THE EFFECT OF 15-LIPOXYGENASE-1 (15-LOX-1) ON ANGIOGENESIS IN COLORECTAL AND PROSTATE CANCER

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ABSTRACT

THE EFFECT OF 15-LIPOXYGENASE-1 (15-LOX-1) ON ANGIOGENESIS IN COLORECTAL AND PROSTATE CANCER

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15-lipoxygenase-1 (15-LOX-1) is one of the lipoxygenases (LOX) that are important for the generation of inflammatory bioactive lipids, which then result in the initiation and resolution of inflammation. The enzyme mainly metabolizes the omega-6 fatty acid, linoleic acid, to 13-(S)-HODE. In colorectal carcinoma, the tumor suppressive role of 15-LOX-1 is highlighted with decreased proliferation, induction of apoptosis, reduced motility and invasion. Oppositely, the expression of 15-LOX-1 was reported to have pro-carcinogenic role in prostate cancer. The effect of 15-LOX-1 expression on angiogenesis in cancer, however, has not been addressed adequately. Therefore, we investigated the expression and secretion of Vascular Endothelial Growth Factor-A (VEGF-A), which acts as a potent vascular permeability factor in the angiogenesis process, in response to 15-LOX-1

expression. We used the colon cancer cell line HCT-116 and prostate cancer cell lines PC-3 and LNCaP as our models. We observed a decreased expression of VEGF-A through decreased transcriptional activity of Hypoxia-Inducible Factor 1α (HIF-1α) along with decreased secretion in 15-LOX-1 expressing HCT-116 cells. Contrary to our expectations, we observed a similar reduction in the expression and secretion of VEGF-A in LNCaP cells but no change in PC-3 cells when these cells were transfected with a 15-LOX-1 vector. To find out 15-LOX-1 mediated signaling mechanisms in angiogenesis, a proteome profiler array was conducted with proteins isolated from Human Umbilical Vein Endothelial Cells (HUVECs) incubated with conditioned media collected from 15-LOX-1 overexpressing LNCaP cells and tissue inhibitor of metalloproteases (TIMP-1) was enriched. Enhanced expression of this anti-angiogenic protein may contribute towards the anti-angiogenic role of 15-LOX-1 in our prostate cancer model. Collectively, this study indicates the anti-tumorigenic and anti-angiogenic roles of 15-LOX-1 both in colorectal and prostate cancer.

Keywords: Angiogenesis, 15-LOX-1, prostate cancer, colon cancer, VEGF-A.

ÖZ

15-LİPOKSİGENAZ-1 (15-LOX-1) ENZİMİNİN KOLON VE PROSTAT KANSERLERİNDE ANJİYOGENEZ ÜZERİNE OLAN ETKİSİNİN İNCELENMESİ

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15-Lipoksigenaz-1 (15-LOX-1) lipoksigenaz (LOX) ailesinden biri olup, inflamasyonun çözümlenmesinde önemli rol oynayan inflamatuar biyoaktif lipidleri oluşturur. 15-LOX-1 çoğunlukla omega-6 yağ asidinden olan linoleic asidi metabolize edip 13-(S)-HODE oluşturur. 15-LOX-1'ın kolon kanserindeki tumor baskılayıcı özellikleri hücre bölünmesini, motiliteyi ve invazyonu azaltıp apoptozu arttırması yönündedir. Buna karşın, 15-LOX-1'ın anjiyogenezdeki etkisi tam olarak bilinememekle birlikte, prostat kanserinde ise bu enzimin, pro-karsinojenik bir etkiye sahip olduğu vurgulanmıştır. Bu nedenle, bu çalışmada 15-LOX-1'ın güçlü bir vasküler geçirgenlik faktörü olan vasküler entodel büyüme faktörünün (VEGF-A) ifadesine ve salınımına olan etkisi kolon kanseri HCT-116 hücre hattı ve prostat kanseri hücreleri PC-3 ile LNCaP'te incelenmiş; HCT-116 hücrelerinde azalış gözlemlenmiştir.

VEGF-A mRNA’sının azalmasi normoksik ve hipoksik koşullarda hipoksiya-indüklü faktör 1-α (HIF1-α)’nün azalan transkripsiyonel aktivitesine bağlı olduğu bulunmuştur. Beklentilerimize karşı olarak, aynı değişimleri LNCaP hücrelerinde gözlemlerken PC-3 hücrelerinde bir değişim gözlemlenmemiştir. 15-LOX-1’ın endotel hücrelerinde hangi sinyal yolağına etki ettiğini görmek amacıyla 15-LOX-1 aşırı ifadeleyen prostat kanseri modelimiz LNCaP hücrelerinden alınan koşullu besi yeri kullanılarak anjiyogenez protein dizisi gerçekleştirilmiştir. Bulgularımızda doku metalloproteaz inhibitörü-1 (TIMP-1) anlamlı olarak artış göstermiştir. Sonuç olarak, prostat kanseri ve kolon kanserinde, 15-LOX-1’ın tümör baskılayıcı ve anjiyogenezi inhibe edici etkisi gözlemlemiştir. Ayrıca, artış gösteren bu anti-anjiyogenik proteinin, prostat kanser modelimizde 15-LOX-1’ın anti-anjiyogenik etkisine katkıda bulunabilecekleri öne sürülmüştür.

Anahtar kelimeler: Anjiyogenez, 15-LOX-1, prostat kanser, kolon kanser, VEGF-A.

To my family

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CHAPTER 1

INTRODUCTION

1.1 Cancer and its Hallmarks

Cancer is the abnormal and uncontrolled growth of cells, invading the tissues around it and eventually spreading out to other parts of the body. According to the data of National Cancer for Health Statistics, in 2016, the predicted new cancer cases will be 1,685,210 and deaths due to cancer will be 595,690 in the United States (R L Siegel, Miller, and Jemal 2016). During their transformation into cancer cells, normal cells acquire certain characteristics, which in turn can sustain their incessant development. Hanahan and Weinberg identified several hallmarks in the development of tumors: 1. sustained proliferative signaling by becoming hyper-responsive to growth factors, 2. evading growth suppressors like Rb (retinoblastoma-associated) and p53, which undergo gain- or loss-of-function mutations, 3. resisting cell death by manipulating pro-apoptotic and anti-apoptotic proteins and signaling pathways, 4. enabling replicative immortality by maintaining telomeric DNA, 5. inducing neoangiogenesis to procure more nutrients and oxygen, 6. activating invasion and metastasis through changes in the shape and

attachment to other cells and extracellular matrix (ECM) in order to be disseminated to discrete organs (Hanahan and Weinberg 2011).

1.1.1 Prostate Cancer

Prostate cancer (PCa) is the second-leading cause of cancer deaths in men with an estimated 26,120 deaths in 2016 in the US. PCa is generally defined as an adenocarcinoma of the prostatic fluid-secreting cells. The disease has no observable symptoms at the beginning; but in later stages can be manifested by interrupted urine flow, repeated and painful urination and finally in cases where the cancer can metastasize to the bone, pain in the hips and spine (American Cancer Society 2016). Like in many other cancer types, the risk factors can be endogenous and/or exogeneous. Endogenous factors include familial history, chromosomal aberrations, altered androgen metabolism, aging and oxidative stress; on the other hand, exogeneous factors are composed of high fat diet, vitamin D deficiency, environmental agents, specifically exposure to endocrine disruptive chemicals. During development of the disease, abnormal growth of the epithelium occurs first and results in benign stage, in other words, benign prostatic hyperplasia (BPH); which may then progress to prostatic intraepithelial neoplasia (PIN) that includes continuous cell proliferation in the lining epithelium prostatic, which requires androgen, then spreading to the tissue itself and invasion of adjacent tissues like the rectum and via the blood or lymphatic systems, followed by metastasis to the bone, lymph nodes and brain (Bostwick et al. 2004). Similar to breast and ovarian cancer, one of the important signs of this disease is germline *BRCA*2 mutations, which causes poor prognosis of this disease (Kote-Jarai et al. 2011; Risbridger et al. 2015). At the molecular level, inactivation of tumor suppressor genes like p53, Rb and PTEN and

hyperactivation of oncogenes like MYC may occur in prostate carcinoma (Schulz 2003). To prevent prostate cancer progression, early diagnosis with newly characterized biomarkers is essential. Well-known markers for PCa include elevated levels of prostatic acid phosphatase (PAP), which is important for liquefaction of semen, and prostate specific antigen (PSA) in the serum. As the techniques improve, new biomarkers such as prostate cancer antigen 3 (PCA3), a long non-coding RNA that is expressed almost in 95% of prostate cancer cases and is a gene fusion product of the ETS oncogene family (*ERG*, ETS-related gene) with the transmembrane protease serine 2 (*TMPRSS2*), as well as some DNA methylation marks for important genes like Glutathione S-transferase P, (*GSTP1*, DNA detoxification) or Adenomatous polyposis coli (*APC*, apoptosis, migration and adhesion) or Ras association domain-containing protein 1 (*RASSF1*, cell cycle regulation) have been reported (J. T. Wei 2015; P. Sharma, Zargar-Shoshtari, and Pow-Sang 2016).

For the characterization and therapeutic applications of PCa, androgen signaling is central. The androgen receptor (AR) is a transcription factor that is activated in the nucleus when bound to the androgen dihydrotestosterone, which results in the regulation of many processes like proliferation, apoptosis, migration, invasion, and differentiation. Its activity is regulated by steroid and peptide hormones; by binding to androgen, it leads to migration of prostate cancer cells, thus leading to metastasis (Culig et al. 2014). As androgen dependency in PCa is very crucial, therapies based on androgen-deprivation through either surgical castration or administration of anti-androgens such as luteinizing hormone-releasing hormone (LHRH) ago-/antagonists to lower the amount of testosterone (Chandrasekar et al. 2015). On the other hand, androgen depletion may evade cure since some prostate carcinoma cells may not express AR because of promoter hypermethylation,

ligand- independent activation of AR, alterations in ligand specificity and affinity due to mutations in AR and having differentially expressed or mutated coactivators (Schulz 2003). These changes result in the relapse of PCa and the development of Castration Resistance Prostate Cancer (CRPC) that is the hormone-resistant form. In CRPC, the effect of the microenvironment is important since cytokines such as IL-6, IL-4 and IL-8 can activate the AR pathway independent of ligand; additionally, IL-6 can boost neuroendocrine cells to secrete survival factors in these patients. Moreover, when there is not androgen, signaling can also activated by the variants of AR. Therefore, a multi-drug combinatorial treatment is proposed to decrease the chance of recurrence (Dutt and Gao 2009).

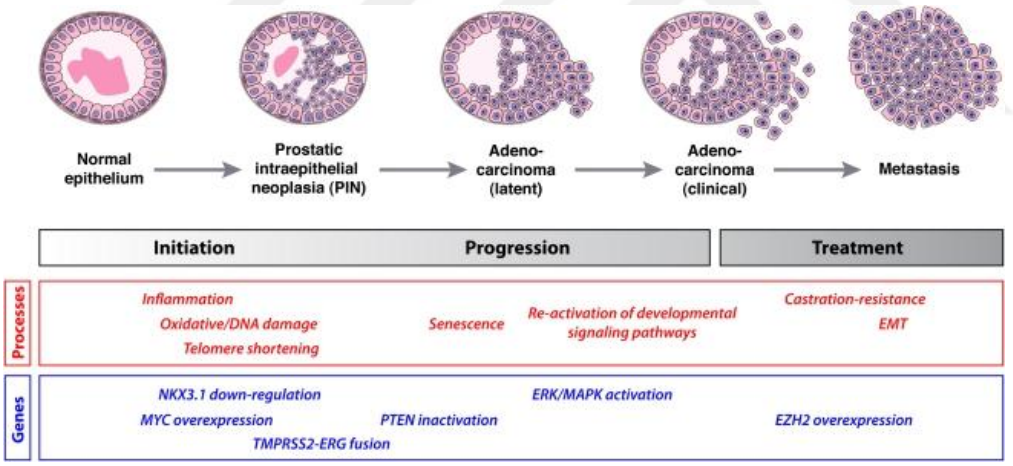


Figure 1.1 Important molecular signs of the prostate cancer progression stages. Adapted from (Shen and Abate-Shen 2010).

1.1.2 Colorectal Cancer

Colorectal cancer (CRC) develops from an uncontrollable growth of cells in the colon or rectum. It manifests in three basic stages: first, abnormal growth of cells in inner lining of the large intestine called polyp, leading to growth on the wall of colon or rectum; second, penetrating either blood or lypmh vessels; and third, spreading out to the other organs. According to American Cancer Society, with 49,190 deaths in 2016, CRC is the third leading cause of cancer-related deaths in the United States; the occurrence and death rates of this disease increases with age. When the gender difference is taken into consideration, CRC incidence is approximately 30-40% more in men than in women, the underlying reasons for which are still not fully understood (R. Siegel, Naishadham, and Jemal 2013; American Cancer Society 2014). The risk factors for CRC can be categorized as uncontrollable and controllable. Controllable or environmental factors are composed of physical inactivity and obesity, heavy alcohol and cigarette consumption, excessive consumption of red meat and low dietary fiber intake. Uncontrollable risk factors include aging, genetic mutations in important tumor suppressor genes like *MLH1*and *MSH2* (MutL homolog 1 & MutS protein homolog 2; which are involved in DNA mismatch repair) and *APC* (Adenomatous polyposis coli; an important negative regulator of cell adhesion), familial history of CRC and adenomatous polyps or suffering from inflammatory bowel disease (Haggar and Boushey 2009).

A well-defined set of progression steps have been defined for CRC that may eventually lead to development of the disease. Physiologically, the order of formation of tumors is the accumulation of dividing cells in the crypts; then this hypergrowth forms dysplastic tissue, finally leading to formation of adenomas and carcinomas. At the molecular scale, the first step is loss of

function of the APC regulatory system which normally suppress the mitogenic protein β-Catenin; therefore, causing adenomatous growth. In later stages, gain of function mutations in the oncogenic RAS family of proteins are observed, where the mutations are haplosufficient for progression. Subsequently, in adenomas, 18q chromosomal loss occurs, where important genes such as DCC (a surface protein with extensive homology to other cell adhesion and surface glycoprotein molecules), SMAD4, and SMAD2 (taking part in the TGF- β pathway, involved in slowing down cell proliferation) reside.

Finally, in order to progress to carcinoma, there is loss of function mutations in p53, one of the important cell cycle stress regulators for induction of apoptosis (Wodarz 2009). The main mutations and the CRC progression are summarized in Figure 1.2.

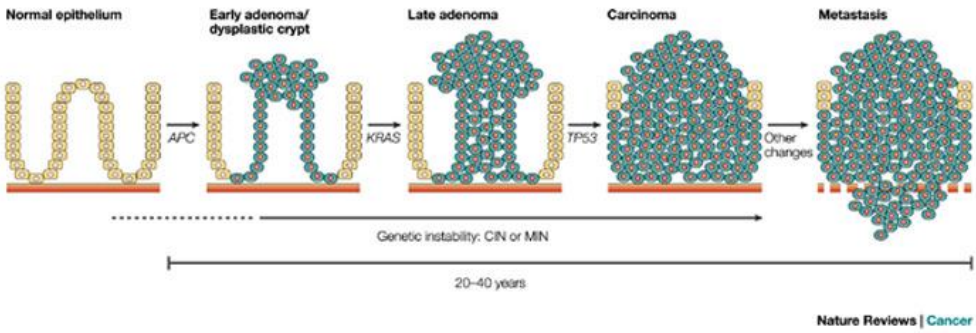


Figure 1.2 Common mutations acquired during different progression stages in colorectal carcinoma; retrieved from (Rajagopalan et al. 2003).

The emergence and progression of CRC are found to be interconnected to many other diverse cellular processes resulting in aberrant growth of cells. Haydon and Jass suggested that besides the mutations of tumor suppressor genes like APC, p53 or oncogene K-Ras, perturbation of the DNA mismatch-repair system due to microsatellite instability (MSI) can also cause CRC, resulting in strand slippage and replication errors. Also, CpG island methylation is another reason for cancers with high MSI (Haydon and Jass 2002). In addition to alterations in important genes, modifications of signaling pathways like the PI(3)-K pathway that is important for controlling cellular growth and invasion are linked to CRC (Parsons et al. 2005). Moreover, aberrant regulations of transcription factor nuclear factor-κB (NF-κB), one of the key regulator of immunity, cell proliferation and death, is also linked to colorectal cancer development and drug-resistance (Hassanzadeh 2011).

1.1.3 Cancer Cell Models in this study

PC-3 is a grade IV adenocarcinoma prostate cancer cell line generated from a bone metastatic site from 62-year-old Caucasian male (ATCC, Figure 1.3 A). These poorly differentiated cells follow an anchorage-independent growth in both monolayers and soft-agar suspension in culture; and can cause subcutaneous tumors in nude mice. Additionally, they are less dependent on serum for their growth and are not responsive to androgens, glucocorticoids, epidermal or fibroblast growth factors (Kaighn et al. 1979).

LNCaP adenocarcinoma cells are from 50-year-old Caucasian male and derived from lymph metastatic site, tumorigenic in nude mice and unlike PC-3, they express PSA, AR and estrogen receptor, resulting in sensitivity to

androgens (ATCC, Figure 1.3 B). In addition to this, they grow very slowly in loosely attached clusters (ATCC).

HCT-116 is an adherent colorectal carcinoma cell line and it grows in monolayers *in vitro*. Also, it is invasive and highly motile *in vitro* (ATCC). These cells are characterized as highly aggressive and non-differentiating, giving rise to no lumen-forming colonies (Yeung et al. 2010).

Additional detailed comparison of some cellular characteristics was given in Table 2.1 in Chapter 2.

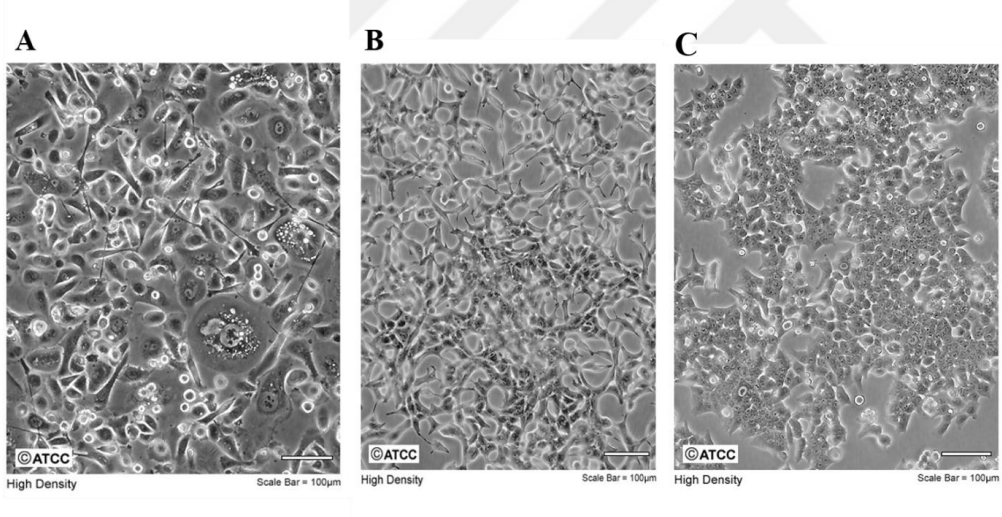


Figure 1.3 Morphologies of PC-3 (A), LNCaP (B) and HCT-116 (C) cells. Adapted from ATCC.

1.2 Inflammation

Inflammation is an immune response against infections or injuries composing of these significant stages: initial recognition via innate immune system receptors; then, production of inflammatory mediators like chemokines, cytokines, vasoactive amines and eicosanoids by macrophages to recruit neutrophils to this place. After proper elimination of infectious agents, a resolution phase is initiated during which homeostasis is restored via the release of pro-resolution mediators such as lipoxins and resolvins by tissue-resident macrophages, inhibiting any further neutrophil recruitment and initiating tissue remodeling (Medzhitov 2008).

However, when it is dysregulated or inefficiently resolved, inflammation becomes chronic. Unlike acute inflammation, chronic inflammation is persistent and involves a continuous low-level inflammatory response; and it may result in complex diseases like cancer as well as autoimmune diseases like inflammatory bowel disease (IBD) in the colon.

1.2.1 Cancer Related Inflammation (CRI)

Chronic or dysregulated state inflammation may lead to the induction of DNA damage and chromosomal stability. Initiation and promotion of cancer may ensue by promoting cell proliferation, angiogenesis and tissue remodeling eventually leading to metastatic spread. Tumor development can be significantly favored by the microenvironment of the tumor cells that contains inflammatory mediators such as cytokines and growth factors as well as innate and adaptive immune cells and stromal cells. In addition to these, in the tumor cells, important signaling pathways like Janus-activated kinase (JAK),

phosphatidylinositol-3-kinase (PI3K/AKT), and mitogen-activated protein kinase (MAPK) are altered to induce proliferation. Additionally, various transcription factors like signal transducer and activator of transcription (STAT) family members, NF-κB, activator protein-1 (AP-1), and hypoxia inducible factor-1α (HIF-1α) are activated to induce proliferation, angiogenesis, and metastasis (Yongzhong Wu et al. 2014). The main events in cancer-related inflammation (CRI) are the infiltration of white blood cells such as tumor-associated macrophages (TAMs), the existence of cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6; and chemokines like CCL2 and CXCL8. Tissue remodeling and angiogenesis are also important factors in CRI. For instance, TAMs start to secrete cytokines, growth factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and matrix-degrading enzymes to feed angiogenesis (Colotta et al. 2009).

Inflammatory cells may release mediators like reactive oxygen and nitrogen species, metalloproteinases (MMPs) and prostaglandin E_2, which can maintain and augment the inflammatory state in the tumor microenvironment. Aside from affecting the tumor microenvironment, inflammatory mediators can also cause chromosomal instability via dysregulated DNA repair pathways like homologous recombination or double strand break repair, which result in a heterogenous cancer population; thus cancer related inflammation can actually be counted as seventh hallmark of cancer (Colotta et al. 2009).

1.2.2 The effect of inflammation on PCa and CRC

In prostate cancer, inflammation is one of the main players in disease progression; infiltration of macrophages and immune suppressor cells were found to be positively linked with PCa. The transcription factor NF-κB is

activated in PCa to produce more tumor-promoting cytokines like IL-6 and TNF-α. In addition to these, inflammasomes like NRLP1 and AIM2 play an important role in the upregulation of pro-inflammatory cytokines (Karan and Dubey 2016) Moreover, it has been reported that single nucleotide polymorphisms (SNPs) in cytokines like IL-10, IL-1β, TNF have correlation with prostate cancer susceptibility and aggressiveness of the cancer (Zabaleta et al. 2009). Furthermore, prostatic intraepithelial neoplasia (PIN) can be triggered by prostatic infections and regions of prostatic atrophy. The microbial species contributing to PCa are generally sexually transmitted infection (STI)-related microorganisms like *Chlamydia* trachomatis and Trichomonas vaginalis, prostatitis-related microbes like *Escherichia* coli and Pseudomonas spp., and viruses like human papilloma virus (HPV). Specifically, STI-infected patients have greater tendency to have high levels of prostate specific antigen (PSA; a marker for PCa) in their sera (Sfanos, Isaacs, and De Marzo 2013).

