EFFECTS OF 15-LIPOXYGENASE-1 ON THE ANGIOGENIC PROPERTIES OF COLORECTAL CANCER CELL LINE SW480

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EFFECTS OF 15-LIPOXYGENASE-1 ON THE ANGIOGENIC PROPERTIES OF COLORECTAL CANCER CELL LINE SW480

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ABSTRACT

EFFECTS OF 15-LIPOXYGENASE-1 ON THE ANGIOGENIC PROPERTIES OF COLORECTAL CANCER CELL LINE SW480

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Colorectal cancer is a disease resulting from the malignant transformation of colon epithelia. As of 2013, it the third leading cause of death among cancer related deaths, in Turkey. Lipoxygenases (LOXs) are a family of fatty acid dioxygenases that convert polyunsaturated fatty acids (PUFAs) to their oxidized form, which are subsequently transformed to bioactive lipid mediators acting in various cell functions. Specifically, 15-Lipoxygenase-1 (15-LOX-1) is a member of the LOX family, oxygenating linoleic acid (LA) preferentially to 13-(S)-HODE as the final product. 15-LOX-1 is known to be important in the resolution of inflammation; the enzyme also has tumor suppressive properties and it can get downregulated in several different cancer types. 15-LOX-1 re-expression in colorectal cancer was shown to decrease metastasis, cell proliferation and invasive capabilities of the cells. We wanted to examine whether re-expression of the enzyme would also lead to a decrease in angiogenic properties. In this context, we assayed the VEGF transcript

and secreted protein levels and treated HUVEC cells with conditioned media from SW480 cells expressing 15-LOX-1 . We observed a decrease in both VEGF transcript levels and secreted protein levels in both normoxic and hypoxic conditions. Additionally, HUVECs incubated with conditioned media collected from 15-LOX-1 transfected SW480 cells showed reduced formation of tube-like structures *in vitro*. In conclusion, 15-LOX-1 re-expression was shown to reduce angiogenic abilities of the colorectal cancer cells.

Keywords: colorectal cancer, 15-lipoxygenase-1, lipoxygenases, angiogenesis, VEGF

ÖZ

15-LİPOKSİGENEZ-1 İFADESİNİN KOLOREKTAL KANSER HÜCRE HATTI SW480'İN ANJİYOGENİK ÖZELLİKLERİNE ETKİSİ

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Kolorektal kanser, kolon epitel hücrelerinin kötü huylu tümöre dönüşmesinden ortaya çıkan bir hastalıktır. Türkiye'de 2013 itibarıyla, kolorektal kanser, kanser nedenli ölümler arasında üçüncü sıradaki ölüm sebebidir. Lipoksigenezler (LOX) çoklu doymamış yağ asitlerini (PUFA) sonrasında hücrede çeşitli görevlerde yer alan oksidize hallerine dönüştüren bir dioksijenaz familyasıdır. Spesifik olarak, 15-Lipoksigenez-1 (15-LOX-1), LOX familyasının bir üyesi olarak, tercihen linoleic asidi (LA) 13-(S)-HODE'ye oksitleyen bir enzimdir. Normal hücrelerde enflamasyonun çözünmesi ya da tümör baskılanması gibi görevleri varken, meme ve kolorektal gibi kanser çeşitlerinde ifadesi azalmaktadır. 15-LOX-1 ifadesinin kolorektal kanserde yeniden oluşturulması metastaz, hücre büyümesi ve hücrelerin invazyon gibi yetilerinde azalmaya sebep olmaktadır. Araştırmamızda, bu enzimin yeniden ifadesinin hücrelerin anjiyogenik özelliklerinde de bir düşüşe yol açıp

açmayacağını incelemek istedik. Bu bağlamda, 15-LOX-1 ile transfekte edilmiş hücrelerden toplanmış şartlandırılmış besiyeri kullanarak ve bu hücrelerden toplanmış RNA'ları analiz ederek VEGF proteini ifadesinin ve salınımının, HUVEC hücrelerinin durumunu gözlemledik. VEGF ifadesinin ve salınımının normal oksijen seviyesinde ve az oksijen seviyesinde azaldığını gözlemledik. Ayrıca, HUVEC hücrelerini şartlandırılmış besiyeri ile muamele ettiğimizde, bu hücrelerin tüp benzeri yapılar oluşturma yetilerinin 15-LOX-1 varlığında azaldığı sonucuna vardık. Sonuç olarak, 15-LOX-1 hücrelerinin yeniden ifadesi kolorektal kanser hücrelerinin anjiyogenik özelliklerinde bir azalmaya yol açmaktadır.

Anahtar kelimeler: kolorektal kanser, 15-lipoksigenez-1, lipoksigenezler, anjiyogenez, VEGF

To the love of my life

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LIST OF ABBREVIATIONS

CRC: Colorectal cancer

FAP: Familial adenomatous polyposis

APC: adenomatous polyposis coli

HNPCC: Hereditary nonpolyposis colorectal cancer

LOXs: Lipoxygenases

5-LOX: 5-Lipoxygenase

8-LOX: 8-Lipoxygenase

12-LOX: 12-Lipoxygenase

15-LOX-1: 15-Lipoxygenase-1

PUFAs: Poly unsaturated fatty acids

LA: Linoleic acid

AA: Arachidonic acid

15-HETE: 15-Hydroxyeicosatetraenoic acid

13-(S)-HODE: 13-S-hydroxyoctadecadienoic acid

PPAR-γ: Peroxisome proliferator-activated receptor gamma

PPAR-α: Peroxisome proliferator-activated receptor alpha

PPAR-δ: Peroxisome proliferator-activated receptor delta

GATA-6: GATA binding protein 6

NuRD: Nucleosome remodeling and histone deacetylase

DNMT-1: DNA methyltransferase 1

VEGF-A: Vascular endothelial growth factor A

VEGF-B: Vascular endothelial growth factor B

VEGF-C: Vascular endothelial growth factor C

VEGF-D: Vascular endothelial growth factor D

VEGF-E: Vascular endothelial growth factor E

VEGFR1: Vascular endothelial growth factor receptor 1

VEGFR2: Vascular endothelial growth factor receptor 2

LB: Luria-Bertani medium

FBS: fetal bovine serum

PBS: Phosphate buffered saline

OD: Optical density

DNTPs: deoxynucleotide triphosphates

NaCl: Sodium chloride

PVDF: Polyvinylidene Fluoride

TBS: Tris Buffered Saline

Tris-HCl: 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride

HCl: Hydrochloric acid

EV: Empty vector

HUVEC: Human umbilical vein endothelial cells

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

CHAPTER I

INTRODUCTION

1.1. Colorectal Cancer

As of 2013, colorectal cancer (CRC) is the third leading cause of death in Turkey among cancer related deaths in both genders (Gültekin & Boztaş, 2014). 40 out of 100,000 people are diagnosed with new cases of CRC and around 24 men and 15 women out of 100,000 people are diagnosed with CRC each year (Gültekin & Boztaş, 2014). In early stages of the development of CRC, mutations commonly lead to deactivation of the tumor suppressor adenomatous polyposis coli (APC) gene (Watson & Collins, 2011). Besides, mutations in the *RAS* gene also contribute to the progression of CRC in the first stages of cancer; adenomas with *RAS* mutations grow faster than adenomas without *RAS* mutations (Fearon & Vogelstein, 1990). *APC* mutations occur as an early event in colorectal cancer and are frequently associated with familial adenomatous polyposis (FAP). FAP is inherited and characterized by the generation of thousands of polyps in the colon, which may transform into colorectal cancer in long term (Galiatsatos & Foulkes, 2006). Mutations in mismatch repair genes can be inherited as an autosomal dominant trait and that can lead to hereditary non-polyposis colorectal cancer (HNPCC); and the incidence of CRC in the offspring of people with HNPCC is up to 80% (Aarnio et al., 1999). Mutations in genes such as *TP53* and *TGFB1* can also contribute to the development of malignancy (Watson & Collins, 2011). Furthermore, studies have shown that intake of red meat and lack of fibers, folate and vitamins in the diet are also correlated with a higher risk of developing CRC (Song, Garrett, & Chan, 2015). Additionally, obesity has been shown to predispose individuals to the development of CRC. Enhanced levels of insulin has been implicated in the development of CRC since it

may increase the levels of insulin related growth factor, thereby leading to the exposure of normal cells to excessive mitogenic signals (X. Zhang, Wu, & Yu, 2016).

1.2. Lipoxygenases

Lipoxygenases (LOXs) are a family of fatty acid dioxygenases that convert polyunsaturated fatty acids (PUFAs) to their oxidized form, which are subsequently transformed to bioactive lipid mediators acting in various cell functions (Kuhn, Banthiya, & Van Leyen, 2015). There are several members of the family that have different functions in the cell depending on the tissue they are expressed in. This family of proteins includes, but is not limited to 5-LOX, 8-LOX (murine), 12-LOX, and 15-LOX-1 (12/15-LOX in mice) or 15-LOX-2 (Kuhn et al., 2015). Each LOX can act on the same polyunsaturated fatty acid, arachidonic acid (AA), which is derived from membrane phospholipids cleaved by phospholipases (PLAs) (Burke & Dennis, 2009). However, the outcome of this enzymatic oxygenation can differ because the preferences of LOXs for different fatty acid substrates and positions at which they oxygenate with the fatty acid are different.

1.3. 15-Lipoxygenase-1

15-LOX-1 is a member of the LOX family that preferentially oxygenates linoleic acid (LA) as its substrate, resulting in the formation of 13-S-hydroxyoctadecadienoic acid (13-S-HODE) (Schneider & Pozzi, 2011). On the other hand, when 15-LOX-1 uses AA as its substrate, the product is 15-S-hydroxyicosatetraenoic acid (15-S-HETE) (Figure 1.1). These bioactive lipids may have very distinct effects in the cells that they are produced in (Schneider & Pozzi, 2011).

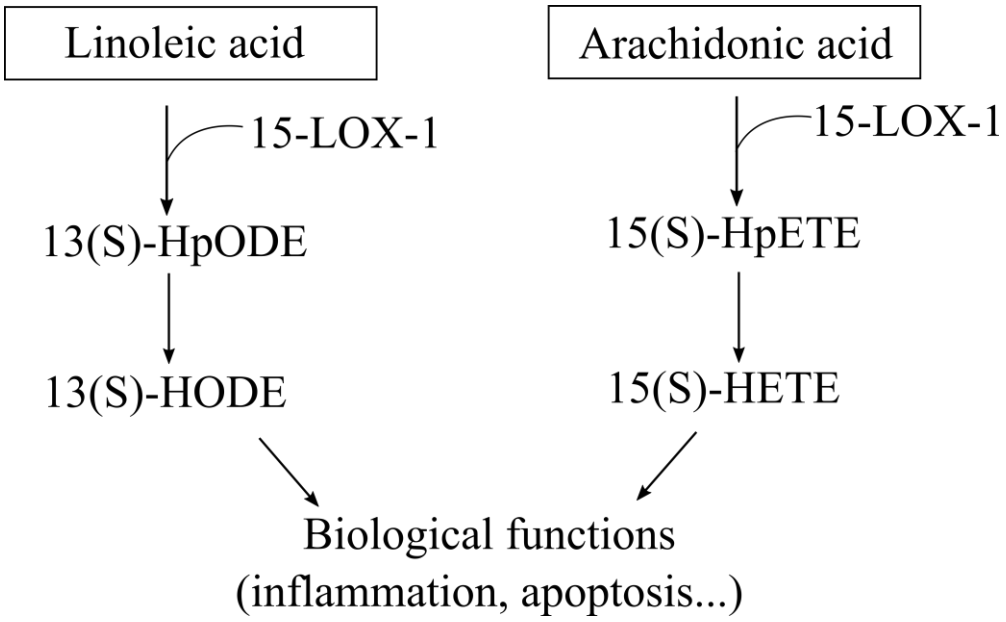

Figure 1.1: A brief summary of 15-LOX-1 pathway.

