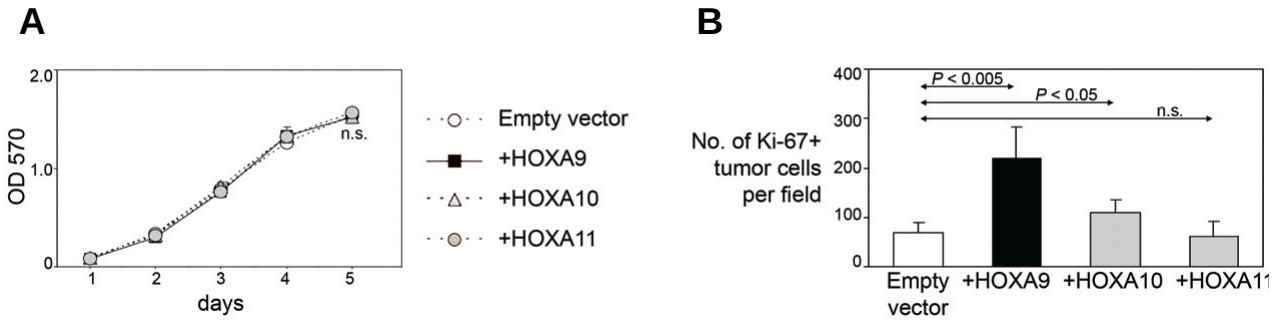


Figure 20. Growth characteristics of MOSEC lines *in vitro* and *in vivo*

(A) Growth rates of cultured MOSEC lines on plastic were measured by MTT assay

(B) Tumor tissues were collected from mice that were inoculated with MOSEC lines. Average numbers of Ki-67+ tumor cells per 200x microscopic field were calculated by scoring five random fields of stained tissue sections of each mouse (n=5 mice per group)



2. HOXA9 expression is associated with a CAF-like gene signature in clinical specimens of EOC

A study by Tothill *et al.* classified tumors in the AOCS cohort into molecular subtypes according to their gene expression signatures and confirmed these subtypes by immunohistochemical analysis.[112] Of these tumor subtypes, the C1 subtype had the poorest outcome and was characterized by a desmoplastic or 'reactive' stromal gene signature.[112] Tumors that were classified as the C1 subtype constituted 39% of cases in the *HOXA9*-High group, but only 4% of the *HOXA9*-Low group in the AOCS cohort (Table 12). Conversely, the frequency of the C4 subtype (characterized by a low stromal response signature) was higher in the *HOXA9*-Low than *HOXA9*-High group (Table 12). CAFs are often characterized by their expression of α–smooth muscle actin (αSMA) and fibroblast activation protein (FAP).[81] Interestingly, expression of *ACTA2* (the gene encoding αSMA) and *FAP* was significantly higher in *HOXA9*-High cases than *HOXA9*-Low cases in the AOCS cohort (*ACTA2, P* = 0.026; *FAP*, $P = 10^{-7}$, Figure 21).

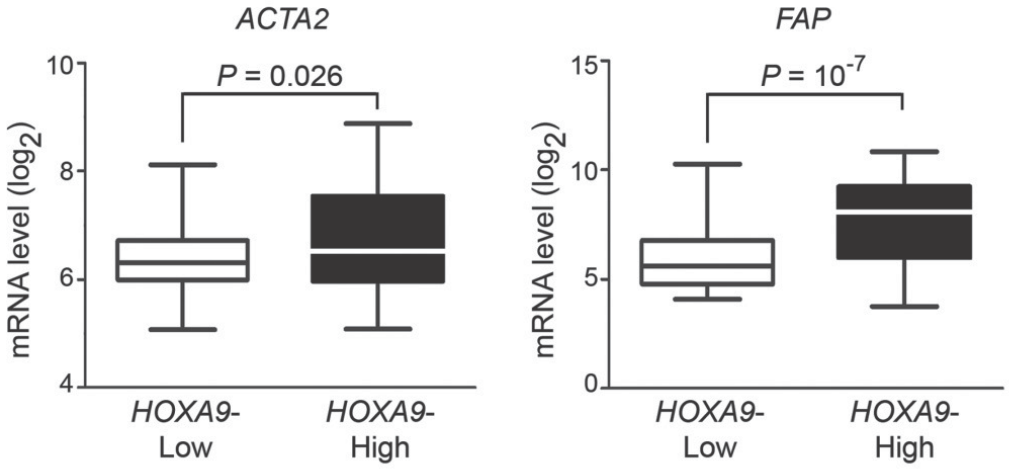
Table 12. Frequency of molecular subtypes classified by Tothill *et al.* among *HOXA9*-Low and *HOXA9*-High cases

Analyzed data from AOCS dataset[112]

Molecular subtype	*HOXA9*-Low cases	*HOXA9*-High cases
C1 (reactive stromal signature)	3/72 (4.2%)	28/72 (38.9%)
C4 (low stromal signature)	14/72 (19.4%)	8/72 (11.1%)

Figure 21. *HOXA9* expression is associated with increased expression of CAF markers in clinical specimens of EOC

Differences in transcript levels of *ACTA2* (the gene encoding αSMA) and *FAP* between *HOXA9*-Low and *HOXA9*-High cases in AOCS dataset. *P*-value was calculated by Mann-Whitney *U*-test. In box-and-whisker plots, horizontal bars indicate the medians, boxes indicate 25th to 75th percentiles, and whiskers indicate minimum and maximum values.



3. HOXA9 expression is associated with increased CAF abundance in mouse xenograft models of EOC

To confirm whether HOXA9 expression in EOC is associated with increased abundance of CAFs in tumors, αSMA expression was evaluated in mouse xenografts. Significantly lower numbers of αSMA+ cells were detected in tumors derived from HOXA9-knockdown SKOV3ip lines (shA9-A and shA9-B) than in tumors derived from +HOXA9 control (Empty vector and Nontargeting) SKOV3ip lines ($P < 0.0001$, Figures 22A and B). Identical results were obtained in tumors derived from ES-2 lines (Figure 22A). Conversely, abundant αSMA+ cells were detected in +HOXA9 MOSEC tumors but not in vector-control, +HOXA10 or +HOXA11 MOSEC tumors (Figure 23). These results suggest that the association between HOXA9 and poor survival could be linked to the ability of HOXA9 to promote a CAF-rich microenvironment.

Figure 22. Knockdown of HOXA9 reduces CAF abundance in mouse xenografts

(**A** and **B**) αSMA expression was evaluated in tumors of mice sacrificed at 20 days after inoculation with SKOV3ip and ES-2 lines. **(A)** The average number of αSMA+ cells per 1000 tumor cells was calculated by scoring 5 random fields of stained tissue sections of each mice (n=5 mice per group) **P*<0.0001. **(B)** Immunofluorescence staining of GFP-expressing tumor cells (green) and αSMA (red) in tumors of mice inoculated with SKOV3ip lines. Nuclei were visualized by staining with DAPI (blue). Scale bar, 100μm.

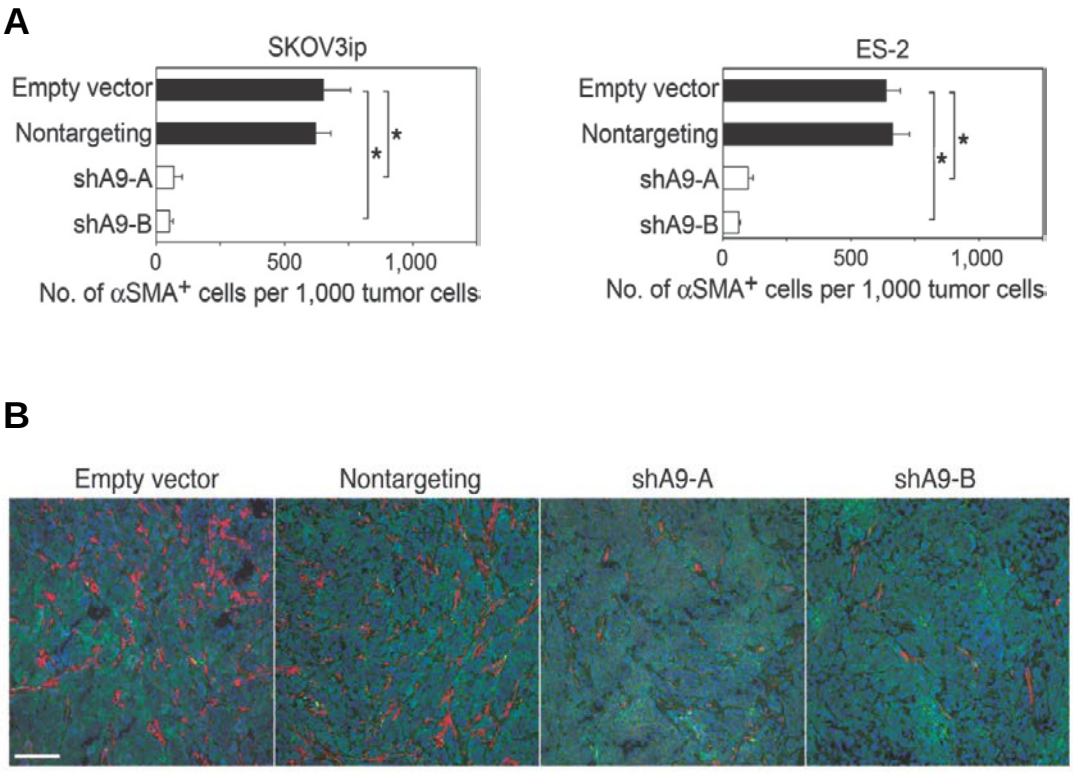
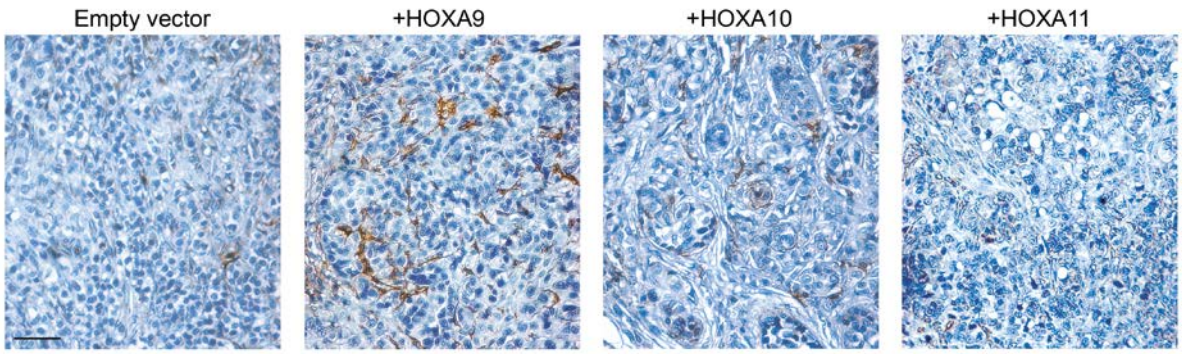


Figure 23. HOXA9, but not HOXA10 or HOXA11, increases CAF abundance in mouse xenografts

αSMA expression was evaluated in tumors of mice sacrificed at 2 months after inoculation with MOSEC lines. Scale bar, 50μm.



4. HOXA9 does not induce CAF-like features in EOC cells

A variety of different cell types have been identified as potential sources of CAFs (Figure 8).[82] Some CAFs can derive from tumor cells that have undergone EMT.[81] HOXA9 might therefore increase the abundance of CAFs in tumors by inducing tumor cells to undergo EMT. To test this possibility, I evaluated whether HOXA9 induces EOC cells to undergo EMT. The expression level of transcription factors that orchestrate EMT, such as *SNAI1* (the gene encoding Snail) and *SNAI2* (the gene encoding Slug), was not altered by HOXA9 in EOC cells *in vitro* or *in vivo* (Figures 24A and B). Virtually no overlap was observed in xenografts between +HOXA9 EOC cells and αSMA-expressing cells (Figure 25), indicating that αSMA+ cells did not originate from tumor cells. These findings indicate that the increased abundance of CAFs in +HOXA9 tumors is unlikely to be due to the trans-differentiation of tumor cells into CAF-like cells.

Figure 24. Effect of HOXA9 on expression of EMT-associated genes

(A) Relative mRNA levels of each indicated gene were assayed by qRT-PCR using human-specific primers in cultured +HOXA9 control (Nontargeting) and HOXA9-Knockdown (shA9-B) SKOV3ip lines, and in omental tumors collected from mice that were inoculated with SKOV3ip lines (n=5 mice per group). **(B)** The ability of human- and mouse-specific primers to discriminate expression of human (tumor) and mouse (stromal) genes was tested using mixtures of RNAs isolated from human EOC cells (SKOV3ip cells) and mouse fibroblasts (L929 cells) at different ratios. Shown is heat map analysis of expression (generated by Tree View software) where the intensity of red color represents the magnitude of expression (log2 scale).

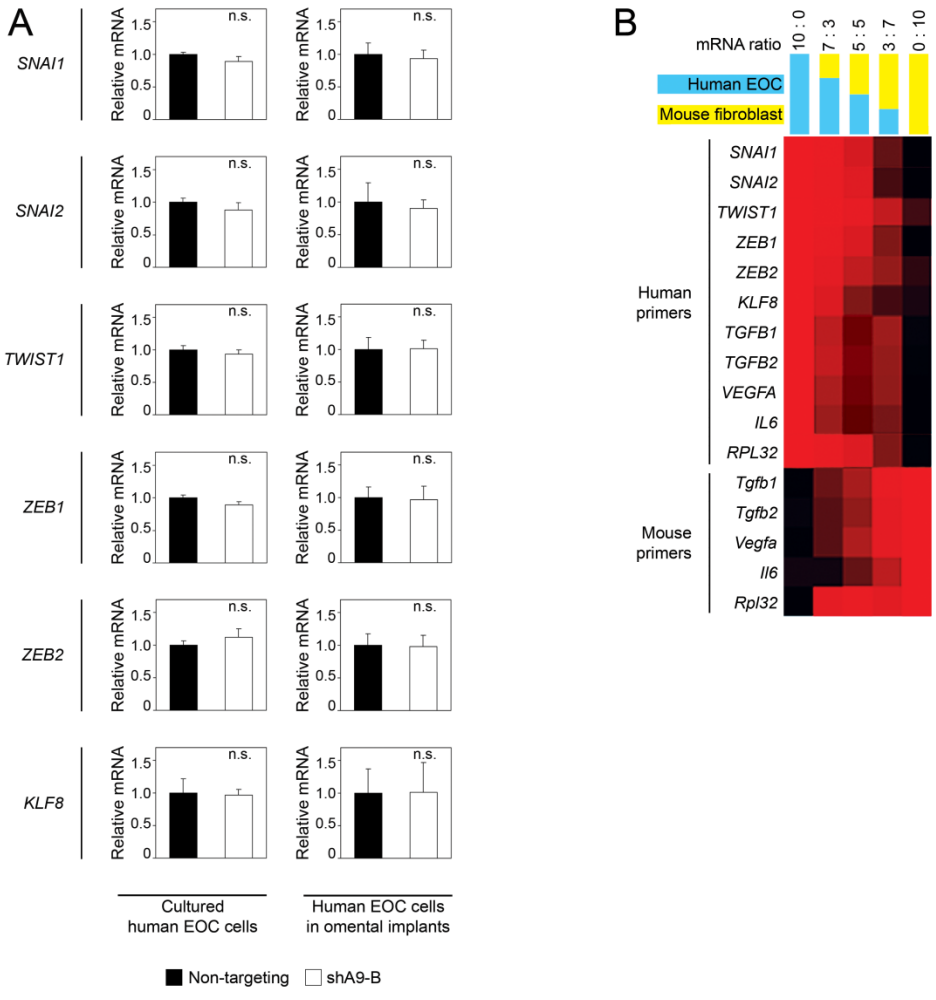
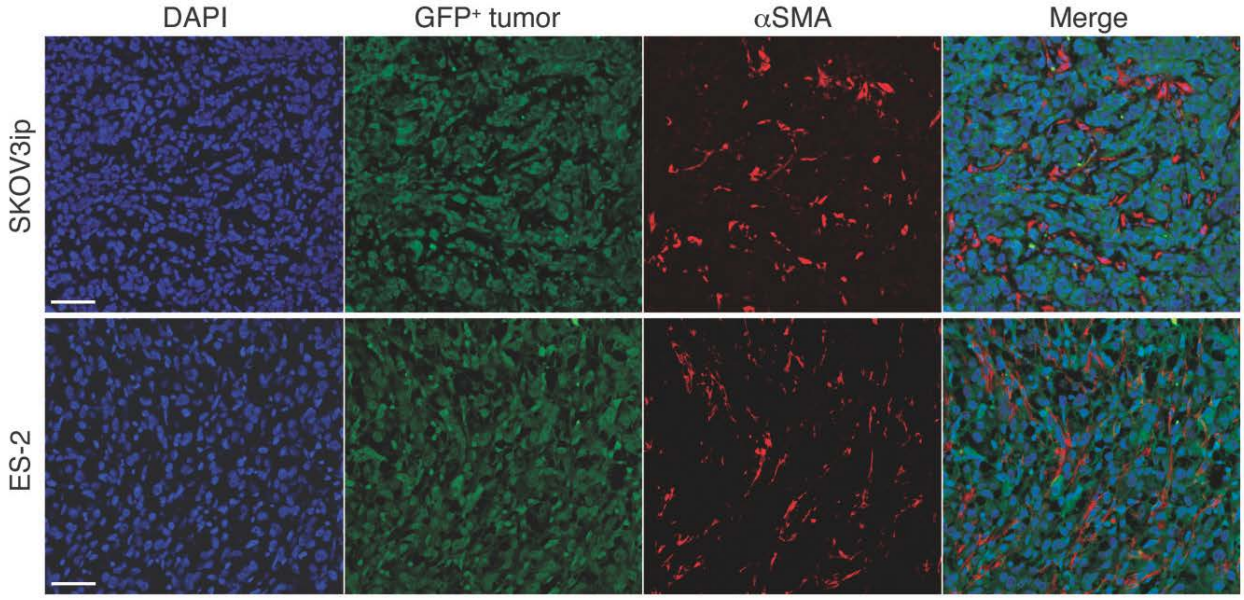


Figure 25. αSMA+ cells in mouse xenografts do not derive from tumor cells

Immunofluorescence staining of GFP-expressing tumor cells (green) and αSMA (red) in tumors of mice inoculated with +HOXA9 control (Nontargeting) SKOV3ip and ES-2 lines. Nuclei were visualized by staining with DAPI (blue). Scale bar, 50μm.