Sporadic colorectal carcinoma also shows a strong link to inflammation. In the intestine, epithelial cells function as a selective barrier between the host and the environment, which contains millions of bacteria; therefore, any damage to this barrier causes infections, chronic inflammatory responses and tumorigenesis. Patients having ulcerative colitis or Crohn's disease are predisposed to CRC due to the upregulation of pro-inflammatory cytokines like IL-6 and IL-23 for initiation and maintaining gut inflammation, upregulation of tumor necrosis factor- alpha (TNF-α) mediated gene mutations, chromosomal instability, micronuclei formation, DNA damage via increased oxidative stress inducing mutations in tumor suppressor APC, hyperactivation of NF-κB to induce proliferation and inhibit apoptosis. Moreover, duration and the extent of colitis in these patients can also affect the development of tumors (Triantafillidis, Nasioulas, and Kosmidis 2009).

Bacterial infections with agents such as *Helicobacter pylori* and *Escherichia coli* has been shown to fuel inflammation and manipulate important host pathways like Wnt and JNK for malignant transformation (Kostic et al. 2013). Furthermore, it has been emphasized that DNA damage in intestinal epithelium can activate innate immune system through the activation of inflammasomes like AIM2 and NLRP3. Among 414 CRC patients, 67% of them showed lower AIM2 in tumor tissue in contrast to normal tissue and it is associated with higher mortality rate (Dihlmann et al. 2014). Transformation to invasive carcinoma may also occur through the acquisition of mutations in the tumor suppressor gene *TP53*; which can in turn activate NF-κB and prolong the inflammatory state (Lasry, Zinger, and Ben-Neriah 2016).

Consequently, it is now known that inflammation and cancer are interconnected, with either one being able to "feed" the other, thereby generating a vicious cycle. It is thus imperative to unravel this relationship and find ways by which the cycle can be broken.

1.2.3 Lipid Mediators of Inflammation

Lipid mediators of inflammation are the products of both omega-6 and omega-3 polyunsaturated fatty acids (PUFA). Membrane phospholipid arachidonic acid (AA) is a major omega-6 PUFA and it is metabolized into leukotrienes (LTs) and prostaglandins (PGs) that are the important players in adaptive immunity and implicated in many inflammatory disorders like septic organ failure, rheumatoid arthritis, asthma and cancer. When AA is utilised by cyclooxygenases like COX-1 or COX-2, it gives rise to prostaglandins, promoting pain and inflammation. On the other hand, metabolism of AA through the lipoxygenase (LOX) pathway leads to the production of

leukotrienes, which can act as inflammatory mediators, or in a temporal manner be further metabolized into precursors that play roles in resolution of inflammation (Funk 2001). In addition to these, metabolism of omega-3 PUFAs, in other words fish oils, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through LOXs results in the production of a series of compounds known as resolvins (Rvs) and lipoxins (Lxs), all of which have roles in the process of resolution of inflammation to halt inflammation and maintain the homeostatic healthy state (Serhan 2007; Mao, Wang, et al. 2015).

The relationship between lipid mediators and cancer is significant (Wang and Dubois 2010). Eicosanoids having pro-inflammatory and pro-cancerous roles promote carcinogenesis via activation of signaling on tumor epithelial cells to induce proliferation, migration, invasion (Tuncer and Banerjee 2015). Additionally, the lipid mediators can manipulate the normal microenvironment to encourage spreading and tumor growth through the secretion of growth factors and inflammatory mediators or even turning this microenvironment into an immune-suppressive state to protect the cancer cells from elimination by the immune system; then, they can spread to other organs.

For instance, high levels of prostaglandin PGE_2 in tumors is reported in many malignancies and is associated with poor prognosis of many cancer types such as colon, lung, breast. PGE_2 induces proliferation by activating oncogenic signaling pathways such as the MAP Kinase and glycogen synthase kinase-3β (GSK3β)–β-catenin pathways. Similarly, PGE_2 promotes CRC cell migration and invasion via the activation of epidermal growth factor receptor (EGFR)–PI3K–Akt signaling. However, stromal cells can secrete the anti-inflammatory prostaglandin PGD_2 that inhibits cell proliferation *in vitro* in

PCa via trans-activating a nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ). In addition to this, pro-inflammatory leukotrienes like LTB_4 and LTD_4 are also reported to induce cell migration and invasion (Wang and Dubois 2010). Therefore, detailed investigation of the roles of these lipid mediators in cancer progression is needed to better understand their role in tumorigenesis and develop better cancer preventive agents. Moreover, the enzymes responsible for the production of these lipid mediators are also deregulated in cancer.

COXs composed of COX-1 and COX-2 are enzymes that synthesize prostaglandins from AA, promoting pain and inflammation. COX-1 is expressed in most of the tissues like kidney, lung, stomach and colon and important in maintenance of integrity of gastric mucosa by producing cytoprotective prostaglandins, such as prostacyclin and PGE; however, COX-2 is only expressed via inflammatory stimuli like cytokines, pathogens to promote acute inflammatory responses (Williams, Mann, and DuBois 1999). Upregulation of COXs, particularly COX-2 is seen in various cancers like breast, colon, lung and prostate and is tied to poor prognosis (Soslow et al. 2000). Thus, blockage of COXs with non-steroidal anti-inflammatory drugs (NSAIDs) is a current therapeutic topic in cancer patients (Harris 2009). In addition to COX, the impact of LOX pathways is also reported in cancer. 5-LOX and 12-LOX have pro-carcinogenic roles, whereas 15-LOX-2 has anti-carcinogenic roles and the role of 15-LOX-1 in cancer is questionable and will be discussed in further sections 4.3 and 5 (Pidgeon et al. 2007a).

1.3 Lipoxygenase (LOX) Metabolism and Cancer

LOX classification depends on which position of the carbon on AA is oxygenated, and this family includes 5-LOX, 8-LOX (murine), 12-LOX, and 15-LOX-1 (12/15-LOX in mice) or 15-LOX-2. Thus, based on the metabolites produced as a result of oxygenation of AA at various positions, the cellular outcomes can be very different. For example, 15-LOX-1 preferably oxygenates linoleic acid (LA) and produces 13-hydroxyoctadecadienoic acid (13-(S)-HODE); however, 15-LOX-2 preferentially acts on AA to produce 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-(S)-HETE) (Tuncer and Banerjee 2015). Both 13-(S)-HODE and 15-(S)-HETE can act as ligands for PPARγ, an orphan nuclear receptor important for inhibiting proliferation and inducing apoptosis and differentiation in several types of cancer (Klil-Drori and Ariel 2013). 5-LOX oxidizes AA and results in the production of 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) which is subsequently metabolized to leukotriene A_4 (LTA_4) (Rådmark and Samuelsson 2009). Leukotrienes are essential mediators of allergic responses and inflammation by acting as potent chemo attractants and activators of immune cells like neutrophils, eosinophils, dendritic cells and macrophages at the site of inflammation (J. N. Sharma and Mohammed 2006; Kuhn, Banthiya, and Van Leyen 2015).

12-LOX has three isoforms that depends on tissue localization, substrate specificities and sequence homology: leukocyte-type, platelet-type and epidermal-type. For example, platelet type 12-LOX forms 12-(S)-HETE from AA but the leukocyte type forms 12-(S)-HETE or 15-(S)-HETE either from AA or LA (Yoshimoto and Takahashi 2002). Studies have shown that both 5-

LOX and murine 12-LOX are normally not expressed in epithelial cells but when there are pro-inflammatory stimuli, constitutive expression of these enzymes occur, which may promote the development and progression of different solid tumors (Pidgeon et al. 2007b). In the context of prostate cancer, blockade of 5-LOX activity has been shown to inhibit proliferation (Matsuyama et al. 2005). Additionally, the expression of 12-LOX is linked to prostate tumor grade and stage, with the highest levels of 12-LOX found in metastatic prostate cancers (Nie et al. 2001). 8-LOX is the murine homolog of the human 15-LOX-2 enzyme. This enzyme has roles that are currently not well defined and has been reported to show controversial roles in the context of cancer (Claus Schneider and Pozzi 2011). Therefore, these different metabolites lead to different outcomes in a cell in terms of neoplastic transformation (Claus Schneider and Pozzi 2011) and which isoform is expressed in which tissue is important for the outcome.

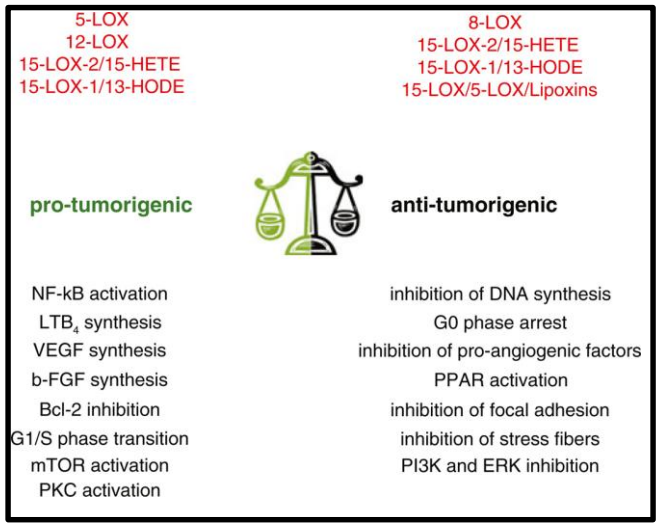


Figure 1.4 Lipoxygenases and their opposing roles in cancer by affecting many important pathways for proliferation, apoptosis, angiogenesis. Adapted from (Claus Schneider and Pozzi 2011).

1.4 15-Lipoxygenase-1 (15-LOX-1)

15-LOX-1 is from LOX family that has gained importance in the context of inflammation and cancer in the past years and has ambiguous roles in different types of cancer. The enzyme is generally expressed in reticulocytes and macrophages (Ford-Hutchinson 1991). The *ALOX15* gene is located on chromosome 17, locus p13.3, and has 14 exons (GenBank: NC_000017). It shares 40% identity with its closest relative, 15-LOX-2 (Brash, Boeglin, and Chang 1997). The enzyme is primarily located in the cytosol, but may be associated with organelles such as the endoplasmic reticulum and mitochondrial membranes (Walther et al. 2002). 15-LOX-1 can oxygenate a broad range of substrates that includes esterified forms of naturally occurring polyenoic fatty acids even when they are esterified in phospholipids in biomembranes or lipoproteins (Walther et al. 2002). Alternatively, it may act on free fatty acids and the oxygenated and thereby more polar products, such as 15-HETE and 13(S)-HODE may be incorporated into the membrane phospholipids (Nigam and Schewe 2000).

1.4.1 15-LOX-1 in Inflammation

Based on the availability of its substrate in the cell, the expression and activity of 15-LOX-1 in immune cells can change. The function of 15-LOX-1 in inflammation is primarily anti-inflammatory and pro-resolution; therefore, after an infection or injury this enzyme is responsible for re-establishing homeostasis. For example, in the presence of IL4, macrophages express 15-LOX-1 and produce 13-(S)-HODE from omega-6 fatty acid LA. This metabolite is ligand for PPAR-γ, and can result in the differentiation of pro-

inflammatory M1 macrophages to an M2 phenotype that is associated with tissue repair and resolution of inflammation (Dioszeghy et al. 2008). In eosinophils, the expression of 15-LOX-1 led to the upregulation in expression of CXCL13, a B cell chemoattractant in macrophages, which is also important for resolution (Tani et al. 2014). In parallel with this, when 12/15-LOX (the mouse isoform of 15-LOX) was administered exogenously, a reduction in neutrophil recruitment was observed (Tani et al. 2014), suggesting an anti-inflammatory role of this enzyme. At the end of acute inflammation, an eicosanoid class switching occurs when the tissue produces lipid mediators suitable for resolution of inflammation, rather than those mediating acute inflammation (Dennis and Norris 2015).

15-LOX-1 can also metabolize omega 3 fatty acids like docosahexaenoic acid (DHA) to form D-series resolvins (RvDs) and protectins (PDs) that act as a pro-resolution agents (Serhan 2014). The expression of 15-LOX-1 in eosinophils was shown to be necessary for the production of protectin D1 (PD1) and the resolution of inflammation (Yamada et al. 2011). Lipoxins (LXs) are pro-resolution mediators produced by combined action of various LOXs and aspirin-acetylated COX-2 in a transcellular manner (Fierro et al. 2003; Serhan et al. 2015). Aspirin not only blocks COX-2 and production of the pro-inflammatory prostaglandins, but also catalyzes the production of 15-*R*-HETE from AA and subsequently lipoxins (Serhan 2014). 15-LOX-1 is also known to lead to the production lipoxin LXA_4 during resolution of inflammation (Serhan et al. 2015). 15-LOX-1 therefore is a key player in halting inflammation and promoting resolution through metabolism of the PUFAs AA, LA, DHA and EPA for homeostasis (Figure 1.4). Chronic inflammation very frequently leads to cancer (Elinav et al. 2013); therefore, the role of an anti-inflammatory enzyme such as 15-LOX-1 needs to be elucidated in cancer. For example, in CRC, 15-LOX-1 is mostly down

regulated and consequently, its metabolite product 13-(S)-HODE levels were decreased and 13-S-HODE inhibits growth and promotes apoptosis in transformed colonic epithelial cells (Imad Shureiqi et al. 1999).

Overexpression of 15-LOX-1 caused decreased proliferation, motility, migration and invasion; on the other hand, lead to increased apoptosis in HCT-116 and HT-29 CRC cell lines (Çimen, Tunçay, and Banerjee 2009). 15-LOX-1 expression has been shown to inhibit the activity of the inflammatory transcription factor NF-κB. Our group showed *in vitro*, overexpression of 15-LOX-1 in HCT-116 and HT-29 CRC cell lines resulted in the degradation of inhibitor of kappa B (IκBα); thereby, decreased transcriptional activity of Nuclear factor kappa B (NF-κB) by inhibiting nuclear translocation of p65 and p50. In addition to these, when HCT-116 cells treated with 13-(S)-HODE, product of 15-LOX-1 and ligand for PPARγ and increased its transcriptional activity. Moreover, selective PPARγ antagonist GW9662 showed exact opposite effects (Cimen, Astarci, and Banerjee 2011). In addition to this, in CRC, 13-S-HODE can also cause inhibition of PPAR-δ (a nuclear receptor that is important in the progression of cancer and chronic inflammatory diseases) activation; resulting in increased apoptosis both *in vivo* and *in vitro* (Imad Shureiqi et al. 2003). Furthermore, IL-6/STAT pathway is linked with colitis associated colon cancer and it is upregulated by PPAR-δ. Targeted transgenic 15-LOX-1 expression in mice intestine, resulted in down regulation of IL-6 and PPAR-δ expression, STAT3 phosphorylation and downstream target expression (Mao, Xu, et al. 2015). Therefore, 15-LOX-1's importance an anti-inflammatory agent can be exploited to ameliorate the state of chronic inflammation which, if left unattended, can promote cancer progression**.**

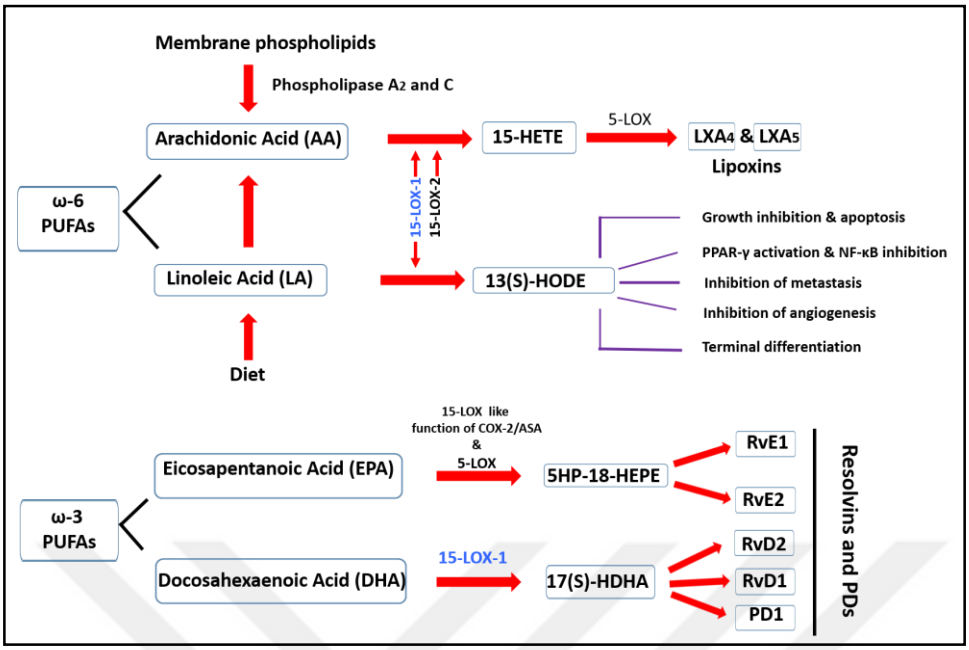


Figure 1.5 Metabolites of 15-LOX-1 and their roles in cancer.

1.4.2 Regulation of 15-LOX-1

Regulation of transcription of *ALOX15* is complex and involves multiple mechanisms at the promoter or chromatin level. The cytokines IL-4 and IL-13 can induce the expression of *ALOX15* in human peripheral monocytes, A549 lung epithelial carcinoma cells, human trachea-bronchial epithelial cells and human colon cancer Caco-2 cells through the transcription factor STAT6 (Kuhn, Walther, and Kuban 2002). The induction of 15-LOX-1 by IL-13 is via the activation of JAK2 and TYK2 tyrosine kinases after STAT6 dimerization and nuclear import (Roy and Cathcart 1999). In the case of IL-4 induction, the up-regulation of the histone acetyltransferase activity of the Creb-binding protein/p300 (CBP/p300), which acetylates both nuclear histones and STAT6 in the lung carcinoma cells was observed

(Shankaranarayanan et al. 2001). Histone acetylation also plays an important role in unmasking the STAT6 binding sites to the promoter of *ALOX15*; after which phosphorylated STAT6 can activate its transcription (Shankaranarayanan et al. 2001). The phosphorylation of STAT1 and STAT3 through p38 MAPK was shown to be important regulators of 15-LOX-1 expression in human primary monocytes after IL-13 treatment (Xu et al. 2003).

The transcription factors GATA-6 and SP1 have also been implicated in the transcriptional regulation of 15-LOX-1. Wittwer *et al.* screened the entire coding region and 3'UTR of *ALOX15* from 44 healthy Caucasians who had higher 15-LOX-1 expression in macrophages due to single nucleotide polymorphism -292C-to-T base exchange, generating a novel SPI1 transcription factor binding site in 15-LOX-1 promoter (Wittwer, Marti-Jaun, and Hersberger 2006). On the other hand, GATA-6 plays suppressive role in 15-LOX-1 expression. For example, GATA-6 knockdown resulted in restoration of 15-LOX-1 expression in CRC cells in which 15-LOX-1 expression is lost. Interestingly, GATA-6 expression is elevated in transformed colon epithelial cells than in normal epithelia (Imad Shureiqi et al. 2007).

In addition to transcriptional regulation, chromatin modifications are also implied in 15-LOX-1 regulation through post-translational modifications of histones such as acetylation and methylation (Bannister and Kouzarides 2011). Nonspecific histone deacetylase inhibitors (HDACi), like sodium butyrate and suberoylanilide hydroxamic acid (SAHA) and also depsipeptide, a selective HDAC1 and HDAC2 inhibitor, were shown to increase 15-LOX-1 transcription in CRC cells (Kamitani, Geller, and Eling 1998; Kamitani et al. 2001; L C Hsi et al., n.d.). Liu *et al.* showed that independent of STAT-6

activation, transcriptional upregulation of 15-LOX-1 in CRC cells required H3K9me2 demethylation by KDM3A and inhibition of histone H3 and H4 acetylation by HDACi (Xiangsheng Zuo, Morris, and Shureiqi 2008).

Complex regulatory mechanisms such as promoter hypermethylation to repress 15-LOX-1 transcriptionally was reported in CRC patients and CRC cell lines, while the promoter methylation was not observed in the healthy control group (Xiangsheng Zuo et al. 2008). Mechanistically, the competition between DNA methyltransferase-1 (DNMT-1) and histone deacetylases HDAC1 and HDAC2 of the NuRD complex to the same region of 15-LOX-1 promoter was shown to be crucial regulation for 15-LOX-1 in CRC cells (Liu et al. 2004; Xiangsheng Zuo et al. 2008).

Chromatin remodeling is also important regulatory mechanism for 15-LOX-1 expression in PCa as well. LNCaP cells having high levels of histone methyltransferase SMYD3 and its inhibition caused decreased 15-LOX-1 expression, proposing the importance of the regulation of 15-LOX-1 promoter in prostate cancer by histone methylation/demethylation (Liu et al. 2012). Contrary to hypermethylation of its promoter in colorectal cancer resulting in decreased expression, hypermethylation was also observed cancer-associated high grade prostatic intraepithelial neoplasia (HGPIN) (U. P. Kelavkar et al. 2007). In this case, surprisingly, hypermethylation of a particular CpG (HpyCH4IV site in the 5′ -flanking region −217 to −474 in the 15-LOX-1 promoter) in the tumor samples and cancer cell lines led to the upregulation of 15-LOX-1 expression (U. P. Kelavkar et al. 2007), suggesting the regulation of this gene in different cancer types result in varying outcomes.

1.5 The effect of 15-LOX-1 on Carcinogenesis

Different metabolites of this enzyme lead to various cellular fates; therefore, understanding whether 15-LOX-1 is pro- or anti-carcinogenic is entirely context dependent. Generally, the substrates of 15-LOX-1 either ω-3 and ω-6 PUFAs have opposite functions in carcinogenesis. Therefore, while oxygenation products of LA and AA (the ω-6 PUFAs) generally possess pro-carcinogenic effects, oxygenation products of eicosapentaenoic acid (EPA) and DHA (ω-3 PUFAs) have pro-resolution anti-carcinogenic properties (Chapkin, McMurray, and Lupton 2007).

The literature suggests an anti-tumorigenic effect of 15-LOX-1 in diverse cancer types like colon, lung and breast. A screening study of 128 cancer cell lines encompassing a wide range of tumor types indicated that terminally differentiated cells have considerably more 15-LOX-1 mRNA levels as compared to cancer cell lines. The levels of 15-LOX-1 detected in cancer cell lines were even lower than that of p16 (INK4A), a tumor suppressor that is commonly lost in cancer cells (Moussalli et al. 2011). In non-small cell lung cancer (NSCLC) patients, decreased expressions of 15-LOX-1 and 15-LOX-2 were shown to result in decreased production of 15(S)-HETE and 13(S)-HODE metabolites respectively. This reduction, in turn, led to decreased activity of PPARγ, a transcription factor that is important for the induction of apoptosis and inhibiting cell growth (Yuan et al. 2010). In breast carcinoma, 15-LOX-1 and 15-LOX-2 staining was shown to be lower in metastatic patients, suggesting the tumor-suppressive capacity of these proteins (Jiang et al. 2006). In pancreatic cancer, a slight or complete loss of 15-LOX-1 expression was observed, while *in vitro* overexpression of the enzyme led to decreased cell growth (Hennig et al. 2007). Examination of normal human

bladder and bladder tumor specimens by immunohistochemistry, Western blot and PCR indicated a significant downregulation of 15-LOX-1 expression in stage T3/T4 bladder tumors compared with controls (Philips et al. 2008). Adenoviral transfection of 15-LOX-1 in a rat model of malignant glioma resulted in the increased production of 13(S)-HODE and caspase-3, thereby enhancing apoptosis, emphasizing the pro-apoptotic role of 15-LOX-1 (Helena Viita et al. 2012). For non-small cell lung cancer cells, exogenous 13(S)-HODE and 15(S)-HETE treatment resulted in increasing the activity of PPARγ, inhibited cell proliferation, induced apoptosis, and activated caspases 9 and 3, emphasizing anti-carcinogenic role of 15-LOX-1 in this cancer type as well (M.-Y. Li et al. 2015).