15-LOX-1 was discovered in rabbit reticulocytes as an enzyme that catalyzes the oxygenation of phospholipids in the cell membrane (Schewe, Halangk, Hiebsch, & Rapoport, 1975). The *ALOX15* gene is located on chromosome 17, p13.3 locus, and has 14 exons (GenBank: NC_000017). The 15-LOX-1 protein is composed of 661 amino acids and consists of the PLAT (Polycystin-1, Lipoxygenase, Alpha-Toxin) domain and the lipoxygenase domain (UniProt Accession Number: P16050). Being a member of lipoxygenase family, 15-LOX-1 has multi-functionality. It can bind to the cell membrane in a calcium dependent manner and act as soon as the phospholipases hydrolyze the phospholipids to generate free fatty acids (Walther, Wiesner, & Kuhn, 2004). It can also act on complex lipoproteins, especially by oxidizing the LDLs (Schewe, 2002). Moreover, it can catalyze the proteolytic cleavage of organelles in reticulocytes during the process of differentiation into a mature red cell (Schewe et al., 1975).

1.4. 15-LOX-1 in disease

15-LOX-1 is expressed in many organs and tissues such as lung (Claesson, 2009), breast (Jiang, Watkins, Douglas-Jones, & Mansel, 2006) and colon (Moussalli et al., 2011). However, in addition to a diverse range of functions in normal cells and tissues, deregulation in 15-LOX-1 expression and/or signaling have been associated with diseases such as cancer, atherosclerosis and obesity. In a study where adipose tissues from 46 obese individuals (with BMI>39 kg/m^2) were analyzed, 15-LOX-1 was shown to be expressed in both diabetic and non-diabetic adipose tissues (Lieb et al., 2014) implying that it can have a role in obesity. 15-LOX-1 was shown to be expressed more in the esophagus of patients with eosinophilic esophagitis than in patients with gastroesophageal reflux disease, which supports the idea that 15-LOX-1 can be used to distinguish between the two diseases (Matoso et al., 2014). In the case of cancers, 15-LOX-1 is usually downregulated when malignant transformation occurs. For instance, in breast cancer cells, both 15-LOX-1 and 15-LOX-2 expressions were shown to decrease when compared to the normal mammary gland cells (Jiang et al., 2006). In contrast, 15-LOX-1 was shown to be overexpressed in lung cancers (Liu et al., 2009), along with it induction in the presence of the cytokine interleukin-4 (IL-4) (Brinckmann et al., 1996). On the other hand, 15-LOX-1 was shown to be downregulated in colorectal cancer cells as compared to the healthy colon epithelia (Moussalli et al., 2011).

1.5. 15-LOX-1 in Inflammation

Inflammation is the sum of biological processes such as the swelling of the tissue, redness, pain and increased heat due to infection or an abnormality in cell signaling. Inflammation is a beneficial process in the context of a pathogenic infection; therefore its resolution is essential after the infection is cleared in order to prevent chronic inflammation and subsequently diseases such as cancer or inflammatory bowel disease (Emily, Weitzman, Shacter, & Weitzman, 2002). A tightly regulated

class switching of eicosanoids helps to change the type of bioactive lipids produced, ranging from pro-inflammatory prostaglandins and leukotrienes at the initial stages of inflammation to the production of resolvins and lipoxins which are pro-resolution mediators for the cessation of inflammation (Levy, Clish, Schmidt, Gronert, & Serhan, 2001; Serhan, 2014).

The 15-LOX-1 product 13-S-HODE can induce the differentiation of the pro-inflammatory M1 type macrophages to M2 type, which are considered to be involved in resolution of inflammation, (Dioszeghy et al., 2008). 12/15-LOX (murine 15-LOX-1) was also shown to induce the chemokine CXCL13 in macrophages; the down-regulation of this chemokine was shown to result in the defective resolution of inflammation in mice (Tani et al., 2014). 15-LOX-1 can also metabolize ω3-fatty acids such as docosahexaenoic acid (DHA) and produce D-series resolvins (RvDs) and protectins (PDs) that can act as agents for resolution of inflammation (Serhan, 2014). Protectin D1 (PD1) was shown to induce resolution after inflammation caused by an influenza virus (Morita et al., 2013). Lipoxins, which also produced from 15-LOX-1 mediated enzymatic oxygenation of PUFA, are important in the resolution of inflammation by reducing the recruitment of neutrophils to the site of infection (Fierro et al., 2003; Serhan, Chiang, Dalli, & Levy, 2015). Specifically, Lipoxin A4 (LXA4) was shown to increase in colon cancer cell lines RKO and LoVo after treatment of these cells with PUFAs such as LA, AA, EPA (eicosapentaenoic acid) and DHA (Zhang, Yu, Ni, Shen, & Das, 2015).

1.6. Regulation of 15-Lipoxygenase-1

The expression of 15-LOX-1 is a tightly regulated. Recruitment of the transcription factor STAT6 and other STAT proteins including STAT1, STAT3 and STAT5 can transcriptionally upregulate 15-LOX-1 in monocytes when the cells are treated with the cytokine IL-13 (Xu et al., 2003). 15-LOX-1 expression has been shown to be almost universally lost in CRC, primarily through epigenetic mechanisms. In the colorectal cancer cell line Caco-2, the downregulation of 15-LOX-1 was achieved

through the activation of GATA-6, which is a transcription factor that is primarily responsible for the differentiation of lung stem cells (Shureiqi et al., 2007; Yang, Lu, Zhang, Whitsett, & Morrisey, 2002). Moreover, a polymorphism that leads to the binding of SP1 on the promoter of the *ALOX15* gene was shown to result in the increased production of 15-LOX-1 (Wittwer, Marti-Jaun, & Hersberger, 2006). Histone acetylation can lead to gene expression by changing the nucleosome structure (Grunstein, 1997). In colon cancer cells, inhibition of the histone deacetylases HDAC1 and HDAC2, which are responsible for removing the acetyl group on histones, was shown to upregulate 15-LOX-1 transcription through enhanced histone acetylation (Zuo, Morris, Broaddus, & Shureiqi, 2009).

1.7. Hallmarks of Cancer

Cancer cells divide, survive and spread around the body. They have constitutively active survival signals, evade anti-proliferative signals, resist death, induce angiogenesis, metastasis and invasion and enable immortality through infinite replication capability (Fig 1.2) (Hanahan & Weinberg, 2011a).

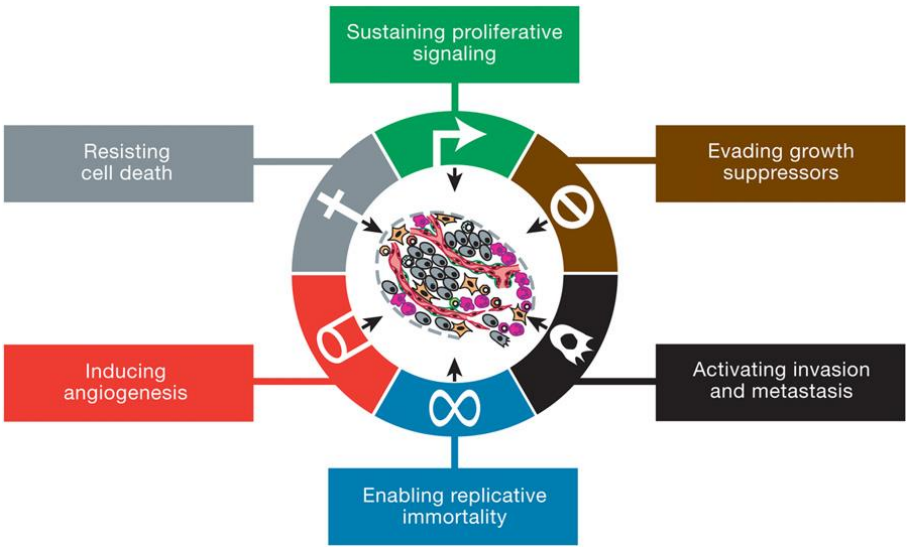

Figure 1.2: Hallmarks of cancer, retrieved from ("Hallmarks of cancer", 2011).

Normal cells have checkpoints in their cell cycles, which control the events for the progression of cell division. Generally, abundance and binding of a growth factor to a receptor gives the proliferation signal. These cells stop dividing when they contact neighboring cells. This cell-to-cell contact inhibits further cell division to keep population under control. However, in cancer cells, cell cycle progression may occur irrespective of whether there is growth signal or contact with other cells or not. Normal (non-transformed) cells require mitogenic membrane receptors phosphorylated and activated in order to progress with cell division. Since cancer cells overexpress cell receptors, these receptors may get phosphorylated by dimerizing randomly without any need for ligand binding (Hanahan & Weinberg, 2011a). In parallel to sustaining proliferative signal, cancer cells also evade growth suppressor genes such as *TP53* and *RB* (retinoblastoma-associated) (Hanahan & Weinberg, 2011a). Between these two, RB, acts as the decision point which integrates intracellular and extracellular signals and decides whether the cell should continue with division or not. In cancer cells, with aberrant RB expression, RB protein may result in an always-activated state which would subsequently cause infinite cell divisions (Hanahan & Weinberg, 2011a). Cancer cells also express anti-apoptotic proteins such as Bcl-x_L, Bcl-w and Mcl-1, which can inhibit pro-apoptotic proteins in the cell, thus leading to a state where the cell is irresponsive to both extrinsic and intrinsic apoptotic pathways (Hanahan & Weinberg, 2011a). Additionally, cancer cells can metastasize by expressing or inducing other cells to express extracellular matrix digesting proteins so that they can intravasate into blood vessels and relocate in another tissue (Hanahan & Weinberg, 2011a). This characteristic allows a cancer cell to change its phenotype from being a stationary cell at the site of the primary tumor to a motile cell that can move to a niche in another organ and generate a secondary tumor. Finally, cancer cells have the capability of inducing angiogenesis, the process of formation of blood vessels from pre-existing ones by the involvement of cytokines which induce endothelial cells to divide and migrate (Risau, 1997).

15-LOX-1 re-expression has been shown to affect a number of these hallmarks of

cancer both in colon cancer cell line models and in a transgenic rodent model where human *ALOX15* is expressed in the colon under the control of the villin promoter. The 15-LOX-1 product, 13-*S*-HODE, was shown to induce apoptosis by down-regulating PPAR-δ, a nuclear receptor responsible for transcriptional activation of several genes related to cell survival (Shureiqi et al., 2003). Therefore, 15-LOX-1 activity can result directly in the induction of apoptosis. In the context of colorectal cancer, other roles have been ascribed to 15-LOX-1 as well. For example, 15-LOX-1 re-expression in colon cancer cell lines was shown to reduce proliferation, induce apoptosis and reduce motility by inactivating nuclear factor kappa B (NF-κB), a master transcription factor that is essential for inflammation and frequently deregulated in CRC (Cimen, Astarci, & Banerjee, 2011; Lawrence, 2009). Additionally, it was shown to reduce metastatic potential of colorectal cancer cells by decreasing the levels of MTA-1(Çimen, Tunçay, & Banerjee, 2009; Tuncer, Tunçay et al, 2016) a master regulator of metastasis that is frequently overexpressed in many different cancer types including CRC (Tunçay, Cimen, Banerjee, 2012) .

1.8. Angiogenesis

Angiogenesis can be defined as the formation of new blood vessels from pre-existing ones as a result of an external stimulus (Fig. 1.3) (Nishida, Yano, Nishida, Kamura, & Kojiro, 2006). Neo-angiogenesis is defined as the process of generating new blood vessels. In embryonic stages, the process of angiogenesis is necessary for the formation of new blood vessels that carry oxygen and nutrients; in adults, it is used in the process of wound healing and is shut down after healing is complete (Hanahan & Weinberg, 2011a). However, in tumor cells, the presence of angiogenic factors results in the vasculature around the tumor cells to broaden and form branches. For tumors beyond a specific size, the formation of new blood vessels is necessary for the tumor cells to have access to nutrients and oxygen. As a result, proliferation and invasive capabilities of the tumor cells increase (Hanahan & Weinberg, 2011).