5. HOXA9 expression in EOC cells induces omental fibroblasts to acquire CAF features

Another important source of CAFs is normal tissue-resident fibroblasts.[88] Because EOC frequently involves the omentum, I investigated whether HOXA9 expression in EOC cells increases the abundance of CAFs by 1) increasing the numbers of CAF precursor cells and 2) inducing normal omental fibroblasts to acquire features of CAFs.

5.1. HOXA9 expression in EOC cells stimulates the expression of CAF markers in normal omental fibroblasts

I initially evaluated whether HOXA9 expression in EOC cells increases proliferation of tissue-resident fibroblasts. Co-culture assays revealed that HOXA9 expression in EOC cells did not affect proliferation of normal omental fibroblasts (Figure 26). In subsequent experiments, I evaluated whether HOXA9 increases the abundance of CAFs by inducing normal omental fibroblasts to acquire CAF features. To accomplish this, normal omental fibroblasts were cultured in medium that had been conditioned by EOC cells that lacked or expressed HOXA9 (Figure 27). *ACTA2* (the gene encoding αSMA) and αSMA protein levels were strongly induced in fibroblasts following incubation (i.e. 'priming') in medium conditioned by +HOXA9 control SKOV3ip cells but not in medium conditioned by HOXA9-knockdown SKOV3ip cells (Figures 28A and B). *FAP* was also more highly induced in fibroblasts following priming in medium conditioned by control SKOV3ip cells than by HOXA9-knockdown SKOV3ip cells (Figure 28A). To confirm these findings, I evaluated omental fibroblasts following priming in medium conditioned by MOSEC cells. Expression of αSMA and FAP was more highly induced in fibroblasts following priming in medium

conditioned by +HOXA9 MOSEC cells than by parental or vector-control MOSEC cells. However, increased induction was not observed when fibroblasts were primed in media conditioned by +HOXA10 or +HOXA11 MOSEC cells (Figures 29A and B).

Figure 26. Effect of HOXA9 expression in EOC cells on growth rates of fibroblasts

Normal human omental fibroblasts (1,000 per well) were seeded with cells of the indicated GFP-expressing +HOXA9 control and HOXA9-knockdown SKOV3ip lines (1,000 per well) in 96-well plates. Numbers of fibroblasts (GFP-negative cells) per well were counted daily over a 5 day time course.

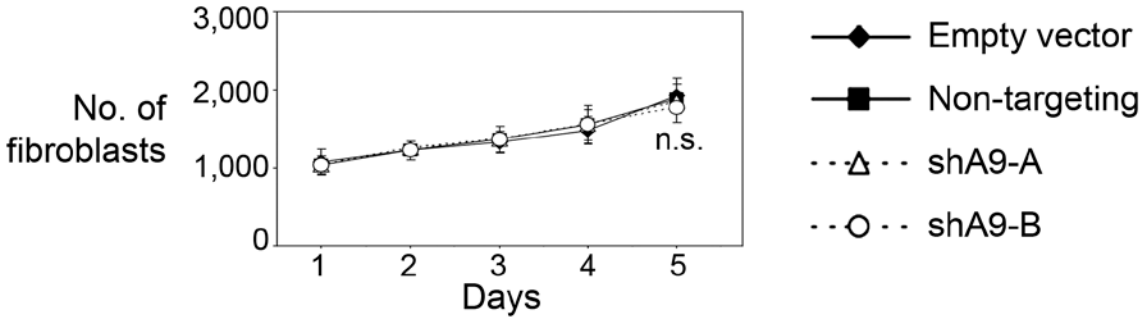


Figure 27. Diagram of fibroblast-priming assays

+HOXA9 control and HOXA9-knockdown SKOV3ip cells were cultured for 2 days to generate tumor-conditioned media (shown in pink). Normal omental fibroblasts were incubated for 5 days in SKOV3ip-conditioned medium (i.e., primed) or nonconditioned medium (i.e., unprimed) and then analyzed by western blot and qRT-PCR. Fresh nonconditioned medium was added to washed fibroblasts. At 2 days thereafter, medium conditioned by fibroblasts (shown in light blue) was analyzed by ELISA and used for incubating control (nontargeting) SKOV3ip cells.

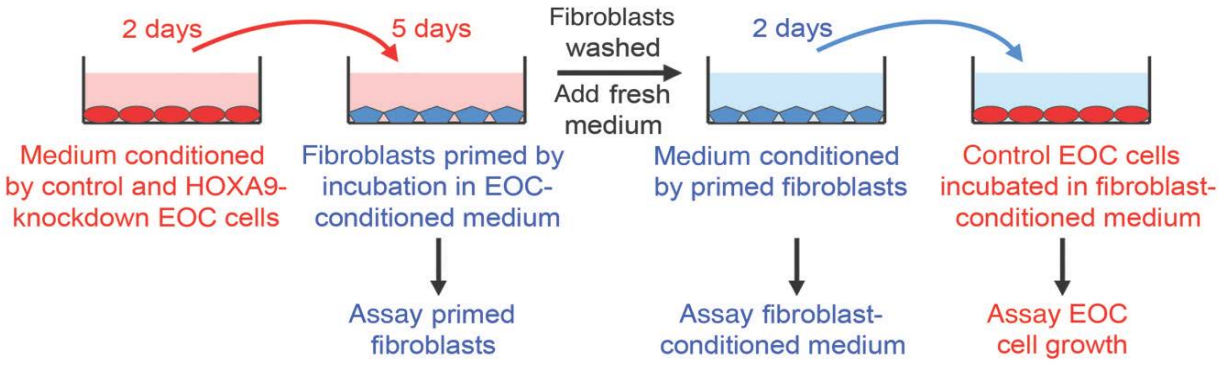


Figure 28. HOXA9 expression in EOC cells induces expression of CAF markers in normal omental fibroblasts

The expression levels of CAF markers were assayed in omental fibroblasts at 5 days after priming in media conditioned by +HOXA9 control and HOXA9-knockdown SKOV3ip cells. **(A)** qRT-PCR analysis of CAF markers. The mRNA level of each gene is expressed relative to its level in unprimed fibroblasts (i.e. incubated in nonconditioned medium). **(B)** Western blot of αSMA levels.

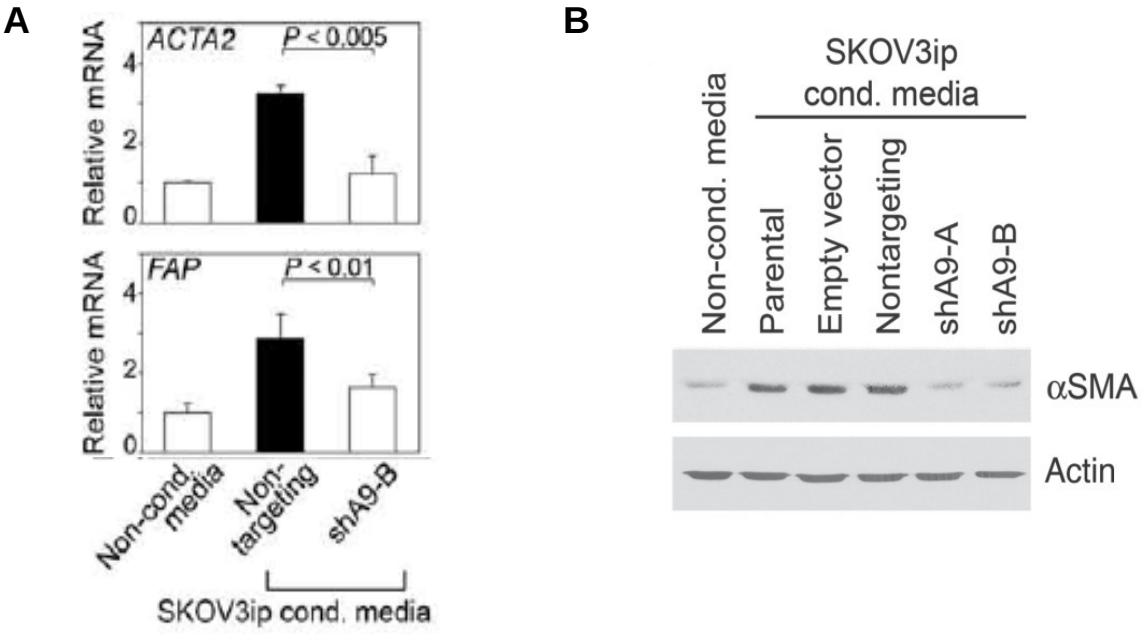
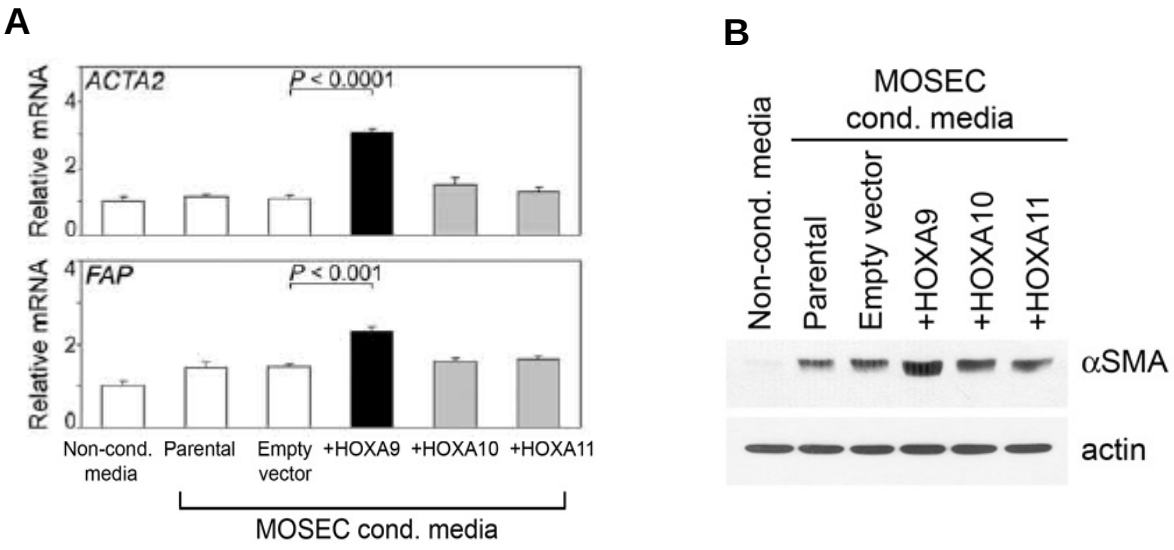


Figure 29. HOXA9, but not HOXA10 and HOXA11, induces expression of CAF markers in normal omental fibroblasts

The expression levels of CAF markers were assayed in omental fibroblasts at 5 days after priming in media conditioned by control and HOX-expressing MOSEC cells **(A)** qRT-PCR analysis of CAF markers. The mRNA level of each gene is expressed relative to its level in unprimed fibroblasts (i.e. incubated in nonconditioned medium). **(B)** Western blot of αSMA levels.



5.2. HOXA9 expression in EOC cells promotes the ability of fibroblasts to stimulate growth of EOC cells

CAFs promote tumor growth by expressing several mitogenic factors for tumor cells such as CXCL12 and IL-6.[88, 89] In subsequent experiments, I evaluated whether HOXA9 expression in EOC cells induces normal omental fibroblasts to acquire both molecular and functional features of CAFs. Both mRNA and protein levels of CXCL12 and IL-6 were highly induced in omental fibroblasts, following priming in medium conditioned by +HOXA9 control SKOV3ip cells, but not in medium conditioned by HOXA9-knockdown SKOV3ip cells (Figures 30A and B). Fibroblasts that had been primed by control SKOV3ip cells were more effective in stimulating tumor cell proliferation than fibroblasts that had been primed by HOXA9-knockdown SKOV3ip cells ($P < 0.001$, Figure 31A). The ability of fibroblasts primed by +HOXA9 tumor-conditioned medium to stimulate tumor cell proliferation was blocked when fibroblast-conditioned medium was depleted of CXCL12 and IL-6 (Figure 31B). These findings indicate that HOXA9 induces tumor-derived factors that stimulate normal fibroblasts to acquire CAF features and to express CXCL12 and IL-6 that act in a paracrine manner to promote tumor cell proliferation.

These findings were also confirmed in omental fibroblasts that were primed in medium conditioned by MOSEC cells. CXCL12 and IL-6 expression was more highly induced in fibroblasts following priming in medium conditioned by +HOXA9 MOSEC cells than by parental or vector-control MOSEC cells. In contrast, fibroblasts that were primed in media conditioned by +HOXA10 or +HOXA11 MOSEC cells showed no significant induction in CXCL12 or IL-6 expression (Figures 32A and B). Tumor cell proliferation was more highly stimulated by fibroblasts that had been primed in medium conditioned by +HOXA9 MOSEC cells than by control MOSEC cells ($P < 0.001$), whereas priming of fibroblasts in media

conditioned by +HOXA10 or +HOXA11 MOSEC cells had little stimulatory effect (Figure 32C). Again, these findings indicate that HOXA9 induces normal fibroblasts to acquire CAF features that stimulate tumor cell proliferation, and that this capability is not shared by HOXA10 or HOXA11.

Figure 30. HOXA9 expression in EOC cells induces expression of growth factors in normal omental fibroblasts

Levels of growth factors in **(A)** unprimed and primed fibroblasts, and **(B)** media conditioned by unprimed and primed fibroblasts. **(A)** qRT-PCR analysis of growth factors. The mRNA level of each gene is expressed relative to its level in unprimed fibroblasts (i.e. incubated in nonconditioned medium). **(B)** ELISA analysis to detect growth factors secreted by unprimed and primed fibroblasts. *P<0.001; #P<0.005.

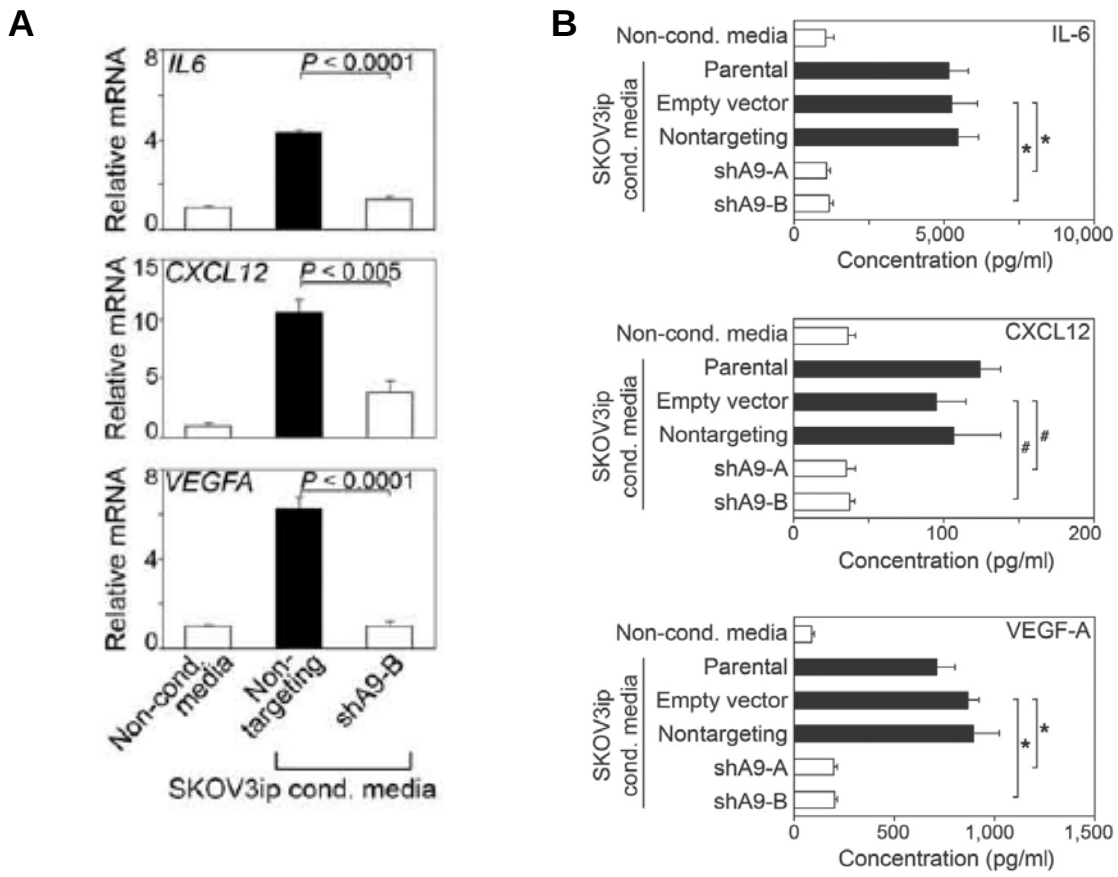


Figure 31. HOXA9 expression in EOC cells promotes the ability of fibroblasts to stimulate growth of EOC cells

(A) Growth rates of control (Nontargeting) SKOV3ip cells incubated in fibroblast-conditioned medium. EOC growth was measured by MTT assay. **(B)** Relative growth of control SKOV3ip cells at 6 days after incubation in medium conditioned by fibroblasts that were initially primed in +HOXA9 control SKOV3ip-conditioned medium, where fibroblast-conditioned medium was left untreated, treated with normal IgG, or depleted by immunoprecipitation with antibodies to CXCL12 and IL-6. **P*<0.001