The role of 15-LOX-1 in hematological malignancies is currently unclear; however, published data suggest an anti-carcinogenic role. No 15-LOX-1 staining was observed by immunohistochemistry in non-Hodgkin lymphoma (NHL), whereas 85% of classical Hodgkin lymphoma (HL) showed 15-LOX-1 expression in tumor samples, concomitant with mRNA levels, suggesting that 15-LOX-1 may be a candidate biomarker for HL (Claesson et al. 2008). Primary mediastinal B-cell lymphoma (PMBCL) tissue samples and the Karpas-1106P cell line were also assayed for 15-LOX-1 expression.

The effect of 15-LOX-1 on colorectal cancer has been investigated extensively with a consistent loss in expression of the protein reported. In a study of 126 samples of primary colorectal carcinoma at stages I-IV, decreased 15-LOX-1 gene expression was observed in human colorectal adenomas and carcinomas compared to the normal mucosa (Heslin et al. 2005). Treatment of colon carcinoma cell line Caco-2 with the metabolite 13(S)-HODE in resulted in decreased cell proliferation (Nixon et al. 2004).

Furthermore, tumors derived from the colon cancer cell line HCT-116 that ectopically express 15-LOX-1 were smaller than the tumors derived from empty vector-expressing HCT-116 cells in athymic nude mice (Nixon et al. 2004). *In vitro* treatment of RKO and HT-29 CRC cell lines with 13(S)-HODE resulted in reduced proliferation and enhanced apoptosis (Imad Shureiqi et al. 1999), implying that a functional enzyme was needed for exerting the tumor-suppressive effects. Re-expression of 15-LOX-1 by adenoviral transfection to HT-29 and LoVo CRC cells inhibited growth of cancer cells in xenografts and down-regulated important anti-apoptotic markers like XIAP and Bcl-xL, suggesting the apoptotic properties of 15-LOX-1 (Yuanqing Wu et al. 2008). Treatment of RKO and HT-29 cells with the non-steroidal anti-inflammatory drugs (NSAIDs) sulindac and NS-39 led to increased 15-LOX-1 expression and subsequent 13(S)-HODE production, cell growth inhibition and increased apoptosis (I Shureiqi et al. 2000). In the same study, the effects of the NSAID treatments became unobservable when 15-LOX-1 was inhibited with caffeic acid (I Shureiqi et al. 2000). In addition, exogenous addition of 13(S)-HODE to these cells led to growth inhibition and apoptosis (I Shureiqi et al. 2000). Treatment with celecoxib, another NSAID that selectively inhibits COX-2, resulted in a two-fold increase in 15-LOX-1 expression and induced apoptosis, suggesting the therapeutic role of celecoxib in colorectal cancer (Heslin et al. 2005). Interestingly, a negative correlation between the expression and activity of 15-LOX-1 and COX-2 has been reported in colonic neoplasia indicating an alteration in the species of bioactive lipids being produced in the malignant tissues from the pro-apoptotic molecules to the more mitogenic prostaglandins (Yuri et al. 2007).

Re-expression of 15-LOX-1 in colon cancer (Çimen, Tunçay, and Banerjee 2009), pancreatic cancer (Hennig et al. 2007), glioblastoma multiform (Linda C Hsi et al. 2011) and breast cancer (Tavakoli Yaraki and Karami Tehrani

2013) have been reported to reduce proliferation and induce apoptosis. 15-LOX-1 over-expression in the colon cancer cell lines HCT-116 and HT-29 was shown to reduce cellular motility, wound healing, migration and invasion *in vitro* (Çimen, Tunçay, and Banerjee 2009). Mechanistically, this was indicated to be through a reduction in the expression of metastasis associated protein-1 (MTA-1) (Çimen, Tunçay, and Banerjee 2009) that is also a member of the Nucleosome Remodeling Deacetylase (NuRD) complex, chromatin remodeling complex that is responsible for the silencing of 15-LOX-1 in CRC (X Zuo et al. 2009). Recently our group found that expression of 15-LOX-1 and MTA-1 negatively correlated in specific subgroups of CRC microarray data sets. Mechanistically, the negative correlation was found to be through the reduced recruitment of NF-κB to the MTA1 promoter. Functionally, MTA-1 overexpression in CRC cells that stably expressed 15-LOX-1 rescued the slower motility (Tuncer et al. 2016). Additionally, the loss of motility in CRC cell lines expressing 15-LOX-1 has also been attributed to a loss of VEGF expression and secretion (Yuanqing Wu et al. 2014).

Opposite to these anti-tumorigenic roles, several studies have suggested a pro-carcinogenic role of 15-LOX-1 signaling in different cancer types, particularly in prostate cancer. 15-LOX-1 expression was shown to be dramatically high in prostate cancer tissues, primarily in high-grade tumors, as compared to normal tissue samples (U. Kelavkar et al. 2006). Same group also showed that mutated p53 is correlated with 15-LOX-1 with also with the degree of malignancy by using 48 prostatectomy specimens in prostate adenocarcinoma, emphasizing the malignant potential of 15-LOX-1(U P Kelavkar et al. 2000), which was in line with an earlier study showing that due to p53 has close chromosomal proximity to 15-LOX-1 and that gain of function mutations of p53 that may lead to more aggressive and worse prognosis than p53-null tumours (deletions impart only a “loss of function”

mutation). Thus, it may be that a missense p53 mutation contributing a "gain of new function" is worse than no p53 gene product at all (U P Kelavkar and Badr 1999). Overexpression of 15-LOX-1 in PC-3 cells *in vitro* resulted in increased Insulin like growth factor receptor (IGF-1R) activity as well as the activity of downstream MAP kinase protein ERK1/2 and Akt/PKB activity. It was also shown that in the presence of 13(S)-HODE the promoter of the receptor of IGF-1 (IGF1-R) was activated while a correlation between IGF-1R expression and 13(S)-HODE levels was shown (Uddhav P Kelavkar and Cohen 2004). In a transgenic mouse model where human 15-LOX-1 was expressed in mouse prostate (human fifteen lipoxygenase-1 in mouse prostate, FliMP), the increase in human 15-LOX-1 in both homozygous FLiMP$^{+/+}$ and hemizygous FLiMP$^{+/-}$ prostates led to the development of mouse prostatic intraepithelial neoplasia (mPIN) characteristics (Uddhav P Kelavkar, Parwani, et al. 2006). Interestingly, bioactive lipids produced from the metabolism DHA by 15-LOX-1 in prostate cancer cells were shown to reduce cell proliferation by activating PPARγ signaling and apoptosis (O'Flaherty et al. 2012). Moreover, the same metabolic products were shown to be important for the activation of syndecan-1 (SDC-1), a protein that is important in cell to matrix interactions, cellular proliferation and migration as well as caspase-3 activity (O'Flaherty et al. 2012). In another *in vivo* study, in order to understand the pro-tumorigenic role of 15-LOX-1 in PCa, it has been shown that switching omega-3 acid diet from omega-6 acid diet increased apoptosis in cancer cells and decreased proliferation, proposing 15-LOX-1 and COX-2 metabolites of fish oils (EPA and DHA) may be used as dietary intervention for PCa prevention (Uddhav P Kelavkar, Hutzley, et al. 2006). In another study, 15-LOX-1 was shown to be expressed more in hepatocellular cancer cell lines compared to normal hepatic cells with a further exacerbation of the expression under hypoxic conditions in the cancer cells. Mechanistically, the

production of 15-HETE, but not 13(S)-HODE, through 15-LOX-1 metabolism led to the activation of phosphoinositide-3 kinase (PI3K)/Akt/heat shock protein 90 pathway resulting in several pro-carcinogenic changes in the cells (Sun et al. 2009). 15-LOX-2, another isoform that is important in the prostate, skin, esophagus, and cornea, has been reported to have anti-carcinogenic effects in prostate cancer (Nie 2007). 15-LOX-2 was shown to regulate the differentiation of normal human prostate epithelial cells and to be upregulated in cells undergoing senescence, underpinning its tumor suppressive role (Tang et al. 2007).

Opposing effects of metabolites generated from 15-LOX-1 and 15-LOX-2 pathways on prostate cancer on epidermal growth factor (EGF) - and IGF-1 signaling in prostate carcinoma cells were shown. While 13(S)-HODE resulted in the upregulation of the MAP kinase ERK1/2 leading to increased PPARγ phosphorylation, 15-HETE led to the exact opposite effect (Linda C. Hsi, Wilson, and Eling 2002). On the whole, the effect of 15-LOX-1 on carcinogenesis appears to rely on the type of cancer, substrates available and metabolites produced; as a result, a further investigation is required to elucidate this complex relationship.

1.6 Angiogenesis

Neo-angiogenesis is by definition the formation of blood vessels from existing ones in order to fulfill the energy and nutrient demands of tumor cells. Tumor cells can also penetrate these blood vessels for metastasis. Tumor associated angiogenesis is generally associated with blood vessels that have irregular and chaotic organization (Goel et al. 2011). In this multi-step

process, in the presence of proangiogenic factors, endothelial cells start to invade and migrate by degrading basement membrane with proteolytic enzymes matrix metalloproteinases (MMPs) and then, form filopodia. These migrating cells, called “tip cells”, explore the environment with their guidance receptors called Roundabouts (ROBOs); then, stalk cells extend the filopodia and proliferate for sprout elongation. Via interacting with cells from other sprouts, tip cells build vessel loops; and finally, blood flow is initiated, along with the establishment of the basement membrane. At this stage, proangiogenic signals may cease and antiangiogenic signals may begin, resulting in more stabilized connections (Figure 1.5) (Potente, Gerhardt, and Carmeliet 2011).

Tip and stalk cells are regulated by the Notch signaling pathway, which cross-talks with vascular endothelial growth factor (VEGF) signaling; tip cells highly express Notch ligand DLL4; while stalk cells express a different Notch ligand JAGGED1 (JAG1) (Eilken and Adams 2010). Contrary to the tip cells, stalk cells are more proliferative and less efficient in production of filopodia and important in forming vascular lumen and basement membrane. They also form junctions with neighboring cells for maintaining the sprout (Phng and Gerhardt 2009). In angiogenesis, angiopoietins such as angiopoietin-1 (ANG1) can stabilize and make these vessels resistant to leakage; on the other hand, angiopoietin-2 (ANG2), being the antagonist of ANG1, work with VEGF to promote proliferation and migration of endothelial cells (Hoong et al. 2004). Moreover, as tumors proliferate, it creates a hypoxic environment, which activates hypoxia-inducible factors (HIFs) that are responsible for promoting angiogenesis by transcriptionally up-regulating (Büchler et al. 2003). Angiostatins and epistatins are the anti-angiogenic factors that block endothelial cells proliferation and migration to stop the angiogenesis process and return to homeostasis (Tahergorabi and Khazaei 2012).

Other growth factors were also implicated in angiogenesis. For instance, EGF (epidermal growth factor) and TGF-α (transforming growth factor-α) were shown to induce proliferation of endothelial cells and angiogenesis in vivo. In addition to these, FGF also stimulates angiogenesis and regulates sprouting by increasing VEGF (Tahergorabi and Khazaei 2012).

Transforming growth factor -β (TGF-β) signaling has a wide variety of functions from proliferation to differentiation, and participated in the regulation of angiogenesis. The activity of TGF-beta in angiogenesis is controversial with data indicating the promotion of angiogenesis *in vivo* but inhibition of cell growth *in vitro* (Bussolino, Mantovani, and Persico 1997). For instance, a negative correlation between the expression of TGF-beta and VEGF-A ha been reported and along with a TGF-beta mediated inhibition of VEGF-A stability, has been reported in CRC patients (Geng et al. 2013). However, in another study, it was proposed that TGF-β1 promotes angiogenesis through autocrine secretion of TGF-α, a cell survival growth factor by activating PI3K/Akt and MAPK pathway in mouse capillary endothelial cells *in vitro* (Viñals and Pouysségur 2001).

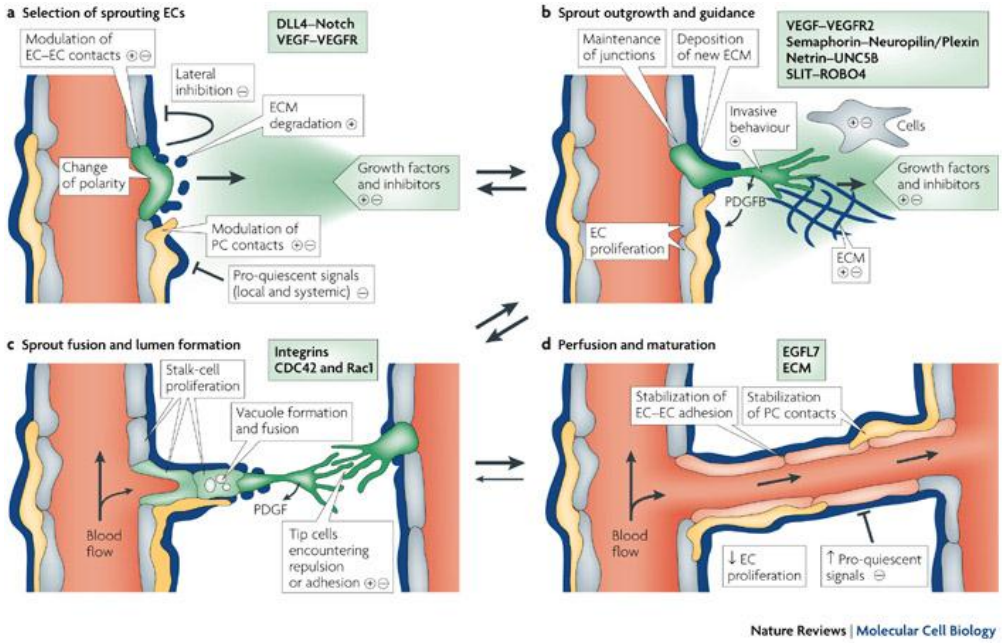


Figure 1.6 Main steps of angiogenesis showing a. sprouting of endothelial cells (ECs) with pro-angiogenic signals (+) like VEGF, which is dependent on the induction of motile and invasive activity, apical–basal polarity modulation, the fine-tuning of cell–cell contacts and local matrix degradation, b. growing of EC sprout by (+) and (-) signals in microenvironment, c. lumen formation from stalk ECs growth and vacuole formation and fusion, d. establishing stabilized vessel by cell junctions, growth factor withdrawal. DLL4, delta-like-4 ligand; EGFL7, epidermal growth factor ligand-7; ROBO4, roundabout homologue-4; VEGFR2, VEGF receptor-2; PDGF, platelet derived growth factor. Retrieved from, (Adams and Alitalo 2007).

1.6.1 Vascular Endothelial Growth Factor (VEGF)

The VEGF family is composed of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. VEGF-A is a dimeric, disulfide-bound glycoprotein, secreted and well-studied isoform. It is important for development of blood vessels during early developmental stages and also important aspect of the wound healing and repair process for providing blood flow to damaged tissue to send oxygen and nutrients for the

growth of reparative cells (Johnson and Wilgus 2014). VEGF monomers have 121, 145, 148, 165, 183, 189, or 206 amino acids, respectively. VEGF-A binds to two tyrosine kinase receptors called VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and promotes angiogenesis by regulating endothelial cell proliferation, migration, vascular permeability, and secretion. VEGFR-2 signaling leads to a strong angiogenic activity, while the soluble VEGFR-1 (sFlt-1) functions as an endogenous VEGF inhibitor by sequestering VEGF with high affinity. Contrary to VEGFR-2, VEGFR-1 has lower tyrosine kinase activity, hence, signaling is weaker. During carcinogenesis, VEGFR-2 knockout mice showed lethal phenotype, contrarily, VEGFR1 knockout mice again showed lethality but due to the hypergrowth of endothelial cells and the disorganized of blood vessels in embryo suggesting opposing roles. In addition to this, both VEGF-A homozygous and heterozygous knockout mice showed lethality, as well. Among the spliced forms of VEGF-A, VEGF-A_{165} has highest biological activity by having higher binding capacity to its co-receptor Neuropilin-1 (Nrp1).

The other forms, namely PlGF and VEGF-B, act through VEGFR-1. PIGF promotes angiogenesis without severe inflammatory response (Shibuya 2013). VEGF-C and VEGF-D are the ligands for VEGFR-3 and regulates mainly lymph angiogenesis and the formation of metastases in lymph nodes. In addition to VEGF-C, VEGF-A also promotes lymph node metastasis by promoting the entrance of tumor cells into lymph nodes and stimulates their growth in metastatic sites. In addition to this, upregulated VEGF-A results in overexpression of the anti-apoptotic protein Bcl-2, not only enhancing endothelial cells' growth but also production of the pro-angiogenic cytokine IL-8 (Nör et al. 2001). Interestingly, the variant VEGF-E is viral-encoded, found in habu snake venom, and induces angiogenesis at the sites of infection of the skin (Shibuya 2013).

1.7 15-LOX-1 in the context of angiogenesis

The role of 15-LOX-1 in angiogenesis appears to be context specific, showing both activation and inhibition of angiogenesis in *in vitro* and *in vivo* models. Anti-angiogenic role of 15-LOX-1 was shown in a hypoxia-induced retinal neovascularization model (RNV) by inhibiting VEGF-A, VEGFR-2 and eNOS in rat microvascular endothelial cells (RMVECs) *in vitro* (Yan et al. 2012). In the same year, another study showed that 15-LOX-1 overexpression in a mouse model of oxygen-induced retinopathy (OIR) inhibited RNV and downregulated VEGF-A expression (Li et al. 2012). The group mechanistically showed that the inhibition of RNV was through the upregulation of PPAR-γ and inhibition of VEGFR-2 expression (Zhi Li et al. 2014). In addition to these, in a rabbit skeletal muscle system, 15-LOX-1 expression inhibited vascular permeability, vasodilation with reduced mRNA expressions of VEGF-A_{165}, PlGF-2 and VEGF0R2 (H. Viita et al. 2008). The mouse ortholog of 15-LOX, 12/15-LOX was reported to take part in retinal microvascular dysfunction that was inhibited with the LOX inhibitor baicalein, suggesting a role in promoting angiogenesis (Al-Shabrawey et al. 2011). The minor product of 15-LOX-1 from AA, 15(S)-HETE was also implicated as a proangiogenic factor by stimulating human dermal microvascular endothelial cell (HDMVEC) tube formation and migration *in vitro* and angiogenesis *in vivo* via the PI3K-Akt-mTOR-S6K1 signaling axis (Zhang, Cao, and Rao 2005). It was also reported that under hypoxic conditions, 15(S)-HETE production was higher in human neonatal vessels than normoxic levels (Setty, Ganley, and Stuart 1985). 15(S)-HETE was also shown to induce angiogenesis in endothelial cells derived from adipose tissue by upregulating the production of CD31 and VEGF through PI3K/Akt/mTOR

signaling in rats (Soumya et al. 2013). On the contrary, another metabolite of 15-(S)-HETE called 15-oxo-ETE inhibited the proliferation of human vascular vein endothelial cells by suppressing DNA synthesis, demonstrating an angiostatic role (C. Wei et al. 2009). Thus, again the metabolites of this enzyme seem to affect the possible outcome.

The effects of 15-LOX-1 expression on angiogenesis have been recently examined in different cancer types and the evidence is controversial, even contradictory. For example, in mouse model of Lewis lung carcinoma, overexpression of 15-LOX-1 in endothelial cells caused inhibition of tumor growth and metastasis (Harats et al. 2005). In another study where HCT-116, LoVo and HT-29 CRC cell lines were used, the restored expression of 15-LOX-1 inhibited VEGF expression and tube formation in endothelial cells via decreasing the stability of HIF1-α, a transcription factor that is upregulated and stabilized in hypoxic conditions to activate VEGF in hypoxia to promote angiogenesis (Yuanqing Wu et al. 2014). However, in prostate cancer, it was suggested that 15-LOX-1 promotes angiogenesis. For instance, 15-LOX-1 overexpression in the human prostate cancer cell line PC-3 was shown to increase the expression of VEGF *in vitro* and increase angiogenesis in subcutaneous xenografts (U P Kelavkar et al. 2001). On the other hand, in a recent study, 15-LOX-1 over-expression in PC-3 cells was shown to reduce angiogenesis through an ubiquitin-mediated enhanced degradation of HIF-1α and a subsequent lower expression of VEGF-A mRNA (Zhong et al. 2014). These conflicting data therefore warrant further studies on the effect of 15-LOX-1 on angiogenesis in cancer.

1.8 Aim of the Study

The role of 15-LOX-1 in angiogenesis in prostate cancer is controversial. Several studies in the past tried to establish a pro-angiogenic role of 15-LOX-1, whereas one very recent study (from the same group) showed an anti-angiogenic role. Therefore, in this study, we aimed to understand and firmly establish the role of 15-LOX-1 in angiogenesis in prostate cancer. Additionally, very few studies have examined the role of 15-LOX-1 signaling in angiogenesis in colorectal cancer. Therefore, based on the literature, in this study, we hypothesized that 15-LOX-1 has a pro-angiogenic role in PCa and an anti-angiogenic role in CRC. We examined angiogenesis in the context of VEGF-A signaling, functional exploration of its effect on tube forming capabilities of endothelial cells and elucidating the underlying pathways by using different PCa and CRC cell line models.

1.9 Contribution to the literature and novelty of the study

We have observed a potent anti-carcinogenic as well as anti-angiogenic role of 15-LOX-1 in both colon and prostate cancers and have proposed a mechanism for this effect in our prostate cancer model. Our data have contributed significantly to the school of thought that 15-LOX-1 mediated signaling plays important role in many different hallmarks of cancer, including angiogenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

Human colorectal cancer cell HCT-116 was recieved from Deutche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Prostate cancer cell line PC-3 and Human Umblical Vein Endothelial Cells (HUVEC) were purchased from ATCC. LNCaP cells were obtained from Ege University. All cell lines used in this study were authenticated at the Genetics Core Facility University of Arizona, USA.

RPMI 1640 medium without phenol red (Biological Industries) containing 10% Fetal Bovine Serum (FBS, Biochrome AG Germany), 1% penicillin-streptomycin (Biochrome, 100units/ml), 2mM L-glutamine (Biochrome, 2mmol/l) was used to grow HCT-116, PC-3 and LNCaP cells. HUVECs were cultured with using CC-4133 EGM Bullet kit (Lonza) medium containing 1% penicillin-streptomycin (Biochrome, 100units/ml) on gelatin-coated flasks/plates to increase their attachment. First, gelatin (Sigma) was diluted in 1:10 ratio with sterile water; then, plates/flasks were covered with it and incubated at 37°C for 1 hour. After removal of excess gelatin, flasks/plates

were kept at 4°C before use, and used within 2 weeks. For all experiments conducted with HUVECs, the passage number of cells was limited to 5. All cell lines were incubated at 37 °C, with 5% CO_2. The tissue culture plastic consumables were obtained either from Corning or Greiner.