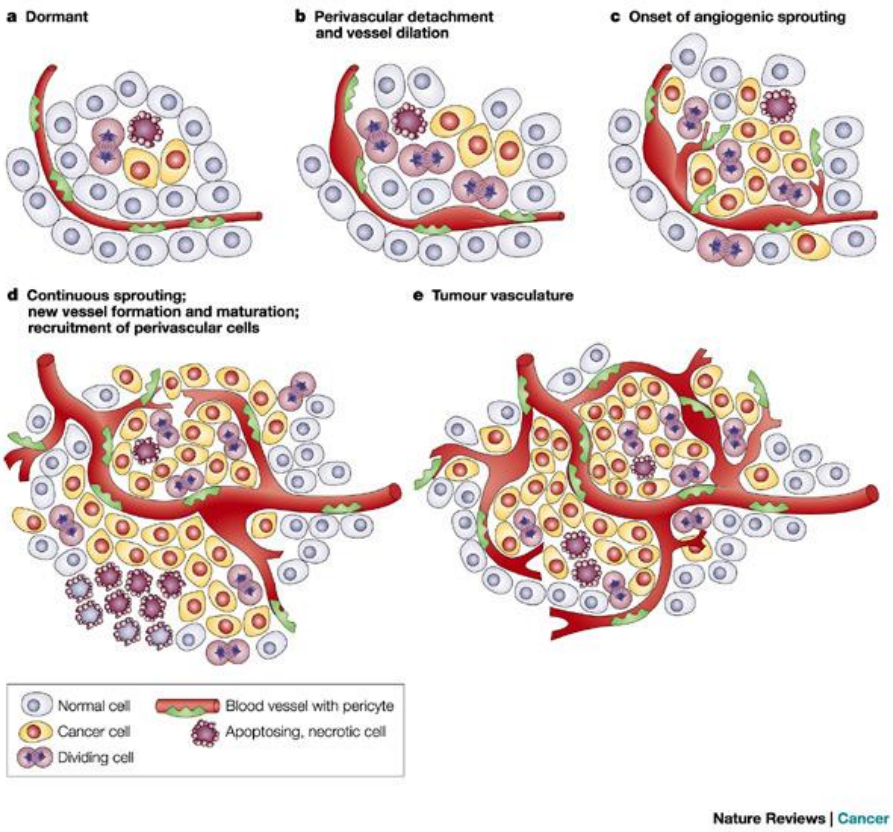

Figure 1.3: Summary of angiogenesis mechanism in cancer; retrieved from ("The classical angiogenic switch", 2003)

There is very limited data available in the literature on the role of 15-LOX-1 in angiogenesis. 15-HETE, the product of oxygenation of AA by 15-LOX-1, was shown to induce angiogenesis in human retinal microvascular endothelial cells (Bajpai et al., 2007). In parallel, 12/15-LOX knockout decreased 15-S-HETE dependent angiogenesis in mice (Kundumani-Sridharan et al., 2010). However, there is little evidence showing the role of 15-LOX-1 in angiogenesis in CRC. In one study, where the human *ALOX15* gene was expressed in mouse model, it was shown that the expression of TNF-α and NO (nitric oxide), both of which induce angiogenesis, were reduced in the presence of 15-LOX-1 (Sainson et al., 2008; Tousoulis, Kampoli, Tentolouris, Papageorgiou, & Stefanadis, 2012; Xiangsheng Zuo et al., 2012).

1.9. Regulation of angiogenesis

Angiogenesis is regulated by a group of growth factors, the most common one being the VEGF (Vascular Endothelial Growth Factor) family. VEGF family consists of 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PGF (Placental Growth Factor) (Prat, Casado, & Cortés, 2007). VEGF-A is the most commonly studied one among the members of the family. Human VEGF-A gene is located on chromosome 6p12 and contains 9 exons within the region (GenBank: GCA_000001405.17). It has four different isoforms produced by alternative splicing: VEGF121, VEGF165, VEGF189, or VEGF206 (Park, Keller, & Ferrara, 1993). Three receptor tyrosine kinases are known to bind to VEGF: VEGFR1, VEGFR2 and VEGFR3 (Stuttfeld & Ballmer-Hofer, 2009). Ligand binding to these receptors (mainly VEGFR2) leads to receptor dimerization and phosphorylation and the initiation of a signaling cascade (Stuttfeld & Ballmer-Hofer, 2009). This pathway is considered to be the core of angiogenesis in tumors (Dvorak, 2002). VEGF-A enhances both endothelial cell growth and migration and vascular permeability by this way (Ellis & Hicklin, 2008). VEGF-A binding to VEGFR1 is considered to be stronger than VEGF-A/VEGFR2 binding and VEGFR1 has lower kinase activity (Hiratsuka, Minowa, Kuno, Noda, & Shibuya, 1998). In some cancers, VEGFR1 activation was shown to induce epithelial to mesenchymal transition (Yi, Feng, Xiang, & Yao, 2011).

1.10. HUVECs

Human Umbilical Vein Endothelial Cells (HUVECs) are a good model to study angiogenesis, immune response, wound healing etc. These cells are isolated from the umbilical cord in the neonatal period and extensively used as a model of endothelial cell. In the current study, the effects of 15-LOX-1 re-expression in colon cancer cell lines on angiogenesis was examined using HUVECs as the model endothelial cells.

1.11. Aims of the study

This study aimed to show the effects of 15-LOX-1 re-expression in the colorectal cancer cell line SW480 on angiogenic properties of the cell. Consistent with the tumor suppressive properties of 15-LOX-1 expression in CRC (Tuncer and Banerjee, 2015), we hypothesized a decrease in angiogenesis with 15-LOX-1 re-expression. To show this, the expression and secretion of VEGF-A was examined. This study, thus, was designed to answer the following question which was missing in the field:

-Does 15-LOX-1 re-expression have an anti- angiogenic effect in CRC and if so what was the mechanism behind this anti-angiogenic effect?

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

HUVEC cells were bought from American Type Culture Collection (USA), SW480 cells were kindly gifted by Assoc. Prof. Dr. Ali Osmay Güre from Bilkent University, Ankara. Fetal Bovine Serum (FBS) used in this study was bought from Biochrom (UK). DMEM medium was bought from Biological Industries (Israel). 15-LOX-1 mouse monoclonal antibody (Cat. No: H00000246-M04) was bought from Abnova (Taiwan). Rabbit polyclonal secondary antibodies, mouse polyclonal secondary antibodies and mouse monoclonal β-actin antibody were bought from Santa Cruz Biotechnology (USA). All other reagents and chemicals were bought from companies as specified.

2.2. Buffers

6X Sample Loading Buffer

12% SDS, 30% β-mercaptoethanol, 0.012% Bromophenol

Blue, 30% glycerol, 0.375 M Tris-HCl pH 6.8

PBS-T

8 g NaCl, 0.27 g KH2PO4, 3.58 g Na2HPO4.12H2O; pH adjusted to 7.4,

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 dH2O.

Transfer Buffer (2 L)

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

SDS-PAGE Buffer

0.1% SDS, 25 mM Tris, 190 mM glycine in 1 L

12% Separating Gel Mix

4.3 ml dH_2O, 3.8 ml 10% SDS+1.5 M Tris-HCl pH 8.8, 5.5 ml of Acrylamide + bisacrylamide (30%), 150 µl APS, 20 µl TEMED.

4% Stacking Gel Mix

4.7 ml dH2O, 2 ml 10% SDS+1.5 M Tris-HCl pH 6.8, 1.2 ml of Acrylamide + bisacrylamide (30%), 50 µl APS, 10 µl TEMED.

2.3. SW480 cell line

SW480 is a commonly used colorectal cancer cell line which was isolated from a 50 year old male patient from a colorectal adenocarcinoma tissue (Leibovitz et al., 1976). It is isolated from a primary tumor, which, later on, metastasized to lymph node and isolated as SW620 cell line (Leibovitz et al., 1976). The cell line has double p53 mutation; that is, both of the alleles of *TP53* gene is mutated (Rochette, Bastien, Lavoie, Guérin, & Drouin, 2005) and a truncated APC (Yang et al., 2006) both of which may have contributed to the malignant transformation of the tissue. The cells are of epithelial origin and grow in small islands in the tissue culture (Leibovitz et al., 1976). SW480 cell line is classified as a Dukes' Type B colorectal

cancer cell line which can be considered as invading into the colon wall without touching the lymph nodes yet (Ahmed et al., 2013).

2.4. Cell culture

SW480 cells were grown in complete DMEM medium containing 10% fetal bovine serum, 2 mM L-Glutamine, 1.5 g/L sodium bicarbonate ($NaHCO_3$) and 1% Penicillin/Streptomycin in an incubator at 37^oC supplemented with 95% air and 5% CO_2. The cells were grown in T-25 flasks or tissue culture plates when necessary. Cells were always grown in monolayers, up to a maximum of 90 % confluency. Cells were washed with ice-cold phosphate buffered saline (PBS, 1X) to remove any debris, cell waste or dead cells before changing the medium.

2.5. Storage of cells

Cells were washed with 1X PBS and detached from the surface of the flask by using a trypsin-EDTA (0.25 %) solution. The trypsin solution was deactivated with complete DMEM medium and cells were centrifuged at 1000 x *g* for 5 min. The supernatant was aspirated, the pellet was resuspended in complete culture medium containing 5% DMSO and aliquoted to cryovials as 1 mL per vial. Then, the cells were kept in a Mr. Frosty box containing isopropanol at -80^oC overnight. This was to ensure that the cells were frozen slowly. Cells were then taken to the vapor phase of liquid nitrogen for long term storage.

2.6. Plasmid isolation and transfection of SW480 cells

The full length 15-LOX-1 cDNA cloned into a pcDNA 3.1 vector was kindly gifted by Dr. Uddhav Kelavkar (Kelavkar et al., 1998). The plasmids were transformed into the *Escherichia coli* TOP10 strain, which were then incubated in Luria Bertani (LB) medium containing 0.1% ampicillin in a microbiology grade incubator at 37^{o}C with shaking at 200 rpm for 16 hours. After this, the bacteria were harvested by centrifuging at 4500 x *g* for 10 min at 4^{o}C. The plasmids were isolated using Qiagen Plasmid Isolation Mini Kit (Qiagen, Germany) according to manufacturer's protocol. Plasmid concentration was determined using a spectrophotometer (BioDrop, UK). For long term storage of the plasmids, they were kept at -20^{o}C.

For transfection, SW480 cells (7.5 x 10^5 cells per well) were seeded the day before transfection in 6-well plates. The next day, the medium was changed with a transfection medium lacking penicillin/streptomycin. In an Eppendorf tube, 200 µL of Opti-MEM medium, 3 µg of plasmid and 3 µL of XtremeGENE-HP Transfection Reagent (Roche, Switzerland) (1:1 ratio of plasmid to transfection reagent) were added without touching the walls of the tubes. This transfection mixture was incubated at room temperature without shaking and then added on top of the cells in a dropwise manner. Cells were incubated in an incubator at 37^{o}C with 5% CO_2 for 24 hours. Following this, the transfection medium was changed with complete culture medium and the cells were returned to the incubator for 48 hours. The reason why the cells were grown for 48 h more is to allow them to produce enough growth factor since we would be collecting conditioned medium for subsequent experiments. After a total of 72 hours, the cells were harvested by means of trypsinization as described earlier. Additionally, to collect the conditioned medium, the culture medium of the cells was collected and centrifuged at 1000 x *g* for 5 min, aliquoted and stored at -80˚C.

2.7. RNA isolation and cDNA synthesis

Cultured cells were collected by trypsinization as described above and pellets of cells were stored at -80^{o}C until RNA isolation was performed. For RNA isolation, the Qiagen RNeasy RNA Isolation Kit (Qiagen, Germany) was used following the manufacturer's protocol. RNA concentration was measured using a spectrophotometer (BioDrop, UK) at 260 nm and the purity of the samples were determined by getting the ratios of RNA to contaminating chemicals (A260 to A230) and RNA to contaminating proteins (A260 to A280). Isolated RNAs were stored in -80^{o}C before going into cDNA synthesis.