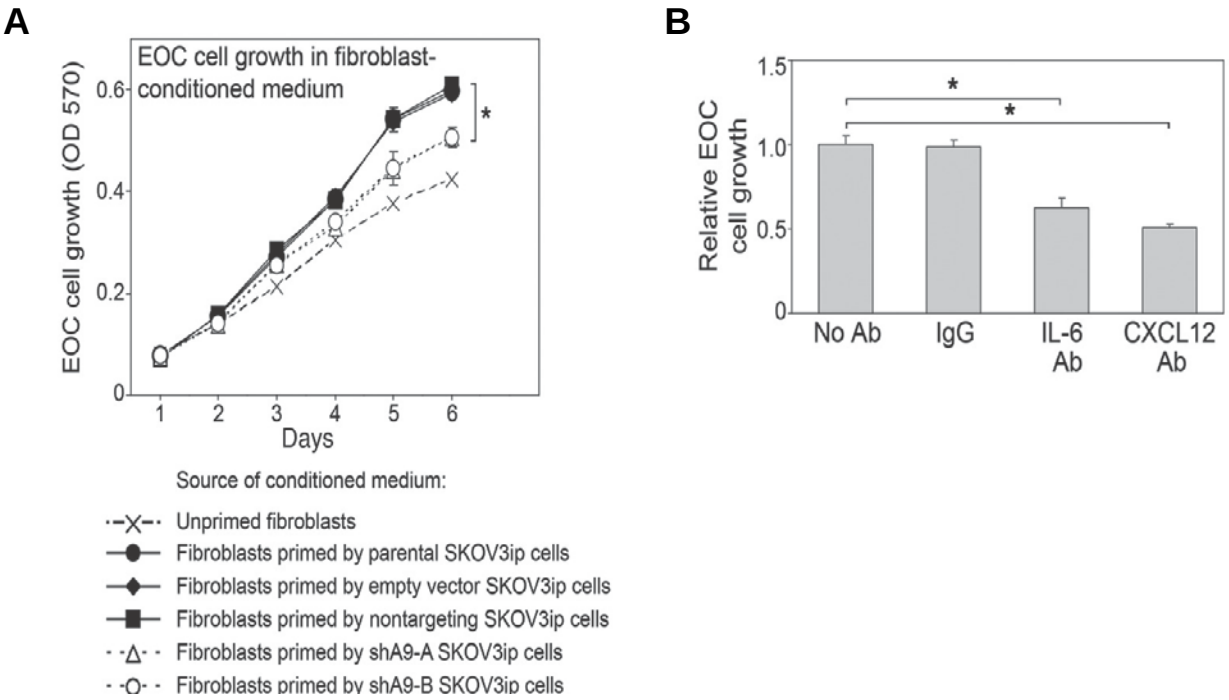
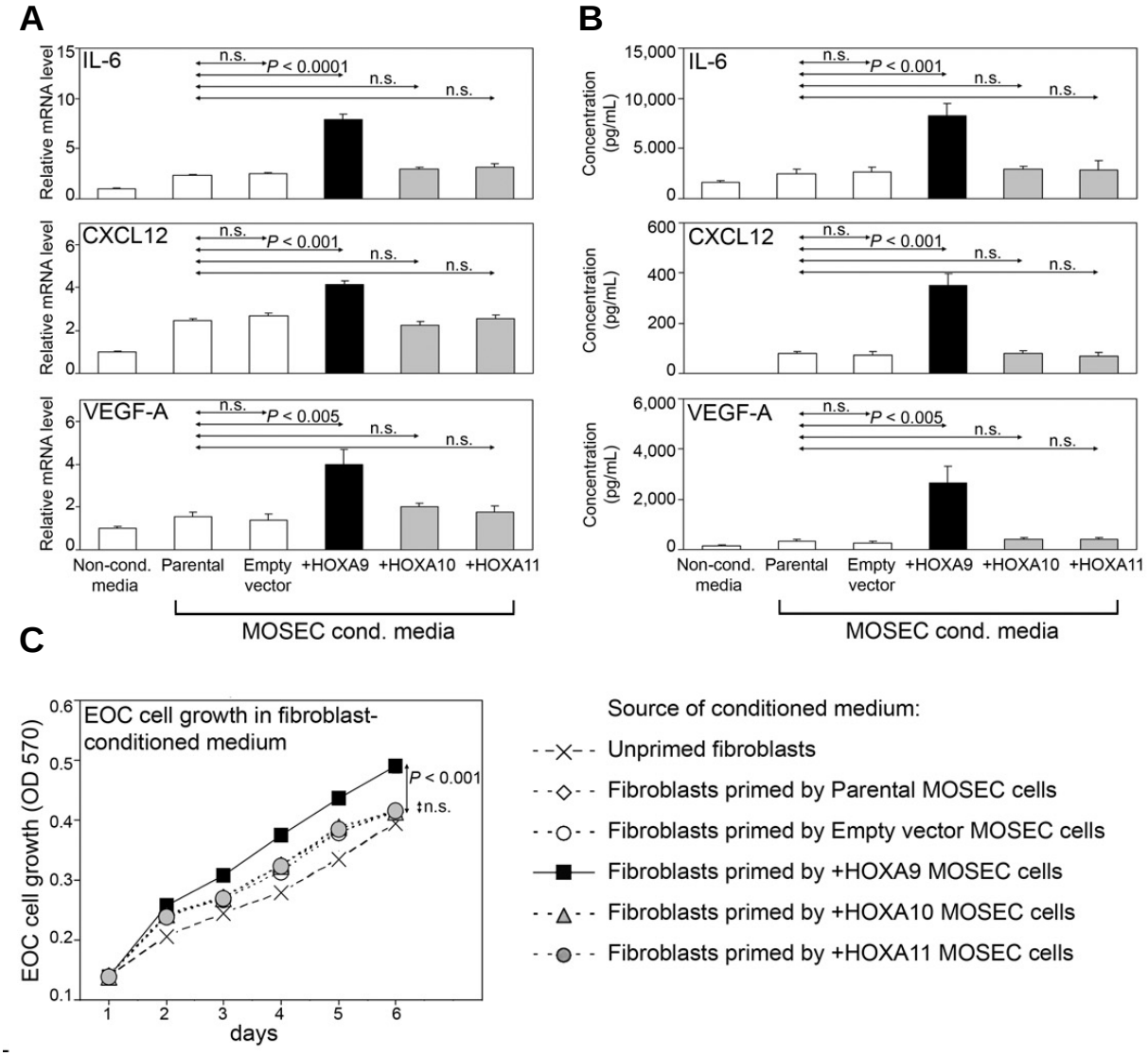


Figure 32. HOXA9, but not HOXA10 and HOXA11, promotes the ability of fibroblasts to stimulate growth of EOC cells

(A) qRT-PCR analysis of growth factors in unprimed and primed fibroblasts that were primed by MOSEC-conditioned medium. The mRNA level of each gene is expressed relative to its level in unprimed fibroblasts. **(B)** ELISA analysis to detect growth factors secreted by unprimed and primed fibroblasts. **(C)** Growth rates of control SKOV3ip cells incubated in medium conditioned by fibroblasts that were primed by MOSEC-conditioned medium or left unprimed.



5.3. HOXA9 expression in EOC cells promotes the ability of fibroblasts to stimulate endothelial cell growth

CAFs promote not only the growth of tumor cells, but also the growth of endothelial cells.[102, 103] CAF abundance in clinical specimens of EOC has been found to strongly correlate with increased microvessel density.[113] In subsequent experiments, I investigated the effect of HOXA9 expression in EOC cells on endothelial cell growth. Firstly, I evaluated microvessel density in mouse xenografts. HOXA9-knockdown SKOV3ip tumors had significantly lower microvessel density than +HOXA9 control SKOV3ip tumors ($P < 0.005$, Figure 33A). However, expression of the genes encoding angiogenic factors IL-6 and VEGF-A in SKOV3ip cells was not altered by HOXA9 *in vitro* or *in vivo* (Figure 33B). On the other hand, IL-6 and VEGF-A expression was highly induced in fibroblasts following priming in medium conditioned by control SKOV3ip cells but not by HOXA9-knockdown SKOV3ip cells (Figures 30A and B). Expression of mouse host cell-derived IL-6 and VEGF-A was significantly higher in omental tumors of control SKOV3ip models than in HOXA9-knockdown tumors (IL-6, $P = 0.03$; VEGF-A, $P = 0.007$; Figure 33B). These data suggest that HOXA9 expression in EOC cells does not directly promote the growth of endothelial cells, but rather activates stromal cells to stimulate endothelial cell growth.

To further evaluate this possibility, growth of endothelial cells was firstly assayed in medium that was conditioned by EOC cells that lacked or expressed HOXA9. Endothelial cells grew at identical rates when cultured in medium conditioned by HOXA9-knockdown or by control SKOV3ip cells (Figure 34A). Secondly, growth of endothelial cells was assayed in medium that was conditioned by fibroblasts that had been primed by EOC cells that lacked or expressed HOXA9. Endothelial cell growth was more highly stimulated by omental fibroblast-derived factors where fibroblasts had been primed in medium

conditioned by control SKOV3ip cells than by HOXA9-knockdown SKOV3ip cells ($P < 0.005$, Figure 34B). The ability of medium conditioned by fibroblasts that were primed by HOXA9-knockdown SKOV3ip cells to stimulate endothelial cell growth was restored when this conditioned medium was reconstituted with IL-6 and VEGF-A at concentrations released by fibroblasts that were primed by +HOXA9 control SKOV3ip cells (Figure 35). These findings strongly suggest that HOXA9 expression in EOC cells promotes tumor microvessel formation via inducing normal omental fibroblasts to express IL-6 and VEGF-A.

These results were confirmed in MOSEC xenografts. As compared to vector-control tumors, microvessel density was significantly higher in +HOXA9 tumors ($P < 0.005$) but not in +HOXA10 or +HOXA11 tumors (Figure 36A). Expression of HOXA9, but not HOXA10 or HOXA11, in MOSEC cells stimulated omental fibroblasts to express IL-6 and VEGF-A (Figures 32A and B) and to promote endothelial cell growth (Figure 36B). These results demonstrated that ability of HOXA9 to stimulate endothelial cell growth is not shared by other Müllerian *HOX* genes.

Figure 33. HOXA9 expression in EOC cells is associated with increased microvessel density

(A) The average number of microvessels per 10^4 tumor cells was calculated in tumors derived from +HOXA9 control (Nontargeting) and HOXA9-knockdown (shA9-B) SKOV3ip lines by scoring five random fields of CD34-stained tissue sections of each mice (n=5 per group). **P*<0.005. **(B)** Relative mRNA levels of *IL6* and *VEGFA* in cultured SKOV3ip cells and those of *IL6, VEGFA* (in human EOC cells) and *Il6* and *Vegfa* (in mouse host cells) in omental tumors of mice that were inoculated with SKOV3ip lines (n=5 mice per group). **P*=0.03; †*P*=0.007. *P* values>0.05 were considered not significant.

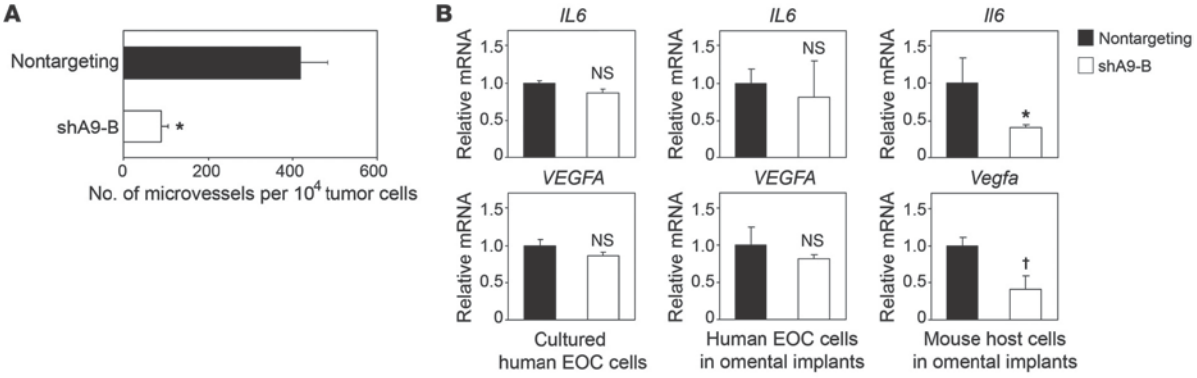
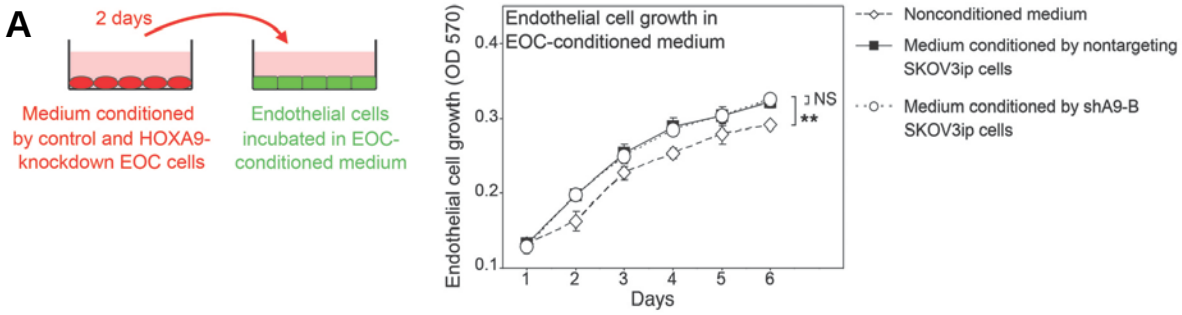


Figure 34. HOXA9 expression in EOC cells promotes the ability of fibroblasts to stimulate endothelial cell growth

(A) Growth rates of mouse endothelial cells incubated in nonconditioned medium and in SKOV3ip-conditioned media. ***P* < 0.005. **(B)** Normal omental fibroblasts were left unprimed or primed with SKOV3ip-conditioned media (shown in pink) for 5 days. Fresh nonconditioned medium was added to washed fibroblasts. Two days thereafter, medium conditioned by fibroblasts (shown in light blue) was collected. Growth rates of endothelial cells incubated in fibroblast-conditioned medium were measured. **P* < 0.005.



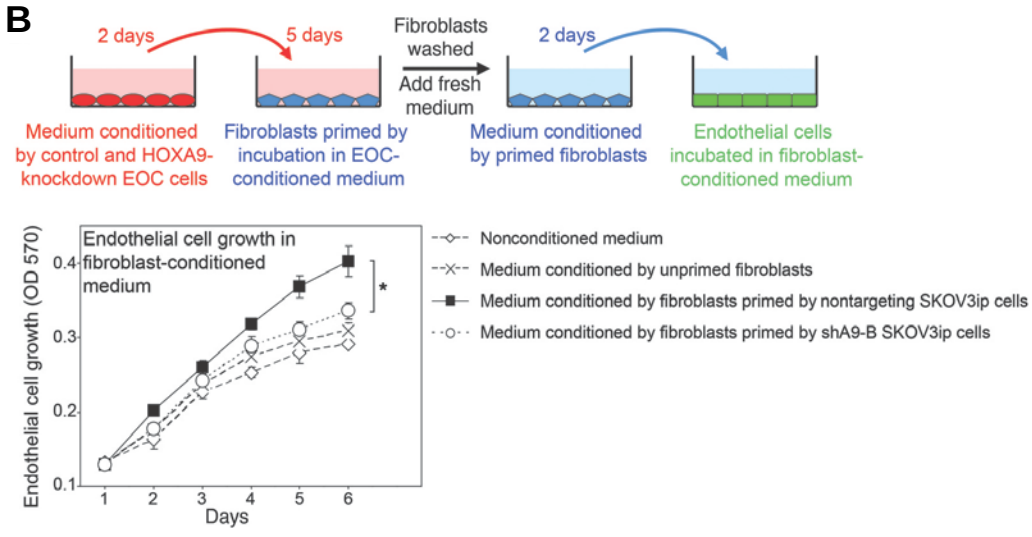


Figure 35. HOXA9 expression in EOC cells promotes endothelial cell growth by inducing normal omental fibroblasts to express IL-6 and VEGF-A

Growth rates of mouse endothelial cells cultured in medium conditioned by omental fibroblasts that were primed by +HOXA9 control (Nontargeting) and HOXA9-knockdown (shA9-B) SKOV3ip cells. Where indicated, recombinant IL-6 and VEGF was added to medium conditioned by fibroblasts that were primed by shA9-B SKOV3ip cells to achieve final concentrations of these growth factors at the same levels as detected in medium conditioned by fibroblasts primed by Nontargeting SKOV3ip cells (5000pg/ml for IL-6, 800pg/ml for VEGF, see Figure 30B).