Table 2.1 Important characteristics of cell lines in this study.

				REFERENCES		
	PC-3	**LNCaP**	**HCT-116**	**PC-3**	**LNCaP**	**HCT-116**
Type	Prostate Adenocarcinoma	Prostate Adenoarcinoma	Colorectal Carcinoma	ATCC		
Aggressiveness	High	Low	High	(Dozmorov et al. 2009)		(Yeung et al. 2010)
Metastatic Behaviour	Bone	Lymph	Liver and Lung	(Carson JP1, Kulik G 1999)		(Rajput et al. 2008)
Growth Factor/Steroid Dependence	Androgen Independent (AR -)	Androgen Dependent (Mutated AR)	Growth Factor Independent	(Carson JP1, Kulik G 1999)		(Boyd et al. 1988)
Molecular Signatures	mutated PTEN and p53	mutated PTEN wild type p53	Mutated RAS, β-catenin and PIK3CA	(Carson JP1, Kulik G 1999; Carroll et al. 1993)		(Ahmed et al. 2013)

2.1.1 Generation of Hypoxic Environment

A hypoxia chamber was obtained from STEMCELL Technologies that consists of a sealed chamber with an inlet for a hypoxic gas mix consisting of 1% O_2, 5% CO_2, 94% N_2. The chamber was filled with this mixture for 4 min at the rate of 20 liters/minute. The oxygen amount in the chamber was controlled with ToxiRAE II, RAE Systems oxygen sensor. Humidity in the chamber was provided with a petri dish containing 25mL sterile water. To maintain a temperature of 37ºC, the hypoxia chamber was placed inside an incubator maintained in 100% air. Prior to use, the cell culture medium was conditioned in the hypoxia chamber overnight. Cells ($1x10^6$ per well) were seeded into 6-well plates and allowed to attach overnight. Before placing the cells in the hypoxia chamber, the medium was replaced with the medium conditioned in the hypoxia chamber. After 1 h of incubation of the cells under hypoxia, the gas in the chamber was re-flushed with fresh hypoxic gas mixture. For all hypoxia experiments, a simultaneous experiment was carried out under normoxic conditions, i.e. in a 5% CO_2 tissue culture incubator at 37ºC. The duration of incubations under hypoxia was optimized based on cell viability and accumulation of HIF-1α protein. For HCT-116 cells, the duration of incubation was set as 6h.

2.1.2 Overexpression of 15-LOX-1 in Cancer Models

The cell lines used in this study, HCT-116, PC-3 and LNCaP, do not express any endogenous 15-LOX-1. HCT-116 cells were stably transfected with either the empty vector (pcDNA3.1) or a 15-LOX-1 expressing vector (pcDNA3.1-15-LOX-1) in previous studies that were conducted in our lab (Çimen, Tunçay, and Banerjee 2009; Cimen, Astarci, and Banerjee 2011).

These cells are continuously under selection pressure with zeocin (Invitrogen; working concentration: 125µg/ml). For some studies, two monoclones were used and the expression of 15-LOX-1 is confirmed for every two passages.

For transient transfection of either the empty vector (pcDNA 3.1) or the 15-LOX-1 vector (pcDNA3.1-15-LOX-1) into PC-3 cells, 4x 10^5 cells/well were seeded onto 6-well plates and allowed to attach for 24h. When the confluency reached 60-70%, the cells were transfected with X-tremeGENE HP (Roche) according to the manufacturer's protocol, at 1:1 plasmid: agent ratio, with 1 µg, 2 µg or 2.5 µg plasmid. After 24 h of transfection, the expression of 15-LOX-1 was confirmed by either qRT-PCR or Western Blotting. Based on the amount of protein and RNA expressed, 1 µg plasmid amount was used for transfections of PC-3 cells for all experiments.

For the transient transfection of LNCaP cells, cells were plated onto 6-well plates and transfected at 60-70% confluency with X-tremeGENE HP (Roche) according to manufacturer's protocol for 24h. The transfection medium was then changed with complete RPMI 1640 medium and cells were grown for a further 48h. For the optimization of transfection, ratios of plasmid: agent of 1:2, 2:1 and 1:1 with 1 µg and 2µg plasmids were tried. Optimal expression on 15-LOX-1 at both proteın and mRNA levels were observed at 1:1 ratio with 2 µg plasmid amount.

2.1.3 Collection of Conditioned Media (CM)

HCT-116 cells stably transfected with the 15-LOX-1 plasmid (HCT-1E7) or the empty vector (HCT-EVE2) were grown until 70% confluency. The medium was refreshed with complete medium not containing zeocin (selection antibiotic). Then, the cells were incubated for a further 72 h at 37°C

at which stage the medium was called Conditioned Medium (CM). CM was collected from each well and centrifuged at 1000 rpm for 20 min to get rid of dead cells. Finally, the CM was aliquoted (200-500µl) and stored at -80°C until use. To collect CM from transiently transfected cell lines, the transfection medium was changed with complete RPMI 1640 24h after transient transfection. 48 h later, the CM were collected, centrifuged, aliquoted and stored as indicated above.

2.2 RNA isolation and cDNA synthesis

Cells were collected in cell culture-grade PBS by either trypsinization or scraping. Then, total RNA was isolated by using RNeasy RNA Extraction Kit (Qiagen, Germany) according to the manufacturer's guidelines. RNA was then treated with DNase I (Thermo Scientific, USA) to eliminate any potential genomic DNA contamination. cDNA synthesis was performed with 1µg of the RNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using oligo-dT primers (provided in the same kit). The cDNAs were kept at -20°C.

2.3 RT-PCR and qRT-PCR

The RT-PCR mixtures contained 0.5µM forward and reverse primers (Table 2.1), 0.2mM dNTP mix, 1.5mM $MgCl_2$, and 1U Taq Polymerase (Thermo Scientific) in a volume of 18µl, into which 2µl of 1µg cDNA (final

concentration: 0.1µg) was added, resulting in a total volume of 20µl. An initial denaturation of 3 min was followed by repeats of denaturation (30 sec, 94-95 °C), and extension (30 sec) and a final extension step (7 min, 72 °C). The annealing temperatures and cycle numbers indicated in Table 2.2. The reactions were carried out in a thermal cycler (Applied Biosystems). β-Actin was used as an internal control. 10µl of PCR product was mixed with 2µl of 6X loading dye and run on a 2% agarose gel at 100V; images were observed and recorded under UV light.

For qRT-PCR mixtures, 0.5µM forward and reverse primers and 10µl of 2X Fast Start SYBR Green Mix were mixed in 18µl volume, then 2µl cDNA (final concentration: 0.01µg) was added. β-actin was used as an internal control. Reactions were carried out in Rotor GeneQ 6000 (Qiagen). Standard curves were prepared for each set of primers used, which were then used to calculate Ct values of the unknown samples. Fold changes with respect to the internal controls were calculated using Pfaffl method (Pfaffl 2004). MIQE guidelines were followed in the RT-qPCR reactions (Bustin et al. 2009).

Table 2.2 List of primers used in this study.

Description	Sequence	Annealing Temp. (°C)/ Cycle Number		Amplicon Length (bp)
VEGF-A	5'-ATCACGAAGTGGTGAAGTTC-3'	53	40	265
	5'-TGCTGTAGGAAGCTCATCTC-3'			
15-LOX-1	5'-GTCTTCCTTCTATGCCCAAGAT-3'	59.5	40	100
	5'-CACAGCCACGTCTGTCTTATAG-3'			
β-Actin	5'-CAGCCATGTACGTTGCTATCCAGG-3'	60.5	40	151
	5'-AGGTCCAGACGCAGGATGGCATG-3'			
GAPDH	5'--3' CGACCACTTTGTCAAGCCCTA	62	40	238
	5'--3' CCCTCTTCAAGGGGTCTAC			

2.4 Analysis of protein expression

2.4.1 Total protein isolation

According to the manufacturer's instructions, total proteins were collected with 100-200 µl M-PER Mammalian Protein Extraction Buffer (Thermo Scientific) from cells in 6-well plates. Protease and phosphatase inhibitors (Roche, Germany) were added to the lysis buffer. After incubation on ice for 15 min, cells were centrifuged at 14000 x *g* for 10 min; then supernatants that had total protein were collected in new Eppendorf tubes.

2.4.2 Quantification of isolated proteins

Quantification of the isolated proteins was carried out using Coomassie Protein Assay Reagent (Thermo Scientific) after dilution with sterile water in 1:5 (protein: water) ratio and incubating with the Coomassie Reagent. Absorbance values were read at 595nm in a microplate reader spectrophotometer (Thermo Scientific). Bovine serum albumin standard curve was used to determine the concentrations.

2.4.3 Western blotting

Electrophoresis was carried out on 10% SDS-polyacrylamide gels containing equal amounts of protein (30-70µg) at 100V. For transfer onto polyvinylidene fluoride membranes (Roche), wet transfer was performed with constant current at 380 mA, 115V for 1 h and 15 min. Blocking of the membranes was conducted either by using 5% skim milk (Sigma Aldrich) in PBS containing

0.1% Tween (Applichem) or 5% BSA (Applichem) in TBS containing 0.1% Tween, for 1 h at room temperature on a shaker. Membranes were incubated with primary antibodies (Table 2.3) at 4 °C overnight on a shaking platform. Next day, membranes were washed with either PBS-T or TBS-T to remove excess primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, on a shaker. Visualization was performed using either WesternBright Sirius HRP Substrate for low abundant proteins (Advansta) or Clarity ECL Substrate (Bio-Rad, USA) with a Chemi-Doc MP (Bio-Rad).

Table 2.3 List of antibodies used in this study.

Description	Origin	Vendor	Catalog Number	Buffer	Dilution
β-Actin	mouse	Santa Cruz Biotech.	sc-47778	PBS-T	1:3000
VEGF	rabbit	Abcam	ab46154	PBS-T	1:1000
VEGFR1	rabbit	Abcam	ab32152	PBS-T	1:1000
VEGFR2	mouse	Santa Cruz Biotech.	sc-6251	PBS-T	1:200
p-VEGFR2 (Tyr1059)	rabbit	Abcam	ab195702	TBS-T	1:500
15-LOX-1	mouse	Abnova	H00000246-m04	PBS-T	1:1000
Bcl-xL	mouse	Santa Cruz Biotech.	sc-8392	TBS-T	1:500
XIAP	mouse	Santa Cruz Biotech.	sc-11426	PBS-T	1:500
anti-rabbit	goat	Santa Cruz Biotech.	sc-2004	PBS-T	1:2000
anti-mouse	rabbit	Santa Cruz Biotech.	sc-2005	PBS-T	1:2000

2.5 MTT Assay

To determine whether transfection of cells with the 15-LOX-1 vector affected cell proliferation, Vybrant® MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay (Life Technologies) was used. Metabolic activity of live cells results in the formation of formazan crystals from MTT. Firstly, $1x10^4$ cells per well were plated in 96-well plates in 100µl complete RPMI 1640 medium. After cells were attached, PC-3 and LNCaP cells were transfected with either empty vector (pcDNA3.1) or 15-LOX-1 vector (pCDNA3.1-15LOX1) as described. After 24h of transfection, medium was changed with fresh media and cells' proliferation were observed with MTT in 24h, 48h and 72h. According to the manufacturer's instructions, 12mM MTT stock solution was diluted with medium in 1:10 ratio and distributed to each well as 100µl, with a final concentration of 1,2mM. Empty wells containing only 100µl of MTT were used as negative controls. Then, they were incubated at 37 °C for 4 h. To solve formazan crystals, 50µl DMSO (AppliChem) was added and incubated at, 37°C for 10 min. Finally, measurements were taken at 540 nm in MultiSkan GO Microplate Spectrophotometer (Thermo Scientific) microplate reader. In this assay, 6 technical replicates and 3 biological replicates were used.

2.6 Cell Counting for Viability of LNCaP Cells

The MTT assay involved transient transfection in individual wells of 96-well plates. This has two inherent problems: 1. Variability because of the

differences in the levels of transfection in each well, 2. The expression of 15-LOX-1 in the 96-well plate could not be confirmed. To circumvent this problem, cells were also transfected with the 15-LOX-1 or empty vector in 6-well plates and counted. The same cells could also be used for total protein collection to ensure the successful transfection of the plasmid.

After transient transfection of LNCaP cells either with empty vector (pcDNA3.1) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) in 6-well plates, attached dead/alive cells and floating dead cells were counted with T20 automated cell counter (Bio-Rad, USA). To discriminate between dead cells from live cells, Trypan Blue (0.4%, Bio-Rad, USA) was used. Live cells can exclude the dye from the cells and therefore appear without any color, while the dead cells lose the ability to exclude the dye and therefore appear blue. After collecting floating dead cells, attached cells were collected by trypsinization, washed and re-suspended in 1 ml PBS in Eppendorf tubes. Next, they were stained with 1:1 (10 µl sample and 10 µl dye) ratio of Trypan Blue and applied on disposable Bio-Rad counting slides. Care was taken to avoid bubbles, which could be mistaken for cells by the machine. The remaining cells were lysed for protein isolation.

2.7 Cell Cycle Analysis

To determine the effects of 15-LOX-1 expression on cell cycle progression, cells were stained with propidium iodide and the cell cycle stages were determined in a flow cytometer. Cells were transfected with either the 15-LOX-1 or the empty vector and $1x10^6$ cells were collected in Eppendorf tubes and fixed with 1 ml cold 70% ethanol overnight at -20°C. Fixed cells were

centrifuged with 300 x *g* for 5 min and washed with 1X PBS twice to remove the fixative. The cells were incubated with propidium iodide (PI, Sigma Aldrich) for 30 min in the dark at room temperature to stain the DNA. The staining solution consisted of 0.1% Triton-X, 0.2 mg/ml RNAse, 15 µg/ml PI stock in 1X PBS. The measurements were carried out in a BD Accuri flow cytometer and the data were analysis using the BD Accuri C6 software.

2.8 VEGF-A Secretion Analysis

To determine if 15-LOX-1 expression in cancer cell lines had any effect on the secretion of VEGF-A_{165} in the conditioned medium (CM), Quantikine Human VEGF Immunoassay ELISA Kit (R&D Systems) was utilized. According to the manufacturer's protocol, CM was added to wells that were coated with VEGF antibody. After three washes, secondary antibody or conjugate was added and incubated for 2 h at room temperature. Next, substrate solution was used for color change and detection. Finally, stop solution was used to terminate the reaction and measurements were taken at 450 nm by microplate reader MultiSkan GO Microplate Spectrophotometer (Thermo Scientific). For background correction, the absorbance values at 540 nm were subtracted.

2.9 Luciferase assay

To understand the VEGF-A promoter activity that is regulated by HIF1-α, the primary hypoxia regulated transcription factor that regulates the expression of VEGF-A, in 15-LOX-1 overexpressing cells. HCT-116 cells ($5x10^4$ cells per well) stably transfected either with the empty vector (pcDNA3.1) or 15-LOX-1 (pcDNA3.1-15-LOX-1) were plated in 48-well plates. After overnight attachment, cells were transfected with pGL3-5xHRE-VEGF-Luc, which contains 5X HIF-1α response elements from the VEGF-A promoter (gift from Dr Ilias Mylonis, School of Medicine, University of Thessally (Shibata et al. 1998)) along with pRL-TK (Renilla vector for normalization) (Promega, Madison, WI, USA). In transfections, the Firefly: Renilla ratio was 250:1 and X-tremeGENE HP was used as a transfection agent. Cells in each well of a 48-well plate were transfected for 24h with a total of 300ng plasmid at 1:2 plasmid: transfection reagent ratio. The cells were then incubated in the hypoxia chamber or under normoxic conditions for an additional 6h. The luciferase assay was conducted with the Dual-Luciferase Assay kit (Promega) according to the manufacturer's instructions in white 96-well plates and luminescence was measured by luminometer (Turner Biosystems, USA).

2.10 *In vitro* Tube Formation Assay

This assay is for determining whether growth factors released into the CM of cells transfected with the 15-LOX-1 or empty vector affected the ability of HUVECs to form tubes and branches. One day before conducting the

experiment, pipette tips and Matrigel (BD Biosciences) that mimics the extracellular matrix were thawed on ice at 4°C. The next day, 45µl of Matrigel for each well was pipetted onto chilled 96- well plates on ice and the plate was incubated at 37°C for 1 hour to solidify the Matrigel. After that, HUVECs were counted and 3 x 10^4 cells/well were re-suspended in a mixture of CM and the HUVEC medium (EBM, Lonza) at a ratio of 1:1. A total of 100 µl of this mix was applied in each well onto the pre-solidified Matrigel. After 6h incubation at 37°C, tubes were photographed with an inverted microscope containing a built-in camera. Images were analyzed by Wimasis Image Analysis Company.

2.11 Angiogenesis Protein Array

To determine whether CM from cancer cell lines overexpressing 15-LOX-1 contained factors that could affect signaling mechanisms in endothelial cells a Proteome Profiler Array, Human Angiogenesis Array Kit (R&D Systems) Angiogenesis array was carried out. This array consists of 55 angiogenesis-related proteins that includes include soluble growth and differentiation factors, extracellular matrix components, proteases, membrane-bound receptors, and intracellular signaling molecules in a single sample. HUVECs were incubated with a 1:1 ratio of CM and EBM for 24 h; then, lysed with a buffer containing 1% NP-40, 20mM Tris-HCl (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA and 1X protease inhibitor cocktail (Roche). 200µg of the HUVEC lysate was used per membrane of the according to manufacturer's protocol. After visualizing the spots, the signals were measured with a Protein Array Analyzer plugin for ImageJ (Caroline a

Schneider, Rasband, and Eliceiri 2012). Intensity files obtained from ImageJ were then uploaded to a custom written Matlab code (Dr. Özlen Konu, Bilkent University). In the background normalization step that contains both global and batch normalization, the mean of the negative spots in each array was subtracted from other spots on the corresponding array; the absolute minimum value of each array plus 1 was added to the spot intensities. The data were then transformed to the log2 space before globally normalizing each array to its own median intensity value. At the last stage, the mean value of the spots in control arrays within the same batch were subtracted from those in the corresponding experiment arrays to represent log fold differences between groups. The consistency between duplicates was used to check the quality of arrays while boxplots were examined to compare data distributions before and after normalizations. Unpaired student t-test was applied on the mean value of the batch corrected duplicate spots. This program can also perform nonparametric hypothesis testing based on Wilcoxon sum rank text or Friedman's tests.

2.12 Statistical analyses

At least three independent biological replicates were used in experiments and Student's t-test or one-way ANOVA tests of GraphPad Prism 6.1 (GraphPad Software Inc., USA) were used to validate significance. $p<0.05$ was accounted as significant.

CHAPTER 3

RESULTS

The primary aim of this thesis was to understand the role of 15-lipoxygenase-1 (15-LOX-1) on angiogenesis in cancer. 15-LOX-1 is a lipid metabolizing enzyme that oxygenates polyunsaturated fatty acids and has been shown to have tumor suppressive properties in many cancer types. However, its role in angiogenesis has not been defined clearly yet. We have chosen prostate cancer, using the cell lines PC-3 and LNCaP, as our model in this study since previous reports indicated that 15-LOX-1 expression in these cell lines were associated with pro-tumorigenic and pro-angiogenic effects. Additionally, the colon cancer cell line HCT-116 cells, where 15-LOX-1 has been shown previously by our group and others to have anti-carcinogenic effects, was used in the current study.

3.1 15-LOX-1 Expression Levels in Cancer Cell Lines

The basal level of 15-LOX-1 expression at the mRNA level was examined in the different cancer cell lines used in this study, i.e. HCT-116, PC-3 and LNCaP. HCT-116 cells stably expressing 15-LOX-1 (HCT116-1E7) that was

generated in our lab previously (Çimen, Tunçay, and Banerjee 2009) was used as a positive control. All of the wild type cancer cells had low *ALOX15* expression at the mRNA level; however, LNCaP cells expressed more *ALOX15* compared to HCT-116 cells (Figure 3.1 A). When we compared the 15-LOX-1 protein levels in the different cell lines, we observed no detectable protein amounts in both PC-3 and LNCaP cell lines (Figure 3.1 B). Therefore, 15-LOX-1 overexpression was chosen as our strategy and optimized accordingly.

Firstly, to express 15-LOX-1 in PC-3 cells, 1:1 plasmid DNA to transfection reagent ratio with increasing plasmid amounts as 1µg, 2µg and 2,5µg were tried and 15-LOX-1 expression was confirmed by Western Blot analysis (Figure 3.1 C). For stable expression, first a kill curve was generated with different concentrations of the antibiotic zeocin and 50µg/ml Zeocin concentration was chosen for the selection of transfected cells (Appendix E). However, even after 2 independent trials, no stably expressing colonies could be obtained. Therefore, the experiments were continued with transient transfection of the 15-LOX-1 plasmid at 1:1 ratio of plasmid to transfection reagent with 1µg plasmid.

For overexpression of 15-LOX-1 in LNCaP cells, 1:2 and 2:1 ratio with 1µg plasmid were tried (Appendix F). Optimal expression and least cell death was optimized at 1:1 ratio and 2µg plasmid. Transfection was confirmed with qRT-PCR and Western Blot (Figure 3.1 C).