To remove any potential genomic DNA contamination, 1 µg RNA was treated with 1 unit DNAse I in 1 µL of 10X DNAse I buffer containing $MgCl_2$ completed to 10 µL with nuclease-free water. The mixture was incubated at 37^{o}C for 30 min. Then, 1 µL of Ethylenediaminetetraacetic acid (EDTA) was added to the mixture to inhibit DNAse I activity by chelating magnesium atoms and the mixture was incubated at 65^{o}C for a further 10 min. After DNAse I treatment, first-strand cDNA synthesis was performed using ThermoFisher Scientific's RevertAid cDNA synthesis kit according to manufacturer's protocol using oligo-dT as primers for Reverse Transcriptase. The cDNA samples obtained were stored at -20^{o}C.

2.8. RT-PCR and qRT-PCR

The mastermix for RT-PCR reactions consisted of 0.5 µM forward and reverse primers, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1X MgCl2 buffer and 1 U Taq Polymerase (Thermo Scientific) in a total volume of 18 µL. To this mixture, 2 µL of cDNA with a final concentration of around 0.1 µg was added to the mixture as the template for the PCR reaction. For the negative control of PCR, no template was added to the mixture and the total volume was 20 µL. Denaturation of DNA strands was carried out at 95^{o}C for 5 minutes and the final extension of the PCR product was done at 72^{o}C for 7 minutes. In between, denaturation, annealing and extension cycles

was carried out according to the tables given below (see Table 2.1). As an internal control for the PCR experiments, β-actin primers were used in a separate mixture; β-actin was used as internal control for all PCR experiments (Kelavkar et al., 2007). All the PCR reactions were performed using a thermal cycler (Applied Biosystems, USA). PCR products were mixed with 6X loading dye (final concentration 1X) and run on an agarose gel having a concentration of 1.5% at 100 V. The images of PCR products were taken using a UV transilluminator.

For qRT-PCR experiments, 0.5 μM of forward and reverse primers and 10 μL 2X FastStart SYBR Green Master (Roche) were added to the PCR mixture and completed to a final volume of 18μl. To this, 2 μL of cDNA (final concentration around 0.01 μg) was added as the template. The PCR was performed using the Rotor-Gene Q thermal cycler (Qiagen) with an initial denaturation temperature of 95^{o}C for 10 minutes and a final extension of 72^{o}C. Annealing temperatures of the cycles are given in the table below. Ct values were calculated based on a standard curve generated for each primer and the results were analyzed using Pfaffl method (Pfaffl, 2004). All the qRT-PCR reactions were carried out based on MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin et al., 2009).

Table 2.1: Table of primers, annealing temperatures and cycle numbers used in qRT-PCR and PCR reactions.

Gene	Sequence		Annealing Temperature	Cycle number
ACTB	Forward	CAGCCATGTAGCTTGCTATCCAGG	60	40
	Reverse	AGGTCCAGACGCAGGATGGCATG		
ALOX15	Forward	GTCTTCCTTCTATGCCCAAGAT	59.5	40
	Reverse	CACAGCCACGTCTGTCTTATAG		
VEGFA	Forward	ATCACGAAGTGGTGAAGTTC	53	40
	Reverse	TGCTGTAGGAAGCTCATCTC		

2.9. Protein isolation

To isolate total proteins, SW480 cells (wild type, or transfected with the appropriate plasmid) were harvested as described before. The cell pellets were resuspended in a mixture of M-PER Mammalian Protein Extraction Kit (Thermo Scientific), protease inhibitors and phosphatase inhibitors. The suspension was centrifuged at 14000 x *g* for 10 minutes at 4^{o}C. The supernatant was collected as the total protein. Protein concentration was measured using Pierce™ Coomassie (Bradford) Protein Assay Kit according to the manufacturer's guidelines and stored at -80^{o}C.

2.10. Western Blotting

The total protein extracts were mixed with a 6X loading buffer containing 30% β-mercaptoethanol, 12% SDS, 30% glycerol, 0.012% Bromophenol Blue and 0.375 M Tris-HCl pH 6.8 and boiled at 95^{o}C for 6 minutes. The boiled proteins were loaded on a 10% SDS-polyacrylamide gel in equal amounts (50 μg) and electrophoresis was carried out at a voltage of 120 V. The separated proteins were transferred to a polyvinylidene fluoride membrane (Roche) at a constant current of 380 mA and voltage of 115 V for 1.5 hours. After transfer, the membrane was blocked with skim milk (5%, Applichem) in PBS containing Tween (0.1%, PBS-T) for 1 hour at room temperature. The skim milk was disposed and the primary antibodies were added in fresh 5% skim milk at the dilutions shown in Table 2 and the membrane was incubated at 4^{o}C overnight. The membrane was washed in PBS-T for 10 minutes three times and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody in 5% skim milk for 1 hour at room temperature. The membrane was washed and incubated with Clarity™ Western ECL Blotting Substrate (BioRad) for 3 minutes at room temperature in the dark. The membrane, then, was dried with filter papers and visualized in Gel Doc™ XR+ System (BioRad).

Table 2.2: Table of used antibodies in Western Blotting.

Protein Name	Catalog number	Vendor	Origin	Dilution	Expected molecular weight
β-actin	sc-69879	Santa Cruz Biotech.	mouse	1:2000	42 kDa
15-LOX-1	H00000246-M04	Abnova	mouse	1:1000	72 kDa
Secondary antibody anti-rabbit	sc-2030	Santa Cruz Biotech.	goat	1:2000	-
anti-mouse	sc-358914	Santa Cruz Biotech.	rabbit	1:2000	-

2.11. MTT Proliferation Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Proliferation Assay was carried out in order to detect number of alive cells by the help of colorimetric changes in cells with the addition of MTT. In this assay, MTT molecules are reduced by the cells, particularly in the mitochondria where dehydrogenase enzymes are highly active. The reduced MTT molecules form purple formazan crystals that can be solubilized with SDS-HCl.

96-well plates were used for this experiment. SW480 cells are seeded as $1x10^4$ cells per well and transfected with either EV or 15-LOX-1 vectors. The transfection mixture was prepared as before and 10 µL was gently applied to each well in a dropwise manner to the wells as per well. After 24h of transfection, the medium was changed with complete DMEM. After 24 h, 48 h and 72 h, the media were aspirated and MTT solution (5 µg MTT in 1 mL PBS) was added to the wells in a volume of 100 µL per well. Four hours after MTT addition, 100 µL of SDS-HCl solution (10% SDS, 0.01 N HCl) was added to each well. After 16 hours, the results were read by the help of a spectrophotometer at 570 nm. A separate 96 well plate was used for each time point.

2.12. Hypoxia

A Hypoxia Incubator Chamber (STEMCELL Technologies, Canada) was used for experiments under hypoxic conditions. The chamber was filled with a gas mixture containing 1% O_2, 5% CO_2 and 94 % N and sealed tightly. A small volume of autoclaved deionized water was placed separately in the chamber to ensure a humidified atmosphere in the chamber. To grow cells under hypoxia, the complete DMEM medium was first placed overnight in the hypoxia chamber to equilibrate the oxygen levels. Next, flasks containing SW480 cells (transfected with the 15-LOX-1 or EV vectors) were incubated in complete medium equilibrated overnight in the hypoxic chamber. The flasks were incubated for 6 hours in the chamber. Incubation under hypoxic conditions longer than 6h resulted in extensive cell death. Additionally, optimization experiments indicated that incubation for 6h was enough to show enhanced levels of VEGF-A mRNA transcripts, used as a positive control in these experiments. The flasks were then taken out and the cells as well as the conditioned medium were harvested immediately for further experiments.

2.13. VEGF secretion assay

Conditioned media collected from both hypoxic and normoxic SW480 cells transfected with either empty vector or 15-LOX-1 vector were assayed for VEGF secretion using an ELISA kit (R&D Systems, Minneapolis, USA) according to manufacturer's guidelines.

2.14. Tube Formation Assay

To determine whether re-expression of 15-LOX-1 in SW480 cells leads to a reduction in angiogenic signaling in endothelial cells, we used HUVEC cells treated

with conditioned media collected from transfected SW480 cells. The aim of the experiment was to see if HUVECs treated with conditioned media would form tube-like structures that resembles the blood vessels or not. In 15-LOX-1 conditioned medium treated HUVEC samples, we expected to see less tubes compared to those treated with EV conditioned media. For the purpose of this experiment, the night before the experiment, pipette tips, 96-well plate and Matrigel (Becton & Dickinson, USA) was put on ice and left at 4ºC to let the Matrigel thaw. The next day, 40 µL Matrigel was added in each well of a 96 well plate and was allowed to warm up at 37ºC for 1 hour. HUVEC cells were counted using a hemocytometer. Endothelial Cell Growth Medium (Lonza, Switzerland) was mixed with either empty vector (EV) conditioned medium or 15-LOX-1 conditioned medium in a dilution of 1:1. Counted cells were resuspended in this mixture and $1x10^4$ cells were added to each well. The cells were incubated at 37ºC for 6 hours and images of tube formation were captured using a microscope (Olympus, Japan). The images were uploaded to Wimasis (Germany) website for automatic analysis of the tubes. The analyzed images contain total tube numbers, branching points and total tube length. Total tube number corresponds to the number of complete, enclosed tubes. Branching points are the points where HUVEC cells sprout from. Total tube length translates into the length of completed tubes in pixels.

2.15. 13-S-HODE assay

13-S-HODE ELISA kit (Enzo, USA) was used to detect 13(S)-HODE levels in the cells. SW480 cells that were transfected with either empty vector or 15-LOX-1 vector were harvested. The cells were lysed by the help of a lysis buffer as described in the kit with a help of a syringe. A 10 µL aliquot of the lysate was saved for the detection of protein using a MicroBCA kit (Thermo Scientific, USA) since this kit allows up to 5% of SDS in the sample, which is a limit beyond our lysis buffer's content. The remaining lysate was centrifuged at 3000 x *g* for 1 min and the supernatant was collected. At this step, 0.2 N HCl was added to the lysate for acidification; acidification is needed to enhance lipid yield (Nishihara & Koga,

1987). Three volumes of saturated ethyl acetate (1:1 dilution of water to ethyl acetate) was added to the samples and they were centrifuged at 100 x *g* for 5 min to clearly separate the organic and the aqueous phases. The organic phase was collected in a separate tube. This phase separation step was repeated three times. The organic phases collected were flash-frozen in liquid nitrogen and lyophilized using a freeze dryer (Labconco, USA). The dried samples were resuspended in 25 µL of absolute ethanol and level of 13-S-HODE was detected with an ELISA kit using the manufacturer's guidelines.

CHAPTER III

RESULTS

In this study, the effect of 15-LOX-1 on angiogenesis in colorectal cancer was examined. To achieve this, a set of experiments was designed using the SW480 colorectal cancer cell line model that ectopically expressed 15-LOX-1 using an expression plasmid and the corresponding empty vector.

3.1. Transient Transfection of SW480 cells with empty vector and 15-LOX-1 vector

SW480 cells were transfected with either the empty pcDNA 3.1 vector or 15-LOX-1 vector using XtremeGENE HP transfection reagent. “Mock” cells were incubated with the transfection reagent only and no plasmid as described in Materials and Methods Section. Protein expression of 15-LOX-1 enzyme was checked with Western Blotting and the expression of 15-LOX-1 was observed when the cells were transfected with the 15-LOX-1 vector only. There was no expression of 15-LOX-1 in either Mock, empty vector or wild type SW480 cells, as expected. In addition, a dose dependent effect was seen whereby cells transfected with 3 μg of plasmid had higher expression than the cells transfected with 2 μg of the plasmid (Fig 3.1).