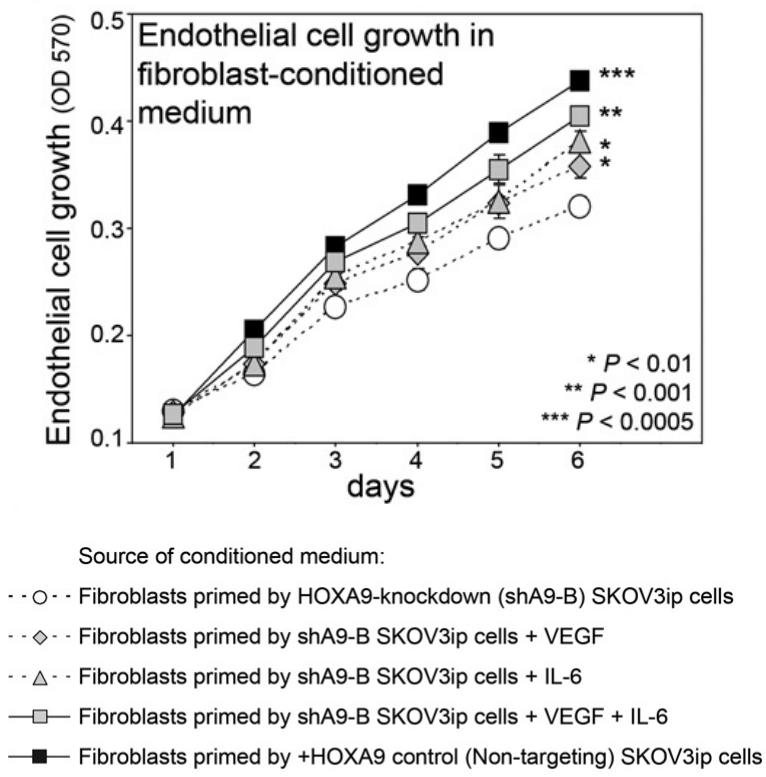
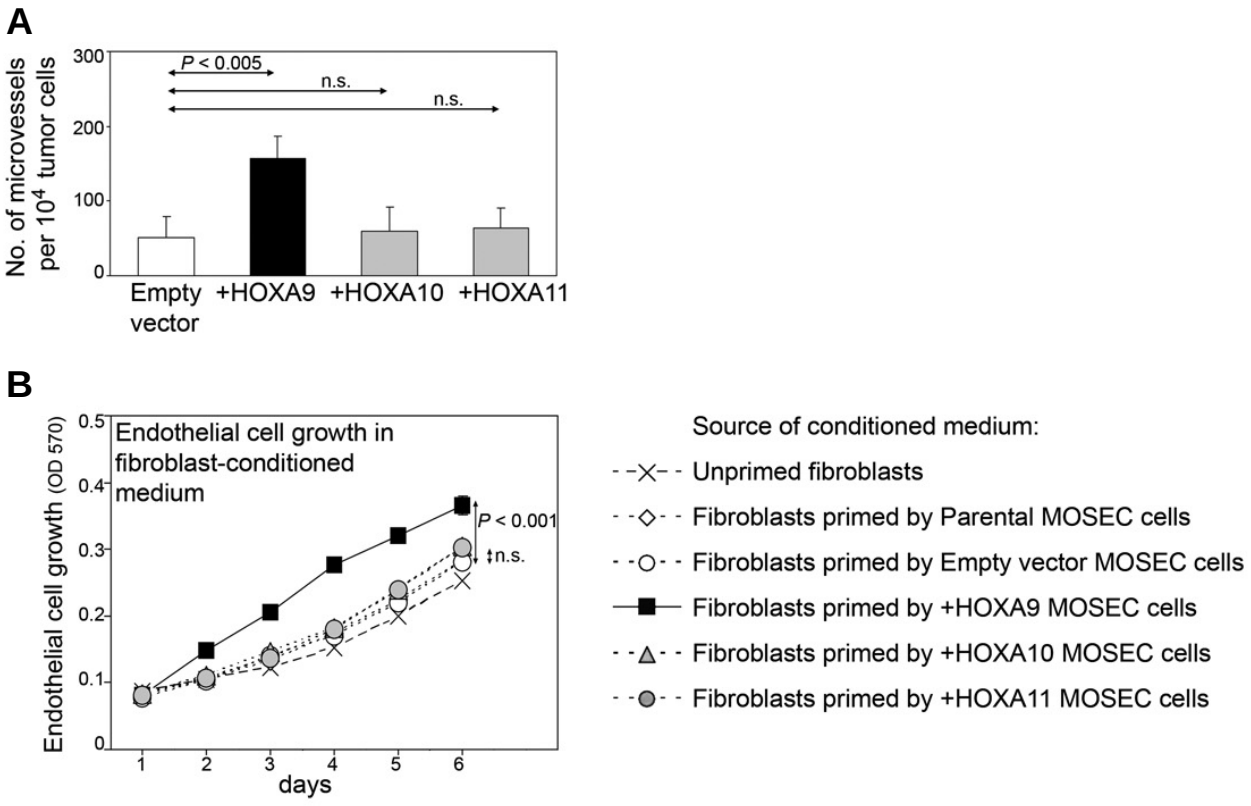


Figure 36. HOXA9, but not HOXA10 and HOXA11, promotes the ability of fibroblasts to stimulate endothelial cell growth

(A) Average numbers of microvessels were calculated in MOSEC-derived tumors by scoring five random fields of CD34-stained tissue sections of each mice (n=5 mice per group). **(B)** Growth rates of mouse endothelial cells cultured in medium conditioned by omental fibroblasts that were primed by MOSEC-conditioned medium or left unprimed. Endothelial cell growth was measured by MTT assays.

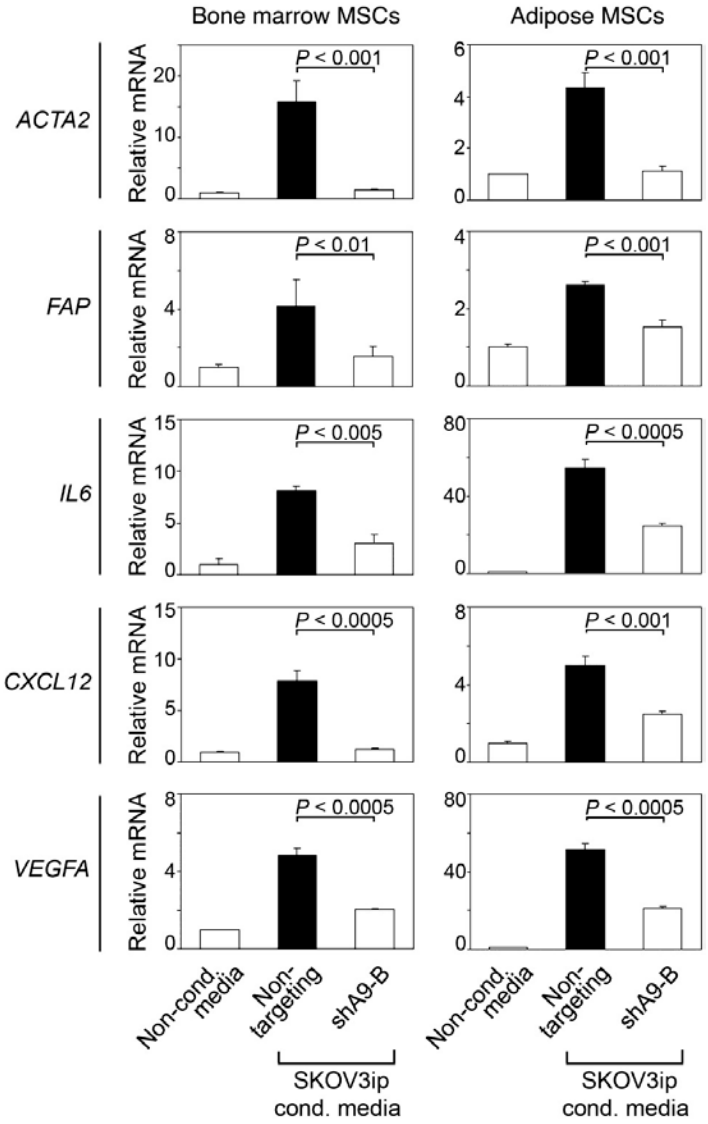


6. HOXA9 expression in EOC cells induces mesenchymal stem cells to acquire CAF features

Mesenchymal stem cells (MSCs) have been studied as a source of CAFs in many types of tumors including EOC (Figure 8).[87, 89, 114] Bone marrow is the most well-characterized source of MSCs, but MSCs also reside in most tissues and are abundant in white adipose tissues.[115, 116] Because EOCs often colonize peritoneal surfaces that overlie connective and adipose tissue, I investigated whether HOXA9 expression in EOC cells induces CAF features in normal bone marrow-derived MSCs and adipose-derived MSCs. As observed in omental fibroblasts*, ACTA2, FAP, IL-6, CXCL12 and VEGFA* expression was highly induced in normal bone marrow- and adipose-derived MSCs following incubation in medium conditioned by +HOXA9 control but not by HOXA9-knockdown SKOV3ip cells (Figure 37). These results suggest that HOXA9 induces tumor-derived factors that stimulate acquisition of CAF features in MSCs as well as in omental fibroblasts.

Figure 37. HOXA9 expression in EOC cells induces CAF features in bone marrow- and adipose-derived MSCs

Levels of *ACTA2, FAP, IL6, CXCL12, and VEGFA* transcripts were assayed in bone marrow MSCs and adipose MSCs at 5 days after incubation in media conditioned by +HOXA9 control (Nontargeting) and HOXA9-knockdown (shA9-B) SKOV3ip cells. The mRNA level of each gene, assayed by qRT-PCR, is expressed relative to its level in MSCs incubated in nonconditioned medium.



C. CONCLUSION

The studies in this chapter provide significant insight into the nature of the cellular process by which HOXA9 promotes EOC growth. Firstly, I demonstrated that HOXA9 did not stimulate autonomous tumor cell growth *in vitro*. On the other hand, HOXA9 expression in EOC cells induced normal omental fibroblasts to express CAF markers. HOXA9 expression in EOC cells also promoted growth of EOC cells and endothelial cells by inducing normal omental fibroblasts to express IL-6, CXCL12, and VEGF-A. My studies also demonstrated that this capability of HOXA9 is not shared by HOXA10 or HOXA11. Similarly, HOXA9 expression in EOC cells induced normal adipose- and bone marrow-derived MSCs to acquire CAF features. Together, my studies raise the possibility that HOXA9 controls expression of a secreted factor(s) that is released by EOC cells and acts in a paracrine manner on normal tissue-resident fibroblasts and MSCs. The molecular mechanisms of HOXA9 in this cellular process will be discussed in Chapter 5.

CHAPTER 5: EFFECTS OF HOXA9 ON EOC GROWTH ARE MEDIATED VIA ITS INDUCTION OF TUMOR-DERIVED TGF-β2

A. RATIONALE

Whereas normal stromal cells tightly restrict the outgrowth of epithelial cells, it has been demonstrated that CAFs promote the growth of various cancers, including pancreatic, breast and gastric cancers.[87-89] However, the molecular mechanisms that mediate the transition of normal fibroblasts into CAFs are not fully understood.

In Chapters 3 and 4, I demonstrated that HOXA9 expression is associated with poor survival in EOC patients and in mouse xenograft models of EOC. The ability of HOXA9 to stimulate EOC growth was found to be primarily due to increased abundance of CAFs in tumors. Specifically, it was found that HOXA9 expression in EOC cells induced CAF features in normal omental fibroblasts and MSCs. The goal of the studies in this chapter is to determine the molecular mechanisms by which HOXA9 induces CAF features in normal fibroblasts and MSCs.

B. RESULTS

1. HOXA9 induces expression of TGF-β2 in EOC cells

Tumor cells express many growth factors that can stimulate fibroblasts.[117-120] To determine the molecular mechanism by which HOXA9 induces CAF features in normal fibroblasts, I initially evaluated the effect of HOXA9 on expression of fibroblast-stimulatory factors in SKOV3ip cells. HOXA9 did not alter expression of genes encoding several fibroblast-stimulatory factors such as TGF-β1, which is known to induce transition of normal tissue-resident fibroblasts and MSCs into CAFs (Figures 38 and 39).[89, 121] However, knockdown of HOXA9 significantly down-regulated levels of *TGF-β2* mRNA and secreted, activated TGF-β2 protein ($P < 0.005$, Figures 39A and B). Conversely, enforced expression of HOXA9, but not HOXA10 or HOXA11, induced TGF-β2 mRNA and protein levels in MOSEC cells (Figures 40A and B). In addition, levels of *HOXA9* transcripts significantly correlated with *TGFB2* transcript levels in EOC tissue specimens ($R = 0.65$, $P = 0.00004$), but no significant correlation was observed for *TGFB1* ($R = 0.11$, $P = 0.54$) (Figure 41). High HOXA9 protein levels were also associated with high TGF-β2 protein levels in clinical specimens of omental implants of EOC patients (Figures 42A and B). These findings strongly raise the possibility that HOXA9 controls TGF-β2 expression in EOC cells.

Figure 38. HOXA9 do not alter expression of genes encoding several fibroblast-stimulatory factors

Relative levels of transcripts encoding the indicated growth factors in +HOXA9 control (Nontargeting) and HOXA9-knockdown (shA9-B) SKOV3ip lines.

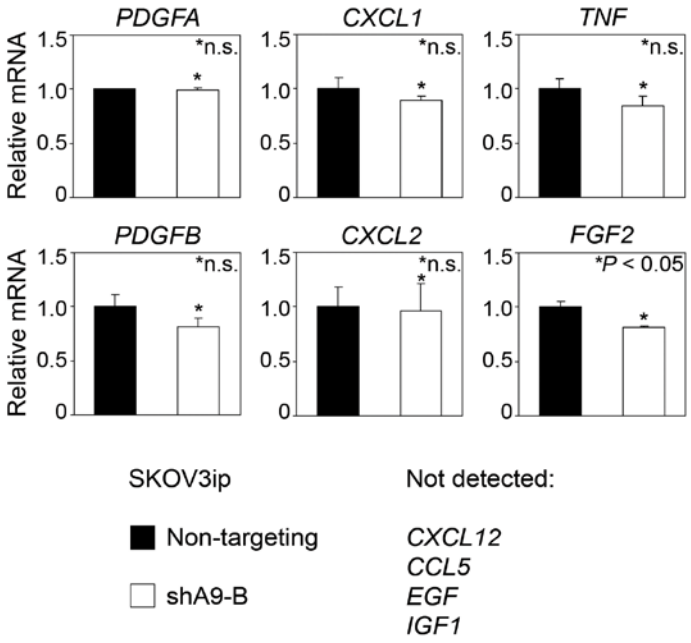


Figure 39. Knockdown of HOXA9 suppresses TGF-β2 expression in EOC cells.

(A) Relative *TGFB1* and *TGFB2* mRNA levels in SKOV3ip lines. **P* < 0.001; ***P* < 0.005.

(B) TGF-β1 and TGF-β2 levels in media conditioned by SKOV3ip lines. **P* < 0.001.

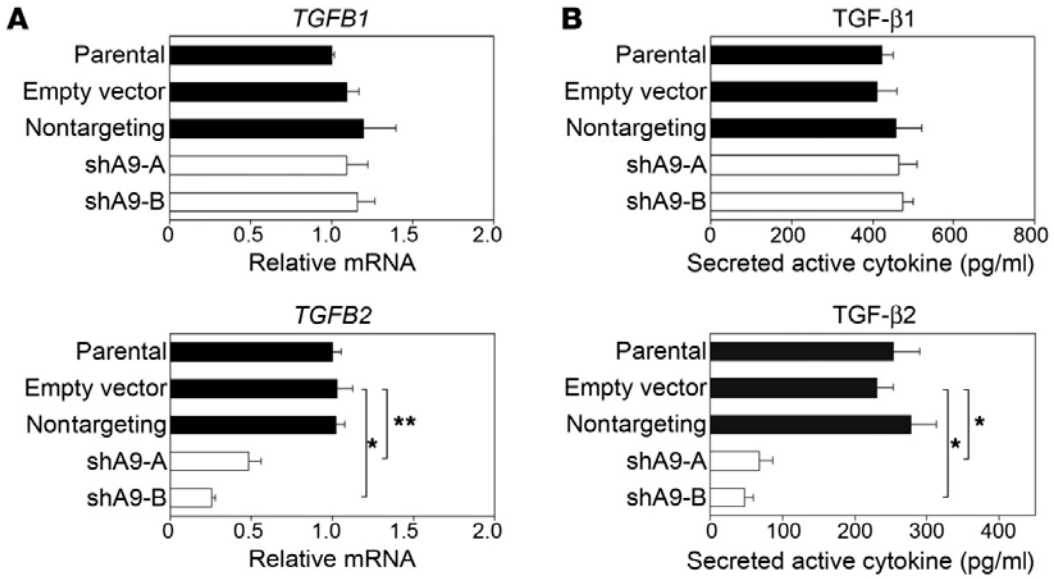


Figure 40. HOXA9, but not HOXA10 and HOXA11, induces TGF-β2 expression in MOSEC cells

(A) Relative level of *Tgfb1* and *Tgfb2* mRNAs in MOSEC cells. **(B)** Levels of TGF-β2 in media conditioned by MOSEC lines.

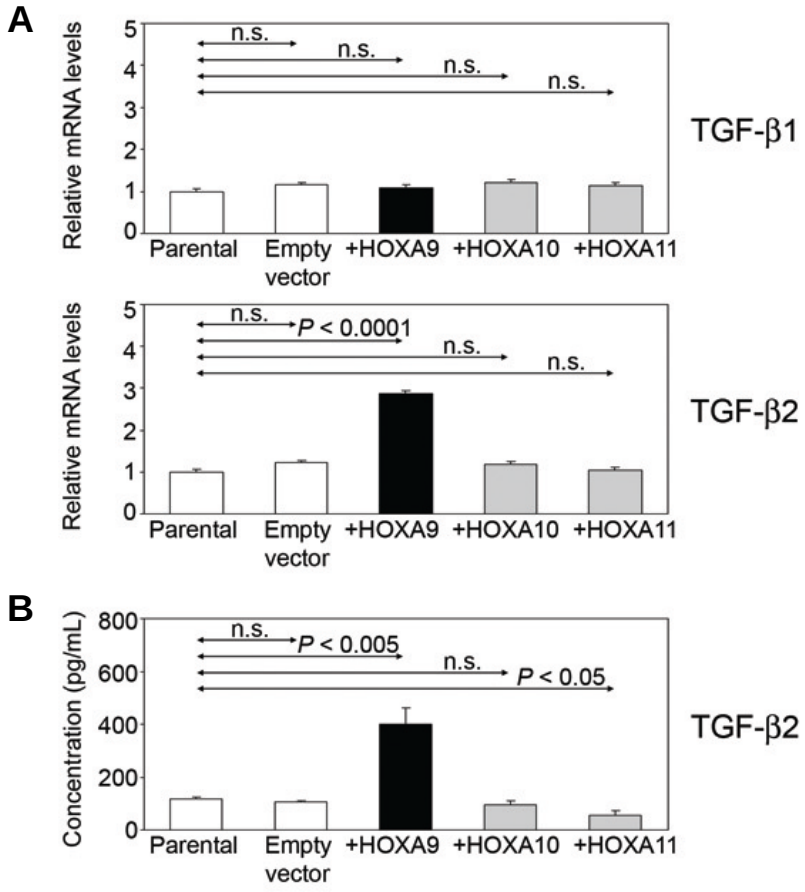


Figure 41. Levels of *HOXA9* transcripts significantly correlate with *TGFB2* transcript levels in EOC tissue specimens

Relative *HOXA9, TGFB1,* and *TGFB2* mRNA levels in primary ovarian tumors of 33 cases. Correlations were determined by Spearman test.