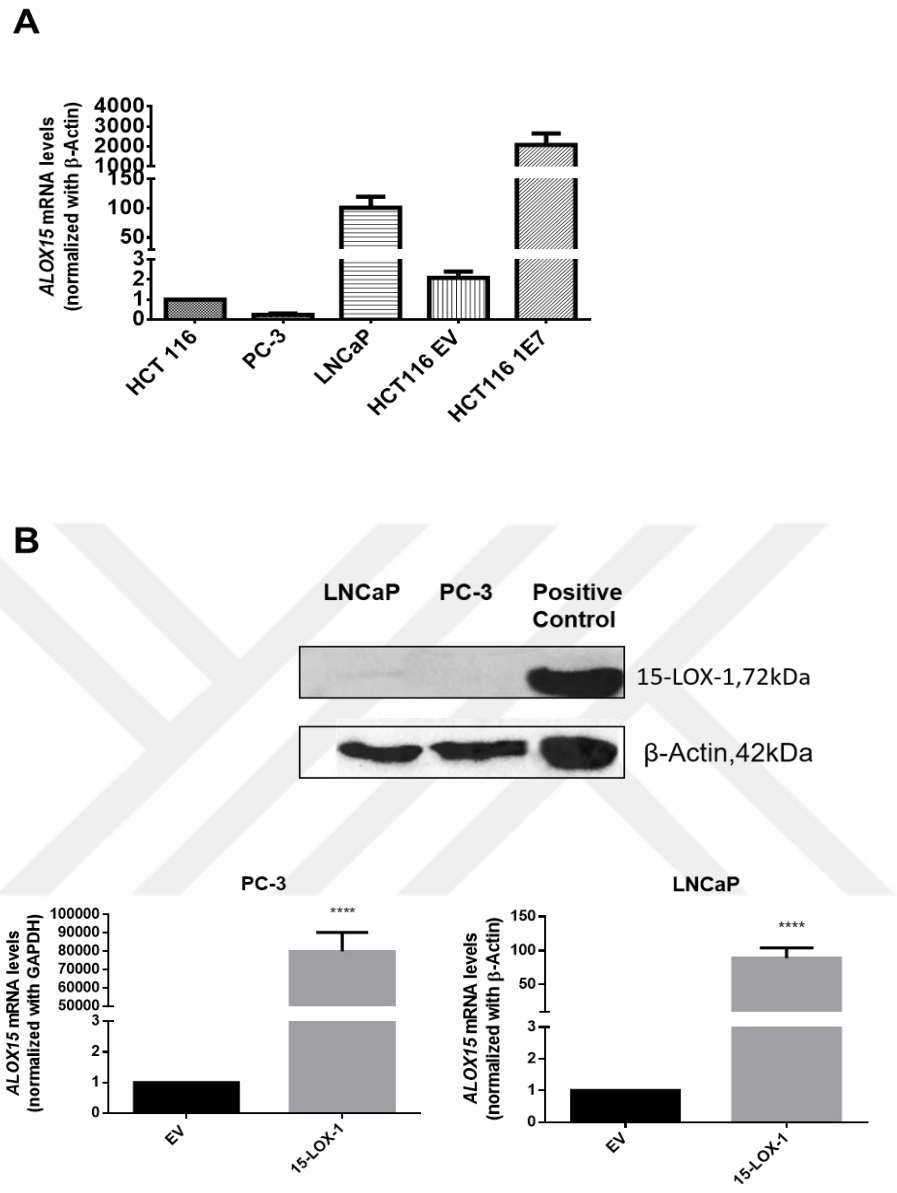


Figure 3.1 15-LOX-1 overexpression in cancer cell lines. (**A)** qRT-PCR analysis of *ALOX15* mRNA levels in wild type colon and prostate cancer cell lines HCT-116, PC-3 and LNCaP. (**B)** Western Blot analysis for comparison of endogenous 15-LOX-1 protein levels in LNCaP and PC-3 wild type cells (upper), qRT-PCR analysis of confirming 15-LOX-1 mRNA levels in PC-3 (lower left) and LNCaP (lower right) cells after they were transiently transfected with either empty vector (EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1).

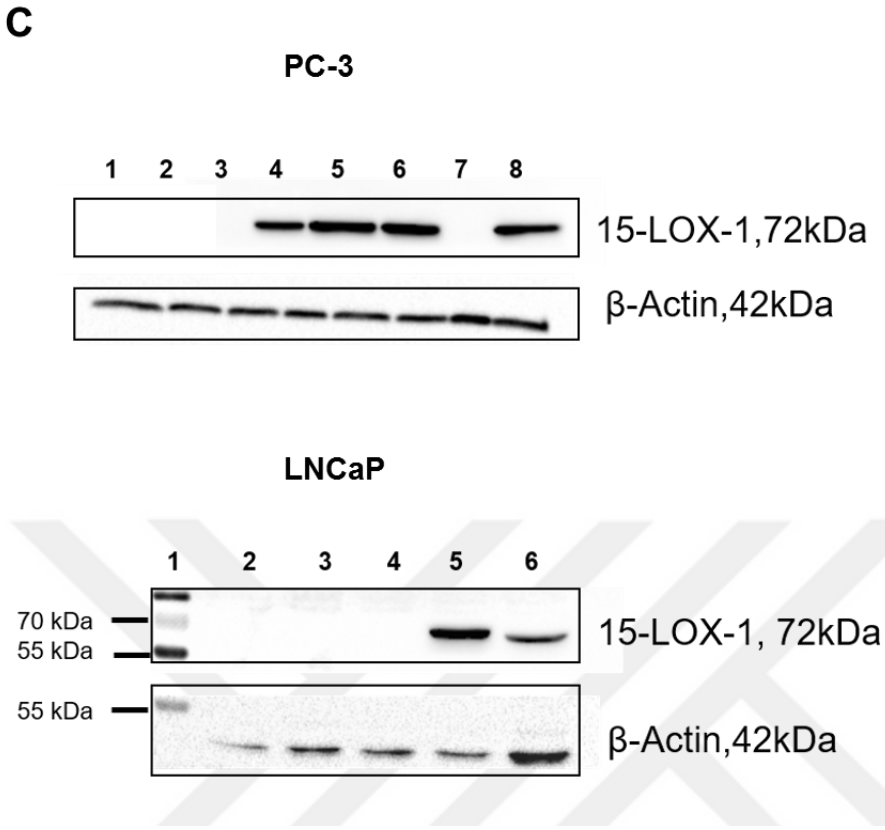


Figure 3.1 15-LOX-1 overexpression in cancer cell lines (Cont'd). (C) Western Blot analysis for confirmation of PC-3 (1. Cells Only, 2. Mock, 3. EV, 4. 1μg 15-LOX-1, 5. 2μg 15-LOX-1, 6. 2,5 μg 15-LOX-1, 7. HCT116-EV, 8. HCT116-1E7) and LNCaP (1. Marker, 2. Cells Only, 3. Mock, 4. EV, 5. 15-LOX-1, 6. HCT116-1E7) cells that are transiently transfected with either empty vector (EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1). Either empty vector (EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) stably overexpressing HCT116-EV and HCT116-1E7 were used as controls in all three sections. GAPDH was used internal control for PC-3 transient transfections in in qRT-PCR analysis and for the rest, β-Actin was used both as internal control and as protein loading control in Western Blotting. Independent experiments were repeated for three times and Student's t-test was used for comparison of two variables; one-way ANOVA tests were used for comparison of more than one variables. $p<0.05$ was considered as significant.

3.2 Cellular Characteristics of 15-LOX-1 Overexpression in Cancer Cells

We examined the effect of 15-LOX-1 expression on cellular characteristics like proliferation, cell cycle and apoptosis in prostate cancer cell lines. Previous studies conducted in our lab showed that overexpression of 15-LOX-1 in colon cancer cell lines resulted in decreased proliferation and enhanced apoptosis (Çimen, Tunçay, and Banerjee 2009). However, similar studies in prostate cancer are limited and controversial. PC-3 cells transiently transfected with the 15-LOX-1 vector showed no effect on proliferation or on cell cycle progression (Figure 3.2 A and B). Similar experiments were also carried out with LNCaP cells, however, owing to the technical difficulties in staining the cell monolayers with propodeum iodide (PI) the cell cycle analysis data with PI staining was inconclusive (data not shown).

To determine the proliferation of LNCaP cells transiently expressing 15-LOX-1, a Trypan blue exclusion assay was carried. Since the growth rate of LNCaP cells is very low, cell proliferation should be measured in these cells at 72h or 96h to ensure at least one round of cell doubling. However, the transient expression of 15-LOX-1 could not be sustained for such a long time. In addition, for MTT or BrdU assays, individual wells of a 96 plate were transfected with the 15-LOX-1 vector or empty vector. This results not only in variations in gene expression, but also confirmation of 15-LOX-1 expression could not be carried out. Therefore, trypan blue exclusion followed by cell counting with automated cell counter (Bio-Rad) was performed. A significant decrease in the number of live cells was seen when 15-LOX-1 was overexpressed in LNCaP cells (Figure 3.2 D right panel) was observed. Floating dead cells were also counted and showed a trend for

increased numbers in the 15-LOX-1 expressing cells; however, the data did not reach significance (Figure 3.2 D right panel). These data imply that 15-LOX-1 may have anti-proliferative effects in LNCaP cells but not in PC-3. To determine whether lower cell viability in LNCaP cells was due to enhanced apoptosis, the apoptosis markers X-linked inhibitor of apoptosis protein (XIAP) and B-cell lymphoma-extra-large (Bcl-xL) (both inhibitors of apoptosis, Czabotar et al. 2014) were checked. However, 15-LOX-1 expression did not result in any change in the levels of either proteins in LNCaP cells (Figure 3.2 E). Future studies will be carried out to determine whether 15-LOX-1 expression altered caspase activity in these cells.

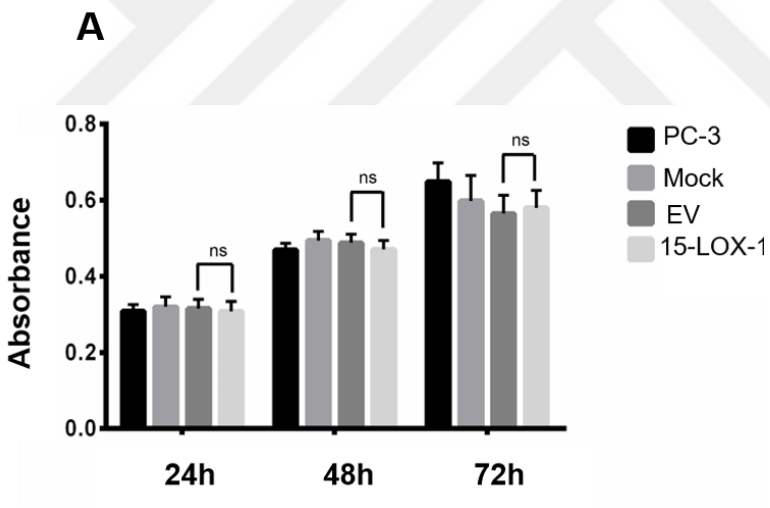
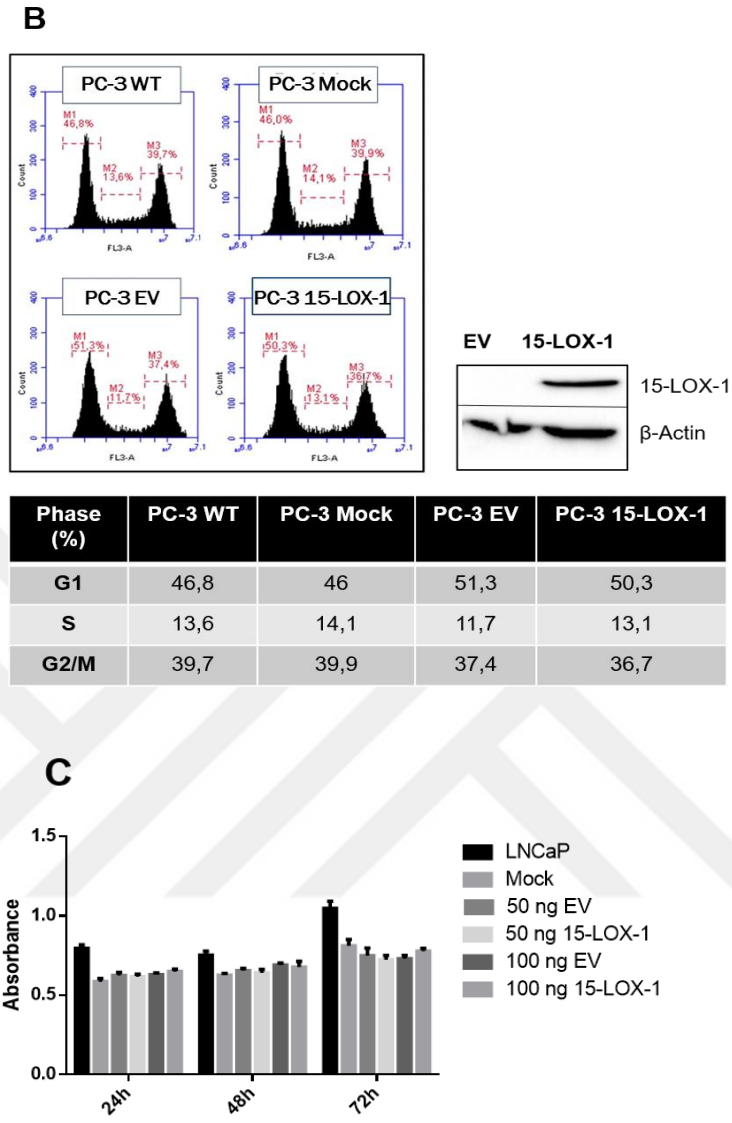


Figure 3.2 Alterations in proliferation, cell cycle and apoptosis with 15-LOX-1 expression in PC-3 and LNCaP cells. (A) PC-3 cells were transfected with the 15-LOX-1 or empty vector plasmids and assayed for proliferation with MTT for 72h at 24h intervals. No alteration in proliferation could be observed.



Phase (%)	PC-3 WT	PC-3 Mock	PC-3 EV	PC-3 15-LOX-1
G1	46,8	46	51,3	50,3
S	13,6	14,1	11,7	13,1
G2/M	39,7	39,9	37,4	36,7

Figure 3.2 Alterations in proliferation, cell cycle and apoptosis with 15-LOX-1 expression in PC-3 and LNCaP cells (Cont'd). (B) Cell cycle analysis of PC-3 cells transiently transfected with the 15-LOX-1 or empty vector. Percentages of the cell cycle stages are shown in the table. No change in cell cycle distribution was seen with 15-LOX-1 expression. The Western blot shows the successful expression of 15-LOX-1 in these cells. **(C)** LNCaP cells were transfected with the 15-LOX-1 or empty vector plasmids and assayed for proliferation with MTT for 72h at 24h intervals. No alteration in proliferation could be observed.

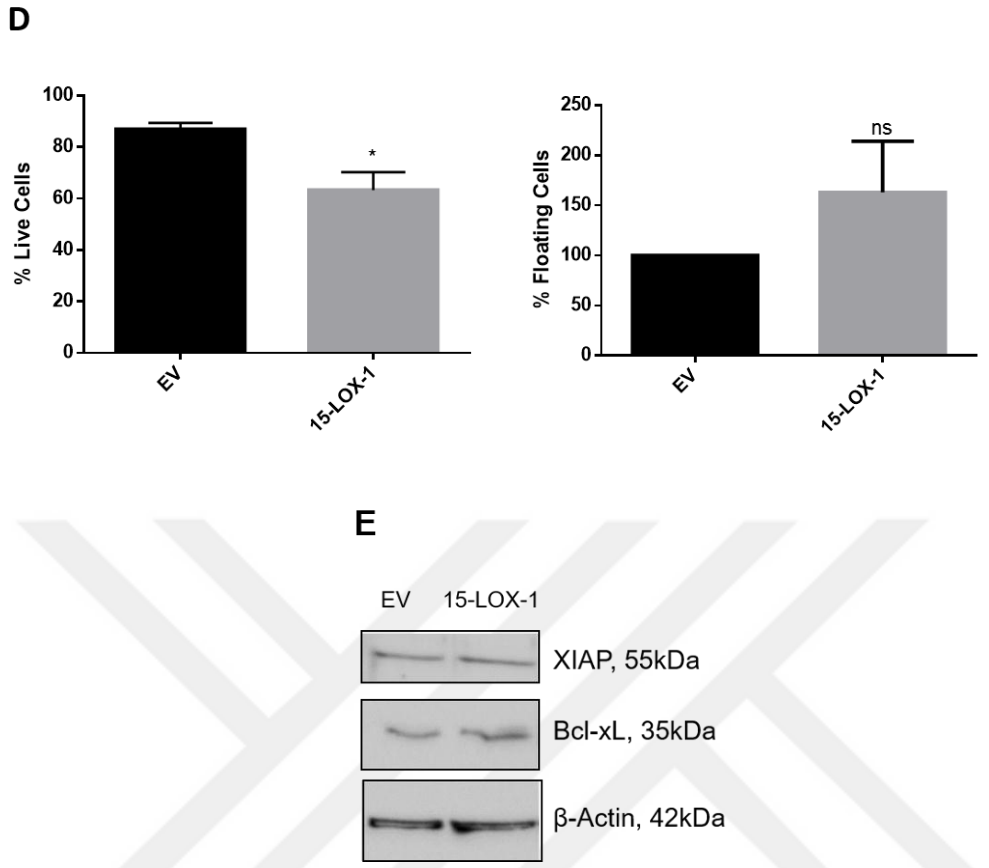


Figure 3.2 Alterations in proliferation, cell cycle and apoptosis with 15-LOX-1 expression in PC-3 and LNCaP cells (Cont'd). (D) Cell counting of LNCaP cells after transient transfection with either the 15-LOX-1 vector or the empty vector showed a significant decrease in the percentage live cells (left) and an increase in the percentage of dead floating cells (right). **(E)** Western Blot analysis of apoptosis markers XIAP and Bcl-xL showing no change in the protein levels of these markers when 15-LOX-1 was expressed. β-actin was used both as loading control. Independent cell cycle analysis of PC-3 with 15-LOX-1 transfections repeated twice and MTT assay for LNCaP transfection was also conducted twice to confirm their slow growth rate. The other results were obtained from three independent biological replicates and Student's t-test was used for comparison of two variables; one-way ANOVA tests were used for comparison of more than one variable. $p<0.05$ was considered as significant.

3.3 The effect of 15-LOX-1 expression on angiogenesis in colon and prostate cancer cells

3.3.1 Expression of VEGF-A at the mRNA level

VEGF is well known factor for growth and differentiation of endothelial cells; the cytokine is also a chemotactic factor for the movement of monocytes (Clauss et al. 1990; Keck et al. 1989). VEGF-A is the isoform that is most active in angiogenesis (Byrne, Bouchier-Hayes, and Harmey 2005), therefore, *VEGFA* mRNA levels were assayed in colon and prostate cancer models that were transfected with either a 15-LOX-1 or empty vector. 15-LOX-1 overexpression in HCT-116 and LNCaP showed a significant reduction in the levels of *VEGFA* mRNA (Figure 3.3 A and C). However, the expression of *VEGFA* did not change in PC-3 cells expressing 15-LOX-1 (Figure 3.3 B).

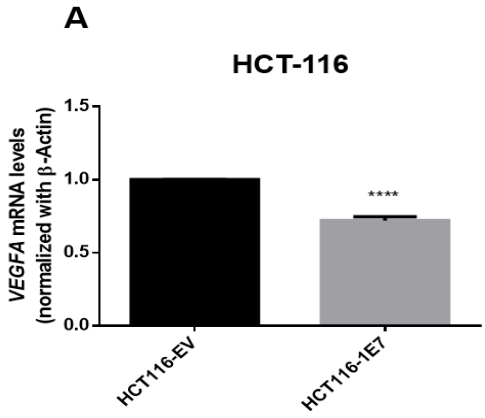


Figure 3.3 Effect of 15-LOX-1 expression on *VEGFA* mRNA levels in colon and prostate cancer cell lines. qRT-PCR analysis of *VEGFA* mRNA levels in HCT-116 cells stably transfected with either EV or 15-LOX-1 **(A)**.

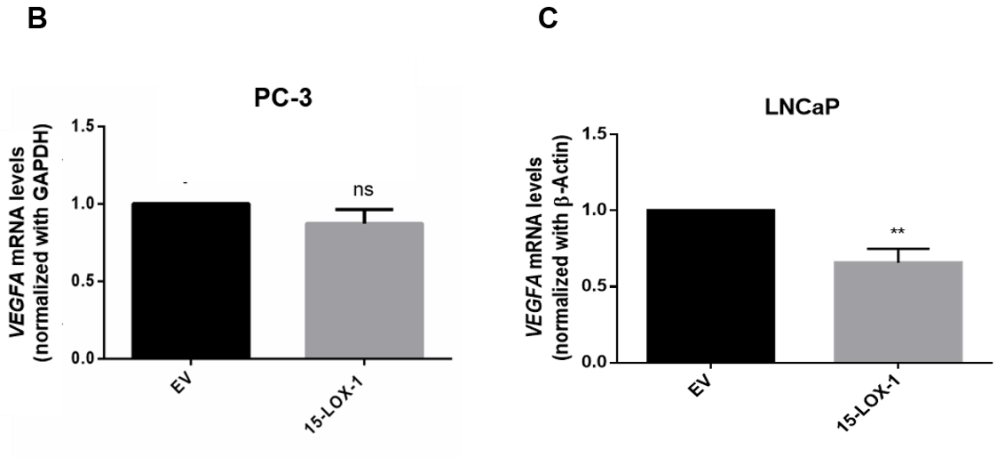


Figure 3.3 Effect of 15-LOX-1 expression on *VEGFA* mRNA levels in colon and prostate cancer cell lines (Cont'd). qRT-PCR analysis of *VEGFA* mRNA levels in cells transfected with either EV or 15-LOX-1 vector in PC-3 cells **(B)** and LNCaP cells **(C)**. Decreased *VEGFA* mRNA levels was observed in HCT-116 and LNCaP cells with 15-LOX-1 expression; however, *VEGFA* mRNA levels didn't change in PC-3 cells with 15-LOX-1 expression. GAPDH was used internal control for PC-3 cells, β-Actin was used as internal control for LNCaP and HCT-116 cells in qRT-PCR assays. Data shown here for HCT-116 cells was combined data from Dr. Sinem Tunçer in our laboratory. All results were obtained from three independent biological replicates. Student's t-test was used as statistical method and $p<0.05$ was considered as significant.

3.3.2 VEGF-A_{165} Secretion

VEGF-A_{165} is one of the spliced variants of VEGF-A and is a well-known secreted mitogen for endothelial cells (Leung et al. 1989). The spliced variants of VEGF-A are discriminated by their heparin and heparin-sulfate binding ability that is important for receptor binding and solubility. Splicing occurs in exon 6 and 7 that encode heparin binding domains. VEGF-A_{165}

lacks exon 6, which prevents its attachment to the extracellular matrix and allows it to diffuse out of the cell. Compared to the other variants, VEGF-A_{165} is the most predominant isoform and strongest stimulator of angiogenesis (Byrne, Bouchier-Hayes, and Harmey 2005).

Conditioned medium (CM) is also described as cell secretome that is composed of proteins such as enzymes, growth factors, cytokines, hormones and soluble mediators secreted by cells into the culture medium. These secreted proteins may be involved in many important cellular aspects like cell growth, differentiation, invasion and angiogenesis by regulating cell-to-cell and cell-to-extracellular matrix interactions (Dowling and Clynes 2011). The method for collection of CM has been described in Materials and Methods.

To determine whether expression of 15-LOX-1 affected the secretion of VEGF-A_{165} into the CM, 15-LOX-1 was stably (HCT-116) or transiently (PC-3 and LNCaP) expressed. CM was collected after 72h and assayed for VEGF-A_{165} using an ELISA kit. Corroborating the mRNA data, 15-LOX-1 expression in both HCT-116 and LNCaP cells resulted in a decrease in the secreted levels of VEGF-A_{165} the CM (Figure 3.4 A and C). However, no change in VEGF secretion was seen in PC- 3 cells transfected with increasing amounts of the 15-LOX-1 plasmid (Figure 3.4 B).

Additionally, colorectal cancer HCT-116 cells showed already almost 5 times higher secretion of VEGF-A_{165} as compared to prostate cancer models, most likely due to differences in the characteristics of their secretome (Figure 3.4 A).