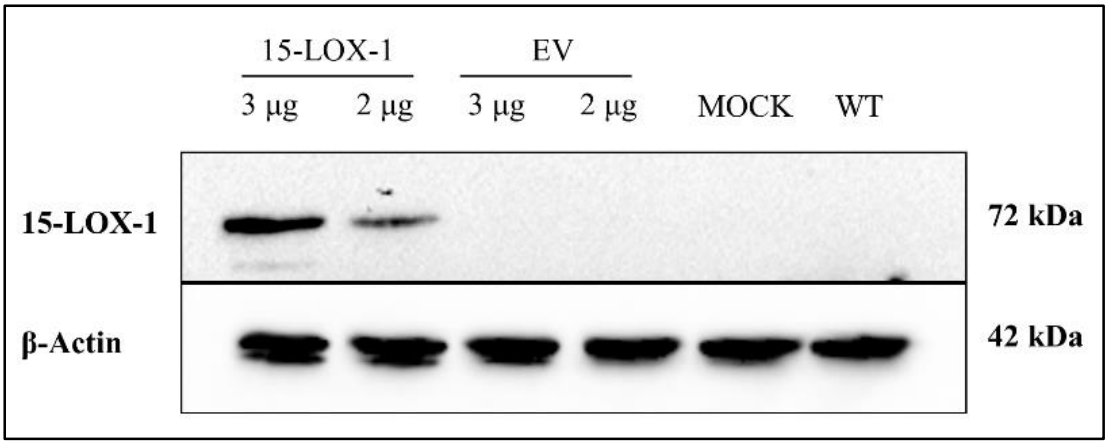

Figure 3.1: Western Blotting image of transient transfection of SW480 with empty vector (EV) or 15-LOX-1 vector using either 3 µg or 2µg of plasmid. There was no expression in EV, Mock or SW480 wild type lanes.

To collect conditioned medium from transfected cells, we performed a time dependent transfection with the same conditions; 24 h of transfection, followed by 48 h of incubation in fresh complete DMEM medium. This allowed enough time for the secretion of various growth factors, other proteins, cytokines and chemicals into the conditioned medium (Dowling & Clynes, 2011). The cells were transfected with EV and 15-LOX-1 vectors (3 µg each). For one group of cells, the transfection medium was changed with complete DMEM after 24 h and then incubated for a further 48h while the transfection medium for the other group was not changed and the transfection continued for 72 h. The cells that received 24 h of transfection followed by 48h of incubation with complete DMEM had similar expression to cells that were incubated in the transfection medium for 72 h (Fig 3.2). Therefore, to allow for the collection of conditioned medium, we decided to continue with 24 h of transfection and 48 h of recovery for subsequent experiments.

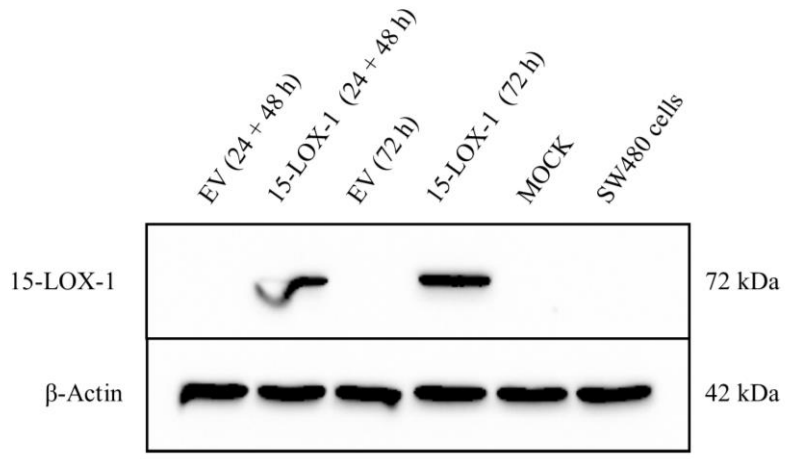

Figure 3.2: Western Blotting image of transient transfection of SW480 with empty vector (EV) or 15-LOX-1 vector, with transfections lasting for either 24 h + 48 h or 72 h. There was no expression in EV, Mock or SW480 wild type lanes. The image is a representative of four different replicates.

3.2. MTT Proliferation Assay

15-LOX-1 re-expression in colon cancer cell lines has previously been shown to decrease survival (Lee, Zuo, & Shureiqi, 2011). We therefore wanted to examine whether the SW480 model used in the current study, would also show less proliferation after transfection with the 15-LOX-1 plasmid. To do so, we performed an MTT proliferation assay with SW480 cells transfected with EV and 15-LOX-1 vectors. A comparison of the absorbance values from the MTT assay, which reflect the number of alive cells, of 15-LOX-1 transfected and EV transfected SW480 cells, indicated a reduction in proliferation at both 48 h and 72 h after transfection. Since the doubling time of SW480 cells is approximately 38 h, we reasoned that the transfection protocol should continue until all the cells went through at least one round of cell division. There was no significant difference between the results of wild type SW480 cells and EV transfected cells, implying that transfection had little effect on the survival of the cells (Fig 3.3).

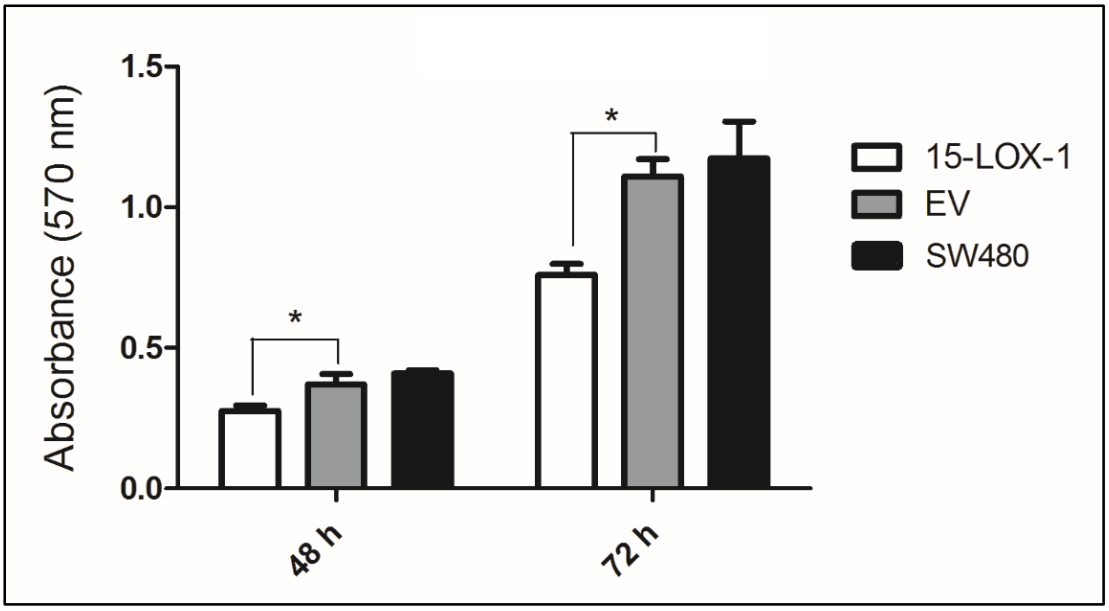

Figure 3.3: MTT proliferation assay done with transfected SW480 cells. There was a significant change between EV transfected and 15-LOX-1 transfected SW480 cells' survival ($P<0.05$, Student's *t*-test). The figure is based on two biological replicates.

3.3. Enzymatic activity of 15-LOX-1: The 13-S-HODE assay

15-LOX-1 is a non heme iron containing enzyme that can oxygenate linoleic acid to 13-S-hydroxyoctadecadienoic acid (13-S-HODE). The presence of 13-S-HODE in the cell indicates that the 15-LOX-1 protein is enzymatically active. Thus, we wanted to see if 15-LOX-1 transfection in SW480 cells resulted in the enzyme translated from the plasmid to produce 13-S-HODE. For this, the lipids were extracted from 15-LOX-1 or EV transfected SW480 cells by organic phase extraction followed by detection of 13-S-HODE by ELISA (Enzo). The kit can detect 13-S-HODE with 100% specificity over 13-R-HODE, a racemic lipid that can also be produced in the cells. As shown in Figure 3.4 we observed a mild increase in 13-S-HODE levels in 15-LOX-1 transfected cells; however, due to large variations in the data observed, the values did not reach statistical significance.

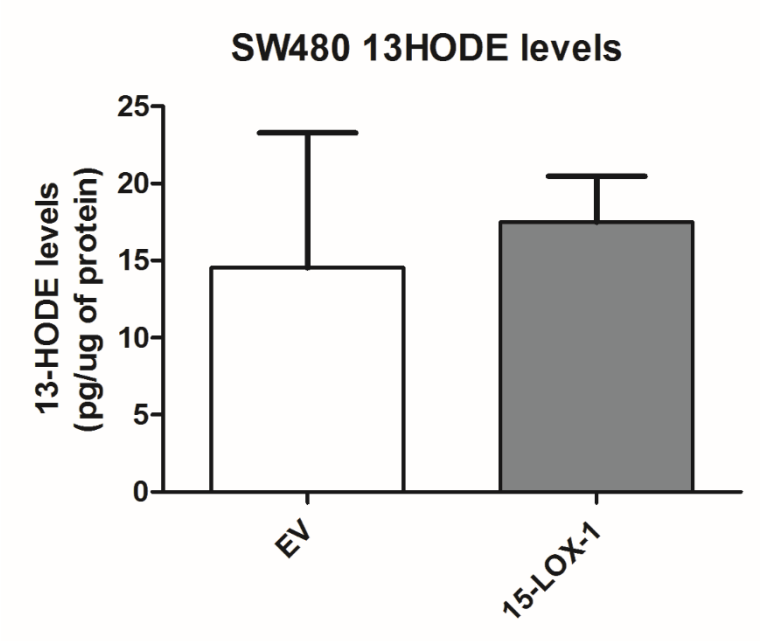

Figure 3.4: 13-(S)-HODE levels detected by an ELISA kit. There is no significant difference between the samples (P>0.05, Student's *t*-test was used); but, the trend is higher for 15-LOX-1 transfected SW480 cells. The figure is based on two independent experiments.

3.4. 15-LOX-1 effect on angiogenesis: VEGF levels

To determine whether re-expression of 15LOX-1 in SW480 cells had any effect on angiogenic properties of these cells, we performed qRT-PCR to determine the levels of vascular endothelial growth factor A (VEGF-A) levels in the presence of 15-LOX-1. Since VEGF-A is described as the most potent angiogenic factor, was shown to be overexpressed in several cancer types and can be induced by hypoxia (Nishida et al., 2006). RNA isolated from SW480 cells transfected with EV or 15-LOX-1 plasmid was converted to cDNA and the expression of VEGF-A at the mRNA level was analyzed. As seen in Figure 3.5, there was a significant reduction in VEGF mRNA expression in cells re-expressing 15-LOX-1, suggesting that 15-LOX-1 may play a role in the process of angiogenesis.

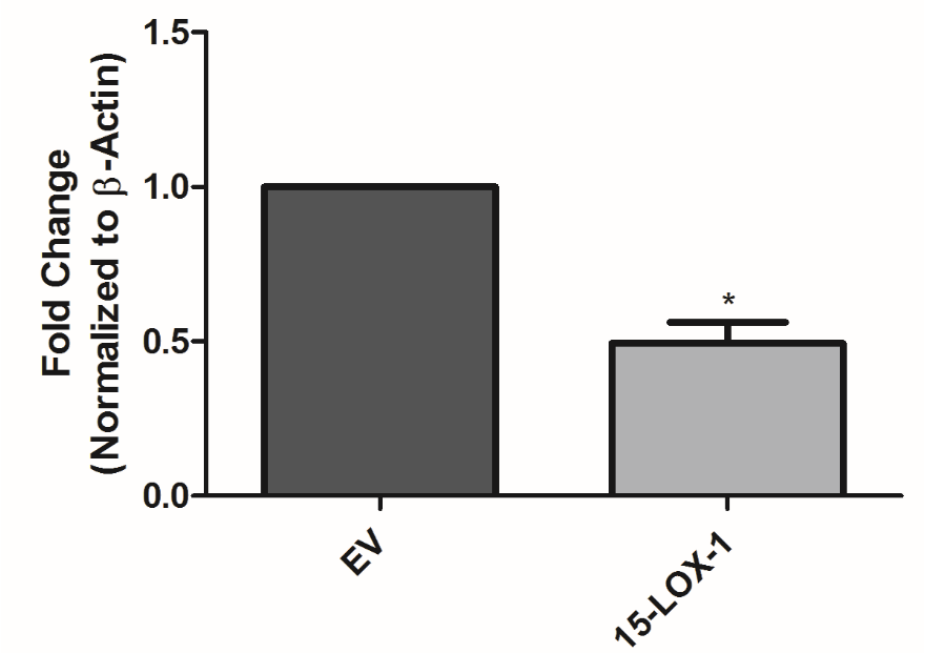

Figure 3.5: VEGF mRNA levels of transfected SW480 cells. The cells were transfected with 3ug pf the 15-LOX-1 plasmid or empty vector for 24h and then recovered for 48h. The cells were then processed for RNA isolation and cDNA synthesis. VEGF mRNA levels were determined by qRT-PCR using gene specific primers. A significant reduction of VEGF transcript in the presence of 15-LOX-1 ($P<0.05$, non-parametric *t*-test) was observed in comparison to the empty vector transfected cells.