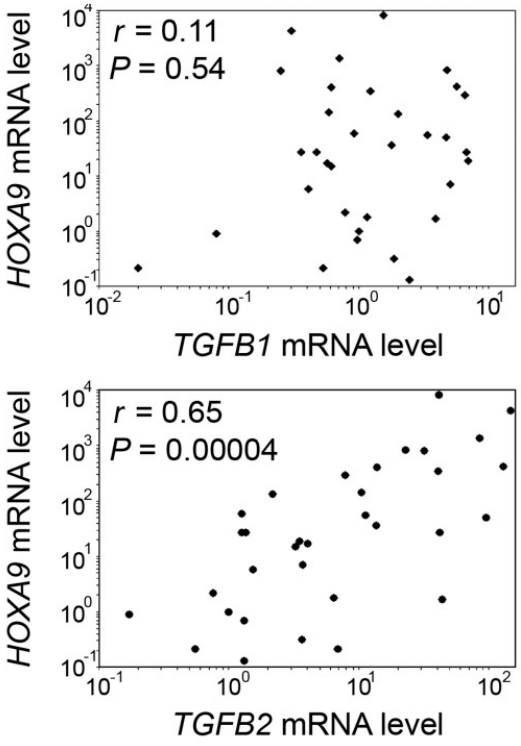
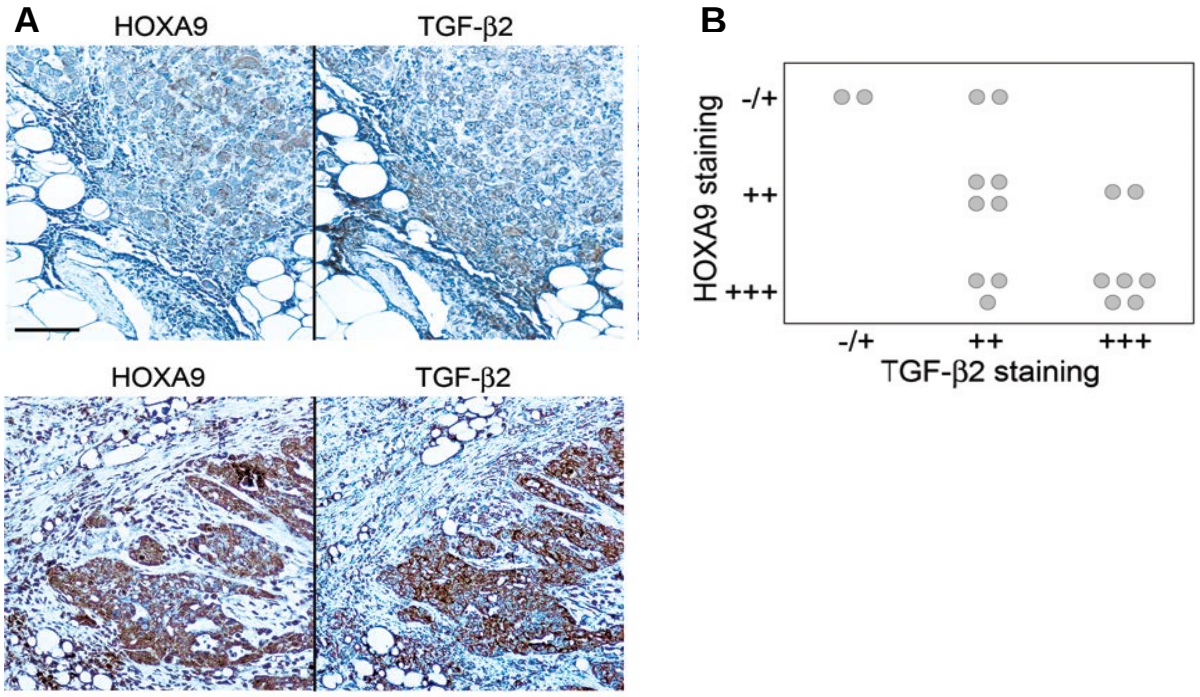


Figure 42. High HOXA9 protein levels are associated with high TGF-β2 protein levels in clinical specimens of EOC

(A) Examples of weak and strong staining of HOXA9 and TGF-β2 in omental tumors of two EOC cases, Bar, 100μm. **(B)** Immunohistochemical staining of HOXA9 and TGF-β2 in sections of omental tumors of 18 EOC cases was scored as nil/weak(-/+), moderate (++) or strong (+++). Each symbol represents an individual case.



2.*TGFB*2 is a direct transcriptional target of HOXA9

Because HOXA9 is a transcription factor, I hypothesized that the gene encoding TGF-β2 is a transcriptional target of HOXA9 in EOC cells. Five putative HOXA9 binding sites were identified in the mouse *Tgfb2* promoter (Figure 43A). Binding of ectopic HOXA9 in MOSEC cells was detected to regions containing two of these sites (S4 and S5) in chromatin immunoprecipitation assays (Figure 43B). Binding of endogenous HOXA9 to the S4 and S5 sites was detected in +HOXA9 control SKOV3ip cells, but no binding was detected in HOXA9-knockdown SKOV3ip cells (Figures 43C and D). These results were confirmed by luciferase reporter assay. Luciferase reporter assays using deletion constructs of the *Tgfb2* promoter demonstrated that the S4 site but not the S5 site was essential for HOXA9-induced promoter activity (Figures 44A and B). Activation by HOXA9 through the S4 binding site was confirmed in reporter assays by using a *Tgfb2* promoter construct in which the S4 site was mutated (Figures 44A and B). These results indicate that the gene encoding TGF-β2 is a direct transcriptional target of HOXA9.

Figure 43. HOXA9 directly binds to *TGFB2* promoter

(A) Representation of the mouse *Tgfb2* promoter. Locations of 5 putative HOXA9-binding sites evaluated by chromatin immunoprecipitation (S1 to S5) relative to the transcription start site (TSS) are indicated. **(B)** Chromatin immunoprecipitation analysis of interactions of FLAG-tagged HOXA9 in MOSEC cells with sites S1 to S5. Immunoprecipitated DNA was assayed by qPCR and is expressed as a percentage of total chromatin input. **(C)** Chromatin immunoprecipitation analysis of interactions of endogenous HOXA9 in SKOV3ip cells with conserved sites S4 and S5 in the human *TGFB2* promoter. The input corresponds to 1% of chromatin solution before immunoprecipitation. Immunoprecipitation using cells expressing FLAG-tag alone or cells expressing HOXA9 shRNA (shA9-B) as well as immunoprecipitation with IgG and amplification of *Gapdh* and *GAPDH* as irrelevant genes are included as negative controls in **B** and **C**. **(D)** qPCR analysis of immunoprecipitated DNA from assays in **C**, expressed as a percentage of total chromatin input.

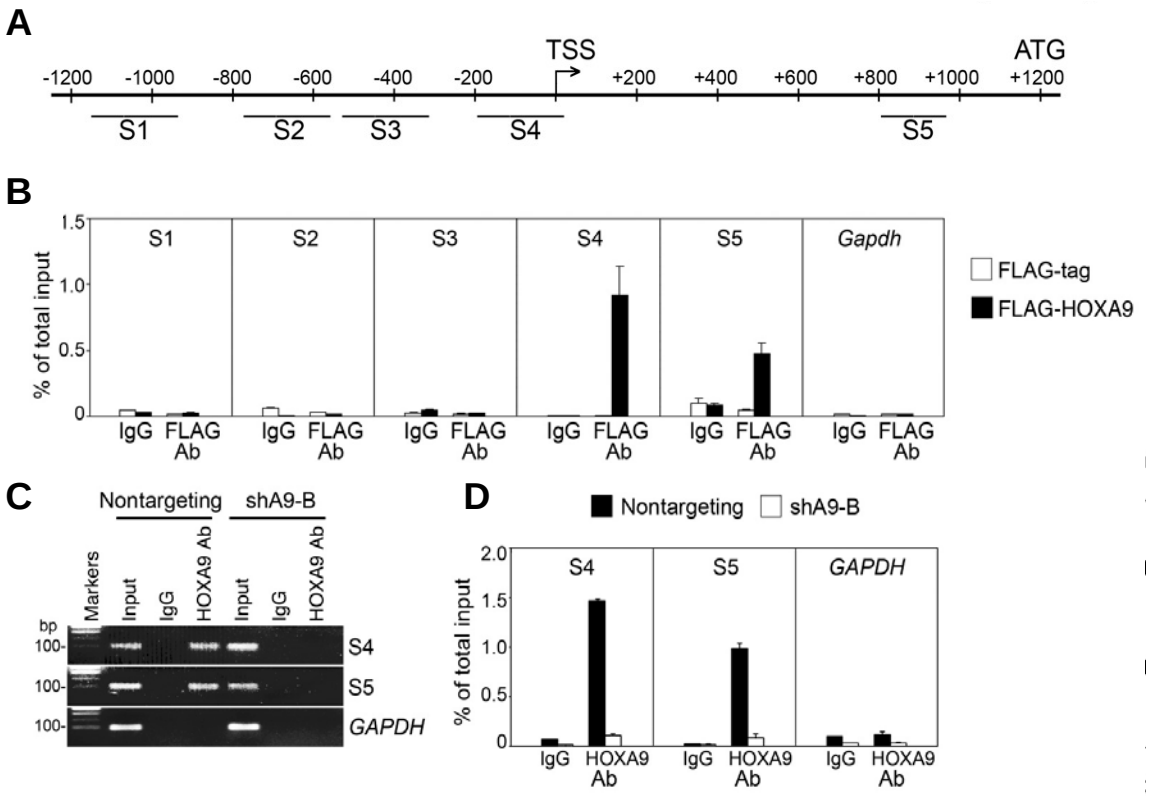
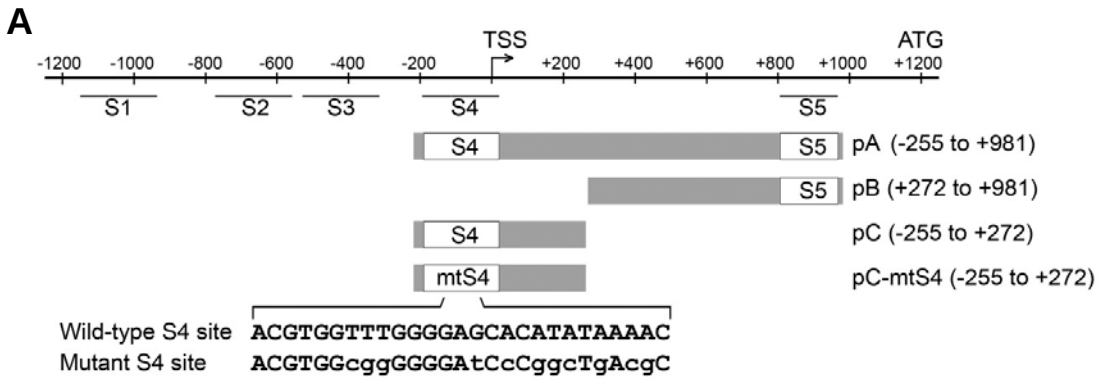
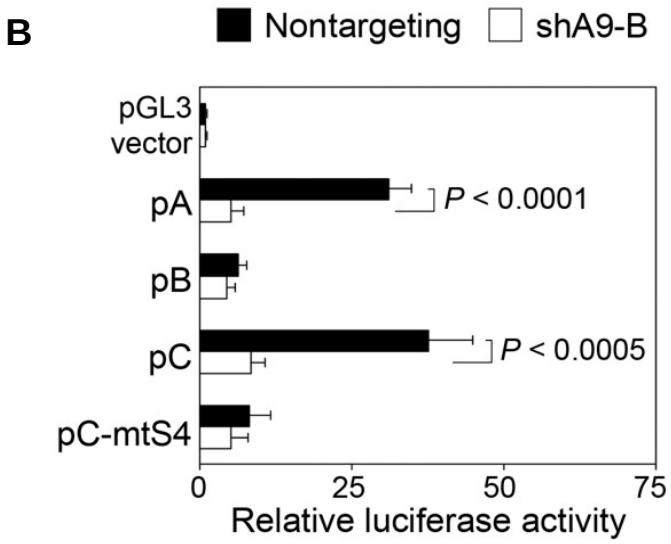


Figure 44. HOXA9 increases promoter activity of *Tgfb2*

(A) Representation of the mouse *Tgfb2* promoter. Locations of 5 putative HOXA9-binding sites evaluated by chromatin immunoprecipitation (S1 to S5) are indicated. Also indicated are the regions evaluated in luciferase reporter assays (pA, pB, pC, pC-mtS4) relative to the transcription start site (TSS). Wild-type sequences within the S4 site (in pA and pC) and mutant sequences (in pC-mtS4) are shown. **(B)** Activity of *Tgfb2* promoter regions shown in **A** was assayed in +HOXA9 control (black bar) and HOXA9-knockdown (white bar) SKOV3ip cells.





3. HOXA9 expression in EOC cells also increases stromal TGF-β expression

TGF-β1 not only induces transition of normal fibroblasts and MSCs into CAFs, but is also highly expressed by CAFs.[121] I therefore investigated whether HOXA9 expression in EOC cells stimulates TGF-β1 expression in omental fibroblasts. Omental fibroblasts that were primed in medium conditioned by +HOXA9 control SKOV3ip cells but not by HOXA9-knockdown SKOV3ip cells had significantly elevated expression of TGF-β1 and also of TGF-β2 ($P < 0.005$, Figures 45A and B). Similarly, TGF-β1 and TGF-β2 were more highly induced in fibroblasts when primed in medium conditioned by +HOXA9 MOSEC cells than by vector-control, +HOXA10 or +HOXA11 MOSEC cells (Figures 46A and B). TGF-β1 and TGF-β2 were also induced in adipose MSCs by +HOXA9 tumor-conditioned medium, whereas TGF-β1 but not TGF-β2 was induced in bone marrow MSCs (Figure 47). Consistent with the ability of HOXA9 to induce TGF-β2 expression in EOC cells *in vitro*, lower levels of human tumor-derived TGF-β2 were detected in omental implants of mice with HOXA9-knockdown tumors as compared to control tumors (Figure 48). Levels of mouse host-derived TGF-β2 and TGF-β1 were also significantly lower in omental implants of mice with HOXA9-knockdown tumors than of mice with control tumors (Figure 48).

TGF-β1 and TGF-β2 expression was induced in fibroblasts following stimulation with recombinant TGF-β1 and TGF-β2 at concentrations released by +HOXA9 tumor cells and by fibroblasts primed by +HOXA9 tumor cells (Figure 49A). TGF-β1 and TGF-β2 expression in fibroblasts was inhibited when +HOXA9 tumor-conditioned medium was depleted of TGF-β2 (Figure 49B). These findings suggest that HOXA9 expression in EOC induces autostimulatory production of TGF-β ligands in the stroma.

Figure 45. HOXA9 expression in EOC cells induces expression of TGF-β1 and TGF-β2 in omental fibroblasts

(A) *TGFB1* and *TGFB2* mRNA levels were assayed in omental fibroblasts at 5 days after priming in media conditioned by +HOXA9 control and HOXA9-knockdown SKOV3ip cells. The mRNA level of each gene is expressed relative to its level in unprimed fibroblasts. **(B)** Level of TGF-β1 and TGF-β2 released by unprimed and primed omental fibroblasts. **P* < 0.005; #*P* < 0.001

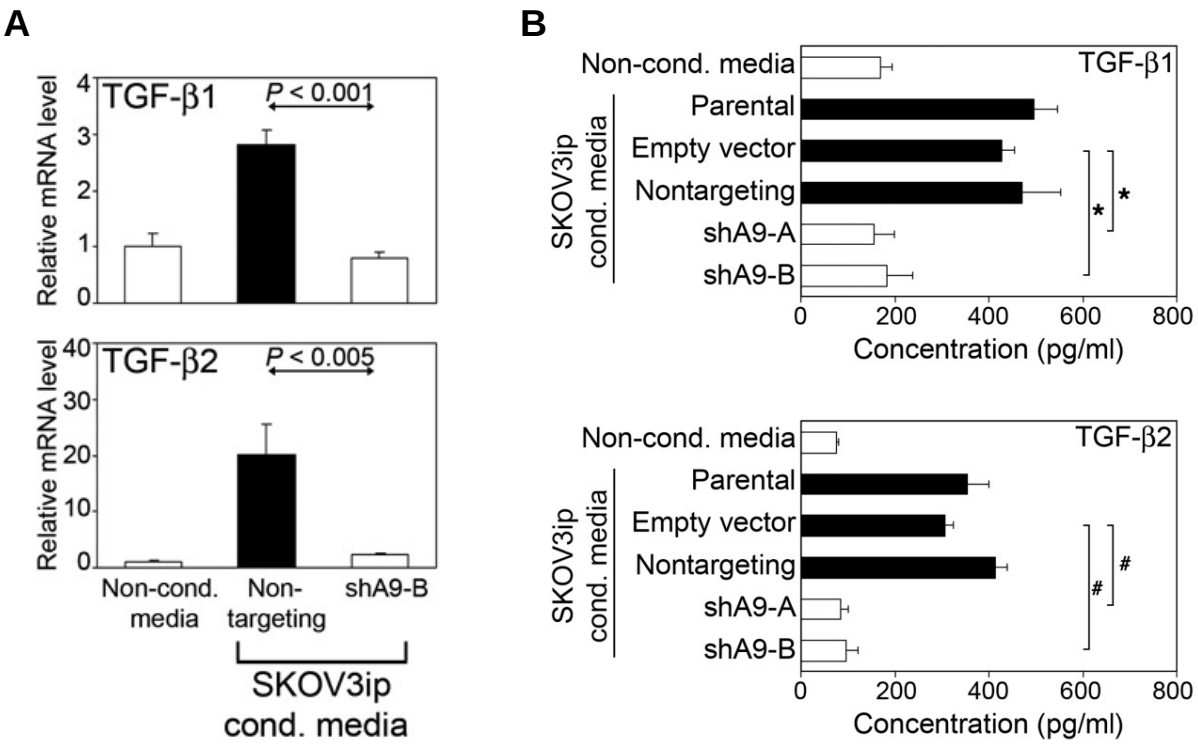


Figure 46. HOXA9, but not HOXA10 and HOXA11, induces expression of TGF-β1 and TGF-β2 in omental fibroblasts

(A) *TGFB1* and *TGFB2* mRNA levels in omental fibroblasts that were primed by MOSEC conditioned medium or left unprimed for 5 days. The mRNA level of each gene is expressed relative to its level in unprimed fibroblasts. **(B)** Level of TGF-β1 and TGF-β2 released by unprimed and primed omental fibroblasts.