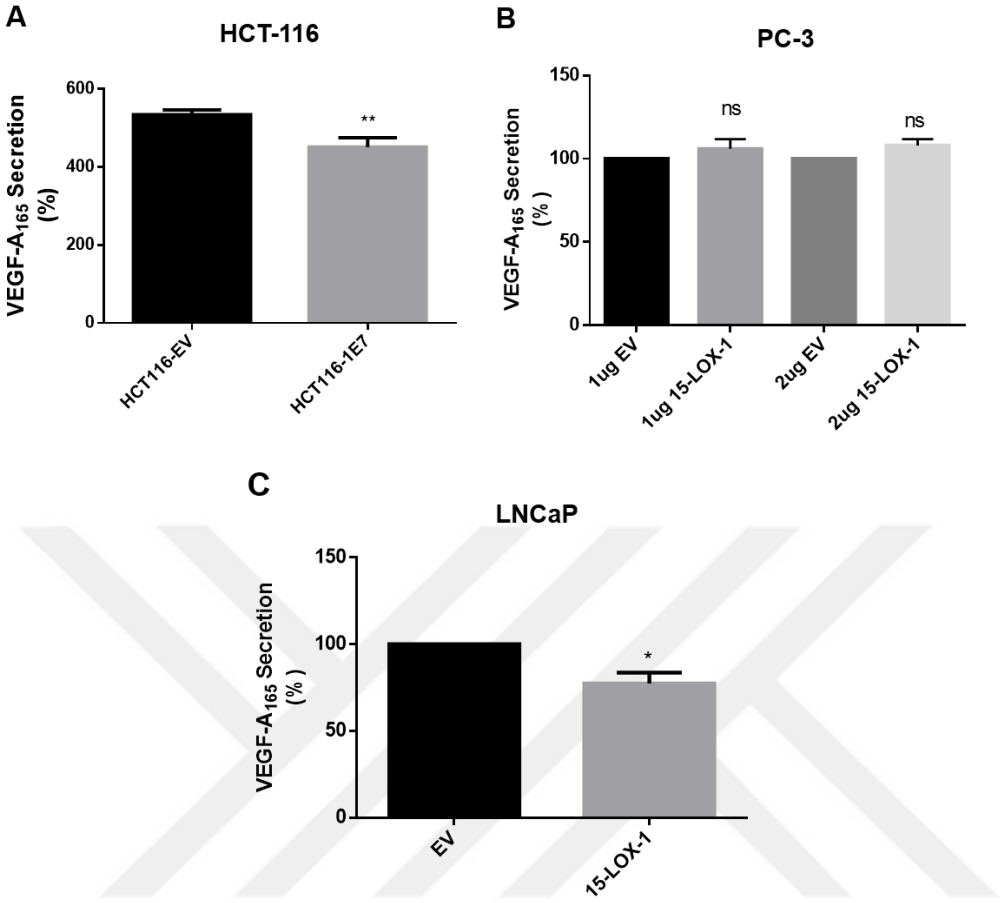


Figure 3.4 Effect of 15-LOX-1 expression on VEGF-A_{165} secretion into the conditioned medium. (**A**) HCT-116 cells stably expressing 15-LOX-1 showed significantly reduced levels of VEGF-A_{165} into the CM (data obtained by Dr. Sinem Tunçer in our laboratory**). (B)** Prostate cancer PC-3 cells transiently transfected with 1μg and 2μg of the empty vector (pcDNA3.1, EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) did not show any difference in the levels of secreted VEGF-A_{165} **(C)**, LNCaP cells transiently transfected with empty vector (pcDNA3.1, EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) showed a significant decrease in the secretion of VEGF-A_{165}. VEGF-A_{165} levels were assayed with Human VEGF Quantikine Elisa Kit (R&D). LNCaP secretion data obtained from two independent biological replicates; others were conducted with three independent biological replicates. Student's t-test was used as statistical method and $p<0.05$ was considered as significant.

3.3.3 Transcriptional activity of HIF-1α under normoxic and hypoxic conditions

Hypoxia is a state of low oxygen availability and is seen frequently in tumors that have grown beyond a certain size. Hypoxia-inducible factor alpha (HIF1-α) is a heterodimeric transcription factor that is the major transcriptional regulator of VEGF-A. This transcription factor binds to the hypoxia response elements (HRE) in the VEGF-A promoter and is activated in hypoxia to enhance the expression of VEGF-A and thereby cell survival and angiogenesis (Pagès and Pouysségur 2005). In normoxic conditions, HIF1-α is expressed in low levels due to the rapid degradation via the ubiquitin protease system (Salceda and Caro 1997). However, when the oxygen levels drop, HIF-1α is stabilized and can enhance the transcription of downstream genes like VEGF. Thus, to examine whether the decreased expression and secretion of VEGF-A in 15-LOX-1 expressing cells was due to HIF1-α mediated regulation of VEGF-A, we conducted a dual luciferase assay. In this, HCT-116 cells stably expressing either the 15-LOX-1 or the empty vector were transfected with a construct that contains 5 copies of the HRE from the VEGF-A promoter.

The cells were then incubated either under normoxic or hypoxic conditions. In both normoxia and hypoxia, 15-LOX-1 expression led to a decrease in HIF-1α transcriptional activity in HCT-116 cells (Figure 3.5). A 15-fold increase in HIF-1α transcriptional activity observed in hypoxia indicated that the cells responded robustly to the hypoxic microenvironment.

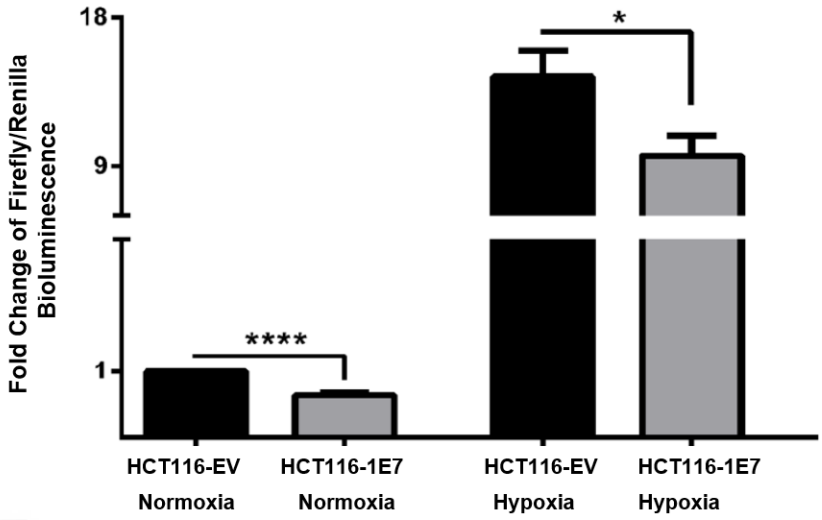


Figure 3.5 15-LOX-1 expression reduces HIF-1α transcriptional activity in both hypoxic and normoxic conditions. A luciferase assay was carried out by transfecting HCT-116 cells stably expressing either 15-LOX-1 (HCT-116-1E7) or the control cells (HCT116-EV) with a 5xHRE VEGF-A construct for 24h and incubating them under normoxia (21% Oxygen) or hypoxia (1% Oxygen) for 6h. The cells were then collected and processed for a dual luciferase assay. Data were normalized with respect to bioluminescence of Renilla luciferase. Data obtained from three independent experiments and one-way ANOVA test was used as statistical method and $p<0.05$ was considered as significant.

3.3.4 Analysis of signaling through VEGF-A in 15-LOX-1 expressing cells

VEGF-A regulates blood vessel development by binding mainly to receptor tyrosine kinases VEGFR-1 and VEGFR-2 and activating signaling pathways through dimerization. VEGFR-1 has poor kinase activity due to a receptor motif in the juxtamembrane domain; the receptor primarily signals for blood vessel morphogenesis and, interestingly, acts as a decoy for VEGF. High

expression of VEGFR-1 is therefore often associated with reduced angiogenesis. On the other hand, VEGFR-2 is the primary VEGF receptor that signals for angiogenesis by regulating endothelial cell migration, proliferation, differentiation, survival, vessel permeability and dilation through seven putative phosphorylation sites in which Tyr951, 1054, 1059, 1175 and 1214 are the most prominent ones (Cébe-Suarez, Zehnder-Fjällman, and Ballmer-Hofer 2006).

To investigate the effect of 15-LOX-1 on VEGF-A signaling in our cancer cell line models, we examined the protein levels of VEGF-A, VEGFR-1, VEGFR-2 and pVEGFR-2 (1059Y) through Western blot in the 15-LOX-1 expressing cells. However, we observed no changes in either the total or phosphorylated protein amounts indicating that the VEGF-A secreted from the epithelial cells most likely did not affect signaling in an autocrine manner (Figure 3.6 A-C).

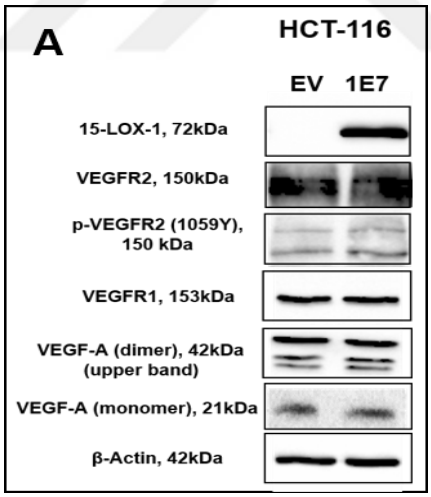


Figure 3.6 VEGF-A does not affect the signaling in autocrine manner in all cancer models. Western Blot analysis of protein expression of VEGFR-1, VEGFR-2, p-VEGFR-2 (1059Y), and VEGF-A in colon (HCT-116, **(A)**) cells expressing 15-LOX-1.

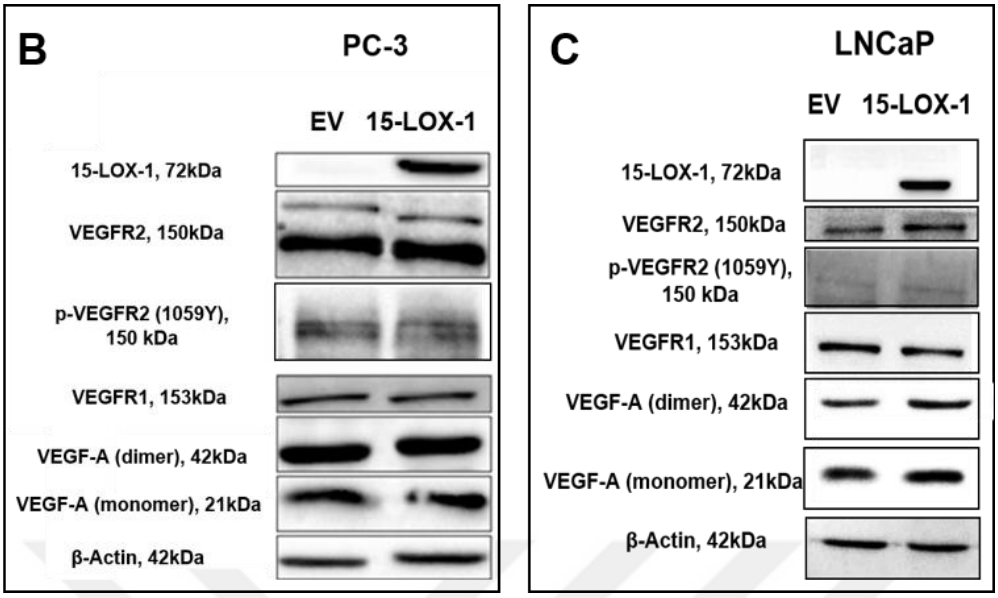


Figure 3.6 VEGF-A does not affect the signaling in autocrine manner in all cancer models (Cont'd). 15-LOX-1 expression did not lead to VEGF mediated autocrine signaling in epithelial cells. Western Blot analysis of protein expression of VEGFR-1, VEGFR-2, p-VEGFR-2 (1059Y), and VEGF-A in prostate cancer (PC-3 **(B)** and LNCaP **(C)**) cells expressing 15-LOX-1. The empty vector transfected cells were used as control β-actin was used as loading control. The experiment was repeated three times independently and a representative Western blot has been shown here.

3.3.5 Functional Changes in Endothelial Cells

Since we did not observe any autocrine signaling through the decreased expression and secretion of VEGF-A, we next examined whether cellular characteristics of endothelial cells could be altered when incubated with CM from 15-LOX-1 expressing cancer cells. Endothelial cells form tubes when incubated with CM, which can be quantified and evaluated according to the number of branch sites/nodes, loops/meshes, or number or length of tubes

formed (DeCicco-Skinner et al. 2014). To assess the effect of 15-LOX-1 on angiogenesis functionally, we conducted this assay by using CM from LNCaP transiently transfected with empty vector (pcDNA3.1, EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1).

This assay was not conducted with PC-3 cells since we did not observe any alteration in VEGF-A mRNA, protein and secretion levels with when the cells were transfected with the 15-LOX-1 vector. Incubation of endothelial cells with CM from LNCaP cells (Figure 3.7 A and B) expressing 15-LOX-1 led to decreased tube formation as well as significant reductions in the number of branch sites/nodes, loops/meshes, or number or length of tubes formed as compared to the control (EV) cells.

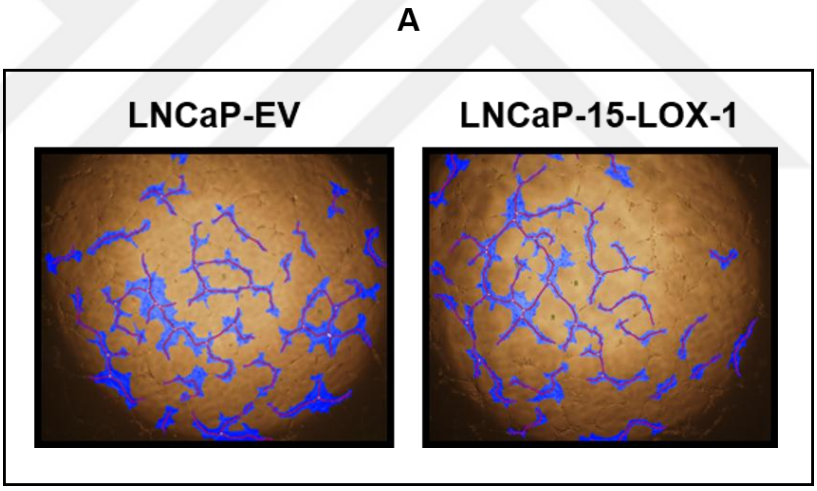


Figure 3.7 Reduced tube formation in endothelial cells incubated with conditioned medium from LNCaP cells expressing 15-LOX-1. Tube formation was analyzed with HUVEC that are plated on Matrigel and incubated for 6h with conditioned media from LNCaP cells transiently expressing 15-LOX-1. Empty vector transfected cells (EV) were used as controls. Representative images of the experiment were shown in **(A)**.

B

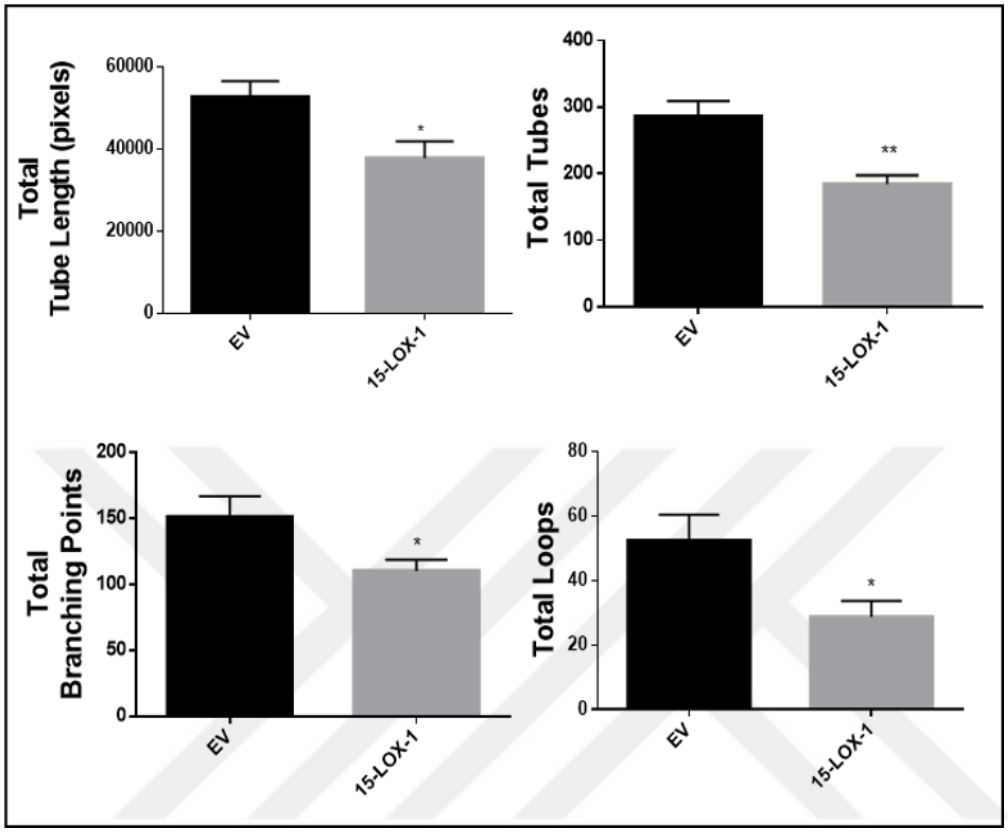


Figure 3.7 Reduced tube formation in endothelial cells incubated with conditioned medium from LNCaP cells expressing 15-LOX-1 (Cont'd). Tube formation was analyzed with HUVEC that are plated on Matrigel and incubated for 6h with conditioned media from LNCaP cells transiently expressing 15-LOX-1. Empty vector transfected cells (EV) were used as controls. Tube charachteristics like total tube length, total tube number, total braching points, total loops were shown in **(B)**.The experiment was repeated 2 times independently. Tube parameters were assayed using a program called WimTube and it is designed by Wimasis GMbH. Student's t-test was used as statistical method and $p<0.05$ was considered as significant.

3.3.6 Downstream Signaling in Endothelial Cells

Angiogenesis is related with several important pathological conditions like cancer, arthritis, asthma, autoimmune diseases and atherosclerosis. The process therefore is closely regulated by keeping a balance between pro and anti-angiogenic signals and involves signaling through integrins, chemokines, angiopoietins, oxygen sensing agents, junctional molecules and other endogenous inhibitors (Bouïs et al. 2006; Chung and Ferrara 2011; Carmeliet and Jain 2000).

To understand the signaling mechanisms that may have contributed to the reduction in tube formation observed with CM from 15-LOX-1 expressing cells, we conducted an angiogenesis proteome profiler array using proteins from endothelial cells that were incubated with conditioned media (CM) from 15-LOX-1 expressing LNCaP cells. Out of 55 different markers in the array, a statistically significant increase was observed in the expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) (Figure 3.8 A and B). Controls included protein from endothelial cells incubated with CM from empty vector transfected cells. TIMP-1 is a 28.5 kDa glycoprotein that inhibits the matrix metalloproteinases (MMPs) by binding to their active sites. Specifically, TIMP-1 is known to inhibit MMP-9, a matrix metalloprotease that is involved in degradation of the extracellular matrix during tissue remodeling in processes like wound healing, embryo growth and menstruation. Thus, the imbalance between MMPs and TIMPS is associated with pathological conditions like cancer metastasis, arthritis, cardiovascular diseases because of the disruption of the homeostasis (Daja et al. 2003; Sang 1998). Dual role of it in the context of carcinogenesis was reported and will be discussed on next section.

The array analysis was carried out by Dr. Özlen Konu and Ayşe Gökçe Keşküş (Bilkent University, Ankara). The analysis performs background correction followed by global and batch normalization of the data and statistically tests for batch corrected differences.

Details of the normalization of the array analysis can be seen in Appendix G. Additionally, the statistical analyses of the expression of the 55 different angiogenesis markers for three independent replicates for the LNCaP model are given in Table G.2 in Appendix G.

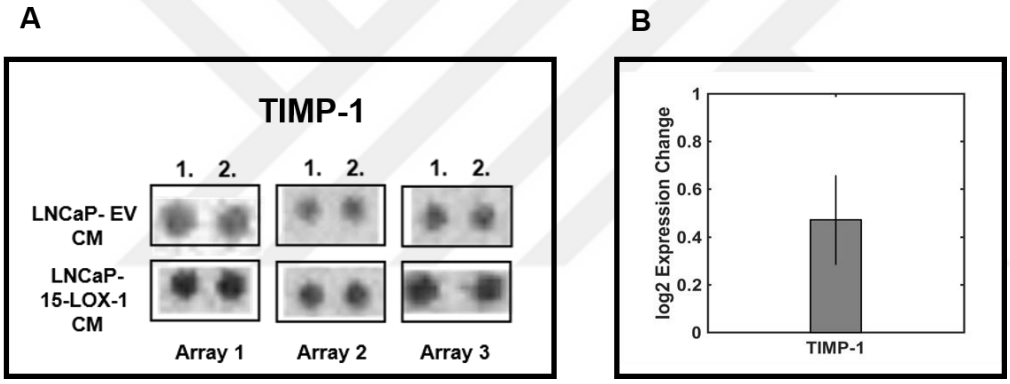


Figure 3.8 Angiogenesis proteome profile array shows enhanced expression of anti-angiogenic protein TIMP-1 in the prostate cancer model. (**A**) In the LNCaP model, the spots indicate enhanced expression of TIMP-1 in three independent arrays using proteins from endothelial cells incubated with CM from LNCaP cells transiently transfected with either the 15-LOX-1 vector or the empty vector (EV). (**B**) Box plot analysis of log2 fold changes of TIMP-1 shows a significant increase in expression in LNCaP 15-LOX-1 CM- endothelial cell model. All array analyses and statistical interpretations were carried out by Dr Özlen Konu and Ayşe Gökçe Keşkuş.

CHAPTER 4

DISCUSSION

15-LOX-1 has been shown in recent years to be important both in the context of inflammation and cancer through cross-talk with pathways involved in proliferation, apoptosis, angiogenesis and motility (Tuncer and Banerjee 2015). Controversies and contradictions still exist since available data indicate that the anti-carcinogenic effects of 15-LOX-1 are cell and context specific, being tumor suppressive in colon cancer and oncogenic in prostate cancer.

In this particular study, to unravel these contradictions, we investigated the effect of 15-LOX-1 on angiogenesis in colon and prostate cancer. To address its effect on angiogenesis in these different cancer models, we examined the expression, signaling and secretion of VEGF-A in these cancer models. In addition, we incubated endothelial cells with conditioned medium from our cancer models and examined the effect of 15-LOX-1 on endothelial tube formation and downstream signaling in these cells as well.

4.1 The relationship between 15-LOX-1 and angiogenesis

The expression of 15-LOX-1 is universally lost in CRC and many other tumors (Heslin et al. 2005). This loss is primarily due to epigenetic regulations, and re-expression of the gene both in cell line models and animal experiments have been shown to have anti-carcinogenic effects (Lee, Zuo, and Shureiqi 2011). Amongst the mechanisms of the anti-carcinogenic effects, the role of 15-LOX-1 in cancer angiogenesis has not been examined in detail. Angiogenesis, or blood vessel formation, is a necessity for a tumor to grow and metastasize via secretion of angiogenic factors including VEGF, the major driver of angiogenesis by cancer cells. Therefore, the secretion of VEGF is a potential therapeutic target in cancer; blocking new vessel formation via VEGF antibodies such as bevacizumab and VEGF receptor inhibitors have been used as therapeutics in different cancers (Folkman 1971; Potente, Gerhardt, and Carmeliet 2011). On the other hand, in clinics, because of resistance, these therapies eventually fail (Potente, Gerhardt, and Carmeliet 2011). Therefore, to inhibit angiogenesis, better understanding of the process and new therapeutic interventions are needed.