In hypoxic conditions, cancer cells tend to release angiogenic inducers such as VEGF, whose expression is regulated by hypoxia inducible factor 1 alpha (HIF-1α) (Wu et al., 2014)We therefore wanted to confirm whether the decrease in VEGF-A levels observed in 15-LOX-1 expressing cells under normoxic conditions (see Figure 3.6) was also retained when the cells were grown under hypoxic conditions. In a separate experimental setup, we examined the levels of VEGF-A in SW480 cells re-expressing 15-LOX-1, however, this time the cells were incubated in a hypoxic environment. For this, the cells were either incubated in a hypoxia chamber containing 1% oxygen or the standard incubator (normoxic conditions) for 6 hours at 37ºC. At the end of the experiment, the cells were harvested for RNA isolation and VEGF levels were determined by qRT-PCR as before. Under normoxic conditions, VEGF levels were seen to decrease with 15-LOX-1 expression. On the other hand, under hypoxic conditions, the VEGF levels in both EV and 15-LOX-1 transfected cells were higher than the normoxic cells (Fig 3.6). This is expected since the expression of VEGF is expected to increase in hypoxic conditions (Wu et al., 2014).

In parallel, we observed a reduction in the levels of VEGF in 15-LOX-1 transfected hypoxic samples as compared to the EV transfected, hypoxic samples. Although, within hypoxia samples, the difference did not reach statistical significance, we observed a trend in 15-LOX-1 transfected cells, which had the lower mRNA levels of VEGF.

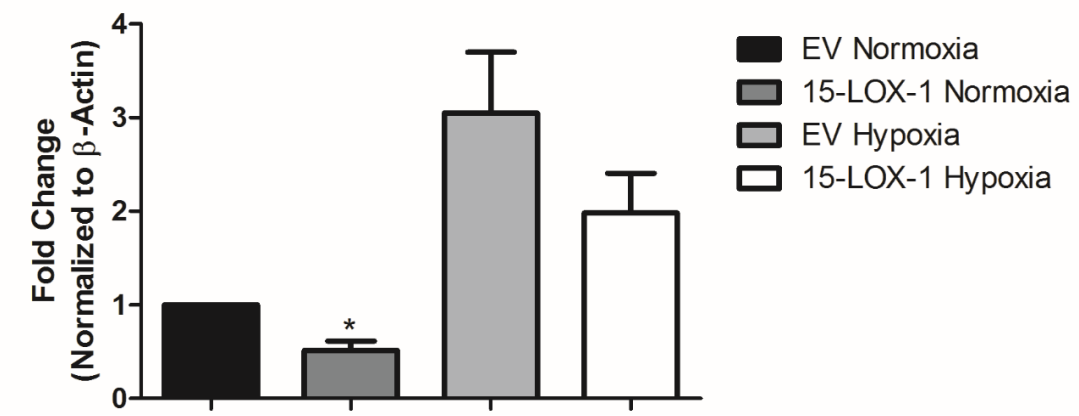

Figure 3.6: VEGF transcript levels in EV or 15-LOX-1 transfected SW480 cells exposed either to hypoxia or to normoxia. There is a significant reduction in normoxic VEGF levels between 15-LOX-1 and EV transfected cells ($P<0.05$, non-paramteric *t*-test), but the reduction in hypoxic samples did not reach statistical significance. The image is a representative of three independent replicates. ($P=0.3408$, Paired t-test was applied.)

The secreted form of VEGF is functional by binding either in an autocrine manner or in a paracrine manner to different receptors (Nishida et al., 2006). Therefore, we examined whether the re-expression of 15-LOX-1 resulted in any alteration in the secretion of VEGF into the conditioned medium, in both normoxic and hypoxic conditions. An ELISA kit that specifically measures secreted VEGF-A was used for this purpose. Similar to the expression of VEGF at the mRNA level, the secretion of VEGF into the conditioned medium was lower when the cells re-expressed 15-LOX-1 both in normoxic and within hypoxic conditions compared to the empty vector transfected cells (Fig 3.7).

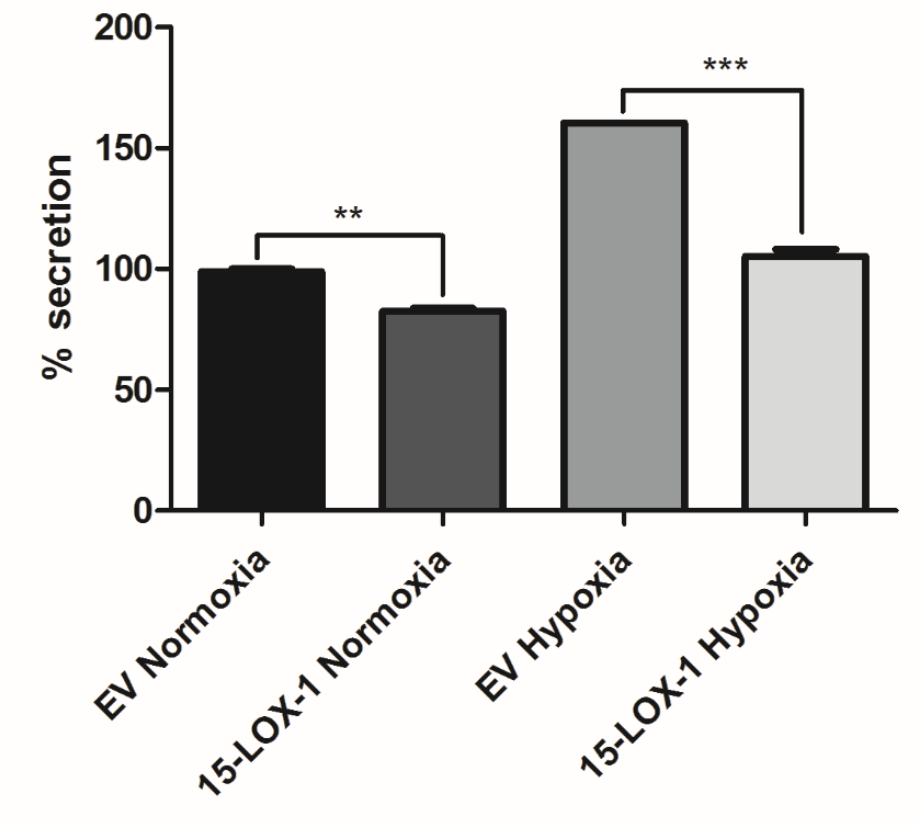

Figure 3.7: VEGF secretion levels quantified by ELISA kit, in both hypoxic and normoxic conditions. SW480 cells that were transfected with either 15-LOX-1 vector or corresponding empty vector were exposed to hypoxia (1% O_2) and normoxia (20% O_2) for 6 h. At the end of exposure, conditioned media from the transfected cells were collected and used for subsequent ELISA experiment according to manufacturer's guidelines. In both hypoxic and normoxic samples, there is a significant reduction in VEGF secretion levels in the cells transfected with 15-LOX-1 (**: $P<0.01$, ***: $P<0.001$, Student's *t*-test).

3.5. Tube formation assay: mimicking the angiogenic environment

Tube formation assay is a technique by which the process of angiogenesis is mimicked. Human Umbilical Vein Endothelial Cells (HUVECs) were incubated in conditioned medium collected from transfected epithelial cells and mixed with endothelial cell medium in a 1:1 ratio. In this experiment, the conditioned medium was collected from SW480 cells transfected with either the 15-LOX-1 plasmid or the empty vector. As a result, we expected HUVECs incubated with EV conditioned medium would form tube-like structures that mimic the formation of new blood vessels. A decrease in the number of tubes formed in HUVECs treated with 15-LOX-

1 conditioned medium when compared to the conditioned medium collected from empty vector transfected cells (Figure 3.8). The photographs were analyzed by an online software that is developed by Wimasis (Germany).

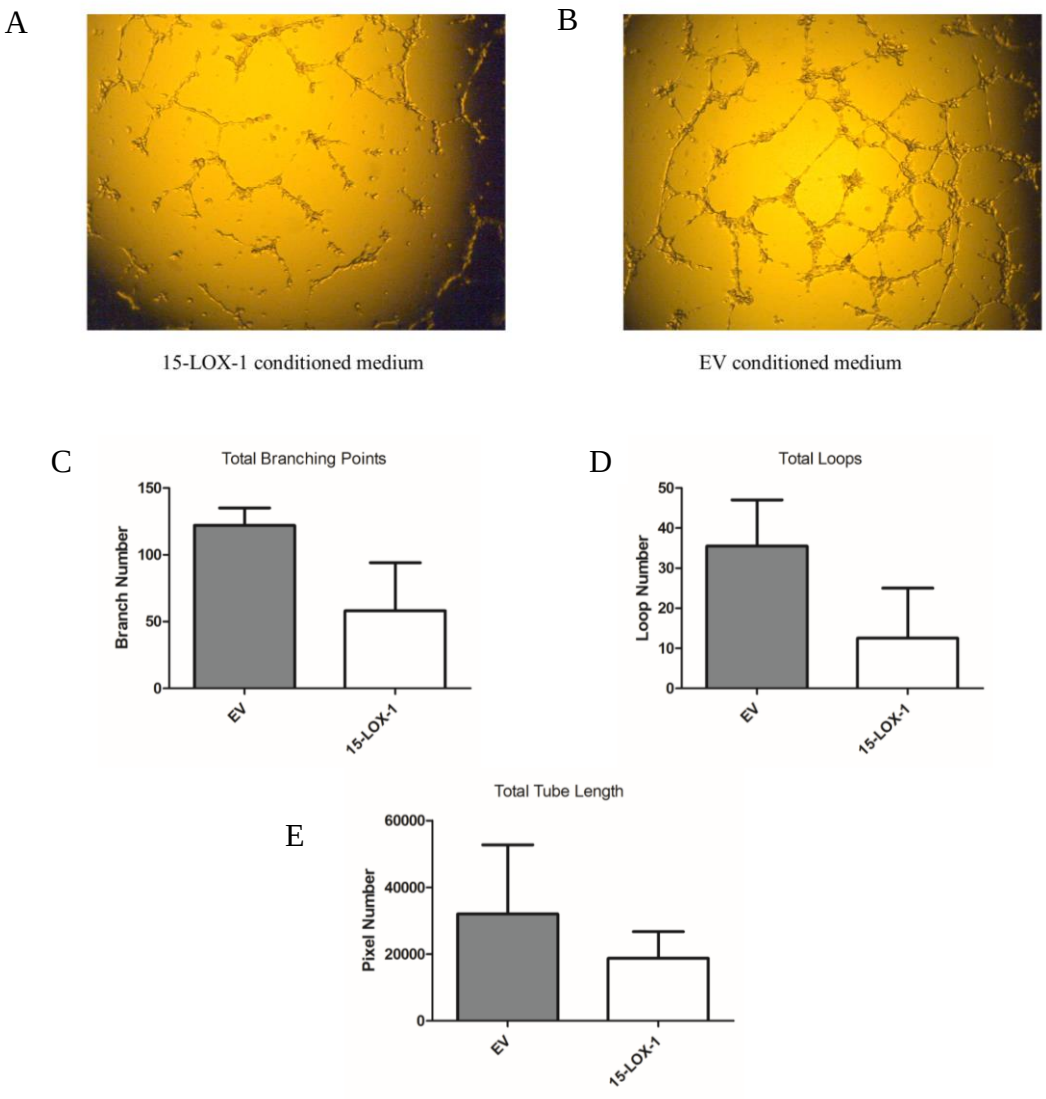

Figure 3.8: HUVECs treated with either EV or 15-LOX-1 conditioned medium forming tubes (A, B). The images are representative of two independent experiments. Numerical analysis of images containing total branching points, total loops and total tube length in pixels (C, D, E) performed by an online software, produced by Wimasis. None of the graphs were statistically significant, but mean of EV samples are always lower than 15-LOX-1 samples (total branching points, $p = 0.2365$; total loops, $p = 0.3084$; total tube length, $p = 0.6100$; Student's *t*-test was used.)