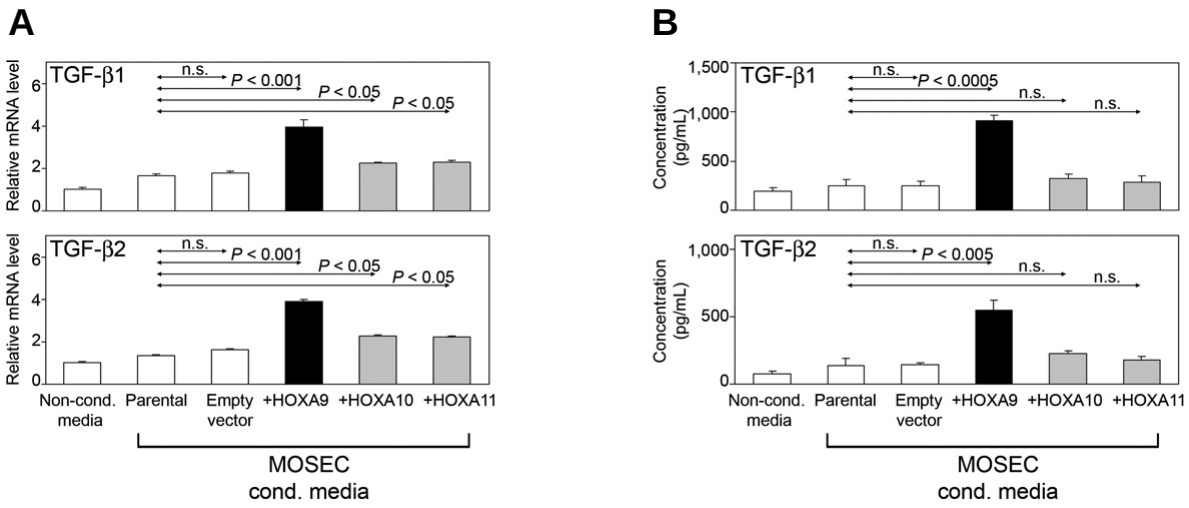


Figure 47. HOX9 expression in EOC cells induces expression of TGF-β1 and TGF-β2 in MSCs

TGFB1 and *TGFB2* mRNA levels were assayed in bone marrow MSCs and adipose MSCs at 5 days after priming in media conditioned by +HOXA9 control and HOXA9-knockdown SKOV3ip cells. The mRNA level of each gene is expressed relative to its level in unprimed MSCs.

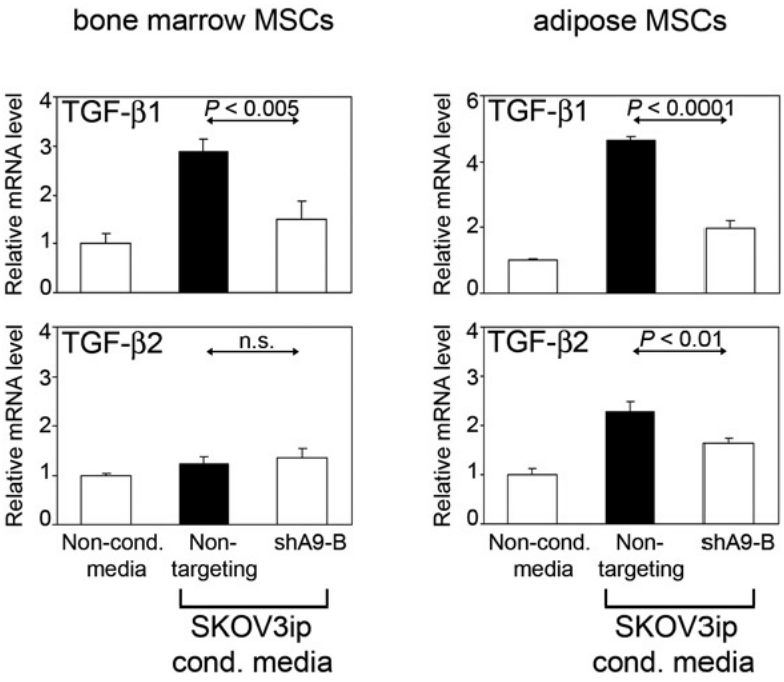


Figure 48. HOXA9 expression in EOC cells induces stromal TGF-β1 and TGF-β2 expression *in vivo*

Relative levels of *TGFB1* and *TGFB2* mRNAs (in human EOC cells) and *Tgfb1* and *Tgfb2* mRNAs (in mouse host cells) in omental tumors of mice that were inoculated with +HOXA9 control (Nontargeting) and HOXA9-knockdown (shA9-B) SKOV3ip lines (n=5 mice per group). Specificity of human- and mouse-specific qRT-PCR primers is shown in Figure 24B. **P* = 0.025; †*P* = 0.018.

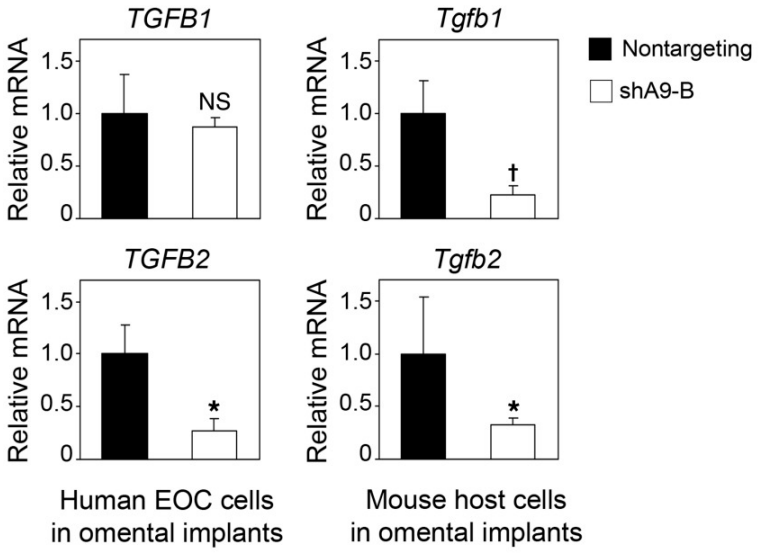
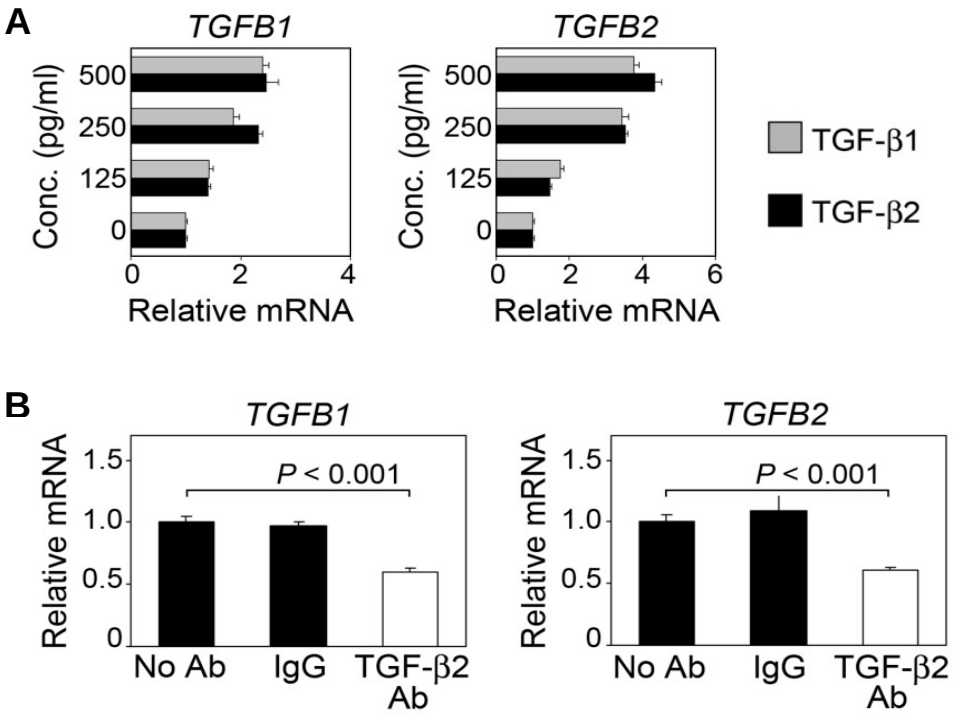


Figure 49. Tumor-derived TGF-β2 induces autostimulatory production of TGF-β ligands in omental fibroblasts

(**A** and **B**) Relative levels of *TGFB1* and *TGFB2* mRNAs in omental fibroblasts at 5 days after incubation with **(A)** recombinant TGF-β1 and TGF-β2 at the indicated concentrations and **(B)** media conditioned by +HOXA9 control SKOV3ip cells, where SKOV3ip-conditioned medium was left untreated, treated with IgG, or depleted by immunoprecipitation with antibody to TGF-β2.



4. Effects of HOXA9 on fibroblasts are mediated by its induction of TGF-β2 expression in EOC cells

4.1. TGF-β2 recapitulates the stimulatory effects of HOXA9 on fibroblasts *in vitro*

In subsequent experiments, I investigated whether TGF-β2 recapitulates the stimulatory effects of HOXA9 on normal omental fibroblasts and whether the effects of HOXA9 could be reversed by inhibiting tumor-derived TGF-β2. TGF-β2 did not alter proliferation of fibroblasts (Figure 50). On the other hand, expression of *ACTA2, FAP, IL-6, CXCL12 and VEGFA* was induced in omental fibroblasts following stimulation with recombinant TGF-β2 at concentrations detected in medium conditioned by +HOXA9 tumor cells (Figure 51A). Conversely, depletion of TGF-β2 from +HOXA9 tumor conditioned medium inhibited induction of CAF markers and growth factors in fibroblasts (Figure 51B).

To determine whether the ability of HOXA9 to activate fibroblasts and thereby promote proliferation of tumor and endothelial cells is mediated by its induction of tumor-derived TGF-β2, I evaluated the effects of specifically inhibiting TGF-β2 in +HOXA9 tumor cells. Stable expression of *TGF-β2* shRNA in SKOV3ip cells (shTGF-β2) decreased the TGF-β2 level to the same low level as seen in HOXA9-knockdown SKOV3ip cells (Figure 52A). In addition, *TGF-β2* cDNA was stably expressed in HOXA9-knockdown SKOV3ip cells (shA9-B + TGF-β2) to test whether reconstituting TGF-β2 in tumor cells restores the effects of HOXA9 (Figure 52A). Neither knockdown nor overexpression of TGF-β2 affected growth rates of SKOV3ip cells cultured in nonconditioned medium (Figure 52B). However, proliferation of tumor cells and of endothelial cells was less effectively stimulated by fibroblast-derived factors when fibroblasts were primed in medium conditioned by TGF-β2-

knockdown SKOV3ip cells than by control SKOV3ip cells ($P < 0.001$, Figures 52C and D). This reduced stimulation was identical to that observed when fibroblasts were primed in medium conditioned by HOXA9-knockdown SKOV3ip cells (Figures 52C and D). Conversely, reconstitution of TGF-β2 in HOXA9-knockdown SKOV3ip cells increased the ability of fibroblasts to stimulate growth of tumor and endothelial cells (Figures 52C and D). These findings indicate that HOXA9 expression in tumor cells promotes the ability of fibroblasts to stimulate growth of tumor and endothelial cells by inducing tumor-derived TGF-β2 that acts in a paracrine manner on fibroblasts.

Figure 50. TGF-β2 does not alter proliferation of fibroblasts

Growth rates of normal omental fibroblasts treated with recombinant TGF-β2 at the indicated concentrations were determined by MTT assay.

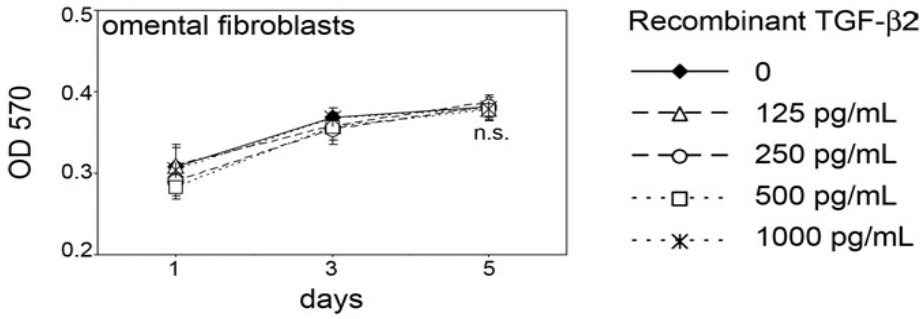


Figure 51. Tumor-derived TGF-β2 induces molecular features of CAFs in normal omental fibroblasts

(**A** and **B**) Relative *ACTA2, FAP, IL6, CXCL12, and VEGFA* mRNA levels in omental fibroblasts at 5 days after incubation with **(A)** recombinant TGF-β1 and TGF-β2 at the indicated concentrations and **(B)** media conditioned by +HOXA9 control SKOV3ip cells, where SKOV3ip-conditioned medium was left untreated, treated with IgG, or depleted by immunoprecipitation with antibody to TGF-β2.

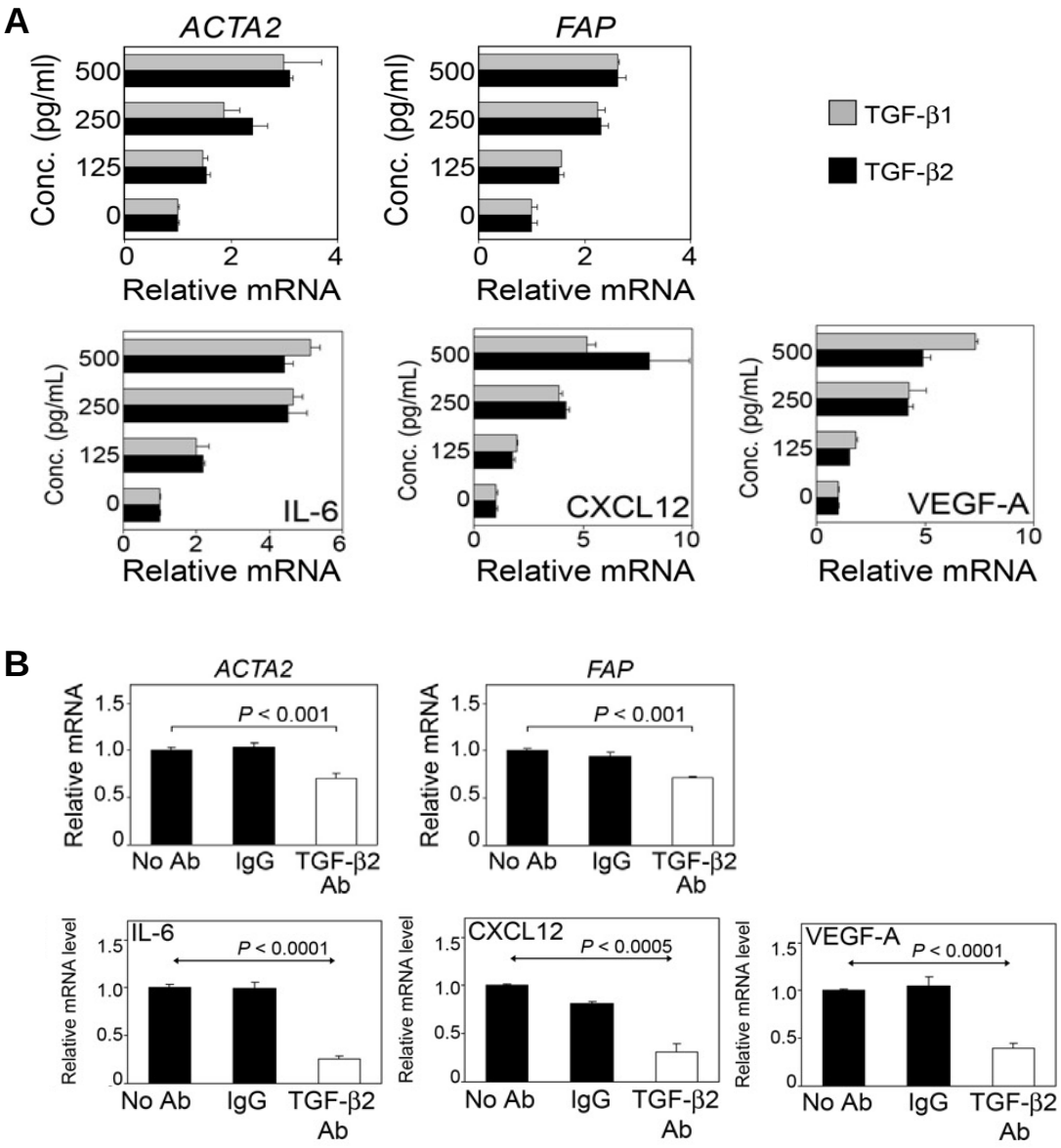
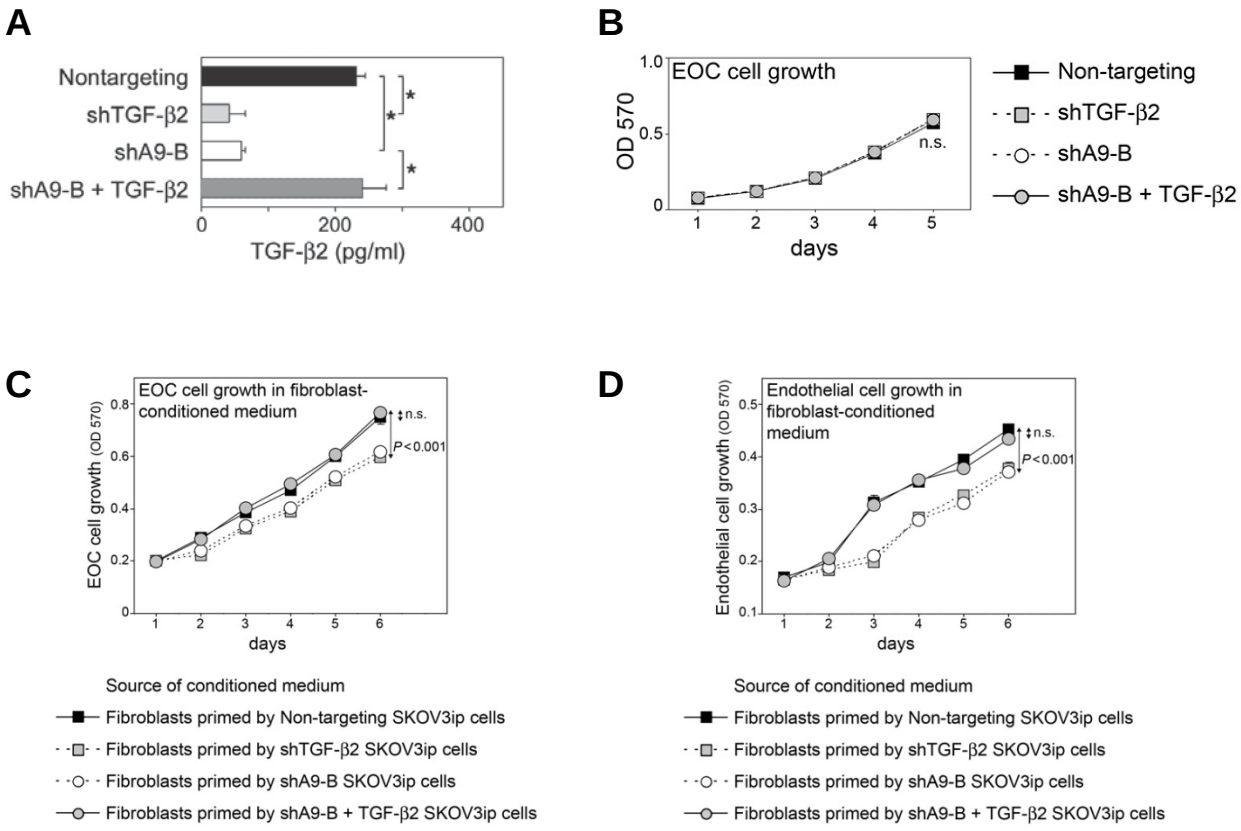