15-LOX-1 has been implicated as a factor that both activates and inhibits angiogenesis in different *in vitr*o and *in vivo* models. The effects of 15-LOX-1 expression on angiogenesis have been recently examined and the evidence is controversial, even contradictory in different cancer models. In a hypoxia-induced retinal neovascularization (RNV) model, 15-LOX-1 showed anti-angiogenic properties by inhibiting the expressions of VEGF-A, VEGF-R2 and eNOS in rat microvascular endothelial cells (RMVECs) *in vitro* (Yan et al. 2012). Mechanistically, the inhibition of RNV in the presence of 15-LOX-

1 was reported to be via the up-regulation of PPAR-γ and down-regulation of VEGFR-2 expressions (Zhi Li et al. 2014). Additionally, in a recent study, the restoration of 15-LOX-1 expression in HCT-116, HT29 and LoVo colon cancer cells inhibited VEGF expression and endothelial cell tube formation through, at least in part, a decrease in the expression and stability of HIF-1α (Wu et al. 2014). However, the signaling mechanisms are currently unknown. Therefore, in this thesis, we mostly focused on VEGF and its signaling, functional changes and downstream signaling in endothelial cells. The results in this study have confirmed existing data (Wu et al. 2014) indicating that 15-LOX-1 expression in colon cancer cell lines can lead to reduced VEGF-A expression and secretion, and decreased endothelial tube formation. On the other hand, the expression of 15-LOX-1 in the colon or prostate cancer models did not lead to any apparent alterations in the protein levels of VEGFR1, VEGFR2 and activation of p-VEGFR2 (1059Y, Figure 3.6).

There are several reasons that may address this loss of signaling:

1. VEGF secreted from the epithelial cells studied in this thesis did not signal in an autocrine manner; therefore, a stronger effect may be expected with paracrine signaling. This indeed turned out to be the case since endothelial cells incubated with conditioned medium (CM) from the epithelial cells showed changes in protein levels of important candidates in angiogenesis.
2. VEGF-A signaling may not be via these receptors in the models that we have studied; alternately, other phosphorylation sites of VEGFR2, such as Tyr951 and Tyr996, present in the kinase-insert domain; Tyr1054, present in the kinase domain; and Tyr1175 and Tyr1214 in the C-terminal tail may have been activated (Hoeben et al. 2004).

3. These receptor proteins can undergo other post-translational modifications like glycosylation (Lee et al. 2015; Holmes et al. 2007); which may affect the activity of the receptors rather than their protein levels.
4. Signaling is a dynamic system, the time point that we collected cells to isolate proteins provided a snapshot of signaling at that time. It is probable that the specific time point that we have collected the proteins may have been incorrect. Also, as opposed to general point of view of the mRNA levels reflecting protein levels, VEGF-A has significant post-transcriptional regulation which may change the correlation between mRNA and protein levels (Arcondéguy et al. 2013; Du et al. 2006).

To date, data about role of 15-LOX-1 in prostate cancer is limited and primarily shown by one research group. 15-LOX-1 was shown to have pro-tumorigenic effects in prostate cancer through transcriptional up-regulation by mutant p53 that acts as an oncogene (Kelavkar & Badr, 1999), enhanced angiogenesis with increased VEGF expression in mouse xenograft models of PC-3 cells (Kelavkar et al, 2001; Kelavkar et al, 2002), and enhanced bone metastasis by upregulation of insulin-like growth factor receptor (IGFR-1) (Kelavkar & Cohen, 2004).

When human 15-LOX-1 is conditionally expressed in mouse prostate, it gave rise to prostatic intraepithelial neoplasia in a FLiMP mouse model (Kelavkar et al, 2006). It should be noted some of these studies were performed on mice and the mouse homolog of 15-LOX-1 (12/15-LOX), has opposing functions with 15-LOX-1, therefore it is difficult to interpret the differential functions of 15-LOX-1 in murine models of neovascularization or tumor angiogenesis. Furthermore, since separate 12-LOX and 15-LOX enzymes do not exist in

rodents (Al-Shabrawey et al. 2011), 12/15-LOX gene knock-outs or knock-ins are likely to give limited and controversial results in human disease models.

We aimed to stably express 15-LOX-1 in PC-3 using the same plasmid that was used by Kelavkar et al. (2011) who could successfully express the protein in PC-3 cells stably. However, we observed very low endogenous levels of 15-LOX-1 in PC-3 cells (Figure 3.1B) and transfection with the 15-LOX-1 plasmid resulted in extensive cell death when cells were transfected with as low as 1μg of the vector; additionally, during the process of selection with zeocin we did not observe any colonies even when the zeocin amount was reduced. We therefore continued with transient transfection of the cells; however, we did not observe any changes in VEGF mRNA, protein and secretion levels (Figure 3.3, 3.4, 3.6) in 15-LOX-1 transfected PC-3 cells. Our observations are contrary to published data. Kelavkar et al. reported high endogenous expression of 15-LOX-1 in PC-3 cells and ***knockdown*** of 15-LOX-1 was reported by the authors to have an anti-carcinogenic, anti-angiogenic effect. These findings may be related to differences in cell passage, overexpression strategy, transfection reagent, technical differences, etc. We are confident of the identity of our cells as they were bought directly from ATCC at the start of this study.

Interestingly, a second prostate cancer cell line that was used in this study, LNCaP, when transfected with the 15-LOX-1 plasmid, showed decreased proliferation, as well as decreased VEGF-A mRNA expression and secretion (Figure 3.3 and 3.4). Conditioned medium collected from the 15-LOX-1 transfected LNCaP cells could also reduce tube formation in endothelial cells (Figure 3.7). Supporting our data, bioactive lipids produced from the metabolism DHA by 15-LOX-1 in LNCaP prostate cancer cells was previously shown to reduce cell proliferation by activating PPARγ signaling and apoptosis (O'Flaherty et al. 2012). Moreover, the same metabolic

products were shown to be important for the activation of syndecan-1 (SDC-1), a downstream target of PPARγ protein that is important in cell to matrix interactions, cellular proliferation and migration as well as caspase-3 activity (O'Flaherty et al. 2012). The induction of apoptosis through the activation of syndecan-1 was via a PDK1/Akt/Bad survival pathway. It should be noted as in the same study silencing of 15-LOX-2, 5-LOX, COX-1, COX-2 or 12-LOX had no discernable effect (O'Flaherty et al. 2012).

In this same study, it was stated that LNCaP had higher 15-LOX-1 expression than PC-3 and the authors used siRNA based strategy for silencing of 15-LOX-1. Similarly, we have also observed relatively high mRNA levels in LNCaP cells (Figure 3.1 A), however, expression at the protein level was very weak when assayed by Western blot (Figure 3.1 B). This may have resulted from differences in the sensitivities of PCR and Western blot, or it may have resulted from post-transcriptional regulation of 15-LOX-1, which has not been examined in 15-LOX-1 to date.

There is no doubt that lipids are crucial in signaling and cellular physiology in both health and disease; thus, products of LOXs mediated metabolism of fatty acids are important in determining disease outcomes by producing pro-inflammatory or pro-resolution mediators.

For instance, diet with omega-3 fatty acids resulted in decreased proliferation and increased apoptosis in prostate cancer by competing with omega-6 fatty acids that are mainly metabolized by 15-LOX-1 and COX-2 (Uddhav P Kelavkar et al. 2009) Also, in prostate cancer cells, a balance between the expression of 15-LOX-1 and 15-LOX-2 appear to be important. 15-LOX-1 been claimed to act as a tumor inducer through the production 13-(S)-HODE while 15-LOX-2 through its metabolite 15-(S)-HETE can act as a tumor suppressor by inhibiting Myc-induced tumor development (Guo and Nie

2014). Therefore, high 15-LOX-1 and low 15-LOX-2 may lead to tumor progression.

15-LOX-1 metabolite 13-(S)-HODE mediated upregulation of MAP kinase and PPARγ phosphorylation was observed in prostate cancer studies, conversely, 15-LOX-2 metabolite 15-(S)-HETE did the exact opposite, implying the opposite roles of the metabolites of 15-LOX-1. In the current study, we have not examined which metabolite of 15-LOX-1 resulted in the reduced angiogenesis observed; therefore, evaluation of the 15-LOX-1 primary and secondary metabolic products and the reactions mediated by these products may help to understand the link between the 15-LOX-1 expression in tumor cells and neovascularization.

LNCaP and PC-3, having different metastatic capabilities and androgen receptor expression, resembles different stages in prostate cancer. LNCaP represents an early stage of the tumor while PC-3 represents late stages; which may have resulted in such different outcomes in angiogenesis. In a high-throughput study where scientists checked the differential expression profiles of PC-3 and LNCaP, PC-3 cells were shown to have unique expression profiles of VEGFC, Il-8 and TGFbeta2 to suppress apoptosis and progress into androgen-independent state.

In LNCaP cells, higher expression of transcription factors like GATA-1, and c-Myc/Max can be responsible for the specific characteristics of these cells that enables the retention of cellular characteristics that are more similar to the normal prostate (Dozmorov et al. 2009). In conjunction with this, two other studies showed that the expression of angiogenic markers such as Angiopoietin-2 and MMP-9 differ in these cell lines as well, explaining the higher invasiveness of PC-3 than LNCaP (Tešan et al. 2008; Aalinkeel et al. 2004). Moreover, the differences in sensitivity to androgen (PC-3 is

insensitive to androgen while LNCaP is sensitive) may have contributed to the different cell behaviors observed in our study. This has not been examined in our study and needs to be addressed with future experiments.

Complicating the story even further, very recently, 15-LOX-1 over-expression in PC-3 cells was shown to reduce angiogenesis through an ubiquitin mediated enhanced degradation of HIF-1α and a consequent lower expression of VEGF-A mRNA (Zhong et al. 2014). It is important to note that this study came from the same group (Kelavkar et al.) that initially defended the notion that 15-LOX-1 expression had angiogenic properties in PC-3 cells. Such conflicting observations may be due to the different biological effects of the many lipid mediators generated by 15-LOX-1 pathway, which have not yet been completely clear (Weibel et al. 2009). Another explanation can be that cells might behave differently when exposed to altered 15-LOX-1 activity. Working with a cancer cell line also bears the risk of rapid accumulation of mutations, which may result in a different response to a given stimulus years after the first study, which may also explain the conflict in Kelavkar's data. In any case, this suggests that 15-LOX-1 is interconnected with a plethora of different pathways, responses of which might change the cellular decision drastically.

4.2 TIMP-1: a potential candidate for the effect of 15-LOX-1 on angiogenic properties of prostate cancer

An angiogenesis array was conducted with proteins from endothelial cells that were exposed to CM from LNCaP cells expressing 15-LOX-1. The primary

candidate that showed altered protein levels in these experiments (observed in three independent replicates) was TIMP-1 (Figure 3.8 A and B). Since neovascularization involves degradation of basement membrane to promote migration and proliferation of endothelial cells, homologous zinc endopeptidases called matrix metalloproteinases (MMPs) are important to degrade peptide bonds of the many extracellular matrix (ECM) proteins that includes collagens, laminins, elastin, and fibronectin. Their roles are regulated through proteins called tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 is a 28.5 kDa glycoprotein that primarily acts on MMP-9 and has conflicting roles in angiogenesis (Sang 1998). In general, MMPs are considered to be stimulators of angiogenesis while TIMPs are the blockers. However, literature suggests that these effects can be context dependent. For instance, immunohistochemistry based analyses showed that loss of TIMP-1 was correlated with prostate cancer recurrence due to failure in regulating MMP-9 (Reis et al. 2015).

Inhibition of TIMP-1 in human dermal microvascular endothelial cells, led to increased angiogenesis and migration (Reed et al. 2003), which supports our observations. Mechanistically, this was exerted by MMP-dependent activation of adhesion molecule VE-cadherin and MMP-independent activation of PTEN with dephosphorylation of FAK, which resulted in decreased F-actin stress fibers that are important for cytoskeletal remodeling (Akahane et al. 2004). However, it has also been reported that TIMP-1 can stimulate cancer associated fibroblasts (CAFs) within prostate and colon cancer tissues to promote proliferation and migration (Gong et al. 2013). Additionally, in colon cancer, TIMP-1 is proposed as prognostic marker whereby increased of the protein can result in increased proliferation, metastasis and decreased apoptosis via FAK-PI3K/AKT and MAPK pathway (Siegel et al. 2016). TIMP-1 was accounted as tumor biomarker and linked with poor prognosis in

breast cancer (Würtz et al. 2008). Complicating its role even further, it has been emphasized that circulating TIMP-1 not the mammary TIMP-1 inhibits growth of mammary carcinomas in transgenic mice (Yamazaki et al. 2004).

As can be seen from these studies, mechanisms of action of MMPs and TIMPs in angiogenesis can vary due to tissue/cell specificity, the stages of endothelial differentiation, and local microenvironmental factors. Thus, any novel mechanistic studies showing potential roles of them in angiogenesis will open new doors to better understanding the role of different proteins in the cancer microenvironment. Our subsequent studies will focus on the role of TIMP-1 in endothelial cells to enlighten this debatable question: ***Is 15-LOX-1 is pro-carcinogenic or anti-carcinogenic in prostate carcinoma?***

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, we investigated the role of 15-LOX-1 in angiogenesis in colon and prostate cancer. The aim was to address several controversial data that exist in the literature suggesting anti-carcinogenic anti-angiogenic roles of 15-LOX-1 in colon cancer and the opposite in prostate cancer. As novel data that was generated from this thesis, we have established that regardless of the cancer model used, 15-LOX-1 expression led to an anti-angiogenic effect, with decreased VEGF-A mRNA levels and secretion from the epithelial cells, reduced endothelial tube formation, and enhanced expression of anti-angiogenic protein TIMP-1 in endothelial cells. Both models showed similar anti-angiogenic effects and prostate cancer model showed alterations in signaling through TIMP-1.

To address the variability of the effects of 15-LOX-1 expression in cancer models in the literature, it is important to appreciate that the effects may be entirely cell type and context specific. Not only the availability of the availability of the enzyme, but also the availability of the substrate needs to be considered as the downstream functions of the wide variety of bioactive lipids produced by this enzyme may vary dramatically. Moreover, the enzyme is also expressed in a number of cells in the stroma such as macrophages and neutrophils, which may completely alter the balance of bioactive lipids

available in the tumor microenvironment. Reductionist studies carried out using cell lines are excellent to elucidate signaling mechanisms, however, when commenting on the effects of the enzyme, it may be important to also consider the role played by the microenvironment.

This particular study conceived the following new questions in the field:

- Variations in the types of bioactive lipids produced in PC-3 vs LNCaP cells may have resulted in the differences in the response of these cells to 15-LOX-1 transfection. The type of bioactive lipids produced in these two models can be examined.
- The relationship between androgen dependence and 15-LOX-1 regulation should be investigated to reveal the different observations with PC-3 and LNCaP in this study.
- The enhanced expression of TIMP-1 in the endothelial cells of the prostate cancer model needs to be confirmed along with the inherent signaling mechanisms.

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APPENDIX A

qRT-PCR STANDARD CURVES

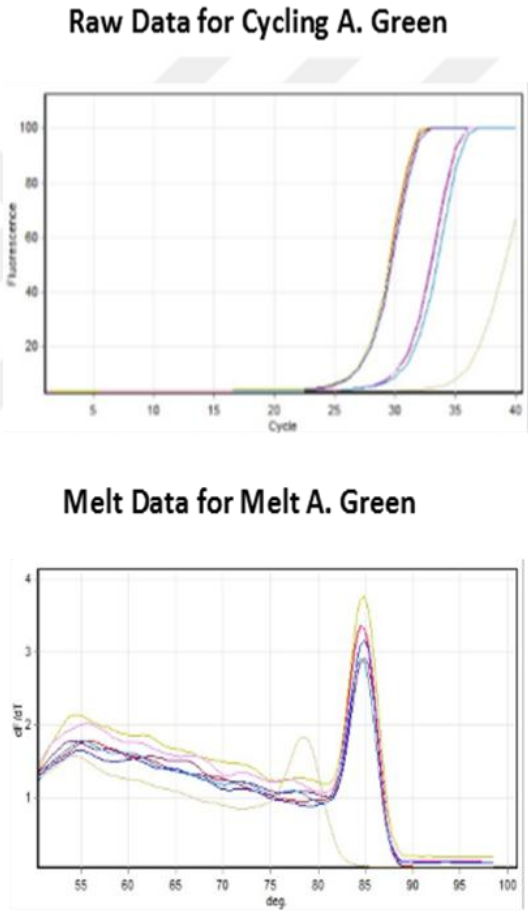
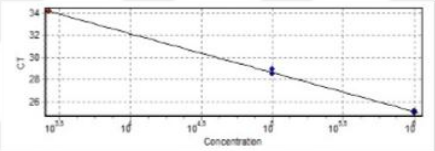


Figure A.1 Standard and melt curves used for β-actin qRT-PCR primers.

Threshold	0,3323
Left Threshold	1,000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0,285*CT + 13,171)
Standard Curve (2)	CT = -3,504*log(conc) + 46,148
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 0,9293
M	-3,50386
B	46,14837
R Value	0,99617
R^2 Value	0,99235
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

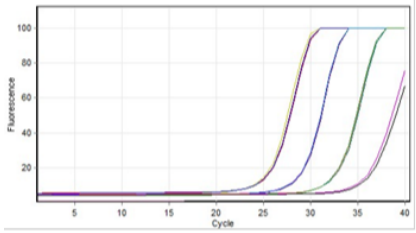
Standard Curve



No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		1:500	Standard	25,02		1000000	1073681,27818643
2		1:500	Standard	25,17		1000000	972394,138874988
3		1:500	Standard	25,19		1000000	957816,440334122
4		1:5000	Standard	28,48		100000	110096,472911426
5		1:5000	Standard	28,49		100000	109725,745825562
6		1:5000	Standard	28,92		100000	82778,5953156149
13		NTC	NTC	34,13		0	2688,49720210606

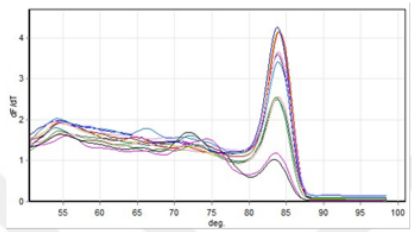
Figure A.1 (Cont'd). Standard and melt curves used for β-actin qRT-PCR primers.

Raw Data for Cycling A. Green

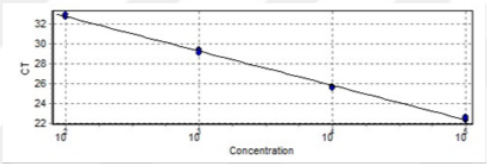


Threshold	0,180
Left Threshold	1,000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0,288*CT + 11,435)
Standard Curve (2)	CT = -3,471*log(conc) + 39,687
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 0,94145
M	-3,4707
B	39,68689
R Value	0,99883
R^2 Value	0,99766
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Melt Data for Melt A. Green



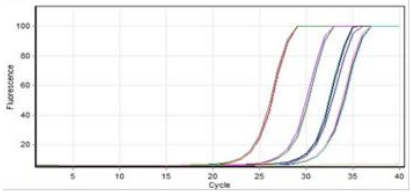
Standard Curve



No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		1-1	Standard	22,64		100000	81718,5069026201
2		1-2	Standard	22,26		100000	105043,77728428
3		1_3	Standard	22,48		100000	90937,6634973294
4		2_1	Standard	25,66		10000	11005,0728136875
5		2_2	Standard	25,65		10000	11099,980642929
6		2_3	Standard	25,54		10000	11907,5124902615
7		3_1	Standard	29,42		1000	910,4126807093
8		3_2	Standard	29,35		1000	949,040368739418
9		3-3	Standard	29,06		1000	1150,0083844794
10		4-1	Standard	32,71		100	102,678954730836
11		4-2	Standard	32,97		100	86,3224658846837
19		NTC	NTC				

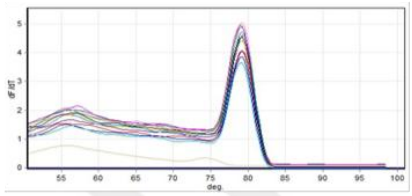
Figure A.2 Standard and melt curves used for VEGF-A qRT-PCR primers.

Raw Data for Cycling A. Green

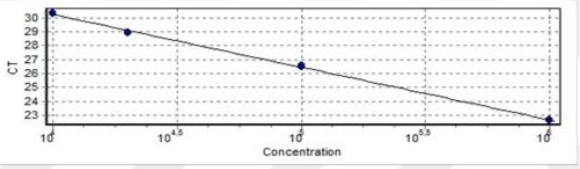


Threshold	0,3283
Left Threshold	1,000
Standard Curve Imported	No
Standard Curve (1)	conc= 10^(-0,263*CT + 11,962)
Standard Curve (2)	CT = -3,799*log(conc) + 45,441
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 0,83334
M	-3,79878
B	45,44089
R Value	0,99927
R^2 Value	0,99854
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Melt Data for Melt A. Green



Standard Curve



No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		10 1	Standard	22,60		1000000	1030293,65006282
2		100 1	Standard	26,56		100000	93447,6528820941
3		500 1	Standard	29,01		20000	21090,0970777733
4		1000 1	Standard	30,26		10000	9923,22814739733
5		10 2	Standard	22,71		1000000	965263,50214159
6		100 2	Standard	26,64		100000	88982,1042480697
7		500 2	Standard	28,91		20000	22470,3816464142
8		1000 2	Standard	30,25		10000	9953,25086548097
9		10 3	Standard	22,56		1000000	1053024,71857274
10		100 3	Standard	26,41		100000	102210,806920393
11		500 3	Standard	29,00		20000	21323,4029337364
12		1000 3	Standard	30,42		10000	9005,61047987783
13		NTC	NTC				

Figure A.3 Standard and melt curves used for 15-LOX-1 qRT-PCR primers

APPENDIX B

*M*INIMUM *I*NFORMATION FOR PUBLICATION OF *Q*UANTITATIVE REAL-TIME PCR *E*XPERIMENTS GUIDELINES

Table B.1 MIQE Guidelines.

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	YES
Number within each group	E	YES
Assay carried out by core lab or investigator's lab?	D	YES
Acknowledgement of authors' contributions	D	NO
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	E	YES
Name of kit and details of any modifications	E	YES
Source of additional reagents used	D	YES
Details of DNase or RNAse treatment	E	YES
Contamination assessment (DNA or RNA)	E	NO
Nucleic acid quantification	E	YES
Instrument and method	E	YES
Purity (A260/A280)	D	YES
Yield	D	YES
RNA integrity method/instrument	E	YES
RIN/RQI or Cq of 3' and 5' transcripts	E	NO
Electrophoresis traces	D	N/A
Inhibition testing (Cq dilutions, spike or other)	E	YES
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	YES
Amount of RNA and reaction volume	E	YES
Priming oligonucleotide and concentration	E	YES
Reverse transcriptase and concentration	E	YES
Temperature and time	E	YES
Manufacturer of reagents and catalogue numbers	D	YES
Cqs with and without RT	D	NO
Storage conditions of cDNA	D	YES
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	E	N/A
Sequence accession number	E	YES
Location of amplicon	D	NO
Amplicon length	E	YES
In silico specificity screen (BLAST, etc)	E	YES
Pseudogenes, retropseudogenes or other homologs?	D	NO
Sequence alignment	D	NO
Secondary structure analysis of amplicon	D	NO
Location of each primer by exon or intron (if applicable)	E	YES
What splice variants are targeted?	E	N/A
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	YES
RTPrimerDB Identification Number	D	N/A
Probe sequences	D	N/A

Table B.1 (Cont'd) MIQE Guidelines.

Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	YES
Purification method	D	NO
qPCR PROTOCOL		
Complete reaction conditions	E	YES
Reaction volume and amount of cDNA/DNA	E	YES
Primer, (probe), Mg++ and dNTP concentrations	E	YES
Polymerase identity and concentration	E	YES
Buffer/kit identity and manufacturer	E	YES
Exact chemical constitution of the buffer	D	N/A
Additives (SYBR Green I, DMSO, etc.)	E	YES
Manufacturer of plates/tubes and catalog number	D	YES
Complete thermocycling parameters	E	YES
Reaction setup (manual/robotic)	D	NO
Manufacturer of qPCR instrument	E	YES
qPCR VALIDATION		
Evidence of optimization (from gradients)	D	YES
Specificity (gel, sequence, melt, or digest)	E	YES
For SYBR Green I, Cq of the NTC	E	YES
Standard curves with slope and y-intercept	E	YES
PCR efficiency calculated from slope	E	YES
Confidence interval for PCR efficiency or standard error	D	N/A
r2 of standard curve	E	YES
Linear dynamic range	E	YES
Cq variation at lower limit	E	YES
Confidence intervals throughout range	D	N/A
Evidence for limit of detection	E	YES
If multiplex, efficiency and LOD of each assay.	E	N/A
DATA ANALYSIS		
qPCR analysis program (source, version)	E	YES
Cq method determination	E	YES
Outlier identification and disposition	E	N/A
Results of NTCs	E	YES
Justification of number and choice of reference genes	E	YES
Description of normalization method	E	YES
Number and concordance of biological replicates	D	YES
Number and stage (RT or qPCR) of technical replicates	E	YES
Repeatability (intra-assay variation)	E	YES
Reproducibility (inter-assay variation, %CV)	D	NO
Power analysis	D	NO
Statistical methods for result significance	E	YES
Software (source, version)	E	YES
Cq or raw data submission using RDML	D	N/A

E: Essential information, D: Desirable information, N/A: Not applicable

APPENDIX C

CONTENTS OF BUFFERS USED FOR WESTERN BLOT EXPERIMENTS

6X Sample Loading Buffer

12% SDS, 30% β-mercaptoethanol, 30% glycerol, 0.012% Bromophenol Blue, 0.375 M Tris-HCl pH 6.8

PBS-T

8 g NaCl, 0.27 g KH_2PO_4, 3.58 g $Na_2HPO_4.12H_2O$; pH adjusted to 7.4, autoclaved, and 0.1% Tween-20 added before use.

TBS-T

50 mM Tris-HCl pH 7.4, 150 mM NaCl; autoclaved, and 0.1% Tween-20 added before use.

10X Blotting Buffer

30.3 g Trizma Base (0.25 M), 144 g glycine (1.92 M); pH adjusted to 8.3 in 1 L dH_2O.

Transfer Buffer (2 L)

400 ml methanol, 200 ml 10X Blotting Buffer, 1400 ml water

SDS-PAGE Buffer

25 mM Tris, 190 mM glycine, 0.1% SDS

	Seperating Gel Mix (10%)	**Stacking Gel Mix (4%)**
30% PAA Mix	1.2 ml	5.6 ml
4X Stacking gel mix	2 ml	----
4X Separating gel mix	-----	3.8 ml
ddH2O	4.7 ml	5.4 ml
10% APS	50 µl	150 µl
TEMED	10 µl	20 µl
Final Volume	8 ml	15

Mild Stripping Buffer

15 g glycine, 1 g SDS, 10 ml Tween-20; pH adjusted to 2.2 in 1 L dH_2O.

Harsh Stripping Buffer

100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl ph 6.8

APPENDIX D

MAPS OF VECTORS USED IN THIS STUDY

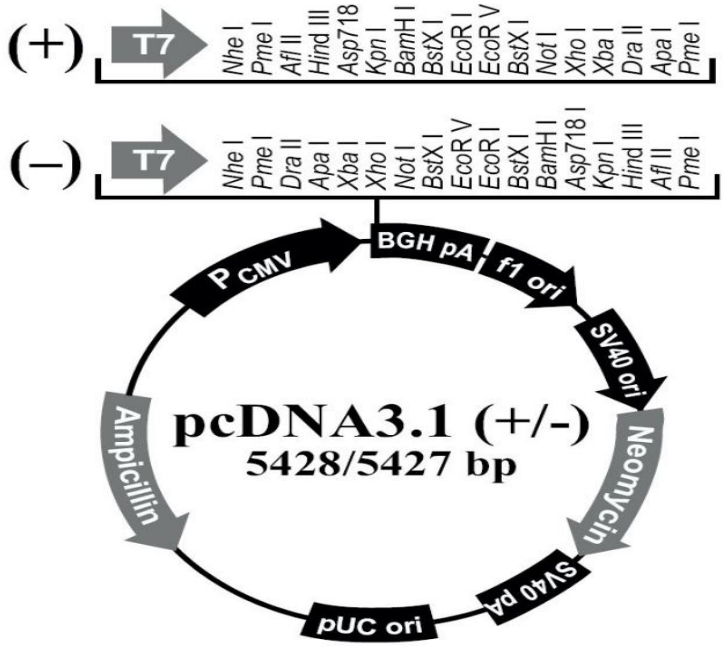


Figure D.1 pcDNA3.1/Zeo (-) 5015 nucleotides Plasmid Map

pcDNA3.1 (-) used as empty vector control and was generated from pcDNA3.1-15-LOX-1 vector (gift from Dr. Uddhav Kelavkar) by cutting it with restriction enzyme EcoRI in our lab's previous studies.

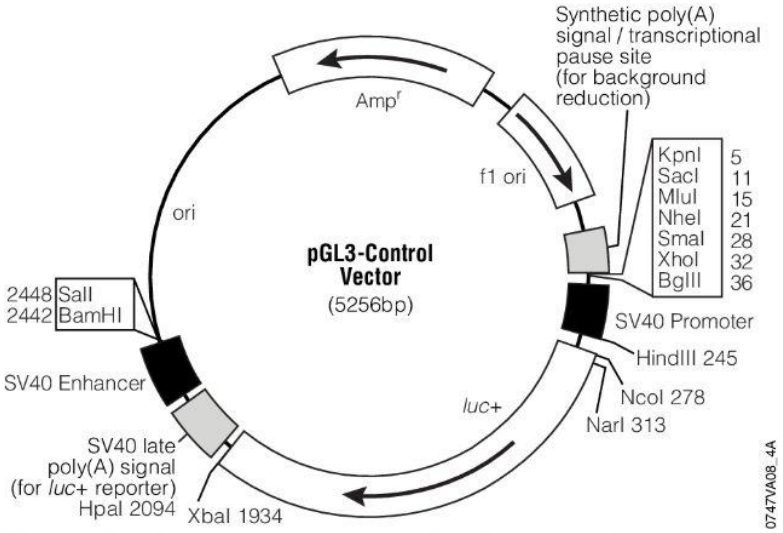


Figure D.2 pGL3 Luciferase Vector Backbone. Into VEGF promoter, 5X HIF-1α Response Elements were cloned. (A generous gift from Dr. Ilias Mylonis, University of Thessaly).

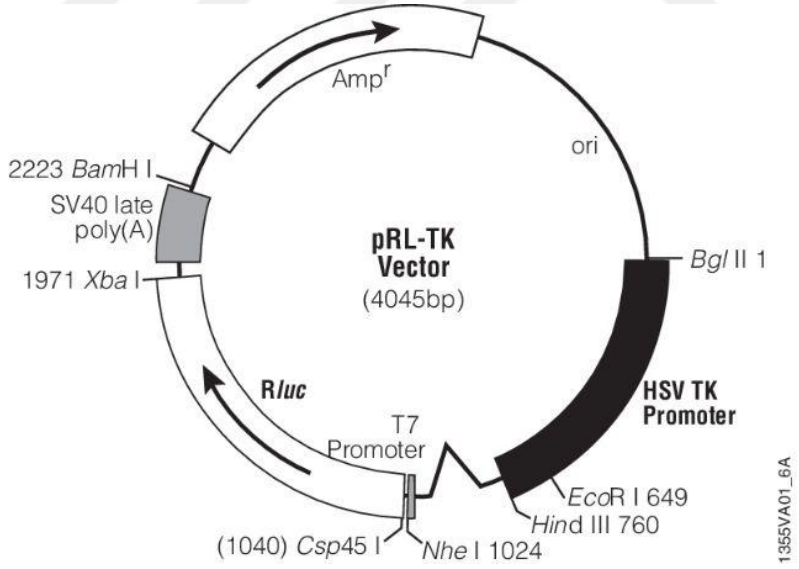


Figure D.3 pRL-TK vector map (Promega). Used as internal control in lusiferase experiment.

APPENDIX E

KILL CURVE ASSAY FOR PC-3 CELLS

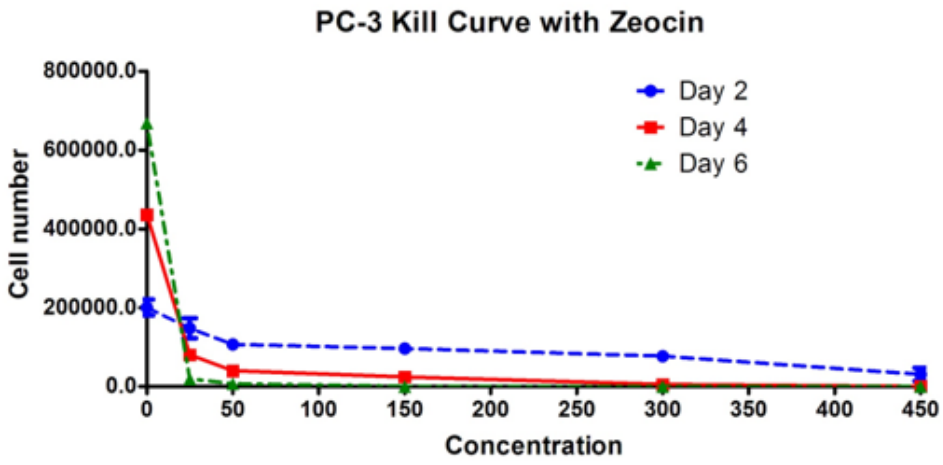


Figure E.1 Zeocin Kill Curve with PC-3 cells. Kill curve was generated by using 50µg/ml, 150 µg/ml, 300 µg/ml, 450 µg/ml zeocin concentrations. Cells were counted for 1 week with 2 day intervals. After transient transfection for 24 h with the conditions above, by using 100 µg/ml, selection process was started. After 1 week of selection, zeocin concentration was lowered to its half amount and waited for colonies to grow.

APPENDIX F

TRANSFECTION OPTIMIZATIONS FOR LNCaP PROSTATE CANCER CELLS

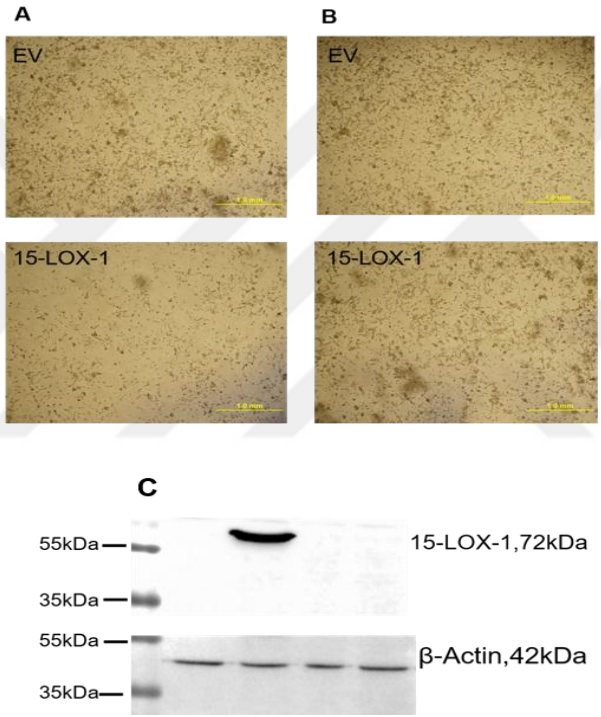


Figure F.1 15-LOX-1 Transient Transfection Optimization with LNCaP Cells. A. 4X image of LNCaP cells transiently transfected for 24 h with 1:2 and 2:1 ratio **(B)** containing 1µg EV (pcDNA3.1) or 15-LOX-1 (pcDNA3.1-15-LOX-1) vector. **C.** Confirmation Western Blotting of 15-LOX-1 in order of proteins: 1.1µg EV with 1:2 ratio, 2. 1µg 15-LOX-1 with 1:2 ratio, 3. 1µg EV with 2:1 ratio, 4. 1µg EV with 2:1 ratio. β-Actin was used as loading control.

APPENDIX G

PROTEOME PROFILER HUMAN ANGIOGENESIS ARRAY

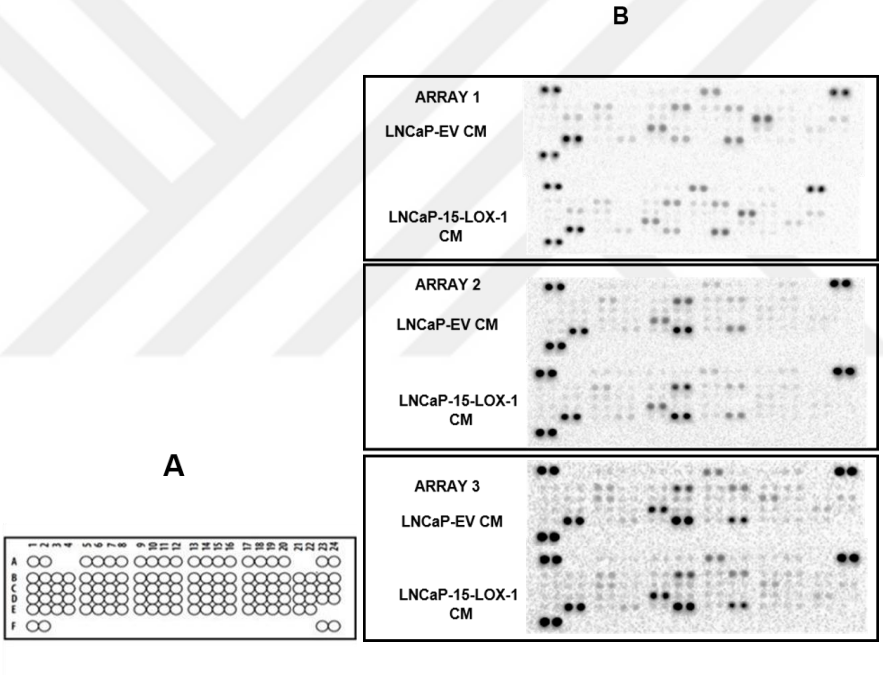


Figure G.1 Proteome Profiler Human Angiogenesis Array and its analysis. (A) Representative image for the array membrane. **(B)** Whole pictures of the all three array membranes of endothelial cells that are treated either with empty vector (pcDNA3.1, EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) LNCaP conditioned media (CM).

C

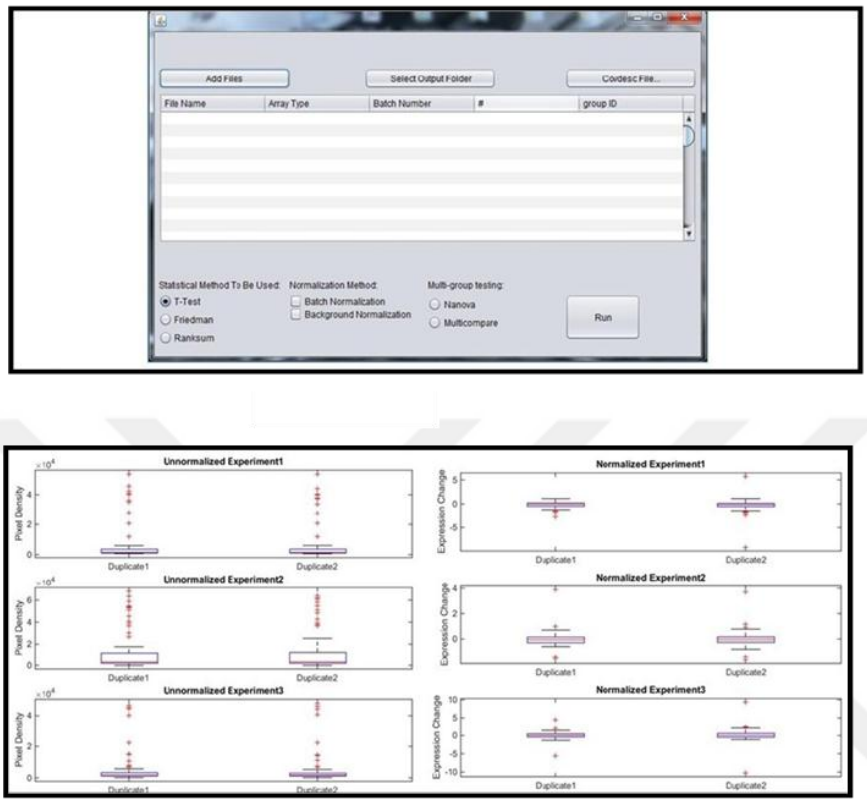


Figure G.1 Proteome Profiler Human Angiogenesis Array and its analysis (Cont'd). (C) Array analysis by Java based GUI programme in Matlab software (upper) in which at the lower part showing spot duplicates of the three arrays before normalization (left: unnormalized experiment) and after normalization (right: normalized experiment) to decrease the difference between spot intensities in the normalization via t-test, Wilcoxon sum rank test (or Friedman's test) and N way ANOVA for comparison of multiple entities (developed by Dr. Özlen Konu and her student Ayşe Gökçe Keşküş from Bilkent University).

Table G.1 p-values and log2 fold changes of all spots in all three arrays of endothelial cells that are treated either with empty vector (pcDNA3.1, EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) LNCaP conditioned media (CM). Genes are ordered according to increasing p values. $p<0.05$ ones were considered to be significant.

Name of the Protein	pValue	log2 fold change (array1)	log2 fold change (array2)	log2 fold change (array3)
Angiostatin_ Plasminogen	0,0314	-0,2854	-0,4630	-0,4927
Leptin	0,0103	0,3706	0,2331	0,1781
TIMP-1	0,0117	0,6436	0,4967	0,2744
Vasohibin	0,0129	0,3518	0,1491	0,3399
EGF	0,0389	-0,1572	-0,4554	-0,6224
Reference Spots	0,0680	0,0521	0,2045	0,0868
PTX3	0,0705	0,4657	0,0636	0,3469
LAP_TGF-β1	0,1338	-0,0233	-0,7872	-0,4318
Reference Spots3	0,1759	-0,1234	-0,2158	0,0125
Thrombospondin-2	0,1982	-0,5814	-0,8932	0,1078
Activin A	0,2007	-1,8401	-0,0704	-0,5248
Angiogenin ANG	0,2066	0,5954	0,0178	0,1681
EG-VEGF	0,2236	1,8721	1,6315	-0,3974
Amphiregulin	0,2249	-0,5552	0,1413	-0,7444
GM-CSF	0,2454	2,1058	0,5045	-0,0108
MCP-1α	0,2739	0,3312	-1,1335	-3,836
Endothelin-1	0,2811	-0,0482	0,1238	0,1855
DPPIV	0,3378	1,3568	-0,0481	0,1316
IGFBP-2	0,3532	-4,1103	-0,2576	0,1161
Endoglin	0,3730	3,6273	-0,1633	0,1764
Serpin E1	0,3937	-3,2909	-0,1423	0,23355
FGF-7	0,3977	0,862311	-0,3743	0,5713
FGF basic	0,4133	-0,4495	9,8548	-0,1429
NC	0,4569	1,5900	-3,6043	-1,7334
PF4	0,4633	-0,4030	0,3717	1,0543
MIP-1α	0,4666	-0,8696	-0,3313	0,3501
FGF-4	0,4773	0,9655	-0,5135	0,5908
Serpin B5	0,4866	-0,8636	-0,5767	0,4927
HGF	0,5063	-0,9166	0,3576	-0,2459

Table G.1 (Cont'd) p-values and log2 fold changes of all spots in all three arrays of endothelial cells that are treated either with empty vector (pcDNA3.1, EV) or 15-LOX-1 vector (pcDNA3.1-15-LOX-1) LNCaP conditioned media (CM).

Name of the Protein	pValue	log2 fold change (array1)	log2 fold change (array2)	log2 fold change (array3)
PDGF-AB_ PDGF-BB	0,5107	0,2116	-0,2076	0,3674
uPA	0,5187	-0,1273	-0,2732	0,1424
Serpin F1	0,5209	0,9970	-0,1833	-0,0318
Reference Spots2	0,5218	0,1353	-0,1076	0,1468
Angiopoietin-1	0,5223	-0,2433	0,3654	0,2767
Artemin	0,5227	0,8246	0,4782	-0,4824
PlGF	0,5424	-0,2522	0,7403	-2,2285
PDGF-AA	0,5479	0,0189	-1,0619	0,2485
GDNF	0,5642	-0,7937	1,9335	0,3494
IL-1β	0,6142	0,5944	-0,8072	-0,4803
Thrombospondin-1	0,6488	0,3931	-0,2706	0,1646
Persephin	0,6721	0,2791	-0,0953	-0,4858
PD-ECGF	0,6749	0,3615	-0,9428	0,0487
HB-EGF	0,7106	-0,5259	0,2531	-0,0013
Endostatin_Collagen XVIII	0,7249	-0,0568	-0,4223	0,2568
FGF acidic	0,7454	0,2692	0,4847	-1,3629
Angiopoietin-2	0,7495	-0,2614	-0,3748	0,3913
TIMP-4	0,7724	0,7239	-0,7725	0,4786
IL-8	0,7818	0,4217	0,2950	-0,469
NRG1-β1	0,7924	0,5802	-0,0688	-0,8639
ADAMTS-1	0,8297	-0,1044	0,6847	-0,8939
IGFBP-1	0,8352	0,7657	-0,6907	0,2074
CXCL16	0,8546	-0,2688	0,6437	-0,5917
MMP-9	0,9017	0,9530	-1,4200	0,1911
Coagulation Factor III	0,9076	-0,3771	0,8331	-0,6227
VEGF	0,9110	-0,8927	0,4734	0,2675
MMP-8	0,9421	-0,0166	-0,5686	0,6680
Prolactin	0,9613	0,3803	0,4301	-0,8766
VEGF-C	0,9998	0,32162	-0,0282	-0,2933

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