CHAPTER IV

DISCUSSION

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This study was aimed to examine the effects of 15-LOX-1 re-expression on angiogenic properties of SW480 cells. Lipoxygenases were first described in plants as enzymes that cause bleaching in wheat. Subsequently they were detected in soybean and many other plants where the enzyme was shown to be involved in pathways leading to the formation of odors with 'grassy notes' (Whitehead, Muller, & Dean, 1995). LOXs can have crucial roles in plants in the context of defense against insects, for example. It was shown that when lipoxygenases are depleted in potato plants using antisense-mediated depletion, it led to an increase in the insect attacks due to the loss of proteinase expression linked to lipoxygenase expression (Royo et al., 1999). In animals, 15-LOX-1, the isoform that oxygenates arachidonic acid at the 15^{th} position, was first isolated from rabbit reticulocytes, where the enzyme was shown to initiate the proteolytic degradation of organelles in the process of differentiation into mature red cells (Kühn & Brash, 1990). Subsequently, a number of effects of 15-LOX-1 expression were reported in inflammatory pathways. For instance, in rabbit, 15-LOX-1 overexpression in macrophages led to a reduction in inflammation and tissue damage (Serhan et al., 2003). 15-LOX-1 was subsequently shown to be expressed in a number of different organs. In the colon, overexpression of 15-LOX-1 resulted in decreased levels of interleukin 1-β (IL-1β), a pro-inflammatory cytokine that can lead to tissue damage in chronic inflammatory diseases (Dinarello, 1996) (Shureiqi et al., 2010).

15-LOX-1 expression is lost in poorly differentiated cancer cells as compared to their well-differentiated counterparts, or cells without tumorigenic characteristics. In a study where 128 cancer cell lines from different cancer types were analyzed, 15-

LOX-1 was shown to be lost universally from all of the cell lines and upregulated during the process of terminal differentiation (Moussalli et al., 2011). This fact supports the idea that 15-LOX-1 is tightly regulated during tumorigenesis. 15-LOX-1 re-expression was shown to induce apoptosis and reduce cell proliferation in colon cancer models (Lee et al., 2011). Additionally, in colon epithelia, 15-LOX-1 expression and 13-S-HODE, the metabolic product formed from the oxygenation of linoleic acid by 15-LOX-1, were decreased at different stages of development of the tumor (Imad Shureiqi et al., 2010).

In this study, we wanted to examine whether 15-LOX-1 re-expression would result in a decreased angiogenic phenotype. To understand this, we selected the SW480 cell line that does not express any 15-LOX-1. We transiently re-expressed 15-LOX-1 in these cells using an expression plasmid. This approach is a commonly used method to re-express 15-LOX-1 in colorectal cancer cells (Cimen, Astarci, & Banerjee, 2011; Çimen et al., 2009; Shureiqi et al., 2005). However, transient transfections do not last long as the stable transfections do. The vector copy number reduces with each cell division and gets lost in the population in a few days. Therefore, long term functional studies are not feasible.

Inhibition of apoptosis, induction of metastasis, immortalization resulting from the disruption of cell cycle checkpoints, loss of tumor suppressor genes and neo-angiogenesis are all considered to be hallmarks of cancer (Hanahan & Weinberg, 2011). Reconstitution of 15-LOX-1 in cancer cells has resulted in reversal of many of these hallmarks. For instance, 15-LOX-1 re-expression with the help of non-steroidal anti-inflammatory drugs (NSAIDs) induced apoptosis in colorectal cancer cells by increasing the levels of 13-(S)-HODE (Shureiqi et al., 2000). 15-LOX-1 expression was also shown to reduce motility and metastatic potential of colon cancer cells (Çimen et al., 2009). Mechanistically, this decrease in metastatic

potential has been attributed to a reduction in the expression of VEGF (Wu et al., 2014) as well as Metastasis-associated protein 1 (MTA1), a master regulator of

carcinogenesis that was shown to be expressed in a reverse correlation with 15-LOX-1 in cancer cell lines and colon cancer patient specimens (Tuncer et al, 2016). There are indications in the literature that 15-LOX-1 expression may be associated with an anti-angiogenic effect. For instance, 15-LOX-1 was shown to reduce VEGF-A and PIGF induced angiogenesis in rabbit skeletal muscle cells (Viita et al., 2008). The 15-LOX-1 metabolic product 13-S-HODE can act as a ligand for the orphan nuclear receptor PPARγ (Cimen et al., 2011). This ligand-receptor interaction was shown to have pro-angiogenic effects on cardiac myofibroblast cells by inducing the expression of VEGF (Chintalgattu et al., 2007). In contrast, another member of PPAR family, PPARα was shown to decrease VEGFR2 (VEGF receptor) levels when activated by agonists (Meissner et al., 2004). However, there is still very limited data on the role 15-LOX-1 in angiogenesis, particularly in colorectal cancer. Thus, we aimed to look at the effects of re-expression of 15-LOX-1 in colorectal cancer cell line SW480 and its role in angiogenesis.

To study angiogenesis, both hypoxic (1% O_2) and normoxic (20% O_2) conditions were used. The rationale is that hypoxic conditions result in stabilization of HIF-1α protein, a transcription factor that upregulates VEGF (Ferrara, 1997). A hypoxia chamber flushed a hypoxic gas mixture was used to incubate the transfected SW480 cells. Because hypoxia naturally stimulates angiogenesis, an overall enhancement in VEGF-A levels was observed in both 15-LOX-1 and EV transfected cells grown in hypoxic conditions. However, 15-LOX-1 transfected cells showed decreased levels of VEGF-A irrespective of whether the cells were grown in hypoxic or normoxic conditions compared to the EV transfected cells. Although the difference did not reach statistical significance ($P = 0.3408$) in the cells grown under hypoxia, it is clear that 15-LOX-1 transfected samples showed decreased trend in the levels of the VEGF transcript. VEGF-A levels in the 15-LOX-1 re-expressing cells showed very high variations and therefore could not reach statistical significance. This may be due to the increasing experimental errors with hypoxia. Hypoxic samples should be

harvested within seconds because hypoxia leads to the stability of normally unstable proteins such as HIF-1α. When cells are taken from hypoxia chamber and harvested

in a normoxic tissue culture hood, HIF-1α levels can decrease dramatically in a very short time (Wu et al., 2014). The small time differences in handling the samples among biological replicates may have resulted in large variations. VEGF expression is regulated primarily by hypoxia and cytokines. Cytokines such as TGF-β (Transforming growth factor – beta) induce VEGF mRNA expression as well (Nam, Park, & Kim, 2010). Although we have not examined the precise mechanism for the reduced expression of VEGF-A in 15-LOX-1 expressing cells, a number of mechanisms have been proposed. In prostate cancer cell lines, 15-LOX-1 expression was shown to enhance the proteolytic degradation of HIF-1α by enhancing its ubiquitination and degradation in the proteasome (Zhong, Wang, Kelavkar, Wang, & Simons, 2014).

Secretion of VEGF into extracellular matrix is a key process of angiogenesis (Park et al., 1993). Secreted VEGF binds to VEGF receptors (VEGFR1 and VEGFR2) and initiate signaling cascades in both epithelial and endothelial cells (Mochizuki & Kwon, 2008). These signaling cascades include the MAPK pathway which result in migration phenotype and leads to proliferation of the cell; this pathway is downstream of VEGFR2 binding of VEGF-A (Koch & Claesson-Welsh, 2012). In epithelial cells, VEGF-A acts as the main component of angiogenic signaling to stimulate the endothelial cells to divide and migrate; in parallel, in endothelial cells, it induces matrix metalloproteinases (MMPs) activity in endothelial cells to digest the extracellular matrix and grow through it (Mochizuki & Kwon, 2008). In this study, the lower transcript levels of VEGF was corroborated by lower levels of the secreted cytokine, both under hypoxic and normoxic conditions. This posits that when 15-LOX-1 is re-expressed in SW480 cells, it not only decreases the VEGF-A transcript levels, but also leads to a decline in the active form of the protein.

Secreted cytokines and other proteins from cancer cells are known to stimulate endothelial cells to grow through the extracellular matrix and form new blood vessels (Nishida et al., 2006). Also, VEGF was shown to be increasing in brown adipose tissue differentiation in mice (Bagchi et al., 2013). To understand whether the re-

expression of 15-LOX-1 in SW480 cells resulted in alterations in secreted angiogenic mediators, we collected conditioned medium from the cells transfected with either the 15-LOX-1 plasmid or the empty vector. Conditioned medium (also called as "cell secretome") can be defined as the collection of secreted proteins in the medium that the cells are growing in (Dowling & Clynes, 2011). Specifically, in conditioned media collected from hypoxic cells, the amount of growth factors such as VEGF and TNF-α is high (Gabrielyan, Knaak, Gelinsky, Arnhold, & Rösen-Wolff, 2014). All experiments were carried out with the same set-up to ensure consistent autocrine signaling. The conditioned medium was then mixed with growth medium for Human Umbilical Vein Endothelial Cells (HUVECs) at a 1:1 ratio. The HUVECs were incubated on Matrigel, a reconstituted extracellular matrix that contains laminin, collagen IV, heparin sulfate proteoglycans, and a number of growth factors (Hughes, Postovit, & Lajoie, 2010) with the conditioned medium from 15-LOX-1 or EV transfected cells. In this 3-dimensional 'tube formation assay', in the presence of appropriate stimuli such as pro-angiogenic factors, HUVECs form tube like structures. Thus, this experiment can experimentally determine the ability of various agents to induce/inhibit angiogenic sprouting. HUVECs incubated with the 15-LOX-1 conditioned medium were observed to produce fewer tubes than the cells treated with EV conditioned medium, indicating that re-expression of 15-LOX-1 resulted in decreased expression and/or secretion of different pro-angiogenic factors or enhanced the secretion or activation of different anti-angiogenic factors that may have resulted in decreased tube formation. This is consistent with both qRT-PCR and VEGF secretion data both of which showed a decrease in the levels of VEGF transcript and secreted form of VEGF, respectively, when 15-LOX-1 was re-expressed. The tube formation data were analyzed for the total tube length, total loops the cells formed and the total number of branching points. Of these three, total tube length corresponds to the pixels on the photo of cells which have formed closed tubes. Total loops refer to the encircled area that the cells formed; that is, the closed tube. Finally, number of branching points indicates how many branches have formed in the assay. When we compared these numbers, HUVECs treated with EV conditioned medium consistently showed higher values in each of these three parameters when compared to cells treated with 15-LOX-1 conditioned medium.

This further proves that the conditioned medium of 15-LOX-1 transfected cells have less growth factors for the endothelial cells to grow and form honeycomb-like structures in the tube formation assay.