Figure 52. Tumor-derived TGF-β2 induces functional features of CAFs in normal omental fibroblasts

(A) TGF-β2 levels released by control (Nontargeting), TGF-β2–knockdown (shTGF-β2), and HOXA9-knockdown (shA9-B) SKOV3ip lines and a HOXA9-knockdown line stably expressing TGF-β2 (shA9-B + TGF-β2). *P < 0.001. **(B)** Growth rates of control (Nontargeting), TGF-β2–knockdown (shTGF-β2), and HOXA9-knockdown (shA9-B) SKOV3ip lines and a HOXA9-knockdown line stably expressing TGF-β2 (shA9-B + TGF-β2), where cell lines were cultured in nonconditioned medium. (**C** and **D**) Growth rates of **(C)** control SKOV3ip cells and **(D)** endothelial cells incubated in medium conditioned by omental fibroblasts that were primed by SKOV3ip-conditioned medium or left unprimed.



4.2. Stimulation of EOC growth by HOXA9 *in vivo* is mediated by its induction of tumor-derived TGF-β2

To validate my *in vitro* findings, I firstly evaluated whether reconstituting TGF-β2 in HOXA9-knockdown EOC cells restores the tumor growth-promoting phenotype of HOXA9 in intraperitoneal mouse xenograft models. As compared to mice inoculated with HOXA9-knockdown SKOV3ip cells, mice that were inoculated with HOXA9-knockdown SKOV3ip cells stably expressing TGF-β2 developed substantially larger tumors (Figure 53A). Reconstitution of TGF-β2 in HOXA9-knockdown tumor cells markedly increased mitotic activity, abundance of αSMA+ cells and microvessel density in tumors to the levels seen in +HOXA9 control tumors (Figure 53B). As compared to mice with HOXA9-knockdown tumors, mice with HOXA9-knockdown tumors that stably expressed TGF-β2 had significantly shorter survival times ($P = 0.003$, Figure 53C). The poor survival rate of these mice was almost identical to that of the +HOXA9 control group (Figure 53C). Conversely, tumor growth was reduced in mice that were inoculated with TGF-β2-knockdown SKOV3ip cells as compared to mice with control tumors (Figure 53A). Mitotic activity, αSMA+ cells and microvessel density were markedly reduced in TGF-β2-knockdown tumors, as were similarly observed in HOXA9-knockdown tumors (Figure 53B). As compared to mice with control tumors, mice with TGF-β2-knockdown tumors had significantly longer survival times ($P = 0.003$, Figure 53C). The higher survival rate of mice with TGF-β2-knockdown tumors and the reduced size of omental tumors in these mice were very similar to observations in the HOXA9-knockdown group (Figures 53A and C).

To confirm these findings, I evaluated the effect of inhibiting TGF-β2 in +HOXA9 MOSEC cells *in vivo* (Figure 54A). As compared to mice inoculated with +HOXA9 MOSEC

cells, mice that were inoculated with +HOXA9 MOSEC cells in which TGF-β2 was inhibited developed smaller intraperitoneal tumors (Figures 54B and C). Knockdown of TGF-β2 in +HOXA9 MOSEC cells substantially reduced mitotic activity, abundance of αSMA+ cells and microvessel density in tumors, almost to the levels seen in vector-control MOSEC tumors (Figure 54D). Together, these results demonstrated that HOXA9 promotes tumor growth by inducing tumor-derived TGF-β2.

In subsequent studies, I evaluated the clinical significance of *TGF-β2* expression in datasets of EOC patients. When EOC patients were stratified according to expression of *TGF-β2* in tumors, high *TGF-β2* expression was significantly associated with poor overall survival (P = 0.005, Figure 55). This association of high *TGF-β2* expression with poor survival in the AOCS dataset was consistent with the association of high *HOXA9* levels with poor survival in the same patient cohort (Figure 10), and with observations in mouse xenograft models.

Figure 53. Reconstitution of tumor-derived TGF-β2 restores the effects of HOXA9-knockdown on growth of SKOV3ip xenografts

(**A** and **B**) Female nude mice were inoculated intraperitoneally with SKOV3ip lines and sacrificed at 4 weeks thereafter. **(A)** Implants viewed under a fluorescence stereoscope. The arrow indicates the omental implant in the control group. Original magnification, ×0.8. **(B)** The average number of Ki-67+ tumor cells, αSMA+ cells, and microvessels in tumors was calculated by scoring 5 random fields of stained tumor tissue sections of each mouse (n = 5 mice per group). $^{*}P < 0.005$; $^{\dagger}P < 0.01$. **(C)** Survival rates of mice inoculated intraperitoneally with SKOV3ip lines (n = 10 per group). Significance values for each group as compared with the Nontargeting control group are indicated.

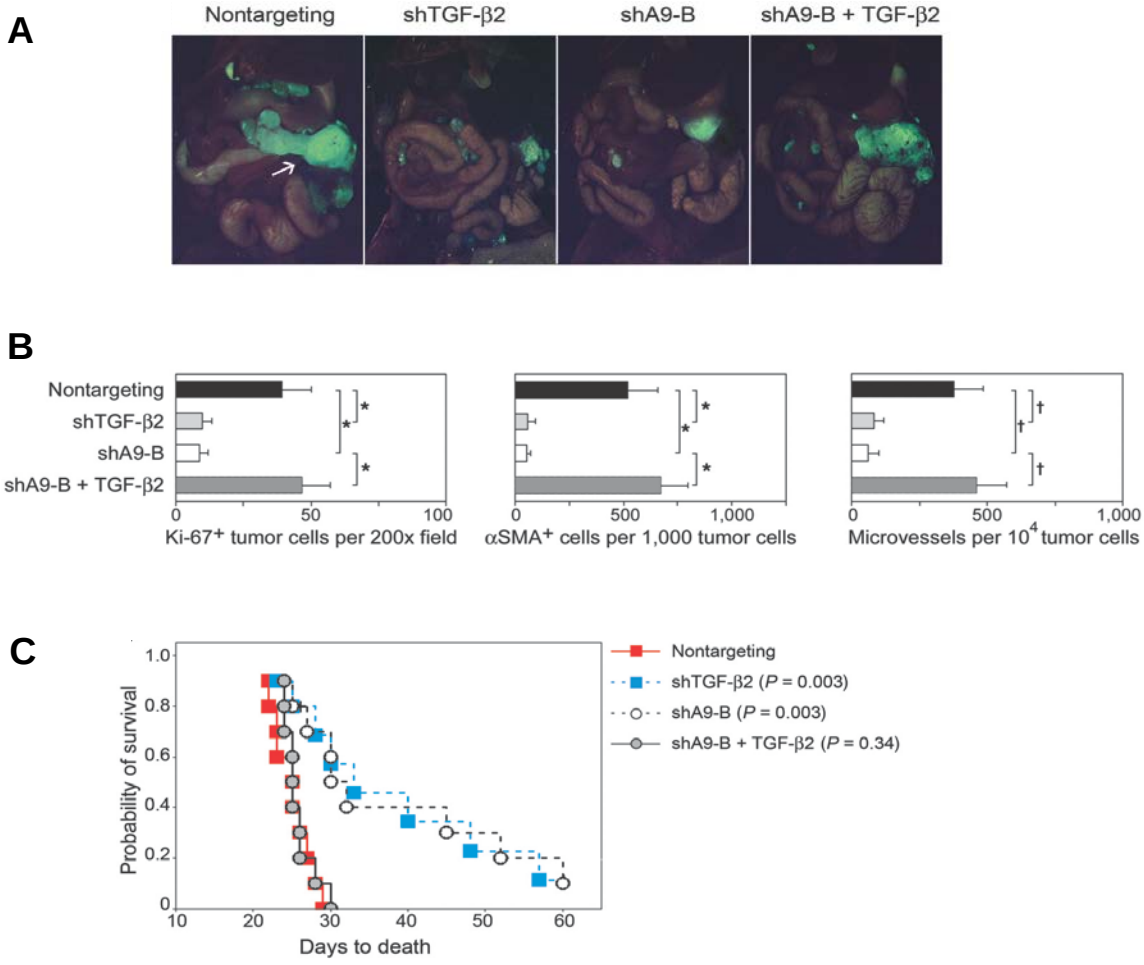

A
Nontargeting
shTGF-β2
shA9-B
shA9-B + TGF-β2
B
Nontargeting
shTGF-β2
shA9-B
shA9-B + TGF-β2
0
50
100
Ki-67+ tumor cells per 200x field
0
500
1,000
αSMA+ cells per 1,000 tumor cells
0
500
1,000
Microvessels per 10^4 tumor cells
C
Probability of survival
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
Days to death
Nontargeting
shTGF-β2 (P = 0.003)
shA9-B (P = 0.003)
shA9-B + TGF-β2 (P = 0.34)

Figure 54. Inhibition of tumor-derived TGF-β2 blocks stimulatory effects of HOXA9 on growth of MOSEC xenografts

(A) TGF-β2 levels released by vector-control and +HOXA9 MOSEC cells, and by +HOXA9 MOSEC cells stably expressing *Tgfb2* shRNA (+HOXA9+shTGF-β2). (**B** and **C**) Female nude mice were inoculated intraperitoneally with MOSEC lines and sacrificed at 2 months thereafter. **(B)** Omental implants are indicated. **(C)** Hematoxylin-eosin-stained tissue sections showing mesenteric implants. Bar, 200μm **(D)** Survival rates of mice inoculated intraperitoneally with MOSEC lines, where the +HOXA9+shTGF-β2 group was compared to vector-control and +HOXA9 groups (n=10 mice per group). **(E)** The average number of Ki-67+ tumor cells, αSMA+ cells, and microvessels in omental implants was calculated by scoring five random fields of stained tumor tissue sections of each mouse (n = 5 mice per group).

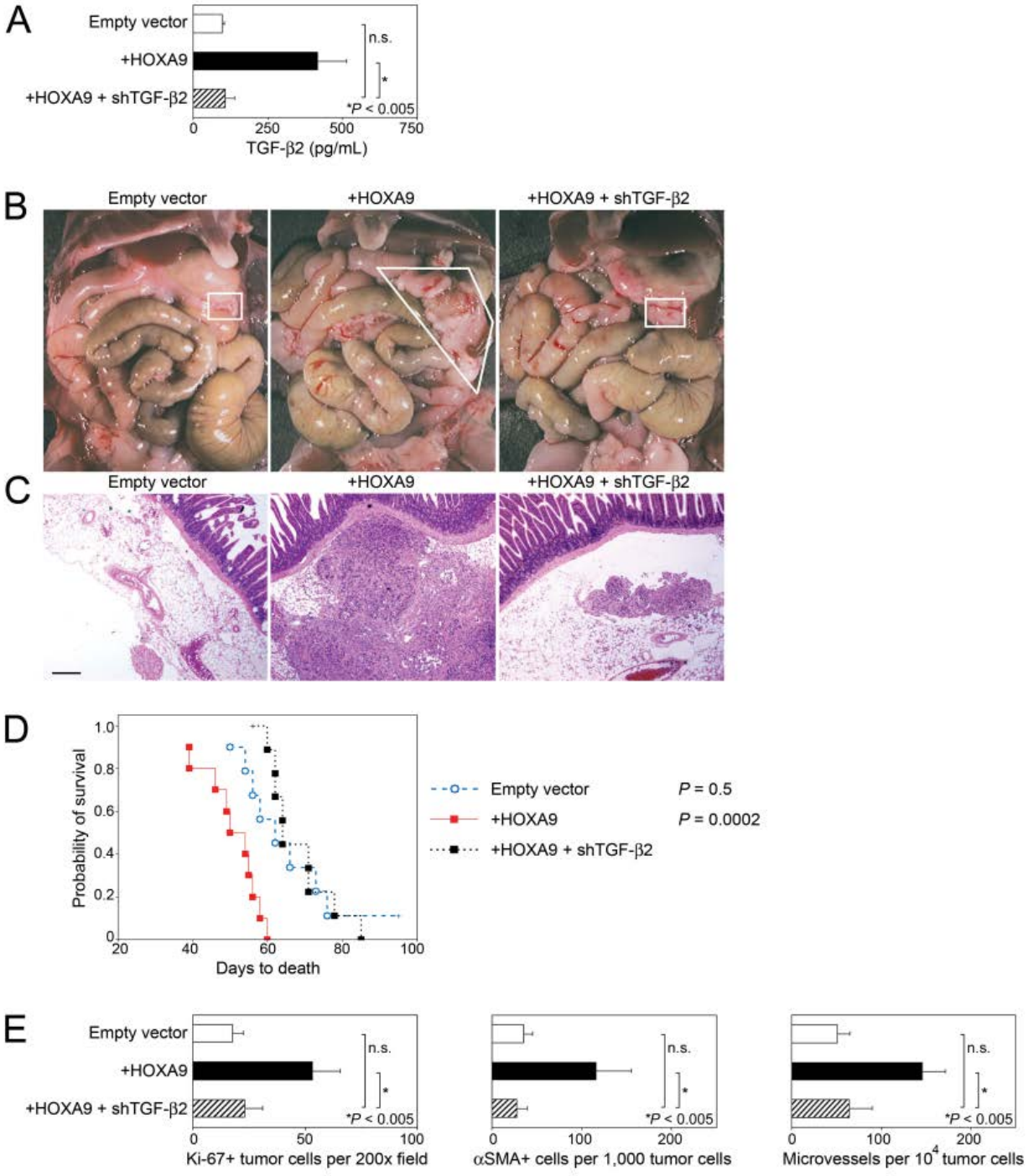
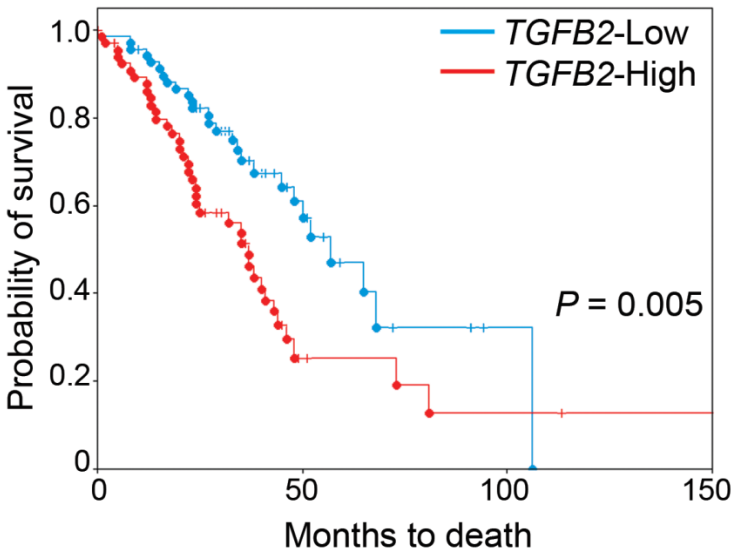

A
Empty vector
+HOXA9
+HOXA9 + shTGF-β2
n.s.
*
*P < 0.005
0
250
500
750
TGF-β2 (pg/mL)
B
Empty vector
+HOXA9
+HOXA9 + shTGF-β2
C
Empty vector
+HOXA9
+HOXA9 + shTGF-β2
D
Probability of survival
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
Days to death
Empty vector
P = 0.5
+HOXA9
P = 0.0002
+HOXA9 + shTGF-β2
E
Empty vector
+HOXA9
+HOXA9 + shTGF-β2
n.s.
*
*P < 0.005
0
50
100
Ki-67+ tumor cells per 200x field
0
100
200
αSMA+ cells per 1,000 tumor cells
0
100
200
Microvessels per 10^4 tumor cells

Figure 55. High *TGFB2* expression is associated with poor overall survival of EOC patients

Kaplan-Meier plot analysis of overall survival times of patients in the AOCS dataset stratified by transcript level of *TGFB2*. *TGFB2* transcript levels were defined as High (≥ upper quartile) and Low (≤ lower quartile) (n = 72 cases per group).



C. CONCLUSION

In this chapter, I determined the molecular mechanisms by which HOXA9 expression in EOC cells induces normal tissue-resident fibroblasts and MCSs to acquire features of CAFs. Firstly, the gene encoding TGF-β2 was identified as a direct transcriptional target of HOXA9 in EOC cells. Tumor-derived TGF-β2 was demonstrated to induce expression of CAF markers and growth factors in normal omental fibroblasts. My studies demonstrated that inhibiting TGF-β2 in +HOXA9 EOC cells substantially blocked the stimulatory effects of HOXA9 on CAFs and tumor growth. Conversely, reconstituting TGF-β2 in HOXA9-knockdown EOC cells restored the CAF-activating, tumor growth-promoting phenotype of HOXA9. Together, my results indicate that HOXA9 promotes EOC growth by inducing tumor-derived TGF-β2 that acts in a paracrine manner to stimulate the ability of fibroblasts to support tumor growth.

CHAPTER 6: DISCUSSION

A.TGF-β2 IS A KEY EFFECTOR OF HOXA9 ON THE GROWTH OF EOC

My studies in Chapter 3 demonstrated that high HOXA9 expression is associated with poor overall survival of EOC patients and that HOXA9 promotes EOC growth in mouse xenograft models. My subsequent studies in Chapter 4 demonstrated that HOXA9 expression in EOC cells promotes tumor growth through a mechanism dependent on host stromal cells and not via a tumor cell-autonomous mechanism. Specifically, my studies showed that HOXA9 expression in EOC cells induces normal omental fibroblasts and MSCs to acquire CAF features that promote growth of tumor cells and endothelial cells. Furthermore, my studies in Chapter 5 demonstrated that the tumor growth-promoting ability of HOXA9 is mediated primarily through its activation of TGF-β2 expression in EOC cells. Several of fibroblast-stimulatory factors, such as PDGF-α and FGF-2, promote fibroblast proliferation but do not induce CAF features.[122] The negligible effect of HOXA9 on expression of these mitogenic factors is consistent with its lack of effect on fibroblast proliferation (Figures 26 and 38). On the other hand, TGF-β2 induced CAF features in normal fibroblasts and MSCs but did not stimulate proliferation of these cells (Figures 50 and 51). Although HOXA9 might control other CAF-activating factors, the significance of HOXA9-induced TGF-β2 is supported by the ability of TGF-β2 antibody to substantially block the stimulatory effect of HOXA9 (Figure 51B). The ability of TGF-β2 to induce CAF features in normal omental fibroblasts could explain why normal cells expressing CAF markers have been detected in omental tissues of EOC patients without overt omental metastasis.[123] Because TGF-β2 is a soluble factor, the observed propensity of +HOXA9

tumors for omental involvement might stem from fertilization of the omental "soil" by tumor-derived TGF-β2.

1. TGF-β2 is a direct transcriptional target of HOXA9

Transforming growth factor-βs (TGF-βs) are multifunctional proteins that control cell proliferation, differentiation and other functions in most cell types.[124] Three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3) have been reported in mammals. Elevated levels of TGF-β1 and TGF-β2 proteins have been reported in EOC patients, but TGF-β3 protein was not detected.[125] TGF-β isoforms share their receptors and activate the same SMAD signaling pathways.[126] These isoforms therefore share many biological functions.[127] In this study, TGF-β1 and TGF-β2 were also found to have a similar ability to induce CAF features in normal omental fibroblasts (Figure 51A). Whereas TGF-β1 is known to induce CAF features in tissue-resident fibroblasts and bone marrow MSCs,[89, 121] this is the first study that demonstrates the significance of TGF-β2 in promoting CAFs. The most pronounced differences in the TGF-βs are their spatially and temporally distinct expression patterns at the mRNA and protein levels. My studies in Chapter 5 demonstrated that HOXA9 promotes expression of TGF-β2, but not expression of TGF-β1 (Figures 39 and 40A). The different isoforms of TGF-β are encoded by different genes located on different chromosomes and their promoter organization is remarkably different.[128, 129] For example, the *TGFB1* gene lacks a classical TATA box, whereas *TGFB2* contains TATA boxes. The *TGFB1* promoter contains AP-1, SP-1, NF-1 binding sites, whereas these sites are absent from the *TGFB2* promoter.[129] My finding that HOXA9 controls transcription of *TGFB2* and not *TGFB1* is consistent with reports that these genes are differentially regulated in developing tissues, regenerating tissues and in pathologic responses.[130-132]

2. TGF-β2 does not act in an autocrine manner in EOC cells

It is well-established that TGF-βs inhibit proliferation of normal epithelial cells by stimulating CDK inhibitor expression which leads to G_1 arrest.[133, 134] However, HOXA9 did not inhibit growth of EOC cells (Figures 19A and 20A). Furthermore, overexpression of TGF-β2 did not inhibit EOC cell growth (Figure 52B). It is also well-established that TGF-βs induce EMT in various cell types.[135, 136] Because HOXA9 induces TGF-β2 expression in EOC cells, it might be expected that +HOXA9 EOC cells undergo EMT. However, HOXA9 did not alter expression of EMT-promoting transcription factors in EOC cells, even those known to be direct targets of TGF-β2[137] (Figure 24). Together, these findings indicate that tumor-derived TGF-β2 does not act in an autocrine manner in EOC cells. This can be explained in several ways. Mutations in genes that encode core components of the TGF-β signaling pathway such as the TGF-β receptors and Smad proteins have been identified in many cancers including EOCs.[138-140] In addition, expression of betaglycan, the co-receptor for TGF-β2, is decreased or lost in 73% of high grade EOCs.[141] Some proteins that are aberrantly expressed in cancers can also block TGF-β signaling. For example, the transcription factor encoded by the homeobox gene *DLX4* has been found to be highly expressed in EOCs and to block both TGF-β-induced growth inhibition and EMT by directly binding to Smad proteins.[142]

3. Tumor-derived TGF-β2 induces autostimulatory production of TGF-βs in the stroma

My studies demonstrated that HOXA9 not only directly induces expression of TGF-β2 in EOC cells, but also leads to increased expression of TGF-β1 and TGF-β2 in omental

fibroblasts and MSCs (Figures 45, 46, and 47). TGF-β auto-induction has been recognized in wound healing.[143] Kojima *et al.* have reported the existence of a TGF-β autocrine signaling loop in the stroma of breast cancer xenograft models.[144] However, this earlier study did not explain how the TGF-β autocrine signaling loop is initiated in the stroma. My studies in Chapter 5 support the existence of a self-sustaining TGF-β autocrine signaling loop in CAFs. Moreover, my findings indicate that tumor-derived TGF-β2 induces normal fibroblasts to acquire CAF features and initiates TGF-β autocrine signaling loop in CAFs (Figure 49). The CAF-stimulatory effects of HOXA9 in EOCs could therefore be amplified by surrounding stroma cells through their auto-induction of TGF-β ligands.

B. HOXA9 EXPRESSION IN EOC CELLS 'EDUCATES' THE STROMA TO BECOME PERMISSIVE FOR TUMOR GROWTH

1. HOXA9 expression in EOC induces CAF features in multiple types of CAF precursor cells

The high abundance of CAFs is associated with poor prognosis in many types of cancers, including EOCs.[145] Recent studies support several potential cellular origins of CAFs (Figure 8). In my study, four different cell types were evaluated as potential CAF precursors: epithelial tumor cells, normal omental fibroblasts, bone marrow-derived MSCs, and adipose-derived MSCs. Because TGF-β signaling induces EMT in many cell types,[146] CAFs in +HOXA9 tumors might derive from epithelial tumor cells. However, HOXA9 did not alter the expression of EMT-promoting transcription factors in EOC cells (Figure 24). Moreover, my study did not identify CAFs that originated from epithelial tumor cells in mouse xenografts (Figure 25). The possibility cannot be entirely excluded that a small proportion of CAFs might derive from EOC cells. However, my findings strongly indicate

that the majority of CAFs in EOC derive from host cells. Furthermore, my studies in Chapter 4 demonstrated that HOXA9 expression in EOC cells induces CAF features in all three types of host-derived cells (i.e. tissue-resident fibroblasts and MSCs, and more distal, bone marrow-derived MSCs).

MSCs reside in most adult tissues, but are particularly abundant in white adipose tissues.[115, 116] Omental, mesenteric and gonadal adipose tissues are major repositories of visceral white adipose tissues and are the most commonly involved sites in EOC.[147-149] My studies in Chapter 4 showed that adipose MSCs can be more effectively induced by +HOXA9 EOC cells than by –HOXA9 EOC cells to acquire CAF features. This finding could explain the greater propensity of +HOXA9 tumors to develop large implants on the omentum, mesentery and broad ligament. Whereas the number of bone marrow MSCs declines with age,[149] visceral white adipose tissue increases with age. Because EOC commonly occurs in post-menopausal women, adipose MSCs might contribute significantly to the EOC stroma.

2. HOXA9 expression in EOC cells induces functional features of CAFs in normal omental fibroblasts

My studies in Chapters 4 and 5 support a model in which HOXA9-induced, tumor-derived TGF-β2 stimulates normal fibroblasts and MSCs to express CXCL12 and IL-6, which in turn acted in a paracrine manner to stimulate tumor cell proliferation (Figures 30 and 32). Stromal-derived CXCL12 and IL-6 are known to promote tumor cell growth and their expression correlates with poor outcomes.[88, 150, 151] Moreover, CXCL12 has been reported to stimulate migration of MSCs toward tumor cells.[89] Increased levels of stromal

CXCL12 might also contribute to CAF abundance in +HOXA9 tumors by stimulating MSC recruitment.

My studies in Chapters 4 and 5 also support a model in which HOXA9, via its induction of tumor-derived TGF-β2, promotes EOC growth by stimulating tumor angiogenesis. Inhibiting TGF-β2 in +HOXA9 EOC cells substantially blocked the stimulatory effect of HOXA9 on tumor microvessel density (Figure 53B). Conversely, reconstituting TGF-β2 in HOXA9-knockdown EOC cells increased tumor microvessel density (Figure 53B). Furthermore, HOXA9 expression in EOC cells induced normal fibroblasts and MSCs to express VEGF-A and IL-6, which in turn acted in a paracrine manner to stimulate endothelial cell growth (Figures 34B and 36B). Because CXCL12 stimulates recruitment of endothelial progenitor cells to tumors,[88] elevated stromal CXCL12 levels might also contribute to increased angiogenesis in +HOXA9 tumors.

C. THE ROLE OF HOXA9 IN TUMOR PROGRESSION

1. HOXA9, but not HOXA10 and HOXA11, promotes EOC growth

Many homeobox genes have been reported to be aberrantly expressed in a wide variety of tumors, but their mechanisms in tumorigenesis are poorly understood. While some homeobox genes have been reported to promote angiogenesis in tumors,[152, 153] most of the homeobox genes studied to date have been found to alter tumor cell proliferation or survival *in vitro.*[47, 154, 155] This implies that homeobox genes primarily modulate tumor growth via tumor cell-autonomous mechanisms. In contrast, my study demonstrates that a homeobox gene that is expressed in tumor cells promotes tumor growth via paracrine effects on stromal cells. The mechanisms that underlie aberrant expression of *HOXA9* in

EOCs are as yet unclear. *HOXA9* expression varies widely among EOC patients in the AOCS and TCGA cohorts. *HOXA9* promoter methylation has been reported in EOCs.[156, 157] However, *HOXA9* promoter methylation has been detected at a significantly lower frequency in late-stage EOC than in early-stage EOC.[157] This is consistent with our findings that HOXA9 promotes progression of EOC.

Aberrant expression of *HOXA10* and *HOXA11* has also been detected in subsets of EOCs.[65] Some *HOX* genes have similar functions.[158] The *HOXA9*, *HOXA10* and *HOXA11* genes share extensive tracts of homology and are thought to have evolved by gene duplication from the same ancestral gene.[158] However, my study demonstrated that the ability of HOXA9 expression in EOC cells to induce normal fibroblasts and MSCs to acquire CAF features is not shared by HOXA10 and HOXA11 (Figures 29 and 32). Interestingly, HOXA10 has both tumor-promoting and tumor-suppressing properties. On one hand, HOXA10 induces the expression of β3-integrin in immortalized ovarian surface epithelial cells and increases their growth by promoting direct contact with fibroblasts.[159] On the other hand, HOXA10 and β3-integrin also inhibit invasiveness in endometrial cancer cells.[160] Consistent with these prior reports, this study showed a modest increase in mitotic activity in +HOXA10 MOSEC xenografts as compared to vector-controls (Figure 20B). However, the expression of *HOXA10* is not associated with poor survival in EOC patients and mouse xenograft model of EOCs (Figures 11 and 14).

2. The role of HOXA9 in other tumors

HOXA9 has been identified as the most highly correlated gene for poor prognosis in acute myeloid leukemia (AML).[161] HOXA9 expression is normally down-regulated in mature

myeloid cells. However, overexpression of HOXA9 in primary bone marrow cells enhances hematopoietic stem cell regeneration, blocks differentiation, expands the myeloid progenitor cell pool, and collaborates with the Meis1a co-factor to induce AML.[162, 163] The exact mechanisms of HOXA9 in leukemogenesis are not fully understood, and only a few HOXA9 target genes have been identified.[164] Interestingly, TGF-β2, like HOXA9, is also preferentially expressed in primitive hematopoietic cells and stimulates the repopulating capacity of hematopoietic stem cells.[165] Therefore, it might be possible that HOXA9 controls expansion of hematopoietic progenitor cells via its induction of TGF-β2.

Although *HOXA9* is a well-characterized oncogene in AML, the role of *HOXA9* in solid tumors is virtually unknown. *HOXA9* is constitutively activated in AML by chromosomal translocation t(7;11)(p15;p15), where the nucleoporin gene *NUP98* is fused to *HOXA9.*[51, 166] However, this chromosomal translocation has not been detected in solid tumors. On the other hand, *HOXA9* has been reported to be down-regulated in breast and lung cancer cells.[167, 168] HOXA9 has been reported to have tumor-suppressing properties in breast cancer by inducing *BRCA1* expression.[167] It is well-established that *HOX* genes exhibit context-dependent functions in normal development.[158] Therefore, HOXA9 might have tumor-promoting or tumor-suppressive properties depending on the specific type of tumor.

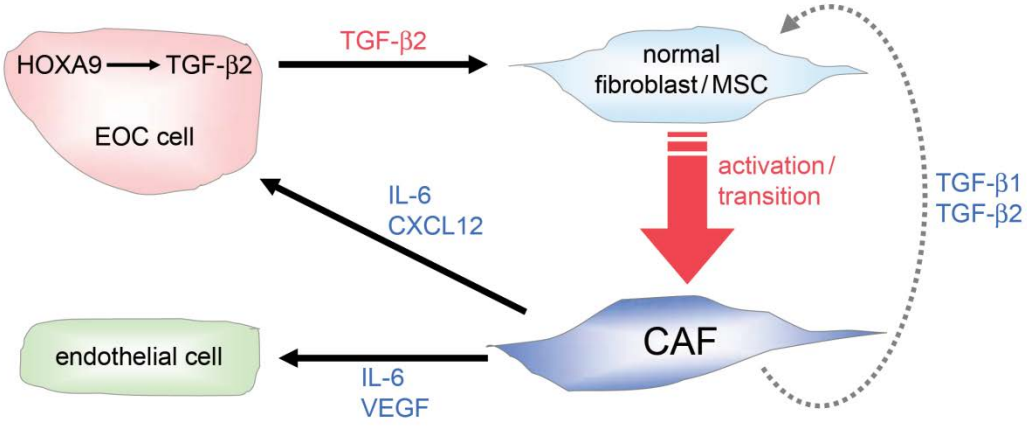
D. CONCLUSION

It is increasingly recognized that crosstalk between tumor cells and surrounding stromal cells is dynamic and essential for tumor growth. Over the past 30 years, numerous genes have been identified as important drivers of autonomous tumor cell growth. However, molecular aberrations in tumor cells that promote interactions between tumor cells and

stromal cells are poorly understood. My study supports a model in which HOXA9 expression in EOC cells "educates" the stroma to become permissive for tumor growth by inducing MSCs and resident fibroblasts to acquire molecular and functional features of CAFs that promote tumor growth and angiogenesis (Figure 56). Whereas targeting HOXA9 is therapeutically challenging, inhibiting its downstream effector TGF-β2 may be a promising therapeutic strategy. Anti-TGF-β2 therapies have been developed to circumvent immunosuppression in cancer patients.[169, 170] In addition, a number of small molecule TGF-β receptor inhibitors have been evaluated in preclinical models and in clinical trials.[171-173] My studies raise the intriguing possibility that anti-TGF-β2 therapies or small molecule TGF-β receptor inhibitors could be used to inhibit CAFs in EOC. This is particularly significant for improving outcomes of patients with advanced-stage EOC which is rarely cured by conventional chemotherapies.

Figure 56. Bidirectional signaling between EOC cells and CAFs

Control of growth factor expression and interactions between EOC cells, CAFs and endothelial cells by HOXA9-induced, tumor-derived TGF-β2.



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❖ **Publications:**

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