CHAPTER V

CONCLUSION

In this study, 15-LOX-1's role on angiogenesis in the context of colorectal cancer was investigated. Our data suggest that 15-LOX-1 re-expression resulted in an inhibition of angiogenesis and provide us with further important data substantiating the tumor suppressive nature of 15-LOX-1. The major findings of this study are as follows:

- 15-LOX-1 re-expression led to decreased levels of VEGF-A both in transcript and secreted protein levels.
- HUVECs incubated in conditioned medium collected from 15-LOX-1 transfected cells formed less tubes compared to the cells treated with conditioned medium from the control cells..

Since the loss of expression of *ALOX15* is primarily of an epigenetic nature rather than through mutations, re-expression of the gene may be a feasible option through the use of chromatin modifiers. Future experiments are needed to determine the mechanism behind the anti-angiogenic effects of 15-LOX-1.

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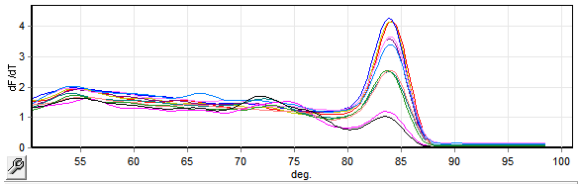
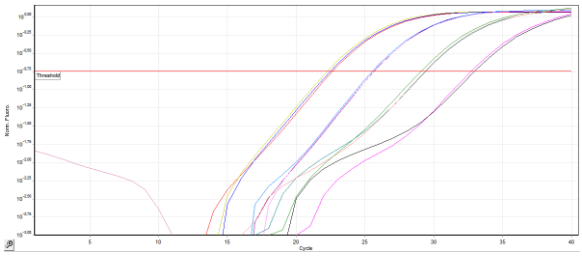
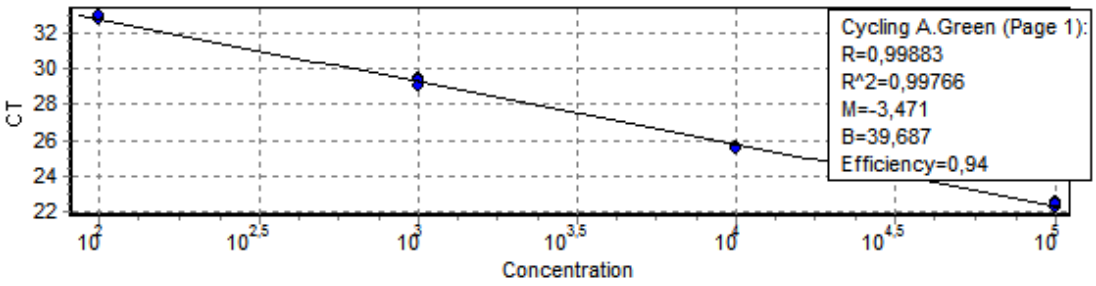
APPENDIX A

qRT-PCR Standard Curves



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





No.	C	Name	Type	Ct	Ct Comment	Given Conc (Cop	Calc Conc (Copie	% Var	Rep. Ct	Rep. Ct Stc	Rep. Ct (95% CI)	Rep. Calc. Conc.	Rep. Calc. Conc. (95%
1		1-1	Standard	22,64		100000	718,5069026211	18,3%	22,64			718,5069026211	[53695,9286553539 ,
2		1-2	Standard	22,26		100000	5043,777284281	5,0%	22,26			5043,777284281	[67557,2008573527 ,
3		1_3	Standard	22,48		100000	937,6634973287	9,1%	22,48			937,6634973287	[59215,3150231134 ,
4		2_1	Standard	25,66		10000	005,0728136876	10,1%	25,66			005,0728136876	[8248,84077983696 ,
5		2_2	Standard	25,65		10000	1099,980642929	11,0%	25,65			1099,980642929	[8317,18998702154 ,
6		2_3	Standard	25,54		10000	907,5124902615	19,1%	25,54			907,5124902615	[8896,95601812884 ,
7		3_1	Standard	29,42		1000	0,412680709301	9,0%	29,42			0,412680709301	[661,661240304368 ,
8		3_2	Standard	29,35		1000	9,040368739424	5,1%	29,35			9,040368739424	[691,402828805746 ,
9		3-3	Standard	29,06		1000	50,00838447939	15,0%	29,06			50,00838447939	[846,684366500123 ,
10		4-1	Standard	32,71		100	2,678954730837	2,7%	32,71			2,678954730837	[62,8572768159803 ,
11		4-2	Standard	32,97		100	6,322465884683	13,7%	32,97			6,322465884683	[52,0054265871541 ,
19		NTC	NTC										

Figure A.1: Standard curves for VEGF qRT-PCR

APPENDIX B

STANDARD CURVE FOR 13-(S)-HODE ASSAY

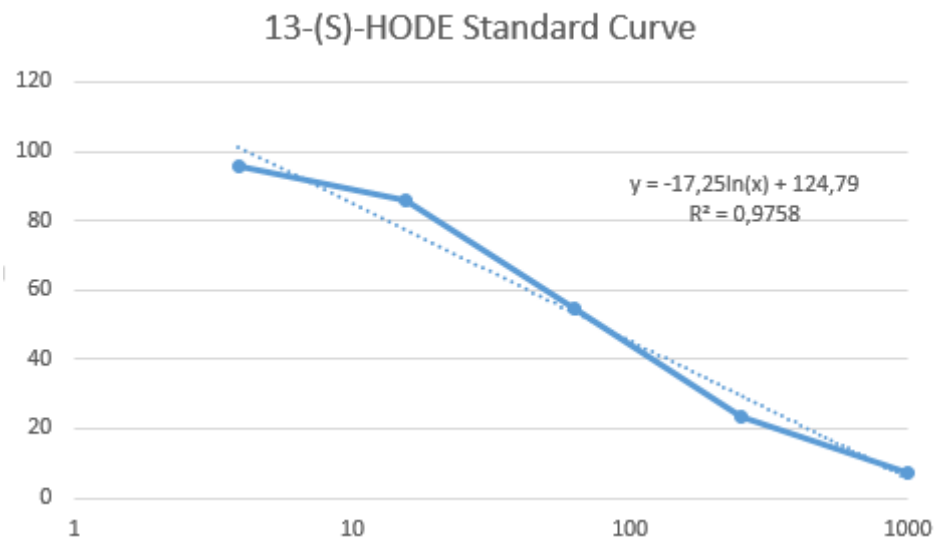


Figure B.1: Standard curve for 13-(S)-HODE assay

APPENDIX C

STANDARD CURVE FOR MICROBCA KIT

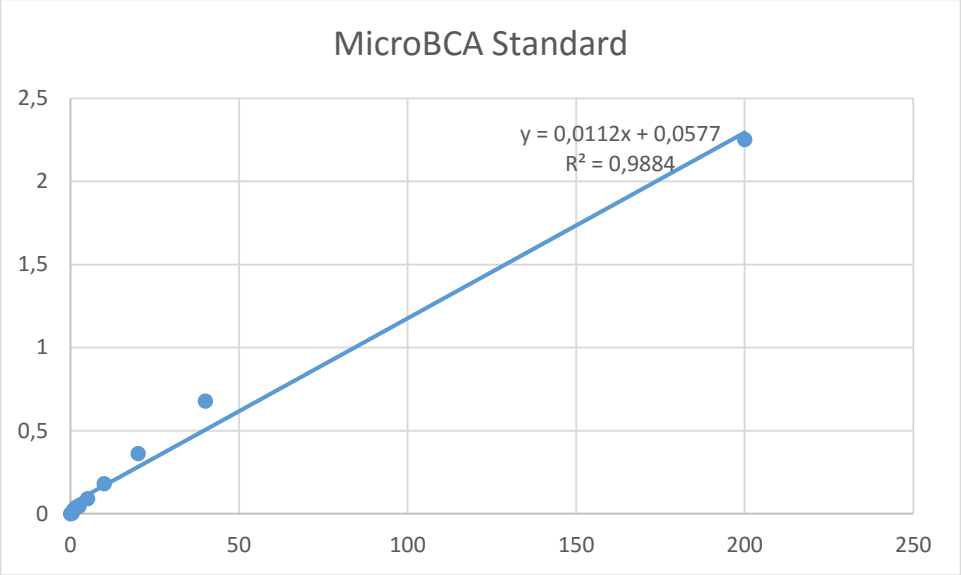


Figure C.1: Standard Curve for MicroBCA kit

APPENDIX D

MAP OF VECTOR USED IN THIS STUDY

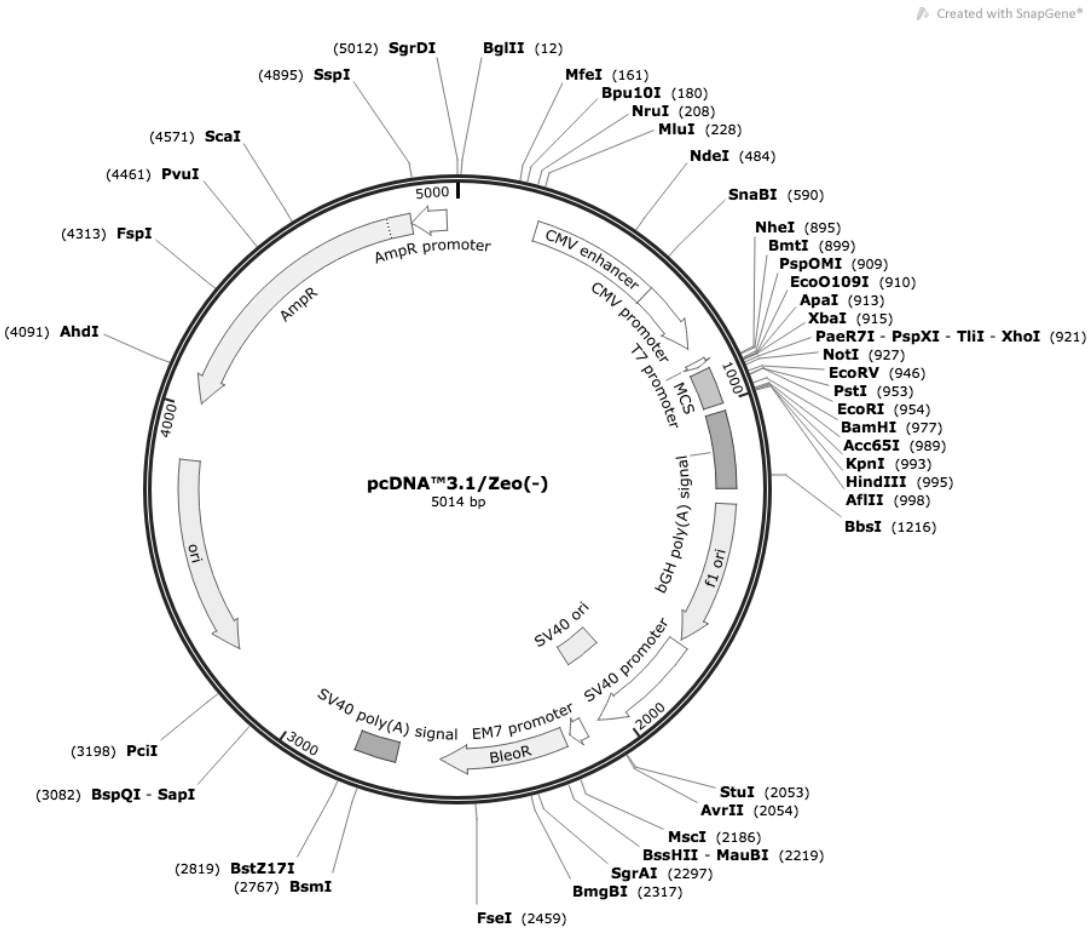


Figure D.1: Map of pcDNA3.1(-) vector used in the study

APPENDIX E

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

Table E.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

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 F – EQUATION FOR FOLD CHANGE CALCULATION IN QRT-PCR EXPERIMENTS

$$\text{Fold change} = \frac{\text{Efficiency}_{\text{target}}^{\Delta Ct(\text{control-sample})}}{\text{Efficiency}_{\text{reference}}^{\Delta Ct(\text{control-sample})}}$$

Ct: Cycle threshold